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Investigação da Variabilidade do Gene *ACKR1* em Doadores de Sangue de Porto Alegre/RS.

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SUMÁRIO

LISTA DE ABREVIATURAS

Sigla	Descrição
DHRN	Doença hemolítica do recém nascido
DNA	Ácido desoxirribonucleico
Fy^{a}	Antígeno Fy^{a}
Fy^{b}	Antígeno Fy^{b}
HCPA	Hospital de Clínicas de Porto Alegre
HLA	Antígeno leucocitário humano
IgG	Imunoglobulina da classe IgG
ISBT	*International Society for Blood Transfusion*
PCR	Reação em cadeia da polimerase
RTH	Reação transfusional hemolítica
SNP	Polimorfismo de um único nucleotídeo
TAD	Teste direto da antiglobulina

RESUMO

Os sistemas de grupos sanguíneos são caracterizados pela presença de antígenos na membrana eritrocitária. Estes antígenos possuem características polimórficas e funcionais originadas a partir de mudanças ao nível genético que variam desde polimorfismos de um único nucleotídeo (SNPs) a trocas gênicas como inversões, deleções, inserções, *splicing* alternativo, dentre outros mecanismos. O sistema de grupo sanguíneo Duffy é clinicamente significativo, podendo estar relacionado com reação hemolítica transfusional e doença hemolítica do recém-nascido. Inicialmente, revisamos as bases moleculares do gene *ACKR1*, que codifica a glicoproteína DARC, responsável pela expressão dos antígenos Duffy. Estudos prévios já identificaram variantes genéticas que determinam a expressão fraca ou nula dos antígenos Fy^a e Fy^b e, no presente trabalho, objetivamos realizar um rastreamento de variantes genéticas que possam estar associadas com discrepâncias entre fenótipo-genótipo em doadores de sangue de Porto Alegre, Rio Grande do Sul. A amostra, composta por 382 doadores voluntários de sangue, foi determinada por hemaglutinação e discriminação alélica dos SNPs c.125G>A, c.265C>T e c.1-67T>C por PCR em tempo real. A partir da análise de correlação entre o fenótipo e o genótipo, nós sequenciamos o gene *ACKR1* em 11 doadores (2,88%) que apresentaram resultados discordantes. Os dados obtidos no sequenciamento foram avaliados *in silico*, através dos programas Poly-Phen2, SIFT e RegulomeDB. Nossa investigação detectou 11 variantes genéticas, das quais 4 (c.-541C>T, c.21+150C>T, c.22-58A>G e c.298G>A SNPs) discutimos o aparente efeito funcional na estrutura e expressão da proteína DARC. Em conclusão, eventos moleculares podem resultar em genótipo e fenótipo eritrocitário sendo aparentemente discrepante. Dessa forma, estudos adicionais, que caracterizem o impacto funcional de variantes genéticas associadas à predição de novos alelos de grupos sanguíneos, são fundamentais na implementação de técnicas moleculares seguras para a genotipagem dos antígenos de pacientes e doadores em bancos de sangue. O aperfeiçoamento das metodologias moleculares pode

contribuir na prática transfusional através do manejo de unidade de sangue apropriadas para cada indivíduo.

ABSTRACT

Blood group systems are characterized by the presence of antigens in the erythrocyte membrane. These antigens have polymorphic and functional characteristics originated from changes at the genetic level that range from single nucleotide polymorphisms (SNPs) to gene exchanges, inversions, deletions, insertions, alternative splicing, among other mechanisms. Duffy blood group system is clinically significant because can cause haemolytic transfusion reactions and haemolytic disease of the fetus and newborn. Initially, we revised the molecular basis of the *ACKR1* gene that encodes the DARC glycoprotein, responsible for Duffy antigens expression. Previous studies have already been identified genetic variants that determine the weak or null Fy^a and Fy^b antigen expression and the present work, we aimed a screening of genetic variants that may be associated with discrepancies between phenotype-genotype in blood donors from Porto Alegre, Rio Grande do Sul. The sample composed by 382 voluntary blood donors was determined by haemagglutination and allelic discrimination of the c.125G>A, c.265C>T and c.1-67T>C SNPs in real-time PCR. From correlation between phenotype-genotype results, we sequenced the entire *ACKR1* gene in 11 (2.88%) donors with inconclusive results. The data obtained in the sequencing were evaluated in silico, through the Poly-Phen2, SIFT and RegulomeDB tools. Our investigation accounted 11 genetic variants from which 4 (c.-541C>T, c.21+150C>T, c.22-58A>G and c.298G>A SNPs) we discuss the apparent functional effect on structure and expression of DARC protein. Overall, molecular events result in the red blood cell genotype and phenotype being apparently discrepant by gathering more information. In conclusion, further studies characterizing the functional impact of genetic variants associated with the prediction of new blood group alleles are critical in the implementation of molecular techniques for secure antigen genotyping of patients and donors in blood banks. The use of effective molecular methodologies can optimize transfusion practices through appropriate blood units for each individual.

APRESENTAÇÃO DA DISSERTAÇÃO

Este trabalho consiste na dissertação de mestrado “Investigação da Variabilidade do Gene *ACKR1* em Doadores de Sangue de Porto Alegre/RS”, a ser apresentada no Programa de Pós-graduação em Biociências da Universidade Federal de Ciências da Saúde de Porto Alegre no ano de 2017. O trabalho foi elaborado a partir da análise de dados de um projeto maior denominado “Biologia Molecular de Grupos Sanguíneos – Impacto na Prática Transfusional”, o qual foi iniciado no ano de 2012.

A presente dissertação é composta de uma introdução sobre os principais conceitos envolvidos na medicina transfusional, que são sistemas de grupos sanguíneos (seção 1), aloimunização eritrocitária (seção 2) e tipagem sorológica e molecular (seção 3). A seguir, o capítulo I compreende o artigo de revisão intitulado *Molecular basis of the Duffy blood group system*, descrevendo uma visão geral do sistema de grupo sanguíneo Duffy e caracterizando as principais variantes alélicas do gene, publicado no periódico *Blood Transfusion*. A descrição da análise experimental desenvolvida durante o mestrado e dos resultados obtidos está apresentada no artigo original, capítulo II: *Identification of ACKR1 variants associated with altered Duffy phenotype expression in blood donors from the Southern Brazil*, o qual será submetido ao periódico *Vox Sanguinis*. As considerações finais apontam perspectivas acerca do refinamento da caracterização do perfil genético no progresso da tipagem molecular sanguínea. Os documentos suplementares estão apresentados na forma de anexos.

INTRODUÇÃO

1.1 Sistemas de grupos sanguíneos

Os sistemas de grupos sanguíneos são caracterizados pela presença ou ausência de antígenos na membrana eritrocitária. Estes antígenos, como parte integrante dos componentes da membrana, possuem características polimórficas e funcionais bem definidas (Issitt & Anstee, 1998). Atualmente, de acordo com a *International Society for Blood Transfusion* (ISBT), estão descritos 346 antígenos eritrocitários, distribuídos em 36 sistemas de grupos sanguíneos (Storry *et al*., 2016).

A maioria dos antígenos de grupos sanguíneos possui um padrão de herança mendeliana. Sua grande diversidade é originada a partir de mudanças ao nível genético que variam desde polimorfismos de um único nucleotídeo (SNPs) a trocas gênicas como inversões, deleções, inserções, *splicing* alternativo, dentre outros mecanismos (Denomme, 2011; Reid *et al*., 2012). Como a prevalência dos antígenos de grupos sanguíneos pode variar entre diferentes populações, este entendimento tem provado ser útil no rastreamento dos padrões de imigração de diferentes grupos étnicos ao longo do tempo (Storry, 2003; Jungbauer, 2011). O conhecimento da distribuição dos antígenos de grupos sanguíneos entre um grupo étnico e outro, é importante também na busca de sangue de fenótipo compatível, entre pacientes e doadores de sangue, em especial, fenótipos raros ou variantes, que são predominantes em determinadas populações (Meny *et al*., 2013).

Os antígenos eritrocitários são formados por sequências específicas de aminoácidos que constituem uma proteína; por carboidratos ligados a proteínas ou a lipídios da membrana eritrocitária (Reid & Yazdanbakhsh, 1998). Em relação aos aspectos funcionais, eles podem ser classificados em: proteínas estruturais, transporte, receptores/moléculas de adesão, enzimas, complemento, proteínas regulatórias e outras, sendo que um antígeno eritrocitário pode apresentar mais do que uma função (Bonifácio & Novaretti, 2009). Os antígenos de grupos sanguíneos têm um papel significativo na medicina transfusional, na incompatibilidade materno-fetal, nas anemias autoimunes e nos transplantes de

orgãos (Issitt & Anstee, 1998). Depois dos antígenos do sistema ABO, os mais significativos clinicamente são os antígenos dos sistemas Rh, Kell, Duffy e Kidd (Klein & Anstee, 2005; Higgins & Sloan, 2008).

1.2 Aloimunização eritrocitária

Os antígenos de grupos sanguíneos apresentam importância na medicina transfusional visto que estão presentes na porção externa da membrana eritrocitária e, por isso, a ausência de um antígeno na membrana pode ocasionar a aloimunização de um receptor, caso o mesmo seja transfundido com concentrados de hemácias que expressem o antígeno em questão (Issitt & Anstee, 1998; Reid, 1995). Além disso, o alto grau de diversidade genética e a presença de recombinações gênicas pode levar a alterações qualitativas e quantitativas na expressão dos antígenos na membrana da hemácia, gerando fenótipos parciais e fracos, que podem também estar associados à aloimunização (Klein & Anstee, 2005). Essa aloimunização se dá uma vez que alguns desses antígenos geram a produção de imunoglobulinas chamadas de anticorpos irregulares, que são os anticorpos formados somente após a exposição, por transfusão ou gestação, ao antígeno correspondente (Daniels *et al*., 2004).

O significado clínico dos anticorpos antieritrocitários depende da frequência do antígeno (variando em diferentes origens étnicas), da sua imunogenicidade e capacidade hemolítica do anticorpo, além de situações clínicas específicas (Issitt, 1994). Considerando as frequências gênicas e a incidência destes anticorpos em várias populações, foi estimado que a probabilidade de um indivíduo produzir um ou mais anticorpos antieritrocitários é de aproximadamente 1% por unidade de sangue transfundida (Giblett, 1961). Contudo, essa frequência pode estar aumentada em determinados grupos de pacientes que recebem transfusão de concentrado de hemácias cronicamente tais como, os pacientes portadores de anemia falciforme, β-talassemia e síndrome mielodisplásica (Giblett, 1977), podendo desenvolver anticorpos contra múltiplos antígenos eritrocitários. Estes anticorpos permanecem na circulação e estão implicados em reações transfusionais hemolíticas (RTH) e na redução do número de bolsas de sangue compatíveis para futuras transfusões (Castilho, 2008).

A obtenção de concentrado de hemácias compatível para pacientes com múltiplos anticorpos ou anticorpos raros pode ser difícil ou até mesmo impossível. O resultado da demora em se encontrar unidades compatíveis ou mesmo a incapacidade de assegurar uma transfusão segura, podem trazer consequências graves aos pacientes e contribuir com a mortalidade (Nickel *et al*., 2016). Na prática transfusional atual, tem-se procurado minimizar as chances de um indivíduo formar aloanticorpos antieritrocitários. Alguns autores estimulam as transfusões intrarraciais (Chou *et al*., 2013). Em contrapartida, outros autores recomendam transfusões fenotipicamente compatíveis com os antígenos eritrocitários mais imunogênicos independente da raça (Vichinsky *et al*., 1990; Vichinsky *et al*., 2001; LaSalle-Williams *et al*., 2011). Apesar de existirem ainda algumas questões não respondidas em relação à resposta imune aos antígenos eritrocitários, a implantação de protocolos seguros e eficientes de fenotipagem e/ou genotipagem de grupos sanguíneos pode reduzir drasticamente os riscos de desenvolvimento de aloanticorpos em pacientes que recebem transfusão sanguínea (Castilho, 2008).

1.3 Tipagem sorológica e molecular

Os procedimentos de fenotipagem são baseados no princípio da hemaglutinação, que é o método clássico para testes de antígenos de grupos sanguíneos e anticorpos. Baseia-se na utilização de métodos sorológicos e tem sido largamente adotada por apresentar boa eficiência, quando bem executado, nos testes de compatibilidade entre pacientes e doadores de sangue (Reid & Lomas-Francis, 2002; Reid, 2007; Strauss & Reid, 2008). Embora seja constantemente utilizada nos bancos de sangue, apresenta limitações técnicas: (1) interpretação subjetiva; (2) procedimentos pouco automatizados; (3) alto custo dos reagentes (em especial soros raros); (4) muitos soros raros não registrados e de origem humana; (5) indisponibilidade de antissoros comerciais para muitos antígenos e (6) falha na identificação de antígenos parciais e com baixa expressão. Além disso, existem limitações clínicas: (1) fenotipagem de pacientes com transfusões recentes; (2) fenotipagem de hemácias de pacientes com autoanticorpos e (3) falha na determinação da zigozidade *RHD* (Reid *et al*., 2000).

Quando se busca doadores negativos para múltiplos antígenos ou antígenos de alta frequência, um pequeno número de doadores pode ser fenotipado ao mesmo tempo, tornando-se um teste trabalhoso para fenotipagem em larga escala e limitando, portanto, o número de estoque de unidades de sangue antígenos-negativos (Reid, 2009).

Enquanto os testes de hemaglutinação avaliam os produtos dos genes de grupos sanguíneos, a genotipagem detecta a sequência do DNA responsável pela expressão destes produtos (os antígenos eritrocitários) (Castilho *et al*., 2002a; Reid & Lomas-Francis, 2002; Daniels, 2005). Entre as indicações para a realização de testes moleculares estão a identificação dos antígenos eritrocitários em pacientes recém-transfundidos, em pacientes com teste direto da antiglobulina (TAD) positivo e a identificação do risco do desenvolvimento de doença hemolítica do recém nascido (DHRN) (Flegel *et al*., 1998; Reid & Lomas-Francis, 2002; Van der Schoot *et al*., 2003). As técnicas moleculares podem ainda identificar a presença de variações em genes que codificam antígenos de grupos sanguíneos fracamente expressos na membrana, contribuindo para a prevenção de possíveis reações transfusionais hemolíticas (Reid, 2007). A utilização das ferramentas de Biologia Molecular é fundamental para a inserção de novas metodologias na rotina laboratorial da imunohematologia.

O interfaciamento das informações dos fenótipos deduzidos dos genótipos, entre o laboratório molecular e os serviços de transfusão é uma alternativa importante na determinação do perfil antigênico e pode auxiliar no esclarecimento dos mecanismos moleculares envolvidos com as diversas variantes de grupos sanguíneos (Castilho *et al*., 2002a, 2002b; Castilho & Pellegrino Jr, 2004; Hillyer *et al*., 2008). Contudo, para se estabelecer protocolos seguros de genotipagem dos antígenos eritrocitários é importante realizar uma triagem sistemática dos alelos e variantes de grupos sanguíneos de determinada população. Em algumas populações ainda existem poucos dados publicados e a diversidade genética dos grupos sanguíneos permanece desconhecida, como é o caso do Rio Grande do Sul.

JUSTIFICATIVA

O grupo sanguíneo Duffy possui um importante significado clínico na medicina transfusional. O anticorpo anti-Fy^a é encontrado principalmente em seguida a uma transfusão e, menos frequentemente, como resultado de uma gravidez. Ele raramente ocorre naturalmente (Rosenfield *et al*., 1950; Algora *et al*., 1991). O anti-Fy^b é aproximadamente 20 vezes menos comum que o anti-Fy^a e está geralmente presente no soro em combinação com outros anticorpos (Marsh *et al*., 1975). Estes anticorpos são predominantemente da subclasse tipo IgG1, com menos contribuição de outras subclasses, por exemplo IgG2 (18%) e IgM (25%) (Hardman & Beck, 1981; Szymanski *et al*., 1982). Os dois anticorpos podem causar reações transfusionais hemolíticas imediatas e tardias (Issitt & Anstee, 1998; Daniels *et al*., 2002). Os antígenos Fy^a e Fy^b expressam-se em células eritróides e não-eritróides, como células endoteliais e, também, em células epiteliais em vários órgãos como cérebro, rins, baço, coração, pulmão, pâncreas e placenta (Girello & Kühn, 2007).

A clonagem dos genes que controlam a expressão dos antígenos eritrocitários, a caracterização estrutural dessas moléculas e a avaliação da sua expressão tecidual diferencial são avanços para o entendimento de aspectos funcionais. Atualmente, estudos têm identificado variantes que determinam a expressão fraca ou nula dos antígenos Fy^a e Fy^b (ISBT), destacando a importância de estabelecer a incidência de variantes e o impacto destas na expressão do fenótipo.

As características de origem étnica e geográfica influenciam na distribuição dos diferentes alelos de antígenos eritrocitários, impactando diretamente na transfusão de hemocomponentes, devido à imunogenicidade desses antígenos (Pellegrino *et al*., 2001). O Brasil possui um território de tamanho continental e seu cruzamento interétnico formou uma das populações mais heterogêneas do mundo (Pena *et al*., 2011). Adicionalmente, o Sul do Brasil é composto predominantemente por uma mistura entre descendentes de europeus, possuindo uma distinta variabilidade genética em relação a outras regiões do país (Pena *et al*., 2011; Salzano & Sans, 2014). O conhecimento do perfil genético, através do

rastreamento de variantes ainda não descritas no gene que codifica o grupo sanguíneo Duffy pode auxiliar na implementação das técnicas moleculares para genotipagem de pacientes e doadores em bancos de sangue. A utilização de metodologias moleculares eficazes pode otimizar as práticas transfusionais, através do direcionamento de unidades de sangue mais compatíveis para cada indivíduo, contribuindo no aumento da segurança e, consequentemente, na qualidade da medicina transfusional.

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OBJETIVOS

Objetivo geral

Investigar a presença de variantes genéticas do grupo sanguíneo Duffy, em doadores de sangue do Sul do Brasil.

Objetivos específicos

- Avaliar a correlação fenótipo-genótipo do grupo sanguíneo Duffy, considerando os polimorfismos c.125G>A (rs12075), c.265C>T (rs34599082) e c.1-67T>C (rs2814778), em 382 doadores de sangue do Hospital de Clínicas de Porto Alegre.
- Realizar um rastreamento molecular para identificação de variações no gene *ACKR1* que possam afetar a expressão dos antígenos eritrocitários do grupo sanguíneo Duffy, nos doadores com discrepância na correlação fenótipo-genótipo.

CAPÍTULO I. *Molecular basis of the Duffy blood group system*

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Molecular basis of the Duffy blood group system

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Abstract

ACKR1, located on chromosome 1q23.2, is the gene that encodes a glycoprotein expressing the Duffy blood group antigens. This gene is transcribed in two mRNA variants yielding two isoforms, encoding proteins with 338 and 336 amino acids. This review provides a general overview of the Duffy blood group to characterise and elucidate the genetic basis of this system. The Fy^a and Fy^b antigens are encoded by co-dominant *FY*A (FY*01)* and *FY*B (FY*02)* alleles, which differ by c.125G>A (rs12075), defining the Fy(a+b−), Fy(a−b+) and Fy(a+b+) phenotypes. The Fy(a−b−) phenotype that occurs in Africans provides an explanation for the apparent absence of *Plasmodium vivax* in this region: this phenotype arises from homozygosity for the *FY*B* allele carrying a point mutation c.1-67T>C (rs2814778), which prevents Fy^b antigen expression only in red blood cells. The same mutation has also been found on the *FY*A* allele, but it is very rare. The Fy(a−b−) phenotype in Europeans and Asians arises from mutations in the coding region of the *FY*A* or *FY*B* allele, preventing Duffy antigen expression on any cell in the body and thus are true Duffy null phenotypes. According to the International Society for Blood Transfusion, ten alleles are associated with the null expression of the Fy antigens. Furthermore, different allelic forms of *FY*B* modify Fy^b antigen expression, which may result in very weak or equivocal serology results. The mostly common found variants, c.265C>T (rs34599082) and c.298G>A (rs13962) - previously defined in combination only with the *FY*B* allele - have already been observed in the *FY*A* allele. Thus, six alleles have been recognised and associated with weak expression of the Fy antigens. Considering the importance of the Duffy blood group system in clinical medicine, additional studies via molecular biology approaches must be performed to resolve and clarify the discrepant results that are present in the erythrocyte phenotyping.

Keywords: Duffy blood group system, *ACKR1* gene, DARC, allele variants.

Introduction

The blood group systems are characterised by the presence or absence of antigens on the erythrocyte membrane, and these antigens are often polymorphic with respect to sequence and function[1]. Currently, according to the International Society for Blood Transfusion (ISBT), there are 346 erythrocyte antigens, dispersed over 36 different blood group systems[2].

The erythrocyte antigens are genetically inherited and defined by specific sequences of amino acids, constituting proteins connected to lipids or carbohydrates[3]. These antigens are important to transfusion medicine because their absence from the red blood cells of an individual can result in alloimmunisation after a transfusion with the respective antigen[1]. Among the various consequences of alloimmunisation, the following stand out: an increased risk of transfusion reactions, reduction of the number of compatible blood bags, destruction of allogeneic erythrocytes, as well as of autologous and foetal erythrocytes, in addition to damage to transplanted tissues[4].

In order to minimise the chances of an individual generating erythrocyte alloantibodies, transfusions must be phenotypically compatible to the most immunogenic antigens[5]. Although phenotyping is essential for the confirmation of the presence of alloantibodies and also for the detection of blood group antigens[6-8], phenotyping suffers from certain technical limitations because it is a subjective test, many antibodies are not commercially available and it is a labour-intensive test, so a relatively small number of donors can be typed for a relatively small number of antigens. There are also certain clinical limitations, including the difficulty of phenotyping recently transfused patients as well as red blood cells coated with IgG, and it can be challenging to distinguish an alloantibody from an autoantibody in antigen-positive people[9]. In these situations, blood group genotyping has proven to be an excellent alternative to phenotypying[10,11].

The main indications for performing such a molecular test in immunohaematology are the identification of erythrocyte antigens in recently transfused patients, in patients with a positive direct antiglobulin test (DAT+) and in situations in which there is a risk of developing haemolytic disease of the foetus and newborn (HDFN)[7,12,13]. Molecular techniques may also be used to identify the presence of variation in genes that encode blood group antigens that are expressed weakly in the membrane, thereby contributing to the prevention of possible haemolytic transfusion reactions[6].

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DNA-based genotyping of the Duffy blood group system can be an important adjunct to traditional phenotyping, especially in clinical situations in which the risk of HDFN is a concern and for locating matched blood for alloimmunised patients. Accordingly, this review provides a general overview of the Duffy blood group to characterise and clarify the genetic basis of the allelic variants of this system.

Duffy blood group system

The Duffy blood group was initially reported by Cutbush in 1950, who described the reactivity of an antibody found in a male, multitransfused, haemophilic patient who had an alloantibody against an antigen, then denoted as Fy^a. This antibody was named anti-Fy^a, in honour of the patient in question[14]. A year later, an antibody was described in the serum of a multiparous woman which defined its antithetical pair, called anti-Fy^b [15]. The Duffy antigens reside in an acidic glycoprotein that spans the membrane seven times. The N-terminal portion forms the extracellular domain and the C-terminal portion forms the intracellular domain.

Biological functions

The Duffy glycoprotein, also known as the Duffy antigen receptor for chemokines (DARC), is a promiscuous receptor that binds chemokines of the C-X-C and C-C classes[16-18]. Examples of C-X-C chemokines are interleukin-8 (IL-8) and melanoma growth stimulatory activity (MGSA), while the C-C chemokines include regulated on activation, normal T expressed and secreted (RANTES) and monocyte chemotactic protein-1 (MCP-1)[16-18]. The main normal function described for DARC is that it effectively sustains homeostatic levels of circulating chemokines and modulates chemokine gradients between tissues and the blood to mediate the influx of neutrophils and monocytes from blood vessels into tissues during immune responses[19,20]. Although the specific mechanisms underlying its functions remain uncertain, there is interest in DARC as an explanatory variable for population-specific differences in disease susceptibility[21], as demonstrated by ongoing research into its role in inflammation-associated pathology and malignancy[21,22], and by the recent, though highly controversial[23], surge in interest around the antigen's role in human immunodeficiency virus infection[24].

Much of the research into this blood group has been concerned with elucidating the characteristic expression patterns among different populations[25]. Interest in the Duffy blood group rose substantially with the recognition of its role as a portal of entry for malarial parasites into human red blood cells[26]. While *Plasmodium falciparum* uses a series of receptors on the surface of human erythrocytes to invade them, *Plasmodium vivax* and *Plasmodium knowlesi* depend on an interaction with the Duffy antigen, meaning that red blood cells lacking the antigen are refractory to invasion by these merozoites[26-29]. The proportion of individuals in African populations who do not express the DARC protein in their erythrocytes is high. The gap in distribution of *P. vivax* in Africa is, therefore, viewed as the consequence of the lack of this protein on red blood cells - suggesting either an adaptive response to the disease or a selective pressure acting on the parasite[30,31]. Furthermore, a genotype-dosage effect on expression of the DARC protein has been described and the level of DARC expression is associated with susceptibility and resistance to infection[32-35]. These results imply that the red cells of heterozygotes for the silent allele bind substantially less *P. vivax* Duffy-binding protein than those of individuals with two active *FY* alleles, indicating that Duffy-negative heterozygosity confers significant protection and may have a selective advantage in areas where *P. vivax* is endemic[36]. However, since 2006 there have been reports of Duffy-negative individuals infected with *P. vivax,* both throughout Africa (Kenya, Madagascar, Mauritania, Cameroon, Angola, Equatorial Guinea, Ethiopia, and Sudan) and in Brazilian Amazon[37-46]. The mechanisms involved in this invasion remain to be elucidated: the hypotheses postulated include expansions of the copy number of gene Duffy-binding protein 1 of the *P. vivax*[47] and the use of alternative ligand-receptor pairs[30,47].

Genetic basis

ACKR1, also known as *DARC* or *FY* (NCBI), is the gene that encodes a transmembranous glycoprotein expressing the Duffy blood group antigens. Its genetic locus was reported to be on chromosome 1, located formerly in the region 1q21-25 by linkage analysis[48]. Later, the position of the gene was refined to 1q23.2. The gene (sequence NC_000001.11, region: 159204013-159206500) is transcribed as two mRNA variants. Chaudhuri *et al*. reported that the first mRNA variant has one exon[49]. Subsequently, Iwamoto *et al.* demonstrated the existence of a spliced mRNA variant that has two exons with the intron encompassing sequences in the initial part of the first mRNA variant[50]. Despite encoding for a shorter protein, the second mRNA variant has a longer transcript than the first because of a longer 5′ untranslated region. These two distinct transcript isoforms are expressed from separate promoters, yielding distinct protein products. The major transcript is derived from exon 1 and exon 2 of *ACKR1*; the minor product is a transcript initiated at the beginning of exon 2. The minor and major transcripts generate, respectively, isoform A (NM_001122951.2/ NP_001116423.1), encoding a protein of 338 amino

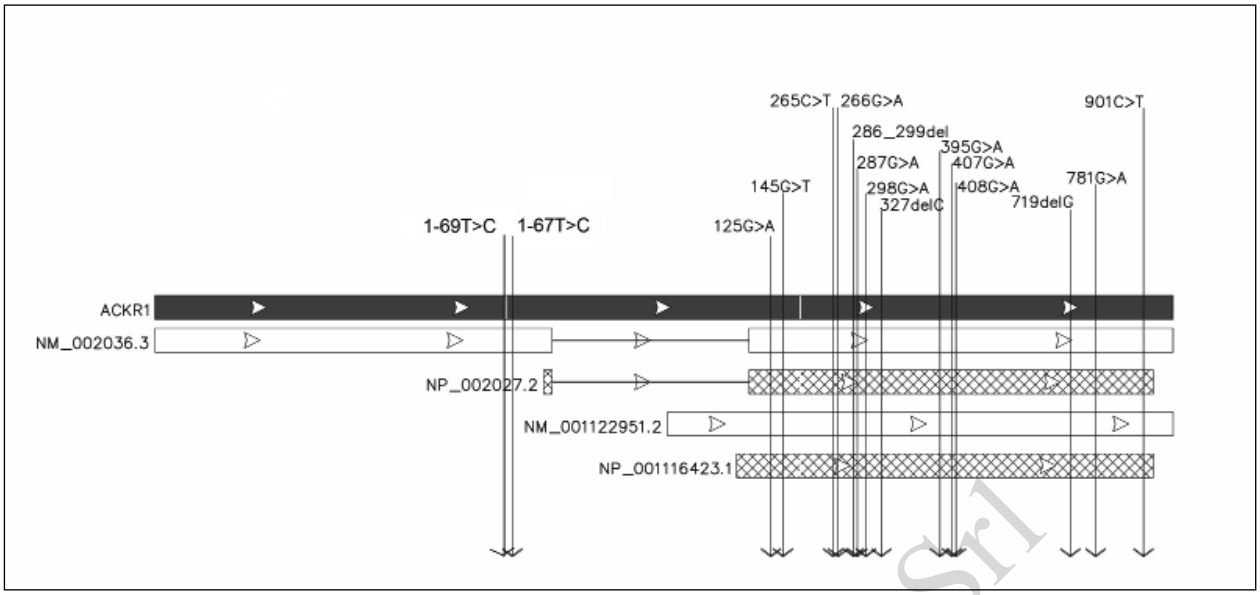

Figure 1 - *ACKR1* gene structure and proteins and mRNA isoforms.
Viewing the figure from top to bottom: the black box represents the *ACKR1* gene, the white box shows the mRNA of isoform B (NM_002036.3) and the dashed box shows the isoform B protein (NP_002027.2); the next white box represents the mRNA of isoform A (NM_001122951.2) and the dashed box shows the protein of isoform A (NP_001116423.1). The arrows indicate the positions of the main genetic variants already described in this gene.

acids and isoform B (NM_002036.3/NP_002027.2), which encodes a protein of 336 amino acids[51] (Figure 1).

The nucleotide and amino acid sequences of *ACKR1* were renumbered after the discovery that the spliced mRNA is the major product of the gene. It was proposed that the first nucleotide of the translation initiation codon of the major spliced mRNA be numbered nucleotide 1. This numbering convention avoids inconsistencies created by differing lengths of the 5-prime untranslated region arising from alternative transcription initiation sites[52]. This isoform B has been chosen as the "canonical" sequence that is known to be relevant for blood group genotyping because is expressed in erythroid lineage cells. All positional information in this review refers to isoform B.

The antithetical antigens, Fy^a and Fy^b, are encoded by co-dominant *FY*A (FY*01)* and *FY*B (FY*02)* alleles, which differ by a single nucleotide polymorphism c.125G>A (rs12075)[49,50]. On the *FY*A* allele, the base is guanine (G), and on the *FY*B* allele the base is adenine (A). This missense mutation produces a codon for glycine in the *FY*A* allele and a codon for aspartic acid in the *FY*B* allele at position 42 of the major product (p.Gly42Asp)[53-55], defining the Fy(a+b−), Fy(a−b+) and Fy(a+b+) phenotypes. Additionally, a number of variants have been identified that cause weak ($+^w$ or **W*) and null (0 or **N*) expression of Duffy Fy^a or Fy^b antigens (Table I). According to the ISBT, there are two mutations associated with weak expression of Fy^a and five mutations associated with weak expression of Fy^b. Seven mutations that cause the null expression of Fy^a have already been observed and three such mutations for Fy^b have been found. Despite the high genetic variability related to Duffy antigen production, some of these variants are more commonly associated with null or weak expression of these antigens than others, and they are described below.

The Fy(a−b−) phenotype, also known as "erythrocyte silent" (Fy^{es}), occurs in African lineages and, depending on the region, has a prevalence of nearly 100% (e.g. in West Africa) and is also found at greater than 80% frequency in African Americans[26,56]. This phenotype arises from homozygosity for an *FY*B* allele carrying a point mutation c.1-67T>C (rs2814778) in the 5' untranslated region[57]. This mutation gives rise to the $FY*B^{ES}$ (*FY*02N.01*) allele, which impairs promoter activity in erythroid cells by disrupting a binding site for the GATA-1 erythroid transcription factor[57]. This mutation prevents Fy^b antigen expression only in red blood cells but not in other tissues[53,58]. As a result, Africans with the Fy(a−b−) phenotype rarely make anti-Fy^b[50,59,60]. The same mutation (previously described as c.-33T>C and c.-46T>C) has been found on the $FY*A^{ES}$ allele (*FY*01N.01*), but only in a heterozygous state in inhabitants of Papua New Guinea and Sudan; it is very rare[35,61]. Písacka *et al.* reported a novel mutation at position c.1-69 in the *FY* promoter that also disrupts the GATA motif and correlates with silencing of the *FY*A* allele, causing a Fy null phenotype[62].

The few documented cases of the Fy(a−b−)

Table I – Variants of the Duffy blood group system.

Fy expression	Allele name	Nucleotide	Region	Amino acid	dbSNP
Fy(a+)	*FY*01*	c.125G	Exon 2	p.Gly42	rs12075
Fy(b+)	*FY*02*	c.125G>A	Exon 2	p.Gly42Asp	rs12075
		Null alleles			
Fy(a) null					
Fy(a−) erythroid cells only	*FY*01N.01*	c.1-67T>C	5'UTR	p.0	rs2814778
Fy(a−)	*FY*01N.02*	c.286_299del	Exon 2	p.Trp96Thrfs	rs587776507
Fy(a−)	*FY*01N.03*	c.408G>A	Exon 2	p.Trp136Ter	−
Fy(a−)	*FY*01N.04*	c.287G>A	Exon 2	p.Trp96Ter	rs750052723
Fy(a−)	*FY*01N.05*	c.327delC	Exon 2	p.Phe109fs	−
Fy(a−)	*FY*01N.06*	c.395G>A	Exon 2	p.Gly132Asp	rs530992295
Fy(a−)	*FY*01N.07*	c.719delG	Exon 2	p.Gly240fs	−
Fy(a−)	−	c.1-69T>C	5'UTR	p.0	−
Fy(b) null					
Fy(b−) erythroid cells only, Fy^{es}	*FY*02N.01*	c.1-67T>C	5'UTR	p.0	rs2814778
Fy(b−)	*FY*02N.02*	c.407G>A	Exon 2	p.Trp136Ter	rs76819093
Fy(b−)	*FY*02N.03*	c.781G>A	Exon 2	p.Gly261Arg	−
		Weak alleles			
Fy(a) weak					
$Fy(a+^{w})$	*FY*01W.01*	c.265C>T	Exon 2	p.Arg89Cys	rs34599082
$Fy(a+^{w})$	*FY*01W.02*	c.265C>T, 298G>A	Exon 2	p.Arg89Cys, Ala100Thr	rs34599082, rs13962
Fy(b) weak					
$Fy(b+^{w})$, Fy^{x}	*FY*02W.01*	c.265C>T, 298G>A	Exon 2	p.Arg89Cys, Ala100Thr	rs34599082, rs13962
$Fy(b+^{w})$, Fy^{x}	*FY*02W.02*	c.145G>T, c.265C>T, 298G>A	Exon 2	p.Ala49Ser, Arg89Cys, Ala100Thr	− rs34599082, rs13962
$Fy(b+^{w})$	*FY*02W.03*	c.266G>A	Exon 2	p.Arg89His	rs371909350
$Fy(b+^{w})$	*FY*02W.04*	c.901C>T	Exon 2	p.Pro301Ser	rs753831902

The nucleotide position is based on NCBI data. fs: frameshift; UTR: untranslated region.

phenotype in Europeans and Asians arise from mutations in the coding region of the *FY*A* or *FY*B* allele[55,63-65]. These mutations, when present in the homozygous state, prevent Duffy antigen expression on any cell in the body and thus are true Duffy null phenotypes. Consequently, these individuals are at risk of being alloimmunised when exposed to red blood cells expressing Fy antigens[66].

Different allelic forms of the Duffy blood group gene modify the antigen's expression level, which may cause problems in blood group phenotyping, leading, specifically, to very weak or equivocal serology typing results[66-69]. The most common variants, c.265C>T (rs34599082) and c.298G>A (rs13962), cause a substitution of arginine to cysteine at position 89 (p.Arg89Cys) and an alanine to threonine substitution at amino acid 100 (p.Ala100Thr) of glycoprotein Duffy, respectively. The aforementioned two variants usually result in the weak expression of Fy^{b} (*FY*02W.01* allele, also referred to as Fy^{x} antigen). This allele has been described mainly among Europeans, with a frequency varying from 2 to 3,5%; but it has not been found in Africans[69-73]. The c.298G>A variant alone does not result in reduced Fy^{b} expression[70]. Another mutation linked to weak expression of the antigen Fy^{b}, c.145G>T, changes the amino acid alanine to serine at position 49 (p.Ala49Ser), generating the *FY*02W.02* allele[69].

Although less common, weak serological reactivity of the Fy^{a} antigen has already been observed and Lopez *et al.* recently investigated this phenotype. They identified two variants of the *FY*A* allele, 265T and 298A, which were consistently present in the donor. Prior to this study, these variants had only been defined in combination with the *FY*B* allele. Reflecting these facts, *FY*01W.02* was the provisional name given to the new allele by the ISBT Working Party on Red Cell Immunogenetics and Blood Group Terminology[74]. One potential explanation for such complexity among Fy antigens could be the co-expression of alternative *ACKR1* gene product

Table II - Phenotype and genotype correlations of the main polymorphisms.

Phenotype	c.125G>A[1]	c.265C>T[2]	c.298G>A[3]	c.1-67T>C[4]	Predicted genotype	Predicted antigen
Fy(a+b−)	G/G	C/C	G/G	T/T	*FY*A/FY*A or FY*01/FY*01*	Fy^a
	G/A	C/C	G/G	T/C	*FY*01/FY*02N.01*	Fy^a/Fy^{es}
Fy(a+b+)	G/A	C/C	G/G	T/T	*FY*A/FY*B or FY*01/FY*02*	Fy^a/Fy^b
	G/A	C/T	G/A	T/T	*FY*01/FY*02W.01*	Fy^a/Fy^x
Fy(a−b+)	A/A	C/C	G/G	T/T	*FY*B/FY*B or FY*02/FY*02*	Fy^b
	A/A	C/T	G/A	T/T	*FY*02/FY*02W.01*	Fy^b/Fy^x
	A/A	T/T	A/A	T/T	*FY*02W.01/FY*02W.01*	Fy^x
	A/A	C/T	G/A	T/C	*FY*02W.01/FY*02N.01*	Fy^x/Fy^{es}
	A/A	C/C	G/G	T/C	*FY*02/FY*02N.01*	Fy^b/Fy^{es}
Fy(a−b−)	A/A	C/C	G/G	C/C	*FY*02N.01/FY*02N.01*	Fy^{es}/Fy^{es}

[1]rs12075 predicts the expression of the Fy^a and Fy^b antigens; [2]rs34599082 determines weak expression of the Fy^b antigen; [3]rs13962 determines weak expression of the Fy^b antigen; [4]rs2814778 prevents expression of Fy^b antigen in red blood cells.

Table III - Allele frequencies according to the 1000 Genomes Project.

dbSNP allele	AFR[1]	AMR[2]	EAS[3]	SAS[4]	EUR[5]
rs12075*G $Fy(a+)$	0.019	0.461	0.923	0.640	0.398
rs12075*A $Fy(b+)$	0.981	0.539	0.077	0.360	0.602
rs34599082*T $Fy(b+^w)$ or $Fy(a+^w)$	0.000	0.007	0.001	0.004	0.013
rs13962*A $Fy(b+^w)$ or $Fy(a+^w)$	0.005	0.094	0.000	0.091	0.184
rs530992295*A $Fy(a^{null})$	0.000	0.000	0.000	0.002	0.000
rs2814778*C $Fy(b^{null})$ or $Fy(a^{null})$	0.964	0.078	0.000	0.000	0.006

[1]AFR: African (from Yoruba in Ibadan, Nigeria; Luhya in Webuye, Kenya; Gambian in Western Divisions in the Gambia; Mende in Sierra Leone; Esan in Nigeria; Americans of African ancestry in the SW USA; African Caribbeans in Barbados); [2]AMR: Admixed Americans (Mexican ancestry from Los Angeles, USA; Puerto Ricans from Puerto Rico; Colombians from Medellin, Colombia; Peruvians from Lima, Peru; [3]EAS: East Asian (Han Chinese in Beijing, China; Japanese in Tokyo, Japan; Southern Han Chinese; Chinese Dai in Xishuangbanna, China; Kinh in Ho Chi Minh City, Vietnam); [4]SAS: South Asian (Gujarati Indians from Houston, Texas, USA; Punjabi from Lahore, Pakistan; Bengali from Bangladesh; Sri Lankan Tamils from the UK; Indian Telugu from the UK); [5]EUR: European (Utah residents with northern and western European ancestry from the Centre d'Etude du Polymorphisme Humain; Tuscany in Italy; Finnish in Finland; British in England and Scotland; Iberian population in Spain).

Table IV - Typical Duffy phenotype frequencies.

Phenotype	Frequencies (%)		
	Europeans	*Africans*	*Asians*
Fy(a+b−)	20	10	89,2
Fy(a−b+)	32	20	1,8
Fy(a+b+)	48	3	9,0
Fy(a−b−)	Rare	67	0

Data compiled from Mourant et al. and De Silva *et al.*[78,79].

isoforms and distinct post-translational modifications between the isoforms acting as immunogens[51]. The phenotypic and genotypic correlations of the main polymorphisms are presented in Table II.

Geographic distribution

It is characteristic of this blood group system that there is a great diversity of distributions of the Duffy antigenic determinants among different ethnic groups. Some of these variants were described in the 1000 Genomes Project database. According to these data, the rs12075*A single nucleotide polymorphism, which determines the *FY*B* ancestral allele (0.541), is more prevalent globally than the rs12075*G variant, which determines the *FY*A* allele (0.459). The *FY*B* allele is common in Africans (0.981), but not in East Asians (0.077). On the other hand, the *FY*A* allele is dominant in East Asians (0.923), but is infrequent in Africans (0.019). Finally, the allele frequencies of the variants that determine the Fy^x and Fy^{es} antigens are highest in Europeans and Africans, respectively (Table III).

The Duffy system is considered one of the most attractive chromosomal loci for evaluating the impact of natural selection in different geographical regions[75,76]. Because the mutation that confers protection from infection by *P. vivax* prevents the expression of the DARC protein only in erythrocytes, it is possible to observe differences in phenotypic and genotypic frequencies of the Fy^b antigen in Africans. Although the most common genotype is *FY*B/FY*B*, almost all of the samples type serologically as Fy(a−b−)[77]. Table IV presents possible phenotypes among different populations.

Clinical significance

The anti-Fy^a antibody is found mainly following transfusion and, less frequently, as a result of pregnancy; it is almost never naturally occuring[80,81].The anti-Fy^b

is about 20 times less common than the anti-Fy^a and is generally present in sera in combination with other antibodies[82].These antibodies are predominantly of the IgG1 type subclass, with lesser contributions from other subclasses, for example, IgG2 (18%) and IgM (25%)[83,84]. Both antibodies cause immediate and delayed haemolytic transfusion reactions[1,85].

The Fy^a and Fy^b antigens are expressed in erythroid and non-erythroid cells, such as endothelial cells, and also in epithelial cells in various organs, including the brain, kidneys, spleen, heart, lungs, pancreas and placenta[86] - lending this system an important role in the inflammatory response, in allograft rejection and, possibly, in histocompatibility. The Duffy blood group is thus a polymorphic system that poses a major challenge for researchers at phenotypic, genotypic and tissue levels[87].

Conclusions

In view of the importance of the Duffy blood group system in clinical medicine, further studies utilising molecular biology approaches must be developed for the purpose of elucidating and characterising new sequence variants. Such molecular typing can help resolve and clarify the equivocal and discrepant results that arise in erythrocyte phenotyping performed by haemagglutination assays. These techniques, used together, contribute to the optimal use of blood units and, therefore, to the quality of transfusion practice.

The Authors declare no conflicts of interest.

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CAPÍTULO II. *Identification of ACKR1 variants associated with altered Duffy phenotype expression in blood donors from the Southern Brazil*

- Artigo a ser submetido:

Identification of ACKR1 variants associated with altered Duffy phenotype expression in blood donors from the Southern Brazil

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Identification of *ACKR1* variants associated with altered Duffy phenotype expression in blood donors from the Southern Brazil

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

SHORT RUNNING TITLE: *ACKR1* variants associated with altered Duffy phenotype expression

Abstract

Background and Objectives: The atypical chemokine receptor 1 gene (*ACKR1*) is responsible for the clinically significant Duffy blood group. The mainly antigens of this system, Fy^a and Fy^b, can be related with null or weak expression of the DARC protein. In the present work, we aimed identify *ACKR1* gene variants in Southern Brazil blood donors from discrepancies between their serological and molecular typing results, and analyze the association of these variants with Duffy phenotype expression.

Materials and Methods: The sample composed by 382 regular repetitive voluntary blood donors in Blood Bank of Hospital de Clínicas de Porto Alegre (HCPA) was determined by haemagglutination and real-time PCR (c.125G>A, c.265C>T and c.1-67T>C SNPs) tests to typing the Fy antigens. Inconclusive correlation between phenotype-genotype analyses was found in 11 (2.88%) donors and the entire *ACKR1* gene was sequenced in these samples.

Results: Our investigation accounted 11 genetic variants from which 4 (c.-541C>T, c.21+150C>T, c.22-58A>G and c.298G>A SNPs) seems to having putative functional effects on structure and expression of DARC undertaken for in silico analysis (SIFT, PolyPhen-2 and RegulomeDB).

Conclusion: In a general way, molecular events result in the red blood cell genotype and phenotype being apparently discrepant. The mechanisms for alterations on gene expression of the variants detected in this study remain to be investigated and our findings should provide new insights into understanding the molecular background of blood typing for support the technical approaches developing in the transfusion medicine.

Key words: Duffy blood group system, DARC protein, Fy phenotype-genotype correlation, *ACKR1* variants

Introduction

Duffy blood group system is clinically significant because can cause haemolytic transfusion reaction (HTR) and haemolytic disease of the fetus and newborn (HDFN) [1]. Duffy antigen receptor for chemokines (DARC) is a transmembraneous glycoprotein found on erythrocytes and other cells, playing a role in inflammation and malaria infection [2]. The *ACKR1* (atypical chemokine receptor 1) gene, also termed *FY*, which encodes the protein is located on chromosome 1q23.2 and have two exons.

Two allelic forms, *FY*01* (*FY*A*) and *FY*02* (*FY*B*), differ by a single nucleotide polymorphism (SNP). The *FY*02* is the reference allele and the *FY*01* allele arises from a c.125A>G (rs12075) on exon 2, which generates an p.Asp42Gly associated with Fy^b and Fy^a antithetical antigens, respectively, defining the Fy(a+b−), Fy(a−b+) and Fy(a+b+) phenotypes [3-5]. Besides this aminoacid substituition, rs2814778, rs34599082 and rs13962 polymorphisms are other common found variants associated with Fy antigen expression. The point mutation c.1-67T>C (rs2814778) in 5' untranslated region (UTR) impairs promoter activity in red blood cell (RBC) preventing the Fy^b antigen expression, also referred to as Fy^{es} [6]. In addition, the missense mutations, c.265C>T (rs34599082) and c.298G>A (rs13962), cause a substitution of arginine to cysteine at position 89 (p.Arg89Cys) and an alanine to threonine substitution at amino acid 100 (p.Ala100Thr) of DARC resulting in the weak expression of Fy^b

antigen, Fy^x [7-9]. Although less common, both variants has already been observed in combination with the *FY*A* allele [10].

Duffy blood group is important in several fields of science including transfusion medicine, immunology and malariology. Therefore, a number of studies have revised the Duffy phenotype, genotype and allele frequencies in different ethnic groups of the world [11] as well as the molecular background of this system [12]. Additionally, *ACKR1* variants have been classified into weak and null types. There are at least three mutations associated with weak expression of Fy^a and five mutations associated with weak expression of Fy^b. Nine mutations that cause the null expression of Fy^a have already been observed and six such mutations for Fy^b have been found according to International Society of Blood Transfusion (ISBT).

The investigation of RBC genetic variants in multi-ethnic populations as Brazil [13,14,15] is of interest to expand the molecular characterization, supporting the quality management in transfusion medicine. The objectives of this study were to identify *ACKR1* gene variants in Southern Brazil blood donors from discrepancies between their serological and molecular typing results, and analyze the association of these variants with Duffy phenotype expression.

Materials and Methods

Subjects

Whole blood samples were collected from 382 regular repetitive voluntary blood donors in Blood Bank of Hospital de Clínicas de Porto Alegre (HCPA), which is located in in the southern Brazilian state of Rio Grande do Sul (30°01′59″S 51°13′48″W), between 2012 and 2015. Considering the ethnic diversity of our population, the random and unrelated sample was clustered by

white (n= 334) and non-white (n= 48) subjects, according to the following phenotypic characteristics: color and texture of hair; skin color in the medial part of the arm; and the shape of the nose and lips [14].

The study protocol was approved by the institutional review board from HCPA (No. 110418) and Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) (No. 1829-12). All blood donors signed a written informed consent.

Serological and molecular RBC typing

Fy phenotypes were determined by haemagglutination using indirect antiglobulin test (IAT) in gel cards (Bio-Rad Laboratories, Hercules, CA), conducted essentially as the blood center practices. Polyclonal antisera commercially available (Lorne Laboratories, Berkshire, United Kingdom) were used to type the Fy^a and Fy^b antigens.

Genomic DNA was extracted from peripheral whole blood by a standard salting-out procedure [16]. Concentration and purity of DNA samples were analyzed by optical density (BioSpec-Nano, Shimadzu, Columbia, MD), diluted to 10 ng/µL and then stored at -20°C for long-term storage. To prediction of Duffy blood group antigens: Fy^a, Fy^b, Fy^x and Fy^{es}, oligonucleotide primers and TaqMan probe for the c.125G>A (rs12075), c.265C>T (rs34599082) and c.1-67T>C (rs2814778) SNPs were synthesized (ID: C_2493442_10, C_11324554_10, C_15769614_10, respectively) by Thermo Fischer Scientific, Waltham, MA. The fluorescence data were analyzed by allelic discrimination assays in real-time polymerase chain reaction (PCR) apparatus (StepOnePlus Real-Time PCR System; Applied Biosystems, Foster City, CA).

Nucleotide sequencing analysis

Genotype results were electronically displayed alongside phenotypes in a database. This comparison created a report of phenotype-genotype discordances. We found discordant results in 11 (2.88%) blood donors, of which nine white and two non-white individuals. The *ACKR1* gene was sequenced in these samples and for sequencing five primer pairs were designed (Primer3 v0.4.0) to cover the entire *ACKR1* gene (Table 1).

PCR amplification was carried out in a thermal cycler (model 2720; Applied Biosystems) with 10 ng of DNA, 10 pmol of each primer, 0.2 mM of dNTP, 1.5 mM $MgCl_2$, 1.0 U Platinum *Taq* DNA polymerase and buffer, in a final volume of 25 μL. The PCR profile used in assays was as follow: 5 minutes of initial denaturation at 94°C, 35 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 72°C for 1 minute each with a final extension step of 7 minutes at 72°C. Annealing temperature for fifth sequenced fragment was 60°C. After PCR clean-up with an enzymatic treatment (ExoSAP-IT), sequencing reactions were conducted with a cycle sequencing kit on a DNA sequencer (BigDye Terminator v3.1 and 3130 Genetic Analyser, respectively, Applied Biosystems) in accordance to default operating parameters.

Bioinformatic analysis

ACKR1 blood donors' sequences were compared on Clustal X v2.1 to the consensus sequence, NCBI RefSeq NG_011626.2 (range 159,204,013-159,206,500) and DNA mutation numbering was based with nucleotide +1 corresponding to the A of the ATG translation initiation codon the coding sequence (CDS) NM_002036.3. The computational tools PolyPhen-2

(Polymorphism Phenotyping v2) [17] and SIFT (Sorting Intolerant from Tolerant) [18] were used to predict possible impact of an amino acid substitution on protein structure and function as well as RegulomeDB [19] was used to identify DNA features and regulatory elements in non-coding regions in *ACKR1* gene undertaken for in silico analysis.

Results

We performed the sequence of 2488 nucleotides of the *ACKR1* gene, consisting in analyses of the 1009 bp in two exons, 948 bp of the 5'-untranslated region (UTR), 50 bp of the 3'-UTR, and 481 bp of the intron adjacent. From 11 blood donor samples with inconclusive correlation between phenotype-genotype analyses (Table 2), our investigation accounted 11 variations distributed throughout the whole gene. Although these polymorphisms had already been described in the Database of Short Genetic Variations (dbSNP – National Center for Biotechnology Information), only two SNPs (c.125G>A and c.1-67T>C) are recognized as functional allele in ISBT. All the variants identified were confirmed by repeating the PCR and sequencing reactions and their characterization are summarized in Table 3. Out of total, two were in the CDS, one in the upstream gene, four in 5'-UTR, and four in the intron region. A map of the gene highlighting the positions of each of these variants is shown in Fig. 1.

Subsequently, in Table 4 was demonstrated for all 11 donors with phenotype-genotype discrepancies: (1) the Duffy phenotype obtained by serological typing, (2) SNP-based typing and (3) the genetic variant detected by sequencing that might explain the equivocal correlations between phenotype-

genotype, since these variants did not be associated, previously, with effect on DARC protein expression. There was 100% concordance for all genotype calls between the Sanger sequencing and the real-time PCR methodology to determine the known c.125G>A, c.265C>T and c.1-67T>C SNPs.

Discussion

In this current study, we performed a screening of genetic variations by direct sequencing of the ACKR1 gene that may be associated with phenotype-genotype discrepancies in blood donors. Analyses on conserved non-coding region have shown that non-coding DNA is involved in biological functions [20]. These non-coding elements can have various regulatory functions within the genome, such as interacting with transcription factors (TFs), miRNA, creating splice sites and acting as exonic splicing enhancers (ESEs) [21]. Despite much less effort has been invested in the functional analysis of non-coding SNPs as compared to the coding regions SNPs, recently, studies have begun to reported mutations in splicing site and promoter region as underlying mechanisms to be involved in the altered expression of the blood group phenotypes [22-24].

We identified in one of the sequenced samples the heterozygous point mutation, c.-541C>T (rs3027013), located in the 5' UTR of *ACKR1*. The variant was classified as "likely to affect binding" by RegulomeDB tool and related to the null expression of the Fy^a antigen in present study, may arising a new functional allele linked to silenced *FY*A* allele. SNPs in gene regulatory regions such as promoter and enhancer regions can affect gene expression by altering binding affinities to transcription factors [25] and we hypothesized that the

aforementioned variant could have functional significance to alter the DARC protein stability and expression.

Additionally, it was detected from intronic region, the c.21+150C>T (rs863002) variant in six donors while five donors presented the c.22-58A>G (rs3027016) variant in heterozygosity, suggesting that these variations might alter the transcription and/or RNA processing of Duffy antigen. On the RegulomeDB both variants also were classified as “likely to affect binding”. These alterations may to modify the protein structure and, consequently, to alter solubility and/or stability, generating either a null or weakened *FY*A* or *FY*B* allele. The precise splicing outcome of the mRNA is controlled by complex interactions and has been known genetic variations that affect splicing regulatory element leading to differences in alternative splicing between human individuals and consequently impact expression level or protein function [26].

Moreover, we also observed the missense variant c.298G>A (rs13962) in heterozygous state in two donors. The variant, when combined with the SNP c.265C>T, usually result in the weak expression of Fy^b antigen [9]. However, in our study, these donors were serological typing as Fy(b+^{w}) carried only the variant 298*A. This Ala100Thr amino acid change was predicted to be “possibly damaging” by PolyPhen-2 and “tolerated” by SIFT software’s, suspecting that this results in quantitatively reduced Fy^b antigen density on the RBC surface.

Thus, our findings indicate that 4 SNPs (c.-541C>T, c.21+150C>T, c.22-58A>G and c.298G>A) of *ACKR1* gene that haven’t been explored much till date seems to having putative functional effects on structure and expression of DARC. Overall, sequence-based typing can improve the accuracy by signaling weak antigen expression for samples phenotypic as Fy(b-) or Fy(a-), probably

not detected by serologic reagents. These discrepancies are mainly associated with single-dose Fy^b or Fy^a expression that are not originally detected as positive, therefore, discrepant results suggest to be related with false-negative historical serologic types. On the other hand, SNP genotyping predict incorrectly the phenotype for cases carrying silenced *FY*A* or *FY*B* allele. This represents an example of caution in molecular typing; that is, simplex PCR tests targeting only the known polymorphisms could lead to erroneous assignment due to the presence of additional mutations, especially in populations in which the genotyping assays are not validated and the prevalence of variant alleles is not comprehensively studied [27]. Variants leading to altered expression of antigens could be missed and this may lead to transfusion mismatches with potentially adverse outcome [27].

In conclusion, molecular events result in the RBC genotype and phenotype being apparently discrepant by gathering more information. The mechanisms for alterations on gene expression of the variants detected in this study remain to be investigated and may be useful for different studies, given the importance of the Duffy blood group system in human health. To date, clinical significance of these phenotypes expressed have yet to be described with regard to either RBC immunogenicity or alloimmunization risk and, thus, would be of interest to better analyze the epitopes associated with these genotypes that predict phenotypes besides their frequencies in different populations. Our findings should provide new insights into understanding the molecular background of blood typing for support the technical approaches developing in the transfusion medicine.

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The dedicated and enthusiastic work performed by Ananda Galvão (*in memory*), is greatly acknowledged. The contribution of each author to the manuscript was as follows: SA, TO and MF: designed research and conceived the project. GW and MMOR: performed the data collection. GH, MMOR, GW, GA, PVVG, MF and SA: carried out the research. GH, MMOR, GW, MF and SA: conducted literature search and analyzed data. GH, MMOR, MF and SA: performed data interpretation and wrote the manuscript; SA had the primary responsibility for final content. All authors had final approval of the submitted and published versions. We are also thankful to FAPERGS (grant number 2373-2551/14-4) for supporting this work.

Web-based resources

The following Web-based resources were used: International Society for Blood Transfusion. Blood Group Allele Tables – FY (ISBT 008), http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/; Primer3 Input, http://bioinfo.ut.ee/primer3-0.4.0; Clustal X, http://www.clustal.org/clustal2/; GenBank, https://www.ncbi.nlm.nih.gov/nuccore/NG_011626.2?from=4294&to=6781&report=genbank; PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/; SIFT, http://sift.bii.a-star.edu.sg/; RegulomeDB, http://regulomedb.org. All URLs were accessed on April, 2017.

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Figure Legends

Fig. 1. Map of *ACKR1* schematic representation. The gene is located at chromosome 1q23.2 and consists of two exons encoding a protein of 336/338 amino acids. The exons (black boxes) are shown schematically along with their 5'-UTR, 3'-UTR, and the intron (black lines). The locations of five amplification reactions covering the complete *ACKR1* are indicated (dashed lines). The positions of the 11 variations (SNPs) are indicated (│).

Table Legends

Table 1

F = forward; R = reverse.

Table 2

n = number of blood donors with the discrepancy phenotype-genotype.

Table 3

Data compiled from Ensembl and NCBI; NA = not available.

* Global minor allele frequency.

† Polymorphism Phenotyping v2: Score 0.00-0.452 = benign, 0.453-0.956 = possibly damaging, 0.957-1.00 = probably damaging.

‡ Sorting Intolerant from Tolerant: Score ≤ 0.05 = damaging, > 0.05 = tolerated.

§ Score 1 = likely to affect binding and linked to expression of a gene target, 2 = likely to affect binding, 3 = less likely to affect binding, and 4, 5 and 6 = minimal binding evidence.

Table 4

* SNP predicted phenotype from c.125G>A, c.265C>T and c.1-67T>C genetic variants

† Blood donor sample ID.

‡ Genetic variants detected in sequencing screening with RegulomeDB score: ≤ 2 and Polymorphism Phenotyping v2 score: ≥ 0.453.

Fig. 1.

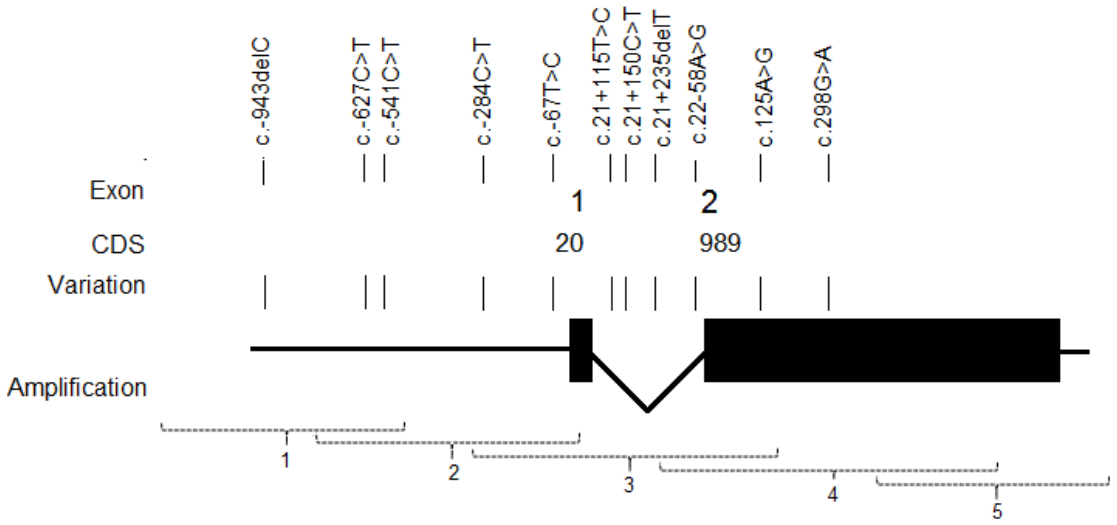
c.-943delC
c.-627C>T
c.-541C>T
c.-284C>T
c.-67T>C
c.21+115T>C
c.21+150C>T
c.21+235delT
c.22-58A>G
c.125A>G
c.298G>A
Exon
1
2
CDS
20
989
Variation
Amplification
1
2
3
4
5

TABLE 1. Oligonucleotide primers used in this study

Primer name	Nucleotide sequence (5’ to 3’)	Amplicon size (bp)
FY-1F	TTGTTCTGACTTGGACACGC	588
FY-1R	TCTGTATGTGAGGGGCTTGG	
FY-2F	CACTCTCACACTGAAACACCC	599
FY-2R	CTTACCCCACGCCCACTG	
FY-3F	TGTAGTCCCAACCAGCCAAA	653
FY-3R	GAGGACAGAGAGGAAGGTCG	
FY-4F	CCCTGCTTTGTCCTTTTCCA	675
FY-4R	GCAGCCACTCCCCAAATTC	
FY-5F	TGCTAGGGTGCCATGCCT	600
FY-5R	TTCAGGTTGACAGGTGGGAA	

TABLE 2. Duffy blood group phenotype–genotype correlations

Observed phenotype	Genotype assignment			Final genotype	SNP predicted phenotype	*n*
	c.1-67T>C	c.125A>G	c.265C>T			
Fy(a-b+)	T/T	A/G	C/C	*FY*01/FY*02*	Fy(a+b+)	3
Fy(a+b-)	T/T	A/G	C/C	*FY*01/FY*02*	Fy(a+b+)	3
Fy(a+b+^{w})	T/T	A/G	C/C	*FY*01/FY*02*	Fy(a+b+)	4
Fy(a-b+^{w})	T/C	A/A	C/C	*FY*02/FY*02N.01*	Fy(a-b+)	1

TABLE 3. Variants characterization

dbSNP	Chr:bp	Position	Class	Region	GMAF*	PolyPhen-2†	SIFT‡	RegulomeDB§
rs397752376	1:159204017	c.-943delC	Deletion	Upstream gene	0.091 (–)	–	–	4
rs3027012	1:159204333	c.-627C>T	SNP	5’ UTR	0.091 (T)	–	–	4
rs3027013	1:159204419	c.-541C>T	SNP	5’ UTR	0.032 (T)	–	–	2b
rs939553080	1:159204676	c.-284C>T	SNP	5’ UTR	–	–	–	–
rs2814778	1:159204893	c.1-67T>C	SNP	5’ UTR	0.266 (C)	–	–	2a
rs7550207	1:159205095	c.21+115T>C	SNP	Intron	0.118 (C)	–	–	4
rs863002	1:159205130	c.21+150C>T	SNP	Intron	0.201 (T)	–	–	2b
rs17838198	1:159205215	c.21+235delT	Deletion	Intron	0.222 (T)	–	–	3a
rs3027016	1:159205403	c.22-58A>G	SNP	Intron	0.057 (G)	–	–	2c
rs12075	1:159205564	c.125A>G	SNP	Exon 2	0.459 (G)	0	0.89	3a
rs13962	1:159205737	c.298G>A	SNP	Exon 2	0.069 (A)	0.763	0.23	5

TABLE 4. Genotypes for each donor with discrepancies between phenotype–genotype for variants with probably functional effects in silico analysis

Observed phenotype	SNP predicted phenotype*	ID†	Genetic variants ‡			
			c.-541C>T	c.21+150C>T	c.22-58A>G	c.298G>A
Fy(a-b+)	Fy(a+b+)	24	C/C	C/T	A/G	G/G
		106	C/T	C/C	A/A	G/G
		374	C/C	C/T	A/A	G/G
Fy(a+b-)	Fy(a+b+)	21	C/C	C/T	A/G	G/G
		91	C/C	C/C	A/G	G/G
		368	C/C	C/C	A/G	G/G
Fy(a+b+^{w})	Fy(a+b+)	233	C/C	C/T	A/A	G/G
		264	C/C	C/C	A/G	G/G
		297	C/C	C/T	A/A	G/G
		339	C/C	C/T	A/A	G/A
Fy(a-b+^{w})	Fy(a-b+)	295	C/C	C/C	A/A	G/A

CONSIDERAÇÕES FINAIS

A descoberta do sistema de grupo sanguíneo ABO por Karl Landsteiner em 1900 foi uma das marcas mais importantes na medicina no século XX (Landsteiner, 1900; Camp & Ellis, 1966). Até a década de 1990, a triagem, a tipagem e o diagnóstico do sangue dependiam inteiramente de técnicas sorológicas. Por mais de um século, a hemaglutinação se mantém como o padrão-ouro para a detecção de antígenos eritrocitários, usada em todos os serviços de sangue. Este clássico método apresenta limitações que podem ser supridas por testes moleculares (Monteiro *et al*., 2011).

A caracterização dos genes e a determinação das bases moleculares de antígenos tornaram possível a detecção de alelos que codificam grupos sanguíneos. Nos últimos anos, pesquisas têm revelado que os sistemas de grupos sanguíneos apresentam uma grande diversidade alélica, semelhante à observada para HLA. De acordo com o banco de dados, *Blood Group Antigen Gene Mutation* (BGMUT), de variações nos genes que codificam antígenos dos sistemas de grupo sanguíneos, mais de 1.500 alelos são conhecidos (Patnaik *et al*., 2014). Atualmente, tem havido um grande progresso no entendimento dos eventos moleculares que levam à diversidade dos antígenos de grupos sanguíneos. Alguns destes mecanismos são: mutação de ponto, deleção, inserção, mecanismos de *splicing* alterados, *crossing over* intragênico, *crossing over* intergênico, conversão gênica e outros rearranjos gênicos, impactando na expressão de proteínas (Cartron *et al*., 1998; Storry & Olsson, 2004; Daniels, 2009). A aplicação deste conhecimento na predição da tipagem sanguínea possui impacto na medicina transfusional (Elizabeth, 2008; Reid, 2009) bem como no diagnóstico de DHRN (Araújo *et al*., 2003; Daniel *et al*., 2009).

A diversidade entre os antígenos é resultado de variações nas sequências dos genes que direta ou indiretamente afetam a expressão ou a natureza do epítopo de antígenos na superfície das hemácias. Para qualquer sistema de grupo sanguíneo, apenas um ou alguns fenótipos comuns predominam na população (Broadberry & Lin, 1996). No entanto, a necessidade de testar a compatibilidade das unidades de sangue na prática transfusional tem levado a

identificação de, até mesmo, variantes extremamente raras de tipos sanguíneos (Patnaik *et al*., 2014). As aparentes discrepâncias entre o fenótipo e genótipo do indivíduo são atribuídas a alterações no gene que afetam sua transcrição quando a genotipagem busca detectar a presença de um gene cuja proteína não está expressa, ocasionando a não expressão do antígeno na superfície das hemácias. Nessas situações, a análise estendida do DNA pela tipagem baseada em sequenciamento é a maneira mais confiável de definir as variações genéticas que possam causar fenótipos inconclusivos (Seltsam & Doescher, 2009).

Presumindo que o genótipo reflete o fenótipo, alguns pesquisadores defendem que a medicina transfusional poderia adotar um processo 'Genótipo ao Fenótipo', onde a base genética definida dos alelos do grupo sanguíneo é reconhecida como seu fenótipo (Avent *et al*., 2015). Desta forma, estudos adicionais que caracterizem o impacto funcional de variantes genéticas associadas a predição de novos alelos de grupos sanguíneos são fundamentais para o desenvolvimento das plataformas de genotipagem, que estão comercialmente disponíveis através de tecnologias de alto rendimento e com poderosa análise de dados, oportunizando os bancos de sangue a tornar rápida e eficiente a tipagem de doadores e pacientes em grande escala.

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ANEXO I – Parecer do Comitê de Ética em Pesquisa HCPA

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
GRUPO DE PESQUISA E PÓS-GRADUAÇÃO

COMISSÃO CIENTÍFICA E COMITÊ DE ÉTICA EM PESQUISA

A Comissão Científica e o Comitê de Ética em Pesquisa do Hospital de Clínicas de Porto Alegre (CEP/HCPA), que é reconhecido pela Comissão Nacional de Ética em Pesquisa (CONEP)/MS e pelo Office For Human Research Protections (OHRP)/USDHHS, como Institutional Review Board (IRB00000921) analisaram o projeto:

Projeto: 110418
Data da Versão do Projeto: 24/10/2011
Data da Versão do TCLE: 24/10/2011

Pesquisadores:
TOR GUNNAR HUGO ONSTEN
SILVANA DE ALMEIDA
ANANDA CRISTINE SANTOS GALVÃO
LEDA MARIA TEIXEIRA DE CAMPOS

Título: Biologia Molecular de Grupos Sanguíneos: Impacto sobre a prática Transfusional.

Este projeto foi APROVADO em seus aspectos éticos e metodológicos, bem como o seu respectivo Termo de Consentimento Livre e Esclarecido, de acordo com as diretrizes e normas nacionais e internacionais de pesquisa clínica, especialmente as Resoluções 196/96 e complementares do Conselho Nacional de Saúde.

- Os membros da Comissão Científica e do Comitê de Ética em Pesquisa não participaram do processo de avaliação dos projetos nos quais constam como pesquisadores.
- Toda e qualquer alteração do projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente ao CEP/HCPA.
- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao CEP/HCPA.
- Somente poderá ser utilizado o Termo de Consentimento Livre e Esclarecido no qual conste o carimbo de aprovação do CEP/HCPA.

Porto Alegre, 16 de dezembro de 2011.

Profª Nadine Clausell
Coordenadora GPPG e CEP/HCPA

ANEXO II – Parecer do Comitê de Ética em Pesquisa UFCSPA

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: INVESTIGAÇÃO DA VARIABILIDADE DO GENE ACKR1 EM DOADORES DE SANGUE DE PORTO ALEGRE/RS

Pesquisador: Silvana de Almeida

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

Versão: 2

CAAE: 49490315.7.0000.5345

Instituição Proponente: Universidade Federal de Ciências da Saúde de Porto Alegre

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.287.791

Apresentação do Projeto:

Trata-se de um projeto de mestrado que objetiva investigar a presença de variações no gene ACKR1 em doadores de sangue do Hospital de Clínicas de Porto Alegre. Serão utilizadas amostras previamente coletadas de 403 doadores voluntários de ambos os sexos que frequentam o Banco de Sangue do Hospital de Clínicas de Porto Alegre (HCPA) e que foram considerados aptos à doação. A amostra compõe o estudo "Biologia Molecular de Grupos Sanguíneos: Impacto sobre a prática Transfusional", avaliado e aprovado pelo Comitê de Ética em Pesquisa da Universidade Federal de Ciências da Saúde de Porto Alegre (Parecer 1829-12) e pelo Comitê de Ética do Hospital de Clínicas de Porto Alegre (Parecer 110418). Todos os participantes já assinaram ao Termo de Consentimento Livre e Esclarecido (TCLE), autorizando a utilização das amostras para futuros estudos sobre o mesmo assunto. As análises fenotípicas dos antígenos do grupo sanguíneo Duffy foram realizadas através da técnica de Hemaglutinação em Gel no Setor de Imunohematologia do Banco de Sangue do Hospital de Clínicas de Porto Alegre. A avaliação das variantes genéticas será realizada no Laboratório de Biologia Molecular da Universidade Federal de Ciências da Saúde utilizando a metodologia de análise PCR-HRM (polymerase chain reaction high-resolution melting).

Objetivo da Pesquisa:

Objetivo geral: Investigar a presença de variações no gene ACKR1 em doadores de sangue do Hospital de Clínicas de Porto Alegre.

Objetivos específicos:

• Determinar a frequência dos polimorfismos, já descritos, 125G>A, 265C>T, 298G>A, -67T>C, em doadores de sangue do Hospital de Clínicas de Porto Alegre.

• Realizar um rastreamento molecular para identificação de novas mutações e polimorfismos no gene ACKR1.

• Avaliar a correlação entre a determinação do genótipo e do fenótipo dos doadores.

Avaliação dos Riscos e Benefícios:

Não há riscos aos sujeitos de pesquisa considerando que as amostras já foram coletadas, de forma consentida pelos doadores, autorizando a utilização do excedente para estudos futuros de mesma natureza. O benefício inclui a ampliação do conhecimento dos polimorfismos envolvidos na transfusão de sangue.

Comentários e Considerações sobre a Pesquisa:

Pesquisa de extrema relevância, derivada de estudo anterior.

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos de apresentação obrigatória foram apresentados de forma adequada em relação aos aspectos éticos. Incluindo as autorizações dos responsáveis pelos locais de realização das análises.

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

A pesquisa está adequada para ser desenvolvida até julho de 2017.

Considerações Finais a critério do CEP:

De acordo com o parecer do Relaor

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_610610E1.pdf	16/10/2015 15:54:05		Aceito

Outros	ParcProjAntUFCSPA18291289211.pdf	23/09/2015 15:53:17	Silvana de Almeida	Aceito
Outros	CartadeAprovaProjAnteriorHCPA.pdf	23/09/2015 15:51:24	Silvana de Almeida	Aceito
Outros	ProjetoAnteriorGenotipagem_CEP_UFCSPA.pdf	23/09/2015 15:50:23	Silvana de Almeida	Aceito
Outros	TermodeCompromissoEntregaRelatorio.pdf	23/09/2015 15:48:09	Silvana de Almeida	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	110418_TCLE_DOADORES.pdf	23/09/2015 15:47:14	Silvana de Almeida	Aceito
Declaração de Instituição e Infraestrutura	TermodeAnuenciaAnexoI.pdf	23/09/2015 15:46:47	Silvana de Almeida	Aceito
Projeto Detalhado / Brochura Investigador	ProjetoMestradoGabrielaHoher_CEP.pdf	23/09/2015 15:45:37	Silvana de Almeida	Aceito
Folha de Rosto	FolhadeRostoGabrielaHoher.pdf	23/09/2015 15:43:11	Silvana de Almeida	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

PORTO ALEGRE, 20 de Outubro de 2015

Assinado por:
Julia Fernanda Semmelmann Pereira Lima
(Coordenador)

ANEXO III – Termo de Consentimento Livre e Esclarecido

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Título do projeto: "Biologia Molecular de Grupos Sanguíneos – Impacto na Prática Transfusional".

I. Por que realizar essa pesquisa e qual o objetivo dela?

Com o aumento do uso de transfusões sanguíneas em cirurgias, transplantes e tratamento clínico do câncer, passou-se a observar um aumento da ocorrência de rejeição ao sangue recebido nos pacientes que necessitam de várias transfusões. O objetivo deste estudo é verificar através de testes genéticos (testes que analisam o DNA) qual o melhor sangue para transfundir determinado paciente.

II. Qual o procedimento que será utilizado?

Uma pessoa treinada coletará 8ml (volume de 2 colheres de chá) de sangue. O tubo com o sangue será identificado apenas por números e será encaminhado ao laboratório para a análise do DNA (DNA é um componente do nosso corpo que herdamos de nossos pais e que é responsável pela determinação de todas as nossas características, como por exemplo a cor da pele, cor dos olhos e cabelos). Também serão acompanhados os resultados dos exames, relacionados ao tratamento, que você realizar antes e após a transfusão com o sangue analisado pelos testes genéticos.

III. O que acontecerá com a minha amostra?

Você poderá autorizar ou não que ao final desse trabalho as amostras de DNA sejam guardadas para, eventualmente, serem utilizadas em futuras pesquisas sobre esse assunto. A utilização em estudos futuros somente será realizada mediante nova aprovação do Comitê de Ética em Pesquisa.

IV. Quais os riscos em participar?

O risco desta pesquisa está associado à coleta de sangue que será realizada em uma das veias do braço, com material descartável, podendo causar desconforto semelhante a uma injeção na veia e em alguns casos deixar uma mancha roxa, que habitualmente melhora em algumas horas ou poucos dias. Para minimizar este risco as coletas serão realizadas apenas por profissionais treinados e capacitados a fazê-lo e será feita juntamente com a doação de sangue.

V. Quais os benefícios para o participante deste estudo?

Com a análise, poderemos saber qual o seu tipo sanguíneo com mais detalhes, o que pode trazer benefícios em relação a transfusões futuras, caso você venha a necessitar, pois poderão ser utilizadas unidades de sangue ainda mais parecidas com o seu tipo sanguíneo, do que é possível atualmente. No entanto, somente em longo prazo poderemos saber todos os benefícios deste estudo .

VI. Com quem você pode esclarecer suas dúvidas?

Você ficará com uma cópia deste Termo e toda a dúvida que você tiver a respeito desta pesquisa, poderá perguntar diretamente para o médico responsável pelo Serviço de Hemoterapia do Hospital de Clínicas de Porto Alegre, Tor Gunnar Hugo Onsten Doutor em Ciências da Saúde, responsável pelo estudo no HCPA, pelo telefone (51) 3359-7650 ou Dra. Silvana de Almeida, responsável pelo estudo na UFCSPA (51) 3303-8763. Dúvidas a respeito da ética dessa pesquisa poderão ser questionadas ao Comitê de Ética em Pesquisa do HCPA (51) 3359-7640, 2º andar, sala 2227 ou CEP da UFCSPA 3303-8804.

VII. Quais são os seus direitos?

A sua participação no estudo é voluntária. Caso você decida não participar, isto não afetará no tratamento normal que você tem direito. Além disso, você tem liberdade para abandonar a pesquisa a qualquer momento sem nenhum prejuízo, devendo apenas contatar o responsável pelo estudo. Os seus registros médicos serão sempre tratados confidencialmente. Os resultados deste estudo poderão ser usados para fins científicos, mas você não será identificado pelo nome. Você não terá nenhum gasto devido à sua participação na pesquisa.

Eu,_____fui informado dos objetivos da pesquisa acima de maneira clara e detalhada. Recebi informação a respeito do procedimento e esclareci minhas dúvidas. Sei que em qualquer momento poderei solicitar novas informações e terei liberdade de retirar meu consentimento de participação na pesquisa. O pesquisador (a) certificou-me de que todos os dados desta pesquisa serão confidenciais, bem como o meu tratamento não será modificado em razão desta pesquisa.

Autorizo/ Não autorizo (sublinhar a opção escolhida) o armazenamento de minha amostra para futuras pesquisas sobre o mesmo assunto. Declaro que recebi cópia do presente Termo de Consentimento.

_____	_____	_____
Assinatura do Doador	Assinatura do Pesquisador	Assinatura da testemunha
_____	_____	_____
Nome do Doador	Nome do Pesquisador	Nome da testemunha

Porto Alegre, ___/___/___

Comitê de Ética em Pesquisa
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ANEXO IV – Diretrizes do periódico *Vox Sanguinis*

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If you cannot submit online please contact the editor:

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Fax: 604-822-7135 or 613-739-2151
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Formação acadêmica/titulação

2015 Mestrado em Biociências.
Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Porto Alegre, Brasil
Título: Investigação da Variabilidade do Gene ACKR1 em Doadores de Sangue de Porto Alegre/RS
Orientador: Silvana de Almeida
Co-orientador: Marilu Fiegenbaum
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
Áreas do conhecimento : Genética Humana e Médica

2015 - 2015 Especialização em Imuno-hematologia.
Universidade Federal do Rio de Janeiro, UFRJ, Rio De Janeiro, Brasil
Título: Bases Moleculares do Sistema Sanguíneo Duffy

2009 - 2013 Graduação em Biomedicina.
Universidade Feevale, FEEVALE, Novo Hamburgo, Brasil
Título: Investigação da alteração de marcadores bioquímicos de nefrotoxicidade induzida pelo uso de quimioterápicos utilizados no tratamento do Câncer de Mama
Orientador: Rejane Giacomelli Tavares/Helena Schirmer

Formação complementar

2016 - 2016 Bioinformática para a Área da Saúde. (Carga horária: 15h).
Hospital de Clínicas de Porto Alegre, HCPA, Porto Alegre, Brasil

2016 - 2016 Minicurso em Bioinformática. (Carga horária: 3h).
Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Porto Alegre, Brasil

2016 - 2016 Sequenciamento de DNA: Métodos e Aplicações. (Carga horária: 15h).

Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

2016 - 2016 Transcriptograma: Ferramenta de Análise de Expressão Gênica. (Carga horária: 8h).
Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Porto Alegre, Brasil

2015 - 2015 VII Curso de Aplicações Clínicas da Bioquímica. (Carga horária: 9h).
Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Porto Alegre, Brasil

2015 - 2015 Excel Intermediário e Avançado. (Carga horária: 27h).
Universidade Feevale, FEEVALE, Novo Hamburgo, Brasil
Bolsista do(a): Universidade Feevale

2015 - 2015 Auditoria/Inspeção em Laboratórios e Unidades NB-3. (Carga horária: 8h).
Associação Nacional de Biossegurança, ANBIO, Rio De Janeiro, Brasil

2014 - 2014 Políticas Públicas em Saúde. (Carga horária: 44h).
Curso Vigor, CV, Brasil

2014 - 2014 Atualização em Informática. (Carga horária: 45h).
Curso Vigor, CV, Brasil

2014 - 2014 Imunohematologia Teórico. (Carga horária: 4h).
Hemorgs, HEMORGS, Brasil

2012 - 2012 III Curso de Extensão Jovem Pesquisador.
Pontifícia Universidade Católica do Rio Grande do Sul, PUCRS, Porto Alegre, Brasil

2012 - 2012 Curso de Língua Inglesa.
Kaplan International Colleges, KIC, Santa Barbara, Estados Unidos

2011 - 2011 PEGAIPB - Instituto de Pesquisas Biomédicas. (Carga horária: 480h).
Pontifícia Universidade Católica do Rio Grande do Sul, PUCRS, Porto Alegre, Brasil

2011 - 2011 Conhecendo o Endnote. (Carga horária: 4h).
Universidade Feevale, FEEVALE, Novo Hamburgo, Brasil

2011 - 2011 Centro de Idiomas - Inglês. (Carga horária: 60h).
Universidade Feevale, FEEVALE, Novo Hamburgo, Brasil

2010 - 2010 Perícia Criminal. (Carga horária: 20h).
Samavit Cursos e Treinamentos Profissionais, SCTP, Brasil

Atuação profissional

1. Fundação Universidade Federal de Ciências da Saúde de Porto Alegre - UFCSPA

Vínculo institucional

2015 - Atual Vínculo: Bolsista, Enquadramento funcional: Mestrado Acadêmico, Regime: Dedicação exclusiva
Outras informações:
Bolsista CAPES do PPG Biociências, a nível de Mestrado, pela UFCSPA.

2. Laboratório Bom Pastor - LBP

Vínculo institucional

2014 - 2014 Enquadramento funcional: Biomédica, Regime: Integral
Outras informações:
Biomédica no Laboratório Bom Pastor, atuando nos setores de Imunologia, Bioquímica e Análises Toxicológicas. Realizando os testes imuno-hematológicos para a transfusão de bolsas de sangue. Além da preparação e análise de amostras biológicas, execução das rotinas de controle de qualidade, manuseio em aparelhos de automação, segregação de resíduos gerados, organização do laboratório e demais atividades na área.

3. Universidade Feevale - FEEVALE

Vínculo institucional

2013 - 2013 Vínculo: Estágio Curricular, Regime: Dedicação exclusiva
Outras informações:
Estágio Curricular em Análises Clínicas nos setores de Coleta e Triagem, Líquidos Corporais, Micologia, Parasitologia, Imunologia, Bacteriologia, Bioquímica e Hematologia no Laboratório Escola e em Laboratório Hospitalar vinculado à Universidade Feevale.

4. Fundação Estadual de Produção e Pesquisa em Saúde - FEPPS

Vínculo institucional

2012 - 2012 Vínculo: Bolsista de Iniciação Científica FAPERGS, Regime: Parcial
Outras informações:
Título do projeto: Diagnóstico Molecular da Infecção pelo Vírus da Hepatite C (HCV) em Pacientes Portadores do Vírus da Imunodeficiência Humana (HIV). Orientador do projeto: Claudia Maria Dornelles da Silva

Projetos

2015 – Atual: Biologia Molecular de Grupos Sanguíneos: Impacto sobre a Prática Transfusional

Os sistemas de grupos sanguíneos são caracterizados por antígenos na membrana eritrocitária, com características funcionais e polimórficas definidas. A utilização de técnicas moleculares na determinação das variantes genéticas poderia contribuir para aumentar a segurança e eficácia dos procedimentos transfusionais de pacientes politransfundidos. Alguns protocolos de avaliação da variabilidade genética de grupos sanguíneos já foram estabelecidos na região Sudeste do Brasil, contudo, torna-se necessária a realização de estudos em diferentes regiões do Brasil, como o Rio Grande do Sul, a qual possui um pool genético ancestral diferente do encontrado nas demais regiões Brasileiras. Os objetivos desse estudo são investigar polimorfismos em genes de grupos sanguíneos em doadores de sangue e pacientes politransfundidos do Hospital de Clínicas de Porto Alegre, descrevendo a frequência destas variantes nesta amostra, bem como, correlacionar os achados genotípicos com os fenotípicos visando otimizar a prática transfusional.

Situação: Em andamento Natureza: Projetos de pesquisa
Integrantes: Gabriela Höher; Gabriela Waskow; Mirelen Moura de Oliveira Rodrigues; Thor Gunnar Hugo Onsten; Marilu Fiegenbaum; Silvana Almeida (Responsável)

2015 – Atual: Investigação da Variabilidade do Gene ACKR1 em Doadores de Sangue de Porto Alegre/RS

ACKR1 é o gene que codifica a glicoproteína que expressa os antígenos do grupo sanguíneo Duffy, localizado no cromossomo 1q21-22. Os principais antígenos do sistema, Fya e Fyb, são codificados por alelos codominantes, definindo os fenótipos Fy(a+b-), Fy(a-b+) e Fy(a+b+). No entanto, algumas variantes já identificadas causam a expressão fraca (+w ou *W) ou nula (0 ou *N) desses antígenos. De acordo com a

International Society for Blood Transfusion (ISBT) existem duas mutações associadas com a expressão fraca do Fya e cinco mutações associadas com a expressão fraca do Fyb. Adicionalmente, sete mutações foram observadas ocasionando a expressão nula do Fya e três para o Fyb. Tendo em vista que o conhecimento das bases genéticas destes antígenos é importante para aumentar a segurança e eficácia dos procedimentos transfusionais, o objetivo deste estudo é investigar a presença de variações no gene ACKR1. Para isso, 382 doadores voluntários de sangue foram analisados para os antígenos Fya e Fyb por fenotipagem e para os polimorfismos rs12075, rs34599082, rs2814778 por PCR em tempo real, utilizando sondas de hidrólise. Após a correlação entre os resultados da análise fenotípica e da análise dos polimorfismos, 17 amostras apresentaram discrepância entre fenótipo e genótipo e terão o gene ACKR1 sequenciado com a finalidade de identificar possíveis novas mutações e polimorfismos que possam estar relacionados com expressão fraca ou nula dos antígenos Fya e Fyb. O conhecimento de variantes genéticas relacionadas à expressão diferencial dos principais antígenos desse sistema, pode no futuro propiciar a melhor seleção de unidades de sangue a serem utilizadas baseadas em análises moleculares.

Situação: Em andamento Natureza: Projetos de pesquisa
Integrantes: Gabriela Höher; Gabriela Waskow; Mirelen Moura de Oliveira Rodrigues; Thor Gunnar Hugo Onsten; Marilu Fiegenbaum; Silvana Almeida (Responsável)

Áreas de atuação

1. Biologia Molecular
2. Genética Humana e Médica
3. Imuno-hematologia

Produção bibliográfica

Artigos completos publicados em periódicos

1. **HÖHER, G.**; FIEGENBAUM, M.; ALMEIDA, S.
Molecular basis of the Duffy blood group system. Blood Transfusion. v.xx, p.xx - xx, 2017.
Palavras-chave: Duffy blood group system , ACKR1 gene, DARC, allele variants

2. MULLER, C. D.; **HÖHER, G.**; MEYER, J. B. F.; CARVALHO, J. F. S.; CAPRARA, J. F.; TRINTINAGLIA, L.; LARA, G. M.
Diagnóstico Sorológico em Doença de Chagas com Ênfase na Reação em Cadeia da Polimerase. Newslab. v.20, p.68 - 72, 2014.

3. **HÖHER, G.**; CAPRARA, J. F.; MEYER, J. B. F.; SANTOS, J. M.; MORAES, J. P.; BAGATINI, P.
Toxocara canis: uma revisão bibliográfica. Newslab. v.21, p.118 - 120, 2014.

Trabalhos publicados em anais de eventos (resumo)

1. RODRIGUES, M. M. O.; WASKOW, G.; **HÖHER, G.**; ONSTEN, T. G. H.; FIEGENBAUM, M.; ALMEIDA, S.
Análise de Antígenos Raros de Grupos Sanguíneos em Doadores de Sangue do Hospital de Clínicas de Porto Alegre In: I Encontro do PPG em Biociências, XIX Encontro de Geneticistas do Rio Grande do Sul e I Encontro Regional Sul da Sociedade Brasileira de Genética Médica, 2016

2. **HÖHER, G.**; WASKOW, G.; RODRIGUES, M. M. O.; ONSTEN, T. G. H.; FIEGENBAUM, M.; ALMEIDA, S.
Investigação da Variabilidade do Gene ACKR1 em Doadores de Sangue de Porto Alegre/RS In: I Encontro do PPGBio, XIX Encontro de Geneticistas do RS e I Encontro Regional Sul da Sociedade Brasileira de Genética Médica, 2016, UFCSPA.

3. WASKOW, G.; RODRIGUES, M. M. O.; **HÖHER, G.**; WAGNER, S. C.; ONSTEN, T. G. H.; FIEGENBAUM, M.; ALMEIDA, S.
Perfil de Alelos Raros de Grupos Sanguíneos em Doadores de Sangue do Hospital de Clínicas de Porto Alegre/RS In: XV Congresso Brasileiro de Biomedicina e III Congresso Internacional de Biomedicina, 2016, Bento Gonçalves.

4. WASKOW, G.; RODRIGUES, M. M. O.; **HÖHER, G.**; HUTZ, M. H.; KURTZ, G. S.; FIEGENBAUM, M.; ALMEIDA, S.
Variabilidade Genética dos Grupos Sanguíneos na População Brasileira In: I Encontro do PPG em Biociências, XIX Encontro de Geneticistas do Rio Grande do Sul e I Encontro Regional Sul da Sociedade Brasileira de Genética Médica, 2016, Porto Alegre.

5. **HÖHER, G.**; TAVARES, R. G.
Investigação da Alteração de Marcadores Bioquímicos de Nefrotoxicidade Induzida pelo Uso de Quimioterápicos Utilizados no Tratamento do Câncer de Mama In: VI Congresso Internacional de Bioanálises, IX Congresso Sulbrasileiro de Biomedicina e XIII Semana Gaúcha de Biomedicina, 2013, Novo Hamburgo.

6. **HÖHER, G.**; CAPRARA, J. F.; MEYER, J. B. F.; SANTOS, J. M.; MORAES, J. P.; BAGATINI, P.; MINOZZO, R.
Toxocara canis: Uma Revisão Bibliográfica In: VI Congresso Internacional de Bioanálises, IX Congresso Sulbrasileiro de Biomedicina e XIII Semana Gaúcha de Biomedicina, 2013, Novo Hamburgo.

7. TRINTINAGLIA, L.; MULLER, C. D.; **HÖHER, G.**; MEYER, J. B. F.; CARVALHO, J. F. S.; CAPRARA, J. F.; LARA, G. M.
Diagnóstico Sorológico em Doença de Chagas com Ênfase na Reação em Cadeia da Polimerase In: V Congresso Internacional de Bioanálises, VIII Congresso Sul-Brasileiro de Biomedicina e XII Semana Gaúcha de Biomedicina, 2012, Novo Hamburgo.

8. HENTSCHKE, M. R.; GUWZINSKI, A.; **HÖHER, G.**; PIZZUTTI, L.; POLI-DE-FIGUEIREDO, C. E.; GADONSKI, G.; COSTA, B. E. P.
Associação do Uso de Anti-Hipertensivos com o Diagnóstico de Doença Hipertensiva Gestacional In: V Simpósio Internacional de Ginecologia, Obstetrícia e Mastologia e 5º Encontro de Ex-Residentes de Ginecologia e Obstetrícia da PUCRS, 2011, Gramado.

Artigos em revistas (Magazine)

1. GUWZINSKI, A.; HENTSCHKE, M. R.; PIZZUTTI, L.; **HÖHER, G.**; POLI-DE-FIGUEIREDO, C. E.; GADONSKI, G.; COSTA, B. E. P.
Uso de Medicamentos anti-hipertensivos em puérperas que desenvolveram Doença Hipertensiva Gestacional. Revista de Saúde/Universidade Católica de Pelotas. Pelotas, p.66, 2011.

Apresentação de trabalho e palestra

1. PIZZUTTI, L.; **HÖHER, G.**; GUWZINSKI, A.; HENTSCHKE, M. R.; POLI-DE-FIGUEIREDO, C. E.; GADONSKI, G.; COSTA, B. E. P.
Relação do Tratamento Medicamentoso com o Diagnóstico Final da Doença Hipertensiva Gestacional, 2011.

Eventos

1. Apresentação de Poster / Painel no(a) **I Encontro do PPG Biociências, XIX Encontro de Geneticistas do RS e I Encontro Regional Sul da Sociedade Brasileira de Genética Médica**, 2016. (Encontro)
Investigação da Variabilidade do Gene ACKR1 em Doadores de Sangue de Porto Alegre/RS.

2. **Biomarcadores em Doenças Neurodegenerativas**, 2015. (Encontro)
.

3. **III Jornada do Serviço de Genética Clínica da UFCSPA**, 2015. (Encontro)
.

4. **IX Congresso Brasileiro de Biossegurança**, 2015. (Congresso)
.

5. **12ª Festa da Vida**, 2013. (Outra)
Realização de tipagem sanguínea.

6. **VI Congresso Internacional de Bioanálises, IX Congresso Sul-Brasileiro de Biomedicina e XIII Semana Gaúcha de Biomedicina**, 2013. (Congresso)
.

7. **III Simpósio de Infecções em Pacientes Transplantados.**, 2012. (Simpósio)
.

8. **V Congresso Internacional de Bioanálises, VIII Congresso Sul-Brasileiro de Biomedicina e XII Semana Gaúcha de Biomedicina**, 2012. (Congresso)
.

9. **VII Jornada Acadêmica de Biomedicina UFCSPA e III Simpósio Habilitações da Biomedicina**, 2011. (Simpósio)
.

10. **Semana Acadêmica do Curso de Biomedicina**, 2010. (Seminário)
.

11. **II Congresso Internacional de Bioanálises, V Congresso Sul-Brasileiro de Biomedicina e IX Semana Gaúcha de Biomedicina**, 2009. (Congresso)
.

12. Apresentação de Poster / Painel no(a) **Salão de Extensão**, 2009. (Exposição)
Atividade de Extensão sobre Poluição Ambiental na Cidade de Novo Hamburgo, no RS.

Organização de evento

1. **HÖHER, G.**
I Encontro do PPG em Biociências, XIX Encontro de Geneticistas do RS e I Encontro Regional Sul da Sociedade Brasileira de Genética Médica, 2016.

2. **HÖHER, G.**
Participação na Comissão de Apoio do IV Congresso Internacional de Bioanálises, VII Congresso Sulbrasileiro de Biomedicina, XI Semana Gaúcha de Biomedicina, 2011.

3. **HÖHER, G.**
Participação na Comissão de Apoio do III Congresso Internacional de Bioanálises, VI Congresso Sulbrasileiro de Biomedicina, X Semana Gaúcha de Biomedicina, 2010.