**SORBONNE-PARIS-CITÉ
UNIVERSITÉ PARIS-DIDEROT
ÉCOLE DOCTORALE ED 561 – HÉMATOLOGIE, ONCOGÈNESE ET
BIOTHÉRAPIES
E
UNIVERSIDADE DE SÃO PAULO
FACULDADE DE MEDICINA DE RIBEIRÃO PRETO
PROGRAMA DE DOUTORADO EM ONCOLOGIA CLÍNICA, CÉLULAS
TRONCO E TERAPIA CELULAR**

KARINA TOZATTO MAIO

IMMUNOGENETICS IN SICKLE CELL DISEASE

Ribeirão Preto, Brazil

2019

KARINA TOZATTO MAIO

Versão Original

Immunogenetics in sickle cell disease

Thesis presented to the Faculty of Medicine of
Ribeirão Preto, University of São Paulo, and
University Paris-Diderot, Sorbonne-Paris-Cité, for
obtention of a joint Doctoral degree in Sciences.

Graduate program in Clinical Oncology, Stem
Cells and Cell Therapy; Doctoral School of
Hematology, Oncogenesis and Biotherapy.

Field: Cell therapy/ Biotherapy

Advisors: Professor Eliane Gluckman

Professor Belinda Simões

Ribeirão Preto

2019

Autorizo a reprodução e divulgação total ou parcial deste trabalho, por qualquer meio convencional ou eletrônico, para fins de estudo e pesquisa, desde que citada a fonte.

FICHA CATALOGRÁFICA

Faculdade de Medicina de Ribeirão Preto

Universidade de São Paulo

T757i

Tozatto-Maio, Karina

Imunogenética na doença falciforme. / Karina Tozatto-Maio –
Ribeirão Preto/Paris, 2019.

84f.; il. 30cm

Orientadores: Belinda Pinto Simões; Eliane Gluckman

Tese de doutorado (Programa de pós-graduação em Oncologia
Clínica, Células-Tronco e Terapia Celular) -- Faculdade de Medicina de Ribeirão
Preto, Universidade de São Paulo - Brasil and Université Paris-Diderot - France,
2019.

1. Doença falciforme. 2. Antígeno leucocitário humano. 3.
Transplante de células tronco hematopoiéticas. 4. Toll-like receptors. 5.
Infecções bacterianas. I. Título.

FINANCIAL SUPPORT

This work was performed with the financial support from:

- Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior – CAPES, process **88881.133635/2016-01**
- Association Eurocord/Monacord
- Association Cordon de Vie

Nome: TOZATTO MAIO, Karina

Título: Immunogenetics in sickle cell disease

*Tese apresentada à Faculdade de Medicina de Ribeirão Preto da
Universidade de São Paulo para obtenção do título de Doutor em
Ciências, Programa de Doutorado em Oncologia Clínica, Células
Tronco e Terapia Celular, Área de Concentração: Terapia Celular.*

Aprovada em: ____/____/____

Banca Examinadora

Prof. Dr. _____

Instituição: _____

Julgamento: _____

Assinatura: _____

Prof. Dr. _____

Instituição: _____

Julgamento: _____

Assinatura: _____

Prof. Dr. _____

Instituição: _____

Julgamento: _____

Assinatura: _____

Prof. Dr. _____

Instituição: _____

Julgamento: _____

Assinatura: _____

Prof. Dr. _____

Instituição: _____

Julgamento: _____

Assinatura: _____

Prof. Dr. _____

Instituição: _____

Julgamento: _____

Assinatura: _____

Prof. Dr. _____

Instituição: _____

Julgamento: _____

Assinatura: _____

Prof. Dr. _____

Instituição: _____

Julgamento: _____

Assinatura: _____

ACKNOWLEDGMENTS

To my parents, Marilda and Mauricio, my sister, Thaís, and my grandma, Léa, the most supportive and cheerful people in the world.

To Benjamin, for all the love and support.

To my thesis directors, Eliane Gluckman, Belinda Simões and Ryad Tamouza, for the opportunity of working with you, all the help, precious advices and care.

To all friends, colleagues, coworkers and employees at Eurocord, Hemocentro FMRP-USP, INSERM 1160, INSERM 955, and Hospital das Clínicas da FMUSP, for all the help and support.

To the universities of São Paulo and Paris-Diderot, for giving me the chance of developing this work.

To Eurocord/Monacord, Association Cordons de Vie and CAPES for the financial support.

To the Pediatric Diseases Working Party of the EBMT and to REDOME, for the collaboration with this work.

To all patients and their families.

ABSTRACT

TOZATTO-MAIO K. Immunogenetics in sickle cell disease. 2019. Doctoral thesis – Faculty of Medicine of Ribeirão Preto, University of São Paulo, and University Paris-Diderot, Sorbonne-Paris-Cité, Ribeirão Preto, 2019.

Sickle cell disease (SCD) is the most common inherited hemoglobinopathy, caused by a single nucleotide polymorphism (SNP) in the beta-globin (*HBB*) gene. This SNP determines the synthesis of S haemoglobin (HbS), which polymerizes under stress conditions, sickling the red blood cell (RBC). Sick RBC are less deformable, more adherent to the endothelium, and more susceptible to haemolysis. SCD complications are explained by the interaction between haemolysis, vaso-occlusion and inflammatory activation, determined by the RBC sickling. Patients with SCD may present several complications, affecting all organs. Clinical presentation is very heterogeneous, ranging from patients who have mild symptoms to patients who die from disease complications. Because inflammation plays a major role in SCD, polymorphisms in inflammatory genes are potential targets to explain this heterogeneity.

Haematopoietic stem cell transplantation (HSCT) is the only curative therapy currently available for SCD, with good results shown after human leukocyte antigen (HLA) identical sibling HSCT. However, most patients will not have a matched sibling donor. Patients with SCD are mostly from African origin, the less represented ethnic group in stem cell donor registries. To date, few studies using local registries were performed to find the probability of having a potential unrelated donor in SCD settings.

This study aimed to assess the role of inflammatory genes encoding Toll-like receptors (TLR) in the occurrence of bacterial infections in patients with SCD, because infection is a leading cause of mortality in SCD, and TLR recognize a wide range of bacteria. Patients included had DNA samples and clinical data available. SNPs were genotyped by real-time polymerase chain reaction (RT-PCR). Four hundred thirty patients, mostly from Brazilian and Sub-Saharan African origin, were divided in two groups: infected (n=235, patients who presented at least one episode of bacterial infection), and non-infected (n=195, patients who never presented bacterial infections). The *T/A* genotype of SNP *rs4696480* in *TLR2* was less frequent in infected patients (50% versus 67%, OR=0.50, 95% CI 0.34-0.75, $p<0.001$). In addition, the *T/T* genotype of this SNP was more frequent among infected patients (15% versus 5%, OR=0.50, 95% CI 0.34-0.75, $p<0.001$). Previous reports in other settings showed that *A/A* carriers had higher secretion of inflammatory markers, while *T* allele was associated with less

occurrence and severity of inflammatory diseases. Hence, *T/A* genotype might express the ideal inflammatory response to defeat bacteria, while the weaker inflammatory response determined by the *T/T* genotype increases susceptibility to bacterial infections in SCD settings.

Our study also aimed to estimate the probability of finding a potential human leukocyte antigen (HLA) allele matched (loci HLA-A, HLA-B and HLA-DRB1) unrelated donor for patients with SCD in international donor searches. In this study, 185 patients were included, 116 from a Brazilian centre, and 69 who underwent related or unrelated HSCT from an HLA identical or non-identical donor in transplant centres reporting to the European Society for Blood and Marrow Transplantation (EBMT). Patients had HLA data available in intermediate or high resolution. HLA haplotypes were estimated using HaploStats software and classified according to ethnicity. Next, we performed donor searches in international stem cell donor registries (WMDA). Although most haplotypes were African, Brazilian patients had more haplotypes from other ethnic groups. However, Brazilian patients and EBMT patients had the same chances of having at least one potential allelic 6/6 donor in donor registries, of 47% and 47% respectively. Most donors were from the National Marrow Donor Program (NMDP) registry (USA) and from the Brazilian donor registry (REDOME). We reported a higher probability of finding a matched unrelated donor than previous studies using local registries, however strategies are needed to ameliorate representativity of ethnic groups in donor registries.

Altogether, our findings on genetic modulation of SCD might contribute to predict potentially severe complications in patients with SCD. Identifying patients at high risk for some complications will help to ameliorate guidelines for diagnosis and management. In addition, given the importance of early referral to HSCT in SCD, predicting the chances of having a potential donor will also influence therapy decisions. Furthermore, our results support the necessity of improving alternative donor sources and new therapies for SCD.

Keywords: sickle cell disease; human leukocyte antigen; stem cell donor registry; Toll-like receptors; bacterial infections

RÉSUMÉ

TOZATTO-MAIO K. Immunogenetics in sickle cell disease. 2019. Thèse de Doctorat - Faculté de Médecine de Ribeirão Preto, Université de São Paulo, et Université Paris-Diderot, Sorbonne-Paris-Cité, Ribeirão Preto, 2019.

La drépanocytose est l'hémoglobinopathie héréditaire la plus fréquente, causée par un polymorphisme unique d'un nucléotide (SNP) dans le gène de la beta-globine (*HBB*). Ce SNP détermine la synthèse de l'hémoglobine S, qui polymérise lorsqu'elle est soumise au stress, et ceci change la forme des hématies drépanocytaires en faucille. Les drépanocytes sont moins déformables, plus adhérents à l'endothélium, et plus susceptibles à l'hémolyse. Les complications cliniques de la drépanocytose peuvent être expliquées par l'interaction entre la vaso-occlusion, l'hémolyse et l'activation inflammatoire résultant de la présence des drépanocytes dans la circulation.

Les patients drépanocytaires peuvent présenter de nombreuses complications, qui touchent tous les organes. La présentation clinique de cette maladie est très hétérogène, variant entre des patients qui ont très peu de symptômes à des patients qui décèdent de la maladie. Sachant que l'inflammation joue un rôle majeur dans la physiopathologie de la drépanocytose, des polymorphismes dans les gènes inflammatoires peuvent être évoqués pour expliquer cette hétérogénéité.

La greffe de cellules souches hématopoïétiques est la seule thérapie curative disponible actuellement pour la drépanocytose, avec des bons résultats démontrés après la greffe d'un donneur apparenté HLA identique. Néanmoins, la plupart des patients n'a pas de donneur apparenté. Les patients drépanocytaires sont d'origine africaine, le groupe ethnique le moins représenté dans les registres de donneurs non apparentés de cellules souches. A ce jour, peu d'études, utilisant des registres locaux, ont été faites pour estimer la probabilité des patients drépanocytaires de trouver un donneur potentiel non apparenté dans les registres internationaux.

Cette étude a eu pour objectif d'évaluer le rôle de quelques gènes inflammatoires liés aux Toll-like récepteurs (TLR) dans la survenue des infections bactériennes chez les patients drépanocytaires, vu que les infections sont une cause majeure de mortalité chez ces patients, et les TLR sont impliqués dans la reconnaissance de plusieurs types de bactéries. Les patients inclus avaient des échantillons d'ADN et des données cliniques disponibles. Les SNPs ont été génotypés par réaction en chaîne par polymérase en temps réel (RT-PCR). Quatre-cents trente patients, la plupart d'origine brésilienne ou africaine subsaharienne, ont été divisés en deux

groupes : infectés (n=235, patients qui ont eu au moins un épisode d'infection bactérienne documentée) et non infectés (n=195, patients qui n'ont jamais présentés d'infections sévères). Le génotype *T/A* du SNP *rs4696480* in *TLR2* a été plus fréquent chez les patients non infectés (50% versus 67%, OR=0.50, 95% CI 0.34-0.75, $p<0.001$). En outre, le génotype *T/T* de ce SNP a été plus fréquent chez les patients infectés (15% versus 5%, OR=0.50, 95% CI 0.34-0.75, $p<0.001$). Des études précédentes ont démontré que les individus *A/A* avaient plus de sécrétion de marqueurs inflammatoires, lorsque l'allèle *T* était associé à moins de fréquence et de sévérité des maladies inflammatoires.

Cette étude a également eu pour objectif d'estimer la probabilité de trouver un donneur potentiel non apparenté, antigène leucocytaire humain (HLA) allélique identique pour les loci HLA-A, HLA-B et HLA-DRB1. Dans cette étude, 185 patients ont été inclus, 116 suivis dans un centre brésilien et 69 greffés d'un donneur apparenté ou non apparenté dans des centres de greffe qui reportent leurs données à la Société Européenne de Greffe de Cellules Souches (EBMT). Les patients inclus avaient des données HLA testés en moyenne ou haute résolution. Les haplotypes HLA ont été estimés à travers un logiciel, HaploStats, et classifiés selon l'ethnicité. Ensuite, nous avons recherché des potentiels donneurs HLA alléliques identiques pour les loci HLA-A, HLA-B et HLA-DRB1 (6/6) dans des registres internationaux (WMDA). La plupart des haplotypes ont été classifiés comme Africains, mais les patients brésiliens avaient plus d'haplotypes d'autres ethnicités que les patients de l'EBMT. Pourtant, la probabilité de trouver au moins un donneur potentiel allélique 6/6 identique était la même chez les patients brésiliens et les patients de l'EBMT, 47% et 47% respectivement. La plupart de donneurs a été trouvée dans le registre national de donneurs des Etats-Unis (NMDP) et dans le registre brésilien de donneurs (REDOME). Même si la probabilité de trouver un donneur non-apparenté identique dans cette étude est plus grande que celle trouvée dans d'autres études, elle reste toujours faible, démontrant que la représentativité des donneurs d'origine africaine dans les registres internationaux devrait toujours être améliorée.

En tout, nos résultats par rapport à la modulation de complications cliniques par des gènes inflammatoires peuvent aider à prévoir quels patients ont le plus de risques de développer des symptômes sévères. Identifier les patients à risque peut améliorer les recommandations pour le diagnostic et le traitement des complications. En outre, vue l'importance d'envoyer les patients drépanocytaires qui ont une indication à être greffés le plus tôt possible au centre de greffe, prévoir la probabilité de trouver un donneur peut aussi influencer les décisions

thérapeutiques. Ainsi, nos résultats supportent le besoin d'améliorer les sources alternatives et les nouvelles thérapies pour la drépanocytose.

Mots-clés: drépanocytose; antigène leucocytaire humain; registre de donneurs de cellules souches; récepteurs Toll-like; infections bactériennes.

RESUMO

TOZATTO-MAIO K. Immunogenetics in sickle cell disease. 2019. Tese de Doutorado - Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, e Universidade Paris-Diderot, Sorbonne-Paris-Cité, Ribeirão Preto, 2019.

A doença falciforme (DF) é a hemoglobinopatia hereditária mais frequente, causada por um polimorfismo de nucleotídeo único (SNP) no gene da betaglobina (*HBB*). A ocorrência desse SNP determina a síntese de hemoglobina S, que polimeriza sob condições de stress, alterando a conformação das hemácias, que adquirem forma de drepanócitos. Os drepanócitos são menos deformáveis, mais aderentes ao endotélio e mais suscetíveis à hemólise. As complicações clínicas da DF podem ser explicadas pela interação entre a vasoclusão, hemólise e ativação inflamatória resultantes da presença dos drepanócitos na circulação.

Os pacientes com DF podem apresentar numerosas complicações, que afetam todos os órgãos. A apresentação clínica da DF é muito heterogênea, variando de pacientes pouco sintomáticos a pacientes que falecem por complicações da doença. Visto que a inflamação tem um papel importante na fisiopatologia da DF, polimorfismos em genes inflamatórios poderiam explicar essa heterogeneidade.

O transplante de células tronco hematopoiéticas (TCPH) é a única terapia curativa disponível atualmente para a DF, com bons resultados demonstrados em TCPH de doador aparentado antígeno leucocitário humano (HLA) idêntico. Não obstante, a maioria dos pacientes não dispõe de doador aparentado HLA idêntico. A DF ocorre em pacientes normalmente de origem africana, o grupo étnico menos representado em registro de doadores de células tronco. Nos dias de hoje, poucos estudos, utilizando registros locais, avaliaram a probabilidade de encontrar potenciais doadores não aparentados para pacientes com DF.

Este estudo teve por objetivo avaliar o papel de genes inflamatórios que codificam receptores Toll-like (TLR) na ocorrência de infecções bacterianas em pacientes com DF, visto que infecção é uma das principais causas de mortalidade em DF, e os TLR reconhecem diversos tipos de bactérias. Os pacientes incluídos no estudo tinham amostras de DNA e dados clínicos disponíveis. Os SNPs foram genotipados por reação em cadeia de polimerase em tempo real (RT-PCR). Quatrocentos e trinta pacientes, a maioria de origem brasileira ou africana subsaariana, foram divididos em dois grupos, infectados (n=235, pacientes que apresentaram ao menos um episódio de infecção bacteriana), e não infectados (n=195, pacientes que nunca tiveram tais infecções). O genótipo T/A do SNP rs4696480 foi menos frequente em pacientes

infectados (50% versus 67%, OR=0.50, 95% CI 0.34-0.75, $p<0.001$). Além disso, o genótipo T/T do mesmo SNP foi mais frequente em pacientes infectados (15% versus 5%, OR=0.50, 95% CI 0.34-0.75, $p<0.001$). Estudos prévios mostraram que indivíduos com genótipo A/A apresentavam mais secreção de marcadores inflamatórios, enquanto o alelo T foi associado a menor ocorrência e menor gravidade de doenças inflamatórias.

Este estudo também objetivou estimar a probabilidade de encontrar um potencial doador não aparentado alélico idêntico para o antígeno leucocitário humano (HLA), considerando os loci HLA-A, HLA-B e HLA-DRB1. Neste estudo, 185 pacientes com SCD foram incluídos, 116 seguidos em um centro brasileiro e 69 que receberam TCPH de doador aparentado ou não aparentado em centros de TCPH que reportam seus dados à Sociedade Européia de Transplante de Células Tronco (EBMT). Os pacientes tinham dados de HLA em resolução intermediária ou alta. Os haplótipos HLA foram estimados através do software HaploStats e classificados conforme a etnia. A seguir, efetuamos a busca de potenciais doadores alélicos idênticos considerando os loci HLA-A, HLA-B e HLA-DRB1 (6/6) em registros de doadores internacionais (WMDA). A maior parte dos haplótipos foi classificada como africana, mas os pacientes brasileiros apresentaram mais haplótipos de outras origens étnicas que os pacientes do EBMT. No entanto, a probabilidade de encontrar pelo um menos um potencial doador alélico idêntico 6/6 foi a mesma para os pacientes brasileiros e pacientes do EBMT, 47% e 47% respectivamente. A maior parte dos doadores foi encontrada no registro nacional de doadores dos Estados Unidos (NMDP) e no registro brasileiro de doadores (REDOME). A probabilidade de encontrar um doador idêntico não aparentado vista em nosso estudo é maior do que previamente publicado, porém estratégias são necessárias para aumentar a representatividade dos doadores de origem africana nos registros internacionais.

De modo geral, os resultados que dizem respeito à modulação de complicações clínicas por genes inflamatórios podem ajudar a prever quais pacientes estão sob maior risco de apresentar complicações mais severas. Identificar tais pacientes pode melhorar as recomendações para profilaxia e tratamento de complicações. Além disso, devido à importância de encaminhar precocemente pacientes com indicação de TCPH, prever a probabilidade de encontrar um doador também pode influenciar decisões terapêuticas. Ainda, tais resultados corroboram a necessidade de melhorar as fontes alternativas de células tronco e de novas terapias para a DF.

Palavras-chave : doença falciforme ; antígeno leucocitário humano ; registro de doadores de células-tronco; receptores Toll-like; infecções bacterianas.

List of figures

Figure 1: <i>Multistep model of inflammatory activation in sickle cell disease</i>	22
Figure 2: <i>Frequency of HLA haplotypes by ethnicity in each population</i>	36

List of tables

Table 1: <i>Univariate analyses of infected versus non infected patients with sickle cell Disease</i>	31
Table 2: <i>Genetic models, using logistic regression, for SNP rs4696480, considering infected x non infected patients</i>	32
Table 3: <i>Frequency of HLA alleles in the whole cohort, Brazilian population and EBMT population. Comparison with the African-American (AFA) population described in the NMDP database</i>	35
Table 4: <i>Probability of finding a donor in international donor search, overall and by population</i>	37

Table of Contents

1. Background and hypothesis.....	17
1.1 Definition of SCD	18
1.2 Epidemiology	18
1.3 Pathophysiology	19
1.4 Treatment	24
1.5 Hypothesis	26
2. Objectives	28
2.1 Primary objectives	29
2.2 Secondary objectives	29
3. Summary of results	30
3.1 Summary of results – manuscript 1	31
3.2 Summary of results – manuscript 2	34
4. Manuscript 1: <i>A Toll-like Receptor 2 Genetic Variant Modulates Occurrence of Bacterial Infections in Patients with Sickle Cell Disease</i>	38
5. Manuscript 2: <i>Human leukocyte antigen matched unrelated donors for patients with sickle cell disease: results of international donor searches</i>	46
6. Conclusion and future perspectives	68
6.1 Conclusion	69
6.2 Future perspectives	70
7. References	72
8. List of publications	82
8.1 Publications related to this work	83
8.2 Other publications	84

1. BACKGROUND AND HYPOTHESIS

1 Background and hypothesis

1.1 Definition of sickle cell disease

Sickle cell disease (SCD) is the most common inherited hemoglobin disorder (PIEL; STEINBERG; REES, 2017), caused by a single nucleotide polymorphism (SNP) in the gene encoding the beta-globin (HBB) chain (STUART; NAGEL, 2004; PIEL; STEINBERG; REES, 2017). Human hemoglobin is a tetramer formed by two alpha-globin chains and two HBB-like chains; hemoglobin A1, the most prevalent in humans after birth, is constituted by two alpha-globin chains and two HBB chains. A SNP, *rs334 A>T*, in the first exon of the *HBB* gene, leads to the synthesis of valine instead of glutamic acid in the sixth position of the beta-globin chain (STUART; NAGEL, 2004; PIEL; STEINBERG; REES, 2017; PAIKARI; SHEEHAN, 2018). This amino acid substitution determines the production of the abnormal hemoglobin S (HbS), which polymerizes under deoxygenation and stress conditions (OTENG-NTIM et al., 2015), causing sickling of the red blood cell (RBC).

Although the inheritance of the HbS mutation is codominant, heterozygotes are healthy and known as S trait carriers (AS). SCD occurs when patients inherit the HbS mutation from both parents, or in association with another mutations in genes encoding globin chains. Most SCD patients are homozygotes for the HbS mutation (sickle cell anemia, SCA, SS), although associations with other inherited hemoglobin mutations can occur, such as S β thalassemia, SC, SD, SE and others. Compound heterozygous patients are also susceptible to manifestations caused by HbS polymerization (RANNEY, 1970).

1.2 Epidemiology

Because HbS is protective against malaria infection, the HbS mutation appeared first in endemic areas such as Africa and India (PIEL et al., 2010), and carriers are mainly from Sub-Saharan, Arabian and Indian genetic admixture (PIEL, 2013). However, currently, the HbS mutation is present worldwide, although its global frequency is underestimated. The World Health Organization estimated that, in 2010, 5,476,000 AS and 312,000 SS individuals were born worldwide (PIEL et al., 2013). It was also reported that 85% of children with SCD are born in Africa, and from these, 50-90% die early because of SCD complications (GROSSE et al., 2011). In Europe, France, United Kingdom, Germany and Sweden are the countries with the highest prevalence of the HbS mutation (PIEL et al., 2014). In France, in 2013, 0.05% of newborns were diagnosed with SCD (<http://www.inserm.fr/thematiques/genetique-genomique-et-bioinformatique/dossiers-d-information/drepanocytose>). In Brazil, the overall prevalence of

the HbS mutation is estimated at 4%; currently, there are 25,000 to 30,000 patients with SCD, and 3500 newborns per year (FELIX AA; SOUZA HM; RIBEIRO SBF, 2010). In the United States of America (USA), around 100,000 people have SCD and 1.5% of newborns carry the HbS mutation (HASSELL, 2010; OJODU et al., 2014). The incidence of SCD is expected to increase worldwide (PIEL; STEINBERG; REES, 2017).

1.3 Pathophysiology

The first description of SCD dates from 1910, when Herrick described a patient originally from the West Indies who presented anemia, palpitations and some infectious complications. Peripheral blood smear showed red cells with a sickling format and nucleated red cells (HERRICK, 2001). In 1924, the hemolysis and vaso-occlusion phenomena were described respectively by Sydenstricker and Graham in patients with SCD (GRAHAM, 1924; SYDENSTRICKER, 1924). In 1949, observing 29 families that had an individual affected by SCD, Neel noticed that all parents of patients with SCD had sickle cells in the blood smear, establishing the basis of the genetic Mendelian inheritance in SCD (NEEL, 1949). At the same time, Pauling and Itano, using electrophoresis methods, showed that sickle cells had a different type of hemoglobin from normal red cells (PAULING; ITANO, 1949), and Harris demonstrated that sickling occurs when HbS is deoxygenated (HARRIS, 1950). Subsequently, Ingram described the amino-acid change from glutamic acid to valine in the mutant beta-globin chain (INGRAM, 1957). In 1978, Dykes proposed the structure of HbS fibers inside the RBC (DYKES; CREPEAU; EDELSTEIN, 1978). It was also found that the polymers remain stable because the abnormal valine present in the HbS binds to a patch in position 85-88 of the adjacent beta globin strain (NAGEL et al., 1979). The mechanism of HbS polymerization was described by Eaton (EATON; HOFRICHTER, 1987).

The first symptoms of SCD occur around six months to one year of age, due to the decrease in fetal hemoglobin (HbF) concentration in the RBC (WATSON, 1948). HbF is composed by two alpha-globin and two gama-globin chains. HbF prevents polymerization of HbS because a glutamine in the gama-globin chain has a weaker interaction with the abnormal valine of HbS, thus impairing the polymer stabilization (NAGEL et al., 1979; STUART; NAGEL, 2004; PAIKARI; SHEEHAN, 2018). Patients can present acute and chronic complications, such as cerebral vasculopathy, ischemic and hemorrhagic stroke, vaso-occlusive crisis, acute chest syndrome, bacterial infections, sickle nephropathy or chronic leg ulcers. Vaso-occlusion and hemolysis, described since 1924 in SCD settings, are the basis of SCD

complications (GRAHAM, 1924; SYDENSTRICKER, 1924; REES; WILLIAMS; GLADWIN, 2010; PIEL; STEINBERG; REES, 2017).

Vaso-occlusion occurs because sickle RBC are less deformable and interact with the endothelium. HbS polymers have a linear form and dispose parallelly into the RBC (DYKES; CREPEAU; EDELSTEIN, 1978). In turn, the RBC acquires a sickle shape because the membrane deforms to accommodate the polymers. When blood transit time in the microcirculation is longer, sickle RBC are more exposed to low oxygen tension levels, HbS polymerization and RBC sickling occurs, clotting the microcirculation (STUART; NAGEL, 2004). However, obstruction alone does not explain the vaso-occlusion phenomenon. Sickle RBC are more adherent to the endothelium (STUART; NAGEL, 2004; SWITZER et al., 2006; CONRAN; BELCHER, 2018); compared with normal RBC, a 20-fold higher shear stress is required to unbind the sickle RBC from the endothelium (SWITZER et al., 2006). Sickle RBC show higher expression of adhesion molecules and bind to the endothelium directly, via vascular-cell adhesion molecule 1 (VCAM-1) (GEE; PLATT, 1995), P-selectin (MATSUI et al., 2001), or interacting with proteins such as thrombospondin (BRITTAIN et al., 1993, 2001). In addition, several studies demonstrated that younger, low-dense, sickle RBCs are also more adherent to the endothelium, leading to the vaso-occlusion model in which low-dense cells adhere to post capillary venules, then trapping of dense cells occurs, resulting in obstruction (reviewed in TURHAN et al., 2002). Turhan and colleagues tested this model in mice, showing that RBCs interacted mostly with adherent leukocytes and only occasionally with the endothelium. They also found that the number of rolling and adherent leukocytes was significantly higher in SS mice compared with controls, which was consistent with the higher leukocyte levels found in SS mice (TURHAN et al., 2002). In addition, previously, Kaul had demonstrated an inflammatory response pattern in SS mice, characterized by an increased number of adherent leukocytes after ischemia/reperfusion compared with controls (KAUL; HEBBEL, 2000). Furthermore, vaso-occlusion also results from damaged vasomotor regulation due to NO degradation, caused by hemolysis, and sickle cell dehydration, caused by disabled cation homeostasis (STUART; NAGEL, 2004). In conclusion, vaso-occlusion is a complex phenomenon driven not only by sickling of RBC, but also by abnormal adhesion due to sickle RBC characteristics, inflammatory activation and impaired vasomotor tonus.

Polymerization of HbS causes destabilization of the RBC membrane, resulting in premature destruction of the sickle RBC and hemolysis (REITER et al., 2002). Intravascular hemolysis ranges from less than 10% to more than 30% of total hemolysis in SCD (KATO;

STEINBERG; GLADWIN, 2017) and overwhelms the hemoglobin degradation pathways (SWITZER et al., 2006). Plasma free hemoglobin consumes nitric oxide (NO) (REITER et al., 2002). NO is produced by endothelial cells, using L-arginine as substrate, and promotes relaxation of the smooth muscle layer in blood vessels (IGNARRO et al., 1987; PALMER; FERRIGE; MONCADA, 1987; REITER et al., 2002; STEINBERG, 2006). When hemolysis occurs, lysed RBC release arginase, which destroys L-arginine, and free cell hemoglobin transforms NO in bio-inactivate nitrate (REITER et al., 2002; STEINBERG, 2006). Therefore, hemolysis reduces NO synthesis and function, impairing vasomotor regulation and enhancing vaso-occlusion. In addition, heme induces TNF- α secretion via Toll-like receptor (TLR) 4 (FIGUEIREDO et al., 2007) and activates neutrophils (GRAÇA-SOUZA et al., 2002), thus triggering inflammatory response.

Altogether, these evidences point to a major role of inflammation in SCD pathophysiology (ZHANG et al., 2016; KATO; STEINBERG; GLADWIN, 2017). Higher levels of leukocytes were associated with increased risk of complications and early mortality in SCD (CASTRO et al., 1994; PLATT et al., 1994; ANYAEGBU et al., 1998; OHENE-FREMPONG et al., 1998; KINNEY et al., 1999; ZHANG et al., 2016). Also, platelets are activated in steady-state SCD, and this activation increases during vaso-occlusive episodes, leading to the secretion of thrombospondin and enhancing sickle RBC adherence to the endothelium (BRITTAIN et al., 1993; ZHANG et al., 2016). Sick monocytes, mediated by tumor necrosis factor alpha (TNF- α), activate endothelial cells, triggering the endothelial nuclear factor kappa B (NF- κ B) and enhancing expression of tissue factor (TF), adhesion molecules and adherence of polymorphonuclear cells (BELCHER et al., 2000; SAFAYA; STEINBERG; KLINGS, 2012). Endothelial activation might also be induced by adhesion of RBC, platelets or heme via TLR4 signaling (ZHANG et al., 2016). Pro-inflammatory cytokines were also assessed in SCD settings. A study found higher levels of endothelin-1 and prostaglandine-E2 during vaso-occlusive crisis; in steady-state, levels were lower but not normal; TNF- α and interleukin (IL)-1 β were not augmented in this cohort (GRAIDO-GONZALEZ et al., 1998). However, other studies found higher levels of TNF- α and IL-1 β in patients during steady state and vaso-occlusive crisis (FRANCIS; HAYWOOD, 1992; MALAVÉ et al., 1993). It was also found that expression of granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-3 correlated with HbF levels (CROIZAT, 1994), and that use of hydroxyurea (HU) regulated expression of TNF- α and IL-10 (LANARO et al., 2009). Pro-inflammatory cytokines activate the NF- κ B pathway, promoting activation of leukocytes

and endothelial cells; NF- κ B activation also increases expression of adhesion molecules (intercellular-cell adhesion molecule 1(ICAM-1), VCAM-1, E-selectin, P-selectin), promoting leukocyte adhesion (ZHANG et al., 2016). Chemokines and coagulation factors are also involved in inflammatory pathways in SCD. A multistep model was proposed, consisting in 1) inflammatory activation of endothelial cells, recruiting neutrophils to post-capillary venules; 2) adhesion of neutrophils, E-selectin signaling, activation of Mac-1 integrin, and capture of circulating sickle RBCs, promoting obstruction; 3) RBC sickling due to ischemia (caused by increase in transit time); enhancing of neutrophil recruitment and vaso-occlusion (ZHANG et al., 2016). The multistep model is represented in figure 1.

Figure 1. Multistep model of inflammatory activation in sickle cell disease. 1) Inflammatory activation of endothelial cells (A), recruiting neutrophils (B) to postcapillary venules; 2) adhesion of neutrophils, inflammatory signaling, and capture of circulating sickle RBCs (C), promoting obstruction; 3) RBC sickling due to ischemia (caused by increase in transit time); enhancing of neutrophil recruitment and vaso-occlusion; (D) platelets.

Although the HbS inheritance is monogenic, SCD has a very heterogeneous clinical presentation. Some patients have mild symptoms, whereas others present severe complications. Compound heterozygous genotypes might be less severe than SCA, but there is heterogeneity within each SCD genotype (PIEL; STEINBERG; REES, 2017). This phenotype variability in SCD still needs to be better understood, and some genetic modifiers were proposed.

HbF is the main modulator of severity in SCD (AKINSHEYE et al., 2011). The gamma-globin genes, *HBG2* and *HBG1*, are in the *HBB-like* gene cluster together with *HBB* and other *HBB-like* genes (PAIKARI; SHEEHAN, 2018). The *HBB-like* gene cluster has a 60kb region in which five different haplotypes (HbS haplotypes) were described in SCD: Bantu, Senegal,

Benin, Cameroon and Arab-Indian, showing that the HbS mutation arose in different geographical regions (PAGNIER et al., 1984; WAINSCOAT et al., 1985; LAPOUMÉROULIE et al., 1992). Some studies established associations between HbS haplotypes and HbF levels in patients with SCD (LABIE et al., 1985; NAGEL et al., 1985, 1987; GREEN et al., 1993; CHANG et al., 1995). Nevertheless, HbF concentration is highly variable among patients who carry the same *HBB* haplotype (VATHIPADIEKAL et al., 2016; PIEL; STEINBERG; REES, 2017; PAIKARI; SHEEHAN, 2018). More recently, other genomic regions that regulate expression of HbF are being investigated, such as *BCL11A* and *HBS1L-MYB* (LETTRE et al., 2008; SANKARAN et al., 2008).

Alpha (α)-thalassemia, when inherited with SCD, has a modulatory effect on SCD symptoms. Individuals with α -thalassemia have less intracellular hemoglobin, which prolongs the delay time for HbS polymerization and RBC sickling (HOFRICHTER; ROSS; EATON, 1974; PIEL; STEINBERG; REES, 2017). In addition, these patients also show less hemolysis (DE CEULAER et al., 1983). However, higher incidence of vaso-occlusive complications has been reported (PIEL; STEINBERG; REES, 2017).

Because inflammation is so important in SCD pathophysiology, several studies assessed the role of inflammatory genes in the phenotypic diversity of SCD. For instance, stroke risk was associated with variants in genes encoding IL-4R, TNF- α , β 2 adrenergic receptor (ADBR2), VCAM-1, human leukocyte antigen (HLA) class I and class II alleles, among other genes (STYLES et al., 2000; TAYLOR et al., 2002; HOPPE et al., 2003, 2004, 2007; SEBASTIANI et al., 2005). Occurrence of viral and bacterial infections were modulated by HLA class I, class II, HLA-E, HLA-G alleles, polymorphisms in genes encoding mannose binding lectin (MBL), chemokines, TLR and Fc-receptor ligands (NORRIS et al., 1996; NEONATO et al., 1999; TAMOUZA et al., 2002, 2007; AL-OLA et al., 2008; CORDERO et al., 2009; DOSSOU-YOVO et al., 2009; DAVID et al., 2018). Polymorphisms in HLA, TNF- α and cytotoxic-T-lymphocyte associated antigen 4 (CTLA-4) (TATARI-CALDERONE et al., 2016; OLIVEIRA et al., 2017; SIPPET et al., 2017) had effects on allo-immunisation; priapism, avascular necrosis and acute chest syndrome were associated with polymorphisms in inflammatory genes (ADEKILE et al., 2005; ELLIOTT et al., 2007; MARTINEZ-CASTALDI et al., 2007). Nevertheless, further studies with larger cohorts are required in these settings to better establish which inflammatory genes and pathways modulate SCD complications. In this context, although the role of TLR4, triggered by heme, in mediating vaso-occlusion, is well established (BELCHER et al., 2014), few studies addressed the influence of TLR in the clinical

manifestations of SCD (GODEFROY et al., 2016; DAVID et al., 2018). TLR are transmembrane and intra-cellular proteins expressed on immune cells that recognize a wide range of pathogen-associated molecular patterns (PAMPs), including several types of virus and bacteria, and endogenous damage-associated molecular patterns (DAMPs). TLR are part of the innate immune system, and when stimulated, activate downstream signalling cascades that include the NF- κ B pathway, promoting transcription of inflammatory cytokines and chemokines (AKIRA; UEMATSU; TAKEUCHI, 2006; OLIVEIRA et al., 2015; NTOUFA et al., 2016).

1.4 Treatment

HU is the only pharmacological therapy established for SCD, because it increases HbF expression (CHARACHE et al., 1995; YAWN et al., 2014). In addition, HU decreases leukocyte counts, modulates expression of adhesion molecules, ameliorates red cell deformability and releases NO when metabolized (CHARACHE et al., 1995; WARE, 2010). The use of HU reduced complications, hospitalizations and mortality in some clinical trials (STEINBERG et al., 2003, 2010; VOSKARIDOU et al., 2010). The current recommendation is to start HU therapy for all children with SCA older than 9 months of age and adolescents, regardless the severity of symptoms (WANG et al., 2011; YAWN et al., 2014). Nevertheless, HU does not prevent several SCD complications (YAWN et al., 2014). Therefore, chronic RBC transfusion exchange is still indicated to decrease the HbS rate and ameliorate or prevent some complications (WARE; HELMS; SWITCH INVESTIGATORS, 2012; YAWN et al., 2014). However, patients who receive chronic RBC transfusions are exposed to RBC allo-immunization and iron overload. Penicillin prophylaxis is indicated in children up to 5 years of age due to loss of spleen function, which impairs opsonization of encapsulated bacteria and increases risk of bacterial infections (YAWN et al., 2014). In addition, immunization against encapsulated bacteria is also recommended in SCD (YAWN et al., 2014). These prophylactic measures are also effective in reducing morbidity and mortality in SCD. Nonetheless, patients with SCD still face decreased life span and quality of life. Furthermore, HU and RBC transfusions are not curative.

Currently, hematopoietic stem cell transplantation (HSCT) is the only curative therapy available for SCD (BERNAUDIN et al., 2007; HSIEH et al., 2009; ANGELUCCI et al., 2014; ARNOLD et al., 2016; GLUCKMAN et al., 2017). In 1984, a pediatric patient with SCD who had acute myeloid leukemia received HSCT from a human leukocyte antigen (HLA) matched sibling donor and was free from vaso-occlusive crisis during follow-up. Hemoglobin

electrophoresis showed a stable sickle cell trait pattern after HSCT (JOHNSON et al., 1984). Subsequently, in Belgium, five children were transplanted for SCD with good results (VERMYLEN et al., 1988). In France, the first HSCT for SCD was performed in 1988, and in 2007, 87 patients had undergone HSCT, with overall survival (OS) of 93% and event-free survival (EFS) of 86% (BERNAUDIN et al., 2007). (GLUCKMAN et al., 2017)

In the largest series reported, 1000 patients with SCD received HSCT from an HLA matched sibling donor between 1986 and 2015 in 23 countries. Median follow-up was 55 months, 5-year OS was 93% and 5-year EFS was 91%, with low cumulative incidences of acute graft versus host disease (aGvHD) and chronic GvHD (cGvHD) (15% and 14% respectively). Age at the time of HSCT, graft source and time when HSCT was performed significantly impacted OS (GLUCKMAN et al., 2017). A recent update, with patients divided by age group at HSCT, confirmed that younger patients showed better results (CAPPELLI et al., 2019). In 2014, a panel of experts proposed indications for HSCT in SCD, such as stroke, lung disease, recurrent vaso-occlusive episodes; it was also suggested that symptomatic children who have a HLA matched sibling donor should be transplanted as soon as possible (ANGELUCCI et al., 2014).

Despite the good results, there are many barriers for the widespread use of HSCT in SCD settings. Lack of financial, social support, parental or patient refusal are some of the issues. However, the main problem is the lack of a HLA matched sibling donor (WALTERS et al., 1996). For instance, in a study that recruited patients with SCD for HSCT, only 14% of patients who met the inclusion criteria had a sibling donor (WALTERS et al., 1996). Another study calculated a probability of having a HLA matched sibling donor as 18% (MENTZER et al., 1994). Lack of matched sibling donor justifies the use of HLA matched unrelated donor (MUD) HSCT for SCD. Although a high overall survival after MUD HSCT has been reported, there is also a high rate of graft rejection and GvHD (SHENOY et al., 2016; CAPPELLI et al., 2018). Therefore, improvements in conditioning and GvHD prophylaxis are required. Unrelated cord blood (UCB) HSCT and haploidentical HSCT are alternative donor sources in patients who lack a MUD. However, these sources were associated with a high rate of graft rejection in HSCT and still need to be better developed (KAMANI et al., 2012; FITZHUGH et al., 2017; DE LA FUENTE et al., 2018).

In addition to HSCT adverse outcomes, donor availability is a major issue for MUD HSCT in SCD. Donors from African origin are underrepresented in donor registries worldwide (DEW et al., 2008; GLUCKMAN, 2013; BARKER et al., 2019). A study using the USA

National Donor Marrow Program (NMDP) database and patients who had HSCT indication assessed the probability of finding a MUD in the NMDP registry, and individuals from African origin had the lowest chances of having a MUD among the 21 ethnic groups tested, with probabilities as low as 16% (GRAGERT et al., 2014). Another prospective study, reporting patients who received HSCT from an unrelated donor divided by ethnic group, showed that patients from African origin had less 8/8 MUD HSCT than other ethnic groups (BARKER et al., 2019). In SCD settings, few studies were performed to evaluate the chances of finding a MUD. A study assessing the probability of patients with SCD and β -thalassemia to find at least one potential donor in the NMDP showed that 60% of patients with SCD had at least one potential HLA matched 6/6 MUD (HLA loci A and B typed at low resolution and DRB1 at intermediate resolution), whereas 80% patients with β -thalassemia had a suitable potential 6/6 MUD (KRISHNAMURTI et al., 2003). Another study, using 85 patients with SCD, performed HLA allelic searches in the NMDP and showed that 20% of patients had a potential allelic 8/8 MUD (loci A, B, DBR1 and DQB1 typed in high resolution) (JUSTUS et al., 2015). Furthermore, a Brazilian study with 126 patients with SCD performed searches in a subset of donors from the Brazilian donor registry (REDOME); in this study, only 8% of patients had a 8/8 potential MUD (loci A, B, DRB1 and DQB1 typed in low resolution). These results outline that the ethnic groups in which SCD occurs are less represented in donor registries, although only single registry searches were performed.

1.5 Hypothesis

Because patients with SCD are clinically heterogeneous, knowing which factors modulate disease heterogeneity might help defining patients at higher risk of developing complications, thus helping preventive management and therapeutic decisions. Inflammation is a major component in the pathophysiology of SCD. There are evidences that expression of inflammatory markers regulates the severity of symptoms in SCD, and that genes encoding inflammatory markers also play a role in the modulation of SCD complications. Therefore, we hypothesize that polymorphisms in genes encoding TLR are associated with occurrence of complications in patients with SCD, explaining their clinical heterogeneity. We also hypothesize that some alleles and genotypes in SNPs in inflammatory genes might have a different frequency in patients with SCD compared with populations of African origin described in genetic databases, such as 1000 genomes.

Furthermore, HSCT is well established as a curative therapy for SCD. However, few patients will have an HLA matched sibling donor. Patients with SCD are from African

background, and a global lack of MUD was reported for individuals from African origin. To date, donor searches reported in SCD settings were performed using single donor registries. We hypothesize that performing donor searches at international level will increase the chances of finding a potential MUD in SCD. In addition, it is important to better predict the probabilities of having a potential allelic MUD in SCD settings, because this can influence therapeutic choices in SCD patients at earlier age.

2. OBJECTIVES

2 Objectives

2.1 Primary objectives

- To establish associations between SNPs in the inflammatory genes *TLR1*, *TLR2*, *TLR6* and *TLR10* and clinical complications in patients with SCD, in genotype and haplotype levels;
- To find the probability of finding a potential allelic HLA MUD in HLA-A, HLA-B and HLA-DRB1 (6/6) donor in international donor registries for patients with SCD.

2.2 Secondary objectives

- To compare allele and genotype frequencies of relevant SNPs in genes *TLR1*, *TLR2*, *TLR6* and *TLR10* in patients with SCD with the frequencies described for the African population from the 1000 genomes database;
- To describe HLA allele and haplotype frequencies for loci *HLA-A*, *HLA-B* and *HLA-DRB1* in patients with SCD.

3. SUMMARY OF RESULTS

3 Summary of results

3.1 Results from manuscript 1

Title: A Toll-like Receptor 2 Genetic Variant Modulates Occurrence of Bacterial Infections in Patients with Sickle Cell Disease.

In this retrospective study, we aimed to find if SNPs in *TLR* genes (*TLR1*, *TLR2*, *TLR6* and *TLR10*) were associated with occurrence of bacterial infections in patients with SCD. All patients included had DNA samples and clinical data available. We genotyped 7 SNPs in these genes by real time polymerase chain reaction (RT-PCR). Patients were divided in two groups: infected, who presented at least one episode of bacterial infection, and non-infected, patients who never had any bacterial infections.

Four hundred thirty patients were included in the study, 235 in the infected group and 195 in the non-infected group. Patients were mostly from Brazilian and Sub-Saharan African origin. Demography data and univariate analyses are shown on table 1.

Table 1. Univariate analyses of infected versus non infected patients with sickle cell disease.

Variable	Non infected (n=195)	Infected (n=235)	P-value
Gender (n=427)			NS
female	108 (56)	135 (58)	
male	85 (44)	99 (42)	
Origin (n=419)			NS
Brazil	90 (47)	103 (45)	
Sub Saharan Africa	83 (44)	96 (42)	
French West Indies	16 (8)	25 (11)	
North Africa	2 (1)	3 (1)	
other	0	1 (1)	
Age >18 y at last follow up (n=401)			NS
no	26 (14)	27 (12)	
yes	154 (86)	192 (88)	
rs3804099 (n=426)			NS
C/C	41 (21)	44 (19)	
C/T	87 (45)	104 (45)	
T/T	65 (34)	85 (36)	
rs4696480 (n=425)			<0.001
T/T	10 (5)	35 (15)	
T/A	127 (67)	117 (50)	
A/A	54 (28)	82 (35)	

Variable	Non infected (n=195)	Infected (n=235)	P-value
rs3804100 (n=416)			NS
T/T	170 (91)	207 (90)	
C/T	17 (9)	20 (9)	
C/C	0	1 (1)	
rs11466653 (n=427)			NS
A/A	175 (91)	215 (92)	
G/A	17 (8)	19 (8)	
G/G	1 (1)	0	
rs11096957 (n=426)			NS
G/G	48 (25)	57 (25)	
G/T	103 (53)	115 (49)	
T/T	42 (22)	61 (26)	
rs4383095 (n=401)			NS
G/G	51 (28)	49 (22)	
A/G	75 (42)	104 (47)	
A/A	53 (30)	69 (31)	
rs5743810 (n=429)			NS
G/G	158 (81)	205 (87)	
A/G	31 (16)	28 (12)	
A/A	5 (3)	2 (1)	

The SNP *rs4696480 T/A* in *TLR2* was significantly associated with occurrence of bacterial infections. The distribution of *rs4696480* genotypes was, in the infected patients, *AA*=82 (35%), *TA*=117 (50%), *TT*=35 (15%); in the non-infected patients, distribution was *AA*=54 (28%), *TA*=127 (67%), *TT*=10 (5%). We tested genetic models for this association. In the over-dominant model, the heterozygous genotype (*TA*) occurred significantly less in infected patients than in non-infected patients compared with *TT+AA* patients (OR=0.50, 95% CI 0.34-0.75, $p<0.001$). Furthermore, in the recessive model, genotype *TT* occurred significantly more in infected patients than in non-infected patients compared with *TA+AA* (OR=3.18, 95% CI 1.53-6.61, $p<0.001$). Genetic models are summarized in table 2.

Table 2. Genetic models, using logistic regression, for SNP *rs4696480*, considering infected x non infected patients.

		Odds ratio	95%CI	P-value
dominant	<i>AAx TA+TT</i>			NS
recessive	<i>TTx TA+AA</i>	3.18	1.53-6.61	<0.001
overdominant	<i>TAx AA+TT</i>	0.5	0.34-0.75	<0.001
log-additive				NS

It was previously reported that individuals carrying the *AA* genotype had higher secretion of cytokines, and *T* allele was associated to less occurrence or severity of autoimmune diseases. The *TA* genotype of SNP *rs4696480* might represent the ideal inflammatory response against bacteria, whereas *TT* individuals would have a weaker response.

3.2 Results from manuscript 2

Title: Human leukocyte antigen matched unrelated donors for patients with sickle cell disease: results of international donor searches.

In this study, we performed international searches to estimate the probability of finding at least one potential allelic HLA MUD donor, considering loci HLA-A, HLA-B and HLA-DRB1 (6/6), for patients with SCD. Also, because potential allelic donors in registries might be unavailable, we also estimated the probability of having 5 or more potential allelic donors. All patients had HLA data available in intermediate or high resolution for loci HLA-A, HLA-B and HLA-DRB1. Using HaploStats, we estimated HLA-A~HLA-B~HLA-DRB1 haplotypes and classified them by ethnic origin (Caucasian, African, Amerindian, Common). Next, we performed searches in international registries to find MUD donors.

One hundred eighty-five patients with SCD were included in this study, 116 followed in one center in Brazil (of whom 23 transplanted from a matched sibling donor), and 69 who underwent related or unrelated HSCT from an HLA identical or non-identical donor in transplant centers reporting to the European Society for Blood and Marrow Transplantation (EBMT). From these, fifty-four patients received HSCT from an identical sibling donor, 10 from a matched unrelated donor and 5 from a mismatched unrelated donor.

The most frequent HLA haplotypes in the whole population were (n=297): *A*01:01 B*08:01 DRB1*03:01* (n=5, 2%); *A*36:01 B*53:01 DRB1*11:01* (n=4, 1%); *A*23:01 B*44:03 DRB1*07:01* (n=4, 1%). In the Brazilian population, the most common HLA haplotypes were *A*01:01 B*08:01 DRB1*03:01* (n=5, 3%); *A*23:01 B*44:03 DRB1*07:01* (n=3, 2%); *A*02:01 B*27:05 DRB1*01:01* (n=3, 2%) and *A*03:01 B*58:02 DRB1*15:03* (n=3, 2%). In the EBMT population, the most frequent HLA haplotypes were *A*36:01 B*53:01 DRB1*11:01* (n=3, 3%); *A*68:02 B*15:10 DRB1*03:01* (n=2, 2%) and *A*23:01 B*07:05 DRB1*11:01* (n=2, 2%). HLA allele frequencies in both populations are summarized in table 3. Distribution of haplotypes by ethnic origin are shown in figure 2.

Table 3. Frequency of HLA alleles in the whole cohort, Brazilian population and EBMT population. Comparison with the African-American (AFA) population described in the NMDP database.

Locus	Alleles							
HLA*A	02:01	03:01	01:01	30:01	30:02	23:01	68:02	24:02
Overall,% (n=364)	16	9	8	7	5	7	5	4
Brazilian patients,% (n=226)	20	11	9	6	6	5	4	3
EBMT patients,% (n=138)	11	6	5	8	4	11	7	7
AFA ranking (frequency,%)	1 (12)	3 (8)	8 (5)	4 (7)	6 (6)	2 (11)	5 (7)	15 (2)
HLA*B	53:01	35:01	08:01	42:01	51:01	07:02	58:01	44:03
Overall,% (n=362)	7	6	5	5	3	4	4	4
Brazilian patients,% (n=224)	7	7	5	5	5	5	3	4
EBMT patients,% (n=138)	7	5	4	8	4	3	6	4
AFA ranking (frequency,%)	1 (11)	3 (6)	9 (4)	5 (5)	16 (2)	2 (7)	11 (4)	6 (5)
HLA*DRB1	07:01	15:03	11:01	03:01	13:01	13:02	11:02	01:02
Overall,% (n=361)	12	9	9	8	5	6	4	4
Brazilian patients,% (n=225)	14	8	8	9	6	5	4	3
EBMT patients,% (n=136)	10	13	10	7	4	8	5	4
AFA ranking (frequency,%)	2 (10)	1 (12)	3 (9)	4 (7)	7 (5)	6 (6)	11 (4)	9 (4)

Abbreviations: AFA: African American; EBMT, European Society for Blood and Marrow Transplantation; HLA, human leukocyte antigen.

Figure 2. Frequency of HLA haplotypes by ethnicity in each population.

Next, we performed donor searches in international donor registries to find at least one potential donor, at least one potential allelic donor and 5 or more potential allelic donors. EBMT patients who received HSCT from MUD were excluded. Table 4 shows the results of donor searches, and P-value comparisons among populations. Most donors were found in the NMDP donor registry, followed by REDOME.

Table 4. Probability of finding a donor in international donor search, overall and by population

	Overall	Brazilian patients (n=116)	EBMT patients (n=59)	P-value
Potential donor				
(%)	138 (79)	99 (85)	42 (71)	
Allelic donor (%)	88 (50)	55 (47)	28 (47)	NS
≥ 5 allelic donors				
(%)	37 (21)	28 (24)	9 (15)	NS

Most HLA haplotypes were from African origin, but Brazilian patients had more haplotypes from other ethnic origins than EBMT patients. Nevertheless, chances of having at least one potential allelic donor were similar in both populations. Although chances of finding a potential allelic MUD in donor registries was higher than previously reported, chances are still low, and efforts to improve ethnic representativity in donor registries are needed.

**4. MANUSCRIPT 1: A Toll-like Receptor 2 Genetic Variant Modulates
Occurrence of Bacterial Infections in Patients with Sickle Cell Disease**

A Toll-like receptor 2 genetic variant modulates occurrence of bacterial infections in patients with sickle cell disease

Karina Tozatto-Maio,^{1,2,3,4}

Robert Girot,⁵ Indou D. Ly,⁶

Vanderson Rocha,^{1,2,4,7}

Ana C. Silva Pinto,³ Ibrahima Diagne,⁸

Yahia Benzerara,⁹

Carla L. Dinardo,⁴

Simone Kashima,³

Itaúá Leston-Araújo,¹⁰

Chantal Kenzey,^{1,2}

Guilherme H. H. Fonseca,⁴

Evandra S. Rodrigues,³

Fernanda Volt,^{1,2}

Luciana R. Jarduli,¹¹

Annalisa Ruggeri,^{1,2,12,13}

Christina M. Mariaselvam,¹⁴

Sandra F. M. Gualandro,⁴

Hanadi Elayoubi,^{1,2} Renato Cunha,³

Barbara Cappelli,^{1,2}

Kelen C. R. Malmegrim,^{3,15}

Belinda P. Simões,³ Eliane Gluckman^{1,2}
and Ryad Tamouza¹⁴

¹Monacord, Centre Scientifique de Monaco, Monaco, Monaco, ²Eurocord, Hôpital Saint Louis, Institut Universitaire d'Hématologie, Université Paris Diderot, Sorbonne Paris Cité, Paris, France, ³Centre for Cell-Based Therapy, Blood Centre of Ribeirão Preto, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, ⁴Department of Haematology, Clinics Hospital, University of São Paulo Medical School, São Paulo, Brazil, ⁵Hôpital Tenon, Hôpitaux Universitaires Est Parisien, Paris, France, ⁶Pediatrics Unit, Cheikh Anta Diop University, Centre Hospitalier National d'Enfants Albert Royer, Dakar, Senegal, ⁷Department of Haematology, Churchill Hospital, University of Oxford, Oxford, UK, ⁸University Gaston Berger, Saint Louis, Senegal, ⁹Département de Bactériologie, Hôpitaux Universitaires Est Parisien, ¹⁰INSERM 1160, Alloimmunity-Autoimmunity-Transplantation, Institut Universitaire d'Hématologie, Université Paris Diderot, Sorbonne Paris Cité, Paris, France, ¹¹School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão

Summary

Despite adequate immunization and penicillin prophylaxis, bacterial infections remain a leading cause of morbidity and mortality in patients with sickle cell disease (SCD). Besides hyposplenism, inflammatory and genetic factors might modulate their susceptibility to bacterial infections. We performed a candidate gene association of single nucleotide polymorphisms (SNPs) located in Toll-like receptor (*TLR*) genes, encoding prominent molecules for innate immune responses, with the occurrence of bacterial infections in patients with SCD. A cohort followed in centres in Brazil, France and Senegal ($n = 430$) was divided in two groups: patients who presented at least one episode of bacterial infection ($n = 235$) and patients who never had bacterial infections ($n = 195$). There were no differences in gender or age distribution among the groups. The frequency of the *TLR2* rs4696480 TA genotype was significantly lower in the infected group (50% vs. 67%, odds ratio [OR] = 0.50, 95% confidence interval [CI] 0.34–0.75, $P < 0.001$), and the TT genotype was significantly higher in the infected group (15% vs. 5%, OR = 3.18, 95% CI 1.53–6.61, $P < 0.001$). Previous reports demonstrated higher secretion of inflammatory factors in cells from AA individuals, lower occurrence and severity of immune diseases in T carriers. The rs4696480 TA genotype might stand between deleterious effects of over inflammatory response (AA genotype) and inefficient responses (TT genotype) to infectious agents in SCD settings.

Keywords: sickle cell disease, bacterial infections, Toll-like receptor, rs4696480, immunogenetics.

Preto, Brazil, ¹²Cellular Therapy &

Immunobiology Working Party of EBMT,

¹³Ospedale Pediatrico Bambino Gesù, Rome,

Italy, ¹⁴INSERM U955, Centre Hospitalier

Universitaire Henri Mondor, Créteil, France and

¹⁵Department of Clinical Analysis, Toxicology

and Food Science, School of Pharmaceutical

Sciences of Ribeirão Preto, University of São

Paulo, Ribeirão Preto, São Paulo, Brazil

Received 23 November 2018; accepted for

publication 28 January 2019

Correspondence: Karina Tozatto-Maio,

Eurocord - 1 avenue Claude Vellefaux 75010

Paris, France.

E-mail: karina.tmaio@usp.br

Sickle cell disease (SCD) is the most commonly inherited haemoglobin disorder (Piel *et al*, 2017). A single nucleotide polymorphism (SNP) in the beta-globin gene (*HBB*) leads to the synthesis of the mutant haemoglobin S (HbS) (Stuart & Nagel, 2004; Yawn *et al*, 2014). When HbS polymerizes under deoxygenation and stress conditions, the red blood cell (RBC) acquires a typical sickle shape (Oteng-Ntim *et al*, 2015). SCD refers to homozygous individuals (SS) and to compound heterozygous of the HbS mutation combined with other haemoglobin traits (HbSC, HbSB and others). Although the HbS mutation is remarkably prevalent in Sub-Saharan, Arabian and Indian populations, the incidence of SCD is increasing in different regions, and it has become a public health issue worldwide (Piel *et al*, 2017).

Patients with SCD show a heterogeneous clinical presentation, ranging from mild clinical symptoms to death due to disease complications (Ballas *et al*, 2010). SCD complications are caused mainly by vaso-occlusion and haemolysis (Yawn *et al*, 2014; Piel *et al*, 2017; Kato *et al*, 2018). Repeated vaso-occlusive episodes in the spleen lead to loss of spleen function and atrophy in most SCD patients (Brousse *et al*, 2014). Impaired spleen function partially explains the susceptibility to bacterial infections observed in SCD, because the vast majority of severe infections occurs before the age of 5 years when the spleen is still, at least partially, functional; however mechanic factors, lower activity of inflammatory pathways, nutrition deficiency and genetic factors might also play a role (Booth *et al*, 2010). Despite adequate vaccination and prophylaxis, infection remains a leading cause of morbidity and mortality among SCD patients (Booth *et al*, 2010).

Patients with SCD present a steady-state inflammatory status (Pitanga *et al*, 2016). Although some studies addressed the role of inflammatory proteins and cells in SCD (Styles *et al*, 2000; Tamouza *et al*, 2002; Hoppe *et al*, 2003; Adekile *et al*, 2005; Wallace *et al*, 2009; Wallace & Linden, 2010; Vingert *et al*, 2015; Godefroy *et al*, 2016; Pitanga *et al*, 2016; Tatari-Calderone *et al*, 2016), few studies have investigated the

potential associations between SCD complications and polymorphisms in genes encoding inflammatory proteins (Norris *et al*, 1996; Neonato *et al*, 1999; Styles *et al*, 2000; Tamouza *et al*, 2002; Hoppe *et al*, 2003; Adekile *et al*, 2005; Fertrin & Costa, 2010; Tatari-Calderone *et al*, 2016; Sippert *et al*, 2017; David *et al*, 2018). In this context, Toll-like receptors (TLRs) are a family of transmembrane and intra-cellular proteins expressed on immune cells that sense pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs). TLRs bind specific related ligands and, by activating downstream signalling cascades, promote transcription of inflammatory cytokines and chemokines (Akira *et al*, 2006; Oliveira *et al*, 2015; Ntoufa *et al*, 2016). TLRs are part of the innate immune system and are at the forefront response against several types of pathogens, including bacteria. Previous reports showed an association between polymorphisms in genes encoding TLR proteins and susceptibility or severity of infection (Sanders *et al*, 2011; van Well *et al*, 2012; Bulat-Kardum *et al*, 2015; Gopalakrishnan & Salgame, 2016). In addition, TLRs might be part of the SCD steady state inflammatory pathways that lead to SCD complications. For instance, TLR4 activation by the haem group, released by intravascular haemolysis, modulates alloimmunisation in this setting (Godefroy *et al*, 2016; Pitanga *et al*, 2016).

Given the high and deleterious burden of infectious events in SCD, and the pivotal role of TLRs in anti-infectious innate immune responses, we assessed the influence of selected genetic variants of *TLR* loci on the susceptibility to bacterial infections in patients with SCD.

Methods

Study population

A total of 430 patients with SCD followed in the Tenon Hospital, Paris, France ($n = 180$, mainly from Sub-Saharan Africa and, to a lesser extent, the French West Indies),

Clinics Hospital of Ribeirão Preto, Ribeirão Preto, Brazil, ($n = 120$), Clinics Hospital of São Paulo, São Paulo, Brazil ($n = 76$), and Albert Royer Hospital, Dakar, Senegal ($n = 54$) were enrolled in the study. All patients, previously diagnosed with SCD by haemoglobin electrophoresis or high performance liquid chromatography, had DNA samples stored and clinical data recorded, which were retrospectively collected from patients records. Bacterial infection was defined as an infectious event ascertained by identification of bacteria in blood or tissue cultures, or by clinical and laboratory findings, occurring at any time from birth to last follow-up. Only infections in the central nervous system (CNS), lower respiratory tract, upper urinary tract, abdomen, bone and joints, blood stream or at any site for tuberculosis were considered. For patients who had undergone haematopoietic stem cell transplantation (HSCT), only bacterial infections that occurred prior to HSCT were considered. Informed consent was obtained according to the declaration of Helsinki. The study was approved by all local ethical board reviews.

Genotyping

DNA was extracted from peripheral blood samples using standard methods (Miller *et al*, 1988). Seven SNPs in *TLR1* (rs4833095), *TLR2* (rs4308099, rs4696480, rs4308100), *TLR6* (rs5743810) and *TLR10* (rs11466653, rs11096957), all located in chromosome 4, were genotyped by mean of TaqMan 5' nuclease assay (Applied Biosystems, Foster City, CA, USA) using pre-designed allele-specific fluorogenic oligonucleotide probes. The polymerase chain reaction (PCR) technique employed was described elsewhere (Oliveira *et al*, 2015). Prior to PCR reaction, DNA was pre-amplified, using the following protocol: primers 40× were diluted with Trizol to 0.2×; for each sample, we added 1.3 µl of DNA at 20 ng/µl, 1 µl of pre-amplification master mix (Fluidigm, San Francisco, CA, USA), 1.25 µl of pooling assay with primers and 1.45 µl of pure water. Plates were centrifuged at 18°C for 1 min, and the thermocycler conditions were 75°C for 2 min, 95°C for 15 s and 60°C for 4 min for 14 cycles. The final product was diluted with Trizol at 1:5 proportion and 1 µl of the final product was used for the PCR reaction.

Comparison with a non-SCD population

For some SNPs, we compared genotype distribution between the study population and the African population described on the 1000 Genomes Project database, comprising 661 healthy adult individuals, mainly from Sub-Saharan African and African-American origin (<http://phase3browser.1000genomes.org/index.html>).

Statistical analysis

Univariate analyses were performed with chi-square or Mann-Whitney tests for categorical and continuous variables

respectively. Associations were measured by odds ratio (OR). Multiple testing was adjusted by the Bonferroni method for significant thresholds of P -value. For SNP associations, corrected significant P -value (cP) was 0.007; for genetic models, cP was 0.01. Linkage disequilibrium between SNPs and haplotype analyses were performed by logistic regression using SNPstats (Solé *et al*, 2006). Analyses were performed on SPSS 21.0 (SPSS Inc, Chicago, IL, USA).

Results

General population

A total of 430 patients with SCD were included in the study. Ethnical origin was known for 419 patients: 193 were Brazilian, 179 Sub-Saharan African, 41 French West Indies, 5 North African and one Indian. Median age at last follow-up was 30 years (range: 2–70) and 346 (87%) patients were older than 18 years (available $n = 399$); 243 (57%) patients were female and 184 (43%) were male (available $n = 427$). SCD genotype was SS in 344 (81%), SC in 46 (11%), SB in 31 (7%) and SD in 2 (1%) (available $n = 423$). Two hundred and nineteen (58%) patients received hydroxycarbamide (available $n = 380$), 294 (79%) received at least one red blood cell (RBC) transfusions (available $n = 374$), and 7 patients underwent HSCT. Eleven patients died during follow-up, mostly from acute chest syndrome and haemorrhagic stroke. In all countries, all patients had access to immunization against encapsulated bacteria and to penicillin prophylaxis at least up to 5 years of age.

Infections

Two hundred and thirty-five (55%) SCD patients had presented at least one episode of bacterial infection, and 195 (45%) SCD patients had not presented any bacterial infections. In the infected group, 135 (58%) were female, 192 (88%) were older than 18 years at last follow-up, 103 were Brazilian, 9 from Sub-Saharan Africa, 25 from French West Indies and 4 from other origin. In the non-infected group, 108 patients (56%) were female, 154 (86%) were older than 18 years at last follow-up, 90 were Brazilian, 83 from Sub-Saharan Africa, 16 from French West Indies and 2 from other origin.

Of the 235 infected patients, 44 (20%) had had more than 3 episodes of bacterial infection. Infections occurred in the lower respiratory tract in 121 cases (52%), bone and joints in 53 cases (23%), upper urinary tract in 47 cases (20%), CNS in 17 cases (7%), abdomen in 13 cases (6%), blood stream in 24 cases (10%); tuberculosis infections ($n = 17$, 7%) were pulmonary in 10 cases and extrapulmonary in 7 cases. In 106 cases an aetiological agent was identified. Most common agents were *Escherichia coli*, *Streptococcus pneumoniae*, *Mycobacterium tuberculosis* and *Salmonella spp.* In univariate analysis, no association was found between occurrence of infections and gender, origin or age distribution. Table I summarizes demography and univariate analysis.

Table I. Univariate analysis of infected *versus* non-infected patients with sickle cell disease.

Variable	Non-infected (<i>n</i> = 235)	Infected (<i>n</i> = 195)	<i>P</i> -value
Gender (<i>n</i> = 427)			
Female	108 (56)	135 (58)	NS
Male	85 (44)	99 (42)	
Origin (<i>n</i> = 419)			
Brazil	90 (47)	103 (45)	NS
Subsaharan Africa	83 (44)	96 (42)	
French West Indies	16 (8)	25 (11)	
North Africa	2 (1)	3 (1)	
Other	0	1 (1)	
Age > 18 years at last follow-up (<i>n</i> = 401)			
No	26 (14)	27 (12)	NS
Yes	154 (86)	192 (88)	
rs3804099 (<i>n</i> = 426)			
C/C	41 (21)	44 (19)	NS
C/T	87 (45)	104 (45)	
T/T	65 (34)	85 (36)	
rs4696480 (<i>n</i> = 425)			
T/T	10 (5)	35 (15)	<0.001
T/A	127 (67)	117 (50)	
A/A	54 (28)	82 (35)	
rs3804100 (<i>n</i> = 416)			
T/T	170 (91)	207 (90)	NS
C/T	17 (9)	20 (9)	
C/C	0	1 (1)	
rs11466653 (<i>n</i> = 427)			
A/A	175 (91)	215 (92)	NS
G/A	17 (8)	19 (8)	
G/G	1 (1)	0	
rs11096957 (<i>n</i> = 426)			
G/G	48 (25)	57 (25)	NS
G/T	103 (53)	115 (49)	
T/T	42 (22)	61 (26)	
rs4383095 (<i>n</i> = 401)			
G/G	51 (28)	49 (22)	NS
A/G	75 (42)	104 (47)	
A/A	53 (30)	69 (31)	
rs5743810 (<i>n</i> = 429)			
G/G	158 (81)	205 (87)	NS
A/G	31 (16)	28 (12)	
A/A	5 (3)	2 (1)	

NS, not significant.

The bold value indicates the significant *P*-value in univariate analysis.

Genetic analysis

All tested SNPs had a call rate greater than 90% and a minimum allele frequency of at least 1%. SNPs rs4696480, rs5743810 and rs4383095 were deviated from Hardy-Weinberg equilibrium (HWE) in the whole study population and in the non-infected group. Given that the whole study population was affected by SCD, and consequently there were no healthy controls in this population, these SNPs were considered for further analyses.

In univariate analysis, SNP rs4696480 in *TLR2* (*n* = 425) was significantly associated with occurrence of infections ($P < 0.001$). The distribution of rs4696480 genotypes was, in the infected patients, AA = 82 (35%), TA = 117 (50%), TT = 35 (15%); in the non-infected patients, distribution was AA = 54 (28%), TA = 127 (67%), TT = 10 (5%). Next, genetic models for this association were tested. In the over-dominant model, the heterozygous genotype (TA) occurred significantly less in infected patients than in non-infected patients compared with TT + AA patients (OR = 0.50, 95% confidence interval [CI] 0.34–0.75, $P < 0.001$). Furthermore, in the recessive model, the TT genotype occurred significantly more in infected patients than in non-infected patients compared with TA + AA (OR = 3.18, 95% CI 1.53–6.61, $P < 0.001$). Among the assessed SNPs, only rs11466653 was in significant linkage disequilibrium with rs4696480. Haplotype analyses were performed, but no association between any haplotype and occurrence of infections were identified. Table I shows genotype distribution and univariate analysis. Table II summarizes the genetic models for rs4696480.

Comparison with non-SCD populations

We compared the genotype distribution of SNP rs4696480 in this SCD population with a population with African origins, comprising adult healthy individuals, described in the 1000 Genomes Project database (*n* = 661). The TA genotype was significantly more frequent in the SCD population (*n* = 244, 57%) than in the 1000 Genomes Project population (*n* = 295, 45%) compared with TT + AA (OR 1.67, 95% CI 1.30–2.13, $P < 0.001$). Moreover, TT genotype was significantly less frequent than AA + TA in the SCD population (*n* = 45, 11%) compared to the 1000 Genomes Project population (*n* = 268, 41%) (OR 0.17, 95% CI 0.12–0.24, $P < 0.001$).

Discussion

We have shown that a polymorphism in the *TLR2* gene is associated with susceptibility to/protection against infections in patients with SCD. More specifically, while the heterozygous rs4696480 TA genotype apparently confers protection against bacterial infections, the TT genotype seems to increase risk of developing such infections.

TLRs are glycoproteins that recognize a wide range of PAMPs and DAMPs, with the consequent induction of inflammatory responses via nuclear factor (NF)-κB family members (Akira *et al*, 2006; Oliveira *et al*, 2015; Ntoufa *et al*, 2016). Currently, 13 TLRs are known. Because they are pattern recognition receptors (PRR), each TLR binds specific related structures (Ntoufa *et al*, 2016). However, some TLRs can recognize several structural unrelated ligands, namely TLR2 and TLR4 (Akira *et al*, 2006) or act as heterodimeric complex extending the repertoire of pattern recognition. TLR2 is highly expressed in monocytes and is capable of recognizing a huge number of proinflammatory inducers, including membrane lipoproteins of gram-

Table II. Genetic models, using logistic regression, for SNP rs4696480, considering infected $\times$ uninfected patients.

	Odds ratio	95% CI	P-value
Dominant			
AA $\times$ TA + TT			NS
Recessive			
TT $\times$ TA + AA	3.18	1.53–6.61	<0.001
Overdominant			
TA $\times$ AA + TT	0.5	0.34–0.75	<0.001
Log-additive			NS

CI, confidence interval; NS, not significant.

positive bacteria, mycobacteria, yeast, mycoplasma, spirochete and parasites (Hornung *et al*, 2002; Eder *et al*, 2004; Veltkamp *et al*, 2007; Oh *et al*, 2009; Kerkhof *et al*, 2010; Gopalakrishnan & Salgame, 2016; Ntoufa *et al*, 2016). TLR2 may function alone or as heterodimers with TLR1, TLR6 and TLR10 towards NF- κ B cascade activation; TLR10 also negatively regulates TLR2 (Moresco *et al*, 2011). Therefore, TLR2 is a key receptor for several exogenous pathogens, playing a pivotal role in immune response against a wide range of pathogens.

The SNP rs4696480 is located in the promoter region of the *TLR2* gene. This SNP was previously studied in the context of inflammatory diseases. Some reports in asthma, atopic dermatitis, inflammatory bowel disease and B hepatitis settings suggested that the presence of a T allele or TT genotype was associated with a lower inflammatory response, lower response to treatment with anti-tumour necrosis factor, and that the presence of an A allele led to a more severe inflammatory response (Eder *et al*, 2004; Oh *et al*, 2009; Kerkhof *et al*, 2010; Bank *et al*, 2014; Lin *et al*, 2018; Loft *et al*, 2018). Nevertheless, other studies did not show any effect of this SNP on the incidence of asthma (Kormann *et al*, 2008; Miedema *et al*, 2012). In addition, a previous case-control study assessed the effect of the S180L SNP in the Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein (TIRAP), part of the downstream signalling of TLR2, in severe bacterial infections in a non-SCD population (Khor *et al*, 2007). Similar to our results, the heterozygous genotype showed a protective effect against infection. Heterozygous transfected cells *in vitro* failed to bind TLR2, reducing activation of the NF- κ B pathway (Khor *et al*, 2007). Based on these findings, we postulate that the rs4696480 TA genotype enables an adequate inflammatory response to be mounted against bacterial infections in SCD patients. The AA genotype might lead to an exacerbated inflammatory reaction, whereas the response determined by the TT genotype is weaker than necessary to defeat bacteria.

In our study, the SNP rs4696480 was in HWE deviation in the whole SCD population and in the non-infected SCD patients. In addition, TA was more frequent, and TT was significantly less frequent in the SCD population compared with the African population described on the 1000 Genomes Project database. Our study population mostly consisted of adult patients. Knowing that bacterial infections are the leading

cause of mortality among SCD children, the HWE and the differences in genotype frequencies might be explained by a positive selective pressure due to the higher occurrence of infections in the SCD population. In other words, we cannot exclude that some patients with SCD carrying the TT genotype might have died in early childhood and could not have been considered in a study such as ours. However, this hypothesis requires further assessment.

Conflicting results were reported by David *et al* (2018). In a cohort of 95 paediatric infected and non-infected homozygous SS patients, the TA genotype was associated with more severe infections, and the AA genotype was protective against respiratory infections. Differences in sample size and age distribution might explain the discrepancy in the results, and could be in line with our observations concerning HWE expectations. Also, in that study, presence of alpha thalassaemia 3.7 kb deletion was associated with less infection occurrence. Previous studies reported a protective effect of alpha thalassaemia against hospitalisations due to infections and respiratory infections in non-SCD settings (Allen *et al*, 1997; Wambua *et al*, 2006). Alpha thalassaemia status was not available for most patients in our SCD cohort; therefore, we were unable to investigate its possible association with infection in our study.

Our study has several limitations: the retrospective nature of collected data and the low sensitivity of bacterial culture methods prevented further analyses on the type of bacteria involved. Similarly, because the study population is comprised mostly of adults, we cannot perform any comparisons on genotype distribution among different age groups. Nevertheless, the strength of this study relies on the large sample size and on the power of the associations found.

Loss of spleen function results in defective opsonization and impairs destruction of encapsulated bacteria. However, hyposplenism does not completely explain the higher occurrence of infections in SCD. Patients with SCD are also at greater risk of bacterial infections caused by non-encapsulated bacteria (Morrissey *et al*, 2015; Sobota *et al*, 2015). In addition, even among patients carrying the same SCD genotype and presenting hyposplenism, occurrence of bacterial infections is highly variable. Our results might contribute to better understand the mechanisms involved in the greater risk of bacterial infections in SCD settings, to ameliorate strategies for prevention and treatment of infections. Further studies, including candidate gene and genome-wide association studies, are needed to evaluate the impact of genetic factors in patients with SCD, with the aim of explaining the heterogeneity of clinical outcomes in this setting and, also, to confirm our findings.

Acknowledgments

This work was supported by the Centre Scientifique de Monaco and the Brazilian Coordination for the Improvement of Higher Education Personnel (CAPES). The authors thank the Centre Scientifique de Monaco, CAPES, all patients and their families.

Conflict of interest

There are no competing interests to report.

Author contributions

KTM, BPS, EG, RG and RT designed the study. KTM, IDL and CK collected clinical data. KTM performed

experiments. CM and ILA advised experiments. KTM performed statistical analysis. RG, YB, ID, IDL, ACSP, SK, ESR, SFMG, GHFF, VR, CLD and LJ provided cases and samples for the study. KTM, FV, AR, EG, BPS, RG, HR, BC and RT wrote the manuscript. All authors edited and approved the manuscript.

References

- Adekile, A., Haider, M.Z., Marouf, R. & Adekile, A.D. (2005) HLA-DRB1 alleles in Hb SS patients with avascular necrosis of the femoral head. *American Journal of Hematology*, **79**, 8–10.
- Akira, S., Uematsu, S. & Takeuchi, O. (2006) Pathogen recognition and innate immunity. *Cell*, **124**, 783–801.
- Allen, S.J., O'Donnell, A., Alexander, N.D., Alpers, M.P., Peto, T.E., Clegg, J.B. & Weatherall, D.J. (1997) alpha+-Thalassemia protects children against disease caused by other infections as well as malaria. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 14736–14741.
- Ballas, S.K., Loeff, S., Benjamin, L.J., Dampier, C.D., Heeney, M.M., Hoppe, C., Johnson, C.S., Rogers, Z.R., Smith-Whitley, K., Wang, W.C. & Telen, M.J. (2010) Definitions of the phenotypic manifestations of sickle cell disease. *American Journal of Hematology*, **85**, 6–13.
- Bank, S., Skytt Andersen, P., Burisch, J., Pedersen, N., Roug, S., Galsgaard, J., Ydegaard Turino, S., Brodersen, J.B., Rashid, S., Kaiser Rasmussen, B., Avlund, S., Bastholm Olesen, T., Jürgen Hoffmann, H., Kragh Thomsen, M., Ostergaard Thomsen, V., Frydenberg, M., Andersen Nexø, B., Sode, J., Vogel, U. & Andersen, V. (2014) Polymorphisms in the inflammatory pathway genes TLR2, TLR4, TLR9, LY96, NFKBIA, NFKB1, TNFA, TNFRSF1A, IL6R, IL10, IL23R, PTPN22, and PPARG are associated with susceptibility of inflammatory bowel disease in a Danish cohort. *PLoS ONE*, **9**, e98815.
- Booth, C., Inusa, B. & Obaro, S.K. (2010) Infection in sickle cell disease: a review. *International Journal of Infectious Diseases*, **14**, e2–e12.
- Brousse, V., Buffet, P. & Rees, D. (2014) The spleen and sickle cell disease: the sick(led) spleen. *British Journal of Haematology*, **166**, 165–176.
- Bulat-Kardum, L.J., Etokebe, G.E., Lederer, P., Balen, S. & Dembic, Z. (2015) Genetic polymorphisms in the Toll-like receptor 10, Interleukin (IL)17A and IL17F genes differently affect the risk for tuberculosis in Croatian population. *Scandinavian Journal of Immunology*, **82**, 63–69.
- David, S., Aguiar, P., Antunes, L., Dias, A., Morais, A., Sakuntabhai, A. & Lavinha, J. (2018) Variants in the non-coding region of the TLR2 gene associated with infectious subphenotypes in pediatric sickle cell anemia. *Immunogenetics*, **70**, 37–51.
- Eder, W., Klimecki, W., Yu, L., von Mutius, E., Riedler, J., Braun-Fahrlander, C., Nowak, D. & Martinez, F.D.; ALEX Study Team. (2004) Toll-like receptor 2 as a major gene for asthma in children of European farmers. *The Journal of Allergy and Clinical Immunology*, **113**, 482–488.
- Fertrin, K.Y. & Costa, F.F. (2010) Genomic polymorphisms in sickle cell disease: implications for clinical diversity and treatment. *Expert Review of Hematology*, **3**, 443–458.
- Godefroy, E., Liu, Y., Shi, P., Mitchell, W.B., Cohen, D., Chou, S.T., Manwani, D. & Yazdankhsh, K. (2016) Altered heme-mediated modulation of dendritic cell function in sickle cell alloimmunization. *Haematologica*, **101**, 1028–1038.
- Gopalakrishnan, A. & Salgame, P. (2016) Toll-like receptor 2 in host defense against Mycobacterium tuberculosis: to be or not to be—that is the question. *Current Opinion in Immunology*, **42**, 76–82.
- Hoppe, C., Klitz, W., Noble, J., Vigil, L., Vichinsky, E. & Styles, L. (2003) Distinct HLA associations by stroke subtype in children with sickle cell anemia. *Blood*, **101**, 2865–2869.
- Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdörfer, B., Giese, T., Endres, S. & Hartmann, G. (2002) Quantitative expression of toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *Journal of Immunology (Baltimore, Md.: 1950)*, **168**, 4531–4537.
- Kato, G.J., Piel, F.B., Reid, C.D., Gaston, M.H., Ohene-Frempong, K., Krishnamurti, L., Smith, W.R., Panepinto, J.A., Weatherall, D.J., Costa, F.F. & Vichinsky, E.P. (2018) Sickle cell disease. *Nature Reviews Disease Primers*, **4**, 18010.
- Kerkhof, M., Postma, D.S., Brunekreef, B., Reijmerink, N.E., Wijga, A.H., de Jongste, J.C., Gehring, U. & Koppelman, G.H. (2010) Toll-like receptor 2 and 4 genes influence susceptibility to adverse effects of traffic-related air pollution on childhood asthma. *Thorax*, **65**, 690–697.
- Khor, C.C., Chapman, S.J., Vannberg, F.O., Dunne, A., Murphy, C., Ling, E.Y., Frodsham, A.J., Walley, A.J., Kyrieles, O., Khan, A., Aucan, C., Segal, S., Moore, C.E., Knox, K., Campbell, S.J., Lienhardt, C., Scott, A., Aaby, P., Sow, O.Y., Grignani, R.T., Sillah, J., Sirugo, G., Peshu, N., Williams, T.N., Maitland, K., Davies, R.J., Kwiatkowski, D.P., Day, N.P., Yala, D., Crook, D.W., Marsh, K., Berkley, J.A., O'Neill, L.A. & Hill, A.V. (2007) A Mal functional variant is associated with protection against invasive pneumococcal disease, bacteremia, malaria and tuberculosis. *Nature Genetics*, **39**, 523–528.
- Kormann, M.S.D., Depner, M., Hartl, D., Klopp, N., Illig, T., Adamski, J., Vogelberg, C., Weiland, S.K., von Mutius, E. & Kabisch, M. (2008) Toll-like receptor heterodimer variants protect from childhood asthma. *The Journal of Allergy and Clinical Immunology*, **122**, 92.e1–92.e8.
- Lin, Y., Gao, Z.-X., Shen, X., Chen, M.-J., Li, Y.-T., Li, S.-L., Lin, H.-L., Zhao, Q.-F., Liu, F. & Niu, J.-J. (2018) Correlation between polymorphisms in toll-like receptor genes and the activity of hepatitis B virus among treatment-naïve patients: a case-control study in a Han Chinese population. *BMC Infectious Diseases*, **18**, 28.
- Loft, N.D., Skov, L., Iversen, L., Gniadecki, R., Dam, T.N., Brandslund, I., Hoffmann, H.J., Andersen, M.R., Dessau, R.B., Bergmann, A.C., Andersen, N.M., Andersen, P.S., Bank, S., Vogel, U. & Andersen, V. (2018) Associations between functional polymorphisms and response to biological treatment in Danish patients with psoriasis. *The Pharmacogenomics Journal*, **18**, 494–500.
- Miedema, K.G.E., Tissing, W.J.E., Te Poele, E.M., Kamps, W.A., Alizadeh, B.Z., Kerkhof, M., de Jongste, J.C., Smit, H.A., de Pagter, A.P., Bierings, M., Boezen, H.M., Postma, D.S., de Bont, E.S.J.M. & Koppelman, G.H. (2012) Polymorphisms in the TLR6 gene associated with the inverse association between childhood acute lymphoblastic leukemia and atopic disease. *Leukemia*, **26**, 1203–1210.
- Miller, S.A., Dykes, D.D. & Polesky, H.F. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research*, **16**, 1215.
- Moresco, E.M.Y., LaVine, D. & Beutler, B. (2011) Toll-like receptors. *Current Biology*, **21**, R488–R493.
- Morrissey, B.J., Bycroft, T.P., Almossawi, O., Wilkey, O.B. & Daniels, J.G. (2015) Incidence and predictors of bacterial infection in febrile children with sickle cell disease. *Hemoglobin*, **39**, 316–319.
- Neonato, M.G., Lu, C.Y., Guilloud-Bataille, M., Lapoumeroulie, C., Nabeel-Jassim, H., Dabit, D., Giro, R., Krishnamoorthy, R., Feingold, J., Besmond, C. & Elion, J. (1999) Genetic polymorphism of the mannose-binding protein gene in children with sickle cell disease: identification of three new variant alleles and relationship to infections. *European Journal of Human Genetics: EJHG*, **7**, 679–686.
- Norris, C.F., Surrey, S., Bunin, G.R., Schwartz, E., Buchanan, G.R. & McKenzie, S.E. (1996) Relationship between Fc receptor IIA polymorphism and infection in children with sickle cell disease. *The Journal of Pediatrics*, **128**, 813–819.

- Ntoufa, S., Vilia, M.G., Stamatopoulos, K., Ghia, P. & Muzio, M. (2016) Toll-like receptors signaling: a complex network for NF- κ B activation in B-cell lymphoid malignancies. *Seminars in Cancer Biology*, **39**, 15–25.
- Oh, D.-Y., Schumann, R.R., Hamann, L., Neumann, K., Worm, M. & Heine, G. (2009) Association of the toll-like receptor 2 A-16934T promoter polymorphism with severe atopic dermatitis. *Allergy*, **64**, 1608–1615.
- Oliveira, J., Etain, B., Lajnef, M., Hamdani, N., Ben-nabi, M., Bengoufa, D., Sundaresh, A., Chaabane, A.B., Bellivier, F., Henry, C., Kahn, J.-P., Charron, D., Krishnamoorthy, R., Leboyer, M. & Tamouza, R. (2015) Combined effect of TLR2 gene polymorphism and early life stress on the age at onset of bipolar disorders. *PLoS ONE*, **10**, e0119702.
- Oteng-Ntim, E., Meeks, D., Seed, P.T., Webster, L., Howard, J., Doyle, P. & Chappell, L.C. (2015) Adverse maternal and perinatal outcomes in pregnant women with sickle cell disease: systematic review and meta-analysis. *Blood*, **125**, 3316–3325.
- Piel, F.B., Steinberg, M.H. & Rees, D.C. (2017) Sickle cell disease. *The New England Journal of Medicine*, **376**, 1561–1573.
- Pitanga, T.N., Oliveira, R.R., Zanette, D.L., Guarda, C.C., Santiago, R.P., Santana, S.S., Nascimento, V.M.L., Lima, J.B., Carvalho, G.Q., Maffili, V.V., Carvalho, M.O.S., Alcântara, L.C.J., Borges, V.M. & Goncalves, M.S. (2016) Sickle red cells as danger signals on proinflammatory gene expression, leukotriene B4 and interleukin-1 beta production in peripheral blood mononuclear cell. *Cytokine*, **83**, 75–84.
- Sanders, M.S., van Well, G.T.J., Ouburg, S., Lundberg, P.S.J., van Furth, A.M. & Morré, S.A. (2011) Single nucleotide polymorphisms in TLR9 are highly associated with susceptibility to bacterial meningitis in children. *Clinical Infectious Diseases*, **52**, 475–480.
- Sippert, E.Â., Visentainer, J.E.L., Alves, H.V., Rodrigues, C., Gilli, S.C.O., Addas-Carvalho, M., Saad, S.T.O., Costa, F.F. & Castilho, L. (2017) Red blood cell alloimmunization in patients with sickle cell disease: correlation with HLA and cytokine gene polymorphisms. *Transfusion*, **57**, 379–389.
- Sobota, A., Sabharwal, V., Fonebi, G. & Steinberg, M. (2015) How we prevent and manage infection in sickle cell disease. *British Journal of Haematology*, **170**, 757–767.
- Solé, X., Guinó, E., Valls, J., Iñiesta, R. & Moreno, V. (2006) SNPStats: a web tool for the analysis of association studies. *Bioinformatics (Oxford, England)*, **22**, 1928–1929.
- Stuart, M.J. & Nagel, R.L. (2004) Sickle-cell disease. *Lancet (London, England)*, **364**, 1343–1360.
- Styles, L.A., Hoppe, C., Klitz, W., Vichinsky, E., Lubin, B. & Trachtenberg, E. (2000) Evidence for HLA-related susceptibility for stroke in children with sickle cell disease. *Blood*, **95**, 3562–3567.
- Tamouza, R., Neonato, M.G., Busson, M., Marzais, F., Girot, R., Labie, D., Elion, J. & Charron, D. (2002) Infectious complications in sickle cell disease are influenced by HLA class II alleles. *Human Immunology*, **63**, 194–199.
- Tatari-Calderone, Z., Gordish-Dressman, H., Fasano, R., Riggs, M., Fortier, C., Campbell, A.D., Charron, D., Gordeuk, V.R., Luban, N.L.C., Vukmanovic, S. & Tamouza, R. (2016) Protective effect of HLA-DQB1 alleles against alloimmunization in patients with sickle cell disease. *Human Immunology*, **77**, 35–40.
- Veltkamp, M., Wijnen, P. A. H.M., van Moorsel, C.H.M., Rijkers, G.T., Ruven, H.J.T., Heron, M., Bekers, O., Claessen, A.M.E., Drent, M., van den Bosch, J.M.M. & Grutters, J.C. (2007) Linkage between Toll-like receptor (TLR) 2 promoter and intron polymorphisms: functional effects and relevance to sarcoidosis. *Clinical and Experimental Immunology*, **149**, 453–462.
- Vingert, B., Tamagne, M., Habibi, A., Pakdaman, S., Ripa, J., Elayeb, R., Galacteros, F., Bierling, P., Ansart-Pirenne, H., Bartolucci, P. & Noizat-Pirenne, F. (2015) Phenotypic differences of CD4(+) T cells in response to red blood cell immunization in transfused sickle cell disease patients. *European Journal of Immunology*, **45**, 1868–1879.
- Wallace, K.L. & Linden, J. (2010) Adenosine A2A receptors induced on iNKT and NK cells reduce pulmonary inflammation and injury in mice with sickle cell disease. *Blood*, **116**, 5010–5020.
- Wallace, K.L., Marshall, M.A., Ramos, S.I., Lannigan, J.A., Field, J.J., Strieter, R.M. & Linden, J. (2009) NKT cells mediate pulmonary inflammation and dysfunction in murine sickle cell disease through production of IFN-gamma and CXCR3 chemokines. *Blood*, **114**, 667–676.
- Wambua, S., Mwangi, T.W., Kortok, M., Uyoga, S.M., Macharia, A.W., Mwacharo, J.K., Weatherall, D.J., Snow, R.W., Marsh, K. & Williams, T.N. (2006) The effect of alpha+-thalassaemia on the incidence of malaria and other diseases in children living on the coast of Kenya. *PLoS Medicine*, **3**, e158.
- van Well, G.T.J., Sanders, M.S., Ouburg, S., van Furth, A.M. & Morré, S.A. (2012) Polymorphisms in Toll-like receptors 2, 4, and 9 are highly associated with hearing loss in survivors of bacterial meningitis. *PLoS ONE*, **7**, e35837.
- Yawn, B.P., Buchanan, G.R., Afenyi-Annan, A.N., Ballas, S.K., Hassell, K.L., James, A.H., Jordan, L., Lanzkron, S.M., Lottenberg, R., Savage, W.J., Tanabe, P.J., Ware, R.E., Murad, M.H., Goldsmith, J.C., Ortiz, E., Fulwood, R., Horton, A. & John-Sowah, J. (2014) Management of sickle cell disease: summary of the 2014 evidence-based report by expert panel members. *JAMA*, **312**, 1033–1048.

5. MANUSCRIPT 2: Human leukocyte antigen matched unrelated donors for patients with sickle cell disease: results of international donor searches.

Human leukocyte antigen matched unrelated donors for patients with sickle cell disease: results of international donor searches.

Journal:	<i>British Journal of Haematology</i>
Manuscript ID	Draft
Manuscript Type:	Ordinary Papers
Date Submitted by the Author:	n/a
Complete List of Authors:	Tozatto-Maio, Karina; Eurocord, Eurocord study office Torres, Margareth; Albert Einstein Jewish Hospital, São Paulo, Brazil, Laboratory of Histocompatibility Dhegaide, Neifi; University of Sao Paulo Faculty of Medicine of Ribeirao Preto, Center for Cell-Based Therapy, Blood Center of Ribeirão Preto Cardoso, Juliana; Instituto Nacional de Cancer, Brazilian Bone Marrow Registry (REDOME) Volt, Fernanda; Hopital Saint-Louis, Eurocord Silva-Pinto, Ana; Hemocentro de Ribeirão Preto, Faculdade de Medicina de Ribeirão Preto, USP Oliveira, Danielli; Instituto Nacional de Cancer, Brazilian Bone Marrow Registry (REDOME) Elayoubi, Hanadi; Eurocord, research Kashima, Simone; Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil, Center for Cell-Based Therapy, Hemotherapy Center of Ribeirão Preto Loiseau, Pascale; Hospital Saint Louis Paris, HLA laboratory Veelken, Hendrik; Leiden University Medical Center, Hematology Ferster, Alina; Hôpital Universitaire Des Enfants Reine Fabiola, Department of Pediatric Hemato-Oncology Cappelli, Barbara; Monacord, research Rodrigues, Evandra; Universidade de Sao Paulo Faculdade de Medicina, Center for Cell Based Therapy Scigliuolo, Graziana; Centre Scientifique de Monaco, Monacord Kenzey, Chantal; Eurocord, Research Ruggeri, Annalisa; Eurocord, Research; Hopital Saint-Antoine, Hématologie-greffe Rocha, Vanderson; University of Sao Paulo Hospital of Clinics, Department of Haematology Corbacioglu, Selim; European Society for Blood and Marrow Transplantation, Paediatric Diseases Working Party Simões, Belinda; School of Medicine of Ribeirão Preto, University of São Paulo, Internal Medicine Tamouza, Ryad; Hopital Henri Mondor, INSERM U955 Gluckman, Eliane; Hopital Saint-Louis, Saint Louis
Key Words:	SICKLE CELL DISEASE, HLA, STEM CELL DONOR REGISTRY, HAEMATOPOIETIC STEM CELL TRANSPLANTATION, UNRELATED DONOR SEARCH

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1

Human leukocyte antigen matched unrelated donors for patients with sickle cell disease: results of international donor searches.

Karina Tozatto-Maio^{1,4}, Margareth Afonso Torres⁵, Neifi Hassan Saloum Degaide³, Juliana Fernandes Cardoso⁶, Fernanda Volt^{1,2}, Ana Cristina Silva Pinto³, Danielli Oliveira⁶, Hanadi Elayoubi^{1,2}, Simone Kashima³, Pascale Loiseau⁷, Joan Hendrick Veelken⁸, Alina Ferster⁹, Barbara Cappelli^{1,2}, Evandra Strazza Rodrigues³, Graziana Maria Scigliuolo^{1,2}, Chantal Kenzey^{1,2}, Annalisa Ruggeri^{1,2,10}, Vanderson Rocha^{1,2,4,11}, Selim Corbacioglu^{12,13}, Belinda Pinto Simões³, Ryad Tamouza¹⁴, and Eliane Gluckman^{1,2}.

¹Monacord, Centre Scientifique de Monaco, Principauté de Monaco, Monaco;

²Eurocord, Hôpital Saint Louis, Institut de recherche Saint Louis, Université Paris Diderot, Sorbonne Paris Cité, Paris, France;

³Center for Cell-Based Therapy, Blood Center of Ribeirão Preto, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil;

⁴Department of Hematology, Clinics Hospital, University of São Paulo Medical School, São Paulo, Brazil;

⁵Laboratory of Histocompatibility, Albert Einstein Jewish Hospital, São Paulo, Brazil

⁶Brazilian Bone Marrow Registry (REDOME), Instituto Nacional do Câncer, Ministério da Saúde, Rio de Janeiro, RJ, Brazil;

⁷Saint Louis Hospital, Assistance Publique Hôpitaux de Paris, Paris, France

⁸Bone Marrow Transplantation Centre Leiden, Leiden University Hospital, Leiden, The Netherlands

⁹Department of Hematology/Oncology, Hôpital Universitaire des Enfants Reine Fabiola, Université Libre de Bruxelles, B-1020 Brussels, Belgium.

¹⁰Ospedale Pediatrico Bambino Gesù, Rome, Italy;

¹¹Department of Hematology, Churchill Hospital, University of Oxford, Oxford, UK

¹²University of Regensburg, Regensburg, Germany.

¹³Pediatric Diseases Working Party, European Blood and Marrow Transplant Society (EBMT)

¹⁴INSERM U955, Centre Hospitalier Universitaire Henri Mondor, Créteil, France

Contact information for correspondence

Karina Tozatto-Maio

karina.tmaio@usp.br

Eurocord - 1 avenue Claude Vellefaux 75010 Paris, France

Phone: [+33 01 42 49 48 23](tel:+330142494823)

Fax : [+ 33 01 42 38 53 90](tel:+330142385390)

Running title: HLA matched unrelated donor searches in sickle cell disease

Key words: sickle cell disease, HLA, stem cell donor registry, hematopoietic stem cell transplantation, unrelated donor search

Word count

Abstract: 198 words

Main text: 3297 words

Tables: 2

Figures: 1

References: 34

Abstract

Sickle cell disease (SCD) is the most common inherited hemoglobinopathy. Hematopoietic stem cell transplantation (HSCT) is the only curative therapy for SCD, but few patients will have a matched sibling donor. Patients with SCD are mostly from African origin, therefore less likely to find a matched unrelated donor in international registries. Using HaploStats, we estimated HLA haplotypes for 185 patients with SCD (n=116 from a Brazilian centre; n=69 from the European Society for Blood and Marrow Transplantation (EBMT) centres) and classified the ethnic origin of haplotypes. Next, we assessed the probability of finding a human leukocyte antigen (HLA) matched unrelated adult donor (MUD), considering loci A, B and DRB1 (6/6), in international registries. Most haplotypes were African, but Brazilians showed higher ethnic admixture than EBMT patients. Nevertheless, chances of finding at least one 6/6 potential allelic donor was 47% for EBMT and 47% for Brazilians. Most potential allelic donors were from the National Marrow Donor Program (NMDP) registry (USA) and from the Brazilian donor registry (REDOME). Although the probability of finding a donor was higher than previously reported, strategies are required to improve ethnic diversity in registries. Moreover, predicting chances of having a MUD might influence SCD management.

Introduction

Sickle cell disease (SCD) is the most common inherited hemoglobinopathy worldwide (Piel *et al*, 2017). SCD is caused by a single nucleotide substitution in the beta globin chain with consequent production of the mutant haemoglobin S (HbS), which polymerizes under stress conditions (Stuart & Nagel, 2004; Yawn *et al*, 2014; Oteng-Ntim *et al*, 2015). HbS polymerization induces haemolysis and vaso-occlusion, which are the key elements in SCD complications (Piel *et al*, 2017). SCD can affect all organs, and some of its associated complications, such as cerebral vasculopathy, renal failure, and acute chest syndrome, have high burden of morbidity and mortality. Although hydroxyurea and red blood cell (RBC) transfusions improved SCD management, these therapies are not curative and do not prevent some severe complications (Angelucci *et al*, 2014). Patients with SCD still face decreased life span and quality of life.

Hematopoietic stem cell transplantation (HSCT) is the only currently available curative therapy for SCD (Gluckman *et al*, 2017; Arnold *et al*, 2016; Angelucci *et al*, 2014; Bernaudin *et al*, 2007). The largest series reported, with 1,000 patients who received HSCT from a human leukocyte antigen (HLA) identical sibling donor, showed good overall survival (OS) and event-free survival (EFS) (Gluckman *et al*, 2017). Nevertheless, few patients will have an identical sibling donor. Therefore, most patients will not benefit from an HLA identical sibling HSCT, justifying the need of considering matched unrelated donor (MUD) HSCT for SCD. Some studies reported a high overall survival after MUD HSCT but a high rate of rejection and graft versus host disease (GvHD) are still major obstacles for long term cure. In addition to the difficulty of finding a matched unrelated donor in this ethnic minority population, improvements in patient selection, conditioning and GvHD prophylaxis are needed (Shenoy *et al*, 2016; Cappelli *et al*, 2018).

Identifying a donor for these patients remains a major concern. SCD originated in Sub-Saharan Africa, Middle East and India (Piel *et al*, 2013; Piel, 2013), and patients are mainly from African origin. These populations are underrepresented in registries worldwide (Gluckman, 2013;

Dew *et al*, 2008; Barker *et al*, 2019). It was reported that patients from African origin have chances as low as 16% of finding an unrelated donor, whereas at least one can be identified for up to 75% of Caucasian patients (Gragert *et al*, 2014). In another recent study, patients with African background had the lowest chances of having a 8/8 MUD (Barker *et al*, 2019). To date, few studies were performed in SCD populations to test their probabilities of finding a matched unrelated donor in local registries (da Silva-Malta *et al*, 2017; Justus *et al*, 2015; Krishnamurti *et al*, 2003).

Because of the importance of HSCT in SCD, and considering the difficulty to identify donors for ethnic minorities, we estimated the HLA haplotypes of two SCD populations of different ethnic background and calculated their probability of finding an allelic matched unrelated donor in international donor searches.

Methods

Study population

The study population was composed by 185 patients with SCD divided in two sub cohorts: one of Brazilian patients who received HSCT from an identical sibling donor or were not submitted to HSCT (n=116), and the other of patients who underwent related or unrelated HSCT from an HLA identical or non-identical donor in transplant centres reporting to the European Society for Blood and Marrow Transplantation (EBMT) (n=69). In Brazil, all patients were followed at the University Hospital of Ribeirão Preto and had intermediate resolution HLA typing available. One patient had two siblings and another patient had one sibling in the same cohort. Siblings were excluded from HLA allele and haplotype frequency estimation. In the EBMT cohort, all patients had intermediate or high resolution HLA typing available.

HLA typing

For the Brazilian patients, intermediate resolution HLA genotyping for loci HLA-A, HLA-B and HLA-DRB1 was performed by polymerase chain reaction (PCR) reverse sequence specific

oligonucleotide (SSO) in intermediate resolution. For the EBMT patients, intermediate and high resolution HLA genotyping was performed by PCR-SSO, sequence based typing (SBT) or next generation sequencing (NGS) for the loci HLA-A, HLA-B and HLA-DRB1.

Alleles and haplotype estimation

For the Brazilian patients, because HLA typing was at intermediate resolution, we decoded NMDP codes using the multiple allele code (MAC) service from the National Marrow Donor Program (NMDP) (<https://hml.nmdp.org/MacUI/>), and estimated alleles based on previously described allele frequencies (Majers *et al*, 2007; Gragert *et al*, 2014). In some cases, the allele was estimated together with haplotype estimation. Hardy-Weinberg equilibrium (HWE) for loci and allele frequencies were assessed by PyPOP software (Lancaster *et al*, 2007). We compared allele frequencies found in our population with the African American population described on NMDP (Gragert *et al*, 2014). Haplotype estimation (loci HLA-A, HLA-B and HLA-DRB1) was made using the HaploStats website (<https://www.haplostats.org/haplostats?execution=e2s1>). Haplostats allows haplotype estimation based on the expectation-maximization (EM) algorithm (Kollman *et al*, 2007). Briefly, the EM algorithm contains two phases: 1) expectation (E), in which the probability of each haplotype is calculated based on the current haplotype estimates; 2) maximization (M), in which the results of the E phase are used to update the haplotype frequency estimates, yielding a new estimate; the E and M phases are repeated until they meet a convergence criterion (Kollman *et al*, 2007; Applied Statistical Genetics with R - For Population-based Association Studies | Andrea S. Foulkes | Springer). The HaploStats database contains information from the NMDP registry on HLA haplotype frequency for the following ethnic groups: Caucasian, African American, Asian, Hispanic and Native American. Based on the described haplotype frequency for each ethnic group, we defined the most likely ethnicity of each estimated haplotype as described elsewhere (Ameen *et al*, 2018); some haplotypes are described at high frequencies, defined as equal or greater than 1/1000 (Kollman *et al*, 2007), in all populations, and were defined as “common”. In 17 cases (9%), family

typing was available and family study agreed with HaploStats estimation in 100%. In 33 (18%) cases, it was not possible to define the haplotype because the probabilities of estimation were very similar.

Unrelated donor search

We performed unrelated donor searches through the world marrow donor association (WMDA) algorithm for adult donors. The matching algorithm was set to match by haplotype. A potential donor was defined as a full matched donor in loci HLA-A, HLA-B and HLA-DRB1 (6/6 HLA match) in low resolution; a potential allelic donor was defined as a full matched donor for the same loci in high resolution. For each patient, we assessed the probability of finding at least one potential donor and one potential allelic donor. Because registered donors are sometimes unavailable for cell donation, it is recommended to perform confirmatory typing from at least five potential allelic donors simultaneously (Spellman *et al*, 2012). For this reason, we also calculated the probability of having at least five potential allelic donors. P-values for comparing the probabilities of finding at least one potential allelic donor, and at least five potential allelic donors, between the EBMT and the Brazilian cohort were calculated by chi-square. Patients who received a MUD HSCT were excluded from donor searches. We did not include mismatched donors in our searches.

Ethical considerations

This study was approved by the EBMT Paediatric Diseases Working Party, by the ethics committee of the Clinics Hospital of Ribeirão Preto, and by the Eurocord scientific committee.

Results

Demography

The Brazilian cohort was composed of 116 Brazilian patients, 64 (55%) females and 52 (45%) males. SCD genotype was homozygote (SS) in 96 (83%) cases, S β thalassemia (S β) in 15 (13%) and hemoglobinopathy SC (SC) in 5 (4%). Twenty-three patients underwent HLA identical related HSCT at the Ribeirão Preto Clinics Hospital. The EBMT cohort was composed of 69 patients, 36 (52%) females

7

and 33 (48%) males. SCD genotype was SS in 47 (82%) cases and S β in 10 (18%) (available n=57). Patients were transplanted in 24 EBMT centres, mainly in France (n=18), Netherlands (n=10), Belgium (n=10) and Italy (n=8). Fifty-four patients received HSCT from an identical sibling donor, 10 from a matched unrelated donor and 5 from a mismatched unrelated donor.

HLA allele and haplotype frequencies

One hundred eighty-two patients, 113 Brazilian and 69 EBMT patients, had allele and haplotype frequencies estimated. Thirty-three different alleles were described for the HLA-A locus, 67 for HLA-B and 43 for HLA-DRB1. In both populations, HWE was neutral for loci A and DBR1, and deviated in loci B. Main HLA allele frequencies found in the whole cohort, in Brazilian patients, EBMT patients, and comparisons with the African population described on NMDP are summarized in table 1.

Two hundred sixty-five HLA A~B~DRB1 haplotypes were observed. Because of the size of the cohort, we did not assess HWE and linkage disequilibrium. The most frequent haplotypes in the whole population were (n=297): A*01:01 B*08:01 DRB1*03:01 (n=5, 2%); A*36:01 B*53:01 DRB1*11:01 (n=4, 1%); A*23:01 B*44:03 DRB1*07:01 (n=4, 1%). In the Brazilian population, the most common haplotypes were A*01:01 B*08:01 DRB1*03:01 (n=5, 3%); A*23:01 B*44:03 DRB1*07:01 (n=3, 2%); A*02:01 B*27:05 DRB1*01:01 (n=3, 2%) and A*03:01 B*58:02 DRB1*15:03 (n=3, 2%). In the EBMT population, the most frequent haplotypes were A*36:01 B*53:01 DRB1*11:01 (n=3, 3%); A*68:02 B*15:10 DRB1*03:01 (n=2, 2%) and A*23:01 B*07:05 DRB1*11:01 (n=2, 2%). Two haplotypes were never previously described: A*23:03 B*08:01 DRB1*03:01 and A*24:03 B*35:43 DRB1*15:01; both patients who carry these haplotypes received HSCT from an identical sibling donor.

HLA haplotype ethnicity

Most HLA haplotypes from the entire population were classified as African haplotypes (n=162/297, 55%); 43 (14%) haplotypes were classified as common, 29 (10%) as Caucasian, 21 (7%) as Amerindian and 43 (14%) could not be classified. Brazilian patients showed greater admixture compared with the EBMT population. In the Brazilian cohort (n=181), the distribution of ethnicity for the estimated haplotypes was: African=81 (45%), common=33 (18%), Caucasian=22 (12%) and Amerindian=17 (9%); 28 (15%) haplotypes could not be classified. In the EBMT cohort (n=116), 81 (70%) haplotypes were African, 9 (8%) were common, 7 (6%) were Caucasian, 4 (3%) were Amerindian and 15 (13%) could not be classified. Figure 1 shows the distribution of haplotype ethnicities among the two populations.

Unrelated donor search

For assessing the probability of finding a matched unrelated donor in an international donor search, we excluded 10 patients from the EBMT cohort who received MUD HSCT. Overall (n=175), 138 (79%) patients had at least one potential donor; from these, 88 (50%) had at least one potential allelic donor and 37 (21%) patients had at least 5 potential allelic donors.

In the Brazilian population (n=116), 99 (85%) had at least one potential donor. Fifty-five of these (47%) had at least one potential allelic donor, of whom 28 (24%) had at least 5 potential allelic donors. The probability of finding at least one potential allelic donor in REDOME was 32%, in NMDP 30%, in European registries 16% and in other registries 9%. In the EBMT cohort, out of 59 patients, 42 (71%) had a potential donor. Twenty-eight patients (47%) had a least one potential allelic donor, and 9 (15%) had 5 or more potential allelic donors. The probability of finding at least one potential allelic donor in REDOME was 22%, in NMDP was 42%, in European registries 24% and 14% in other registries. The differences in the probabilities of finding a potential allelic donor and 5 or more potential allelic donors were not significant between the two groups. Table 2 summarizes donor search probabilities.

Haplotype ethnicity and donor search

Among the 28 Brazilian patients with 5 or more potential allelic donors, 18 patients had at least one common haplotype, 9 patients had an African haplotype, 3 patients a Caucasian haplotype and 4 patients an Amerindian haplotype. Conversely, among the 61 patients with no donors available, 38 patients had at least one African haplotype, 9 patients had one Indian haplotype, 8 patients a Caucasian haplotype and only 6 had a common haplotype.

In the EBMT cohort, among the 9 patients with 5 or more potential allelic donors, 3 had an African haplotype, 3 a Caucasian haplotype, 2 a common haplotype and 2 an Amerindian haplotype. Interestingly, the 2 patients with an Amerindian haplotype had 32 and 12 potential allelic donors in the REDOME database; one had a donor in NMDP and none had donors in other registries. Considering the patients who did not have any MUD (n=31), 23 carried at least one African haplotype; 21 of these patients had two African haplotypes. Two patients had an Amerindian haplotype. The two patients who carried the never described haplotypes were also in the group with no donors. Furthermore, considering the 10 patients who underwent matched unrelated HSCT, 9 had exclusively African haplotypes, however in all these cases the haplotypes were frequently described in the HaploStats database.

Discussion

We showed the differences in allele and haplotype frequencies among two SCD cohorts and the probability of patients with SCD to find an unrelated donor. To date, this is the largest report on HLA frequencies in SCD and the largest unrelated donor search reported in these settings.

The HbS mutation appeared first in Africa and India. The original distribution of the HbS mutation coincides with the main areas affected by malaria, because HbS is protective against infection by the plasmodium parasite (Piel *et al*, 2013; Shelton *et al*, 2015). SCD was originally not seen in Northern European and Amerindian populations (Piel *et al*, 2014). However, because of slavery routes from Africa to America, SCD was brought to the American and European continent.

Brazil received the largest number of African slaves in the American continent from 1501 to 1875 (<http://www.slavevoyages.org/assessment/estimates>), and interethnic relationships have been occurring since then. This is in line with our observations that, despite the African origin of the SCD population, Brazilian patients had almost half of their HLA haplotypes classified as common, Caucasian or Amerindian. Although the first description of SCD dates from 1910, in a patient from the French West Indies (Herrick, 2001), the disease probably arrived in the American continent during the colonial period. A previous study that analysed the HLA haplotypes of donors registered in the REDOME had also shown a great level of admixture in that population, and haplotype frequency was similar among different ethnic groups (Torres *et al*, 2017). Another study that evaluated REDOME donors also pointed a similar frequency of haplotypes among the ethnic groups, and a high allele variability, showing the diversity of the HLA composition of the Brazilian registry (Halagan *et al*, 2018). In addition, a study composed by Brazilian patients with SCD indicated high genetic admixture based on low resolution HLA typing (da Silva-Malta *et al*, 2017).

By contrast, although migratory movements from Africa to Europe have always existed, migration became more intense in the last century. Therefore, ethnic admixture is less marked in the SCD population that was transplanted in EBMT centres, which explains the higher rate of African haplotypes, and lower rate of other ethnicity haplotypes, found in these centres.

Despite the differences in the HLA composition of the two populations, chances of finding an unrelated 6/6 potential allele matched donor in international donor search were the same in both groups. Moreover, the probability of having 5 or more potential allelic donors was worse in the EBMT group, but it was low in both groups. This is worrisome because, for several reasons, potential donors might be unavailable, and most patients with few potential allelic donors end up having no donors at all. Although Brazilian patients showed more Caucasian and common HLA haplotypes, most had at least one African haplotype, and most EBMT patients had 2 African haplotypes.

Donors from African origin are underrepresented in donor registries (Gluckman, 2013; Dew *et al*, 2008; Barker *et al*, 2019). In 2014, a study analysed the probability of finding a matched donor in the NMDP registry, and according to the ethnicity reported that African origin groups (African American, African, Black Central or South American and Black Caribbean) had 16 to 19% of chances of finding an allele matched 8/8 adult donor, the lowest rates among the 21 racial/ethnic groups analysed (Gragert *et al*, 2014). Another study reported that patients with previously undescribed HLA haplotypes, of which non-white subjects were the majority, had 1.2% of probability of finding a MUD among NMDP donors (Olson *et al*, 2017). More recently, Barker *et al* prospectively analysed the access to HLA matched unrelated donor transplants in a racial distinct population of patients with haematological malignancy or aplastic anaemia; in a subgroup of 78 African American patients, only 17 received a 8/8 MUD, 17 a 7/8 unrelated donor, and 46 an unrelated cord blood transplant (Barker *et al*, 2019). In SCD settings, in 2003, it was reported that patients with SCD would have 60% of chances of having one potential 6/6 donor or umbilical cord blood unit in the NMDP registry (Krishnamurti *et al*, 2003). However, another study performing allelic search for 85 patients in NMDP found that 20% of patients had a potential 8/8 MUD (Justus *et al*, 2015), and a Brazilian study with 126 patients with SCD showed that only 8% of the cohort had a 6/6 potential donor (low resolution typing) in a small subset of REDOME donors (da Silva-Malta *et al*, 2017). It is difficult to compare these results, because the level of resolution and the number of loci tested were different. Nevertheless, the probabilities of finding a donor were higher in our study than in previous reports, likely due to the international search, which is more representative of real donor search in the clinical settings than single-registry searches. Although we did not test locus DQB1, this locus would not have greatly influenced our results because of the high linkage disequilibrium described between DRB1 and DQB1 (Begovich *et al*, 1992).

In our study, overall, the highest probability of finding a donor was in the NMDP registry. NMDP is the largest donor registry in the world, with a great diversity of ethnicities represented. As expected, REDOME accounted for most matchings in the Brazilian SCD cohort. Interestingly, 15

patients from Brazil found donors only in REDOME, probably because of the unique genetic admixture of Africans, Caucasians and South American Amerindians that form the Brazilian population. Since 2012, REDOME implemented strategies to improve the representativity of all ethnic groups that compose the Brazilian population (Torres *et al*, 2017). Also, one third of patients found donors in European registries, notably in the German registry, reflecting also the results of strategies to ameliorate ethnic diversity throughout international registries. However, the probability of having a MUD remains low in patients with SCD. In addition, Africans show a high degree of genetic diversity (Campbell & Tishkoff, 2008), and a higher frequency of different HLA haplotypes (Gragert *et al*, 2014), which might hamper representativity. In this population, in the absence of an identical donor, alternative donor sources, such as cord blood and haploidentical HSCT, might be considered.

The main limitation of our study is the size of the cohort, which prevented us from performing further HLA analyses. Also, because it was not possible to estimate alleles and haplotypes from low resolution HLA typing, only patients typed in intermediate or high resolution could be included. However, to date, our study has the largest SCD cohort to have allele donor search for SCD performed in international settings.

Although our results show a higher probability of finding donors for patients with SCD compared with previous reports, the overall probabilities of having at least one allelic donor, and at least five donors, remain low. Further efforts are needed to keep improving donor availability in this population. Furthermore, our study might contribute to establish specific algorithms for donor search in SCD. This is particularly important because performing HSCT at earlier age is associated with better outcomes in SCD (Cappelli *et al*, 2019), and knowing which patients are less likely to find a MUD might influence therapy management, and even indications of new treatments such as gene therapy.

Acknowledgments: this work was supported by the Centre Scientifique de Monaco and by the Brazilian Coordination for the Improvement of Higher Education Personnel (CAPES). The authors thank the Centre Scientifique de Monaco, CAPES, all patients and their families. The authors also thank the Brazilian Bone Marrow Donor Registry, the Paediatric Diseases Working Party of EBMT and the following centres: **Austria** – St. Anna Kinderspital, Vienna; **Belgium** – Children’s University Hospital, Brussels; Cliniques Universitaires St. Luc, Brussels; **Brazil** – Hospital das Clinicas da Faculdade de Medicina de Ribeirão Preto, Ribeirão Preto; **Canada** – Centre Hospitalier Universitaire St Justine, Montréal; University Hospital, Leuven; **England** – St Mary’s Hospital, London; Birmingham Children’s Hospital, Birmingham; **France** - Hôpital Saint Louis, Paris; Hôpital Necker, Paris; Nouvel Hôpital Civil, Strasbourg; Hôpital Jeane de Flandre, Lille; **Germany** – Universitaetsklinikum Muenster, Muenster; Klinikum der Johann-Wolfgang Goethe Universitaet, Frankfurt; **Italy** – Clinica di Oncoematologia Pediatrica, Padova; Azienda Ospedaliero Universitaria, Bologna, Italy; Clinica Pediatrica Università di Milano Bicocca, Monza; Ospedale Infantile Regina Margherita, Torino; Centro Trapianti di Midollo Osseo, Cagliari; Fondazione IRCCS Policlinico San Matteo, Pavia; **Netherlands** – Leiden University Hospital, Leiden, Netherlands; **Spain** - Hospital Vall d’Hebron, Barcelona; **Turkey** – Acibadem University Atakent Hospital, Istanbul.

Authorship statement: KTM, EG, RT, BPS and VR designed the study. KTM, HE, FV, BC and GMS collected clinical and HLA data. ACSP, RC, ESR, SK, PL, JHV, AF and SC provided cases and DNA. NHSG performed experiments. JFC and DO performed donor search. KTM and MAT performed analyses. KTM wrote the manuscript. FV, AR, VR, BPS, RT, JFC, DO, MAT and EG revised the manuscript. All authors edited and approved the manuscript.

Competing interests: the authors have no competing interests.

References

Ameen, R., Shemmari, S.A. & Askar, M. (2018) Next-generation sequencing characterization of HLA in multi-generation families of Kuwaiti descent. *Human Immunology*, **79**, 137–142.

Angelucci, E., Matthes-Martin, S., Baronciani, D., Bernaudin, F., Bonanomi, S., Cappellini, M.D., Dalle, J.-H., Di Bartolomeo, P., de Heredia, C.D., Dickerhoff, R., Giardini, C., Gluckman, E., Hussein, A.A., Kamani, N., Minkov, M., Locatelli, F., Rocha, V., Sedlacek, P., Smiers, F., Thuret, I., et al (2014) Hematopoietic stem cell transplantation in thalassemia major and sickle cell disease: indications and management recommendations from an international expert panel. *Haematologica*, **99**, 811–820.

Applied Statistical Genetics with R - For Population-based Association Studies | Andrea S. Foulkes | Springer Available at: <https://www.springer.com/gp/book/9780387895536> [Accessed April 16, 2019].

Arnold, S.D., Bhatia, M., Horan, J. & Krishnamurti, L. (2016) Haematopoietic stem cell transplantation for sickle cell disease - current practice and new approaches. *British Journal of Haematology*, **174**, 515–525.

Barker, J.N., Boughan, K., Dahi, P.B., Devlin, S.M., Maloy, M.A., Naputo, K., Mazis, C.M., Davis, E., Nhaissi, M., Wells, D., Cooper, C., Ponce, D.M., Kernan, N., Scaradavou, A., Giralt, S.A., Papadopoulos, E.B. & Politikos, I. (2019) Racial disparities in access to HLA-matched unrelated donor transplants: a prospective 1312-patient analysis. *Blood Advances*, **3**, 939–944.

Begovich, A.B., McClure, G.R., Suraj, V.C., Helmuth, R.C., Fildes, N., Bugawan, T.L., Erlich, H.A. & Klitz, W. (1992) Polymorphism, recombination, and linkage disequilibrium within the HLA class II region. *Journal of Immunology (Baltimore, Md.: 1950)*, **148**, 249–258.

Bernaudin, F., Socie, G., Kuentz, M., Chevret, S., Duval, M., Bertrand, Y., Vannier, J.-P., Yakouben, K., Thuret, I., Bordigoni, P., Fischer, A., Lutz, P., Stephan, J.-L., Dhedin, N., Plouvier, E., Margueritte, G., Bories, D., Verlhac, S., Esperou, H., Coic, L., et al (2007) Long-term results of related myeloablative stem-cell transplantation to cure sickle cell disease. *Blood*, **110**, 2749–2756.

Campbell, M.C. & Tishkoff, S.A. (2008) African genetic diversity: implications for human demographic history, modern human origins, and complex disease mapping. *Annual Review of Genomics and Human Genetics*, **9**, 403–433.

Cappelli, B., Scigliuolo, G.M., Volt, F., Corbacioglu, S., Fuente, J. de la, Dhedin, N., Pondarré, C., Foell, J., Boyle, F.O., Lankester, A., Calore, E., Varotto, S., Zecca, M., Al-Seraihy, A., Aljurf, M., Fasth, A., Bonanomi, S., Addari, M.C., Locatelli, F., Ferster, A., et al (2018) Alternative Donor Hematopoietic Stem Cell Transplantation for Sickle Cell Disease in Europe. *Blood*, **132**, 4645–4645.

Cappelli, B., Volt, F., Tozatto-Maio, K., Scigliuolo, G.M., Ferster, A., Dupont, S., Pinto Simões, B., Al-Seraihy, A., Aljurf, M.D., Almohareb, F., Belendez, C., Matthes, S., Dhedin, N., Pondarre, C., Dalle, J.-H., Bertrand, Y., Vannier, J.P., Kuentz, M., Lutz, P., Michel, G., et al (2019) Risk factors and outcomes according to age at transplantation with an HLA-identical sibling for sickle cell disease. *Haematologica*.

Dew, A., Collins, D., Artz, A., Rich, E., Stock, W., Swanson, K. & van Besien, K. (2008) Paucity of HLA-identical unrelated donors for African-Americans with hematologic malignancies: the need for new donor options. *Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation*, **14**, 938–941.

- Gluckman, E. (2013) Allogeneic transplantation strategies including haploidentical transplantation in sickle cell disease. *Hematology. American Society of Hematology. Education Program*, **2013**, 370–376.
- Gluckman, E., Cappelli, B., Bernaudin, F., Labopin, M., Volt, F., Carreras, J., Pinto Simões, B., Ferster, A., Dupont, S., de la Fuente, J., Dalle, J.-H., Zecca, M., Walters, M.C., Krishnamurti, L., Bhatia, M., Leung, K., Yanik, G., Kurtzberg, J., Dhedin, N., Kuentz, M., et al (2017) Sickle cell disease: an international survey of results of HLA-identical sibling hematopoietic stem cell transplantation. *Blood*, **129**, 1548–1556.
- Gragert, L., Eapen, M., Williams, E., Freeman, J., Spellman, S., Baitty, R., Hartzman, R., Rizzo, J.D., Horowitz, M., Confer, D. & Maers, M. (2014) HLA match likelihoods for hematopoietic stem-cell grafts in the U.S. registry. *The New England Journal of Medicine*, **371**, 339–348.
- Halagan, M., Oliveira, D.C., Maers, M., Fabreti-Oliveira, R.A., Moraes, M.E.H., Visentainer, J.E.L., Pereira, N.F., Romero, M., Cardoso, J.F. & Porto, L.C. (2018) The distribution of HLA haplotypes in the ethnic groups that make up the Brazilian Bone Marrow Volunteer Donor Registry (REDOME). *Immunogenetics*, **70**, 511–522.
- Herrick, J.B. (2001) Peculiar elongated and sickle-shaped red blood corpuscles in a case of severe anemia. 1910. *The Yale Journal of Biology and Medicine*, **74**, 179–184.
- Justus, D., Perez-Albuern, E., Dioguardi, J., Jacobsohn, D. & Abraham, A. (2015) Allogeneic donor availability for hematopoietic stem cell transplantation in children with sickle cell disease. *Pediatric Blood & Cancer*, **62**, 1285–1287.
- Kollman, C., Maers, M., Gragert, L., Müller, C., Setterholm, M., Oudshoorn, M. & Hurley, C.K. (2007) Estimation of HLA-A, -B, -DRB1 haplotype frequencies using mixed resolution data from a National Registry with selective retyping of volunteers. *Human Immunology*, **68**, 950–958.
- Krishnamurti, L., Abel, S., Maers, M. & Flesch, S. (2003) Availability of unrelated donors for hematopoietic stem cell transplantation for hemoglobinopathies. *Bone Marrow Transplantation*, **31**, 547–550.
- Lancaster, A.K., Single, R.M., Solberg, O.D., Nelson, M.P. & Thomson, G. (2007) PyPop update--a software pipeline for large-scale multilocus population genomics. *Tissue Antigens*, **69 Suppl 1**, 192–197.
- Maers, M., Gragert, L. & Klitz, W. (2007) High-resolution HLA alleles and haplotypes in the United States population. *Human Immunology*, **68**, 779–788.
- Olson, J.A., Gibbens, Y., Tram, K., Kempenich, J., Novakovich, J., Buck, K. & Dehn, J. (2017) Identification of a 10/10 matched donor for patients with an uncommon haplotype is unlikely. *HLA*, **89**, 77–81.
- Oteng-Ntim, E., Meeks, D., Seed, P.T., Webster, L., Howard, J., Doyle, P. & Chappell, L.C. (2015) Adverse maternal and perinatal outcomes in pregnant women with sickle cell disease: systematic review and meta-analysis. *Blood*, **125**, 3316–3325.
- Piel, F.B. (2013) [Sickle-cell disease: geographical distribution and population estimates]. *Medecine Sciences: M/S*, **29**, 965–967.

Piel, F.B., Patil, A.P., Howes, R.E., Nyangiri, O.A., Gething, P.W., Dewi, M., Temperley, W.H., Williams, T.N., Weatherall, D.J. & Hay, S.I. (2013) Global epidemiology of sickle haemoglobin in neonates: a contemporary geostatistical model-based map and population estimates. *Lancet (London, England)*, **381**, 142–151.

Piel, F.B., Steinberg, M.H. & Rees, D.C. (2017) Sickle Cell Disease. *The New England Journal of Medicine*, **376**, 1561–1573.

Piel, F.B., Tatem, A.J., Huang, Z., Gupta, S., Williams, T.N. & Weatherall, D.J. (2014) Global migration and the changing distribution of sickle haemoglobin: a quantitative study of temporal trends between 1960 and 2000. *The Lancet. Global Health*, **2**, e80-89.

Shelton, J.M.G., Corran, P., Risley, P., Silva, N., Hubbart, C., Jeffreys, A., Rowlands, K., Craik, R., Cornelius, V., Hensmann, M., Molloy, S., Sepulveda, N., Clark, T.G., Band, G., Clarke, G.M., Spencer, C.C.A., Kerasidou, A., Campino, S., Auburn, S., Tall, A., et al (2015) Genetic determinants of anti-malarial acquired immunity in a large multi-centre study. *Malaria Journal*, **14**, 333.

Shenoy, S., Eapen, M., Panepinto, J.A., Logan, B.R., Wu, J., Abraham, A., Brochstein, J., Chaudhury, S., Godder, K., Haight, A.E., Kasow, K.A., Leung, K., Andreansky, M., Bhatia, M., Dalal, J., Haines, H., Jaroscak, J., Lazarus, H.M., Levine, J.E., Krishnamurti, L., et al (2016) A trial of unrelated donor marrow transplantation for children with severe sickle cell disease. *Blood*, **128**, 2561–2567.

da Silva-Malta, M.C.F., Rodrigues, P.S., Zuccherato, L.W., de Souza, F.C.B., Domingues, E.M.F.L., Souza, V.R., Tarazona-Santos, E. & Martins, M.L. (2017) Human leukocyte antigen distribution and genomic ancestry in Brazilian patients with sickle cell disease. *HLA*, **90**, 211–218.

Spellman, S.R., Eapen, M., Logan, B.R., Mueller, C., Rubinstein, P., Setterholm, M.I., Woolfrey, A.E., Horowitz, M.M., Confer, D.L., Hurley, C.K., National Marrow Donor Program & Center for International Blood and Marrow Transplant Research (2012) A perspective on the selection of unrelated donors and cord blood units for transplantation. *Blood*, **120**, 259–265.

Stuart, M.J. & Nagel, R.L. (2004) Sickle-cell disease. *Lancet (London, England)*, **364**, 1343–1360.

Torres, L., da Silva Bouzas, L.F., Almada, A., de Sobrino Porto, L.C.M. & Abdelhay, E. (2017) Distribution of HLA-A, -B and -DRB1 antigenic groups and haplotypes from the Brazilian bone marrow donor registry (REDOME). *Human Immunology*, **78**, 602–609.

Yawn, B.P., Buchanan, G.R., Afenyi-Annan, A.N., Ballas, S.K., Hassell, K.L., James, A.H., Jordan, L., Lanzkron, S.M., Lottenberg, R., Savage, W.J., Tanabe, P.J., Ware, R.E., Murad, M.H., Goldsmith, J.C., Ortiz, E., Fulwood, R., Horton, A. & John-Sowah, J. (2014) Management of sickle cell disease: summary of the 2014 evidence-based report by expert panel members. *JAMA*, **312**, 1033–1048.

Table I. Frequency of the most common HLA alleles in the whole cohort, Brazilian population and EBMT population compared with the AFA population described on NMDP registry

HLA*A	02:01	03:01	01:01	30:01	30:02	23:01	68:02	24:02
Overall frequency(n=364)	16%	9%	8%	7%	5%	7%	5%	4%
Brazilian patients (n=226)	20%	11%	9%	6%	6%	5%	4%	3%
EBMT patients (n=138)	11%	6%	5%	8%	4%	11%	7%	7%
AFA ranking (frequency)	1 (12%)	3 (8%)	8 (5%)	4 (7%)	6 (6%)	2 (11%)	5 (7%)	15 (2%)
HLA*B	53:01	35:01	08:01	42:01	51:01	07:02	58:01	44:03
Overall (n=362)	7%	6%	5%	5%	3%	4%	4%	4%
Brazilian patients (n=224)	7%	7%	5%	5%	5%	5%	3%	4%
EBMT patients (n=138)	7%	5%	4%	8%	4%	3%	6%	4%
AFA ranking (frequency,%)	1 (11%)	3 (6%)	9 (4%)	5 (5%)	16 (2%)	2 (7%)	11 (4%)	6 (5%)
HLA*DRB1	07:01	15:03	11:01	03:01	13:01	13:02	11:02	01:02
Overall (n=361)	12%	9%	9%	8%	5%	6%	4%	4%
Brazilian patients (n=225)	14%	8%	8%	9%	6%	5%	4%	3%
EBMT patients (n=136)	10%	13%	10%	7%	4%	8%	5%	4%
AFA ranking (frequency,%)	2 (10%)	1 (12%)	3 (9%)	4 (7%)	7 (5%)	6 (6%)	11 (4%)	9 (4%)

Abbreviations: AFA: African American; EBMT, European Society for Blood and Marrow Transplantation; HLA, human leukocyte antigen; NMDP: National Marrow Donor Program.

Table II. Probability of finding a donor in international donor search, overall and by population

	Overall	Brazilian patients (n=116)	EBMT patients (n=59)	P-value
Potential donor (%)	138 (79)	99 (85)	42 (71)	
Allelic donor (%)	88 (50)	55 (47)	28 (47)	NS
≥ 5 allelic donors (%)	37 (21)	28 (24)	9 (15)	NS

Abbreviations: EBMT, European Society for Blood and Marrow Transplantation.

Figure I. Frequency of haplotypes by ethnicity in each population.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

6 Conclusions and future perspectives

6.1 Conclusions

In the first paper, we demonstrated that the heterozygous genotype of a SNP in the *TLR2* gene was associated with less occurrence of bacterial infection in patients with SCD, and that a homozygous genotype of the same SNP occurred more in infected patients. Previous reports had already demonstrated how alleles and genotypes in this SNP can alter the expression of inflammatory markers, incidence and severity of inflammatory diseases. We also demonstrated a difference between allele and haplotype frequency in our population compared with the African population described in the 1000 genomes database.

TLR2 is a key receptor that recognizes a wide range of pathogens, playing a pivotal role in innate immune response against infections. Patients with SCD are more susceptible to bacterial infections, which are a leading cause of mortality in SCD settings, and this susceptibility still needs to be better understood. Our study points out a modulatory role of a SNP in the *TLR2* gene in a potentially severe complication in patients with SCD. In addition, the difference of genotype frequencies compared with a non-SCD population of African origin described in the 1000 genomes database might indicate a positive pressure selection shaped by infections in the SCD population for this SNP. Currently, penicillin prophylaxis in SCD is recommended until the age of 5 years. Predicting which patients are at a greater risk of bacterial infections might help tailoring prophylaxis recommendations, clinical decisions concerning antimicrobial therapy and even, when HSCT is indicated, infection management post HSCT. Our study also might give the basis for prospective evaluations to better establish a cause-effect association between this SNP and protection or susceptibility against bacteria.

In the second paper, we performed international donor searches to find a HLA 6/6 matched unrelated potential allelic donor for patients with SCD from different geographical origins. We demonstrated that, despite the differences in HLA ethnicity among the Brazilian cohort and the SCD cohort, both groups had the same chances of finding at least one potential allelic MUD in international registries, and that although Brazilian patients were more likely to have at least 5 donors, the difference was not statistically significant.

Identical related HSCT is well established for SCD, however, because a limited number of patients will have a matched sibling donor, MUD HSCT in SCD settings is expected to improve over the next years. Patients with SCD are mostly from African origin, and several studies had previously demonstrated that individuals from African origin are less likely to find

a MUD in donor registries. Although we reported higher probabilities of finding a potential allelic MUD in international registries than previously described, the low representativity of donors from African origin remains worrisome. Therefore, our study outlines the importance of implementing strategies to improve donor availability for patients from African origin. In addition, because earlier age is associated with better outcomes after HSCT for SCD, early referral to HSCT centers is important. For patients who lack an HLA matched sibling donor, predicting the odds of having a MUD might help clinicians with therapeutic decisions and patient management. Furthermore, our findings contribute to justify the need for developing alternative HSCT sources, such as haploidentical HSCT, unrelated cord blood HSCT, and more recently, gene therapy. Finally, although the size of our cohort did not allow further HLA analyses, since it was previously reported that some HLA alleles and haplotypes were associated with SCD complications, our study also might give the basis for evaluating the influence of HLA alleles and haplotypes in SCD heterogeneity.

We showed that a polymorphism in an inflammatory gene might modulate the severity of SCD complications, and that although the probability of finding a MUD for SCD patients increased lately, further strategies are needed to improve donor availability. Importantly, knowing the genetic profile of patients might contribute to help therapeutic decisions in SCD settings.

6.2 Future perspectives

We intend to continue this work. Our next objectives are to evaluate the effect of SNPs in other inflammatory genes on clinical complications of SCD, to better describe HLA allele and haplotype frequency in SCD patients using a larger cohort, to further evaluate their probability of finding a MUD, and to analyse whether HLA alleles or haplotypes influence SCD complications and HSCT outcomes.

For the first objective, we will perform genotyping of SNPs in genes encoding natural killer (NK) cell receptors, CTLA-4, major histocompatibility class I polypeptide-related sequence A (MICA) and the non-classical HLA loci HLA-E and HLA-G. We will analyse the following clinical complications: stroke, acute chest syndrome, chronic leg ulcers, osteonecrosis and alloimmunisation. We will describe genotype frequencies for the SNPs and haplotype frequencies for the gene clusters; perform analyses of association with the clinical complications described; and compare genotype and haplotype frequencies with the population from African origin described in the 1000 genomes database.

For the second objective, because we need to increase the cohort, we will perform HLA genotyping of the non-transplanted patients for whom we have DNA samples available (the cohort described in the first paper). We will also include additional transplanted and non-transplanted patients followed at the two Brazilian centres, and transplanted patients reported to EBMT with HLA data available. We will describe allele and haplotype frequencies of the SCD cohort, perform comparisons between different populations and with the population from African origin described in the NMDP database.

For further estimations of MUD search, we will perform international searches in adult donors and cord blood registries and describe the probability for the overall cohort and for groups divided by geographic origin. We hypothesize that increasing the cohort might increase the probabilities of finding a MUD. Furthermore, we are also checking with REDOME how we can help improving representativity of donors from African origin.

For analysing the effect of classical HLA on clinical and HSCT outcomes, we will collect clinical and HSCT data for all new patients included in the cohort. We will analyse the association between classical HLA haplotypes and the clinical complications mentioned above. For HSCT complications, endpoints will be occurrence of aGvHD, cGvHD, graft failure, infections and transplant related mortality. If we find associations between HLA and adverse outcomes, knowing which patients are at a greater risk of such adverse events might help to decide if it is worth to perform HSCT.

7. REFERENCES

7 References

- ADEKILE, A. et al. HLA-DRB1 Alleles in Hb SS Patients with Avascular Necrosis of the Femoral Head. **American Journal of Hematology**, v. 79, n. 1, p. 8–10, maio 2005.
- AKINSHEYI, I. et al. Fetal Hemoglobin in Sickle Cell Anemia. **Blood**, v. 118, n. 1, p. 19–27, 7 jul. 2011.
- AKIRA, S.; UEMATSU, S.; TAKEUCHI, O. Pathogen Recognition and Innate Immunity. **Cell**, v. 124, n. 4, p. 783–801, 24 fev. 2006.
- AL-OLA, K. et al. Evidence for HLA Class II Susceptible and Protective Haplotypes for Osteomyelitis in Pediatric Patients with Sickle Cell Anemia. **Tissue Antigens**, v. 71, n. 5, p. 453–457, maio 2008.
- ANGELUCCI, E. et al. Hematopoietic Stem Cell Transplantation in Thalassemia Major and Sickle Cell Disease: Indications and Management Recommendations from an International Expert Panel. **Haematologica**, v. 99, n. 5, p. 811–820, maio 2014.
- ANYAEGBU, C. C. et al. Peripheral Blood Neutrophil Count and Candidacidal Activity Correlate with the Clinical Severity of Sickle Cell Anaemia (SCA). **European Journal of Haematology**, v. 60, n. 4, p. 267–268, abr. 1998.
- ARNOLD, S. D. et al. Haematopoietic Stem Cell Transplantation for Sickle Cell Disease - Current Practice and New Approaches. **British Journal of Haematology**, v. 174, n. 4, p. 515–525, 2016.
- BARKER, J. N. et al. Racial Disparities in Access to HLA-Matched Unrelated Donor Transplants: A Prospective 1312-Patient Analysis. **Blood Advances**, v. 3, n. 7, p. 939–944, 9 abr. 2019.
- BELCHER, J. D. et al. Activated Monocytes in Sickle Cell Disease: Potential Role in the Activation of Vascular Endothelium and Vaso-Occlusion. **Blood**, v. 96, n. 7, p. 2451–2459, 1 out. 2000.
- BELCHER, J. D. et al. Heme Triggers TLR4 Signaling Leading to Endothelial Cell Activation and Vaso-Occlusion in Murine Sickle Cell Disease. **Blood**, v. 123, n. 3, p. 377–390, 16 jan. 2014.
- BERNAUDIN, F. et al. Long-Term Results of Related Myeloablative Stem-Cell Transplantation to Cure Sickle Cell Disease. **Blood**, v. 110, n. 7, p. 2749–2756, 1 out. 2007.
- BRITTAIN, H. A. et al. Thrombospondin from Activated Platelets Promotes Sickle Erythrocyte Adherence to Human Microvascular Endothelium under Physiologic Flow: A Potential Role for Platelet Activation in Sickle Cell Vaso-Occlusion. **Blood**, v. 81, n. 8, p. 2137–2143, 15 abr. 1993.
- BRITTAIN, J. E. et al. Integrin-Associated Protein Is an Adhesion Receptor on Sickle Red Blood Cells for Immobilized Thrombospondin. **Blood**, v. 97, n. 7, p. 2159–2164, 1 abr. 2001.
- CAPPELLI, B. et al. Alternative Donor Hematopoietic Stem Cell Transplantation for Sickle Cell Disease in Europe. **Blood**, v. 132, n. Suppl 1, p. 4645–4645, 21 nov. 2018.

CAPPELLI, B. et al. Risk Factors and Outcomes According to Age at Transplantation with an HLA-Identical Sibling for Sickle Cell Disease. **Haematologica**, 24 abr. 2019.

CASTRO, O. et al. The Acute Chest Syndrome in Sickle Cell Disease: Incidence and Risk Factors. The Cooperative Study of Sickle Cell Disease. **Blood**, v. 84, n. 2, p. 643–649, 15 jul. 1994.

CHANG, Y. C. et al. An Analysis of Fetal Hemoglobin Variation in Sickle Cell Disease: The Relative Contributions of the X-Linked Factor, Beta-Globin Haplotypes, Alpha-Globin Gene Number, Gender, and Age. **Blood**, v. 85, n. 4, p. 1111–1117, 15 fev. 1995.

CHARACHE, S. et al. Effect of Hydroxyurea on the Frequency of Painful Crises in Sickle Cell Anemia. Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia. **The New England Journal of Medicine**, v. 332, n. 20, p. 1317–1322, 18 maio 1995.

CONRAN, N.; BELCHER, J. D. Inflammation in Sickle Cell Disease. **Clinical Hemorheology and Microcirculation**, v. 68, n. 2–3, p. 263–299, 2018.

CORDERO, E. a. A. et al. HLA-G Polymorphism Influences the Susceptibility to HCV Infection in Sickle Cell Disease Patients. **Tissue Antigens**, v. 74, n. 4, p. 308–313, out. 2009.

CROIZAT, H. Circulating Cytokines in Sickle Cell Patients during Steady State. **British Journal of Haematology**, v. 87, n. 3, p. 592–597, jul. 1994.

DAVID, S. et al. Variants in the Non-Coding Region of the TLR2 Gene Associated with Infectious Subphenotypes in Pediatric Sickle Cell Anemia. **Immunogenetics**, v. 70, n. 1, p. 37–51, 2018.

DE CEULAER, K. et al. Alpha-Thalassemia Reduces the Hemolytic Rate in Homozygous Sickle-Cell Disease. **The New England Journal of Medicine**, v. 309, n. 3, p. 189–190, 21 jul. 1983.

DE LA FUENTE, J. et al. Haploidentical Bone Marrow Transplantation with Post-Transplantation Cyclophosphamide Plus Thiotepa Improves Donor Engraftment in Patients with Sickle Cell Anemia: Results of an International Learning Collaborative. **Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation**, 28 nov. 2018.

DEW, A. et al. Paucity of HLA-Identical Unrelated Donors for African-Americans with Hematologic Malignancies: The Need for New Donor Options. **Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation**, v. 14, n. 8, p. 938–941, ago. 2008.

DOSSOU-YOVO, O. P. et al. Effects of RANTES and MBL2 Gene Polymorphisms in Sickle Cell Disease Clinical Outcomes: Association of the g.In1.1T>C RANTES Variant with Protection against Infections. **American Journal of Hematology**, v. 84, n. 6, p. 378–380, jun. 2009.

DYKES, G.; CREPEAU, R. H.; EDELSTEIN, S. J. Three-Dimensional Reconstruction of the Fibres of Sickle Cell Haemoglobin. **Nature**, v. 272, n. 5653, p. 506, abr. 1978.

EATON, W. A.; HOFRICHTER, J. Hemoglobin S Gelation and Sick Cell Disease. **Blood**, v. 70, n. 5, p. 1245–1266, nov. 1987.

ELLIOTT, L. et al. Genetic Polymorphisms Associated with Priapism in Sick Cell Disease. **British Journal of Haematology**, v. 137, n. 3, p. 262–267, maio 2007.

FELIX AA; SOUZA HM; RIBEIRO SBF. Aspectos epidemiológicos e sociais da doença falciforme. *Rev Bras Hematol Hemoter.* 2010;32(3):203-208. [s.d.]

FIGUEIREDO, R. T. et al. Characterization of Heme as Activator of Toll-like Receptor 4. **The Journal of Biological Chemistry**, v. 282, n. 28, p. 20221–20229, 13 jul. 2007.

FITZHUGH, C. D. et al. At Least 20% Donor Myeloid Chimerism Is Necessary to Reverse the Sick Phenotype after Allogeneic HSCT. **Blood**, v. 130, n. 17, p. 1946–1948, 26 2017.

FRANCIS, R. B.; HAYWOOD, L. J. Elevated immunoreactive tumor necrosis factor and interleukin-1 in sick cell disease. **Journal of the National Medical Association**, v. 84, n. 7, p. 611–615, jul. 1992.

GEE, B. E.; PLATT, O. S. Sick Reticulocytes Adhere to VCAM-1. **Blood**, v. 85, n. 1, p. 268–274, 1 jan. 1995.

GLUCKMAN, E. Allogeneic Transplantation Strategies Including Haploidentical Transplantation in Sick Cell Disease. **Hematology. American Society of Hematology. Education Program**, v. 2013, p. 370–376, 2013.

GLUCKMAN, E. et al. Sick Cell Disease: An International Survey of Results of HLA-Identical Sibling Hematopoietic Stem Cell Transplantation. **Blood**, v. 129, n. 11, p. 1548–1556, 16 2017.

GODEFROY, E. et al. Altered Heme-Mediated Modulation of Dendritic Cell Function in Sick Cell Alloimmunization. **Haematologica**, v. 101, n. 9, p. 1028–1038, 2016.

GRAÇA-SOUZA, A. V. et al. Neutrophil Activation by Heme: Implications for Inflammatory Processes. **Blood**, v. 99, n. 11, p. 4160–4165, 1 jun. 2002.

GRAGERT, L. et al. HLA Match Likelihoods for Hematopoietic Stem-Cell Grafts in the U.S. Registry. **The New England Journal of Medicine**, v. 371, n. 4, p. 339–348, 24 jul. 2014.

GRAHAM, G. S. A CASE OF SICKLE CELL ANEMIA WITH NECROPSY. **Archives of Internal Medicine**, v. 34, n. 6, p. 778–800, 1 dez. 1924.

GRAIDO-GONZALEZ, E. et al. Plasma Endothelin-1, Cytokine, and Prostaglandin E2 Levels in Sick Cell Disease and Acute Vaso-Occlusive Sick Crisis. **Blood**, v. 92, n. 7, p. 2551–2555, 1 out. 1998.

GREEN, N. S. et al. Senegal Haplotype Is Associated with Higher HbF than Benin and Cameroon Haplotypes in African Children with Sick Cell Anemia. **American Journal of Hematology**, v. 44, n. 2, p. 145–146, out. 1993.

GROSSE, S. D. et al. Sick Cell Disease in Africa: A Neglected Cause of Early Childhood Mortality. **American Journal of Preventive Medicine**, v. 41, n. 6 Suppl 4, p. S398-405, dez. 2011.

HARRIS, J. W. Studies on the Destruction of Red Blood Cells. VIII. Molecular Orientation in Sick Cell Hemoglobin Solutions. **Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)**, v. 75, n. 1, p. 197–201, out. 1950.

HASSELL, K. L. Population Estimates of Sick Cell Disease in the U.S. **American Journal of Preventive Medicine**, v. 38, n. 4 Suppl, p. S512-521, abr. 2010.

HERRICK, J. B. Peculiar Elongated and Sick-Cell Shaped Red Blood Corpuscles in a Case of Severe Anemia. 1910. **The Yale Journal of Biology and Medicine**, v. 74, n. 3, p. 179–184, jun. 2001.

HOFRICHTER, J.; ROSS, P. D.; EATON, W. A. Kinetics and Mechanism of Deoxyhemoglobin S Gelation: A New Approach to Understanding Sick Cell Disease. **Proceedings of the National Academy of Sciences of the United States of America**, v. 71, n. 12, p. 4864–4868, dez. 1974.

HOPPE, C. et al. Distinct HLA Associations by Stroke Subtype in Children with Sick Cell Anemia. **Blood**, v. 101, n. 7, p. 2865–2869, 1 abr. 2003.

HOPPE, C. et al. Gene Interactions and Stroke Risk in Children with Sick Cell Anemia. **Blood**, v. 103, n. 6, p. 2391–2396, 15 mar. 2004.

HOPPE, C. et al. Confirmation of an Association between the TNF(-308) Promoter Polymorphism and Stroke Risk in Children with Sick Cell Anemia. **Stroke**, v. 38, n. 8, p. 2241–2246, ago. 2007.

HSIEH, M. M. et al. Allogeneic Hematopoietic Stem-Cell Transplantation for Sick Cell Disease. **The New England Journal of Medicine**, v. 361, n. 24, p. 2309–2317, 10 dez. 2009.

IGNARRO, L. J. et al. Endothelium-Derived Relaxing Factor from Pulmonary Artery and Vein Possesses Pharmacologic and Chemical Properties Identical to Those of Nitric Oxide Radical. **Circulation Research**, v. 61, n. 6, p. 866–879, dez. 1987.

JOHNSON, F. L. et al. Bone-Marrow Transplantation in a Patient with Sick-Cell Anemia. **The New England Journal of Medicine**, v. 311, n. 12, p. 780–783, 20 set. 1984.

JUSTUS, D. et al. Allogeneic Donor Availability for Hematopoietic Stem Cell Transplantation in Children with Sick Cell Disease. **Pediatric Blood & Cancer**, v. 62, n. 7, p. 1285–1287, jul. 2015.

KAMANI, N. R. et al. Unrelated Donor Cord Blood Transplantation for Children with Severe Sick Cell Disease: Results of One Cohort from the Phase II Study from the Blood and Marrow Transplant Clinical Trials Network (BMT CTN). **Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation**, v. 18, n. 8, p. 1265–1272, ago. 2012.

- KATO, G. J.; STEINBERG, M. H.; GLADWIN, M. T. Intravascular Hemolysis and the Pathophysiology of Sickle Cell Disease. **The Journal of Clinical Investigation**, v. 127, n. 3, p. 750–760, 1 mar. 2017.
- KAUL, D. K.; HEBBEL, R. P. Hypoxia/Reoxygenation Causes Inflammatory Response in Transgenic Sickle Mice but Not in Normal Mice. **The Journal of Clinical Investigation**, v. 106, n. 3, p. 411–420, ago. 2000.
- KINNEY, T. R. et al. Silent Cerebral Infarcts in Sickle Cell Anemia: A Risk Factor Analysis. The Cooperative Study of Sickle Cell Disease. **Pediatrics**, v. 103, n. 3, p. 640–645, mar. 1999.
- KRISHNAMURTI, L. et al. Availability of Unrelated Donors for Hematopoietic Stem Cell Transplantation for Hemoglobinopathies. **Bone Marrow Transplantation**, v. 31, n. 7, p. 547–550, abr. 2003.
- LABIE, D. et al. Common haplotype dependency of high G gamma-globin gene expression and high Hb F levels in beta-thalassemia and sickle cell anemia patients. **Proceedings of the National Academy of Sciences of the United States of America**, v. 82, n. 7, p. 2111–2114, abr. 1985.
- LANARO, C. et al. Altered Levels of Cytokines and Inflammatory Mediators in Plasma and Leukocytes of Sickle Cell Anemia Patients and Effects of Hydroxyurea Therapy. **Journal of Leukocyte Biology**, v. 85, n. 2, p. 235–242, fev. 2009.
- LAPOUMÉROULIE, C. et al. A Novel Sickle Cell Mutation of yet Another Origin in Africa: The Cameroon Type. **Human Genetics**, v. 89, n. 3, p. 333–337, maio 1992.
- LETTRE, G. et al. DNA Polymorphisms at the BCL11A, HBS1L-MYB, and Beta-Globin Loci Associate with Fetal Hemoglobin Levels and Pain Crises in Sickle Cell Disease. **Proceedings of the National Academy of Sciences of the United States of America**, v. 105, n. 33, p. 11869–11874, 19 ago. 2008.
- MALAVÉ, I. et al. Levels of Tumor Necrosis Factor Alpha/Cachectin (TNF Alpha) in Sera from Patients with Sickle Cell Disease. **Acta Haematologica**, v. 90, n. 4, p. 172–176, 1993.
- MARTINEZ-CASTALDI, C. et al. Association of Genetic Polymorphisms in the TGF- β Pathway with the Acute Chest Syndrome of Sickle Cell Anemia. **Blood**, v. 110, n. 11, p. 2247–2247, 16 nov. 2007.
- MATSUI, N. M. et al. P-Selectin Mediates the Adhesion of Sickle Erythrocytes to the Endothelium. **Blood**, v. 98, n. 6, p. 1955–1962, 15 set. 2001.
- MENTZER, W. C. et al. Availability of Related Donors for Bone Marrow Transplantation in Sickle Cell Anemia. **The American Journal of Pediatric Hematology/Oncology**, v. 16, n. 1, p. 27–29, fev. 1994.
- NAGEL, R. L. et al. Structural bases of the inhibitory effects of hemoglobin F and hemoglobin A2 on the polymerization of hemoglobin S. **Proceedings of the National Academy of Sciences of the United States of America**, v. 76, n. 2, p. 670–672, fev. 1979.

NAGEL, R. L. et al. Hematologically and Genetically Distinct Forms of Sickle Cell Anemia in Africa. The Senegal Type and the Benin Type. **The New England Journal of Medicine**, v. 312, n. 14, p. 880–884, 4 abr. 1985.

NAGEL, R. L. et al. The Hematologic Characteristics of Sickle Cell Anemia Bearing the Bantu Haplotype: The Relationship between G Gamma and HbF Level. **Blood**, v. 69, n. 4, p. 1026–1030, abr. 1987.

NEEL, J. V. The Inheritance of Sickle Cell Anemia. **Science (New York, N.Y.)**, v. 110, n. 2846, p. 64–66, 15 jul. 1949.

NEONATO, M. G. et al. Genetic Polymorphism of the Mannose-Binding Protein Gene in Children with Sickle Cell Disease: Identification of Three New Variant Alleles and Relationship to Infections. **European journal of human genetics: EJHG**, v. 7, n. 6, p. 679–686, set. 1999.

NORRIS, C. F. et al. Relationship between Fc Receptor IIA Polymorphism and Infection in Children with Sickle Cell Disease. **The Journal of Pediatrics**, v. 128, n. 6, p. 813–819, jun. 1996.

NTOUFA, S. et al. Toll-like Receptors Signaling: A Complex Network for NF-KB Activation in B-Cell Lymphoid Malignancies. **Seminars in Cancer Biology**, v. 39, p. 15–25, 2016.

OHENE-FREMPPONG, K. et al. Cerebrovascular Accidents in Sickle Cell Disease: Rates and Risk Factors. **Blood**, v. 91, n. 1, p. 288–294, 1 jan. 1998.

OJODU, J. et al. Incidence of Sickle Cell Trait--United States, 2010. **MMWR. Morbidity and mortality weekly report**, v. 63, n. 49, p. 1155–1158, 12 dez. 2014.

OLIVEIRA, J. et al. Combined Effect of TLR2 Gene Polymorphism and Early Life Stress on the Age at Onset of Bipolar Disorders. **PloS One**, v. 10, n. 3, p. e0119702, 2015.

OLIVEIRA, V. B. et al. -318C/T Polymorphism of the CTLA-4 Gene Is an Independent Risk Factor for RBC Alloimmunization among Sickle Cell Disease Patients. **International Journal of Immunogenetics**, v. 44, n. 5, p. 219–224, out. 2017.

OTENG-NTIM, E. et al. Adverse Maternal and Perinatal Outcomes in Pregnant Women with Sickle Cell Disease: Systematic Review and Meta-Analysis. **Blood**, v. 125, n. 21, p. 3316–3325, 21 maio 2015.

PAGNIER, J. et al. Evidence for the Multicentric Origin of the Sickle Cell Hemoglobin Gene in Africa. **Proceedings of the National Academy of Sciences of the United States of America**, v. 81, n. 6, p. 1771–1773, mar. 1984.

PAIKARI, A.; SHEEHAN, V. A. Fetal Haemoglobin Induction in Sickle Cell Disease. **British Journal of Haematology**, v. 180, n. 2, p. 189–200, 2018.

PALMER, R. M.; FERRIGE, A. G.; MONCADA, S. Nitric Oxide Release Accounts for the Biological Activity of Endothelium-Derived Relaxing Factor. **Nature**, v. 327, n. 6122, p. 524–526, 11 jun. 1987.

PAULING, L.; ITANO, H. A. Sick Cell Anemia, a Molecular Disease. **Science (New York, N.Y.)**, v. 109, n. 2835, p. 443, 29 abr. 1949.

PIEL, F. B. et al. Global Distribution of the Sick Cell Gene and Geographical Confirmation of the Malaria Hypothesis. **Nature Communications**, v. 1, p. 104, 2 nov. 2010.

PIEL, F. B. et al. Global Epidemiology of Sick Cell Haemoglobin in Neonates: A Contemporary Geostatistical Model-Based Map and Population Estimates. **Lancet (London, England)**, v. 381, n. 9861, p. 142–151, 12 jan. 2013.

PIEL, F. B. [Sick-cell disease: geographical distribution and population estimates]. **Medicine Sciences: M/S**, v. 29, n. 11, p. 965–967, nov. 2013.

PIEL, F. B. et al. Global Migration and the Changing Distribution of Sick Cell Haemoglobin: A Quantitative Study of Temporal Trends between 1960 and 2000. **The Lancet. Global Health**, v. 2, n. 2, p. e80-89, fev. 2014.

PIEL, F. B.; STEINBERG, M. H.; REES, D. C. Sick Cell Disease. **The New England Journal of Medicine**, v. 376, n. 16, p. 1561–1573, 20 2017.

PLATT, O. S. et al. Mortality in Sick Cell Disease. Life Expectancy and Risk Factors for Early Death. **The New England Journal of Medicine**, v. 330, n. 23, p. 1639–1644, 9 jun. 1994.

RANNEY, H. M. Interactions of Other Hemoglobin Variants with Sick-Cell Hemoglobin. **The New England Journal of Medicine**, v. 283, n. 26, p. 1462–1463, 24 dez. 1970.

REES, D. C.; WILLIAMS, T. N.; GLADWIN, M. T. Sick-Cell Disease. **Lancet (London, England)**, v. 376, n. 9757, p. 2018–2031, 11 dez. 2010.

REITER, C. D. et al. Cell-Free Hemoglobin Limits Nitric Oxide Bioavailability in Sick-Cell Disease. **Nature Medicine**, v. 8, n. 12, p. 1383–1389, dez. 2002.

SAFAYA, S.; STEINBERG, M. H.; KLINGS, E. S. Monocytes from Sick Cell Disease Patients Induce Differential Pulmonary Endothelial Gene Expression via Activation of NF- κ B Signaling Pathway. **Molecular Immunology**, v. 50, n. 1–2, p. 117–123, fev. 2012.

SANKARAN, V. G. et al. Human Fetal Hemoglobin Expression Is Regulated by the Developmental Stage-Specific Repressor BCL11A. **Science (New York, N.Y.)**, v. 322, n. 5909, p. 1839–1842, 19 dez. 2008.

SEBASTIANI, P. et al. Genetic Dissection and Prognostic Modeling of Overt Stroke in Sick Cell Anemia. **Nature Genetics**, v. 37, n. 4, p. 435–440, abr. 2005.

SHENOY, S. et al. A Trial of Unrelated Donor Marrow Transplantation for Children with Severe Sick Cell Disease. **Blood**, v. 128, n. 21, p. 2561–2567, 24 2016.

SIPPERT, E. Â. et al. Red Blood Cell Alloimmunization in Patients with Sick Cell Disease: Correlation with HLA and Cytokine Gene Polymorphisms. **Transfusion**, v. 57, n. 2, p. 379–389, 2017.

STEINBERG, M. H. Pathophysiologically Based Drug Treatment of Sickle Cell Disease. **Trends in Pharmacological Sciences**, v. 27, n. 4, p. 204–210, abr. 2006.

STUART, M. J.; NAGEL, R. L. Sickle-Cell Disease. **Lancet (London, England)**, v. 364, n. 9442, p. 1343–1360, 9 out. 2004.

STYLES, L. A. et al. Evidence for HLA-Related Susceptibility for Stroke in Children with Sickle Cell Disease. **Blood**, v. 95, n. 11, p. 3562–3567, 1 jun. 2000.

SWITZER, J. A. et al. Pathophysiology and Treatment of Stroke in Sickle-Cell Disease: Present and Future. **The Lancet. Neurology**, v. 5, n. 6, p. 501–512, jun. 2006.

SYDENSTRICKER, V. P. FURTHER OBSERVATIONS ON SICKLE CELL ANEMIA. **Journal of the American Medical Association**, v. 83, n. 1, p. 12–17, 5 jul. 1924.

TAMOUZA, R. et al. Infectious Complications in Sickle Cell Disease Are Influenced by HLA Class II Alleles. **Human Immunology**, v. 63, n. 3, p. 194–199, mar. 2002.

TAMOUZA, R. et al. HLA-E*0101 Allele in Homozygous State Favors Severe Bacterial Infections in Sickle Cell Anemia. **Human Immunology**, v. 68, n. 10, p. 849–853, out. 2007.

TATARI-CALDERONE, Z. et al. Protective Effect of HLA-DQB1 Alleles against Alloimmunization in Patients with Sickle Cell Disease. **Human Immunology**, v. 77, n. 1, p. 35–40, jan. 2016.

TAYLOR, J. G. et al. Variants in the VCAM1 Gene and Risk for Symptomatic Stroke in Sickle Cell Disease. **Blood**, v. 100, n. 13, p. 4303–4309, 15 dez. 2002.

TURHAN, A. et al. Primary role for adherent leukocytes in sickle cell vascular occlusion: A new paradigm. **Proceedings of the National Academy of Sciences of the United States of America**, v. 99, n. 5, p. 3047–3051, 5 mar. 2002.

VATHIPADIEKAL, V. et al. A Candidate Transacting Modulator of Fetal Hemoglobin Gene Expression in the Arab-Indian Haplotype of Sickle Cell Anemia. **American Journal of Hematology**, v. 91, n. 11, p. 1118–1122, nov. 2016.

VERMYLEN, C. et al. Bone Marrow Transplantation in Five Children with Sickle Cell Anaemia. **Lancet (London, England)**, v. 1, n. 8600, p. 1427–1428, 25 jun. 1988.

WAINSCOAT, J. S. et al. A Genetic Marker for Elevated Levels of Haemoglobin F in Homozygous Sickle Cell Disease? **British Journal of Haematology**, v. 60, n. 2, p. 261–268, jun. 1985.

WALTERS, M. C. et al. Barriers to Bone Marrow Transplantation for Sickle Cell Anemia. **Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation**, v. 2, n. 2, p. 100–104, maio 1996.

WANG, W. C. et al. Hydroxycarbamide in Very Young Children with Sickle-Cell Anaemia: A Multicentre, Randomised, Controlled Trial (BABY HUG). **Lancet (London, England)**, v. 377, n. 9778, p. 1663–1672, 14 maio 2011.

WARE, R. E. How I Use Hydroxyurea to Treat Young Patients with Sick Cell Anemia. **Blood**, v. 115, n. 26, p. 5300–5311, 1 jul. 2010.

WARE, R. E.; HELMS, R. W.; SWITCH INVESTIGATORS. Stroke With Transfusions Changing to Hydroxyurea (SWiTCH). **Blood**, v. 119, n. 17, p. 3925–3932, 26 abr. 2012.

WATSON, J. The Significance of the Paucity of Sick Cells in Newborn Negro Infants. **The American Journal of the Medical Sciences**, v. 215, n. 4, p. 419–423, abr. 1948.

YAWN, B. P. et al. Management of Sick Cell Disease: Summary of the 2014 Evidence-Based Report by Expert Panel Members. **JAMA**, v. 312, n. 10, p. 1033–1048, 10 set. 2014.

ZHANG, D. et al. Neutrophils, Platelets, and Inflammatory Pathways at the Nexus of Sick Cell Disease Pathophysiology. **Blood**, v. 127, n. 7, p. 801–809, 18 fev. 2016.

8. LIST OF PUBLICATIONS

8 List of publications

8.1 Publications related to this study

TOZATTO-MAIO, K. et al. A Toll-like Receptor 2 Genetic Variant Modulates Occurrence of Bacterial Infections in Patients with Sickle Cell Disease. **British Journal of Haematology**, 25 mar. 2019. doi: 10.1111/bjh.15875. [Epub ahead of print]

TOZATTO-MAIO, K. et al. A Polymorphism in Toll-like Receptor 2 Gene Is Associated with Occurrence of Bacterial Infections in Sickle Cell Disease Patients. **Blood**, v. 132, n. Suppl 1, p. 3652–3652, 21 nov. 2018.

TOZATTO-MAIO, K. et al. Association of Toll-Like Receptor 2 Gene Polymorphism With The Incidence Of Bacterial Infections In Sickle Cell Disease. 2017; 22nd European Haematology Association Congress. [s.d.]Disponível em: <<http://learningcenter.ehaweb.org/eha/2017/22nd/181271/karina.association.of.toll-like.receptor.2.gene.polymorphism.with.the.html>>.

TOZATTO-MAIO, K. et al. Sickle Cell Disease: Management and Complications of Patients Followed in a Brazilian University Hospital. Congresso Brasileiro de Hematologia (Hemo) 2017.

CAPPELLI, B. et al. Risk factors and outcomes according to age at transplantation with an HLA-identical sibling donor for sickle cell disease. *Haematologica*. *In press*.

CAPPELLI, B. et al. Alternative Donor Hematopoietic Stem Cell Transplantation for Sickle Cell Disease in Europe. **Blood**, v. 132, n. Suppl 1, p. 4645–4645, 21 nov. 2018

CAPPELLI, B. et al. Risk Factors and Outcomes According to Age at Transplant with an HLA Identical Sibling for Sickle Cell Disease. [s.d.]Disponível em: <<https://ash.confex.com/ash/2017/webprogram/Paper101419.html>>.

8.2 Other publications (2015-2019)

TOZATTO-MAIO, K. et al. Cord Blood Unit Dominance Analysis and Effect of the Winning Unit on Outcomes after Double-Unit Umbilical Cord Blood Transplantation in Adults with Acute Leukemia: A Retrospective Study on Behalf of Eurocord, the Cord Blood Committee of Cellular Therapy, Immunobiology Working Party, and the Acute Leukemia Working Party of the European Group for Blood and Marrow Transplantation. **Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation**, v. 24, n. 8, p. 1657–1663, ago. 2018a.

PAVIGLIANITI, A. et al. Outcomes of Advanced Hodgkin Lymphoma after Umbilical Cord Blood Transplantation: A Eurocord and EBMT Lymphoma and Cellular Therapy & Immunobiology Working Party Study. **Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation**, v. 24, n. 11, p. 2265–2270, nov. 2018b.

PAVIGLIANITI, A. et al. Low Body Mass Index Is Associated with Increased Risk of Acute GVHD after Umbilical Cord Blood Transplantation in Children and Young Adults with Acute Leukemia: A Study on Behalf of Eurocord and the EBMT Pediatric Disease Working Party.

Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation, v. 24, n. 4, p. 799–805, 2018a.

RAFII, H. et al. Family Cord Blood Banking for Sickle Cell Disease: A Twenty-Year Experience in Two Dedicated Public Cord Blood Banks. **Haematologica**, v. 102, n. 6, p. 976–983, 2017.