Immunogenetics in sickle cell disease

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Thèse de doctorat en Biothérapies

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Présentée et soutenue publiquement à l’Hemocentro de Ribeirão Preto le 26 juin 2019

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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
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Tozatto-Maio, Karina

Immunogenetics in sickle cell disease. / Karina Tozatto-Maio – Ribeirão Preto/Paris, 2019.

84 p.; il. 30cm

Advisors: Belinda Pinto Simões; Eliane Gluckman

Thesis (Doctorate degree - Graduate Program in Clinical Oncology, Stem cells and Cell Therapy) -- Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo - Brasil and Université Paris-Diderot - France, 2019.
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.

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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.
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). Sickle 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 vasooclusão, 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 TCPP de doador aparentado ou não aparentado em centros de TCPP 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 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 TCPP, 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.
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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).²

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 Sβ 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/drepanocyte). 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 Syndenstricker 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 into 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-alpha 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). Sickle monocytes, mediated by tumor necrosis factor alpha (TNF-α), activate endothelial cells, triggering the endothelial nuclear factor kappa B (NF-κB) 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)-1β were not augmented in this cohort (GRAIDO-GONZALEZ et al., 1998). However, other studies found higher levels of TNF-α and IL-1β 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-κB pathway, promoting activation of leukocytes.
and endothelial cells; NF-κB 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; WAINSSCOAT 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, β2 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-alpha and cytotoxic-T-lymphocyte associated antigen 4 (CTLA-4) (TATARI-CALDERONE et al., 2016; OLIVEIRA et al., 2017; SIPPERT 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-κB 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, DRB1 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)          |         |
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.
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     | 02:01 | 03:01 | 01:01 | 30:01 | 30:02 | 23:01 | 68:02 | 24:02 |
|-----------|-------|-------|-------|-------|-------|-------|-------|-------|
| HLA*A     |       |       |       |       |       |       |       |       |
| 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     | 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.
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
5. MANUSCRIPT 2: Human leukocyte antigen matched unrelated donors for patients with sickle cell disease: results of international donor searches.
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.
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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

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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.

RAFIH, 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.
9. Annexe : résumé en Français

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.

Cette étude a eu deux questions principales et a été divisé dans deux parties. Pour la première partie, nous avons eu comme objectif d’évaluer le rôle de quelques gènes inflammatoires liés aux Toll-like récepteurs (TLR) dans la survenue des infections bactériennes sévères 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. Sept SNPs dans le gène TLR (TLR1 rs4833095, TLR2 rs3804099, TLR2 rs3804100, TLR2 4696480, TLR6 rs5743810, TLR10 rs11466653, TLR10 rs11096957) ont été génotypés par réaction en chaîne par polymérase en temps réel (RT-PCR) TaqMan 5’ nuclease assay. Quatre-cents trente patients, d’origine brésilienne, africaine subsaharienne, d’Amérique Centrale et d’Afrique du Nord ont été divisés en deux groupes : infectés (n=235, patients qui ont eu au moins un épisode d’infection bactérienne sévère documentée) et non infectés (n=195,
patients qui n’ont jamais présentés d’infections sévères). La médiiane d’âge des patients était de 30 ans (range 2-70), 57% étaient des femmes et le génotype drépanocytaire le plus fréquent était HbSS (81%). Il n’y avait pas de variable démographique significativement associée à la survenue d’infections. Le génotype T/A du SNP TLR2 rs4696480 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). En outre, nous avons fait des comparaisons entre la fréquence des génotypes du SNP rs4696480 chez les patients drépanocytaires de notre cohorte et chez les individus d’origine africaine décrits dans la base de données 1000 génomes. Le génotype T/A était significativement plus fréquent chez les drépanocytaires que dans la population 1000 génomes (OR 1.67, 95% CI 1.30-2.13, p<0.001), lorsque le génotype T/T était significativement moins fréquent chez les drépanocytaires comparé à la population 1000 génomes (OR 0.17, 95% CI 0.12-0.24, 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 de maladies inflammatoires. Cela peut indiquer que le génotype T/A détermine la réponse inflammatoire idéale, lorsque la réponse inflammatoire du génotype T/T n’est pas suffisante pour protéger contre les bactéries, et la réponse inflammatoire du génotype A/A est peut-être exacerbée. Ainsi, sachant que les infections bactériennes sont une cause majeure de mortalité chez les enfants drépanocytaires, la déviation de l’équilibre de Hardy Weinberg de ce SNP dans cette cohorte, composée majoritairement d’adultes, peut indiquer un avantage adaptatif du génotype hétérozygote dans cette population.

Cette étude a également eu pour objectif d’estimer la probabilité de trouver un donneur potentiel non apparenté, identique pour les loci HLA-A, HLA-B et HLA-DRB1. 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.

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 rapportent leurs données à la Société Européenne de Greffe de Cellules Souches (EBMT). Les patients inclus avaient des données HLA testées en moyenne ou haute résolution. Les haplotypes HLA ont été estimés à travers le logiciel HaploStats. Nous avons recherché des donneurs potentiels HLA
alléliques identiques pour les loci HLA-A, HLA-B et HLA-DRB1 (6/6) dans des registres internationaux (WMDA). Nous avons aussi classifié les haplotypes selon l’ethnicité la plus probable, basés sur la fréquence des haplotypes dans les groupes ethniques décrits dans le National Marrow Donor Program (NMDP) des Etats-Unis. Les patients de l’EBMT ont eu 70% des haplotypes classifiés comme Africains, 6% Caucasiens, 3% Amérindiens, 8% communs (haplotypes avec une fréquence > 1/1000 dans tous les populations) et 12% non classifiés. Les patients brésiliens ont eu 45% d’haplotypes Africains, 12% Caucasiens, 9% Amérindiens, 18% communs et 16% non classifiés. Pourtant, la probabilité de trouver au moins un donneur potentiel allélique 6/6 identique était la même chez les patients brésiliens et chez les patients de l’EBMT, 47% et 47% respectivement. La probabilité d’avoir au moins 5 donneurs potentiels alléliques 6/6 identiques était 15% pour les patients de l’EBMT et 24% pour les brésiliens, mais cette différence n’était pas significative.

Malgré les différences de composition ethnique, la probabilité de trouver un donneur était identique entre les deux groupes car la présence d’au moins un haplotype africain diminue les chances d’avoir un donneur identique. La plupart de donneurs a été trouvée dans le registre national de donneurs des Etats-Unis (NMDP), dans le registre brésilien de donneurs (REDOME) et dans le registre allemand de donneurs (ZKRD). 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.

Nous avons démontré qu’un SNP dans le gène TLR module la survenue d’infections bactériennes chez les patients drépanocytaires, et que le génotype hétérozygote T/A du SNP TLR2 rs4696480 est associé à moins d’infections, alors que les patients qui ont le génotype T/T ont plus d’infections. Nous avons aussi proposé que la haute fréquence d’hétérozygotes dans la population drépanocytaire peut indiquer un avantage adaptatif de ce SNP chez ces patients. En outre, nous avons démontré que, malgré les différences de composition ethnique entre les patients drépanocytaires brésiliens et ceux greffés dans des centres EBMT, leur probabilité de trouver au moins un donneur potentiel allélique identique est la même. Cela indique le besoin d’améliorer la représentativité des groupes ethniques touchés par la drépanocytose dans les registres internationaux de donneurs de cellules souches.

Au total, nos résultats montrent que la modulation de complications cliniques par des gènes inflammatoires peut 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 à un centre de greffe, et de prévoir la probabilité de trouver un donneur.

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