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Implicações das variantes genéticas nas manifestações fenotípicas da doença falciforme

SÃO JOSÉ DO RIO PRETO
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Tese apresentada ao Programa de Pós-Graduação em Biociências, como parte dos requisitos para a obtenção do título de doutor em Biociências, Área de Genética Molecular, junto ao Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista “Júlio de Mesquita Filho”, Campus de São José do Rio Preto, SP.

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"A ciência é uma maneira de fazer perguntas sobre o universo e encontrar respostas baseadas em evidências, não em crenças."

(SAGAN, 1996, p. 27)

RESUMO

A doença falciforme (DF) é um conjunto de desordens genéticas caracterizadas pela produção de uma hemoglobina anormal, conhecida como hemoglobina S (Hb S). Essa mutação no gene da beta-globina leva à deformação dos glóbulos vermelhos em forma de foice, resultando em bloqueios vasculares e complicações graves, incluindo a vaso-oclusão. A anemia falciforme (HbSS), forma homozigótica em que a Hb S é herdada de ambos os pais, manifesta-se com sintomas como fadiga, dor intensa, anemia crônica e complicações neurológicas. Embora a DF não esteja ligada aos cromossomos sexuais, as manifestações clínicas podem variar entre homens e mulheres. A coexistência com outras doenças e variações genéticas pode agravar o quadro clínico, destacando a importância de investigar os mecanismos moleculares envolvidos. O tratamento foca no alívio sintomático, prevenção de complicações e melhora da qualidade de vida, com medidas como transfusões sanguíneas e medicamentos específicos. Este estudo investigou a relação entre marcadores moleculares e as manifestações fenotípicas de doentes falciformes. A análise genética revelou associações significativas entre genótipos e fenótipos, incluindo a ligação dos marcadores moleculares *TNFa* e *H63D* com a gravidade da DF em homens. Além disso, demonstramos que os genótipos AA+AG do gene *SLC44A2* estão associados à uma susceptibilidade na formação de úlceras nas pernas, bem como ao aumento de valores absolutos de monócitos, neutrófilos e plaquetas. Notavelmente, esse gene também codifica a base molecular de um grupo sanguíneo raro, conhecido como COST. Outro achado relevante foi a associação significativa entre o gene *CD36* e níveis elevados de HDL em pacientes com DF. Esses resultados fornecem insights valiosos para a compreensão da complexidade molecular da DF e para o desenvolvimento de abordagens terapêuticas mais eficazes.

Palavras-chave: Hemoglobina S; Variantes genéticas; inflamação; Crises vaso-oclusivas; Úlceras de pernas

ABSTRACT

Sickle cell disease (SCD) is a group of genetic disorders characterized by the production of an abnormal hemoglobin known as hemoglobin S (Hb S). This mutation in the beta-globin gene leads to the deformation of red blood cells into a sickle shape, resulting in vascular blockages and severe complications, including vaso-occlusion. Sickle cell anemia (HbSS), the homozygous form in which Hb S is inherited from both parents, manifests with symptoms such as fatigue, severe pain, chronic anemia, and neurological complications. Although SCD is not linked to sex chromosomes, clinical manifestations can vary between males and females. The coexistence of other diseases and genetic variations can worsen the clinical presentation, highlighting the importance of investigating the underlying molecular mechanisms. Treatment focuses on symptom relief, prevention of complications, and improving patients' quality of life, including blood transfusions and specific medications. This study investigated the relationship between molecular markers and phenotypic manifestations in sickle cell patients. Genetic analysis revealed significant associations between genotypes and phenotypes, including the connection of the molecular markers *TNFa* and *H63D* with the severity of SCD in males. Additionally, we demonstrated that the AA+AG genotypes of the *SLC44A2* gene are associated with susceptibility to leg ulcer formation, as well as increased absolute counts of monocytes, neutrophils, and platelets. Notably, this gene also encodes the molecular basis of a rare blood group known as COST. Another significant finding was the association between the *CD36* gene and elevated HDL levels in SCD patients. These results provide valuable insights for understanding the molecular complexity of SCD and for developing more effective therapeutic approaches.

Keywords: Hemoglobins; Genetic variants; inflammation; Vaso-occlusive crises; Leg ulcers

SUMÁRIO

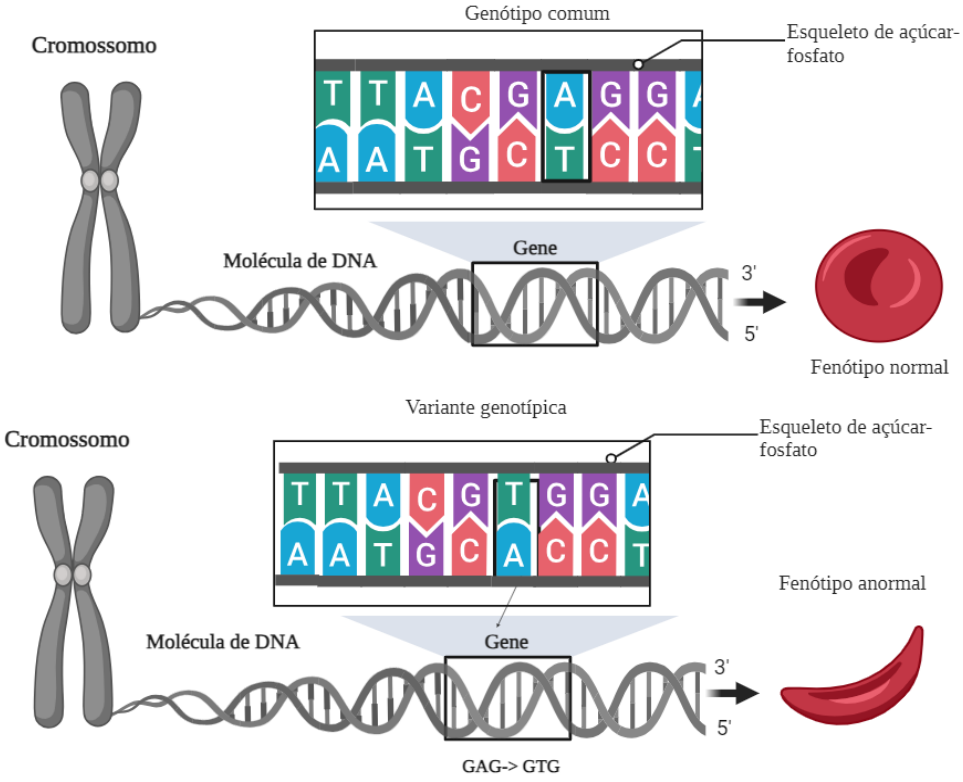
INTRODUÇÃO

1 INTRODUÇÃO

A hemoglobina (Hb) é uma proteína tetramérica composta por diferentes subunidades de globina, cada qual associada ao grupo heme. Sua função primária é transportar oxigênio (O_2) do pulmão para os tecidos. A doença falciforme (DF) é um grupo de doenças hereditárias causadas por uma mutação no gene que codifica a subunidade da beta globina, resultando na presença de hemoglobina S (Hb S). As formas de manifestação da DF incluem anemia falciforme (Hb SS), Hb SC e Hb Sβ-talassemias. (Kato *et al.,* 2018).

A anemia falciforme (AF) é uma hemoglobinopatia hereditária resultante da homozigose de uma mutação pontual no gene da globina beta (Figura 1), levando à substituição do ácido glutâmico por valina na sexta posição da cadeia. Isso resulta em uma hemoglobina alterada, com propriedades físico-químicas diferentes, apresentando fenótipo anormal em indivíduos Hb SS. Nos indivíduos heterozigotos (Hb AS), ocorre a presença tanto de hemoglobina normal (Hb A) quanto de hemoglobina alterada (Hb S), o que geralmente resulta em um fenótipo clinicamente assintomático, mas com a possibilidade de manifestações leves sob condições de estresse fisiológico, como hipóxia ou desidratação. (Franklin Bunn, 1997; Kato *et al.,* 2018).

Figura 1: Mutação do gene da beta globina e fenótipo de um indivíduo homozigoto para Hb S no sangue.

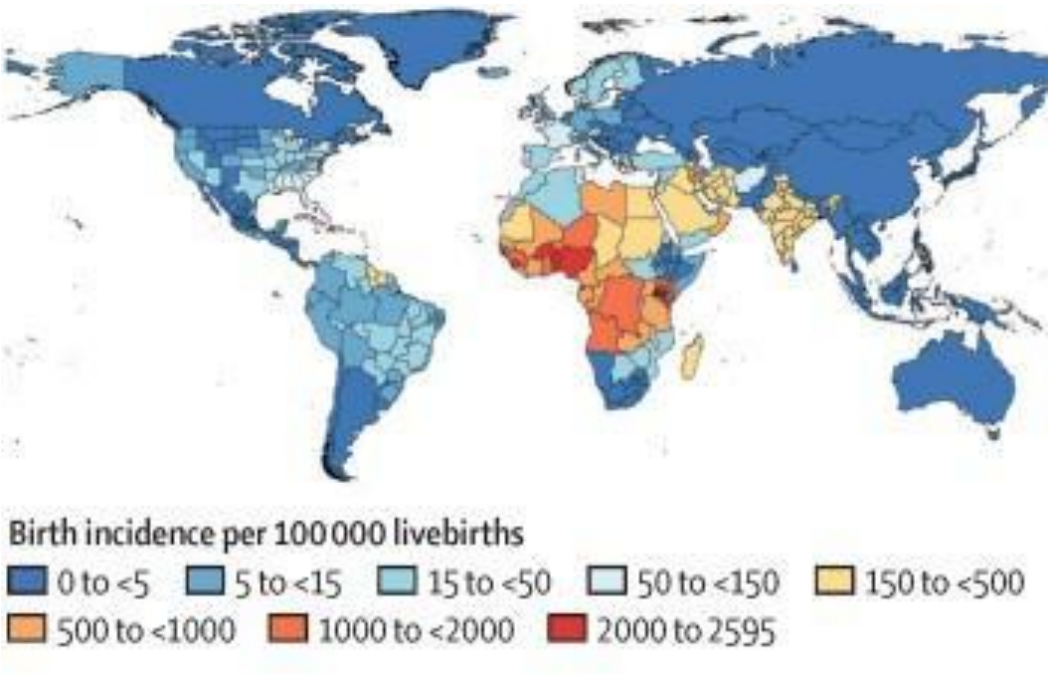


Esquema mostrando o genótipo e fenótipo normais observados para o gene da beta globina, e o genótipo anormal para esse gene após a troca do par de bases (mutação GAG para GTG), resultando em um formato alterado do eritrócito, característico na anemia falciforme. Fonte: Elaborado pelo autor, 2024.

A incidência em recém-nascidos de portadores de doenças falciformes varia globalmente (Figura 2), conforme a região e os grupos étnicos (Cavalcanti; Maio, 2011; Ojodu *et al.,* 2014; Thomson *et al*.,2023) (Figura2). Por exemplo, entre os afro-americanos dos Estados Unidos da América, um a cada 360 recém-nascidos portam uma DF (Wang *et al.,* 2015). Por outro lado, no Brasil, a incidência de DF em recém-nascidos varia substancialmente entre os estados, fato explicado pela heterogeneidade étnica da população brasileira (Santos; Domingos; Castro, 2021). Dados de 2013 do Ministério da Saúde mostraram que a cada 650 nascimentos no estado da Bahia um era portador da DF, ao passo que no estado do Rio de Janeiro, esse valor era um em 1.300. Recentemente um estudo feito por Santos, Domingos e Castro, (2021) identificou que um em 10.226 indivíduos do Rio Grande do Sul são

portadores de DF, número inferior àquele fornecido pelo Ministério da Saúde (Brasil, 2013). Em 2016, em todo o país, 1.071 recém-nascidos tinham DF e mais de 60.000 eram heterozigotos para o alelo da Hb S (Braga *et al.,* 2016). A prevalência do alelo Hb S no Brasil varia consideravelmente entre as regiões e com ocorrência estimada em mais de dois milhões de pessoas, sendo oito mil afetados com a forma homozigótica (Rodrigues *et al.,* 2010).

Figura 2: Incidência de nascimento de homens e mulheres com doença falciforme

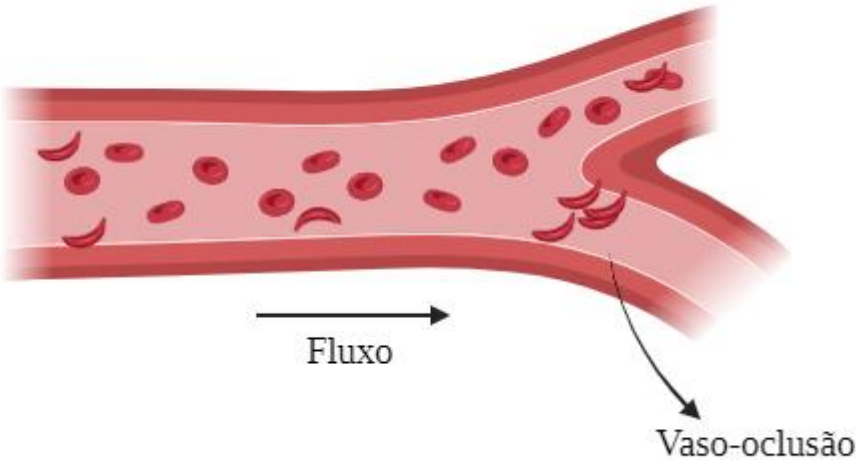


O mapa apresenta as taxas de nascimentos para cada 100.000 nascimentos em 2021 no mundo (Thomson *et al.,* 2023).

Além da distribuição geográfica da AF, a heterogeneidade esta presenta nas características clinicas da doença embora a base molecular da Hb S resida na substituição de um único aminoácido. Indivíduos com AF vivenciam diversas manifestações fenotípicas (Stuart; Nagel, 2004; Conran; Embury, 2021) como asplenia funcional, síndrome torácica aguda, hipertensão pulmonar, acidente vascular cerebral, dano cumulativo de múltiplos órgãos, baixa expectativa de vida e principalmente a vaso-oclusão (Figura 3). A vaso-oclusão é um fenômeno complexo, que tem como um dos seus iniciadores a polimerização da Hb S, sendo essa a principal ocorrência fisiopatológica na AF (Steinberg, 1998; Vekilov, 2007). A

polimerização altera a forma e as propriedades físicas dos eritrócitos, causando anemia hemolítica e bloqueio do fluxo sanguíneo, particularmente em pequenos vasos, o que pode danificar os órgãos (KATO *et al.,* 2018).

Figura 3: Mecanismo de vaso-oclusão ocasionado pelo eritrócito falciforme.



Glóbulos vermelhos falciformes obstruindo um vaso sanguíneo. Essa condição pode ocorrer quando há um impedimento no fluxo normal do sangue: Fonte: Elaborado pelo autor, 2024.

1.1 Anemia falciforme: Inflamação e Vaso oclusão

O estado inflamatório é uma resposta adaptativa das células do organismo para combater infecções ou lesões teciduais e restaurar a homeostase dos órgãos (Medzhitov, 2010; Shen; Kreisel; Goldstein, 2013). Em pacientes com AF, a inflamação crônica é resultado de vários fatores que interagem entre si, formando um ciclo inflamatório permanente. O endotélio vascular desempenha um papel crucial no processo inflamatório e na vaso-oclusão (Zago; Pinto, 2007). Em particular, na AF, a vaso-oclusão é um fenômeno complexo que resulta da interação entre vários componentes, como eritrócitos falciformes, células endoteliais, leucócitos e plaquetas (Kato *et al.,* 2018). A ativação das células endoteliais ocorre devido ao contato direto com eritrócitos falciformes, bem como a presença de heme, hemoglobina livre e espécies reativas de oxigênio (ROS) induzidas pela hipóxia (Sultana *et al.,* 1998). Uma

vez ativadas, as células endoteliais produzem mediadores inflamatórios, como interleucina-1β, interleucina-6 e fator de necrose tumoral (TNF), que levam a um estado inflamatório crônico que, na AF acarreta em uma menor expressão de proteínas anti-inflamatórias bem como maior expressão de citocinas pró-inflamatórias (Revisado em Kany; Vollrath; Relja, 2019) (Kaul *et al.,* 2000; Oliveira *et al.,* 2011; Torres *et al.,* 2016; Kany; Vollrath; Relja, 2019).

Outras moléculas que têm sido estudadas no contexto da AF são as quimiocinas, que são proteínas que atraem células do sistema imunológico para o local da inflamação. A quimiocina CXCL10, por exemplo, está aumentada em pacientes com anemia falciforme e tem sido associada à vaso-oclusão e a uma piora na função pulmonar (Lard *et al.,* 2006). Além disso, o TNF-α é uma citocina pró-inflamatória que pode contribuir para a disfunção endotelial e a vaso-oclusão na anemia falciforme (Reiter *et al.,* 2018).

Ao contrário do formato globular típico, os eritrócitos falciformes são células sanguíneas anormais, que possuem uma morfologia distorcida em forma de foice. Estudos têm demonstrado que esses eritrócitos possuem uma maior capacidade de aderir às células endoteliais do que os eritrócitos normais (Wagner; Eckman; Wick, 2004; Murphy *et al.,* 2005;). Diversos fatores podem contribuir para a ocorrência desse evento, como por exemplo uma maior expressão de moléculas de adesão celular na superfície, como P-selectina (Zago; Pinto, 2007) a integrina α4β1 (Brittain; Parise, 2008), que se ligam às moléculas de adesão expressas pelas células endoteliais normais promovendo assim, a aderência dos eritrócitos falciformes às células endoteliais. A adesão excessiva de neutrófilos ao endotélio vascular pode contribuir para a progressão da vaso-oclusão na AF levando a uma série de problemas como trombose e crises vaso oclusivas, que são características da DF (Rees *et al.,* 2010; Koehl *et al*.,2017). A adesão de neutrófilos é mediada por moléculas de adesão como a integrina β2 (também conhecida como CD18), (Diamond *et al.,* 1993), selectina E (Martens *et al.,* 1995) e molécula de adesão intercelular 1 (ICAM-1) (Kucukal *et al.,* 2020).

A adesão de eritrócitos falciformes e neutrófilos pode ser influenciada por fatores como inflamação e estresse oxidativo, que são comuns em indivíduos com DF (Wang; Zennadi, 2021; Adegoke *et al.,* 2021). Esses fatores podem aumentar a expressão de moléculas de adesão nas células sanguíneas e no endotélio, facilitando

a interação entre elas e levando a processos inflamatórios e trombose. De fato, estudos mostram que a adesão de neutrófilos ao endotélio pode ser um processo crucial na patogênese de diversas doenças relacionadas ao sistema circulatório (Zapponi *et al.,* 2015) e também na AF (Koehl *et al.,* 2017)

A compreensão dos mecanismos moleculares subjacentes à adesão de eritrócitos falciformes e neutrófilos ao endotélio é fundamental para o desenvolvimento de novas terapias relacionadas a doenças do sistema circulatório e imunológico. Estudos pré-clínicos têm demonstrado o potencial de inibidores de moléculas de adesão, como o natalizumabe, no tratamento de doenças inflamatórias e autoimunes, incluindo a DF e outras doenças associadas à inflamação e trombose (SILVA *et al.,* 2016).

As transfusões de eritrócitos e sangue são amplamente utilizadas no tratamento da DF com o objetivo de reduzir os efeitos da hemoglobina S (HbS) e diminuir as crises vaso-oclusivas (Darshana; Rees; Premawardhena, 2021). No entanto, é importante destacar que a sobrecarga de ferro resultante dessas transfusões, em conjunto com mutações no gene *H63D* (High FE2+), podem desempenhar um papel significativo na mortalidade e morbidade dos pacientes com DF (Boga *et al.,* 2007; Mónaco *et al.,* 2023). O gene *HFE* regula o metabolismo do ferro no organismo, e a presença de mutações pode contribuir para uma maior absorção e acúmulo de ferro. Portanto, a interação entre as transfusões de sangue, a sobrecarga de ferro e as mutações do gene *HFE* é um aspecto importante a ser considerado no manejo da DF. (Terz *et al*.,2016).

Além disso, estudos mostram que a hidroxicarbamida (HC), um agente antineoplásico, pode atuar inibindo moléculas de adesão (Gambero et al., 2006; Chaar et al., 2014; Yasara et al., 2021). Desse modo, é essencial continuar investigando a biologia da adesão de células sanguíneas, como eritrócitos falciformes, plaquetas e neutrófilos, e os genes envolvidos nos processos inflamatórios e nas crises vaso-oclusivas, a fim de aprimorar a compreensão da doença e identificar novos alvos terapêuticos.

1.2 Anemia falciforme em Homens

A incidência da doença falciforme não está relacionada ao gênero, pois é transmitida como uma doença autossômica recessiva. No entanto, existem na literatura relatos de diferenças relacionadas ao sexo no que diz respeito a mortalidade e morbidade em portadores de AF (Ceglie *et al.,* 2019). Por exemplo, o estudo de Platt *et al.* (1994) mostrou maior mortalidade de pacientes que apresentam Hb SS no sexo masculino, com idade média de óbito de 42 anos para homens e 48 para mulheres. Assim, apesar da doença não estar ligada aos cromossomos sexuais, as manifestações clínicas entre os sexos podem ser diferentes.

Um importante modulador do tônus vasomotor é o óxido nítrico (ON) que age limitando a agregação plaquetária, inibindo a lesão de isquemia-reperfusão e modulando a expressão de moléculas de adesão endoteliais (Kim-shapiro; Gladwin, 2018). Fenômenos vasculares relacionados às células falciformes como o aumento do estresse que causa cisalhamento e respostas compensatórias à lesão vascular crônica normalmente promovem aumento da produção endotelial de ON, mas esse sistema é prejudicado no sexo masculino (Ceglie *et al.,* 2019). Sabe-se que a produção de ON é facilitada por estrogênios que pode estar ligado ao controle transcricional da hemoglobina fetal, o que poderia contribuir para diferenças de gênero na expressão da hemoglobina fetal entre os sexos masculino e feminino. Terapias que restauram a bioatividade do ON ou reduzem seu consumo (ou aumentam a vasodilatação induzida por ON) podem ser particularmente benéficas em pacientes com anemia falciforme, especialmente em homens (Gladwin *et al.,* 2003).

Uma outra diferença observada está relacionada às crises de dor. Ceglie *et al.,* (2019) mostraram que os homens tendem a apresentar mais crises de dor ao ano do que mulheres, e com dores mais persistentes (Udezue; Girshab, 2004). Lamarre *et al.,* (2013), apontam para diferenças nas complicações clínicas evidenciando que estas são mais graves em homens. Além disso, sabe-se que homens podem apresentar mais chances de ter síndrome torácica aguda (Masese, *et al.,* 2021). Além do mais, homens com anemia falciforme são mais propensos a desenvolver complicações renais e consequentemente apresentarem uma maior mortalidade isso pode estar relacionado ao fato de que os homens têm maiores níveis de hemoglobina

e hemácias no sangue, o que pode culminar em uma maior obstrução dos vasos sanguíneos nos rins (Ataga *et al.,* 2022).

Outra importante diferença é a disfunção sexual, que mostrou-se ser mais agravante em homens com Hb SS que em mulheres (Idris *et al.,* 2020). Dados de análise multivariada apontaram que homens apresentam maiores chances de ter complicações relacionadas à síndrome torácica aguda, cardiovascular e músculo esquelético e menor chance de depressão quando comparados com mulheres Hb SS (Masese *et al.,* 2021). Com isso, é possível observar que o gênero tem um importante papel no fenótipo clinico da AF.

1.3 Transfusão sanguínea na Anemia Falciforme

A transfusão de eritrócitos desempenha um papel crucial no tratamento de complicações agudas e crônicas em pacientes com anemia falciforme. A transfusão ameniza a anemia, diminui a porcentagem relativa de eritrócitos falciformes na circulação e reduz a hemólise (Howard,2016). Todos esses efeitos são benéficos e têm se mostrado eficazes na prevenção primária de acidentes vasculares cerebrais (AVC) (Verduzco; Nathan, 2009) e infartos silenciosos em crianças em risco (Dolatkhah; Dastgiri, 2020). Os eritrócitos podem ser administrados como uma simples transfusão aditiva ou por meio de uma transfusão por troca.

A transfusão por troca é indicada quando a concentração inicial de hemoglobina for alta ou se houver a necessidade de uma diminuição rápida na porcentagem de HbS sem aumentar o hematócrito e a viscosidade do sangue, especialmente em pessoas com sintomas neurológicos agudos. No entanto, a transfusão crônica de sangue resulta em sobrecarga de ferro, sendo, portanto, obrigatória a quelação de ferro em pacientes submetidos a transfusões crônicas para evitar danos aos órgãos devido ao acúmulo de ferro (Rees *et al.,* 2010).

A aloimunização é outra complicação relacionada à transfusão que ocorre em 18 a 37% dos pacientes transfundidos com AF. A taxa de sensibilização diminui substancialmente quando o pareamento antigênico profilático (PAP) é utilizado, mesmo em pacientes submetidos a transfusões crônicas (Campbell-lee *et al.,* 2018; Rosse *et al.,* 1990). Assim, torna-se imprescindível possuir um conhecimento aprofundado dos antígenos dos grupos sanguíneos para a condução adequada de transfusões em qualquer paciente.

Os antígenos dos grupos sanguíneos são determinados pela presença de proteínas ou açúcares na superfície dos glóbulos vermelhos de cada indivíduo. Em julho de 2023, a ISBT-GIWP *International Society of Blood Transfusion - Working Party on Granulocyte Immunobiology and Genetics* (Sociedade Internacional de Transfusão Sanguínea) oficialmente reconheceu 45 sistemas de grupos sanguíneos e 365 antígenos eritrocitários distintos, dos quais 28 deles estão incorporados nos 45 sistemas de grupos sanguíneos e foram caracterizados geneticamente. Dentre esses antígenos, 46% são considerados de alta incidência (encontrados em ≥99% da população global, denominados antígenos "públicos"), 35% são de baixa incidência (≤1% da população global) e são chamados de antígenos "privados", enquanto os últimos 19% possuem uma prevalência equilibrada (entre 1% e 99%). Outros antígenos são agrupados como séries e coleções, uma vez que sua base bioquímica, genética ou sorológica são desconhecidas (série 200) como por exemplo o Cost cujos fenótipos são Cs^a e Cs^b. Antígenos de alta incidência (série 901) têm uma prevalência superior a 90%, ao passo que antígenos de baixa incidência (série 700) têm uma prevalência inferior a 1%. Em algumas situações, antígenos não se enquadram nessas categorias, e sua especificidade permanece desconhecida. Esses são designados como anticorpos anti-PNI (*Public Non Identific),* enquanto os antígenos correspondentes são denominados PNIs. Essa circunstância implica um desafio na transfusão, uma vez que devido a sua raridade, é extremamente difícil encontrar doadores de sangue compatíveis para esses pacientes, a menos que seja de seus próprios irmãos sem o mesmo antígeno (com 1/4 de chance). Contudo, mesmo nesses casos, a compatibilidade com os sistemas ABO e Rh não é garantida.

1.4 Genes *SLC44A2* e *CD36*

O gene *SLC44A2*, localizado no cromossomo 19p13.2, codifica uma proteína de membrana que se expressa em diversas células do corpo humano, incluindo as células sanguíneas. De acordo com Bowens, Sullivan e Curtis (2012), variantes alélicas do gene estão associadas a fenótipos de aloimunização sanguínea, como os antígenos de neutrófilos humanos (HNA) HNA-3a e HNA-3b, que são de grande importância clínica, pois podem causar complicações em transfusões sanguíneas e

na gravidez por questões de incompatibilidade a esses antígenos entre mãe e feto (Lopes *et al.,* 2018). Os HNA são um conjunto de glicoproteínas que se encontram na superfície dos neutrófilos humanos e desempenham um papel relevante na autoimunidade e aloimunidade (Flesch; Reil, 2018). Em decorrência do seu polimorfismo, existem diversas variedades de alo e isoantígenos.

Segundo o ISBT-GIWP, há quatorze alelos HNA responsáveis por cinco sistemas de antígenos HNA (Flesch *et al.,* 2016). Esses alelos são variações alternativas de um gene, diferenciando-se principalmente pelas trocas de nucleotídeos que codificam as respectivas glicoproteínas. Cada glicoproteína HNA pode transportar um ou mais epítopos HNA, que são especificamente reconhecidos pelos anticorpos. O termo "epítopo" é utilizado para definir o determinante antigênico ou região de ligação do anticorpo em uma molécula imunogênica, e é preferido ao termo "antígeno", que é o termo genérico para uma molécula que induz a imunização, como é o caso do HNA (Flesch; Reil, 2018).

Os aloantígenos HNA-3a e HNA-3b são expressos em neutrófilos, linfócitos, plaquetas e em vários tecidos humanos, como pulmão, fígado, cólon e, também, no ouvido interno (Bayat *et al.,* 2013; Flesch *et al.,* 2013), sendo o principal determinante genético envolvido em sua expressão o gene *SLC44A2* (Curtis *et al.,* 2010). A diferença entre esses dois aloantígenos reside na troca em *SLC44A2*455G>A* (rs2288094), resultando na mudança de Arg152Gln dentro do primeiro *loop* extracelular de CTL2 (*choline transporter- like protein 2*), sendo que o anticorpo HNA-3a somente se liga na presença de Arg152, enquanto o HNA-3b necessita de Gln152 (Curtis *et al.,* 2010, Flesch *et al.,* 2016;).

Recentemente foi descoberto que a expressão de *SLC44A2* em eritrócitos está ligada à presença de dois novos antígenos do grupo sanguíneo humano RIF e VER, sendo estes classificados como o 39° sistema de grupos sanguíneos humanos (Koehl *et al*.,2023).

O gene *CD36 (rs3211938),* localizado na região q11.2 do cromossomo 7, possui 15 éxons (XU *et al.,* 2014) e é responsável por codificar a proteína transmembrana CD36, também conhecida como glicoproteína plaquetária IV (GPIV), que é expressa em diversas células do corpo humano, incluindo plaquetas, células endoteliais e monócitos (Silverstein, 2009). A proteína CD36 desempenha múltiplas funções biológicas, incluindo transporte de ácidos graxos, sinalização celular, adesão

de células, angiogênese e fagocitose (Yang; Silverstein, 2019). Além disso, vários estudos têm investigado a relação entre o gene *CD36* e doenças cardiovasculares, tais como aterosclerose, infarto agudo do miocárdio e acidente vascular cerebral. De acordo com uma revisão realizada em 2022, polimorfismos no gene *CD36* podem estar associados ao risco de doenças coronarianas e cardiometabólicas (Yazdanpanah *et al.,* 2022). Em relação à AF, um estudo buscou determinar se um polimorfismo no gene *CD36* estaria implicado no estado hemolítico e em eventos clínicos em pacientes tunisianos. Os autores observaram que alguns polimorfismos poderiam predizer crises hemolíticas mais graves nesses pacientes, entretanto a amostra utilizada no estudo foi pequena (Kalai *et al.,* 2016). É importante destacar que esses achados não contrastam com resultados obtidos em estudos mais antigos (Lee *et al.,* 2001).

Dessa forma, é essencial investigar a relação entre os genes *SLC44A2* e *CD36* e a AF. Até o momento, não há trabalhos que abordem os epítopos do gene *SLC44A2* e sua relação com a AF. Embora haja evidências de possíveis conexões entre *CD36* e a AF, não se tem estudos que mostrem a frequência desse gene na população brasileira e nem com pacientes com AF, o que indica a necessidade de pesquisas para investigar essas relações. Portanto, analisar a interação desses genes com a gravidade e a patologia da AF pode contribuir para a compreensão da natureza complexa dessas interações e para o desenvolvimento de novas terapias para a doença.

JUSTIFICATIVA

2 JUSTIFICATIVA

Os progressos notáveis na agenda global de saúde têm sido evidentes no âmbito das doenças negligenciadas (Oliveira, 2018). No entanto, apesar do impacto considerável dessas enfermidades na saúde, historicamente, elas foram negligenciadas pelas agendas nacionais e internacionais (Morel, 2006), recebendo atenção inadequada. Assim, as doenças negligenciadas apresentam algumas características como: distribuição mundial; afetam milhões de pessoas; maior prevalência entre as populações mais pobres e desfavorecidas; morbidade grave, diminuição da qualidade de vida e aumento da mortalidade (Hotez, 2011; Oms, 2010; Ware, 2013).

Dentre as doenças negligenciadas está a AF que ceifa a vidas de milhares de crianças como, por exemplo, na África, onde essa doença corresponde de 50% a 90% da mortalidade de crianças com até cinco anos (Makani *et al.,* 2011; Piel *et al.,* 2010). Tais mortes são decorrentes da precarização do sistema de saúde como a falta de triagens neonatais e tratamento precoce (Wang, 2015). No Brasil, segundo o Programa Nacional de Triagem Neonatal do Ministério da Saúde (PNTN/MS), em 2019, foram diagnosticados 1.214 casos de doença falciforme, e caso não recebam o devido tratamento em tempo adequado, apenas 20% destas crianças poderão chegar aos cinco anos de idade. Além disso, entre 2015 e 2019 o Brasil registrou 3.320 óbitos decorrentes da DF cuja média de idade foi 32 anos (Cançado, *et al*.,2021).

Diversos trabalhos têm buscado elucidar os mecanismos genéticos e imunopatológicos da AF (Azevedo; Malmegrim, 2020; Guarda *et al.,* 2020. Williams; Thein, 2018); no entanto, como qualquer doença negligenciada, ainda faltam estudos, tanto clínicos quanto pré-clínicos, que ajudem a preencher as lacunas que norteiam esse tema como por exemplo: 1) aspectos genéticos da doença e a relação dos genes dessa doença com os de outras doenças; 2) mecanismos fisiopatológicos e moleculares da doença; 3) monitoramento do efeito da doença em órgãos como coração, cérebro, rins e função cognitiva; e por fim, 4) Técnicas/prognóstico que contribuam para uma melhor acessibilidade dos pacientes a um tratamento personalizado.

Sabe-se que a dor é uma das maiores dificuldades enfrentadas pelos portadores de anemia falciforme, afetando cerca de 70% dos pacientes (Dickerhoff; Ruecker, 1995; Yusuf *et al.,* 2010). Essa dor é resultado da vaso-oclusão, um processo em que diversos grupos celulares, incluindo glóbulos vermelhos, leucócitos, células endoteliais, plaquetas e neutrófilos, participam ativamente. Somado a essas questões, o gênero pode trazer informações importantes sobre a manifestação da doença, especialmente em homens, visto que algumas manifestações tendem a afetar mais pacientes do sexo masculino (Ceglie *et al.,* 2019).

A AF é uma doença genética caracterizada por uma inflamação crônica e vaso-oclusão recorrente, resultando em uma variedade de complicações clínicas. A compreensão dos marcadores moleculares associados à AF é de suma importância para o avanço do conhecimento e tratamento dessa doença. Em primeiro lugar, a identificação desses marcadores moleculares pode fornecer uma previsão mais precisa do risco de complicações em pacientes com anemia falciforme. Alguns desses marcadores, como HbF (hemoglobina fetal) elevada, polimorfismos nos genes *BCL11A* e *HBS1L-MYB* e variantes genéticas no gene *HBB*, têm sido associados a um aumento do risco de desenvolvimento de complicações específicas, tais como derrames e síndrome torácica aguda. Ao identificar esses marcadores em pacientes, torna-se possível uma intervenção precoce e personalizada, com o objetivo de prevenir essas graves complicações. Além disso, o estudo de marcadores moleculares pode levar à identificação de novos alvos terapêuticos para o tratamento da anemia falciforme. A compreensão dos mecanismos moleculares subjacentes à doença pode contribuir para identificar proteínas ou moléculas específicas que podem ser alvo de medicamentos. Esses medicamentos podem ajudar a prevenir ou reduzir a gravidade das complicações associadas à anemia falciforme e melhorar a qualidade de vida dos pacientes. Dessa forma, hipotetizamos que os polimorfismos de alguns dos genes estudados aqui, como *SLC44A2*, *CD36* e *HFE*, pode estar associado às manifestações clínicas, o que poderia afetar a gravidade da doença.

OBJETIVOS

3 OBJETIVOS

3.1 Objetivo geral

Analisar a influência de marcadores moleculares nas manifestações fenotípicas da doença falciforme.

3.2 Objetivos específicos

- Investigar a relação entre os polimorfismos dos genes *TNFα* (-308 G>A), *TGFβ* (-509 C>T), *eNOS* (-786 T>C), *HFE – C282Y* (282 G>A), *H63D* (63 G>A), *S65C* (65 A>T) e marcadores bioquímicos na gravidade da doença em pacientes do sexo masculino com anemia falciforme.

- Examinar a associação dos polimorfismos nos genes *SLC44A2* e *CD36* com as complicações fenotípicas da doença falciforme

- Investigar se as variantes de *SLC44A2* corresponde a base molecular do antígeno de grupo sanguíneo raro Cs(a–)

MATERIAL E MÉTODOS

4 MATERIAL E MÉTODOS

A presente tese consistiu de dois eixos de investigação. O primeiro eixo analisou o impacto de marcadores genéticos, bioquímicos e hematológicos no perfil inflamatório de homens com anemia falciforme, além de investigar a relação do gene *HFE* com a razão de neutrófilos/linfócitos em pacientes tratados ou não com HC. Este eixo foi conduzido no Laboratório de Hemoglobinas e Genética das Doenças Hematológicas (LHGDH) da UNESP, no campus de São José do Rio Preto.

Já o segundo eixo objetivou avaliar a relação dos polimorfismos do gene *SLC44A2* e *CD36* com as manifestações fenotípicas em pacientes com anemia falciforme, incluindo tanto aqueles que fazem uso de HC quanto os que não fazem. Essa análise foi realizada em colaboração com o laboratório de excelência *Biologie Intégrée du Globule Rouge*, localizado na Université Paris Cité, em Paris, França.

4.1 Casuística

A casuística foi constituída por 364 pacientes com doença falciforme, sendo 182 mulheres (50%) e 182 homens (50%) com idade média de 25 anos, que faziam acompanhamento no Instituto Estadual de Hematologia Arthur de Siqueira Cavalcanti, HEMORIO, no Rio de Janeiro, a partir de um grupo inicial de 500 indivíduos. Para este estudo, excluímos pacientes fumantes, consumidores de bebidas alcoólicas, grávidas e menores de 18 anos. Estes critérios foram válidos até quatro semanas antes da coleta das amostras de sangue. Além disso, foram incluídos na amostra pacientes que usaram HC por mais de 90 dias.

Após a seleção dos pacientes, estes foram convidados a doar uma amostra de sangue após a assinatura do TCLE (em anexo). A coleta de sangue foi realizada em punção venosa pela equipe do HEMORIO e o sangue foi armazenado em tubos contendo EDTA a 5%. Em seguida, as amostras foram enviadas para o laboratório de Hemoglobinas e Genética das Doenças Hematológicas da UNESP, onde foram triadas para a confirmação de AF e rastreamento dos polimorfismos de nucleotídeos únicos (SNPs).

Os dados referentes ao uso de hidroxicarbamida, episódios de dor, crises vaso-oclusivas, problemas oftalmológicos, cardíacos, urinários, pulmonares e hepatobiliares foram obtidos por meio de consulta aos prontuários dos pacientes. Após todos esses dados, os pacientes foram classificados conforme a gravidade da doença e, para isso, utilizamos o software *Sickle Cell Disease Severity Calculator*, disponível em http://bios.ugr.es/dss-calculator/index.php (Sebastiani *et al.,* 2007) adaptado para a população brasileira por Belini-Junior *et al.,* (2015).

Este estudo foi conduzido em acordo com os Protocolos Clínicos e Diretrizes Terapêuticas para Pacientes com DF no Brasil (BRASIL., 2015) e aprovado pelo comitê de ética da UNESP, com o seguinte protocolo CAAE (Certificado de Apresentação para Apreciação Ética) 62525922.1.0000.5466.

Para confirmar a presença de hemoglobinopatias de todas as amostras deste estudo foram usadas as seguintes técnicas padronizadas pelo laboratório (LHGDH) (Bonini, 2006):

A etapa inicial das técnicas consistiu no preparo de um hemolisado com saponina (Naoum, 1999) Para preparar o reativo de hemólise, utilizou-se 1g de Saponina P.A. dissolvida em 100mL de água destilada. Em seguida, 50µL de sangue foram adicionados a 50µL do reativo para hemólise em uma placa de Kline. Os dois componentes foram cuidadosamente homogeneizados para garantir uma mistura uniforme. Posteriormente, a mistura foi utilizada na realização das eletroforeses alcalina e ácida.

Após o preparo do hemolisado procedemos à execução da eletroforese em pH alcalino (Marengo-Rowe, 1965) e ácido (Vella, 1968). A eletroforese alcalina, foi empregada para identificar e quantificar as diferentes formas de hemoglobina em uma amostra, detectando tanto as hemoglobinas normais quanto a maioria das hemoglobinas anormais, revelando suas mobilidades eletroforéticas distintas.

Para esta etapa, preparamos o Tampão TRIS-EDTA-BORATO (TEB) de pH 8,6, essencial para a eletroforese alcalina. A composição deste tampão incluiu 10,2g de Tris hidroximetil aminometano, 0,6g de ácido etilenodiaminotetracético e 3,2g de ácido bórico, misturados e completados para 1000mL com água destilada. Após o ajuste de pH, o tampão foi armazenado adequadamente na geladeira.

Adicionalmente, preparamos o Corante *Ponceau* para a etapa de coloração durante a análise pós-eletroforese. Esta solução foi composta por 0,5g de *Ponceau* S,

5,0 g de ácido tricloroacético, completados para 100 mL com água destilada. Este corante foi utilizado para corar as fitas durante a análise e, em seguida, as fitas foram incubadas por 5 a 8 minutos no corante, seguidas por uma incubação de 30 minutos em solução descorante.

Após a preparação de todos os reagentes, utilizamos fitas de acetato de celulose para a eletroforese. Inicialmente, as fitas foram lavadas em duas etapas: 15 minutos embebidas em água destilada e 20 minutos embebidas no tampão TEB. Posteriormente, as fitas foram cuidadosamente secadas com papel absorvente e colocadas na cuba de eletroforese contendo o mesmo tampão TEB para a corrida. Para conectar as fitas ao tampão, utilizamos um perflex para garantir que a corrente elétrica percorresse toda a fita. Em seguida, aplicamos o hemolisado na fita com uma distância de 1 cm das bordas laterais. A eletroforese foi conduzida a 300V até que as bandas fossem devidamente separadas, levando aproximadamente 30 minutos. Foram realizadas duas análises: uma fita permaneceu sem coloração e a outra foi corada com *Ponceau,* sendo as fitas incubadas conforme descrito.

A eletroforese em pH ácido também foi usada, cujo princípio é separar as moléculas de hemoglobina com base em suas cargas elétricas, utilizando um gel contendo um tampão ácido. Devido às diferentes cargas elétricas das moléculas de hemoglobina, elas migram em velocidades distintas no meio de separação sob a influência de um campo elétrico. A identificação das diferentes hemoglobinas ocorre pela observação de suas posições de migração em relação a padrões de referência conhecidos. Essa técnica é especialmente valiosa para identificar hemoglobinas anormais, mesmo em quantidades diminutas no sangue, e também para caracterizar, de maneira semiquantitativa, a presença de Hb Fetal. Para isso, procedemos com a preparação do Tampão Fosfato pH 6,2 cuja composição incluiu 2,02g de Na2HPO4, 7,66g de NaH2PO4.H2O, completados para 1000mL com água destilada. Após a mistura, o pH foi ajustado e o tampão foi armazenado de forma apropriada na geladeira. Para o preparo do Gel de Ágar-Fosfato, 500mg de ágar-ágar foram misturados com 25mL do tampão fosfato pH 6,2 em um bécker. A mistura foi levada ao micro-ondas por 1 minuto, com agitação a cada 25 segundos. Posteriormente, 5mL do gel foram pipetados em uma lâmina de microscópio, aguardando a solidificação. As amostras do hemolisado foram então aplicadas cuidadosamente na porção média da lâmina. Utilizou-se um perflex para conectar a lâmina (com o gel) ao compartimento

eletrolítico. A eletroforese foi conduzida a 100V por 30 minutos, garantindo a devida separação das bandas. As lâminas foram subsequentemente analisadas, tanto coradas quanto sem o corante *Ponceau*, proporcionando uma avaliação abrangente das hemoglobinas presentes nas amostras

Uma outra técnica que também foi empregada foi o teste de Resistência Globular Osmótica em solução de NaCl a 0,36% que teve por objetivo avaliar a capacidade das células sanguíneas, especialmente os glóbulos vermelhos, de resistir à hemólise em um ambiente hipertônico. A solução utilizada possui uma concentração de NaCl considerada isotônica, similar à do meio intracelular das células sanguíneas. Este teste desempenha um papel crucial no diagnóstico de algumas anemias, como a anemia falciforme e talassemias.

Os reagentes para a preparação da Solução Estoque - NaCl a 10% - pH 7,4 foram combinados, incluindo 9,0g de NaCl, 1,36g de Na2HPO4 e 0,28g de NaH2PO4.H2O. A mistura foi completada para 100mL com água destilada, sendo ajustado o pH após a preparação. Para a Solução de Trabalho a 0,36%, 36mL de NaCl 10% foram misturados com 1000mL de água destilada. Essa solução foi então utilizada para avaliar a resistência globular osmótica. No procedimento, 2mL da Solução de NaCl a 0,36% foram adicionados a um tubo de hemólise, seguido pela adição de 10uL de sangue total. O tubo foi agitado vagarosamente e deixado em repouso por 10 minutos. Após esse período, uma folha branca com linhas pretas foi posicionada a dois centímetros do tubo. Se as linhas pretas permanecessem visíveis, o resultado seria negativo para a hemólise. Por outro lado, se as linhas se tornassem opacas ou invisíveis, o resultado seria positivo, indicando a ocorrência de hemólise.

Para quantificar as frações globínicas de Hb A2, Hb A, e Hb F as amostras de todos os pacientes foram submetidas a análises cromatográficas através da Cromatografia Líquida de Alta Performance (HPLC) utilizando o sistema *Premier Resolution* da *Trinity Biotech*. Esse método oferece a capacidade de discriminar variantes estruturais da globina beta, tais como Hb S, Hb C, Hb E, Hb D-Los Angeles e Hb O-Arab, além de permitir a análise detalhada das diferentes frações de hemoglobina.

Após a conclusão de todas as análises laboratoriais, foram também empregados métodos moleculares, iniciando-se com a extração de DNA por meio da técnica de Fenol clorofórmio (Sambrook; Fritsch; Manatis, 1989). A técnica permite

extrair o DNA a partir de sangue total. É um processo que envolve a separação das células do sangue das outras moléculas presentes na amostra, seguido da quebra das membranas celulares por meio de um detergente para liberar o DNA. O Fenol é adicionado à solução de lisado celular para separar as proteínas e outras moléculas celulares do DNA. No final do processo o DNA é precipitado em álcool absoluto. A primeira etapa consistiu no preparo do Tampão 1 para lise celular de glóbulos vermelhos, Tampão 2 para lise celular de glóbulos brancos, Proteinase K (20 mg/mL), Fenol, Clorofórmio: álcool isoamílico (24:1), Etanol 70%, e KCl 2M. O processo teve início com a coleta de amostras de sangue periférico com EDTA, transferindo-as para microtubos de 2 mL e completando-as com 1,5 mL do Tampão 1. Após agitação e centrifugação a 6500 rpm por 5 minutos, o sobrenadante foi descartado, sendo o procedimento repetido duas vezes antes de adicionar 450 µl do Tampão 2, 25 µl de SDS a 10%, e 5 µl de Proteinase K 20 mg/mL ao precipitado.

O microtubo foi homogeneizado e incubado em banho-maria por três horas a 42°C. Em seguida, foram adicionados 500 µl de fenol, o material foi homogeneizado e centrifugado por 5 minutos a 7000 rpm. A fase superior foi transferida para outro microtubo, adicionando 500 µl da solução de clorofórmio e álcool isoamílico na proporção 24:1. O material foi homogeneizado novamente e centrifugado por 5 minutos a 7000 rpm, repetindo o processo mais uma vez.

O sobrenadante foi transferido para um tubo contendo 50 µl de solução de KCl 2M gelada, adicionando 500 µl de etanol 100% gelado para a precipitação do DNA. O DNA foi centrifugado por 30 segundos a 13000 rpm, o sobrenadante foi descartado, e o DNA no fundo do tubo foi lavado com 200 µl de etanol 70% gelado. Após a evaporação do etanol, o DNA foi solubilizado com 50 µl de água ultra pura e armazenado em freezer.

Uma vez obtido o DNA de todos os pacientes, foi empregado a PCR-RFLP para detecção de HbS (Saiki et al., 1985- adaptado) e para as mutações do gene *HFE*.

Para a detecção de HbS uma região específica do DNA que contém a mutação Hb S foi amplificada por meio de reação em cadeia da polimerase (PCR). Em seguida, a amostra foi submetida à digestão com uma enzima de restrição específica, que corta o DNA em um local diferente, dependendo da presença ou ausência da mutação Hb S. O produto foi analisado por eletroforese em gel, permitindo a visualização da presença ou ausência do alelo Hb S.

A região do códon 6 foi amplificada utilizando os primers P277 (*sense)* 5’ GGC AGA GCC ATC TAT TGC TTA 3’ e P278 (*antisense*) 5’ ACC TTA GGG TTG CCC ATA AC 3’. A mistura da reação de PCR foi preparada com um volume final de 25,0µl, contendo 13,0µl de água, 1,0µl de cada primer a uma concentração de 10,0µM, 1,0µl de dNTP a 1,25mM cada, 1,5µl de Taq Polimerase de 1U, 2,5µl de tampão sem MgCl2, 3,0µl de MgCl2 a 50mM e 2,0µl de DNA a 100ng/µl. A reação de amplificação seguiu as seguintes condições: 35 ciclos de 30 segundos a 94ºC para desnaturação, 30 segundos a 55ºC para anelamento, 1 minuto a 72ºC para extensão e um ciclo final de 10 minutos a 72ºC.

Após a amplificação do fragmento de 382 pb, procedeu-se à digestão utilizando a enzima Dde I a 37°C durante 3 horas. A reação foi realizada com 10,0µl do produto da PCR, 13,1µl de água, 2,1µl de tampão e 1,0µl da enzima Dde I. A mutação no códon 6 (GAG → GTG) eliminou um sítio de restrição, o que resultou na geração de três fragmentos (201 pb, 88 pb e 87 pb) para o alelo normal e dois fragmentos (288 pb e 88 pb) para o alelo mutante. A eletroforese em gel de agarose a 1,5% foi realizada sob uma corrente constante de 80 V por 30 minutos, seguida da visualização dos resultados sob luz UV, após a coloração do gel com brometo de etídio.

Para as mutações do gene *HFE* a detecção da mutação *H63D (G>A)*, que afeta o códon 63 da proteína, utilizando a técnica de reação em cadeia da polimerase seguida de análise de restrição de polimorfismo de fragmento único (PCR-RFLP). A amplificação foi realizada com os primers L63 (*sense*) 5´ ACA TGG TTA AGG CCT GTT GC 3’ e R63 (*antisense*) 5´ ATC CCC AGC CTT GTT AAC TG 3’, seguindo o seguinte procedimento descritivo: Foi preparada uma mistura de reação em um tubo de microcentrífuga com os seguintes componentes: 10,1 µl de água ultrapura, 2,5 µl de tampão sem $MgCl_2$ (10x), 2 µl de $MgCl_2$ (50x), 3,2 µl de dNTP (1,25x), 2,5 µl de PrimerL63 (10 µM), 2,5 µl de PrimerR63 (10 µM), 0,2 µl de Taq (5U) Invitrogen ou Ludwig, 2 µl de DNA molde (100ng/µl)

A mistura foi homogeneizada e adicionada em um tubo PCR, contendo o DNA molde previamente diluído em água ultrapura. A reação foi colocada em um termociclador com as seguintes condições de amplificação: desnaturação inicial: 95ºC por 3 minutos, ciclos de amplificação: 95ºC por 45 segundos para desnaturação, 60ºC por 30 segundos para anelamento dos primers, 72ºC por 60 segundos para extensão da fita por adição dos dNTPs, extensão final: 72ºC por 5 minutos. Foram realizados

32 ciclos de amplificação. Após a etapa de amplificação, o fragmento de 496 pares de base (pb) foi submetido à eletroforese em gel de agarose a 2%, sob corrente constante de 80 Volts por 20 minutos. Em seguida, o fragmento foi digerido usando uma mistura contendo Tampão 1X (1,0 µl), *FastDigest* Bcl I (1FDU/µL) (0,3 µl), água destilada (8,7 µl) e produto da PCR (10 µl), em banho-maria a 37ºC (Fermentas), por um período de cinco minutos. A mutação no códon 63 (CAT → GAT) do éxon 2, eliminou um sítio de restrição da enzima Bcl I, resultando em 2 fragmentos de 352 pb e 144 pb para o alelo normal e 1 fragmento de 496 pb para o alelo mutante. A digestão foi então analisada por eletroforese em gel de agarose a 2%, sob corrente constante de 80 V por 40 minutos, e os fragmentos foram visualizados sob luz UV após a coloração com brometo de etídio.

A detecção da mutação S65C (A>T), que altera o códon 65 da proteína, foi realizada por reação em cadeia da polimerase, seguida de análise de restrição de polimorfismo de fragmento único (PCR-RFLP). A sequência de primers utilizada para a amplificação foi: o L63 (sense): 5´ ACA TGG TTA AGG CCT GTT GC 3' e o R63 (antisense): 5´ ATC CCC AGC CTT GTT AAC TG 3'. Para um volume de 25 µl, foram adicionados 10,1 µl de H_2O, 2,5 µl de tampão sem $MgCl_2$ (10X), 2,0 µl de $MgCl_2$ (50 mM), 3,2 µl de dNTP1 (25 mM), 2,5 µl de primer L63 (10 µM), 2,5 µl de primer R63 (10 µM), 0,2 µl de Taq (5U) Invitrogen ou Ludwig e 2,0 µl de DNA molde (100 ng/µL). A reação de amplificação seguiu as seguintes condições: 30 ciclos de 45 segundos a 95ºC para a abertura da dupla-hélice do DNA, 30 segundos a 60ºC para o anelamento dos primers e 60 segundos a 72ºC para a extensão da fita por adição dos dNTPs; seguido por uma extensão final da dupla hélice dos DNAs recém-sintetizados por 5 minutos a 72ºC, totalizando 32 ciclos. Em seguida, o fragmento foi digerido usando uma mistura contendo Tampão 1X (1,0 µl), *FastDigest* Hinf I (1FDU/µL) (0,3 µl), água destilada (8,7 µl) e produto da PCR (10 µl), em banho-maria a 37ºC (Fermentas), por um período de cinco minutos. A mutação no códon 65 (AGT > TGT), localizada no éxon 2, resulta na eliminação de um sítio de restrição para a enzima Hinf I. Após a digestão, o alelo normal produz quatro fragmentos de 274 pb, 147 pb, 69 pb e 6 pb, enquanto o alelo mutante gera três fragmentos de 274, 216 e 6 pb. A análise da digestão foi realizada por eletroforese em gel de agarose a 2% sob corrente constante de 80 V por 40 minutos. Os fragmentos foram visualizados sob luz UV após coloração com brometo de etídio.

Por fim, a mutação (G>A) *C282Y*, que afeta o códon 282 da proteína, foi detectada por PCR seguida de análise de restrição de polimorfismo de fragmento único (PCR-RFLP). Para amplificar o DNA, foram utilizados os seguintes primers: L282 (sense): 5´ GGG TAT TTC CTT CCT CCA ACC 3' e R282 (antisense): 5´ CTC AGG CAC TCC TCT CAA CC 3'. A reação de amplificação foi realizada em um volume total de 25 µl, contendo água (10,1 µl), tampão sem MgCl2 (10X) (2,5 µl), MgCl2 (250 mM) (2,0 µl), dNTPs (3,2 µl), primer L282 (10 µM) (2,5 µl), primer R282 (10 µM) (2,5 µl), Taq DNA polimerase (5U) (0,2 µl) e DNA molde (100 ng/µl) (2,0 µl). O protocolo de amplificação incluiu uma desnaturação inicial a 95ºC por três minutos, seguida de 30 ciclos de amplificação a 95ºC por 45 segundos, 60ºC por 30 segundos e 72ºC por 60 segundos, e um ciclo final de extensão a 72ºC por cinco minutos. O produto de amplificação, um fragmento de 441 pb, foi analisado por eletroforese em gel de agarose a 1,5%, seguido de digestão com a enzima Rsa I, utilizando uma mistura contendo tampão 1X, *FastDigest* Rsa I, água destilada e produto de PCR. A digestão foi realizada em banho-maria a 37ºC por cinco minutos. A mutação no códon 282 (TGC > TAC), localizada no éxon 4, origina um sítio de restrição para a enzima Rsa I. Após a digestão com a enzima, o alelo normal gera dois fragmentos de 296 e 145 pb, enquanto que o alelo mutante gera três fragmentos de 296, 116 e 29 pb. A digestão foi analisada por eletroforese em gel de agarose a 2%, sob corrente constante de 80 V por 40 minutos e visualizados sob luz UV, após coloração com brometo de etídio.

Para os genes *SLC44A2* e *CD36* foram empregados ensaio de genotipagem *TaqMan* (Steffernsen, Baech e Nielsen, 2015), para a determinação dos polimorfismos *HNA-3a* e *HNA-3b* do gene *SLC44A2* (rs2288904), empregando-se duas sondas com diferentes marcadores de fluorescência: FAM (6-carboxifluoresceína) e VIC (4,7,2'-tricloro-7'-fenil-6-carboxifluoresceína) (Kutyavin et al*.,* 2000). As duas sondas *TaqMan* foram utilizadas no mesmo mix de PCR, permitindo a genotipagem em um único tubo para cada sistema bialélico. Durante a fase de extensão do primer da PCR, a atividade exonucleásica 5'-3' da Taq polimerase cliva e libera um dos corantes ligados às sondas, resultando em um aumento na fluorescência. No final da PCR, a intensidade de emissão de cada fluoróforo é medida. Após a extração dos DNAs genômicos, preparou-se o seguinte mix para a determinação dos polimorfismos. TaqMan Genotyping Master Mix (2x) (Applied Biosystems™): 12,50 µl, TaqMan SNP

Genotyping Assay, human (40x) (Applied Biosystems™): 1,25 µl, amostra de DNA [20-25] ng/µL: 2 µl

O ensaio realizado empregou um mix *SNP TaqMan* que consiste em primers e sondas projetados pela *Thermo Fisher Scientific*, cujas sequências dos primers não foram disponibilizadas pela empresa. A técnica foi conduzida em placas de PCR contendo 96 poços, dos quais 3 foram destinados para os controles sendo: positivo homozigoto (*HNA-3a/a*), positivo homozigoto (*HNA-3b/b*), positivo heterozigoto (*HNA-3a/b*), e 1 para controle negativo. Após adicionar as amostras na placa de PCR, esta foi selada com um filme plástico e centrifugada a 900g por 1 minuto. Em seguida, a placa foi inserida no termociclador, no qual ocorreu a amplificação do material genético sob as seguintes condições: 50°C por 2 minutos, 95°C por 10 minutos, seguido de 40 ciclos de desnaturação a 95°C por 15 segundos e extensão a 60°C por 1 minuto. Após a amplificação, a placa foi incubada a 4°C. O mesmo procedimento ocorreu para o gene *CD36 (rs3211938) e foi usado um controle* homozigoto *(TT)* normal*,* homozigoto *(GG)* mutante e um heterozigoto *(TG).*

A interpretação dos resultados foi realizada utilizando o instrumento ABI Prism 7900HT, com o auxílio do software SDS versão 2.3.

4.2 Análises estatísticas

Para avaliar a normalidade dos dados, foram aplicados dois testes específicos: o teste de Shapiro-Wilk e o teste de Kolmogorov-Smirnov. Para verificar possíveis diferenças estatísticas entre as variáveis independentes, optou-se pelo teste de Kruskal-Wallis, um teste não-paramétrico utilizado para comparar mais de duas amostras independentes. Além disso, para explorar possíveis correlações entre as amostras, foi aplicado o teste de correlação de Spearman, um teste não-paramétrico adequado para medir a relação entre duas variáveis contínuas. Para avaliar a associação entre duas variáveis categóricas, o teste qui-quadrado foi utilizado. Todas as análises estatísticas foram conduzidas no software RStudio, com um nível de significância estabelecido em $p < 0,05$. Os gráficos foram gerados por meio do pacote ggplot2, uma biblioteca popular para visualização de dados no ambiente R.

RESULTADOS

5 RESULTADOS

Os resultados desta tese estão organizados em quatro capítulos, abordando os objetivos propostos. O primeiro capítulo explora o papel dos marcadores moleculares, bioquímicos e hematológicos na gravidade da anemia falciforme em homens. No segundo capítulo, elucidamos o impacto do gene *SLC44A2* nas manifestações fenotípicas em pacientes com doença falciforme. No terceiro capitulo buscamos investigar se o gene *SLC44A2* corresponde base molecular do antígeno de neutrófilo humano raro COST. Por fim, o último capítulo investiga a associação do gene *CD36* com as manifestações fenotípicas da doença falciforme.

CAPÍTULO 1

5.1 The Severity of sickle cell anemia in men - The action of molecular and biochemical markers

O primeiro objetivo desta Tese consistiu em examinar a possível relação entre os polimorfismos dos genes *TNFα* (-308 G>A), *TGFβ* (-509 C>T), *eNOS* (- 786 T>C), *HFE - C282Y* (282 G>A), *H63D* (63 G>A), *S65C* (65 A>T), marcadores bioquímicos e a gravidade da anemia falciforme em pacientes do sexo masculino.

Os resultados estão apresentados no artigo publicado na revista *International Journal of Blood Research and Disorders* conforme destaque abaixo:

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ORIGINAL ARTICLE

The Severity of Sickle Cell Anemia in Men - The Action of Molecular and Biochemical Markers

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Abstract

Background: Sickle cell disease is characterized by a very heterogeneous clinical course among patients with the same mutations for sickle cell hemoglobin (HbS). Sickle cell anemia (SCA) is a hereditary hemoglobinopathy caused by the homozygosity of a point mutation in the beta-globin gene, which leads to the substitution of glutamic acid for valine in the sixth position. Thus, this mutation results in an individual with the abnormal Hb phenotype and different physicochemical properties being known as HbS. The molecular basis for the formation of HbS is known, however, the mutation alone (*HBB*, *glu6val*, *rs334*) is not enough to explain the heterogeneous phenotype found in patients with SCD, other factors such as HbF levels, genetic polymorphisms, drug use how hydroxyurea and environmental factors can help to better understand this hemoglobinopathy.

Objective: To study how these factors modulate sickle cell anemia expression, the present work aimed toinvestigate the association of molecular, hematological, and biochemical markers with the severity of sickle cell anemia**.**

Materials and methods: 182 male patients homozygous for the Hb S genotype were selected. Disease severity was calculated from the "Sickle Cell Disease Severity Calculator". In this study, hematological (Ferritin), biochemical (TBARS-Thiobarburic acid-reactive species) and molecular markers - SNP were used in the genes: [*TNFα(-308 G > A)*, *TGFβ (-509 C > T)*, *eNOS* (*-786 T > C*) and *HFE - H63D* (*63 G > A*), *C282Y* (*282 G > A)* and *S65C* (*65 A > T*)].

Results: Some associations were stronger, for example with polymorphisms of *TNF-α (-308 G > A*) genes with the most predominant genotype being the homozygous mutant polymorphism [*GG*]. Patients with Moderate and severe severity had pain attacks with a frequency of two attacks per year on average. In contrast, the frequency observed in Mild patients was 3-5/year. The *eNOS* gene polymorphism *(-786 T > C*) showed a higher frequency of the [*TT*] polymorphism being associated with stroke. The use of hydroxyurea, as well as the levels of Ferritin, showed to be great agents associated with the degree of severity. Of the polymorphisms of the HFE gene studied, only the H63D (*63 G > A*) presented a mean associated with the degrees of severity, with the frequency of the *W/H63D* genotype being predominant in patients with a moderate degree.

Conclusion: This work showed that the degree of severity in SCA is associated with *TNFα*, Stroke, Pain Crises, *TGFβ*, TBARS, Number of infections/year, *eNOS*, Ferritin, and mutations in the *HFE* gene (*H63D*, *C282Y*, and *S65C*).

Keywords

Genetic markers, Sickle cell anemia, Severity, TBARS

Abbreviations

Hb: Hemoglobin; SCA: Sickle cell anemia; SCD: Sickle cell disease; Il: Interleukin; TNF-α: Tumor necrosisfactor α; HU: Hydroxyurea; TGF-β: Transforming growth factor-beta; eNOS: Endothelial nitric oxide synthase; TBARS: Thiobarbituric acid reactive substances; HFE: Homeostatic iron regulator

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Introduction

Hemoglobin (Hb) is a heterotetramer consisting of four alpha and beta subunits, each, composed of a protein fraction called globin and a prosthetic group, the heme [1,2]. Mutations in the globin gene cause changes in the synthesis of alpha and beta chains, producing structurally different proteins, characterized by variant hemoglobins [3]. One of these variants is Hb S. Its formation is caused by a point mutation in the beta-globin gene (*HBB*), which leads to the substitution of glutamic acid for valine in the sixth position in the chain. When the gene for Hb S occurs in homozygosis (Hb SS), individuals have a severe disease condition called sickle cell anemia (SCA). Thus, this mutation results in an individual with an abnormal Hb phenotype and different physicochemical properties [4].

The incidence of newborns, worldwide, with sickle cell disease (SCD), varies according to region and ethnicity. Among African Americans in the United States of America, 1 in 360 newborns is a carrier of the gene [5]. In Brazil, the incidence of SCD in newborns varies substantially between states, a fact explained by the ethnic heterogeneity of the Brazilian population [6]. In the state of Rio de Janeiro, the incidence among live births is 1 in 1,200, which is one of the states with the highest representation of children born with SCD [7] Therefore, neonatal screening for hemoglobinopathies with a focus on sickle cell anemia is essential for early diagnosis, which in turn provides a better understanding and management of the genetics and pathophysiology of the disease.

The pathophysiological basis and underlying inflammatory mechanisms, as well as evolutionary genetics, are still gaps that require further studies in patients with SCA. It is known that the striking complication of sickle cell anemia is pain, typically described as acute, chronic, neuropathic, due to comorbidity or a combination of two or more of them [8] and resulting from Vaso-occlusive processes [2,9]. However, other manifestations are observed such as functional asplenia, acute chest syndrome, pulmonary hypertension, stroke, cumulative damage to multiple organs, and low life expectancy [10-12]. Polymerization of Hb S is the main pathophysiological occurrence in SCA [13,14] in which there is a change in the physical properties of erythrocytes that causes them to acquire a sickle shape, resulting in hemolytic anemia and blockage of blood flow, particularly in small vessels, that can damage organs [4].

Sickle Cell Anemia: Inflammation and Vaso-Occlusion

The inflammatory state is a set of adaptive responses of the organism's cells as a result of infection or tissue injury, to restore organ function and homeostasis [15-17]. In patients with SCA, the chronic inflammatory state is a consequence of several factors that articulate and feed each other, forming a permanent inflammatory cycle. The vascular endothelium is an important factor in the inflammatory process and vaso-occlusion in SCA [18,19] in which interactions between erythrocytes and endothelial cells, leukocytes, and platelets play a central role [4]. Thus, during the vaso-occlusion process, neutrophils may be essential in adhering to the endothelium, promoting the attachment of sickle erythrocytes to these cells, reducing blood flow, and promoting vaso-occlusion [20]. The expression of some integrins, with together fibrinogen and leukocytes, can stimulate effector functions such as phagocytosis, degranulation, and the production of pro-inflammatory cytokines/chemokines, such as interleukin 6 (IL-6), tumor necrosis factor α (TNF- α) and IL-1 [21].

A chemotherapy drug that significantly contributes to reducing the number of vaso-occlusive crises is hydroxyurea, which stops cell division [22-24] to compensate for low oxygen levels. In addition, it promotes a decrease in the number of circulating neutrophils and a reduction in the expression of erythrocyte adhesion molecules [25].

Sebastiani and contributors [26] proposed a predictive model (https://www.bu.edu/sicklecell/) to assess the severity of the Hb S genotype in American patients. This model was designed using Bayesian network modeling, in which the risk of death is estimated at five years as a disease severity score, which, in turn, can be used in studies involving clinical and laboratory variables and has shown high specificity and sensitivity. This tool was used in Brazilian populations [27] to classify patients with SCD according to the age range of the score established by the calculator.

Based on this context, this study aimed to verify the association of molecular, hematological, and biochemical markers with the severity, determined by the calculator in male patients with the Hb SS genotype.

Materials and Methods

Sample

From a universe of 1500 blood samples from patients with SCD, 500 were randomly chosen and classified according to the Severity calculator.

Inclusion and exclusion criteria

From the initial sample (500) we excluded those who were: 1) Smokers; 2) Alcohol drinkers; 3) Pregnant 4) Patients with stroke; 4) Patients with pain, 5) Patients with a hemolytic crisis. The time established for the exclusion criteria was four weeks before the date of sample collection.

In the selection of patients who used hydroxyurea, those who used the drug for more than 90 days with a mean of 41.1 months were selected, with the mean

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dose per patient being 26 mg/kg/day. To eliminate possible biases arising from different Hb genotypes, only homozygotes (Hb SS) were selected, performed on a total of 182 male patients, aged between 18 and 71 years and included. This manuscript is in According to the Clinical Protocols and Therapeutic Guidelines for Patients with SCD in Brazil [28].

Research Instruments

Disease severity calculator

To classify the disease severity scores in the selected patients, the "Sickle Cell Disease Severity Calculator" tool was used, which ranks patients according to their clinical phenotype (Mild, Moderate, Severe). The model considers the risk of death as a score, ranging from 0 (least severe) to 1 (most severe) [27].

Hemoglobin phenotypes and genotypes

Two electrophoretic procedures were used to identify the patient's phenotype: In cellulose acetate at pH 8.6 and on agar at pH 6.2. Data of the Hb fraction quantification were obtained using high-performance liquid chromatography (HPLC) with the Trinity® Premier Resolution Analytical Columns equipment. Genotype was confirmed by PCR-RFLP [29].

Biomarkers

Due to their importance in pathophysiology, the following parameters were selected for this study: Hematological (Ferritin), biochemical (TBARS-Thiobarburic acid-reactive species) and molecular markers - SNP in following genes: [*TNFα (-308 G > A), TGFβ (-509 C > T), eNOS (-786 T > C)* and *HFE - C282Y (282 G > A), H63D (63 G > A)* and *S65C (65 A > T)*].

Hematological data and information onthe use of HU, clinical complications, blood transfusions, and exposure to environmental interferences were obtained by consulting the medical records and also the hematology service administration system (SASH) by the clinicians of the service, protecting the patient confidentiality.

Ferritin is a protein responsible for iron storage in the body and, for this biomarker, we considered reference values in healthy male patients of 23 to 336 ng/mL. For this, ferritin levels were considered normal (23 to 336 ng/mL), high (337 to 1000 ng/mL) and elevated (< 1000 ng/mL). The values obtained in the biochemical tests were determined in fasting venous blood samples by routine methods.

The biochemical marker TBARS was estimated through the levels of lipid peroxidation evaluated in heparinized plasma according to the protocol proposed by Uchiyama and Mihara [30] and values up to 440 ng/mL were considered normal [31].

The detection of the *-308G/A (rs1800629)* mutation of the *TNFα* gene was performed by the PCR RFLP method followed by restriction analysis. The fragment obtained from PCR was 117 bp. The mutation in the *-308G/A* promoter region eliminates the restriction site of the *NcoI* Enzyme. Therefore, when the amplified fragment was submitted to enzymatic digestion, the normal allele generated two fragments: 97 bp and 20 bp, and the mutant allele only produced one fragment: 117bp [32].

The detection of the *-509C/T (rs1800469)* mutation of the *TGFβ* gene (*-509 C > T*) was performed by RFLP PCR followed by restriction analysis. The fragment obtained from PCR was 406 bp. The mutation in the *-509C/T* promoter region eliminates the restriction site of the *Bsu36I* Enzyme. Therefore, when the amplified fragment was submitted to enzymatic digestion, the normal allele generated two fragments: 223pb and 183pb, and the mutant allele only generated one fragment: 406pb [33].

The detection of the *-786 T/C (rs2070744)* mutation of the *eNOS* gene was performed by RFLP PCR followed by restriction analysis. The fragment obtained from PCR was 180 bp. The mutation in the promoter region *-786T/C* eliminates the restriction site of the MspI Enzyme, therefore, when the amplified fragment was submitted to enzymatic digestion, the normal allele generated two fragments: 138 bp and 42 bp, and the mutant allele three fragments: 92 bp, 46 bp and 42 bp [34].

The frequencies of the three variants of the *HFE* gene (*C282Y*, *H63D,* and *S65C*) were analyzed by the PCR RFLP method. To detect the *C282Y* mutation, the sequence of primers used were: L282 (sense): 5' GGG TAT TTC CTT CCT CCA ACC 3' and R282 (antisense): 5' CTC AGG CAC TCC TCT CAA CC 3'. The following primers were used to detect *H63D* and *S65C* mutations: L63 (sense): 5' ACA TGG TTA AGG CCT GTT GC 3' and R63 (antisense): 5' ATC CCC AGC CTT GTT AAC TG 3', according to protocols previously established [29].

Statistical analysis

IBM SPSS Statistics 20 and *RStudio®* software were used for the analysis. Data were presented in percentage, according to the frequency presented. We used the chi-square test of independence test between the level of severity and the dependent variables to verify whether or not there was an association. Due to the expected frequency of some variables to present low values, Fisher's exact test was also performed. The degree of association was assessed using Cramer's V test. Continuous variables were categorized for carrying out the tests. The results were arranged in descriptive tables and bar graphs. A significance level of 5% ($p \leq 0.05$) was considered for all analyses.

Results and Discussion

Our results were compiled in Table 1.

The degree of disease severity was significantly

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Table 1

Severity						
Related Factors	Mild N = 60 (33.0%)	Moderate N = 82 (45.1%)	Severe N = 40 (22.0%)	χ^2	**F-test**	**φ**
BIOCHEMICAL MARKERS						
TBARS [Normal]	18 (30.0%)	0 (0.0%)	0 (0.0%)	76.34*	63.44	0.458
TBARS [High]	0 (0.0%)	0 (0.0%)	10 (25.0%)			
TBARS [Elevated]	42 (70.0%)	82 (100.0%)	30 (75.0%)			
CLINICAL COMPLICATIONS						
Stroke [Yes]	21 (35.0%)	82(100.0%)	40 (100.0%)	100.92*	103.53	0.745
Stroke [No]	39 (65.0%)	0 (0.0%)	0 (0.0%)			
Pain Crises [0-2]	15 (25.0%)	82 (100.0%)	22 (55.0%)	186.0*	173.04	0.715
Pain Crises [3-5]	45 (75.0%)	0 (0.0%)	0 (0.0%)			
Pain Crises [≥ 6]	0 (0.0%)	0 (0.0%)	18 (45.0%)			
Number of Infections [0-2]	40 (66.7%)	82 (100.0%)	40 (100.0%)	45.6	43.36	0.501
Number of Infections [3-5]	20 (33.3%)	0 (0.0%)	0 (0.0%)			
Number of Infections [≥ 6]	0 (0.0%)	0 (0.0%)	0 (0.0%)			
HEMATOLOGICAL MARKERS						
Ferritin [Low]	0 (0.0%)	4 (4.9%)	0 (0.0%)	272.3*	258.37	0.865
Ferritin [Normal]	60 (100.0%)	18 (22.0%)	0 (0.0%)			
Ferritin [High]	0 (0.0%)	3 (3.7%)	40 (100.0%)			
Ferritin [Elevated]	0 (0.0%)	57 (69.5%)	0 (0.0%)			
MOLECULAR MARKERS						
TNF-α [AA]	0 (0.0%)	0 (0.0%)	0 (0.0%)	134.31*	118.17	0.857
TNF-α [GA]	0 (0.0%)	0 (0.0%)	31 (75.6%)			
TNF-α [GG]	60 (100.0%)	2 (100.0%)	9 (22.0%)			
TGF-β [CC]	0 (0.0%)	60 (73.2 %)	40 (100.0%)	124.4*	166.51	0.585
TGF-β [CT]	44 (73.3%)	22 (26.8%)	0 (0.0%)			
TGF-β [TT]	16 (26.8%)	0 (0.0%)	0 (0.0%)			
eNOS [CC]	0 (0.0%)	0 (0.0%)	5 (12.5%)	107.1*	129.1	0.543
eNOS [CT]	0 (0.0%)	34 (41.5%)	35 (87.5%)			
eNOS [TT]	60 (100.0%)	48 (58.5%)	0 (0.0%)			
H63D [H63D/H63D]	0 (0.0%)	0 (0.0%)	2 (4.9%)	114.87*	142.0	0.560
H63D [W/H63D]	0 (0.0%)	55 (67.1%)	39 (97.5%)			
H63D [W/W]	60(100.0%)	27 (32.9%)	0 (0.0%)			
C282Y [W/C282Y]	0 (0.0%)	0 (0.0%)	3 (9.8%)	14.63*	9.21	0.278
***C282Y* [W/W]**	60 (100.0%)	82 (100.0%)	37 (92.5%)			
***S65C* [W/S65C]**	0 (0.0%)	0 (0.0%)	1 (2.5%)	3.57*	2.79	0.140
***S65C* [W/W]**	60 (100.0%)	82 (100.0%)	39 (97.5%)			

*Statistically significant $p < 0.05$, df = 4; n = 182 patients; $\varphi \in [0.1, 0.3]$: Weak association; $\varphi \in [0.4, 0.5]$: Medium association; $\varphi > 0.5$: strong association; n = 182 patient is.

associated with all the variables presented in the table above ($p > 0.05$).

The degree of association between severity, clinical manifestation, and the investigated markers is shown in Figure 1.

Some associations were stronger, for example, with the polymorphisms of the *TNF-α* genes [*AA*], [*GA*], and [*GG*], The most predominant genotype in the groups studied was the homozygous mutant polymorphism [*GG*] (Figure 2) in individuals who had a mild degree of severity.

This high frequency of the [*GG*] genotype in mild patients was explained by recent work [35] that showed that the *TNFα* gene polymorphism (*-308 G > A*), specifically the [*GG*] genotype, is more frequent in healthy patients than in cancer patients, also an inflammatory condition, and that this genotype is linked to low production of *TNF-α*, so, the [*GG*] genotype

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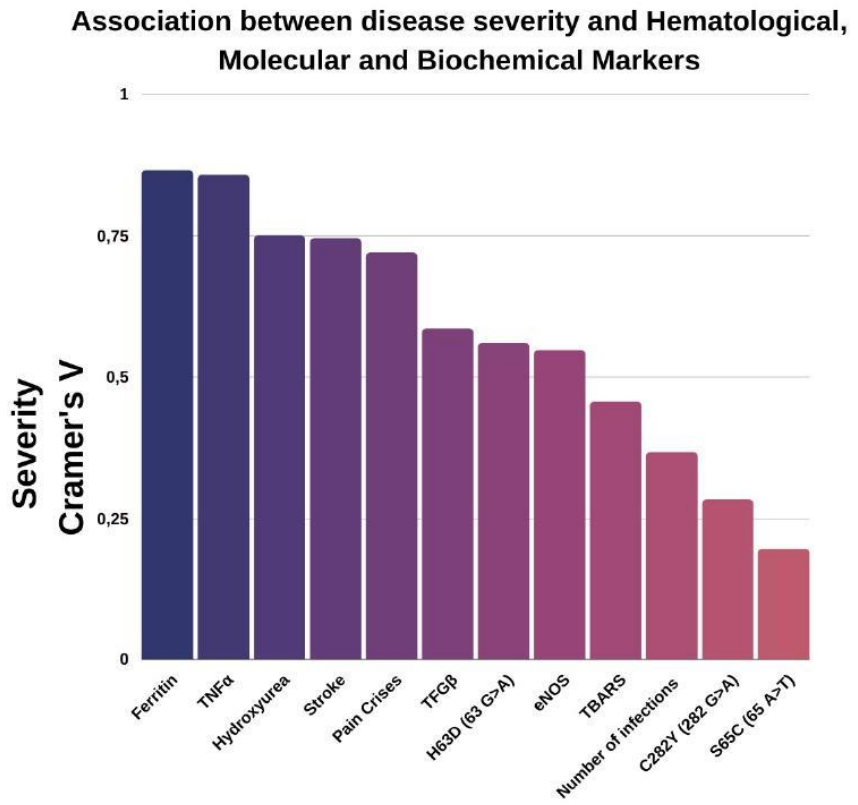

Figure 1: Association between severity score calculated by Cramer's V test and data from hematological, biochemical, and molecular markers. High values for Cramer's V2 indicate a stronger relationship between the variables, and lower values for V2 indicate a weak relationship. A value of 0 indicates that an association does not exist. A value of 1 indicates that there is a very strong association between the variables.

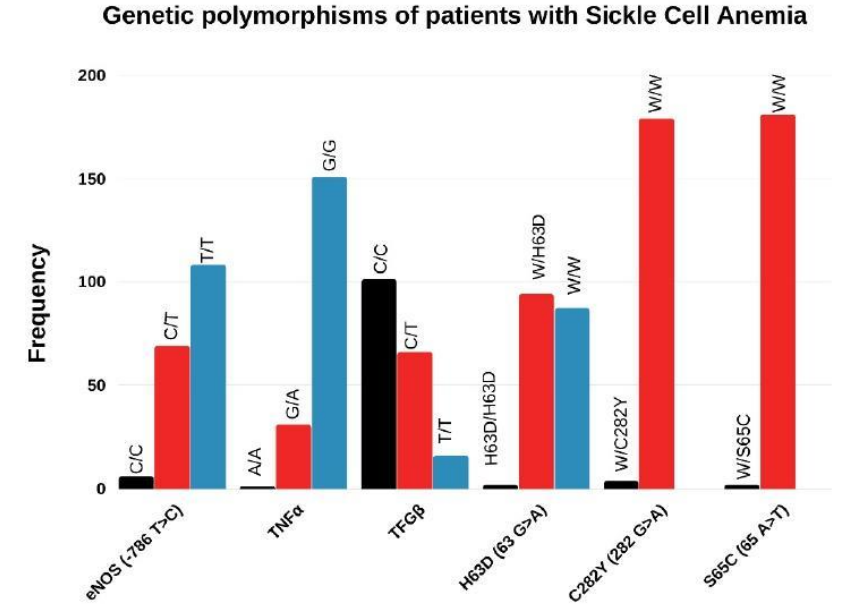

Figure 2: Demonstration of the frequency of genetic polymorphisms in which *eNOS* genes. *C/C*: Wild-type homozygote, *C/T*: Heterozygous, T/T: Homozygous mutant, *TNFα*: *A/A*: Wild-type homozygote, G/A: Heterozygous, *G/G*: Homozygous mutant, *TGFβ*: *C/C*: Homozygous wild, *C/T* : Heterozygous, *T/T*: Mutant heterozygote, *H63D/H63D:* Mutant homozygote, *W/H63D*: Heterozygous, *W/W:* Wild homozygote, *W/S65C:* heterozygous, *W/W*: Wild homozygote.

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would confer greater protection against the severity of the disease. The second most frequent polymorphism [*GA*] was observed in those patients who had a severe phenotype by the calculator. The heterozygous polymorphism [*GA*] is associated with the promotion of the activation of adhesion molecules in neutrophils and vascular endothelium [36] thus being able to be a risk factor for the occurrence of crises in SCA.

Our frequency of polymorphisms data corroboratesthose obtained in the Cavalcanti and contributors study [37], which from 25 patients with SCA in the northeast region of Brazil observed that SCA patients tend to have a higher production of *TNF-α*, a fact that can be explained by the chronic inflammatory processes even in patients in steady-state [38-40]; with the frequencies of polymorphisms [*GG*] > [*GA*] > [*AA*] > fact also observed in other studies [36,41]. These results are important as this high frequency/prevalence of the [*GG*] polymorphism can be used as a severe prognosis biomarker of serum *TNF-α* production.

Another fact with a strong association was pain crises, classified in frequencies of 0-2 and 3-5/year and greater than six/year. In our work, we observed that patients with a moderate severity had pain attacks with a frequency of 2 attacks per year on average. In contrast, the frequency observed in Mild patients was 3-5/year. This observation can be explained by the greater use of Hydroxyurea in patients with a moderate severity degree. The use of HU may be associated with peripheral pain sensitization [42,43] and other analgesic drugs [44]. It is known that one of the greatest difficulties faced by patients with sickle cell anemia is the pain (common in 70% of patients) [45,46] and these crises are be caused by vaso-occlusive processes, a complex phenomenon in which the interactions between erythrocytes and endothelial cells, leukocytes and platelets play a central role [4].

The analysis of the *eNOS* gene polymorphism (*-786 T > C*) showed a higher frequency of the polymorphism [*TT*] a wild-type genotype, followed by the heterozygote [*CT*], and finally, a lower frequency forthe variant allele [*CC*] in this patients. Similar data were reported by [47] who studied the impact of these polymorphic variants on nitric oxide activity in patients with sickle cell anemia. From these data, we can suggest that *eNOS* gene polymorphisms (*-786 T > C*) are important in the pathophysiology of SCA. There was an association with stroke (Figure 1) and these episodes were observed in patients with Moderate and Severe scores severity (Figure 3).

It is known that stroke is a major driver of mortality and morbidity in patients with sickle cell disease [48] a fact that could be explained by two hypotheses, the first is that the high rate of strokes would be due to the viscosity of sickle cell red cells, which would culminate in thrombosis and consequently ischemia [49]; however, this would not be enough to explain the origin of strokes in the great vessels, which are marked episodes in this disease. The second hypothesis concerns the connection of sickle cells to the vascular endothelium, where activation occurs by direct contact of sickle erythrocytes, heme, free Hb, and ROS induced by hypoxia/reperfusion [50]. This process influences the production of inflammatory mediators, such as IL-1β, IL-6, and tumor necrosis factor (TNF), which lead to a chronic inflammatory state that, in sickle cell disease, leads to a lower expression of anti-inflammatory proteins and increased expression of pro-inflammatory

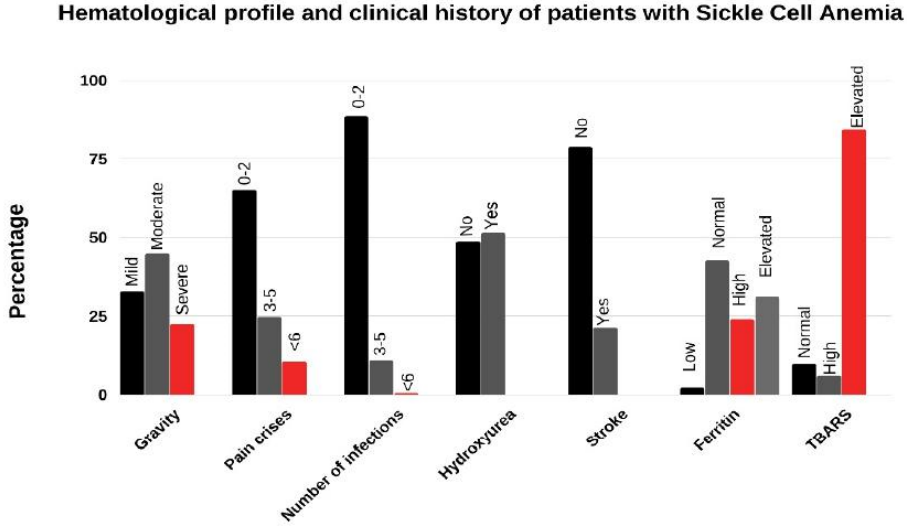

Figure 3: Percentage of the main components of the hematological profile (clinical complications, biochemical and hematological markers) of male patients with SCA as well as the percentage of patients with different degrees of severity according to the calculator. N = 182 patients observed.

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cytokines [51-54].

This stroke mortality rate could be explained by the high number of leukocytes, as well as the increase in the levels of adhesion molecules and inflammatory markers such as VCAM-1 [55,56]. Furthermore, it has been shown that *TNF-α 308* [*GA*] is associated with an increased risk of stroke in patients with SCA [38] the same findings were observed in our results. Thus, if there was a high frequency of TNF-α 308 [GA] we would tend to have a higher percentage of stroke patients. A review carried out by the author [57] showed that there is controversy regarding the genetic, clinical, and laboratory factors associated with the development of stroke in individuals with SCD, a fact that could be explained by the lack of uniformity and standardization in the definition of events other than cerebrovascular diseases (ischemic stroke, TIA, hemorrhagic stroke, silent infarctions, vascular stenosis detected by ARM and moya-moya disease) making it difficult to interpret and compare the results of the studies.

The use of hydroxyurea, as well as the levels of Ferritin, were shown to be major agents associated with the degree of severity (Figure 1). Patients with moderate (48 patients) and severe (40 patients) scores of severity had the highest drug use. Normal ferritin levels (23 to 336 ng/mL) were predominant in patients with a mild score; high ferritin levels (337 to 1000 ng/mL) were predominant in severely ill patients, and very elevated levels (< 1000 ng/mL) in moderately severe patients. It is known that the use of HU can improve the clinical conditions of patients with SCD by 98% [58-60] acting to increase fetal hemoglobin [61,62] by activating nitric oxide-dependent on soluble guanylyl cyclase [63]. It has been observed that the use of HU for two years can decrease high serum ferritin levels [64] in patients with sickle cell anemia. A similar study in beta-thalassemia patients reached the same results [58,64,65]. Interestingly, the high level of TBARS, prevalent in patients with moderate severity, could infer oxidative stress in the cellular environment, which results in the formation of highly reactive and unstable lipid hydroperoxides due to iron overload. Thus, although TBARS shows a median association with severity, it probably acts as an additional result of the increased value of iron and ROS in SCA.

Of the polymorphisms of the *HFE* gene studied, only the *H63D* showed a mean associated with degrees of severity, its frequency being predominant in patients with a moderate degree. The *HFE* gene is located on the short arm of chromosome 6 (6.p21.3) and collaborates in the regulation of iron metabolism, being a cofactor for several physiological and biological processes, and with a fundamental role in the manifestation of hereditary hemochromatosis (HH), an autosomal recessive disease characterized by excess iron in the blood [66]. The mutation to the *C282Y* polymorphism is a consequence of the transition from tyrosine to cysteine in codon 282 of exon 4. The *H63D* polymorphism results from the transversion of histidine by aspartic acid in the protein and the mutation occurs in exon 2 of codon 63. A mutation for the *S65C* polymorphism is responsible for the substitution of serine for a cysteine, due to the exchange in exon 2 [67]. These are the most frequent mutations for this gene in the Brazilian population [68-70].

The most frequent symptoms of iron overload are usually: fatigue, arthralgia/arthritis, abdominal pain, decreased libido, weight loss, hepatomegaly, splenomegaly, and arthropathy [71]. However, as iron deposition occurs gradually and cumulatively, the onset of symptoms occurs after the third decade of life, making early diagnosis of the disease difficult [72].

The HH diagnosis is confirmed by the presence of mutations *H63D, C282Y,* and *S65C* and is indicated for individuals with iron overload due to high serum ferritin concentrations, > 200 µg/L in women and > 300 µg/L in men. However, HH is mostly associated with homozygosity for the *C282Y* genotype, evidencing the clinical disease, and like the mutations for *S65C* and *H63D* has reduced penetrance, despite the mutation for *H63D* being more frequent when in compound heterozygosity with the *C282Y* gene (*C282Y /H63D* and *C282Y/S65C*) shows only moderate iron overload [73].

The HFE proteins resulting from the mutations alter cellular iron homeostasis and are related to several inflammatory and neuronal diseases. Especially in the brain, microglia and macrophages are responsible for maintaining iron homeostasis and inflammatory modulation associated with the pathogenic process of multiple diseases [74].

Thus, the genotypes of polymorphisms in the *HFE* gene fundamentally impact macrophage functions such as proliferation and survival and may increase the pro-inflammatory state [74] and consequently the release of mediators. This may be the mechanism by which *HFE* variants impact sickle cell disease status.

The genotypes in the *HFE* gene influence the severity of sickle cell anemia, especially the W/H63D genotype, more frequently in patients with moderate and severe degrees (Figure 1). Ferritin is high in inflammatory processes, such as those caused by hemochromatosis and hemolytic anemia [71]. Thus, the use of HU contributes to the improvement of severity in severe cases caused by genotypes in the *HFE* gene with high levels of ferritin, by reducing the number of leukocytes and the adhesion of red blood cells in SCA [59,75] preventing a worsening of iron absorption. In this way, the damage caused by oxidative stress would be reduced, with a consequent reduction in the level of TBARS. Since stress is a factor that may be involved in leukocyte recruitment, this is due to the release of iron,

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which initiates the hemoglobin oxidation pathway with damage to sickle cell erythrocytes [27] the combined results of polymorphisms and markers intensify vaso-occlusion and severity in SCA.

Conclusions

Our work showed that the degree of severity in SCA is associated with TNFα, Stroke, Pain Crises, TGFβ, TBARS, Number of infections, eNOS, Ferritin, and mutations in the *HFE* gene (*H63D*, *C282Y*, and *S65C*).

Furthermore, we did not find an association between the hydroxyurea use and the severity of the disease, with evidence of greater use of the hydroxyurea by patients with severe clinical conditions.The use of HU with an impact on the improvement of pain crises.

Some polymorphisms were more frequent in different degrees of severity, such as *TNFα* [*GG*] in patients with mild severity, and this genotype may provide greater protection against disease severity. In turn, the heterozygous polymorphism [*GA*] of *TNFα* may be a risk factor for the occurrence of crises in SCA; the stroke episodes were observed in patients with the moderate and severe scores; the high levels of TBARs displayed in patients with moderate severity; as well as the high levels of Ferritin and, finally, the polymorphisms in the *HFE* gene associated with moderate severity.

The association found between TBARS and severity evidences a probable relationship between the increased value of iron and ROS observed in patients with SCA.

Few studies and relative data regarding the association of these variables with disease severity were performed. Our data allow us to delineate a profile based on the severity of the disease with the most frequent clinical conditions in this segment of the population. The investigation of markers and their prognoses presented here allow health professionals to devise strategies for more effective support to patients, improving the quality of life of these individuals.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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Authors Contribution

1. Jonathan de Oliveira Rios: Supported research planning and data collection in the field, laboratory and data analysis, as well as the writing of the article.

2. Thais Fernandes Ribeiro: Supported research planning and data collection in the field, as well as the writing of the article.

3. Gabriel Felipe Arantes Bertochi: Supported the statistical part and writing of the article.

4. Clarisse Lopes de Castro Lobo: Supported the writing and clinical aspects, of reading and writing of the article.

5. Claudia Regina Bonini Domingos: Supported research planning and field data collection, laboratory and data analysis, as well as supporting the writing of the article.

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CAPÍTULO 2

5.2 Neutrophil alloantigen HNA-3b is associated with higher risk of leg ulcers in sickle cell disease

Esse capitulo é resultado dos estudos desenvolvidos no Laboratório "*Biologié Intégrée du Globule Rouge*" em Paris França, sob a orientação do Dr. Slim Azouzi e teve como foco estudar a associação gene *SLC44A2* nas manifestações fenotípicas da doença falciforme. O artigo foi submetido na revista *The American Journal of Hematology* (fator de impacto 10.1).

The neutrophil alloantigen HNA-3b is associated with higher risk of leg ulcers in sickle cell disease

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ABSTRACT

Sickle cell disease (SCD) is a common hemolytic disorder with a broad range of complications, including vaso-occlusive crisis (VOC), acute chest syndrome (ACS), pain, stroke, and leg ulcers. *SLC44A2* encodes the choline transporter-like protein identified as a receptor for Von Willebrand factor (VWF) and involved in the interaction with the activated $\alpha_{II}b\beta_3$ in platelets. Several recent genome-wide association studies linked the human neutrophil antigen 3a/b polymorphism (HNA-3a/b) in *SLC44A2* (rs2288904-G/A) to venous thrombosis (VT) risk. We examined this polymorphism to test the hypothesis that *HNA-3b* allele is associated with a specific risk of adverse outcomes among a cohort of 840 patients with SCD from different geographic regions. Unexpectedly, although *HNA-3b* allele is associated with a 30% decreased risk of VT in general population, this allele is associated with an increased total number of platelets and monocytes, as well as a higher susceptibility to leg ulcers in SCD patients. No relationship was identified between this polymorphism and VOC and thrombosis. These findings are important for understanding the mechanisms involved in specific complications of SCD, contributing to the development of therapeutic strategies targeted at specific disease phenotypes.

INTRODUCTION

Sickle cell disease (SCD; ORPHA232; OMIM 603903) is a common hereditary red cell anemia related to a point mutation on *HBB* gene that results in the synthesis of the pathological hemoglobin S (HbS or sickle Hb). This condition results in a variety of clinical complications, including chronic hemolytic anemia, acute and chronic pain, damage to target organs and a variety of other phenotypic manifestations.[1,2] SCD is a chronic and invalidating disorder, with elevated mortality in both pediatric and adult population.[3] In the last decades, progresses have been made on the pathogenesis of SCD disease, but the therapeutic tools are still limited to transfusion regimes and/or hydroxyurea (HU) treatment used as fetal Hb inducer or bone marrow transplantation on highly selected SCD patients.[4-6] The introduction of these strategies has significantly increased SCD patients' survival.[7] However, the impact of HU and transfusion regimes on both acute and chronic organ damages, such as cerebrovascular, kidney or leg ulcers, is still limited. This points out the urgent

requirement to understand how genetic and environmental factors influence the variety of clinical complications in this disease.

The *SLC44A2* gene, located on chromosome 19p13.2, encodes an ubiquitous transmembrane protein that belongs to the family of choline transporter-like proteins. The identification of *SLC44A2* as a susceptibility locus for venous thrombosis (VT) has generated heightened interest in this protein who's the physiological function is not yet fully understand. Broad genome-wide association studies have identified the single nucleotide polymorphism (SNP) in *SLC44A2* (*rs2288904-A*), which is linked to protection against VT.[8] This polymorphism defines the expression of the human neutrophil antigen 3a (HNA-3a, 455G; Arg152) or HNA-3b (455A, Gln152).[9,10] The HNA-3a encoding allele was demonstrated in Caucasians with a frequency of 0.79, in African Americans with 0.93 and in Chinese with 0.74.[11-13] Neutrophils homozygous for the *rs2288904-A* SNP have an impaired ability to interact with the von Willebrand factor (VWF) and have also a reduced ability to release NETs under venous shear stress rates.[14,15]

VWF regulates vascular permeability and PMN extravasation into inflamed tissues ,[16,17] and has been implicated in the pathogenesis of SCD VOC.[18-21] We postulated that HNA-3a/b polymorphism would affect the clinical complications in SCD. In this study, we determined the prevalence of HNA-3a antigen in patients with SCD from 5 countries and we tested whether this antigen associated with specific clinical complication.

METHODS

Biological material and collections

Study Population: For this study, we considered five populations from distinct geographical regions (Brazil, France, Guadeloupe, Mali, and Senegal), comprising patients with SCD in HbSS and HbSC genotypes. The sample included a total of 840 individuals, including 200 HbSS individuals from Brazil, 154 HbSS from Guadeloupe, 113 HbSS from Senegal, 97 HbSS from Mali, 112 HbSS individuals from France, and 164 HbSC individuals from the Brazilian population. Exclusion criteria were applied as described, excluding those who used tobacco, alcohol, individuals under 10 years of age, and those with hydroxyurea treatment for less than 90 days, as well as pregnant

women. All patients had been previously diagnosed with SCD by hemoglobin electrophoresis or high-performance liquid chromatography (HPLC), with clinical records available for data collection and stored DNA samples. The ethics regulations on research involving humans were followed by each of the participating centers, consistent with the Declaration of Helsinki. For the Brazilian population, the study was approved under CAAE 62525922.1.0000.5466; for the Malian population, under protocol 008/MSHP-CNESS/2014; and for the Senegalese population, under protocol 35/MSAS/DPRS.

Analysis of Clinical Complications: We investigated the potential influence of the SNP *rs2288904* on the occurrence of six clinical complications: thrombosis, vaso-occlusive crisis, stroke, leg ulcers, cholelithiasis, femoral head osteonecrosis, renal deficiency, and sickle cell retinopathy of any grade. For each complication, a case was defined as the patient presenting the specific complication, and a control as the patient from the same study cohort who did not present the complication. Stroke was defined as a new finding on neurological examination and new imaging on cerebral computed tomography (CT). Cholelithiasis was documented by abdominal ultrasonography or CT. Femoral head osteonecrosis was documented by radiography, CT, or magnetic resonance imaging (MRI). Retinopathy was documented by examination performed by an ophthalmologist; both non-proliferative and proliferative retinopathy were included due to shared pathophysiological bases. Leg ulcers were defined as persistent pain, local swelling, and color change (in the observed area), often accompanied by a sensation of weight, which did not decrease after treatment and was also confirmed by imaging. Additionally, the association of the SNP with hematological parameters was investigated.

Genotyping

Genomic DNA was extracted from blood samples using the commercially available DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD, USA), following the manufacturer's instructions. For real-time PCR, purified DNA samples from 840 donors were analyzed using TaqMan (Applied Biosystems, Foster City, CA, USA) real-time PCR to detect allelic polymorphisms (HNA3-a and HNA3b) in *SLC44A2* (rs2288904). Two probes with different fluorescence markers, FAM (6-carboxyfluorescein) and VIC (4,7,2'-trichloro-7'-phenyl-6-carboxyfluorescein), were used. Both TaqMan probes were incorporated into a single PCR mixture, allowing genotyping in a single tube for

each biallelic system. The PCR reaction mixture for a sample volume of 10 µL included 5 µL of TaqMan Genotyping Master Mix (2x) (Applied Biosystems™), 0.5 µL of TaqMan SNP Genotyping Assay, human (40x) (Applied Biosystems™), and 3 µL of DNA, followed by volume complementation with ultrapure water. Amplification was performed using the Bio-Rad CFX96 Touch real-time PCR thermocycler, under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 35 cycles of denaturation at 95°C for 15 seconds and extension at 60°C for 1 minute, with a final incubation at 4°C. Genotype analysis for each donor was performed using Bio-Rad CFX Manager 3.0 software.

Statistical analysis

The evaluation of the association between the SNP and phenotypic manifestations was conducted using R Studio software version 3.4.4. Chi-square and Mann-Whitney tests were employed, with a significance level set at $p < 0.05$. Interquartile ranges were represented. The ggplot2 package was used for graphical creation.

RESULTS

In this study, we aimed to understand the genetic structure of sickle cell disease populations from different ethnic groups using the SNP rs2288904 and subsequently compare it with the phenotypic manifestations of these patients. For this purpose, we calculated allelic frequencies and Hardy-Weinberg equilibrium. Genotypic analyses revealed significant variations in allelic frequency and genotypic distribution among different ethnic groups (MS -Table 1).

Our results show a predominance of the wild-type allele GG in all populations. Additionally, the absence of the mutant homozygote AA was observed in the French population. This variation of the mutant allele can be more prominently observed in the Brazilian SS population, where the allelic frequency of allele A was higher than all others (15%). Analysis of Hardy-Weinberg equilibrium revealed equilibrium in all populations except for the Brazilian SC population ($p=0.001$) in the HbSC Brazilian population, indicating potential selective pressures such as genetic drift, bottleneck effect, or historical events influencing the genetic distribution of the *SLC44A2* gene.

To verify whether the patients in this study were indeed undergoing treatment with HU, we analyzed hematological and clinical parameters (MS -Table 2). We observed that patients receiving the medication showed an increase in fetal

hemoglobin (HbF) and an improved mean corpuscular volume (MCV), along with a decrease in the number of reticulocytes. There was also a reduction in anemia. Furthermore, these patients exhibited a decrease in the number of thrombotic

After confirming that the patients were indeed undergoing treatment with the medication, we stratified these patients based on their treatment status and investigated whether the *SLC44A2* genotype influenced the clinical and hematological parameters in the Brazilian population of HbSS patients (Table 1).

Our results showed a significant difference in the occurrence of leg ulcers between the GG and AG+AA genotypes ($p = 0.03$). In the GG group, 12 out of 93 patients were identified with leg ulcers, while in the AG+AA group, 10 out of 27 cases were recorded. This could suggest a possible association between the AG+AA genotype and the development of leg ulcers in sickle cell anemia patients without the use of HU. The analysis of other clinical parameters did not reveal significant associations. However, there was a trend ($p=0.089$) in patients with the AG+AA genotype to present a higher number of vaso-occlusive crises.

In the analysis of patients undergoing HU treatment (Table 2)., no associations between phenotype and genotype were observed.

Patients with GG and AG + AA genotypes showed similar results regarding the evaluated clinical parameters. There was no significant difference between the GG and AG + AA groups regarding clinical and hematological parameters; similar analyses were conducted for the Brazilian SC and French SS populations; however, no association was found between genotype and medication. Therefore, our results suggest that, in the context of hydroxyurea treatment, the *SLC44A2* gene genotype does not play a significant role in the manifestation of these clinical parameters in patients with SCD. The lack of significant association may be attributed to various factors, such as sample size, genetic heterogeneity of the studied population, or the complexity of the interaction between genotype and treatment effects. Thus, we hypothesized that any association between genotype and clinical manifestations of patients would be more pronounced in complete cohorts, encompassing both HU-treated and untreated patients, leading us to investigate all individuals from different populations. The data are compiled in Table 3 The results of the analysis of the total cohort revealed significant associations between *SLC44A2* genotypes and the phenotypic manifestations of patients with SCD. Notably, hematological parameters,

especially platelet and monocyte counts, demonstrated a strong association with different genotypes. We observed that patients with AG+AA genotypes had significantly higher platelet counts compared to those with the GG genotype. Additionally, our results were consistent across three distinct cohorts, including Brazilian SS and SC cohorts and the Guadeloupe SS population, indicating the replicability of the findings. Another parameter that showed a significant difference was the monocyte count, also observed in the three Brazilian SS and SC cohorts and the Guadeloupe SS population. Similar to platelets, the monocyte count was higher in individuals with AG+AA genotypes compared to the wild-type allele. These findings suggest a consistent association between *SLC44A2* genotypes and hematological parameters. The results could not be investigated in the Mali SS, Senegal SS, and France SS cohorts since hematological parameters were not available.

Initially, this work aimed to investigate the association of thrombosis with the *SLC44A2* gene; however, our results did not show any association with this comorbidity in the study populations but a strong association of this polymorphism gene with leg ulcers. This association was observed in the Brazilian SS ($p = 0.01$) and SC ($p = 0.019$) populations, as well as in the Mali/Senegal SS population ($p = 0.03$) and the French SS population ($p = 0.02$).

This association suggests a genotypic influence on the predisposition to leg ulcers in patients with the AG+AA genotype. To gain a better insight into these results, we aggregated all populations in this study and observed that the higher the number of individuals with the AA+AG genotype in the population, the higher the prevalence of leg ulcers (Figure 1)

DISCUSSION

In this work, we reported the association of GG and AA+AG genotypes with the phenotypic manifestations of SCD such as increased monocyte and platelet counts. One possible explanation for these findings may be found in the *SLC44A2* gene, which is related to platelet activation. Previous studies, reported that the decrease and release of ATP, observed in the murine *SLC44A2*(KO) model, may lead to increased platelet activation.[22] This information is relevant to our study because patients with AG+AA genotype, who likely have functional expression of the *SLC44A2* gene, exhibited a higher platelet count. This suggests that the presence of the A allele may

be associated with higher gene activity and, consequently, a higher platelet count. Additionally, a study demonstrated that neutrophils can bind to activated platelet αIIbβ3 protein through *SLC44A2*.[15] Although a direct relationship between monocyte activation and the *SLC44A2* gene has not been demonstrated, it is known that these cells express this gene.[8] Therefore, it is possible that the expression of *SLC44A2* in monocytes may indirectly influence the monocyte count, leading to a higher count in patients with AG+ AA genotype.

A meta-analysis involving more than 65,000 individuals identified that carriers of the *SLC44A2* gene, especially HNA-3a, were more susceptible to developing venous thromboembolism.[8] In sickle cell anemia, under hypoxic conditions, erythrocytes undergo a conformational change in their structure, resulting in a sickle shape that is more prone to the formation of cellular aggregates in the bloodstream, increasing viscosity and, consequently, reducing blood flow. This reduction can lead to the formation of thrombi and thrombotic events, such as venous thromboembolism and vaso-occlusive crises.[23] Although we did not find an association between *SLC44A2* genotypes and thrombosis, we reported an association with leg ulcers.

Leg ulcers are the most common manifestations of SCD. The pathogenesis is complex and may include mechanical obstruction, bacterial infection, local thrombi, and functional nitric oxide deficiency, leading to endothelial dysfunction.[24,25] To date, there are no studies in the literature that indicate the relationship of HNA3b with the susceptibility to leg ulcers in any disease.

The results of this study revealed different patterns of association between genotypes and investigated phenotypes in the rare variants of the *SLC44A2* gene. It is noteworthy that several significant associations were found between the genotypes present in the rare variant of the *SLC44A2* gene and the analyzed phenotypes, suggesting a possible role played by this gene in determining these characteristics. This finding is particularly significant considering the possibility that sickle cell disease, a well-characterized monogenic condition, may be associated with this variant.

In the literature, it is widely known that hydroxyurea plays an important role in inducing an increase in HbF, resulting in modifications in the clinical phenotype of patients.[26] Recently, a study covering 728 SNPs revealed that 50 SNPs from 17 distinct genes are associated with changes in HbF in SCA patients treated with HU, and these changes are known to affect baseline HbF levels.[26] Consequently, erythrocytes

expressing higher levels of HbF have increased survival, reducing the occurrence of hemolysis.[27] Additionally, the medication increases hemoglobin and MCV levels, reduces neutrophil, monocyte, and reticulocyte levels, and affects the expression of adhesion molecules in the endothelium and nitric oxide generation. These hematological changes decrease the risk of vaso-occlusion in sickle cell anemia patients.[27-28] In our study populations, we showed that patients undergoing medication treatment experienced improvements in their clinical conditions.

The HNA-3a and HNA-3b antigens result from a single nucleotide polymorphism at position 154 of the first extracellular loop of the choline transporter-like protein 2 (CTL2).[9,29] The HNA-3a allele is characterized by the substitution of arginine at this specific position, while the HNA-3b allele has glutamine. The HNA-3 antigen is located on the CTL2 protein, a glycoprotein encoded by the *SLC44A2* gene, with a molecular mass between 70-95 kDa.[29,30] In addition to being expressed in neutrophils, the HNA-3 antigen is also found in monocytes, lymphocytes, and platelets.[15] Recent studies have demonstrated its expression in human erythroid precursors as well as mature erythrocytes.[31] CTL2 is a membrane protein that has 10 transmembrane domains and 5 extracellular loops. There are two distinct isoforms, named CTL2-P1 and CTL2-P2, both capable of binding to HNA-3a antibodies. However, only the CTL2-P2 isoform exhibits limited choline transport activity.[30]

The HNA-3a antigen present in plasma can trigger severe acute lung injury related to transfusion (TRALI), which is one of the leading causes of transfusion-related mortality .[32] For instance, a study showed that TRALI was responsible for 33.3% of transfusion-related fatalities.[33] Furthermore, a study evidenced that HNA antibodies are present in 42% of TRALI cases, with 12% of these cases being specific to HNA-3a.[34]

In this study, we provide an overview of the prevalence of these antigens in patients with sickle cell disease across different populations.

The allelic frequencies of the HNA-3 antigen vary considerably among different populations. For instance, a study conducted in various Chinese cities revealed that the frequencies of the HNA-3a and HNA-3b antigens were 0.76 and 0.24, respectively, with HNA-3b being less prevalent.[35] When examining other populations, we observed a correlation between allelic frequencies and geographical origin. In the case of Iranians, the frequencies were 0.63 and 0.37 for HNA-3a and HNA-3b, respectively,[36]

while in English Caucasians, the frequencies were 0.76 and 0.23.[37] In the context of the Brazilian Caucasian population, the frequency of HNA-3a was 0.95;[38] however, Lopes et al.,[39] found frequencies of 0.81 and 0.19 for HNA-3a and HNA-3b, respectively, in HbSS individuals. Our results are in agreement with the literature, indicating that the frequencies of the HNA-3 phenotype observed in the studied populations are comparable to those reported in Africans, African Americans, and Caucasians, but differ from those observed in the Eastern population. This fact reflects the different layers of miscegenation present in these populations.

CONCLUSIONS

Our results indicate that the *SLC44A2* gene may modulate certain manifestations of sickle cell disease, such as an increased susceptibility to leg ulcers, as well as an increase in the number of monocytes and platelets

These findings provide relevant insights into understanding the complexity of the genetics of the studied phenotypes and underscore the importance of further exploring the underlying molecular mechanisms of the associations observed in the rare variant of the *SLC44A2* gene, as well as examining other genetic variations in relation to the phenotypes in question.

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Tables and Figures

Table 1 Comparison of clinical parameters among *SLC44A2* gene genotypes of Brazilian SS patients without the use of HU.

	GG	AG+ AA	P values
n	43	15	
Age (years)	23 (14 – 31)	23 (9 – 32)	0.74
Sex ratio (M/F)	19/24	9/6	0.37
Retinopathy (Yes/No)	12/31	5/10	0.74
Cholelithiasis (Yes/No)	28/15	6/9	0.13
Renal deficiency (Yes/No)	13/30	3/12	0.52
Leg ulcers (Yes/No)	9/34	6/9	0.18
ONTF (Yes/No)	9/34	0/15	***0.09***
Thrombosis (Yes/No)	12/31	2/13	0.71
VOC (number)	3 (2 – 4)	3 (2 – 4)	0.98
Infection events (number)	2 (2 – 3)	3 (2 – 3)	0.46

Comparative analysis of clinical manifestations, including the occurrence of complications and clinical measures, among different genotypes (AA AG+GG) of the *SLC44A2* gene in SCA patients who were not undergoing treatment with HU. Significance was considered for $p<0.05$

Table 2 Comparison of clinical parameters among *SLC44A2* gene genotypes of Brazilian SS patients with the use of HU.

	GG	AG + AA	P values
n	105	37	
Age (years)	23 (17 – 31)	21 (15 – 29)	0.34
Sex ratio (M/F)	50/55	14/23	0.34
Retinopathy (Yes/No)	9/96	4/33	0.74
Cholelithiasis (Yes/No)	32/73	15/22	0.31
Renal deficiency (Yes/No)	21/84	6/31	0.81
Leg ulcers (Yes/No)	12/93	10/27	**0.03**
ONFH (Yes/No)	10/95	2/35	0.73
Thrombosis (Yes/No)	13/91	3/34	0.56
VOC (number)	3 (2 – 3)	3 (2 – 4)	***0.089***
Infection events (number)	2 (2 – 3)	2 (2 – 3)	0.29

The values display the number of patients and proportions for each category, as well as the p-values (statistical significance $p<0.05$) to determine if there are significant differences between the groups. Clinical parameters include age, sex ratio, retinopathy, cholelithiasis, renal deficiency, leg ulcers, avascular necrosis of the femoral head, thrombosis, number of vaso-occlusive crises (VOC), and infection events.

Table 3 Comparison of hematological and clinical parameters between GG and AG/AA genotypes in the total cohort of all studied SS and SC populations.

	Brazil SS			Brazil SC			Guadeloupean SS			Mali/Senegal SS			France GrEx SS		
	GG	AA+AG	**P**	GG	AA+AG	**P**	GG	AA+AG	**P**	GG	AA+AG	**P**	GG	AA+AG	P
n	148	52		139	25		132	17		179	31		100	12	
Age (years)	23 (15 – 31)	21.5 (14 – 29)	0.38	25 (10 – 37)	28 (11 – 46)	0.59	15 (10 – 39)	21 (8 – 42)	0.94	31 (16 – 59)	29(15 – 62)	0.57	30 (17 – 66)	30 (19 – 43)	0.18
Sex ratio (M/F)	69/79	23/29	0.87	70/69	13/12	0.69	83/52	8/9	0.29	86/93	19/12	0.24	40/60	6/6	0.54
HU (yes/no)	43/105	15/37	0.98	12/124	3/20	0.45	59/76	8/9	0.63	-	-	-	58/42	7/5	0.98
Hb (g/dL)	8.9 (8.1 – 9.8)	9.2 (8.3 – 9.8)	0.61	11.6 (10.6 – 12.8)	12.1 (11.5 – 12.8)	0.07	8.2 ± 1.2	8.8 ± 1.2	0.09	-	-	-	8.4 (6.0 – 13.8)	8.3 (6.6– 10.2)	0.94
WBC (10^9/dL)	12.1 (4.5 – 23.3)	13.4 (5.3 – 35.2)	0.06	8.5 (3.8 – 14.3)	9.0 (4.7 – 12.6)	0.34	9.2 ± 3.2	11.3 ± 4.6	**0.02**	-	-	-	-	-	-
Neutrophils (10^9/dL)	4881 (3608 – 6200)	4628 (2975 – 6221)	0.58	4499 (3315– 5735)	5158 (4120 – 6100)	**0.018**	3.9 (2.9 – 5.5)	5.3 (3.7 – 9.6)	**0.01**	-	-	-	-	-	-
Platelets (10^9/dL)	446 (345 – 555)	501 (402 – 632)	**0.007**	249 (167 – 332)	314 (228 – 404)	**0.007**	381.2 ± 127.5	458.4 ± 213.0	**0.03**	-	-	-	-	-	-
Monocytes (10^9/dL)	0.9 (0.51 – 1.50)	1.14 (0.73 – 1.90)	**0.02**	0.56 (0.41 – 0.71)	0.68 (0.56 – 0.79)	**0.016**	1.0 (0.69 – 1.30)	0.98 (0.81 – 1.23)	0.78	-	-	-	-	-	-
Retinopathy (Yes/No)	21/127	9/43	0.12	27/112	12/13	**0.041**	-	-	-	10/159	2/29	0.98	29/71	1/11	0.17
Cholelithiasis (Yes/No)	60/88	21/31	0.99	5/134	1/24	0.97	-	-	-	-	-	-	71/29	4/8	**0.01**
Renal deficiency (Yes/No)	34/114	9/43	0.44	9/130	6/19	**0.01**	-	-		-	-		23/77	4/8	0.47
Leg ulcer (Yes/No)	21/127	16/36	**0.01**	4/135	4/21	**0.019**	-	-	-	19/160	8/23	**0.03**	4/96	3/9	**0.02**
HTAP (Yes/No)	-	-		-	-	**-**	-	-	**-**		-	-			
Cardiopathy (Yes/No)	5/143	3/49	0.43	-	-		-	-	-		-	-	16/84	1/11	0.68
Thrombosis (Yes/No	25/122	5/47	0.26	11/128	0/25	0.21	-	-	-		-				
ONTF (Yes/No)	19/129	2/50	0.11	6/133	0/25	0.59	-	-	-	31/148	7/24	--	22/78	1/11	0.45
VOC (Yes/No)	146/2	50/2	0.27	-	-	-	-	-	-	-	-	-	46/54	6/6	0.98
Stroke (Yes/No)	-	-	-	3/136	2/23	0.16	-	-	-	-	-	-	23/77	7/5	**0.01**
Hospitalization (Yes/No)	140/8	49/3	0.45	26/113	6/19	0.58	-	-	-	-	-	-	23/77	3/9	0.99
Infection events (Yes/No)	142/6	51/1	0.67	16/123	7/18	**0.05**	-	-	-	-	-	-	83/17	7/5	**0.05**

The table presents data from five populations of individuals with sickle cell disease (HbSS and HbSC) in different countries: Brazil, Guadeloupe, Mali/Senegal, and France. The information is divided into various categories: Genotype: GG: Homozygous for the wild-type allele (G variant) of the *SLC44A2* gene. AA + AG: Combination of homozygotes for the mutant allele (AA) and heterozygotes (AG) for the *SLC44A2* gene.

Demographic characteristics: n: Number of individuals in the population. Age (years): Mean age (and range) of individuals in the population. Sex ratio (M/F): Proportion of males and females in the population.

Clinical characteristics: HU (yes/no): Use or non-use of hydroxyurea. Hb (g/dL): Mean hemoglobin level (and range) in the population. Leukocytes (10x9/dL): Mean white blood cell count (and range) in the population. Neutrophils (10x9/dL): Mean neutrophil count (and range) in the population. Platelets (10x9/dL): Mean platelet count (and range) in the population. Monocytes (10x9/dL): Mean monocyte count (and range) in the population.

Other complications: Retinopathy (yes/no): Presence or absence of retinopathy. Cholelithiasis (yes/no): Presence or absence of cholelithiasis. Renal insufficiency (yes/no): Presence or absence of renal insufficiency. Leg ulcer (yes/no): Presence or absence of leg ulcer. Pulmonary arterial hypertension (PAH) (yes/no): Presence or absence of pulmonary arterial hypertension. Cardiopathy (yes/no): Presence or absence of cardiopathy. Thrombosis (yes/no): Presence or absence of thrombosis. Osteonecrosis of the femoral head (ONFH) (yes/no): Presence or absence of osteonecrosis of the femoral head. Vaso-occlusive crises (VOC) (yes/no): Presence or absence of vaso-occlusive crises. Stroke (yes/no): Presence or absence of stroke. Infection events (yes/no): Presence or absence of infection events.

P: p-value for the chi-square test comparing the frequencies of genotypes GG, AA, and AG. --: Data not available.

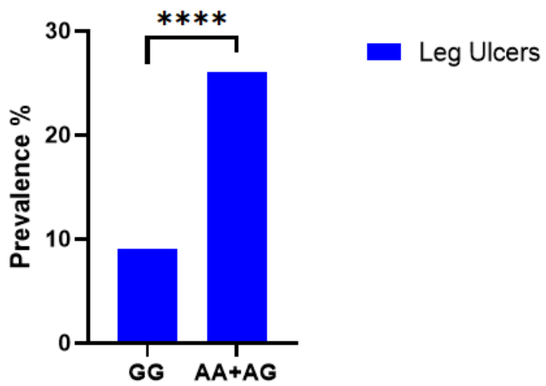


Figure 1. Distribution of leg ulcer frequency in patients with SCD stratified according to the *SLC44A2*. The image depicts a bar chart illustrating the frequency of leg ulcers in patients with SCD (HbSS/HbSC) across all populations in this study, stratified by genotype. The chart shows that the frequency of leg ulcers is significantly higher in patients with AG+AA genotype (19.05%) compared to patients with GG genotype (2.96%) p <0.001, n=840.

. SUPLEMENTARY MATERIALS:

MS-Table 1: Allelic Frequencies of the *SLC44A2* Gene in SCD Populations

Gene	SNP	N	Population	Genotype	Number observed	Allele frequency A G	Homozygoty Expected	Heterozygoty Expected	Homozygoty Rare Expected	H.W.E Ch2
SLC44A2	rs2288904	164	Brazilian HbSC	AA GG AG	1 139 24	0.08 0.92	139	23	1.03	0.001
		200	Brazilian HbSS	AA GG AG	8 148 34	0.15 0.85	144.5	51	4.5	8.9
		54	Guadeloupe HbSS	AA GG AG	1 135 17	0.06 0.94	134	16.9	0.53	0.32
		97	Mali HbSS	AA GG AG	1 81 15	0.09 0.91	80	15	0.7	0.10
		113	Senegal HbSS	AA GG AG	2 98 13	0.08 0.92	96	15	0.3	3.3
		112	France HbSS	AA GG AG	0 100 12	0.06 0.94	100	11	0.3	0.35

Genotype distribution of the *SLC44A2* polymorphism in different SCD populations. The observations include the total number of individuals (N) in each population, the frequency of genotypes AA, GG, and AG, the observed number for each genotype, the allelic frequency for alleles A and G, and the frequency of homozygotes for the rare allele, heterozygotes, and homozygotes.

Table 2: Comparison of hematological parameters and complications associated with SCA in the Brazilian SS population with and without HU treatment.

	SS patients without HU	SS patients with HU	P value
n	142	58	
Age (years)	22.0 (16.7 – 30.0)	23.0 (13.7 – 31.2)	0.46
Sex ratio(M/F)	64/78	28/30	0.75
Hb (g/dL)	8.8 (7.8 – 9.6)	9.4 (8.7 – 10.1)	**0.0002**
MCV (fl)	84.6 (73.3 - 89.6)	94.9 (88.7 – 102.0)	**<0.0001**
HbF (%)	5.7 (3.3 – 8.3)	19.9 (18.1 – 21.8)	**<0.0001**
RET (%)	10.8 (8.3 – 12.8)	8.3 (5.7 – 10.6)	**<0.0001**
LDH (U/L)	988.0 (802.5 – 1236.0)	840.0 (664.5 – 1094.0)	**0.01**
Bilirubin	3.1 (1.9 – 4.7)	2.1 (1.5 – 3.4)	**0.0012**
PNN	5.1 (4.1 – 6.3)	4.3 (2.9 – 6.1)	**0.005**
PLT	584.5 (368.8 – 584.5)	403.0 (332.8 – 504.0)	**0.019**
Retinopathy (Yes/No)	13/129	17/41	**0.0007**
Cholelithiasis (Yes/No)	47/95	34/24	**0.0014**
Renal deficiency (Yes/No)	27/115	16/42	0.19
Leg ulcers (Yes/No)	22/120	15/43	0.11
ONFH (Yes/No)	12/130	9/49	0.22
Thrombosis (Yes/No)	26/116	4/54	**0.04**
ASS (Yes/No)	1/141	4/54	**0.03**
VOC (number)	3 (2 – 3)	3 (2 – 4)	0.51
Infection events (number)	2 (2 – 3)	2 (2 – 3)	0.54

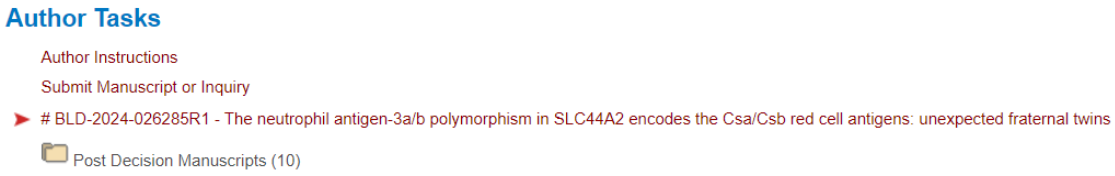
Comparison of hematological parameters and complications associated with SCA between patients who received HU and those who did not. The values presented are means (and interquartile ranges) for hematological parameters and frequencies for the analyzed complications. Significance was considered for $p<0.05$.

CAPÍTULO 3

5.3 The neutrophil antigen-3a/b polymorphism in *SLC44A2* encodes the Cs^{a} and Cs^{b} red cell antigens: unexpected fraternal twins

Esse capitulo é resultado dos estudos desenvolvidos no Laboratório "*Biologié Intégrée du Globule Rouge*" em Paris França, sob a orientação do Dr. Slim Azouzi e teve como objetivo elucidar a base molecular do antígeno de grupo sanguíneo raro Cs(a–).

O artigo foi submetido na revista *Blood* (fator de impacto 21,0) e foi aceito com pequenas revisões.



The neutrophil antigen-3a/b polymorphism in *SLC44A2* encodes the Cs^{a} and Cs^{b} red cell antigens: unexpected fraternal twins

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Short title: COST and HNA-3 antigens: unexpected fraternal twins

Key points:

- The HNA-3a/b polymorphism underlies the molecular basis of the Cs^a/Cs^b red cell antigens
- Anti-Cs^a and anti-HNA-3a recognize different epitopes on the SLC44A2 protein.

Abstract

The Cs^a blood group antigen was identified more than 50 years ago but its molecular basis has yet to be elucidated. All our recent genomic investigation has failed to resolve the molecular basis of this enigmatic antigen. By investigating the association of the HNA-3a/b polymorphism (rs2288904-G/A) in *SLC44A2* with clinical features of Sickle Cell Anemia (SCA), we incidentally discovered that rare subjects with the homozygous HNA-3b/b genotype also carry the uncommon Cs(a–) phenotype. We genotyped this SNP in a cohort of 25 Cs(a–) subjects and found that all of them showed a HNA-3b/b genotype. This result suggests that the high-prevalence allele with rs2288904 (HNA-3a; 455G) encoding Arg152 encodes the high-prevalence Cs^a. Accordingly, anti-Cs^a does not react with $SLC44A2_{null}$ RBCs, *SLC44A2* knockout K562 cells, and K562 cells expressing HNA-3b, confirming that the Cs^a and Cs^b antigens are carried on this protein. Furthermore, mass spectrometry analysis of SLC44A2 from neutrophils and RBCs, along with serological investigation, showed that, despite HNA-3a and Cs^a having the same molecular basis, anti-HNA-3a and anti-Cs^a recognize different epitopes on the SLC44A2 protein. Overall, our data resolve the genetic bases of the Cs(a–) and Cs(b–) blood phenotypes, with new insights on the anti-HNA-3a specificity.

Introduction

Unraveling the genetic bases of blood groups is crucial to ensure the immunological safety of red blood cell (RBC) transfusion and to implement novel genotyping assays. The recent development of genomic approaches has allowed the elucidation of numerous enigmatic antigens, including the recent VER and RIF antigens within the CTL2 blood group system[1]. Both antigens are carried by the choline transporter-like protein 2, CTL2 or SLC44A2, which also express the diallelic HNA-3a and HNA-3b antigens associated with a single nucleotide polymorphism (SNP) at position 455G>A (rs2288904-G/A), respectively[2]. Genome-wide association studies linked expression of HNA-3b (455A, Gln152) on the SLC44A2 protein with a decreased risk of venous thromboembolism (VTE) in humans[3]. Notably, this antigen plays a critical role in the interaction of neutrophils with platelets and the von Willebrand factor[4,5].

In 1965, Giles et al. described an antibody to a high-prevalence antigen in three unrelated patients, that did not correspond to any known specificity at that time. It was decided to name this antigen Cs^a, after the name of the two first antibody makers. The Cs(a–) blood phenotype is infrequent in the Caucasian population (2%) and -Cs^a is not uncommon[6]. Many years later, its antithetical low-prevalence antigen, Cs^b, was identified. Both antigens were included to the so-called COST blood group collection in 1988, with the following antigen numbers: 205001 and 205002, respectively. However, the presumptive protein carrier for the Cs^a and Cs^b antigens and the genetic basis of the Cs(a–) blood phenotype remain unknown. ^{51}Cr-labeled red cell survival study reported that anti-Cs^a does not cause significant destruction of transfused Cs(a+) red cells, indicating that anti-Cs^a had no clinical significance in the event of an

incompatible transfusion[7]. Although anti-Cs^a is considered to be clinically insignificant and a typical high-titer low avidity (HTLA) antibody, it causes problems upon red cell antibody screen and identification because of the risk of masking clinically relevant alloantibodies[8]. Here, we elucidate the molecular basis of the long-established blood group phenotype Cs(a–) and we demonstrate that the erythroid Cs^a antigen corresponds to the neutrophil alloantigen HNA-3a carried by SLC44A2 glycoprotein. Interestingly, we also demonstrated that anti-Cs^a and anti-HNA-3a recognize different epitopes in this protein.

Methods

Study design

Fresh peripheral blood samples were obtained from Cs(a–) and healthy donors from the Etablissement Français du Sang, and SCD patients after obtaining written informed consent.

The study was approved by a medical ethics committee (GR-Ex/CPP-DC2016-2618/CNIL-MR01).

Genomic experiments

Whole exome sequencing (WES), Sanger sequencing and genotyping assays of genomic DNA were detailed in the supplementary methods. List primers were cited in Supplemental Table S1.

Cell engineering and flow cytometry

K562 cells were genetically modified by Crispr-Cas9 to generate K562 *SLC44A2* KO and K562 HNA-3b cell lines (Supplemental Figure S1). Primers are listed in Supplemental Table S1. Cs^a and HNA-3a antigen expression were analyzed by flow

cytometry using an anti-Cs^a eluate and serum containing an anti-HNA-3a alloantibody, respectively.

Results

To unravel the genetic basis of the Cs^a and Cs^b antigens, DNAs from seven unrelated Cs(a–) individuals were subjected to WES. Homozygous variants from WES were filtered using several gnomAD minor allele frequency (MAF) cutoff thresholds, but no shared variants were identified among all Cs(a–) individuals. Despite our intense efforts to solve this enigma, we eventually abandoned our investigation with respect to this blood group and we pursuited our research on other red cell antigens. Motivated by the association of SLC44A2 polymorphisms with VTE and vascular diseases[3,9,10], we assessed the involvement of the HNA-3a/b antigens in the clinical features of patients with sickle cell disease (SCD) in whose VTE and stroke are frequently encountered complications[11]. Incidentally, TaqMan SNP genotyping for the *SLC44A2* polymorphism (rs2288904, NM_001145056.2) in a cohort of SCD patients and extended phenotyped donors of RBC panel revealed that rare subjects with the homozygous HNA-3b/b genotype (rs2288904-A) also carry the uncommon Cs(a–) red cell phenotype (Figure 1A). Inspired by this surprising coincidence, we hypothesized that rs2288904-A and Cs^a antigen expression are closely associated. Thus, we genotyped this SNP in a cohort of 25 Cs(a–) and 50 Cs(a+) subjects. All the Cs(a–) individuals were homozygous for the low-frequency 455A allele, encoding the HNA-3b phenotype whereas all Cs(a+) subjects displayed the 455G allele encoding the HNA-3a phenotype (Figure 1B). The strong association between the Cs(a–) phenotype and the rs2288904 ($P< 2.2\times10^{-16}$) unambiguously confirmed the link between HNA-3a/b and Cs^a/Cs^b phenotypes. This result was also confirmed by Sanger sequencing of

exon 7 of *SLC44A2* from individuals with different HNA-3 and COST phenotypes, validating that c.G455A variant is associated with both the neutrophil HNA-3b and erythroid Cs(a–) phenotypes (Figure 1C). Using flow cytometry analysis, we tested the reactivity of an anti-Cs^{a} eluate, purified from the plasma of alloimmunized Cs(a–) patients, with a selection of RBCs with the rare VER– blood type, namely VER– (SLC44A2$_{null}$), HNA-3a and HNA-3b phenotypes. As expected, anti-Cs^{a} did not react with SLC44A2$_{null}$ and HNA-3b RBCs, confirming that the Cs^{a} antigen is carried by the SLC44A2 protein (Figure 1D). To obtain direct evidence that the Cs^{a} antigen is encoded by the c.G455A SNP in *SLC44A2*, we generated a panel of K562 cells expressing either SLC44A2/HNA-3a (Arg152) or SLC44A2/HNA-3b (Gln152) antigens using the CRISPR/Cas9 genome editing mediated by HDR. Sequencing of a selected clone and flow cytometry analysis using an anti-RIF eluate confirmed that both cell lines expressed similar levels of SLC44A2 (Figure S1D). As shown in Figure 1D, K562 cells expressing HNA-3a were readily recognized by anti-Cs^{a} but this antibody failed to elicit any significant staining of K562 expressing HNA-3b. In good agreement with the results obtained with VER– RBCs (SLC44A2$_{null}$), anti-Cs^{a} did not react with *SLC44A2* knockout K562 (Figure 1D).

We then verified how WES approach failed to detect the *SLC44A2* variant among Cs(a–) individuals. Integrative Genomics Viewer analysis revealed that the GRCh37 human reference genome used for variant calling unexpectedly correspond to the rs2288904-A allele encoding the Cs(a–) phenotype (Figure 1E). We next evaluated whether the choice of reference assembly had the same impact for the detection of rs2288904-A by WES. We found that both GRCh37 and GRCh38 references were unable to detect this variant, confirming the limit of WES in the detection of common genetic variants[12]. Taken together, our results demonstrate that

the Cs^a antigen is encoded by c.G455 in *SLC44A2*, which corresponds to the genetic basis of the HNA-3a antigen. As a result, the Cs^a and Cs^b red cell antigens leave the COST collection and join the 39th erythroid blood group system, CTL2.

HNA-3a antibodies can result in severe, sometimes fatal transfusion-related acute lung injury (TRALI) in transfused patients. TRALI is a clinical syndrome defined by hypoxia and pulmonary edema, occurring during or within 6 hours of blood transfusion in the absence of alternate causes of pulmonary injury[13]. Current testing for anti-HNA-3a includes cumbersome techniques such as the granulocyte agglutination and granulocyte indirect immunofluorescence tests. In our cohort, anti-Cs^a is usually detected in pregnant women or in RBC transfused patients using the indirect antiglobulin red cell agglutination test (IAT) (Table S2). We typed neutrophils from Cs(a–) and healthy blood donors serologically for the HNA-3a antigen on their neutrophils by flow cytometry and by a granulocyte aggregation test using plasma with HNA-3a specific antibodies. Anti-HNA-3a was able to bind Cs(a+) neutrophils but not Cs(a–) neutrophils (Figure 2A). Furthermore, flow cytometry analysis using three different anti-Cs^a eluates showed no reaction with HNA-3b neutrophils (Figure 2B), but a strong reactivity with HNA-3a neutrophils. Consistently, the granulocyte test aggregation demonstrated that Cs^a-specific antibodies induce large granulocyte aggregates, in a similar manner to that observed with anti-HNA-3a (Figure 2C). These data confirm that both anti-HNA-3a and anti-Cs^a recognize the same antigen in neutrophils. Next, we addressed whether anti-HNA-3a is able to bind Cs(a+) antigen in RBCs. The reactivity of five different anti-HNA-3a antibodies (with three of them having caused TRALI) with RBCs were evaluated using the IAT. Unexpectedly, none of these antibodies agglutinated RBCs, regardless of their Cs^a/Cs^b phenotype (Figure 2D and E), indicating that anti-HNA-3a does not recognize the Cs^a antigen in RBCs.

Although Cs^a and HNA-3a antigens are encoded by the p.Arg152 in SLC44A2 (Figure 3A), it seems that the antigens carried by the neutrophil cells and red blood cells are structurally different. Since we recently showed that erythroid cells and neutrophils expressed the same *SLC44A2* isoform[1], we hypothesized that epitope difference between anti-Cs^a and anti-HNA-3a is probably due to different post-translational modifications, including disulfide bonds and N-glycosylation sites in this protein (Figure 3A)[14]. Since it has been shown that SLC44A2 glycosylation changes peptide detection by mass-spectrometry[2], we then compared SLC44A2-derived peptides from neutrophils (n=18) and RBC membranes (n=8). Twenty-one peptides were identified from neutrophils, while only nine peptides were detected from RBCs (Figure 3B, Supplemental Table S3). Interestingly, the HNA-3a-carrying peptide is detected in neutrophils but not in RBCs, which strongly indicates a structural difference of SLC44A2 between both cell types. In addition, the 3D analysis of SLC44A2 model showed that the Arg152 residue is located in the first loop and interacts with the Asp153 amino acid by hydrogen bond (Figure 3C). This interaction seems to be stabilized by a disulfide bond Cys137-Cys156 (Figure 3C), which is crucial for the anti-HNA-3a binding to neutrophils[15]. By contrast, the anti-Cs^a reactivity on neutrophils (Figure 3D) and RBCs (data not shown) are not altered by reduction with dithiothreitol (DTT). Several studies demonstrated that the first loop SLC44A2 peptides containing Arg152 are recognized by only about half of the HNA-3a antibodies identified in donors whose blood products caused TRALI[16]. Our findings strongly indicate that anti-Cs^a could correspond to a sub-group of anti-HNA-3a antibodies, although the involvement of anti-Cs^a in TRALI remains to be proven.

Acknowledgments

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Authorship Contributions

B.K., T.P., and S.A. conceived the project. B.K., and S.A. designed and coordinated the study. R.D. designed and performed Crispr/Cas9 experiments and performed the flow cytometry experiments. A.S. performed serological and flow cytometry experiments. J.D.O.R., and M.T., performed genotyping of HNA-3a/b polymorphism. C.M., and S.A. analyzed the whole exome sequencing data. E.F.G., performed and analyzed proteomic experiments. A.R., E.L., D.V., J.B., G.L., and T.P. performed immunohematological analyses and provided essential materials, M.R., C.L.V.K., O.H., D.C., and S.S., provided advice and critical review. B.K. and S.A. analyzed data and wrote the paper, and all authors contributed to review of the final manuscript.

Footnotes

[#] R.D. and A.S. contributed equally to this work

[#] B.K. and T.P. contributed equally to this work

Disclosure and competing interest statement

The authors declare that they have no conflict of interest.

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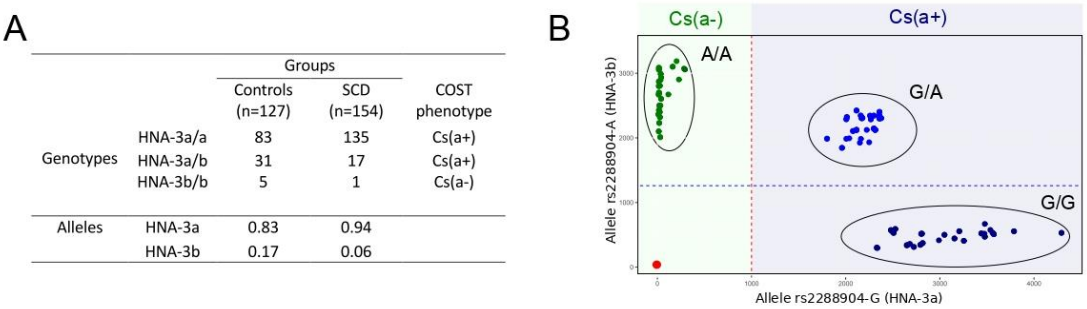
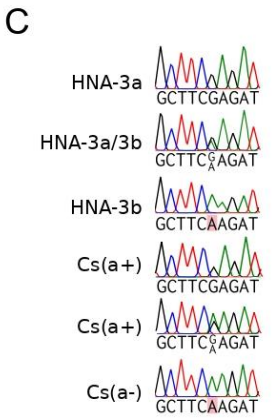
Figure Legends

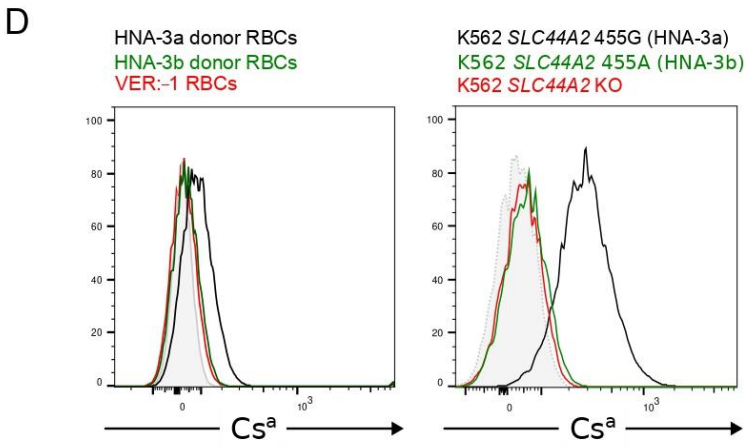
Figure 1: Homozygosity for the p.Arg152Gln variation in the SLC44A2 transporter is responsible for the Cs(a–) blood phenotype.

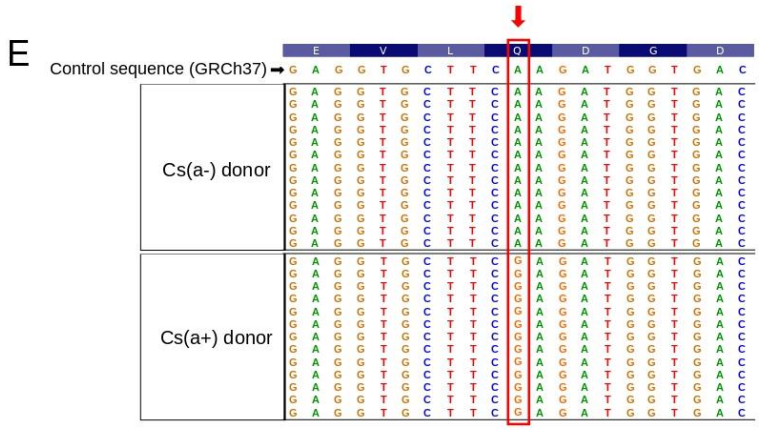
A

		Groups		
		Controls (n=127)	SCD (n=154)	COST phenotype
Genotypes	HNA-3a/a	83	135	Cs(a+)
	HNA-3a/b	31	17	Cs(a+)
	HNA-3b/b	5	1	Cs(a-)
Alleles	HNA-3a	0.83	0.94	
	HNA-3b	0.17	0.06	





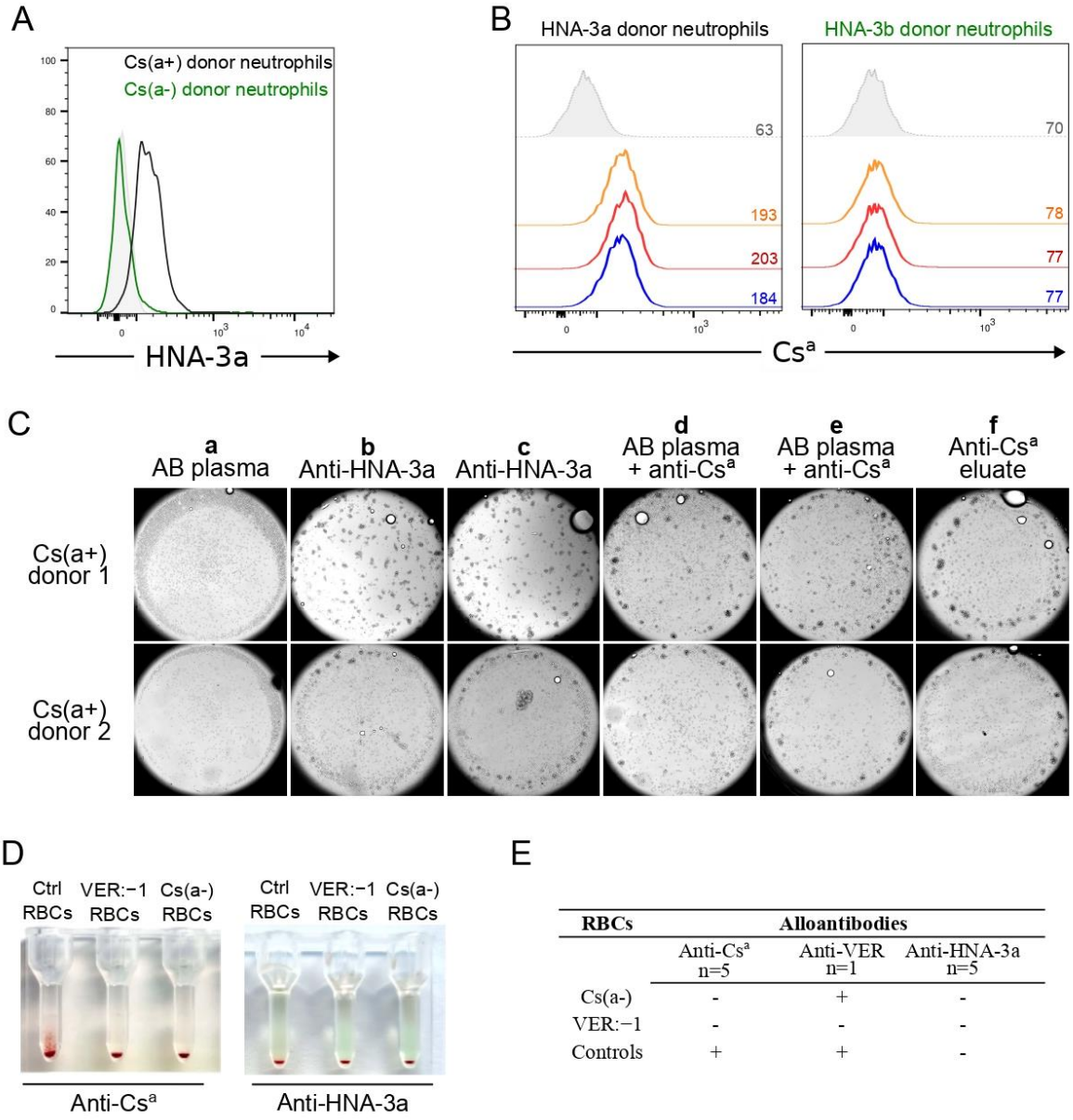




A. Genotypic and allelic frequencies of HNA-3 in blood donors and SCD patients.

B. TaqMan rs2288904 genotyping in Cs(a–) (n= 25) and Cs(a+) (n=50) individuals by TaqMan SNP genotyping assay. The association between the homozygous *AA* allele and Cs(a-) phenotype was analyzed by pearson's chi-squared test ($P< 2.2\times10^{-16}$). **C.** Sanger sequencing of the *SLC44A2* in HNA-3a/a, HNA-3a/b, HNA-3b/b, and Cs(a–) individuals showing the homozygosity for the A allele of rs2288904 in individuals with the Cs(a–) and HNA-3b phenotypes. **D.** RBC surface expression of the Cs^a antigen in HNA-3a, HNA-3b and $SLC44A2_{null}$ (VER–) individuals. The gray profile corresponds to RBCs incubated with only the secondary antibody. **E.** Cell surface expression of the Cs^a antigen in K562 *SLC44A2* c.455G (HNA-3a), K562 *SLC44A2* c.455A (HNA-3b) and K562 *SLC44A2* KO cells. The gray profile corresponds to K562 cells incubated with only the secondary antibody. **F.** Genome sequencing read mapping alignment data from Cs(a–) and Cs(a+) individuals showing the A allele of rs2288904 in the GRCh37 reference.

Figure 2: Anti- HNA-3a and anti-Cs^a recognize different epitopes on SLC44A2

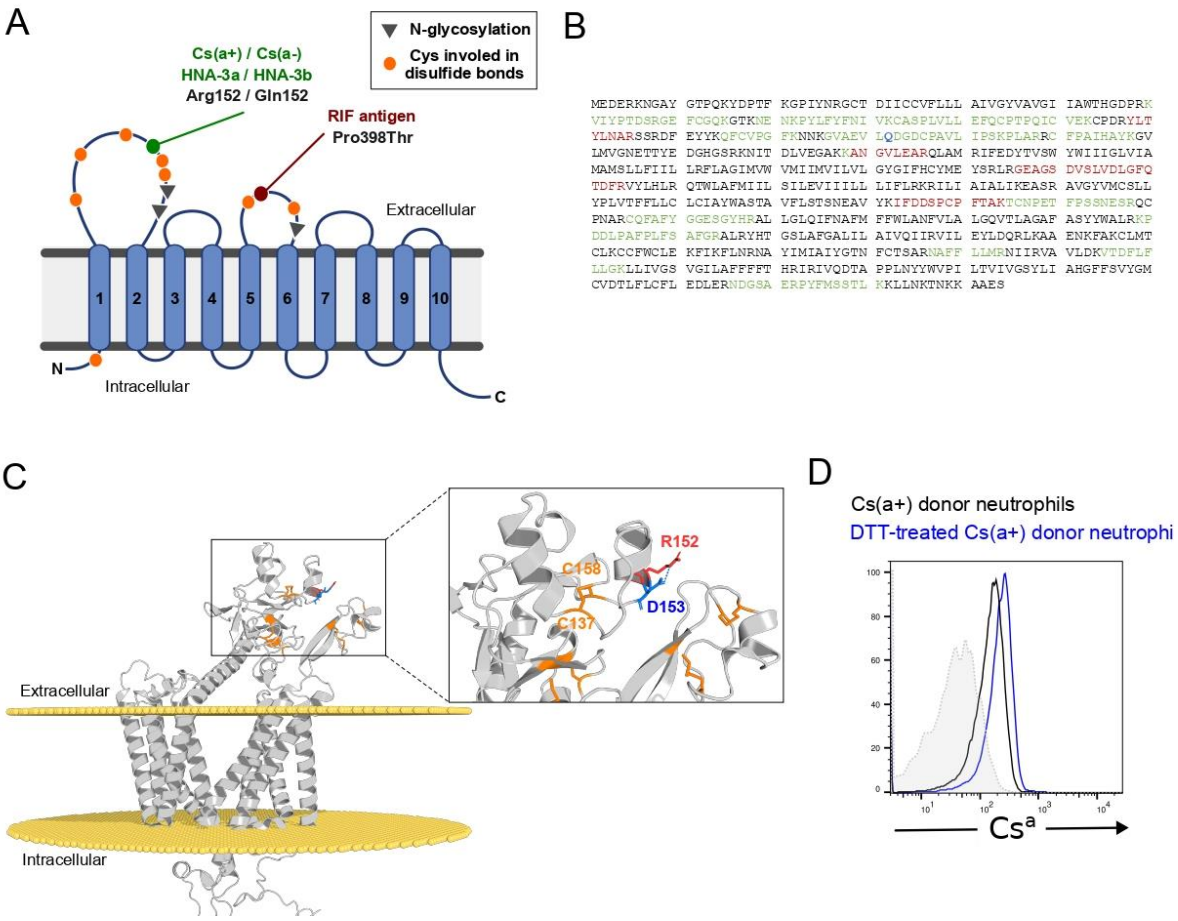


RBCs	Alloantibodies		
	Anti-Cs^a n=5	Anti-VER n=1	Anti-HNA-3a n=5
Cs(a-)	-	+	-
VER:−1	-	-	-
Controls	+	+	-

A. Flow cytometry analysis of HNA-3a antigen expression on granulocytes. Granulocytes from Cs(a+) (Black line) or Cs(a–) (green line) donors were incubated with plasma containing anti-HNA-3a and plasma without HNA-3a–specific antibodies (gray profile)**.** The figure is representative of 5 different anti-HNA-3a sources. **B.** Granulocyte aggregation test. HNA-3a-positive granulocytes from two donors were

incubated with the indicated antibodies: two different human plasma containing anti-HNA-3a (**b** and **c**), two different anti-Cs^a in plasma AB (**d** and **e**), and anti-Cs^a eluate without plasma. Plasma AB was used as negative control (**a**). **C**. Cell surface expression of the Cs^a antigen on granulocytes from HNA-3a (left panel) or HNA-3b (right panel) donors. Anti-Cs^a alloantibodies purified from three alloimmunized Cs(a–) patients were used (orange, red and blue profile). The gray profile corresponds to granulocytes incubated with only the secondary antibody. The geometric mean of fluorescence value is indicated for each flow cytometry histogram. **D**. Indirect antiglobulin test (IAT) using column agglutination of RBCs. Control, VER− or Cs(a–) papain-treated RBCs were incubated with plasma containing an anti-Cs^a alloantibody (left panel) or serum containing an anti-HNA-3a alloantibody (right panel). This figure is representative of several IAT using different anti-HNA-3a and anti-Cs^a antibodies. **E**. Cross-match between RBCs and alloantibodies from CTL2 variants and control.

Figure 3. Structural characterization of HNA-3a and Cs^a antigens



A. Predicted topology of the human SLC44A2 (isoform P1). The location of the HNA-3a/b, Cs^a/Cs^b, and RIF antigens are highlighted. Transmembrane domains are numbered 1–10. Orange dots indicate Cysteine residues likely to be involved in disulfide bonds and grey triangles indicate the predicted N-glycosylation sites in the extracellular loops[14]. **B.** SLC44A2 peptides identified by mass spectrometry of neutrophils and RBC membranes. SLC44A2-dervied peptides from neutrophils were showed with green. Common peptides between neutrophils and RBCs were indicated in red. **C.** Structural model of SLC44A2. The ionic interaction between Arg152 (red) and Asp153 (Blue) was stabilized by the Cys137-Cys156 disulfide bonds (Orange) The

H-bond between the ionic groups of Arg152 and Asp153 is denoted by the blue broken line. **D.** Impact of DTT treatment on anti-Cs^a reactivity on neutrophils.

Supplemental Methods

Blood samples

Samples from Cs(a–) individuals were obtained from the cryopreserved rare reference material collection from the National Reference Center for Blood Group (CNRGS, Paris, France, Biocollection DC-2016-2872). Fresh blood with different HNA-3 phenotypes were obtained from healthy blood donors from the Etablissement Français du Sang (EFS, Paris, France). Blood samples were collected in accordance with French blood donation regulations and ethics and the French Public Health Code (Article L.1221-1). Blood samples from 154 SCA patients were collected in EDTA and informed consent was obtained from adult patients and from parents or legal guardians for children (aged <18 years). The study was approved by a medical ethics committee (GR-Ex/CPP-DC2016-2618/CNIL-MR01).

Serological tests

Standard hemagglutination tests were used for assessment of the reactivity of several anti-Cs^a and anti-HNA-3a with RBCs. Anti-Cs^a alloantibodies were sourced from alloimmunized patients with the uncommon Cs(a–) phenotype. HNA-3a antisera were obtained from blood donors previously reported to be implicated in TRALI. Indirect antiglobulin gel tests (ID-Card LISS/Coombs; DiaMed, Bio-Rad, Hercules, CA) were performed at 37°C on papain-treated RBCs diluted in a low-ionic strength solution (LISS). Anti Cs^a eluates were prepared using the GAMMA ELU-KIT II device (Immucor Inc, Norcross, GA). The anti-Cs^a specificity was checked by using Cs(a–) and Cs(a+) RBCs, as well as the absence of contaminating ABO antibodies.

Whole Exome Sequencing

Genomic DNA was extracted from whole blood using standard methods. Exome sequencing was performed on genomic DNA using a SureSelect Human All Exon kit (Agilent Technologies) for targeted enrichment and Illumina HiSeq2000 for sequencing. WES and data analysis were performed as previously described[1].

HNA-3 genotyping and nucleotide sequencing

A TaqMan real-time PCR was established for genotyping of DNA samples. Purified genomic DNA samples were analyzed using TaqMan real-time PCR to detect allelic polymorphisms (HNA3-a and HNA-3b) in SLC44A2 (rs2288904). Two probes with different fluorescence markers, FAM (6-carboxyfluorescein) and VIC (4,7,2'-trichloro-7'-phenyl-6-carboxyfluorescein), were utilized. Both TaqMan probes were incorporated into a single PCR mixture, enabling genotyping in a single tube for each biallelic system. The PCR reaction mixture for a 10 µL sample volume included 5 µL of TaqMan Genotyping Master Mix (2x) (Applied Biosystems™), 0.5 µL of TaqMan SNP Genotyping Assay, human (40x) (Applied Biosystems™), and 3 µL of DNA, followed by volume completion with ultrapure water. Amplification was carried out using the CFX96 Touch Real-Time PCR thermocycler (Bio-Rad), under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 35 cycles of denaturation at 95°C for 15 seconds and extension at 60°C for 1 minute, with a final incubation at 4°C. Genotype analysis for each donor was performed using Bio-Rad CFX Manager 3.0 software. For Sanger sequencing, genomic DNA from blood samples or K562 cells was extracted by using the MagNA Pure System (Roche Life Science, Penzberg, Germany) or PureLink™ Genomic DNA Kit (Invitrogen), respectively. Exons 4, 6 and 7 of *SLC44A2* gene were amplified from genomic DNA

and the PCR products were sequenced by Sanger (Eurofins Genomic). PCR and sequencing primers are listed in the supplemental Table S1.

Cell culture

K562 cells were cultured in Iscove modified Dulbecco medium, 25 mM N-2-hydroxyethylpiperazine-N9 -2-ethanesulfonic acid, and GlutaMAX (Gibco, Carlsbad, CA) supplemented with 10% de-complemented (56°C, 30 minutes) fetal bovine serum, and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) at 37°C under a humidified atmosphere containing 5% carbon dioxide. Authentication of our K562 cell line based on genetic characterization was performed by using polymerase chain reaction (PCR) single-locus technology (PowerPlex 21 PCR Kit; Promega, Madison, WI) by Eurofins Genomics (Ebersberg, Germany).

Generation of K562 *SLC44A2* klnockout and K562 HNA-3b cell lines using Crispr-Cas9 technology

RNA guides were designed with CRISPOR (http://crispor.tefor.net/). For the generation of K562 *SLC44A2* KO cell lines, RNA guides targeting two different exons of SLC44A2 were selected (Supplemental Table S1). For the generation of K562 HNA-3b cell line, RNA guide targeting exon 7 was selected (Supplemental Table S1). sgRNA was synthesized by assembly PCR and *in-vitro* transcription using Precision gRNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. Cas9 RNP assembly was carried out by incubating 10 µg of recombinant Cas9 protein (TrueCut™ Cas9 Protein v2; Invitrogen) with 6 µg of in-vitro transcribed sgRNA for 15 min in PBS. K562 cells (5x106) were washed in PBS and suspended in 100 µl of Cell Line Nucleofector™ Kit V buffer (Lonza) and mixed with Cas9 RNP complex. For the generation of K562 HNA-3b, 1µL of 100µM ssODN template (Supplemental Table S1) was added. The mixture was then electroporated using Nucleofector IIb (program T-

016; Lonza). Two days after the transfection, INDEL and knock-in events in the targeted exons were assessed by sequencing the target sites and analyzed by ICE (https://ice.synthego.com/) (Supplementary Figure S1A). Edited pools of K562 were sorted into a 96 well plate at single cell densities using MA900 Cell Sorter (SONY) and grown to confluence (approximately two weeks). For the screening, the targeted exons of each clone were PCR amplified and sanger sequenced (Supplementary Figure S1B and S1C).

Flow cytometry

Thawed RBCs or K562 cells were washed 3 times in phosphate buffered saline (PBS) (Gibco) and then resuspended in low-ionic strength buffer supplemented with 0.5% bovine serum albumin (BSA) and incubated with anti-Cs^{a} or anti-HNA-3a (1:2) for 60 min. Cells were then washed with PBS and resuspended PBS supplemented with 0.5% BSA and incubated with an anti-human IgG-PE for 20 min. A FACSCanto II flow cytometer (BD Bio- sciences, Franklin Lakes, NJ) and FlowJo software (FlowJo LLC, Ashland, OR) were used for data acquisition and analysis, respectively.

Neutrophil labeling and aggregation tests

Human neutrophils were isolated from fresh whole blood (< 4h after blood sampling) using the MACSxpress Neutrophil Isolation Kit followed by a MACSxpress Erythrocyte Depletion Kit (Miltenyi Biotec). The binding of anti-HNA 3a or anti-Cs^{a} to neutrophils was performed by flow cytometry and by granulocyte agglutination tests, per current recommendations of the International Granulocyte Workshop.

Liquid Chromatography-coupled Mass spectrometry analysis (nLC-MS/MS)

RBC membranes and neutrophil lysates were prepared from mature RBCs and neutrophils, respectively, isolated from Cs(a+) donors as described[2,3]. A total of 50 µg of proteins per sample were reduced and alkylated using TCEP 20 mM and

chloroacetamide 50 mM and digested by filter-assisted sample preparation (FASP) using trypsin. Resulting peptides were fractionated on strong cation exchange chromatography (SCX) StageTips into five fractions. nLC-MS/MS analyses were performed on a Dionex U3000 RSLC nano-LC- system coupled to a TIMS-TOF Pro mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). After drying, peptides were solubilized in 45 µL of 0.1% TFA containing 2% acetonitrile (ACN). One µL of each fraction were loaded, concentrated and washed for 3min on a C_{18} reverse phase column (5µm particle size, 100 Å pore size, 300 µm inner diameter, 0.5 cm length, from Thermo Fisher Scientific). Peptides were separated on an Aurora C18 reverse phase resin (1.6 µm particle size, 100Å pore size, 75µm inner diameter, 25cm length mounted to the Captive nanoSpray Ionisation module, from IonOpticks, Middle Camberwell Australia) with a 170 min run time with a gradient ranging from 98% of solvent A containing 0.1% formic acid in milliQ-grade H2O to 35% of solvent B containing 80% acetonitrile, 0.085% formic acid in mQH2O. The mass spectrometer acquired data throughout the elution process and operated in DDA PASEF mode with a 1.16 cycle time, with Timed Ion Mobility Spectrometry (TIMS) enabled. Ion accumulation and ramp time in the dual TIMS analyzer were set to 100 ms each and the ion mobility range was set from 1/K0 = 0.6 Vs cm^{-2} to 1.6 Vs cm^{-2}. Precursor ions for MS/MS analysis were isolated in positive polarity with PASEF in the 100-1.700 m/z range by synchronizing quadrupole switching events with the precursor elution profile from the TIMS device.

nLC-MS/MS data analysis

The mass spectrometry data were processed using Maxquant Sofware version 1.6.17.0.[4] The database used was a concatenation of human sequences from the TrEMBL database (Uniprot, release sept 2022) and an incremented list of

contaminants. The enzyme specificity was trypsin. Carbamidomethylation of cysteins was set as constant modification and acetylation of protein N-terminus and oxidation of methionines were set as variable modifications. False discovery rate (FDR) was kept below 1% on both peptides and proteins. Label-free protein quantification (LFQ) was done using both unique and razor peptides. At least two ratio counts were required for LFQ. All experiments were analyzed simultaneously with the 'match between runs' option.

Structural model of SLC44A2

The model was generated based on the reference protein sequence (Uniprot accession number Q8IWA5-3) of the *SLC44A2* gene. The HNA-3a polymorphism was obtained by substituting glutamine at position 152 with arginine. The structure was modeled using AlphaFold2[5] via the ColabFold interface[6], and the membrane positioning was calculated using the ANVIL method[7]. The insertion of the protein into a lipid bilayer with a lipid composition corresponding to that of red blood cells was performed using CHARMM-GUI[8]. The images of the model and the calculation of polar interactions were performed using PyMOL Version 2.5.7.

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CAPÍTULO 4

5.4 Variants of *CD36* gene are implicated in increased cholesterol levels in sickle cell disease

Esse capitulo é resultado dos estudos desenvolvidos no Laboratório "*Biologié Intégrée du Globule Rouge*" em Paris França, sob a orientação do Dr. Slim Azouzi e teve como foco estudar a associação do gene *CD36* nas manifestações fenotípicas da doença falciforme.O artigo será submetido para publicação na revista *The American Journal of Hematology* (fator de impacto 10.1).

INTRODUCTION

Sickle cell disease (SCD; ORPHA232; OMIM 603903) is a common hereditary red cell anemia related to a point mutation on *HBB* gene that results in the synthesis of the pathological hemoglobin S (HbS or sickle Hb). This condition results in a variety of clinical complications, including chronic hemolytic anemia, acute and chronic pain, damage to target organs and a variety of other phenotypic manifestations.[1,2] SCD is a chronic and invalidating disorder, with elevated mortality in both pediatric and adult population.[3] In the last decades, progresses have been made on the pathogenesis of SCD disease, but the therapeutic tools are still limited to transfusion regimes and/or hydroxyurea (HU) treatment used as fetal Hb inducer or bone marrow transplantation on highly selected SCD patients.[4-6] The introduction of these strategies has significantly increased SCD patients' survival.[7] However, the impact of HU and transfusion regimes on both acute and chronic organ damages, such as cerebrovascular, kidney or leg ulcers, is still limited. This points out the urgent requirement to understand how genetic and environmental factors influence the variety of clinical complications in this disease.

The *CD36* gene, located on chromosome 7 in the region 7q21.11. CD36 is a broadly expressed scavenger receptor that interacts with a variety of ligands, including lipid substances such as long-chain fatty acids and both native and oxidized lipoproteins, as well as non-lipid ligands like thrombospondin 1 (TSP1) and collagen.[8] This receptor is particularly abundant in vascular endothelial cells, especially in the heart, skeletal muscles, and adipose tissue.[9,10] In these tissues, CD36 facilitates the efficient high-affinity uptake of fatty acids (and contributes to their cellular metabolism.[11] Recently, a study found that the G allele of the SNP *rs3211938* (G/T) is strongly associated with endothelial alterations.[10] It was demonstrated that this allele is absent in European and Asian populations but is quite frequent in African populations.[10] Most research to date has focused on the role of this SNP in lipid pathways; however, in the context of sickle cell disease, there are no studies linking

this variant to the phenotypic manifestations of the disease, presenting an excellent opportunity for further investigation.

METHODS

Biological material and collections

Study Population: For this study, we considered five populations from distinct geographical regions (Brazil, France, Guadeloupe, Mali, and Senegal), comprising patients with SCD in HbSS and HbSC genotypes. The sample included a total of 840 individuals, including 200 HbSS individuals from Brazil, 154 HbSS from Guadeloupe, 113 HbSS from Senegal, 97 HbSS from Mali, 112 HbSS individuals from France, and 164 HbSC individuals from the Brazilian population. Exclusion criteria were applied as described, excluding those who used tobacco, alcohol, individuals under 10 years of age, and those with hydroxyurea treatment for less than 90 days, as well as pregnant women. All patients had been previously diagnosed with SCD by hemoglobin electrophoresis or high-performance liquid chromatography (HPLC), with clinical records available for data collection and stored DNA samples. The ethics regulations on research involving humans were followed by each of the participating centers, consistent with the Declaration of Helsinki. For the Brazilian population, the study was approved under CAAE 62525922.1.0000.5466; for the Malian population, under protocol 008/MSHP-CNESS/2014; and for the Senegalese population, under protocol 35/MSAS/DPRS.

Analysis of Clinical Complications: We investigated the potential influence of the SNP *rs3211938 (G/T)* on the occurrence of six clinical complications: thrombosis, vaso-occlusive crisis, stroke, leg ulcers, cholelithiasis, femoral head osteonecrosis, renal deficiency, and sickle cell retinopathy of any grade. For each complication, a case was defined as the patient presenting the specific complication, and a control as the patient from the same study cohort who did not present the complication. Stroke was defined as a new finding on neurological examination and new imaging on cerebral computed tomography (CT). Cholelithiasis was documented by abdominal ultrasonography or CT. Femoral head osteonecrosis was documented by radiography, CT, or magnetic resonance imaging (MRI). Retinopathy was documented by

examination performed by an ophthalmologist; both non-proliferative and proliferative retinopathy were included due to shared pathophysiological bases. Leg ulcers were defined as persistent pain, local swelling, and color change (in the observed area), often accompanied by a sensation of weight, which did not decrease after treatment and was also confirmed by imaging. Additionally, the association of the SNP with hematological parameters was investigated.

Genotyping

Genomic DNA was extracted from blood samples using the commercially available DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD, USA), following the manufacturer's instructions. For real-time PCR, purified DNA samples from 840 donors were analyzed using TaqMan (Applied Biosystems, Foster City, CA, USA) real-time PCR to detect allelic polymorphisms (G/T) in *CD36* (*rs3211938*). Two probes with different fluorescence markers, FAM (6-carboxyfluorescein) and VIC (4,7,2'-trichloro-7'-phenyl-6-carboxyfluorescein), were used. Both TaqMan probes were incorporated into a single PCR mixture, allowing genotyping in a single tube for each biallelic system. The PCR reaction mixture for a sample volume of 10 µL included 5 µL of TaqMan Genotyping Master Mix (2x) (Applied Biosystems™), 0.5 µL of TaqMan SNP Genotyping Assay, human (40x) (Applied Biosystems™), and 3 µL of DNA, followed by volume complementation with ultrapure water. Amplification was performed using the Bio-Rad CFX96 Touch real-time PCR thermocycler, under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 35 cycles of denaturation at 95°C for 15 seconds and extension at 60°C for 1 minute, with a final incubation at 4°C. Genotype analysis for each donor was performed using Bio-Rad CFX Manager 3.0 software.

Statistical analysis

The evaluation of the association between the SNP and phenotypic manifestations was conducted using R Studio software version 3.4.4. Chi-square and Mann-Whitney tests were employed, with a significance level set at $p < 0.05$. Interquartile ranges were represented.

RESULTS

In this study, we aimed to understand the genetic structure of sickle cell disease populations from different ethnic groups using the SNP *rs3211938* and subsequently compare it with the phenotypic manifestations of these patients. For this purpose, we

calculated allelic frequencies and Hardy-Weinberg equilibrium. Genotypic analyses revealed significant variations in allelic frequency and genotypic distribution among different ethnic groups (MS -Table 1).

In the *CD36* gene, specifically the *rs3211938* (G/T) SNP, genotype frequencies were analyzed across various populations of patients with different forms of sickle cell disease (HbSS and HbSC). Overall, the T allele was predominant in all populations studied, while the G allele appeared at a significantly lower frequency. Homozygosity for the T allele was much more common than homozygosity for the G allele in most groups, with heterozygosity (TG) showing variability between populations. Notably, the G allele was more frequent in African populations (Mali, Senegal, and Guadeloupe), but much rarer in Brazilian. The analysis of Hardy-Weinberg equilibrium (H.W.E.) indicated that most populations were largely in equilibrium, with some deviations observed in specific groups (HbSS patients from Mali and Senegal). These deviations suggest the potential influence of selective pressures, such as genetic drift, population bottlenecks, or historical events, on the genetic distribution of the *CD36* gene in these populations.

We then tested all cohorts, but focused specifically on the Brazilian HbSS cohort to assess the influence of the allele in patients who were either on hydroxyurea treatment or not (MS Table 2 and 3). Our results did not show any significant effect of the alleles on the clinical complications of patients, regardless of whether they were undergoing hydroxyurea treatment.

Therefore, we decided to investigate each population without distinguishing between those who were or were not undergoing treatment (Table 1). The results of this analysis reveal significant clinical differences between groups of sickle cell anemia patients from different cohorts and genotypes. In the Brazil SS population, patients with the TT genotype had significantly lower hemoglobin levels compared to the GG+TG group ($p=0.01$), highlighting a relevant variation in Hb levels between genotypes. Additionally, lactate dehydrogenase (LDH) levels, a marker of hemolysis, were higher in patients with the GG+TG genotype compared to the TT group, both in the Brazil SS cohort ($p=0.02$) and the Brazil SC cohort ($p=0.004$), indicating increased hemolytic activity associated with the GG+TG genotype.

In the Brazil SS population group, a significant difference in neutrophil count was observed, with higher levels in the GG+TG group compared to the TT group

(p=0.01), a trend that was also seen in the Brazil SC group (p=0.006). These data demonstrate important variations in neutrophil levels between genotypes. Regarding monocytes, the Brazil SS population showed a highly significant difference, with the GG+TG group having lower counts compared to the TT group (p<0.001). In the Mali/Senegal SS cohort, a notable difference was observed in the prevalence of retinopathy, which was significantly more common in the GG+TG group (p<0.001). Additionally, this cohort also showed a higher incidence of pulmonary hypertension in the TT group (p=0.005) and a higher prevalence of leg ulcers in the GG+TG group (p=0.01).

DISCUSSION

Several studies have sought to elucidate the molecular mechanisms underlying sickle cell disease. In this study, we aimed to analyze the *rs3211938* SNP and its impact on the phenotypic manifestations of sickle cell disease. *CD36*, a scavenger receptor located on the surface of monocytes, erythrocytes, and platelets, has been implicated as a receptor for *P. falciparum*-infected red blood cells.[12,13] Variants of *CD36* are also known to be involved in cerebral malaria, although studies have not found an association with severe malaria syndromes.[14,15] The minor allele, G, has been associated with cerebral malaria and confers resistance to severe hemolytic anemia.[16,17]

CD36 is a marker of erythroid progenitor differentiation and may also be involved in the macrophage-mediated clearance of red blood cells.[18,19] Additionally, CD36 plays a role in various biological pathways, such as lipid metabolism/transport, atherosclerosis, hemostasis, and inflammation.[20] Some studies have shown that *CD36* is associated with platelet count, HDL cholesterol, and C-reactive protein levels in African Americans, as well as malaria resistance in African populations.[21-23] Our results show an association of this gene with HDL, as well as neutrophil and monocyte counts; however, unlike other studies, our findings did not show an association with platelets. We also found that the T allele was predominant in the populations studied, and the French population had a minor allele frequency of less than 1%, which aligns with reports that the minor allele is absent in European populations but can reach up to 30% in African populations.[24]

In SS individuals, serum HDL is generally elevated.[25] During painful vaso-occlusive crises, HDL may further increase in some patients due to hyperhemolysis,

as shown by studies of red blood cell survival.[26,27] In the context of sickle cell patients carrying the GG/TG allele, there may be an even greater increase in LDH levels, which in turn could affect other phenotypic complications of the disease.

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CONCLUSION

Our results indicate a significant association between the *rs3211938* SNP and phenotypic manifestations of sickle cell disease, particularly the GG/TG alleles, which were linked to elevated LDH levels. These findings suggest that monitoring patients with homozygous SS genotypes who carry these alleles may be crucial, as increased LDH levels could signal a worsening of the clinical condition. Early identification of these alleles could therefore aid in more effective disease management, helping to prevent complications related to heightened hemolysis.

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Tables

Table 1 Comparison of hematological and clinical parameters between TT and GG/TG genotypes in the total cohort of all studied SS and SC populations.

	Brazil SS			Brazil SC			Guadeloupean SS			Mali/Senegal SS			France GrEx SS		
	TT	GG+TG	P	TT	GG+TG	P	TT	GG+TG	P	TT	GG+TG	P	TT	GG+TG	P
n	171	19		131	9		150	4		188	22		101	11	
Age (years)	24 (8 – 58)	27 (12 – 50)	0.35	28 (8 – 75)	25 (11 – 39)	0.46	23(10–38)	31 (13 – 48)	0.51	29 (12 – 62)	27 (11– 49)	0.52	30 (17 – 66)	28 (18 – 41)	0.98
Sex ratio (M/F)	78/93	9/10	0.96	63/68	5/4	0.98	89/61	2/2	0.98	95/93	10/12	0.82	39/62	4/7	0.97
HU (yes/no)	47/124	5/14	0.94	12/119	2/7	0.22	64/83	3/1	0.32	-	-	-	57/44	8/3	0.35
Hb (g/dL)	8.7 (5.2 – 10.7)	9.4 (7.9 – 10.7)	**0.01**	11.6 (8.6 – 14.2)	11.9 (10.3 – 13.7)	0.46	8.2 (7.4 – 9.2)	8.9 (7.3 – 9.1)	0.78	-	-	-	8.5 (6.0 – 13.8)	8.1 (7.0–10.2)	0.24
WBC (10^9/dL)	12.6 (4.5 – 35.2.)	12.8. (6.3 – 19.5)	0.83	8.5 (3.8 – 14.3)	9.0 (4.7 – 12.6)	0.34	-	-	-	-	-	-	-	-	-
LDH (U/L)	542.3 (417–663)	613.6 (536 –687)	**0.02**	579 (265 – 968)	780 (500 – 981)	**0.004**	513(362 – 653)	385(321 – 559)	0.58						
Neutrophils (10^9/dL)	4818(4400 – 8200)	5859 (2663 – 7720)	**0.01**	4300 (1400– 9370)	5270 (4330 – 6250)	**0.006**	4.6 (2.9 – 5.7)	4.7 (2.3 – 6.2)	0.92	-	-	-	-	-	-
Platelets (10^9/dL)	476(150 – 1370)	413 (232 – 786)	0.13	257 (85 – 564)	256 (104 – 463)	0.99	396 (410 – 690)	385 (278 – 488)	0.72	-	-	-	-	-	-
Monocytes (10^9/dL)	1.12 (0.1 – 2.8)	0.53 (0.33 – 0.81)	**<0.001**	0.58 (0.2 – 1.4)	0.53 (0.33 – 0.81)	0.42	2.6 (1.4 – 3.5)	3.3 (2.5 – 4.2)	0.30	-	-	-	-	-	-
Retinopathy (Yes/No)	29/142	1/18	0.31	32/99	4/5	0.23	-	-	-	14/174	8/14	**<0.001**	28/73	1/10	0.28
Cholelithiasis (Yes/No)	7/168	1/18	0.57	3/128	0/9	0.25	-	-	-	-	-	-	68/33	5/6	0.18
Renal deficiency (Yes/No)	36/135	6/13	0.37	12/119	2/7	0.22	-	-		-	-		27/74	1/10	0.28
Leg ulcer (Yes/No)	34/137	3/16	0.98	7/124	1/8	0.44	-	-	-	20/168	7/15	**0.01**	6/95	1/10	0.52
HTAP (Yes/No)	-	-		-	-	-	-	-	-	47/141	0/22	**0.005**			
Cardiopathy (Yes/No)	7/164	1/18	0.57	-	-		-	-	-	-	-	-	16/85	2/9	0.96
Thrombosis (Yes/No	27/144	1/18	0.31	10/121	1/8	0.53	-	-	-	-	-	-			
ONTF (Yes/No)	20/151	0/19	0.22	6/125	0/9	0.98	-	-	-	32/156	6/16	0.24	20/81	2/9	0.97
VOC (Yes/No)	118/53	14/5	0.79	77/54	1/8	**0.01**	-	-	-	-	-	-	47/54	5/6	0.99
Stroke (Yes/No)	-	-	-	5/126	0/9	0.98	-	-	-	-	-	-	26/75	3/8	0.98
Hospitalization (Yes/No)	125/46	14/5	0.98	17/114	0/9	0.60	-	-	-	-	-	-	24/77	2/9	0.99
Infection events (Yes/No)	121/50	14/5	0.97	22/109	1/8	0.99	-	-	-	-	-	-	81/20	9/2	0.95

The table presents data from five populations of individuals with sickle cell disease (HbSS and HbSC) in different countries: Brazil, Guadeloupe, Mali/Senegal, and France. The information is divided into various categories: Genotype: TT: Homozygous for the wild-type allele (G variant) of the *CD36* gene. GG + TG: Combination of homozygotes for the mutant allele (GG) and heterozygotes (TG) for the *CD36 gene*.

Demographic characteristics: n: Number of individuals in the population. Age (years): Mean age (and range) of individuals in the population. Sex ratio (M/F): Proportion of males and females in the population.

Clinical characteristics: HU (yes/no): Use or non-use of hydroxyurea. Hb (g/dL): Mean hemoglobin level (and range) in the population. Leukocytes (10x9/dL): Mean white blood cell count (and range) in the population. Neutrophils (10x9/dL): Mean neutrophil count (and range) in the population. Platelets (10x9/dL): Mean platelet count (and range) in the population. Monocytes (10x9/dL): Mean monocyte count (and range) in the population.

Other complications: Retinopathy (yes/no): Presence or absence of retinopathy. Cholelithiasis (yes/no): Presence or absence of cholelithiasis. Renal insufficiency (yes/no): Presence or absence of renal insufficiency. Leg ulcer (yes/no): Presence or absence of leg ulcer. Pulmonary arterial hypertension (PAH) (yes/no): Presence or absence of pulmonary arterial hypertension. Cardiopathy (yes/no): Presence or absence of cardiopathy. Thrombosis (yes/no): Presence or absence of thrombosis. Osteonecrosis of the femoral head (ONFH) (yes/no): Presence or absence of osteonecrosis of the femoral head. Vaso-occlusive crises (VOC) (yes/no): Presence or absence of vaso-occlusive crises. Stroke (yes/no): Presence or absence of stroke. Infection events (yes/no): Presence or absence of infection events.

P: p-value for the chi-square test comparing the frequencies of genotypes GG, TT, and TG. --: Data not available.

. SUPLEMENTARY MATERIALS:

MS-Table 1: Allelic Frequencies of the *CD36* Gene in SCD Populations

Genotype distribution of the *CD36* polymorphism in different SCD populations. The observations

Gene	SNP	N	Population	Genotype	Number observed	Allele frequency A G	Homozygoty Expected	Heterozygoty Expected	Homozygoty Rare Expected	H.W.E Ch2
CD36	*rs3211938*	164	Brazilian HbSC	TT GG TG	155 1 8	0.97 0.03	154.5	9.6	0.15	5.01
		200	Brazilian HbSS	TT GG TG	181 4 15	0.95 0.05	177	21	0.6	18.9
		54	Guadeloupe HbSS	TT GG TG	160 4 22	0.89 0.11	157.6	27.6	1.2	7.6
		97	Mali HbSS	TT GG TG	78 1 18	0.90 0.10	78	17	1	0.001
		113	Senegal HbSS	TT GG TG	110 0 3	0.98 0.01	110	2.9	0.01	0.020
		112	GrExHbSS	TT GG TG	103 0 9	0.96 0.04	103	8.6	0.18	0.19

Qinclude the total number of individuals (N) in each population, the frequency of genotypes TT, GG, and TG, the observed number for each genotype, the allelic frequency for alleles T and G, and the frequency of homozygotes for the rare allele, heterozygotes, and homozygotes.

Table 2: Comparison of hematological parameters and complications associated with SCA in the Brazilian SS population without HU treatment stratified by genotype

	TT	TG + GG	P values
n	128	14	
Age (years)	24 (16 – 29)	28 (18 – 41)	0.06
Sex ratio (M/F)	56/72	8/6	0.50
Retinopathy (Yes/No)	12/116	1/13	0.98
Cholelithiasis (Yes/No)	41/87	6/8	0.60
Renal deficiency (Yes/No)	24/104	3/11	0.98
Leg ulcers (Yes/No)	20/108	2/12	0.99
ONFH (Yes/No)	12/116	0/14	0.48
Thrombosis (Yes/No)	16/112	0/14	0.33
VOC (number)	3 (2 – 3)	2 (2 – 3)	**0.04**
Infection events (number)	2 (2 – 3)	2 (2 – 3)	0.80
Hospitalization (number)	2 (2 – 3)	2 (1 – 3)	0.04

Comparison of complications associated with SCA between patients who received HU and those who did not. The values presented are means (and interquartile ranges) for hematological parameters and frequencies for the analyzed complications. Significance was considered for $p<0.05$.

Table 3: Comparison of hematological parameters and complications associated with SCA in the Brazilian SS population with HU treatment stratified by genotype

	TT	TG, GG	P values
n	53	5	
Age (years)	23 (12 – 33)	24 (18 – 29)	0.37
Sex ratio (M/F)	26/27	3/2	0.98
Retinopathy (Yes/No)	17/36	0/5	0.32
Cholelithiasis (Yes/No)	32/21	2/3	0.68
Renal deficiency (Yes/No)	13/40	3/2	0.68
Leg ulcers (Yes/No)	14/39	1/4	0.98
ONTF (Yes/No)	9/44	0/5	0.72
Thrombosis (Yes/No)	11/42	1/4	0.98
VOC (number)	3 (1 – 4)	3 (2 – 3)	0.37
Infection events (number)	2 (2 – 3)	1 (1 – 2)	0.12
Hospitalization (number)	2 (2 – 3)	2(2– 3)	0.50

Comparison of complications associated with SCA between patients who received HU. The values presented are means (and interquartile ranges) for hematological parameters and frequencies for the analyzed complications. Significance was considered for $p<0.05$.

CONCLUSÃO GERAL

6 CONCLUSÃO GERAL

Neste estudo, investigamos a relação entre marcadores moleculares e o processo inflamatório em pacientes com anemia falciforme, identificando associações genotípicas relevantes com fenótipos clínicos. Destacamos a correlação entre o polimorfismo *TNFα* (-308 G > A) e crises de dor, assim como o polimorfismo *eNOS* (-786 T > C) [TT], associado a maior risco de AVC. Além disso, o uso de hidroxicarbamida e níveis elevados de ferritina foram identificados como fatores cruciais para a gravidade da doença. O polimorfismo H63D (63 G > A) também foi vinculado a diferentes graus de severidade clínica. Outro achado importante foi a associação dos alelos AA+AG do gene *SLC44A2* com uma maior predisposição à formação de úlceras de perna e elevações nos níveis de plaquetas, neutrófilos e monócitos, além de sua implicação na expressão de um grupo sanguíneo raro, com o genótipo homozigoto HNA-3b/b apresentando o fenótipo Cs(a–). Por fim, mostramos que uma variante do gene *CD36* está associada ao aumento de HDL, um fator que pode influenciar positivamente o perfil lipídico em pacientes com anemia falciforme.

Essas descobertas são de grande importância para a compreensão mais aprofundada da fisiopatologia da anemia falciforme, pois revelam novos biomarcadores que podem ajudar a estratificar os pacientes de acordo com o risco de complicações graves, como crises dolorosas e AVCs. O conhecimento dessas associações genotípicas oferece oportunidades para o desenvolvimento de estratégias de medicina personalizada, permitindo que tratamentos sejam ajustados de acordo com o perfil molecular de cada paciente. Por exemplo, a identificação precoce de pacientes com polimorfismos relacionados a maior gravidade pode orientar o uso mais direcionado de terapias como a hidroxicarbamida, ajudando a reduzir a incidência de complicações severas.

Além disso, a descoberta de polimorfismos como o do gene *SLC44A2*, associado a úlceras de perna e distúrbios hematológicos, pode levar ao desenvolvimento de novas abordagens terapêuticas para essas complicações, que são de difícil manejo clínico. A identificação de variantes raras de grupos sanguíneos também tem implicações importantes para transfusões seguras e para a prevenção de reações adversas durante o tratamento.

A descoberta da variante do gene *CD36*, que influencia os níveis de HDL, abre a possibilidade de explorar intervenções que melhorem o perfil lipídico desses pacientes, potencialmente reduzindo o risco cardiovascular, um aspecto importante que afeta a longevidade e qualidade de vida dos indivíduos com anemia falciforme.

Esses achados não apenas ampliam o conhecimento científico sobre os mecanismos moleculares envolvidos na doença, mas também podem servir de base para políticas de saúde pública voltadas à melhoria do manejo clínico e à personalização do cuidado. A implementação dessas descobertas nos sistemas de saúde pode contribuir para a identificação precoce de pacientes de alto risco, otimizando intervenções terapêuticas e melhorando os desfechos clínicos a longo prazo.

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ANEXOS

Termo de Consentimento Livre e Esclarecido - TCLE

(Conselho Nacional de Saúde, Resolução 466/2012/Resolução 510/2016)

Você está sendo convidado a participar como voluntário do projeto de pesquisa "*Associação de polimorfismos que modulam o inflamassoma na gravidade da anemia falciforme em homens*" sob responsabilidade do aluno de doutorado Jonathan de Oliveira Rios, regularmente matriculada no Programa de Pós-Graduação em Biociências. O estudo será realizado com amostras de sangue com um grupo controle e outro experimental para entendermos a relação de alguns marcadores genéticos no estresse oxidativo (composto que podem causar envelhecimento precoce nas células do corpo) e na gravidade de pacientes que fazem o uso do medicamento HC, também será investigado o papel dos glóbulos brancos (neutrófilos) e suas estruturas em pacientes do sexo masculino. Para facilitar o entendimento da nossa pesquisa, talvez será necessário o acesso às informações de dados clínicos presentes no prontuário médico. Haverá um risco considerado mínimo, caracterizado pela possibilidade de mancha roxa no local da picada da agulha durante coleta da amostra de sangue. De modo a minimizar o hematoma, a coleta será realizada pelos enfermeiros do hemocentro, capacitados e com muita experiência, e o participante deverá pressionar a região por alguns segundos, além disso, não dobrar o braço, usar blusas com mangas mais frouxas e evitar carregar peso podem ajudar a reduzir os hematomas. Você poderá consultar o pesquisador responsável em qualquer época, pessoalmente ou pelo telefone pessoal (17) 981462469, ou da instituição (17) 32212392, para esclarecimento de qualquer dúvida. Você está livre para, a qualquer momento, deixar de participar da pesquisa. Todas as informações por você fornecidas e os resultados obtidos serão mantidos em sigilo, e estes últimos só serão utilizados para divulgação em reuniões e revistas científicas. Você será informado de todos os resultados obtidos, independentemente do fato de estes poderem mudar seu consentimento em participar da pesquisa. Você não terá quaisquer benefícios ou direitos financeiros sobre os eventuais resultados decorrentes da pesquisa, nem custos pessoais. Você tem direito à assistência e à indenização nos casos de danos decorrentes de sua participação na pesquisa, o que é garantido pelo Código Civil,

Lei 10.406 de 2002, Artigos 927 a 954 e Resolução CNS nº 510 de 2016, Artigo 9º, inciso 6. Este estudo é importante porque seus resultados fornecerão informações para entender como alguns marcadores genéticos influenciam na gravidade dos pacientes masculinos com anemia falciforme e também como os glóbulos brancos agem na inflamação desses pacientes. O material biológico cedido não será armazenado para pesquisas futuras.

Diante das explicações, se você concorda em participar deste projeto, forneça os dados solicitados e coloque sua assinatura a seguir.

Nome:_____R.G.
_____ Endereço:
_____Fone:
,_____de_____de 20

_____ _____
Assinatura do Participante Assinatura do Pesquisador(a) responsável

OBS.: Termo apresenta duas vias, uma destinada ao participante e a outra ao pesquisador.

Nome Pesquisador(a): Jonathan de Oliveira Rios	Cargo/Função: Doutorando
Instituição: Unesp- Ibilce- São José do Rio Preto	
Endereço: Rua Cristóvão Colombo, 2265	
Telefone: 17-981462469	
Projeto submetido ao Comitê de Ética em Pesquisa do IBILCE/UNESP Rua Cristóvão Colombo, 2265. Bairro: Jardim Nazareth. São José do Rio Preto/SP – Fone 17-3221.2480 e 3221.2545	

Parecer de aprovação CEP

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PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Associação de polimorfismos que modulam o inflamassoma na gravidade da anemia falciforme em homens

Pesquisador: JONATHAN DE OLIVEIRA RIOS

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

Versão: 5

CAAE: 62525922.1.0000.5466

Instituição Proponente: UNESP - CAMPUS DE SÃO JOSÉ DO RIO PRETO

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 5.908.274

Apresentação do Projeto:

Trata a presente notificação de resposta a pendências referentes a um projeto de pesquisa a ser desenvolvido neste Instituto sob responsabilidade Jonathan de Oliveira Rios, aluno do Programa de Pós-Graduação em Biociências deste Instituto, nível Doutorado, sob orientação da Profª. Drª. Claudia Regina Bonini Domingos.

Objetivo da Pesquisa:

Previamente apresentados.

O projeto de pesquisa tem como objetivos:

- Investigar o impacto de polimorfismos genéticos no perfil leucocitário, no estresse oxidativo e na gravidade da anemia falciforme em homens com e sem uso de Hidroxiureia.
- Investigar o impacto das armadilhas extracelulares de neutrófilos (NETs – mecanismo de reposta imunológica inata contra patógenos, que resulta em um processo de morte celular de neutrófilos, denominado netose) mediado pelo inflamassoma (complexo multiproteico intracelular que atua na regulação da imunidade) em homens com anemia falciforme.

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Continuação do Parecer: 5.908.274

Avaliação dos Riscos e Benefícios:

Previamente apresentados.

Comentários e Considerações sobre a Pesquisa:

Em documento anterior, o pesquisador mencionou que haverá no atual estudo o uso de fontes secundárias de dados a partir de prontuários médicos dos participantes da pesquisa e que algumas dessas informações seriam provenientes de um projeto de pesquisa anterior (N° 0030.0.229.000-10). Considerando que o projeto de pesquisa citado não está registrado na Plataforma Brasil por ser anterior à criação da mesma, este Comitê solicitou ao pesquisador que apresentasse o parecer de aprovação do projeto de pesquisa mencionado, bem como o termo de consentimento aplicado aos participantes.

O pesquisador apresenta, agora, o parecer de aprovação da pesquisa N° 0030.0.229.000-10, intitulada "Determinantes genéticos na resposta ao uso de hidroxiureia na anemia falciforme", sob responsabilidade do pesquisador Edis Bellini Junior, colaborador do grupo de pesquisa, bem como o termo de consentimento utilizado, o qual menciona que haverá armazenamento de material biológico e que o participante poderia ser contatado para autorizar sua utilização em novos projetos. Entende-se que o mesmo deve ser feito em relação aos dados de prontuários médicos desses indivíduos, portanto, é de responsabilidade do pesquisador solicitar nova autorização para esses indivíduos para que possam participar do projeto de pesquisa atual.

Considerações sobre os Termos de apresentação obrigatória:

Os termos de apresentação obrigatória foram devidamente apresentados, conforme acima explicitado.

Conclusões ou Pendências e Lista de Inadequações:

Não há.

Considerações Finais a critério do CEP:

Deliberação ad referendum do Comitê de Ética em Pesquisa do IBILCE/UNESP, de 23 de fevereiro de 2023.

Dispõe sobre a análise do Projeto de Pesquisa CAAE: 62525922.1.0000.5466, “Associação de

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polimorfismos que modulam o inflamassoma na gravidade da anemia falciforme em homens", pesquisador JONATHAN DE OLIVEIRA RIOS.

Tendo em vista a urgência que o assunto requer e a manifestação da relatora, manifesto pela aprovação, ad referendum do Comitê de Ética em Pesquisa, do Projeto de Pesquisa CAAE: 62525922.1.0000.5466, "Associação de polimorfismos que modulam o inflamassoma na gravidade da anemia falciforme em homens", pesquisador JONATHAN DE OLIVEIRA RIOS.

Comitê de Ética em Pesquisa do Instituto de Biociências, Letras e Ciências Exatas do Câmpus de São José do Rio Preto, 23 de fevereiro de 2023.

Profa. Dra. Natália Soares Janzantti
Vice-Coordenadora no exercício da Coordenação do Comitê de Ética em Pesquisa - IBILCE/UNESP

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_2002504.pdf	14/02/2023 15:39:05		Aceito
Parecer Anterior	PB_PARECER_CONSUBSTANCIADO.pdf	14/02/2023 15:38:31	JONATHAN DE OLIVEIRA RIOS	Aceito
Outros	ParecerdeaprovacaoeTCLE.pdf	14/02/2023 15:37:04	JONATHAN DE OLIVEIRA RIOS	Aceito
Outros	Respostaaoparecer.pdf	20/12/2022 18:18:23	JONATHAN DE OLIVEIRA RIOS	Aceito
Outros	TermoSigiloConfcolaborador.pdf	20/12/2022 18:16:55	JONATHAN DE OLIVEIRA RIOS	Aceito
Outros	TermodeSigiloeConfidencialdadejonatha Rios.pdf	20/12/2022 18:15:37	JONATHAN DE OLIVEIRA RIOS	Aceito
Projeto Detalhado	ProjetoDoutorado.pdf	15/12/2022	JONATHAN DE	Aceito

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Continuação do Parecer: 5.908.274

/ Brochura Investigador	ProjetoDoutorado.pdf	08:47:32	OLIVEIRA RIOS	Aceito
Declaração de concordância	CartaAnuencia.pdf	05/10/2022 08:30:42	JONATHAN DE OLIVEIRA RIOS	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLEAdultos.pdf	04/10/2022 20:56:07	JONATHAN DE OLIVEIRA RIOS	Aceito
Folha de Rosto	folha.pdf	18/08/2022 20:05:54	JONATHAN DE OLIVEIRA RIOS	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

SAO JOSE DO RIO PRETO, 24 de Fevereiro de 2023

Assinado por:
Natalia Soares Janzantti
(Coordenador(a))

Endereço: Rua Cristóvão Colombo, 2265 - Prédio principal, Ala 1, 1° andar, sala 9.
Bairro: JARDIM NAZARETH **CEP:** 15.054-000
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