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ACIDENTE VASCULAR CEREBRAL NA HEMOGLOBINOPATIA SC (*HBB* GLU6VAL E GLU6LYS): AVALIAÇÃO DE MARCADORES DE PROGNÓSTICO

RAYRA PEREIRA SANTIAGO

Salvador – Bahia

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“ACIDENTE VASCULAR CEREBRAL NA HEMOGLOBINOPATIA SC (*HBB* GLU6VAL E GLU6LYS): AVALIAÇÃO DE MARCADORES DE PROGNÓSTICO”

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RESUMO

O acidente vascular cerebral (AVC) é uma complicação clínica grave da doença falciforme (DF). Poucos estudos avaliaram a velocidade do fluxo sanguíneo cerebral utilizando o Doppler transcraniano (DTC) e marcadores preditores do AVC na hemoglobinopatia SC (HbSC) e, desta forma, as velocidades consideradas de risco para os indivíduos com esta hemoglobinopatia são baseadas em velocidades descritas para a anemia falciforme (AF) e para a Sβ talassemia (HbS/β). Assim, o objetivo do presente estudo foi identificar marcadores preditores do AVC em indivíduos com HbSC, estabelecendo subfenótipos da doença pela associação de biomarcadores genéticos, hematológicos, bioquímicos e imunológicos com o valor da velocidade do fluxo sanguíneo cerebral. Para tanto, foi realizado um estudo transversal, onde foram investigados 68 indivíduos com HbSC. A velocidade média máxima do fluxo sanguíneo cerebral nas artérias cerebral média, carótida anterior e cerebral anterior foi determinada utilizando o DTC. Os marcadores hematológicos, bioquímicos e imunológicos foram avaliados por métodos automatizados e os marcadores genéticos que pudessem estar relacionados ao AVC foram identificados pelas técnicas de reação em cadeia da polimerase e *Restriction Fragment Length Polymorphism* além da avaliação de polimorfismos de nucleotídeo único na plataforma Illumina. A velocidade média máxima observada nos indivíduos com HbSC apresentou correlação negativa com marcadores hematológicos (hemácias, hemoglobina, hematócrito) e bilirrubina direta e correlação positiva com monócitos e ferritina. Os indivíduos com velocidades do fluxo sanguíneo cerebral superiores a descrita por Deane e colaboradores (2008) apresentaram menores valores de *Red Cell Distribution Width* (RDW) e óxido nítrico (NO), já os indivíduos com velocidades do fluxo sanguíneo cerebral superiores a Vieira e colaboradores (em submissão) apresentaram níveis inferiores de hemoglobina e hematócrito e superiores de ferritina. Usando o percentil 75 da velocidade do fluxo sanguíneo cerebral foi possível verificar que os indivíduos com velocidades superiores a 125,75 cm/s possuíam valores diminuídos de hemoglobina, hematócrito, RDW e NO e valores aumentados de ferritina. O perfil genético indicou que o polimorfismo no gene da *MTHFR* C677T e o genótipo selvagem para a talassemia alfa -3,7kb exerciam um efeito protetor em relação ao AVC e, portanto, podem vir a ser utilizados como indicadores preditivos de AVC nos indivíduos com HbSC. A velocidade de 125,75 cm/s pode ser a mais adequada para se avaliar os indivíduos com HbSC, porém são necessários mais estudos para identificar a associação dessa velocidade com o risco de AVC. A avaliação dos dados de sequenciamento de nova geração em indivíduos com HbSC e com o perfil de DTC anormal vs normal permitiu identificar que os genes *DOCK6* rs2278426, *TYR* rs1042602, *CYP4F2* rs2108622, *MST1* rs3197999, *OR51B5/6* rs5006884, *THADA* rs7578597, *FUT2* rs602662, *MTHFR* rs1801133, *TSEN15* rs1046934, *CFB* rs12614 e *ABCG5* rs6756629 podem ser

preditores para a ocorrência do AVC. Os resultados deste trabalho sugerem que os indivíduos com HbSC e valor de DTC aumentados apresentam subfenótipo específico, caracterizado por um perfil hemolítico e inflamatório e com um perfil genético bem definido. Desse modo, sugerimos que a busca por marcadores preditores do AVC em indivíduos com HbSC é de grande relevância, uma vez que foi possível associar marcadores laboratoriais e genéticos com os resultados obtidos pelo DTC.

Palavras chaves: Doença SC, Doppler transcraniano, acidente vascular cerebral, subfenótipos.

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ABSTRACT

Stroke is a serious clinical complication of sickle cell disease (SCD). Only few studies have evaluated the rate of cerebral blood flow by transcranial Doppler (TCD) and stroke predictor markers on hemoglobinopathy SC (HbSC), thus, velocity considered as risk for stroke that is used to diagnose HbSC individuals are based on velocities described for the sickle cell anemia (SCA) and Sβ thalassemia. The objective of this study was to identify predictors markers of stroke in individuals with HbSC, establishing subphenotypes disease by the association of genetic biomarkers, hematological, biochemical and immunological with the value of the velocity of cerebral blood flow. For that, we conducted a cross-sectional study, which were investigated 68 HbSC individuals. The average maximum rate of cerebral blood flow in the middle cerebral artery, anterior cerebral artery and anterior carotid artery was determined using the DTC. Hematological, biochemical and immunological markers were evaluated by automated methods and genetic markers that could be related to stroke were identified by polymerase chain reaction techniques and restriction fragment length polymorphism, in addition to the evaluation of single nucleotide polymorphisms was used the Illumina platform. The maximum average velocity observed in HbSC individuals, in turn, was negatively correlated with hematological markers (erythrocytes, hemoglobin, hematocrit) and direct bilirubin and positive correlation with monocytes and ferritin. Individuals with TCD velocities greater than what was described by Deane and colleagues (2008) showed lower RDW and nitric oxide, as individuals with higher TCD velocities than described by Vieira and colleagues (under submission) showed lower hemoglobin and hematocrit and higher ferritin levels. Using the 75th percentile of TCD velocity we have found that individuals with a velocities exceeding 125.75 cm / s have diminished values of hemoglobin, hematocrit, RDW and NO and ferritin increased values. The genetic profile indicated that the polymorphism in gene of MTHFR C677T and the absence of alpha thalassemia -3,7kb exert a protective effect in relation to stroke. We have found that the velocity of 125.75 cm/s was may be the most appropriate to evaluate individuals with HbSC, but more studies are needed to identify the association of this velocity with the risk of stroke. The evaluation of next-generation sequencing data in individuals with HbSC with abnormal TCD profile vs Normal identified that the *DOCK6* rs2278426, *TYR* rs1042602, *CYP4F2* rs2108622, *MST1* rs3197999, *OR51B5/6* rs5006884, *THADA* rs7578597, *FUT2* rs602662, *MTHFR* rs1801133, *TSEN15* rs1046934, *CFB* rs12614 and *ABCG5* rs6756629 SNPs are predictors of stroke. These results suggest that individuals with HbSC and increased TCD value present specific sub-phenotype, characterized by hemolytic and inflammatory status and with a well-defined genetic profile. Thus, we suggest that the search for predictors markers for of stroke in individuals with HbSC is

of great importance, since it was possible to associate laboratory and genetic markers with the results obtained from the TCD.

Key words: SC disease, transcranial Doppler, stroke, subphenotypes.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS, SIGLAS

ACM	Artéria Cerebral Média
AF	Anemia falciforme
AVC	Acidente Vascular Cerebral
BEN	Haplótipo ligado ao grupo de genes da globina beta do tipo Benin
BD	Bilirrubina direta
BI	Bilirrubina indireta
CAM	Haplótipo ligado ao grupo de genes da globina beta do tipo Camarões
CAR	Haplótipo ligado ao grupo de genes da globina beta do tipo Bantu ou República Central Africana
CSSCD	Grupo de estudo cooperativo em doença falciforme, do inglês *Cooperative study of sickle cell disease*
DF	Doença falciforme
DTC	Doppler Transcraniano
Hb	Hemoglobina
HbAC	Heterozigoto para a hemoglobina C
HBB	Gene da globina beta
HbC	Hemoglobina C
HbF	Hemoglobina Fetal
HbS	Hemoglobina S
HbSC	Hemoglobinopatia SC
HbSS	Anemia falciforme
Hm	Contagem de hemácias
Ht	Hematócrito
HU	Hidroxiuréia
K^+	Potássio
K-Cl	Co-transporte de potássio e cloro
LDH	Lactato Desidrogenase
RDW	Amplitude de distribuição dos eritrócitos, do inglês *Red Cell Distribution Width*
NO	Óxido Nítrico
SAUDI	Haplótipo ligado ao grupo de genes da globina beta do tipo Índia-Arábia Saudita
SEN	Haplótipo ligado ao grupo de genes da globina beta do tipo Senegal

SNP	Polimorfismos de único nucleotídeo, do inglês *Single Nucleotide Polymorphism*
STA	Síndrome Torácica Aguda
STOP	Ensaio de prevenção do acidente vascular cerebral na doença falciforme, do inglês *Stroke Prevention Trial in sickle cell disease*
SUS	Sistema Único de Saúde
VMMAX	Velocidade Média Máxima

LISTA DE SÍMBOLOS

β^C	Alelo beta C
β^S	Alelo beta S
Α	Alfa
Β	Beta
Δ	Delta
ε	Épsilon
Γ	Gama
γ^A	Gama A
γ^G	Gama G
Ψα	Pseudo alfa
Ψξ	Pseudo zeta
ξ	Zeta

SUMÁRIO

1 REVISÃO DE LITERATURA

1.1 HEMOGLOBINA

A hemoglobina (Hb) é uma proteína esferoide globular presente no interior dos eritrócitos dos mamíferos que tem como função principal o transporte de oxigênio por todo o organismo. A molécula de Hb é formada por quatro subunidades, cada uma composta pela cadeia polipeptídica denominada globina e pelo grupamento prostético heme, que é composto pelo complexo Protoporfirina IX - $Ferro^{++}$. As cadeias polipeptídicas da Hb se agrupam duas a duas, sendo um par de cadeias do tipo alfa (α – alfa e ξ - zeta) e outro de cadeias tipo não alfa (β - beta, δ - delta, γ - gama e ε - epsilon), que em associação formam os tipos de Hb encontradas nos diferentes estágios de desenvolvimento do indivíduo, desde o período embrionário até a fase adulta (WEATHERALL e PROVAN, 2000; NETO e PITOMBEIRA, 2003; MARENGO-ROWE, 2006).

A síntese das cadeias globínicas é regulada por genes, que se encontram agrupados em cromossomos diferentes, sendo que o agrupamento α está localizado no cromossomo 16 e contém o gene *zeta* (*HBZ*), que codifica a cadeia ξ globínica, o pseudogene *pseudo zeta* (*HBZP1*), o gene *mu* (*HBM*), o pseudogene *pseudo alfa-1* (*HBAP1*), os genes *alfa-2* (*HBA2*) e *alfa-1* (*HBA1*) que são responsáveis pela codificação das cadeias alfa e o gene *theta* (*HBQ1*). O grupo de genes da globina β está localizado no cromossomo 11 e contem cinco genes orientados na posição 5'→ 3' na seguinte ordem, *épsilon* (*HBE1*), *gama-G* (*HBG2*), *gama-A* (*HBG1*), *delta* (*HBD*) e *beta* (*HBB*). Os genes da Hb humana estão dispostos ao longo do cromossomo na mesma ordem em que são expressos durante o desenvolvimento ontogênico para produzir os diferentes tetrâmeros (LITCHMAN e WILLIAMS, 2006; NETO e PITOMBEIRA, 2003; HIGGS et al., 2012). Alterações nos genes que codificam as cadeias globínicas podem dar origem as hemoglobinopatias.

1.2 HEMOGLOBINOPATIAS

As hemoglobinopatias constituem um grupo de doenças genéticas, caracterizadas por alterações na porção proteica da molécula da Hb, em decorrência de alterações em genes da globina e que envolvem mudanças estruturais e de síntese na molécula. As alterações estruturais são decorrentes de substituições, deleções e inserções de um ou mais aminoácidos ou da fusão de duas cadeias polipeptídicas diferentes e as alterações de síntese da Hb, conhecidas como talassemia, que envolvem a redução ou a ausência da síntese de um ou mais tipos de cadeias polipeptídicas, a depender do mecanismo e genótipo envolvido (BUNN, 1994; BUNN, 1997; WEATHERALL e PROVAN, 2000).

As Hbs variantes podem ser decorrentes de mutações nos genes da globina que ocasionam alterações de aminoácidos nas cadeias globínicas, que podem ocorrer tanto na cadeia tipo α quanto nas cadeias tipo não α, e podem provocar alterações na estrutura secundária e terciária do tetrâmero formado (CLARKE, 2000). As Hbs variantes S (HbS) e C (HbC) possuem frequência mundial elevada, principalmente em regiões acometidas pela malária (HANNEMANN et al., 2011).

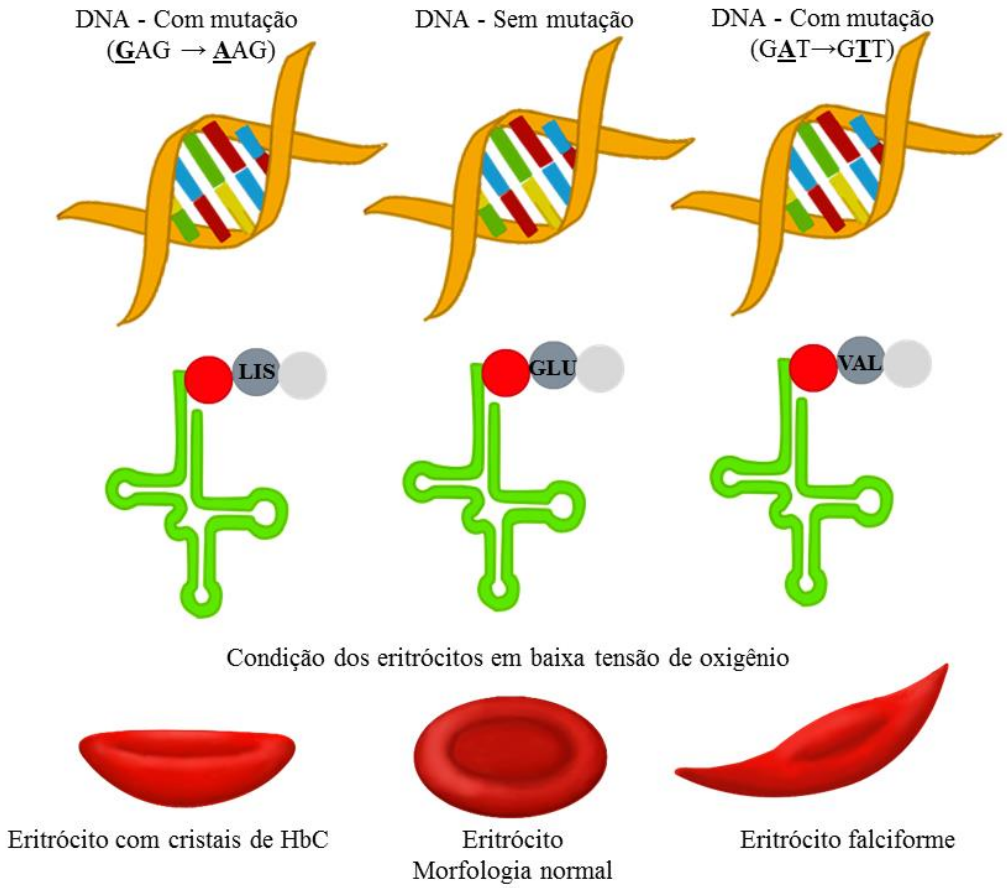
A anemia falciforme (AF) é uma doença genética de herança autossômica recessiva caracterizada pela presença do alelo beta S (β^S) em homozigose, caracterizando o genótipo HbSS. Os indivíduos com essa doença possuem numerosas complicações que podem afetar quase todos os órgãos e sistemas, com morbidade elevada, redução da capacidade de trabalho e da expectativa de vida (STEINBERG, 2001; RAPHAEL, 2005).

A HbS é resultante de uma mutação de ponto, com mudança de base no sexto códon do gene da globina β (*HBB*) onde uma adenina é substituída por uma timina (G<u>A</u>T→G<u>T</u>T), ocasionando a substituição de aminoácido na cadeia polipeptídica β, na posição β^6, onde o ácido glutâmico é substituído por valina, levando a perda líquida de uma carga negativa na molécula (HANNEMANN et al., 2011). Essa alteração tem pouco efeito quando a HbS está em um ambiente vascular com tensão elevada de oxigênio, porém quando a concentração de HbS em ambiente vascular esta sob tensão reduzida de oxigênio, as propriedades físico-químicas da molécula se alteram, causando a formação de polímeros insolúveis que distorcem os eritrócitos e alteram o seu formato para um formato de foice (Figura 1) (NETO e PITOMBEIRA, 2003; MARENGO-ROWE, 2006).

A doença falciforme (DF) é caracterizada pela presença da HbS associada a outras Hbs variantes (C e D, por exemplo), como na doença SC ou hemoglobinopatia SC (HbSC), ou a hemoglobinopatias de síntese, como a talassemia beta (STEINBERG, 2001). A HbSC (*HBB* GLU6VAL E GLU6LYS**)** é ocasionada pela associação da HbS com a HbC, onde os indivíduos apresentam a doença mais branda que os indivíduos com AF, devido a concentração intracelular reduzida de HbS (STEINBERG e SEBASTIANI, 2012; NAGEL e STEINBERG, 2003).

A HbC resulta da mutação de base no sexto códon do gene *HBB* onde uma guanina é substituída por adenina (GAG → AAG), ocasionando a substituição do ácido glutâmico por lisina na cadeia polipeptídica β, na posição β^6, levando a perda líquida de duas cargas negativas (HANNEMANN et al., 2011; NAGEL et al., 2003). A HbC produz agregados amorfos que levam à formação de cristais tetragonais quando esta presente em ambiente vascular com tensão de oxigênio elevada, porém, menos numerosos que os encontrados nas formas desoxigenadas da HbC, onde os cristais formam agregados em forma de macro-fita torcida, resultando numa variedade maior de agregados de fibras não ramificadas (Figura 1) (NAGEL et al., 2003).

Os cristais formados pela HbC oxigenada dissociam-se antes que possam gerar qualquer dano a microcirculação; entretanto, os eritrócitos falcizados que abrigam a forma desoxigenada da HbS aderem ao endotélio das vénulas pós-capilares, formando agregados heterocelulares com leucócitos, que contribuem para a obstrução capilar, resultando em hipóxia local, aumento na formação de polímeros de HbS, e a propagação da vaso-oclusão na vasculatura adjacente. Nesse contexto, destaca-se também a participação dos neutrófilos que contribuem para o aumento da inflamação na microvasculatura e diminuição de mediadores vasodilatadores, como o óxido nítrico (NO), que atuam na desregulação do tônus vasomotor (NAGEL et al., 2003; STUART e NAGEL, 2004; MARENGO-ROWE, 2006).



Fonte: Elaborada por Luciana Fiuza

Figura 1. Alterações morfológicas ocasionadas nos eritrócitos devido à presença das Hbs variantes C e S.

1.3 HEMOGLOBINOPATIA SC

A HbSC é uma condição onde os indivíduos são heterozigotos duplos, que herdam o alelo da globina β^S de um parental e o alelo da globina β^C de outro, fazendo com que em seus eritrócitos coexistam em concentrações similares de HbS e HbC (BUNN et al., 1982; COLELLA et al., 2015). A HbSC é uma condição de heterozigose para a HbC, assim como o heterozigoto para a HbC (HbAC), contudo a combinação entre HbS e HbC dá origem a condição patológica, enquanto que o indivíduo HbAC é assintomático (NAGEL et al., 2003). A principal razão para isso é que a HbC desencadeia a formação dos polímeros intracelulares de HbS, ao favorecer a desidratação dos eritrócitos (LIONNET et al., 2012; HANNEMANN et al., 2011).

Os eritrócitos que contêm a HbC têm como característica um volume de efluxo de potássio (K^+) elevado, fazendo com que haja a redução intracelular de cátions e do teor de água

como ocorre na HbSC. A atividade elevada do co-transporte de potássio e cloro (K-Cl) leva à perda de K^+ e água fazendo com que os eritrócitos que contêm a HbC fiquem desidratados, aumentado a concentração de hemoglobina intracelular e elevando a concentração de hemoglobina corpuscular média (CHCM) (NAGEL et al., 2003; HANNEMANN et al., 2011). A alteração no efluxo de K^+ e a desidratação dos eritrócitos com as Hb S e C propiciam o aumento do CHCM e polimerização da HbS, com diminuição no atraso da polimerização, amplificando o efeito da presença de 50 % de HbS nesses eritrócitos, responsável pelas características clinicas observadas (NAGEL et al., 2003; HANNEMANN et al., 2011). Tem sido relatada também a capacidade da HbS em acelerar a cristalização da HbC, influenciando de forma negativa para a clínica dos indivíduos com genótipo HbSC, sendo que a reidratação destes eritrócitos pode reverter estes eventos (LAWRENCE et al., 1991). Esses mecanismos são os responsáveis pela fisiopatologia observada na HbSC, que apesar de pouco estudada, apresenta manifestações clínicas importantes.

1.4 MANIFESTAÇÕES CLÍNICAS NA HEMOGLOBINOPATIA SC

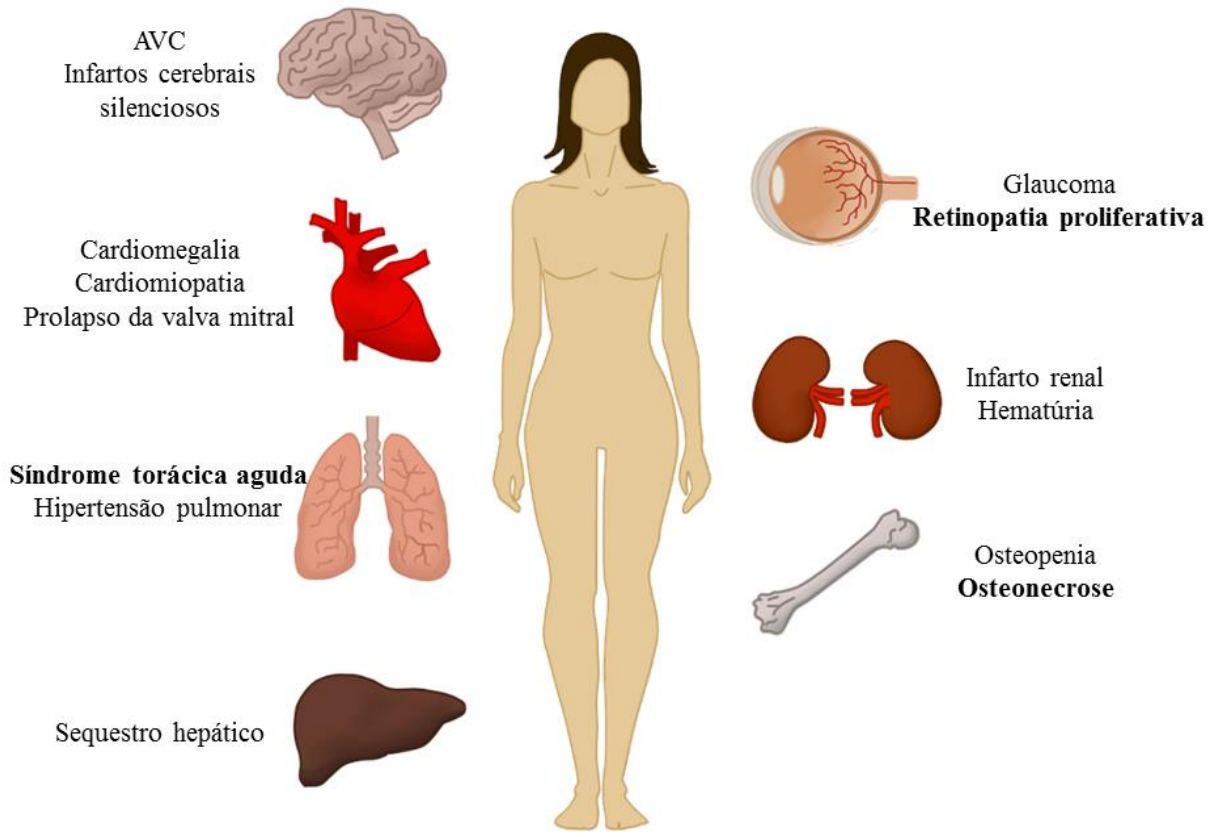
Os indivíduos com DF apresentam quadro clínico heterogêneo, com grande variabilidade nas manifestações clínicas, sendo estas influenciadas por diversos fatores, tais como idade, gênero, características genéticas, fatores ambientais e socioeconômicos (LYRA et al., 2005; BRASIL, 2008).

As principais manifestações clínicas encontradas na AF ocorrem em resposta à falcização intravascular, hemólise e leucocitose, bem como a associação entre os eritrócitos falciformes e outros componentes do sangue e incluem três conjuntos de sinais e sintomas. O primeiro conjunto é referente à anemia hemolítica; o segundo, à síndrome dolorosa e o terceiro refere-se às complicações que afetam os principais órgãos e sistemas (TAYLOR et al., 2008; BALLAS et al., 2010; DOMINGOS et al., 2014).

As complicações hematológicas mais comuns nesses indivíduos são a anemia aguda exacerbada, hiper-hemólise, sequestro esplênico agudo, crises aplásticas, complicações relacionadas à transfusão, síndrome de hiperviscosidade, hemólise imune e hemossiderose transfusional; já entre as síndromes dolorosas mais frequentes estão os quadros de dor, episódios vaso-oclusivos e neuropatias (BALLAS et al., 2010).

Entre as complicações que afetam os principais órgãos destacam-se o dano neurológico, como o acidente vascular cerebral (AVC) e o infarto cerebral silencioso; complicações oftalmológicas, como glaucoma e retinopatia proliferativa; complicações cardíacas, como cardiomegalia, cardiomiopatia e prolapso da valva mitral; complicações pulmonares, como síndrome torácica aguda (STA) e hipertensão pulmonar; complicações gastrointestinais, como colelitíase e sequestro hepático; complicações renais e genitourinárias, como falha renal aguda, hematúria e priapismo; complicações esplênicas, como sequestro esplênico agudo e asplenia funcional; complicações musculoesqueléticas, como necrose avascular, úlceras de pernas e osteopenia; e distúrbios de crescimento e desenvolvimento representados pelo atraso no crescimento (BALLAS et al., 2009). Todas as complicações encontradas em indivíduos com AF podem ocorrer em grau menor nos indivíduos HbSC (NAGEL et al., 2003; LIONNET et al., 2012).

A hemólise é menos intensa na HbSC de modo que a anemia é menos significativa e as complicações da hemólise, como os episódios de aplasia e a colelitíase, são menos frequentes e graves; porém, os indivíduos com HbSC possuem risco aumentado para a ocorrência de STA, osteonecrose óssea e retinopatia proliferativa, sendo que esta última afeta mais de 70 % dos adultos (Figura 2) (NAGEL et al., 2003; REES et al., 2015). Além disso, crianças com HbSC apresentam risco 100 vezes maior em desenvolver AVC quando comparadas a população saudável (REES et al., 2015).



Fonte: Elaborada por Luciana Fiuza

Figura 2. Manifestações clínicas encontradas na doença falciforme com foco especial nas manifestações verificadas com maior frequência nos indivíduos com HbSC.

1.5 ACIDENTE VASCULAR CEREBRAL

O AVC foi definido pelo *Cooperative Study of Sickle Cell Disease* (CSSCD) como uma síndrome neurológica aguda secundária a oclusão de uma artéria ou hemorragia, que tem como resultado a isquemia, bem como sintomas e sinais neurológicos (BALLAS et al., 2010). Essa síndrome foi descrita pela primeira vez na DF em 1923, 13 anos após a primeira descrição da doença (SYDENSTRICKED et al., 1923; VERDUZCO e NATHAN, 2009).

O AVC isquêmico é resultante da interrupção do fluxo circulatório normal, que pode ser devido a redução na pressão de perfusão, da obstrução dos pequenos ou grandes vasos, ou de ambos; já o AVC hemorrágico é decorrente de sangramento cerebral provocado pelo rompimento de uma artéria ou vaso sanguíneo, ou pode ser um fenômeno secundário à ocorrência de infartos em zonas de fronteira arterial ou de obstrução vascular transitória (ROBINS e COTRAN, 2010).

Na DF, o AVC resulta de um estreitamento progressivo dos vasos sanguíneos de médio e grande porte que abastecem o cérebro, particularmente na artéria cerebral média (ACM) e na artéria carótida interna (ACI), e em menor medida as artérias cerebrais anteriores (ACA). O mecanismo exato desse estreitamento progressivo é desconhecido, mas pode estar relacionado à deficiência de óxido nítrico (NO) funcional resultante da hemólise que ocasiona o aumento da Hb plasmática livre (OHENE-FREMPONG et al.,1998; DEANE et al., 2007).

O AVC é a principal causa de óbito em crianças e adultos com DF (LEIKIN et al., 1989; PLATT et al., 1994). Uma criança com DF tem o risco 333 vezes maior de desenvolver AVC do que crianças saudáveis e sem doença cardíaca (OHENE-FREMPONG et al.,1998; BRODERICK et al., 1993).

O AVC ocorre em 11% dos indivíduos com AF até a idade dos 20 anos, sendo que as taxas na infância são particularmente elevadas (ADAMS et al., 2005). O CSSCD descreveu a incidência de 0,61 eventos por 100 indivíduos/ano para a ocorrência de AVC em indivíduos com AF de todas as faixas etárias (OHENE-FREMPONG et al., 1998). As taxas de AVC foram ainda mais elevadas em crianças com AF menores de 10 anos, com a taxa de 1,02 por 100 indivíduos/ano dos dois aos 5 anos e a taxa de 0,79 por 100 indivíduos/ano em crianças de 6 a 9 anos (ADAMS et al.,1998). Apesar da incidência de AVC ser maior nos indivíduos com AF, este também pode ocorrer em indivíduos com outros genótipos da DF (LOBO et al., 2011). De acordo com a publicação da CSSCD, a incidência de AVC é de 0,61 por 100 indivíduos/ano em

indivíduos com AF; 0,17 por 100 indivíduos/ano na hemoglobinopatia HbSC; 0,11 por 100 indivíduos/ano na HbS/β+ talassemia e de 0,10 por 100 indivíduos/ano na HbS/β0 talassemia. Entre os indivíduos com HbSC, o AVC acontece em aproximadamente 2%, 4%, e 10% dos indivíduos com idades inferiores a 20, 30 e 45 anos, respectivamente (OHENE-FREMPONG et al., 1998; BRODERICK et al., 1993).

Os eventos cerebrovasculares podem trazer sequelas graves em cerca de 7% das crianças com DF, com a possibilidade de episódios novos (0,7% por ano) durante os primeiros 20 anos de vida. Os episódios aparecem isolados ou associados com infecção, desidratação, crises dolorosas agudas, crises aplásicas, priapismo, entre outros (OLIVEIRA et al., 2008).

As manifestações clínicas neurológicas nos indivíduos com DF são diversas podendo ser desde focais, como hemiparesias, hemianestesias, deficiência de campos visuais, afasias e paralisias dos nervos cranianos até a ocorrência de crises epilépticas generalizadas e coma que podem levar o indivíduo a óbito (OHENE-FREMPONG et al.,1998). Para identificar os indivíduos com o risco elevado para o AVC, atualmente se utiliza o Doppler transcraniano (DTC) como método diagnóstico padrão (ADAMS, 2005).

1.6 DOPPLER TRANSCRANIANO

O uso do DTC para identificar indivíduos com DF com o risco aumentado para o AVC foi introduzido no início de 1990 e validado em uma série de estudos e demonstrou na triagem *Stroke Prevention Trial in Sickle Cell Disease* (STOP) ser extremamente útil na identificação de indivíduos com risco para esta complicação devastadora (ADAMS et al., 1992; ADAMS, 2005; BALLAS et al., 2010).

O DTC é ideal para a triagem de doenças de grandes vasos em indivíduos com DF, por ser um método seguro, não invasivo, com custo relativamente baixo e bem tolerado pelas crianças (AASLID et al.,1982; LEY-POZO et al.,1990). O DTC tem a capacidade de detectar infartos cerebrais silenciosos e isso permite prever problemas futuros no sistema nervoso central (ADAMS, 2005).

O DTC é usado para medir a velocidade do fluxo nas grandes artérias intracranianas do círculo de Willis, a qual é influenciada por diversos fatores, dos quais os principais são: a diferença no gradiente de pressão ao longo do vaso, o comprimento do vaso, a área de secção

transversal (calibre) e a viscosidade sanguínea (HOKAZONO et al., 2011). A estenose pode ser identificada pelo aumento da velocidade que resulta do diâmetro arterial reduzido, pois a velocidade do sangue é diretamente relacionada ao fluxo sanguíneo cerebral e inversamente relacionada ao diâmetro do vaso sanguíneo. Na DF há o aumento da velocidade do fluxo sanguíneo devido à anemia grave (ADAMS et al., 1992); assim, uma vez que a velocidade do fluxo sanguíneo se apresenta elevada ao ser medida pelo DTC, isso torna o método diagnóstico poderoso como um preditor para o AVC, cujo risco aumenta em proporção direta com o aumento da velocidade média máxima (VMMAX) (ADAMS et al., 2004).

O estudo de Adams e colaboradores (1992) definiu os valores para a velocidade do fluxo sanguíneo cerebral em indivíduos com AF em idades entre 2 e 16 anos. Os indivíduos com AF e VMMAX de até 170 cm/s são considerados normais; os de VMMAX de 170 a 200 cm/s são considerados condicionais; e os que possuem VMMAX maiores que 200 cm/s são considerados críticos ou anormais e possuem risco alto para desenvolver AVC. Neste mesmo estudo foi demonstrado que indivíduos com DF têm velocidade de fluxo sanguíneo cerebral médio 40 a 50% maior em vasos do polígono de Willis do que indivíduos controles saudáveis, e ainda que crianças com DF que apresentam velocidade de fluxo cerebral acima do percentil 95 possuíam risco maior para ocorrência de AVC.

O tempo indicado para que o exame do DTC seja repetido dependerá do resultado obtido. Se o DTC inicial for normal (VMMAX <170 cm/s), o seguimento deve ser realizado anualmente; se condicional baixa (VMMAX 170-184 cm/s), o exame deve ser realizado a cada 6 meses; se condicional alta (VMMAX 185-199 cm/s), o exame deve ser realizado a cada 3 meses; e dentro de 1 mês se anormal (VMMAX ≥ 200cm/s); se uma velocidade de DTC anormal for confirmada, é recomendado o regime crônico de transfusão de hemocomponentes (HANKINS et al., 2008).

O DTC é um teste muito sensível, mas somente moderadamente específico, pois 60% das crianças não tratadas com risco elevado para o AVC, indicado pelo DTC, não são acometidas por nenhum episódio de AVC (ADAMS et al., 1997). Além disso, existem outras limitações associadas ao DTC: AVC pode ocorrer em indivíduos que apresentam resultados de DTC normais; há uma variabilidade alta intra-sujeito; o acesso ao DTC e à terapia de transfusão crônica são limitados, especialmente em países em desenvolvimento como é o caso do Brasil (BELISARIO et al., 2015; ADAMS et al., 1997; ADAMS et al., 2004; BRAMBILLA et al.,2007).

Outra limitação é a ausência de estudos robustos para a determinação da velocidade do fluxo sanguíneo cerebral em indivíduos com HbSC que até o momento permanecem sendo diagnosticados pelas velocidades descritas por Adams e colaboradores (1992) para indivíduos com AF, podendo gerar diagnósticos falsos. Deane e colaboradores (2007) descreveram que as velocidades do fluxo sanguíneo cerebral em indivíduos com HbSC são menores que as descritas para indivíduos com AF e determinaram que em indivíduos com HbSC as velocidades superiores a 128 cm/s já deveriam ser consideradas como anormais. Outro estudo realizado em 1875 indivíduos com DF, reafirmou que as velocidades dos indivíduos com HbSC são inferiores a dos indivíduos com AF, e determinou que para indivíduos com HbSC as velocidades superiores a 143,5 cm/s já deveriam ser consideradas como anormais (VIEIRA, submetido).

Devido às limitações anteriormente citadas faz-se necessária a busca de variáveis preditoras para ocorrência de AVC em indivíduos com DF em especial nos indivíduos com HbSC, bem como indicar padrões para as velocidades do fluxo sanguíneo cerebral nesses indivíduos.

1.7 MARCADORES PREDITORES DO AVC

Os dados clínicos dos indivíduos com DF têm sido cada vez mais associados a valores anormais de DTC, bem como ao risco aumentado de desenvolver AVC. Alguns autores descreveram que anemia grave pode representar risco adicional para o desenvolvimento do AVC. Assim também como tem sido sugerido que o aumento do fluxo sanguíneo cerebral e a velocidade do fluxo associados à anemia crônica causam distúrbios que podem levar a danos cerebrovasculares (ADAMS et al., 1994; PROHOVNIK et al., 1989; LEITE et al., 2012). A contagem elevada de leucócitos parece também ser um fator de risco para várias complicações associadas a DF, como crises dolorosas, STA, AVC e óbito (BALKARAN et al.,1992; LEITE et al., 2012).

Os marcadores de hemólise como contagem de reticulócitos, concentrações de bilirrubina indireta (BI) e lactato desidrogenase (LDH) foram associadas a susceptibilidade elevada ao AVC (DOMINGOS et al., 2014).

O estudo de Ohene-Frempong e colaboradores (1998) mostrou que os níveis aumentados de hemoglobina fetal (HbF) não estão associados a um efeito protetor em relação ao AVC; no

entanto, outros trabalhos mostram que esta hemoglobina apresenta efeito inibitório no risco de AVC (BALKARAN et al.,1992; OHENE-FREMPONG et al., 1991). A HbF tem sido inversamente relacionada a frequência de outras manifestações vaso-oclusivas na DF (KATO et al., 2007).

Vários marcadores genéticos têm sido associados na literatura ao risco de desenvolver AVC, porém os resultados alcançados são controversos e não conseguiram elucidar completamente o efeito da heterogeneidade genética dos indivíduos no desenvolvimento do AVC.

Os haplótipos ligados ao gene da globina β estão associados a origens geográficas e étnicas diferentes e são denominados conforme a região geográfica africana na qual se originaram. Assim, é possível encontrar os haplótipos do grupo Senegal (SEN), Benin (BEN), Bantu ou República Central Africana (CAR), Camarões (CAM) e o Índia-Arábia Saudita (SAUDI) (LABIE,1984; GONÇALVES et al., 2003). Esses haplótipos têm sido descritos por exercerem influência no curso clínico dos indivíduos com AF, sendo o haplótipo BEN associado a concentrações intermediárias de HbF e a gravidade moderada da doença; o CAR a concentrações diminuídas de HbF e quadro clinico mais grave; o SEN e SAUDI a concentrações elevadas de HbF e curso clínico menos grave da doença (POWARS et al.,1991; RAHGOZAR et al., 2000). De acordo com Domingos e colaboradores (2014), os indivíduos com o genótipo CAR/CAR possuem risco três vezes maior de desenvolver o AVC que indivíduos sem esse genótipo, porém outros estudos não verificaram associação entre o tipo dos haplótipos e o AVC (BELISÁRIO et al., 2010; BELISÁRIO et al., 2015).

O efeito da talassemia alfa na incidência do AVC é controverso, enquanto muitos estudos relatam que a sua co-herança reduz o risco de AVC, atuando como um fator protetor em grande parte devido à melhoria das concentrações de Hb (OHENE-FREMPONG et al.,1998; GILL et al.,1995; ADAMS et al.,1994; DOMINGOS et al., 2014), outros não encontraram resultados significativos (BALKARAN, et al.,1992; LEITE et al., 2012; KATO et al., 2007).

Estudos recentes têm associado à presença dos polimorfismos nos genes da enzima metilenotetrahidrofolato redutase *(MTHFR)* 677C>T (rs1801133), da protrombina (*PT*) 20210G>A (rs1799963), do fator V de Leiden (*FV*) 1691G>A (rs6025) e da enzima oxido nítrico sintase endotelial (*NOS3*) -786T>C (rs2070744), com o risco de desenvolvimento de AVC (LI e QUI, 2014; WANG et al., 2013; NIU et al., 2013; PEREIRA et al., 2007; BERNAUDIN et al.,

2008; CASAS et al., 2004), porém outros estudos não encontram essa associação (DOMINGOS et al., 2014). Outros genes como o da molécula de adesão celular vascular (*VCAM*), do receptor da interleucina 4 (*IL4R*) e do adrenoreceptor beta 2 (*ADRB2*) também já foram associados ao risco de AVC em indivíduos com AF (TAYLOR et al., 2002; HOPPE et al., 2004).

O estudo de Sebastiani e colaboradores (2005) utilizou a análise de rede bayesiana e encontrou 31 SNPs em 12 genes que interagem com a HbF para modular o risco do AVC, sendo alguns deles envolvidos na via do fator de transformação do crescimento β (TGF- β) e selectina P (SELP).

Vários estudos realizados em indivíduos com DF apresentaram informações escassas sobre os riscos de AVC na HbSC. Assim, é necessária a busca de marcadores preditores além do DTC para o desenvolvimento de AVC em indivíduos com HbSC, contribuindo para o estabelecimento de critérios para o monitoramento e tratamento desses indivíduos.

2 JUSTIFICATIVA

Os indivíduos com HbSC apresentam as Hbs variantes mais frequentes no Brasil, a HbS e a HbC, ambas com frequência elevada no estado da Bahia. Esses indivíduos apresentam quadro clínico heterogêneo, no qual é possível verificar a presença de todas as complicações encontradas em indivíduos com AF, embora na HbSC elas ocorram em gravidade e frequência menores e mais tardiamente em relação a AF (NAGEL et al., 2003; LIONNET et al., 2012).

Esses indivíduos também apresentam manifestações clínicas que vão desde crises vaso oclusivas e dolorosas, AVC, priaprismo até lesões crônicas em órgãos diversos. O AVC é uma complicação clínica que apresenta consequências debilitantes em indivíduos com DF, deixando sequelas importantes, com custo elevado para o Sistema Único de Saúde (SUS), uma vez que estes indivíduos são frequentemente internados e desenvolvem alterações cognitivas importantes, fato que repercute no número de indivíduos jovens dependentes da previdência social e sem condições para desenvolver atividades escolares e laborais (LOUREIRO e ROZENFELD, 2005; CANÇADO e JESUS, 2007).

Atualmente, o DTC, que é um método diagnóstico não invasivo e seguro, é utilizado para avaliar o risco para ocorrência do AVC pela determinação da VMMAX do fluxo cerebral em indivíduos falciformes dos 2 aos 16 anos de idade, possibilitando a realização de tratamento profilático através de regime transfusional crônico; entretanto, na literatura ainda não existem estudos específicos para a determinação dessa velocidade nos indivíduos com HbSC, que atualmente são diagnosticados utilizando os parâmetros descritos para indivíduos com AF, o que pode gerar resultados falsos, fazendo com que esses indivíduos venham a ter o AVC sem a chance de evitá-lo pela terapia transfusional ou pelo uso de hidroxiureia (HU), fármaco atualmente utilizado para o tratamento sintomático de indivíduos com DF em estado grave (AASLID et al.,1982; LEY-POZO et al.,1990; ADAMS, 2005).

Estudos mostram que há associação entre alguns marcadores clínicos em indivíduos com DF e o DTC com valores anormais como, por exemplo, o genótipo, a presença da talassemia alfa, anemia crônica, contagem de leucócitos e concentração de HbF, embora diversos dados permaneçam controversos (ADAMS et al., 1994; PROHOVNIK et al., 1989; LEITE et al., 2012; BALKARAN et al.,1992; DOMINGOS et al., 2014; OHENE-FREMPONG et al., 1991;

OHENE-FREMPONG et al.,1998). Além disso, não existem estudos na literatura relacionando apenas os indivíduos com HbSC a biomarcadores e valores de DTC anormais.

Assim, a determinação das velocidades específicas e de biomarcadores para os indivíduos com HbSC é de extrema necessidade, visando evitar a ocorrência do AVC nesses indivíduos, podendo fornecer ferramentas para uma terapêutica mais individualizada, que seja capaz de contribuir para o controle deste fenômenos, com redução no número de internações, melhoria no suporte clínico e na qualidade de vida desses indivíduos.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Identificar marcadores preditores do AVC em indivíduos com HbSC, estabelecendo subfenótipos da doença pela associação de biomarcadores genéticos, hematológicos e bioquímicos e imunológicos com as velocidades do fluxo sanguíneo cerebral.

3.2 OBJETIVOS ESPECÍFICOS

- Investigar os perfis hematológico, bioquímico e imunológico em indivíduos com HbSC, associando-os aos subfenótipos clínicos da doença baseados nas alterações do fluxo sanguíneo cerebral;
- Investigar marcadores genéticos em indivíduos com HbSC, associando-os aos subfenótipos clínicos da doença baseados nas alterações do fluxo sanguíneo cerebral;
- Correlacionar os fatores estudados ao curso clínico da HbSC, identificando biomarcadores preditores do AVC nos indivíduos com as alterações do fluxo sanguíneo cerebral.

4 MANUSCRITOS

4.1 MANUSCRITO 1

Título: A proposal of predictor markers of stroke for hemoglobin SC disease based on laboratorial biomarkers and Transcranial Doppler

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Situação: A ser submetido

Objetivo: (referente aos três objetivos específicos da dissertação):

- Investigar os perfis hematológico, bioquímico e imunológico em indivíduos com HbSC, associando-os aos subfenótipos clínicos da doença baseados nas alterações do fluxo sanguíneo cerebral;
- Investigar marcadores genéticos em indivíduos com HbSC, associando-os aos subfenótipos clínicos da doença baseados nas alterações do fluxo sanguíneo cerebral;
- Correlacionar os fatores estudados ao curso clínico da HbSC, identificando biomarcadores de preditores do AVC nos indivíduos com as alterações do fluxo sanguíneo cerebral.

Principais resultados: Neste artigo os marcadores hematológicos e bioquímicos: hemácias, hemoglobina, hematócrito, RDW, monócito, óxido nítrico, ferritina e bilirrubina direta foram identificados como candidatos a marcadores preditores para o acidente vascular cerebral (AVC) na hemoglobinopatia SC (HbSC). O polimorfismo no gene *MTHFR* C677T e a ausência da talassemia alpha -3,7kb demonstraram exercer um efeito protetor em relação ao AVC e podem estar associados como fatores preditores de AVC nesses indivíduos. Foi também sugerida uma nova velocidade média para predizer o AVC em indivíduos HbSC que foi a de 125,75 cm/s, sendo essa velocidade menor que as já descritas por Deane e colaboradores (2007) e por Vieira e

colaboradores (em submissão). Essa nova velocidade mostrou estar associada com alterações hematológicas e bioquímicas para as velocidades já descritas.

A proposal of predictor markers of stroke for hemoglobin SC disease based on laboratorial biomarkers and Transcranial Doppler

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List of the tables and figures

Key Words: SC disease, stroke, Transcranial Doppler, gene polymorphisms.

Subject Terms: Biomarkers, Genetics, Cerebrovascular disease/Stroke

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Abstract

Background and Purpose There is a lack of studies about Transcranial Doppler (TCD) velocities values for hemoglobin SC disease (HbSC) that use the established for sickle cell anemia. This study aims to identify predictor markers of stroke in HbSC individuals.

Methods We study 68 HbSC individuals, and the TCD was performed using time-averaged maximum mean velocity (TAMMV). Hematological, biochemical, immunological, nitric oxide (NO) metabolites and genetic analyses were performed.

Results – The TAMMV was correlated with red blood cells (RBC), hemoglobin, hematocrit, monocytes, direct bilirubin and ferritin. We found higher RDW and NO concentrations in HbSC individuals with TAMMV lower than the cut off value of 128cm/s. We found higher concentration of hemoglobin and hematocrit in HbSC individuals with TAMMV lower than the cut off value of 143.50 cm/s, and increase of ferritin levels in individuals with TAMMV greater than 143.50 cm/s. Hemoglobin, hematocrit, RDW and NO levels were higher in HbSC individuals with TAMMV lower than 125.75 cm/s, and ferritin in individuals with TAMMV above 125.75 cm /s. Multivariate analyses suggest a protective effect on stroke risk associated to the *MTHFR* 677C>T gene polymorphism and absence of thalassemia alpha -3.7kb.

Conclusions - We suggest that RBC, hemoglobin, hematocrit, RDW, monocyte, direct bilirubin, NO, ferritin, T allele of *MTHFR* 677C>T gene and absence of thalassemia alpha -3.7kb are predictors markers for stroke in HbSC individuals. We also suggest additional studies to validate the TAMMV of 125.75 cm/s as a cut-off point for stroke risk in HbSC individuals.

Introduction

Clinical complications in sickle cell anemia (SCA), the most severe type of sickle cell disease (SCD), occur in less severity in HbSC individuals.[1, 2] However, HbSC individuals have an increased risk of acute chest syndrome (ACS), osteonecrosis and proliferative retinopathy, and HbSC individuals have 100 times greater risk of stroke when compared to the healthy population.[1, 3]

The incidence of stroke is 0.61 per 100 individuals/year in SCA individuals, and 0.17 per 100 individuals/year in HbSC, according to the *Cooperative Study of Sickle Cell Disease* (CSSCD).[4, 5] Adams and colleagues[6] established reference values of Transcranial Doppler (TCD) to identify SCA individuals with stroke risk; however, they did not establish reference values for the TCD velocities related to stroke risk in HbSC individuals.[6-8] The lack of studies to determine reference values for TCD velocities in HbSC is still a limitation, using the velocities for SCA described by Adams and his colleagues. [6] Deane and his colleagues[9] described that TCD velocities in HbSC individuals are lower than those described for SCA and they determined that HbSC velocities exceeding 128 cm/s should be considered abnormal. Another study showed that the cerebral velocities of HbSC individuals are lower than those described in SCA individuals, and that velocities higher than 143.5 cm/s should be considered abnormal.[10]

Studies show an association between abnormal TCD in SCD and the fetal hemoglobin (HbF) concentration, genotypes, co-inheritance of alpha-thalassemia, chronic anemia, leukocyte count, and polymorphisms in methylenetetrahydrofolate reductase (*MTHFR*) 677C>T (rs1801133), factor V Leiden (*FV*) 1691G>A (rs6025) and vascular cell adhesion molecule (*VCAM*) 833T>C (rs1041163) and *VCAM* 1238G>C, although many data remains controversial.[6, 11-13] To our knowledge, there are no published studies based on specific biomarkers and abnormal TCD values for HbSC individuals.

The absence of TCD velocities values and of specific biomarkers for HbSC, and also the high risk of SCD individuals to develop neurological clinical manifestations [13, 14] have contribute to the search of biomarkers for stroke risk, especially among HbSC individuals, since 54 000 HbSC babies are born every year and they are exposed to stroke risk.[15, 16]

Thus, the aim of this study was to identify stroke predictor markers in HbSC through the association of genetic, hematological, immunological and biochemical data with cerebral blood flow velocities.

Materials and Methods

Subjects

We studied 68 HbSC individuals, with an average age of 6.96 ± 3.90 years, a median of 6.00, and the 25th percentile of 4.00 and 75th percentile of 9.00, whom 40 (58.82 %) were female, attending the Pediatric Cerebrovascular Disease Outpatient Center at the Hospital Universitario Professor Edgard Santos of the Universidade Federal da Bahia.

Since all individuals were younger than 18 years their legal guardians signed the consent term of patient participation. Inclusion criteria were: age between 2-17 years; hemoglobin profile of HbSC and be in the steady state of the disease. Non-inclusion criteria were: hemoglobin profile different from HbSC pattern; blood transfusion in the past three months, chronic blood therapy regimens, and previous stroke event.

The study was approved by the Research Board of the Hospital Universitario Professor Edgard Santos of the Universidade Federal da Bahia under 287,768/2013 number and followed the standards of Good Clinical Practice Complex (Good Clinical Practice – GCP). All procedures were in accordance with the 1964 Helsinki declaration and its later amendments.

Transcranial Doppler measurements

The Transcranial Doppler (TCD) was performed in all HbSC subjects, always by the same professional and equipment, and the time-averaged maximum mean velocity (TAMMV) in the middle cerebral arteries (MCA) was assessed by 2 MHz probe, and distal intracranial internal carotid (ICA) through the transtemporal window using the Doppler-BoxTMX (Compumedics Germany GmbH, Singen, Hohentwiel, Germany).[6, 9]

Hematological and biochemical data

Hematological data were obtained using electronic cell counter Ruby Cell Dyn (Abbott Diagnostics, Lake Forest, Illinois, USA) and hemoglobin profile was performed by high performance liquid chromatography (HPLC) using the Variant II equipment - Bio-Rad (Hercules, California, EUA).

Biochemical analyses included the lipid profile, dosage of total proteins and fractions, total bilirubin and fractions, lactate dehydrogenase (LDH), alanine transaminase (ALT) and aspartate transaminase (AST), renal profile and iron were performed by immunochemistry assay (A25 BIOSYSTEMS SA, Barcelona, Catalunya, Spain). The dosage of ferritin was performed at Access 2 (Beckman Coulter Inc, CA, USA) and C-reactive protein and alpha-1 antitrypsin measurements were performed in the Immage equipment (Beckman Coulter Inc, Pasadena, California, USA).

Nitric oxide metabolite

The dosage of nitric oxide (NO) was based on the colorimetric test of Griess and the results were expressed as micromolar concentration of nitrite in the samples.[17]

Genetic analysis

The methylenetetrahydrofolate reductase (*MTHFR*) 677C>T (rs1801133), factor V Leiden (*FV*) 1691G>A (rs6025), *prothrombin* 20210G>A (rs1799963), vascular cell adhesion molecule (*VCAM*) 833T>C (rs1041163) and *VCAM* 1238G>C gene polymorphisms were investigated by polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLP) techniques.[18, 19] Beta S (β^S) haplotypes were determined by PCR-RFLP, [20] and the $\alpha 2^{3.7Kb}$ thalassemia was investigated by the allele specific PCR.[21-23]

Statistics analysis

Statistical analyses were performed using SPSS version 18.0 software (IBM, New York, NY, USA) and Graphpad Prism version 6.0 (Graphpad Software, San Diego, CA, USA). P values <0.05 were considered to be significant. Baseline characteristics summarize means and proportions of selected variables. We use the Shapiro-Wilk test to determine the quantitative variables distribution, and the Spearman's rank correlation coefficient to measures the strength of a linear relation between paired data. The Mann-Whitney test and independent t-test were used for analysis of two numerical variables, in accordance with variables distribution. Multivariate binary logistic regression analysis was performed to investigate a possible interaction of TAMMV with genetic, hematological, immunological and biochemical data. The JMP software was used for assembling the correlation graphs with the support of the University of Pennsylvania.

Results

Baseline characteristics of HbSC individuals, including the mean ± standard deviation (SD) of TAMMV values and laboratory data as well as 25th, 50th, and 75th percentile values were shown in Table I in the online-only Data Supplement.

The median of TAMMV in the HbSC individuals was 111.50 cm/s, the 25th percentile was 101.50 cm/s, the 75th percentile was 125.75 cm/s and the 95th percentile was 156.00 cm/s. The TAMMV was significantly correlated to red blood cells (RBC), hemoglobin, hematocrit, monocytes, direct bilirubin and ferritin (Figure 1). The figure 1A shows an outlier who had TAMMV of 204.00 cm/s and abnormal TCD.

According to previous protocol for SCD established by Adams and his colleagues,[6] we found two individuals with low TCD, 64 with normal TCD, one with abnormal TCD and one with inconclusive TCD. However, this classification is not suitable for HbSC individuals.

Using a cut off value of 128 cm/s, as defined by Deane and his colleagues,[9] we found 53 HbSC individuals with TAMMV lower than 128 cm/s and 15 individuals with TAMMV above to 128 cm/s. We compared the hematological, biochemical and immunological laboratory profile of these two groups and found significant increase of RDW and NO values in HbSC individuals with TAMMV lower than 128 cm/s (Figure 2 and Table II in the online-only Data Supplement).

Vieira and his colleagues[10] defined a cut off value of 143.50 cm/s for HbSC individuals in the same population of this study. Using this cut off, we found 60 HbSC individuals with TAMMV lower than 143.50 cm/s and 8 individuals with TAMMV above to 143.50 cm/s. By comparing hematological, biochemical and immunological laboratory profiles between these two groups, we found significant increase of hemoglobin and hematocrit concentration in HbSC individuals with TAMMV lower than 143.50 cm/s, and in the levels of ferritin in HbSC individuals with TAMMV higher than 143.50 cm/s (Figure 3 and Table III in the online-only Data Supplement).

Values of TAMMV at 75th percentile and 95th percentile were used in order to evaluate hematological, biochemical and immunological profile. Using the 75th percentile, which is 125.75 cm/s, we had 51 individuals with TAMMV below 125.75 cm/s and 17 individuals with TAMMV above 125.75 cm/s. Comparing the hematological, biochemical and immunological data of these two groups, we found significant differences in hemoglobin, hematocrit, RDW and NO levels, which were elevated in HbSC individuals with TAMMV lower than 143.50 cm/s; and in the ferritin levels, which were elevated in HbSC individuals with TAMMV above 143.50 cm/s (Table 1 and Figure I in the online-only Data Supplement). Using the 95th percentile, which was 156.00 cm/s, we found 64 individuals with TAMMV below 156.00 cm/s and 4 individuals with TAMMV above 156.00 cm/s. Comparing the hematological, biochemical and immunological data of these two groups, we found significant differences in hemoglobin and hematocrit levels, which were elevated in HbSC individuals with TAMMV lower than 156.00 cm/s; and in the monocyte count and ferritin levels that were elevated in HbSC individuals with TAMMV above 156.00 cm/s (Figure 4 and Table IV in the online-only Data Supplement).

When the genetic data was evaluated, we found 43 individuals with wild-type genotype and 24 heterozygous for the *MTHFR* 677C> T polymorphism; 67 individuals with wild-type genotype and one heterozygous for the *FV* 1691G>A polymorphism; 57 individuals with wild-type genotype and 10 heterozygous and one recessive homozygous for the *VCAM* 833T>C; 61 individuals with wild-type genotype and 7 heterozygous for the *VCAM* 1238T>C polymorphisms and 68 individuals with wild-type genotype for *PT* 20210G>A. We found 53 individuals with wild-type genotype of thalassemia alpha-3.7kb and 13 heterozygous. The haplotype analyses showed 35 individuals with CAR haplotype and 27 non-CAR haplotype.

The multivariate analysis investigates the interaction of hemoglobin, hematocrit, RDW, Ferritin, NO, thalassemia alpha-3.7kb, CAR haplotype, and *MTHFR* 677C>T, *FV* 1691G>A, *VCAM* 833T>C and *VCAM* 1238G>C gene polymorphisms on TAMMV 75th percentile (Table 2).

Discussion

The present study investigated the association of genetic, hematological, immunological and biochemical data with TAMMV, aiming to find possible stroke predictor markers in HbSC individuals.

Our correlation results show that HbSC individuals with high TAMMV had low RBC, hemoglobin, hematocrit and direct bilirubin levels. Thus, these individuals had a more severe anemia than the individuals with a low TAMMV. These data are consistent with previous studies that associated hemolysis markers with the susceptibility of stroke in SCA.[24] Some authors have reported that severe anemia can be an additional risk factor for the development of stroke. Likewise, it has been suggested that the increase in cerebral blood flow and the flow velocity associated with chronic anemia cause disturbances in the flow, which may lead to cerebrovascular damage. [6, 11, 25]

In addition, our data indicate that HbSC individuals with high TAMMV had higher monocyte counts and ferritin levels. Our findings are supported by previous reports that described that elevated white blood cell counts also seem to be a risk factor for a wide range of complications associated with SCD including stroke, pain crisis and ACS. This can be explained by the adverse effect of neutrophils on the vascular endothelium.[12] High ferritin levels are described during inflammatory and infectious processes. The observation that high ferritin values are present in individuals with elevated TAMMV can be associated with a chronic inflammatory state and a chronic hemolytic event.[26]

Using a cut off value of 128 cm/s defined by Deane and his colleagues,[9] we found that low RDW and NO levels were associated with HbSC individuals with TAMMV greater than 128 cm/s. These data can be explained by intravascular hemolysis, where free hemoglobin is released into the vascular microenvironment and reacts rapidly degrading NO, with simultaneous arginase release in plasma. This cascade of events results in reactive oxygen species formation and leads to vasoconstriction in individuals with SCA.[27] This data is also consistent with previous finding [28] that demonstrate that continuous NO production is important for maintaining cerebral blood flow in an experimental model of stroke.

Using a cut off value of 143.50 cm/s previously defined by Vieira and his colleagues[10], our results suggest that low hemoglobin and hematocrit concentrations were associated with HbSC individuals with TAMMV higher than 143.50 cm/s and that high ferritin levels were associated with HbSC individuals with TAMMV higher than 143.50 cm/s. These data indicate that individuals with TAMMV higher than 143.50 cm/s had severe anemia and a state of inflammation and hemolytic process as discussed above.

When the 75th percentile was used in order to evaluate the laboratorial data, we found a combination of markers in the TAMMV defined by Deane and his colleagues[9] and Vieira and his colleagues[10]. Our results show that low hemoglobin, hematocrit, RDW and NO concentration were associated to HbSC individuals with TAMMV higher than 125.75 cm/s and that high ferritin levels were associated to HbSC individuals with TAMMV higher than 125.75 cm/s. Thus, HbSC individuals with TAMMV lower than described by Deane and Vieira already present hematological and biochemical alterations found when we use the velocities described on those studies.[9, 10] Our result, in turn, suggests that the TAMMV of 125.75cm/s would be ideal for HbSC individuals screening.

When the 95th percentile was used in order to evaluate the laboratorial data, we found that low hemoglobin and hematocrit concentration and high monocyte count and ferritin levels were associated with HbSC individuals with TAMMV higher than 156.00 cm/s. These data indicate that individuals with TAMMV higher than 156.00 cm/s had severe anemia and a state of chronic inflammation, hemolytic process and increased monocyte counts.

The results of multivariate analysis corroborate the influence of the hemoglobin, hematocrit, RDW, ferritin, NO, thalassemia alpha-3.7kb, CAR haplotype, and *MTHFR* 677C>T, *VCAM* 833T>C and *VCAM* 1238G>C gene polymorphisms in TAMMV higher than 125.75 cm/s and the influence of the *VCAM* 1238G>C and *MTHFR* 677C>T gene polymorphisms, hematocrit, monocyte, ferritin, thalassemia alpha -3.7kb and hemoglobin on TAMMV higher than 156.00 cm/s. Recent studies have individually associated the presence of *MTHFR* 677C>T, *PT* 20210G>A and *FV* 1691G>A polymorphisms with a great risk of stroke development in SCA individuals[29-34] but in the multivariate analysis using the TAMMV higher than 125.75 the presence of polymorphisms on *MTHFR* 677C>T gene may have a protective effect in relation to TAMMV in HbSC individuals. One possible explanation for this finding is that in HbSC individuals, anemia is less severe than that found in SCA, so even if those individuals are heterozygous for *MTHFR* gene, as found in this study, causing enzyme reduced activity, these individuals may metabolize folate enough to supply their body demands. The absence of thalassemia alpha -3.7kb shows a protective effect in relation to TAMMV. This is in agreement with previous studies that individually analyzed thalassemia alpha -3.7kb, and found no significant association regarding the TAMMV.[12] The HbSC individuals have a high blood viscosity and the presence of thalassemia alpha -3.7kb is associated with increased risk of the viscosity-vaso-occlusive phenotypes, like acute painful episodes, osteonecrosis and ACS, hence,

the absence of thalassemia alpha -3.7kb is associated with improved clinical status in these individuals.[35]

Our results of multivariate analysis confirm the influence of the haplotype CAR in the high TAMMV. This is in agreement with previous study[19] that individually analyzing the haplotype CAR and found association with increased stroke risk.[26]

Conclusion

We suggest that RBC, hemoglobin, hematocrit, RDW, monocyte, direct bilirubin, NO and ferritin are predictor markers of stroke in HbSC individuals. Our data shows that these markers are involved with inflammation and hemolysis in SCD. The polymorphisms on the *MTHFR* 677C>T gene and the absence of thalassemia alpha -3.7kb may be predictor markers of stroke. Based on our results, we also suggest a new TAMMV of 125.75 cm/s that should be investigated in additional studies as a cut-off point for stroke risk in HbSC individuals. This TAMMV is lower than the TAMMV proposed by Deane et al [9] and Vieira et al[10] and have shown to be associated with hematological and biochemical changes found in both velocities 128 cm/s and 143.50 cm/s respectively.

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Disclosures

None.

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Figure legends

Figure 1. Correlations between the TAMMV and biomarkers in HbSC individuals. (A) Red blood cells (RBC), Hemoglobin (Hb), Hematocrit (Ht) and Direct Bilirubin (DB) are negatively correlated with TAMMV; Monocyte and Ferritin are positively correlated with TAMMV.

Figure 2. **Association of hematological, biochemical and immunological data among HbSC individuals with TAMMV defined by Deane and colleagues (2007).** (A) HbSC individuals with TAMMV lower than 128 cm/s have high RDW level (*p*-value calculated using Mann Whitney); (B) HbSC individuals with TAMMV lower than 128 cm/s have high NO metabolites levels (*p*-value calculated using t test).

Figure 3. Association of hematological, biochemical and immunological data among HbSC individuals with TAMMV defined by Viera and colleagues (2015). (A) HbSC individuals with TAMMV lower than 143.50 cm/s have higher haemoglobin levels (p-value calculated using t test); (B) HbSC individuals with TAMMV lower than 143.50 cm/s have higher haematocrit levels (p-value calculated using t test); (C) HbSC individuals with TAMMV higher than 143.50 cm/s have higher ferritin level (p-value calculated using Mann Whitney).

Figure 4. Association of hematological, biochemical and immunological data in HbSC individuals with TAMMV defined using the 95th percentile. A) HbSC individuals with TAMMV lower than 156.00 cm/s have high haemoglobin levels (p-value calculated using t test); (B) HbSC individuals with TAMMV lower than 156.00 cm/s have high hematocrit levels (p-value calculated using t test); (C) HbSC individuals with TAMMV higher than 156.00 cm/s have high monocyte (p-value calculated using Mann Whitney); (D) HbSC individuals with TAMMV higher than 156.00 cm/s have high ferritin level (p-value calculated using Mann Whitney).

Figure 1

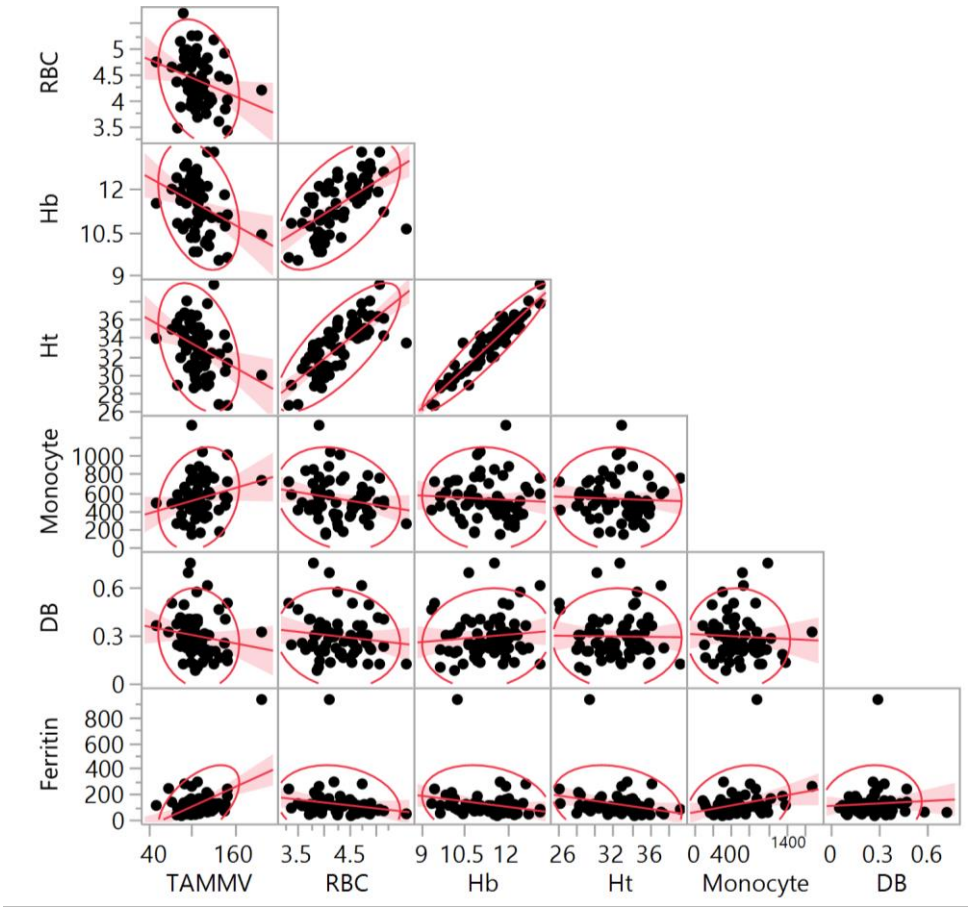


Figure 2

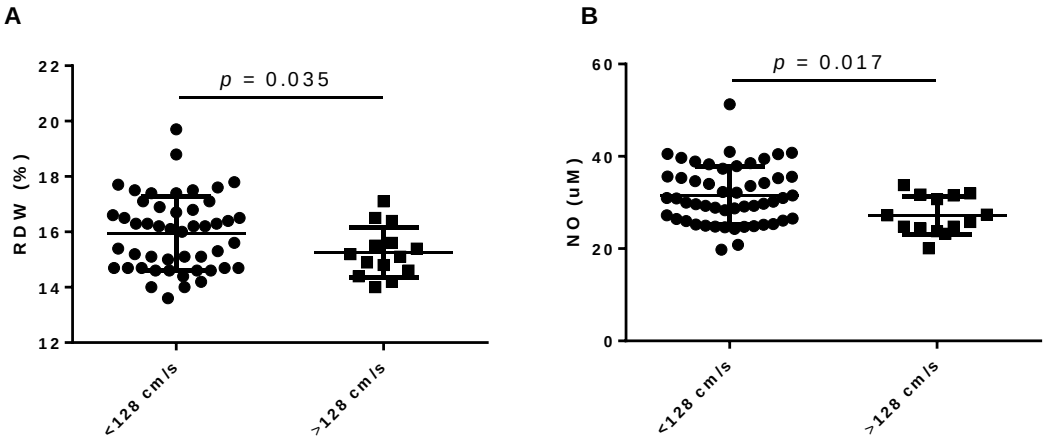

A
p = 0.035
RDW (%)
22
20
18
16
14
12
<128 cm/s
>128 cm/s
B
p = 0.017
NO (uM)
60
40
20
0
<128 cm/s
>128 cm/s

Figure 3

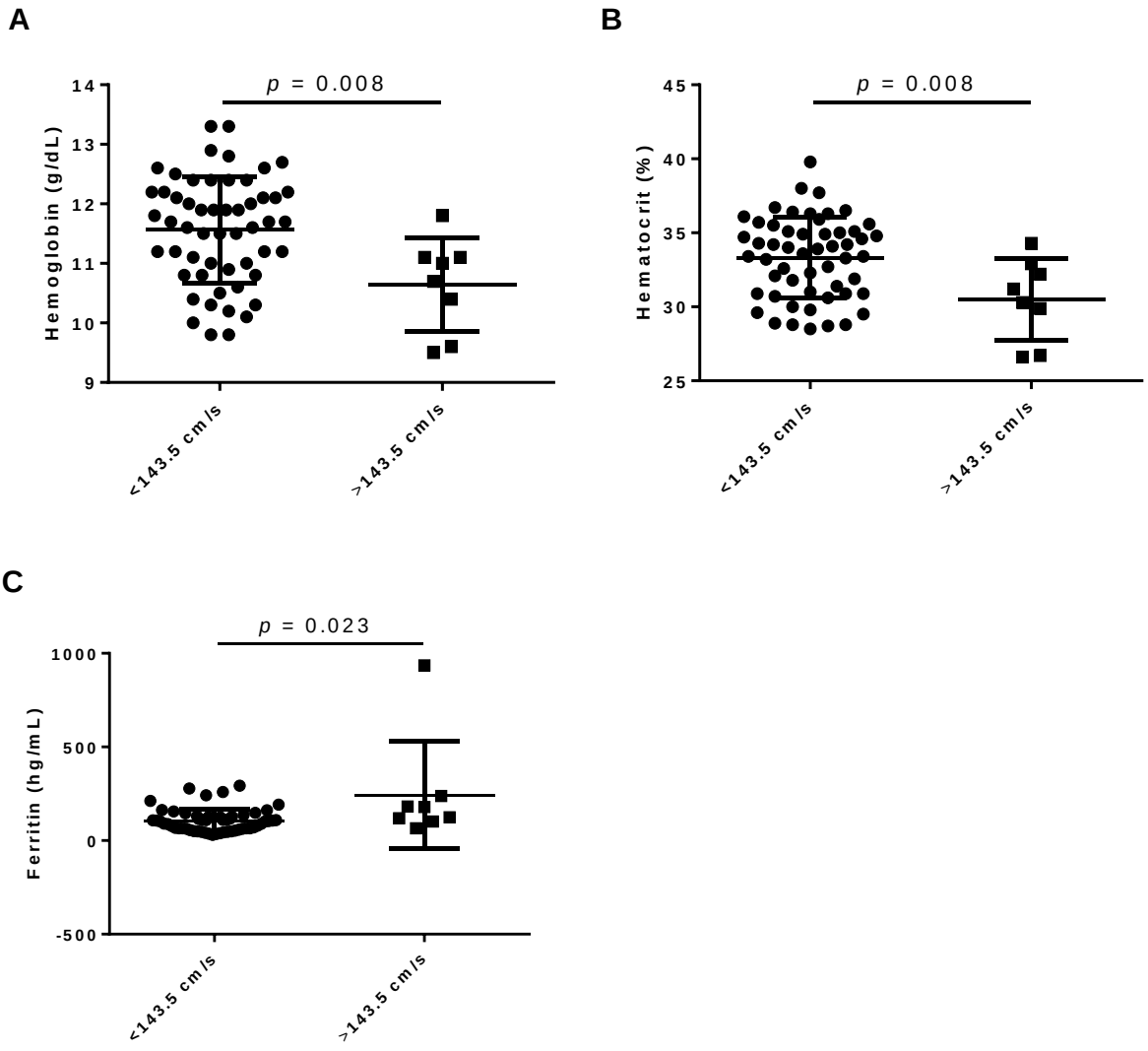

A
p = 0.008
Hemoglobin (g/dL)
14
13
12
11
10
9
<143.5 cm/s
>143.5 cm/s
B
p = 0.008
Hematocrit (%)
45
40
35
30
25
<143.5 cm/s
>143.5 cm/s
C
p = 0.023
Ferritin (hg/mL)
1000
500
0
-500
<143.5 cm/s
>143.5 cm/s

Figure 4

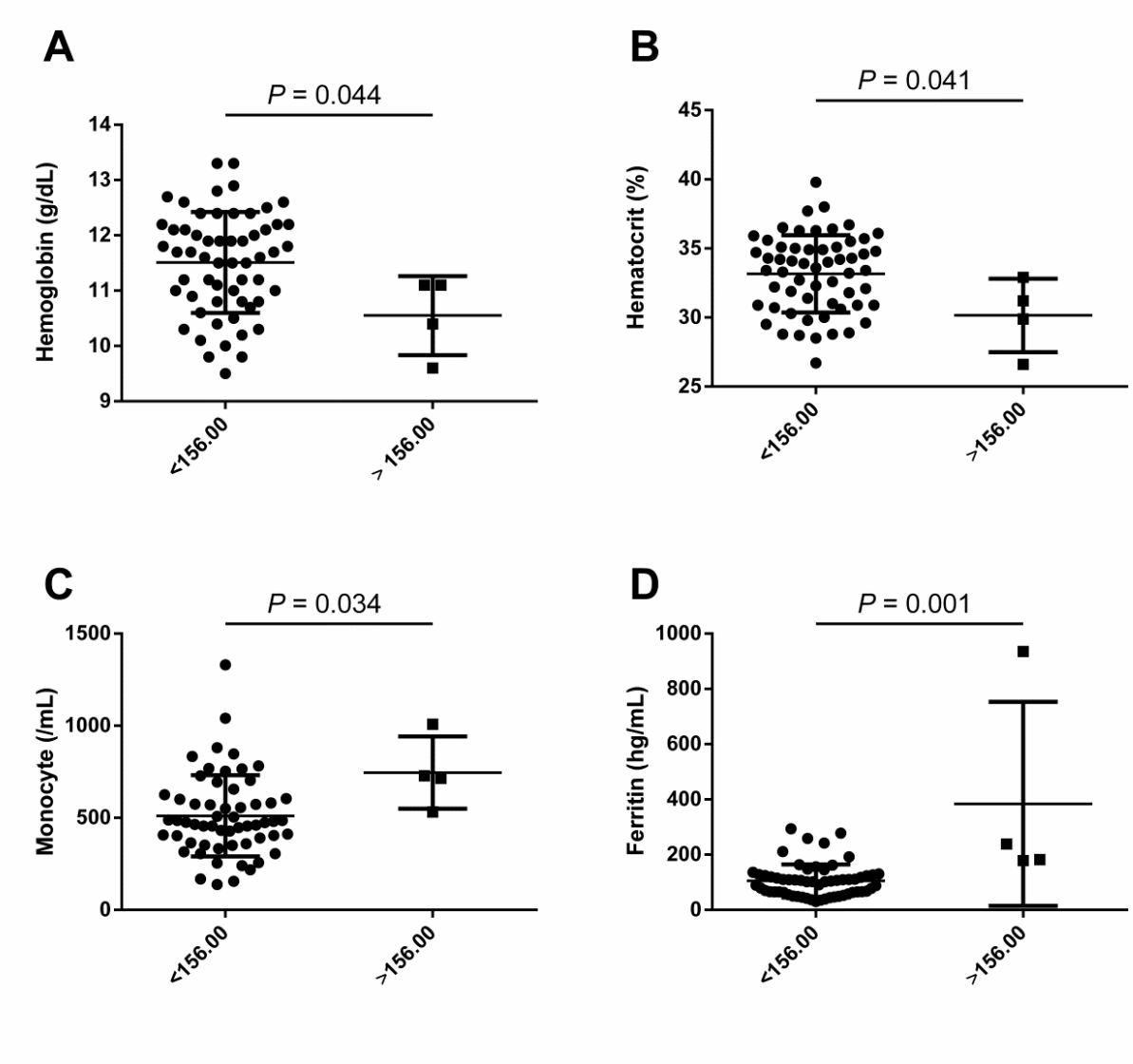

A
P = 0.044
Hemoglobin (g/dL)
14
13
12
11
10
9
<156.00
> 156.00
B
P = 0.041
Hematocrit (%)
45
40
35
30
25
<156.00
>156.00
C
P = 0.034
Monocyte (/mL)
1500
1000
500
0
<156.00
>156.00
D
P = 0.001
Ferritin (hg/mL)
1000
800
600
400
200
0
<156.00
>156.00

Table 1. Comparison of laboratory data of HbSC individuals with TAMMV velocities defined using a cut off value of 125.75 cm/s (75th percentile)

Laboratory value	TAMMV <125.75 cm/s		TAMMV ≥ 125.75 cm/s		*p* value*
	N	Mean ± SD	N	Mean ± SD	
Hemolysis markers					
RBC, x10^{12}/L	46	4.44 ± 0.48	16	4.18 ± 0.49	0.073
Hemoglobin, g/dL	46	11.58 ± 0.81	16	11.04 ± 1.12	**0.042**
Hematocrit, %	46	33.40 ± 2.45	16	31.68 ± 3.59	**0.037**
MCV, fL	46	75.63 ± 5.63	16	75.87 ± 3.77	0.877
MCH, ρg	46	26.30 ± 2.53	16	26.48 ± 1.58	0.783
MCHC, g/dL	46	34.90 ± 0.76	16	34.95 ± 0.79	0.532
RDW (%)	46	16.00 ± 1.34	16	15.19 ± 0.90	**0.010**
Reticulocyte Count, %	45	4.05 ± 1.94	16	3.74 ± 1.62	0.787†
Total bilirubin, mg/dL	51	1.27 ± 1.15	17	1.08 ± 0.89	0.237†
Direct bilirubin, mg/dL	51	0.30 ± 0.13	17	0.27 ± 0.14	0.288†
Indirect bilirubin, mg/dL	51	0.97 ± 1.08	17	0.80 ± 0.77	0.357†
LDH, U/L	50	584.92 ± 201.47	15	531.67 ± 110.50	0.528†
NO metabolite, uM	50	31.56 ± 6.36	16	27.75 ± 4.20	**0.029**
Iron metabolism					
Serum Iron, mcg/dL	49	69.30 ± 22.89	16	77.88 ± 22.91	0.180†
Ferritin, ηg/mL	47	102.82 ± 62.63	17	177.50 ± 202.97	**0.029**†

RBC: Red blood cells; MCV: Mean cell volume; MCH: Mean cell hemoglobin; SD: standard deviation. *p-value using t test †p-value using Mann-Whitney **Standard error

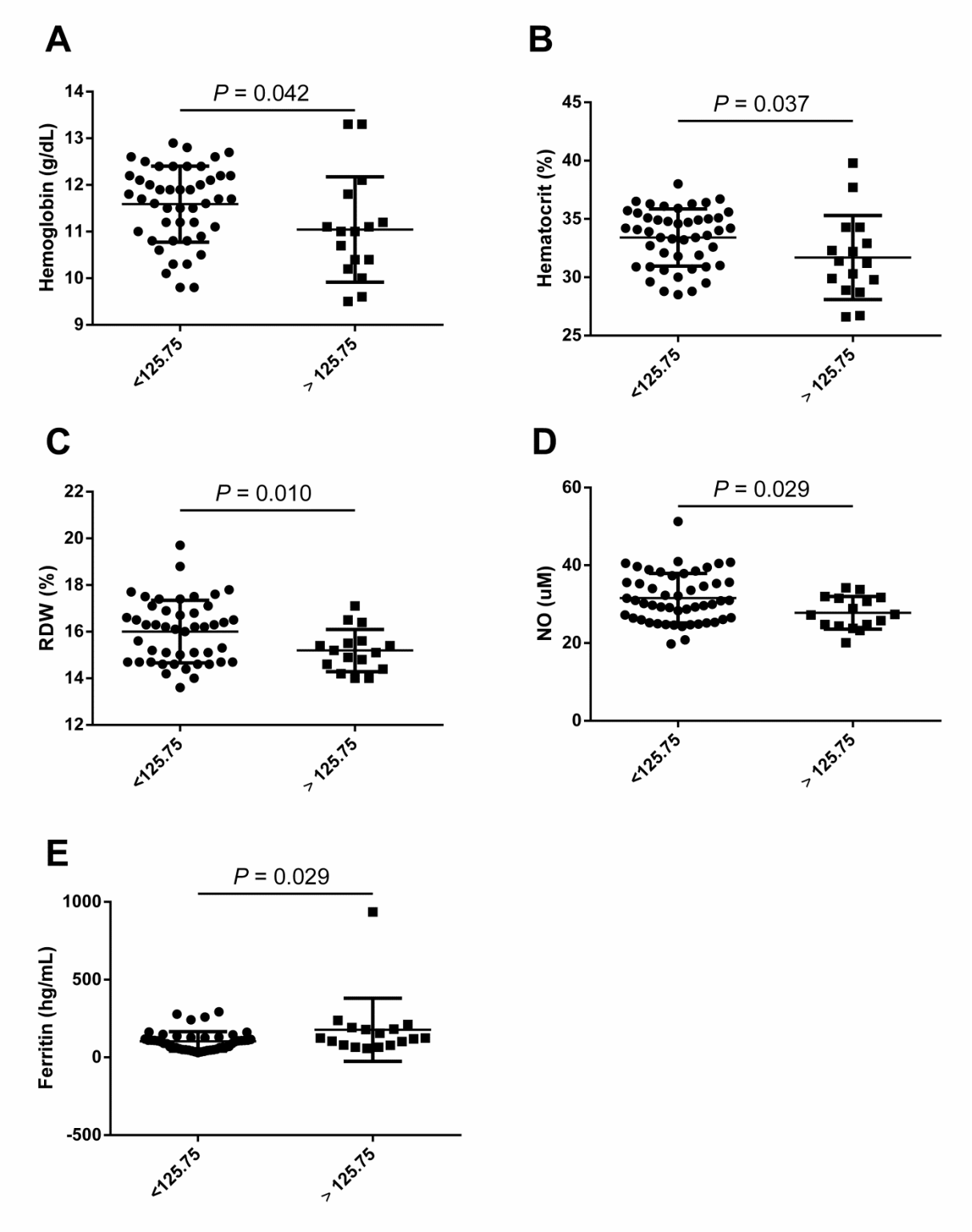
Table 2. Multivariable model associating hematologic and biochemical data and gene polymorphisms in TAMMV 75th percentile.

Variables	B	S.E.	Wald	*P* value	OR	95% C.I.		R Square	*P* model
						Lower	Upper		
Model 1									
Hematocrit	2.95	1.47	4.02	**0.045**	19.25	1.07	346.20		
RDW	-3.79	1.50	6.36	**0.012**	0.02	0.00	0.42		
Ferritin	2.09	1.33	2.45	0.117	8.13	0.59	111.87		
NO	3.33	1.58	4.42	**0.036**	27.99	1.25	625.23		
Absence of alpha Talassemia -3.7kb	-1.53	1.21	1.59	0.206	0.21	0.02	2.32	0.432	**0.004**
CAR Haplotype	1.59	1.06	2.25	0.133	4.90	0.61	39.13		
MTHFR C677T	-1.85	1.12	2.71	0.100	0.15	0.01	1.42		
VCAM T833C	1.73	1.22	2.01	0.155	5.66	0.51	61.91		
VCAM G1238C	3.41	1.94	3.10	0.078	30.50	0.68	1368.45		

B: beta coefficient; S.E.: standard error; OR: Odds Ratio; C.I.: confidence interval.

Supplemental Figures

Supplemental figure I



Supplemental Figure I: Association of hematological, biochemical and immunological data among HbSC patients with TAMMV defined using the 75th percentile. A) HbSC patients with TAMMV lower than 125.75 cm/s have high haemoglobin levels (p-value calculated using t test); (B) HbSC patients with TAMMV lower than 125.75 cm/s have high hematocrit levels (p-value calculated using t test); (C) HbSC patients with TAMMV lower than 125.75 cm/s have high RDW (p-value calculated using t test); (D) HbSC patients with TAMMV lower than 125.75 cm/s have high NO metabolite levels (p-value calculated using t test); (E) HbSC patients with TAMMV higher than 125.75 cm/s have high ferritin level (p-value calculated using Mann Whitney).

Supplemental Tables

Supplemental Table I. Baseline characteristics of HbSC patients, including anthropometric, TCD, hematological, biochemical and immunological data.

Laboratory value	N	Mean ± SD	Percentile values		
			25th	50th	75th
TCD					
TAMMV	68	114.31 ± 22.72	101.50	111.50	125.75
Hemolysis markers					
RBC, $x10^{12}$/L	62	4.37 ± 0.49	3.98	4.33	4.75
Hemoglobin, g/dL	62	11.44 ± 0.92	10.80	11.55	12.12
Hematocrit, %	62	32.96 ± 2.86	30.85	33.35	35.02
MCV, fL	62	75.70 ± 5.19	72.12	75.10	79.32
MCH, ρg	62	26.34 ± 2.31	24.87	25.95	27.85
MCHC, g/dL	62	34.76 ± 1.01	34.00	34.90	35.32
RDW (%)	62	15.79 ± 1.28	14.70	15.55	16.62
Reticulocyte Count, %	61	3.97 ± 1.85	2.75	3.50	4.95
Total bilirubin, mg/dL	68	1.22 ± 1.09	0.60	0.90	1.33
Direct bilirubin, mg/dL	68	0.29 ± 0.13	0.20	0.28	0.36
Indirect bilirubin, mg/dL	68	0.93 ± 1.01	0.38	0.61	0.95
LDH, U/L	65	572.63 ± 185.09	442.00	554.00	663.50
NO metabolite, uM	66	30.63 ± 6.10	25.31	29.86	34.77
Hemoglobin pattern					
Fetal haemoglobin, %	68	2.91 ± 2.28	1.50	2.30	4.02
S haemoglobin, %	68	52.09 ± 2.53	50.40	51.80	53.85
C haemoglobin, %	68	40.59 ± 2.47	39.32	40.70	42.37
A_2 haemoglobin, %	68	4.33 ± 1.37	3.62	4.20	4.80
Leukocytes					
WBC, x 10^9/L	62	8249.31 ± 2233.32	6734.50	8080.00	9670.00
Neutrophil count, x 10^9/L	62	4269.73 ± 1724.12	2753.00	4011.00	5561.50
Segmented count, x 10^9/L	62	4267.71 ± 1724.47	2753.00	4011.00	5561.50
Eosinophil count , x 10^9/L	62	434.29 ± 333.50	208.75	349.50	598.00
Basophil count , x 10^9/L	62	74.84 ± 90.46	0	54.50	106.00
Lymphocyte count, x 10^9/L	62	2860.61 ± 1068.08	2064.25	2537.50	3522.00
Monocyte count , x 10^9/L	62	526.18 ± 225.19	383.50	483.00	664.75
Platelets					
Platelet count, $x10^3$/mL	62	251.16 ± 87.93	183.00	230.50	305.75
Platelet Volume Average, fL	62	7.41 ± 1.84	6.00	7.00	8.52
Glucose					
Glucose, mg/dL	66	75.29 ± 12.21	68.00	75.00	84.25
Lipid metabolism					

Total Cholesterol, mg/dL	67	136.24 ± 28.17	121.00	133.00	147.00
HDL-C, mg/dL	65	41.20 ± 9.90	34.00	40.00	48.00
LDL-C, mg/dL	65	80.37 ± 23.52	67.30	78.00	91.20
VLDL-C, mg/dL	68	14.00 ± 4.75	10.20	13.50	17.40
Triglycerides, mg/dL	68	70.00 ± 23.76	51.00	67.50	87.00
Liver					
ALT, U/L	68	16.04 ± 8.67	11.00	14.00	19.00
AST, U/L	68	30.40 ± 10.00	23.00	28.50	36.75
Total protein, g/dL	67	7.28 ± 0.53	6.92	7.26	7.64
Albumin, g/dL	67	4.34 ± 0.32	4.00	4.40	4.60
Globulin, g/dL	67	2.94 ± 0.59	2.50	3.00	3.40
Albumin /Globulin Ratio	67	1.55 ± 0.41	1.20	1.40	1.90
Iron metabolism					
Serum Iron, mcg/dL	65	71.41 ± 23.02	53.45	66.60	88.35
Ferritin, ηg,/mL	64	122.66 ± 120.13	64.00	105.75	134.72
Kidney					
Urea nitrogen, mg/dL	68	19.10 ± 5.57	15.00	19.00	23.00
Creatinine, mg/dL	68	0.52 ± 0.13	0.43	0.50	0.58
Inflammation					
CRP, mg/L	42	3.33 ± 3.11	1.39	2.31	4.35
AAT, mg/dL	48	132.42 ± 32.70	120.50	139.50	150.75
Haptoglobin, mg/dL	48	11.16 ± 17.88	5.83	5.83	5.83

RBC: Red blood cells; MCV: Mean cell volume; MCH: Mean cell hemoglobin; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; VLDL-C: Very low-density lipoprotein cholesterol; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; CRP: C reactive protein; AAT: Alpha 1-antitrypsin; NO: nitric oxide; SD: standard deviation.

Supplemental Table II. Comparison of laboratory data of HbSC patients with TAMMV velocities defined using a cut off value of 128 cm/s, as described by Deane et al., 2007.

Laboratory value	TAMMV <128 cm/s		TAMMV ≥128 cm/s		*p* value*
	N	Mean ± SD	N	Mean ± SD	
Hemolysis					
RBC, $x10^{12}$/L	48	4.41 ± 0.48	14	4.23 ± 0.51	0.237
Hemoglobin, g/dL	48	11.55 ± 0.82	14	11.09 ± 1.18	0.104
Hematocrit, %	48	33.26 ± 2.50	14	31.92 ± 3.76	0.123
MCV, fL	48	75.77 ± 5.66	14	75.44 ± 3.24	0.860†
MCH, ρg	48	26.37 ± 2.53	14	26.25 ± 1.37	0.869
MCHC, g/dL	48	34.75 ± 1.08	14	34.80 ± 0.76	0.873
RDW (%)	48	15.94 ± 1.34	14	15.26 ± 0.90	**0.035**
Reticulocyte Count, %	47	3.94 ± 1.97	14	4.07 ± 1.44	0.487†
Total bilirubin, mg/dL	53	1.26 ± 1.13	15	1.11 ± 0.95	0.209†
Direct bilirubin, mg/dL	53	0.29 ± 0.13	15	0.28 ± 0.14	0.336†
Indirect bilirubin, mg/dL	53	0.96 ± 1.06	15	0.82 ± 0.82	0.304†
LDH, U/L	51	588.90 ± 201.46	14	513.36 ± 87.94	0.303†
NO metabolite, uM	52	31.56 ± 6.26	14	27.21 ± 4.09	**0.017**
Hemoglobin pattern					
Fetal haemoglobin, %	53	2.93 ± 2.32	15	2.84 ± 2.20	0.745†
S haemoglobin, %	53	52.10 ± 2.67	15	52.06 ± 2.02	0.988†
C haemoglobin, %	53	40.72 ± 2.32	15	40.14 ± 3.00	0.559†
A_2 haemoglobin, %	53	4.27 ± 1.49	15	4.56 ± 0.80	0.270†
Leukocytes					
WBC, x 10^9/L	48	8185.98 ± 2259.70	14	8466.43 ± 2208.48	0.683
Neutrophil count, x 10^9/L	48	4224.71 ± 1742.35	14	4424.07 ± 1714.53	0.707
Segmented count, x 10^9/L	48	4223.83 ± 1743.98	14	4418.14 ± 1710.67	0.714
Eosinophil count , x 10^9/L	48	420.23 ± 266.87	14	482.50 ± 511.05	0.625†
Basophil count , x 10^9/L	48	67.31 ± 76.92	14	100.64 ± 126.81	0.384†
Lymphocyte count, x 10^9/L	48	2867.21 ± 1092.40	14	2838.00 ± 1018.75	0.893†
Monocyte count , x 10^9/L	48	509.42 ± 229.04	14	583.64 ± 209.04	0.134†
Platelets					
Platelet count, $x10^3$/mL	48	248.38 ± 86.60	14	260.71 ± 95.07	0.434†
Platelet Volume Average, fL	48	7.59 ± 1.89	14	6.80 ± 1.59	0.138†
Glucose					
Glucose, mg/dL	52	74.87 ± 12.66	14	76.86 ± 10.68	0.592
Lipid metabolism					
Total Cholesterol, mg/dL	52	138.37 ± 29.04	15	128.87 ± 24.41	0.253

HDL-C, mg/dL	51	41.71 ± 10.43	14	39.36 ± 7.72	0.436
LDL-C, mg/dL	51	82.24 ± 24.51	14	73.57 ± 18.68	0.224
VLDL-C, mg/dL	53	14.16 ± 4.46	15	13.41 ± 5.80	0.592
Triglycerides, mg/dL	53	70.83 ± 22.31	15	67.07 ± 29.00	0.592
Liver					
ALT, U/L	53	16.98 ± 9.41	15	12.73 ± 4.00	0.072†
AST, U/L	53	31.21 ± 10.81	15	27.53 ± 5.73	0.343†
Total protein, g/dL	52	7.30 ± 0.54	15	7.21 ± 0.49	0.571
Albumin, g/dL	52	4.34 ± 0.32	15	4.34 ± 0.30	0.980
Globulin, g/dL	52	2.96 ± 0.59	15	2.86 ± 0.62	0.558
Albumin /Globulin Ratio	52	1.54 ± 0.40	15	1.61 ± 0.46	0.672†
Iron metabolism					
Serum Iron, mcg/dL	51	69.45 ± 22.61	14	78.55 ± 23.92	0.207†
Ferritin, ηg,/mL	49	105.08 ± 63.24	15	180.08 ± 215.91	0.060†
Kidney					
Urea nitrogen, mg/dL	53	19.40 ± 5.01	15	18.07 ± 7.34	0.173†
Creatinine, mg/dL	53	0.52 ± 0.14	15	0.53 ± 0.07	0.219†
Inflammation					
CRP, mg/L	32	3.15 ± 2.32	10	3.93 ± 5.01	0.673†
AAT, mg/dL	37	132.35 ± 34.00	11	132.69 ± 29.37	0.589†
Haptoglobin, mg/dL	37	9.92 ± 12.60	11	15.36 ± 30.09	0.675†

RBC: Red blood cells; MCV: Mean cell volume; MCH: Mean cell haemoglobin; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; VLDL-C: Very low-density lipoprotein cholesterol; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; CRP: C reactive protein; AAT: Alpha 1-antitrypsin; NO: nitric oxide; SD: standard deviation. *p-value using t test †p-value using Mann-Whitney.

Supplemental Table III. Comparison of laboratory data of HbSC patients with TAMMV velocities defined using a cut off value of 143.50 cm/s as described by Vieira et al. (under submission)

Laboratory value	TCD <143.5 cm/s		TCD ≥143.5 cm/s		*p* value*
	N	Mean ± SD	N	Mean ± SD	
Hemolysis markers					
RBC, $x10^{12}$/L	54	4.41 ± 0.48	8	4.09 ± 0.49	0.087
Hemoglobin, g/dL	54	11.56 ± 0.89	8	10.65 ± 0.78	**0.008**
Hematocrit, %	54	33.32 ± 2.71	8	30.51 ± 2.76	**0.008**
MCV, fL	54	75.83 ± 5.41	8	74.77 ± 3.47	0.522†
MCH, ρg	54	26.37 ± 2.41	8	26.15 ± 1.67	0.798
MCHC, g/dL	54	34.73 ± 1.05	8	34.95 ± 0.79	0.579
RDW (%)	54	15.81 ± 1.34	8	15.67 ± 0.86	0.900†
Reticulocyte Count, %	53	3.91 ± 1.93	8	4.32 ± 1.28	0.295†
Total bilirubin, mg/dL	60	1.24 ± 1.13	8	1.12 ± 0.76	0.753†
Direct bilirubin, mg/dL	60	0.29 ± 0.13	8	0.30 ± 0.14	0.834†
Indirect bilirubin, mg/dL	60	0.94 ± 1.05	8	0.82 ± 0.63	0.849†
LDH, U/L	57	578.26 ± 194.90	8	532.50 ± 85.01	0.826†
NO metabolite, uM	58	31.07 ± 6.17	8	27.46 ± 4.79	0.141†
Hemoglobin pattern					
Fetal haemoglobin, %	60	2.96 ± 2.24	8	2.53 ± 2.71	0.216†
S haemoglobin, %	60	52.16 ± 2.62	8	51.62 ± 1.68	0.487†
C haemoglobin, %	60	40.50 ± 2.46	8	41.23 ± 2.62	0.458†
A_2 haemoglobin, %	60	4.30 ± 1.42	8	4.60 ± 0.96	0.475†
Leukocytes					
WBC, x 10^9/L	54	8196.24 ± 2170.72	8	8607.50 ± 2760.33	0.631
Neutrophil count, x 10^9/L	54	4228.02 ± 1729.54	8	4551.25 ± 1775.18	0.625
Segmented count, x 10^9/L	54	4225.70 ± 1729.89	8	4551.25 ± 1775.18	0.622
Eosinophil count , x 10^9/L	54	403.63 ± 258.74	8	641.25 ± 637.30	0.629†
Basophil count , x 10^9/L	54	70.30 ± 78.08	8	105.50 ± 154.65	0.672†
Lymphocyte count, x 10^9/L	54	2882.24 ± 1061.35	8	2714.63 ± 1176.56	0.450†
Monocyte count , x 10^9/L	54	519.61 ± 222.92	8	570.50 ± 251.16	0.515†
Platelets					
Platelet count, $x10^3$/mL	54	245.56 ± 85.24	8	289.00 ± 103.38	0.156†
Platelet Volume Average, fL	54	7.53 ± 1.93	8	6.57 ± 0.77	0.215†
Glucose					
Glucose, mg/dL	59	75.46 ± 12.31	7	73.86 ± 12.24	0.746
Lipid metabolism					
Total Cholesterol, mg/dL	59	138.05 ± 27.76	8	122.88 ± 29.47	0.238†

HDL-C, mg/dL	58	41.38 ± 9.99	7	39.71 ± 9.77	0.678
LDL-C, mg/dL	58	82.14 ± 23.44	7	65.77 ± 20.04	0.122†
VLDL-C, mg/dL	60	14.30 ± 4.63	8	11.72 ± 5.34	0.125†
Triglycerides, mg/dL	60	71.52 ± 23.16	8	58.63 ± 26.73	0.125†
Liver					
ALT, U/L	60	16.50 ± 9.08	8	12.63 ± 3.06	0.150†
AST, U/L	60	30.93 ± 10.30	8	26.38 ± 6.45	0.261†
Total protein, g/dL	59	7.30 ± 0.54	8	7.09 ± 0.46	0.300
Albumin, g/dL	59	4.34 ± 0.32	8	4.31 ± 0.32	0.764
Globulin, g/dL	59	2.96 ± 0.59	8	2.77 ± 0.61	0.407
Albumin /Globulin Ratio	59	1.54 ± 0.40	8	1.66 ± 0.52	
Iron metabolism					
Serum Iron, mcg/dL	58	70.66 ± 22.85	7	77.62 ± 25.34	0.568†
Ferritin, ηg,/mL	56	105.41 ± 61.45	8	243.40 ± 284.93	**0.023**†
Kidney					
Urea nitrogen, mg/dL	60	19.32 ± 5.47	8	17.50 ± 6.48	0.391
Creatinine, mg/dL	60	0.52 ± 0.14	8	0.52 ± 0.07	0.594†
Inflammation					
CRP, mg/L	37	3.10 ± 2.24	5	5.07 ± 7.07	0.678†
AAT, mg/dL	42	133.73 ± 33.21	6	123.26 ± 29.82	0.237†
Haptoglobin, mg/dL	42	9.54 ± 11.87	6	22.52 ± 40.89	0.867†

RBC: Red blood cells; MCV: Mean cell volume; MCH: Mean cell haemoglobin; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; VLDL-C: Very low-density lipoprotein cholesterol; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; CRP: C reactive protein; AAT: Alpha 1-antitrypsin; NO: nitric oxide; SD: standard deviation. *p-value using t test †p-value using Mann-Whitney.

Supplemental Table IV. Comparison of laboratory data of HbSC patients with TAMMV velocities defined using a cut off value of 156.00 cm/s (95th percentile).

Laboratory value	TAMMV <156.00 cm/s		TAMMV ≥156.00 cm/s		*p* value*
	N	Mean ± SD	N	Mean ± SD	
Hemolysis markers					
RBC, x10^{12}/L	58	4.40 ± 0.49	4	3.99 ± 0.43	0.116
Hemoglobin, g/dL	58	11.51 ± 0.91	4	10.55 ± 0.71	**0.044**
Hematocrit, %	58	33.15 ± 2.79	4	30.15 ± 2.66	**0.041**
MCV, fL	58	75.70 ± 5.32	4	75.60 ± 3.20	1.000†
MCH, ρg	58	26.33 ± 2.36	4	26.47 ± 1.71	0.911
MCHC, g/dL	58	34.74 ± 1.02	4	35.00 ± 0.98	0.632
RDW (%)	58	15.82 ± 1.31	4	15.40 ± 0.67	0.709†
Reticulocyte Count, %	57	3.98 ± 1.90	4	3.82 ± 1.12	0.833†
Total bilirubin, mg/dL	64	1.24 ± 1.11	4	0.96 ± 0.66	0.521†
Direct bilirubin, mg/dL	64	0.29 ± 0.13	4	0.28 ± 0.16	0.831†
Indirect bilirubin, mg/dL	64	0.94 ± 1.03	4	0.68 ± 0.50	0.660†
LDH, U/L	61	576.07 ± 188.69	4	520.25 ± 122.00	0.683†
NO metabolite, uM	62	30.07 ± 6.23	4	30.47 ± 4.16	0.907†
Hemoglobin pattern					
Fetal haemoglobin, %	64	2.92 ± 2.22	4	2.72 ± 3.55	0.342†
S haemoglobin, %	64	52.07 ± 2.56	4	52.42 ± 2.12	0.773†
C haemoglobin, %	64	40.58 ± 2.46	4	40.67 ± 3.06	0.990†
A_2 haemoglobin, %	64	4.34 ± 1.41	4	4.17 ± 0.43	0.870†
Leukocytes					
WBC, x 10^9/L	58	8132.02 ± 2217.45	4	9950.00 ± 1967.23	0.116
Neutrophil count, x 10^9/L	58	4175.29 ± 1697.18	4	5639.25 ± 1744.20	0.101
Segmented count, x 10^9/L	58	4173.14 ± 1697.44	4	5639.00 ± 1744.20	0.100
Eosinophil count , x 10^9/L	58	413.40 ± 314.88	4	737.25 ± 496.89	0.101†
Basophil count , x 10^9/L	58	68.55 ± 75.97	4	166.00 ± 211.87	0.351†
Lymphocyte count, x 10^9/L	58	87.52 ± 149.09	4	28.00 ± 56.00	0.533†
Monocyte count , x 10^9/L	58	511.05 ± 220.52	4	745.50 ± 196.45	**0.034**†
Platelets					
Platelet count, x10^3/mL	58	245.24 ± 83.80	4	337.00 ± 115.25	0.068†
Platelet Volume Average, fL	58	7.49 ± 1.87	4	6.22 ± 0.74	0.180†
Glucose					
Glucose, mg/dL	62	75.40 ± 12.23	4	73.50 ± 13.62	0.765
Lipid metabolism					
Total Cholesterol, mg/dL	63	136.16 ± 28.64	4	137.50 ± 22.51	0.949†

HDL-C, mg/dL	62	41.10 ± 9.84	3	43.33 ± 13.42	0.706
LDL-C, mg/dL	62	80.83 ± 23.85	3	71.00 ± 14.46	0.463†
VLDL-C, mg/dL	64	13.99 ± 4.73	4	14.05 ± 5.88	0.987
Triglycerides, mg/dL	64	69.98 ± 23.65	4	70.25 ± 29.40	0.983
Liver					
ALT, U/L	64	16.36 ± 8.84	4	11.00 ± 1.41	0.074†
AST, U/L	64	30.58 ± 10.10	4	27.50 ± 8.88	0.660†
Total protein, g/dL	63	7.27 ± 0.54	4	7.34 ± 0.34	0.825
Albumin, g/dL	63	4.34 ± 0.32	4	4.37 ± 0.38	0.969
Globulin, g/dL	63	2.93 ± 0.60	4	2.95 ± 0.47	0.974
Albumin /Globulin Ratio	63	1.56 ± 0.42	4	1.52 ± 0.37	1.000
Iron metabolism					
Serum Iron, mcg/dL	62	71.12 ± 22.95	3	77.40 ± 28.88	0.712†
Ferritin, ηg,/mL	60	105.25 ± 59.63	4	383.75 ± 369.04	**0.001**†
Kidney					
Urea nitrogen, mg/dL	64	19.19 ± 5.50	4	17.75 ± 7.54	0.426
Creatinine, mg/dL	64	0.52 ± 0.13	4	0.50 ± 0.09	0.870†
Inflammation					
CRP, mg/L	40	3.02 ± 2.21	2	9.55 ± 11.23	0.455†
AAT, mg/dL	46	132.49 ± 33.42	2	131.00 ± 0.00	0.543†
Haptoglobin, mg/dL	46	9.22 ± 11.38	2	55.91 ± 70.83	0.348†

RBC: Red blood cells; MCV: Mean cell volume; MCH: Mean cell haemoglobin; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; VLDL-C: Very low-density lipoprotein cholesterol; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; CRP: C reactive protein; AAT: Alpha 1-antitrypsin; NO: nitric oxide; SD: standard deviation. *p-value using t test †p-value using Mann-Whitney.

4.2 MANUSCRITO 2

Título: Genome wide association study of sickle cell disease individuals with stroke risk

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Situação: A ser submetido

Objetivo:

- Investigar marcadores genéticos em indivíduos com HbSC e associar ao risco de AVC na hemoglobinopatia SC

Principais resultados:

Entre os indivíduos com AF, ao estudamos a condição de DTC anormal vs normal, encontramos o total de cinco SNPs; entre a condição de DTC anormal vs condicional encontramos o total de três SNPs, e entre a condição de DTC anormal vs condicional alto encontramos cinco SNPs. Entre o total de indivíduos com AF encontramos dois SNPs comuns. Entre os indivíduos com HbSC, ao estudarmos a condição de DTC anormal vs baixo encontramos o total de oito SNPs, entre os indivíduos com DTC anormal vs normal (<128cm/s) encontramos seis SNPs, e entre DTC anormal vs normal (>128 cm/s) nós encontramos sete SNPs, e entre esses indivíduos com HbSC encontramos um SNP em comum.

Genome wide association study of sickle cell disease individuals with stroke risk

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ABSTRACT

The association of genetic markers and stroke risk in sickle cell disease (SCD), is controversial. Therefore, is important the investigation of single nucleotide polymorphisms (SNPs) and cerebral blood flow velocities (CBV) in SCD. We selected four individuals with sickle cell anemia (SCA) and four with hemoglobin SC disease (HbSC) in according to CBV achieved by transcranial Doppler (TCD). SNPs were developed in an Illumina HiScan platform and the analysis was performed on SNPnexus online tool, and the network of protein-protein interactions was constructed in the STRING database. We found five unique SNPs in the SCA groups of abnormal TCD vs normal TCD; three unique SNPs in the abnormal TCD vs conditional TCD; and five unique SNPs in the abnormal vs high conditional TCD. We found two common SNPs in the SCA individuals. In the HbSC genotype, we found a total of eight unique SNPs in the abnormal TCD vs low TCD groups; six unique SNPs in the abnormal TCD vs normal (<128 cm/s) TCD groups, seven unique SNPs in the abnormal TCD vs normal (>128 cm/s) TCD groups, and one common SNP in all HbSC groups. We suggest that the DOCK6 rs2278426, TYR rs1042602, CYP4F2 rs2108622, MST1 rs3197999, OR51B5/6 rs5006884, THADA rs7578597, FUT2 rs602662, MTHFR rs1801133, TSEN15 rs1046934, CFB rs12614 and ABCG5 rs6756629 SNPs may be associated with high CBV on HbSC individuals and that the SLCO1B1 rs4149056, PRIM1 rs2277339, APOB rs676210, TYK2 rs12720356, TSEN15 rs1046934, CYP4F2 rs2108622 and MST1 rs3197999 SNPs may be associated with CBV on SCA individuals.

Key words: Genes, stroke, sickle cell anemia, hemoglobin SC disease, transcranial Doppler.

INTRODUCTION

The sickle cell disease (SCD) is characterized by the presence of hemoglobin S (HbS) associated with other hemoglobin variants (C and D, for example) as is found in the hemoglobin SC disease (HbSC), or with globin chain synthesis defects, such as in thalassemia. The sickle cell anemia (SCA) is the most severe form of SCD, in which the beta S allele (β^S) is in homozygosis, featuring HbSS genotype (Bunn 1997; Steinberg 2001).

Among the most common clinical manifestations in SCD we can highlight the vaso-occlusive events, stroke, acute chest syndrome, priapism, pulmonary hypertension, retinopathy, hemolytic anemia, splenic sequestration, osteonecrosis, infections and ulcers, among others (Kato et al. 2007; Sonati and Costa 2008; Steinberg 2009).

Stroke is the main cause of death in children and adults with SCD (Leikin et al. 1989; Platt et al. 1994). A child with SCD has 333 times increased risk of developing stroke, when compared to a healthy child or any heart disease (Ohene-Frempong et al. 1998). This cerebrovascular event can have serious consequences in about 7% of children with SCD, with the possibility of new episodes (0.7% per year) during the first 20 years of life (Oliveira et al. 2008).

Recent studies have associated the presence of some gene polymorphisms with the stroke risk in SCA individuals. Those polymorphisms were the methylenetetrahydrofolate reductase enzyme (*MTHFR*) *C677T* (rs1801133), prothrombin (*PT*) *G20210A* (rs1799963), factor V Leiden (*FV*) *G1691A* (rs6025) and nitric oxide (NO) endothelial synthase enzyme (*NOS3*) *T-786C* (rs2070744) (Bernaudin and Verlhac 2008; Casas et al. 2004; Li and Qin 2014; Niu et al. 2013; Pereira et al. 2007; Wang et al. 2013). Despite this, another study did not found this association (Domingos et al. 2014). Other genes such as vascular cell adhesion molecule (*VCAM*), interleukin 4 receptor (*IL4R*) and the adrenoreceptor beta2 (*ADRB2*) have also been associated with the risk of stroke in SCA individuals (Hoppe et al. 2004; Taylor et al. 2008).

Several genetic markers have been previously associated with the risk of developing stroke, but many of the results achieved are controversial and could not completely elucidate the effect of individuals' genetic heterogeneity in the development of stroke. Thus, it is necessary to increase the knowledge about gene polymorphisms' association with a possible risk of stroke in SCD individuals.

METHODS

Subjects

Individuals were from the Pediatric Cerebrovascular Disease Outpatient Center at the Hospital Universitario Professor Edgard Santos of the Universidade Federal da Bahia that attends about 420 children a year to perform the TCD that was evaluated in the equipment Doppler-Box^{TM}X (Compumedics Germany GmbH, Singen, Hohentwiel, Germany). To this study we selected four individuals with SCA and four individuals with HbSC based on the TCD values in according to the velocities described by Adams et al (1997)(Adams et al. 1997) and matched by sex and age. Baseline characteristics of SCA and HbSC patients, including 25th, 50th, and 75th percentile values of laboratory data were shown in the Table 1.

As exclusion criteria were considered individuals with a prior overt stroke event, those on hydroxyurea therapy, and those who received any blood transfusion in the last three months before the sample collection or on chronic blood therapy regimens. All enrolled individuals had their hemoglobin profile confirmed by high performance liquid chromatography (HPLC) (Bio-Rad, Hercules, California, EUA).

This study was approved by the Research Board of the Secretaria de Saúde do Estado da Bahia (SESAB) 054/2011, and all parents or guardians provided written informed consent in accordance with the Helsinki Declaration of 1975 and its revisions.

Transcranial Doppler measurements

The transcranial Doppler (TCD) was performed in all subjects included in the study. TCDs were always performed by the same professional, and the same equipment was used in all measurements. Briefly, a 2 MHz probe was used to assess the mean blood flow velocity in the middle cerebral arteries (MCA) and distal intracranial internal carotid (ICA) through the transtemporal window, according to the protocol established by the Stroke Prevention Trial in Sickle Cell Anemia (STOP) study, considering the highest velocity found (TAMMV) (Adams et al. 1997).

DNA extraction and Genotyping

Genomic DNA was extracted from peripheral blood using QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Vestfália, Germany) according to the manufacturer's recommendations. The DNA concentration was evaluated using the NanoDrop ND-1000 machine (Thermo Fisher Scientific, Wilmington, Delaware, USA).

The DNA (400 ng) was used for SNP analysis in an Illumina Human Omni5-4 v.1.1 BeadChip kit (Illumina Inc., San Diego, California, USA). This type of SNP array consists of >4.3 million SNPs selected from the International HapMap Project and the 1000 Genome Project. Raw intensities were analyzed in GenomeStudio Software, using default parameters of normalization to generate X and Y intensity values for generic A and B alleles respectively.

Genotyping analysis

To perform genotyping analysis we used GenomeStudio software to select SNPs identified in NCBI SNP database (rs – Reference SNP cluster ID). We select SNPs showing different genotypes when individuals were pairwise compared according to TCD speed. For SCA group,

the patient with abnormal TCD (224 cm/s) was compared to the individuals with normal (132 cm/s), low conditional (174 cm/s) and high conditional (191 cm/s) TCD. For HbSC group, the patient with abnormal TCD (204 cm/s) was compared to individuals with low TCD (62.8 cm/s), normal TCD with average velocity less than 128 cm/s (95.5 cm/s), and normal TCD with average velocity greater than 128 cm/s (156 cm/s) (Figure 1).

The above selected SNPs were then subjected to SNPnexus online tool (www.snp-nexus.org), for coding variant classification using the UCSC category. This tool allowed us to select SNPs mapped in gene coding regions and with a corresponding non-synonymous amino acid change. The non-synonymous coding SNPs selected in this second step were then analyzed with SIFT and PolyPhen categories, this time to predict the effect of the nucleotide change on protein function.

From SIFT category analysis, we selected SNPs with a highly confident prediction of having a damaging effect on the protein function (Kumar et al. 2009). PolyPhen classification was used to select SNPs appraised as probably damaging by this prediction tool (Adzhubei et al. 2010). Then we selected the common SNPs within the above described criteria for SIFT and POLYPHEN. The next step consisted of running these selected SNPs in a third link category of SNP-nexus: the NHGRI-EBI Catalog of published genome-wide association studies (http://www.ebi.ac.uk/gwas/) (Welter et al. 2014), which comprises a quality controlled, manually curated, literature-derived collection of all published genome-wide association studies assaying at least 100,000 SNPs and all SNP-trait associations with p-values $< 1.0 \times 10^{-5}$.

Additionally, we used STRING database (http://www.string-db.org/) to construct a network of protein-protein interactions. STRING prediction was performed with the corresponding genes from the SNPs found in the intersection of SIFT and PolyPhen filtering. String database analyzes the known and predicted interactions between proteins. The interactions include direct (physical)

and indirect (functional) associations. STRING integrates data from these sources into a final score that corresponds to the confidence of the interaction (Liu et al. 2015). For our analyses, we used a confidence score threshold of 0.9, which corresponds to the highest confidence score for predicted interactions and all the sources available: neighborhood, gene fusion, co-occurrence, co-expression, experiments, database and textmining. After the network was constructed, we excluded the nodes with no predicted interaction links.

RESULTS

Among the 483862, 466818 and 476873 SNPs with different genotypes when SCA individuals were pairwise compared according to TCD, we identified 7576, 7146 and 7316 as unique rs SNPs predicted by UCSC to be mapped in genomic coding regions, for the 3 comparisons of SCA individuals, respectively: abnormal TCD vs normal TCD, abnormal TCD vs conditional TCD and abnormal TCD vs conditional high TCD. These unique SNPs corresponded to 6372, 6102 and 6275 unique genes in the same TCD-SCA groups described above (Table 2).

Using the UCSC category, we were able to classify those unique SNPs with predicted location in coding regions in synonymous and non-synonymous for corresponding amino acid change. We found 3698, 3851 and 3528 unique, non-synonymous SNPs in the TCD-SCA groups respectively. These unique SNPs corresponded to 3310, 3085 and 3201 unique genes in the same groups (Table 2).

Using the SIFT category we selected the SNPs with a highly confident prediction to be damaging. The PolyPhen classification was used to select SNPs predicted to be probably damaging, and we found 551, 505 and 598 unique SNPs in the same comparison of TCD-SCA individuals respectively. The next step was to select the common SNPs within these two criteria

(probably damaging in PolyPhen and highly confident predicted damage in SIFT) to perform Genome-wide association study (GWAS) classification (Table 2).

The same strategy used for TCD-SCA was performed to TCD-HbSC groups of individuals: abnormal TCD vs low TCD, abnormal TCD vs normal TCD with average velocity less than 128 cm/s and abnormal TCD vs normal TCD with average velocity high than 128 cm/s. Among the 475752, 480082 and 468363 SNPs with different genotypes when HbSC individuals were pairwise compared according to TCD, we identified 7465, 7565 and 7076 as unique rs SNPs predicted by UCSC to be located in genomic coding regions respectively. Corresponding to these SNPs, we identified 6361, 6427 and 6134 unique genes in the same groups described above.

The UCSC category was used to classify those SNPs with predicted location in coding regions in synonymous and non-synonymous regarding corresponding amino acid change. We found 3654, 3722 and 3847 unique non-synonymous SNPs in the same TCD-HbSC groups of individuals respectively. We identified 3279, 3363 and 3507 unique genes that corresponded to these non-synonymous SNPs in the same HbSC individuals respectively.

Next, SIFT and Polyphen classification was used to select SNPs predicted to have a damaging phenotype. Using the SIFT category we selected the SNPs with a highly confident prediction to have a damaging effect on the corresponding protein: 429, 447 and 418 unique SNPs were found in the TCD-HbSC groups of individuals respectively. The PolyPhen classification was used to select SNPs probably damaging to protein function and we found 517, 539 and 508 unique SNPs in the TCD-HbSC groups respectively. As described to SCA groups, the next step was to select the common SNPs within these two criteria (probably damaging in PolyPhen and highly confident predicted damage in SIFT) to perform GWAS classification.

The results obtained utilizing the GWAS showed that each pair of individuals had a different number of SNPs identified. In the SCA groups, in the abnormal TCD vs normal TCD comparison

a total of five unique SNPs were found. For abnormal TCD vs conditional TCD comparison, three unique SNPs were found. Finally, in the conditions abnormal vs high conditional TCD, five unique SNPs were found. We found two common SNPs in the SCA groups. In the HbSC genotype, in the condition abnormal TCD vs low TCD we found a total of eight unique SNPs; in the condition abnormal TCD vs normal TCD (< 128cm/s) we found a total of six unique SNPs and in the condition abnormal TCD vs normal TCD (> 128cm/s) we found a total of seven unique SNPs. One common SNP was found among these groups (Table 3).

The network of protein-protein interactions for the 3 conditions of SCA individuals and HbSC individuals are shown in the figure 2 and 3 respectively.

DISCUSSION

The SCD is characterized by a large clinical heterogeneity. SCD individuals present a large spectrum of clinical complications ranging from vaso-occlusive crisis to even more severe complications such as stroke. The stroke is a significant clinical complication in SCD, as individuals can develop from hemiparesis to even coma (Ohene-Frempong et al. 1998). Few studies have been conducted to identify gene polymorphisms associated with the risk of stroke in SCD, especially in individuals with HbSC that end up being neglected, not even having adequate TCD reference values for diagnosis of stroke risk. Thus, this study aimed to identify genes associated with the risk of stroke in individuals with SCA and HbSC.

Gene variants associated with a disease could reveal novel mechanisms into the pathophysiological processes. The microarray results identified common SNPs among SCA individuals with different TCD velocities: rs2277339, in the *primase DNA polypeptide 1* (49kDa) (*PRIM1*) gene and rs4149056, in the solute carrier *organic anion transporter family, member 1B1* (*SLCO1B1*) gene. Regarding the HbSC individuals, one common SNP was found among TCD

groups, rs6756629, in the *ATP-binding cassette, sub-family G (WHITE), member 5* (*ABCG5*) gene.

The *PRIM1* is an enzyme involved in the initiation of DNA polymer synthesis and a subunit of the nuclear DNA primase. This enzyme was associated with human osteosarcoma, glioma, neuroblastoma, chronic lymphocytic leukemia and sarcomas in general (Yotov et al. 1999), but in SCD it was not yet studied.

The *SLCO1B1* gene is located on chromosome 12 and has 15 exons with 190 common variants. It encodes a 691 amino acid protein with 12 transmembrane helixes, SLC (solute carrier organic anion transporter family member 1B1) transporters are primarily involved in the uptake of small molecules into cells. The membrane-bound sodium-independent organic anion transporter protein is involved in active cellular influx of endogenous substrates (bile acids), xenobiotics and a wide range of drugs (statins, antibiotics, angiotensin-converting-enzyme inhibitors) (Nagy et al. 2015). *SLCO1B1* polymorphisms are important in the process of statin pharmacokinetics. A study carried out on the Roman and Hungarian population suggested that the haplotypes identified are relevant for statin therapy and could also modulate the clinical outcome (Nagy et al. 2015). Although statins are not currently used on the clinical practice to treat SCD individuals, a pilot-study was carried out in order to evaluate the anti-inflammatory effect of statin in SCD. Two different doses were administered during 39 days, and it was shown that NO levels were increased, and interleukin (IL)-6 and high-sensitivity C-Reactive Protein were decreased (Hoppe et al. 2011). Thus, *SLCO1B1* polymorphisms could be relevant to evaluate the efficacy of statins therapeutic approach in SCD.

Polymorphisms on *SLCO1B1* and *SLCO1B3* genes were also associated with high bilirubin levels (Lin et al. 2015). These SLC transporters are responsible for the uptake of conjugated bilirubin — the product of heme metabolic catabolism — into hepatocytes. From there, it is excreted into the bile. Common polymorphisms in transporter genes that are involved in the hepatic bilirubin elimination pathway may lead to hyperbilirubinaemia and jaundice (Lin et al. 2015). In SCD individuals the elevated bilirubin levels is a frequent laboratorial hallmark. Thus, besides the *SLCO1B1* polymorphism being associated with statins metabolism, the bilirubin metabolism is equally important on the individuals' clinical outcome. To our records, there are no studies evaluating the *SLCO1B1* polymorphisms and SCD or stroke risk, therefore more information is necessary to define its role.

The ABCG5 is a half-transporter belonging to the G subfamily of ABC proteins. Like the other three members of the human G subfamily of ABC transporters (ABCG1, ABCG2, and ABCG4), the ATPase catalytic domains of G5 and G8 are located N-terminal to the transmembrane domain. Mutations in either ABCG5 or ABCG8 cause sitosterolemia, an autosomal recessive disorder characterized by the accumulation of both plant-derived (primarily sitosterol) and animal derived (cholesterol) sterols in plasma and tissues (Graf et al. 2003). To our records, there are no studies regarding ABCG5 transporters in SCD. Lipid metabolism in SCD individuals may be altered and it has been suggested that the individuals can have a specific dyslipidemic sub phenotype characterized by low high-density lipoprotein-cholesterol (HDL-C) with hypertriglyceridemia and high very-low density lipoprotein-cholesterol (VLDL-C) (Seixas et al. 2010), thus the association of *ABCG5* gene polymorphism and clinical outcome of SCD individuals may be related.

In the HbSC individuals with abnormal TCD vs normal TCD (<128 cm/s) we found six unique SNPs, the *dedicator of cytokinesis 6* (*DOCK6*) rs2278426, *tyrosinase* (*TYR*) rs1042602, *cytochrome P450, family 4, subfamily F, polypeptide* (*CYP4F2*) rs2108622, *macrophage stimulating 1 (hepatocyte growth factor-like)* (*MST1*) rs3197999, *olfactory receptor, family 51, subfamily B, member* (*OR51B6*) rs5006884 and *thyroid adenoma associated* (*THADA*) rs7578597 SNPs.

The Angiopoietin-like (*ANGPTL*) genes encode a family of proteins associated with lipid metabolism. One of these *ANGPTL* genes, the *ANGPTL8* gene is located in the corresponding intron of *DOCK6* gene. In three populations, the polymorphism rs2278426 *DOCK6* was associated with lower plasma LDL-C and HDL-C levels (Quagliarini et al. 2012). To our records, there are no studies evaluating the *DOCK6* polymorphism and SCD or stroke risk, but this gene can be useful to explain the dyslipidemic sub phenotype found in SCD individuals.

The *TYR* gene encodes the tyrosinase, an enzyme that catalyzes steps of melanin production pathway. The allele A of *TYR* polymorphism rs1042602 is associated with light skin and eye color, and mutations in this gene are associated with vitiligo and cutaneous melanoma (Wilde et al. 2014). There are no studies evaluating the *TYR* polymorphism and stroke risk or SCD.

The *CYP4F2* gene encodes a member of the cytochrome P450 (CYP) superfamily of cysteinato-heme enzymes that is responsible for the metabolism of xenobiotics and a host of endobiotics, for example, the arachidonic acid. Arachidonic acid metabolites derived from CYP are associated with cerebrovascular pathology. The *CYP4F2* acts as an enzyme in the metabolism of leukotriene B4, a potent mediator of inflammation, and 20-hydroxyeicosatetraenoic acid, which plays an important role in the regulation of vascular tone in the brain and such as a potent constrictor of cerebral arteries. In a study in Japanese men with cerebral infarction was found an association between this condition and the *CYP4F2* polymorphism (Fu et al. 2008). This polymorphism is

also associated with interindividual variability in the warfarin dose. Warfarin is an anticoagulant used to prevent heart attacks, strokes, and blood clots. Individuals with variant genotype require higher doses of warfarin than individuals with wild-type genotype (Borgiani et al. 2009). To our records, there are no studies evaluating the *CYP4F2* polymorphism and stroke risk in SCD.

The *MST1* gene encodes a protein that contains four domains and a serine protease domain. Despite the presence of the serine protease domain, the encoded protein may not have any proteolytic activity. This gene has been associated in the literature with pathological conditions such as inflammatory bowel disease, primary sclerosing cholangitis, Crohn's disease and ulcerative colitis, but in SCD it was not yet characterized (Franke et al. 2010; Jostins et al. 2012; McGovern et al. 2010a; Melum et al. 2011).

OR51B5/6 rs5006884 SNP is part of the olfactory receptor gene cluster that might play a regulatory role in gamma-globin gene expression, as three loci of this gene are known to affect fetal hemoglobin (HbF) levels. This SNP has the same frequency on regular hemoglobin profile population (HbAA) and SCA individuals, but shows significantly increased frequency in SCA individuals with high HbF levels when compared to SCA individuals with low HbF. Thus, this mutation is associated with alterations in HbF, which is an important prognostic marker of stroke risk and other clinical manifestations in SCD individuals (Akinsheye et al. 2012; Galarneau et al. 2010; Solovieff et al. 2010; Wonkam et al. 2014).

The *THADA* gene is a protein coding gene related Type 2 diabetes and metabolic syndrome. In a study of DeMenna and colleagues (2014) the *THADA* rs7578597 SNP was significantly associated with obesity, glycemic, and lipid phenotypes (DeMenna et al. 2014; Zeggini et al. 2008). There are no studies regarding *THADA* gene and SCD.

In the HbSC individuals with abnormal TCD vs normal TCD (>128 cm/s) we found seven unique SNPs, the *fucosyltransferase 2* (*FUT2*) rs602662, *methylenetetrahydrofolate reductase* (*MTHFR*)

rs1801133, *TSEN15 tRNA splicing endonuclease subunit* (*TSEN15*) rs1046934, *MST1* rs3197999, *OR51B6* rs5006884, *complement factor B* (*CFB*) rs12614 and *THADA* rs7578597 SNPs.

The *FUT2* gene regulates the expression of ABH antigens in tissues and body fluids other than blood cells, encoding an alpha 1,2-fucosyltransferase capable of transferring L-fucose to carbon 2 of galactose (beta, 1-3) *N*-acetyl D-glucosamine–containing glycans (Anstee 2010). The *FUT2* rs602662 SNP was described to be involved in the folate pathway vitamin levels and in pathologic conditions such as primary sclerosing cholangitis and Crohn's disease. The *FUT2* rs602662 SNP or *FUT2* gene was not yet associated with SCD, according to the current literature (Folseraas et al. 2012; Hazra et al. 2009; McGovern et al. 2010b).

The *MTHFR* rs1801133 SNP encoding the enzyme called methylenetetrahydrofolate reductase involved in folate metabolism. This enzyme converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. This reaction is required for the multistep process that converts the amino acid homocysteine to another amino acid, methionine. This SNP was associated with pathologic conditions such as risk of coronary artery disease, homocysteine metabolism, nonsyndromic cleft lip with or without cleft palate, antenatal risk factors of white matter abnormalities, risk for adult stroke and stroke (Aguiar et al. 2015; Marseglia et al. 2015; Pare et al. 2009; van Meurs et al. 2013; Zhang et al. 2015). The *MTHFR* rs1801133 SNP variant allele was associated to vascular disease, vascular complications and stroke in SCA (Couto et al. 2004; Hatzlhofer et al. 2012). In this study we found the wild-type allele of *MTHFR* rs1801133 in HbSC individuals with abnormal TCD different from what was verified in SCA.

The *TSEN15* rs1046934 SNP encodes a subunit of the tRNA splicing endonuclease, which catalyzes the removal of introns from tRNA precursors and is associated with clinical manifestations such as height, neurogenetic disorders, atrial fibrillation and hipoplasia

pontocerebellar, but we did not find any data related to SCD in the literature (Alazami et al. 2015; Cassandrini et al. 2010).

The rs12614 in the *CFB* gene is associated to the immune response and encodes the complement factor B, a component of the alternative pathway of complement activation. The factor B circulates in the blood as a single chain polypeptide. This gene belongs to a cluster of genes involved in the regulation of the immune reaction. Polymorphisms in this gene are associated with a reduced risk of age-related macular degeneration and severe bacterial sepsis, because this gene acts as a downstream effector of toll-like receptors (TLR) signaling (Yu et al. 2011; Zou et al. 2013). There are no studies regarding *CFB* gene and SCD.

In the SCA individuals with abnormal TCD vs normal TCD we found five unique SNPs, the *apolipoprotein B* (*APOB*) rs676210, *tyrosine kinase 2* (*TYK2*) rs12720356, *TSEN15* rs1046934, *CYP4F2* rs2108622 and *MST1* rs3197999.

A 10 year follow-up prospective study which aimed to determine if the risk of stroke was related to the balance between the proatherogenic apoB lipoprotein particles and the antiatherogenic apoA-I particles as is the case for myocardial infarction, it was found that high apoB and low apoA-I values were significantly related to the risk of stroke (Walldius and Jungner 2004). The *APOB* rs676210 SNP has been associated with LDL-C and was associated with pathologic conditions such as atherosclerosis and hypertriglyceridemia (Wojczynski et al. 2010). The *APOB* gene was associated with diabetes mellitus in SCD, but the SNP associated is different from the SNP we found in this study (ZHANG et al., 2015) and can be useful to understand the dyslipidemic sub phenotype found in SCD individuals.

The *TYK2* gene has been associated with the hyperimmunoglobulin E syndrome and encodes a member of the tyrosine kinase and the Janus kinases protein families. This protein acts in the interferon signaling pathways and promulgate cytokine signals. Thus, this gene is associated with

infections (Casanova and Abel 2004), which is an important clinical feature in SCD. Despite this, there are no studies regarding *TYK2* and SCD.

The data in the literature regarding the clusters identified for different TCD groups of SCA individuals and HbSC individuals are shown in Tables 4 and 5 respectively. However, olfactory receptor cluster and MTHFR – MTRR cluster deserve our attention because they were found in different TCD groups of SCA and HbSC individuals.

There is a big cluster of olfactory receptor proteins identified in all TCD groups of SCA and HbSC individuals. Importantly, olfactory receptor genes' SNPs located on chromosome 1 regulate HbF levels (Solovieff et al. 2010). Higher HbF levels were associated with a reduced rate of acute painful episodes, fewer leg ulcers, less osteonecrosis, less frequent acute chest syndromes, and reduced disease severity (Steinberg 2009).

Another important cluster was MTHFR and MTRR (5-methyltetrahydrofolate-homocysteine methyltransferase reductase). MTHFR catalyzes the conversion of 5,10-ethylenetetrahydrofolate to 5-methyltetrahydrofolate, a co- substrate for homocysteine remethylation to methionine and MTRR is involved in the reductive regeneration of cob(I)alamin cofactor required for the maintenance of methionine synthase in a functional state. Both proteins are thus involved in the metabolism of homocysteine. High homocysteine levels are associated with cardiovascular diseases and with stroke in children with SCD (Akar et al. 2001; Houston et al. 1997).

In the present study we observed that the genetic contribution to stroke in SCD is polygenic. We identified SNPs in genes involved in lipid metabolism, inflammation, immune response, metabolic syndrome, xenobiotics metabolism, pharmacokinetics, DNA synthesis, bilirubin levels, hemoglobin gamma gene expression, and homocysteine metabolism. Importantly, the SNPs found here are mainly associated with lipid metabolism and inflammation, which led us to

suggest that stroke, is associated with an inflammatory and dyslipidemic profile in SCD. SCD individuals were shown to have a specific dyslipidemic sub phenotype characterized by low HDL-C with hypertriglyceridemia and high VLDL-C and these lipid changes can directly activate inflammatory pathways in these individuals, which may culminate in an event such as stroke (Seixas et al. 2010).

Genes described here in their majority have not been previously studied in SCD, but represent a possible pathway to study new prognostic markers of stroke.

CONCLUSION

We suggest that the *DOCK6* rs2278426, *TYR* rs1042602, *CYP4F2* rs2108622, *MST1* rs3197999, *OR51B5/6* rs5006884, *THADA* rs7578597, *FUT2* rs602662, *MTHFR* rs1801133, *TSEN15* rs1046934, *CFB* rs12614 and *ABCG5* rs6756629 SNPs may be candidate variants to be further investigated in HbSC individuals with high speed of cerebral blood flow. We suggest that the *SLCO1B1* rs4149056, *PRIM1* rs2277339, *APOB* rs676210, *TYK2* rs12720356, *TSEN15* rs1046934, *CYP4F2* rs2108622 and *MST1* rs3197999 as candidate SNPs to be further investigated in SCA with high speed of cerebral blood flow.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflicts of interest: The authors declare that they have no conflict of interest.

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

Informed consent: Informed consent was obtained from all individual participants included in the study.

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Figure legends

Figure 1. Algorithm for the selection of SCA individuals and HbSC individuals with different stroke risk.

Figure 2. Network of protein-protein interactions for the 3 conditions of SCA individuals. (A) Abnormal TCD vs High Conditional TCD; (B) Abnormal TCD vs Conditional TCD; (C) Abnormal TCD vs Normal TCD. A red line indicates the presence of fusion evidence; a green line - neighborhood evidence; a blue line - coocurrence evidence; a purple line - experimental evidence; a yellow line - textmining evidence; a light blue line - database evidence; a black line - coexpression evidence.

Figure 3. Network of protein-protein interactions for the 3 conditions of HbSC individuals. (A) Abnormal TCD vs Low TCD; (B) Abnormal TCD vs Normal <128cm/s TCD; (C) Abnormal TCD vs Normal >128cm/s TCD. A red line indicates the presence of fusion evidence; a green line - neighborhood evidence; a blue line - coocurrence evidence; a purple line - experimental evidence; a yellow line - textmining evidence; a light blue line - database evidence; a black line - coexpression evidence.

Figure 1

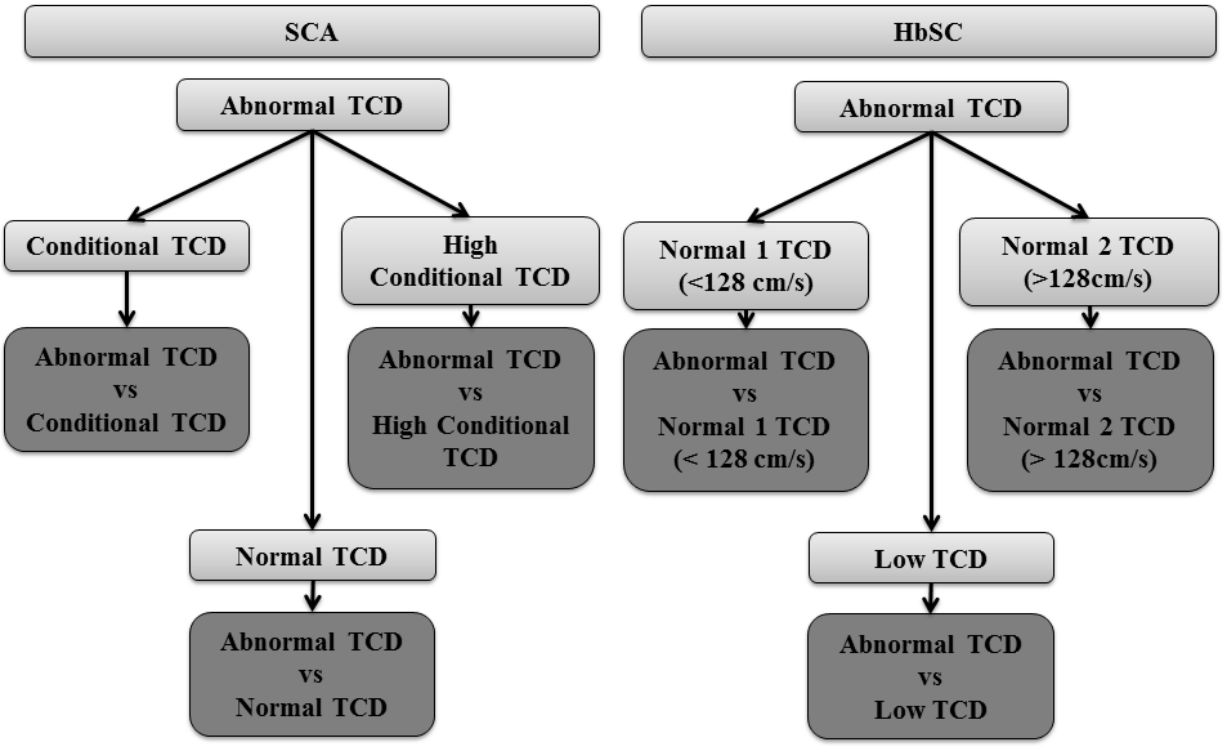
SCA
Abnormal TCD
Conditional TCD
High Conditional TCD
Abnormal TCD vs Conditional TCD
Abnormal TCD vs High Conditional TCD
Normal TCD
Abnormal TCD vs Normal TCD
HbSC
Abnormal TCD
Normal 1 TCD (<128 cm/s)
Normal 2 TCD (>128cm/s)
Abnormal TCD vs Normal 1 TCD (< 128 cm/s)
Abnormal TCD vs Normal 2 TCD (> 128cm/s)
Low TCD
Abnormal TCD vs Low TCD

Figure 2

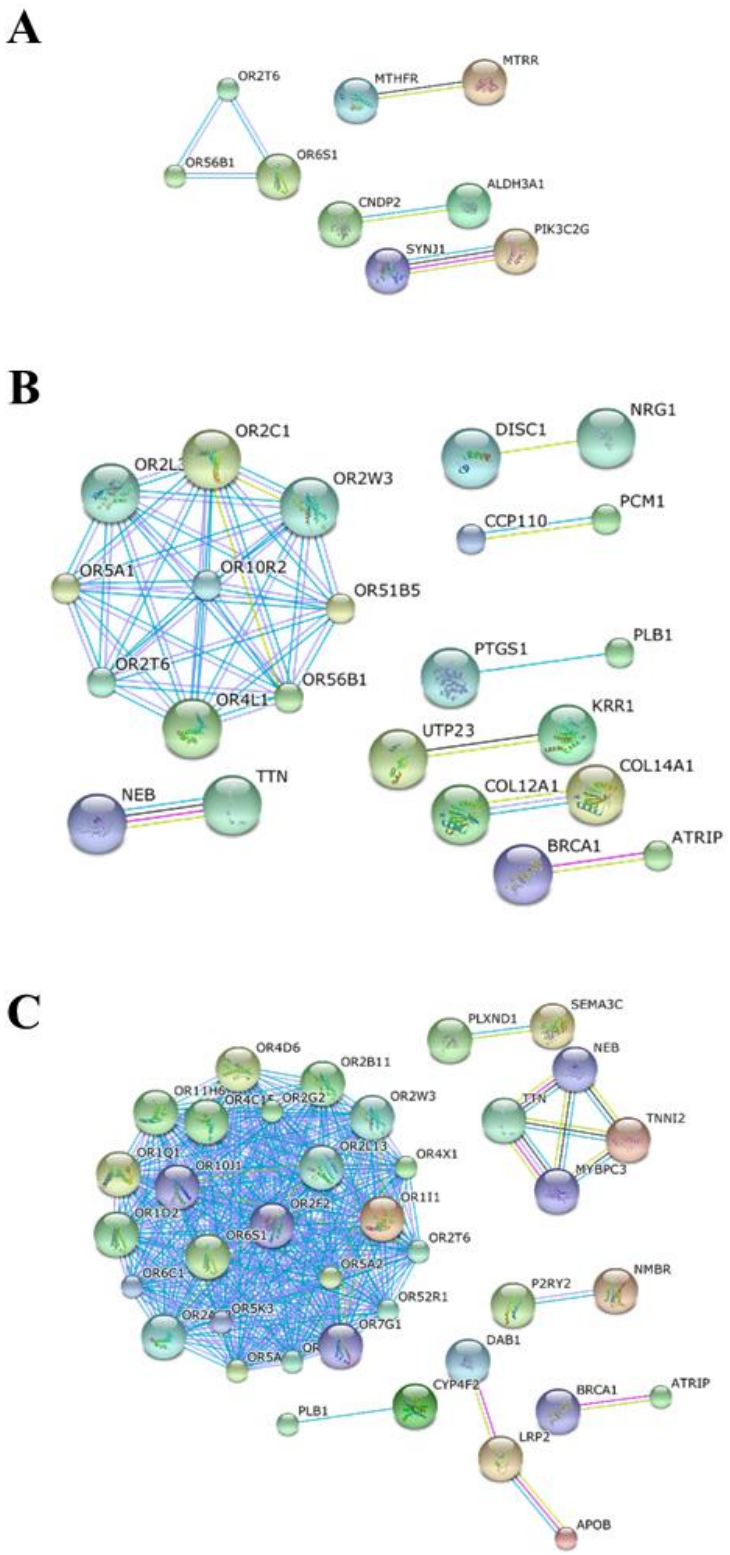
A
OR2T6
MTHFR
MTRR
OR56B1
OR6S1
CNDP2
ALDH3A1
PIK3C2G
SYNJ1
B
OR2C1
OR2L3
OR2W3
DISC1
NRG1
CCP110
PCM1
OR5A1
OR10R2
OR51B5
PTGS1
PLB1
OR2T6
OR56B1
OR4L1
KRR1
UTP23
COL14A1
COL12A1
NEB
TTN
BRCA1
ATRIP
C
PLXND1
SEMA3C
NEB
OR4D6
OR2B11
OR11H6
OR4C15
OR2G2
OR2W3
TTN
TNNI2
OR1Q1
OR10J1
OR2L13
OR4X1
MYBPC3
OR1D2
OR2F2
OR1I1
OR6S1
OR2T6
OR6C1
OR5A2
P2RY2
NMBR
OR52R1
OR2A
OR5K3
OR7G1
DAB1
CYP4F2
BRCA1
ATRIP
PLB1
LRP2
APOB

Figure 3

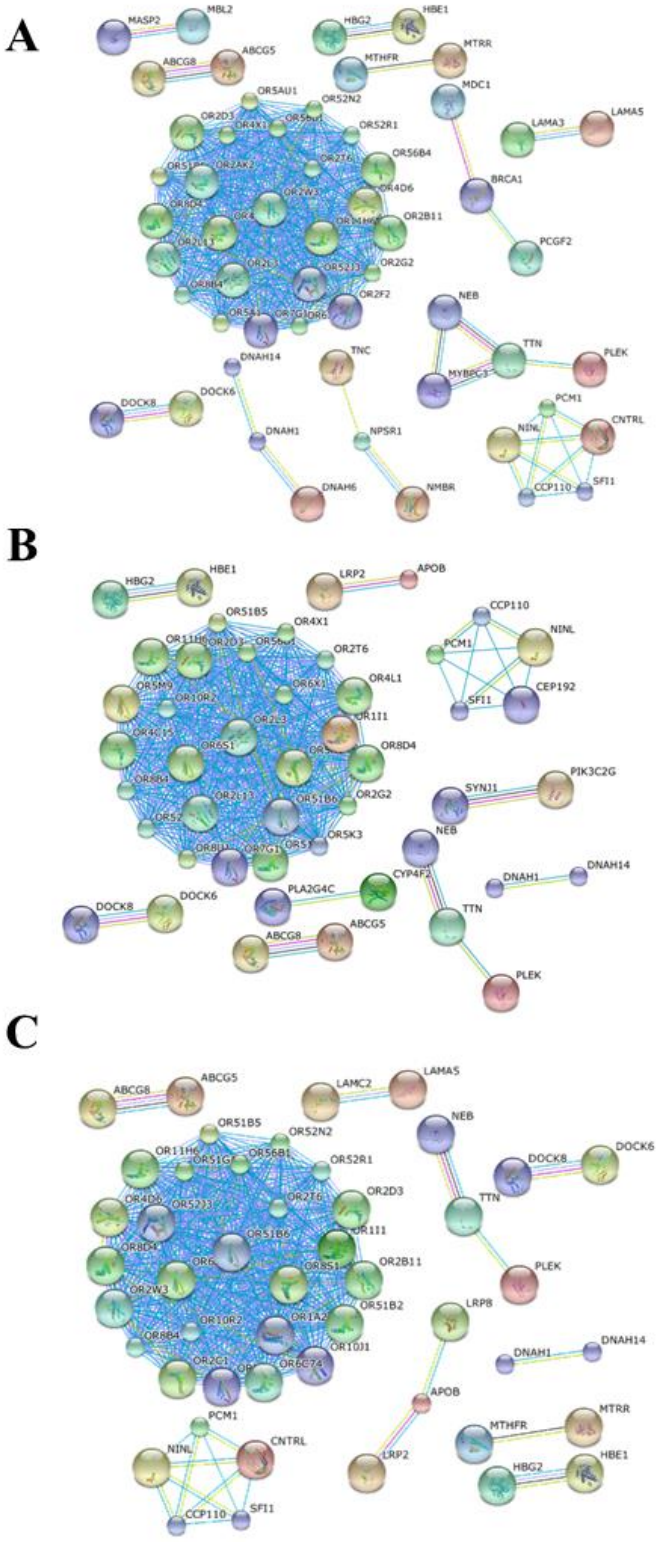
A
MASP2
MBL2
HBG2
HBE1
ABCG5
ABCG8
MTHFR
MTRR
MDC1
OR5AU1
OR52N2
OR2D3
OR4X1
OR52R1
LAMA3
LAMA5
OR56B4
OR2T6
OR4D6
BRCA1
OR2W3
OR2B11
PCGF2
OR2G2
OR2F2
NEB
TTN
PLEK
TNC
DNAH14
MYBPC3
PCM1
DOCK8
DOCK6
NINL
CNTRL
DNAH1
NPSR1
CCP110
SFI1
DNAH6
NMBR
B
HBG2
HBE1
LRP2
APOB
OR51B5
OR4X1
CCP110
NINL
PCM1
OR2T6
OR4L1
CEP192
SFI1
OR1I1
OR8D4
PIK3C2G
SYNJ1
OR2G2
NEB
OR5K3
DNAH1
DNAH14
CYP4F2
DOCK8
DOCK6
PLA2G4C
TTN
ABCG8
ABCG5
PLEK
C
ABCG8
ABCG5
LAMC2
LAMA5
NEB
OR51B5
OR52N2
DOCK8
DOCK6
OR52R1
OR2D3
TTN
OR2T6
OR1I1
OR2B11
PLEK
OR51B2
LRP8
OR1A2
DNAH1
DNAH14
OR10J1
APOB
PCM1
MTRR
MTHFR
CNTRL
NINL
LRP2
HBE1
HBG2
CCP110
SFI1

Table 1. Baseline characteristics of SCA and HbSC individuals, including hematological, biochemical and immunological data.

Laboratory value	SCA	HbSC
	50^{th} (25^{th} - 75^{th})	**50^{th} (25^{th} - 75^{th})**
Hemolysis markers		
RBC, $x10^{12}$/L	2.73 (2.28 – 2.96)	4.50 (4.24 – 4.76)
Hemoglobin, g/dL	8.75 (7.52 – 9.15)	11.35 (10.57 – 11.75)
Hematocrit, %	24.50 (21.42 – 24.95)	33.50 (30.65 – 34.62)
MCV, fL	90.75 (82.77 – 94.72)	73.10 (71.02 – 75.25)
MCH, ρg	32.80 (29.02 – 34.10)	25.00 (24.27 – 25.50)
MCHC, g/dL	35.95 (34.70 – 36.60)	33.95 (33.75 – 34.60)
RDW (%)	20.00 (19.27 – 20.57)	15.40 (14.97 – 16.95)
Total bilirubin, mg/dL	2.49 (2.10 – 4.87)	0.97 (0.59 – 1.00)
Direct bilirubin, mg/dL	0.44 (0.30 – 0.67)	0.30 (0.21 – 0.31)
Indirect bilirubin, mg/dL	2.15 (1.45 – 4.44)	0.66 (0.38 – 0.70)
LDH, U/L	1271.00 (1047.50 – 1490.75)	457.00 (395.25 – 512.00)
Hemoglobin pattern		
Fetal haemoglobin, %	7.60 (5.00 – 10.87)	0.65 (0.32 – 1.50)
Leukocytes		
WBC, x 10^9/L	15000.00 (10076.25 – 19650.00)	8942.50 (8128.75 – 10675.00)
Neutrophil count, x 10^9/L	6754.50 (3736.50 – 11131.50)	5718.50 (4354.00 – 7351.50)
Eosinophil count , x 10^9/L	686.00 (538.25 – 1125.50)	286.50 (125.25 – 425.25)
Lymphocyte count, x 10^9/L	5167.50 (4797.75 – 5613.75)	2325.00 (1915.00 – 2846.00)
Monocyte count , x 10^9/L	1507.00 (525.75 – 2897.00)	601.50 (382.00 – 938.00)
Platelets		
Platelet count, $x10^3$/mL	353.50 (328.50 – 504.50)	260.00 (190.50 – 319.00)
Platelet Volume Average, fL	6.45 (5.82 – 6.85)	7.75 (6.82 – 9.27)
Glucose		
Glucose, mg/dL	82.00 (69.75 – 85.25)	83.50 (76.25 – 87.75)
Lipid metabolism		
Total Cholesterol, mg/dL	105.00 (102.25 – 131.75)	143.50 (122.75 – 165.75)
Triglycerides, mg/dL	68.00 (61.25- 86.00)	81.00 (69.00 – 100.50)
Liver		
ALT, U/L	15.00 (10.50 – 21.75)	10.50 (7.00 – 13.50)
AST, U/L	46.50 (34.50 – 75.75)	17.00 (16.25 – 27.50)
Total protein, g/dL	7.42 (6.47 – 7.92)	7.20 (6.90 – 7.43)
Albumin, g/dL	3.85 (3.57 – 4.50)	4.30 (4.05 – 4.63)
Globulin, g/dL	3.75 (2.07 – 4.15)	2.65 (2.60 – 3.30)
Albumin /Globulin Ratio	1.00 (0.92 – 2.42)	1.60 (1.22 – 1.75)
Kidney		
Urea nitrogen, mg/dL	16.50 (14.50 – 18.50)	14.50 (14.00 – 15.75)
Creatinine, mg/dL	0.49 (0.34 – 0.60)	0.54 (0.50 – 0.61)

Table 2. SNP filtering statistics

SNP	SCA			HbSC		
	Abnormal TCD *vs* Normal TCD	**Abnormal TCD *vs* Conditional TCD**	**Abnormal TCD *vs* High conditional TCD**	**Abnormal TCD *vs* Low TCD**	**Abnormal TCD *vs* Normal TCD (< 128cm/s)**	**Abnormal TCD *vs* Normal TCD (> 128cm/s)**
Coding variants						
Total SNPs	19520	18568	19113	19262	19462	18482
Unique rs SNPs	7576	7146	7316	7465	7565	7076
Unique genes	6372	6102	6275	6361	6427	6134
Non-synonymous rs coding variants						
Unique SNPs	3698	3851	3528	3654	3722	3847
Unique genes	3310	3085	3201	3279	3363	3507
Polyphen						
Total unique SNPs	3392	3134	3232	3347	3410	3173
Probably damaging SNPs	551	505	518	517	539	508
SIFT						
Total unique SNPs	3317	3078	3174	3293	3344	3120
High confidence, damaging SNPs	424	393	425	429	447	418

Table 3. Comparison of SNPs and genes among TCD groups of patients with SS and SC genotype

TCD groups of patients	SS genotype		TCD groups of patients	SC genotype	
	SNPs	**Genes**		**SNPs**	**Genes**
Abnormal vs. Conditional High	rs602662	FUT2	*Abnormal vs. Low*	rs6136	SELP
	rs1042602	TYR		rs2278426	DOCK6
	rs1801133	MTHFR		rs1801133	MTHFR
	rs1046934	TSEN15		rs676210	APOB
	rs2257205	RNF43		rs17279437	SLC6A20
				rs2277339	PRIM1
				rs2257205	RNF43
				rs2108622	CYP4F2
Abnormal vs. Conditional	rs757978	FARP2	*Abnormal vs. Normal 1*	rs2278426	DOCK6
	rs3197999	MST1		rs1042602	TYR
	rs5006884	OR51B5/6		rs2108622	CYP4F2
				rs3197999	MST1
				rs5006884	OR51B5/6
				rs7578597	THADA
Abnormal vs normal	rs676210	APOB	*Abnormal vs Normal 2*	rs602662	FUT2
	rs12720356	TYK2		rs1801133	MTHFR
	rs1046934	TSEN15		rs1046934	TSEN15
	rs2108622	CYP4F2		rs3197999	MST1
	rs3197999	MST1		rs5006884	OR51B5/6
				rs12614	CFB
				rs7578597	THADA
Common	rs4149056	SLCO1B1	*Common*	rs6756629	ABCG5
	rs2277339	PRIM1			

Table 4. Descriptions of proteins clusters identified in analyses of SCA patients with different TCD velocities.

TCD status	Cluster	Description of cluster	Link	Reference
Common				
	Olfactory receptor (OR) proteins	Olfactory receptor genes showed SNPs with distinct genotypes between the groups compared in this study. This is very important, since olfactory receptor genes' SNPs located on chromosome 1 regulate HbF levels. Higher HbF levels were associated with a reduced rate of acute painful episodes, fewer leg ulcers, less osteonecrosis, less frequent acute chest syndromes, and reduced disease severity.	Co-mentions in the literature	SOLOVIEFF, 2010 STEINBERG et al., 2009
Abnormal TCD vs High conditional TCD				
	MTHFR and MTRR	MTHFR catalyzes the conversion of 5,10-ethylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteine remethylation to methionine and MTRR is involved in the reductive regeneration of cob(I)alamin cofactor required for the maintenance of methionine synthase in a functional state. Both proteins are thus involved in the metabolism of homocysteine. High homocysteine levels are associated with cardiovascular diseases and with stroke in children with SCD.	Co-mentions in the literature and evidences of co-expression	HOUSTON, 1997 AKAR et al., 2001
	CNDP2 and ALDH3A1	CNDP2 protein is found in dopaminergic neurons and associated with Parkinson's disease pathogenesis and neurodegeneration. The ALDH3A1 is involved in the detoxification of alcohol-derived acetaldehyde and in the metabolism of corticosteroids, biogenic amines, neurotransmitters, and lipid peroxidation. The CNDP2 and ALDH3A1 proteins interact in the beta-alanine and histidine metabolisms.	Co-mentions in the literature and in databases	LICKER et al., 2012 JANG et al., 2014
	SYNJ1 and PIK3C2G	These proteins are associated with phospholipid binding motifs that mediate translocation of proteins to membranes, and mutations on them may affect the membrane trafficking. SYNJ1 is associated with neuropathological mechanisms in Parkinson's disease.	Co-mentioned in the literature and experimental evidence	OLGIATI, 2014

Abnormal TCD vs conditional TCD

DISC1 and NRG1	DISC1 and NRG1 are multifunctional proteins associated with schizophrenia and play roles in neurodevelopmental processes, such as proliferation, migration, and differentiation of progenitor cells.	Co-mentions in databases	SESHADRI et al., 2010
CCP110 and PCM1	CCP110 and PCM1 proteins were described with interacting partners of CEP290 protein; this protein is responsible for the recruitment to the cilium. Mutations in these proteins can lead to the dysfunction or absence of primary cilia and altered cellular motility.	Co-mentions in the literature and in databases	ASH et al., 2014
PTGS1 and PLB1	PTGS1 and PLB1 proteins are associated with arachidonic acid metabolism. PTGS1 enzymes catalyze the conversion of arachinodate to prostaglandin.	Co-mentions in the literature	KORBECKI et al., 2015
UTP23 and KRR1	These proteins are part of processome protein complex that assembles cotranscriptionally onto the nascent pre-ribosomal RNA.	Co-mentions in the literature, and evidences of co-expression	SLOAN et al., 2014
COL12A1 and COL14A1	COL12A1 and COL14A1 are members of the fibril-associated collagens with interrupted triple helices collagen family and have a similar structure and function as type XII collagen. Alterations in these proteins can lead to cancer and myopathy.	Co-mentions in the literature and in databases	RIBBANS et al., 2013
BRCA1 and ATRIP	BRCA1 and ATRIP are essential components of the DNA damage checkpoint and play a role in maintaining genomic stability, besides this, these proteins can also act as tumor suppressors. These proteins interaction is critical to the function of ATR/ATRIP function in the DNA damage checkpoint.	Co-mentions in the literature and experimental evidences	VENERE et al., 2007
NEB and TTN	NEB and TTN are involved in muscle homeostasis.	Co-mentions in the literature, experimental and co-expression evidences	SCHEUERMANN et al., 2004

Abnormal TCD vs normal TCD

PLXND1 and SEMA3C	These proteins form a Semaphorin-Plexin complex signaling and play critical roles for cellular aspects such as organogenesis, including cell migration, proliferation and survival.	Co-mentions in the literature and in databases	GAY et al., 2011
NEB, TNNI2, MYBPC3 and TTN	NEB, TNNI2, MYBPC3 and TTN proteins are key structural proteins of the sarcomere, they are all involved in muscle homeostasis.	Co-mentions in the literature, co-expression and experimental data	HWANG & SYKES, 2015
P2RY2 and NMBR	P2RY2 and NMBR proteins are involved in proliferation cellular, such as the phosphoinositide mediated signaling and G protein signaling coupled to IP3 phospholipase C activating pathways.	Co-mentions in the literature	ZAZA et al., 2014
BRCA1 and ATRIP	BRCA1 and ATRIP are essential components of the DNA damage checkpoint and play a role in maintaining genomic stability, besides this, these proteins can also act as tumor suppressors. These proteins interaction is critical to the function of ATR/ATRIP function in the DNA damage checkpoint.	Co-mentions in the literature and experimental evidences	VENERE et al., 2007
DAB1, LRP2 and APOB	DAB1 decreases the endocytosis rate of LDL receptor because this protein interfering in the endocytosis complex formation. Thus, this protein can lead to lipid alterations such is verified in LRP2 and APOB proteins which were also associated with lipid alterations like hypertriglyceridemia.	Co-mentions in the literature and experimental data	SHEN et al., 2012 DUIT et al., 2010
PLB1 and CYP4F2	PLB1 and CYP4F2 proteins play a role in the arachidonic acid metabolism pathway.	Co-mentions in databases	KEGG, (http://www.genome.jp/kegg/)

MTRR: 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; CNDP2: CNDP dipeptidase 2 (metallopeptidase M20 family); ALDH3A1: aldehyde dehydrogenase 3 family member A1; SYNJ1: synaptojanin 1; PIK3C2G: phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 gamma; DISC1: disrupted in schizophrenia 1; NRG1: neuregulin 1; CCP110: centriolar coiled-coil protein 110kDa; PMC1: pericentriolar material 1; PTGS1: prostaglandin-endoperoxide synthase 1; PLB1: phospholipase B1; UTP23: UTP23, small subunit processome component, homolog (yeast); KRR1: KRR1, small subunit processome component, homolog (yeast); COL12A1: collagen type XII alpha 1; COL14A1: collagen type XIV alpha 1; BRCA1: breast cancer 1; ATRIP: ATR interacting protein; NEB: nebulin; TTN: titin; PLXND1: plexin D1; SEMA3C: semaphorin 3C; TNNI2: troponin I type 2; MYBPC3: myosin binding protein C, cardiac; P2RY2: purinoceptor 2; NMBR: neuromedin B receptor; DAB1: disabled-1.

Table 5. Descriptions of proteins clusters identified in analyses of HbSC patients with different TCD velocities.

TCD status	Cluster	Description of cluster	Link	Reference
Common				
	Olfactory receptor (OR) proteins	Olfactory receptor genes showed SNPs with distinct genotypes between the groups compared in this study. This is very important, since olfactory receptor genes' SNPs located on chromosome 1 regulate HbF levels. Higher HbF levels were associated with a reduced rate of acute painful episodes, fewer leg ulcers, less osteonecrosis, less frequent acute chest syndromes, and reduced disease severity.	Co-mentions in the literature	SOLOVIEFF, 2010 STEINBERG et al., 2009
	PMC1, CNTRL, SFI1, CCP110 and NINL	The cluster formed by PCM1, NINL, CNTRL, CCP110 and SFI1 are all linked because they function in cell division, especially centrosome duplication and mitotic spindle assembly.	Co-mentions in the literature and databases	SCHATTEN, 2008
	HBG2 and HBE1	HBG chains make up the fetal hemoglobin F, in combination with alpha chains and HBE is a beta-type chain of early mammalian embryonic hemoglobin. The presence of HBG is particularly important because these chains make up HbF, associated with disease severity.	Co-mentioned in the literature and in databases	STEINBERG et al., 2001
	ABCG8 and ABCG5	ABCG8 and ABCG5 are ATP-binding cassette transporters that appear to be indispensable for the selective transport of dietary cholesterol.	Co-mentioned in the literature and in databases	YU et al., 2013
	NEB, TTN and PLEK	MYBPC3, TTN, NEB and PLEK are all involved in muscle homeostasis.	Co-mentions in the literature	ROSADO et al., 2014
	DOCK8 and DOCK6	DOCK6 and DOCK8 are members of the family of dedicator of cytokinesis, who belong to atypical Rho guanine nucleotide exchange factors for Rac and/or Cdc42 GTPases. These proteins play pivotal roles in various processes of brain development.	Co-mentioned in the literature and in databases Experimental data	SHI, 2013
	DNAH1 and DNAH14	DNAH1 and DNAH14 are members of the dyneins family, who are composed of chains presents in the microtubule-associated	Co-mentions in the literature	KHELIFA et al., 2014; MAITI et al., 2000

	motor protein complexes. These proteins were associated with non-syndromic male infertility due to sperm motility disorder and related pathways such as respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins.		

Abnormal TCD vs low TCD

MASP2 and MBL2	MBL2 participates in innate immune defense and MASP2 is involved in the mannan-binding lectin pathway of complement activation.	co-mentioned in the literature	GOELDNER, 2014
MTHFR and MTRR	MTHFR catalyzes the conversion of 5,10-ethylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteine remethylation to methionine and MTRR is involved in the reductive regeneration of cob(I)alamin cofactor required for the maintenance of methionine synthase in a functional state. Both proteins are thus involved in the metabolism of homocysteine. High homocysteine levels are associated with cardiovascular diseases and with stroke in children with SCD.	Co-mentions in the literature and evidences of co-expression	HOUSTON, 1997 AKAR et al., 2001
BRCA1, MDC1 and PCGF2	BRCA1 and MDC are both involved in DNA repair and PCGF2 is a transcriptional repressor that acts as tumor suppressor, although itis not functionally linked to BRCA1.	Co-mentioned in the literature	SHENG et al., 2014 BATTAGLIA, 2014
LAMA3 and LAMA5	LAMA3 and LAMA5 are alpha lamininins that mediate the attachment, migration and organization of cells into tissues during embryonic development by interacting with other extracellular matrix components. Laminin is one of the predominant components of subendothelial matrix and it has been described that SS RBC bind soluble laminin markedly more than do AA RBC.	Co-mentions in the literature and in databases	UDANI, 1998
NPSR1, TNC and NMBR	NPSR1, TNC and NMBR were linked to childhood asthma and allergic disease.	Co-mentions in the literature	BEGIN, 2014

Abnormal TCD vs normal (<128cm/s)

LRP2 and APOB	LRP2 protein was associated with hypercholesterolemia and the study of Mii and colleagues (2007) confirms the association between LRP2 and high levels of total cholesterol and low density lipoprotein in human plasma. APOB is also associated with lipid alterations such as hypertriglyceridemia.	Co-mentioned in the literature and experimental evidence	MII et al., 2007
SYNJ1 and PIK3C2G	These proteins are associated with phospholipid binding motifs that mediate translocation of proteins to membranes, and mutations on them may affect the membrane trafficking. SYNJ1 is associated with neuropathological mechanisms in Parkinson's disease.	Co-mentioned in the literature and experimental evidence	OLGIATI, 2014
PLA2GAC and CYP4F2	PLA2GAC is an enzyme required for the prostaglandin E2 (PGE2) synthesis, an inflammatory mediator and CYP4F2 enzyme degrades leukotriene B4, a potent mediator of inflammation.	Co-mentioned in the literature	BARTOSH et al., 2013 FREITAG et al., 2014

Abnormal TCD vs normal (>128cm/s)

LAMC2 and LAMA5	LAMC2 also mediate the attachment, migration and organization of cells into tissues during embryonic development by interacting with other extracellular matrix components like the LAMA3 and LAMA5.	Co-mentioned in the literature	GUDJONSSON et al., 2002
LRP2, APOB and LRP8	LRP8 is a member of the LDL receptor related protein family and such as LRP2. The LRP8 such as the LRP2 and APOB proteins was associated with lipid alterations like hypertriglyceridemia.	Co-mentioned in the literature	SHEN et al., 2012
MTHFR and MTRR	MTHFR catalyzes the conversion of 5,10-ethylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteine remethylation to methionine and MTRR is involved in the reductive regeneration of cob(I)alamin	Co-mentions in the literature and evidences of co-expression	HOUSTON, 1997 AKAR et al., 2001

	cofactor required for the maintenance of methionine synthase in a functional state. Both proteins are thus involved in the metabolism of homocysteine. High homocysteine levels are associated with cardiovascular diseases and with stroke in children with SCD.

PMC1: Pericentriolar material 1; CNTRL: centriolin; SFI1: Sfi1 homolog, spindle assembly associated; CCP110: centriolar coiled coil protein 110kDa; NINL: ninein-like; HBG2: hemoglobin, gamma G; HBE1: hemoglobin, epsilon 1; NEB: nebulin; TTN: titin; PLEK: pleckstrin; DOCK8: dedicator of cytokinesis 8; DOCK6: dedicator of cytokinesis 6; DNAH1: dynein, axonemal, heavy chain 1; DNAH14: dynein, axonemal, heavy chain 14; MASP2: mannan-binding lectin serine peptidase 2;
MBL2: mannose-binding lectin protein C2; BRCA1: breast cancer 1, early onset; MDC1: mediator of DNA-damage checkpoint 1; PCGF2: polycomb group ring finger 2; LAMA3: alpha lamininins 3; LAMA5: alpha lamininins 5; NPSR1: neuropeptide S receptor 1; TNC: tenascin; NMBR: neuromedin B receptor; LRP2: LDL receptor related protein 2; SYNJ1: synaptojanin 1; PIK3C2G: phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 gamma; LAMC2: laminin subunit gamma 2; LRP8: LDL receptor related protein 8.

5 DISCUSSÃO

Assim como outras manifestações clínicas da DF, o AVC esta associado a mudanças no formato dos eritrócitos, fenômeno central na patologia da DF, e com as interações com as células sanguíneas circulantes e com o endotélio, fato que pode contribuir para o aparecimento de disfunção endotelial, secreção de citocinas pró-inflamatórias, oclusão vascular e isquemia tecidual (FASANO et al., 2014). Os eritrócitos falcizados impedem o fluxo sanguíneo através das artérias e capilares por diversos mecanismos, incluindo alterações na reologia, ativação endotelial e proliferação, que levam à estenose arterial e oclusão. Diversos mecanismos fisiopatológicos já foram identificados como relacionados à ocorrência do AVC na DF, incluindo o aumento da adesividade das hemácias, ativação endotelial, respostas inflamatórias e desregulação da coagulação (FASANO et al., 2014). O AVC em crianças com DF é primariamente embólico; entretanto, resulta da estenose que eventualmente limita o fluxo cerebral abaixo de um limiar que conduz ao infarto (FASANO et al., 2014). O tratamento com HU não reduz o risco de AVC nem previne a ocorrência de um segundo evento com a mesma eficácia que o regime transfusional. No entanto, a HU pode ser uma alternativa aceitável à terapia transfusional para crianças com DTC com velocidades maiores que o normal e que não apresentem ainda vasculopatia cerebral (FASANO et al., 2014).

Historicamente, crianças com DF com vasculopatia cerebral eram diagnosticadas quando apresentavam sintomas clássicos de AVC, sendo que evoluíam com frequência para um déficit neurológico permanente. Nos últimos 15 anos a implementação do DTC levou a prevenção primária do AVC (FASANO et al., 2014; ADAMS, 2007), sendo que a triagem pelo DTC e o tratamento das crianças com risco elevado reduziu a incidência do primeiro AVC de aproximadamente 0,8 eventos/100 indivíduos por ano para aproximadamente 0,2/100 indivíduos por ano (FASANO et al., 2014; FULLERTON et al., 2004; McCARVILLE et al., 2008). Atualmente, o DTC é a única ferramenta prognóstica disponível para determinar o risco de AVC em crianças com DF (BELISÁRIO et al., 2015). Considerando o risco elevado para o desenvolvimento de AVC em crianças com DF, a realização de estudos que busquem por abordagens novas que auxiliem o DTC como ferramenta diagnóstica poderá trazer um suporte maior como marcadores preditores dos eventos.

O manuscrito 1 da sessão apêndice buscou estabelecer a velocidade para o diagnóstico do AVC em indivíduos com HbSC. Para esse fim foram comparadas as características do fluxo sanguíneo cerebral em indivíduos com AF e com HbSC usando o DTC. Em comparação ao artigo publicado por Kwiatkowski e colaboradores (2011) que avaliaram o DTC em 85 crianças e adolescentes com DF, foi encontrada um numero maior de DTC anormal em nosso estudo; esse resultado discordante pode ser explicado pelo tamanho amostral do nosso estudo que avaliou um número maior de indivíduos com DF. Deane e colaboradores (2008) avaliaram o DTC em 47 indivíduos com HbSC e encontraram a velocidade média máxima de 104,90 cm/s.

Este estudo avaliou uma quantidade maior de crianças e adolescentes com HbSC e ao estabelecermos a comparação entre a velocidade encontrada nos dois grupos foi possível verificarmos que os indivíduos com AF apresentavam VMMAX maiores que os indivíduos com HbSC. A média da velocidade em indivíduos com HbSC foi` de 104,90 cm/s com desvio padrão de 19,3 cm/s; ao adicionarmos dois desvios padrões encontramos a velocidade de 143,50 cm/s, levando a conclusão de que indivíduos com DTC superiores a 143,50 cm/s devem ser considerados anormais. No estudo de Deane e colaboradores (2008) a VMMAX encontrada foi inferior à verificada neste trabalho, no qual consideramos que acima do percentil 98 que corresponde a 128 cm/s, o paciente já deveria ter o DTC considerado anormal.

Os dados clínicos dos indivíduos com DF têm sido cada vez mais associados a valores anormais de DTC, bem como ao risco aumentado de desenvolver o AVC. Assim, o manuscrito 1 do presente trabalho investigou marcadores preditores para ocorrência do AVC em indivíduos com HbSC através da associação de biomarcadores genéticos, hematológicos, bioquímicos e imunológicos com a VMMAX.

Nossas correlações mostraram que indivíduos com HbSC e VMMAX elevada tiveram contagem diminuída de hemácias (Hm) e concentrações diminuídas de hemoglobina (Hb), hematócrito (Ht) e de bilirrubina direta (BD). Dessa forma, os indivíduos com VMMAX elevadas apresentaram a anemia mais grave do que a que é apresentada por indivíduos com VMMAX baixa. Esses dados são consistentes com estudos prévios que associaram marcadores de hemólise como a contagem de Hm, reticulócitos, concentração da Hb, bilirrubina indireta (BI) e LDH com a suscetibilidade ao AVC na AF (DOMINGOS et al., 2014). Alguns autores sugerem que a anemia grave pode ser um risco adicional para o desenvolvimento do AVC. Do mesmo modo, já foi sugerido que o aumento no fluxo sanguíneo cerebrovascular e a velocidade do fluxo com a

anemia crônica causam distúrbios no fluxo sanguíneo que podem levar a lesão cerebrovascular (ADAMS et al., 1994; PROHOVNIK et al., 1989; LEITE et al., 2012).

Além disso, foi possível verificar que indivíduos HbSC com VMMAX elevada tem contagem maior de monócitos e níveis elevados de ferritina. Esses achados estão de acordo com estudos prévios que descrevem que a contagem elevada de leucócitos também pode ser um fator de risco para diversos tipos de complicações associadas com a DF, como crises de dor, STA e AVC. Isso pode ser explicado pelo efeito ativador do contato entre os neutrófilos e o endotélio vascular (BALKARAN et al., 1992; LEITE et al., 2012). As interações celulares entre os monócitos e o endotélio vascular desempenham papel importante nas doenças inflamatórias, e, portanto, podem modular a vasculopatia persistente presente nos indivíduos com AF. Os leucócitos são capazes de interagir com o endotélio vascular, com os eritrócitos falcizados circulantes e com as plaquetas (SAFAYA et al., 2012). Mesmo em indivíduos com AF em estado-estável, a leucocitose constitui um fator de risco para STA, AVC e mortalidade precoce (JOHNSON e TELEN, 2008).

Níveis de ferritina elevados podem ser observados durante processos inflamatórios e infecciosos. A associação entre o estado inflamatório e hemolítico corrobora com a observação de que níveis mais elevados de ferritina estão presentes em indivíduos com VMMAX elevada (ROGERS, 1996).

Usando um valor de *cut off* de 128 cm/s definido por Deane e colaboradores (2007), foi identificado que a redução de RDW e dos níveis de NO estavam associados em indivíduos com HbSC com TAMMV maior que 128 cm/s. Esses dados podem ser justificados pela hemólise intravascular, onde a hemoglobina livre é liberada no microambiente vascular e reage rapidamente degradando o NO, com simultânea liberação de arginase no plasma. Essa cascata de eventos resulta na produção de espécies reativas de oxigênio e leva a vasoconstrição em indivíduos com AF (ROTHER et al., 2005). Acredita-se que a concentração de hemoglobina em indivíduos com DF em estado estável seja em torno de 4μM, e que essa concentração seja capaz de depletar o NO numa taxa de $9x10^{7}M^{-1}s^{-1}$ ($4x10^{-6}M$). Essa taxa é suficiente para depletar quase todo o NO produzido por células endoteliais em regiões vasculares adjacentes à parede vascular, atenuando, portanto, a vasodilatação, que é modulada pela concentração de NO na camada de células musculares lisas vasculares (WOOD et al., 2008; JEFFERS et al., 2006).

Esses dados são semelhantes ao de French e colaboradores (1994) que avaliando um modelo experimental de AVC em ratos identificaram que a produção contínua de NO é importante pare a manutenção do fluxo sanguíneo cerebral. O NO é constitutivamente produzido por diferentes tipos celulares (plaquetas, eritrócitos e neurônios) e o NO produzido por células endoteliais é idealmente definido como regulador das interações célula-célula necessárias para a modulação do tônus vasomotor; acredita-se também que a enzima óxido nítrico sintase neuronal também desempenhe papel semelhante (WOOD et al., 2008; GHALAYINI, 2004).

Utilizando um valor de *cut off* de 143,5 cm/s definido por Vieira e colaboradores (manuscrito em submissão), nossos resultados sugerem que concentrações diminuídas de Hb e Ht estavam associadas a indivíduos com HbSC com VMMAX maior que 143,5 cm/s, bem como níveis elevados de ferritina. Esses dados indicam que indivíduos com VMMAX maior que 143,5 cm/s possuem anemia mais grave e estado inflamatório e hemolítico como foi discutido previamente.

Quando avaliamos os dados laboratoriais utilizando o percentil 75 da VMMAX nós encontramos a combinação de marcadores associados a VMMAX definida por Deane e colaboradores (2007) e por Vieira e colaboradores (em submissão). Os dados encontrados sugerem que a diminuição de Hb, Ht, RDW e níveis de NO estariam associados a indivíduos com VMMAX maior que 125,75 cm/s, bem como níveis elevados de ferritina. Dessa forma, indivíduos com HbSC e com velocidades mais baixas que aquelas descritas por Deane e colaboradores (2007) e Vieira e colaboradores (em submissão), já possuíam as alterações bioquímicas e hematológicas encontradas nas velocidades propostas por esses dois autores.

Quando o percentil 95 da VMMAX foi utilizado com o intuito de avaliar os dados laboratoriais, foram identificados que níveis diminuídos de Hb e Ht e contagem elevada de monócitos e dos níveis de ferritina estavam associados aos indivíduos com VMMAX maior que 156 cm/s. Esses dados indicam que indivíduos com VMMAX maior que 156 cm/s possuíam anemia mais grave com estado inflamatório e hemolítico crônico e contagem de monócitos aumentada.

Os resultados da análise multivariada corroboram a influência da Hb, Ht, RDW, ferritina, NO, talassemia alfa-3,7kb, haplótipo CAR e dos polimorfismos nos genes *MTHFR* 677C>T, *VCAM* 833T>C e *VCAM* 1238 G>C em indivíduos com a TAMMV maior que 125,75 cm/s; e a influência dos polimorfismos nos genes *VCAM* 1238 G>C e *MTHFR* 677C>T e de Ht, monócito,

ferritina, talassemia alfa-3,7kb e Hb em indivíduos com TAMMV maior que 156 cm/s. Estudos recentes associaram, individualmente, a presença de polimorfismos nos genes das enzimas *MTHFR* 677 C>T, *PT* 20210 G>A e *FV* 1691G>A com risco maior para o desenvolvimento de AVC (LI e CHI, 2013;. WANG et al., 2013;. NIU et al., 2013; PEREIRA et al., 2007; BERNAUDIN et al., 2008;. CASAS et al., 2004; BANERJEE et al., 2007); contudo, na análise multivariada, a presença do polimorfismo no gene da enzima *MTHFR* 677 C>T, possivelmente, possui efeito protetor em relação à velocidade do VMMAX. A falta da co-herança com a talassemia alfa-3,7kb mostrou ter um efeito protetor em relação à velocidade do VMMAX. Tal dado está em acordo com estudos anteriores que ao avaliarem, individualmente, a talassemia alfa-3,7kb não encontraram resultados significantes em relação à velocidade do VMMAX (BALKARAN, et al, 1992;. LEITE et al, 2012;. KATO et al., 2007).

Ao realizarmos a análise multivariada com o percentil 95 e o polimorfismo no gene *VCAM* 1238G>C foi observado o efeito protetor em relação à velocidade do VMMAX. Este resultado está em concordância com o estudo feito por Taylor e colaboradores (2002) que identificaram que o polimorfismo *VCAM* 1238 G>C tem efeito protetor em relação ao risco de AVC. Em estudo realizado em uma população também brasileira, não foi observada associação entre o polimorfismo *VCAM* 1238G>C e AVC ou doença cerebrovascular (BELISÁRIO et al., 2015).

Alguns marcadores genéticos como os descritos no manuscrito 1 têm sido associados na literatura como preditores do AVC, mas os resultados ainda assim permanecem controversos e não conseguem elucidar completamente a heterogeneidade genética dos indivíduos no desenvolvimento do AVC. Assim, a fim de compreender a influência genética dos indivíduos com DF no AVC, o manuscrito 2 do presente estudo buscou identificar uma ampla gama de SNPs preditores para o AVC em indivíduos com AF e HbSC.

As variantes genéticas associadas a doenças podem revelar mecanismos novos nos processos fisiopatológicos. Os resultados do sequenciamento de nova geração identificaram dois SNPs e genes comuns entre as diferentes velocidades de fluxo sanguíneo cerebral em indivíduos com AF, sendo eles, a polipeptídeo da DNA primase 1 (*PRIM1*) rs2277339 e a soluto transportador membro da família transportador de ânion orgânico 1 B1 (*SLCO1B1*) rs4149056. Em relação aos indivíduos com HbSC, um único SNP e gene comum foi encontrado entre os grupos de indivíduos com DTC anormal vs baixo, DTC anormal vs normal (<128 cm/s) e DTC

anormal vs normal (>128 cm/s), a *ATP-binding cassette, sub-family G (WHITE) (ABCG5)* rs6756629.

O polipeptídeo da DNA primase 1 é uma enzima envolvida no início da síntese de polímeros de DNA e uma subunidade da primase do DNA nuclear. Esta enzima foi associada a condições patológicas como, por exemplo, osteossarcoma humano, glioma, neuroblastoma, leucemia linfocítica crónica e sarcomas em geral, mas na DF esse gene ainda não foi estudado (YOTOV et al., 1999).

O gene *SLCO1B1* codifica uma proteína transmembrana, a SLC (soluto transportadora membro da família transportador de ânion orgânico 1B1) que está envolvida, principalmente, na captação de pequenas moléculas em células. O transportador independente de sódio ligado à membrana está envolvido no influxo celular ativo de substratos endógenos (ácidos biliares), xenobióticos e uma vasta gama de medicamentos (estatinas, antibióticos, inibidores de ACE) (NAGY et al., 2015). Os polimorfismos no gene *SLCO1B1* são importantes no processo de farmacocinética da estatina. Um estudo realizado em Roma e na Hungria sugeriu que os haplótipos identificados são relevantes sobre a terapia com estatinas e também podem modular o resultado clínico (NAGY et al., 2015). Embora as estatinas não sejam atualmente utilizadas na prática clínica para o tratamento de indivíduos com DF, um estudo-piloto foi realizado para avaliar o efeito anti-inflamatório da estatina. Duas doses diferentes foram administradas durante 39 dias, e foi demonstrado que os níveis de NO foram aumentados, e IL-6 e hsCRP foram diminuídos (HOPPE et al., 2011). Assim, polimorfismos no gene *SLCO1B1* podem ser relevantes para avaliar a eficácia de abordagens terapêuticas as quais os indivíduos são submetidos.

Os polimorfismos nos genes da *SLCO1B1* e *SLCO1B3* também foram associados a níveis elevados de bilirrubina (LIN et al., 2015). Os transportadores SLC são responsáveis pela absorção de bilirrubina conjugada - o produto do catabolismo do heme - para os hepatócitos. Polimorfismos comuns nos genes dos transportadores que estão envolvidos na via de eliminação hepática da bilirrubina podem levar a hiperbilirrubinemia e icterícia (LIN et al., 2015). Em indivíduos com DF, os níveis de bilirrubina são um marco laboratorial frequente. Assim, além do polimorfismo no gene da *SLCO1B1* estar associado ao metabolismo das estatinas, o metabolismo da bilirrubina é igualmente importante sobre os resultados clínicos dos indivíduos. Não há na literatura estudos que tenham associado os polimorfismos no gene *SLCO1B1* e DF ou risco de AVC; portanto, mais informações são necessárias para definir o seu papel.

As mutações em qualquer um dos genes da *ABCG5* e *ABCG8* causam sitosterolemia, uma desordem autossômica recessiva caracterizada pela acumulação de colesterol (GRAF et al., 2003). Não foram encontrados estudos sobre os transportadores *ABCG5* na DF. O metabolismo lipídico em indivíduos com DF pode estar alterado, isso foi sugerido por Seixas e colaboradores (2010) que identificaram que os indivíduos podem apresentar um subfenótipo dislipidêmico específico caracterizado por níveis diminuídos de lipoproteínas de alta densidade (HDL-C) com hipertrigliceridemia e elevação de lipoproteínas de muito baixa densidade (VLDL-C), assim a associação do polimorfismo no gene *ABCG5* e a evolução clínica de indivíduos com DF podem estar relacionados.

Nos indivíduos com HbSC e grupo de DTC anormal vs DTC normal (< 128 cm/s) foram encontrados seis SNPs únicos, o dedicador de citocinese 6 (*DOCK6*) rs2278426, tirosinase (*TYR*) rs1042602, citocromo P450, família 4, subfamília F (*CYP4F2*) rs2108622, estimulante de macrófagos 1 (*MST1*) rs3197999, membro do receptor olfativo, família 51, subfamília B, membro (*OR51B6*) rs5006884 e o gene associado ao adenoma da tireoide (*THADA*) SNPs rs7578597.

Os genes semelhantes a angiopoietina (*ANGPTL*) codificam uma família de proteínas associadas com o metabolismo lipídico. Um desses genes *ANGPTL*, o gene *ANGPTL8* está localizado no íntron do gene *DOCK6*. Em três populações, o polimorfismo *DOCK6* rs2278426 foi associado com níveis plasmáticos mais baixos de HDL-C e LDL-C (QUAGLIARINI et al., 2016). Na literatura não há estudos que avaliem o polimorfismo *DOCK6* e a DF ou risco de AVC, mas este gene pode ser útil para explicar os subfenótipos dislipidêmicos encontrados em indivíduos com DF.

O gene *TYR* codifica a tirosinase, uma enzima que catalisa reações da via de produção de melanina. O alelo A do polimorfismo *TYR* rs1042602 está associado com a presença de pele e olhos claros, e mutações nesse gene estão associadas com vitiligo e melanoma cutâneo (WILDE et al., 2014). Não existem estudos avaliando o polimorfismo *TYR* e o risco de AVC ou DF.

O gene *CYP4F2* codifica um membro da superfamília do citocromo P450 (CYP) de enzimas cisteinato-heme que é responsável pelo metabolismo de xenobióticos e uma série de endobióticos como, por exemplo, o ácido araquidônico. Metabolitos do ácido araquidônico derivados de CYP estão associados com alterações vasculares cerebrais. O *CYP4F2* atua como uma enzima no metabolismo do leucotrieno B4, um potente mediador da inflamação, e do ácido

20-hidroxieicosatetraenóico, que desempenha papel importante na regulação do tónus vascular no cérebro e atua como um potente constritor das artérias cerebrais. Em estudo realizado em homens japoneses com infarto cerebral foi encontrada a associação entre essa condição e o polimorfismo no gene *CYP4F2* (FU et al., 2008). Este polimorfismo é também associado com a variabilidade interindividual na dose de varfarina. Varfarina é um anticoagulante usado para prevenir ataques cardíacos, AVC e coágulos sanguíneos. Os doentes com o genótipo variante tendem a necessitar de doses mais elevadas de varfarina que os indivíduos com o genótipo selvagem (BORGIANI et al., 2009). Não foram encontrados estudos que associaram o polimorfismo no gene C*YP4F2* ao risco de AVC na DF.

O gene *MST1* codificada uma proteína que contém quatro domínios e um domínio de protease de serina. Apesar da presença do domínio de serina-protease, a proteína codificada pode não ter qualquer atividade proteolítica. Este gene foi associado na literatura a condições patológicas, tais como doença inflamatória do intestino, colangite esclerosante primária, doença de Crohn e colite ulcerosa, mas na DF não foi caracterizada (JOSTINS et al., 2012; MELUM et al., 2010; MCGOVERN et al., 2010; FRANKE et al., 2010).

O SNP rs5006884 no gene OR51B5/6 pode desempenhar papel regulador na expressão do gene da gama-globina e foram descritos 3 loci deste gene que tem a capacidade de afetar as concentrações de HbF. Os indivíduos com AF possuem frequência elevada dessa mutação em relação à população em geral. A frequência deste SNP em indivíduos com AF e HbF elevada é significativamente maior do que no grupo de indivíduos com HbF diminuída; assim, essa mutação está associada com alteração na HbF e é um importante marcador de prognóstico para o risco de AVC e outras manifestações clínicas em indivíduos com DF (SOLOVIEFF et al., 2010;. AKINSHEYE et al., 2012; GALARNEAU et al., 2016; WONKAM et al., 2014).

O gene *THADA* é um gene de proteína codificante e está relacionado com a diabetes tipo 2 e a síndrome metabólica. Em estudo de DeMenna e colaboradores (2014), o SNP rs7578597 foi significativamente associado com a obesidade, glicemia e fenótipo lipídico (ZEGGINI et al., 2008; DEMENNA et al., 2014). Não existem estudos a respeito gene *THADA* na DF.

Nos indivíduos com HbSC com DTC anormal vs DTC normal (> 128 cm/s) foram encontrados 7 SNPs únicos, o fucosiltransferase 2 (*FUT2*) rs602662, o metilenotetrahidrofolato redutase (*MTHFR*) rs1801133, endonuclease splicing RNAt TSEN15 (*TSEN15*) rs1046934, o

MST1 rs3197999 , o *OR51B6* rs5006884, o fator de complemento B (*CFB*) rs12614 e o *THADA* rs7578597.

O gene *FUT2* regula a expressão de antígenos ABH em tecidos e células do sangue, este gene codifica uma alfa 1,2-fucosiltransferase capaz de transferir a L-fucose ao carbono 2 de galactose (beta, 1-3) N-acetil D contendo glucosamina-glicanos (ANSTEE, 2010). O SNP rs602662 deste gene foi descrito por estar envolvido nos níveis de folato e vitamina e em condições patológicas, tais como colangite esclerosante primária e doença de Crohn. Este SNP ou o gene não foi associado com a DF (HAZRA et al., 2009;. MCGOVERN et al., 2010; FOLSERAAS et al., 2012).

O SNP rs1801133 está localizado no gene da *MTHFR* que codifica a enzima chamada metilenotetrahidrofolato redutase que está envolvida no metabolismo do folato. Esta enzima converte a molécula de 5,10-metilenotetrahidrofolato em uma molécula de 5-metiltetrahidrofolato. Esta reação é necessária para a via que converte a homocisteína em outro aminoácido, a metionina. Este SNP foi associado com condições patológicas, como o risco de doença arterial coronariana, lábio leporino não sindrômico com ou sem fenda palatina, fatores de risco pré-natal de anormalidades na substância branca e o risco de AVC (MEURS et al., 2013; PARE et al., 2016; AGUIAR et al., 2015; MARSEGLIA et al., 2016; ZHANG et al., 2014). Este SNP já foi associado na literatura ao risco de AVC na DF (HATZLHOFER et al., 2012;. COUTO et al., 2004).

O SNP do *TSEN15* rs1046934 codifica a subunidade que catalisa a remoção de íntrons de precursores RNAt e está associada a manifestações clínicas como peso, doenças neurogênicas, fibrilação atrial e hipoplasia pontocerebelar, mas em DF não foram ainda estudados (ALAZAMI et al., 2015; CASSANDRINI et al., 2010).

O SNP rs12614 do gene *CFB* associado a resposta imune foi também descrito, sendo que este codifica para o fator do complemento B, um componente da via alternativa de ativação do complemento. O fator B circula no sangue como um polipeptídio de cadeia única. O agrupamento deste gene inclui vários genes envolvidos na regulação da reação imune. Os polimorfismos neste gene estão associados com um risco reduzido de degeneração macular relacionada com a idade e sepse bacteriana grave, pois este gene atua como efetor na via de sinalização dos receptores semelhantes Toll (TLR) (YU et al., 2011; ZOU et al., 2016). Não existem estudos relacionados ao gene *CFB* e a DF.

Nos indivíduos com AF com DTC anormal vs DTC normal foram encontrados 5 SNPs únicos, apolipoproteína B (*APOB*) rs676210, tirosina quinase 2 (*TYK2*) rs12720356, *TSEN15* rs1046934, *CYP4F2* rs2108622 e *MST1* rs3197999.

Um estudo prospectivo, que teve como objetivo determinar se o risco de AVC estava relacionado ao equilíbrio entre partículas pró-aterogênicas de lipoproteínas apoB e as partículas antiaterogênicas apoA-I, identificou nos indivíduos apoB elevada e valores diminuídos de apoA-I que foram significativamente relacionados com o risco de AVC (WALLDIUS et al., 2006). O SNP rs676210 no gene da *APOB* tem sido associado com colesterol de lipoproteína de baixa densidade (LDL) e foi também associado a condições patológicas conhecidas como aterosclerose e hipertrigliceridemia (WOJCZYNSKI, et al., 2010). O gene *APOB* foi associado com diabetes mellitus na DF, mas o SNP associado foi diferente do que identificamos no presente estudo (ZHANG et al., 2015).

O gene *TYK2* tem sido associado a síndrome de hiperimunoglobulina E e codifica um membro da tirosina quinase e da família de proteínas Janus quinase. Esta proteína atua nas vias de sinalização de interferon e de citocinas. Assim, este gene está associado com a ocorrência de infecções (CASANOVA et al., 2004). Não existem estudos sobre o gene *TYK2* e sua associação na DF.

Os dados na literatura sobre os agrupamentos identificados para diferentes grupos de DTC de indivíduos com AF e indivíduos com HbSC estão apresentados nas tabelas 3 e 4 do manuscrito 2, respectivamente. No entanto, o agrupamento do receptor olfatório e da MTHFR - MTRR merecem destaque, visto que foram encontrados no diferentes grupos DTC de indivíduos com AF e com HbSC.

Há um grande agrupamento de proteínas do receptor olfatório identificadas em todos os grupos DTC de indivíduos com AF e com HbSC. Esses SNPs e genes dos receptores olfatórios estão localizados no cromossomo 1 regulam os níveis de HbF (SOLOVIEFF et al., 2010). Os níveis de HbF mais elevados foram associados a taxa reduzida de episódios dolorosos agudos, úlceras de perna, osteonecrose e síndrome torácica aguda, reduzindo assim a gravidade da doença (STEINBERG et al., 2009).

Outro agrupamento importante foi o MTHFR e MTRR. A enzima MTHFR catalisa a conversão de 5,10-ethylenetetrahydrofolate a 5-metiltetrahidrofolato, um co-substrato para a remetilação da homocisteína para metionina e a MTRR está envolvida na regeneração redutiva de

cofactor cob (I) alamina necessário para a manutenção da síntese de metionina num estado funcional. Assim, ambas as proteínas estão envolvidas no metabolismo da homocisteína. Altos níveis de homocisteína estão associados a doenças cardiovasculares e com AVC em crianças com AF (HOUSTON et al, 1997;. AKAR et al., 2001).

No presente estudo observou-se que a contribuição genética para o AVC é poligênica. Identificamos SNPs em genes envolvidos no metabolismo lipídico, inflamação, resposta imune, síndrome metabólica, metabolismo xenobióticos, farmacocinética, síntese de DNA, produção de bilirrubina, expressão do gene gama da hemoglobina e no metabolismo da homocisteína. É importante ressaltar que os SNPs encontrados aqui estão associados principalmente com o metabolismo lipídico e a inflamação, o que nos levou a sugerir que o AVC na DF está associado ao perfil inflamatório e dislipidêmico. Indivíduos com DF apresentam subfenótipo dislipidêmico específico caracterizado por níveis diminuídos de HDL-C com hipertrigliceridemia e níveis elevados de VLDL-C e essas alterações lipídicas podem ativar diretamente vias inflamatórias nestes indivíduos, o que pode culminar no AVC (SEIXAS et al., 2010).

A maioria dos genes descritos por este trabalho não foi previamente estudado na DF, mas representam uma possível via para estudar novos marcadores preditores de AVC.

6 CONCLUSÕES

- A velocidade do fluxo sanguíneo cerebral encontrada nos indivíduos com HbSC foi inferior a verificada em estudos anteriores para indivíduos com AF;
- A velocidade média máxima de 125,75 cm/s pode ser a mais adequada para avaliar os indivíduos com HbSC, porém são necessários mais estudos para identificar a associação dessa velocidade com o risco de AVC;
- Os marcadores hematológicos e bioquímicos, como contagem diminuída de Hm, níveis diminuídos de Hb, Ht, RDW, BD e NO, contagem elevada de monócitos e níveis elevados de ferritina podem ser usados como marcadores preditivos para a ocorrência de AVC em indivíduos com HbSC;
- O polimorfismo no gene da *MTHFR* 677C>T e a ausência da talassemia alfa -3,7kb estiveram associados ao efeito protetor em relação ao AVC e, por isso, podem vir a ser utilizados como preditores do AVC nos indivíduos com HbSC;
- Os SNPs nos genes *DOCK6* rs2278426, *TYR* rs1042602, *CYP4F2* rs2108622, *MST1* rs3197999, *OR51B5/6* rs5006884, *THADA* rs7578597, *FUT2* rs602662, *MTHFR* rs1801133, *TSEN15* rs1046934, *CFB* rs12614 e *ABCG5* rs6756629 podem ser preditores para a ocorrência do AVC na HbSC e os SNPs dos genes *SLCO1B1* rs4149056, *PRIM1* rs2277339, *APOB* rs676210, *TYK2* rs12720356, *TSEN15* rs1046934, *CYP4F2* rs2108622 e *MST1* rs3197999 podem ser preditores para a ocorrência do AVC na AF.

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A - MANUSCRITOS

Artigos produzidos em colaboração durante o período do mestrado e que não entraram no corpo da dissertação.

A.1 – MANUSCRITO I

Título: Transcranial Doppler in hemoglobin SC disease

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Situação: Submetido à *Pediatric Blood & Cancer*, *Manuscript ID*: PBC150948

Objetivo:

Avaliar a velocidade média de fluxo sanguíneo cerebral na artéria cerebral média dos indivíduos com HbSC.

Principais resultados:

A velocidade média máxima (VMMAX) nas artérias cerebrais médias (ACM) e na carótida interna intracranial distal (CIID), localizadas no lado esquerdo e direito, foram de 134,3 ± 32,0 cm/s e 134,4 ± 32,6 cm/s em indivíduos com anemia falciforme (AF) e 105,2 ± 20,6 cm/s e 104,7 ± 20.0 cm/s em indivíduos com doença SC (HbSC). A média da VMMAX entre as ACM/CIID esquerda e a direita foi de 134,5 ± 30,5 cm/s no grupo com AF e 104,9 ± 19,3 cm/s nos indivíduos com HbSC. Assim, os indivíduos com HbSC tem velocidades menores de Doppler Transcraniano que os indivíduos com AF. A VMMAX superior a 143,5 cm/s pode ser considerada como anormal nos indivíduos com HbSC.

Title: Transcranial Doppler in hemoglobin SC disease

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Number of Tables and Figures: 1 table and 1 figure

Running title: Transcranial Doppler velocity in HbSC disease

Key words: Transcranial Doppler; Stroke; Sickle Cell Disease; SC Disease.

Abbreviations	Full term
SCD	Sickle cell disease
TCD	Transcranial Doppler
HbSC	Hemoglobin SC disease
SCA	Sickle cell anemia
TAMM	Time-averaged maximum mean velocity
ICA	Intracranial internal carotid
MCA	Middle cerebral arteries
SD	Standard deviation
STOP	Stroke Prevention in Sickle Cell Anemia study

Abstract

Background: Stroke is a severe clinical disorder in sickle cell disease (SCD), and few studies have evaluated Transcranial Doppler (TCD) flow velocities in hemoglobin SC disease (HbSC), and the guidelines for stroke risk are based on sickle cell anemia (SCA) or HbS/β thalassemia evaluation.

Procedure: In this study, we compare cerebral blood flow in individuals with SCD stratified by genotypes. A total of 1664 pediatric individuals with SCD underwent TCD velocity screening, and the time-averaged maximum mean speed in the middle cerebral arteries and the distal internal carotid arteries were determined.

Results: The mean time-averaged maximum mean velocity (TAMM) in the left and right in the distal intracranial internal carotid (ICA) and middle cerebral arteries (MCA) (ICA/MCA) was 134.3 ± 32.0 cm/s and 134.4 ± 32.6 cm/s in individuals with SCA, and 105.2 ± 20.6 cm/s and 104.7 ± 20.0 cm/s in the individuals with HbSC respectively. A mean TAMM between right and left ICA/MCA was 134.5 ± 30.5 cm/s in the SCA group, and 104.9 ± 19.3 cm/s in the HbSC group. Notably, our data show that TCD velocities were significantly lower among the individuals with HbSC compared to SCA.

Conclusion: This results indicating that the results of the TCD velocities obtained in the SCA genotype cannot be extrapolated to individuals with HbSC. Therefore, additional studies are warranted to establish the pattern of increased risk for stroke in HbSC genotype, which would support preventive strategies and clinical monitoring.

INTRODUCTION

Stroke is a common clinical manifestation in sickle cell disease (SCD) children one year or older. [1-5] However, there are differences in stroke incidence among the SCD genotypes, with a rate of 0.61/100 individuals/year for sickle cell anemia (SCA) individuals, 0.17/100 individuals/year for HbSC, 0.11/100 individuals/year for HbS/β^+ thalassemia, and 0.11/100 individuals/year for HbS/β^0 thalassemia. [3]

The Transcranial Doppler (TCD) monitors the cerebral mean blood flow velocities of individuals with SCD allowing the identification of those with an increased risk to developing stroke. [6-10] The stratification of stroke risk can be determined by measuring the average maximum velocity or the time-averaged maximum mean velocity (TAMM) in the distal intracranial internal carotid (ICA) and middle cerebral arteries (MCA). Values ≥ 200 cm/s are considered high risk, whereas values < 170 cm/s are considered low risk; speeds ≥ 170 cm/s and < 200 cm/s are considered conditional. [6] Approximately 46-90 % of untreated individuals with SCA have a risk of recurrent strokes, which is particularly common after the first episode. [11,12] Despite the high incidence of stroke in individuals with HbSC compared with the pediatric population without SCD, [3,4] few studies have evaluated flow velocities by TCD in this patient subset, using TCD values for measuring risk stratification that are obtained from SCA or HbS/β thalassemia individuals. [3,6,11] Therefore, theoretically, these values may not extrapolate well to individuals with HbSC. The aim of this study is to compare the characteristics of cerebral blood flow among individuals with SCA and HbSC using TCD.

METHODS

Individuals with SCD evaluated from August 2011 to May 2015 in the Pediatric Cerebrovascular Disease Outpatient Center at the Hospital Universitario Professor Edgard Santos of the Universidade Federal da Bahia were included in the study. Only genotype HbSS and HbSC between 2 to 16 years old were included. Individuals with a prior overt stroke event, on hydroxyurea therapy, with a simple transfusion in the last three months or on chronic blood therapy regimens were not included in the study. One examiner performed the TCD in all individuals using the same device (Doppler, probe 2 Mhz model, Ezdop, Germany). The TAMM

was determined in the ICA and MCA, and the highest velocity was considered; if TAMM in all arteries were between 70 cm/s and 170 cm/s, the examination was considered normal; the TAMM ≥ 170 cm/s, but less than 200 cm/s in any one artery was considered conditional; the TAMM ≥ 200 cm/s in an artery was considered abnormal, and the TAMM < 70 cm/s was considered low. The failure to detect the flux wave during the examination was characterized as inadequate. Individuals were analyzed and stratified according to SCD genotypes (SCA and HbSC).

The Independent Student's T test was used to compare the means among the groups of quantitative variables with normal distribution. The results were considered significant if the P value was less than 0.05. The data analysis was performed using SPSS version 21 (SPSS Inc., Chicago, IL, USA).

This study was approved by the Research Board of the Secretaria de Saúde do Estado da Bahia (SESAB) 054/2011, and all parents or guardians provided written informed consent in accordance with the Helsinki Declaration of 1975 and its revision.

RESULTS

A total of 2774 individuals with SCD were evaluated with TCD examination from August 2011 to April 2015 in the Pediatric Cerebrovascular Disease Center at the Hospital Universitario Professor Edgard Santos of the Universidade Federal da Bahia. Were excluded 1110 individuals (some had more than one exclusion criteria): SD genotype: 10; sickle cell beta thalassemia: 70; age less than two or more than 16 years old: 75; prior overt stroke: 116, blood transfusion therapy: 183; hydroxyurea use: 656. A total of 1664 SCD were investigated, with a mean ± standard deviation (SD) age of 6.5 ± 3.8 years, and 48.6 % of females; 1106 (66.5 %) individuals with SCA were investigated, with a mean age of 6.8 ± 3.9, and 47 % were females. In addition, 558 (33.5 %) individuals with HbSC were investigated, with a mean age of 6.0 ± 3.5 years, and 51.6 % were females.

The mean TAMM was 124.5 ± 31.8 cm/s and 124.4 ± 32.2 cm/s in the right and left ICA/MCA respectively. The mean time-averaged maximum mean velocity in the left and right ICA/MCA was 134.3 ± 32.0 cm/s and 134.4 ± 32.6 cm/s in the individuals with SCA, and 105.2 ± 20.6 cm/s

and 104.7 ± 20.0 cm/s in the individuals with HbSC respectively. A mean TAMM between right and left ICA/MCA was 134.5 ± 30.5 cm/s in the SCA group, and 104.9 ± 19.3 cm/s in the HbSC group (Figure 1). These differences were statistically significant ($p<0.001$).

In a smaller number of these individuals were assessed severities markers like hemoglobin and hematocrit. We evaluated 68 individuals with HbSC and 79 individuals with HbSS. The TAMM was correlated with hemoglobin and hematocrit in both genotypes. In the HbSC genotype were found a negative correlation between TAMM and hemoglobin (R=-0.3390, p=0.007); and between TAMM and hematocrit (R=-0.3470, p=0.0057) (Figure 2A, 2B). In the HbSS genotype were found a negative correlation between TAMM and hemoglobin (R= -0.2310, p=0.0447) and between TAMM and hematocrit (R= -0.2649, p=0.0208) (Figure 2C, 2D).

DISCUSSION

Few studies evaluating TCD examination of a large number of individuals with SCD. [13-15] Adams et al. [13] published the TCD results from the Stroke Prevention in Sickle Cell Anemia study (STOP), which was a clinical trial that included 5613 SCD children; they found that 67 % of individuals with SCD exhibited normal TCD results, 17.6 % exhibited conditional TCD results, 9.3 % exhibited abnormal results, and 6.1 % exhibited inadequate TCD evaluation. Another important study was performed by Enninful-Eghan et al. [14] which evaluated the occurrence of stroke and the response to transfusion therapy in 475 individuals with SCD over eight years of follow-up prior to TCD examination, and in 530 individuals with SCD over eight years of follow-up with TCD. However, after some loss, the analysis of 404 individuals with SCD revealed 14.4 % with conditional TCD, 12.5 % with abnormal and 0.7 % with an inconclusive TCD evaluation. Additionally, the use of blood transfusion was successful among the individuals followed with TCD, which was also demonstrated by Kwiatkowski et al [15]

Our results differed from previous report developed in 85 Brazilian children and teenagers with SCD, which found lower numbers of abnormal TCD. [16] However, the difference between these two studies may be explained by the sample size, once our study evaluated a larger number of individuals with SCD.

Deane et al. [17] evaluated 47 TCD tests from individuals with HbSC, and showed a TAMM velocity in the MCA of 94 cm/s. In our study, individuals with HbSC had an average TAMM velocity of 104.9 ± 19.3 cm/s in the MCA/ICA. Rees's study did not identify individuals with high speeds according to the STOP protocol. [6] This study analyzed a greater number of children and teenagers with HbSC, and the comparison of individuals with SCA showed that the TAMM velocities in individuals with HbSC were significantly lower, in accord to previous report from our group. [18] Using rates of stroke risk in individuals with SCA, only 0.7 % of individuals with HbSC presented a high risk of stroke (Table I). However, the differences in the mean TAMM velocities in the both MCA/ICA of SCA (134.5 ± 30.5cm/s) and HbSC (104.9 ± 19.3 cm/s) individuals may suggest a specific risk velocity for this genotype.

The average TAMM velocity in the MCA/ICA in individuals with HbSC was 104.9 cm/s, with a SD of 19.3 cm/s, which allows us to consider a normal rate (two standard deviations) in individuals with HbSC values above 143.5 cm/. In this case, velocities greater than 143.5 cm/s should be considered a cutoff point for individuals with HbSC. According to this parameter, 39 (7.0 %) individuals in our study would have high values. In the study conducted by Deane et al., [17] the TAMM velocity in the 98^{th} percentile was 128 cm/s and the authors could not assign stroke risk to this population.

In the present study, less than 1.6% of individuals with HbSC presented TAMM higher than 170cm/s. However, the mean MCA/ICA velocities were different between SCA and HbSC. A TAMM higher than 143.5 cm/s can be considered as abnormal, but if it means an increase in stroke risk is unknown. It is necessary new studies to determinate the stroke risk TAMM for individuals with HbSC.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

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Figure legends

Figure 1. Time-averaged maximum mean velocity in the internal carotid artery and middle cerebral artery (ICA/MCA) in children and adolescents with sickle cell disease (HbSS and HbSC) ($P<0.0001$; Independent Student's T test).

Figure 2. Correlations of maximum TAMM with markers of severity in individuals with HbSS and HbSC. A: Correlation between hemoglobin and TAMM in HbSC individuals; (B) Correlation between hematocrit and TAMM in HbSC individuals; (C) Correlation between hemoglobin and TAMM in HbSS individuals; (D) Correlation between hematocrit and TAMM in HbSS individuals.

Figure 1

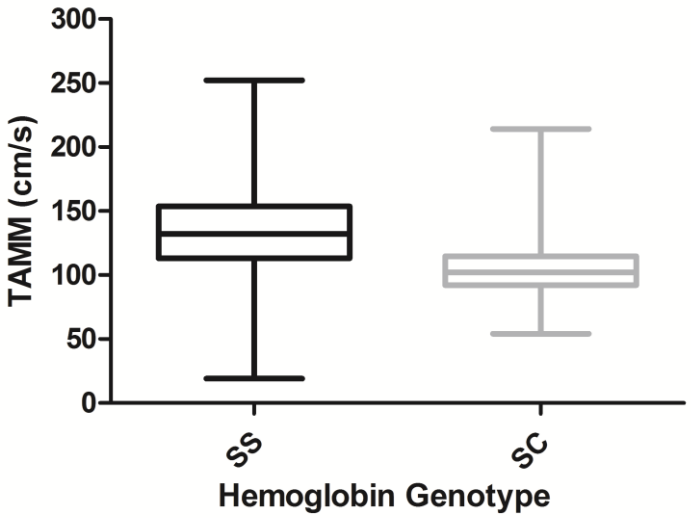

Figure 2

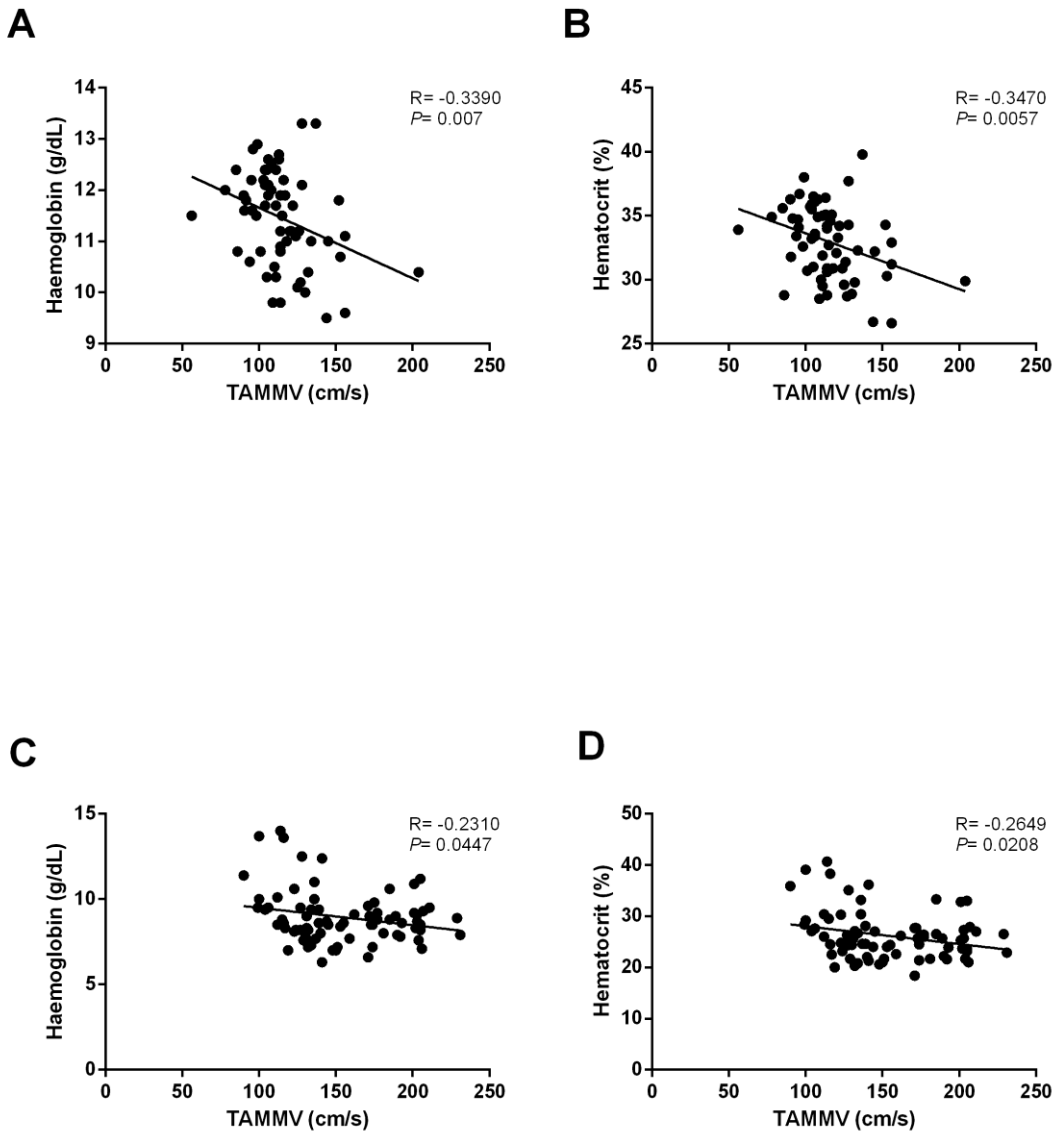
A
R= -0.3390
P= 0.007
Haemoglobin (g/dL)
TAMMV (cm/s)
B
R= -0.3470
P= 0.0057
Hematocrit (%)
TAMMV (cm/s)
C
R= -0.2310
P= 0.0447
Haemoglobin (g/dL)
TAMMV (cm/s)
D
R= -0.2649
P= 0.0208
Hematocrit (%)
TAMMV (cm/s)

Table I. Differences in Transcranial Doppler (TCD) ultrasound screening among sickle cell anemia (HbSS) and sickle cell SC disease (HbSC).

	Transcranial Doppler Result N (%)					
Genotype	Normal	Conditional	Abnormal	Inconclusive	Low	Total
	N (%)	N (%)	N (%)	N (%)	N (%)	
HbSS	832(75.2)	158(14.3)	80 (7.2)	19(1.7)	17(1.5)	1106
HbSC	536(96.1)	5(0.9)	4(0.7)	6(1.1)	7(1.3)	558
Total	1368(82.2)	163(9.8)	84(5.0)	25(1.5)	24(1.4)	1664(100)

A.2 – MANUSCRITO II

Título: Association of homocysteine and inflammatory-related molecules in sickle cell anemia

Autores: Wendell Vilas-Boas, Bruno Antonio Veloso Cerqueira, Camylla V. B. Figueiredo, Rayra Pereira Santiago, Caroline C. da Guarda, Thassila Nogueira Pitanga, Sanzio Silva Santana, Angela Maria Dias Zanette, Marilda de Souza Gonçalves.

Situação: Publicado na *Hematology*. DOI: http://dx.doi.org/10.1179/1607845415Y.0000000048

Objetivo:

O objetivo do estudo foi investigar o papel da homocisteína (Hci), citocinas relacionadas às células Th17, moléculas de adesão, e o estado inflamatório observado em indivíduos com anemia falciforme (AF).

Principais resultados:

Nós encontramos associações significativas entre os níveis de Hci e expressão elevada de interleucina-17 (IL-17) e fator de crescimento transformador-beta (TGF-β) entre os indivíduos, e uma correlação significativa e positiva entre Hci e molécula de adesão celular vascular solúvel (sVCAM). Indivíduos com AF tiveram elevados níveis de IL-17 quando comparados com indivíduos controles.

Association of homocysteine and inflammatory-related molecules in sickle cell anemia

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Objective: Investigate the role of homocysteine (Hcy), Th17-related cytokines, and adhesion molecules in the inflammatory state seen in the sickle cell anemia (SCA).
Methods: We studied the Hcy, interleukin (IL)-17, and transforming growth factor β (TGF-β) cytokine levels of 62 SCA patients, as well as the expression levels of inflammatory and endothelial activation markers.
Results: We found significant associations between Hcy levels and increased expression of IL-17 and TGF-β among SCA patients, and a positive significant correlation between Hcy and soluble vascular cellular adhesion molecules (sVCAM). SCA individuals had raised IL-17 levels when compared with controls.
Discussion: These results suggest a possible role of Hyc in the induction of TGF-β and IL-17. Other authors proposed that Hcy may contribute to the initiation and progression of vascular disease by monocyte activation, resulting in the secretion of cytokines that amplify the inflammatory response. The role of Hcy in cytokine production and oxidative stress in the endothelium may explain the increase of sVCAM expression and, the vascular activation currently described among the SCA individuals with the highest Hcy serum levels. The chronic inflammation was observed in hyperhomocysteinemic mice, with an increased expression of VCAM-1 and plasma levels of tumor necrosis factor-alpha, showing an association of this inflammatory molecule and vascular changes.
Conclusion: Our findings suggest that the increased levels of IL-17,Hcy and sVCAM contributes contributes to the vascular inflammation and activation presented by SCA patients, which probably have an important role in vaso-occlusion. On the basis of the presented data, IL-17 and Hcy might be considered as important components in the pathogenesis of SCA.

Keywords: Sickle cell anemia, Cytokines, Soluble adhesion molecules, Homocysteine, IL-17

Introduction

Sickle cell anemia (SCA) is associated with a pro-inflammatory state, characterized by an elevated baseline leukocyte count, known to be correlated with several specific complications and mortality of the disease, and by increased plasma levels of pro-inflammatory cytokines, such as interleukin (IL)-6 and IL-8.[1–4] Inflammation, white blood cell adhesion to vascular endothelium, and consequent endothelial damage contribute to SCA pathogenesis.[5,6] The chronic inflammatory state, which is different from one patient to another, may partly account for the variability of the disease expression.

Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are cytokine-inducible, single-chain glycoproteins belonging to the immunoglobulin superfamily. The ICAM-1 is constitutively expressed on vascular endothelium and cells of the immune system and is upregulated in response to various stimuli, including cytokines synthesized during inflammation.[7,8]

The VCAM-1 is present in activated endothelial cells after stimulation with cytokines, such as IL-1β, tumor necrosis factor-alpha (TNF-α), and IL-4.[9,10] The VCAM-1 serves as a counter receptor for integrin very late activation antigen-4 (α4β1), and is important

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in the recruitment of leukocytes to inflammatory sites.[11] The VCAM-1 also participates in the cellular interactions involved in lymphocyte activation, and both ICAM-1 and VCAM-1 also can be found as soluble forms circulating in plasma although the physiological roles of these soluble cell adhesion molecules are not yet clearly known.[12–15]

The IL-17 is a pro-inflammatory T cell-derived cytokine with numerous biological actions. It has been reported that IL-17 plays an essential role in microbial host protection by connecting lymphoid and myeloid host defenses.[16] Although considered to be dependent on IL-23 for differentiation from naive T cells into Th17 cells, current work has shown that initial differentiation is dependent on transforming growth factor beta (TGF-β), with subsequent Th17 lineage expansion dependent on IL-23.[17] The role of the Th17 response in vascular cell alterations is not well known.

The homocysteine (Hcy), a sulfur-containing amino acid, is found at low concentration in blood and cells and is an important intermediate molecule involved in the biosynthesis of methionine and cysteine.[18] The high plasma concentration of Hcy is a well-established risk factor for several disorders including cardiovascular disease, stroke, venous thrombosis, and arteriosclerosis.[19,20]

The hyperhomocysteinemia has an important role in vascular disorders and may act through increasing cytotoxic activity, especially for endothelial cells; elevating hydrogen peroxide (H_2O_2) levels and decreasing nitric oxide (NO) synthesis, inducing cytokine production to stimulate the inflammatory state, activation of procoagulant factors, and dysregulation of lipid metabolism via oxidative modification of low-density lipoprotein cholesterol with enhanced atherogenesis. Higher levels of Hcy have also been implicated in causing changes in the rheological properties of blood, such as decreasing antithrombin III and tissue plasminogen activator, and increasing factor VII and C-protein.[21,22] Additionally, Hcy has been associated with enhanced interaction between endothelial cells and leukocytes.[23]

The possibility that Hcy may contribute to the ischemic phenomena present in SCA has attracted some interest in total plasma Hcy. Lowenthal *et al.*[24] showed that the median plasma concentration of Hcy among SCA subjects was 1.5-fold higher than that of controls. Additionally, sickle cell disease (SCD) patients have higher plasma concentrations of Hcy in spite of higher plasma folate levels and vitamin B_{12} concentrations compared with those observed in healthy individuals.

Since SCA patients are prone to ischemic complications, the search for mechanism is particularly challenging because the pathogenesis of vaso-occlusion involves several molecules and processes across multiple length and timescales, including polymerization and melting of hemoglobin, vascular inflammation, adhesion molecules, cytokines, and still unclear factors such as the contribution of Hcy. We hypothesized that the plasma Hcy may contribute to the increased endothelial activation and inflammatory states seen in SCA individuals, and we performed a study to assess serum concentrations of Hcy, inflammatory markers, such as IL-18, IL-23, IL-17, TGF-β, and soluble adhesion molecules (sICAM and sVCAM) in SCA patients.

Materials and methods

Subjects

We studied 62 patients (28 men and 34 women; mean age of 21 ± 14) from Northeast, Bahia, Brazil who were diagnosed with SCA and were followed at the hematology ambulatory from the Fundação de Hematologia e Hemoterapia do estado da Bahia (HEMOBA). All patients maintained a steady-state, transfusion-free treatment course with the use of 1 mg of oral folate supplementation. Patients had no other systemic diseases that could have potentially altered their inflammatory functions. Blood samples were obtained during regular clinical visits. The study was approved by the Fundação Oswaldo Cruz's Human Research Board and is in accordance with the Declaration of Helsinki of 1975, as revised in 2000. All subjects signed informed consent forms. Forty age-matched control individuals were included for the IL-17 analysis.

Biochemical analyses of Hcy

Serum concentrations of Hcy were measured by a chemiluminescence immunoassay using the automatic device IMMULITE 2000 (Diagnostic Products Corporation, Los Angeles, CA, USA), according to the manufacturer's instructions.

Cytokine and soluble adhesion molecule measurements

The IL-18, IL-23, IL-7, and TGF-β serum levels were measured using Cytokine ELISA OptEIA kits (BD Pharmingen, San Diego, CA, USA) and soluble adhesion molecules (sICAM and sVCAM) were characterized using ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's recommendations.

Statistical analysis

Baseline characteristics were summarized as proportions and means of selected variables. The Kolmogorov–Smirnov test was performed to determine the distribution of quantitative variables. Bivariate correlation analyses were carried out to verify correlations between pairs of variables using Pearson's or Spearman's rank correlations (r) and two-tailed test was used. The non-parametric

Table 1 Steady-state hematologic markers, soluble adhesion molecules, and cytokine values of the SCA patient group

	Mean	SD
Hematologic markers		
RBC ($\times 10^6$/μl)	2.69	0.67
Hemoglobin (g/dl)	8.07	1.60
Hematocrit (%)	24.42	5.26
Platelets ($\times 10^3$/μl)	368	178
Leukocytes (10^6/ml)	10.9	3.7
Reticulocytes count (%)	6.9	3.1
Cytokines		
IL-18 (pg/ml)	3556.81	1279
TGF-β (pg/ml)	1588.78	430.88
IL-23(pg/ml)	101.52	180.47
IL-17(pg/ml)	3.9	7.8
Soluble adhesion molecules		
sICAM (ng/ml)	393.37	95.74
sVCAM (ng/ml)	516.06	226.94

SD, standard deviation.

Kruskal–Wallis test was used to compare two or more groups as measured by interval variables. Test analyses were considered significant if *P*-values obtained were less than 0.05. Data analyses were conducted using the software programs STATA 10 (StataCorp, TX, USA) and GraphPad Prism 5 (Graphpad Software, San Diego, CA, USA).

Results

Data of hematological markers, soluble adhesion molecules, and cytokine of SCA patients in steady-state are represented in Table 1.

Hcy concentration, cytokine profile, and adhesion molecules

The median of Hcy concentration in the 62 SCA patients was 7.3 μmol/l (±3.3 μmol/l). Considering the classification commonly used for the degree of homocysteinemia as normal (5–14 μmol/l) and hyperhomocysteinemia as mild (15–30 μmol/l), moderate (31–100 μmol/l), and severe (>100 μmol/l), hyperhomocysteinemia was not found among the studied patient group.[13] The association between cytokines and Hcy serum levels revealed a significant positive association between IL-17, quantified in 32/62 SCA patients, and TGF-β quantified in 13/62 SCA patients ($P = 0.019$, $r = 0.38$ and $P = 0.027$, $r = 0.610$, respectively). However, the IL-18 (13/62) and IL-23 (13/62) levels were not associated with Hcy concentration in this group (Fig. 1).

To assess the hypothesis that Hcy is related to vascular activation, analysis was performed with soluble adhesion molecules. The association between soluble vascular and intercellular adhesion molecules (sVCAM and sICAM) with Hcy showed a significant positive correlation with sVCAM ($P = 0.014$, $r = 0.396$), but not with sICAM (Fig. 2). When we considered the mean concentrations of Hcy in the 50th and 75th percentiles as a cutoff point, we can also report a statistically significant association between the sVCAM concentration and Hcy levels in these patients ($P = 0.021$, $\chi^2 = 5.28$ and $P = 0.031$, $\chi^2 = 4.64$, respectively).

Analysis of IL-17 levels in the 25th and 75th percentiles of Hcy, in the SCA patients, confirms the positive correlation between them ($P = 0.047$) (Fig. 3).

IL-17 levels are increased in SCA patient group

SCA patients presented raised levels of IL-17 cytokine compared with control group ($P = 0.011$) (Fig. 4).

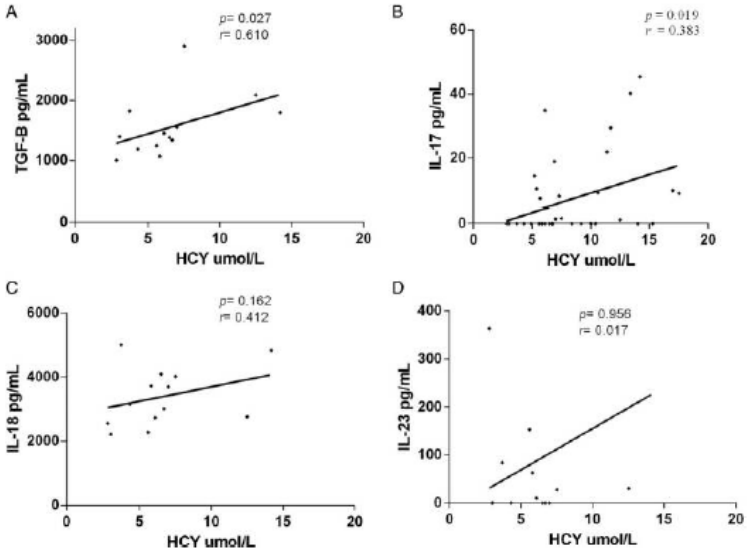

Figure 1 Hcy levels and correlation with TGF-β, IL-17, IL-18, and IL-23 cytokines in SCA patients.

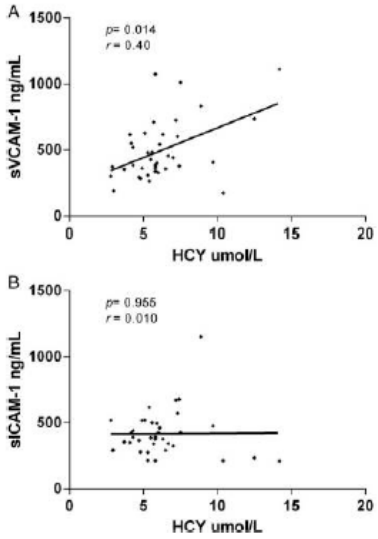

Figure 2 Hcy levels and correlation with soluble adhesion molecules in SCA patients.

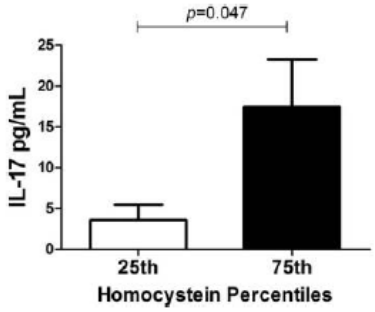

Figure 3 Hcy percentiles and levels of IL-17 in SCA individuals.

Hcy concentration, cytokines, or adhesion molecules with any clinical manifestations

We performed the analyses of Hcy, adhesion molecules, and cytokine data associated with patient's clinical profile and there were not significant results. Interestingly, we noted that one patient who had acute chest syndrome (ACS) also had high-Hcy level (14 µmol/l) compared with mean Hcy in the group (7.3 µmol/l).

Discussion

A chronic inflammatory state in vascular tissue is recognized to contribute to thrombotic and vaso-occlusive events in SCA. Since Hcy has been associated with vascular complications in the pathology of other diseases, it may contribute to the vascular complications presented by patients with SCD.[24]

In our study, we investigated Hcy levels and markers of vascular activation, including cytokines and soluble

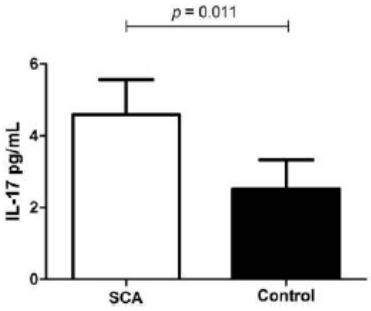

Figure 4 Levels of IL-17 in SCA individuals and controls.

adhesion molecules in SCA patients. Our results related to Hcy analyses showed that the studied group did not present increased levels of Hcy (7.3 µmol/l) when compared with previous results from Lowenthal *et al.*[24] that found a mean concentration of 12.6 µmol/l among SCD patients, or when compared with the common classification criterion for hyperhomocysteinemia (>14 µmol/l).[25] However, Balasa *et al.*[26] found Hcy levels even lower (5.8 µmol/l) in SCA patients and there was no difference between controls. Despite the use of folic acid by our patients, this supplementation did not influence Hcy levels, confirm previous report about no association of hyperhomocysteinemia and folic acid supplementation among SCA patients.[27] In this work, We did not realize Hcy dosage in the control group to compare with Hcy levels in patients with SCA, but previous work by our team carried out a cross-sectional study comprising 143 healthy neonates, from the same population, that showed Hcy mean levels about 7.4 µmol/l in this group, showing that in the SCA group investigates, the Hcy levels were not elevated when compared with individuals from the same population.[28]

The effect of Hcy on cytokine production is not well understood. We found a positive association among Hcy and IL-17 and TGF-β. The IL-17 promotes inflammation by induction of various pro-inflammatory cytokines and chemokines, recruitment of neutrophils, enhancement of antibody production, and activation of T cells.[29] The TGF-β is a pleiotropic cytokine involved in several human diseases, including cardiovascular disease, and also can induce IL-17 production.[30] Our results suggest a possible role of Hcy in the induction of TGF-β and IL-17. This hypothesis is supported by Su *et al.*, [31] who proposed that Hcy may contribute to the initiation and progression of vascular disease by monocyte activation, resulting in the secretion of cytokines that amplify the inflammatory response. We also found an elevated basal level of IL-17 in SCA patients when compared with controls, representing the chronic inflammatory state in these individuals.

A new field of research in SCA is rising on Th17 cells. These IL-17 producing cells are involved in the inflammatory response and a little information is available regarding studies in SCA. Thus, it becomes important to investigate the role of cytokines in endothelial dysfunction and activation, once the role of other molecules, such as IL-1 and TNF-α, is yet not very established. Our team has previously demonstrated a significant and positive association of TGF-β and circulating arginase levels in SCA patients, showing a vascular relevance of this cytokine and the need to clarify the role of these molecules in SCA.[32]

We found lower levels of IL-17 and TGF-β in this study when compared with previous reports.[21,22] These discrepancies can be due to genetic differences in the studied populations. More importantly, the IL-17 control group in this work is age-matched and from the same population as the SCA patients. Many patients and controls had undetectable IL-17, so levels of this cytokine appear to be usually very low.

We believe that the role of Hcy in cytokine production and oxidative stress in the endothelium may explain our findings related to the increase of sVCAM expression and, consequently, with the vascular activation currently described among the SCA individuals with the highest Hcy serum levels. In agreement with our results, Hofmann *et al.*[33] observed evidence of chronic inflammation in hyperhomocysteinemic mice, including an increased expression of VCAM-1 and elevated plasma levels of TNF-α, showing an association of this inflammatory molecule and vascular changes. Hcy could be considered an emerging potential target of inflammation in SCA and it may be significant in the investigation of vaso-occlusion. The ACS is a combination of radiographic evidence of new pulmonary infiltrates and respiratory symptoms, and is a frequent cause of hospitalization in SCA, also, it likely involves alterations of normal homeostatic functions of vascular endothelium in the lungs.[34] We did not find other studies that related to our finding of increased Hcy in ACS, indicating the importance of further investigation of this potential link.

We conclude that the combination of IL-17, Hcy, and sVCAM may be involved in vascular damage in SCA and this might be implicated in the disease pathogenesis. Additional studies including SCA patients under vaso-occlusive crises are warranted in order to confirm our findings and show the behavior of these molecules in crisis condition. More importantly, we suggest the development of a longitudinal study in order to answer more accurately the influence of these molecules on vascular damage in SCA.

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Disclaimer statements

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Conflicts of interest The authors stated that there are no conflicts of interest regarding the publication of this article.

Ethics approval The study was approved by the Fundação Oswaldo Cruz's Human Research Board and is in accordance with the Declaration of Helsinki of 1975, as revised in 2000. All subjects signed informed consent forms.

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A.3 – MANUSCRITO III

Título: Endothelial Nitric Oxide Synthase (-786T>C) and endothelin-1 (5665G>T) gene polymorphisms as a vascular dysfunction risk factors in sickle cell anemia

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Situação: Aceito com alterações pela revista Gene Regulation and Systems Biology

Objetivo: O objetivo do estudo foi investigar os polimorfismos ET-1 5665G>T e eNOS -786T>C, níveis de moléculas de adesão solúveis (sVCAM-1 e sICAM-1), marcadores bioquímicos e quadro clínico dos indivíduos com AF.

Principais resultados: Nossos resultados indicaram que os indivíduos com AF que carregavam o alelo recessivo do polimorfismo em eNOS (C) tiveram os maiores níveis de sVCAM (p=0,028). Encontramos uma maior ocorrência de síndrome torácica aguda (STA) nos portadores do alelo recessivo da ET-1 5665G>T. A análise multivariada confirmou a influência do gene ET-1 na STA e a associação do polimorfismo em eNOS e ocorrência de infecções.

Endothelial Nitric Oxide Synthase (-786T>C) and *endothelin-1* (5665G>T) gene polymorphisms as a vascular dysfunction risk factors in sickle cell anemia

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Gene Regulation and Systems Biology

We believe that our manuscript is suitable to be published on the Gene Regulation and Systems Biology because we confirm the information about the association of the minor allele of the *ET-1* 5665G>T gene polymorphism and acute chest syndrome and brought information about the *eNOS* -786T>C minor allele (C) association with the highest levels of sVCAM-1 among sickle cell anemia (SCA) patients. We also access new data about both gene polymorphisms throughout the multivariate analysis, suggesting that these gene polymorphisms may contribute to modify important systemic markers related to the SCA outcome and, probably, with mechanisms involved in leukocyte activation, endothelial dysfunction and vascular occlusion in SCA.

Abstract

Sickle cell anemia (SCA) patients have vascular complications, and polymorphisms in Endothelin-1 (*ET-1*) and Nitric oxide (*eNOS*) genes were associated with ET-1 and Nitric oxide disturbance. We investigate the association of *ET-1* 5665G>T and *eNOS* -786T>C polymorphisms with soluble adhesion molecules (sVCAM-1 and sICAM-1), biochemical markers and medical history. We studied 101 SCA patients, and carriers of *eNOS* minor allele (C) had the highest levels of sVCAM-1, and *ET-1* minor allele carriers had more occurrence of acute chest syndrome (ACS). The multivariate analysis suggested the influence of the *ET-1* gene on ACS outcome and an association of the *eNOS* gene with upper respiratory tract infection. We suggest that *eNOS* and *ET-1* gene polymorphisms can influence SCA pathophysiology, and that *eNOS* variant in SCA patients might be important to NO activity and vascular alteration. We found an association of the *ET-1* minor allele in ACS and, showing the importance of genetic screening in SCA.

Keywords: Sickle cell anemia; eNOS; Entothelin-1; Gene polymorphisms.

Introduction

A single amino acid substitution in the hemoglobin (Hb) molecule is the molecular basis for sickle cell anemia (SCA). However, the disease clinical evolution is heterogeneous and involves multiple factors. The SCA is a vascular disease, and it is already known that genetic differences associated to endothelial function contribute to its phenotypic diversity[1].

Endothelin-1 (ET-1) and nitric oxide (NO) are endothelium-derived mediators essential for maintaining vascular homeostasis. The correct balance between NO and ET-1 production seems to be essential in preventing vascular endothelial dysfunction[2,3].

The endothelin (ET) is an endothelium-derived molecule and an important vasoconstrictor. Among the three isoforms of ET, ET-1 is the only isoform produced by endothelial cells. Various stimuli such as thrombin, inflammatory mediators and hypoxia increase ET-1 levels that plays a pivotal role in vascular function regulation and acts through the smooth muscle producing vasoconstriction, cell growth and cell adhesion[2,3]. Because of the role of ET-1 in vascular pathophysiology, polymorphic gene coding ET-1 increase vascular reactivity in several vascular disorders. A single nucleotide polymorphism (SNP) in the *ET-1* gene involving a G-to-T replacement at nucleotide 5665 in exon 5 was correlated to increased susceptibility of acute chest syndrome (ACS) in SCA individuals[4].

The NO is synthesized by a family of NO synthase (NOS) and the dominant NOS isoform in the vasculature is the endothelial NOS (eNOS), an enzyme that can metabolize L-arginine and generate NO[5,6]. The NO plays an important role in the pathogenesis of several diseases such as SCA, and has vasodilator and anti-thrombogenic properties that if impaired can contribute to the vasoconstriction that coupled with adhesion of circulating cells may lead to occlusion of micro vessels[5,7]. The *eNOS* polymorphic variant -786 T>C is associated with decreased NO production

because of the reduction of *eNOS* gene expression and consequently the molecule activity. This condition results in vasoconstriction, platelet aggregation and thrombosis[3,8]. The reduced or impaired NO production may results in endothelial cell activation and up regulation of adhesion molecules. Thus, shedding of soluble adhesion molecules into blood plasma can serve as markers either of endothelial dysfunction or of inflammation, with endothelial activation, a clinical situation present in SCA individuals[6,8]. Recent studies have suggested the importance of several SNPs, including the *eNOS* and *ET-1* genes, as risks markers for stroke, leg ulceration, pulmonary hypertension, priapism and osteonecrosis in sickle cell disease patients[10].

The aim of this study was to investigate the *eNOS* -786T>C (rs2070744) and *ET-1* 5665G>T (rs5370) gene polymorphisms in SCA individuals and controls associating their presence with levels of soluble intercellular adhesion molecule 1 (sICAM-1) and soluble vascular cell adhesion molecule 1 (sVCAM-1), biochemical markers and medical history.

Methods

Subjects

We studied 101 SCA patients (mean age of 15.6 ± 12.11) from Northeast Brazil attending the outpatient clinic of the Fundação de Hematologia e Hemoterapia da Bahia (HEMOBA). All SCA patients were in the steady state of the disease that was characterized as a time of three months without any acute clinical events and without using blood therapy 4 months prior to blood sampling. As exclusion criteria were considered the presence of infectious diseases, Hb profiles other than SCA, and inflammatory episodes during the blood collection. Determination of *eNOS* polymorphism was possible in 60 of these patients, due to sample availability. One hundred and eight healthy Brazilian subjects with normal Hb profiles were included as a control group for *ET-1* polymorphism and 81 for *eNOS* polymorphism.

This study was approved by the Centro de Pesquisas Gonçalo Moniz da Fundação Oswaldo Cruz (FIOCRUZ) Research Board, and all patients and their guardians provided written informed consent, in accordance with the Declaration of Helsinki of 1975, and its revisions. Clinical information was collected from the patients's records.

Polymorphisms genotyping

The *ET-1* 5665G>T (rs5370) and *eNOS* -786T>C (rs2070744) gene polymorphisms were investigated by the polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLP) techniques as previously described[8,11].

Soluble adhesion molecule measurements

Soluble adhesion molecules sICAM-1and sVCAM were estimated using ELISA Kits (R&D Systems, Minneapolis, USA), according to the manufacturer's recommendations.

Biochemical and hematological analyses

Serum concentrations of bilirubin, lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol and fractions, and triglyceride levels, and C-reactive protein (CRP) were determined using commercially available biochemical kits (LABTEST, Minas Gerais, Brazil). Electronic cell counter Coulter (Coulter Corporation, FL, USA) was used to quantify hematological parameters. Hemoglobin (Hb) pattern and its concentration were estimated by high performance liquid chromatography (HPLC) (BIO-RAD, CA, USA).

Statistical Analysis

Baseline characteristics were summarized as proportions and means of selected variables. The Kolmogorov-Smirnov test determines the distribution of quantitative variables. The Spearman's rank correlation coefficient measures the strength of a linear relationship between paired data. Non-parametric tests of Mann-Whitney and Kruskal-Wallis compare two or more groups of *ET-1* and *eNOS* alleles, and sVCAM-1 and sICAM-1 levels measured as quantitative variables. The Chi Square statistic test compares the tallies of categorical variables between two independent groups. Multivariate analyses were performed to show a possible interaction of *ET-1* 5665G>T gene polymorphism, sVCAM-1 and LDH levels as a risk factor on ACS outcome, and of *eNOS* -786T>C gene polymorphisms, white blood cell (WBC), LDH and CRP on infection outcome. Tests analyses were significant if p values were less than 0.05. Data analyses were conducted using the software programs STATA 10 (StataCorp, Texas, USA) and GraphPad Prism 5 (GraphPad Software, San Diego, CA).

Results

Polymorphisms frequencies

The *ET-1* 5665G>T polymorphism was analyzed in 101 SCA patients and 108 healthy individuals, while the *eNOS* -786T>C was investigated in 60 SCA patients and 81 healthy controls. Our results showed frequencies of 66.3% (67/101) for wild-type genotype (GG), 33.6% (34/101) of heterozygous (GT) and 2.9% (3/101) of homozygous for the variant allele (TT) of *ET-1* 5665G>T gene polymorphism in SCA patients (Table 1). The *eNOS* -786T>C gene polymorphism analysis showed 56.7% (34/60) for wild-type genotype (TT), 36.7% (22/60) of heterozygous (TC) and 6.5% (4/60) of homozygous for the variant allele (CC) in SCA patients (Table 1). Both polymorphisms were in Hardy-Weinberg equilibrium. In the control group, the frequency of *ET-1* 5665G>T gene polymorphism was 60.2% (65/108) for wild-type genotype, 32.4% (35/108) of heterozygous and 7.4% (8/108) of homozygous for variant allele (Table 1). The frequency of *eNOS* -786T>C was 54.3% (44/81) for wild-type genotype, 42% (34/81) of heterozygous and 3.7% (3/81) of homozygous for variant allele (Table 1).

Adhesion molecules and polymorphisms

We associated gene polymorphisms and serum levels of soluble adhesion molecules (sVCAM-1 and sICAM-1), and found that patients' carriers of the minor allele of *eNOS* gene polymorphism had the highest levels of sVCAM-1. The Table 2 shows the genotypes of *ET-1* and *eNOS* gene polymorphisms and means of serum levels of the studied soluble adhesion molecules.

Polymorphism and clinical data

Genotypes frequencies were compared between SCA patients with and without clinical events. The Table 3 summarizes the association between clinical data and presence of polymorphisms in

ET-1 and *eNOS* genes. When the allele frequencies were evaluated we found an association of the ACS in patients carries of the minor allele of the *ET-1* 5665G>T ($p< 0.001$) (Figure 1). We emphasize that in the total of SCA patients group included in the present study, be carrier of the minor allele T of the *ET-1* 5665G>T was associated with ACS. However, we did not find this association among the carrier of the variant genotype TT.

Biochemical data

Biochemical data were assessed in SCA patients. Analyses of the 51 SCA show that patients in percentile 25 % and 75 % showed an association with presence of the minor allele *ET-1* 5665G>T and levels of direct bilirubin and total cholesterol. Patients carrying the minor allele T had higher direct bilirubin (≥ 0.4 mg/dL) ($p= 0.021$, Fisher's exact test), as well as a higher concentration of total cholesterol (≥ 169.7 mg/dL) ($p= 0.03$, Fisher's exact test). Patients carrying the minor allele (T) had higher levels of direct bilirubin (≥ 0.4 mg/dL) ($p= 0.012$, unpaired t test) (Figure 2). Others biochemical data did not show differences with gene polymorphisms, including the *eNOS* gene polymorphism. However, sVCAM-1 was negatively correlated to total cholesterol levels ($p= 0.027$, $r= -0.243$) and ALT levels ($p= 0.005$, $r= -0.307$) (Figure 3).

Multivariate analysis

The multivariate analysis approach model investigate the interaction of the *ET-1* 5665G>T gene polymorphism, sVCAM-1, and LDH levels on ACS outcome (Table 4), and of the *eNOS* -786T>C gene polymorphism, WBC count, and LDH and CRP levels on upper respiratory tract infection (Table 5).

Discussion

This study shows a new interesting result regarding the association of *eNOS* -786T>C gene polymorphism and sVCAM-1 levels. Sickle cell anemia patient`s carrier of the minor allele (C) had higher sVCAM-1 levels, suggesting a contribution of this polymorphism on vascular inflammation. Based on the information that *eNOS* polymorphic variant is related to decreased NO production because of the reduction of gene promoter activity[12,13], decreasing NO production in the minor allele carries (C) is supposed to up-regulate vascular adhesion molecules (as sVCAM-1), the anti-inflammatory role of NO on vascular environment and, consequently, increase the endothelial damage. It is known that NO inhibits platelet activation and the expression of endothelial adhesion molecules, thus, participating in healthy endothelial function and the maintenance of blood flow[14,15].

These results suggest a role of these molecules on SCD mechanism. In addition to endothelial dysfunction, SCA patients also has a decrease of vasodilators responses to NO donors such as sodium nitroprusside or nitroglycerin[16,17], molecules that promote vascular smooth muscle relaxation. This phenomenon is related to vascular cell dysfunction and NO resistance where a portion of exogenous NO is scavenged by reactive oxygen species or free serum heme before it can stimulate vascular smooth muscle[18]. The *eNOS* -786T>C minor allele can be associated with enhancement of the NO resistance state in the SCA individuals[3,8].

Also, in the current work, the *ET-1* 5665G>T minor allele was associated with the occurrence of ACS in SCA patients, confirming previous results[4]. The ACS is a combination of radiographic evidence of new pulmonary infiltrates and respiratory symptoms, and is a frequent cause of hospitalization in SCA patients[19]. Pathophysiology of events leading to ACS progress in SCA were not determined but were considered similar to those observed in other organ systems. The ACS likely involves alterations of normal homeostatic functions of vascular endothelium in the

lungs[20]. In addition to adherence, interaction of plasma factors and/or sickle red blood cells (RBCs) with endothelial cells may modify endothelial production of vasoactive mediators[2]. The plasma ET-1 levels were clearly elevated during the initial period of ACS and decreased by third day of hospitalization[21]. This suggests a contribution of ET-1 on ACS events probably by deregulating the mediators balance because of *ET-1* 5665G>T minor allele presence, once this gene polymorphism is related to abnormal vascular reactivity and ET-1 plasma levels[12,22]. In the present study, we found the association of the minor allele of *ET-1* 5665G>T with ACS, but not the homozygous state of the minor allele, and we emphasize that further study including a higher number of SCA patients is necessary to confirm the association with these genotype as high-risk of ACS among these patients.

It was suggested that an imbalance between ET-1 and NO may contribute to changes in endothelial tone observed in the SCA[3] and, consequently, the presence of those polymorphisms can break such balance by abnormal expression or activity of these mediators contributing to the vascular impairment.

In this study we found negative significant correlation of sVCAM-1 and total cholesterol and ALT. Low total cholesterol levels was associated to the severity of hypertension, intracerebral hemorrhage, followed by the magnetic resonance imaging changes[23]; also, a decrease in high-density lipoprotein cholesterol (HDL-c), which may have influence in the total cholesterol levels, have been related as an independent marker of endothelial activation, and also with the increase of inflammatory and oxidative stress molecules, such as sVCAM-1[24]. The negative correlation with ALT levels may suggest that the increase of sVCAM-1 in this studied SCA group was not associated with hepatocellular damage[25].

Our results of multivariate analysis described a possible influence of the *ET-1* gene in ACS outcome, and the association of *eNOS* gene with upper respiratory tract infection, showing a

pivotal role of vascular mediators, like ET-1 and NO, in SCA pathophysiology, and also an interaction of the investigated gene with molecules and cells commonly involved in hemolytic and inflammatory process. Further studies will clarify the role of *ET-1* and *eNOS* gene polymorphisms and will advance our understanding of the altered endothelial state and clinical complications in SCA patients. It would be interesting to show whether -786C minor allele has a reduced promoter activity and eventually less eNOS transcription, and whether endothelial cells with -786C minor allele has lower levels of eNOS and eventually NO production.

Conclusion

We suggest that *eNOS* -786T>C and *ET-1* 5665G>T gene polymorphisms may participate of the SCA pathophysiology. Our data show that *eNOS* variant in SCA patients might be important to NO activity and anti-inflammatory vascular process. Also, the ACS, a major clinical feature in SCA, which leads to patient morbidity and mortality, was associated with the *ET-1* minor allele showing the importance of the screening of genetic biomarkers, and their mechanisms.

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Author Contributions

WVB carried out the polymorphism typing, statistical analysis, participated of the study design and drafted the manuscript; CVBF carried out the polymorphism typing and participated of the study design; TNP, RPS, SSS and CCG helped to draft the manuscript; MOS and AMDZ helped in the sample collection and contributed to the experimental work; BAVC performed the biochemical and statistical analyses, participated of the study design and drafted the manuscript; MSG participated in the design and coordination of the study.

Disclosures and Ethics

The study was approved by the Fundação Oswaldo Cruz's Human Research Board and is in accordance with the Declaration of Helsinki of 1975, and further revision. All subjects signed informed consent forms.

Conflict of Interests

The authors declare no conflict of interests.

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Tables

Table 1. Frequencies of *ET-1* 5665G> T and *eNOS* -786T> C gene polymorphisms among healthy individuals and SCA patients.

Polymorphism	***Genotype***	***Frequencies*** ***Healthy individuals (%)***	***Frequencies*** ***SCA patients (%)***
***ET-1* 5665G>T**	GG	60.2 (65/108)	66.3 (67/101)
	GT	32.4 (35/108)	33.6 (34/101)
	TT	7.4 (8/108)	2.9 (3/101)
***eNOS* -786 T>C**	TT	54.3 (44/81)	567(34/60)
	TC	42 (34/81)	36.7 (22/60)
	CC	3.7 (3/81)	6.5 (4/60)

Table 2. Association of soluble adhesion molecules levels (sVCAM-1 and sICAM-1) and *ET-1* 5665G>T and *eNOS* -786T>C gene polymorphisms.

	N	***Mean (±SD)***	
		sVCAM-1 (ng/mL)	**sICAM-1 (ng/mL)**
ET-1	51		
Allele G	32	622.93 (±393)	425.71 (±165)
Allele T	19	574.09 (±373)	407.06 (±131)
****p* value**		0.815	0.693
eNOS	38		
Allele T	23	420.39 (±161)	439.97 (±193)
Allele C	15	584.09 (±238)	411.61 (±133)
****p* value**		**0.028**	0.906

* Mann-Whitney test; sICAM-1: soluble intercellular adhesion molecule 1; sVCAM-1: soluble vascular cell adhesion molecule 1.

Table 3. *ET-1* 5665G>T and *eNOS* -786T>C gene polymorphisms association with clinical events among SCA patients.

Clinical Data	***ET-1***			***eNOS***		
	Genotype GG	***Genotype GT and TT***	***p value***	***Genotype TT***	***Genotype TC and CC***	****p value***
Transfusion	14/21	7/21	0.580	8/15	7/15	0.689
Leg Ulcers	7/9	2/9	0.302	1/5	4/5	0.188
Acute Chest Syndrome	3/8	5/8	0.114	1/3	2/3	0.329
Splenic Sequestration	1/3	2/3	0.268	1/3	2/3	0.435
Avascular Necrosis	¾	1/4	0.569	0/1	1/1	0.376
Retinopathy	2/2	0/2	0.418	1/2	1/2	0.829
Splenectomy	1/3	2/3	0.268	1/3	2/3	0.435
Hepatomegaly	¾	1/4	0.569	0/2	2/2	0.127
Stroke	1/1	0/1	0.654	-	-	-
Osteomyelitis	2/2	0/2	0.418	2/2	0/2	0.403
Hand Foot Syndrome	1/1	0/1	0.654	1/1	0/1	0.650
Infection	10/16	6/16	0.517	6/14	8/14	0.231
Pneumonia	4/8	4/8	0.255	4/8	4/8	0.920
Cholelithiasis	6/6	0/6	0.054	2/3	1/3	0.801
Aplastic Crisis	0/1	1/1	0.346	1/1	0/1	0.650

* Chi Square statistic test, and ** Fisher exact test; *ET-1*: Endothelin-1; *eNOS*: Endothelial Nitric Oxide Synthase.

Table 4. The multivariate model of the association of *ET-1* 5665G>T gene polymorphism, sVCAM-1 and lactate dehydrogenase (LDH) levels in Acute Chest Syndrome.

Variable	B	SE	T	*P value*
Model 1				
***ET-1* 5665G>T**	0.351	0.127	2.772	**0.011**
sVCAM-1 (ng/mL)	0.058	0.122	0.472	0.641
Model 2				
***ET-1* 5665G>T**	0.358	0.126	2.843	**0.009**
sVCAM-1 (ng/mL)	0.057	0.121	0.467	0.645
LDH (U/L)	0.165	0.145	1.136	0.268

B: coefficient; SE: standard error.

Table 5. The Multivariable Model of the association of *eNOS* -786T>C gene polymorphism, white blood cells (WBC) count, lactate dehydrogenase (LDH), and C-reactive protein (CRP) levels on upper respiratory tract infection.

Variable	B	SE	T	P value
Model 1				
eNOS -786T>C	0.420	0.187	2.249	**0.037**
WBC (x 10^9/L)	0.373	0.190	1.959	0.066
Model 2				
eNOS -786T>C	0.268	0.176	1.517	0.149
WBC (x 10^9/L)	0.502	0.177	2.838	**0.012**
CRP (mg/L)	0.332	0.182	1.823	0.087
LDH (U/L)	0.443	0.209	2.125	0.05

B: coefficient; SE: standard error.

Figure legends

Figure 1. Presence of *ET-1* 5665G>T alleles and occurrence of acute chest syndrome among SCA patients.

Figure 2. SCA patients carrying the minor allele (T) of the polymorphism *ET-1* 5665G>T had higher levels of direct bilirubin (≥ 0.4 mg/dL) (p= 0.012).

Figure 3. Correlation between soluble vascular cellular adhesion molecule 1 (sVCAM-1), total cholesterol and alanine aminotransferase (ALT) among 83 SCA patients.

Figures

Figure 1

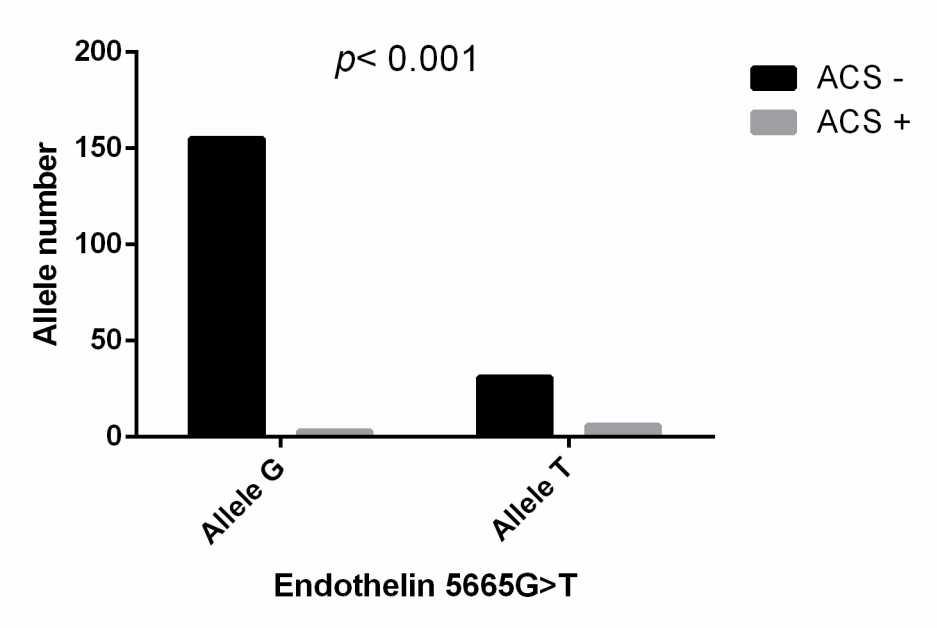


Figure 2

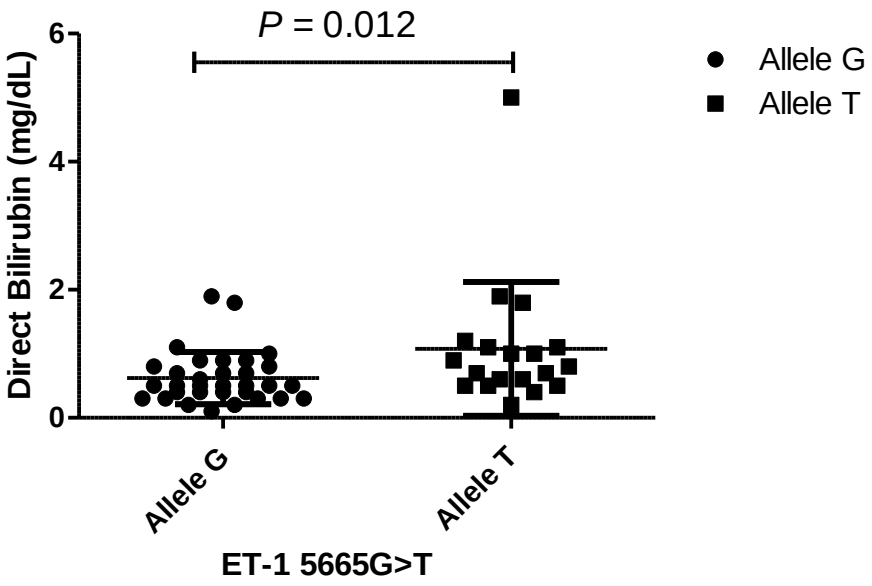
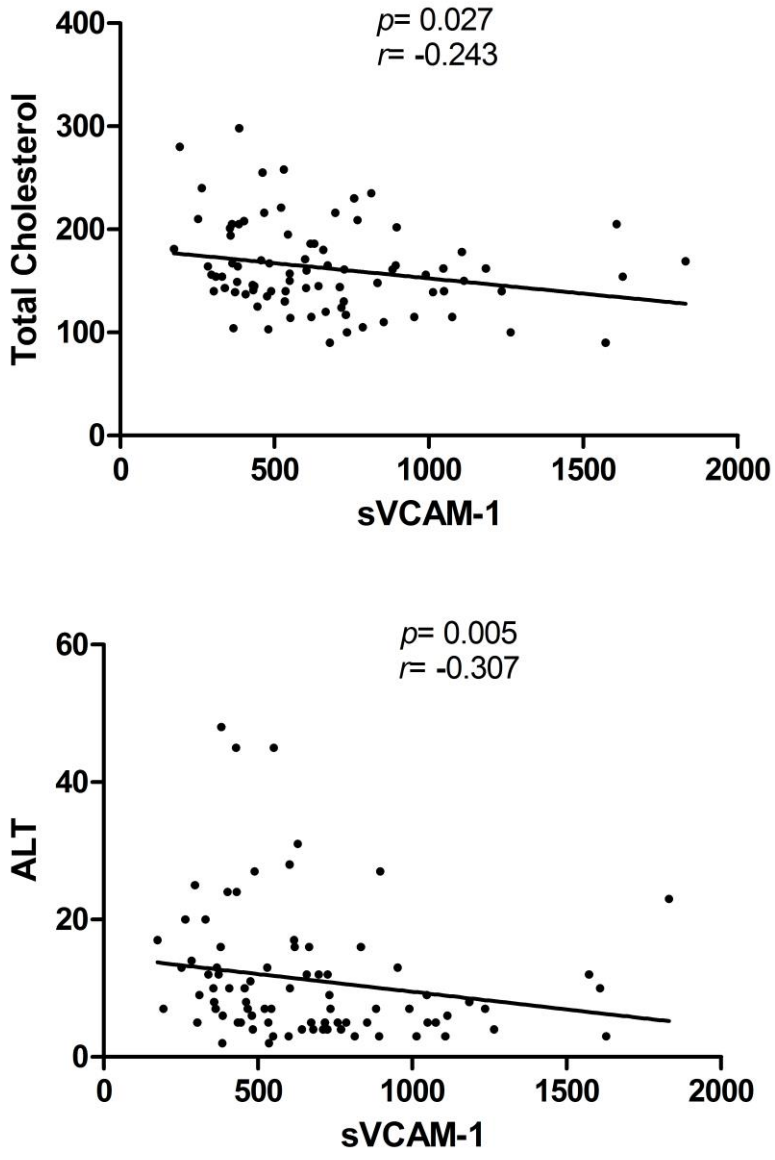

P = 0.012
Direct Bilirubin (mg/dL)
6
4
2
0
Allele G
Allele T
Allele G
Allele T
ET-1 5665G>T

Figure 3


p= 0.027
r= -0.243
Total Cholesterol
400
300
200
100
0
0
500
1000
1500
2000
sVCAM-1
p= 0.005
r= -0.307
ALT
60
40
20
0
0
500
1000
1500
2000
sVCAM-1

B - TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

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TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Identificação de Pacientes Portadores de doença falciforme com risco aumentado para o desenvolvimento de acidente vascular cerebral através da realização do Doppler Transcraniano e perfil clínico associado.

Durante a leitura do documento abaixo, fui informado (a) que posso interromper para fazer qualquer pergunta, com objetivo de tirar dúvidas, para o meu melhor esclarecimento.

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Eu,....., fui procurado (a) pela Dra. Isa Menezes Lyra (CRM-BA 9567) e Dr. Camilo Vieira (CRM-BA 16.549), sobre o Projeto de pesquisa com o título acima citado realizado pelo Serviço de Hematologia Pediátrica do CPPHO/ UFBA.

Nesse estudo, o (a) **MENOR**....., de ..... anos de idade, portador de doença falciforme sob a minha inteira responsabilidade) foi selecionado por comparecer para a realização do doppler transcraniano. Os médicos supracitados me explicaram que serão coletadas informações relacionadas à doença do (a) menor sob a minha responsabilidade, para tentar identificar alguns aspectos importantes na evolução clínica da doença.

Outra explicação dada pelos referidos médicos foi que o projeto de pesquisa, caso haja permissão para participar, consta da colheita de 5 a 10 ml de sangue para realização de exames laboratoriais para tentar identificar alguns aspectos importantes da doença que poderão beneficiar, não somente o (a) menor do qual sou responsável, como também toda a população portadora desta doença, caso os resultados sejam significantes ou não. O sangue será colhido em uma veia do braço, através de seringa e agulha esterilizadas e que depois do uso serão descartadas em caixas coletoras específicas de material perfuro cortante. Também foi esclarecido que a sensação de desconforto durante o ato de colher o sangue na veia do braço varia de pessoa para pessoa e a criança pequena pode chorar. Pode haver a formação de um hematoma (ou "calombo de sangue") no local de retirada do sangue no braço, devido ao sangue que saiu da veia, mas que esse problema é passageiro, na grande maioria das pessoas, e que pode ser resolvido colocando compressas com água gelada de quatro a seis vezes por dia.

Entendo também que eu tenho permissão para a qualquer momento revogar o meu consentimento e retirar o menor do estudo sem sofrer nenhuma punição ou perda de direitos. Entretanto, exames adicionais poderão ser solicitados, caso o médico que o assiste julgue-os necessários para a saúde e bem estar da criança. Minha recusa em permitir que meu filho ou tutelado participe do estudo não resultará em punições ou perdas de benefícios aos quais ele (a) tenha direito.

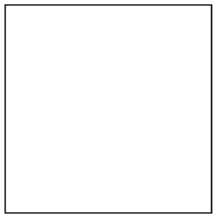
Assinatura do responsável _____

Eu presenciei a explicação acima descrita, confirmando a oportunidade concedida ao responsável de formular perguntas e testemunho a assinatura do seu responsável neste documento.
COMO TENHO DIFICULDADE PARA LER (□ sim ou não □), O ESCRITO ACIMA, ATESTO TAMBÉM QUE O (A) Dr(a).....QUANDO DA LEITURA PAUSADA DESSE DOCUMENTO, ESCLARECEU AS MINHAS DÚVIDAS E COMO TEM O MEU CONSENTIMENTO PARA PARTICIPAR DO ESTUDO, CONCORDEI COLOCAR ABAIXO A MINHA IMPRESSÃO DO DEDO POLEGAR.

Salvador, _____ de _____ de 201_

NOME: ..... Assinatura ⇒

Ou impressão digital ou datiloscópica



Testemunhas:

1. NOME: .....Assinatura .....

2. NOME: .....Assinatura .....

Assinatura do Investigador: .....

Se necessário entra em contato pelo telefone (71) 3283-8306

C - QUESTIONÁRIO

UFBA

IDENTIFICAÇÃO DE PACIENTES PORTADORES DE DOENÇA FALCIFORME COM RISCO AUMENTADO PARA O DESENVOLVIMENTO DE ACIDANTE VASCULAR CEREBRAL ATRAVÉS DA REALIZAÇÃO DO DOPPLER TRANSCRANIANO E PERFIL CLÍNICO ASSOCIADO.

FIOCRUZ

QUESTIONÁRIO PARA PACIENTES

Nome: {NOME} _____ Sigla: {sig} _____ Telefone: ( ) _____

Endereço: _____

Idade: {I} _____ Peso {P}:_____ Altura: {A} _____ Data de Nasc.: ____/____/____

Gênero: {GENER} ( ) Masculino [0] ( )Feminino [1]

Velocidade do Doppler: {VD} Esquerda:_____ Direita:_____

( ) Baixa [0] ( ) Normal [1] ( ) Condicional [2] ( ) Anormal [3]

01. Qual a sua cor? {cor} ( ) Branca[0] ( ) Negra[1] ( ) Parda[2] ( ) Amarela[3] ( ) Indígena[4]

02. Você estuda? {EST} ( ) NÃO [0] ( ) SIM [1]

03. Nível de escolaridade: {NESC} ( ) Alfabetiz.[0] ( ) Até 4 FM[1] ( ) Até 8 FM[2] ( ) Até 3 MD[3]

04. Número de irmãos: {NIRM} ( ) 0 [0] ( ) 1 [1] ( ) 2 [2] ( ) 3 [3] ( ) 4 ou + [4]

05. Familiares com DF? {FDFALC} ( ) Nenhum[0] ( ) Pai [1] ( ) Mãe [2] ( ) Irmão [3]

06. Idade primeira menstruação: {IPM} ( ) Não menst.[0] ( ) 09-11[1] ( ) 12-14 [2] ( ) 15-17 [3]

Já engravidou? {ENGRA} ( ) NÃO [0] ( ) SIM [1]

Está grávida? {GRA} ( ) NÃO [0] ( ) SIM [1]

Usa anticoncepcional? {ANTICO} ( ) NÃO [0] ( ) SIM [1]

Menstruação é regular? {MREG} ( ) NÃO [0] ( ) SIM [1]

07. Idade do 1° diagnóstico de Doença ( ) <6 m [0] ( ) 6m - 4anos [1] ( ) 5 - 9anos [2]

Falciforme: {ID} ( ) 10 - 14anos [3] ( ) 15 - 17anos [4]

08. Já esteve internado? {INTER} ( ) NÃO [0] ( ) SIM [1]

Se SIM, quantas vezes? {QINTER} ( ) 1 [0] ( ) 2-5 [1] ( ) 6-10 [2] ( ) 11 ou + [3]

Qual especialidade? {ESPEC} ( ) Cardiologia [0] ( ) Oftalmologia [1] ( ) Neurologia [2]

( ) Infectologia [3] ( ) Pneumologia [4] ( ) Cirurgia [5]

( ) Angiologia [6] ( ) Nefrologia [7] ( ) Clínica da Dor [8]

( ) Outras [9]

09. Já teve pneumonia? {PNEU} ( ) NÃO [0] ( ) SIM [1]

Se SIM, quantas vezes? {QPNEU} ( ) 1[0] ( ) 2-3[1] ( ) 4-6[2] ( ) 7 ou + [3]

Se SIM, teve febre? {FEBRE} ( ) NÃO [0] ( ) SIM [1]

Anormalidade no RX? {ARX} ( ) NÃO [0] ( ) SIM [1]

Quando internado, usou medicação? {MPNEU} ( ) NÃO [0] ( ) SIM [1]

Quais? {DESCMPNEU} _____

10. Teve ou tem esplenomegalia? {ESPLE} ( ) NÃO [0] ( ) SIM [1]

Em que período? {PERIOESPLE} ( ) <6m[0] ( ) 6m-1ano [1] ( ) 2-3a [2] ( ) 4-5a [3] ( ) >6a [4]

Teve crise de seqüestro esplênico? {SEQESPLE} ( ) NÃO [0] ( ) SIM [1]

Se SIM, quantas vezes? {QSEQESPLE} _____

11. Faz uso profilático de Penicilina? {PROP} ( ) NÃO[0] ( ) SIM [1]

Se SIM, qual? {QPEN} ( ) Penicilina V oral [0] ( ) Penicilina benzatina [1]

Se Sim, há quanto tempo? {QTPEN} ( ) até 1 ano [0] ( ) + de 1 ano a 3 anos [1]

( ) + 3 anos a 5 anos [2] ( ) + 5 anos a 7 anos [3]

( ) + de 7 anos [4]

12. Já teve AVC? {AVC} ( ) NÃO [0] ( ) SIM [1]
Se SIM, quantas vezes? {QAVC} ( ) 1 [0] ( ) 2 [1] ( ) 3 [2] ( ) 4 ou + [3]
Se SIM, sequelas do AVC? {SEQAVC} ( ) NÃO [0] ( ) SIM [1]
Já fez ressonância magnética? {RESSOMAG} ( ) NÃO [0] ( ) SIM [1]
Alguma alteração? {ALTRESSOMAG} ( ) NÃO [0] ( ) SIM [1]
13. Esplectomizado? {ESPLECTO} ( ) NÃO [0] ( ) SIM [1]
Esplenectomia: {TIPOESPLECTO} ( ) Total [0] ( ) Parcial [1]
14. Apresenta asma? {ASMA} ( ) NÃO [0] ( ) SIM [1]
Se SIM, quantas crises nos últimos 06 meses? {QASMA} ( ) 0[0] ( ) 1-3[1] ( ) 4-7[2] ( ) 8ou+[3]
Faz uso regular de nebulização?{NEBU} ( ) [0] NÃO ( ) SIM [1]
15. Tem crises de dor? {CRISDOR} ( ) [0] NÃO ( ) SIM [1]
Se SIM, quantas crises nos últimos 06 meses? {QCRISDOR} ( ) 0[0] ( ) 1-3[1] ( ) 4-7[2] ( ) 8ou+[3]
Quando foi a última crise? {ULTCRISDOR} ( ) <1mês [0] ( ) 1-3m [1] ( ) 4m ou+[2]
Usa medicação para a dor? {MDOR} ( ) NÃO [0] ( ) SIM [1]
Prescrita por um médico? {PRESMDOR} ( ) NÃO [0] ( ) SIM [1]
Assistido por especialista em dor? {ESPECDOR} ( ) NÃO [0] ( ) SIM [1]
16. Faz tratamento com hidroxiuréia? {HIDROXI} ( ) NÃO [0] ( ) SIM [1]
Usa há quanto tempo? {QTEMH} _____
Modo de utilização {MUTILH} _____
17. Faz uso de alguma medicação? {MEDIC} ( ) NÃO [0] ( ) SIM [1]
Se SIM, qual? {DESCMEDIC} _____
Com que frequência? {FREQMEDIC} ( ) Diário [0] ( ) Dias alternados[1] ( ) Semanal[2]
( ) Quinzenal[3] ( ) Mensal [4] ( ) Bimestral[5] ( ) Semestral [6]
18. Vaso-Oclusão: {VO} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QVO} _____
Fez uso de alguma medicação? {MVO} ( ) NÃO [0] ( ) SIM [1]
19. Faz consultas periódicas com oftalmo? {CONSOFTAL} ( ) NÃO [0] ( ) SIM [1]
20. Retinopatia: {RETIN} ( ) NÃO [0] ( ) SIM [1]
Se SIM, fez uso de alguma medicação? {MRETIN} ( ) NÃO [0] ( ) SIM [1]
21. Infecções: {INFEC} ( ) NÃO[0] ( ) SIM [1]
Quais? {DESCINFEC} ( ) Rinite [0] ( ) Sinusite [1] ( ) Otite [2]
( ) Faringite [3] ( ) Amigdalite [4] ( ) Outros [5]
Fez uso de alguma medicação? {MINFEC} ( ) NÃO[0] ( ) SIM [1]
22. Priapismo: {PRIAP} ( ) NÃO [0] ( ) SIM [1]
N° de vezes: {QPRIAP} ( ) Até 4 [0] ( ) 05-09 [1] ( ) 10 ou +[2]
Fez uso de alguma medicação? {MPRIAP} ( ) NÃO [0] ( ) SIM [1]
23. Úlcera maleolar: {ULCMALEO} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QULCMALEO} _____
Idade da primeira úlcera: {IDULC} ( ) Até 4 anos [0] ( ) 5-9 [1] ( ) 10 ou + [2]
Tratou a úlcera? {TRATULC} ( ) NÃO [0] ( ) SIM [1]
Qual tratamento? {QUALTRAT} _____
24. Síndrome torácica aguda: {SDTOR} ( ) NÃO [0] ( ) SIM [1]
Quantas vezes? {QSDTOR} ( ) Até 2 [0] ( ) 03-05 [1] ( ) 06 ou + [2]
25. Alterações ósseas: {ALTOSSEA} ( ) NÃO [0] ( ) SIM [1]
Quais? {DESCALTOSSEA} _____
26. Insuficiência Renal Aguda: {INSRENAG} ( ) NÃO [0] ( ) SIM [1]

Quantas vezes? {QINSRENAG} ( ) Até 2 [0] ( ) 03-05 [1] ( ) 06 ou + [2]

27. Insuficiência Renal Crônica: {INSRENCRO} ( ) NÃO [0] ( ) SIM[1]

Idade do diagnóstico: {IDINSRENCRO} ( ) Até 5 anos [0] ( ) 06-11[1] ( ) 12 ou + [2]

28. Alterações cardíacas: {INSCARD} ( ) NÃO [0] ( ) SIM[1]

Qual alteração? {QUALALTCA} _____

Idade diagnóstico: {IDINSCARD} ( ) Até 5 anos [0] ( ) 06-11 [1] ( ) 12 ou + [2]

Fez eletrocardiograma? {ELETRO} ( ) NÃO [0] ( ) SIM [1]

Fez ecocardiograma? {ECOCARD} ( ) NÃO [0] ( ) SIM [1]

29. Sequestro hepático: {SEQHEP} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QSEQHEP} _____

30. Insuficiência respiratória: {INSRESP} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QINSRESP} _____

31. Distúrbio do sono? {DISTSONO} ( ) NÃO [0] ( ) SIM [1]

32. Litíase biliar: {LITIBILI} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QLITIBILI} _____

33. Cirurgia: {CIRURG} ( ) NÃO [0] ( ) SIM [1]

Quais? {QUALCIRURG} _____

Se SIM, fez uso de profilaxia antibiótica? {PROFANTIB} ( ) NÃO [0] ( ) SIM [1]

34. Completou o calendário vacinal? {CALVAC} ( ) NÃO [0] ( ) SIM [1]

Fez uso das seguintes vacinas? {USOVAC} ( ) 7 valente [0] ( ) 23 valente [1]

( ) Meningo [2] ( ) Haemophilus [3]

35. Faz uso de hemoderivados? {HEMODER} ( ) NÃO [0] ( ) SIM [1]

Se SIM, quantas vezes ao ano? {QHEMODER} _____

36. Possui outra patologia? {PATOLOG} ( ) NÃO [0] ( ) SIM [1]

Quais? {DESCPATOLOG} ( ) Hipertensão [0] ( ) Diabetes [1] ( ) Obesidade [2] ( ) Outras [3]

37. Você trabalha? {TRAB} ( ) NÃO [0] ( ) SIM [1]

Tipo de profissão: {QTRAB} _____

Se SIM, manipula alguma substância química? {SUBQUIM} ( ) NÃO [0] ( ) SIM [1]

Qual? {QSUBQUIM} _____ Frequência? {FREQSUBQUI} _____

Manipula diretamente esta subst? {MANIDIRE} ( ) NÃO [0] ( ) SIM [1]

38. Pratica esportes? {ESPOR} ( ) NÃO [0] ( ) SIM [1]

39. Faz uso de bebida alcoólica? {BEBE} ( ) NÃO [0] ( ) SIM [1]

Se SIM, que frequência? {FREQBEBE} _____

40. Você fuma? {FUMA} ( ) NÃO [0] ( ) SIM [1]

Se SIM, que frequência? {FREQFUMA} _____

41. Faz uso de alguma droga? {DROGA} ( ) NÃO [0] ( ) SIM [1]

Em caso de SIM, que frequência? {FREQDROGA} _____

42. Além dos seus pais quantos membros da família ou parentes são apegados a você? {APEG}

( ) 01[0] ( ) 02 – 03 [1] ( ) 04 – 06[2] ( ) 07 – 10[3] ( ) nenhum[4]

43. Quantos amigos você têm aproximadamente? {AMIGO}

( ) 01[0] ( ) 02 – 03 [1] ( ) 04 – 06[2] ( ) 07 – 10[3] ( ) nenhum[4]

44. Com que frequência você se reúne com seus parentes, amigos ou vizinhos? {REUNI}

( ) Diariamente ou quase todos os dias [0] ( ) Várias vezes na semana [1]

( ) Várias vezes no mês [2] ( ) Várias vezes por ano [3] ( ) Quase nunca [4]