UNIVERSIDADE FEDERAL DE MINAS GERAIS
Instituto de Ciências Biológicas
Programa de Pós-Graduação em Genética

POLIMORFISMOS E HAPLÓTIPOS DE GENES ENVOLVIDOS NA REGULAÇÃO DA HEMOGLOBINA FETAL BASAL E INDUZIDA PELA HIDROXIUREIA EM PACIENTES PEDIÁTRICOS COM ANEMIA FALCIFORME

Rahyssa Rodrigues Sales

Belo Horizonte
2022

RAHYSSA RODRIGUES SALES

POLIMORFISMOS E HAPLÓTIPOS DE GENES ENVOLVIDOS NA REGULAÇÃO DA HEMOGLOBINA FETAL BASAL E INDUZIDA PELA HIDROXIUREIA EM PACIENTES PEDIÁTRICOS COM ANEMIA FALCIFORME

Tese apresentada ao Programa de Pós-Graduação em Genética, do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais como requisito parcial para obtenção de título de Doutora em Genética.

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ATA DE DEFESA DE TESE

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Às oito horas do dia **30 de agosto de 2022**, reuniu-se, a Comissão Examinadora de Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho intitulado: **"“Polimorfismos e Haplótipos de genes envolvidos na regulação da Hemoglobina Fetal basal e induzida pela Hidroxiureia em pacientes pediátricos com Anemia Falciforme"**, requisito para obtenção do grau de Doutora em **Genética.** Abrindo a sessão, o Presidente da Comissão, **Marcelo Rizzatti Luizon**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	**Instituição**	**CPF**	**Indicaçã**
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Renan Pedra de Souza	UFMG	064.488.066-01	APROVA

Pelas indicações, a candidata foi considerada: APROVADA

O resultado final foi comunicado publicamente à candidata pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora.

Belo Horizonte, 30 de agosto de 2022.

Marcelo Rizzatti Luizon

Claudia Regina Bonini Domingos

Pamela Souza Almeida Silva

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UNIVERSIDADE FEDERAL DE MINAS GERAIS
Programa de Pós-Graduação em Genética
Instituto de Ciências Biológicas

FOLHA DE APROVAÇÃO

“Polimorfismos e Haplótipos de genes envolvidos na regulação da Hemoglobina Fetal basal e induzida pela Hidroxiureia em pacientes pediátricos com Anemia Falciforme"

Rahyssa Rodrigues Sales

Tese aprovada pela banca examinadora constituída pelos Professores:

Marcelo Rizzatti Luizon
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Belo Horizonte, 30 de agosto de 2022

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Resumo

A anemia falciforme (AF) é uma doença autossômica recessiva causada por uma mutação de ponto no gene da beta globina, que leva à produção de hemoglobina variante, a HbS. A polimerização da HbS desencadeia hemólise intravascular e vaso-oclusão, que dão origem às manifestações sistêmicas da doença. A hemoglobina fetal (HbF) inibe a polimerização da HbS e, portanto, é modificadora dos desfechos clínicos e diminui a gravidade da AF. A Hidroxiurea (HU) é usada no tratamento da AF e sua eficácia clínica é relacionada à indução da HbF. Entretanto, existe grande variabilidade no aumento dos níveis de HbF ou toxicidade em resposta à HU entre pacientes com AF. Neste trabalho investigamos o efeito de polimorfismos de nucleotídeo único (Single Nucleotide Polymorfism-SNPs) em genes candidatos à concentração de HbF basal ou induzida pela HU, e sua associação com parâmetros hematológicos e clínicos em uma coorte de 250 pacientes pediátricos com AF diagnosticadas pelo Programa de Triagem Neonatal de Minas Gerais com acompanhamento retrospectivo pelo Hemocentro de Belo Horizonte da Fundação HEMOMINAS por no mínimo por 8 anos. No Capítulo 1 examinamos o efeito de SNPs em genes candidatos à concentração basal de HbF, em parâmetros hematológicos e na incidência de complicações clínicas da AF na coorte de 250 pacientes pediátricos com AF acompanhada pelo HEMOMINAS. Encontramos associação independente dos SNPs rs4671393 (gene *BCL11A*), rs9399137 e rs4895441 (região intergênica *HBS1L-MYB*, HMIP-2) com a concentração de HbF. SNPs associados com HbF foram associados também à contagem reduzida de reticulócitos e menor incidência de síndrome torácica aguda e necessidade de transfusão sanguínea. No Capítulo 2 estimamos haplótipos compostos por alelos de SNPs funcionais de *BCL11A* (TAT; rs1427407, rs766432 e rs4671393) e HMIP-2 (CGC; rs9399137, rs4895441 e rs9494145). Esses haplótipos foram associados com parâmetros hematológicos menos severos e taxa reduzida de complicações clínicas quando comparados com o respectivo haplótipo selvagem. Adicionalmente, observamos que *BCL11A* e HMIP-2 parecem interagir na regulação dos níveis de HbF. No Capítulo 3 conduzimos uma revisão sistemática da literatura para responder se SNPs influenciam as alterações nos níveis de HbF em pacientes com AF tratados com HU. Encontramos evidência

de que SNPs do íntron 2 de *BCL11A* influenciam a resposta da HbF em pacientes com AF. Foi conduzid uma análise de enriquecimento de vias a partir dos genes com SNPs associados encontrados na revisão sistemática e identificamos vias biológicas potencialmente envolvidas nos mecanismos de ação da HU. No Capítulo 4 selecionamos dentre os 250 pacientes pediátricos com AF aqueles que iniciaram tratamento com HU durante o período retrospectivo de acompanhamento de 8 anos (n = 110), para testar o efeito de SNPs na resposta à HU. Em um modelo de regressão linear múltipla ajustado pela idade, gênero, tempo de acompanhamento, dose de HU e HbF basal, identificamos os SNPs rs766432, rs4671393 e rs7599488 de *BCL11A* como preditores do ∆HbF (HbF final – HbF basal) nos pacientes com AF tratados com HU. O SNP rs4671393 também foi associado com maior risco de incidência de transfusão sanguínea nestes pacientes com AF tratados com HU. Em conclusão, SNPs do gene BCL11A e da região intergênica HMIP-2 influenciam HbF basal e são associados com quadro clínico menos severo da AF em pacientes não tratados com HU. SNPs de *BCL11A* também parecem influenciar a resposta da HbF nos pacientes com AF tratados com HU, mas sua utilidade clínica ainda precisa ser avaliada.

Palavras-chave: Anemia Falciforme, Hemoglobina Fetal (HbF), *BCL11A*, *HBS1L-MYB*, Hidroxiureia, Farmacogenética.

Abstract

Sickle cell anemia (SCA) is an autosomal recessive disease caused by a point mutation in the beta globin gene. It produces the HbS variant of hemoglobin. The polymerization of HbS triggers intravascular hemolysis and vaso-occlusion, the systemic manifestations of the disease. Fetal hemoglobin (HbF) inhibits the HbS polymerization, thereby reducing the SCA severity. Hydroxyurea (HU) is used in the treatment of SCA and its clinical efficacy is related to the induction of HbF. However, there is variability in the increase of HbF levels or toxicity in response to HU among patients with SCA. We investigated the effect of single nucleotide polymorphism (SNPs) on baseline and HU-induced HbF concentration, and their association with hematological and clinical outcomes in a cohort of 250 pediatric patients with SCA. The patients were diagnosed by the Newborn Screening Program of Minas Gerais from the Blood Center of Minas Gerais state, the HEMOMINAS Foundation. There is a retrospective cohort with 8 years of follow-up at least. In Chapter 1, we examined the effect of SNPs of candidate genes on baseline HbF concentration, hematologic parameters, and in the incidence of clinical complications of SCA in a cohort of 250 pediatric patients with SCA. The SNPs rs4671393 (*BCL11A* gene), rs9399137 and rs4895441 (*HBS1L-MYB* intergenic region, HMIP-2) were found to be independently associated with HbF concentration. SNPs associated with HbF were also found to be associated with reduced reticulocyte count and lower incidence of acute chest syndrome and blood transfusion. In Chapter 2, we estimated haplotypes composed by functional alleles of *BCL11A* (TAT; rs1427407, rs766432 and rs4671393) SNPs and HMIP-2 (CGC; rs9399137, rs4895441 and rs9494145). These haplotypes were found to be associated with less severe hematological parameters and reduced rate of clinical complications when compared to their respective wild-type haplotypes. Additionally, we observed that the *BCL11A* and HMIP-2 *loci* seems to interact in the regulation of HbF levels. In Chapter 3 we conducted a systematic review of the literature to answer whether genetic polymorphisms influence the HbF changes in patients with SCA treated with HU. We found evidence that SNPs of the intron 2 of *BCL11A* influences the HbF response in patients with SCA. A pathway enrichment analysis was conducted from the genes with associated SNPs found in the systematic review and we identified biological pathways

potentially involved in the mechanisms of action of HU. In Chapter 4, we selected from the cohort of 250 pediatric patients with SCA, those who started treatment with HU during the retrospective follow-up (n = 110). We tested the effect of SNPs of the *BCL11A* gene and HMIP-2 intergenic region on the HbF changes in response to HU. In a multiple linear regression model adjusted for age, gender, follow-up time, HU dosage, and baseline HbF, we identified *BCL11A* SNPs (rs766432, rs4671393, and rs7599488) as predictors of ∆HbF (final HbF – baseline HbF) in pediatric patients with SCA treated with HU. The rs4671393 SNP was also found to be associated with a higher risk of blood transfusion incidence in these AF patients treated with HU. In conclusion, SNPs of the *BCL11A* gene and the HMIP-2 intergenic region influence baseline HbF and were found to be associated with a less severe clinical outcomes in patients with SCA. SNPs of the *BCL11A* gene seems to influence the HbF response in patients with SCA treated with HU, but their clinical useful remains to be evaluated.

Key-words: Sickle Cell Anemia (SCA), Fetal hemoglobin (HbF), *BCL11A*, *HBS1L-MYB*, Hydroxyurea, Pharmacogenetics

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Lista de Abreviaturas

AF: Anemia Falciforme

ATP: Adenosina Trifosfato

BCL11A: Linfoma de Células B 11A (do inglês, B-Cell Lymphoma 11A)

CEU: População de Utah, com residentes da Europa Ocidental e do Norte do estudo "*The 1000 Genomes*"

DMT: Dose Máxima Tolerada

DNA: Ácido Desoxirribonucleico (do inglês, *Deoxyribonucleic Acid*)

ENCODE: *Encyclopedia of DNA Elements*

eDAMPs: Moléculas de Padrão Molecular associadas ao Dano/Perigo de Eritrócitos Moléculas de Padrão Molecular associadas ao Dano/Perigo (do inglês, *erythrocyte Danger-associated Molecular Pattern Molecules*)

eNOS: Sintase Endotelial do Óxido Nítrico

eQTLs: *Locus* de Herança Quantitativa (do inglês, E*xpression Quantitative Trait Loci*)

GTEx: *The Genotype Tissue Expression*

GWAS: Estudo de Associação Genômica Ampla (do inglês *Genome Wide Association Studies*)

HbF: Hemoglobina Fetal

HBG1: Subunidade Gama 1 da Hemoglobina (do inglês, *Hemoglobin Subunit Gamma 1*)

HBG2: Subunidade Gama 2 da Hemoglobina (do inglês, *Hemoglobin Subunit Gamma 2*)

HBS1L: *HBS1L Like Translational GTPase*

HbS: Hemoglobina S (do inglês, *Sickle Hemoglobin*)

HPLC: Cromatografia Líquida de Alta Eficiência (do inglês, *High-performance Liquid Chromatography*)

HU: Hidroxiureia

LCR: Região de Controle do *Locus* (do inglês, *Locus Control Region*)

LD: Desequilíbrio de Ligação (do inglês, *Linkage Desequilibrium*)

MAF: Frequência do Menor Alelo (do inglês, *Minor Allele Frequency*)

MYB: *MYB Proto-Oncogene, Transcription Factor*

NO: Óxido Nítrico

OR: *Odds Ratio*

PTN-MG: Programa de Triagem Neonatal de Minas Gerais

QTL: *Locus* de Herança Quantitativa (do inglês *Quantitative trait Loci*)

ROS: Radicais Livres de Oxigênio

STA: Síndrome Torácica Aguda

SEA: Sequestro Esplênico Agudo

SUS: Sistema Único de Saúde

SNP: Nucleotídeo de Polimorfismo Único, do inglês (*Single Nucleotide Polymorphisms*)

SpO2: Saturação Periférica de Oxigênio

TA: Termo de Assentimento

TCLE: Termo de Consentimento Livre e Esclarecido

VCAM-1: Molécula de Adesão Celular Vascular (do inglês, *Vascular Cell Adhesion Molecule-1*)

VEGF: Fator de Crescimento Vascular Endotelial (do inglês, *Vascular Endothelial Growth Factor*)

YRI: População de Yoruba, em Ibadan, Nigéria do estudo “*The 1000 Genomes*”

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1. Introdução

1.1 Anemia Falciforme: epidemiologia e impacto na saúde pública

A anemia falciforme (AF) é uma das doenças genéticas mais comuns no Brasil e constitui um problema de saúde pública. Segundo dados do Programa Nacional de Triagem Neonatal do Ministério da Saúde, estima-se a prevalência de 25.000 a 30.000 indivíduos com AF e a incidência de 3.500 novos casos por ano no Brasil (CANÇADO; JESUS, 2007). Em Minas Gerais, a incidência da AF é de um a cada 2.400 nascidos vivos (FERNANDES *et al.*, 2010), uma parcela significativa no cenário nacional.

A doença falciforme (DF) compõe um grupo amplo de doenças genéticas, a doença falciforme, que é caracterizada pela concentração de hemoglobina S (HbS; $\alpha_2\beta^S{}_2$) variante superior a 50% (NAOUM, 2000). O alelo β^S é uma mutação *missense* (rs334) no sétimo códon do gene da beta globina, codificando uma valina residual no lugar do ácido glutâmico, aminoácido codificado normalmente nessa posição (STEINBERG, 2008). A proteína variante HbS é produzida como resultado da mudança do aminoácido. A AF é a homozigose do alelo β^S, genótipo mais prevalente no Brasil e de maior gravidade clínica (HABARA; STEINBERG, 2016; SERJEANT, 1997).

A AF é doença que exige cuidados especiais. Na ausência de tratamento adequado e em tempo oportuno, estima-se que cerca de 20% das crianças com AF não alcançam os 5 anos de idade (FEDERAL, [*s. d.*]). A AF raramente apresenta sinais clínicos em neonatos, o que se justifica pela alta concentração de hemoglobina fetal (HbF) ao nascimento. Portanto, o diagnóstico precoce pela triagem neonatal constitui peça fundamental para melhoria da expectativa e qualidade de vida dos pacientes (RODRIGUES *et al.*, [*s. d.*]). Estes pacientes são então encaminhados para centros especializados, onde iniciam tratamento adequado, com intervenções que incluem antibioticoterapia profilática e vacinação especial adicional ao calendário nacional de vacinação (BRAGA, 2007; DE MONTALEMBERT; TSHILOLO, 2007).

Além do diagnóstico precoce, a instituição da Política Nacional de Atenção Integral às Pessoas com Doença Falciforme e outras Hemoglobinopatias em

2005 (hoje consolidada na Portaria n° 2/2017 GM/MS) no âmbito do Sistema Único de Saúde (SUS) tem mudado a história natural da doença falciforme no Brasil.

1.2 Fisiopatologia da AF: o papel central da hemólise intravascular

A AF cursa como doença multissistêmica, embora seja uma doença monogênica (REES *et al.*, 2010a). A polimerização da HbS é o evento primário na fisiopatologia da AF, responsável pelos desfechos clínicos (KATO *et al.*, 2018; REES *et al.*, 2010a). Em condições de hipóxia, a oxi-HbS perde o oxigênio e se torna deoxi-HbS, dando início à polimerização (NAOUM, 2000). A deoxi-HbS promove a formação entre aminoácidos de cadeias adjacentes da β^S-globina (**Figura 1**). Três fatores determinam a extensão da polimerização da HbS: o grau de desoxigenação, a concentração intracelular de HbS e a concentração intracelular de HbF (GALIZA NETO; PITOMBEIRA, 2003a; NAOUM, 2000).

A polimerização da HbS promove alteração morfológica nas hemácias, que assumem o formato de foice. O grau de saturação de oxigênio da HbS é determinante para a polimerização e retorno a forma discóide (GALIZA NETO; PITOMBEIRA, 2003a; NAOUM, 2000). Ao circularem nos capilares teciduais, as hemácias passam por ciclos repetidos de isquemia e reperfusão (HABARA; STEINBERG, 2016). Embora possam reassumir o formato discóide quando reoxigenadas, as alterações morfológicas causadas pela falcização diminuem a sobrevida das hemácias (STEINBERG, 2008). A taxa de hemólise, frequentemente, excede a capacidade de produção de novas hemácias pela medula óssea, resultando em anemia hemolítica crônica e alta contagem de reticulócitos, liberados de forma prematura (QUINN, 2013).

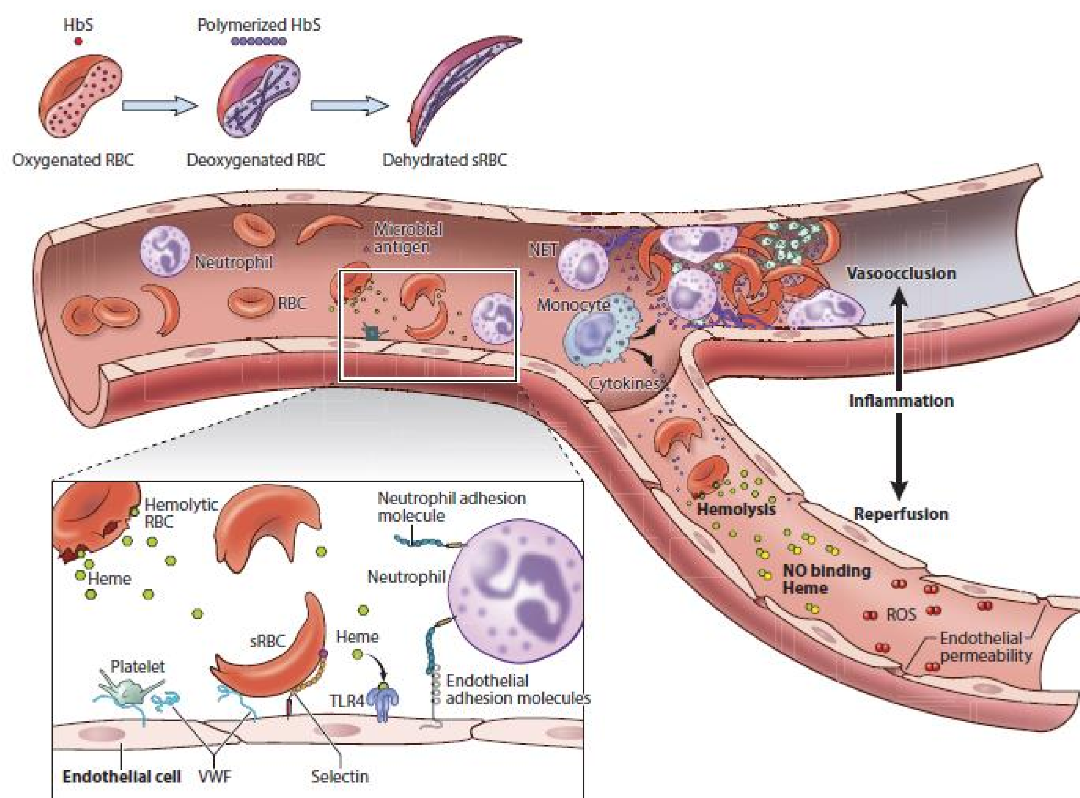

Figura 1: Fisiopatologia da anemia falciforme.

Quando desoxigenada, a HbS se polimeriza, levando à falcização das hemácias. O processo diminui a sobrevida das hemácias. Hemólise promove vasculopatia por meio da liberação de substâncias que ativam o endotélio vascular. As células falciformes desencadeiam oclusão microvascular através de interações com neutrófilos e plaquetas ativados e adesão ao endotélio vascular, processo que ocasiona isquemia e hipóxia tecidual, seguida de vasodilatação e lesão por reperfusão. A liberação de hemoglobina e arginase no plasma reduz a biodisponibilidade de óxido nítrico (NO), o que agrava a vaso-oclusão. Fonte: Williams and Thein, 2018*(WILLIAMS; THEIN, 2018)*.

A hemólise persistente promove vasculopatia por uma série de mecanismos, todos eles causados pela liberação de substâncias que apresentam propriedades patológicas no endotélio vascular. Estas moléculas têm sido reconhecidas como eDAMPs (do inglês, *erythrocyte Danger-associated Molecular Pattern molecules*), que são moléculas liberadas durante a lesão tecidual maciça e que podem ativar vias da imunidade inata (**Figura 2**) (POTOKA; GLADWIN, 2015).

Primeiramente, pequenas quantidades de hemoglobina liberadas no plasma são suficientes para depletar o óxido nítrico (NO), um potente vasodilatador e inibidor plaquetário, e prejudicar completamente a sinalização do

NO no endotélio (REES *et al.*, 2010a). Arginase-1 também é liberada, a enzima metaboliza l-arginina em ornitina, reduzindo a biodisponibilidade de l-arginina, que é essencial para a síntese de NO pela sintase endotelial do óxido nítrico (eNOS)(POTOKA; GLADWIN, 2015; STEINBERG, 2008). A hemoglobina livre também pode levar à formação de radicais livres de oxigênio (ROS), por meio da peroxidação lipídica, que por fim levam à disfunção endotelial(POTOKA; GLADWIN, 2015). Possivelmente, a formação de ROS pela hemoglobina livre ativa oxidases, como xantina oxidase e NADPH oxidase, que promovem estresse oxidativo vascular (ASLAN *et al.*, 2001).

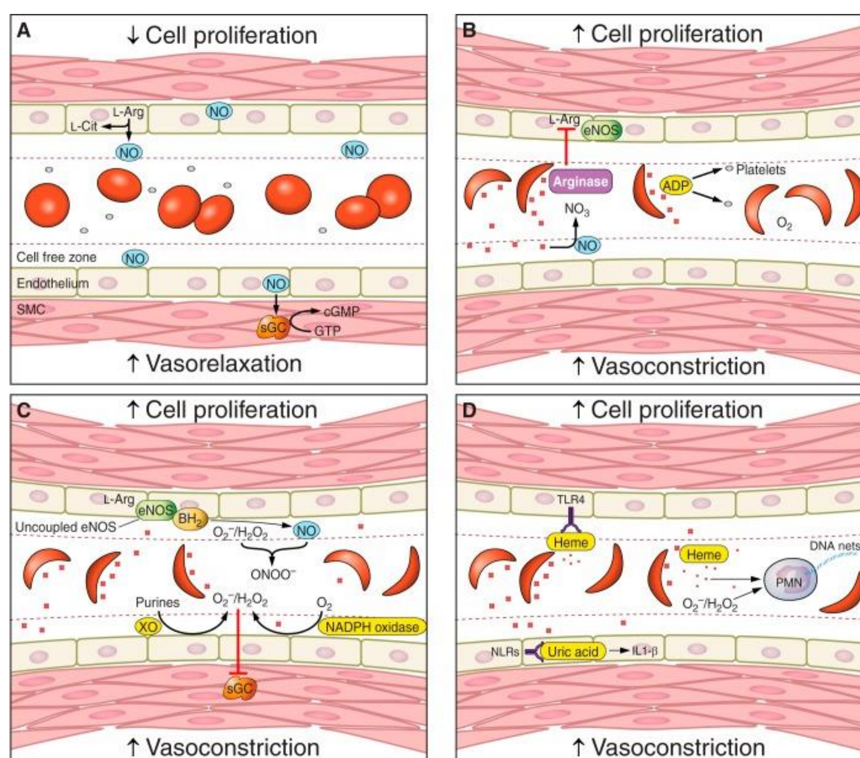

Figura 2: Hemólise intravascular

Efeitos da liberação das eDAMPs provenientes da hemólise intravascular que contribuem para disfunção endotelial e vasculopatia) Homeostase vascular e da formação de óxido nítrico (NO, do inglês Nitric Oxide); **B**) Arginase modula o desbalanço na biodisponibilidade de NO, e ativação plaquetária; C) Formação de ROS e extresse oxidativo. **D**) Liberação de heme, ATP, ácido úrico e inflamação estéril. Fonte: Potoka e Gladwin, 2015 (POTOKA; GLADWIN, 2015).

A alta taxa de hemólise também está associada a uma alta taxa de produção de novas hemácias, que liberam seus núcleos fornecendo DNA significativo para o metabolismo, o que leva ao aumento das concentrações de ácido úrico. Os cristais de ácido úrico podem se ligar aos receptores do tipo NOD e ativar potencialmente a imunidade inata (MARTINON *et al.*, 2006). O ATP liberado durante a hemólise também ativará essa via. Heme, ATP e ácido úrico

podem então ser consideradas como novas eDAMPs, que conduzem à inflamação estéril (GLADWIN; OFORI-ACQUAH, 2014).

Quando as hemácias falcizadas perdem o seu poder deformatório, que as impossibilita de circular nos capilares da microcirculação, a possibilidade de aderência ao endotélio e de vaso-oclusão aumenta (NAOUM, 2000; REES *et al.*, 2010a). Quando aderidas à superfície do vaso, as hemácias falcizadas favorecem a formação de trombos na microcirculação e na macrocirculação, o que desencadeia hipóxia tecidual e leva mais moléculas de HbS ao estado de deoxi-HbS (NAOUM, 2000; REES *et al.*, 2010a).Os episódios de isquemia nos vasos sanguíneos levam a formação de mais estresse oxidativo, aumentando a expressão de moléculas de adesão celular endotelial, a síntese de citocinas inflamatórias e o recrutamento de leucócitos, o que exacerba uma situação circulatória já desfavorável (REES *et al.*, 2010a).

Os processos fisiopatológicos descritos acima desencadeiam as manifestações clínicas da AF, que envolvem principalmente hemólise intravascular e vaso-oclusão. Os desfechos clínicos de crise álgica, síndrome torácica aguda (STA), acidente vascular cerebral, sequestro esplênico, priapismo, retinopatia e osteonecrose são originados principalmente pelo processo vaso-oclusivo. Por outro lado, crise aplástica, colelitíase, anemia e icterícia são originadas principalmente pela hemólise (STEINBERG, 1999).

A **Figura 3** ilustra a etiologia da STA, uma complicação grave da AF, cuja etiologia envolve vaso-oclusão pulmonar e na medula óssea, seguida de processo inflamatório (REES *et al.*, 2010a). A STA é uma das principais causas de morte em crianças com doença falciforme, como mostrado em uma coorte oriunda do Programa de Triagem Neonatal de Minas Gerais (FERNANDES *et al.*, 2017).

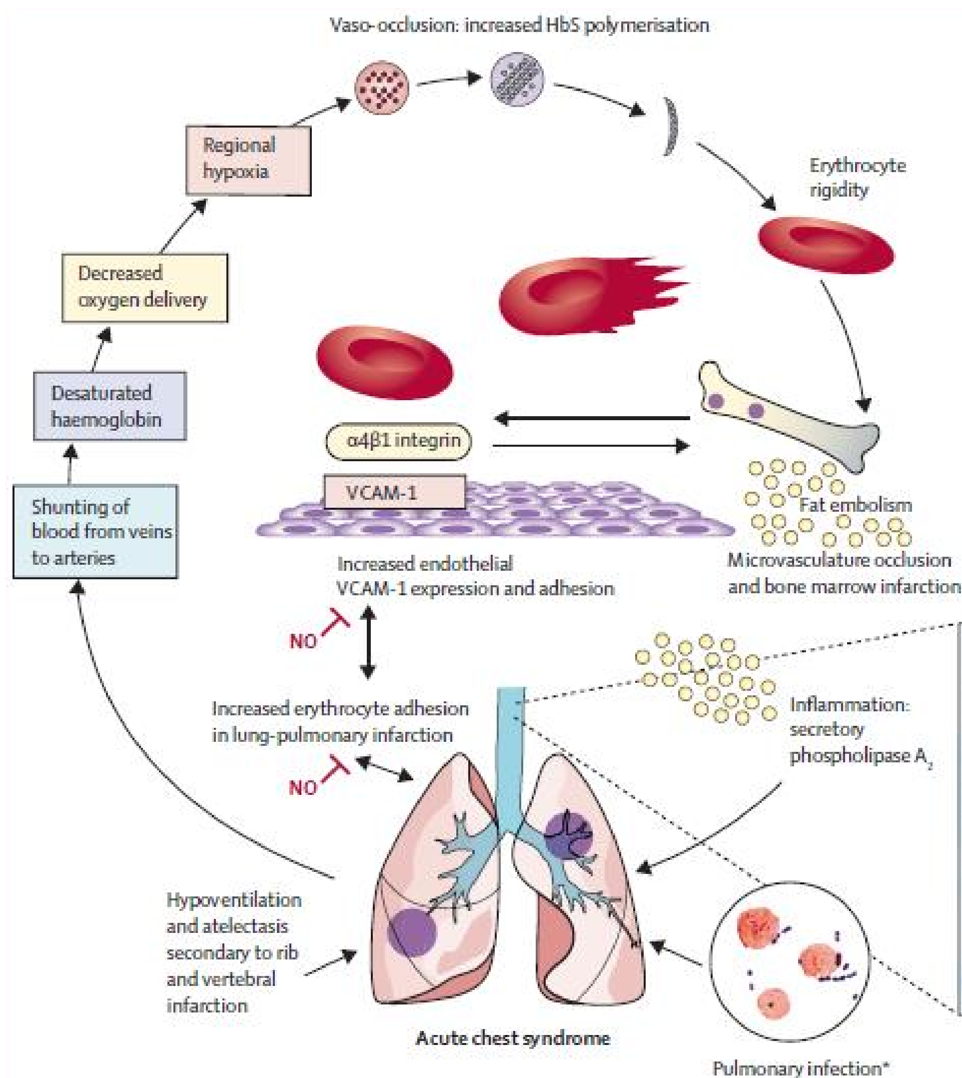

Figura 3: Fisiopatologia da Síndrome Torácica Aguda

Síndrome Torácica Aguda na anemia falciforme. Infecções ou outros estímulos inflamatórios ocasionam hipóxia pulmonar e expressão aumentada de moléculas de adesão endotelial. Polimerização da HbS e vaso-oclusão aumentam a hipóxia pulmonar e a inflamação em um ciclo constante. Hemoglobina plasmática livre, reduz a biodisponibilidade de óxido nítrico (NO) e altera a expressão de VCAM-1 (Vascular Cell Adhesion Molecule-1). A vaso-oclusão e o infarto da medula óssea podem causar embolia gordurosa, prejudicando a circulação pulmonar. A secreção aumentada de fosfolipase A2 como resposta à inflamação pode aumentar ainda mais a expressão de moléculas de adesão na vasculatura pulmonar e causar mais vaso-oclusão. Fonte: modificado de Rees, et.al. 2010 (REES et al., 2010b).

Sequestro esplênico agudo (SEA) é outro desfecho responsável por alta taxa de mortalidade na AF (FERNANDES *et al.*, 2010). O SEA pode ser definido como diminuição da concentração de hemoglobina maior ou igual a 2 g/dl comparada ao valor basal do paciente, acompanhada de sinais sugestivos do aumento da atividade eritropoética e aumento súbito das dimensões do baço, normalmente acompanhado de choque hipovolêmico (KHATIB *et al.*, 2009). O

baço é um grande repositório de células fagocíticas mononucleares na polpa vermelha e tecido linfóide na polpa branca, combinando função de defesa imunológica com controle de qualidade das hemácias senescentes ou alteradas(KHATIB *et al.*, 2009). Contudo, o baço é o primeiro órgão injuriado pela fisiopatologia da AF, com evidência de hipoesplenismo antes de um ano de idade na maioria das crianças com AF (BROUSSE *et al.*, 2014).

Com a baixa deformabilidade das hemácias falcizadas associada a alta viscosidade, a polpa vermelha do baço começa a congestionar logo nos primeiros meses de vida, levando a asplenia funcional. Hemorragias e infartos também ocorrem e o baço começa a diminuir, podendo haver substituição por tecido fibroso, um evento conhecido como “autoesplenectomia” (KHATIB *et al.*, 2009). Na ausência de medidas profiláticas, a disfunção esplênica na AF contribui muito para a alta taxa de mortalidade na primeira infância devido a devastadora infecção bacteriana (ONWUBALILI, 1983; OVERTURF; POWARS, 1980). O súbito acúmulo de sangue em um baço dilatado, o chamado SEA, apresenta poucos sinais de alerta antes do início da hipotensão vital, o que provavelmente ocasiona a alta taxa de mortalidade decorrente dessa morbidade (KHATIB *et al.*, 2009). Além disso, a taxa de recorrência é consideravelmente alta (~50%) (EMOND *et al.*, 1985).

1.3 Hemoglobina fetal: principal modificadora da gravidade clínica da AF

A **hemoglobina fetal (HbF)** apresenta impacto significativo na gravidade clínica da AF e é a principal modificadora dos fenótipos da AF. A HbS tende a formar extensa rede de polímeros dentro das hemácias, quando desoxigenada (STEINBERG, 2008). O processo de polimerização da HbS constitui o evento molecular primário que inicia a cascata fisiopatológica que dá origem às manifestações sistêmicas da AF (NAOUM, 2000). A HbF pode atenuar a fisiopatologia da AF, por inibir a polimerização da HbS. A concentração intracelular de HbF modifica a taxa e a extensão da polimerização da HbS, mitigando a gravidade clínica da AF (GALIZA NETO; PITOMBEIRA, 2003b; NAOUM, 2000). Pacientes com AF apresentam concentração consideravelmente maior de HbF que a população geral sem AF, o que pode ser resultado de seleção positiva nesses pacientes (BAUER; ORKIN, 2015; LETTRE; BAUER, 2016a).

A HbF ($\alpha^2\gamma^2$) é a hemoglobina predominante durante o período fetal (SANKARAN *et al.*, 2008). Após o nascimento, a HbF é gradativamente substituída pela hemoglobina do adulto (HbA) e, no caso de pacientes com AF, pela hemoglobina variante HbS. Este processo é mediado por reguladores transcricionais e envolve a formação de uma alça na fita de DNA linear, de modo que a região de controle do *locus* (LCR, do inglês *Locus Control Region*) se ligue em sequências conservadas no agrupamento de genes da β-globina, ativando sequencialmente os genes ao longo do desenvolvimento humano (**Figura 4**) (BAUER; ORKIN, 2015). Nesse período, ocorre um progressivo silenciamento dos genes da γ-globina (*HBG1* e *HBG2*) e ativação do gene da β-globina (*HBB*) (BAUER; ORKIN, 2015; SANKARAN *et al.*, 2008).

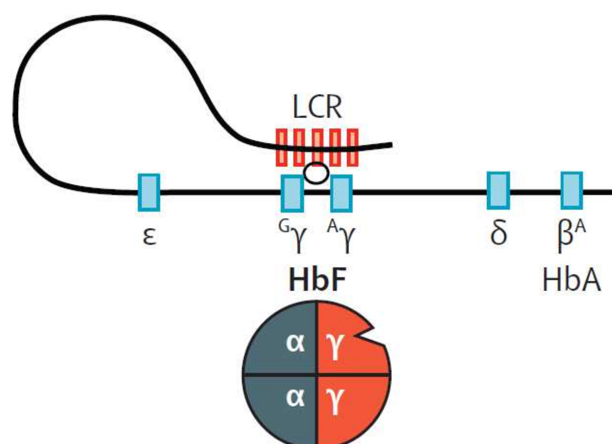

Figura 4: Regulação da expressão no agrupamento de genes da beta globina

Expressão gênica sequencial mediada pela LCR no agrupamento de genes da β-globina. Lettre and Bauer, 2016 (LETTRE; BAUER, 2016a).

Importante destacar que concentrações elevadas de HbF foram associadas à diminuição de episódios de dor aguda, úlceras de pernas e taxa de hemólise, além do aumento da sobrevida das hemácias e da longevidade em pacientes com AF (BAILEY *et al.*, 1992; FRANCO *et al.*, 2006; NOLAN *et al.*, 2005; SERJEANT *et al.*, 1996; WEBB *et al.*, 1989).

Embora ainda controverso com relação à achados prévios (KINNEY *et al.*, 1999; OHENE-FREMPONG *et al.*, 1998), estudos mais recentes têm sugerido efeito protetor da HbF também em doenças cerebrovasculares. Um estudo prospectivo que acompanhou crianças Holandesas com AF (HbSS) e Sβ^0-talassemia observou a associação de baixas concentrações de HbF com infartos cerebrais silenciosos definidos por maior hiperintensidade na substância branca em imagem de ressonância magnética, sugerindo o efeito protetor da HbF contra infartos silenciosos (VAN DER LAND *et al.*, 2016). Em outro estudo prospectivo, envolvendo uma coorte de recém nascidos com AF (HbSS) e Sβ^0-talassemia, a HbF foi identificada como fator protetor para macrovasculopatia (SOMMET *et al.*, 2016). Por fim, um estudo envolvendo pacientes adultos com AF (HbSS) mostrou que a baixa porcentagem de HbF foi independentemente associada com lesões silenciosas da substância branca em imagens cerebrais (CALVET *et al.*, 2017). Adicionalmente, a HbF foi identificada como o fator prognóstico mais forte para as complicações da AF em um recente estudo longitudinal prospectivo e multicêntrico que acompanhou crianças com AF por 19 meses (BROUSSE *et al.*, 2018).Finalmente, outro estudo prospectivo que envolveu crianças com AF em

tratamento com hidroxiureia, um fármaco indutor da HbF, mostrou que pacientes que alcançaram no mínimo 20% de HbF apresentaram uma taxa significativamente reduzida de hospitalizações (ESTEPP *et al.*, 2017).

1.4. Genes candidatos à regulação da HbF

Estudos de associação genômica ampla (GWAS, do inglês *Genome Wide Association Studies*) indicaram potenciais moduladores da fisiopatologia da DF, por influenciarem a concentração de HbF (MENZEL *et al.*, 2007a; MTATIRO *et al.*, 2014; SOLOVIEFF *et al.*, 2010). Hoje sabemos que os principais *loci* envolvidos na regulação da expressão dos genes *γ-globina* são o gene *HBG2* (Subunidade Gama 2 da Hemoglobina), o gene *BCL11A* (Linfoma de Células B 11A), e a região intergênica *(HMIP-2),* localizada entre os genes *HBS1L* e *MYB* (revisado em (MENZEL; THEIN, 2019)). Juntos, esses *loci* de herança quantitativa (QTL, do inglês *Quantitative trait Loci*), respondem por 20-50% da variância da HbF em pacientes com AF (THEIN *et al.*, 2009).

O gene *BCL11A* está localizado no cromossomo 2p16 e codifica uma proteína dedo de zinco (*zinc finger*), que se liga ao DNA (BAUER; ORKIN, 2015). A proteína BCL11A se liga em vários locais ao longo do cluster da *β-globina*, incluindo a região de controle do *locus* e elementos acentuadores da transcrição (BAUER; ORKIN, 2015). Análises de nocaute gênico em eritrócitos mostraram que *BCL11A* é uma repressora da expressão do gene *γ-globina* e que sua perda evita o silenciamento desse gene, que normalmente ocorre durante o desenvolvimento tardio do feto (SANKARAN *et al.*, 2008, 2009). Consistentemente com a função silenciadora do gene *γ-globina*, BCL11A é expressa em precursores eritróides (MENZEL *et al.*, 2007b). Os SNPs de *BCL11A*, associados com concentração elevada de HbF em pacientes com AF, localizam-se principalmente no íntron 2 do gene, conforme evidenciado na **Figura 5**.

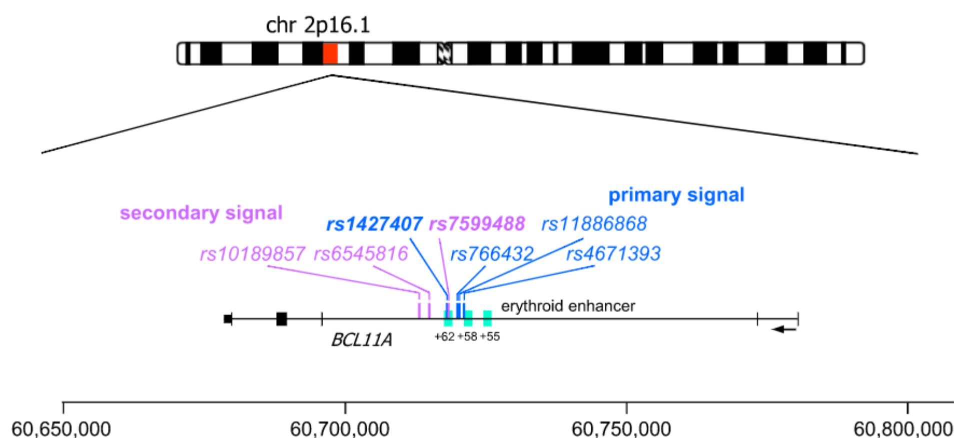

Figura 5: SNPs de *BCL11A*

Localização cromossômica dos SNPs de *BCL11A* associados com níveis elevados de HbF em pacientes com AF. Fonte: Menzel e Thein, 2019 (MENZEL; THEIN, 2019).

A região HMIP localizada no cromossomo 6q23.3, entre os genes *HBS1L e MYB* foi detectada pela primeira vez em uma grande família indiana com Persistência Hereditária de HbF e quadro clínico mais brando da doença falciforme (MENZEL *et al.*, 2014). O gene *HBS1L* codifica uma GTPase transacional e o gene *MYB é* um oncogene de mieloblastose, que codifica o fator de transcrição c-MYB (AL, [*s. d.*]; THEIN *et al.*, 2007). A **Figura 6** mostra SNPs localizados em HMIP-2, associados com concentração elevada de HbF.

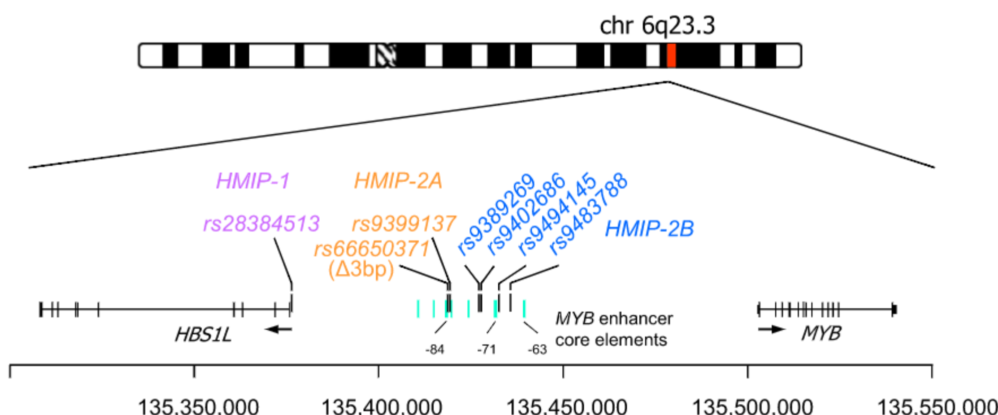

Figura 6: SNPs de HMIP-2

Localização cromossômica dos polimorfismos de HMIP associados com níveis elevados de HbF em pacientes com AF. Fonte: Menzel e Thein, 2019 (MENZEL; THEIN, 2019).

1.5 Hidroxiureia: fármaco usado no tratamento de pacientes com AF

A Hidroxiureia (HU) é um análogo hidroxilado da ureia, testado em um modelo experimental em 1928 por Rosenthal, que sugeriu sua característica mielossupressora (YAHOUÉDÉHOU *et al.*, 2018). Devido ao seu potencial anti-polimerização, causado principalmente pelo aumento dos níveis de HbF, a HU foi aprovada pela primeira vez em 1999 para tratamento de pacientes com doença falciforme (SEGAL *et al.*, 2008; WARE *et al.*, 2011).

A eficácia clínica da HU está relacionada (mas não restrita) com a indução da produção de HbF. A HU mata seletivamente as células da medula óssea e aumenta a número de hemácias produzindo HbF, inibindo a polimerização intracelular da HbS e prevenindo o processo de falcização nas hemácias, diminuindo assim o número de células falcizadas (MCGANN; WARE, 2015). Hemácias com elevada concentração HbF têm sobrevida mais longa, atenuando assim a hemólise (STEINBERG, 1999). Além disso, a HU aumenta os níveis de hemoglobina total; reduz neutrófilos, monócitos e reticulócitos; altera a expressão de moléculas de adesão no endotélio e geração de NO, alterações hematológicas que diminuem o risco de vaso-oclusão em pacientes com AF (MCGANN; WARE, 2015; RIGANO *et al.*, 2018; STEINBERG, 1999).

O protocolo da HU inicia-se com dose mínima de 15 mg/kg/dia, seguido do escalonamento até a Dose Máxima Tolerada (DMT) (SUS, 2018). A DMT é definida como a maior dose capaz de promover a melhora mais proeminente no curso clínico e laboratorial da AF, sem a ocorrência de toxicidade hematológica. A DMT não deve exceder 35 mg/kg/dia. Os benefícios laboratoriais e clínicos da HU mostraram-se otimizados quando dimensionados para a DMT. A maioria dos pacientes com AF mostram um aumento significativo na concentração de HbF sob DMT (MCGANN; WARE, 2015). Entretanto, tanto a indução da HbF quanto a DMT é variável entre os pacientes (MCGANN; WARE, 2015; WARE *et al.*, 2011).

O escalonamento de dose de HU é um processo trabalhoso que requer monitoramento de risco de citopenias, principalmente a neutropenia, e a resposta clínica ao tratamento com HU pode demorar até seis meses após atingir a DMT (EVIDENCE-BASED MANAGEMENT OF SICKLE CELL DISEASE: EXPERT PANEL, 2014, [*s. d.*]). Portanto, pacientes graves com indicação clínica

para HU podem vivenciar um longo período de exposição até deduzir que o tratamento com HU é pouco eficaz, quando for o caso.

Importante ressaltar que a HU, além de ser amplamente utilizada no tratamento de pacientes com AF, é atualmente preconizada e disponibilizada pelo SUS para os pacientes com indicação clínica do tratamento.

1.5 Farmacogenética da resposta à Hidroxiurea em pacientes com AF

Os benefícios clínicos da HU no quadro clínico e hematológico de pacientes com AF são amplamente reconhecidos na literatura científica (SEGAL *et al.*, 2008; STEINBERG, 1999). Entretanto, existe variabilidade na resposta ao tratamento entre os pacientes, tanto com relação à DMT quanto ao aumento da concentração de HbF induzido pela HU (MCGANN; WARE, 2015; WARE *et al.*, 2011).

A porcentagem de HbF alcançada com a DMT de HU atinge 10-15% em alguns pacientes, mas pode chegar a 40% em outros pacientes (WARE *et al.*, 2011). Além disso, enquanto alguns pacientes toleram altas doses de HU, como 30-35 mg/kg/dia, outros pacientes desenvolvem mielossupressão grave mesmo em doses mais baixas de HU (LETTRE *et al.*, 2008). Esses achados clínicos sugerem que importantes diferenças na farmacocinética e farmacodinâmica, além de fatores genéticos podem contribuir para a variabilidade fenotípica da resposta à terapia com HU (MCGANN; WARE, 2015). No entanto, o efeito de polimorfismos genéticos no aumento dos níveis de HbF em resposta à terapia com HU em pacientes com AF é ainda pouco compreendido.

2. Justificativa

O estudo clínico que tem sido desenvolvido apresenta grande relevância clínica e potencial aplicabilidade, uma vez que se ancora em uma coorte de pacientes pediátricos com AF que recebem tratamento em um centro de referência no tratamento de doenças hematológicas e tem sido acompanhada pelo grupo de pesquisa por mais de 8 anos (AR *et al.*, 2015; BELISÁRIO *et al.*, 2015, 2016, 2017; SALES *et al.*, 2020).

Vale ressaltar que, embora nosso estudo clínico inclua apenas pacientes homozigotos para o alelo β^S (HbSS) – genótipo de maior gravidade clínica – por uma questão de homogeneização da amostra, os achados desta proposta podem ser clinicamente relevantes para o grupo mais amplo, reconhecido como doença falciforme e eventualmente outras hemoglobinopatias, como a talassemia beta, por exemplo.

A Hidroxiurea (HU) é o fármaco atualmente adotado e disponibilizado pelo SUS para tratamento dos pacientes com AF. A HU tem vários efeitos fisiológicos, incluindo o aumento da expressão de HbF (KATO *et al.*, 2018). Na evolução clínica, a HU reduz consideravelmente as crises de dor vaso-oclusivas, episódios de síndrome torácica aguda, necessidade de transfusão sanguínea e a taxa de mortalidade dos pacientes com AF (HUSAIN *et al.*, 2017).

Apesar da comprovada eficácia da HU para o tratamento de crianças e adultos com AF, existe uma variabilidade interindividual fenotípica substancial em relação a eficácia e toxicidade em reposta a HU. Tal variabilidade pode ser devida às limitações de adesão ao tratamento ou de disponibilidade do fármaco, mas também pode ser explicada por fatores genéticos (WARE *et al.*, 2011), razão para a nossa abordagem relacionada à farmacogenética.

A associação de SNPs de *BCL11A* com os níveis de HbF foram demonstradas na literatura (BHANUSHALI *et al.*, 2015; MENZEL; THEIN, 2019; WONKAM, 2017). Entretanto, o efeito de SNPs na variação da HbF em resposta ao tratamento com HU ainda não é bem compreendido.

Portanto, nossa abordagem farmacogenética poderia auxiliar no manejo dos pacientes com AF, por meio da determinação da resposta ao fármaco em grupos de pacientes com diferentes perfis genéticos. Isso poderia acarretar em

maior assertividade na escolha do tratamento dos pacientes por parte dos médicos e em redução de gastos com o fármaco fornecido pelo SUS naqueles pacientes que apresentam baixa eficácia ao tratamento com HU.

O protocolo de HU é realizado a partir de uma dose mínima de 15 mg/kg/dia seguido do escalonamento da mesma até a DMT. O escalonamento da dose de HU é um processo trabalhoso que requer monitoramento do risco de citopenias, principalmente neutropenia. A resposta clínica ao tratamento com HU pode levar até seis meses após atingir o DMT (EVIDENCE-BASED MANAGEMENT OF SICKLE CELL DISEASE: EXPERT PANEL, 2014, [*s. d.*]). Uma vez que existe variabilidade na resposta ao tratamento com HbF nos pacientes com AF, pacientes graves com indicação clínica para HU podem vivenciar um longo tempo de exposição até deduzir que o tratamento com HU é pouco eficaz em seu tratamento.

3 Objetivos

Objetivo Geral:

Examinar se polimorfismos genéticos (SNPs) de genes candidatos aos níveis de HbF, com foco em *BCL11A* e na região intergênica *HBS1L-MYB* (HMIP-2), e se haplótipos formados pela combinação de seus alelos, são associados com a concentração de HbF e podem predizer complicações hematológicas e clínicas, bem como a resposta ao tratamento com o fármaco Hidroxiureia em uma coorte de pacientes pediátricos com Anemia Falciforme.

Objetivos específicos:

1. Examinar o efeito de SNPs em genes candidatos à concentração de HbF basal, e sua associação com parâmetros hematológicos e risco de incidência de complicações clínicas em uma coorte de 250 pacientes pediátricos com AF;
2. Realizar a anotação funcional *in silico* dos SNPs associados com HbF para selecionar SNPs funcionais envolvidos na regulação de HbF;
3. Estimar a frequência de haplótipos formados pela combinação de alelos dos SNPs funcionais e verificar se podem predizer complicações hematológicas e desfechos clínicos na coorte de pacientes pediátricos com AF;
4. Conduzir revisão sistemática da literatura para investigar se existem polimorfismos genéticos que influenciam a alteração nos níveis de HbF em pacientes com AF tratados com HU;
5. Examinar o efeito de SNPs previamente associados com HbF basal ou identificados pela revisão sistemática na resposta ao tratamento com HU na coorte de pacientes pediátricos com AF;

4 Materiais e Métodos

Os materiais e métodos utilizados no desenvolvimento do estudo e os resultados serão apresentados em formato de quatro **Capítulos**, constituídos pelos artigos científicos gerados durante o período de doutoramento, sejam eles publicados ou submetidos para publicação em revistas científicas indexadas.

4.1 Capítulo 1: Identificação de polimorfismos genéticos associados à HbF basal em uma coorte de pacientes pediátricos com AF

4.1.1 Determinação da casuística

Estudo de coorte retrospectiva envolvendo pacientes com AF (genótipo HbSS) diagnosticados pelo Programa de Triagem Neonatal de Minas Gerais (PTN-MG) entre 1999-2008, que realizam acompanhamento clínico na Fundação Centro de Hematologia e Hemoterapia de Minas Gerais (HEMOMINAS), localizado na cidade de Belo Horizonte (HBH). Durante esse período, 472 crianças com o perfil hemoglobínico FS ao nascimento (genótipos SS, S/HPFH - Persistência Hereditária de Hemoglobina Fetal - ou Sβ^0-talassemia) foram triadas e encaminhadas para o ambulatório do HBH. Os seguintes critérios de seleção dos participantes foram aplicados:

- Pacientes com genótipo homozigoto para o alelo β^S (HbSS) confirmado por biologia molecular;
- Pacientes com no mínimo uma determinação da concentração de HbF após 5 anos de idade e antes do início de regime de hipertransfusão crônica ou terapia com HU; Resultados com menos de três meses após transfusão sanguínea irregular foram descartados.
- Assinatura do Termo de Consentimento Livre e Esclarecido (TCLE) pelos pais e do Termo de Assentimento (TA) pelas próprias crianças, quando aplicável.

Os seguintes critérios de exclusão foram aplicados:

- Pacientes que vieram a óbito por qualquer razão;
- Pacientes transferidos para acompanhamento em outros ambulatórios durante o período de acompanhamento;

4.1.2 Variáveis clínicas e hematológicas estudadas

As seguintes variáveis laboratoriais foram analisadas:

- Concentração relativa de hemoglobina fetal (%)
- Concentração total de hemoglobina (g/dL)
- Leucometria basal (10^9/L)

- Contagem relativa de reticulócitos (%)
- Contagem de plaquetas (10^9/L)
- Saturação periférica de oxigênio (SpO2, %)

Com relação ao valor basal de HbF, foi utilizado o resultado do último exame após os cinco anos de idade, desprezando-se os valores após transfusões sanguíneas (90 dias) ou eventos clínicos significativos (crise de dor, infecção grave, sequestro esplênico agudo, crise aplástica e síndrome torácica aguda). Para as crianças que entraram em regime de transfusão crônica ou que iniciaram tratamento com hidroxiureia, foi considerado o último exame até a data anterior ao início das respectivas terapêuticas. A quantificação da concentração relativa total de HbF foi realizada utilizando-se o método de eletroforese de Hb em pH alcalino ou HPLC.

Os valores de hemoglobina total, leucometria basal e contagem de reticulócitos foram determinados por contador eletrônico de células (Coulter T890, Coulter, Hialeah, FL, EUA ou CELL-DYN Ruby, Abbott Laboratories, Santa Clara, CA, USA). Para a contagem de reticulócitos, usou-se o método azul de cresil brilhante à microscopia óptica. Esses exames são realizados normalmente para acompanhamento clínico dos pacientes na Fundação HEMOMINAS, estando os resultados disponíveis no prontuário médico.

Para determinação do valor basal de cada parâmetro hematológico, foi considerada a média de todos os valores de exames, após os dois anos de idade, desprezando-se os valores de exames realizados com menos de 90 dias após transfusões sanguíneas ou eventos clínicos significativos (crise de dor, infecção grave, sequestro esplênico agudo e crise aplástica). Nas crianças em regime de hipertransfusão ou sob tratamento com HU, os valores foram considerados até a data anterior ao início das respectivas condutas terapêuticas. Apenas os episódios clínicos antes do início do tratamento com HU ou, no mínimo 90 dias após algum evento de transfusão irregular foram contabilizados.

Síndrome torácica aguda (STA) foi definida como um novo infiltrado pulmonar, incluído alterações inflamatórias pulmonares, embolia gordurosa ou infarto pulmonar por oclusão microvascular ou tromboembolismo. Dor foi um sintoma subjetivo reportado pelo paciente ou pelos cuidadores, que levaram a

administração de analgésicos/narcóticos. Episódios de infecção (infecção das vias aéreas superiores, pneumonia/STA, infecção do trato urinário, osteomielite, etc.) foram consideradas apenas quando reconhecidas pelo médico responsável pelo atendimento clínico e que demandaram hospitalização. Sequestro esplênico agudo (SEA) foi definido como aumento rápido e maciço do tamanho do baço devido ao aprisionamento de hemácias falcizadas e outros constituintes do sangue, causando uma queda abrupta na concentração de hemoglobina.

4.1.3 Genotipagem dos polimorfismos genéticos

A **Tabela** sumariza a metodologia para genotipagem dos SNPs estudados.

Polimorfismo	Sequência do Primer	Condições da PCR	Concentração de $MgCl_2$	Endonuclease / RFLP	Padrão de clivagem da RFLP
HBBP1 rs10128556	F: 5’GAACAGAAGTTGAGATAGAGA3’ R: 3’ACTCAGTGGTCTTGTGGGCT5’	94°C. 5 min 94°C. 45 sec; 57°C. 45 sec; 72°C. 45 sec X 35 cycles 72°C. 10 min	1.5 mM	10U *HincII* ; 37°C. *1 hour*	CC (701); CT (701 + 361 + 340) e TT (361 + 340)
BCL11A rs7557939	F: 5’ACCATGGCCTGGTCACCA3’ R: 3’TCCCCCCACTAGCTCAGAAAT5’	95°C. 5 min 94°C. 45 sec; 64°C. 30 sec; 72°C. 30 sec X 30 cycles 72°C. 5 min	2.0 mM	2U *BlpI* ; 37°C. *1 hour*	AA (698); AG (698 + 568 + 130) e (GG 568 + 130)
BCL11A rs4671393	F: 5’ACCATGGCCTGGTCACCA3’ R: 3’TCCCCCCACTAGCTCAGAAAT5’	95°C. 5 min 94°C. 45 sec; 64°C. 30 sec; 72°C. 30 sec X 30 cycles 72°C. 5 min	2.0 mM	-	-
BCL11A rs7599488	F: 5’CACTCATCCCATGCACCACT3’ R: 3’TAGCTGAAGGGGGCCAAAAG5’	95°C. 5 min 94°C. 45 sec; 65°C. 30 sec; 72°C. 30 sec X 30 cycles 72°C. 5 min	1,5 mM	2U *FspI*; 37°C. *1 hour*	TT (739); TC (739 + 607 + 132) e (CC 607 + 132)
HMIP2A rs93991737 rs35786788	F: 5’ GCGGTTCCCTCAGAAGACAC3’ R: 3’ATGCTTGCCAAAGTGCTTCTG5’	95°C. 5 min 95°C. 30 sec; 61°C. 30 sec; 72°C. 30 sec X 30 cycles 72°C. 5 min	2.0 mM	-	-

HMIP2B rs9402686	F: 5’GTGCTCGTTGAAAATGGGGG3’ R: 3’ATACGTGGCGTGACCATGAA5’	95°C. 5 min 94°C. 45 sec; 65°C. 30 sec; 72°C. 30 sec X 30 cycles 72°C. 5 min	2.0 mM	5U *BslI* ; 37°C. *1 hour*	GG (270 + 200); GA (470 + 270 + 200) e AA (470)
HMIP2B rs4895441	F: 5’AGTTGGCCAGAGCACACTTG3’ R: 3’TCTCCCTGTCCCCAGATACT5’	95°C. 5 min 94°C. 45 sec; 65°C. 30 sec; 72°C. 30 sec X 30 cycles 72°C. 5 min	2.0 mM	5U *RsaI* ; 37°C. *1 hour*	AA (207 + 164); AG (207 + 164 + 102 + 62) e GG (207 + 102 + 62)

4.1.4 Aspectos éticos

O protocolo de estudo referente aos Capítulos 1, 2 e 4 foi aprovado pelos Comitês de Ética em Pesquisa da Fundação HEMOMINAS - instituição de execução do projeto – e da Universidade Federal de Minas Gerais - instituição coparticipante (CAAE: 4204.8215.6.0000.5118; **Anexo 1**). Os pacientes foram incluídos no estudo apenas mediante assinatura do TCLE pelos responsáveis e do TA pelo próprio paciente, quando aplicável. O Biorrepositório de amostras vinculado ao projeto já foi instituído e é gerenciado atendendo ao disposto nas Resoluções CNS 441/11 e CNS 466/12.

4.1.5 Análises estatísticas

Para as variáveis contínuas com distribuição normal foi utilizado o teste t de *Student* e o teste ANOVA para comparação de médias entre dois e três grupos, respectivamente. Para as variáveis contínuas com distribuição não-normal foi utilizado o teste de Mann-Whitney ou Kruskal-Wallis para comparação de médias entre dois ou três grupos, respectivamente. A distribuição normal ou não-normal dos dados contínuos foi verificada utilizando-se o teste de Kolmogorov-Smirnov. Foram considerados como estatisticamente significativos os testes em que a probabilidade de erro alfa foi igual ou inferior a 0,05. As análises estatísticas foram realizadas utilizando o programa SPSS, versão 20.0 (SPSS Inc.; Chicago, IL, USA). A taxa de incidência por 100 pacientes-ano, bem como a razão dessas taxas entre os grupos comparados foi calculada utilizando o programa *OpenEpi*, um programa online de estatísticas epidemiológicas de código aberto voltado para saúde pública (https://www.openepi.com/PersonTime2/PersonTime2.htm).

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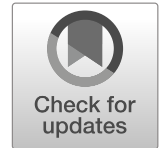

Functional polymorphisms of *BCL11A* and *HBS1L-MYB* genes affect both fetal hemoglobin level and clinical outcomes in a cohort of children with sickle cell anemia

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Abstract

Fetal hemoglobin (HbF) ameliorates clinical severity of sickle cell anemia (SCA). The major loci regulating HbF levels are *HBB* cluster, *BCL11A*, and HMIP-2 (*HBS1L-MYB*). However, the impact of noncoding single-nucleotide polymorphisms (SNPs) in these loci on clinical outcomes and their functional role on regulating HbF levels should be better elucidated. Therefore, we performed comprehensive association analyses of 14 noncoding SNPs in five loci with HbF levels and with clinical outcomes in a cohort of 250 children with SCA from Southeastern Brazil, and further performed functional annotation of these SNPs. We found SNPs independently associated with HbF levels: rs4671393 in *BCL11A* (β-coefficient = 0.28), rs9399137 in HMIP-2A (β-coefficient = 0.16), and rs4895441 in HMIP-2B (β-coefficient = 0.15). Patients carrying minor (HbF-boosting) alleles for rs1427407, rs93979137, rs4895441, rs9402686, and rs9494145 showed reduced count of reticulocytes ($p < 0.01$), while those carrying the T allele of rs9494145 showed lower white blood cell count ($p = 0.002$). Carriers of the minor allele for rs9402686 showed higher peripheral saturation of oxygen ($p = 0.002$). Patients carrying minor alleles in *BCL11A* showed lower risk of transfusion incidence rate ratio (IRR ≥ 1.3; $p < 0.0001$). This effect was independent of HbF effect ($p = 0.005$). Carriers of minor alleles for rs9399137 and rs9402686 showed lower risk of acute chest syndrome (IRR > 1.3; $p \leq 0.01$). Carriers of the reference allele for rs4671393 showed lower risk of infections (IRR = 1.16; $p = 0.01$). In conclusion, patients carrying HbF-boosting alleles of *BCL11A* and HMIP-2 were associated with milder clinical phenotypes. Higher HbF concentration may underlie this effect.

Keywords *BCL11A* gene · Fetal hemoglobin · Single-nucleotide polymorphisms (SNPs) · *HBB* gene · *HBS1L-MYB* gene · Clinical outcomes · Sickle cell anemia

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Introduction

Sickle cell anemia (SCA) is a monogenic hemoglobin disorder caused by homozygous A-to-T transversion at codon 7 in the *HBB* gene (*HBB*:c.20A > T, p.E6V) [1]. Pathophysiology of SCA is characterized by multisystem damage, including chronic hemolytic anemia, vaso-occlusion, and tissue and organ damage in several degrees [2]. SCA derives directly from the polymerization of deoxy sickle hemoglobin (HbS; $\alpha_2\beta^S{}_2$), and there is a large clinical heterogeneity in patients with SCA [2]. Notably, fetal hemoglobin (HbF; $\alpha_2\gamma_2$) in higher levels ameliorates clinical outcome and hematological parameters of SCA, since it reduces HbS concentration and inhibits copolymerization between hemoglobin tetramers [3, 4]. Hydroxyurea is currently used for the treatment of SCA, and its clinical and hematological effects primarily result from induction of HbF expression [5].

The hemoglobin expression is regulated during human development [6]. The switch from fetal to adult hemoglobin occurs soon after birth and higher persistent concentration of HbF is often observed in patients with SCA compared to individuals without SCA [3]. Despite the clinical benefits of higher HbF concentration in reducing SCA severity, the basis for the genetic regulation of HbF production is not fully understood. The first two trans-acting *loci* that seemed to control γ-chain expression were located at X-linked chromosome (Xp22.2) and at 6q23, flanked by *HBS1L* and *MYB* genes [7–9][1–3]. Genome-wide association studies (GWAS) identified *BCL11A* as able to influence HbF concentration in healthy subjects and in patients with β-thalassemia and sickle cell disease (SCD) [10–12]. Notably, *XmnI* site (rs7482144 SNP at *HBB* cluster), *BCL11A*, and *HBS1L-MYB* intergenic polymorphisms (designated HMIP-2) have been recognized as the major quantitative trait loci (QTL) for HbF [10–14].

Genetic studies have confirmed the association of single-nucleotide polymorphisms (SNPs) at *HBB* gene cluster, *BCL11A*, and *HBS1L-MYB* (HMIP-2) loci with HbF levels in several populations [15–18]. However, their causal effects on HbF are difficult to interpret because all of these SNPs are located in noncoding DNA regions. The effects of some polymorphisms at these loci on the clinical severity of SCA have already been demonstrated [13, 15–19]. Estimates of the relative incidence of clinical events in patients with or without these QTL have not been determined for each clinical outcome in SCA. Additionally, there are few studies examining these polymorphisms in Brazilian patients [15, 19–21].

In the present study, we performed comprehensive genetic association analyses and functional annotation of SNPs in the major loci affecting HbF levels, namely *HBBP1*, *OR51B5/6*, *BCL11A*, and HMIP-2. We found novel associations of these SNPs with clinical outcomes and hematological parameters that are relevant for SCA management and confirmed the effect of SNPs in the *BCL11A* and HMIP-2 on HbF levels in a cohort of patients with SCA from Minas Gerais, Southeastern Brazil.

Materials and methods

Subjects and study design

This was a retrospective cohort study involving 250 pediatric patients with SCA diagnosed by the Newborn Screening Program of Minas Gerais state, Southeastern Brazil, between 1999 and 2008, and followed up at Center of Hematology and Hemotherapy of Minas Gerais–HEMOMINAS, in Belo Horizonte, capital of Minas Gerais. The flow chart of patient recruitment is shown in Fig. 1.

The following eligibility criteria were applied: (1) patients with the homozygous β^S allele confirmed by molecular methods and (2) patients with a least one determination of HbF concentration when 5 years old or later and before starting chronic blood transfusion or hydroxyurea. Laboratory results

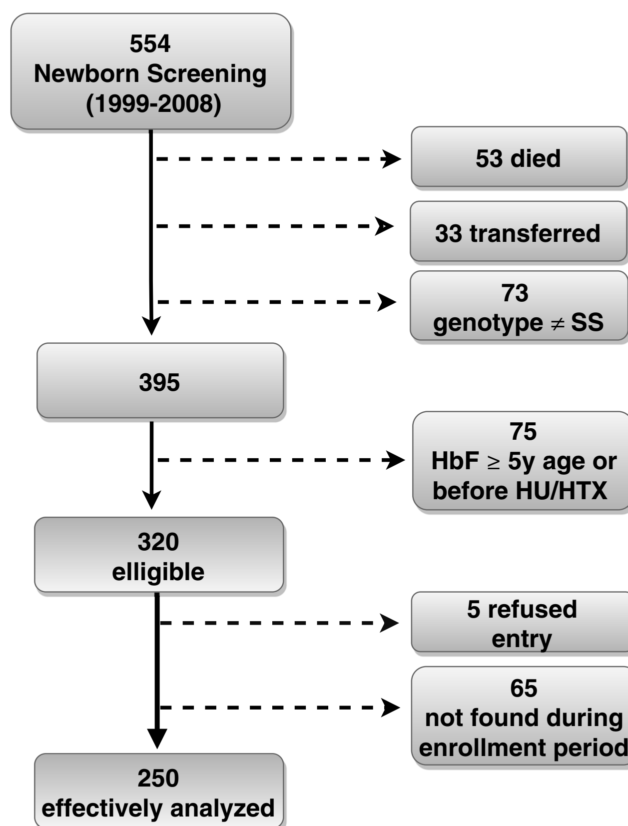

Fig. 1 Study population flow chart. Cohort of children diagnosed by the Newborn Screening Program of Minas Gerais state, Southeastern Brazil, between 1999 and 2008. Dashed lines indicate patients who were excluded from the study due to different reasons. Out of 320 eligible patients, we were able to recruit 250 children into the study. Patients who died ($n = 53$) were excluded from the study because the majority was less than 5 years old or because informed consent from parents was impossible to be obtained; HU/HTX: hydroxyurea or hypertransfusion.

up to 3 months after a blood transfusion were disregarded. The following exclusion criteria were applied: (1) patients with genotypes other than homozygous β^S allele, (2) transference to another blood center in Minas Gerais or in other states, (3) death during the retrospective follow-up, and (4) patients or guardians who refused to entry into the study.

Research Ethics Committee from the involved institutions approved the study protocol (certification number: 4204.8215.6.0000.5118). Written informed consent was obtained from every child and its guardians in accordance with “Declarations of Helsinki” guidelines. Age appropriate assent was also obtained. Venous blood samples were collected from patients at clinical attendance.

Clinical and hematological data

Clinical and hematological data were retrieved from medical records of the patients. Clinical episodes were counted before the start of hydroxyurea or chronic transfusion therapy and at least 90 days after a blood transfusion administration.

The definition of clinical events was based on the guidelines published by the Comprehensive Sickle Cell Centers [22]. Acute chest syndrome (ACS)/pneumonia is an acute illness characterized by fever and/or respiratory symptoms, accompanied by a pulmonary infiltrate on a chest x-ray. Because few children had a previous x-ray to be compared to, the adjective “new” was dropped from the original definition. Pain is a subjective symptom reported by patients/family that led to analgesic/narcotic administration in hospital or emergency room. Episodes of infection (upper airway infection, pneumonia/ACS, urinary tract infection, osteomyelitis, etc) were only considered as those recognized by a doctor and requiring hospital admission.

Total hemoglobin concentration, white blood cell (WBC), and platelet counts were determined using electronic cell counter (Coulter T890, Coulter, Hialeah, FL, EUA or CELL-DYN Ruby, Abbott Laboratories, Santa Clara, CA, USA). Quantification of HbF relative concentration was performed by hemoglobin electrophoresis at alkaline pH or high-performance liquid chromatography (HPLC). Reticulocyte count was determined using bright cresyl blue under optical microscopy. Pulse oximetry measured the peripheral oxygen saturation (SpO_2). Baseline values were the average of all exams during steady state, after 2 years of age and at least 3 months after blood transfusion or relevant clinical events, for instance, pain crisis and infection. Administration of hydroxyurea or chronic transfusion protocol interrupted acquisition of new data to calculate baseline values. Baseline of HbF relative concentration for each patient was the last available test after the age of 5 years.

Genetic data

Genomic DNA extraction from venous blood sample of patients was performed using commercial kit (QIAamp, DNA Blood Mini Kit; Qiagen; Hilden, Germany). DNA sequencing, real-time q-PCR, or restriction fragment length polymorphism (RFLP) methods were used to allelic discrimination of the polymorphisms. The authors designed the primer sequences and standardized PCR-RFLP conditions (Supplementary Table1).

DNA fragments containing rs9399137, rs3578678 (HMIP-2A), and rs4671393 (*BCL11A*) SNPs were sequenced by Sanger method using ABI Prism 3730 Analyzer (Applied Biosystems; Foster City, CA, USA). Sequences were analyzed using Bio Edit Sequence Alignment software v.7.2. Real Time q-PCR detected rs5006884 (*OR51B5/6*), rs766432, rs1427407, rs11886868 (*BCL11A*), and rs9494145 (HMIP-2B) SNPs using TaqMan™ SNP Genotyping Assay from Thermo Fisher Scientific. PCR-RFLP with HincII was used to genotype rs10128556 (*HBBP1* pseudogene).

Statistical analyses

Nominal variables were expressed by percentage and continuous variables as mean and standard deviation. Incidence of clinical events was reported by rates to 100 patient-years (pt-yrs), with 95% confidence intervals.

Incidence rate ratio (IRR) of clinical episodes for genotype groups was calculated on OpenEpi online software, PersonTime2/PersonTime2 option (http://www.openepi.com/PersonTime2/PersonTime2.htm).

Kolmogorov–Smirnov test was used to evaluate the distribution of continuous variables. The unpaired *t* test (ANOVA for more than two groups) and Mann–Whitney *U* test (Kruskal-Wallis for more than two groups) were used to compare different groups for continuous variables that were normally and non-normally distributed, respectively. Univariate association between polymorphisms and outcomes was evaluated using two-tailed Chi-square or Fisher’s exact test for categorical variables.

The independent association of polymorphisms with HbF levels was analyzed using multiple linear regression analyses. Through the stepwise method, the covariates were removed one by one and the final model included only those that were statistically significant ($P < 0.05$). Child’s gender and age at HbF determination were retained as covariates, since they influence HbF levels [6, 23]. Statistical analyses were conducted on SPSS 21.0 software (SPSS Inc.; Chicago, IL, USA).

Pairwise linkage disequilibrium (LD) among SNPs was characterized by D' and r^2 using HaploView software [24]. The Bonferroni method of correction was applied on genetic association tests to maintain the risk of false positive outcomes

(type I error) at a reasonable level [25]. Since many of the SNPs studied are part of LD blocks, families of non-independent null hypothesis were defined on the basis of the genomic position of SNPs [25]. Once five genomic regions were studied, namely *HBBP1*, *ORB51B5/6*, *BCL11A*, HMIP-2A, and HMIP-2B, the significance level after correction was considered as $\alpha_{Bonf} = 0.05/5 = 0.01$.

Results

Clinical, laboratory, and genetic profile of the cohort

Out of 250 patients with SCA enrolled in this study (Fig. 1), 125 (50%) were males, with a minimum of five and a maximum of 16 years of age (mean 8.86 ± 0.19). Hematological parameters and clinical outcomes of the cohort are summarized in Table 1. Baseline HbF levels were available for 240 patients enrolled in the study. The global mean (SEM) of HbF was 13.63% (0.44) (range: 2.6–37%). HbF relative concentration was significantly higher in females compared to males ($p = 0.018$). The means of HbF for each genotype group are summarized in Supplementary Table 2.

The distribution of genotypes for all SNPs studied showed no deviation from the Hardy-Weinberg equilibrium (HWE) (data not shown). Genotype frequencies found in our cohort and their respective allele frequencies for populations of The Trans-Omics for Precision Medicine (TOPMed) Program and for The 1000 Genomes Project are shown in Supplementary Table 3. Different allele frequencies were found between our cohort and data for the African population (YRI, Yoruba in Ibadan, Nigeria) from *The 1000 Genomes*.

Pairwise linkage disequilibrium (LD) among the SNPs genotyped in our cohort are shown in the Supplementary Fig. 1 and Supplementary Fig. 2. A high LD ($D' = 1$; $r^2 = 1$) was found between nearby rs9399137 and r35786788 SNPs in HMIP-2A (Supplementary Fig. 2). Two polymorphisms close to rs9399137 and rs35786788 were found in the sequenced fragments from HMIP-2A: rs7776196 (upstream of rs9399137; MAF: G = 0.0435) and an insertion of A base plus double C deletion (indel CC > A) between rs9399137 and rs35786788, corresponding to rs796512567 or to the equivalent rs796983051. To date, there was no allele frequency reported for this indel in dbSNP. In our cohort, the frequency of the minor allele delCCinsA was 0.224. The LD between rs9399137/rs35786788 and indel mutation had $D' = 1$ and $r^2 = 0.50$.

Association of genetic variants with HbF levels

SNPs in *HBBP* or *OR51B5/6* (rs10128556 and rs5006884, respectively) were not found to be associated with HbF levels (Table 2).

Except for rs7599488, all SNPs located in *BCL11A* were significantly associated with HbF concentration. Minor alleles of rs766432, rs1427407, and rs4671393 were significantly associated with increased HbF levels ($p < 0.001$). Notably, the *BCL11A* rs4671393 explained 12% of the variation in HbF levels in our cohort (Table 2).

Regarding HMIP-2, all minor alleles of SNPs were significantly associated with higher HbF levels, except for rs7776196 (Table 2). The C allele for rs9337137 and the A allele for rs35786788 (these two SNPs had $D' = 1$ and $r^2 = 1$) were associated with higher HbF values ($p < 0.001$), and they explain 7% of variation in HbF levels. Noteworthy, this study is the first to report that delCCinsA (rs796512567) was associated with higher HbF levels ($p = 0.002$). However, it is located between the SNPs rs9337137 and rs35786788, and these SNPs are in complete LD. Moreover, delCCinsA was not found to be an independent factor in multivariate analysis. The minor alleles for rs4895441, rs9402686, and rs9494145 were also found to be associated with increased HbF levels ($p < 0.001$).

In the multivariate analysis, three SNPs remained independently associated with HbF concentration: rs4671393 in *BCL11A* (β-coefficient = 0.28), rs9399137 in HMIP-2A (β-coefficient = 0.16), and rs4895441 in HMIP-2B (β-coefficient = 0.15).

Table 1 Hematological and clinical characteristics of the studied cohort

Hematological variables[a]	Mean (SEM)	95% CI		Clinical variables[a]	Rate per 100 patient-years	95% CI	
		Upper	Lower			Lower	Upper
Hb F (%)	13.63 (0.44)	12.77	14.49	Transfusion	41.4	38.9	44
Hb (g/dL)	7.91 (0.06)	7.80	8.03	Acute chest syndrome	26.2	24.2	28.3
WBC (10^9/L)	15.38 (0.21)	14.97	15.80	Painful episode requiring medical attention	38.5	36.1	41
Reticulocyte (%)	14.65 (0.26)	14.14	15.16	Infection requiring hospitalization	50	47.2	52.9
Platelet (10^9/L)	412.86 (7.03)	399.00	426.70				
SpO_2 (%)	93.60 (0.22)	93.17	94.03				

[a] For hematological and clinical variables, $n = 250$, except for HbF ($n = 240$) and SpO2 ($n = 220$)

Table 2 Association between genetic polymorphisms and relative concentration of fetal hemoglobin in 240 Brazilian children with SCA

Locus	Position[a] on chromosome	SNP ID	Allele change	MAF	Effect size	Variance explained (%)	*p* value	*p* value (group I × group II)[d]
Chromosome 2								
BCL11A	60490908	rs1427407	G > T	0.2	0.309	9.6	*< 0.001*	*< 0.001*
BCL11A	60491212	rs7599488	C > T	0.37	−0.04	0.2	0.98	0.96
BCL11A	60492835	rs766432	A > C	0.22	0.267	7	*< 0.001*	*< 0.001*
BCL11A	60493111	rs11886868	T > C	0.35	0.211	4.5	*0.003*	*0.004*
BCL11A	60493816	rs4671393[c]	G > A	0.24	0.346	12	*< 0.001*	*< 0.001*
BCL11A	60494212	rs7557939	A > G	0.35	0.181	3	*0.01*	*.007*
Chromosome 6								
HMIP-2A	135097850	rs7776196	A > G	0.1	−0.009	0	0.94	0.78
HMIP-2A	135097880	rs9399137[bc]	T > C	0.13	0.268	7.2	*< 0.001*	*< 0.001*
HMIP-2A	135097900-1	rs796512567	delCCinsA	0.22	0.215	4.6	*0.002*	*0.009*
HMIP-2A	135097904	rs35786788[b]	G > A	0.13	0.268	7.2	*< 0.001*	*< 0.001*
HMIP-2B	135105435	rs4895441[c]	A > G	0.09	0.266	7.1	*0.001*	*< 0.001*
HMIP-2B	135106679	rs9402686	G > A	0.14	0.265	7	*< 0.001*	*< 0.001*
HMIP-2B	135111414	rs9494145	T > C	0.13	0.246	6.1	*0.001*	*< 0.001*
Chromosome 11								
HBBP1	5242453	rs10128556	C > T	0.012	−0.09	0.9	–	0.89
OR51B5/6	5352021	rs5006884	C > T	0.08	0.006	0	–	0.20

Significance level was 0.05/5 = 0.01 after Bonferroni correction (significant values in italics)

[a] GRCh38.p7

[b] Total linkage disequilibrium ($D' = 1$; $r^2 = 1$)

[c] SNPs that remained independently associated with HbF at the final model of multiple linear regression

[d] Group I: genotype without minor allele; group II: genotypes with minor allele in homozygosis or heterozygosis

Association of SNPs with clinical outcomes and hematological parameters

All the SNPs found to be associated with increased HbF levels were also associated with higher total Hb concentration ($p < 0.01$), except for the delCCinsA. Lower reticulocyte count was observed in subjects carrying at least one minor allele for rs1427407 ($p = 0.009$), rs93979137 ($p = 0.004$), rs4895441 ($p = 0.005$), rs9402686 ($p = 0.001$), and rs9494145 ($p = 0.003$) (Fig. 2a). Subjects carrying at least one minor allele for rs9494145 showed lower WBC ($p = 0.004$; Fig. 2b). Additionally, SpO_2 was higher in carriers of the minor allele for rs9402686 ($p = 0.007$; Fig. 2c). We found no associations with platelet count (Supplementary Table 5). Considering the three SNPs that remained significantly associated with HbF concentration in the multivariate analysis (see above), none of them had independent impact on total Hb concentration, after adjusting for the HbF relative concentration.

Association tests between genotypes and the incidence of clinical events are shown in Table 3. Patients carrying *BCL11A* alleles associated with lower HbF levels had a significant higher ratio of blood transfusion per 100 pt-yrs than those with minor alleles associated with higher HbF levels, except for rs7599488. The highest rate ratio was recorded for rs4671393, 1.62 (95% CI, 1.42–1.85; $p = 1 \times 10^{-7}$). This effect was independent of HbF relative concentration when a regression model was adjusted for the effect of HbF ($p = 0.005$). Additionally, patients who were GG homozygotes for rs4671393 showed higher risk of infection than patients carrying at least one minor A allele (IRR = 1.16 (1.03–1.3); $p = 0.01$). When adjusted for the HbF concentration, this association was not significant anymore. No association of HMIP-2 genotypes with transfusion incidence was detected in the present study (Table 3).

Reference homozygous patients for rs93999137 (HMIP-2A) and rs9402686 (HMIP-2B) exhibited risk of acute chest syndrome 1.3 times greater than those carrying minor alleles that enhance HbF levels (Table 3). These effects were also not statistically independent from that asssociated with HbF concentration.

Table 4 summarizes the association of rs4671393, rs9399137, and rs4895441 (SNPs that remained independently associated with HbF concentration; see above) with some clinical outcomes and hematological parameters.

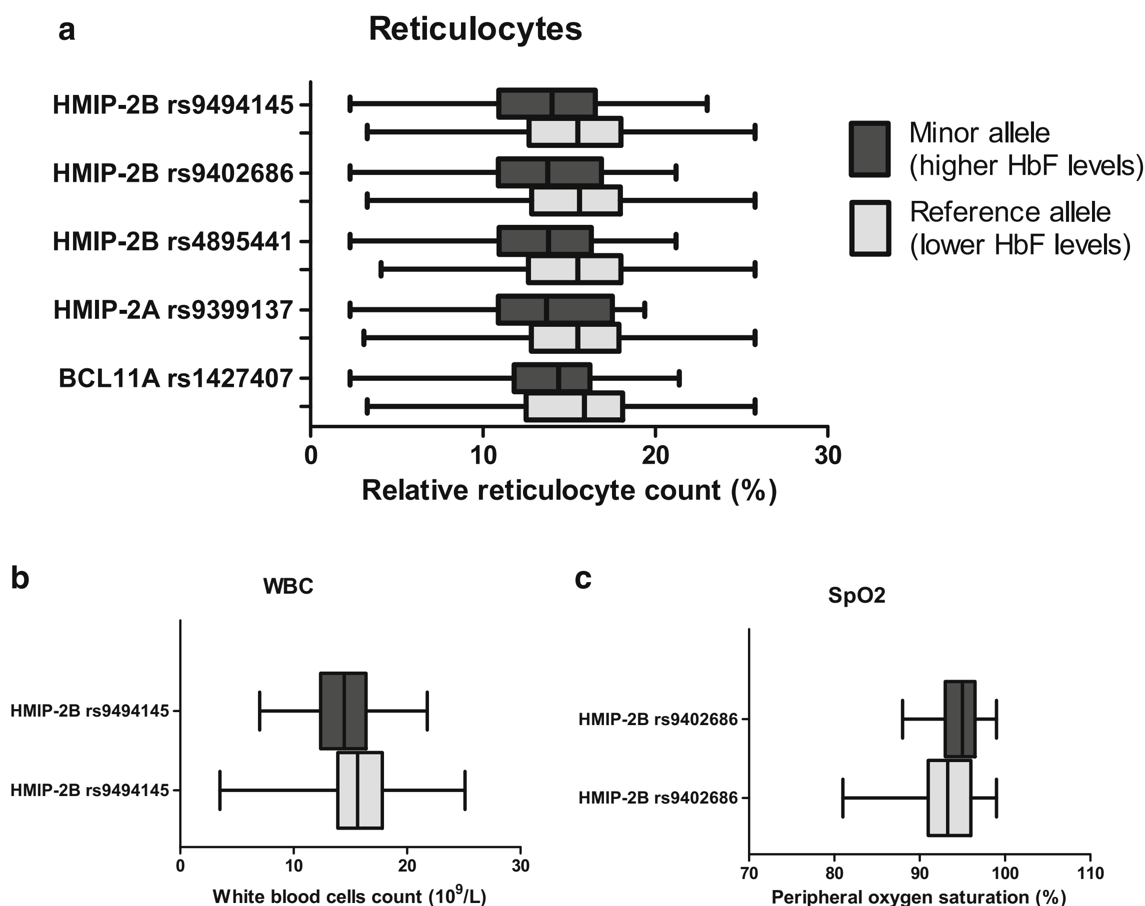

Fig. 2 Comparative boxplot of SNPs significantly associated with hematological parameters. **a** Relative reticulocyte count. **b** White blood cell count. **c** Peripheral oxygen saturation. As usual, vertical bars represent, from left to right, the minimum, the first quartile, the median, the third quartile, and the maximum values

Discussion

The main novel findings reported here were that (1) three SNPs were independently associated with HbF concentration (rs4671393 in *BCL11A*, rs9399137 in HMIP-2A, and rs4895441 in HMIP-2B), considering SNPs as part of genomic regions of high LD, and (2) HbF-boosting alleles decrease risk of many severe clinical outcomes of SCA; most of the effect seems to be dependent on the effect of HbF concentration.

The SNPs in the *BCL11A* and HMIP-2 explained almost 20% of the variance of HbF concentration in our cohort. BCL11A is a well-known silencer of the γ-genes that represses HbF production, as reviewed elsewhere [26]. Therefore, it is possible that *BCL11A* expression is reduced by changing motifs required for the binding of transcription factors [27, 28]. Lowering *BCL11A* expression would lead to an increase of HbF level, because it has already been shown that silencing activity of *BCL11A* is dose-dependent. The SNPs rs766432, rs1427407, and rs11886868 had high effects on the HbF concentration in our cohort (β-coefficient = 0.26, 0.31, and 0.21, respectively), and they explained 5–10% of variation. In line with our findings, higher effect was also found in Tanzanian, British and in the Cooperative Study of Sickle Cell Disease (CSSCD) [10, 29]. The rs1427407 SNP falls in +62 DNAse I Hypersensitive Site, an erythroid enhancer of *BCL11A*, and overlaps a peak of GATA1 and TAL1 transcription factor binding. Notably, the minor T allele for rs1427407 disrupts the consensus sequence enriched for GATA1 and TAL1 transcription factors [30].

Regarding the polymorphisms at HMIP-2, the SNPs rs4895441 and rs9397137 were independently associated with HbF levels in the multivariate model. According to its score 1f at RegulomeDB, rs4895441 is suggested to motif match for transcriptional factor binding, overlap with DHSs and it was correlated with expression of *HBG1* and *HBG2* (genes codifying γ-chain of HbF) as quantitative trait locus (eQTLs), which are genomic loci associated with variation in gene expression levels. Thus, functional annotation suggested that rs48954441 may be a functional SNP, although its associations with HbF level are controversial [10, 19, 31–35]. Conversely, many studies confirmed the effect of rs9399137 on HbF levels [10, 16, 18, 20, 21, 29, 31, 36–40]. However, its role on regulating HbF levels is not clear. According to The

Table 3 Incidence rate ratio of some clinical outcomes between genotypes of polymorphisms at major loci affecting HbF levels in 250 Brazilian children with SCA

SNPs	Transfusion		Acute chest syndrome		Pain requiring care in hospital or emergency room		Infection requiring hospital admission	
	Rate ratio (I/II)[a] 95% CI	*p* value	Rate ratio (I/II)[a] 95% CI	*p* value	Rate ratio (I/II)[a] 95% CI	*p* value	Rate ratio (I/II)[a] 95% CI	*p* value
BCL11A								
rs1427407	*1.49 (1.3–1.7)*	*1×10^{-7}*	1.12 (0.95–1.32)	0.17	0.94 (0.83–1.07)	0.39	1.08 (0.98–1.23)	0.2
rs7599488	0.96 (0.85–1.09)	0.55	1 (0.86–1.17)	0.96	1.02 (0.9–1.16)	0.77	0.99 (0.89–1.11)	0.9
rs766432	*1.53 (1.34–1.75)*	*1×10^{-7}*	1.13 (0.96–1.32)	0.16	0.85 (0.75–0.97)	0.02	1.1 (0.5–1.14)	0.13
rs11886868	*1.3 (1.15–1.47)*	*2×10^{-5}*	1.17 (1–1.4)	0.06	0.9 (0.79–1.03)	0.11	1.12 (1–1.12)	0.06
rs4671393	*1.62 (1.42–1.85)*	*1×10^{-7}*	1.17 (1–1.37)	0.05	0.99(0.87–1.12)	0.85	*1.16 (1.03–1.3)*	*0.01*
rs7557939	*1.32 (1.17–1.49)*	*1×10^{-5}*	1 (0.96–1.32)	0.96	0.86 (0.75–0.98)	0.03	1.06 (0.95–1.19)	0.29
HMIP-2A								
rs7776196	1.05 (0.89–1.23)	0.62	0.89 (0.73–1.09)	0.28	*1.28 (1.07–1.54)*	*0.008*	0.98 (0.85–1.14)	0.81
rs9399137	1.13 (0.97–1.3)	0.12	*1.37 (1.13–1.66)*	*0.001*	0.99 (0.85–1.15)	0.91	1.17 (1.02–1.33)	0.03
rs796512567	1.11 (0.98–1.3)	0.1	1.2 (1–1.4)	0.03	1.12 (0.98–1.3)	0.09	1.13 (1–1.3)	0.05
HMIP-2B								
rs4895441	1.01 (0.86–1.18)	0.99	0.96 (0.79–1.17)	0.74	0.97 (0.82–1.14)	0.72	0.86 (0.75–0.98)	0.03
rs9402686	1.05 (0.92–1.2)	0.51	*1.33 (1.11–1.6)*	*0.002*	1.05 (0.91–1.21)	0.49	1.12 (0.98–1.26)	0.09
rs9494145	1.14 (0.99–1.31)	0.08	1.16 (0.97–1.39)	0.12	1.1 (0.95–1.3)	0.2	1.15 (1–1.3)	0.04
OR51B5/6								
rs5006884	1.03 (0.87–1.22)	0.78	0.78 (0.64–0.95)	0.017	0.92 (0.78–1.1)	0.37	0.87 (0.75–1)	0.07
*HBBP*1								
rs10128556	2.08 (0.99–4.37)	0.048	1.53 (0.68–3.41)	0.39	0.67 (0.43–1.04)	0.11	0.75 (0.5–1.14)	0.23

[a] Group I: genotype without minor allele; group II: genotypes with minor allele in homozygosis or heterozygosis. Significance level was 0.05/5 = 0.01 after Bonferroni correction (significant values in italics). For comparison of observational times between Groups I and II, see Supplementary Table 4

Genotype-Tissue Expression project (GTEx) data, the minor allele (C) of rs9399137 is associated with decreased expression of *HBS1L* in several tissues, but not in whole blood or spleen, and expression of *HBS1L* has been associated with the number of F-cells [14]. Moreover, SNPs associated with HbF higher levels display positive correlation with transcription of *HBS1L* and *MYB* [14]. It was proposed that *MYB* regulates KLF1, a transcriptional factor that trans-activates *BCL11A*, binding to its promoter [26]. However, the most plausible explanation for the association between rs9399137 with HbF is the LD with a 3-base indel (rs66650371), itself a significant functional motif in HMIP-2A that modulates HbF [41].

The SNP rs10128556 was not found to be associated with HbF concentration in this study, which could be due to the low frequency of the T minor allele (MAF = 0.01) in the present study. Noteworthy, rs10128556 SNP has been previously associated with HbF levels and it showed high LD with rs7482144 (*XmnI*) [13], which is found at the Senegal beta globin haplotype known by the increased HbF levels [42]. According to GTEx database, the rs10128556 SNP was correlated with *HBG2* expression in spleen and whole blood as eQTL. *HBG2* expression increases with each copy of its minor allele in whole blood and spleen tissues. Notably, the spleen is used to expand the erythropoetic capacity under erythroid stress conditions such as hypoxia or anemia [43].

We found no association of rs5006884 (*OR51B5/6*) with HbF concentration in our cohort. The SNP rs5006884 was associated with HbF levels by GWAS in CSSCD patients and in a replication set of black patients, but not replicated in a cohort of patients with beta thalassemia or hemoglobin E from Hong Kong and Thailand [44]. However, this association has shown stronger effect in males. Therefore, since females show higher HbF levels in our cohort, the putative sex specific regulation may be contributing to this lack of association. However, this hypothesis remains to be tested.

The HbF-boosting alleles modified clinical outcomes and hematological parameters in children with SCA. Reticulocyte is a precursor of red blood cells, and reticulocytosis is a result of

Table 4 Incidence rate ratio of some clinical outcomes and comparison of hematological variables for the three polymorphisms that were statistically significant in the multivariate model for fetal hemoglobin in 250 Brazilian children with SCA

SNP		*BCL11A* rs4671393	HMIP-2A rs9399137	HMIP-2B rs4895441
Clinical data				
Transfusion	Rate ratio (I/II)[a]	*1.62 (1.42–1.85)*	1.13 (0.97–1.3)	1.01 (0.86–1.18)
	p value	*1 × 10⁻⁷*	0.12	0.99
Acute chest syndrome	Rate ratio (I/II)[a]	1.17 (1–1.37)	*1.37 (1.13–1.66)*	0.96 (0.79–1.17)
	p value	0.05	*0.001*	0.74
Pain requiring care in hospital or emergency room	Rate ratio (I/II)[a]	0.99(0.87–1.12)	0.99 (0.85–1.15)	0.97 (0.82–1.14)
	p value	0.85	0.91	0.72
Infection requiring hospital admission	Rate ratio (I/II)[a]	*1.16 (1.03–1.3)*	1.17 (1.02–1.33)	0.86 (0.75–0.98)
	p value	*0.01*	0.03	0.03
Hematological data				
Total Hb concentration (g/dL)	Mean ± SEM (I/II)[a]	7.7 ± 0.1/8.2 ± 0.1	7.8 ± 0.1/9.2 ± 0.2	7.8 ± 0.1/8.3 ± 0.2
	95% CI (I/II)[a]	7.5–7.8/8.0-8.4	7.7–7.9/7.7–8.5	7.7–7.9/7.9–8.7
	p value	*< 0.001*	*0.004*	*0.001*
WBC count (× 10^9/L)	Mean ± SEM (I/II)[a]	15.7 ± 0.3/15.2 ± 0.4	15.6 ± 0.3/15.3 ± 0.5	15.7 ± 0.3/14.9 ± 0.6
	95% CI (I/II)[a]	15.2–16.3/14.5-16.0	15.1–16.1/14.3–16.3	15.2–16.2/13.7–16.0
	p value	0.286	0.135	0.074
Reticulocyte count (%)	Mean ± SEM (I/II)[a]	15.0 ± 0.3/14.5 ± 0.5	15.1 ± 0.3/13.6 ± 0.6	15.1 ± 0.3/13.2 ± 0.7
	95% CI (I/II)[a]	14.2–15.6/13.6-15.4	14.5–15.7/12.4–14.8	14.6–15.7/11.8–14.7
	p value	0.355	*0.004*	*0.005*
Platelet count (× 10^9/L)	Mean ± SEM (I/II)[a]	426.2 ± 10.7/398.7 ± 11.1	406.5 ± 8.2/442.3 ± 19.5	411.9 ± 8.5/427.0 ± 19.1
	95% CI (I/II)[a]	405.1–447.4/376.5-420.9	390.3–422.7/403.0–481.6	395.1–428.8/388.3–465.7
	p value	0.06	0.182	0.697

[a] Group I: genotype without minor allele; group II: genotypes with minor allele in homozygosis or heterozygosis. Significance level was 0.05/5 = 0.01 after Bonferroni correction (significant values in italics); mean ± SEM: mean values for group I and group II ± standard error of the mean; 95% CI (I/II): 95% confidence interval for the mean values of group I and group II

bone marrow compensatory activity in response to chronic hemolytic anemia [45]. Therefore, reduction of reticulocyte count indicates a mild hemolytic profile. Reticulocyte count was lower in subjects carrying minor allele of rs9399137, rs4895441, rs9402686, rs9494145, and rs1427407. Previously, rs1427407 was also associated with lower reticulocyte count in the BABY HUG cohort [40]. WBC and SpO2 are also relevant indicators of the severity of sickle cell disorders. Significantly, decreased WBC and higher SpO2 were shown to be associated with the minor alleles of rs9494145 and rs9402686.

Transfusion is a key therapy in the management of SCA for treating and avoiding several complications of the disease [22]. Therefore, the increased overall rate of transfusion observed (> 40 per 100 pt-yrs) denotes the clinical severity of the group of patients enrolled in this study. Notably, we found association of all minor *BCL11A* SNPs with lower risk of transfusion. Therefore, our findings suggest that HbF-boosting alleles at *BCL11A* ameliorate the clinical phenotype of SCA. Notably, this effect was independent of HbF relative concentration. Noteworthy, these effects may be clinically relevant for the broad spectrum of hemoglobinopathies, since genetic studies have reported associations of the SNPs not only with SCA [10–12].

Incidence of ACS was high considering average age of the subjects (8.9 years old). ACS is one of the main causes of death in Brazilian children with SCD from Minas Gerais state, according to data of Newborn Screening Program [46]. Notably, the minor alleles for rs9399137 and rs9402686 SNPs showed lower incidence rate ratio for ACS in our cohort.

Painful episode incidence was almost 80 per 100 pt-yrs and half of the events required medical care. Acute painful episode is the most important cause of hospitalization of patients with SCA [47]. Higher pain rates correlate with early death in patients with SCA over the age of 20 [48]. We were not able to find that HbF-boosting alleles were associated with a reduction of painful crisis. Contradictory findings have been reported [40]. The pain rate was shown to be inversely related to HbF levels in a previous study [48]. Interestingly, the minor

allele G for rs7776196 SNP in the HMIP-2A was the only one associated with a lower incidence rate ratio of painful episode. In the present study, even this SNP was not found to be associated with HbF concentration. Although multiple testing correction was applied, a type 1 error is the most likely explanation here, since there was no effect on other hematological/clinical variable and, furthermore, functional information do not presently justify this finding.

The use of medical records as the source of retrospective data is one of the limitations of the present study, because data were generated for clinical follow-up and not for research purpose. In addition, the sample size represents slightly more than half of the patients diagnosed by our Newborn Screening during the period of the study. However, 30% of those patients initially eligible had to be excluded from the study because they did not have HbF quantification after 5 years of age or did not have it before hydroxyurea or chronic transfusion therapy was begun. Although this criterion is surely important to access baseline level of HbF, it may characterize selection bias, as far as it excludes patients with likely more severe clinical profile, requiring adoption of these therapies before the age of 5 years. Likewise, exclusion of children who died before the study was started may have biased the results in the same direction. Conversely, patients who did not come for routine care at the outpatient clinic during the period of the study most probably represent milder forms of SCA.

In conclusion, our comprehensive genetic association analyses including 14 SNPs at five main loci affecting HbF levels demonstrated that three SNPs were independently associated with higher HbF concentration (minor alleles of rs4671393 in BCL11A, rs9399137 in HMIP-2A, and rs4895441 in HMIP-2B). HbF-boosting alleles decrease risk of many severe clinical outcomes of SCA, but this effect seems to be dependent on the association of these minor alleles with high HbF concentration. Because of the limitations of the present study, a full evaluation of genetic polymorphisms as prognostic markers would require a newborn cohort that is genetically characterized and followed prospectively over several years.

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Compliance with ethical standards

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent Informed consent was obtained from all patients for being included in the study.

References

1. Steinberg MH (2008) Sickle cell Anemia, the first molecular disease: overview of molecular etiology, pathophysiology, and therapeutic approaches. Sci World J 8:1295–1324. https://doi.org/10.1100/tsw.2008.157
2. Habara A, Steinberg MH (2016) Minireview: genetic basis of heterogeneity and severity in sickle cell disease. Exp Biol Med 241(7):689–696. https://doi.org/10.1177/1535370216636726
3. Lettre G, Bauer DE (2016) Fetal haemoglobin in sickle-cell disease: from genetic epidemiology to new therapeutic strategies. Lancet 387(10037):2554–2564
4. Steinberg MH, Sebastiani P (2012) Genetic modifiers of sickle cell disease. Am J Hematol 87(8):795–803. https://doi.org/10.1002/ajh.23232
5. Green NS, Barral S (2014) Emerging science of hydroxyurea therapy for pediatric sickle cell disease. Pediatr Res 75(0):196–204. https://doi.org/10.1038/pr.2013.227
6. Sankaran VG, Orkin SH (2013) The switch from fetal to adult hemoglobin. Cold Spring Harb Perspect Med 3(1):a011643–a011643. https://doi.org/10.1101/cshperspect.a011643
7. Close J, Game L, Clark B, Bergounioux J, Gerovassili A, Thein SL (2004) Genome annotation of a 1.5 Mb region of human chromosome 6q23 encompassing a quantitative trait locus for fetal hemoglobin expression in adults. BMC Genomics 5(1):1
8. Craig JE, Rochette J, Fisher CA, Weatherall DJ, Marc S, Lathrop GM, Demenais F, Thein S (1996) Dissecting the loci controlling fetal haemoglobin production on chromosomes 11p and 6q by the regressive approach. Nat Genet 12(1):58–64. https://doi.org/10.1038/ng0196-58
9. Dover G, Smith K, Chang Y, Purvis S, Mays A, Meyers D, Sheils C, Serjeant G Fetal hemoglobin levels in sickle cell disease and normal individuals are partially controlled by an X-linked gene located at Xp22.2.11
10. Lettre G, Sankaran VG, Bezerra MAC, Araújo AS, Uda M, Sanna S, Cao A, Schlessinger D, Costa FF, Hirschhorn JN, Orkin SH (2008) DNA polymorphisms at the BCL11A, HBS1L-MYB, and beta-globin loci associate with fetal hemoglobin levels and pain crises in sickle cell disease. Proc Natl Acad Sci U S A 105(33):11869–11874. https://doi.org/10.1073/pnas.0804799105
11. Menzel S, Garner C, Gut I, Matsuda F, Yamaguchi M, Heath S, Foglio M, Zelenika D, Boland A, Rooks H, Best S, Spector TD, Farrall M, Lathrop M, Thein SL (2007) A QTL influencing F cell production maps to a gene encoding a zinc-finger protein on chromosome 2p15. Nat Genet 39(10):1197–1199. https://doi.org/10.1038/ng2108
12. Uda M, Galanello R, Sanna S, Lettre G, Sankaran VG, Chen W, Usala G, Busonero F, Maschio A, Albai G, Piras MG, Sestu N, Lai S, Dei M, Mulas A, Crisponi L, Naitza S, Asunis I, Deiana M, Nagaraja R, Perseu L, Satta S, Cipollina MD, Sollaino C, Moi P, Hirschhorn JN, Orkin SH, Abecasis GR, Schlessinger D, Cao A (2008) Genome-wide association study shows BCL11A associated with persistent fetal hemoglobin and amelioration of the phenotype of beta-thalassemia. Proc Natl Acad Sci U S A 105(5):1620–1625. https://doi.org/10.1073/pnas.0711566105
13. Galarneau G, Palmer CD, Sankaran VG, Orkin SH, Hirschhorn JN, Lettre G (2010) Fine-mapping at three loci known to affect fetal

hemoglobin levels explains additional genetic variation. Nat Genet 42(12):1049–1051. https://doi.org/10.1038/ng.707
14. Thein SL, Menzel S, Peng X, Best S, Jiang J, Close J, Silver N, Gerovasilli A, Ping C, Yamaguchi M, others (2007) Intergenic variants of HBS1L-MYB are responsible for a major quantitative trait locus on chromosome 6q23 influencing fetal hemoglobin levels in adults. Proc Natl Acad Sci 104(27):11346–11351
15. Laurentino MR, Barbosa MC, Santos TEJ, Perdigão ACB, Araújo FMC, Lemes RPG (2018) Analysis of BCL11A gene polymorphisms and hemolysis parameters in patients with sickle-cell disease. J Bras Patol Med Lab 54(3). https://doi.org/10.5935/1676-2444.20180025
16. Mtatiro SN, Makani J, Mmbando B, Thein SL, Menzel S, Cox SE (2015) Genetic variants at HbF-modifier loci moderate anemia and leukocytosis in sickle cell disease in Tanzania. Am J Hematol 90(1):E1–E4. https://doi.org/10.1002/ajh.23859
17. Rumaney MB, Ngo Bitoungui VJ, Vorster AA, Ramesar R, Kengne AP, Ngogang J, Wonkam A (2014) The co-inheritance of alpha-thalassemia and sickle cell anemia is associated with better hematological indices and lower consultations rate in Cameroonian patients and could improve their survival. PLoS One 9(6):e100516. https://doi.org/10.1371/journal.pone.0100516
18. Wonkam A, Ngo Bitoungui VJ, Vorster AA, Ramesar R, Cooper RS, Tayo B, Lettre G, Ngogang J (2014) Association of variants at BCL11A and HBS1L-MYB with hemoglobin F and hospitalization rates among sickle cell patients in Cameroon. PLoS One 9(3): e92506. https://doi.org/10.1371/journal.pone.0092506
19. Cardoso GL, Diniz IG, Martins da Silva ANL, Cunha DA, da Silva Junior JS, Carvalho Uchôa CT, dos Santos SEB, Trindade SMS, Cardoso MSO, Guerreiro JF (2014) DNA polymorphisms at BCL11A, HBS1L-MYB and Xmn1-HBG2 site loci associated with fetal hemoglobin levels in sickle cell anemia patients from Northern Brazil. Blood Cell Mol Dis 53(4):176–179. https://doi.org/10.1016/j.bcmd.2014.07.006
20. Friedrisch JR, Sheehan V, Flanagan JM, Baldan A, Ginter Summarell CC, Bittar CM, Friedrisch BK, Wilke II, Ribeiro CB, Daudt LE, da Rocha Silla LM (2016) The role of BCL11A and HMIP-2 polymorphisms on endogenous and hydroxyurea induced levels of fetal hemoglobin in sickle cell anemia patients from southern Brazil. Blood Cells Mol Dis 62:32–37. https://doi.org/10.1016/j.bcmd.2016.11.002
21. Leonardo FC, Brugnerotto AF, Domingos IF, Fertrin KY, de Albuquerque DM, Bezerra MAC, Araújo AS, Saad STO, Costa FF, Menzel S, Conran N, Thein SL (2016) Reduced rate of sickle-related complications in Brazilian patients carrying HbF-promoting alleles at the BCL11A and HMIP-2 loci. Br J Haematol 173:456–460. https://doi.org/10.1111/bjh.13961
22. Ballas SK, Lieff S, Benjamin LJ, Dampier CD, Heeney MM, Hoppe C, Johnson CS, Rogers ZR, Smith-Whitley K, Wang WC, Telen MJ (2010) Definitions of the phenotypic manifestations of sickle cell disease. Am J Hematol 85(1):6–13. https://doi.org/10.1002/ajh.21550
23. Garner CP, Tatu T, Best S, Creary L, Thein SL (2002) Evidence of genetic interaction between the β-globin complex and chromosome 8q in the expression of fetal hemoglobin. Am J Hum Genet 70(3): 793–799
24. Barrett JC, Fry B, Maller J, Daly MJ (2005) Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics (Oxford, England) 21(2):263–265. https://doi.org/10.1093/bioinformatics/bth457
25. O'Brien PC (1983) The appropriateness of analysis of variance and multiple-comparison procedures. Biometrics 39(3):787–794
26. Bauer DE, Orkin SH (2015) Hemoglobin switching's surprise: the versatile transcription factor BCL11A is a master repressor of fetal hemoglobin. Curr Opin Genet Dev 33:62–70. https://doi.org/10.1016/j.gde.2015.08.001
27. Boyle AP, Hong EL, Hariharan M, Cheng Y, Schaub MA, Kasowski M, Karczewski KJ, Park J, Hitz BC, Weng S, Cherry JM, Snyder M (2012) Annotation of functional variation in personal genomes using RegulomeDB. Genome Res 22(9):1790–1797. https://doi.org/10.1101/gr.137323.112
28. Schaub MA, Boyle AP, Kundaje A, Batzoglou S, Snyder M (2012) Linking disease associations with regulatory information in the human genome. Genome Res 22(9):1748–1759. https://doi.org/10.1101/gr.136127.111
29. Makani J, Menzel S, Nkya S, Cox SE, Drasar E, Soka D, Komba AN, Mgaya J, Rooks H, Vasavda N, Fegan G, Newton CR, Farrall M, Thein SL (2011) Genetics of fetal hemoglobin in Tanzanian and British patients with sickle cell anemia. Blood 117(4):1390–1392. https://doi.org/10.1182/blood-2010-08-302703
30. Bauer DE, Kamran SC, Lessard S, Xu J, Fujiwara Y, Lin C, Shao Z, Canver MC, Smith EC, Pinello L, Sabo PJ, Vierstra J, Voit RA, Yuan G-C, Porteus MH, Stamatoyannopoulos JA, Lettre G, Orkin SH (2013) An erythroid enhancer of BCL11A subject to genetic variation determines fetal hemoglobin level. Science 342(6155): 253–257. https://doi.org/10.1126/science.1242088
31. Creary LE, Ulug P, Menzel S, McKenzie CA, Hanchard NA, Taylor V, Farrall M, Forrester TE, Thein SL (2009) Genetic variation on chromosome 6 influences F cell levels in healthy individuals of African descent and HbF levels in sickle cell patients. PLoS One 4(1):e4218. https://doi.org/10.1371/journal.pone.0004218
32. Green NS, Barral S (2011) Genetic modifiers of HbF and response to hydroxyurea in sickle cell disease. Pediatr Blood Cancer 56(2): 177–181. https://doi.org/10.1002/pbc.22754
33. Ngo D, Bae H, Steinberg MH, Sebastiani P, Solovieff N, Baldwin CT, Melista E, Safaya S, Farrer LA, Al-Suliman AM, Albuali WH, Al Bagshi MH, Naserullah Z, Akinsheye I, Gallagher P, Luo H-y, Chui DHK, Farrell JJ, Al-Ali AK, Alsultan A (2013) Fetal hemoglobin in sickle cell anemia: genetic studies of the Arab-Indian haplotype. Blood Cells Mol Dis 51(1):22–26. https://doi.org/10.1016/j.bcmd.2012.12.005
34. Nguyen TKT, Joly P, Bardel C, Moulsma M, Bonello-Palot N, Francina A (2010) The XmnI Gγ polymorphism influences hemoglobin F synthesis contrary to BCL11A and HBS1L-MYB SNPs in a cohort of 57 β-thalassemia intermedia patients. Blood Cell Mol Dis 45(2):124–127. https://doi.org/10.1016/j.bcmd.2010.04.002
35. Roy P, Bhattacharya G, Mandal A, Dasgupta UB, Banerjee D, Chandra S, Das M (2012) Influence of BCL11A, HBS1L-MYB, HBBP1 single nucleotide polymorphisms and the HBG2 XmnI polymorphism on HbF levels. Hemoglobin 36(6):592–599. https://doi.org/10.3109/03630269.2012.735626
36. Akinsheye I, Solovieff N, Ngo D, Malek A, Sebastiani P, Steinberg MH, Chui DHK (2012) Fetal hemoglobin in sickle cell anemia: molecular characterization of the unusually high fetal hemoglobin phenotype in African Americans. Am J Hematol 87(2):217–219. https://doi.org/10.1002/ajh.22221
37. Bae HT, Baldwin CT, Sebastiani P, Telen MJ, Ashley-Koch A, Garrett M, Hooper WC, Bean CJ, Debaun MR, Arking DE, Bhatnagar P, Casella JF, Keefer JR, Barron-Casella E, Gordeuk V, Kato GJ, Minniti C, Taylor J, Campbell A, Luchtman-Jones L, Hoppe C, Gladwin MT, Zhang Y, Steinberg MH (2012) Meta-analysis of 2040 sickle cell anemia patients: BCL11A and HBS1L-MYB are the major modifiers of HbF in African Americans. Blood 120(9):1961–1962. https://doi.org/10.1182/blood-2012-06-432849
38. Bhanushali AA, Patra PK, Pradhan S, Khanka SS, Singh S, Das BR (2015) Genetics of fetal hemoglobin in tribal Indian patients with sickle cell anemia. Transl Res 165(6):696–703. https://doi.org/10.1016/j.trsl.2015.01.002
39. Bitoungui VJN, Ngogang J, Wonkam A (2015) Polymorphism at BCL11A compared to HBS1L-MYB loci explains less of the variance in HbF in patients with sickle cell disease in Cameroon. Blood

Cells Mol Dis 54(3):268–269. https://doi.org/10.1016/j.bcmd.2014.11.010
40. Sheehan VA, Luo Z, Flanagan JM, Howard TA, Thompson BW, Wang WC, Kutlar A, Ware RE, Investigators BH (2013) Genetic modifiers of sickle cell anemia in the BABY HUG cohort: influence on laboratory and clinical phenotypes. Am J Hematol 88(7):571–576. https://doi.org/10.1002/ajh.23457
41. Farrell JJ, Sherva RM, Chen Z-Y, Luo H-Y, Chu BF, Ha SY, Li CK, Lee ACW, Li RCH, Li CK, Yuen HL, So JCC, Ma ESK, Chan LC, Chan V, Sebastiani P, Farrer LA, Baldwin CT, Steinberg MH, Chui DHK (2011) A 3-bp deletion in the HBS1L-MYB intergenic region on chromosome 6q23 is associated with HbF expression. Blood 117(18):4935–4945. https://doi.org/10.1182/blood-2010-11-317081
42. Powars DR (1991) Beta s-gene-cluster haplotypes in sickle cell anemia. Clinical and hematologic features. Hematol Oncol Clin North Am 5(3):475–493
43. Socolovsky M (2007) Molecular insights into stress erythropoiesis. Curr Opin Hematol 14(3):215–224. https://doi.org/10.1097/MOH.0b013e3280de2bf1
44. Solovieff N, Milton JN, Hartley SW, Sherva R, Sebastiani P, Dworkis DA, Klings ES, Farrer LA, Garrett ME, Ashley-Koch A, others (2010) Fetal hemoglobin in sickle cell anemia: genome-wide association studies suggest a regulatory region in the 5′ olfactory receptor gene cluster. Blood 115(9):1815–1822
45. Brugnara C, Zelmanovic D, Sorette M, Ballas SK, Platt O (1997) Reticulocyte hemoglobin: an integrated parameter for evaluation of erythropoietic activity. Am J Clin Pathol 108(2):133–142
46. Fernandes APPC, Avendanha FA, Viana MB (2017) Hospitalizations of children with sickle cell disease in the Brazilian Unified Health System in the state of Minas Gerais. J Pediatr 93(3):287–293. https://doi.org/10.1016/j.jped.2016.07.005
47. Ballas SK, Lusardi M (2005) Hospital readmission for adult acute sickle cell painful episodes: frequency, etiology, and prognostic significance. Am J Hematol 79(1):17–25. https://doi.org/10.1002/ajh.20336
48. Platt OS, Thorington BD, Brambilla DJ, Milner PF, Rosse WF, Vichinsky E, Kinney TR (1991) Pain in sickle cell disease. N Engl J Med 325(1):11–16. https://doi.org/10.1056/NEJM199107043250103

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4.2 Capítulo 2: Haplótipos de *BCL11A* e HBS1L-MYB associados com concentração elevada de HbF na predição de desfechos clínicos da AF

A casuística estudada neste capítulo foi retirada da casuística descrita no capítulo 1. Vale ressaltar que o protocolo de estudo aprovado pelos Comitês de Ética em Pesquisa da Fundação HEMOMINAS e da UFMG, mencionado no capítulo anterior já contemplam os dados e análises realizadas neste capítulo (**Anexo 1**). A partir dos 250 participantes do estudo anterior, foram excluídos os indivíduos aparentados, restando 220 não aparentados.

As variáveis clínicas, hematológicas e genéticas estudadas seguiram o mesmo protocolo de determinação e análise de dados descrito no capítulo 1.

As frequências dos haplótipos foram estimadas usando o pacote Haplo.stats (versão 1.7.9; http://www.r-project.org). A função *haplo.em* foi utilizada para calcular estimativas de probabilidade dos haplótipos usando o algoritmo de inserção progressiva, que progressivamente insere blocos de *loci* em haplótipos de comprimentos crescentes. A função *haplo.score* foi usada para calcular a pontuação estatística de cada haplótipo para testar sua associação nos modelos aditivo, recessivo e dominante. O valor de significância (p=0.05) foi ajustado para correção de múltiplos testes, dividindo o valor p pelo número de haplótipos estimados na casuística. A função *haplo.cc* foi utilizada para calcular a razão de chances (Odds ratio - OR) e intervalos de confiança de 95% para cada haplótipo. O poder das análises foi calculado utilizando a função *haplo.power.cc,* que especifica os coeficientes haplótipos de haplótipo como odds ratio (OR) e depois converte em coeficientes de regressão logística de acordo com log (OR).

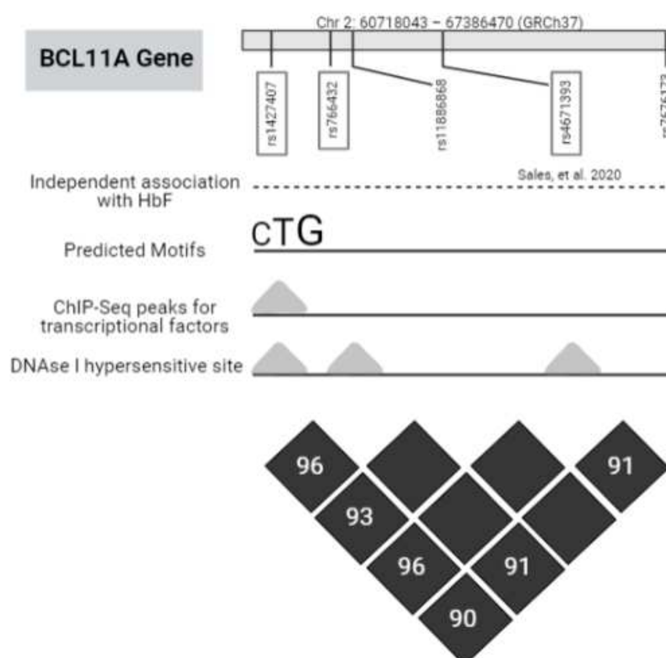
Para ambos os tipos de testes, o poder depende da quantidade de variação na característica explicada pelos haplótipos ou coeficiente de correlação, r^2. Em vez de especificar diretamente os coeficientes do haplótipo, calculamos o vetor de coeficientes baseados em um valor R2.

Anotação funcional *in silico* dos SNPs previamente estudados foi realizada utilizando bancos de dados públicos: The *Genotype Tissue Expression* (*GTEx*; https://gtexportal.org/home/) foram acessados para verificar se os genótipos dos SNPs correlacionam com expressão tecido-específica, ou seja, se

os SNPs são eQTLs (*expression Quantitative Trait Loci*). Dados do *Encyclopedia of DNA Element*s (*ENCODE)* e do *RegulomeDB* (http://www.regulomedb.org/) foram acessados para a buscar por SNPs em regiões regulatórias em *BCL11A* e HMIP-*2*. Finalmente, nós usados utilizamos o navegador genômico *UCSC Genome Browser* (https://genome.ucsc.edu/cgi-bin/hgTracks), que integra múltiplas fontes de dados funcionais, para buscar por regiões acentuadoras (*enhancers)* para os genes envolvidos na via de regulação da HbF proposta (BAUER; ORKIN, 2015).

Na seleção de SNPs para as análises de haplótipos foram priorizados SNPs que foram independentemente – pelo desequilíbrio de ligação alélica - associados e/ou que apresentaram características funcionais biologicamente compatíveis com a via de regulação da HbF (**Figura 7A** e **Figura 7B**). Selecionamos ainda, aqueles SNPs que apresentaram alto LD ($r^2 \geq 0{,}8$) com SNPs funcionais importantes, mas que não foram genotipados em nossa coorte. Tendo em vista que estimativas de ancestralidade genômica mostraram maior contribuição Europeia (39,7%) e Africana (47,3%) em pacientes com doença falciforme do estado de Minas Gerais(DA SILVA *et al.*, 2011), nós verificamos as medidas de LD nas populações de CEU (População de Utah, com residentes da Europa Ocidental e do Norte) e YRI (População de Yoruba, em Ibadan, Nigéria), utilizando dados do *The 1000 Genomes*.

A



B

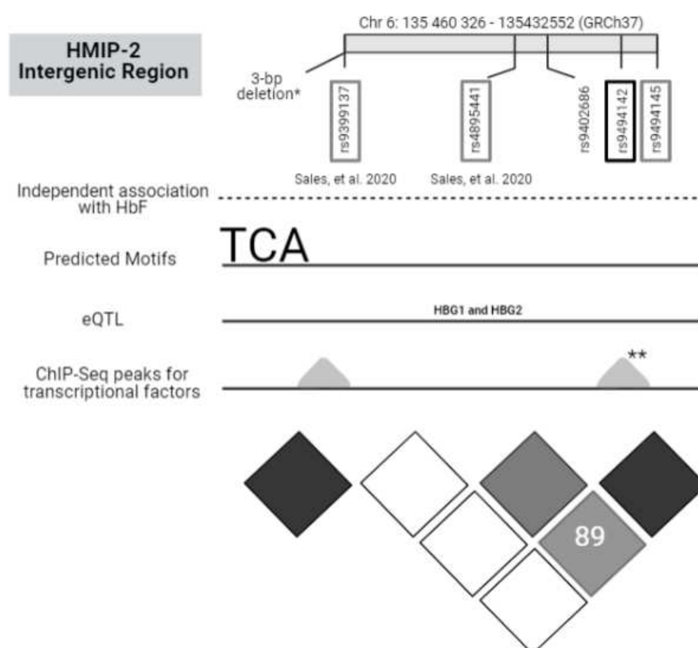


Figura 7: Esquema representativo da seleção de SNPs funcionais

(A) no íntron 2 do gene *BCL11A* [rs1427407, rs766432 e rs4671393; Chr 2 60718043 – 67386470 (GRCh37)] e na região intergênica (B) HMIP-2 [rs9399137, rs4895441 e rs9494145; Chr 6 135460326 - 135432552 (GRCh37)] a partir do conjunto de SNPs previamente associados com a concentração de HbF (Capítulo 1). Informações regulatórias na região genômica dos SNPs foram compilados nas três linhas contínuas. Os quadrados na parte inferior da figura mostram o desequilíbrio de ligação (LD) entre SNPs vizinhos, que foi calculado usando dados de genótipo dos Yoruba em Ibadan, Nigéria (YRI) da fase 3 de The 1000 Genomes. Os números dentro dos quadrados indicam o D' expresso como percentil. Quadrados cinza escuro indicam pares em LD forte com escores LOD para LD ≥ 2, quadrados cinza claro D′ < 1 com LOD ≥ 2 e quadrados

brancos D′ < 1,0 e LOD < 2. Os asteriscos indicam SNPs funcionais (deleção de 3 pb e rs9494142) que não foram genotipados em nossa coorte, mas estão em LD com SNPs genotipados.

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ARTICLE

Fetal hemoglobin-boosting haplotypes of *BCL11A* gene and *HBS1L-MYB* intergenic region in the prediction of clinical and hematological outcomes in a cohort of children with sickle cell anemia

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Single nucleotide polymorphisms (SNPs) of *BCL11A* gene and *HBS1L-MYB* intergenic region (named HMIP-2) affect both fetal hemoglobin (HbF) concentration and clinical outcomes in patients with sickle cell anemia (SCA). However, no previous study has examined the interaction among these SNPs in the regulation of HbF. We examined whether HbF-boosting haplotypes combining alleles of functional SNPs of *BCL11A* and HMIP-2 were associated with clinical outcomes and hematological parameters, and whether they interact to regulate HbF in a cohort of Brazilian children with SCA. The minor haplotype of *BCL11A* ("TCA", an allele combination of rs1427407, rs766432, and rs4671393) was associated with higher HbF, hemoglobin and lower reticulocytes count compared to reference haplotype "GAG". The minor haplotype of HMIP-2 ("CGC", an allele combination of rs9399137, rs4895441, and rs9494145) was associated with higher HbF and hemoglobin compared to reference haplotype "TAT". Subjects carrying minor haplotypes showed reduced rate of clinical complications compared to reference haplotypes. Non-carriers of both minor haplotypes for *BCL11A* and HMIP-2 showed the lowest HbF concentration. Subjects carrying only the minor haplotype of *BCL11A* showed significantly higher HbF concentration than non-carriers of any minor haplotype, which showed no significant difference compared to subjects carrying only the minor haplotype of HMIP-2. Interestingly, subjects carrying both minor haplotypes of *BCL11A* ("TCA") and HMIP-2 ("CGC") showed significantly higher HbF levels than subjects carrying only the minor haplotype of *BCL11A*. Our novel findings suggest that HbF-boosting haplotypes of *BCL11A* and HMIP-2 can predict clinical outcomes and may interact to regulate HbF in patients with SCA.

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INTRODUCTION

Sickle cell anemia (SCA) is a monogenic disorder caused by the homozygosity of a point mutation in the *HBB* (hemoglobin subunit beta) gene, which produces the variant of hemoglobin S (HbS). SCA is the most common and clinically severe form of sickle cell disease [1], and it has a complex pathophysiology, starting with the polymerization of an abnormal deoxy sickle hemoglobin (HbS, $\alpha_2\beta^S{}_2$) [2]. Notably, the level of fetal hemoglobin (HbF, $\alpha_2\gamma_2$) is the major modifier of clinical phenotypes of SCA, since HbF acts as a potent anti-sickling agent and ameliorates the clinical severity of SCA [3].

Genome-wide association studies (GWAS) have identified the main loci involved in the regulation of HbF production [4–6], namely the *BCL11A* gene (BAF chromatin remodeling complex subunit BCL11A), the intergenic region located between the *HBS1L* (HBS1 like translational GTPase) and *MYB* (MYB proto-oncogene, transcription factor), and the globin gene cluster itself. A further GWAS have identified single nucleotide polymorphisms (SNPs) of *BCL11A* gene significantly associated with the proportion of HbF-containing erythrocytes in individuals of African ancestry with sickle cell disease [7]. Notably, SNPs located in the intron 2 of *BCL11A* gene and in the *HBS1L-MYB* intergenic region (named HMIP-2) were associated with increased HbF concentration and with milder clinical phenotypes in pediatric patients with SCA [8]. These HbF-boosting alleles were also associated with less severe hematological parameters and lower incidence of common clinical episodes of SCA [9, 10]. Recently, a systematic review provided evidence that SNPs located in the intron 2 of *BCL11A* affect HbF levels in response to hydroxyurea treatment in patients with SCA [11].

Haplotypes are the combination of alleles for different genetic polymorphisms that occur in the same gene or chromosomal

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region and that tend to be inherited together [12]. Noteworthy, haplotypes carry more genetic information than the analysis of single SNPs and their use may provide higher prediction power in genetic association studies [13–15]. For example, previous analyses were focused on typical and atypical haplotypes classified according to six polymorphic sites in the β-globin cluster and their relationship with pathophysiology of SCA [16]. However, although *BCL11A* and *HBSIL-MYB* act in the same pathway in the regulation of HbF [3], no previous study has examined whether they interact in the regulation of HbF. It is possible that patients with SCA who co-inherit specific haplotypes of *BCL11A* gene and HMIP-2 intergenic region may exhibit different HbF levels, which may be associated with different clinical outcomes in SCA.

In the present study, we examined whether haplotypes combining HbF-boosting alleles of functional SNPs of *BCL11A* gene and HMIP-2 intergenic region are associated with hematological parameters and with the incidence of clinical outcomes in children with SCA. Moreover, we examined whether the haplotypes of *BCL11A* gene and HMIP-2 intergenic region interact in the regulation of HbF levels.

MATERIALS AND METHODS

Subjects and study design

This study was conducted accordingly with the Declaration of Helsinki and was approved by the Institutional Review Board from the Hemominas Foundation and the Federal University of Minas Gerais (certification number: 4204.8215.6.0000.5118). Written informed consent and age-appropriate assent were obtained from every child included in the study and from their parents/guardians.

This study involved a retrospective cohort of pediatric patients with SCA diagnosed between 1999 and 2008 by the Newborn Screening Program of Minas Gerais state, Southeastern Brazil, and followed up at Center of Hematology and Hemotherapy of Minas Gerais–HEMOMINAS, in Belo Horizonte, capital of Minas Gerais. The patient recruitment and eligibility criteria were previously described [8]. For the present study, we excluded those related subjects from our cohort of 250 pediatric patients with SCA [8] and, accordingly, we selected 220 unrelated children with SCA for the haplotype analyses.

Hematological and clinical data

Hematological parameters and clinical outcomes were retrieved from the medical records of the patients. The hematological parameters tested were relative HbF concentration, total hemoglobin concentration (total Hb), white blood cell count (WBC), reticulocytes, platelets and peripheral oxygen saturation (SpO2), which were determined according to protocol previously described by our group [8].

Clinical episodes were defined based on the guidelines from the Comprehensive Sickle Cell Centers [17]. Acute chest syndrome (ACS)/ pneumonia was defined as an acute event characterized by fever and/or respiratory symptoms, accompanied by a pulmonary infiltrate on a chest x-ray. Because few children had a previous x-ray to be compared to, the adjective "new" was dropped from the original definition. Pain was defined as an acute episode reported by patients/family that led to analgesic/ narcotic administration in hospital or emergency room. Definition of infection included upper airway infection, pneumonia/ACS, urinary tract infection and osteomyelitis. The episodes of infection were only considered as those recognized by a physician and requiring hospital admission. Acute splenic sequestration (ASS) was defined as a tender, rapidly enlarging, and sometimes massive spleen due to the trapping of sickled erythrocytes and other blood constituents, which caused a precipitous fall in Hb level.

Functional annotation of SNPs associated with HbF levels

First, we performed the identification of functional SNPs previously associated with HbF levels. We searched for regulatory information in the human genome according to the approach to identify "functional SNPs", as proposed elsewhere [18]. These authors described a scenario in which a lead SNP associated with a phenotype in a GWAS is in strong linkage disequilibrium (LD) with a functional SNP, which overlaps predicted binding motifs of transcriptional factors and DNAseI hypersensitivity peaks, as well as is in strong LD with a third SNP that is associated with gene expression as an expression Quantitative Trait Loci (eQTL). If neither the lead SNP from a GWAS nor the eQTL SNP overlaps with a functional region, then the functional SNP is more likely to be the SNP that plays a biological role in the phenotype [18].

Next, we selected SNPs of *BCL11A* gene and HMIP-2 intergenic region that explain a greater fraction of the variance in the HbF levels in our cohort of children with SCA [8]. We performed the functional annotation of these SNPs using publicly available databases: The Genotype Tissue Expression (GTEx [19]; https://gtexportal.org/home/) was used to assess whether the SNPs genotypes correlate with tissue-specific expression, which means that the SNPs may be eQTLs. Data from The Encyclopedia of DNA Elements (ENCODE) Project, including DNase I hypersensitivity sites and Chromatin immunoprecipitation followed by sequencing (ChIP-seq) peaks for transcription factor binding sites, and RegulomeDB [20] (http://www.regulomedb.org/) were accessed to search for SNPs located within putative regulatory regions in the *BCL11A* gene and the HMIP-2 intergenic region. Finally, we searched for enhancer regions in the genes involved in the proposed HbF regulatory pathway [3] at the UCSC Genome Browser (https://genome.ucsc.edu/), which integrates multiple sources of functional data.

Genetic data and haplotype association analysis

Venous blood samples were collected from patients at clinical attendance. Genomic DNA extraction from venous blood samples of patients in our cohort and SNP genotyping were performed as previously described [8].

The haplotypes frequencies were estimated using the Haplo.stats package (version 1.8.9; https://cran.r-project.org/web/packages/haplo.stats/). The function haplo.em computes maximum likelihood estimates of haplotype probabilities on unrelated subjects, with linkage phase unknown, using the progressive insertion algorithm, which progressively inserts batches of loci into haplotypes of growing lengths, as described in detail elsewhere [21–23]. Because linkage phase is unknown on unrelated subjects, there may be more than one pair of haplotypes, so posterior probabilities of pairs of haplotypes for each subject are also computed [23]. The haplotype frequencies formed by the combination of functional SNPs selected from the *BCL11A* gene and HMIP-2 intergenic region are shown in Supplementary Tables S1 and S2. The reference and minor haplotypes were defined as the haplotypes that combined the wild-type alleles and minor alleles of the functional SNPs, respectively. Regarding *BCL11A*, the possible haplotypes that combined alleles of the functional SNPs rs1427407 (G > T), rs766432 (A > C), and rs4671393 (G > A) were: H1 (GAA); H2 ("GAG", Reference haplotype); H3 (GCA); H4 (GCG); H5 (TAG); H6 ("TCA", Minor haplotype); and H7 (TCG) (Supplementary Table S1**)**. Regarding HMIP-2, the possible haplotypes that combined alleles of the functional SNPs rs9397137 (T > C), rs4895441 (A > G), and rs9494145 (T > C) were: H1 (CAC); H2 (CAT); H3 ("CGC", Minor haplotype); H4 (CGT); H5 (TAC); H6 ("TAT", Reference haplotype); H7 (TGC); and H8 (TGT) (Supplementary Table S2).

We then tested the association of the haplotypes combining functional SNPs of *BCL11A* or HMIP-2 with hematological parameters and with the incidence of clinical outcomes. Haplotypes that had frequencies below 5% in our cohort were excluded from the association analyses. For these analyses, we named as "TCA+" and "TCA−" the groups of subjects that carry and not carry the "TCA" minor haplotype of *BCL11A* gene, respectively. Similarly, we named as "CGC+" and "CGC−" the groups of subjects that carry and not carry the "CGC" minor haplotype of HMIP-2 intergenic region, respectively.

Statistical analyses

Continuous variables were reported as mean and standard deviation. Incidence of clinical episodes was reported by relative rates to 100 patient-years (pt-yrs), with 95% confidence intervals. The incidence rate ratio (IRR) of clinical episodes was calculated on OpenEpi online software, PersonTime2/PersonTime2 option (http://www.openepi.com/PersonTime2/PersonTime2.htm).

For continuous variables with normal distribution, Student's *t* test and ANOVA test were used to compare means between two and three groups, respectively. The distribution of continuous variables was evaluated by using the Kolmogorov–Smirnov test. The Mann–Whitney or Kruskal–Wallis tests were used to compare means between two or three groups, respectively in testing the association with continuous variables with non-normal distribution. Cumulative incidence of ASS was estimated by using a

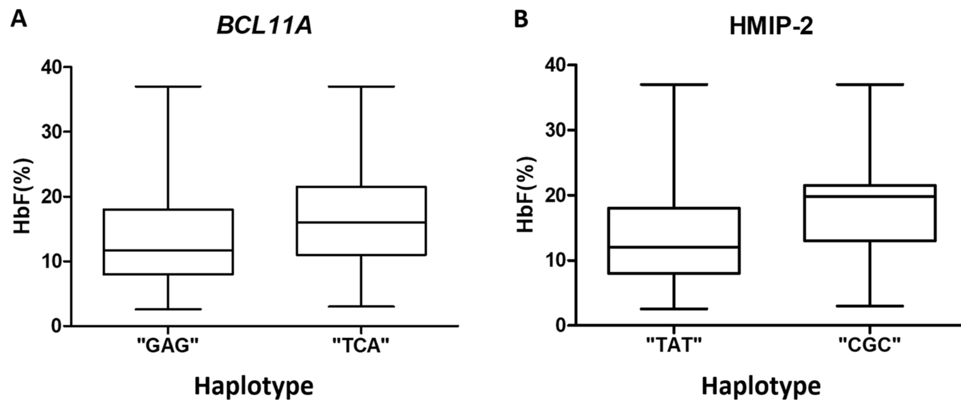

Fig. 1 Comparative boxplot of HbF concentration between the reference and minor haplotypes of *BCL11A* gene and HMIP-2 intergenic region. **A** The minor TCA haplotype of *BCL11A* (16.61 ± 7,56; $n = 73$ haplotypes) showed increased HbF concentration ($P = 0.0006$) when compared with reference GAG haplotype (13.28 ± 6,68; $n = 208$). **B** The minor CGC haplotype of HMIP-2 (17,94 ± 7,91; $n = 25$) showed increased HbF concentration ($P = 0.003$), when compared with GAG reference haplotype (13.45 ± 6,88; $n = 216$)

Kaplan–Meier method (function: one minus survival) and the log rank test was used to compare the incidence between haplotype groups. The tests were considered statistically significant when the probability of an alpha error was equal to or less than 0.05. Haplotype association tests were performed using the SPSS software, version 21.0 (SPSS Inc.; Chicago, IL, USA).

We compared the mean of HbF levels between minor and reference haplotype using Student's *T* test. Due to the low frequency of the minor haplotypes in our cohort, we performed the association tests with the hematological parameters and the incidence clinical outcomes by comparing the subjects carrying the minor haplotypes for *BCL11A* gene and HMIP-2 intergenic region with the non-carriers of these minor haplotypes.

Pairwise LD among SNPs was calculated as D′ and r^2 using HaploView software version 4.2 [24], and data from the 1000 Genomes Phase 3 for Africans (YRI, Yoruba in Ibadan, Nigeria) and Europeans (CEU, Utah Residents with European Ancestry).

Finally, in order to examine whether the haplotypes of *BCL11A* gene and HMIP-2 intergenic region interact in the regulation of HbF levels, we compared the mean of HbF levels between the combinations of haplotypes. Haplotype groups were defined by the presence of minor haplotype of each locus.

RESULTS

Clinical and laboratory profile of the cohort

The present study included 220 children with SCA, 105 (47.7%) were males. The mean age at the time of enrollment was 9.8 ± 2.9 years, with a minimum of 5.2 and a maximum of 16.1 years. The hematological parameters and the incidence of clinical outcomes of pediatric patients with SCA are summarized in Supplementary Table S3. The mean of the HbF concentration was 13.5% ± 6.9%, minimum of 2.6% and maximum of 37%. The mean ± standard error of the mean (95% confidence interval, lower - upper) of hematological parameters in the 220 children with SCA (192 for SpO_2) were: HbF, 13.5 ± 0.46 (%; 12.6–14.4); total hemoglobin concentration, 7.9 ± 0.62 (g/dL; 7.7–7.9); WBC count, 15.5 ± 0.23 (10^9/L; 15–15.9); relative reticulocyte count, 14.9 ± 0.26 (%; 14.3–15.3); platelet count, 413.9 ± 7.33 (10^9/L; 399.4–428.2); SpO_2, 93.4 ± 0.24 (%; 92.9–93.9). The rate per 100 patient-years (95% confidence interval) of the clinical outcomes in the 220 children with SCA are: transfusion, 41.8 (39.2–44.6); ACS, 25.9 (23.9–28.2); Painful episode requiring medical attention 37.7 (35.2–40.4); Infection requiring hospitalization 54.3 (45.1–64.8).

Functional annotation of SNPs related to HbF regulation

We selected three SNPs from both the *BCL11A* gene (rs1427407, rs766432, and rs4671393) and the HMIP-2 intergenic region (rs9399137, rs4895441, and rs9494145) based on the fraction of the variance on HbF levels these SNPs explain and whether they were associated to the regulatory information in the human genome (see Methods).

Supplementary Fig. 1A shows an overview of the approach used to select functional SNPs in the intron 2 of *BCL11A* gene [rs1427407, rs766432, and rs4671393; Chr 2 60718043–67386470 (GRCh37)], by joining those SNPs that were independently associated with HbF levels. The intron 2 has an erythroid enhancer for *BCL11A* gene, encoding a master repressor of HbF [3]. Both rs1427407 and rs766432 SNPs lie within an open chromatin region (+62 DNAse I hypersensitive site) and overlap with ChIP-seq peaks of GATA1 and TAL1 transcription factor binding sites [25]. Notably, the minor T allele for the rs1427407 SNP (T > G) disrupts a consensus sequence [CTG(n9)] enriched for GATA1 and TAL1 transcription factors, which are more likely to binding in the presence of G-allele in primary erythroblast samples, suggesting that both SNPs modulate critical cis-regulatory elements [25]. Finally, the rs4671393 SNP was found to be independently associated with HbF concentration in our cohort [8] (Supplementary Fig. 1A).

For the selection of functional SNPs in the HMIP-2 intergenic region (rs9399137, rs4895441, and rs9494145; Chr 6 135460326–135432552 (GRCh37)] (Supplementary Fig. 1B), the rs9399137 SNP was found to be both independently associated with HbF levels in our cohort and in LD within the region nearby the 3-bp deletion polymorphism [8], which has enhancer-like activity [26]. Without the deletion, the DNA fragment enhances the promoter activity of *HBG2* gene (hemoglobin subunit gamma) by 3.4-fold, which encodes γ-globin chain of HbF. With the 3-bp deletion, the enhancement of the *HBG2* promoter activity is increased by 5.4-fold [26]. Moreover, the rs4895441 SNP was found to be independently associated with HbF levels in our cohort [8], and it was found to be an eQTL for *HBG1* e *HBG2* genes according to RegulomeDB (score 1 f). Finally, the rs9494145 SNP was found in strong LD with rs9494142 SNP in African (D′ = 1 and $r^2 = 0.53$)] and European (D′ = 1 and $r^2 = 0.81$)] populations from "The 1000 Genomes phase 3". Notably, the rs9494142 is located at an enhancer for *MYB* gene, a site of several DNA-binding erythroid (K562 cell line) transcriptional factors, as supported by ChIP-seq data available in https://regulomedb.org/regulome-search?regions=chr6%3A135431639–135431640&genome=GRCh37/thumbnail=chip).

Haplotypes association analyses

The frequencies for the seven haplotypes of *BCL11A* gene and eight haplotypes of HMIP-2 intergenic region found from the combination of alleles from three SNPs in each locus are shown

Table 1. Distribution of hematological parameters according to haplotype groups of *BCL11A* gene and HMIP-2 intergenic region in 220 children with sickle cell anemia

Hematological parameters	*BCL11A* haplotypes					HMIP-2 haplotypes				
	"TCA−"		"TCA+"		*p* value[a]	"CGC−"		"CGC+"		*p* value[a]
	Mean ± SEM	95% CI	Mean ± SEM	95% CI		Mean ± SEM	95% CI	Mean ± SEM	95% CI	
HbF (%)	12.01 ± 0.5	11.03–12.99	16.61 ± 0.88	14.85–18.38	**<0.001**	12.97 ± 0.47	12.04–13.9	17.94 ± 1.58	14.68–21.21	**0.001**
Total Hb (g/dL)	7.6 ± 0.07	7.4–7.7	8.3 ± 0.1	8.1–8.5	**<0.001**	7.7 ± 0.06	7.6–7.9	8.3 ± 0.2	7.8–8.7	**0.005**
WBC (10^9/L)	15.8 ± 0.2	15.2–16.3	15.1 ± 0.4	14.3–16.0	0.182	15.6 ± 0.2	15.1–16.1	15.1 ± 0.6	13.7–16.5	0.379
Reticuloytes (%)	15.3 ± 0.3	14.7–16.0	13.9 ± 0.4	13.0–14.8	**0.010**	14.9 ± 0.2	14.4–15.5	14.2 ± 0.7	12.7–15.8	0.211
SpO2 (%)	93.2 ± 0.2	92.6–93.7	93.9 ± 0.3	93.1–94.6	0.155	93.3 ± 0.2	92.8–93.8	94.1 ± 0.4	93.1–95.0	0.44
Platelets (10^9/L)	421.3 ± 9.3	402.9–439.8	408.9 ± 14.0	380.9–437.0	0.457	410.8 ± 7.9	395.2–426.5	461.8 ± 26.5	406.9–516.8	0.117

"TCA−" (non-carriers of "TCA" haplotype): $n = 147$; "TCA+" (carriers of "TCA" haplotype): $n = 73$
"CGC−" (non-carriers of "CGC" haplotype): $n = 195$; "CGC+" (carriers of "CGC" haplotype): $n = 25$
Data are expressed as mean values ± standard error of the mean
CI Confidence interval, *HbF* Fetal hemoglobin, *SpO_2* peripheral oxygen saturation, *WBC* white blood cell count
[a]Statistically significant *p* values are highlighted in bold

Table 2. Incidence rates per 100 person-year and incidence rate ratios of clinical outcomes according to haplotype groups of *BCL11A* gene and HMIP-2 intergenic region in 220 children with sickle cell anemia

Clinical outcomes	*BCL11A* haplotypes				HMIP-2 haplotypes			
	"TCA−"	"TCA+"		*p* value[a]	"CGC−"	"CGC+"		*p* value[a]
	Incidence rate per 100 person-year (95% CI)		IRR ("TCA−/TCA+")		Incidence rate per 100 person-year (95% CI)		IRR ("CGC−/CGC+")	
Transfusion	47.5 (43.9–51.2)	31.2 (27.3–35.5)	1.52 (1.31–1.77)	**<0.001**	43 (40.10–46.0)	33.3 (26.7–41.2)	1.30 (1.03–1.61)	**0.021**
ACS	27.6 (24.9–30.5)	22.9 (19.6–26.7)	1.2 (1.0–1.44)	**0.043**	27.3 (25.0–29.8)	16.3 (11.7–22.0)	1.70 (1.22–2.30)	**0.001**
Pain	36.6 (33.5–39.9)	39.9 (35.5–44.8)	0.92 (0.8–1.06)	0.235	38.9 (36.1–41.8)	29.5 (23.2–36.9)	1.32 (1.04–1.67)	**0.018**
Infection	54.1 (50.3–58.1)	43.3 (38.7–48.3)	1.25 (1.10–1.42)	**0.001**	52.0 (48.8–55.3)	38.8 (31.5–47.1)	1.34 (1.10–1.65)	**0.003**

"TCA−" (non-carriers of "TCA" haplotype): $n = 147$; "TCA+" (carriers of "TCA" haplotype): $n = 73$
"CGC−" (non-carriers of "CGC" haplotype): $n = 195$; "CGC+" (carriers of "CGC" haplotype): $n = 25$
ACS Acute chest syndrome, *CI* Confidence interval, *IRR* Incidence Rate Ratio
[a]Statistically significant *p* values are highlighted in bold

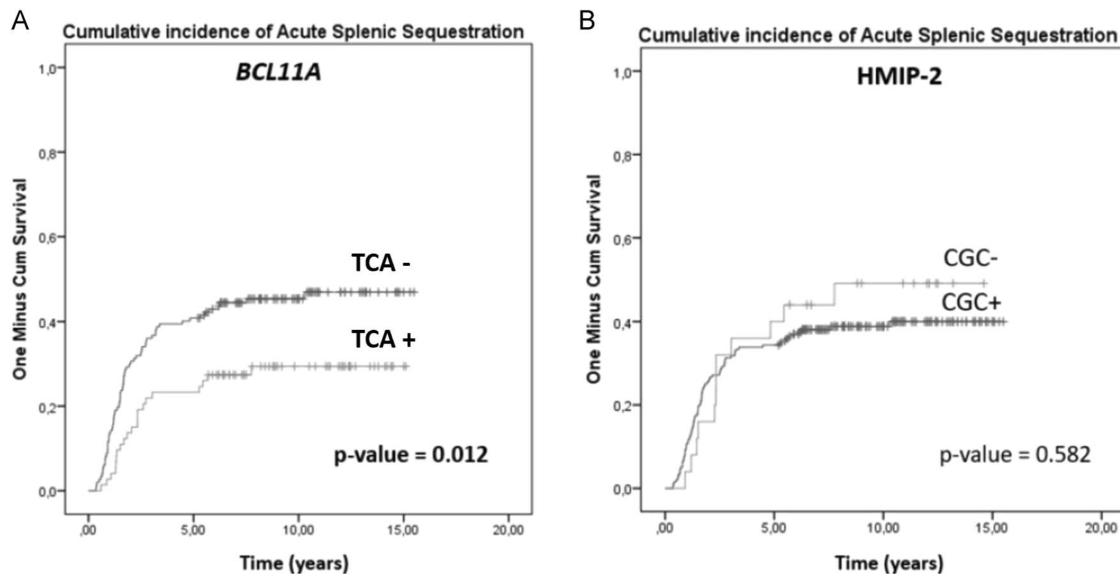

Fig. 2 Cumulative incidence of acute splenic sequestration (ASS) according to haplotype groups of BCL11A gene and HMIP-2 intergenic region. **A** The cumulative incidence of ASS was higher ($P = 0.012$) in children who were non-carriers of "TCA" haplotype (light grey; 46.9%, SE 4.3%) than in those carrying the "TCA" haplotype (dark grey; 29.4%, SE 5.5%). **B** There was no difference in the cumulative incidence of ASS between haplotype groups of HMIP-2 ($P = 0.58$)

in Supplementary Tables S1 and S2. We compared the average of HbF concentration between the minor and reference haplotypes for both *BCL11A* and HMIP-2 (Fig. 1). The total number of haplotypes was calculated as twice the number of homozygous subjects plus the number of heterozygotes subjects for each haplotype. The minor haplotypes for both the *BCL11A* gene (16.61 ± 7.56; $n = 73$ haplotypes) and the HMIP-2 intergenic region (17.94 ± 7.91; $n = 25$ haplotypes) showed increased HbF concentration when compared to the respective reference haplotypes (*BCL11A*: 13.28 ± 6.68; $n = 208$ haplotypes; $P = 0.0006$, Fig. 1A) and (HMIP-2: 13.45 ± 6.88; $n = 216$ haplotypes; $P = 0.003$; Fig. 1B).

We further compared the average of the relative HbF concentration between carriers of one or two copies of the minor *BCL11A* haplotype (referred as "TCA+") with those non-carriers ("TCA−"; Table 1). Children carrying "TCA+" showed increased HbF concentration when compared to those "TCA−" (12.0% vs. 16.6%; $P < 0.001$). Similarly, we compared the average of relative HbF concentration between carriers of one or two copies of the minor HMIP-2 haplotype (refereed as "CGC+") with those non-carriers ("CGC−"; Table 1). Children carrying "CGC+" showed increased HbF concentration when compared to "CGC−" (13.0% vs. 17.9 %; $P = 0.001$).

The average of hematological parameters in the haplotype groups of *BCL11A* gene and HMIP-2 intergenic region are shown in Table 1. Children with SCA carrying one or two copies of the minor haplotype of *BCL11A* gene ("TCA+") showed higher total Hb concentration (8.3 g/dl vs. 7.6 g/dL; $P < 0.001$) and lower relative reticulocyte count (13.9% vs. 15.3%; $P = 0.01$) as compared to subjects not carrying this minor haplotype ("TCA−"). Moreover, children with SCA carrying one or two copies of the minor haplotypes of HMIP-2 intergenic region ("CGC+") showed higher total Hb concentration when compared to subjects not carrying this minor haplotype ("CGC−") (8.3 g/dl *vs.* 7.7 g/dL; $P < 0.005$). There was no difference in the WBC, platelet count and SpO2 between haplotype groups of *BCL11A* gene or HMIP-2 intergenic region.

Table 2 shows the comparison of the incidence of clinical outcomes between haplotype groups of *BCL11A* gene and HMIP-2 intergenic region. The red cell transfusion incidence per 100 pt/yrs was higher in subjects not carrying the minor haplotype of *BCL11A* gene ("TCA−") than in those subjects carrying one or two copies of the minor haplotype ("TCA+") [IRR = 1.52 (1.31–1.77); $P < 0.001$)]. Moreover, the incidence of ACS [IRR = 1.20 (1.00–1.44); $P < 0.043$)] and infection [IRR = 1.25 (1.10–1.42); $P < 0.001$)] were higher in children with SCA carrying the haplotype "TCA−" than in those subjects carrying the haplotype "TCA+". Finally, children with SCA carrying the haplotype "TCA−" showed higher cumulative incidence of ASS than those subjects carrying the haplotype "TCA+" ($P = 0.012$; Fig. 2A).

The risk of transfusion [IRR = 1.30 (1.03–1.61); $P < 0.021$)], ACS [IRR = 1.70 (1.22–2.30); $P = 0.001$)], pain crisis [IRR = 1.32 (1.04–1.67); $P = 0.018$)] and infection [IRR = 1.34 (1.10–1.65); $P < 0.003$)] were higher in children with SCA who were non-carriers of the minor haplotype of HMIP-2 intergenic region ("CGC−") than in those subjects carrying one or two copies of minor haplotypes ("CGC+"). There was no difference in cumulative incidence of ASS when compared the haplotype groups of HMIP-2 intergenic region ($P = 0.58$; Fig. 2B).

Children with SCA who were non-carriers of minor haplotypes for both *BCL11A* gene and HMIP-2 intergenic region showed the lowest mean (± SEM) value of HbF level ("TCA−/CGC−"; 11.99 ± 0.51; Fig. 3). Subjects carrying only the minor haplotype of *BCL11A* gene showed significantly higher mean HbF value ("TCA+/CGC−"; 15.29 ± 0.95; $P = 0.002$; Fig. 3) than non-carriers of minor haplotypes for *BCL11A* and HMIP-2. However, regarding the HMIP-2 intergenic region, there was no significant difference in HbF mean concentration between those children with SCA carrying only the minor haplotype of HMIP-2 ("TCA−/CGC+" = 12.27 ± 1.98) and non-carriers of minor haplotypes for both *BCL11A* and HMIP-2 ($P = 0.77$). Interestingly, children with SCA carrying both minor haplotypes of *BCL11A* gene and HMIP-2 intergenic region showed the highest mean HbF concentration ("TCA+/CGC+"; 21.73 ± 1.71), which was significantly higher than all other haplotype groups taken together ($P < 0.01$; Fig. 3).

DISCUSSION

We reported here the main novel findings. First, HbF-boosting haplotypes formed by minor alleles of functional SNPs of *BCL11A* gene and HMIP-2 intergenic region were associated with less severe hematological phenotypes and lower incidence of clinical complications in children with SCA. Second, these HbF-boosting

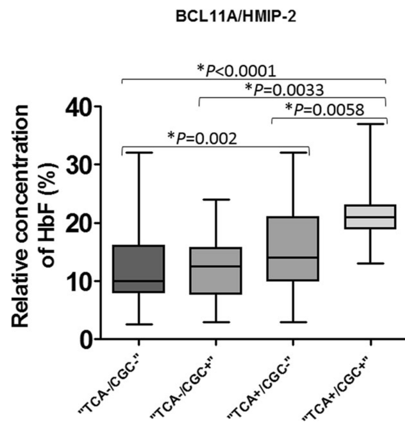

Fig. 3 Comparative boxplot of HbF concentration between combinations of the haplotype groups of BCL11A and HMIP-2. A The mean HbF concentration (±SEM) among non-carriers of minor haplotypes ("TCA-CGC−") was 11.99 ± 0.51. The mean HbF concentration among those carrying only minor haplotype of BCL11A ("TCA+/CGC−") was 15.29 ± 0.95 while among those carrying only minor haplotype of HMIP-2 ("TCA−/CGC+") was 12.27 ± 1.98. Patients carrying both minor haplotype of BCL11A and HMIP-2 ("TCA+/CGC+") showed the highest mean HbF concentration (21.73 ± 1.71). The asterisks indicate statistically significant differences between haplotype groups

haplotypes of both *BCL11A* and HMIP-2 may interact to regulate HbF levels in children with SCA.

HbF is the predominant fraction of hemoglobins during the fetal life due to its high affinity for oxygen. It consists of two alpha and two gamma chains, the latter encoded by γ-globin genes (*HBG1* and *HBG2*) in the β-globin gene cluster [27]. Noteworthy, the HbF concentration vary among patients with SCA and among erythrocytes of each individual [1]. HbF prevents HbS from polymerizing in situations of low oxygenation and, therefore, is considered an important modifier of the clinical course of patients with SCA [28]. With the partial inhibition of polymerization, the events of vaso-occlusion, as well as hemolytic anemia are reduced, which alleviates the complications of SCA [29].

BCL11A directly acts on the β-globin cluster, thereby inhibiting the expression of γ-globin genes (*HBG1* and *HBG2*) and reducing HbF production at the expense of adult hemoglobin. The intron 2 of *BCL11A* is a key locus in HbF regulation since it harbors functional elements linked to its repressive function [3]. Polymorphisms overlapping functional elements at intron 2 of *BCL11A* disrupt conserved sequences and affect the repression of γ-globin genes, thereby increasing HbF production [27, 30]. Similarly, the intergenic region located between *HBS1L* and *MYB* genes (HMIP-2) harbors functional elements linked to the regulation of *HBS1L* and *MYB* expression [26, 27]. MYB is a transcription factor acting as an upstream regulator in the HbF regulation pathway. Polymorphisms located at HMIP-2 are associated with the expression of both *MYB* and γ-globin genes [3]. Noteworthy, the functional annotation and the selection of SNPs for haplotype analyses in the present study capture a wide extension of both the intron 2 of *BCL11A* (Chr 2 60718043–67386470 (GRCh37) and the HMIP-2 intergenic region (Chr 6 135460326–135432552 (GRCh37) (Supplementary Fig. 1).

Vaso-occlusive episodes and chronic hemolysis are the main pathophysiological features that underlie the clinical manifestations in SCA, with consequences that result in a systemic disease that can affect any organ [31]. This study is the first to show that HbF-boosting haplotypes of both *BCL11A* gene and HMIP-2 intergenic region are related to less severity in children with SCA, with reduction of some vaso-occlusive and hemolytic complications, such as pain and ACS. Vaso-occlusion is a process by which normal tissue perfusion is precluded by sickle erythrocytes [32]. Obstruction might first occur in the small post-capillary venules by sickle cells and their adhesion to the vascular endothelium, with the subsequent accumulation of dense sickled erythrocytes, leukocytes, and platelets [1]. Sickle cell pain crises are critical vaso-occlusive complications, causing hospitalization in almost 95% of cases [33], harming the quality of patients' life.

Beyond the first year of life, acute episodes of pain are the main symptoms of SCA [34, 35]. Such episodes of pain most commonly occur in the extremities, chest, abdomen, and back and result from vaso-occlusive episodes with ischemia–reperfusion injury [1, 35]. Among patients with hemoglobinopathies, the number, severity and frequency of painful episodes are quite variable [36]. On average, subjects with HbSC and HbS-β^{+} thalassemia have half the number of painful episodes compared with patients with SCA. Painful episodes, which last from hours to many days, usually occur with little warning and a clear precipitating event is often not found. No useful laboratory test can tell if a painful episode is occurring and the patient history is the best clue [1]. Mutations in the *HBG* disrupting binding of major repressors of HbF (BCL11A and ZBTB7A) were highlighted as genetic predictors to vaso-occlusive complications, among the risk factors recently reviewed [30, 37]. Here, we did not find association between HbF-boosting haplotypes of *BCL11A* gene and the incidence of pain crisis. However, we found that subjects carrying the HbF-boosting haplotype of HMIP-2 intergenic region ("CGC") had low incidence of pain crisis.

It is clinically relevant to note that episodes of pain crisis precede ACS in 10–20% of cases [1]. ACS is a typical example of organ injury in sickle cell disease and a major cause of hospitalization and death among patients [1]. The risk of ACS was also significantly lower in subjects carrying HbF-boosting haplotypes of *BCL11A* gene ("TCA") or HMIP-2 intergenic region ("CGC").

High reticulocyte count is the most important predictor of acute cerebral ischemia and high-risk transcranial Doppler in a newborn cohort, as reported by our group of researchers [38]. In the present study, we showed that subjects carrying HbF-boosting haplotypes of *BCL11A* gene ("TCA") had lower reticulocyte count. In addition, subjects carrying the HbF-boosting haplotypes of *BCL11A* gene ("TCA") or HMIP-2 intergenic region ("CGC") had lower incidence of transfusion because the ongoing hemolytic process may have been reduced.

The primary causes of death before 10 years of age in patients with SCA include infections and ASS [31]. The loss of spleen function increases the susceptibility of children with SCA to bacterial infection, which is notably reduced by penicillin prophylaxis and immunization. Once again, we found that children with SCA carrying the HbF-boosting haplotypes of *BCL11A* gene ("TCA") or HMIP-2 intergenic region ("CGC") had lower incidence risk of infections. Moreover, we observed that children with SCA carrying the HbF-boosting haplotype of *BCL11A* gene ("TCA") had a lower cumulative incidence of ASS, which suggests that this haplotype might be a genetic predictor of ASS.

We have previously reported that HbF-boosting alleles of SNPs in the *BCL11A* gene and HMIP-2 intergenic region were associated with increased HbF levels and milder hematological parameters in children with SCA [8]. In the present study, while the haplotype analyses showed significant associations with HbF levels (Table 1), they did not improve the strength of associations with HbF levels found when compared to those previously reported in our analysis of single SNPs [8]. Noteworthy, we show here that haplotype analyses we were able to capture associations with reduced risk of clinical complications in children with SCA that were not found in our analysis of single SNPs [8]. Our findings are in agreement with previous studies showing that the use of

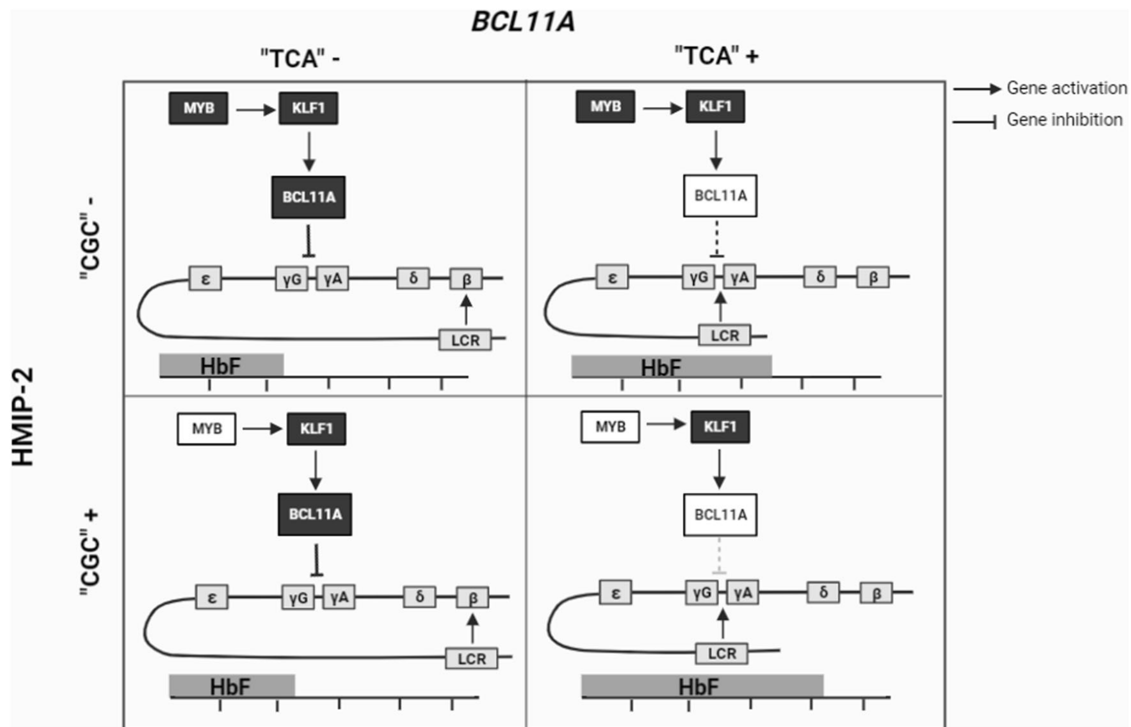

Fig. 4 Proposed scheme of HbF regulation by interaction between *BCL11A* gene and HMIP-2 intergenic region based on the combination of alleles of the HbF-boosting haplotypes. BCL11A is a key repressor of γ-globin genes, decreasing HbF production in the switching to adult hemoglobin. KLF1 activates *BCL11A* gene and its expression is in turn activated by MYB. Inheritance of minor alleles overlapping functional elements in the intron 2 of *BCL11A* can disrupt conserved sequence and decreases the repression of γ-globin, increasing HbF production. Minor alleles of HMIP-2 can decrease *MYB* gene expression. Inheritance of minor alleles of HMIP-2 by itself seems not to be able to increase HbF production. Co-inheritance of minor alleles of *BCL11A* and HMIP-2 seems to act synergistically increasing even more HbF production

haplotypes may provide higher prediction power in genetic association studies [13–15].

In this study, we combined haplotypes formed by functional SNPs from intron 2 of *BCL11A* gene and HMIP-2 intergenic region that are associated with increased HbF concentration. We found that children with SCA carrying only the HbF-boosting haplotype of HMIP-2 intergenic region did not show HbF levels higher than those subjects not carrying none of these HbF-boosting haplotypes. However, children carrying both HbF-boosting haplotype of *BCL11A* gene and HMIP-2 intergenic region showed higher HbF levels than those carrying only the HbF-boosting of *BCL11A* gene. These findings suggest that wild-type alleles of functional SNPs of *BCL11A* gene can display epistatic effect on HbF-boosting alleles of HMIP-2 intergenic region, while in the co-inheritance minor alleles of HMIP-2 intergenic region can exhibit synergism with minor alleles of *BCL11A* gene in decreasing the repression of γ-globin genes, as proposed in the Fig. 4. It makes biological sense, since polymorphisms of HMIP-2 intergenic region seem to act in the HbF regulation pathway by affecting *MYB* gene expression. MYB is upstream in the HbF regulation, while *BCL11A* acts directly on β-globin cluster [3]. Nevertheless, this hypothesis needs to be confirmed by further experimental assays.

Interestingly, an allelic score combining alleles of a *BCL11A* (rs11886868) with alleles of HMIP intergenic region (rs9399137 or 9389369) was previously proposed [39]. The results showed that HbF levels in patients is more elevated the higher is the number of HbF-boosting alleles (ranging from zero to 4 alleles), while the rate of clinical complications of the disease is lower (priapism, elevated tricuspid regurgitation velocity, leg ulcer, osteonecrosis and stroke). These findings suggest that the co-inheritance of minor alleles of *BCL11A* gene and HMIP-2 intergenic region may have a synergistic effect on HbF concentration, thereby extending the clinical benefit to the patient.

Additionally, other factors may influence the effect of these alleles, including alpha-thalassemia deletion status. Another HbF-boosting allele combination score calculated ranging from zero to six minor alleles for the SNPs rs1427407 (*BCL11A* gene), rs28384513 (HMIP-2 intergenic region) and *Xmn*I, rs7482144 (*HBB* gene) was tested in a study of Senegalese adults and children with SCA [40]. A positive impact of the score was observed only in patients without alpha-thalassemia deletion, who showed reduced rate of vaso-occlusive crises, lower leukocyte count and C-reactive protein level. Conversely, the combination of alpha thalassemia homozygotes (−3.7 kb) with at least 3 HbF-boosting alleles had a higher rate of vaso-occlusive crises. Moreover, age at first vaso-occlusive crisis was delayed in patients with one or more alpha-thalassemia deletions and at least two HbF-boosting alleles.

It is important to note the limitations of the present study. The haplotype analysis was partially impaired by the small number of homozygote subjects for the HbF-boosting haplotypes ($n = 7$ for the "TCA" haplotype of *BCL11A* gene and $n = 3$ for the "CGC" haplotype of HMIP-2 intergenic region). Therefore, we had to merge subjects who were homozygotes and heterozygotes for the minor haplotypes. Although there may be differences in clinical phenotypes between children who are homozygotes and heterozygotes to the HbF-boosting haplotypes of *BCL11A* gene and the HMIP-2 intergenic region, we were not able to evaluate them separately due to the relatively small sample size in our study. In addition, it should be clear that other SNPs in the intron 2 of *BCL11A* gene and the HMIP-2 intergenic region may account for the variance on HbF levels, which were not examined in the present study. For example, the SNP rs9494142 of HMIP-2 is a widely replicated HbF modifier variant [41, 42].

In conclusion, we found that HbF-boosting haplotypes formed by the minor alleles for the functional SNPs of *BCL11A* gene and

HMIP-2 intergenic region were associated with less severe hematological parameters and reduced rate of clinical complications in pediatric patients with SCA, and these HbF-boosting haplotypes of *BCL11A* gene and HMIP-2 intergenic region may interact to regulate HbF levels in children with SCA. Our novel findings suggest that the identified HbF-boosting haplotypes of *BCL11A* gene and HMIP-2 intergenic region may constitute a useful tool to predict clinical severity of SCA.

REFERENCES

1. Steinberg MH. Sickle cell anemia, the first molecular disease: overview of molecular etiology. Pathophysiol, Therapeutic Approaches Sci World J. 2008;8:1295–324.
2. Habara A, Steinberg MH. Minireview: genetic basis of heterogeneity and severity in sickle cell disease. Exp Biol Med. 2016;241:689–96.
3. Bauer DE, Orkin SH. Hemoglobin switching's surprise: the versatile transcription factor BCL11A is a master repressor of fetal hemoglobin. Curr Opin Genet Dev. 2015;33:62–70.
4. Lettre G, Sankaran VG, Bezerra MAC, Araújo AS, Uda M, Sanna S, et al. DNA polymorphisms at the BCL11A, HBS1L-MYB, and beta-globin loci associate with fetal hemoglobin levels and pain crises in sickle cell disease. Proc Natl Acad Sci USA. 2008;105:11869–74.
5. Menzel S, Garner C, Gut I, Matsuda F, Yamaguchi M, Heath S, et al. A QTL influencing F cell production maps to a gene encoding a zinc-finger protein on chromosome 2p15. Nat Genet. 2007;39:1197–99.
6. Thein SL, Menzel S, Peng X, Best S, Jiang J, Close J, et al. Intergenic variants of HBS1L-MYB are responsible for a major quantitative trait locus on chromosome 6q23 influencing fetal hemoglobin levels in adults. Proc Natl Acad Sci. 2007;104:11346–51.
7. Bhatnagar P, Purvis S, Barron-Casella E, DeBaun MR, Casella JF, Arking DE, et al. Genome-wide association study identifies genetic variants influencing F-cell levels in sickle-cell patients. J Hum Genet. 2011;56:316–23.
8. Sales RR, Belisario AR, Faria G, Mendes F, Luizon MR, Viana MB. Functional polymorphisms of BCL11A and HBS1L-MYB genes affect both fetal hemoglobin level and clinical outcomes in a cohort of children with sickle cell anemia. Ann Hematol. 2020;99:1453–63.
9. Sedgewick AE, Timofeev N, Sebastiani P, So JCC, Ma ESK, Chan LC, et al. BCL11A is a major HbF quantitative trait locus in three different populations with beta-hemoglobinopathies. Blood Cells, Molecules Dis. 2008;41:255–58.
10. Uda M, Galanello R, Sanna S, Lettre G, Sankaran VG, Chen W, et al. Genome-wide association study shows BCL11A associated with persistent fetal hemoglobin and amelioration of the phenotype of beta-thalassemia. Proc Natl Acad Sci USA. 2008;105:1620–25.
11. Sales RR, Nogueira BL, Tosatti JAG, Gomes KB, Luizon MR. Do genetic polymorphisms affect fetal hemoglobin (HbF) levels in patients with sickle cell anemia treated with hydroxyurea? a systematic review and pathway analysis. Front in Pharmacol. 2022;12:779497.
12. Crawford DC, Nickerson DA. Definition and clinical importance of haplotypes. Annu Rev Med. 2005;56:303–20.
13. Clark AG. The role of haplotypes in candidate gene studies. Genet Epidemiol. 2004;27:321–33.
14. Morris RW, Kaplan NL. On the advantage of haplotype analysis in the presence of multiple disease susceptibility alleles. Genet Epidemiol. 2002;23:221–33.
15. Schaid DJ. Evaluating associations of haplotypes with traits. Genet Epidemiol. 2004;27:348–64.
16. Okumura JV, Silva DGH, Torres LS, Belini-Junior E, Venancio LPR, Carrocini GCS, et al. Atypical beta-S haplotypes: classification and genetic modulation in patients with sickle cell anemia. J Hum Genet. 2019;64:239–48.
17. Ballas SK, Lieff S, Benjamin LJ, Dampier CD, Heeney MM, Hoppe C, et al. Definitions of the phenotypic manifestations of sickle cell disease. Am J Hematol. 2010;85:6–13.
18. Schaub MA, Boyle AP, Kundaje A, Batzoglou S, Snyder M. Linking disease associations with regulatory information in the human genome. Genome Res. 2012;22:1748–59.
19. Consortium GT. The Genotype-Tissue Expression (GTEx) project. Nat Genet. 2013;45:580–5.
20. Boyle AP, Hong EL, Hariharan M, Cheng Y, Schaub MA, Kasowski M, et al. Annotation of functional variation in personal genomes using RegulomeDB. Genome Res. 2012;22:1790–97.
21. Luizon MR, Belo VA, Palei AC, Amaral LM, Lacchini R, Sandrim VC, et al. Effects of NAMPT polymorphisms and haplotypes on circulating visfatin/NAMPT levels in hypertensive disorders of pregnancy. Hypertension Research: Official. J Jpn Soc Hypertension. 2015;38:361–66.
22. Metzger IF, Luizon MR, Lacchini R, Ishizawa MH, Tanus-Santos JE. Effects of endothelial nitric oxide synthase tagSNPs haplotypes on nitrite levels in black subjects. Nitric Oxide. 2013;28:33–8.
23. Schaid DJ, Rowland CM, Tines DE, Jacobson RM, Poland GA. Score tests for association between traits and haplotypes when linkage phase is ambiguous. Am J Hum Genet. 2002;70:425–34.
24. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinforma (Oxf, Engl). 2005;21:263–65.
25. Bauer DE, Kamran SC, Lessard S, Xu J, Fujiwara Y, Lin C, et al. An erythroid enhancer of BCL11A subject to genetic variation determines fetal hemoglobin level. Science 2013;342:253–57.
26. Farrell JJ, Sherva RM, Chen Z-Y, Luo H-Y, Chu BF, Ha SY, et al. A 3-bp deletion in the HBS1L-MYB intergenic region on chromosome 6q23 is associated with HbF expression. Blood 2011;117:4935–45.
27. Menzel S, Thein SL. Genetic modifiers of fetal haemoglobin in sickle cell disease. Mol Diagn Ther. 2019;23:235–44.
28. Lettre G, Bauer DE. Fetal haemoglobin in sickle-cell disease: from genetic epidemiology to new therapeutic strategies. Lancet. 2016;387:2554–64.
29. Steinberg MH. Predicting clinical severity in sickle cell anaemia. Br J Haematol. 2005;129:465–81.
30. Martyn GE, Wienert B, Yang L, Shah M, Norton LJ, Burdach J, et al. Natural regulatory mutations elevate the fetal globin gene via disruption of BCL11A or ZBTB7A binding. Nat Genet. 2018;50:498–503.
31. Fernandes APPC, Januário JN, Cangussu CB, Macedo DLD, Viana MB. Mortality of children with sickle cell disease: a population study. J Pediatr. 2010;86:279–84.
32. Kaul DK, Fabry ME, Nagel RL. The pathophysiology of vascular obstruction in the sickle syndromes. Blood Rev. 1996;10:29–44.
33. Ballas SK, Lusardi M. Hospital readmission for adult acute sickle cell painful episodes: frequency, etiology, and prognostic significance. Am J Hematol. 2005;79:17–25.
34. Bonds DR. Three decades of innovation in the management of sickle cell disease: the road to understanding the sickle cell disease clinical phenotype. Blood Rev. 2005;19:99–110.
35. Williams TN, Thein SL. Sickle cell anemia and its phenotypes. Annu Rev Genomics Hum Genet. 2018;19:113–47.
36. Platt OS, Thorington BD, Brambilla DJ, Milner PF, Rosse WF, Vichinsky E, et al. Pain in sickle cell disease. N Engl J Med. 1991;325:11–16.
37. Darbari DS, Sheehan VA, Ballas SK. The vaso-occlusive pain crisis in sickle cell disease: definition, pathophysiology, and management. Eur J Haematol. 2020;105:237–46.
38. Belisario AR, Sales RR, Toledo NE, Muniz MB, Velloso-Rodrigues C, Silva CM, et al. Reticulocyte count is the most important predictor of acute cerebral ischemia and high-risk transcranial Doppler in a newborn cohort of 395 children with sickle cell anemia. Ann Hematol. 2016;95:1869–80.
39. Leonardo FC, Brugnerotto AF, Domingos IF, Fertrin KY, de Albuquerque DM, Bezerra MA, et al. Reduced rate of sickle-related complications in Brazilian patients carrying HbF-promoting alleles at the BCL11A and HMIP-2 loci. Br J Haematol. 2016;173:456–60.
40. Gueye Tall F, Martin C, Ndour EHM, Renoux C, Ly ID, Connes P, et al. Combined and differential effects of alpha-thalassemia and HbF-quantitative trait loci in Senegalese hydroxyurea-free children with sickle cell anemia. Pediatr Blood Cancer. 2019;66:e27934.
41. Gardner K, Fulford T, Silver N, Rooks H, Angelis N, Allman M, et al. g(HbF): a genetic model of fetal hemoglobin in sickle cell disease. Blood Adv. 2018;2:235–39.
42. Menzel S, Rooks H, Zelenika D, Mtatiro SN, Gnanakulasekaran A, Drasar E, et al. Global genetic architecture of an erythroid quantitative trait locus, HMIP-2. Ann Hum Genet. 2014;78:434–51.

AUTHOR CONTRIBUTIONS

RRS, BLN, ARB, GF, FM, MBV, and MRL made substantial contributions to the conception or design of the work, acquired data, and all authors analyzed and interpreted the results. RRS, BLN, ARB, MBV, and MRL drafted the manuscript. and all authors revised it for important intellectual content. All authors read and approved the final version of the manuscript for submission.

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COMPETING INTERESTS

The authors declared no competing interests.

ADDITIONAL INFORMATION

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4.3 Capítulo 3: Revisão sistemática da literatura

Revisão sistemática da literatura foi conduzida para identificar polimorfismos genéticos associados à alteração nos níveis de HbF em pacientes com AF tratados com HU.

A revisão sistemática foi conduzida de acordo com o *Cochrane Handbook for Systematic Reviews of Interventions.* A estratégia de busca foi definida com base na pergunta PECO: Participantes (P) = Pacientes com AF (genótipo HbSS) tratados com HU; Exposição (E) = Alelos menos frequentes dos polimorfismos genéticos candidatos; Controle (C) = Alelos mais frequentes dos polimorfismos genéticos candidatos Desfechos (O) = Nível de HbF. As buscas foram realizadas nas bases de dados EMBASE, Medline/PubMed, Cochrane, CINAHL, Scopus, e Web of Science e literatura cinzenta. A qualidade dos estudos foi avaliada seguindo a abordagem do Instituto *Joanna Briggs* para a síntese de evidências.

As etapas de seleção e extração de dados dos estudos primários foram realizadas de forma independente pela doutoranda Ms.C Rahyssa Rodrigues Sales e pela então mestranda deste Programa de Pós-graduação em Genética, Ms.C Bárbara Lisboa Nogueira. As divergências foram resolvidas pela Dr. Jéssica Abdo Gonçalves Tosatti, então doutoranda do Programa de Pós-Graduação em Análises Clínicas e Toxicológicas da Faculdade de Farmácia da UFMG.

A partir do conjunto de genes encontrados na revisão sistemática que possuiam polimorfismos associados à resposta da HbF em pacientes com AF tratados com HU, foi realizada análise de enriquecimento de vias biológicas utilizando o software online *Enrich* (XIE *et al.*, 2021) e a base de dados do *Reactome Pathway Knowledgebase* (JASSAL *et al.*, 2020).

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SYSTEMATIC REVIEW
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Do Genetic Polymorphisms Affect Fetal Hemoglobin (HbF) Levels in Patients With Sickle Cell Anemia Treated With Hydroxyurea? A Systematic Review and Pathway Analysis

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Hydroxyurea has long been used for the treatment of sickle cell anemia (SCA), and its clinical effectiveness is related to the induction of fetal hemoglobin (HbF), a major modifier of SCA phenotypes. However, there is substantial variability in response to hydroxyurea among patients with SCA. While some patients show an increase in HbF levels and an ameliorated clinical condition under low doses of hydroxyurea, other patients present a poor effect or even develop toxicity. However, the effects of genetic polymorphisms on increasing HbF levels in response to hydroxyurea in patients with SCA (Hb SS) have been less explored. Therefore, we performed a systematic review to assess whether single-nucleotide polymorphisms (SNPs) affect HbF levels in patients with SCA treated with hydroxyurea. Moreover, we performed pathway analysis using the set of genes with SNPs found to be associated with changes in HbF levels in response to hydroxyurea among the included studies. The systematic literature search was conducted on Medline/PubMed, EMBASE, Cochrane Central Register of Controlled Trials, Cumulative Index to Nursing and Allied Health Literature (CINAHL), Scopus, and Web of Science. Seven cohort studies were included following our inclusion and exclusion criteria. From the 728 genetic polymorphisms examined in the included studies, 50 different SNPs of 17 genes were found to be associated with HbF changes in patients with SCA treated with hydroxyurea, which are known to affect baseline HbF but are not restricted to them. Enrichment analysis of this gene set revealed reactome pathways with the lowest adjusted *p*-values and highest combined scores related to VEGF ligand–receptor interactions (R-HSA-194313; R-HSA-195399) and the urea cycle (R-HSA-70635). Pharmacogenetic studies of response to hydroxyurea therapy in patients with SCA are still scarce and markedly heterogeneous regarding candidate genes and SNPs examined for association with HbF changes and outcomes, suggesting that further studies are needed. The reviewed findings highlighted that similar to baseline HbF, changes in HbF levels upon hydroxyurea therapy are likely to

be regulated by multiple loci. There is evidence that SNPs in intron 2 of *BCL11A* affect HbF changes in response to hydroxyurea therapy, a potential application that might improve the clinical management of SCA.

Systematic Review Registration: (https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=208790).

Keywords: *BCL11A* gene, fetal hemoglobin (HbF), genetic polymorphisms, hydroxyurea (HU) therapy, pathway analysis, pharmacogenetics, sickle cell anemia (SCA), systematic review

INTRODUCTION

Sickle cell anemia (SCA) is a global health problem, and approximately 300,000 infants are born with SCA every year (Azar and Wong, 2017). SCA is defined as a monogenic hemoglobin disorder caused by homozygosis for A-to-T transversion at codon 7 (c.20A > T, p.E7V) in the hemoglobin subunit beta (*HBB*) gene (den Dunnen and Antonarakis, 2000; Steinberg, 2008). The pathophysiology of SCA is directly related to polymerization of deoxygenated hemoglobin (HbS; α2βS2), leading to a cascade of pathologic events including erythrocyte sickling, vaso-occlusion, and hemolysis (Kato et al., 2018). It is important to note that higher levels of fetal hemoglobin (HbF; α2γ2) ameliorate clinical outcomes and hematological parameters of SCA, since it reduces HbS concentration and inhibit copolymerization between hemoglobin tetramers (Kato et al., 2018). Notably, higher persistent HbF concentration is often observed in patients with SCA than in subjects without SCA (Lettre and Bauer, 2016).

Hydroxyurea (HU) was approved by the U.S. Food and Drug Administration for the treatment of adults with severe SCA in 1998, and it has been associated with improved survival for both adults and children with SCA, as reviewed elsewhere (McGann and Ware, 2015). The clinical effectiveness of HU is related to the induction of the production of HbF, but it is not restricted to it. HU selectively kills cells in the bone marrow and increases the number of erythroblasts producing HbF, which inhibits the intracellular polymerization of HbS and prevents the sickling process in erythrocytes, thereby decreasing the number of sickled cells (McGann and Ware, 2015). Erythrocytes with high HbF have longer survival, thereby attenuating hemolysis (Steinberg, 1999). Furthermore, HU increases the hemoglobin levels; reduces neutrophils, monocytes, and reticulocytes; and alters the expression of adhesion molecules in the endothelium and the generation of nitric oxide. These hematological changes decrease the risk of vaso-occlusion in patients with SCA (Steinberg, 1999; McGann and Ware, 2015; Rigano et al., 2018).

Because HU has dose-related effects, the laboratory and clinical benefits of HU were shown to be optimized when dimensioned for the maximum tolerated dose (MTD). Almost all patients with SCA show a significant increase in HbF concentration at the MTD (McGann and Ware, 2015). The American Society of Hematology guideline panel suggests HU therapy with at least 20 mg/kg/day at a fixed dose or the MTD (DeBaun et al., 2020). However, there is substantial interpatient variability both in the MTD itself and in the percentage of HbF (% HbF) achieved (Ware et al., 2011; McGann and Ware, 2015). For example, the % HbF achieved with the MTD of HU reaches 10–15% in some patients, but it can reach 40% in other patients (Ware et al., 2011). Moreover, while some patients tolerate high HU doses, such as 30–35 mg/kg/day, others develop severe myelosuppression even at lower doses (Lettre et al., 2008). These findings suggest that important individual differences on pharmacokinetics and pharmacodynamics, and genetic factors contribute to the phenotypic variability in both the dosing and response to HU therapy (McGann and Ware, 2015). However, the effect of genetic polymorphisms on increasing HbF levels in response to HU therapy in patients with SCA has been less explored.

Therefore, the aim of the present study was to perform a systematic review to assess whether genetic polymorphisms affect HbF levels in patients with SCA treated with HU. In addition, we performed pathway analysis using the set of genes which had single-nucleotide polymorphisms (SNPs) that were found to be associated with changes in HbF levels in response to HU therapy among the studies included in the systematic review.

MATERIALS AND METHODS

This study was conducted according to the Cochrane Handbook for Systematic Reviews of Interventions (Higgins et al., 2020), and the results were reported in accordance with the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) checklist (Stroup et al., 2000). The protocol of the current study was registered on the International Prospective Register of Systematic Reviews [PROSPERO (CRD42020208790); https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=208790].

Search Strategy

The search strategy was defined based on the PECO question: Participants (P) = Sickle cell anemia patients (HbSS); Exposition (E) = Minor alleles; Control (C) = Major alleles of genetic polymorphisms and; Outcomes (O) = Fetal hemoglobin levels. A literature review was conducted by searching the electronic databases EMBASE, Medline/PubMed (Medical Literature Analysis and Retrieve System Online), Cochrane Central Register of Controlled Trials (CENTRAL), Cumulative Index to Nursing and Allied Health Literature (CINAHL), Scopus, and Web of Science (WoS) to identify studies published until July 2021. The initial search included the Medical Subject Headings (MeSH) entry terms: “Anemia, Sickle Cell”; and “Hydroxyurea”; and “Polymorphism, Genetic” or “Amplified Fragment Length Polymorphism Analysis” or “Polymorphism,

Single Nucleotide," or "Polymorphism, Restriction Fragment Length"; and "Fetal Hemoglobin," which were then included for a high-sensitivity search strategy in Medline/PubMed (**Supplementary Table S1**).

The same terms were used to search for gray literature and conference proceedings. The reference lists of included articles were also checked to identify additional relevant citations. All potentially eligible studies were considered for review, regardless of the language and publication date.

Inclusion and Exclusion Criteria

The inclusion criteria were restricted to studies that described the pharmacogenetics of response to HU therapy in patients with SCA measured by HbF levels (primary outcome). We included only cohort studies that examined patients with the SS genotype, with a minimum age of three y at the time of HU initiation and with a minimum period of six months of HU therapy.

We excluded studies that did not differentiate patients with SCA from patients with another sickle cell disease (SCD), studies that focused on haplotypes and not on individualized SNPs, and studies that did not examine whether SNPs affect HbF levels in patients with SCA treated with HU. Review articles, conference proceedings, case reports, and commentary studies were also excluded.

Study Selection and Data Extraction

Initially, the studies retrieved from the databases were input into a single electronic library, and duplicates were excluded using EndNote® software. Two reviewers (R.R.S. and B.L.N.) independently analyzed the titles and abstracts of the articles retrieved, reviewed the full text of the published articles, and used a standard data extraction protocol. Any disagreements between the two reviewers were resolved by a third reviewer (J.A.G.T.).

The extracted data from selected studies included study design, country, sample size, follow-up duration, median/mean age of participants, gender of patients, eligibility criteria, median/mean of HU dose, changes in HbF levels after HU therapy, genes, and polymorphisms associated with the primary outcome. The associated genes found in the included studies were used for pathway analysis.

Assessment of Bias Across Studies

The quality assessment of included studies was carried out independently by two reviewers (R.R.S. and B.L.N.), following the approach of the Joanna Briggs Institute for the synthesis of evidence (Moola et al., 2020), and any disparity between the two reviewers was resolved by a third reviewer (J.A.G.T.). The approach indicates the application of critical assessment tools used in systematic reviews, in which the checklist for cohort studies is applied (Moola et al., 2020). The instrument is structured around eleven questions, in which the selected studies were evaluated: 1) the two groups were similar and recruited from the same population; 2) how they were similarly measured to designate exposed and unexposed groups as people; 3) exposure was measured in a valid and reliable manner; 4) confounding factors have been identified; 5) the instrument was created to deal with confounding factors; 6) the groups were free of the outcome at the beginning of the study; 7) the results were measured in a valid and reliable way; 8) the follow-up time was reported and long enough for the results to occur; 9) the follow-up was complete and, if not, whether the reasons for the loss of follow-up were obtained and explored; (10) the instrument was used to deal with incomplete follow-up; and 11) statistical statistics was applied. The answer options for signaling questions are 1) yes, 2) no, 3) unclear, and 4) not applicable (Moola et al., 2020).

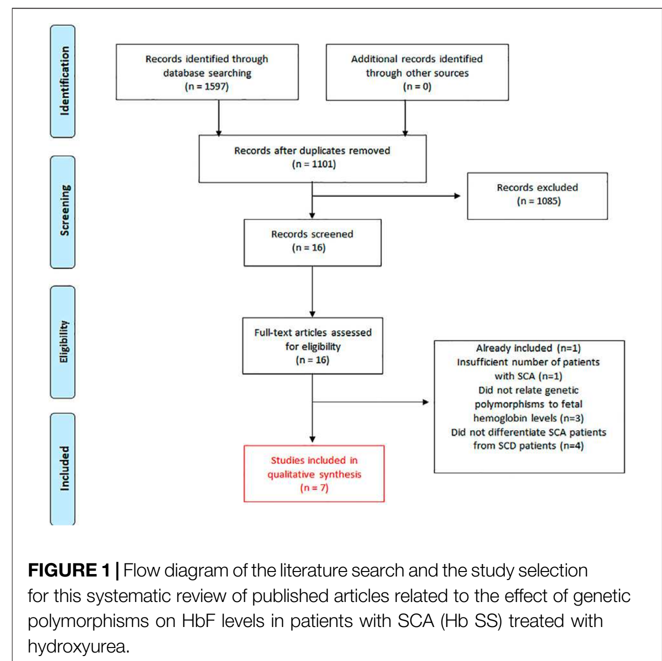

FIGURE 1 | Flow diagram of the literature search and the study selection for this systematic review of published articles related to the effect of genetic polymorphisms on HbF levels in patients with SCA (Hb SS) treated with hydroxyurea.

Gene Set Enrichment Analysis and Pathway Analysis

After the data extraction, we manually curated the genes which had SNPs that were found to be associated with changes in HbF levels in patients with SCA treated with HU among the seven studies included in the systematic review (listed in **Table 2**). Next, we interrogated this gene set for significant well-curated signaling pathways obtained from the Reactome Pathway Knowledgebase (Jassal et al., 2020). The pathways found were sorted both by the adjusted p-values ranking <0.05, which were calculated using a Benjamini–Hochberg method (Benjamini and Hochberg, 1995), and the combined scores according to the gene set enrichment analysis web server Enrichr (Kuleshov et al., 2016; Xie et al., 2021).

RESULTS

Study Selection

We identified 1,597 records in the initial search (**Figure 1**). After the exclusion of duplicates, 1,101 articles were selected for title and abstract analyses. Of these, 1,085 articles were subsequently excluded due to the following reasons (as stated before in the "exclusion criteria"): 1) studies that focused on haplotypes rather than individualized SNPs; 2) studies that did not differentiate patients with SCA from patients with another SCD; or 3) studies

TABLE 1 | Characteristics of the seven cohort studies included in the systematic review, which examined the effects of genetic polymorphisms on fetal hemoglobin (HbF) levels in patients with SCA treated with hydroxyurea (HU).

Author, data; country	Sample (n)	Average age (years)*	Gender (M/F)	Dose of HU (mean ± SD; mg/kg/day)	Time of follow-up on HU therapy (months)	HbF changes	HbF measurement	Number of genes (SNPs) studied	Multiple test correction
Friedrisch et al. (2016); Brazil	111	21 ± 14 (from 4 to 54)	38/62	23 ± 7.6	Minimum of 6	Δ MTD HbF (%)[a]	Capillary electrophoresis	3 (6)	Not applied
Ware et al. (2011); United States	88	9.6 ± 4.8	57/31	23.9 ± 5.1	Minimum of 6	Δ MTD HbF (%)[b]	HPLC	Not informed (331)	Applied
Aleluia et al. (2017); Brazil	42	15.2 ± 11.1	70/71	15 (47.6%) 20 (23.8) 25 (26.2%)**	Mean of 13.4 ± 9.7	Not informed	HPLC	3 (6)	Not applied
Green et al. (2013); United States	38	12.5 ± 4.9	57/60	25.3 ± 3.0	Minimum of 6	Δ HbF (%)[c]	HPLC	9 (20)	Applied
Kumkhaek et al. (2008); United States	32	Not informed	Not informed	Not informed	Minimum of 8	Δ HbF (% and g/dl)[b]	HPLC	1 (20)	Not applied
Sheehan et al. (2014); United States	Discovery cohort (171)	10.4 ± 4.5	Not informed	25.1 ± 4.5	Minimum of 6	Δ MTD HbF (%)[b]; Final HbF	HPLC	Whole exome	Unclear
	Validation cohort (130)	8.1 ± 4.0	Not informed	27.1 ± 4.3	Minimum of 6	Δ MTD HbF (%)[b]; Final HbF	HPLC	24 (25)	Unclear
Ma et al. (2007); United States	137	Not informed	Not informed	Not informed	Minimum of 21	Δ HbF (% and g/dl)[a]	Alkali denaturation	29 (320)	Not applied

Abbreviations: HbF, fetal hemoglobin; M, male; F, female. All selected studies were part of cohort studies.
**age at the time of hydroxyurea initiation.*
***dose (case percentage).*
[a](ΔHbF = MTD HbF—baseline HbF).
[b](ΔHbF = final HbF—baseline HbF).
[c](ΔHbF = maximum HbF—baseline HbF).

that did not focus on SNPs related to HbF levels in patients with SCA treated with HU. Subsequently, 16 full-text articles were thoroughly assessed for inclusion. Following review, nine articles were removed due to the following reasons (**Figure 1**): One cohort study had an insufficient number of patients with SCA (Sclafani et al., 2016). Three studies did not assess whether the SNPs affected HbF levels (Italia et al., 2010; Zhu et al., 2017; Yahouedehou et al., 2020). Four studies did not differentiate patients with SCA from patients with SCD (Borg et al., 2012; Gravia et al., 2016; Chondrou et al., 2017; Elalfy et al., 2017). One study was part of an oral session and their results were later published in an original article already included in this systematic review (Wyszynski et al., 2004). Finally, seven cohort studies were included in this systematic review (Ma et al., 2007; Kumkhaek et al., 2008; Ware et al., 2011; Green et al., 2013; Sheehan et al., 2014; Friedrisch et al., 2016; Aleluia et al., 2017) (**Figure 1**).

Characteristics of the Included Studies

Out of the seven included studies, five studies had data from the United States and two studies had data from Brazil. Sample size in the included studies ranged from 42 to 174 patients with SCA. The publication date ranged from 2007 to 2018, and the sample mean age ranged from 8.1 to 21 y. The mean dose of HU ranged from 19 to 27.1 ± 4.3 mg/kg/day. The mean duration of treatment with HU ranged from 13.4 to 102 months (**Table 1**). Two studies calculated the change in HbF levels for each patient from baseline to the MTD (delta HbF) (Ware et al., 2011; Friedrisch et al., 2016), while other four studies used the increment in HbF after treatment with HU (final HbF) (Ma et al., 2007; Kumkhaek et al., 2008; Green et al., 2013; Sheehan et al., 2014), and one study calculated from the baseline to maximum HbF during treatment with HU (Aleluia et al., 2017).

Overall, 728 genetic polymorphisms were assessed for their association with changes in HbF levels in patients with SCA under treatment with HU, and 11 candidate genes were the most examined in the seven included studies. Four studies examined *BCL11A* and the *HBSIL-MYB* intergenic region (Ware et al., 2011; Green et al., 2013; Sheehan et al., 2014; Friedrisch et al., 2016; Aleluia et al., 2017). Three studies focused on arginase 1 and 2 (*ARG1* and *ARG2*) genes (Ma et al., 2007; Ware et al., 2011; Green et al., 2013). Two studies evaluated the secretion-associated Ras-related GTPase 1A (*SAR1A*) gene (Ma et al., 2007; Green et al., 2013). Two studies examined the *XmnI* gene (Ware et al., 2011; Friedrisch et al., 2016). The Fms-related receptor tyrosine kinase 1 (*FLT1*), hydroxyacid oxidase 2 (*HA O 2*), nitric oxide synthase 1 (*NOS1*), and olfactory receptor family 51 subfamily B member 5 and 6 (*OR51B5/6*), genes were mentioned only once by two studies (Ma et al., 2007; Aleluia et al., 2017).

Regarding the quality assessment according to the Joanna Briggs Institute checklist (**Supplementary Table S2**), one of the seven articles answered affirmative in all the 11 questions. Five studies responded affirmative to ten out of the 11 questions. One

study answered affirmative to six out of the 11 questions, while three of the questions were negative and two were not applicable.

Pharmacogenetics of Response to HU Therapy in Patients With SCA

Among the included studies, a cohort study involving 137 adult African-Americans with SCA from the Multicenter Study of Hydroxyurea in Patients With Sickle Cell Anemia (MSH) examined the association of 320 tagging SNPs from 29 candidate genes with changes in HbF concentrations (measured by %, g/dL and F-cell count) after two years of HU treatment (Ma et al., 2007). Candidate genes were involved in the regulation of DNA transcription, cell proliferation/ differentiation, and drug metabolism functions. The daily dose of HU started at 15 mg/kg and increased by 5 mg/kg each week up to the MTD, which means 35 mg/kg, unless toxicity was established. The authors found 17 SNPs to be associated with % HbF change and 20 SNPs to be associated with change of absolute HbF (g/dl), most of them being located in introns or untranslated genomic regions. The SNPs found to be associated with the higher mean of squared were rs2182008 in the *FLT1* gene and rs10483801 in the *ARG2* gene, which is involved in the metabolism of HU. This MSH cohort study performed analysis with age, sex, and *β-globin* haplotypes as covariates and showed several SNPs in other genes as predictors for HbF response. In the random forest algorithm, the SNP rs21822998 in *FLT1* and the SNP rs9376173 in the phosphodiesterase 7B (*PDE7B*) gene had a higher mean of squared residuals as predictors for % HbF and absolute HbF, respectively (Ma et al., 2007).

The hypothesis for a later pharmacogenetic study (Kumkhaek et al., 2008) was supported by an experimental study on the molecular mechanisms underlying the increase in HbF levels induced by HU (Tang et al., 2005). The authors searched for differential gene expression in human adult erythroid cells and identified a small guanosine triphosphate (GTP)–binding protein, whose secretion is associated with Ras-related (SAR) protein, as a specific gene induced by HU. SAR was shown to play a key role in *γ*-globin (*HBG*) gene induction by promoting cell apoptosis and G1/S-phase arrest by the reduction of PI3K and extracellular signal–regulated kinase phosphorylation and increasing p21 and GATA-binding protein 2 expression (Tang et al., 2005). From these experimental findings, variations of the *SAR1A* gene were hypothesized to explain differences in individual responses to HU treatment (Kumkhaek et al., 2008). The authors tested whether 20 variants in the upstream promoter region, exon 1, and intron 1 of *SAR1A* were associated with HbF changes in response to HU compared to baseline in 32 adults with SCA from the Sickle Cell Pulmonary Hypertension Screening Study, prospectively followed up during two y of HU therapy. The SNP rs231099 was found to be associated with the change in % HbF, and the SNPs rs2310991, rs76901216, rs76901216, and rs4282891 were found to be associated with the change in absolute HbF (g/dl). The intronic SNP rs4282891 showed stronger association, which is phylogenetically conserved in vertebrates (Kumkhaek et al., 2008).

The Hydroxyurea Study of Long-Term Effects (HUSTLE) was a prospective clinical trial for pediatric patients with SCA receiving HU designed to understand the interpatient variability in the responses and toxicities to HU (Ware et al., 2011). A candidate gene study was conducted to carry out pharmacogenetic analyses for the HU end points of % HbF and the MTD. The dose administrated in patients who were included before beginning HU therapy (new cohort; $n = 88$) started with 15 mg/kg/day, and it was escalated every eight weeks to a maximum dose of 30 mg/kg/ day or the MTD, with careful monitoring of blood counts every two weeks. If hematologic toxicity occurred twice at the same dose, the MTD was set at 2.5 mg/kg below the toxic dose. Pharmacogenetic analyses included 331 SNPs in candidate genes that were selected based on their presumed pharmacogenetic and pharmacodynamic effects of HU. The *ARG1* rs175999586 and *ARG2* rs2295644 SNPs were associated with the change in % HbF between baseline and MTD. The SNP rs1427407 of the *BCL11A* gene was associated with the MTD, but none was associated with the MTD after adjustment for baseline % HbF (Ware et al., 2011).

The association of several SNPs with HbF levels induced by HU was also examined in a multi-site observational study of 117 pediatric patients (5–21 y), which was mainly composed of SCA patients (93% of HbSS and 7% of Sβ0-thalassemia) (Green et al., 2013). SNPs of *BCL11A*, *HBSIL-MYB*, *HBB*, hemoglobin subunit beta (*HBE*), *OR51B6*, glucagon-like peptide 2 receptor (*GLP2R*), *SAR1A*, *ARG1*, and *ARG2* genes, which were reported as associated with baseline HbF levels, were also examined for their association with HbF under HU therapy ("maximum HbF" and "delta HbF," from baseline to maximum). Clinical indications for HU therapy were comparable across sites (nearly all for repetitive painful crises and/or acute chest episodes) at least for six months. Stable dosing was reached at three months or near maximal dose by absolute neutrophil count criteria, excluding data from subjects on less than 20 mg/kg/day, even for dose-limiting toxicity. The SNPs of *BCL11A* (rs766432, rs11886868, rs4671393, and rs7557939), *HBE* (rs7130110), and *GLP2R* (rs12103880) were associated with maximum HbF under HU. Only the SNP rs7130110 of *HBE* was associated with delta (Δ) HbF (Green et al., 2013).

A cohort composed of 171 patients from the HUSTLE study and 51 patients from the Stroke with Transfusions Changing to Hydroxyurea (SWiTCH) (called "discovery") was examined to identify genetic predictors of HbF response to HU, with focus on protein coding regions (Sheehan et al., 2014). Whole-exome sequencing was performed in two prospective pediatric cohorts with robust HbF phenotype data and a standardized dose escalation regimen to the MTD, which were genotyped for SNPs of *BCL11A* (rs1427407, rs4671393, rs11886868, and rs7599488) and *HBSIL-MYB* (rs9399137 and rs9402686). HbF responses to HU were measured by maximum % HbF at the MTD ("final HbF") or the change in % HbF from baseline to final ("delta HbF"). The patients had baseline HbF measured after three y of age. The HU therapy initiated with 20 mg/kg, and then dose was escalated to mild myelosuppression. The average age of the patients at the time of HU initiation was 10.4 ± 4.5 y. However, they found no associations of *BCL11A* or *HBSIL-MYB* variants with HbF response. Whole-exome sequencing identified 13 and 12 variants associated with final HbF and delta HbF (p-value $< 5 \times 10^{-4}$), respectively. Although these

associations did not achieve the genome-wide significance level (p-value < 1.3×10^{-6}), they did provide suggestive signals (Sheehan et al., 2014). By using functional prediction methods, the authors identified that 13 variants associated with HbF response to HU were predicted to introduce an amino acid change, inducing damage in the protein structure or function (Sheehan et al., 2014). These 13 variants were then genotyped in a validated cohort composed of 130 unrelated children with SCA receiving HU at Texas Children's Hospital Hematology Center for at least six months prior to the MTD. One variant (P840R; rs61743453) in the spalt-like transcription factor 2 (*SALL2*) gene was associated with higher delta HbF and with final HbF in the discovery and the validated cohorts, respectively. A meta-analysis combining the discovery and validation cohorts (n = 301) found that the P840R variant was associated with both delta HbF ($p = 8.30 \times 10^{-4}$) and final HbF ($p = 1.48 \times 10^{-4}$) (Sheehan et al., 2014).

A cohort of 121 patients with SCA under regular HU therapy for at least six months at the Sickle Cell Center of the Clinical Hospital from Porto Alegre (Southern Brazil) was examined for the effect of genetic variants at the major loci modifier of baseline HbF on HU-induced HbF levels (Friedrisch et al., 2016). Patients who received any other drugs stimulating HbF (e.g., erythropoietin) or blood transfusion within three months prior to the study were excluded. HbF measurements were obtained before HU (baseline HbF) and at the MTD (MTD HbF), and the change from baseline to the MTD (delta HbF) was calculated for each patient. The association tests were performed by linear regression analyses adjusted for age at start HU, gender, and absolute neutrophil count at MTD. SNPs of hemoglobin subunit gamma 2 (*HBG2*) (rs7482144), *BCL11A* (rs1427407, rs4671393, and rs11886868), and *HBS1L-MYB* (rs9399137 and rs9402686) were assessed, and patients with variations in SNPs of *BCL11A* had a favorable probability of producing more HbFs in response to HU treatment (Friedrisch et al., 2016).

A cross-sectional study of 141 patients with SCA, including 99 patients under HU treatment, followed up at the Sickle cell Disease Reference Center in Itabuna (Northeastern Brazil) was examined for the role of *HBB* haplotypes and SNPs at quantitative trait loci (QTL) associated with baseline HbF in regulating HbF response to HU (Aleluia et al., 2017). HbF measures were not performed in patients with clinical manifestations of vaso-occlusive crisis or transfusions in the last three months. Patients were genotyped for *β^S-globin* gene cluster haplotypes and SNPs of *BCL11A* (rs6732518 and rs766432), *HBS1L-MYB* (rs11759553 and rs3595442), and *OR51B5/6* (rs4910755 and rs7483122). Almost 48% of the patients received 15 mg/kg/day, while 23.8% received 20 mg/kg/day and 26.2% received 25 mg/kg/day. The only patient who received the maximum dose of 35 mg/kg/day was excluded from the analysis. Multiple linear regression analysis adjusted for gender and age were used to investigate the association of SNPs with HbF induction, and the authors concluded that homozygous subjects for the minor allele of rs766432 of *BCL11A* had higher increases in HbF (Aleluia et al., 2017).

In summary, seven studies involving patients with SCA treated with HU identified 50 SNPs of 17 different genes to be associated with HbF changes from baseline to HU (**Table 2**; **Figure 2**). Five out of the seven included studies examined SNPs of *BCL11A*, of which four (80%) found SNPs to be associated with HbF changes (Ware et al., 2011; Green et al., 2013; Friedrisch et al., 2016; Aleluia et al., 2017). These studies confirmed the associations of the *BCL11A* SNPs rs1427407 (Ware et al., 2011; Friedrisch et al., 2016), rs4671393, rs11886868 (Green et al., 2013; Friedrisch et al., 2016), rs766432 (Green et al., 2013; Aleluia et al., 2017), and rs7557939 (Green et al., 2013). In addition, two studies found associations for SNPs of *ARG1* (rs17599586, rs2781667, and rs1799586) and *ARG2* (rs2246012, rs2295644, rs10483801, and rs10483802) (Ma et al., 2007; Ware et al., 2011). Among them, only the SNP rs1799586 of *ARG1* was found to be associated with HbF changes in the two studies (Ma et al., 2007; Ware et al., 2011).

Gene Set Enrichment Analysis and Pathway Analysis

Reactome pathways were obtained from the enrichment analysis using the set of genes that had SNPs found to be associated with changes on HbF levels in patients with SCA under HU therapy (**Figure 3**; **Supplementary Table S3**). The reactome pathways with both lowest adjusted p-values and highest combined scores were related to VEGF binding, namely, "VEGF ligand–receptor interactions" (R-HSA-194313; adjusted p-value = 0.0002847; combined score = 4,826.43) and "VEGF binds to VEGFR leading to receptor dimerization" (R-HSA-195399; adjusted p-value = 0.0002847; and combined score = 4,826.43). Moreover, we obtained the reactome pathway "urea cycle" (R-HSA-70635; adjusted p-value = 0.0003048; combined score = 3,461.84)] (**Figure 3**; **Supplementary Table S3**). The reactome pathway "nitric oxide stimulates guanylate cyclase" (R-HSA-392154; **Figure 3**) ranked fourth but with a lower combined score (200.68; p-value = 0.02105; **Supplementary Table S3**).

DISCUSSION

Genetic variability in response to HU therapy is scarcely explored, despite almost 50 y of HU use and 30 y of the treatment of patients with SCA (Ware et al., 2011). Notably, the literature regarding the effects of genetic polymorphisms on HbF levels in patients with SCA (Hb SS) treated with HU is remarkably scarce. In this systematic review, only seven studies met the inclusion criteria.

Importantly, patient-specific factors, including age, concomitant diseases, diet, and genetic factors, contribute to the interindividual variability in drug efficacy and risk of adverse reactions, and genetic polymorphisms explain around 20–30% of the interindividual variability in drug response (Lauschke et al., 2017). Indeed, the knowledge of how genetic variation contributes to variation in drug response has been expanded (Gong et al., 2021), and guidelines for the clinical implementation of pharmacogenetics have been published (Relling et al., 2020).

Single-Nucleotide Polymorphisms and HbF Modulation

As expected, some genes previously associated with baseline HbF and known for acting in the HbF regulation pathway were found

TABLE 2 | Genes and chromosomes for the 50 different SNPs found to be associated with changes on HbF [described as delta (Δ) % HbF, Δ HbF (g/dl), maximum tolerated dose (MTD) % HbF, or maximum HbF] in response to hydroxyurea therapy in the seven cohort studies included in the systematic review. *The SNP rs17599586 of *ARG1* and three SNPs of *BCL11A* (rs1427407, rs4671393, and rs11886868) were found to be associated by two different cohort studies.

Gene	Chromosome	SNP	HbF response	Reference
ARG1	6	*rs17599586	Δ % HbF; Δ HbF (g/dL)	Ma et al. (2007)
ARG1	6	rs2781667	Δ % HbF; Δ HbF (g/dL)	Ma et al. (2007)
ARG1	6	*rs17599586	Δ % HbF	Ware et al. (2011)
ARG2	14	rs2246012	Δ HbF (g/dl)	Ma et al. (2007)
ARG2	14	rs2295644	Δ % HbF	Ware et al. (2011)
ARG2	14	rs10483801	Δ % HbF; Δ HbF (g/dl)	Ma et al. (2007)
ARG2	14	rs10483802	Δ % HbF; Δ HbF (g/dl)	Ma et al. (2007)
ASS	9	rs7860909	Δ % HbF; Δ HbF (g/dl)	Ma et al. (2007)
ASS	9	rs10793902	Δ % HbF	Ma et al. (2007)
ASS	9	rs10901080	Δ % HbF	Ma et al. (2007)
ASS	9	rs543048	Δ HbF (g/dl)	Ma et al. (2007)
BCL11A	2	rs766432	Δ % HbF	Aleluia et al. (2017)
BCL11A	2	*rs1427407	MTD % HbF; Δ % HbF	Friedrisch et al. (2016)
BCL11A	2	*rs4671393	MTD % HbF	Friedrisch et al. (2016)
BCL11A	2	*rs11886868	MTD % HbF; Δ % HbF	Friedrisch et al. (2016)
BCL11A	2	rs766432	Maximum HbF	Green et al. (2013)
BCL11A	2	*rs11886868	Maximum HbF	Green et al. (2013)
BCL11A	2	*rs4671393	Maximum HbF	Green et al. (2013)
BCL11A	2	rs7557939	Maximum HbF	Green et al. (2013)
BCL11A	2	*rs1427407	MTD, mg/kg	Ware et al. (2011)
FIGF	X	rs6632521	Δ % HbF	Ma et al. (2007)
FLT1	13	rs9319428	Δ % HbF; Δ HbF (g/dl)	Ma et al. (2007)
FLT1	13	rs2182008	Δ % HbF; Δ HbF (g/dl)	Ma et al. (2007)
FLT1	13	rs3751395	Δ HbF (g/dl)	Ma et al. (2007)
FLT1	13	rs8002446	Δ % HbF; Δ HbF (g/dl)	Ma et al. (2007)
FLT1	13	rs2387634	Δ HbF (g/dl)	Ma et al. (2007)
GLP2R	17	rs12103880	Maximum HbF	Green et al. (2013)
HA O 2	1	rs10494225	Δ % HbF	Ma et al. (2007)
HBE	11	rs7130110	Maximum HbF; Δ % HbF	Green et al. (2013)
MAP3K5	6	rs9376230	Δ % HbF	Ma et al. (2007)
MAP3K5	6	rs9483947	Δ % HbF	Ma et al. (2007)
NOS1	12	rs7309163	Δ HbF (g/dl)	Ma et al. (2007)
NOS1	12	rs816361	Δ % HbF	Ma et al. (2007)
NOS1	12	rs7977109	Δ % HbF; Δ HbF (g/dL)	Ma et al. (2007)
NOS2A	17	rs1137933	Δ % HbF	Ma et al. (2007)
NOS2A	17	rs944725	Δ % HbF	Ma et al. (2007)
PDE7B	6	rs2327669	Δ HbF (g/dl)	Ma et al. (2007)
PDE7B	6	rs11154849	Δ HbF (g/dl)	Ma et al. (2007)
PDE7B	6	rs9376173	Δ HbF (g/dl)	Ma et al. (2007)
PDE7B	6	rs1480642	Δ HbF (g/dl)	Ma et al. (2007)
PDE7B	6	rs487278	Δ HbF (g/dl)	Ma et al. (2007)
PIR	X	rs2071182	Δ HbF (g/dl)	Ma et al. (2007)
SALL2	14	rs61743453	Δ % HbF	Sheehan et al. (2014)
SAR1	10	rs2310991	Δ % HbF; Δ HbF (g/l)	Kumkhaek et al. (2008)
SAR1	10	rs76901216	Δ HbF (g/dl)	Kumkhaek et al. (2008)
SAR1	10	rs76901220	Δ HbF (g/dl)	Kumkhaek et al. (2008)
SAR1	10	rs4282891	Δ HbF (g/dl)	Kumkhaek et al. (2008)
TOX	8	rs2693430	Δ HbF (g/dl)	Ma et al. (2007)
TOX	8	rs826729	Δ % HbF	Ma et al. (2007)
TOX	8	rs765587	Δ % HbF; Δ HbF (g/dl)	Ma et al. (2007)
TOX	8	rs9693712	Δ % HbF; Δ HbF (g/dl)	Ma et al. (2007)
TOX	8	rs172652	Δ % HbF	Ma et al. (2007)
TOX	8	rs380620	Δ % HbF; Δ HbF (g/dl)	Ma et al. (2007)
TOX	8	rs12155519	Δ HbF (g/dl)	Ma et al. (2007)

to be associated with HbF changes in response to HU therapy in the included studies. There is evidence that SNPs of intron 2 of *BCL11A* affect HbF changes in patients with SCA treated with HU. Five out of the seven included studies examined the role of the three main QTLs associated with baseline HbF levels: *BCL11A*, *XmnI*, and *HBS1L-MYB* intergenic region (Ware et al., 2011; Green et al., 2013; Sheehan et al., 2014; Friedrisch et al., 2016; Aleluia et al., 2017). Noteworthy, BCL11A is a negative regulator of HbF expression. Subjects with variation in any of the established SNPs of *BCL11A* are known to have

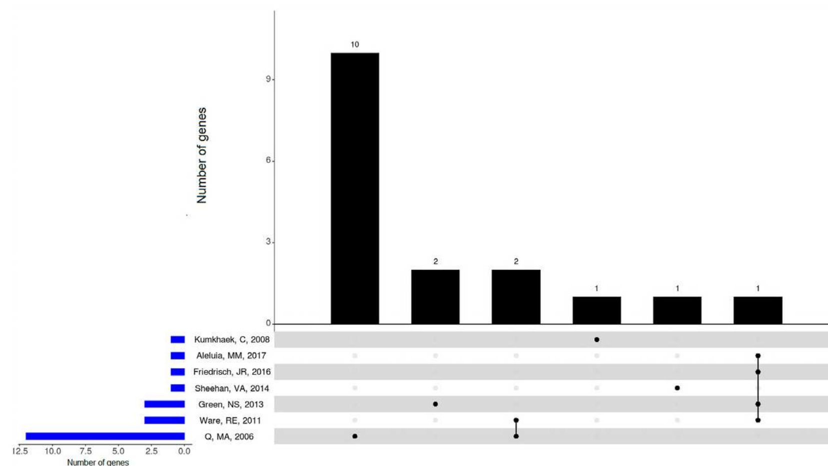

FIGURE 2 | Overlapping genes with SNPs associated with changes on HbF levels in the included studies involving patients with SCA (Hb SS) under HU therapy. *BCL11A*, *ARG1*, and *ARG2* genes showed overlap between included studies. Upset plot showing the total number of genes with SNPs found to be associated with changes on HbF levels identified in each study (horizontal bars), and the number of genes exclusive to one study or shared by different studies (vertical bars). Black dots below vertical bars indicate genes quantified.

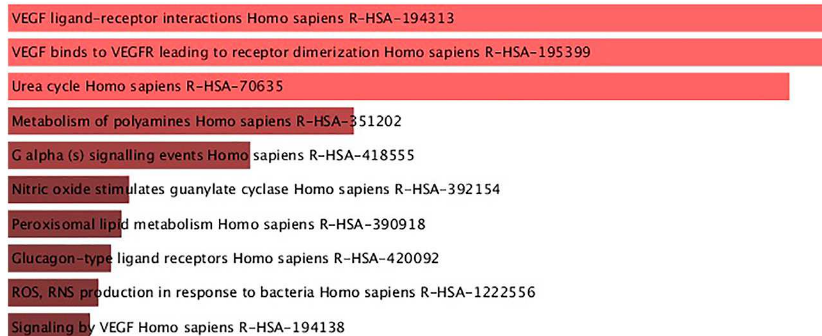

FIGURE 3 | Reactome pathways obtained from enrichment analysis of the set of genes with SNPs found to be associated with HbF changes in patients with SCA treated with hydroxyurea. Pathways are ordered from top to bottom according to the lowest adjusted *p*-values, and light red bars indicate adjusted *p*-values < 0.001.

decreased *BCL11A* expression, which results in increased HbF production (Lettre et al., 2008; Bauer et al., 2013). *HBS1L-MYB* genes are expressed in the erythroid precursor cells (Lettre et al., 2008; Bauer et al., 2013). *HBS1L* encodes a protein with apparent GTP-binding activity and is involved in a variety of cellular processes, while *MYB* encodes a transcription factor for erythroid differentiation in hematopoiesis (Thein et al., 2007). The *HBS1L-MYB* intergenic region is known to contain several common QTLs associated with HbF levels and a long-range erythroid enhancer that regulates *MYB* expression by chromatin looping (Stadhouders et al., 2014). Finally, the *XmnI* restriction site at -158 position of the *HBG* gene is associated with an increased expression of γ-globin and higher HbF production (Sripichai and Fucharoen, 2016). Together, they account for approximately 20–50% of the variation in HbF levels in patients with SCA and β-thalassemia, and even in healthy adults (Galarneau et al., 2010).

Four out of five studies that examined SNPs of *BCL11A* found associations with HbF response (Ware et al., 2011; Green et al., 2013; Sheehan et al., 2014; Friedrisch et al., 2016). These SNPs (rs1427407, rs4671393, rs11886868, rs766432, and rs7557939) are located in intron 2 of *BCLL1A*, which is a region marked by functional elements. These SNPs are located nearby several DNase hypersensitive sites, which indicate a genomic region of open chromatin. Noteworthy, the critical SNP rs1427407 (G > T) falls within a +62 DNase I hypersensitive site, an erythroid enhancer of *BCL11A*, and overlaps a peak of GATA1 and TAL1 transcription factor–binding sites. Notably, the minor T-allele for the SNP rs1427407 disrupts the G nucleotide of a consensus sequence [CTG (n9)] enriched for GATA1 and TAL1 transcription factors. GATA1- and TAL1-binding sites are more frequent in the G-allele than T-allele in the primary erythroblast samples (Bauer et al., 2013). In agreement with our findings regarding the effect of SNPs of *BCL11A* on HbF response, a recent

functional *in vitro* study based on gene editing comparative analysis showed that *BCL11A* is the most clinically relevant approach focused on HbF resurgence (Lamsfus-Calle et al., 2020). This functional information supports the effect of *BCL11A* SNPs on baseline HbF, but the way it affects the response to HU remains to be elucidated. Biological network analysis integrating effects of HU on gene expression in erythroid precursors could highlight pathways involved in this process.

The *XmnI* variant in the *HBBP1* gene was also examined. One study found the SNP rs7482144 to be associated only with an increase in baseline HbF (Ware et al., 2011) but not with HbF changes to HU, while other studies excluded this SNP because it had a very low allele frequency (0.45%, only one heterozygous subject) in a Brazilian cohort (Friedrisch et al., 2016).

The SNPs rs9399137 and rs9402686 are located in the *HBS1L-MYB* intergenic region but were not found to be associated with the increase in HbF in two included studies (Sheehan et al., 2014; Friedrisch et al., 2016). *SAR1A*, a gene belonging to the small GTPase superfamily that encodes a GTP-binding protein called SAR1A, has been reported to be associated with *HBG* expression. The SNP rs2310991 of *SAR1A* was associated with the change in absolute HbF concentration (Kumkhaek et al., 2008). Conversely, other studies found no association of rs2310991 with posttreatment HbF levels (Kumkhaek et al., 2008; Green et al., 2013).

Our findings may potentially guide the selection of candidate gene regulatory sequences within these genomic regions to be validated by *in vitro* functional assays in cells treated with HU, such as luciferase reporter assays. Further studies may examine whether the variation in these SNPs would affect the activity of the gene regulatory element, such as an enhancer or a silencer. Therefore, the present findings can contribute to guide further functional studies, which may advance the research focused on genomics of HbF changes in response to HU therapy.

Signaling Pathways Underlying HbF Changes in Response to HU Therapy

Pathway analysis using genes with SNPs found to be associated with HbF changes in patients with SCA treated with HU in the included studies revealed pathways underlying HbF changes in response to HU. For example, we found enrichment of the reactome pathway “urea cycle” (R-HAS-70635; **Figure 3**), which is directly related to arginine (Friebe and Koesling, 2003). Indeed, cytosolic arginase 1 is a canonical enzyme of the urea cycle. Arginase 2 was described to play a role in the regulation of the urea cycle arginine metabolism and in downregulation of nitric oxide synthesis (Mori, 2007). Arginase isoforms encoded by *ARG1* and *ARG2* genes were also related to the increase in HbF levels induced by HU (Ware et al., 2011). The SNPs rs175999586 and rs2295644 of *ARG1* and *ARG2* were associated with the changes in HbF, respectively. Notably, rs2295644 has been implicated in kidney disease, so it could affect the renal clearance of HU and possibly the dose of the MTD (Ware et al., 2011). Another *ARG2* SNP (rs10483801) was also associated with the absolute HbF change (Ma et al., 2007).

We also found the enrichment of the reactome pathway “nitric oxide stimulates guanylate cyclase” (R-HSA-392154; **Figure 3**). Noteworthy, HU was suggested to act as a nitric oxide donor in patients with SCA (King, 2004). Nitric oxide is synthetized from L-arginine, stimulates vasodilatation of the endothelium and disaggregation of platelet aggregates, and inhibits platelet activation, an important modulator of SCA pathophysiology (Radomski et al., 1987). Moreover, HU was shown to modulate the nitric oxide signaling pathway in erythrocytes, rheology of erythrocytes, and oxidative stress through its effects on HbF and possibly on nitric oxide bioavailability (Nader et al., 2018).

A complex regulatory environment determines the HbF concentration in the blood, as well as chromosome remodeling, transcription factors, erythropoiesis modulation, gene regulatory elements linked to the β-globin gene cluster, and the kinetics of erythroid cell differentiation and differential red cell survival (Ma et al., 2007). Therefore, there is a large opportunity for the genetic modulation of HbF production. Consistent with this complex regulation apparatus, even with the restricted number of studies, our systematic review suggests that there is huge heterogeneity in genetic elements modulating the HbF levels in response to HU treatment. Unfortunately, some genetic associations with HbF response have not been reproduced by other studies, and further investigations are needed to conclude their use in predicting HbF response to HU.

Dosing and monitoring regimens of HU have yet to be determined (Ware, 2010). The best results from treatment with HU are found when the dose is escalated to the MTD, improving laboratory variables and reducing clinical complications. The dose escalation of HU is a labored process that requires risk monitoring of cytopenias, mainly neutropenia, and the clinical response to treatment with HU may take up to six months after reaching the MTD (NIH US, 2014). Therefore, severe patients with clinical recommendation for HU might have to experience a long exposure time until deducing that the treatment with HU is ineffective. Therefore, the prediction of HbF induction in response to treatment with HU by using SNPs in the intron 2 of *BCL11A* gene may have potential clinical applicability in the management of SCA.

The induction of HbF is a powerful mechanism of action of HU. However, since several other mechanisms of actions are known, further research is needed to conclude whether such SNPs are able to predict a subgroup of patients as “responders” to HU. Noteworthy, these SNPs were previously associated with increased baseline HbF levels, milder hematological parameters, and lower risk of clinical complications (Sales et al., 2020). Interestingly, not all these associations were dependent on HbF. Therefore, future studies should evaluate if the SNPs located in intron 2 of the *BCL11A* gene are also able to distinguish patients who show a reduced rate of clinical complications when treated with HU from those patients who do not show this reduction. This evidence is of huge importance to assess the cost-effectiveness of the use of pharmacogenetic tests for these SNPs in the SCA management.

Some studies do not meet the inclusion criteria of this review due to the different genotypes of the study subjects (Borg et al., 2012; Chondrou et al., 2017; Elalfy et al., 2017). They involve other β-type hemoglobinopathies, and known differences in their hematological parameter could bias the review. However, these studies highlight specific points regarding pathways related to the HbF regulation. Two studies suggested that *KFL1* expression and the SNP rs3191333 of *KFL1* play a role in the HbF regulation and are biomarkers of HU response in β-type hemoglobinopathies. It makes biological sense, since KLF1 regulates *BCL11A* expression and the γ- to β-globin gene switching (Zhou et al., 2010). Further studies can confirm their influence in HU therapy in patients with SCA (Hb SS).

Another study suggests that the vascular endothelial growth factor (*VEGFA*) gene is a biomarker in β-type hemoglobinopathies severity and efficacy of HU therapy (Chondrou et al., 2017). These findings are in agreement with a study included in this systematic review that found SNPs in the *FLT1* gene, encoding VEGF receptor 1, associated with HbF changes by HU therapy in Hb SS patients (Ma et al., 2007). Interestingly, we found enrichment of two reactome pathways related to VEGF ligand–receptor interactions (R-HSA-194313 and R-HSA-195399; **Figure 3**). The binding of VEGF ligands to VEGFR receptors in the cell membrane triggers intracellular signaling cascades, which results in proliferation, survival, migration, and increased permeability of vascular endothelial cells (Matsumoto and Mugishima, 2006). It is important to SCA pathophysiology, since endothelial dysfunction plays a key role in sickle cell vasculopathy, as reviewed elsewhere (Wood et al., 2008).

Our systematic review highlighted the role of SNPs on HbF induction upon HU therapy. However, it is important to note that this is one of the several mechanisms underlying response to HU. Indeed, a previous systematic review reported on the molecular mechanisms of HbF induction by HU in SCD (Pule et al., 2015). The reviewed findings pointed out three main pathways: epigenetic modifications, signaling pathways involving HU-mediated response, and posttranscriptional pathways, focusing on regulation by small non-coding RNAs (miRNAs). In this context, several miRNAs were identified as differentially expressed in patients with SCD under HU treatment, most of them being functionally related to genes known to regulate HbF, including *BCL11A* (Mnika et al., 2019). Notably, an experimental study showed that downregulation of *BCL11A*, *MYB*, and *KLF1* induces *γ-globin* expression by miRNA-mediated mechanisms, and miR-26b directly interacted with the $3'$-untranslated region of *MYB* (Pule et al., 2016). Since miRNAs have been associated with a multitude of regulatory mechanisms, their functions may add to the complex mechanisms underlying response to HU.

In summary, the regulation of HbF involves both *cis*- and *trans*-regulatory elements, which interact in a complex network. HU promotes the induction of HbF, and the mechanisms by which it interacts with genetic modifiers of HbF affecting drug response are not fully understood. In this context, SNPs located within gene regulatory elements can have a major effect on differences in drug response (Luizon and Ahituv, 2015). A proposed schematic diagram to HbF regulation in response to HU is shown in **Figure 4**, including the functional findings of genes found in this systematic review as candidates to modify the HbF response to HU in patients with SCA.

Confounding Factors

The first clinical manifestations of SCA appear along with the replacement of HbS by HbF (Rumaney et al., 2014). After 10 y, age is no longer an indicator of red cell deformability in patients with SCA; instead, this hemorheological parameter is mainly affected by the level of HbF, sex, and HU treatment (Thein et al., 2007).

The level of HbF is the best predictor of clinical severity of SCA (Wonkam et al., 2014). However, there is no threshold or value that characterizes the high and low baseline HbF levels for response to HU treatment. It was established that subjects who start with baseline HbF values between 5 and 10% can have a 2- to 3-fold HbF increase, whereas subjects with very low baseline HbF can have a 10-fold increase after treatment with HU (Wonkam et al., 2014). In the MSH cohort study, the baseline HbF was not predictive to HbF response to HU. On the other hand, baseline HbF was found as a predictor of the direction of association to % HbF at MTD (Ware et al., 2011). However, the change in HbF outcome in patients with SCA treated with HU was largely heterogeneous among the seven included studies, which examined different HbF outcomes in response to HU. Changes in HbF upon HU therapy was calculated as absolute HbF (g/dl), % HbF, and F-cell count from baseline until the MTD or a defined time of therapy (about 2 y). Although the % HbF and the amount of F cells are highly correlated, some patients with high levels of HbF develop severe complications of SCA probably due to the heterogeneous distribution of HbF among erythrocytes (Khandros et al., 2020). The number of F cells with polymer-inhibiting concentrations of HbF is likely to be a more accurate predictor of clinical benefits of HU therapy than HbF levels. However, the distribution of HbF among F cells is often unavailable, mainly in health centers of least developed countries. Using HbF under the MTD to calculate delta HbF probably provides the maximum level that the patient can achieve.

Patients with SCA experience several acute clinical events involving pronounced changes in hematological parameters (Novelli and Gladwin, 2016). Moreover, they commonly receive blood transfusion for treating and avoid a range of complications, which introduces biases on evaluating the association with hematological variables, including HbF. However, three of the seven included studies did not describe whether strategies were used to deal with these established confounding factors, which introduces bias in our analyses and constitute a limitation of this systematic review.

Although clinical experience of HU therapy for patients with SCA has been related for more than 25 y, there is still much questioning about the pharmacokinetics, pharmacodynamics,

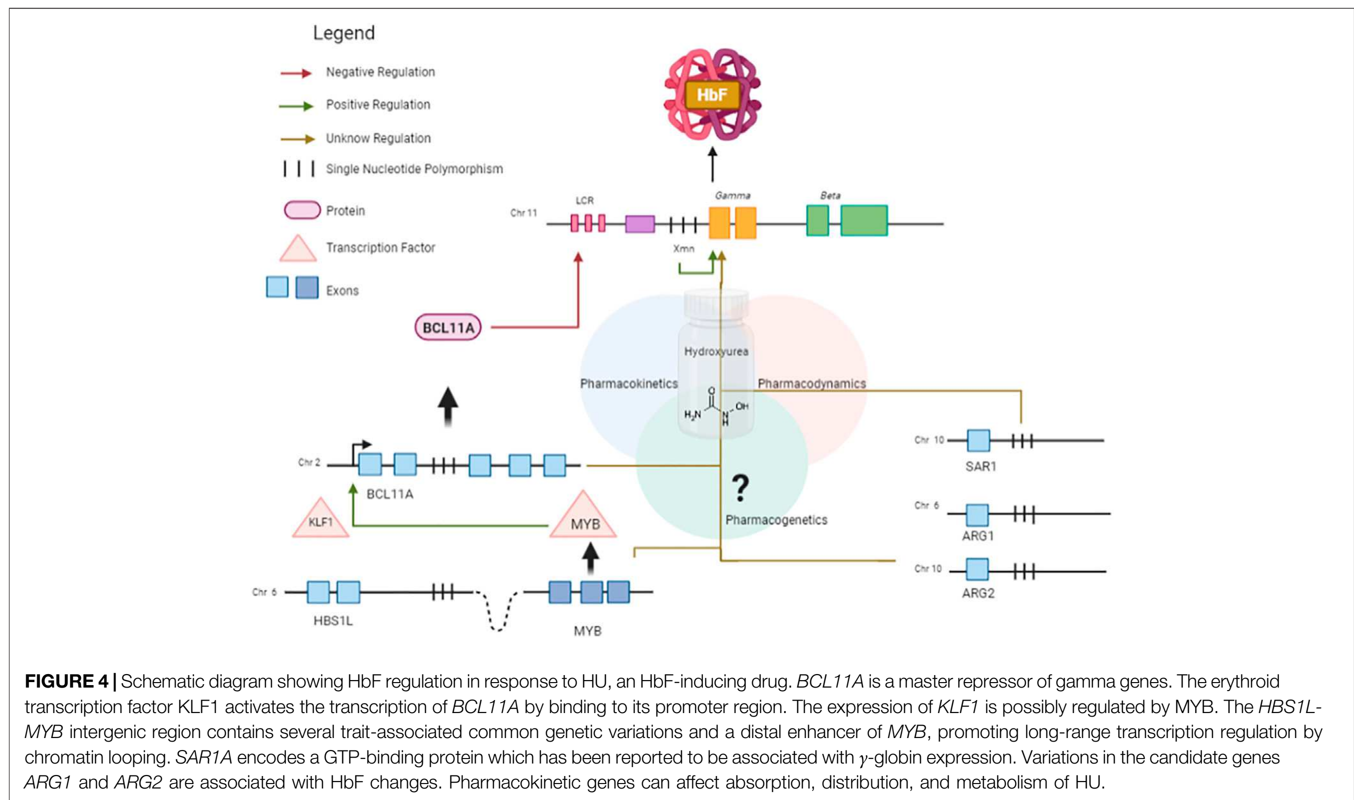

FIGURE 4 | Schematic diagram showing HbF regulation in response to HU, an HbF-inducing drug. *BCL11A* is a master repressor of gamma genes. The erythroid transcription factor KLF1 activates the transcription of *BCL11A* by binding to its promoter region. The expression of *KLF1* is possibly regulated by MYB. The *HBS1L-MYB* intergenic region contains several trait-associated common genetic variations and a distal enhancer of *MYB*, promoting long-range transcription regulation by chromatin looping. *SAR1A* encodes a GTP-binding protein which has been reported to be associated with γ-globin expression. Variations in the candidate genes *ARG1* and *ARG2* are associated with HbF changes. Pharmacokinetic genes can affect absorption, distribution, and metabolism of HU.

and pharmacogenetics of HU (Ware et al., 2011). To better understand the interpatient variability, polymorphisms in genes encoding drug-metabolizing enzymes and solute transporters were recently examined to learn their role in HU bioavailability and metabolism (Yahouedehou et al., 2020). The authors found evidence for the involvement of enzymes of the CYP450 family and catalases in the metabolism of HU, and the association between urea transporter-B (UTB) and response to HU in erythroid cells. SNPs in the *CYP2D6* (rs3892097), *CAT* (rs7943316 and rs1001179), and *SLC14A1* (rs2298720) genes were found to be linked to reduced metabolism or the elimination of HU, which may increase its therapeutic effects in patients with SCA (Yahouedehou et al., 2020). Unfortunately, this study did not examine the association between the SNPs with HbF response to HU, and thus, it was not included in the systematic review.

There was great heterogeneity in the patients' age in the included studies. For example, one study examined patients aged 4–54 y (mean 21 ± 14 y) (Friedrisch et al., 2016). The average age of initiation of HU was 9.6 ± 4.8 y in another study (Ware et al., 2011).

The present study has layers of complexity linked to the multifactorial characteristic of the disease. The heterogeneity of HU dose, patient age, HbF outcomes in response to HU, and candidate genes brought limitations to the search and contributed to the result being only seven included studies. These findings highlight that the pharmacogenetics of response to HU in patients with SCA is a fertile field for further investigations.

CONCLUSION

The literature about the pharmacogenetics of response to HU therapy in patients with SCA is highly heterogeneous regarding the chosen candidate genes and SNPs examined for the possible association with changes in HbF levels, and regarding the HbF outcomes measured during HU therapy. Nevertheless, the findings of the studies included in this systematic review point out two main conclusions. First, as well as the baseline HbF, changes in HbF levels in response to HU therapy are likely to be regulated by genetic variations on multiple loci. Second, there is evidence that SNPs located in intron 2 of the *BCL11A* gene affect HbF changes in patients with SCA treated with HU. However, further studies are needed to test whether such SNPs may also predict the success of the treatment in ameliorating other hematological parameters and reducing the incidence of clinical complications.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**; further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

RS, BN, JT, KG, and ML made substantial contributions to the conception or design of the work; RS, BN, and JT acquired the data,

and all authors analyzed and interpreted the data for the work; RS, BN, and ML drafted the manuscript, and all authors revised it critically for important intellectual content; and all authors read and approved the final version of the manuscript for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.779497/full#supplementary-material

REFERENCES

Aleluia, M. M., Santiago, R. P., Da Guarda, C. C., Fonseca, T. C., Neves, F. I., Quinto, R. S., et al. (2017). Genetic Modulation of Fetal Hemoglobin in Hydroxyurea-Treated Sickle Cell Anemia. *Am. J. Hematol.* 92, E70–E72. doi:10.1002/ajh.24680

Azar, S., and Wong, T. E. (2017). Sickle Cell Disease: A Brief Update. *Med. Clin. North. Am.* 101, 375–393. doi:10.1016/j.mcna.2016.09.009

Bauer, D. E., Kamran, S. C., Lessard, S., Xu, J., Fujiwara, Y., Lin, C., et al. (2013). An Erythroid Enhancer of BCL11A Subject to Genetic Variation Determines Fetal Hemoglobin Level. *Science* 342, 253–257. doi:10.1126/science.1242088

Benjamini, Y., and Hochberg, Y. (1995). Controlling the False Discovery Rate: a Practical and Powerful Approach to Multiple Testing. *J. R. Stat. Soc. Ser. B Methodol.* 57, 289–300. doi:10.1111/j.2517-6161.1995.tb02031.x

Borg, J., Phylactides, M., Bartsakoulia, M., Tafrali, C., Lederer, C., Felice, A. E., et al. (2012). KLF10 Gene Expression Is Associated with High Fetal Hemoglobin Levels and with Response to Hydroxyurea Treatment in β-hemoglobinopathy Patients. *Pharmacogenomics* 13, 1487–1500. doi:10.2217/pgs.12.125

Chondrou, V., Kolovos, P., Sgourou, A., Kourakli, A., Pavlidaki, A., Kastrinou, V., et al. (2017). Whole Transcriptome Analysis of Human Erythropoietic Cells during Ontogenesis Suggests a Role of VEGFA Gene as Modulator of Fetal Hemoglobin and Pharmacogenomic Biomarker of Treatment Response to Hydroxyurea in β-type Hemoglobinopathy Patients. *Hum. Genomics* 11, 24. doi:10.1186/s40246-017-0120-8

DeBaun, M. R., Jordan, L. C., King, A. A., Schatz, J., Vichinsky, E., Fox, C. K., et al. (2020). American Society of Hematology 2020 Guidelines for Sickle Cell Disease: Prevention, Diagnosis, and Treatment of Cerebrovascular Disease in Children and Adults. *Blood Adv.* 4, 1554–1588. doi:10.1182/bloodadvances.2019001142

den Dunnen, J. T., and Antonarakis, S. E. (2000). Mutation Nomenclature Extensions and Suggestions to Describe Complex Mutations: a Discussion. *Hum. Mutat.* 15, 7–12. doi:10.1002/(SICI)1098-1004(200001)15:1<7:AID-HUMU4>3.0.CO;2-N

Elalfy, M. S., El Sherif, N. H., Kamal, T. M., and Aly, N. H. (2017). Klf10 Gene, a Secondary Modifier and a Pharmacogenomic Biomarker of Hydroxyurea Treatment Among Patients with Hemoglobinopathies. *J. Pediatr. Hematol. Oncol.* 39, e155–e162. doi:10.1097/MPH.0000000000000762

Friebe, A., and Koesling, D. (2003). Regulation of Nitric Oxide-Sensitive Guanylyl Cyclase. *Circ. Res.* 93, 96–105. doi:10.1161/01.RES.0000082524.34487.31

Friedrisch, J. R., Sheehan, V., Flanagan, J. M., Baldan, A., Summarell, C. C., Bittar, C. M., et al. (2016). The Role of BCL11A and HMIP-2 Polymorphisms on Endogenous and Hydroxyurea Induced Levels of Fetal Hemoglobin in Sickle Cell Anemia Patients from Southern Brazil. *Blood Cell Mol. Dis.* 62, 32–37. doi:10.1016/j.bcmd.2016.11.002

Galarneau, G., Palmer, C. D., Sankaran, V. G., Orkin, S. H., Hirschhorn, J. N., and Lettre, G. (2010). Fine-mapping at Three Loci Known to Affect Fetal Hemoglobin Levels Explains Additional Genetic Variation. *Nat. Genet.* 42, 1049–1051. doi:10.1038/ng.707

Gong, L., Whirl-Carrillo, M., and Klein, T. E. (2021). PharmGKB, an Integrated Resource of Pharmacogenomic Knowledge. *Curr. Protoc.* 1, e226. doi:10.1002/cpz1.226

Gravia, A., Chondrou, V., Kolliopoulou, A., Kourakli, A., John, A., Symeonidis, A., et al. (2016). Correlation of SIN3A Genomic Variants with β-hemoglobinopathies Disease Severity and Hydroxyurea Treatment Efficacy. *Pharmacogenomics* 17, 1785–1793. doi:10.2217/pgs-2016-0076

Green, N. S., Ender, K. L., Pashankar, F., Driscoll, C., Giardina, P. J., Mullen, C. A., et al. (2013). Candidate Sequence Variants and Fetal Hemoglobin in Children with Sickle Cell Disease Treated with Hydroxyurea. *PLoS One* 8, e55709. doi:10.1371/journal.pone.0055709

Higgins, J. P. T., Thomas, J., Chandler, J., Cumpston, M., Li, T., Page, M. J., et al. (Editors) (2020). *Cochrane Handbook for Systematic Reviews of Interventions Version 6.2*. Cochrane. Available at: www.training.cochrane.org/handbook.

Italia, K. Y., Jijina, F. F., Jain, D., Merchant, R., Nadkarni, A. H., Mukherjee, M., et al. (2010). The Effect of UGT1A1 Promoter Polymorphism on Bilirubin Response to Hydroxyurea Therapy in Hemoglobinopathies. *Clin. Biochem.* 43, 1329–1332. doi:10.1016/j.clinbiochem.2010.08.006

Jassal, B., Matthews, L., Viteri, G., Gong, C., Lorente, P., Fabregat, A., et al. (2020). The Reactome Pathway Knowledgebase. *Nucleic Acids Res.* 48, D498–D503. doi:10.1093/nar/gkz1031

Kato, G. J., Piel, F. B., Reid, C. D., Gaston, M. H., Ohene-Frempong, K., Krishnamurti, L., et al. (2018). Sickle Cell Disease. *Nat. Rev. Dis. Primers* 4, 18010. doi:10.1038/nrdp.2018.10

Khandros, E., Huang, P., Peslak, S. A., Sharma, M., Abdulmalik, O., Giardine, B. M., et al. (2020). Understanding Heterogeneity of Fetal Hemoglobin Induction through Comparative Analysis of F and A Erythroblasts. *Blood* 135, 1957–1968. doi:10.1182/blood.2020005058

King, S. B. (2004). Nitric Oxide Production from Hydroxyurea. *Free Radic. Biol. Med.* 37, 737–744. doi:10.1016/j.freeradbiomed.2004.02.073

Kuleshov, M. V., Jones, M. R., Rouillard, A. D., Fernandez, N. F., Duan, Q., Wang, Z., et al. (2016). Enrichr: a Comprehensive Gene Set Enrichment Analysis Web Server 2016 Update. *Nucleic Acids Res.* 44, W90–W97. doi:10.1093/nar/gkw377

Kumkhaek, C., Taylor, J. G., Zhu, J., Hoppe, C., Kato, G. J., and Rodgers, G. P. (2008). Fetal Haemoglobin Response to Hydroxycarbamide Treatment and Sar1a Promoter Polymorphisms in Sickle Cell Anaemia. *Br. J. Haematol.* 141, 254–259. doi:10.1111/j.1365-2141.2008.07045.x

Lamsfus-Calle, A., Daniel-Moreno, A., Antony, J. S., Epting, T., Heumos, L., Baskaran, P., et al. (2020). Comparative Targeting Analysis of KLF1, BCL11A, and HBG1/2 in CD34+ HSPCs by CRISPR/Cas9 for the Induction of Fetal Hemoglobin. *Sci. Rep.* 10, 10133. doi:10.1038/s41598-020-66309-x

Lauschke, V. M., Milani, L., and Ingelman-Sundberg, M. (2017). Pharmacogenomic Biomarkers for Improved Drug Therapy-Recent Progress and Future Developments. *AAPS J.* 20, 4. doi:10.1208/s12248-017-0161-x

Lettre, G., and Bauer, D. E. (2016). Fetal Haemoglobin in Sickle-Cell Disease: from Genetic Epidemiology to New Therapeutic Strategies. *Lancet* 387, 2554–2564. doi:10.1016/S0140-6736(15)01341-0

Lettre, G., Sankaran, V. G., Bezerra, M. A., Araújo, A. S., Uda, M., Sanna, S., et al. (2008). DNA Polymorphisms at the BCL11A, HBS1L-MYB, and Beta-Globin Loci Associate with Fetal Hemoglobin Levels and Pain Crises in Sickle Cell Disease. *Proc. Natl. Acad. Sci. U S A.* 105, 11869–11874. doi:10.1073/pnas.0804799105

Luizon, M. R., and Ahituv, N. (2015). Uncovering Drug-Responsive Regulatory Elements. *Pharmacogenomics* 16, 1829–1841. doi:10.2217/pgs.15.121

Ma, Q., Wyszynski, D. F., Farrell, J. J., Kutlar, A., Farrer, L. A., Baldwin, C. T., et al. (2007). Fetal Hemoglobin in Sickle Cell Anemia: Genetic Determinants of Response to Hydroxyurea. *Pharmacogenomics J.* 7, 386–394. doi:10.1038/sj.tpj.6500433

Matsumoto, T., and Mugishima, H. (2006). Signal Transduction via Vascular Endothelial Growth Factor (VEGF) Receptors and Their Roles in Atherogenesis. *J. Atheroscler. Thromb.* 13, 130–135. doi:10.5551/jat.13.130

McGann, P. T., and Ware, R. E. (2015). Hydroxyurea Therapy for Sickle Cell Anemia. *Expert Opin. Drug Saf.* 14, 1749–1758. doi:10.1517/14740338.2015.1088827

Mnika, K., Mazandu, G. K., Jonas, M., Pule, G. D., Chimusa, E. R., Hanchard, N. A., et al. (2019). Hydroxyurea-Induced miRNA Expression in Sickle Cell Disease Patients in Africa. *Front. Genet.* 10, 509. doi:10.3389/fgene.2019.00509

Moola, S., Munn, Z., Tufanaru, C., Aromataris, E., Sears, K., Sfetcu, R., et al. (2020). "Chapter 7: Systematic Reviews of Etiology and Risk," in *JBI Manual for Evidence Synthesis*. Editors E. Aromataris and Z. E. Munn (JBI). doi:10.46658/jbimes-20-08

Mori, M. (2007). Regulation of Nitric Oxide Synthesis and Apoptosis by Arginase and Arginine Recycling. *J. Nutr.* 137, 1616S–1620S. doi:10.1093/jn/137.6.1616S

Nader, E., Grau, M., Fort, R., Collins, B., Cannas, G., Gauthier, A., et al. (2018). Hydroxyurea Therapy Modulates Sickle Cell Anemia Red Blood Cell Physiology: Impact on RBC Deformability, Oxidative Stress, Nitrite Levels and Nitric Oxide Synthase Signalling Pathway. *Nitric Oxide* 81, 28–35. doi:10.1016/j.niox.2018.10.003

NIH US (2014). Evidence-Based Management of Sickle Cell Disease: Expert Panel Report. NHLBI Publications and Resources: U. S. Department of Health and Human Services: National Institute of Health, National Heart, Lung, and Blood Institute. Available at: https://www.nhlbi.nih.gov/health-topics/evidence-based-management-sickle-cell-disease (Accessed September 18, 2021).

Novelli, E. M., and Gladwin, M. T. (2016). Crises in Sickle Cell Disease. *Chest* 149, 1082–1093. doi:10.1016/j.chest.2015.12.016

Pule, G. D., Mowla, S., Novitzky, N., Wiysonge, C. S., and Wonkam, A. (2015). A Systematic Review of Known Mechanisms of Hydroxyurea-Induced Fetal Hemoglobin for Treatment of Sickle Cell Disease. *Expert Rev. Hematol.* 8, 669–679. doi:10.1586/17474086.2015.1078235

Pule, G. D., Mowla, S., Novitzky, N., and Wonkam, A. (2016). Hydroxyurea Down-Regulates BCL11A, KLF-1 and MYB through miRNA-Mediated Actions to Induce γ-globin Expression: Implications for New Therapeutic Approaches of Sickle Cell Disease. *Clin. Transl. Med.* 5, 15. doi:10.1186/s40169-016-0092-7

Radomski, M. W., Palmer, R. M., and Moncada, S. (1987). Endogenous Nitric Oxide Inhibits Human Platelet Adhesion to Vascular Endothelium. *Lancet* 2, 1057–1058. doi:10.1016/s0140-6736(87)91481-4

Relling, M. V., Klein, T. E., Gammal, R. S., Whirl-Carrillo, M., Hoffman, J. M., and Caudle, K. E. (2020). The Clinical Pharmacogenetics Implementation Consortium: 10 Years Later. *Clin. Pharmacol. Ther.* 107, 171–175. doi:10.1002/cpt.1651

Rigano, P., De Franceschi, L., Sainati, L., Piga, A., Piel, F. B., Cappellini, M. D., et al. (2018). Italian Multicenter Study of Hydroxyurea in Sickle Cell Anemia, IReal-Life Experience with Hydroxyurea in Sickle Cell Disease: A Multicenter Study in a Cohort of Patients with Heterogeneous Descent. *Blood Cell Mol. Dis.* 69, 82–89. doi:10.1016/j.bcmd.2017.08.017

Rumaney, M. B., Ngo Bitoungui, V. J., Vorster, A. A., Ramesar, R., Kengne, A. P., Ngogang, J., et al. (2014). The Co-inheritance of Alpha-Thalassemia and Sickle Cell Anemia Is Associated with Better Hematological Indices and Lower Consultations Rate in Cameroonian Patients and Could Improve Their Survival. *PLoS One* 9, e100516. doi:10.1371/journal.pone.0100516

Sales, R. R., Belisário, A. R., Faria, G., Mendes, F., Luizon, M. R., and Viana, M. B. (2020). Functional Polymorphisms of BCL11A and HBS1L-MYB Genes Affect Both Fetal Hemoglobin Level and Clinical Outcomes in a Cohort of Children with Sickle Cell Anemia. *Ann. Hematol.* 99, 1453–1463. doi:10.1007/s00277-020-04079-2

Sclafani, S., Pecoraro, A., Agrigento, V., Troia, A., Di Maggio, R., Sacco, M., et al. (2016). Study on Hydroxyurea Response in Hemoglobinopathies Patients Using Genetic Markers and Liquid Erythroid Cultures. *Hematol. Rep.* 8, 6678. doi:10.4081/hr.2016.6678

Sheehan, V. A., Crosby, J. R., Sabo, A., Mortier, N. A., Howard, T. A., Muzny, D. M., et al. (2014). Whole Exome Sequencing Identifies Novel Genes for Fetal Hemoglobin Response to Hydroxyurea in Children with Sickle Cell Anemia. *PLoS One* 9, e110740. doi:10.1371/journal.pone.0110740

Sripichai, O., and Fucharoen, S. (2016). Fetal Hemoglobin Regulation in β-thalassemia: Heterogeneity, Modifiers and Therapeutic Approaches. *Expert Rev. Hematol.* 9, 1129–1137. doi:10.1080/17474086.2016.1255142

Stadhouders, R., Aktuna, S., Thongjuea, S., Aghajanirefah, A., Pourfarzad, F., Van Ijcken, W., et al. (2014). HBS1L-MYB Intergenic Variants Modulate Fetal Hemoglobin via Long-Range MYB Enhancers. *J. Clin. Invest.* 124, 1699–1710. doi:10.1172/JCI71520

Steinberg, M. H. (1999). Management of Sickle Cell Disease. *N. Engl. J. Med.* 340, 1021–1030. doi:10.1056/NEJM199904013401307

Steinberg, M. H. (2008). Sickle Cell Anemia, the First Molecular Disease: Overview of Molecular Etiology, Pathophysiology, and Therapeutic Approaches. *Sci. World J.* 8, 1295–1324. doi:10.1100/tsw.2008.157

Stroup, D. F., Berlin, J. A., Morton, S. C., Olkin, I., Williamson, G. D., Rennie, D., et al. (2000). Meta-analysis of Observational Studies in Epidemiology: a Proposal for Reporting. Meta-Analysis of Observational Studies in Epidemiology (MOOSE) Group. *JAMA* 283, 2008–2012. doi:10.1001/jama.283.15.2008

Tang, D. C., Zhu, J., Liu, W., Chin, K., Sun, J., Chen, L., et al. (2005). The Hydroxyurea-Induced Small GTP-Binding Protein SAR Modulates Gamma-Globin Gene Expression in Human Erythroid Cells. *Blood* 106, 3256–3263. doi:10.1182/blood-2003-10-3458

Thein, S. L., Menzel, S., Peng, X., Best, S., Jiang, J., Close, J., et al. (2007). Intergenic Variants of HBS1L-MYB Are Responsible for a Major Quantitative Trait Locus on Chromosome 6q23 Influencing Fetal Hemoglobin Levels in Adults. *Proc. Natl. Acad. Sci. U S A.* 104, 11346–11351. doi:10.1073/pnas.0611393104

Ware, R. E., Despotovic, J. M., Mortier, N. A., Flanagan, J. M., He, J., Smeltzer, M. P., et al. (2011). Pharmacokinetics, Pharmacodynamics, and Pharmacogenetics of Hydroxyurea Treatment for Children with Sickle Cell Anemia. *Blood* 118, 4985–4991. doi:10.1182/blood-2011-07-364190

Ware, R. E. (2010). How I Use Hydroxyurea to Treat Young Patients with Sickle Cell Anemia. *Blood* 115, 5300–5311. doi:10.1182/blood-2009-04-146852

Wonkam, A., Ngo Bitoungui, V. J., Vorster, A. A., Ramesar, R., Cooper, R. S., Tayo, B., et al. (2014). Association of Variants at BCL11A and HBS1L-MYB with Hemoglobin F and Hospitalization Rates Among Sickle Cell Patients in Cameroon. *PLoS One* 9, e92506. doi:10.1371/journal.pone.0092506

Wood, K. C., Hsu, L. L., and Gladwin, M. T. (2008). Sickle Cell Disease Vasculopathy: a State of Nitric Oxide Resistance. *Free Radic. Biol. Med.* 44, 1506–1528. doi:10.1016/j.freeradbiomed.2008.01.008

Wyszynski, D. F., Baldwin, C. T., Cleves, M., Farrell, J. J., Bisbee, A., Kutlar, A., et al. (2004). Genetic Polymorphisms Associated with Fetal Hemoglobin Response to Hydroxyurea in Patients with Sickle Cell Anemia. *Blood* 104, 108. doi:10.1182/blood.V104.11.108.108

Xie, Z., Bailey, A., Kuleshov, M. V., Clarke, D. J. B., Evangelista, J. E., Jenkins, S. L., et al. (2021). Gene Set Knowledge Discovery with Enrichr. *Curr. Protoc.* 1, e90. doi:10.1002/cpz1.90

Yahouédéhou, S. C. M. A., Neres, J. S. D. S., Da Guarda, C. C., Carvalho, S. P., Santiago, R. P., Figueiredo, C. V. B., et al. (2020). Sickle Cell Anemia: Variants in the CYP2D6, CAT, and SLC14A1 Genes Are Associated with Improved Hydroxyurea Response. *Front. Pharmacol.* 11, 553064. doi:10.3389/fphar.2020.553064

Zhou, D., Liu, K., Sun, C. W., Pawlik, K. M., and Townes, T. M. (2010). KLF1 Regulates BCL11A Expression and Gamma- to Beta-Globin Gene Switching. *Nat. Genet.* 42, 742–744. doi:10.1038/ng.637

Zhu, X., Hu, T., Ho, M. H., Wang, Y., Yu, M., Patel, N., et al. (2017). Hydroxyurea Differentially Modulates Activator and Repressors of γ-globin Gene in Erythroblasts of Responsive and Non-responsive Patients with Sickle Cell Disease in Correlation with Index of Hydroxyurea Responsiveness. *Haematologica* 102, 1995–2004. doi:10.3324/haematol.2017.175646

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4.4 Capítulo 4: Farmacogenética da HU na AF

A casuística do estudo foi selecionada a partir da coorte de pacientes com AF descrita por Sales, 2020 (SALES *et al.*, 2020). Assim como descrito no Capítulo 1, os dados e análises realizadas neste capítulo já estão contemplado no protocolo de estudo aprovado pelos comitês de ética das instituições envolvidas (**Anexo 1**). A partir da coorte de 250 pacientes com AF, foram selecionados os que iniciaram tratamento com HU durante o período de acompanhamento da coorte e que atendem aos seguintes critérios de inclusão:

Critérios de inclusão:

- Pacientes que iniciaram tratamento com HU até 31/12/2018;
- Pacientes que possuem determinação da concentração basal de HbF antes do tratamento com HU com mínimo de 90 dias após evento de transfusão ou eventos clínicos significativos (quando for o caso);
- Pacientes que possuem determinação da concentração de HbF após período mínimo de seis meses de tratamento com HU e com, no mínimo, 90 dias após evento de transfusão ou eventos clínicos significativos (quando for o caso);

Critérios de exclusão

- Pacientes que iniciaram regime de hipertransfusão crônica antes do início do tratamento com HU;
- Pacientes que tiveram o tratamento com HU suspenso por um período superior a 90 dias durante o acompanhamento;
- Pacientes que possuíam em seu prontuário médico relatos de falta de adesão ao tratamento com HU;
- Pacientes que não possuem descrição detalhada no prontuário médico das doses de HU administradas durante o período de tratamento;

A partir da aplicação dos critérios de inclusão/exclusão, foram selecionados 110 pacientes em tratamento com HU. O número absoluto de pacientes excluídos com base nos critérios está descrito no Fluxograma de seleção de pacientes (**Figura 8**).

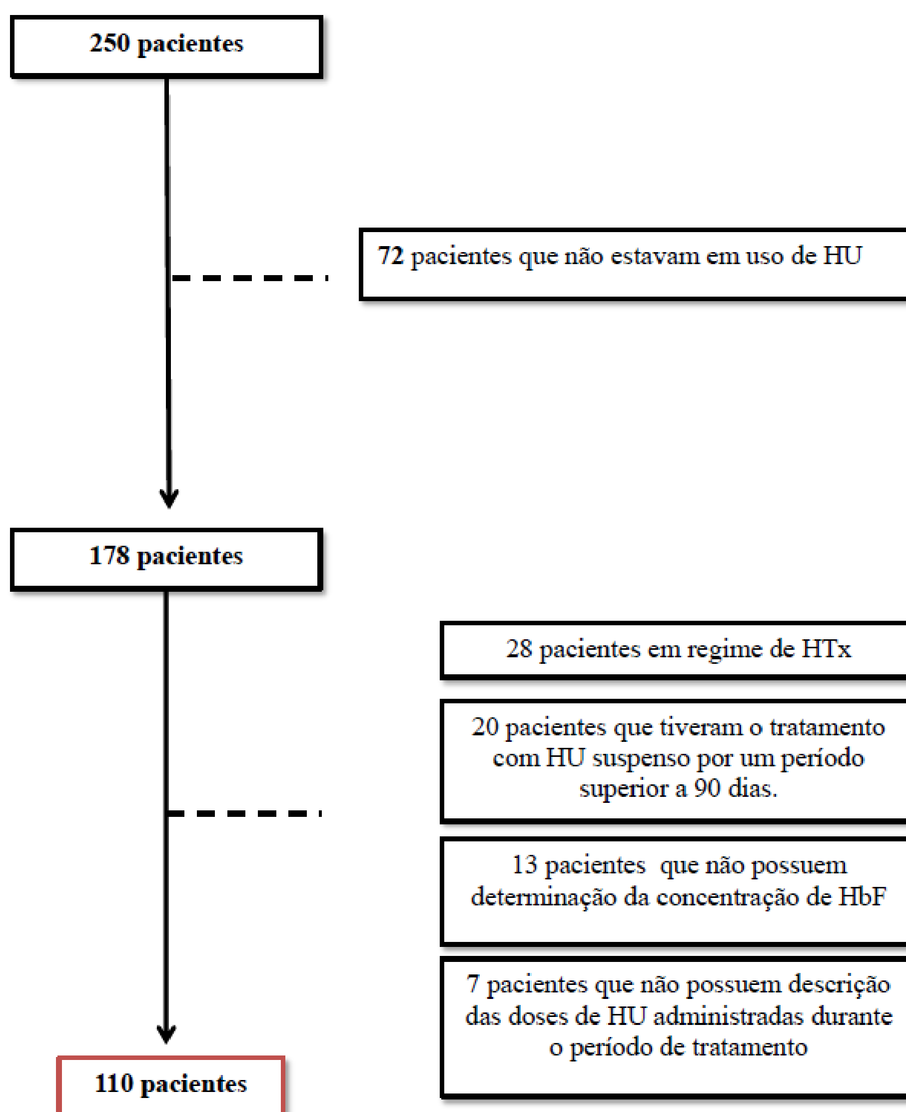

Figura 8: Fluxograma de seleção de pacientes tratados com HU de acordo com os critérios estabelecidos

A quantificação da concentração relativa total de HbF foi realizada utilizando-se o mesmo método descrito no **Capítulo 1**. Para determinação da HbF basal foi considerado o resultado do último exame após os cinco anos de idade, desprezando-se os valores após transfusões sanguíneas (90 dias) ou eventos clínicos significativos (crise de dor, infecção grave, sequestro esplênico agudo, crise aplástica e síndrome torácica aguda).

A HbF final foi determinada pelo último exame de quantificação realizado durante o período de acompanhamento (mínimo de 6 meses), a depender da disponibilidade de dados no prontuário juntamente.

Para determinação da alteração na concentração de HbF da basal para a final foi calculado o DeltaHbF (ΔHbF), que corresponde à subtração do valor de HbF Final pelo valor da HbF basal.

Para determinação da dose de HU foi coletada a data de início do tratamento com HU, assim como, a dose inicial e o peso do paciente. Por se tratarem de crianças em fase de desenvolvimento o peso foi criteriosamente registrado de acordo com sua redução ou aumento e observada ou realizada correção de dose (mg/kg/dia), quando necessário. Por essa razão e também porque o protocolo de tratamento com HU é preconizado para AF com escalonamento de dose até a Dose Máxima Tolerada (DMT), a dose média de cada paciente foi calculada como média ponderada pelo tempo de cada dose.

Durante o período de acompanhamento dos pacientes, as doses e o tempo permanecido em cada uma delas foram registrados (**Figura 8**). Períodos de interrupção de fornecimento da dose de HU pelo SUS e/ou desabastecimento da Farmácia de Minas do estado ocorreram durante o acompanhamento o que, consequentemente, acarretou a perda de adesão de alguns pacientes. Portanto, os pacientes que interromperam o tratamento com HU por três meses ou mais foram excluídos do estudo.

O protocolo de tratamento com HU, a concentração relativa total de HbF e a ocorrência de eventos de neutropenia e transfusão sanguínea durante o tratamento foram coletados dos prontuários médicos dos pacientes. Neutropenia foi definida como contagem de neutrófilos inferior a 2.000/nm^3.

A genotipagem dos SNPs testados foi descrita no Capítulo 1.

Para avaliação da associação independente dos SNPs com o aumento da HbF (Delta HbF), foi usada análise de regressão linear múltipla seguindo o método *stepwise*. As variáveis genéticas foram retiradas a cada rodada, em ordem decrescente do valor de significância. As covariáveis gênero, idade, dose, período de tratamento e HbF basal foram mantidas até o final da análise, pois são características inerentes aos pacientes ou que tem seu impacto comprovado no desfecho. Foram considerados como estatisticamente significativos os testes em que a probabilidade de erro alfa foi igual ou inferior a 0,05.

TITLE PAGE

Baseline fetal hemoglobin and single nucleotide polymorphisms of *BCL11A* gene predict HbF changes in children with sickle cell anemia treated with Hydroxyurea

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Abstract

Increased fetal hemoglobin (HbF) levels reduces clinical severity of sickle cell anemia (SCA). Hydroxyurea (HU) is used for the treatment of SCA, and its clinical effectiveness is related but not restricted to induction of HbF. Notably, there is variability in the response to HU therapy among SCA patients. However, the effect of single genetic polymorphisms (SNPs) on HU-induced HbF changes in patients with SCA remains is not fully understood. Here, we characterized the time of follow-up of HU therapy and HbF changes from baseline to final HbF (ΔHbF%) in a retrospective cohort of 110 children with SCA (HbSS) treated with HU. Next, we examined whether SNPs of *BCL11A* gene and *HBS1L-MYB* intergenic region (named HMIP-2) can predict HbF changes, and their association with the risk of transfusion and neutropenia. Multiple linear regression model adjusted by age, gender, HU dosage and time of follow-up identified baseline HbF (p=0.002; β-coefficient=0.277) and the minor alleles of rs7599488 (T; p=0.011; β-coefficient=0.236), rs766432 (C; p= 0.002; β-coefficient=-0.439) and rs4671393 (A; p=0.003; β-coefficient=0.416) SNPs of *BCL11A* as independent predictors for ΔHbF%. Subjects carrying minor allele of rs4671393 [AG + AA: 13.9 (9.0 – 20.5) per 100 patient-years], exhibited higher risk [IRR = 0.51 (95% CI 0.28-0.92); p = 0.03] of transfusion compared to the subjects homozygous for the reference allele [GG: 7.1 (4.3 – 10.9) per 100 patient-years]. Patients who did not had neutropenia showed a significant higher ΔHbF% (p = 0.034). In conclusion, our findings suggests that baseline HbF and SNPs of *BCL11A* are predictors of HU response in children with SCA.

Key-words: *BCL11A* gene; fetal hemoglobin; hydroxyurea; pharmacogenetics; single nucleotide polymorphisms; sickle cell anemia.

Introduction

Sickle cell anemia (SCA) is a monogenic hemoglobin disorder which express a wide spectrum of clinical phenotypes[1]. Despite seven decades since the discovery of the causative molecular mechanisms of SCA, to mitigate its high morbidity and mortality remains a challenge[2,3].

Fetal hemoglobin (HbF) is a major main predictor of clinical severity of SCA, displaying protective effect on several clinical outcomes, as reviewed elsewhere[4]. Genome wide association studies (GWAS) identified several single nucleotide polymorphisms (SNPs) associated with HbF levels in SCA and highlighted the cluster of beta globin genes, the *BCL11A* gene and the *HBS1L-MYB* intergenic region as three main loci involved in the HbF regulation[5–9].

Hydroxyurea (HU) is a HbF-inducer drug used to treat SCA, which reduces morbidity and mortality of patients with SCA[10]. HU ameliorates the hematological and clinical outcomes of SCA by affecting HbF expression, but it is not restricted to these effects[11]. In the bone marrow, HU selectively kills the cells increasing the number of erythroblasts with high HbF expression, thereby reducing the number of circulating sickled cells[12]. High HbF levels increases the erythroblasts survival decreasing hemolysis. HU also increases the levels of total hemoglobin and reduces neutrophils, monocytes and reticulocytes. In the endothelium, HU alters the expression of adhesion molecules and the nitric oxide generation, thereby attenuating endothelium dysfunction[13]. Taken together, these hematological changes decrease the risk of vaso-occlusion complications related to SCA.

Notably, there is substantially interindividual variability on response and toxicity to HU among patients with SCA. While some patients achieved 10–15% in HbF changes, others achieve 40%[14]. In addition, while some patients tolerate the higher HU doses (30–35 mg/kg/day), others develop severe myelosuppression even at low doses[14]. A recent systematic review focusing on HbF response to HU therapy in patients with SCA highlighted the scarce and heterogeneous literature regarding candidate genes and SNPs, HU dose, patient age, and HbF outcomes in response to HU[15]. Notably, there is evidence that SNPs located in intron 2 of *BCL11A* gene affect HbF changes in patients with SCA treated with HU[15]. However, further studies are needed to examine whether

such SNPs may predict the success of HU therapy in ameliorating other hematological parameters and reducing the incidence of clinical complications.

In this study, we first characterized the time of follow-up of HU therapy, along with the HbF changes from baseline to final HbF in a cohort of pediatric patients with SCA treated with HU. Next, we examined the effects of the *BCL11A* and HMIP-2 SNPs on baseline HbF and HbF changes upon HU therapy. Finally, we examined whether these SNPs are associated with the incidence risk of transfusion and neutropenia in this cohort.

Materials and Methods

This retrospective cohort study involves Brazilian pediatric patients with SCA (HbSS) treated with HU recruited from the Center of Hematology and Hemotherapy of Minas Gerais (HEMOMINAS), in Belo Horizonte, capital of Minas Gerais, Southeastern Brazil. The 110 children who started HU therapy until December/2018 were selected from the 250 children enrolled according to eligibility criteria described in a previous study[16]..

The study protocol is in accordance with Declaration of Helsinki and was approved by the Institutional Review Board from the HEMOMINAS (certification number: 4204.8215.6.0000.5118). Written informed consent were obtained from every child and parents/guardians.

HbF relative concentrations were determined by using hemoglobin electrophoresis in alkaline pH or high-performance liquid chromatography (HPLC). Baseline HbF relative concentration for each patient was the last available test after the age of 5 years and before HU therapy. Measurements performed until ninety days after blood transfusion or relevant clinical events (pain crisis, infection requiring hospitalization, acute splenic sequestration, aplastic crises or acute chest syndrome) were not considered. Children who started regular hypertransfusion therapy before or during the HU therapy was excluded from the study or had their follow-up time ended immediately before the hypertransfusion therapy start. Final HbF was defined as the last HbF measurement after a minimal of 6 months of HU therapy. The delta (ΔHbF%) was calculated subtracting baseline HbF value from the Final HbF value.

The HU dosage was calculated as time weighted average in mg/kg/day. Children who had interruption of HU therapy for a minimal of three months were excluded from the study or had their follow-up time ended immediately before the interruption. Neutropenia was defined as a neutrophil count $<2000/nm^3$. Genomic DNA extracted from the venous blood samples of patients and the SNP genotyping were performed as previously described[16].

The mean of HbF relative concentration was compared by univariate linear regression. A multiple linear regression model was constructed to identify a set of genetic predictors of HbF changes (ΔHbF%) among the candidate SNPs. The model was adjusted by age, gender, HU dosage, time of follow-up and baseline HbF. Genetic variables (SNPs genotype groups) were included as covariates. Through the stepwise method, the covariates were removed one by one and the final model included only those that were statistically significant ($P < 0.05$). Statistical analyses were conducted on SPSS 21.0 software (SPSS Inc.; Chicago, IL, USA).

The incidence of clinical outcomes was reported by relative rates to 10 patient-years (pt-yrs), with 95% confidence intervals. The incidence rate ratio (IRR) of clinical outcomes between genotype groups was compared using *OpenEpi* online software, PersonTime2/PersonTime2 option (http://www.openepi.com/PersonTime2/PersonTime2.htm) by Fisher's Exact Test. The tests were considered statistically significant when the probability of an alpha error was equal to or less than 0.05.

Results

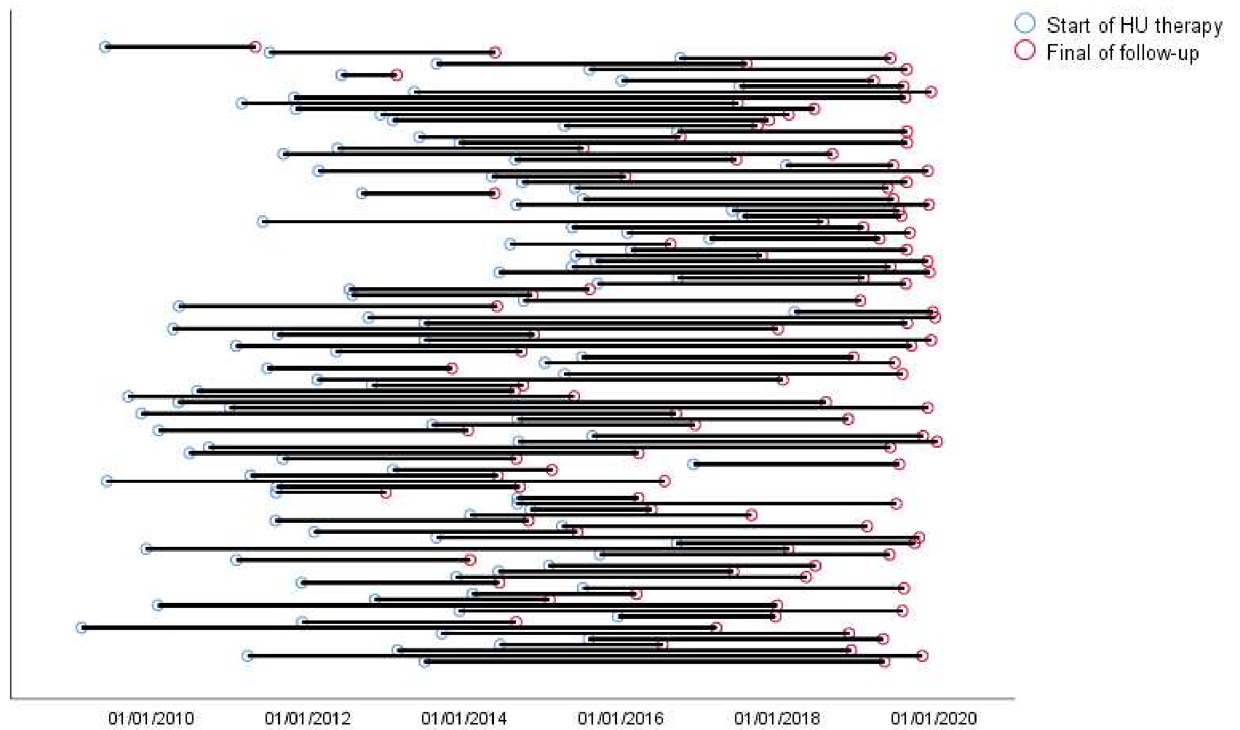
From the 110 children with SCA in HU therapy enrolled from HEMOMINAS Foundation, 59 (53.6%) were females. **Table 1** shows the data related to HU therapy in the cohort. **Figure 1A** shows the time of follow-up for each subject treated with HU as a timeline graph. **Figure 1B** shows the changes on HbF relative concentration from baseline to minimal of six months of HU therapy for each subject, which was the basis for the calculation of delta HbF% (ΔHbF%).

Table 1. Hydroxyurea treatment in a cohort of 110 pediatric patients with SCA.

Cohort characteristics	Mean ± standart error	95% CI	
		Upper	Lower
Age (start; years)	10,01 ± 2,8	9,3	10,4
Age (final; years)	14,2 ± 3,1	13,3	17,2
Time of follow-up (years)	4,2 ± 2.03	1401,3	1681,1
HU dosage (mg/kg/day)	24,4 ± 4,4	23,6	25,3
Baseline HbF (%)	12,1 ± 6,0	10,9	13,2
Final HbF (%)	18,9 ± 8,7	17,3	20,6
Delta HbF (%)	6,88 ± 8,2	5,3	8,4

Table 2 summarizes the association tests between baseline HbF, final HbF (after minimal of six month of HU therapy) and the ΔHbF% with the candidate SNPs of *BCL11A* and HMIP-2 as well as the respective β-coefficient. The minor alleles of the rs1427407 (T; p = 0.001), rs766432 (C; p = 0.030) and rs4671393 (A; p = 0.003) SNPs of *BCL11A* gene and the minor alleles of the rs4895441 (G; p = .008), rs9402686 (A; p = 0.004) and the rs9494145 (C; p = 0.026) SNPs of HMIP-2 were associated with increased baseline HbF relative concentration. There was no SNP associated with final HbF relative concentration, but the rs9399137 showed a marginally significative p-value (0.061). The minor alleles of the rs1427407 (p = 0.037) and the rs7599488 (p = 0.001) were associated with increased ΔHbF%, while the minor allele of rs766432 (p = 0.006) was associated with reduced ΔHbF%. The allele and genotype frequencies of the SNPs are described on the **Supplementary Table 1**.

A



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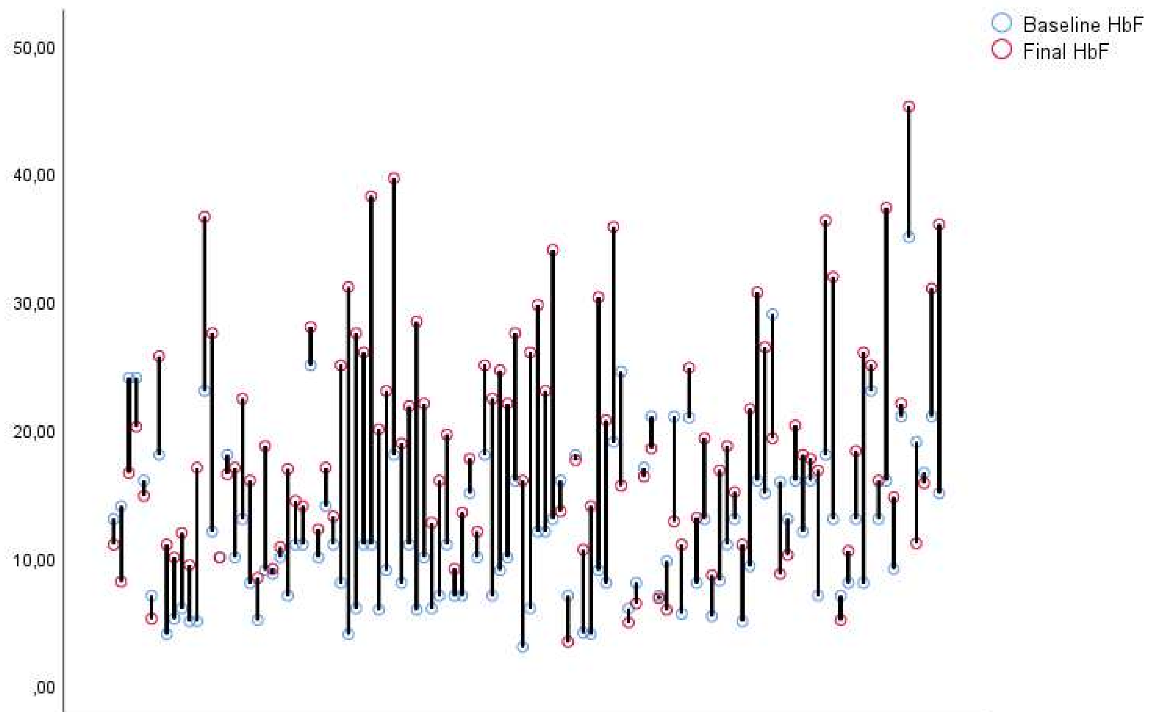


Figure 1. **A)** Follow-up time of each pediatric patient of the cohort of 110 patients with SCA treated with HU. **B)** HbF changes of each pediatric patient of the cohort.

Table 2. Association tests between SNPs and baseline, final and delta HbF in the cohort of 110 patients with SCA treated with HU

Locus, SNPs (alleles)	Genotype groups	Baseline HbF p-value	β	Final HbF p-value	β	Delta HbF p-value	β
BCL11A							
rs1427407 (G>T)	GG x GT + TT	**0.001**	0.306	0.795	0.025	**0.037**	-0.199
rs7599488 (C>T)	CC x CT + TT	0.603	-0.050	0.009	0.247	**0.001**	0.301
rs766432 (A>C)	AA x AC + CC	**0.030**	0.207	0.303	-0.099	**0.006**	-0.258
rs11886868 (T>C)	TT x TC + CC	0.052	0.186	0.421	0.077	0.563	-0.056
rs4671393 (G>A)	GG x GA +AA	**0.003**	0.281	0.206	0.122	0.416	-0.078
rs7557939 (A>G)	AA x AG + GG	0.091	0.162	0.659	0.043	0.436	-0.075
HMIP-2							
rs9399137 (T>C)	TT x TC + CC	0.088	0.163	0.061	0.179	0.469	0.070
rs4895441 (A>G)	AA x AG + GG	**0.008**	0.250	0.114	0.151	0.808	-0.023
rs9402686 (G>A)	GG x GA + AA	**0.004**	0.078	0.169	0.162	0.811	-0.023
rs9494145 (T>C)	TT x TC + CC	**0.026**	0.212	0.598	0.051	0.287	-0.102

Table 3. Multiple linear regression adjusted model of delta HbF primary endopoint in the cohort of 110 patients with SCA treated with HU

Covariates	β	Explained variance (%)	p-value
Constant	-		0.082
Age	-0.248	6.0	0.007
Gender	0.078	0.6	0.358
HU dosage	0.027	-	0.763
Time of follow-up	-0.212	4.4	0.013
Baseline HbF	-0.277	7.6	0.002
BCL11A rs7599488	0.236	5.5	0.011
BCL11A rs766432	-0.439	19.3	0.002
BCL11A rs4671393	0.416	17.3	0.003

From all the candidate SNPs, the multiple linear regression model adjusted by age, gender, HU dosage and time of HU therapy identified baseline HbF ($p = 0.002$), and the SNPs rs7599488 ($p = 0.011$), rs766432 ($p = 0.002$) and rs4671393 (A; $p = 0.003$) of *BCL11A* gene as independent predictors for ΔHbF%. The minor alleles of rs7599488 (T) and rs4671393 (A) were associated with increased ΔHbF%, while the increase baseline HbF and the minor allele of rs766432 (C) was associated with reduced ΔHbF% as independent variables. The final model, the β-coefficient and the explained variance for each predictor is showed in **Table 3**.

The incidence rate per 100 patient-years of transfusion and neutropenia per genotype of the SNPs is described in **Supplementary Table 2**. Association tests between genotypes and these clinical outcomes as well as the incidence rate ratio of subjects carrying minor alleles compared to reference homozygous are shown in **Table 4**. Notably, subjects carrying the minor allele of the *BCL11A* SNP rs4671393 [AG + AA: 13.9 (9.0 – 20.5) per 100 patient-years], an allele independently associated with increased ΔHbF%, exhibited higher risk of transfusion [IRR = 0.51 (0.28 – 0.92); $p = 0.03$] as compared to the subjects homozygous for the reference allele [GG: 7.1 (4.3 – 10.9) per 100 patient-years]. There was a marginally significant association ($p = 0.07$) of the minor allele of rs1427407 SNP [GT + TT: 7.6 (3.6 – 14.0) versus GG: 14.5 (10.7 – 19.2) per 100 patient-years] with a lower risk of neutropenia. Additionally, a significantly higher ΔHbF was found in children who did not experienced neutropenia (Mann-Whitney U test; $p = 0.34$) (**Figure 2**).

Table 4. Incidence rate ratio (IRR) of transfusion and neutropenia per 10 patients-year according to SNP genotype in the cohort of 110 patients with SCA treated with HU

SNPs	**Transfusion**		**Neutropenia**	
	Rate Ratio (I/II)* 95% CI	p-value	Rate Ratio (I/II)* 95% CI	p-value
BCL11A				
rs1427407	0.66 (0.36-1.19)	0.22	1.91 (0.97-3.78)	0.07
rs7599488	0.81 (0.44-1.5)	0.62	0.72 (0.42-1.25)	0.29
rs766432	0.66 (0.37-1.19)	0.22	1.39 (0.78-2.48)	0.32
rs11886868	1.31 (0.73-2.35)	0.44	0.82 (0.49-1.40)	0.55
rs4671393	0.51 (0.28-0.92)	**0.03**	1.42 (0.82-2.48)	0.26
rs7557939	1.40 (0.78-2.51)	0.32	0.70 (0.41-1.21)	0.20
HMIP-2				
rs9399137	1.08 (0.50-2.32)	>0.99	0.67 (0.37-1.20)	0.23
rs4895441	0.58 (0.28-1.21)	0.22	0.56 (0.30-1.05)	0.11
rs9402686	9.73 (7.10-13.02)	0.47	0.63 (0.36-1.09)	0.13
rs9494145	1.01 (0.49-2.11)	0.99	1.26 0.95-1.62)	0.22

* Group I: genotype without minor allele; Group II: genotypes with minor allele in homozygosis or heterozygosis. Abbreviations: CI, Confidence Interval.

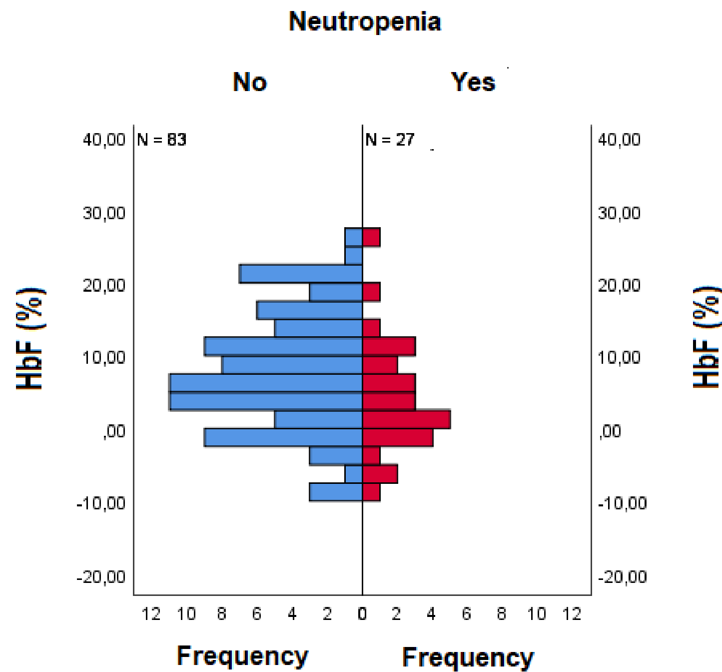


Figure 2. Distribution of ΔHbF according to neutropenia occurrence in the cohort of 110 children with SCA treated with HU. A significantly higher ΔHbF was found in children who did not experienced neutropenia (Mann-Whitney U test; p = 0.34). Average points: 59.17(Yes) and 44.20 (No).

DISCUSSION

The main novel findings reported here were that (1) The SNPs rs7599488, rs766432 and rs4671393 of intron 2 of *BCL11A* gene were identified as predictors of HbF changes upon HU therapy in a multiple linear regression analysis adjusted for age, gender, HU dosage, time of follow-up and baseline HbF in a Brazilian cohort of pediatric patients with SCA; (2) Subjects carrying minor allele of rs4671393 of *BCL11A* exhibited significantly higher risk of transfusion incidence as compared to the subjects homozygous for the reference alleles.

This is a well-characterized cohort of pediatric patients with SCA from Minas Gerais state, Southeastern Brazil, which has been followed up for more than a decade[16–20]. The SNPs have been previously examined for percentage of explained variance in baseline HbF, and for their association with hematologic parameters and clinical outcomes[16]. In the present study, our focus was on a subcohort of children with SCA treated with HU who had retrieved reliable data

from the medical records about the time of follow-up of HU therapy, along with the HbF changes from baseline to final HbF upon HU therapy. Noteworthy, this subcohort is strictly composed by pediatric patients with 5-18 years, as opposed to the other two cohorts among the seven cohorts studies included in a recent systematic review [15]. Our primary endpoint is delta HbF, from baseline to final HbF after a minimum of six months of uninterrupted HU therapy. The dose of HU was carefully retrieved and adjusted by the weight (even when there was no weight adjustment by physicians) since the cohort is composed by pediatric patients in growth phase. Moreover, the dose was adjusted by the time of each dose since the HU because the dose escalation int the protocol of HU therapy. We also retrieved the incidence of neutropenia and transfusion episodes during HU therapy analyzed as secondary endpoints of the retrospective study.

Three SNPs (rs1427407, rs7599488 and rs766432) of the *BCL11A* gene were associated with ΔHbF% in the univariate analyses. After adjusting by baseline HbF, the rs1427407 SNP did not reached significance level and the rs7599488, rs766432 and rs4671393 SNPs were found as predictor of ΔHbF% in a model adjusted by age, gender, dose of HU, time of therapy and baseline HbF. These results corroborate findings from a recent systematic review of seven cohort studies which raised the evidence that SNPs in the intron 2 of *BCL11A* influences the HbF response in patients with SCA upon HU therapy[15].

The SNP rs1427407 was found to be associated with HbF% under maximum tolerated dose (MTD) and with ΔHbF% in a cohort of 111 Brazilian adults and children with SCA treated for a minimum of six months with an average dose of 23 mg/kg/day of HU[21]. Similarly, rs1427407 was also found to be associated with HbF% under MTD in a cohort of children with SCA from United States treated for a minimum of 6 months with about 24 mg/kg/day of HU[14]. From the functional perspective, the SNP rs1427407 is a critical cis-regulatory element of the intron 2 of *BCL11A*. It falls in an erythroid enhancer of *BCL11A* (+62 DNAse I Hypersensitive Site) and disrupt a consensus sequence [CTG(n9)] enriched for GATA1 and TAL1 transcription factor binding[22], which are indeed more frequent in the G-allele than T-allele in the sample of primary erythroblasts[22]. Therefore, lack of association of rs1427407 SNPs with ΔHbF% after adjustments can be due

to the lower frequency of its minor allele (T allele) compared to the other associated SNPs of *BCL11A*.

The rs7599488 and rs766432 SNPs of the *BCL11A* were identified as predictor of HbF changes corroborating previous finding of a cohort of children with SCA treated for a minimum of six months with an average dose of 25 mg/kg/day of HU[23]. We found the minor allele of rs7599488 (T allele) to be associated with lower baseline HbF and higher ΔHbF%, the same direction observed by Green et al., even that the association with baseline HbF has not reached the level of significance[23]. However, while we found the minor allele of rs766432 to be associated with lower ΔHbF%, Green et al. found it to be associated with higher ΔHbF%.

The minor allele (A allele) of rs4671393 SNP of the *BCL11A* was identified as predictor of increased ΔHbF% in our smaller cohort, which is corroborated by previous findings[23]. This SNPs was also identified as a predictor of baseline HbF in the larger cohort of Brazilian children with SCA from Minas Gerais state before the starting HU therapy[16].

There is no association between SNPs of HMIP-2 and ΔHbF% in our cohort. This results is agreement with the previous systematic review which did not found evidence that HMI-2 influences the HbF changes in patients with SCA upon HU therapy[15].

Notably, subjects carrying the A allele of the SNP rs4671393 of *BCL11A* gene showed significant higher risk of transfusion incidence than non-carriers of the A allele, which was associated with higher ΔHbF% in the adjusted multivariate model. However, the HU therapy reduces the transfusion frequency in sickle cell patients, as reviewed elsewhere[24]. In addition, the same A allele was previously associated with higher baseline HbF, lower risk of transfusion (independent of HbF effect) and infections per 100 patients-year in the larger cohort untreated with HU[16]. This could be a type II error, or maybe the protective effect of A allele on transfusion is hidden by HU.

The HU displays a myelosuppressive effect, selectively killing cells of the bone marrow[25]. In children, myelotoxicity has been characterized as a hemoglobin level lower than 5 g/dL or when there is a 20% reduction compared

to the basal hemoglobin concentration, neutrophils lower than 2.0 x 10^9/L, reticulocyte count lower than 80 x 10^9/L and platelets less than 80 x 10^9/L. Subjects carrying the minor T allele of the SNP rs1427404 of *BCL11A* showed higher incidence of neutropenia per 10 patients-year than non-carriers of the T allele, but it did not reach the significance level (p = 0,07). Probably, we had no sampling power to find the association. The T allele of the SNP rs1427404 was previously found to be associated with higher baseline HbF, lower reticulocyte count and lower risk of transfusion[16]. Here, we reported its association with higher baseline and delta HbF. Maybe, non-carriers of the T allele (GG genotype) show more severe SCA due to low HbF, thereby requiring more frequent dose advancement attempts and increasing the neutropenia frequency. Indeed, patients with SCA who did had neutropenia showed a significant lower ΔHbF% that may have been caused by dose retreat as response to neutropenia.

Several confounding factors contributing to interindividual variability in response to HU in patients with SCA and may introducing bias in analyzing the effect of SNPs on HbF changes: (1) The daily dose of HU is a critical aspect of the treatment: once HU is a myelosuppressive drug, cytopenia is an expected effect, which needs to be carefully monitored. Therefore, dose adjustment can be difficult in some patients and advance and retreat of doses are frequent; (2) The adherence to the treatment protocol. The HU is provided in capsules containing 500 mg, which usually need to be diluted in water to adjust the daily dose for children to ingest only a fraction of the solute; (3) The access to HU is still an important barrier for many patients. In Brazil, HU is provided by the public health system but there may be temporary interruption of supply. Therefore, to mitigate the bias caused by these real-world limitations in our observational study, we properly addressed these factors during the patient's follow-up and adjusted for them in the multivariate analyses.

Other limitations should be taken into account when analyzing the results of this study. The use of medical records as the source of retrospective data is one of the limitations, because the data were generated for clinical follow-up and not for research purpose. Moreover, the limited sample size may have resulted in type I error.

In conclusion, our findings suggests that baseline HbF and of SNPs of *BCL11A* gene are predictors of HU response in pediatric patients with SCA.

References

1. Williams, T. N. & Thein, S. L. Sickle Cell Anemia and Its Phenotypes. *Annu. Rev. Genomics Hum. Genet.* **19**, 113–147 (2018).
2. L, P. & Ha, I. Sickle cell anemia a molecular disease. *Science* **110**, (1949).
3. Kato, G. J. *et al.* Sickle cell disease. *Nat. Rev. Dis. Primer* **4**, 18010 (2018).
4. Akinsheye, I. *et al.* Fetal hemoglobin in sickle cell anemia: molecular characterization of the unusually high fetal hemoglobin phenotype in African Americans. *Am. J. Hematol.* **87**, 217–219 (2012).
5. Thein, S. L. *et al.* Intergenic variants of HBS1L-MYB are responsible for a major quantitative trait locus on chromosome 6q23 influencing fetal hemoglobin levels in adults. *Proc. Natl. Acad. Sci.* **104**, 11346–11351 (2007).
6. Uda, M. *et al.* Genome-wide association study shows BCL11A associated with persistent fetal hemoglobin and amelioration of the phenotype of -thalassemia. *Proc. Natl. Acad. Sci.* **105**, 1620–1625 (2008).
7. Solovieff, N. *et al.* Fetal hemoglobin in sickle cell anemia: genome-wide association studies suggest a regulatory region in the 5′ olfactory receptor gene cluster. *Blood* **115**, 1815–1822 (2010).
8. Bhatnagar, P. *et al.* Genome-wide association study identifies genetic variants influencing F-cell levels in sickle-cell patients. *J. Hum. Genet.* **56**, 316–323 (2011).
9. Mtatiro, S. N. *et al.* Genome Wide Association Study of Fetal Hemoglobin in Sickle Cell Anemia in Tanzania. *PLoS ONE* **9**, e111464 (2014).
10. Charache, S. *et al.* Hydroxyurea: effects on hemoglobin F production in patients with sickle cell anemia. *Blood* **79**, 2555–2565 (1992).
11. 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. *N. Engl. J. Med.* **332**, 1317–1322 (1995).

12. McGann, P. T. & Ware, R. E. Hydroxyurea therapy for sickle cell anemia. *Expert Opin. Drug Saf.* **14**, 1749–1758 (2015).
13. Potoka, K. P. & Gladwin, M. T. Vasculopathy and pulmonary hypertension in sickle cell disease. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **308**, L314-324 (2015).
14. Ware, R. E. *et al.* Pharmacokinetics, pharmacodynamics, and pharmacogenetics of hydroxyurea treatment for children with sickle cell anemia. *Blood* **118**, 4985–4991 (2011).
15. Sales, R. R., Nogueira, B. L., Tosatti, J. A. G., Gomes, K. B. & Luizon, M. R. Do Genetic Polymorphisms Affect Fetal Hemoglobin (HbF) Levels in Patients With Sickle Cell Anemia Treated With Hydroxyurea? A Systematic Review and Pathway Analysis. *Front. Pharmacol.* **12**, 779497 (2021).
16. Sales, R. R. *et al.* Functional polymorphisms of BCL11A and HBS1L-MYB genes affect both fetal hemoglobin level and clinical outcomes in a cohort of children with sickle cell anemia. *Ann. Hematol.* **99**, 1453–1463 (2020).
17. Belisário, A. R. *et al.* Association between ENPP1 K173Q and stroke in a newborn cohort of 395 Brazilian children with sickle cell anemia. *Blood* **126**, 1259–1260 (2015).
18. Belisário, A. R. *et al.* Interleukin-10 haplotypes are not associated with acute cerebral ischemia or high-risk transcranial Doppler in a newborn cohort of 395 children with sickle cell anemia. *Rev. Bras. Hematol. E Hemoter.* **39**, 108–114 (2017).
19. Belisário, A. R. *et al.* Reticulocyte count is the most important predictor of acute cerebral ischemia and high-risk transcranial Doppler in a newborn cohort of 395 children with sickle cell anemia. *Ann. Hematol.* **95**, 1869–1880 (2016).
20. Ar, B. *et al.* Association of alpha-thalassemia, TNF-alpha (-308G>A) and VCAM-1 (c.1238G>C) gene polymorphisms with cerebrovascular disease in a newborn cohort of 411 children with sickle cell anemia. *Blood Cells. Mol. Dis.* **54**, (2015).

21. Friedrisch, J. R. *et al.* The role of BCL11A and HMIP-2 polymorphisms on endogenous and hydroxyurea induced levels of fetal hemoglobin in sickle cell anemia patients from southern Brazil. *Blood Cells. Mol. Dis.* **62**, 32–37 (2016).
22. Bauer, D. E. *et al.* An Erythroid Enhancer of BCL11A Subject to Genetic Variation Determines Fetal Hemoglobin Level. *Science* **342**, 253–257 (2013).
23. Green, N. S. *et al.* Candidate Sequence Variants and Fetal Hemoglobin in Children with Sickle Cell Disease Treated with Hydroxyurea. *PLoS ONE* **8**, e55709 (2013).
24. Wong, T. E., Brandow, A. M., Lim, W. & Lottenberg, R. Update on the use of hydroxyurea therapy in sickle cell disease. *Blood* **124**, 3850–3857 (2014).
25. Steinberg, M. H. Management of Sickle Cell Disease. *N. Engl. J. Med.* **340**, 1021–1030 (1999).

Supplementary Material

Supplementary Table 1. Genotype frequencies in the cohort of 110 patients with SCA

BCL11A Chromosome-2 position	SNP ID	Allele change	MAF*	Genotype groups**	HWE***
60490908	rs1427407	G>T	0,2	GG (72); GT+TT (38)	0,4676
60491212	rs7599488	C>T	0,373	CC (46); CT+TT (64)	0,3393
60492835	rs766432	A>C	0,223	AA (67); AC+CC (43)	0,9194
60493111	rs11886868	T>C	0,341	TT (47); TC+CC (63)	0,9478
60493816	rs4671393	G>A	0,241	GG (64); GA+AA (46)	0,8945
60494212	rs7557939	A>G	0,341	AA (46); AG+GG (64)	0,6224
HMIP-2 Chromosome-6 position	**SNP ID**	**Allele change**	**MAF***	**Genotype groups****	**HWE*****
135097880	rs9399137	T>C	0,095	TT (89); TC+CC (21)	0,6967
135105435	rs4895441	A>G	0,064	AA (96); AG+GG (14)	1,0
135106679	rs9402686	G>A	0,123	GG (83); GA+AA (27)	0,3242
135111414	rs9494145	T>C	0,1	TT (88); TC+CC (22)	0,6232

* MAF, *Minor Allele Frequency*;

** Absolute number;

*** HWE, Hardy-Weinberg Equilibrium (p > 0,05).

Supplementary Table 2. Incidence rate of genotype groups in the cohort of 110 patients with SCA

SNPs	Transfusion		Neutropenia	
	Group I	**Group II**	**Group I**	**Group II**
BCL11A				
rs1427407	0.85 (0.56-1.22)	1.29 (0.75-2.07)	1.45 (1.07-1.92)	1.76 (0.36-1.40)
rs7599488	0.86 (0.49-1.39)	1.05 (0.70-1.51)	1.02 (0.61-1.59)	1.41 (1.01-1.93)
rs766432	0.83 (0.53-1.22)	1.25 (0.76-1.93)	1.39 (1.00-1.88)	1.00 (0.57-1.62)
rs11886868	1.12 (0.71-1.68)	0.85 (0.54-1.30)	1.12 (0.71-1.68)	1.36 (0.95-1.89)
rs4671393	0.71 (0.43-1.09)	1.93 (0.90-2.05)	1.42 (1.01-1.93)	0.99 (0.59-1.58)
rs7557939	1.16 (0.73-1.75)	0.83 (0.52-1.26)	1.01 (0.62-1.56)	1.44 (1.02-1.97)
HMIP-2				
rs9399137	0.99 (0.70-1.36)	0.92 (0.39-1.80)	1.15 (0.83-1.54)	1.71 (0.96-2.83)
rs4895441	0.89 (0.63-1.23)	1.53 (0.70-2.90)	1.14 (0.83-1.50)	2.04 (1.05-1.50)
rs9402686	1.05 (0.74-1.44)	0.74 (0.32-1.45)	1.10 (0.78-1.51)	1.75 (1.05-2.74)
rs9494145	0.98 (0.68-1.35)	0.96 (0.44-1.83)	1.14 (0.82-1.54)	1.71 (0.98-2.78)

Abbreviations: * Group I: genotype without minor allele; Group II: genotypes with minor allele in homozygosis or heterozygosis.

5 Discussão

A hemoglobina fetal (HbF) é a fração predominante de hemoglobinas durante a vida fetal, devido à sua alta afinidade pelo oxigênio (SANKARAN *et al.*, 2008). A HbF é composta pelas cadeias alfa e gama, sendo a cadeia gama codificada pelos genes da γ-globina (*HBG1* e *HBG2*) no agrupamento de genes da β-globina (SANKARAN *et al.*, 2008). Importante destacar que a concentração de HbF varia entre os pacientes com anemia falciforme (AF) e entre os eritrócitos de cada indivíduo (STEINBERG, 2008). A HbF inibe a polimerização da HbS em situações de baixa oxigenação e, portanto, é considerada um importante modificador da evolução clínica de pacientes com AF (LETTRE; BAUER, 2016b). Devido à inibição parcial da polimerização da HbS, os eventos fisiopatológicos de vaso-oclusão, bem como a anemia hemolítica são reduzidos, o que ameniza as complicações da AF (STEINBERG, 2005).

BCL11A, uma proteína dedo de zinco, se liga diretamente no agrupamento de genes da β-globina, inibindo a expressão dos genes γ-globina e consequentemente a produção de HbF, concomitantemente ao aumento da expressão da hemoglobina adulta (BAUER; ORKIN, 2015). O íntron 2 do gene *BCL11A* é um *locus* chave na regulação de HbF, pois abriga elementos funcionais ligados à sua função como repressor (BAUER *et al.*, 2013). Polimorfismos que sobrepõe elementos funcionais no íntron 2 de *BCL11A* abolem sequências conservadas que afetam a expressão de BCL11A e, consequentemente, dos genes γ-globina, aumentando assim a produção de HbF (MARTYN *et al.*, 2018; MENZEL; THEIN, 2019). De forma semelhante, a região intergênica localizada entre os genes *HBS1L* e *MYB* (HMIP-2) abriga elementos funcionais ligados à regulação da expressão dos mesmos (FARRELL *et al.*, 2011; MENZEL; THEIN, 2019). MYB é um fator de transcrição que atua como um trans regulador na via de regulação da HbF.

Neste trabalho, nós examinamos os efeitos de polimorfismos genéticos na concentração de HbF, parâmetros hematológicos e na incidência de desfechos clínicos em uma coorte de 250 pacientes pediátricos com AF do estado de Minas Gerais, acompanhadas pelo Hemocentro de Belo Horizonte, Fundação Hemominas. O **Capítulo 1** compreende as análises de associação de polimorfismos genéticos individuais, no qual observamos SNPs do gene *BCL11A*

e da região intergênica HMIP-2 associados à concentração elevada de HbF, parâmetros hematológicos menos severos e a menor incidência de complicações clínicas nestes pacientes pediátricos com AF (SALES *et al.*, 2020).

No **Capítulo 2** (artigo submetido para publicação) reportamos os resultados das análises de haplótipos formados pela combinação de alelos dos SNPs funcionais na mesma coorte de pacientes pediátricos com AF, mas excluindo os 30 indivíduos aparentados (n= 220). Por carregar mais informação genética, as análises de haplótipos fornecem maior poder de predição em estudos de associação genética (CLARK, 2004; MORRIS; KAPLAN, 2002; SCHAID, 2004).

De fato, nas análises de haplótipos foram observadas mais associações significativas com desfechos clínicos da AF, quando comparamos às análises de SNPs individuais. Nós observamos que haplótipos formados por SNPs funcionais do gene *BCL11A* (“TCA”, que combina os alelos menos frequentes de rs1427407, rs766432 e rs4671393, respectivamente) e da região intergênica HMIP-2 (“CGC”, que combina alelos menos frequentes de rs9399137, rs4895441, e rs9494145, respectivamente) foram associados com todos os desfechos clínicos examinados, sendo eles, necessidade de transfusão sanguínea, síndrome torácica aguda, dor, infecção e ainda sequestro esplênico agudo. Por outro lado, quanto aos parâmetros hematológicos, as análises de haplótipos não capturaram associações de SNPs de HMIP-2 com contagem de reticulócitos, leucócitos ou saturação periférica de oxigênio, como observado na análise de SNPs individuais (SALES *et al.*, 2020).

Ao combinarmos haplótipos formados por SNPs funcionais do íntron 2 do gene *BCL11A* e da região intergênica HMIP-2 que estão associados ao aumento da concentração de HbF, nós observamos que crianças com AF portadoras do haplótipo “CGC” de HMIP-2, mas não do haplótipo “TCA” de *BCL11A* apresentaram concentração média de HbF semelhante aqueles indivíduos que não carregam nenhum desses haplótipos. Por sua vez, portadoras dos haplótipos “TCA” de *BCL11A* e “CGC” de HMIP-2 apresentaram concentração de HbF significativamente maior quando comparados aqueles indivíduos que não carregam nenhum desses haplótipos. No entanto, crianças com AF portadoras tanto do haplótipo “TCA” de *BCL11A* quanto do haplótipo “CGC” de

HMIP-2 apresentaram níveis de HbF mais elevados do que aquelas que carregavam apenas o haplótipo "TCA" de *BCL11A*. Esses achados sugerem que os alelos ancestrais dos SNPs de *BCL11A* podem exibir efeito epistático nos alelos alterados de HMIP-2 na regulação da HbF. Na co-herança, os alelos menos frequentes de *BCL11A* e HMIP-2 podem ter efeito sinérgico. A hipótese de interação entre estes *loci* pode ter sentido biológico, pois polimorfismos da região HMIP-2 parecem atuar na via de regulação da HbF afetando a expressão do gene *MYB*, um fator de transcrição que regula a expressão de *BCL11A* que, por sua vez, reprime a expressão dos genes γ-globina e reduz substancialmente a concentração de HbF (BAUER; ORKIN, 2015). Todavia, outros estudos com ensaios experimentais são necessários para confirmar essa hipótese.

Os efeitos dos SNPs/haplótipos de *BCL11A* e HMIP-2 na incidência de complicações clínicas da AF podem ser consequência da concentração elevada de HbF, que foi observada nos pacientes portadores dos alelos menos frequentes dos SNPs analisados. Todavia, no **Capítulo 1**, observamos que a associação dos SNPs de *BCL11A* com a incidência de transfusão sanguínea foi independente da HbF (SALES *et al.*, 2020). Diferentes elementos regulatórios foram caracterizados na grande região entre os genes *HBS1L* e *MYB*, em sobreposição com SNPs associados com HbF (FARRELL *et al.*, 2011; MENZEL; THEIN, 2019). Portanto, haplótipos formados por SNPs distais de HMIP-2 capturam maior informação genética desta região intergênica. O mesmo pode ser dito de *BCL11A*, pois apesar dos SNPs associados com HbF estarem mais restritos ao intron 2 do gene, trata-se de uma região extensa contendo assinaturas funcionais e um acentuador eritróide que afeta a expressão de *BCL11A*, e, consequentemente o nível de HbF (BAUER *et al.*, 2013). Experimentos funcionais alelo-específicos mostraram alterações sutis na ligação de fatores de transcrição, expressão de *BCL11A* e nível de HbF (BAUER *et al.*, 2013).

Nós também examinamos o efeito de SNPs de *BCL11A* e HMIP-2 sobre a concentração de HbF em pacientes com AF tratados com hidroxiureia (HU), fármaco amplamente usado no tratamento da AF que aumenta a concentração de HbF, dentre outros efeitos. Para isso, selecionamos um subgrupo de 110 pacientes tratados com HU inclusos na coorte de pacientes pediátricos com AF

que vinha sendo acompanhada pela Fundação Hemominas. O desenho do estudo também foi retrospectivo e o acompanhamento de cada paciente foi iniciado a partir da data de início do tratamento com HU. Concomitantemente ao estudo clínico, nós conduzimos uma Revisão Sistemática da literatura, no intuito de identificar possíveis lacunas na literatura em que nosso estudo clínico pudesse ajudar a elucidar.

A Revisão Sistemática (**Capítulo 3)** foi estruturada para avaliar a influência de polimorfismos genéticos na alteração dos níveis de HbF em pacientes com HU (crianças ou adultos) tratados com HU. Foram incluídos na revisão sete estudos observacionais de coorte de pacientes com AF (genótipo SS) maiores que 3 anos de idade e que receberam tratamento com HU pelo período mínimo de seis meses. Ao todo, 50 polimorfismos de 17 genes foram encontrados como associados ao aumento da concentração de HbF em pacientes tratados com HU (SALES *et al.*, 2021). Na análise de sobreposição dos genes associados, *BCL11A*, arginase 1 (*ARG1*) e arginase 2 (*ARG2*) foram identificados com associação replicada no conjunto dos sete estudos de coorte inclusos. SNPs do íntron 2 do gene *BCL11A* tiveram associação replicada na maioria dos estudos inclusos. Nossa Revisão Sistemática forneceu evidência de que tais SNPs podem influenciar a resposta da HbF em pacientes com AF tratados com HU (SALES *et al.*, 2021).

A partir do conjunto de genes que tiveram SNPs associados à resposta da HbF nos pacientes tratados com HU, realizamos análise de enriquecimento de vias biológicas usando o banco de dados do Reactome (*The Reactome Pathway Knowledgebase*). Nós encontramos enriquecimento para vias relacionadas ao Fator de Crescimento Vascular Endotelial (VEGF; “interação ligante-receptor de VEGF” e “ligação VEGF-VEGFR”), para a via “ciclo da ureia” e via “óxido nítrico estimulando guanilato ciclase”. Arginase 1 e arginase 2 catalisam a hidrólise da arginina para produzir ornitina e uréia. A arginase 1 citosólica é a enzima canônica do ciclo da uréia. A arginase 2 é mitocondrial, mas também catalisa a produção de ureia a partir da arginina e pode ter papel substancial em pacientes sem a arginase 1 (MOLECULAR MEDICINE, 2001). A arginase plasmática é intrinsecamente relacionada à produção de óxido nítrico (POTOKA; GLADWIN, 2015).

Importante reconhecer que nossas análises de vias são limitadas pela disponibilidade dos estudos inclusos na Revisão Sistemática que examinaram a influência de SNPs na resposta ao tratamento com HU em pacientes com AF (SALES *et al.*, 2021). Nesse contexto, análises mais abrangentes poderão elucidar as múltiplas vias moleculares de ação da HU, que por fim levam ao aumento da expressão da HbF.

A Revisão Sistemática também apontou algumas lacunas e limitações na literatura da área. Em primeiro lugar, os estudos que avaliaram o efeito dos SNPs sobre a HbF de pacientes com AF tratados com HU são bastante heterogêneos, o que levou ao número limitado de sete estudos que atenderam aos critérios de inclusão (FRIEDRISCH *et al.*, 2016; GREEN *et al.*, 2013; KUMKHAEK *et al.*, 2008; MA *et al.*, 2007; MM *et al.*, 2017; SHEEHAN *et al.*, 2014; WARE *et al.*, 2011). Notadamente, existe heterogeneidade no protocolo da terapia com HU, na dose e escalonamento para dose máxima tolerada (DMT), na idade dos pacientes, nos genes candidatos examinados, no tratamento estatístico dos fatores de confusão e, principalmente, nos desfechos de HbF examinados como delta (Δ) % HbF, Δ HbF (g/dl), DMT, % HbF, ou HbF máxima (SALES *et al.*, 2021). Portanto, o efeito de polimorfismos genéticos no aumento dos níveis de HbF em resposta a HU em pacientes com AF ainda precisa ser explorado.

O **Capítulo 4** reporta os resultados do estudo clínico envolvendo um subgrupo de 110 pacientes pediátricos com AF tratados com HU acompanhados pela Fundação Hemominas. Até o momento, nós examinamos a associação de SNPs do gene *BCL11A* e da região intergênica HMIP-2 com alteração nos níveis de HbF basal, HbF induzida pela HU e na incidência de transfusão e neutropenia neste subgrupo.

Três SNPs do intron 2 do gene *BCL11A* (rs766432, rs7599488 e rs4671393) foram identificados como preditores da variação na concentração de HbF em resposta ao tratamento com HU nos pacientes pediátricos com AF. Esses dados foram gerados a partir de um modelo de regressão linear múltipla ajustado pela idade, gênero, tempo de tratamento, dose de HU e HbF basal. Não houve associação dos SNPs da região intergênica HMIP-2 com a alteração da concentração de HbF nestes pacientes pediátricos com AF tratados com HU. Os

achados de associação corroboram os resultados da Revisão Sistemática (SALES *et al.*, 2021).

A indução de HbF é um poderoso mecanismo de ação de HU. No entanto, uma vez que vários outros mecanismos de ação são conhecidos, estudos adicionais são necessários para examinar se os SNPs de *BCL11A* são capazes de identificar subgrupos de resposta clínica ao tratamento com HU.

O alelo A do SNP rs4671393 do gene *BCL11A* foi associado com maior incidência de transfusão. Todavia, as análises de associação com a incidência de complicações clínicas (até o momento neutropenia e transfusão) são preliminares, tendo em vista que temos previsão de expansão do tempo de acompanhamento da coorte, o que contribuirá para a produção de dados mais acurados do acompanhamento clínico da coorte.

Trata-se de uma coorte bem caracterizada de pacientes pediátricos com AF do estado de Minas Gerais, sudeste do Brasil, que tem sido acompanhada pelo grupo de pesquisa por mais de uma década (AR *et al.*, 2015; BELISÁRIO *et al.*, 2015, 2016, 2017, 2017; SALES *et al.*, 2020). Os SNPs foram previamente examinados quanto à porcentagem de variância explicada de HbF basal(SALES *et al.*, 2020). A dose de HU foi cuidadosamente coletada e, quando necessário ajustada pelo peso mesmo quando o ajuste não foi descrito pelo médico no prontuário. Além disso, a dose foi ajustada pelo tempo de cada dose de HU devido ao seu protocolo de escalonamento de dose. Adicionalmente, os principais fatores de confusão observados foram levados em consideração nas análises de associação por meio da construção de um modelo de regressão multivariada.

Entretanto, é importante destacar as principais limitações do estudo. Primeiro, devido ao desenho observacional e retrospectivo do estudo, todos os dados hematológicos e clínicos da coorte foram coletados dos prontuários médicos dos pacientes. Esses dados não foram gerados para os fins propostos na pesquisa, podendo configurar viés de informação. Adicionalmente, o tamanho amostral não foi determinado pelo cálculo formal de tamanho amostral, mas pela disponibilidade de acesso e/ou consentimento dos pacientes pediátricos com AF e seus responsáveis. Ainda, pode ter havido perda de seguimento diferenciada por conta de transferências para outros centros de acompanhamento, óbito, e

encaminhamentos por complicações clínicas graves ou para transplante de medula óssea. Essas limitações são aplicáveis tanto ao estudo dos pacientes não tratados quanto ao estudo dos pacientes tratados com HU. Uma limitação específica do acompanhamento dos pacientes pediátricos com AF tratados com HU é a falta da averiguação da adesão dos pacientes ao tratamento com HU, devido à coleta retrospectiva dos dados.

6 Conclusões

- SNPs do gene *BCL11A* e da região intergênica *HBS1L-MYB* (HMIP-2) parecem exercer efeito na concentração basal de HbF em pacientes pediátricos com AF, com alta variância explicada.
- SNPs de *BCL11A* e de HMIP-2 foram associados a parâmetros hematológicos menos severos e à menor incidência de complicações clínicas nos pacientes pediátricos com AF, seja de forma dependente ou independente da HbF.
- Haplótipos formados pela combinação de alelos de SNPs funcionais de *BCL11A* e de HMIP-2 parecem ser melhores preditores das complicações clínicas que os SNPs individuais nos pacientes pediátricos com AF.
- A Revisão Sistemática forneceu evidência de que SNPs do íntron 2 do gene *BCL11A* podem influenciar a resposta da HbF em pacientes com AF tratados com HU
- SNPs de *BCL11A* podem predizer a resposta da HbF ao tratamento com HU em pacientes pediátricos com AF, o que não foi observado para os SNPs da região intergênica HMIP-2.
- Parece existir associação de SNP de *BCL11A* com a necessidade de transfusão sanguínea nos pacientes pediátricos com AF tratados com HU.

9 Referências bibliográficas

AL, R. S. et. **Dynamic long-range chromatin interactions controlMybproto-oncogene transcription during erythroid development | EndNote Click**. [*S. l.: s. n.*], [*s. d.*]. Disponível em: https://click.endnote.com/viewer?doi=10.1038%2Femboj.2011.450&token=WzM2MDY0NTMsIjEwLjEwMzgvZW1ib2ouMjAxMS40NTAiXQ.Nuxmtj5r9zBewnSB9qlftalID7w. Acesso em: 2 ago. 2022.

AR, B.; FL, N.; RS, R.; NE, T.; AL, C.; C, V.-R.; FO, D.; CM, S.; MB, V. Association of alpha-thalassemia, TNF-alpha (-308G>A) and VCAM-1 (c.1238G>C) gene polymorphisms with cerebrovascular disease in a newborn cohort of 411 children with sickle cell anemia. **Blood cells, molecules & diseases**, [*s. l.*], v. 54, n. 1, 2015. Disponível em: https://pubmed.ncbi.nlm.nih.gov/25175566/. Acesso em: 28 jul. 2022.

ASLAN, M.; RYAN, T. M.; ADLER, B.; TOWNES, T. M.; PARKS, D. A.; THOMPSON, J. A.; TOUSSON, A.; GLADWIN, M. T.; PATEL, R. P.; TARPEY, M. M.; BATINIC-HABERLE, I.; WHITE, C. R.; FREEMAN, B. A. Oxygen radical inhibition of nitric oxide-dependent vascular function in sickle cell disease. **Proceedings of the National Academy of Sciences of the United States of America**, [*s. l.*], v. 98, n. 26, p. 15215–15220, 2001.

BAILEY, K.; MORRIS, J. S.; THOMAS, P.; SERJEANT, G. R. Fetal haemoglobin and early manifestations of homozygous sickle cell disease. **Archives of Disease in Childhood**, [*s. l.*], v. 67, n. 4, p. 517–520, 1992.

BAUER, D. E.; KAMRAN, S. C.; LESSARD, S.; XU, J.; FUJIWARA, Y.; LIN, C.; SHAO, Z.; CANVER, M. C.; SMITH, E. C.; PINELLO, L.; SABO, P. J.; VIERSTRA, J.; VOIT, R. A.; YUAN, G.-C.; PORTEUS, M. H.; STAMATOYANNOPOULOS, J. A.; LETTRE, G.; ORKIN, S. H. An Erythroid Enhancer of BCL11A Subject to Genetic Variation Determines Fetal Hemoglobin Level. **Science**, [*s. l.*], v. 342, n. 6155, p. 253–257, 2013.

BAUER, D. E.; ORKIN, S. H. Hemoglobin switching's surprise: the versatile transcription factor BCL11A is a master repressor of fetal hemoglobin. **Current Opinion in Genetics & Development**, [*s. l.*], v. 33, p. 62–70, 2015.

BELISÁRIO, A. R.; SALES, R. R.; TOLEDO, N. E.; MUNIZ, M. B. de S. R.; VELLOSO-RODRIGUES, C.; SILVA, C. M.; VIANA, M. B. Reticulocyte count is the most important predictor of acute cerebral ischemia and high-risk transcranial Doppler in a newborn cohort of 395 children with sickle cell anemia. **Annals of Hematology**, [*s. l.*], v. 95, n. 11, p. 1869–1880, 2016.

BELISÁRIO, A. R.; SALES, R. R.; TOLEDO, N. E.; VELLOSO-RODRIGUES, C.; SILVA, C. M.; VIANA, M. B. Association between ENPP1 K173Q and stroke in a newborn cohort of 395 Brazilian children with sickle cell anemia. **Blood**, [*s. l.*], v. 126, n. 10, p. 1259–1260, 2015.

BELISÁRIO, A. R.; SALES, R. R.; TOLEDO, N. E.; VELLOSO-RODRIGUES, C.; SILVA, C. M.; VIANA, M. B. Interleukin-10 haplotypes are not associated with acute cerebral ischemia or high-risk transcranial Doppler in a newborn cohort of 395 children with sickle cell anemia. **Revista Brasileira De Hematologia E Hemoterapia**, [*s. l.*], v. 39, n. 2, p. 108–114, 2017.

BHANUSHALI, A. A.; PATRA, P. K.; NAIR, D.; VERMA, H.; DAS, B. R. Genetic variant in the BCL11A (rs1427407), but not HBS1-MYB (rs6934903) loci associate with fetal hemoglobin levels in Indian sickle cell disease patients. **Blood Cells, Molecules & Diseases**, [*s. l.*], v. 54, n. 1, p. 4–8, 2015.

BRAGA, J. A. P. Medidas gerais no tratamento das doenças falciformes. **Revista brasileira de hematologia e hemoterapia**, [*s. l.*], 2007. Disponível em: http://www.repositorio.unifesp.br/handle/11600/3876. Acesso em: 1 dez. 2016.

BROUSSE, V.; BUFFET, P.; REES, D. The spleen and sickle cell disease: the sick(led) spleen. **British Journal of Haematology**, [*s. l.*], v. 166, n. 2, p. 165–176, 2014.

BROUSSE, V.; EL HOSS, S.; BOUAZZA, N.; ARNAUD, C.; BERNAUDIN, F.; PELLEGRINO, B.; GUITTON, C.; ODIÈVRE-MONTANIÉ, M.-H.; MAMES, D.; BROUZES, C.; PICARD, V.; NGUYEN-KHOA, T.; PEREIRA, C.; LAPOUMÉROULIE, C.; PISSARD, S.; GARDNER, K.; MENZEL, S.; LE VAN KIM, C.; COLIN-ARONOVICZ, Y.; BUFFET, P.; MOHANDAS, N.; ELIE, C.; MAIER-REDELSPERGER, M.; EL NEMER, W.; DE MONTALEMBERT, M. Prognostic factors of disease severity in infants with sickle cell anemia: A comprehensive longitudinal cohort study. **American Journal of Hematology**, [*s. l.*], v. 93, n. 11, p. 1411–1419, 2018.

CALVET, D.; TUILIER, T.; MÉLÉ, N.; TURC, G.; HABIBI, A.; ABDALLAH, N. A.; MAJHADI, L.; HEMERY, F.; EDJLALI, M.; GALACTEROS, F.; BARTOLUCCI, P. Low fetal hemoglobin percentage is associated with silent brain lesions in adults with homozygous sickle cell disease. **Blood Advances**, [*s. l.*], v. 1, n. 26, p. 2503–2509, 2017.

CANÇADO, R. D.; JESUS, J. A. Sickle cell disease in Brazil. **Revista Brasileira de Hematologia e Hemoterapia**, [*s. l.*], v. 29, n. 3, p. 204–206, 2007.

CLARK, A. G. The role of haplotypes in candidate gene studies. **Genetic Epidemiology**, [*s. l.*], v. 27, n. 4, p. 321–333, 2004.

DA SILVA, M. C. F.; ZUCCHERATO, L. W.; LUCENA, F. C.; SOARES-SOUZA, G. B.; VIEIRA, Z. M.; PENA, S. D. J.; MARTINS, M. L.; TARAZONA-SANTOS, E. Extensive admixture in Brazilian sickle cell patients: implications for the mapping of genetic modifiers. **Blood**, [*s. l.*], v. 118, n. 16, p. 4493–4495, 2011.

DE MONTALEMBERT, M.; TSHILOLO, L. [Is therapeutic progress in the management of sickle cell disease applicable in sub-Saharan Africa?]. **Medecine Tropicale: Revue Du Corps De Sante Colonial**, [*s. l.*], v. 67, n. 6, p. 612–616, 2007.

EMOND, A. M.; COLLIS, R.; DARVILL, D.; HIGGS, D. R.; MAUDE, G. H.; SERJEANT, G. R. Acute splenic sequestration in homozygous sickle cell disease: Natural history and management. **The Journal of Pediatrics**, [*s. l.*], v. 107, n. 2, p. 201–206, 1985.

ESTEPP, J. H.; SMELTZER, M. P.; KANG, G.; LI, C.; WANG, W. C.; ABRAMS, C.; AYGUN, B.; WARE, R. E.; NOTTAGE, K.; HANKINS, J. S. A clinically meaningful fetal hemoglobin threshold for children with sickle cell anemia during hydroxyurea therapy. **American Journal of Hematology**, [*s. l.*], v. 92, n. 12, p. 1333–1339, 2017.

EVIDENCE-BASED MANAGEMENT OF SICKLE CELL DISEASE: EXPERT PANEL, 2014. [*s. l.*], p. 161,

FARRELL, J. J.; SHERVA, R. M.; CHEN, Z.; LUO, H.; CHU, B. F.; HA, S. Y.; LI, Chi Kong; LEE, A. C. W.; LI, R. C. H.; LI, Chi Keung; YUEN, H. L.; SO, J. C. C.; MA, E. S. K.; CHAN, L. C.; CHAN, V.; SEBASTIANI, P.; FARRER, L. A.; BALDWIN, C. T.; STEINBERG, M. H.; CHUI, D. H. K. A 3-bp deletion in the HBS1L-MYB intergenic region on chromosome 6q23 is associated with HbF expression. **Blood**, [*s. l.*], v. 117, n. 18, p. 4935–4945, 2011.

FEDERAL, S. de S. do D. Anemia falciforme, uma doença que exige cuidados especiais. *Em*: SECRETARIA DE SAÚDE DO DISTRITO FEDERAL. [*s. d.*]. Disponível em: https://www.saude.df.gov.br/anemia-falciforme-uma-doenca-que-exige-cuidados-especiais/. Acesso em: 28 jan. 2022.

FERNANDES, A. P. P. C.; AVENDANHA, F. A.; VIANA, M. B. Hospitalizations of children with sickle cell disease in the Brazilian Unified Health System in the state of Minas Gerais. **Jornal de Pediatria**, [*s. l.*], v. 93, n. 3, p. 287–293, 2017.

FERNANDES, A. P. P. C.; JANUÁRIO, J. N.; CANGUSSU, C. B.; MACEDO, D. L. de; VIANA, M. B. Mortality of children with sickle cell disease: a population study. **Jornal de Pediatria**, [*s. l.*], v. 0, n. 0, 2010. Disponível em: http://www.jped.com.br/conteudo/Ing_resumo.asp?varArtigo=2090&cod=&idSecao=1. Acesso em: 27 set. 2016.

FRANCO, R. S.; YASIN, Z.; PALASCAK, M. B.; CIRAOLO, P.; JOINER, C. H.; RUCKNAGEL, D. L. The effect of fetal hemoglobin on the survival characteristics of sickle cells. **Blood**, [*s. l.*], v. 108, n. 3, p. 1073–1076, 2006.

FRIEDRISCH, J. R.; SHEEHAN, V.; FLANAGAN, J. M.; BALDAN, A.; SUMMARELL, C. C. G.; BITTAR, C. M.; FRIEDRISCH, B. K.; WILKE, I. I.; RIBEIRO, C. B.; DAUDT, L. E.; DA ROCHA SILLA, L. M. The role of BCL11A and HMIP-2 polymorphisms on endogenous and hydroxyurea induced levels of fetal hemoglobin in sickle cell anemia patients from southern Brazil. **Blood Cells, Molecules, and Diseases**, [*s. l.*], v. 62, p. 32–37, 2016.

GALIZA NETO, G. C. de; PITOMBEIRA, M. da S. Aspectos moleculares da anemia falciforme. **Jornal Brasileiro de Patologia e Medicina Laboratorial**, [*s. l.*], v. 39, n. 1, 2003a.

GALIZA NETO, G. C. de; PITOMBEIRA, M. da S. Aspectos moleculares da anemia falciforme. **Jornal Brasileiro de Patologia e Medicina Laboratorial**, [*s. l.*], v. 39, p. 51–56, 2003b.

GLADWIN, M. T.; OFORI-ACQUAH, S. F. Erythroid DAMPs drive inflammation in SCD. **Blood**, [*s. l.*], v. 123, n. 24, p. 3689–3690, 2014.

GREEN, N. S.; ENDER, K. L.; PASHANKAR, F.; DRISCOLL, C.; GIARDINA, P. J.; MULLEN, C. A.; CLARK, L. N.; MANWANI, D.; CROTTY, J.; KISSELEV, S.; NEVILLE, K. A.; HOPPE, C.; BARRAL, S. Candidate Sequence Variants and Fetal Hemoglobin in Children with Sickle Cell Disease Treated with Hydroxyurea. **PLoS ONE**, [*s. l.*], v. 8, n. 2, p. e55709, 2013.

HABARA, A.; STEINBERG, M. H. Minireview: Genetic basis of heterogeneity and severity in sickle cell disease. **Experimental Biology and Medicine**, [*s. l.*], v. 241, n. 7, p. 689–696, 2016.

HUSAIN, M.; HARTMAN, A. D.; DESAI, P. Pharmacogenomics of sickle cell disease: steps toward personalized medicine. **Pharmacogenomics and Personalized Medicine**, [*s. l.*], v. 10, p. 261–265, 2017.

JASSAL, B.; MATTHEWS, L.; VITERI, G.; GONG, C.; LORENTE, P.; FABREGAT, A.; SIDIROPOULOS, K.; COOK, J.; GILLESPIE, M.; HAW, R.; LONEY, F.; MAY, B.; MILACIC, M.; ROTHFELS, K.; SEVILLA, C.; SHAMOVSKY, V.; SHORSER, S.; VARUSAI, T.; WEISER, J.; WU, G.; STEIN, L.; HERMJAKOB, H.; D'EUSTACHIO, P. The reactome pathway knowledgebase. **Nucleic Acids Research**, [*s. l.*], v. 48, n. D1, p. D498–D503, 2020.

KATO, G. J.; PIEL, F. B.; REID, C. D.; GASTON, M. H.; OHENE-FREMPONG, K.; KRISHNAMURTI, L.; SMITH, W. R.; PANEPINTO, J. A.; WEATHERALL, D. J.; COSTA, F. F.; VICHINSKY, E. P. Sickle cell disease. **Nature Reviews Disease Primers**, [*s. l.*], v. 4, p. 1–22, 2018.

KHATIB, R.; RABAH, R.; SARNAIK, S. A. The spleen in the sickling disorders: an update. **Pediatric Radiology**, [*s. l.*], v. 39, n. 1, p. 17–22, 2009.

KINNEY, T. R.; SLEEPER, L. A.; WANG, W. C.; ZIMMERMAN, R. A.; PEGELOW, C. H.; OHENE-FREMPONG, K.; WETHERS, D. L.; BELLO, J. A.; VICHINSKY, E. P.; MOSER, F. G.; GALLAGHER, D. M.; DEBAUN, M. R.; PLATT, O. S.; MILLER, S. T. Silent cerebral infarcts in sickle cell anemia: a risk factor analysis. The Cooperative Study of Sickle Cell Disease. **Pediatrics**, [*s. l.*], v. 103, n. 3, p. 640–645, 1999.

KUMKHAEK, C.; TAYLOR, J. G.; ZHU, J.; HOPPE, C.; KATO, G. J.; RODGERS, G. P. Fetal haemoglobin response to hydroxycarbamide treatment and sar1a promoter polymorphisms in sickle cell anaemia. **British Journal of Haematology**, [*s. l.*], v. 141, n. 2, p. 254–259, 2008.

LETTRE, G.; BAUER, D. E. Fetal haemoglobin in sickle-cell disease: from genetic epidemiology to new therapeutic strategies. **The Lancet**, [*s. l.*], v. 387, n. 10037, p. 2554–2564, 2016a.

LETTRE, G.; BAUER, D. E. Fetal haemoglobin in sickle-cell disease: from genetic epidemiology to new therapeutic strategies. **The Lancet**, [*s. l.*], v. 387, n. 10037, p. 2554–2564, 2016b.

LETTRE, G.; SANKARAN, V. G.; BEZERRA, M. A. C.; ARAÚJO, A. S.; UDA, M.; SANNA, S.; CAO, A.; SCHLESSINGER, D.; COSTA, F. F.; HIRSCHHORN, J. N.; OTHERS. DNA polymorphisms at the BCL11A, HBS1L-MYB, and β-globin loci associate with fetal hemoglobin levels and pain crises in sickle cell disease. **Proceedings of the National Academy of Sciences**, [*s. l.*], v. 105, n. 33, p. 11869–11874, 2008.

MA, Q.; WYSZYNSKI, D. F.; FARRELL, J. J.; KUTLAR, A.; FARRER, L. A.; BALDWIN, C. T.; STEINBERG, M. H. Fetal hemoglobin in sickle cell anemia: genetic determinants of response to hydroxyurea. **The Pharmacogenomics Journal**, [*s. l.*], v. 7, n. 6, p. 386–394, 2007.

MARTINON, F.; PÉTRILLI, V.; MAYOR, A.; TARDIVEL, A.; TSCHOPP, J. Gout-associated uric acid crystals activate the NALP3 inflammasome. **Nature**, [*s. l.*], v. 440, n. 7081, p. 237–241, 2006.

MARTYN, G. E.; WIENERT, B.; YANG, L.; SHAH, M.; NORTON, L. J.; BURDACH, J.; KURITA, R.; NAKAMURA, Y.; PEARSON, R. C. M.; FUNNELL, A. P. W.; QUINLAN, K. G. R.; CROSSLEY, M. Natural regulatory mutations elevate the fetal globin gene via disruption of BCL11A or ZBTB7A binding. **Nature Genetics**, [*s. l.*], v. 50, n. 4, p. 498–503, 2018.

MCGANN, P. T.; WARE, R. E. Hydroxyurea therapy for sickle cell anemia. **Expert Opinion on Drug Safety**, [*s. l.*], v. 14, n. 11, p. 1749–1758, 2015.

MENZEL, S.; GARNER, C.; GUT, I.; MATSUDA, F.; YAMAGUCHI, M.; HEATH, S.; FOGLIO, M.; ZELENIKA, D.; BOLAND, A.; ROOKS, H.; BEST, S.; SPECTOR, T. D.; FARRALL, M.; LATHROP, M.; THEIN, S. L. A QTL influencing F cell production maps to a gene encoding a zinc-finger protein on chromosome 2p15. **Nature Genetics**, [*s. l.*], v. 39, n. 10, p. 1197–1199, 2007a.

MENZEL, S.; GARNER, C.; GUT, I.; MATSUDA, F.; YAMAGUCHI, M.; HEATH, S.; FOGLIO, M.; ZELENIKA, D.; BOLAND, A.; ROOKS, H.; BEST, S.; SPECTOR, T. D.; FARRALL, M.; LATHROP, M.; THEIN, S. L. A QTL influencing F cell production maps to a gene encoding a zinc-finger protein on chromosome 2p15. **Nature Genetics**, [*s. l.*], v. 39, n. 10, p. 1197–1199, 2007b.

MENZEL, S.; ROOKS, H.; ZELENIKA, D.; MTATIRO, S. N.; GNANAKULASEKARAN, A.; DRASAR, E.; COX, S.; LIU, L.; MASOOD, M.; SILVER, N.; GARNER, C.; VASAVDA, N.; HOWARD, J.; MAKANI, J.; ADEKILE, A.; PACE, B.; SPECTOR, T.; FARRALL, M.; LATHROP, M.; THEIN, S. L. Global genetic architecture of an erythroid quantitative trait locus, HMIP-2. **Annals of Human Genetics**, [*s. l.*], v. 78, n. 6, p. 434–451, 2014.

MENZEL, S.; THEIN, S. L. Genetic Modifiers of Fetal Haemoglobin in Sickle Cell Disease. **Molecular Diagnosis & Therapy**, [*s. l.*], v. 23, n. 2, p. 235–244, 2019.

MM, A.; RP, S.; CC, da G.; TC, F.; FI, N.; RS, Q.; CV, F.; SC, Y.; RM, O.; JR, F.; BA, C.; CG, B.; JN, M.; MH, S.; M, de S. G. Genetic modulation of fetal hemoglobin in hydroxyurea-treated sickle cell anemia. **American journal of hematology**, [*s. l.*], v. 92, n. 5, 2017. Disponível em: https://pubmed.ncbi.nlm.nih.gov/28195442/. Acesso em: 28 jul. 2022.

MOLECULAR MEDICINE: THE METABOLIC AND MOLECULAR BASES OF INHERITED DISEASE, VOLS 1-4. **JAMA**, [*s. l.*], v. 286, n. 18, p. 2329, 2001.

MORRIS, R. W.; KAPLAN, N. L. On the advantage of haplotype analysis in the presence of multiple disease susceptibility alleles. **Genetic Epidemiology**, [*s. l.*], v. 23, n. 3, p. 221–233, 2002.

MTATIRO, S. N.; SINGH, T.; ROOKS, H.; MGAYA, J.; MARIKI, H.; SOKA, D.; MMBANDO, B.; MSAKI, E.; KOLDER, I.; THEIN, S. L.; MENZEL, S.; COX, S. E.; MAKANI, J.; BARRETT, J. C. Genome wide association study of fetal hemoglobin in sickle cell anemia in Tanzania. **PloS One**, [*s. l.*], v. 9, n. 11, p. e111464, 2014.

NAOUM, P. C. Interferentes eritrocitários e ambientais na anemia falciforme. **Revista Brasileira de Hematologia e Hemoterapia**, [*s. l.*], v. 22, n. 1, p. 5–22, 2000.

NOLAN, V. G.; BALDWIN, C.; MA, Q.; WYSZYNSKI, D. F.; AMIRAULT, Y.; FARRELL, J. J.; BISBEE, A.; EMBURY, S. H.; FARRER, L. A.; STEINBERG, M. H. Association of single nucleotide polymorphisms in klotho with priapism in sickle cell anaemia. **British Journal of Haematology**, [*s. l.*], v. 128, n. 2, p. 266–272, 2005.

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

ONWUBALILI, J. K. Sickle cell disease and infection. **The Journal of Infection**, [*s. l.*], v. 7, n. 1, p. 2–20, 1983.

OVERTURF, G.; POWARS, D. Infections in sickle cell anemia: pathogenesis and control. **Texas Reports on Biology and Medicine**, [*s. l.*], v. 40, p. 283–292, 1980.

POTOKA, K. P.; GLADWIN, M. T. Vasculopathy and pulmonary hypertension in sickle cell disease. **American Journal of Physiology. Lung Cellular and Molecular Physiology**, [*s. l.*], v. 308, n. 4, p. L314-324, 2015.

QUINN, C. T. Sickle Cell Disease in Childhood. **Pediatric Clinics of North America**, [*s. l.*], v. 60, n. 6, p. 1363–1381, 2013.

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

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

RIGANO, P.; FRANCESCHI, L. D.; SAINATI, L.; PIGA, A.; PIEL, F. B.; CAPPELLINI, M. D.; FIDONE, C.; MASERA, N.; PALAZZI, G.; GIANESIN, B.; FORNI, G. L. Real-life experience with hydroxyurea in sickle cell disease: A multicenter study in a cohort of patients with heterogeneous descent. **Blood Cells, Molecules, and Diseases**, [*s. l.*], v. 69, p. 82–89, 2018.

RODRIGUES, D. de O. W.; FERREIRA, M. C. B.; CAMPOS, E. M. S.; PEREIRA, P. M.; OLIVEIRA, C. M.; TEIXEIRA, M. T. B. História da triagem neonatal para doença falciforme no Brasil - capítulo de Minas Gerais. [*s. l.*], v. 22, n. 1, p. 1–128,

SALES, R. R.; BELISÁRIO, A. R.; FARIA, G.; MENDES, F.; LUIZON, M. R.; VIANA, M. B. Functional polymorphisms of BCL11A and HBS1L-MYB genes affect both fetal hemoglobin level and clinical outcomes in a cohort of children with sickle cell anemia. **Annals of Hematology**, [*s. l.*], v. 99, n. 7, p. 1453–1463, 2020.

SALES, R. R.; NOGUEIRA, B. L.; TOSATTI, J. A. G.; GOMES, K. B.; LUIZON, M. R. Do Genetic Polymorphisms Affect Fetal Hemoglobin (HbF) Levels in Patients With Sickle Cell Anemia Treated With Hydroxyurea? A Systematic Review and Pathway Analysis. **Frontiers in Pharmacology**, [*s. l.*], v. 12, p. 779497, 2021.

SANKARAN, V. G.; MENNE, T. F.; XU, J.; AKIE, T. E.; LETTRE, G.; VAN HANDEL, B.; MIKKOLA, H. K. A.; HIRSCHHORN, J. N.; CANTOR, A. B.; ORKIN, S. H. Human fetal hemoglobin expression is regulated by the developmental stage-specific repressor BCL11A. **Science (New York, N.Y.)**, [*s. l.*], v. 322, n. 5909, p. 1839–1842, 2008.

SANKARAN, V. G.; XU, J.; RAGOCZY, T.; IPPOLITO, G. C.; WALKLEY, C. R.; MAIKA, S. D.; FUJIWARA, Y.; ITO, M.; GROUDINE, M.; BENDER, M. A.; TUCKER, P. W.; ORKIN, S. H. Developmental and species-divergent globin switching are driven by BCL11A. **Nature**, [*s. l.*], v. 460, n. 7259, p. 1093–1097, 2009.

SCHAID, D. J. Evaluating associations of haplotypes with traits. **Genetic Epidemiology**, [*s. l.*], v. 27, n. 4, p. 348–364, 2004.

SEGAL, J. B.; STROUSE, J. J.; BEACH, M. C.; HAYWOOD, C.; WITKOP, C.; PARK, H.; WILSON, R. F.; BASS, E. B.; LANZKRON, S. Hydroxyurea for the treatment of sickle cell disease. **Evidence Report/Technology Assessment**, [*s. l.*], n. 165, p. 1–95, 2008.

SERJEANT, G. R. Sickle-cell disease. **The Lancet**, [*s. l.*], v. 350, n. 9079, p. 725–730, 1997.

SERJEANT, G.; SERJEANT, B.; STEPHENS, A.; ROPER, D.; HIGGS, D.; BECKFORD, M.; COOK, J.; THOMAS, P. Determinants of haemoglobin level in steady-state homozygous sickle cell disease. **British Journal of Haematology**, [*s. l.*], v. 92, n. 1, p. 143–149, 1996.

SHEEHAN, V. A.; CROSBY, J. R.; SABO, A.; MORTIER, N. A.; HOWARD, T. A.; MUZNY, D. M.; DUGAN-PEREZ, S.; AYGUN, B.; NOTTAGE, K. A.; BOERWINKLE, E.; GIBBS, R. A.; WARE, R. E.; FLANAGAN, J. M. Whole Exome Sequencing Identifies Novel Genes for Fetal Hemoglobin Response to Hydroxyurea in Children with Sickle Cell Anemia. **PLoS ONE**, [*s. l.*], v. 9, n. 10, p. e110740, 2014.

SOLOVIEFF, N.; MILTON, J. N.; HARTLEY, S. W.; SHERVA, R.; SEBASTIANI, P.; DWORKIS, D. A.; KLINGS, E. S.; FARRER, L. A.; GARRETT, M. E.; ASHLEY-KOCH, A.; TELEN, M. J.; FUCHAROEN, S.; HA, S. Y.; LI, C.-K.; CHUI, D. H. K.; BALDWIN, C. T.; STEINBERG, M. H. Fetal hemoglobin in sickle cell anemia: genome-wide association studies suggest a regulatory region in the 5′ olfactory receptor gene cluster. **Blood**, [*s. l.*], v. 115, n. 9, p. 1815–1822, 2010.

SOMMET, J.; ALBERTI, C.; COUQUE, N.; VERLHAC, S.; HAOUARI, Z.; MOHAMED, D.; FRANÇOIS, M.; MISSUD, F.; HOLVOET, L.; ELMALEH, M.; ITHIER, G.; DENJEAN, A.; ELION, J.; BARUCHEL, A.; BENKERROU, M. Clinical and haematological risk factors for cerebral macrovasculopathy in a sickle cell disease newborn cohort: a prospective study. **British Journal of Haematology**, [*s. l.*], v. 172, n. 6, p. 966–977, 2016.

STEINBERG, M. H. Management of sickle cell disease. **The New England Journal of Medicine**, [*s. l.*], v. 340, n. 13, p. 1021–1030, 1999.

STEINBERG, M. H. Predicting clinical severity in sickle cell anaemia. **British Journal of Haematology**, [*s. l.*], 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. **The Scientific World Journal**, [*s. l.*], v. 8, p. 1295–1324, 2008.

SUS, C. N. de I. de T. no. Protocolo clínico e diretrizes terapêuticas: doença falciforme. [*s. l.*], 2018. Disponível em: http://conitec.gov.br/images/Relatorios/2018/Relatorio_PCDT_DoencaFalciforme.pdf. Acesso em: 2 ago. 2022.

THEIN, S. L.; MENZEL, S.; LATHROP, M.; GARNER, C. Control of fetal hemoglobin: new insights emerging from genomics and clinical implications. **Human Molecular Genetics**, [*s. l.*], v. 18, n. R2, p. R216–R223, 2009.

THEIN, S. L.; MENZEL, S.; PENG, X.; BEST, S.; JIANG, J.; CLOSE, J.; SILVER, N.; GEROVASILLI, A.; PING, C.; YAMAGUCHI, M.; WAHLBERG, K.; ULUG, P.; SPECTOR, T. D.; GARNER, C.; MATSUDA, F.; FARRALL, M.; LATHROP, M. Intergenic variants of HBS1L-MYB are responsible for a major quantitative trait locus on chromosome 6q23 influencing fetal hemoglobin levels in adults. **Proceedings of the National Academy of Sciences**, [*s. l.*], v. 104, n. 27, p. 11346–11351, 2007.

VAN DER LAND, V.; MUTSAERTS, H. J. M. M.; ENGELEN, M.; HEIJBOER, H.; ROEST, M.; HOLLESTELLE, M. J.; KUIJPERS, T. W.; NEDERKOORN, P. J.; CNOSSEN, M. H.; MAJOIE, C. B. L. M.; NEDERVEEN, A. J.; FIJNVANDRAAT,

K. Risk factor analysis of cerebral white matter hyperintensities in children with sickle cell disease. **British Journal of Haematology**, [*s. l.*], v. 172, n. 2, p. 274–284, 2016.

WARE, R. E.; DESPOTOVIC, J. M.; MORTIER, N. A.; FLANAGAN, J. M.; HE, J.; SMELTZER, M. P.; KIMBLE, A. C.; AYGUN, B.; WU, S.; HOWARD, T.; SPARREBOOM, A. Pharmacokinetics, pharmacodynamics, and pharmacogenetics of hydroxyurea treatment for children with sickle cell anemia. **Blood**, [*s. l.*], v. 118, n. 18, p. 4985–4991, 2011.

WEBB, D. K.; DARBY, J. S.; DUNN, D. T.; TERRY, S. I.; SERJEANT, G. R. Gall stones in Jamaican children with homozygous sickle cell disease. **Archives of Disease in Childhood**, [*s. l.*], v. 64, n. 5, p. 693–696, 1989.

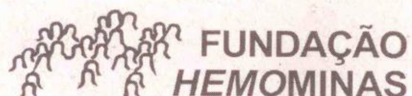
WILLIAMS, T. N.; THEIN, S. L. Sickle Cell Anemia and Its Phenotypes. **Annual Review of Genomics and Human Genetics**, [*s. l.*], v. 19, n. 1, p. 113–147, 2018.

WONKAM, A. Is there a role for pharmacogenetics in the treatment of sickle cell disease?. **Pharmacogenomics**, [*s. l.*], v. 18, n. 4, p. 321–325, 2017.

XIE, Z.; BAILEY, A.; KULESHOV, M. V.; CLARKE, D. J. B.; EVANGELISTA, J. E.; JENKINS, S. L.; LACHMANN, A.; WOJCIECHOWICZ, M. L.; KROPIWNICKI, E.; JAGODNIK, K. M.; JEON, M.; MA'AYAN, A. Gene Set Knowledge Discovery with Enrichr. **Current Protocols**, [*s. l.*], v. 1, n. 3, p. e90, 2021.

YAHOUÉDÉHOU, S. C. M. A.; ADORNO, E. V.; DA GUARDA, C. C.; NDIDI, U. S.; CARVALHO, S. P.; SANTIAGO, R. P.; ALELUIA, M. M.; DE OLIVEIRA, R. M.; GONÇALVES, M. de S. Hydroxyurea in the management of sickle cell disease: pharmacogenomics and enzymatic metabolism. **The Pharmacogenomics Journal**, [*s. l.*], v. 18, n. 6, p. 730–739, 2018.

Anexo 1: Declaração do Comitê de Ética em Pesquisa da Fundação HEMOMINAS



COMITÊ DE ÉTICA EM PESQUISA

DECLARAÇÃO

Belo Horizonte, 18 de março de 2016.

Declaro que o projeto de pesquisa **"Avaliação da modulação dos níveis de hemoglobina F em crianças com anemia falciforme triadas pelo Programa de Triagem Neonatal de Minas Gerais e acompanhadas no Hemocentro de Belo Horizonte da Fundação Hemominas" (CAAE 42048215.6.0000.5118)**, coordenado pelo pesquisador Dr. André Rolim Belisário, foi **APROVADO** em 24/04/2015 pelo Comitê de Ética em Pesquisa da Fundação Hemominas.

Daniel Gonçalves Chaves
Coordenador
Comitê de Ética em Pesquisa
Fundação Hemominas

Daniel Gonçalves Chaves
Coordenação do Comitê de Ética em Pesquisa
Fundação Hemominas

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