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Investigação de polimorfismos em genes relacionados à resposta ao estresse oxidativo e inflamação na susceptibilidade à ocorrência da doença cerebrovascular em pacientes com anemia falciforme

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2015

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"Não importa quanto a vida possa ser ruim, sempre existe algo que você pode fazer, e triunfar. Enquanto há vida, há esperança"

(Stephen Hawking)

Resumo

O acidente vascular cerebral (AVC) é uma complicação grave da doença falciforme e uma das principais causas de morte em crianças e adultos. Isoladamente, o AVC é responsável por 20% dos óbitos de crianças falciformes entre 5-10 anos; entretanto, poucos marcadores de risco foram identificados. Espécies reativas de oxigênio (ERO) e processos inflamatórios crônicos, ao reduzirem a vida útil das hemácias falcizadas e alterarem os níveis de citocinas inflamatórias, respectivamente, podem modular o desenvolvimento do AVC, caracterizando os polimorfismos genéticos relacionados ao estresse oxidativo e inflamação como possíveis moduladores desse evento clínico. O objetivo deste trabalho foi avaliar os polimorfismos em genes relacionados à resposta ao estresse oxidativo (genes *SOD2* e *GPx3*) e processos inflamatórios (genes *IL1RN*, *IL6* e *IL10*), verificando se existe associação com a susceptibilidade à ocorrência da doença cerebrovascular em pacientes portadores de AF acompanhados no HEMOPE. A amostra foi constituída por 270 pacientes com anemia falciforme, que, de acordo com o desenvolvimento da doença cerebrovascular, foram classificados em caso (71), controle (140) e faixa de risco (59). A pesquisa dos polimorfismos foi realizada por PCR em tempo real utilizando o sistema TAQMAN®, com exceção do polimorfismo VNTR no gene da IL-1RN, que foi realizada por PCR seguido da análise das repetições. Em nosso estudo, o polimorfismo *SOD2* (Val-16Ala) (rs4880) apresentou influência como modulador genético para a prevalência do AVC (p=0,0137).

Palavras-chave: Anemia falciforme; doença cerebrovascular; estresse oxidativo; inflamação

Abstract

Stroke is a devastating complication of sickle cell anemia (SCA), and is one of the leading causes of death in both adults and children with SCA, being responsible for 20% of mortalities in SCA children aged 5-10 years. Evidence suggests that some genetic polymorphisms could be related to stroke development in SCA. Reactive oxygen species (ROS), by reducing the life of sickle red blood cells, and chronic inflammatory processes, by changing the levels of inflammatory cytokines, can modulate stroke development. Genetic polymorphisms related to oxidative stress and inflammation could modulate this clinical event. Here, we evaluated polymorphisms related to oxidative stress response (*SOD2* and *GPx3* genes) and inflammatory (*IL1RN*, *IL6* and *IL10* genes), and evaluated their relationship on stroke development in SCA patients followed at HEMOPE. The study was drawn from a cohort of 270 patients with SCA which, according to the development of cerebrovascular disease, were classified in case (71), control (140) and stroke risk (59) groups. Polymorphisms genotyping were performed by qPCR using TaqMan® assays, except for the VNTR polymorphism in the *IL-1RN* gene, which was performed by PCR followed by repetition analysis. Our findings suggest that *SOD2* (Val-16Ala) (rs4880) polymorphism modulates the prevalence of stroke (p=0.0137).

Key words: Sickle cell anemia; cerebrovascular disease; oxidative stress; inflammation

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Item	Definição
%	Porcentagem
≤	Menor ou igual que
α	Alfa
β	Beta
γ	Gama
β^S	Globina beta falciforme
≥	Maior ou igual que
A	Adenina
AF	Anemia falciforme
Ala	Alanina
AVC	Acidente Vascular Cerebral
C	Citosina
CAT	Catalase
cm/s	Centímetros por segundo
Cu	Cobre
Da	Dalton
DTC	Doppler Transcraniano
ERO	Espécie reativa de oxigênio
G	Guanina
G6PD	Glicose-6-fosfato desidrogenase
GMPc	Guanosina monofosfato cíclica
Glu	Ácido glutâmico ou glutamato

GPx	Glutationa peroxidase
GPx3	Gene da glutationa peroxidase 3
H_2O	Água
H_2O_2	Peróxido de Hidrogênio
HBB	Gene da globina beta
HbF	Hemoglobina fetal
HBG	Gene da globina gama
HbS	Hemoglobina S
HEMOPE	Fundação de Hematologia e Hemoterapia de Pernambuco
HU	Hidroxiuréia
IL10	Gene da interleucina 10
IL1RN	Gene do antagonista ao receptor de interleucina 1
IL6	Gene da interleucina 6
K	Kilo
Mn	Manganês
MnSOD	Superóxido dismutase dependente de manganês
O_2^-	Superóxido
OH•	Hidroxila
ON	Óxido nítrico
Pb	Pares de bases
pH	Potencial Hidrogeniônico
SNC	Sistema Nervoso Central
SNP	*Single nucleotide polymorphism*
SOD	Superóxido dismutase

SOD2	Gene da superóxido dismutase 2
T	Timina
TNF-α	Fator de Necrose Tumoral alfa
Val	Valina
VNTR	*Variable number of tandem repeat*
X	Número de vezes
Zn	Zinco
IL-10	Interleucina 10
IL-6	Interleucina 6
GPx-3	Glutationa peroxidase 3
IL-1Ra	Antagonista ao receptor de interleucina 1
IL-1β	Interleucina 1 do tipo beta

Sumário

1. Introdução

A anemia falciforme (AF), doença autossômica recessiva de distribuição mundial, é causada por uma mutação pontual no gene da globina β. Devido a uma substituição do ácido glutâmico pela valina na 6ª posição do gene da cadeia β globínica (β^6 GAG→GTG; $glu^6 \rightarrow val^6$), ocorre a formação de uma hemoglobina anormal, a hemoglobina S (HbS). Em condições específicas, como baixas concentrações de oxigênio, diminuição do pH e baixas concentrações de hemoglobina fetal (HbF), a HbS forma um polímero que se deposita na membrana das hemácias, modificando a forma dos eritrócitos, tornando-os falciformes.

As manifestações clínicas da AF são baseadas nos episódios de vaso-oclusão proveniente das hemácias falcizadas, que impedem o fluxo sanguíneo principalmente nos pequenos vasos, além dos episódios hemolíticos, que diminuem a disponibilidade de óxido nítrico. São elas: crises álgicas, crises hemolíticas, úlceras de membros inferiores, síndrome torácica aguda, sequestro esplênico, necrose asséptica de fêmur e úmero, retinopatia, insuficiência renal crônica, priapismo, acidente vascular cerebral (AVC), entre outros.

O AVC é um evento neurológico agudo secundário à oclusão de uma artéria ou a uma hemorragia, com posterior isquemia tecidual e/ou sinais e sintomas neurológicos. Na AF, 25% dos pacientes apresentam manifestações cerebrovasculares, entretanto os fatores que predispõem o desenvolvimento desse evento não estão bem estabelecidos. Deste modo, a busca de métodos mais específicos de prognóstico melhorariam os tratamentos profiláticos, como as transfusões crônicas e o uso da hidroxiuréia, nos pacientes de alto risco para o AVC.

Fatores genéticos que predispõem pacientes portadores de AF a desenvolver um AVC não estão bem estabelecidos. Marcadores clássicos da AF, tais como o haplótipo β^S e a coerança entre a AF e a talassemia α, parecem estar modulando os fenótipos da AF. Entretanto, polimorfismos de base única em genes relacionados com vias de inflamação, regulação vascular, resposta ao estresse oxidativo, adesão celular e hemostasia aparecem como possíveis candidatos para predizer o desenvolvimento do AVC na AF.

Espécies reativas de oxigênio (ERO) podem causar danos significativos nos eritrócitos, proporcionando a ocorrência de novos eventos hemolíticos. Sendo assim, alterações genéticas relacionadas às enzimas envolvidas nos mecanismos antioxidantes, como a superóxido dismutase dependente de manganês (MnSOD) e a glutationa peroxidase (GPx), podem modular o desenvolvimento das diversas manifestações clínicas na AF, como o AVC.

Além disso, pacientes com AF apresentam um aumento de mediadores inflamatórios, adesivos e trombóticos, caracterizando a AF como uma doença de caráter inflamatório crônico. Desse modo, a ativação de citocinas pró-inflamatórias e anti-inflamatórias pode estar relacionada ao desenvolvimento e evolução do AVC, classificando os polimorfismos em genes inflamatórios, como o *IL1RN*, *IL6* e *IL10*, como possíveis moduladores desse evento clínico.

Devido ao grave risco e alta incidência do AVC na AF e considerando que pacientes com AF apresentam quadros clínicos variados, a identificação de marcadores genéticos que atuem como fatores moduladores das complicações cerebrovasculares na AF pode apresentar um grande impacto na melhoria da sobrevida e na qualidade de vida dos pacientes.

2. Revisão da Literatura

2.1 Anemia Falciforme

2.1.1 Prevalência

A anemia falciforme (AF) é uma das desordens hereditárias mais comuns no mundo, em que 2% da população mundial apresenta a doença, além de nascerem entre 300.000-400.000 crianças falciformes a cada ano (Rusanova et al., 2011). No Brasil, estima-se que de 5-6% da população seja portadora do traço falciforme e que, a cada ano, nascem entre 700-1000 crianças portadoras da AF (Lyra et al., 2005).

No estado de Pernambuco, um em cada 23 recém-nascidos vivos possui o traço falciforme e um em cada 1400 nasce com a doença falciforme (Cançado & Jesus, 2007). Bandeira e cols. (1999), ao realizarem uma triagem em sangue de cordão umbilical, encontraram uma frequência de 5,1% de recém-nascidos portadores do traço falciforme no estado de Pernambuco.

2.1.2 Fisiopatologia

A anemia falciforme (AF), doença autossômica recessiva de distribuição mundial, é uma hemoglobinopatia causada por uma mutação pontual no gene da globina β, que promove a substituição do ácido glutâmico pela valina no 6º códon da cadeia polipeptídica (*HBB*; β^6 GAG→GTG; $glu^6 \rightarrow val^6$), levando à formação de uma hemoglobina anormal (HbS). Em condições de baixas concentrações de oxigênio, diminuição do pH e baixas concentrações de hemoglobina fetal (HbF), a HbS sofre uma polimerização devido a interação entre os resíduos hidrofóbicos dessa molécula, formando estruturas filamentosas que se depositam nas

hemácias, modificando sua forma e tornando-as falciformes (Rees et al., 2010). O acúmulo de polímeros de HbS dentro das hemácias falcizadas resulta em uma lesão celular e, em larga escala, os eritrócitos danificados promovem os efeitos hemolíticos e vaso-oclusivos, caracterizando o fenótipo principal da AF (Steinberg, 2008) (Figura 1).

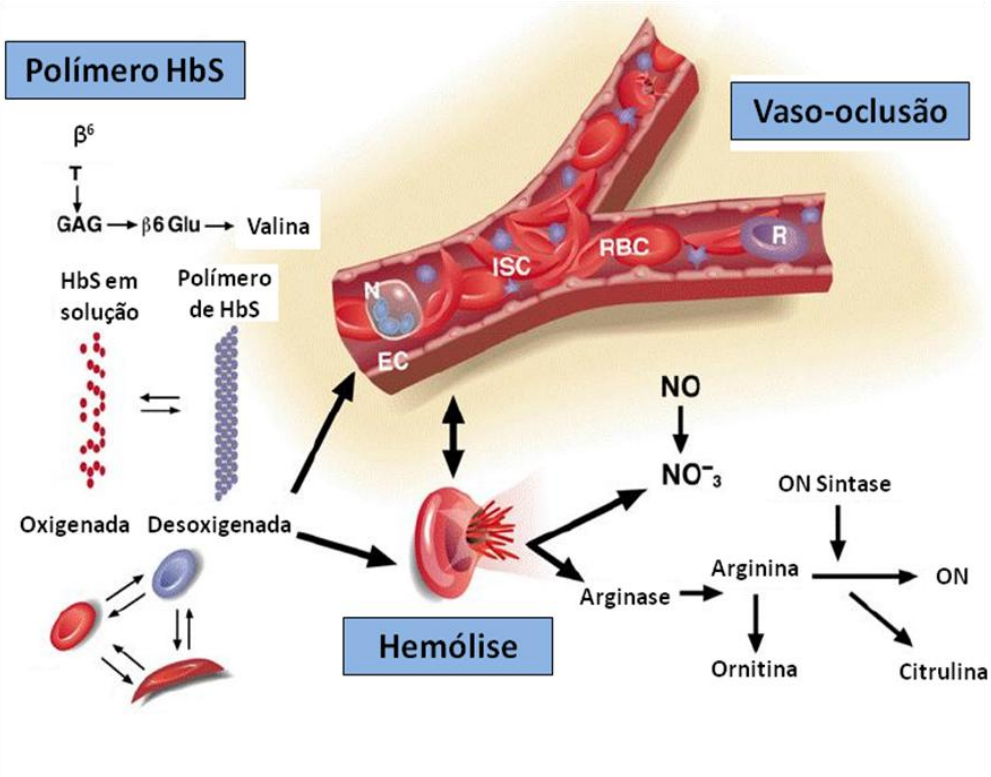

Figura 1: Fisiopatologia da anemia falciforme. Mutação na 6ª posição no gene da globina β, levando a formação de uma hemoglobina anômala, a HbS, que sofre uma polimerização em baixas concentrações de oxigênio. O polímero de HbS danifica o eritrócito, diminuindo sua vida útil (hemólise) e aumentando o consumo de óxido nítrico, além de promoverem uma vasoclusão (Steinberg, 2008).

Pacientes falciformes, por desenvolverem hemácias falcizadas que apresentam um tempo de vida mais curto, cursam com um elevado grau de hemólise. Devido à hemólise crônica, esses pacientes apresentam altos níveis de hemoglobina plasmática que sequestram o óxido nítrico, diminuindo sua

disponibilidade, levando ao desenvolvimento de algumas manifestações clínicas da doença falciforme, como a osteonecrose e o AVC. Na AF, a hemólise ocorre extravascularmente através de um reconhecimento das hemácias danificadas por células do sistema reticuloendotelial. Entretanto, esse processo também pode ocorrer dentro dos vasos, podendo corresponder até 30% da hemólise total de um paciente falciforme (Steinberg, 2008).

Acredita-se que o processo de vaso-oclusão é resultado de um complexo cenário envolvendo interações de diferentes tipos celulares, incluindo células falcizadas, reticulócitos, células endoteliais, leucócitos, plaquetas, além de citocinas e fatores teciduais (Capellini, 2007; Morris, 2008; Lanaro et al., 2009; Sakamoto et al., 2013). Vaso-oclusões recorrentes, processos de isquemia-reperfusão e consequente ativação do endotélio vascular induzem a contínuas respostas inflamatórias na anemia falciforme, que se propagam por níveis elevados de citocinas inflamatórias, diminuição da biodisponibilidade do óxido nítrico e estresse oxidativo (Conran et al., 2009). Desse modo, a ocorrência de eventos vaso-oclusivos, em associação com o quadro hemolítico, tem papel determinante na origem dos sinais e sintomas presentes no paciente com AF, como o AVC (Ballas & Mohandas, 1996).

2.1.3 Acidente Vascular Cerebral

O AVC pode ser definido como um evento neurológico agudo secundário à oclusão de uma artéria ou a uma hemorragia, com consequente isquemia e/ou sinais e sintomas neurológicos. Em pacientes com AF, crianças entre 1-9 anos são consideradas como a faixa etária mais predisponente para desenvolver o AVC (Ohene-Frempong et al., 1998). Além disso, os acidentes vasculares

cerebrais isquêmicos apresentam uma grande incidência em pacientes falciformes menores de 20 anos, com um pico entre 7 e 11 anos, além de acometer adultos maiores de 30 anos. Já o acidente vascular cerebral hemorrágico acomete principalmente adultos falciformes entre 20-30 anos (Kato et al., 2009) (Figura 2).

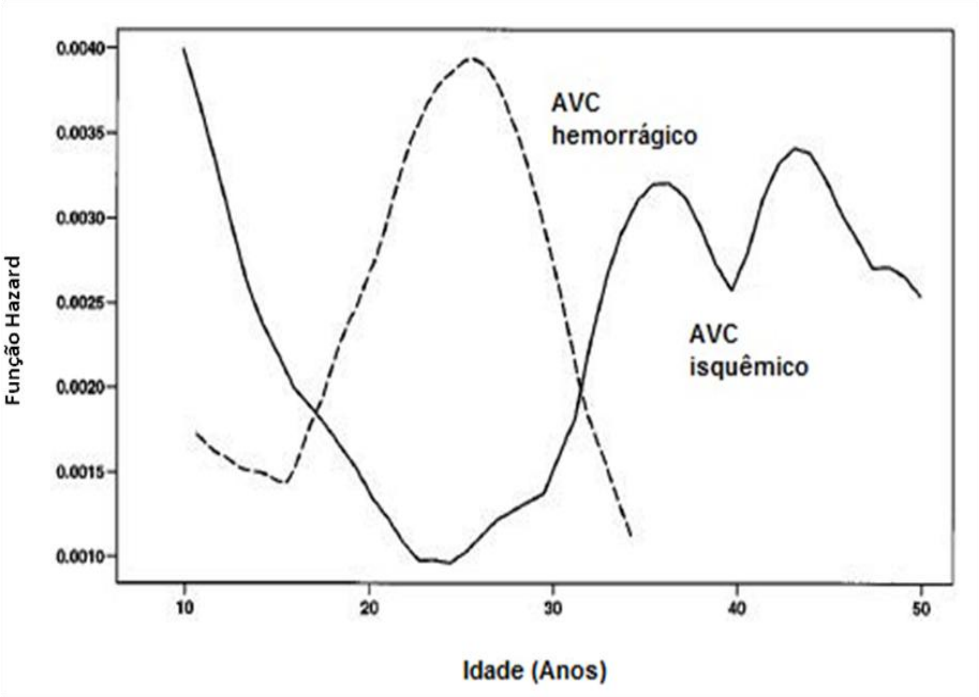

Figura 2: Taxas de risco de acidente vascular cerebral isquêmico e hemorrágico em pacientes falciformes, de acordo com a idade. (——) AVC isquêmico; (-----) AVC hemorrágico (Ohene-Frempong et al., 1998).

A AF pode contribuir para o desenvolvimento do AVC por criar uma fonte persistente de lesão endotelial em consequência a hipóxia, aumentar a tensão de cisalhamento, promover uma maior adesão das hemácias falcizadas ao endotélio, além da inflamação gerada pela lesão de reperfusão. Estes efeitos levam a produção de citocinas, promovendo uma disfunção endotelial, aumento de mediadores inflamatórios, adesivos e trombóticos, além de inibir a produção de

mediadores citoprotetores, como o óxido nítrico (Phelan & Faller, 1996; Solovey et al., 1997; Fasano et al., 2015).

Na AF, o acidente vascular cerebral é uma das principais causas de óbito em crianças e adultos. O AVC, isoladamente, é responsável por 20% dos óbitos de crianças com doença falciforme entre 5-10 anos; além disso, 70% das crianças que desenvolvem o AVC apresentam déficit motor e significante déficit cognitivo (Ohene-Frempong et al., 1998; Zhou et al., 2011). Ademais, crianças portadoras de anemia falciforme possuem um risco 300x maior de desenvolver um acidente vascular cerebral, tornando assim a AF a maior causadora de AVC durante a infância. A recorrência do AVC é frequente e acontece em cerca de 70% destes pacientes, geralmente nos três primeiros anos após o primeiro acidente cerebrovascular (Hoppe et al., 2007; Zhou et al., 2011).

Fatores que predispõem pacientes com anemia falciforme a essa complicação não estão bem estabelecidos (Sarnaik & Ballas, 2001). Apesar da etiologia do AVC não ser bem compreendida, estudos sugerem na existência de um componente genético, além da mutação pontual na globina β. Os principais candidatos para a avaliação da predisposição do AVC em pacientes falciformes incluem genes envolvidos na lesão endotelial, trombose e inflamação (Hoppe et al., 2007).

Por apresentar uma alta incidência na AF, são necessários testes prognósticos que possam identificar precocemente pacientes que possuam alto risco de desenvolver o AVC. A identificação precisa pode contribuir para um tratamento preventivo, diminuindo a ocorrência de acidentes vasculares cerebrais primários em portadores de AF (Flanagan et al., 2011; Bernaudin et al., 2014).

2.2 Prevenção da Doença Cerebrovascular Falciforme

2.2.1 Doppler Transcraniano (DTC)

Dentre os pacientes com AF que desenvolveram o AVC, 70-90% apresentam vasculopatia estenótica dos grandes vasos cerebrais (Hsu et al, 2003). A ultrassonografia através do Doppler Transcraniano (DTC), método não invasivo que determina as velocidades de fluxo sanguíneo das artérias cerebrais, identifica as crianças com risco elevado para o desenvolvimento do AVC pela detecção precoce dessa vasculopatia, permitindo que se faça a profilaxia primária da ocorrência desse evento (Adams et al., 1992; Flanagan et al., 2011; Connes et al., 2013). O risco do AVC é diretamente proporcional ao aumento da velocidade média nas artérias cerebrais, como as artérias carótidas internas distais e cerebrais médias proximais (Adams et al., 2004; Flanagan et al., 2011).

Crianças com AF, por apresentarem ossos cranianos mais finos e janelas acústicas maiores, têm velocidade de fluxo cerebral detectada de modo mais fácil que os adultos pelo DTC. Entretanto, cerca de 5% das crianças apresentam DTC inadequado, por não se conseguir mensurar o fluxo sanguíneo (Adams et al., 2004; Hoppe, 2005). Porém, aproximadamente 19% das crianças falciformes, mesmo com DTC normal, podem vir a desenvolver um AVC (Adams et al., 2004).

Devido à anemia, crianças falciformes apresentam DTC ainda maiores (Velocidade de fluxo 130-140 cm/s) do que crianças sem AF (Velocidade de fluxo 90-100 cm/s). Velocidades de fluxo cerebral acima de 140 cm/s podem indicar uma estenose cerebral ou apenas um aumento geral no fluxo sanguíneo cerebral (HOPPE, 2005). Crianças falciformes com DTC alterado (Velocidade de fluxo ≥ 200 cm/s) apresentam 44x mais chances de desenvolver um AVC do que as que tem DTC normal (Velocidade de fluxo ≤ 170 cm/s). Entretanto, o valor de 200

cm/s não é absoluto, visto que pacientes que apresentam DTC em faixa condicional (Velocidade de fluxo 170-199 cm/s) também apresentam elevado risco de desenvolver o AVC (Flanagan et al., 2011) (Figura 3).

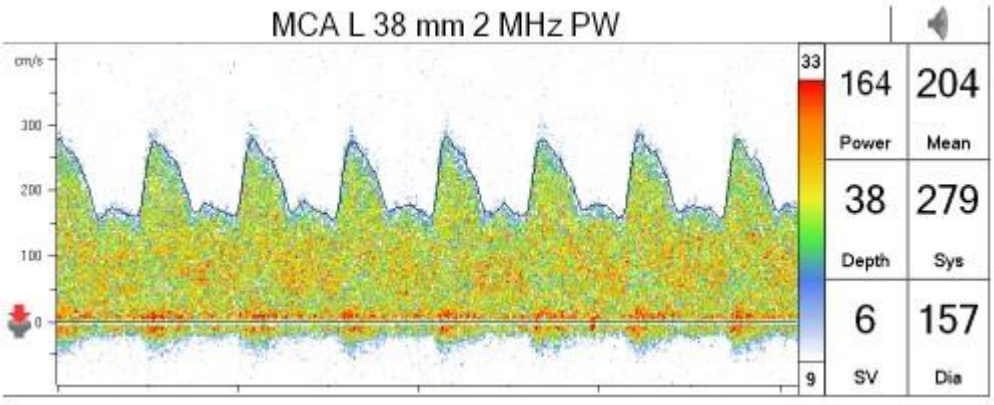

Figura 3: DTC na faixa alterada (Velocidade de fluxo média de 204 cm/s) de criança com anemia falciforme. (Fonte: HEMOMINAS).

2.2.2 Hidroxiuréia (HU)

Atualmente, a hidroxiuréia (HU), agente quimioterápico leve, inicialmente utilizado nas doenças onco-hematológicas, é a única droga aprovada capaz de modificar o curso clínico da doença, por melhorar os parâmetros hematológicos e diminuir o número de crises dolorosas e hospitalizações dos pacientes com AF (Charache et al., 1995; Rosse et al., 2000; Wang et al., 2011). Os efeitos benéficos da HU, um inibidor da fase S do ciclo celular, são atribuídos a sua capacidade de aumentar a produção de hemoglobina fetal (HbF), codificada pelo gene da globina γ (*HBG*), em células progenitoras eritróides através de uma via dependente de GMPc, aumentando a concentração final de HbF na hemácia falcizada e inibindo, assim, a polimerização da HbS (Cokic et al., 2003). Além disso, alguns estudos sugerem que a HU pode promover benefícios por mecanismos não relacionados a indução de HbF, como um efeito anti-inflamatório

na diminuição do número de leucócitos, citocinas e moléculas de adesão e um aumento da produção de óxido nítrico (Zimmerman et al., 2004; Cokic et al., 2006, Platt, 2008; Green & Barral, 2014; Yawn et al., 2014).

A hemoglobina fetal (HbF) é a molécula mais estudada como modulador genético na AF. Por não participar do polímero de HbS e, consequentemente, diminuir a formação deste mesmo polímero, o aumento dos níveis de HbF pode melhorar o curso clínico do paciente falciforme. Pacientes com AF apresentam índices de HbF variando entre 1-30% (média de 8%), modulados, em parte, pelos haplótipos da globina β. No entanto, ter o conhecimento do nível de HbF de um paciente falciforme é insuficiente para prever as possíveis complicações clínicas. Alguns pacientes apresentam graves complicações da doença mesmo apresentando níveis de HbF em torno de 20% (Wyszinski et al., 2004; Akinsheye et al., 2011).

Crianças com AF apresentam uma maior sobrevida após o tratamento com HU, principalmente pela diminuição do desenvolvimento de síndrome torácica aguda e infecções. Além disso, uma diminuição dos níveis de reticulócitos e neutrófilos, fatores de risco já estabelecidos da doença falciforme, tem sido descrita após o tratamento com essa droga (Lobo et al., 2013). Ademais, estudos prévios já demonstraram que a terapia com HU tem sido associada com uma diminuição das velocidades de fluxo sanguíneo nas artérias cerebrais, mensurado pelo DTC, e com uma menor taxa de recorrência do AVC (Ali et al., 2011; Lagunju et al., 2015).

Por se tratar de um agente quimioterápico, o uso da HU foi questionado inicialmente devido aos possíveis efeitos adversos que poderiam ser causados a um longo prazo. Entretanto, vários estudos de acompanhamento de pacientes

falciformes que utilizaram a droga foram realizados, não se encontrando associação entre a HU e possíveis efeitos neoplásicos (Steinberg et al., 2003; Ballas et al., 2009; Steinberg et al., 2010).

Sendo assim, o uso da HU tem sido cada vez mais incentivado em pacientes com AF (Steinberg et al., 2003; Steinberg et al., 2010). Apesar de conter alguns efeitos adversos temporários, como leucopenia e plaquetopenia, que poderiam predispor os pacientes a infecções e sangramentos, o risco do uso da HU em pacientes falciformes é aceitável quando comparado com o risco de pacientes falciformes não tratados (Brawley et al., 2008).

2.2.3 Transfusões Crônicas

A identificação de pacientes com risco para o desenvolvimento do AVC por um método de triagem, como o DTC, permite a administração precoce de transfusões profiláticas, beneficiando o portador de AF (Steinberg, 2005). Manter o nível de HbS em torno dos 30% é recomendado como prevenção do AVC primário e secundário em crianças de 2-16 anos, com o uso de terapias baseadas em transfusões crônicas. Apesar de sua eficácia em prevenir o AVC, transfusões crônicas apresentam riscos associados, como infecções, aloimunizações e sobrecarga de ferro, necessitando de uma terapia quelante (Vichinsky, 2001).

Em pacientes com AF e velocidades de fluxo elevadas no DTC, transfusões crônicas e regulares de concentrado de hemácias (entre 21 e 30 dias) reduzem em 90% o risco de ocorrer um primeiro AVC, além de diminuir a taxa hemolítica e o nível de hemoglobina plasmática livre (Lezcano et al., 2006). Entretanto, estudos têm demonstrado que a descontinuidade das transfusões, mesmo após vários anos, pode reverter as velocidades de fluxo cerebrais para

valores pré-transfusionais, favorecendo o desenvolvimento do AVC (Steinberg, 2005).

A limitação do DTC em identificar todos os portadores de AF que irão desenvolver um AVC, associada com a dificuldade de comprometimento dos pacientes com programas crônicos de transfusão por tempo indeterminado, expõe a necessidade de marcadores mais sensíveis e específicos para inferir o risco do AVC (Flanagan et al., 2011).

2.3 Moduladores Genéticos

2.3.1 Estresse Oxidativo

Espécies reativas de oxigênio (ERO) podem causar danos significativos nos eritrócitos, reduzindo seu período de vida útil, em especial nos pacientes com AF (Amer & Fibach, 2005). Como consequência da hemólise, há uma menor biodisponibilidade do óxido nítrico (ON), um importante agente antioxidante, além de apresentar um aumento da liberação de agentes oxidantes, como o grupo heme da hemoglobina (Kato et al., 2009). Desse modo, o estresse oxidativo resultante pode levar à rigidez e à instabilidade da membrana dos eritrócitos, proporcionando a ocorrência de novos eventos hemolíticos (Schacter et al. 1988; Amer et al. 2006).

Apesar de os processos bioquímicos normais do corpo humano levarem à formação de EROs, existem vários mecanismos de defesa para neutralizar a ação desses radicais livres (Airede & Ibrahim, 1999). Esses mecanismos incluem enzimas antioxidantes, como a superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e glicose-6-fosfato desidrogenase (G6PD), que são consideradas como o sistema defensivo primário das células humanas, dentre

elas, os eritrócitos (Fridovich & Freeman, 1986; Gizi et al., 2011). No entanto, pacientes com AF apresentam baixos níveis/atividade dos mecanismos antioxidantes, sendo mais susceptíveis aos efeitos do estresse oxidativo (Schacter et al., 1988; Sultana et al., 1998; Kaul et al., 2004; Amer et al., 2006) (Figura 4).

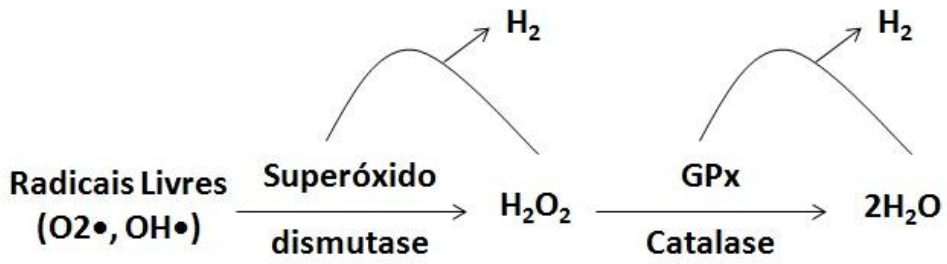

Figura 4: Ação das enzimas antioxidantes para a neutralização dos radicais livres. (Fonte: Adaptado de http://www.hemoglobinopatias.com.br/d-falciforme/fisio-falci.htm).

2.3.1.1 Superóxido dismutase

A superóxido dismutase (SOD) faz parte da primeira linha de defesa antioxidante dos organismos contra o estresse oxidativo, dismutando o superóxido (O_2^-) a água (H_2O) e peróxido de hidrogênio (H_2O_2), protegendo as células contra os danos causados por esse radical (Sanders et al., 1995; Han et al., 2012). A SOD atua em conjunto com as enzimas CAT, GPx e peroxirredoxinas, as quais eliminam o H_2O_2 (Halliwell & Gutteridge, 2007). De modo geral, a SOD está presente no meio intracelular, apresentando pequenas concentrações no plasma, fluido espinhal e linfa. Em humanos, a SOD apresenta três isoformas, sendo uma com cobre (Cu) e zinco (Zn) em seu sítio catalítico e outra com Cu e Zn em seu sítio ativo. Por fim, a terceira isoforma da SOD, denominada de superóxido dismutase dependente de manganês (MnSOD), é

mitocondrial e apresenta um átomo de manganês (Mn) em seu sítio ativo (Fattman et al., 2003).

O gene *SOD2*, que codifica a MnSOD, está localizado no braço longo do cromossomo 6 (6q25.3) e possui uma região polimórfica no peptídeo sinal, no códon 16, posição -9, onde ocorre uma transição T→C, resultando na substituição do aminoácido valina (Val) por alanina (Ala) (Val16Ala) (rs4880) (Rosenblum et al., 1996). A presença do alelo variante Ala está relacionado a uma maior atividade da MnSOD (Suresh et al. 2003), promovendo uma diminuição da gravidade e dos sintomas da AF (Schacter et al., 1985). Entretanto, alguns estudos sugerem que, devido ao aumento da formação de H_2O_2 e radicais hidroxila (OH•), pacientes que apresentem uma menor capacidade de remover esses radicais por falhas em outras enzimas, como a GPx ou CAT, possam apresentar um efeito negativo para os níveis elevados de MnSOD (Shimoda-Matsubayashi et al., 1996; Sutton et al., 2005).

2.3.1.2 Glutationa peroxidase

A glutationa peroxidase plasmática (GPx-3) é uma importante enzima antioxidante sintetizada pelo gene *GPx3*, localizado no cromossomo 5, sendo responsável por remover H_2O_2 produzidos durante o metabolismo normal ou após um estresse oxidativo (Maddipati & Marnett, 1987; Takahashi et al., 1987). Além disso, a GPx-3 contribui para manutenção da biodisponibilidade do ON, agente vasodilatador que pode ser inativado na presença de EROs (Voestsch et al., 2007).

Das cinco isoformas conhecidas, a GPx-3 é a única encontrada no meio extracelular, e sua deficiência tem sido associada a um aumento do risco de

desenvolvimento do AVC infantil em populações não falciformes (Freedman et al., 1996; Kenet et al, 1999). Desse modo, alterações genéticas no *GPx3* poderiam modular o desenvolvimento do AVC, e polimorfismos na região promotora desse gene, como -568 (T→C) (rs8177404), -518 (T→C) (rs8177406) e – 65 (T→C) (rs8177412), podem reduzir o nível transcricional, favorecendo o desenvolvimento de complicações vaso-oclusivas como o AVC (Voestsch et al., 2007).

2.3.2 Inflamação

A adesão de leucócitos ao endotélio vascular e a lesão endotelial subsequente, com produção de citocinas e proteínas de fase aguda, podem apresentar um papel significativo nas crises vaso-oclusivas da AF (Chiang & Frenette, 2005). Além disso, um elevado número de leucócitos é considerado um fator de risco da doença, por ter sido associado com um aumento de morte precoce (Turhan et al., 2002).

Processos inflamatórios crônicos, seguidos de lesões de isquemia-reperfusão, apresentam um papel fundamental no desenvolvimento de isquemias cerebrais (Marousi et al., 2011). A ativação de citocinas pró-inflamatórias e anti-inflamatórias podem estar relacionadas ao desenvolvimento e evolução do AVC, caracterizando os polimorfismos em genes inflamatórios como possíveis moduladores desse evento clínico (Marousi et al., 2011; Park et al., 2011).

2.3.2.1 Antagonista ao receptor de interleucina 1

O antagonista ao receptor de interleucina 1 (IL-1Ra), citocina anti-inflamatória que se liga ao receptor tipo I, protege as células endoteliais contra a

apoptose induzida pela interleucina 1 do tipo beta (IL-1β), caracterizando o IL-1Ra como um possível agonizador contra as desordens inflamatórias (Tong et al., 2011). É codificada pelo gene *IL1RN,* localizado no braço curto do cromossomo 2 (2p13), e apresenta um polimorfismo do tipo *variable number tandem repeat* (VNTR) de 86pb no íntron 2, cujos alelos podem variar entre duas a seis repetições (Tabela 1) (Gromadzka et al., 2007; Tong et al., 2011).

Tabela 1: Identificação dos cinco alelos do polimorfismo do tipo *variable number tandem repeat* (VNTR) de 86pb no íntron 2 do gene *IL1RN* (Tarlow et al., 1993).

Alelo	Repetições	Pares de Base
A1	4R	410pb
A2	2R	240pb
A3	5R	500pb
A4	3R	325pb
A5	6R	595pb

Por estar envolvida no processo inflamatório, acredita-se que polimorfismos no gene *IL1RN*, como o *variable number tandem repeat (VNTR)* de 86pb no intron 2, possam alterar o desenvolvimento de doenças que apresentem um componente inflamatório, como o AVC (Tong et al., 2011).

2.3.2.2 Interleucina 10

A interleucina 10 (IL-10), proteína de 36kDa codificada pelo gene *IL10* que está localizado no cromossomo 1 (1q21-32), é uma citocina anti-inflamatória secretada por linfócitos e monócitos, responsável por contrabalancear os efeitos do fator de necrose tumoral alfa (TNF-α) e outras moléculas pró inflamatórias

(Munshi et al., 2010). No cérebro, o mecanismo de ação da IL-10 envolve a inibição da expressão de citocinas pró-inflamatórias (IL-1α e β, IL-6, TNF-α) e de seus respectivos receptores, além da indução da síntese da IL-1Ra (Seitz et al., 1995; Strle et al., 2001).

A IL-10, pelo papel anti-inflamatório no sistema nervoso central (SNC), parece ser um candidato à modulação do AVC. Desse modo, um polimorfismo na posição -1082 (G/A) do gene *IL10* (rs1800896), que está relacionado com uma alta/baixa produção de IL-10, respectivamente, tem sido bastante analisado como um possível modulador desse evento clínico (Turner et al., 1997; Munshi et al., 2010).

2.3.2.2 Interleucina 6

A interleucina 6 (IL-6) é uma citocina pró-inflamatória codificada pelo gene *IL6*, localizado no cromossomo 7 (7p21). É uma citocina multifuncional sintetizada por vários tipos celulares, porém, células endoteliais, fibroblastos e monócitos são os maiores produtores da IL-6 durante inflamações sistêmicas (Heinrich et al., 1990; Ma et al., 2011; Chakraborty et al. 2013). No SNC, sua produção está envolvida na patogênese de várias desordens neurológicas, como o AVC (Van Wagoner & Benveniste, 1999).

Estudos demonstraram que alterações nessa citocina, como um polimorfismo na posição -174 (G/C) do gene *IL6* (rs1800795), podem influenciar o desenvolvimento do AVC, com o alelo G sendo responsável por uma maior atividade da IL-6 do que o alelo C (Ma et al., 2011; Chakraborty et al. 2013). Além disso, esse polimorfismo está associado com a extensão do AVC isquêmico em

pacientes jovens, com o alelo G sendo um fator de risco para essa condição (Fishman et al., 1998; Greisenegger et al., 2003; Flex et al., 2004).

3. Objetivos

3.1 Geral

Investigar a associação dos polimorfismos em genes relacionados à resposta ao estresse oxidativo e à processos inflamatórios e correlacionar esses achados com a susceptibilidade à ocorrência da doença cerebrovascular em pacientes com anemia falciforme acompanhados no serviço de hematologia da fundação HEMOPE, no estado de Pernambuco.

3.2 Específicos

1. Investigar a associação dos polimorfismos nos gene *SOD2* (rs4880) e *GPx3* (rs8177404, rs8177406 e rs8177412), relacionados à resposta ao estresse oxidativo, com a susceptibilidade à ocorrência da doença cerebrovascular em pacientes com anemia falciforme;
2. Investigar a associação polimorfismos nos genes *IL1RN*, *IL6* (rs1800795) e *IL10* (rs1800896), relacionados à inflamação, com a susceptibilidade à ocorrência da doença cerebrovascular em pacientes com anemia falciforme.

4. Capítulo I

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***SOD2* Val16Ala polymorphism is associated with stroke development in a sickle cell anemia Brazilian population**

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ABSTRACT

Sickle cell anemia (SCA) is caused by a single β-globin gene mutation coding for the sickle β-hemoglobin chain, leading to the formation of sickle hemoglobin, and their patients can present different clinical courses, varying from death in childhood to recurrent painful vasoocclusive crises and multiple organ damage in adults. Of the many severe consequences of the disease, stroke is one of the leading causes of death in both adults and children with SCA, and most cases are associated with vasculopathy affecting the middle cerebral and distal internal carotid arteries. As SCA patients are susceptible to increased oxidative stress due to the constant hemolysis of sickle red blood cells (RBCs), it has been suggested that some genetic factors related to oxidative stress could influence the development of cerebrovascular disease. Here, we genotyped four published single nucleotide polymorphisms in *SOD2* and *GPx-3* genes and evaluated their possible prognostic impact in a large series of SCA patients with well-defined stroke phenotypes. Out of the 270 unrelated SCA patients included in the study, the majority of patients (n = 62, 92%) presented ischemic stroke while only three and two patients presented silent infarcts and hemorrhagic stroke, respectively. Genetically, only the homozygous TT genotype from the *SOD2* Val16Ala polymorphism (odds ratio [OR]: 0.4947; 95%CI: 0.2840 to 0.8616; p = 0.0137) were associated with decreased stroke risk. In summary, our findings suggest that SOD2 Val16Ala polymorphism is associated with the prevalence of stroke in our SCA population. Other studies in a different population of SCA are needed to determine the significance of our findings.

KEY WORDS: Sickle cell disease, Stroke, Oxidative stress, SOD2, Genetic modulators.

INTRODUCTION

Sickle cell anemia (SCA) is an inherited monogenic and multisystem disease associated with episodes of acute illness and progressive organ damage, which is not only influenced by genetic features, but also environmental, social and economic factors (Stuart & Nagel, 2004; Steinberg, 2009; Rees et al., 2010; Cajado et al., 2011). The primary defect in sickle cell anemia is hemoglobin S (HbS), originated by amino acid substitution of glutamic acid for valine at the sixth position of the β-globin chain due to single nucleotide substitution (GAG→GTG) in codon 6 of the β-globin gene on chromosome 11p15.5. This mutation causes red blood cells (RBCs) to acquire a sickle shape under conditions of hypoxia, resulting in a very large range of phenotypes such as anemia, cell adhesion, vaso-occlusion, severe pain, organ failure and stroke (Driss et al., 2009; Chui & Dover, 2001).

Of the many severe consequences of SCA, stroke is one of the most disabling and a leading cause of death in both children and adults. By the age of 20, stroke occurs in about 11% of patients with sickle cell anemia (Ohene-Frempong 1998; Platt, 2005). The most frequent cause of brain infarction in these patients is blockage of the intracranial internal carotid and middle cerebral arteries (Stockman et al., 1972; Rothman et al., 1986). These lesions can be detected by TCD ultrasonography because blood-flow velocity is inversely related to arterial diameter (Adams et al., 1992; Adams 2005). Nevertheless, TCD is restricted to children, and it does not accurately identify all SCA patients who will develop cerebrovascular complications. Therefore, the discovery of new sensitive and specific markers to determine stroke risk in SCA patients is required.

RBCs are continuously exposed to both endogenous and exogenous sources of reactive oxygen species (ROS) that can damage the RBC and impair its function (Mohanty et al., 2014), and, due to the constant hemolysis of sickle RBCs, SCA patients are susceptible to increased oxidative stress (Hebbel et al., 1982). Also, ROS defense mechanisms are affected in SCA, as the levels/activities of nonenzymatic antioxidants (vitamins A, C, and E) and enzymatic antioxidants (glutathione peroxidase and superoxide dismutase) have been shown to be reduced in individuals with SCA (Amer et al., 2006; Natta et al., 1990; Schacter et al., 1988; Conran et al., 2009). Therefore, oxidative stress occurs as the result of the imbalance between enhanced generation of reactive oxygen species and low cellular content of antioxidants (Fasola et al., 2007).

Chronic oxidative stress constitutes a critical factor in endothelial dysfunction, inflammation, and multiple organ damage in SCA, playing a role in the disease physiopathology (Hebbel et al., 2004; Sogut et al., 2011). Plasma glutathione peroxidase (GPx-3) is a major antioxidant enzyme in plasma which scavenges hydrogen peroxide and organic (lipid) hydroperoxides produced during normal metabolism or after oxidative stress (Maddipati & Marnett, 1987; Takahashi et al., 1987), and it's deficiency was associated with stroke risk in a clinical study (Kenet et al., 1999). Furthermore, manganese superoxide dismutase (MnSOD), encoded by the SOD2 gene, is translocated into the mitochondrial matrix, where it scavenges superoxide radicals to protect the cells from oxidative stress (Shimoda-Matsubayashi et al., 1996), and the *SOD2* Val16Ala polymorphism could compromise the ability to neutralize superoxide radicals. Therefore, the identification of genetic variations related to oxidative stress could elucidate the different SCA phenotypes and their effects on patient outcome. In the present study, we genotyped four published single nucleotide polymorphisms (SNPs) (*SOD2* Val16Ala (rs4880), *GPx-3* T-568C (rs8177404), *GPx-3* T-518C (rs8177406), *GPx-3* T-65C (rs8177412)) and evaluated their possible prognostic impact on the development of stroke in SCA patients.

MATERIAL and METHODS

Patients

Between March 2013 and November 2014, peripheral blood (PB) samples from 270 unrelated patients with SCA (SS homozygotes), who had been attending at the Hematology and Hemotherapy Foundation of Pernambuco (HEMOPE), Brazil, were collected and genomic DNA was extracted using the Puregene kit (Gentra System, Minneapolis, MN, USA), according to the manufacturer's protocol. Also, imaging exams (TCD, X-ray, CT and/or Magnetic Resonance Imaging, MRI) and clinical data were obtained from the patients' medical records. According to stroke development, we classified our patients in three groups. Patients presenting a documented, primary stroke event (n = 71) on clinical records, clinically evaluated by a neurologist and confirmed with an imaging exam (MRI or CT) were called "Stroke positive". As the "Stroke negative" group, was selected a non-stroke group of SCA patients who had not received prior hydroxyurea treatment (n = 140); none of these participants had a history of clinical stroke, elevated

TCD velocities, or any evidence of silent infarcts, based on their baseline brain MRI. Finally, pediatric SCA patients (n = 59) with abnormally high flow velocities (TCD velocities ≥ 170 cm/s), and without any evidence of stroke, constituted the "Stroke-risk" group. In accordance with the Declaration of Helsinki, informed consent was obtained from all patients and/or parents. This study was approved by the local Research Ethics Board (#413.574).

SNP genotyping

After genomic DNA extraction, the SOD2 Val16Ala, GPx-3 T-568C, GPx-3 T-518C and GPx-3 T-65C polymorphisms were genotyped by real-time PCR using TaqMan® SNP Genotyping Assay for SNP rs4880 (C___8709053_10), rs8177404 (C__31986209_10), rs8177406 (C__31986207_10) and rs8177412 (C__25964717_20), respectively.

Statistical analyses

Patient baseline characteristics were descriptively reported. Statistical analysis was performed using SPSS Statistics 17.0 (IBM Corporation, Somers, NY, USA) and STATA Statistical Software 9.0 (STATA, College Station, TX, USA), with the level of significance set to 5%. Chi-square and Fisher's exact test was employed to compare associations between categorical variables.

RESULTS

Seventy one SCA patients presented a documented, primary stroke event, which was confirmed with an imaging exam. The majority of patients (n = 66, 92.9%) presented ischemic stroke, while only three and two patients presented silent infarcts and hemorrhagic stroke, respectively. Therefore, analysis taking in account stroke etiology could not be performed. All patients, allocated to either the control or stroke-risk groups, remained free of clinical stroke during the entire study.

All of the main data are summarized in Table I. Genotyping results reveals an influence of SOD2 Val16Ala polymorphism on stroke development on SCA. Patients with the homozygous TT genotype had more than two times less risk of developing stroke, compared with the homozygous CC genotype (odds ratio [OR]: 0.4947; 95%CI: 0.2840 to

0.8616; p = 0.0137). In contrast, polymorphisms in the GPx-3, which had previously been shown to be associated with stroke risk, failed to reveal a difference in our study.

Upon the observation of the striking similarities between the stroke positive and stroke-risk groups in their clinical and laboratory features, these two groups were pooled together. Their combined results were also compared with the control group. Except for the allelic frequency from the *GPx3* T-65C (rs8177412), which started to reveal a significant difference (odds ratio [OR]: 1.707; 95%CI: 1.049 to 2.778; p = 0.0307), all other results remained similar after pooling (Supplementary Table I).

DISCUSSION

Increasing evidence has indicated a role of oxidative stress in patients with SCA, which was also related to the severity of their clinical features (Rusanova et al., 2010; Aslan, 2007). It has been shown in a human study group that the SOD activity is lower in SCA patients than in general population and it's inversely proportional to the degree of symptom severity (Schacter et al., 1985). Analyses of associations between SOD genotypes and SOD activity levels on plasma demonstrated that the "Ala" allele from the *SOD2* Val16Ala was responsible for high levels of the enzyme (Iida et al., 2008). Additionally, the presence of the "Val" allele, instead of the "Ala" allele, results in less efficient transport of MnSOD into the mitochondrial matrix (Sutton et al., 2003). However, our study found an association between the homozygous TT genotype and a protective effect on stroke development. Similar results demonstrating an association between high levels of SOD and the "Val" allele was found by Bastaki et al. (2006) and Martin et al. (2009), when they evaluated the MnSOD activity in erythrocytes and in cryopreserved human hepatocytes, respectively.

Plasma glutathione peroxidase (GPx-3) is a major antioxidant enzyme in plasma that scavenges hydrogen peroxide and organic (lipid) hydroperoxides produced during normal metabolism or after oxidative insult (Maddipati & Marnett, 1987; Takahashi et al., 1987). Of the 5 known GPx isoforms, GPx-3 is the only one found in the extracellular space and it's deficiency has been associated clinically with an increased risk of childhood stroke in general population (Freedman et al., 1996; Kenet et al., 1999). Taken together, this evidence points to the *GPx-3* gene as a compelling candidate gene for thrombotic cerebrovascular disease risk (Voetsch et al., 2007). However, other studies could not find

an association between *GPx-3* polymorphisms and stroke development in general population (Grond-Ginsbach et al., 2007; Grond-Ginsbach et al., 2009), similarly to our study, in SCA. Due to the small sample size in Voetsch (n=23) work and with no other study to ratify his findings, it's highly probable that his study was a victim of statistical error. (Grond-Ginsbach et al., 2007; Grond-Ginsbach et al., 2009).

Similarly to previous findings (Domingos et al., 2014), our results demonstrate a molecular similarity between the patients who present risk of stroke development and those who actually developed a stroke. Therefore, these patients might have had a documented stroke event if they have not the stroke risk diagnosed by the TCD and an early medical intervention, reducing the gravity of SCA and decreasing the risk of stroke development (Brawley et al., 2008; Lanzkron et al., 2008; Lezcano et al., 2006).

In summary, *SOD2* Val16Ala polymorphism is associated with the prevalence of stroke in our SCA population. However, it´s interesting to point out that other studies might be necessary to determine the significance of our findings. Furthermore, experimental studies to evaluate the SOD activity would be helpful to determine the role of *SOD2* Val16Ala polymorphisms in the functional protein levels and their prognostic impact on the prevalence and predictability of stroke development in SCA.

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CONFLICT-OF-INTEREST DISCLOSURE

The authors declare no conflict of interest.

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Table I. Molecular features related to oxidative stress of SCA patients, according to cerebral vascular disease status.

Characteristics patients	All patients (n = 270)		Stroke positive (n = 71)		Stroke-risk (n = 59)		Stroke negative (n = 140)		*p*-value
	No.	%	No.	%	No.	%	No.	%	
***SOD2* Val16Ala (rs4880)**									
Allele									
T	283	52.4	66	46.5	59	50.0	158	56.4	Ref
C	257	47.6	76	53.5	59	50.0	122	43.6	0.1294
Codominant									
TT	73	27.0	14	19.7	12	20.3	47	33.6	Ref
TC	137	50.8	38	53.5	35	59.4	64	45.7	0.0501
CC	60	22.2	19	26.8	12	20.3	29	20.7	0.1506
Dominant									
TT	73	27.0	14	19.7	12	20.3	47	33.6	Ref
TC + CC	114	73.0	57	80.3	47	79.7	93	66.4	0.0429*
Recessive									
TT + TC	147	77.8	52	73.2	47	79.7	111	79.3	Ref
CC	60	22.2	19	26.8	12	20.3	29	20.7	0.5623
Overdominant									
TT + CC	147	49.2	33	46.5	24	40.6	76	54.3	Ref
TC	137	50.8	38	53.5	35	59.4	64	45.7	0.1852
***GPx-3* T-568C (rs8177404)**									
Allele									
T	461	85.4	123	86.6	107	90.7	231	82.5	Ref
C	79	14.6	19	13.4	11	9.3	49	17.5	0.0960
Codominant									
TT	197	73.0	53	74.6	48	81.4	96	68.6	Ref
TC	67	24.8	17	24.0	11	18.6	39	27.8	0.3111
CC	6	2.2	1	1.4	0	0.0	5	3.6	0.2130
Dominant									
TT	197	73.0	53	74.6	48	81.4	96	68.6	Ref
TC + CC	73	27.0	18	25.4	11	18.6	44	31.4	0.1672
Recessive									
TT + TC	264	97.8	70	98.6	59	100.0	135	96.4	Ref
CC	6	2.2	1	1.4	0	0.0	5	3.6	0.2554
Overdominant									
TT + CC	203	75.2	54	76.0	48	81.4	101	72.2	Ref
TC	67	24.8	17	24.0	11	18.6	39	27.8	0.3814
***GPx3* T-518C (rs8177406)**									
Allele									
T	460	85.2	123	86.6	106	89.8	231	82.5	Ref
C	80	14.8	19	13.4	12	11.1	49	17.5	0.1460
Codominant									
TT	197	73.0	53	74.6	47	79.7	97	69.3	Ref
TC	66	24.4	17	24.0	12	20.3	37	26.4	0.5527
CC	7	2.6	1	1.4	0	0.0	6	4.3	0.1453
Dominant									
TT	197	73.0	53	74.6	47	79.7	97	69.3	Ref
TC + CC	73	27.0	18	25.4	12	20.3	43	30.7	0.3006
Recessive									
TT + TC	263	97.4	70	98.6	59	100.0	134	95.7	Ref

CC	7	2.6	1	1.4	0	0.0	6	4.3	0.1692
Overdominant									
TT + CC	204	75.6	54	76.0	47	79.7	103	73.6	Ref
TC	66	24.4	17	24.0	12	20.3	37	26.4	0.6549
***GPx3* T-65C (rs8177412)**									
Allele									
T	459	85.0	123	86.6	107	90.7	229	81.8	Ref
C	81	15.0	19	13.4	11	9.3	51	18.2	0.0625
Codominant									
TT	196	72.6	53	74.6	48	81.4	95	67.8	Ref
TC	67	24.8	17	24.0	11	18.6	39	27.9	0.2964
CC	7	2.6	1	1.4	0	0.0	6	4.3	0.1345
Dominant									
TT	196	72.6	53	74.6	48	81.4	95	67.8	Ref
TC + CC	74	27.4	18	25.4	11	18.6	45	32.2	0.1349
Recessive									
TT + TC	263	97.4	70	98.6	59	100.0	134	95.7	Ref
CC	7	2.6	1	1.4	0	0.0	6	4.3	0.1692
Overdominant									
TT + CC	203	75.2	54	76.0	48	81.4	101	72.1	Ref
TC	67	24.8	17	24.0	11	18.6	39	27.9	0.3814

* Indicates statistically significant differences.

Supplementary Table I. Molecular features related to oxidative stress of SCA patients, according to cerebral vascular disease status (2 groups).

Characteristics patients	All patients (n = 270)		Stroke positive + risk (n = 130)		Stroke negative (n = 140)		OR (CI 95%)	*p*-value
	No.	%	No.	%	No.	%		
***SOD2* Val16Ala (rs4880)**								
Allele								
T	283	52.4	125	48.1	158	56.4		Ref
C	257	47.6	135	51.9	122	43.6		0.0579
Codominant								
TT	73	27.0	26	20.0	47	33.6		Ref
TC	137	50.8	73	56.2	64	45.7	0.4850 (0.2702 to 0.8706)	0.0199*
CC	60	22.2	31	23.8	29	20.7		0.0787
Dominant								
TT	73	27.0	26	20.0	47	33.6		Ref
TC + CC	114	73.0	104	80.0	93	66.4	0.4947 (0.2840 to 0.8616)	0.0137*
Recessive								
TT + TC	147	77.8	99	76.2	111	79.3		Ref
CC	60	22.2	31	23.8	29	20.7		0.5606
Overdominant								
TT + CC	147	49.2	57	43.8	76	54.3		Ref
TC	137	50.8	73	56.2	64	45.7		0.0900
***GPx-3* T-568C (rs8177404)**								
Allele								
T	461	85.4	230	88.5	231	82.5		Ref
C	79	14.6	30	11.5	49	17.5		0.0522
Codominant								
TT	197	73.0	101	77.7	96	68.6		Ref
TC	67	24.8	28	21.5	39	27.8		0.2040
CC	6	2.2	1	0.8	5	3.6		0.1185
Dominant								
TT	197	73.0	101	77.7	96	68.6		Ref
TC + CC	73	27.0	29	22.3	44	31.4		0.1011
Recessive								
TT + TC	264	97.8	129	99.2	135	96.4		Ref
CC	6	2.2	1	0.8	5	3.6		0.2155
Overdominant								
TT + CC	203	75.2	102	78.5	101	72.2		Ref
TC	67	24.8	28	21.5	39	27.8		0.2604
***GPx3* T-518C (rs8177406)**								
Allele								
T	460	85.2	229	88.1	231	82.5		Ref
C	80	14.8	31	11.9	49	17.5		0.0704
Codominant								
TT	197	73.0	100	76.9	97	69.3		Ref
TC	66	24.4	29	22.3	37	26.4		0.3938
CC	7	2.6	1	0.8	6	4.3		0.1187
Dominant								
TT	197	73.0	100	76.9	97	69.3		Ref
TC + CC	73	27.0	30	23.1	43	30.7		0.1721
Recessive								
TT + TC	263	97.4	129	99.2	134	95.7		Ref
CC	7	2.6	1	0.8	6	4.3		0.1219

Overdominant								
TT + CC	204	75.6	101	77.7	103	73.6		Ref
TC	66	24.4	29	22.3	37	26.4		0.4797
***GPx3* T-65C (rs8177412)**								
Allele								
T	459	85.0	230	88.5	229	81.8		Ref
C	81	15.0	30	11.5	51	18.2	1.707 (1.049 to 2.778)	0.0307*
Codominant								
TT	196	72.6	101	77.7	95	67.8		Ref
TC	67	24.8	28	21.5	39	27.9		0.2029
CC	7	2.6	1	0.8	6	4.3		0.0650
Dominant								
TT	196	72.6	101	77.7	95	67.8		Ref
TC + CC	74	27.4	29	22.3	45	32.2		0.0770
Recessive								
TT + TC	263	97.4	129	99.2	134	95.7		Ref
CC	7	2.6	1	0.8	6	4.3		0.1219
Overdominant								
TT + CC	203	75.2	102	78.5	101	72.1		Ref
TC	67	24.8	28	21.5	39	27.9		0.2604

n, number of individuals; OR, odds ratio; CI, confidence intervals; * Indicates statistically significant differences.

5. Capítulo II

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_IL1RN_, _IL6_ and _IL10_ polymorphisms are not associated with stroke development in a Brazilian population with sickle cell anemia

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***IL1RN*, *IL6* and *IL10* polymorphisms are not associated with stroke development in a Brazilian population with sickle cell anemia**

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To the Editor,

Sickle cell anemia (SCA) is a severe debilitating hematological disorder associated with a high degree of morbidity and mortality, and stroke is one of the most catastrophic complication in SCA (Ohene-Frempong et al., 1998). Nowadays, it is well established that sickle cell disease vaso-occlusion induces a chronic inflammatory state in a sickle cell disease individual, and is propagated by elevated levels of circulating inflammatory cytokines (Conran et al., 2009). This was evidenced by the elevation in proinflammatory, and the reduction in anti-inflammatory cytokine levels in SCD patients compared to healthy individuals (Lanaro et al., 2009). Recently, ischemic stroke (IS) has also been recognized as an inflammation-related disease in which inflammatory cytokines play an important role (Kriz et al., 2009; Tong et al., 2010; Tong et al., 2011), and genetic variation in the genes encoding the inflammatory mediators, such as *IL6 G-174C* (rs1800795), *IL10 G-1082A* (rs1800896) and the penta-allelic polymorphism in intron 2 of the human *IL1RN* gene is caused by 86-bp tandem repeats (VNTR), has been reported to influence the etiology of ischemic stroke (Chakraborty et al., 2013; Trompet et al., 2007; Titov et al., 2012; Sultana et al., 2011; Tong et al., 2010). Although previous studies have already analyzed the relationship between polymorphisms in inflammatory genes and clinical development of ischemic stroke, to best of our knowledge, no study involving patients with SCA was developed so far. In the present study, we genotyped three published inflammatory single nucleotide polymorphisms (SNPs) (*IL6 G-174C* (rs1800795), *IL10 G-1082A* (rs1800896) and the 86-bp tandem repeats (VNTR) polymorphism of *IL1RN*) and evaluated their possible prognostic impact on the development of ischemic stroke in SCA patients.

The present study was drawn from a cohort of 270 unrelated patients with SCA (SS homozygotes), who had been attending at the Haematology and Haemotherapy Foundation of Pernambuco (HEMOPE), Brazil, since January 2000. Between March 2013 and August 2014, peripheral blood (PB) samples were collected, and genomic DNA was extracted using the Puregene kit (Gentra System, Minneapolis, MN, USA), according to the manufacturer's protocol. Additionally, clinical data and imaging exams (TCD, X-ray, CT and/or Magnetic Resonance Imaging, MRI) were obtained from the patients' medical records. Therefore, imaging exams, clinical histories, and adequate DNA samples availability constituted the inclusion criteria of this study. Documented, primary ischemic stroke events (n = 71) in patients were confirmed with an imaging exam (MRI or CT), clinically evaluated by a neurologist. As a control, a non-stroke group was selected of SCA patients who had not received prior hydroxyurea treatment (n = 140); none of these participants had a history of clinical stroke, elevated TCD velocities, or any evidence of silent infarcts, based on their baseline brain MRI. Finally, pediatric SCA patients (n = 59) with abnormally high flow velocities (TCD velocities ≥ 170 cm/s), and without any evidence of stroke, constituted a third group of patients. In accordance with the Declaration of Helsinki, informed consent was obtained from all patients and/or parents. This study was approved by the local Research Ethics Board (#413.574). After genomic DNA extraction, we genotyped the *IL-6* G-174C and *IL-10* G-1082A polymorphisms by real-time PCR using TaqMan® SNP Genotyping Assay for SNP rs1800795 (C___1839697_20) and SNP rs1800896 (C___1747360_10), respectively. The penta-allelic polymorphism in intron 2 of the human *IL1RN* gene was also determined, as previously described (Tarlow et al., 1993). Patient baseline characteristics were descriptively reported. Statistical analysis was performed using SPSS Statistics 17.0 (IBM Corporation, Somers, NY, USA) and

STATA Statistical Software 9.0 (STATA, College Station, TX, USA), with the level of significance set to 5%. Patients presenting with a documented, primary stroke event on clinical records were called "Stroke ^{positive}"; patients presenting risk to develop stroke, but without evidence of a stroke event, were called "Stroke-risk"; patients without records of stroke were defined as "Stroke ^{negative}". Clinical and laboratory features were then compared. Chi-square and Fisher's exact test was employed to compare associations between categorical variables.

All of the main clinical and laboratory features are summarized in Table I. Genotyping results showed that *IL6 G-174C* (rs1800795) (p = 0.5982), *IL10 G-1082A* (rs rs1800896) (p = 0.2973) and the 86-bp tandem repeats (VNTR) polymorphism of *IL1RN* (p = 0.9280), which had previously been shown to be associated with stroke risk (Chakraborty et al., 2013; Trompet et al., 2007; Titov et al., 2012; Sultana et al., 2011; Tong et al., 2010) in general population, were not associated in our study, similarly than the results found by Ma et al., 2011; Balcerzyk et al., 2012; Marousi et al., 2010. Upon the observation of the striking similarities between the stroke ^{positive} and stroke-risk groups in their clinical and laboratory features, these two groups were pooled together and their combined results were also compared with the control group, but still there was no significantly different association. (Supplementary Table I).

Inflammation promotes the adherence of sickle erythrocytes to the endothelium (Buchanan et al., 2004), and several studies have shown an increased levels of cytokines in serum even during the steady state of SCA (Pathare et al., 2004; Taylor et al., 1997; Bourantas et al., 1998). Therefore, the identification of genetic associations with inflammation is an important strategy for the elucidation of different SCA phenotypes and their effects on patient outcome. Although the role of the studied cytokines in ischemia

development had already been published in general population (Chakraborty et al., 2013; Trompet et al., 2007; Titov et al., 2012; Sultana et al., 2011; Tong et al., 2010), it is conceivable that the same association may be applied to the SCA patients, but no association was found in our study. Recently, Vicari et al. (2014) found no significant difference between the *IL6 G-174C* (rs1800795) and stroke development in a SCA population.

As far as we know, our study was the first analyzing the *IL10* G-1082A (rs rs1800896) and the 86-bp tandem repeats (VNTR) polymorphism of *IL1RN* in stroke development on a SCA cohort. Other studies to confirm the lack of influence from inflammatory polymorphisms in a different population of SCA are necessary to ratify our results.

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Table I. Molecular features related to inflammation of SCA patients, according to cerebral vascular disease status.

Characteristics patients	All patients (n = 270)		Stroke positive (n = 71)		Stroke-risk (n = 59)		Stroke negative (n = 140)		*p*-value
	No.	%	No.	%	No.	%	No.	%	
IL1RN VNTR 86pb#									
Allele									
4R	488	94.2	130	94.2	106	94.6	252	94.0	Ref
2R	30	5.8	8	5.8	6	5.4	16	6.0	0.9732
Codominant									
4R/4R	243	93.8	65	94.2	53	94.6	125	93.3	Ref
4R/2R	2	0.8	0	0.0	0	0.0	2	1.5	------$
2R/2R	14	5.4	4	5.8	3	5.4	7	5.2	0.9888
Dominant									
4R/4R	243	93.8	65	94.2	53	94.6	125	93.3	Ref
4R/2R + 2R/2R	16	6.2	4	5.8	3	5.4	9	6.7	0.9280
Recessive									
4R/4R + 4R/2R	245	94.6	65	94.2	53	94.6	127	94.8	Ref
2R/2R	14	5.4	4	5.8	3	5.4	7	5.2	0.9853
Overdominant									
4R/4R + 2R/2R	257	99.2	69	100.0	56	100	132	98.5	Ref
4R/2R	2	0.8	0	0.0	0	0.0	2	1.5	------$
***IL-6* G-174C (rs1800795)**									
Allele									
G	417	77.2	102	71.8	94	79.7	221	78.9	Ref
C	123	22.8	40	28.2	24	20.3	59	21.1	0.2010
Codominant									
GG	158	58.5	36	50.7	36	61.0	86	61.4	Ref
GC	101	37.4	30	42.3	22	37.3	49	35.0	0.4497
CC	11	4.1	5	7.0	1	1.7	5	3.6	0.2006
Dominant									
GG	158	58.5	36	50.7	36	61.0	86	61.4	Ref
GC + CC	112	41.5	35	49.3	23	39.0	54	38.6	0.2973
Recessive									
GG + GC	259	95.9	66	93.0	58	98.3	135	96.4	Ref
CC	11	4.1	5	7.0	1	1.7	5	3.6	0.2800
Overdominant									
GG + CC	169	62.6	41	57.7	37	62.7	91	65.0	Ref
GC	101	37.4	30	42.3	22	37.3	49	35.0	0.5889
***IL10* G-1082A (rs1800896)**									
Allele									
G	181	33.5	48	33.8	37	31.4	96	34.3	Ref
A	359	66.5	94	66.2	81	68.6	184	65.7	0.8493
Codominant									
GG	26	9.6	9	12.7	5	8.4	12	8.6	Ref
GA	129	47.8	30	42.2	27	45.8	72	51.4	0.4694
AA	115	42.6	32	45.1	27	45.8	56	40.0	0.7660
Dominant									
GG	26	9.6	9	12.7	5	8.4	12	8.6	Ref
GA + AA	244	90.4	62	87.3	54	91.6	128	91.4	0.5982
Recessive									
GG + GA	155	57.4	39	44.9	32	54.2	84	60.0	Ref
AA	115	42.6	32	45.1	27	45.8	56	40.0	0.6684
Overdominant									

GG + AA	141	52.2	41	57.8	32	54.2	68	48.6	Ref
GA	129	47.8	30	42.2	27	45.8	72	51.4	0.4248

[#] Due to the low frequency of some alleles, we used only the two more frequent (2R and 4R); [$] Chi-square test was not valid; * Indicates statistically significant differences.

Supplementary Table I. Molecular features related to inflammation of SCA patients, according to cerebral vascular disease status (2 Groups).

Characteristics patients	All patients (n = 270)		Stroke positive + risk (n = 130)		Stroke negative (n = 140)		OR (95%CI)	*p*-value
	No.	%	No.	%	No.	%		
IL1RN VNTR 86pb#								
Allele								
4R	488	94.2	236	94.4	252	94.0		Ref
2R	30	5.8	14	5.6	16	6.0		1.0000
Codominant								
4R/4R	243	93.8	118	94.4	125	93.3		Ref
4R/2R	2	0.8	0	0.0	2	1.5		-------$
2R/2R	14	5.4	7	5.6	7	5.2		1.0000
Dominant								
4R/4R	243	93.8	118	94.4	125	93.3		Ref
4R/2R + 2R/2R	16	6.2	7	5.6	9	6.7		0.7993
Recessive								
4R/4R + 4R/2R	245	94.6	118	94.4	127	94.8		Ref
2R/2R	14	5.4	7	5.6	7	5.2		1.000
Overdominant								
4R/4R + 2R/2R	257	99.2	125	100.0	132	98.5		Ref
4R/2R	2	0.8	0	0.0	2	1.5		-------$
***IL-6* G-174C (rs1800795)**								
Allele								
G	417	77.2	196	75.4	221	78.9		Ref
C	123	22.8	64	24.6	59	21.1		0.3559
Codominant								
GG	158	58.5	72	55.4	86	61.4		Ref
GC	101	37.4	52	40.0	49	35.0		0.3740
CC	11	4.1	6	4.6	5	3.6		0.7562
Dominant								
GG	158	58.5	72	55.4	86	61.4		Ref
GC + CC	112	41.5	58	44.6	54	38.6		0.3254
Recessive								
GG + GC	259	95.9	124	95.4	135	96.4		Ref
CC	11	4.1	6	4.6	5	3.6		0.7630
Overdominant								
GG + CC	169	62.6	78	60.0	91	65.0		Ref
GC	101	37.4	52	40.0	49	35.0		0.4505
***IL10* G-1082A (rs1800896)**								
Allele								
G	181	33.5	85	32.7	96	34.3		Ref
A	359	66.5	175	67.3	184	65.7		0.7158
Codominant								
GG	26	9.6	14	10.8	12	8.6		Ref
GA	129	47.8	57	43.8	72	51.4		0.3951
AA	115	42.6	59	45.4	56	40.0		0.8318
Dominant								
GG	26	9.6	14	10.8	12	8.6		Ref
GA + AA	244	90.4	116	89.2	128	91.4		0.6804
Recessive								
GG + GA	155	57.4	71	54.6	84	60.0		Ref
AA	115	42.6	59	45.4	56	40.0		0.3906

Overdominant							
GG + AA	141	52.2	73	56.2	68	48.6	Ref
GA	129	47.8	57	43.8	72	51.4	0.2248

[#] Due to the low frequency of some alleles, we used only the two more frequent (2R and 4R); [$] Fisher's exact test was not valid; * Indicates statistically significant differences.

6. Discussão geral

Vários estudos sugerem que alterações genéticas podem influenciar o desenvolvimento do AVC (Driscoll et al., 2003; Kwiatkowski et al., 2003; Hoppe et al., 2004; Voetsch et al., 2007), e seus efeitos podem modificar o curso clínico da AF (Sarnaik & Ballas, 2001; Hoppe et al., 2004; Sebastiani et al., 2010). A natureza multifatorial da AF envolve episódios de vaso-oclusão e crises hemolíticas, além da ativação de mediadores inflamatórios, disfunção endotelial e estresse oxidativo (Conran et al., 2009). Em pacientes com AF, o estresse oxidativo pode causar danos aos eritrócitos, reduzindo sua vida média (Amer & Fibach, 2005). Desse modo, alterações genéticas relacionadas às enzimas envolvidas nos mecanismos antioxidantes, como a SOD e a GPx-3, podem contribuir para o esclarecimento dos fatores relacionados à evolução clínica da AF.

Como dito anteriormente, o polimorfismo *SOD2* Val16Ala (rs4880) pode levar a uma mudança conformacional na estrutura secundária da enzima MnSOD, afetando a localização e seu transporte para dentro da mitocôndria (Shimoda-Matsubayashi et al., 1996; Sutton et al., 2003). Em nosso estudo de associação, um efeito protetor contra o AVC foi demonstrado apenas ao genótipo Val-Val do polimorfismo *SOD2* Val-16Ala (rs4880), possivelmente caracterizando esse alelo como o responsável por altos níveis enzimáticos da MnSOD. Resultados semelhantes foram encontrados por Bastaki e cols. (2006), os quais, ao avaliarem eritrócitos humanos de pacientes saudáveis, observaram que os genótipos Val-Val e Val-Ala eram responsáveis por altos níveis da enzima MnSOD; e por Martin e cols. (2009), ao observarem que a presença do alelo Ala reduzia

significativamente a atividade catalítica da MnSOD em hepatócitos humanos. Entretanto, Sutton e cols. (2003) observaram que na presença do alelo variante (Ala), existe um aumento de 30 a 40% na atividade da MnSOD. Além disso, Shimoda-Matsubayashi e cols. (1996) e Sutton e cols. (2005) sugerem que, devido ao aumento da formação de peróxido de hidrogênio (H_2O_2) e radicais hidroxila (OH•), pacientes que apresentaram uma menor capacidade de remover esses radicais por falhas em outras enzimas, como a glutationa peroxidase ou catalase, podem apresentar um efeito negativo para os níveis elevados de MnSOD. Portanto, fica evidente que os resultados na literatura acerca da atividade da SOD são controversos. Assim, novos entendimentos sobre o real impacto do polimorfismo *SOD2* (Val-16Ala) (rs4880) e de variações presentes em outras enzimas envolvidas no sistema antioxidante ainda são necessários.

Em relação aos polimorfismos na região promotora do *GPx3*, Voetsch e cols. (2007), ao analisarem uma população não falciforme, demonstraram uma associação entre esses polimorfismos e um aumento do risco de desenvolvimento do AVC. No entanto, nosso trabalho avaliando AVC em pacientes falciformes, assim como outros trabalhos em populações europeias, não conseguiram reproduzir os trabalhos de Voetsch (Grond-Ginsbach et al., 2007; Grond-Ginsbach et al., 2009). Apesar de Voetsch e cols. (2007) demonstrarem que apenas o haplótipo do gene *GPx3* conseguiria modular o AVC, o mesmo trabalho demonstrou que os polimorfismos no *GPx3* estão em um alto desequilíbrio de ligação. Desse modo, a falta de associação de um polimorfismo com um alto desequilíbrio de ligação indica que a análise haplotípica tem uma grande chance de também não apresentar associação. Sendo assim, é teoricamente possível

que esse polimorfismo não exerça nenhum papel no desenvolvimento do AVC em pacientes com AF.

A anemia falciforme é reconhecida como uma doença que apresenta um quadro inflamatório crônico (Steinberg, 2006) e, como em outras doenças crônicas, o equilíbrio entre citocinas pró-inflamatórias e anti-inflamatórias está comprometido (Hibbert et al. 2005). Em pacientes falciformes, níveis aumentados e diminuídos de moléculas pró-inflamatórias e anti-inflamatórias, respectivamente, já foram descritos quando comparados com populações não falciformes (Bourantas et al., 1998; Makis et al., 2000; Hibbert et al. 2005). Apesar disso, os efeitos das moléculas inflamatórias na AF ainda são pouco esclarecidos (Lanaro et al., 2009). Em nosso estudo, nenhuma associação foi encontrada entre os polimorfismos envolvendo os genes relacionados à inflamação e o desenvolvimento do AVC; resultado semelhante ao encontrado por outros estudos ao avaliarem pacientes não falciformes (Cvetkovic et al., 2005; Trompet et al., 2007; Zee et al., 2008; Marousi et al., 2011; Ma et al., 2011). Até onde sabemos, nosso estudo foi o primeiro a analisar o polimorfismo *IL10* G-1082A (rs1800896) e o VNTR de 86pb no intron 2 do *IL1RN* e sua possível associação no desenvolvimento do AVC em uma população com AF.

O gene *IL1RN*, responsável por codificar a citocina anti-inflamatória IL1-Ra, apresenta um polimorfismo do tipo VNTR de 86pb no íntron 2, sendo o ‘alelo 2R’ o menor dentre todos, com 2 repetições. Entretanto, enquanto alguns estudos envolvendo pacientes coreanos e italianos demonstraram que a presença do alelo "2R" tem sido associada com um risco maior para o desenvolvimento do AVC (Lee et al., 2003; Tuttolomondo et al., 2012), outros trabalhos classificaram o alelo "2R" como um fator protetor para o desenvolvimento dessa manifestação clínica

(Gromadzaka et al., 2007; Tong et al., 2011). Além disso, a ausência de associação entre o VNTR de 86pb no intron 2 do *IL1RN* e o desenvolvimento do AVC, resultado encontrado em nosso estudo envolvendo pacientes com AF, também já foi descrita por Cvetkovic e cols (2005), ao analisarem pacientes suecos não falciformes. Desse modo, é provável que os resultados conflitantes ocorram devido as diferentes contribuições alélicas para cada população e, consequentemente, dos diferentes grupos étnicos estudados.

O polimorfismo *IL6* G-174C (rs1800795) apresenta um efeito negativo na transcrição gênica, sendo o genótipo "CC" associado com baixos níveis de IL-6 (Fishman et al., 1998) e, consequentemente, com um papel protetor no desenvolvimento do AVC, visto que, na resposta aguda pós-isquêmica, níveis elevados de IL-6 foram identificados (Fuller & Zhang, 2001). Entretanto, as associações descritas com esse polimorfismo em populações não falciformes são bastante variáveis. Titov e cols (2012) e Chakraborty e cols. (2013) analisaram pacientes russos e indianos, respectivamente, e encontraram associação entre o *IL6* G-174C (rs1800795) e o desenvolvimento do AVC, resultados que não foram corroborados por Balding e cols. (2004) e Tuttolomondo e cols. (2012), ao analisarem pacientes irlandeses e italianos, respectivamente. Em nossa coorte, nenhuma associação entre o polimorfismo *IL6* G-174C (rs1800795) com o desenvolvimento do AVC foi encontrada. Recentemente, Vicari e cols. (2014) não encontraram associação entre o desenvolvimento do AVC e o *IL6* G-174C (rs1800795) em pacientes falciformes do estado de São Paulo. Desse modo, com os diferentes resultados encontrados, é provável que outros genes e proteínas influenciem o fenótipo inflamatório geral do AVC, e que o polimorfismo *IL6* G-174C (rs1800795) não seja suficiente para predizer esse evento clínico.

Alguns estudos demonstraram que o polimorfismo na posição -1082 (G/A) do gene *IL10* (rs1800896), por apresentar um efeito funcional na quantidade de IL-10 secretada, aparece como candidato para modular o AVC (Van der Poll et al., 1997; Turner et al., 1997). Em populações não falciformes, uma diminuição dos níveis de IL-10 tem sido associada com o desenvolvimento do AVC (Munshi et al., 2010; Sultana et al., 2011), destacando assim o papel neuroprotetor da IL-10 (Tuttolomondo et al., 2012). Entretanto, nenhuma associação foi encontrada entre o polimorfismo *IL10* -1082 G/A (rs1800896) e o desenvolvimento do AVC em nossa população de pacientes com AF, resultado semelhante ao encontrado por Marousi e cols. (2011) e Trompet e cols. (2007), ao avaliarem pacientes saudáveis. Entretanto, novas avaliações sobre as moléculas inflamatórias e anti-inflamatórias e suas variações genéticas ainda são necessárias, visto que, por exemplo, alguns estudos determinam que apenas o haplótipo *IL10* GCC (G na posição -1082, C na posição -819 e C na posição -592) tem a capacidade de modular os níveis de IL-10 (Yilmaz et al., 2005; Trompet et al., 2007) e, consequentemente, o desenvolvimento do AVC.

Estudos experimentais para avaliar a atividade enzimática da SOD seriam úteis para determinar o papel do polimorfismo *SOD2* Val16Ala (rs4880) nos níveis plasmáticos dessa molécula, e consequentemente, o real impacto prognóstico sobre a prevalência e previsibilidade do desenvolvimento AVC em pacientes com AF. Entretanto, para a realização dessas análises, é necessário a realização de um trabalho prospectivo, visto que os pacientes com AF sofrem inúmeras intervenções clínicas após o desenvolvimento do AVC, como o uso de hidroxiuréia e transfusões crônicas, para evitar um novo evento clínico. Além disso, a dosagem das citocinas seria importante para confirmar a não influência

dos polimorfismos avaliados em nosso estudo no desenvolvimento do AVC em pacientes com AF.

7. Conclusões gerais

A partir dos resultados obtidos, foi possível concluir que:

- O genótipo TT do polimorfismo *SOD2* (Val-16Ala) (rs4880) caracterizou uma aparente proteção para a ocorrência do AVC em nossa população de pacientes com AF.
- Os polimorfismos *GPx-3* (T-568C (rs8177404), T-518C (rs8177406), T-65C (rs8177412)), IL-6 G-174C (rs1800795), IL10 G-1082A (rs1800896) e o *VNTR* de 86pb no *IL1RN* não foram associados com a prevalência do AVC em nossa população de pacientes com AF.

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9. Anexo 1

UNIVERSIDADE FEDERAL DE PERNAMBUCO CENTRO DE CIÊNCIAS DA SAÚDE / UFPE-

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Investigação de polimorfismos em genes relacionados ao estresse oxidativo e inflamação no desenvolvimento da doença cerebrovascular em pacientes com anemia falciforme

Pesquisador: Igor de Farias Domingos

Área Temática:

Versão: 1

CAAE: 21722613.7.0000.5208

Instituição Proponente: CENTRO DE CIÊNCIAS BIOLÓGICIAS

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 413.574

Data da Relatoria: 02/10/2013

Recomendações:

Nenhuma.

Conclusões ou Pendências e Lista de Inadequações:

Nenhuma.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

O Colegiado aprova o parecer do protocolo em questão e o pesquisador está autorizado para iniciar a coleta de dados.

Projeto foi avaliado e sua APROVAÇÃO definitiva será dada, após a entrega do relatório final, na PLATAFORMA BRASIL, através de ¿Notificação ¿ e, após apreciação, será emitido Parecer Consubstanciado .

RECIFE, 02 de Outubro de 2013

Assinador por:
GERALDO BOSCO LINDOSO COUTO
(Coordenador)

10. Anexo 2

Normas revista "Neuromolecular Medicine" - Fator de Impacto: 3,885;

Qualis/CAPES Ciências Biológicas I: A1

Instructions for Authors

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

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Online Submission

Authors should submit their manuscripts online. Electronic submission substantially reduces the editorial processing and reviewing times and shortens overall publication times. Please follow the hyperlink “Submit online” on the right and upload all of your manuscript files following the instructions given on the screen.

Title Page

The title page should include:

- The name(s) of the author(s)
- A concise and informative title
- The affiliation(s) and address(es) of the author(s)
- The e-mail address, telephone and fax numbers of the corresponding author

Abstract

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

Text Formatting

Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.

- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.
Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.
Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section before the reference list. The names of funding organizations should be written in full.

Terminology

Please always use internationally accepted signs and symbols for units (SI units).

Scientific Style

- Nomenclature: Insofar as possible, authors should use systematic names similar to those used by Chemical Abstract Service or IUPAC.
- Generic names of drugs and pesticides are preferred; if trade names are used, the generic name should be given at first mention.

References

Citation

Cite references in the text by name and year in parentheses. Some examples:

- Negotiation research spans many disciplines (Thompson 1990).
- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott 1991; Barakat et al. 1995; Kelso and Smith 1998; Medvec et al. 1999).

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

Reference list entries should be alphabetized by the last names of the first author of each work.

- Journal article

Harris, M., Karper, E., Stacks, G., Hoffman, D., DeNiro, R., Cruz, P., et al. (2001). Writing labs and the Hollywood connection. Journal of Film Writing, 44(3), 213–245.

- Article by DOI

Slifka, M. K., & Whitton, J. L. (2000) Clinical implications of dysregulated cytokine production. Journal of Molecular Medicine, doi:10.1007/s001090000086

- Book

Calfee, R. C., & Valencia, R. R. (1991). APA guide to preparing manuscripts for journal publication. Washington, DC: American Psychological Association.

- Book chapter

O'Neil, J. M., & Egan, J. (1992). Men's and women's gender role journeys: Metaphor for healing, transition, and transformation. In B. R. Wainrib (Ed.), Gender issues across the life cycle (pp. 107–123). New York: Springer.

- Online document

Abou-Allaban, Y., Dell, M. L., Greenberg, W., Lomax, J., Peteet, J., Torres, M., & Cowell, V. (2006). Religious/spiritual commitments and psychiatric practice. Resource document. American Psychiatric Association. http://www.psych.org/edu/other_res/lib_archives/archives/200604.pdf. Accessed 25 June 2007.

Journal names and book titles should be italicized.

Tables

- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.

Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

11. Anexo 3

Normas revista "Immunology Letters" - Fator de Impacto: 2,367; Qualis/CAPES

Ciências Biológicas I: B1

Types of paper

Research Articles - these are full length manuscripts that describe significant experimental findings. There is no page limit but articles may be returned for shortening at the Editors' discretion.

Current Views - these are (mini)-reviews which are usually invited by the Executive Editor responsible for Review articles.

Review articles - full reviews, also usually invited by the Executive Editor for Reviews, will cover a particular issue in depth with a comprehensive review of the literature. The liberal use of Figures is strongly encouraged. It is expected that a full length review will provide an up to date, comprehensive treatise on the subject matter with detailed citations to direct readers to the appropriate original work.

When submitting a Review, it is important to select the correct article type, also for Special Issue papers.

Letters to the Editor - these are notes sent to the Editor-in-Chief, raising a topic for discussion, providing an opinion or an hypothesis, or commentaries on previously published work, etc. Letters to the Editor do not need to follow the general arrangement instructions given below and should normally not exceed 800 words.

Contact details for submission

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Preparation

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Curriculum Vitae

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Formação complementar

2013 - 2013 Proficiência Técnica de Laboratório em Hematologia.
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2013 - 2013 Curso de curta duração em PCR em Tempo Real: Princípios Básicos e Aplicações.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil

Produção bibliográfica

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Orientações e supervisões concluídas

Trabalhos de conclusão de curso de graduação

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2. Manuela Andrade Costa. **Investigação dos Polimorfismos no Gene TGFBR3 (rs2038931, rs7526590) na Susceptibilidade à Ocorrência do Priapismo em Pacientes com Anemia Falciforme**. 2014. Curso (Biomedicina) - Universidade Federal de Pernambuco

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