UNIVERSIDADE FEDERAL DA BAHIA
FACULDADE DE MEDICINA
FUNDAÇÃO OSWALDO CRUZ
INSTITUTO GONÇALO MONIZ

Curso de Pós-Graduação em Patologia Humana e Experimental

TESE DE DOUTORADO

CEREBROVASCULOPATIA EM PACIENTES PEDIÁTRICOS COM ANEMIA FALCIFORME (HbSS): DIAGNÓSTICO PRECOCE E IDENTIFICAÇÃO DE BIOMARCADORES

CORYNNE STEPHANIE AHOUEFA ADANHO

Salvador– Bahia

2018

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Tese apresentada ao Curso de Pós-Graduação em Patologia Humana e Experimental para obtenção do grau de Doutor.

Salvador– Bahia

2018

Titulo da Tese: **"CEREBROVASCULOPATIA EM PACIENTES PEDIÁTRICOS COM ANEMIA FALCIFORME (HbSS): DIAGNÓSTICO PRECOCE E IDENTIFICAÇÃO DE BIOMARCADORES."**

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FONTES DE FINANCIAMENTO

"O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Código de Financiamento 001”

Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)
COSAU 2012. N º400005/2013-1 APQ

Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB)
(SUS0034/2013 and 8133/2014)

Instituto Gonzalo Moniz (IGM)-FIOCRUZ-BA

A minha família, por todo o amor recebido desde que nasci.

“At times our own light goes out and is rekindled by a spark from another person. Each of us has cause to think with deep gratitude of those who have lighted the flame within us. “

Albert Schweitzer

AGRADECIMENTOS

A Deus, por sua misericordia e sua bondade infinita por mim e minha familia durante esse caminho cuidando de meus passos! Que Ele seja louvado e adorado a cada batimento de meu coração por ter me sustentado e colocado pessoas maravilhas no meu caminho, para me ajudar e me reconfortar cada vez que preciso.

Aos meus pais, Parfait Adanho e Hilda Adonon, foi muito difficil estar longe de voces durantes esses 4 anos e 5 meses sem poder abraça-los fisicamente, mas sempre me senti amada, apoiada, através dos conselhos, ensinamentos e orações quotidianas. Obrigada por todo sacrificio que fizeram para que eu pudesse chegar aqui hoje! **Obrigado pela força que vocês me dão!!!!** Que honra a minha ter vocês como pais!!! Deus vos guarde e vos proteja sempre!!!

Aos meus irmãos queridos Axel, Ariel e Auréle obrigada pelo amor, pelas orações e confiança imbalável em minha capacidade! Por conseguir me fazer rire vero lado positivo das circunstâncias e sempre conseguir me reconfortar e ter coragem nos momentos difficeis. Obrigada por sempre me motivar e acreditarem mim. Deus abençõe os caminhos de vocês sempre e nos guarde sempre unidos!!!!

A minha avó Celine (*in memoriam)*, eu sei que de onde ela está, ela está muito feliz em me ver concluir esta etapa da minha vida. Obrigada pelo incentivo para que eu vá atrás de meus sonhos ! Que Deus te proporcione um descanço eterno!!!

Obrigada minha família por tudo.

Amooo vocês!!!

A Leonardo Gómez Bernal,obrigada por tudo que me ensinou durante a nossa convivência.Obrigada pelo respeito. Obrigada pelos cuidados que teve por mim. Obrigada por ser tão especial. Esse último ano do doutorado foi desafiante e teria sido muito mais sem seu apoio constante e sua presença a meu lado. São poucas palavras para expressar toda a minha gratidão e tudo que sinto. Deus te abençoe além de suas expectativas e te proteja sempre!!!

A Dona Terezinha Lima Santos, desde meus primeiros dias aqui no Brasil você foi como uma mãe para mim. Você fez de seus amigos os meus também. Jamais poderei esquecer as refeições deleitosas dadas de coração e os nossos passeios. Obrigada por me acolher na sua casa com tanta generosidade quando eu não tinha onde ir morar e por fazer com que me sentisse na minha casa. Aprendi muito com você sobre a beleza da generosidade e da amizade. Obrigada pelos ensinamentos, cuidados e conselhos que me deu. Que Deus te abençoe sempre e cuide de você.

Á minha querida orientadora, Dra. Marilda Gonçalves. Coordenadora do projeto CNPQ/Brasil-África. Pró, minha gratidão toda pela iniciativa e a todos os esforços envidados para fazer esse projeto se concretizar. Obrigada pela oportunidade de estar aqui no Brasil realizando esse sonho de criança de fazer pesquisa! Pelo carinho e cuidado por mim e meus amigos desde o inicio de nossa jornada aqui no Brasil. Obrigada pela generosidade da senhora em me ajudar tanto na minhas questões pessoais e de trabalho. Obrigada pela paciência durante todos esses anos. Obrigada pelos ensinamentos recebidos e pela exigência na minha formação.Obrigada pelo incentivo e por ser modelo de ética e profissionalismo! Que Deus abençoe a senhora sempre!

A Dra Isa Lyra minha coorientadora e Dra Ivana Leite do HUPES pela colaboração durante esse trabalho. Obrigada portodo carinho e ensinamento recebidos. Deus vos abençoe assim como toda a vossa equipe de trabalho.

A Dr. Camilo Viera por ter realizado todos exames de Doppler desse estudo. Obrigada pela disposição, ensinamentos e pelo seu professionalismo durante esse período. Deus abençoe a sua carriera e seus projetos.

Ao Professor Mohamed Chérif Rahimy e ao Professor Ambaliou Sanni da República do Benim, pela confiança, iniciativa e todo esforço consentido e porterme permitido fazer parte deste projeto, assim como meus amigos. Que Deus vos abençoe assim como a suas familias.

Aos membros da banca de qualificação que contribuiram muito para o aprimoramento desse estudo. Agradeço particularmente a Dra. Maria da Conceição

Chagas e a Mestre Ana Clara Campos pela preciosa ajuda nas análises de Classe latente.

Aos amigos e amigas dos laboratórios LIGHT e LPA, Carol, Karol, Camylla, Luciana, Rayra, Suelen, Karina, Luciana, Mateus, Rodrigo, Cleverson e as Dra(s). Junia, Milena e Elisangela,. Apprendi muito vivenciando com cada um de vocês. Vocês foram importante nesse trabalho. Obrigada por toda ajuda e a paciência comigo durante esses anos de convivência. Vocês facilitaram muito minha integração. Um agradecimento especial á Sanzio e Jeanne pela amizade e pela disponibilidade quando precisei de ajuda para coletaras amostras dos pacientes e pelos conselhos sábios compartilhados sobre as dificuldades atravessadas. Que Deus abençoe a todos segundo a Sua generosidade.

Ao meus queridos amigos do *coffee club* Dra. Dalila Zanette, Dr. VitorMafili e Dra. Fernanda Amarante pelos inumeráveis momentos de apoio e conselhos ao redor da xicará de café/chá. Vocês não imaginam quanto estou grata poresses momentos. Também agradeço pela ajuda e apoio de todo tipo que me proporcionam durante os momentos dificeis que passei. Vocês foram sempre disponíveis para me dar coragem e me dizer que tudo vai dar certo. Deus abençoe cada um de vocês sempre e vos recompense!!!!

Dra. Thassila Pitanga, obrigada pela ajuda, a aprendizagem e o treinamento durante o doutorado e, especialmente, durante o tirocinio docente. Obrigado por todos os ensinamentos recebidos. Obrigada também por todos os conselhos que me deu durante o doutorado. Que Deus te abençoe e a sua familia sempre.

Agradeço aos professores que gentilmente aceitaram compor a banca de avaliação deste trabalho, pelas sugestões e correções que contribuirão para a sua melhoria do presente. Sejam abençoados.

Aos amigos e amigas do Benin e da Nigeria que fizeram parte dessa aventura e com os quais compartilhei bastante essa parte da minha vida. Bartholomew, Francine, Ferreol, Jeanne, Roukiyath, Marie Laurence, Modeste e Uche. Irmãs e irmãos, grata pela ajuda de todos vocês nos momentos em que precisei. Um grande obrigada pela convivência fraternal, por todos os ensinamentos recebidos e pelos momentos alegres

e também pelos momentos mais dificeis compartilhados. Que Deus abençoe o caminho profissional e a vida de cada um de nós.

Não posso deixar de agradecer as senhoras da Rua irmã Dulce e irmãs da capela do Bom pastor que me acolheram com muita generosidade e carinho desde minha chegada na comunidade. Cada uma de você contribui para que me sentisse em familia. Que Deus abençoe cada uma de vocês e suas respectivas famílias, levarei para toda vida cada uma de vocês no meu coração e serei eternamente grata.

Ao Coral Santo Antônio, especialmente, Ana Elena Sodré que me introduziu com tanta generosidade e muito carinho. Sinto-me muito honrada por ter participado do coral e por ter convivido com artistas tão talentosos. As apresentaçoes foram lindas e tenho certeza que levamos alegria a várias pessoas da terceira idade. Vocês são maravilhosos!!!! Deus abençoe a todos vocês!!!

A meus amigos e amigas da Comunidade Shalom especialmente do grupo Shekina, Deliane, Rafael, Cacau, Lidiane, Marta e Muriel, obrigada por toda a amizade e o apoio incondicional. Quanto tenho agradecer pela amizade de vocês e pela alegria contagiante dos encontros nas quintas-feiras. Que bom ter vocês na minha vida para louvar a Deus !!!

A Dr Washington Luis Conrado dos Santos e sua esposa Dra. Nadia Khouri pela generosidade e o carinho comigo e os meus amigos. Quantas vezes nos acolheram na casa de voces e para participar do natal com a familia de vocês. Sempre vi em voces um modelo de casal cristão generoso e de profissionais competentes. Que Deus vos guarde e vos proteja sempre, assim como a toda familia!!!!

A Jean Tadeu Brito, e todos o técnicos e profissionais da Faculdade de Farmácia que foram essenciais na parte laborarorial da nossa pesquisa.

À IGM FIOCRUZ, por abrir suas portas e pela infraestrutura física e pessoal que possibilitaram a execução deste trabalho.

Ao pessoal da Plataforma de Sequenciamento do IGM/Fiocruz-BA.

Ao pessoal da Biblioteca de Ciências Biomédicas Eurydice Pires de Sant’Anna do IGM/Fiocruz-BA.

A Coordenação de Ensino do Curso de Pós-Graduação do PGPAT, pela administração e sempre pela disposição em ajudaros alunos.

A todo o pessoal da administração que sempre com muita boa vontade e muito carinho me atenderam em todos os momentos em que precisei de apoio; Rita, Jane, Taiane e Carol, Jurema, Dona Eugênia, do LASP-LIGHT, assim como Elze, Avani e Karina. Que Deus abençoe a todas.

Aos estudantes do PGPAT, particularmente à turma 2014.1 e seguinte pela amizade, carinho e ajuda durante os primeiros meses de minha chegada no Brasil.

A Sandra e Nellya por todas as conversas, apoio, amizade verdadeira, conselhos trocados durante esses anos,mesmo a distançia. pelo Whatapps

A Agnes, Daiana, Ana Carolina,Raquel e Mélie que cada uma me trouxeram sorrisos. Grata ter cruzados a vida de vocês.

Um agradecimento também aos profissionais Ulisses Reis e Blenda Ranieri que me ajudaram a superar momentos de desafios que passei.

Aos professores do curso de patologia Humana e experimental porterem sido os responsáveis poresta conquista pelo ensinamento de muita qualidade recebido aqui.

A todos os pacientes que aceitaram participar desse estudo e ao pessoal do ambulatório da HUPES e do CERDOFI , sem os quais tudo isso não teria sido realizado;

Ao CNPq, pela bolsa de estudo (processo no 400005/2013-1), obrigada pela disponibilidade financeira concedida, que foi de fundamental importância para realização da pós-graduação. Ao FAPESB e CAPES, pelo apoio financeiro concedido na execução desse estudo.

Obrigada Brasil e especialmente a Bahia por ter abrigado tanto momentos de felicidade e de crescimento.

ADANHO Corynne Stephanie Ahouefa. Cerebrovasculopatia em pacientes pediátricos com anemia falciforme(hbss): diagnóstico precoce e identificação de biomarcadores. 2018. 170 f. Tese (Doutorado em Patologia) - Universidade Federal da Bahia. Instituto de Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, 2018.

RESUMO

INTRODUÇÃO:O Acidente vascular cerebral (AVC) é uma das maiores complicações da Anemia Falciforme (AF). Ele afeta cerca de dez por cento dos indivíduos com menos de vinte anos e pode causar danos cerebrais permanentes. A hidroxiuréia (HU) é atualmente usada para tratar complicações da SCA e também para prevenir eventos de AVC. No presente trabalho, avaliamos a correlação entre marcadores laboratoriais e velocidades do Doppler Transcraniano (DTC) que é atualmente, o método padrão ouro para detecção de AVC em crianças com AF .O doppler transcraniano (DTC) mede as velocidades médias no tempo (VMMAX) na artéria carótida interna e nas artérias cerebrais médias. A estimativa da velocidade do fluxo sanguíneo arterial acima de 200 centímetros por segundo está associado ao risco elevado de AVC. **OBJETIVO** O objetivo principal deste estudo foi investigar marcadores bioquímicos, hematológicos e genéticos na AF pediátrica associado a possibilidade de ocorrência de AVC, correlacionando-os as velocidades DTC. **MATERIAL E MÉTODO** 163 pacientes com AF foram incluídos no estudo, com ou sem evento de AVC prévio, entre 2 e 18 anos no Complexo Hospitalar Universitário Professor Edgard Santos (Com-HUPES/UFBA) e no Centro de Referência de Doença Falciforme (CERDOFI), respectivamente localizados em Salvador e em Itabuna. Comparamos o fluxo sanguíneo cerebral em pacientes estratificados por: TCD1 - definido como normal, com VMMAX inferior a 170 cm / s; TCD2 - condicional, com VMMAX acima de 170cm / s, mas menorque 199cm / s; TCD3 - alto risco, com VMMAX maior ou igual a 200 cm / s. A análise da Classe Latente (LCA) foi realizada com base nos marcadores laboratoriais e foram modelados os perfis de classe inflamatória hemólise e perfil de lipidemico. Avaliamos o possível impacto prognóstico no desenvolvimento de altas velocidades em pacientes com AF de características clínicas e hematológicas. parametros tratados ou não com HU e dos seguintes genes: Metilenotrihidrofolato redutase (*MTHFR*), familia de receptores olfactivos 51 Membro da subfamilia B 6 (*OR51B6*), familia do citocromo P450 4

membros F da subfamilia 2 (*CYP4F2*), membro da familia do transportadorde anions do do portador de soluto B1 (*SLCOB1*) e Apolipoproteína B (*APOB*), bem como genótipo de α-talassemia e haplótipo de cluster gênico de βS-globina. **RESULTADO E CONCLUSÕES.** Não encontramos, em nosso estudo, o efeito dos genes analisados sobre a prevenção do AVC. Entretanto, estudos adicionais incluindo um número maior de pacientes que estiveram risco ou que desenvolveram AVC, são necessários para entender melhora influência desses genes no risco de AVC em pacientes com AF**.** Observamos correlações significativas (com Spearman r<-0,2 e p <0,05) entre VMMAX esquerdo e direito e parâmetros laboratoriais. O presente estudo mostra observamos correlação positiva (com Spearman r≤0,6 e p <0,05) entre NOx e VLDL (Very Low Density Lipoprotein) e triglicérides (TG) sugerindo que o óxido Nítrico (NO) pode serconsiderado um parâmetro relevante em relação à fisiopatologia do AVC em pacientes com AF. A correlação com os parâmetros do VMMAX e dos lipídios é negativa e significativa com o VLDL (r=-0,1743;p=0,0378) e triglicérides (r=-0176;p=0,0354).Os resultados também mostram correlações significativas entre DTC-VMMAX e marcadores laboratoriais de rotina no monitoramento dos pacientes pediátricos com AF. Também se observou que o tratamento com hidroxiureia (HU) teve um impacto muito interessante nos parâmetros laboratoriais pois, pacientes tratados com HU mostraram diminuição da contagem de leucócitos e linfócitos em todos os grupos estratificados, mas apenas de forma significativa comparando o grupo de alto risco tratado e aqueles não tratados. Na análise da Classe Latente (LCA) realizada,sessenta e dois porcento (62%) da nossa população foi encontrada na subclasse mais inflamatória e 52% da população como hipolipidêmico. Com base nessa observação, sugerimos que, em acidente vascular cerebral, será interessante avaliar o potencial terapêutico de lipídios direcionados na prevenção de risco nesse grupo populacional. Além disso, sugerimos que o subfenótipo dislipidêmico na população estudada deve ser considerado como de risco alto para o AVC.

Palavras-chave: Anemia falciforme; Acidente vascular cerebral; Ultrassonografia Doppler Transcraniana; Oxído nitrico; SNP.

ADANHO Corynne Stephanie Ahouefa. Cerebrovasculopathy in pediatric patients with sickle cell anemia (HBSS): early diagnosis and biomarkersearch. 2018. 170 f. Tese (Doutorado em Patologia) - Universidade Federal da Bahia. Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, 2018.

ABSTRACT

INTRODUCTION: Stroke is one of the major complications of Sickle Cell Anemia (SCA). It affects about ten percent of individuals under the age of twenty and can cause permanent brain damage. Hydroxyurea (HU) is currently used to treat complications of ACS and also to prevent stroke events. In the present work, we evaluated the correlation between laboratory markers and Transcranial Doppler velocities (TCD), which is currently the gold standard method for detecting strokes in children with SCA. Transcranial Doppler (TCD) measures Time-averaged maximum mean velocity (TAMMV) in the internal carotid artery and in the middle cerebral arteries. Estimation of arterial blood flow velocity above 200 centimeters per second is associated with a high risk of stroke. **OBJECTIVE:**The main objective of this study was to investigate biochemical, hematological and genetic markers in pediatric SCA patients associated with the risk of stroke, correlating them with TDC velocities. **MATERIAL AND METHOD**: 163 patients with SCA were included in the study, with or without a previous stroke, between 2 and 18 years old at the University Hospital Complex Professor Edgard Santos (Com-HUPES / UFBA) and the Reference Center for Sickle Cell Disease (CERDOFI), respectively located in Salvador and Itabuna. We compared the cerebral blood flow in patients stratified by: TCD1 - defined as normal, with TAMMV lower than 170 cm / s; TCD2 - conditional, with TAMMV above 170cm/s, but lower than 199cm / s; TCD3 - high risk, with TAMMV greater than or equal to 200 cm / s. The Latent Class (LCA) analysis was performed based on the laboratory markers and the profiles were modeled from inflammatory class hemolysis and lipidemic profile. We evaluated the possible prognostic impact in the development of high velocities in patients with clinical and hematological characteristics. parameters treated or not with HU and of the following genes: Methylenetrihydrofolate reductase (*MTHFR*), family of olfactory receptors 51 Member of subfamily B 6 (*OR51B6*), family of cytochrome P450 4 F members of subfamily 2 (*CYP4F2*), member of the transporter family anions of the solute carrier B1 (*SLCOB1*) and Apolipoprotein B (*APOB*), as well as α-thalassemia and

haplotype genotype of βS-globin gene cluster. **RESULTS AND CONCLUSIONS**. We did not find in our study, the effect of genes analyzed on stroke prevention. However, additional studies including a larger number of patients who were at risk or who developed strokes are needed to understand the improved influence of these genes on the risk of stroke in patients with AF. We observed significant correlations (with Spearman r <-0.2 and p <0.05) between left and right TAMMV and laboratory parameters. The present study shows a positive correlation (with Spearman r≤0.6 and p <0.05) between NOx and VLDL (Very Low Density Lipoprotein) and triglycerides (TG) suggesting that nitric oxide (NO) can be considered a relevant parameter in relation to the pathophysiology of stroke in patients with FA. The correlation with the parameters of TAMMV and lipids was negative and significant with VLDL (r = -0.1743; p = 0.0378) and triglycerides (r = -0176; p = 0.0354). The results also show significant correlations between TCD-TAMMV and routine laboratory markers in the monitoring of pediatric patients with SCA. It was also observed that hydroxyurea (HU) treatment had a very interesting impact on laboratory parameters since patients treated with HU showed a decrease in leukocyte and lymphocyte counts in all stratified groups but only in a significant way comparing the high risk group treated and those not treated. In the Latent Class (LCA) analysis, sixty-two percent (62%) of our population was found in the most inflammatory subclass and 52% of the population as hypolipidemic. Based on this observation, we suggest that in stroke, it will be interesting to evaluate the therapeutic potential of lipids directed at risk prevention in this population group. In addition, we suggest that the dyslipidemic subphenotype in the studied population should be considered as high risk for stroke.

Keys-word: Sickle cell anemia; Stroke; Transcranial doppler; Nitric oxide;SNP

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A1AT	alfa 1 antitripsina
ACA	artérias cerebrais anteriores
ACI	artérias carótida Interna
ACM	artérias cerebrais médias
AcoA	artéria comunicante anterior
AcoP	artéria comunicante posterior
ACP	artéria cerebral posterior
AF	anemia falciforme
ALT	alanina amino transferase
ATP	adenosina trifosfato
ARM	angioressonância magnética
AST	aspartato amino transferase
ATP	adenosina trifosfato
AVC	acidente vascular cerebral
BEN	haplótipo de tipo Benin
CAR	haplótipo de tipo Bantu
CEP	comitê de ética em pesquisa
CHCM	concentração de hemoglobina corpuscular média
Com-HUPES	complexo Hospitalar Universitário Professor Edgard Santos
CVA	acidente vascular cerebral (do inglês: *Cerebral VascularAccident*)
DF	doença falciforme
DNA	acído Desoxiribonucleico
DTC	doppler transcraniano

EDTA	ácido etileno diamino tetracético
EROs	espécies reativas de oxigênio
FDA	agência de alimentos e administração de droga (do inglês: *Food and drug Administration)*
Fe^{2+}	átomo de ferro ferroso
FIOCRUZ	fundação Oswaldo Cruz
Hb	hemoglobina
HbA	Hemoglobina A
HBB	gene da globina beta
HbF	hemoglobina fetal
HbS	hemoglobina S
HCM	hemoglobina corpuscular média
HDL	lipoproteína do colesterol de alta densidade (do inglês: *high-density lipoprotein*)
HPLC	cromatografia liquída de alta eficiência
Ht	hematócrito
HU	hidroxiuréia
LDH	Desidrogenase lática
LDL	lipoproteína do colesterol de baixa densidade (do inglês: *low-density lipoprotein*)
MCH	hemoglobina Corpuscular Média (do inglês: *mean corpuscular hemoglobin*)
MHz	Mega Herz
MS	Ministério da Saúde
N	Átomo de Azoto
NO	Óxido nítrico
NO_2^-	Íon nitrito

NO_3^-	Íon nitrato
NOS	Enzima óxido nítico sintase
OMS	Organização Mundial da Saúde
PCR	Reação em cadeia da polimerase (do inglês: *polymerase chain reaction*
P-CR	Proteína-C reativa
PCR-RFLP	PCR seguido de Análise do comprimento de fragmentos de restrição (do inglês: *restriction fragment length polymorphism*)
PHHF	Persistência Hereditária de HbF
RBC	*Hemácias* (do inglês: *Red Blood Cells*)
SEN	Haplótipo de tipo Senegal
STOP	Avaliação de prevenção do AVC na doença *falciforme* (do inglês: *Stroke Prevention Trial in Sickle Cell Disease*)
STA	Síndrome torácica aguda
Tal α	Talassemia alfa
VMMAX	Média de velocidade máxima em função do tempo (do inglês: *Time-averaged maximum mean velocity*)
TCD	Dopplertranscraniano (do inglês: *Transcranial Doppler*)
TCLE	Termo de consentimento livre e esclarecido
TG	Triglicérideos
UV	Ultravioleta
VCM	Volume corpuscular médio
VLDL:	Lipoproteína do colesterol de muita baixa densidade (do inglês: *very low-density lipoprotein*)
WBC:	*White Blood Cells*
WHO:	Organização Mundial de Saúde (do inglês: *World Health Organization*)
α:	Alfa
β:	Beta
γ:	Gama

SUMÁRIO

1. INTRODUÇÃO

A anemia falciforme (AF) (do latim *falci-*, foice e *-forme*, formato de) é conhecida na África há séculos, com diferentes nomes, onde inspirou muitas lendas nas diferentes populações onde era encontrada (KONOTEY-AHULU, 1974; ONWUBALILI, 1983; STUART e NAGEL, 2004). Nos Estados Unidos da América, Lebby em1846, e Hodenpyl em 1898, definiram a AF como uma “doença estranha” que causava atrofia no baço (SIDDIQI; JORDAN;pARKER, 2013). Em 1910, James Herrick identificou, pela primeira vez, os eritrócitos em foice, em um estudante oriundo de Granada, solucionando parte dos questionamentos provocados pela doença. Desde então, aprendeu-se muito sobre a doença falciforme (DF) através de estudos laboratoriais e clínicos desenvolvidos durante o século XX (HERRICK 2014).Vernom Ingram (INGRAM, 1958) descobriu que as características clínicas apresentadas pelos pacientes com DF eram decorrentes da mutação no gene *HBB*, que codifica a cadeia beta (β) da globina, com substituição do ácido glutâmico porvalina na sexta posição da cadeia β (Glu6Val, rs334) (**FIGURA 1**) (PIEL, STEINBERG, e REES 2017) ;STUART e NAGEL, 2004).

A hemoglobina S (HbS) é uma variante da hemoglobina normal (HbA), sendo esta última, presente nos indivíduos saudáveis.O indivíduo com AF possui herança autossômica recessiva caracterizada pela presença da HbS e de hemácias em forma de foice quando em presença de ambiente com redução de oxigênio ou hipóxia. Quando a HbS está associada a outras hemoglobinas variantes ou de hemoglobinopatias de síntese, a terminologia é de DF (KATO et al., 2018; WIYEH et al., 2018).A AF, diferentemente de outros genótipos da DF, apresenta sintomas clínicos mais complexos e mais graves, tais como crises álgicas, síndrome torácica aguda (STA), aumento da sensibilidade a infecções, acidente vascular cerebral (AVC), entre outros. Segundo estimativas (PIEL, STEINBERG, e REES 2017; KATO et al., 2018), cerca de 300.000 indivíduos nascem mundialmente com a AF.Em 2015, foi estabelecido que esses nascimentos ocorriam, principalmente, em três países, Nigéria, República Democrática do Congo e na Índia (PIEL *et al.* 2013;pIEL, STEINBERG, e REES 2017).O AVC é uma complicação importante da AF, sendo que cerca de 11%

das crianças com a doença estão em risco de apresentaro evento antes dos 20 anos de idade (OHENE-FREMPONG *et a*l., 1998; BREWIN, KAYA, e CHAKRAVORTY 2017). O Doppler transcraniano (DTC) foi estabelecido desde os estudos de ADAMS (ADAMS *et al*,. 1988; ADAMS *et al.,* 1992), como ferramenta para identificar pacientes com risco para ocorrência de AVC e para prevenção do primeiro evento. O DTC por insonância ou ultrassom, avalia a velocidade média máxima nas artérias carótidas e identifica os pacientes que possuem velocidade aumentada e determina indicadores que estimam o risco de desenvolvimento de AVC (BREWIN, KAYA, e CHAKRAVORTY 2017; KATO *et al*., 2018). Apesardo exame de DTC sermuito sensível na detecção do risco de AVC, ele possui falhas e, de fato, cerca de 60% dos indivíduos que apresentam alto risco de AVC, detectado pelo DTC, poderão não apresentaro evento (ADAMS et al., 1997). Como tratamento para o AVC, a hidroxiuréia (HU) é uma das drogas utilizadas no tratamento da AF, sendo comprovadamente associada a melhoria dos sintomas em decorrência da redução na frequência de eventos agudos e na prevenção e reversão de algumas complicações, como os eventos de AVC e de vaso-oclusão (WARE et al., 2016).

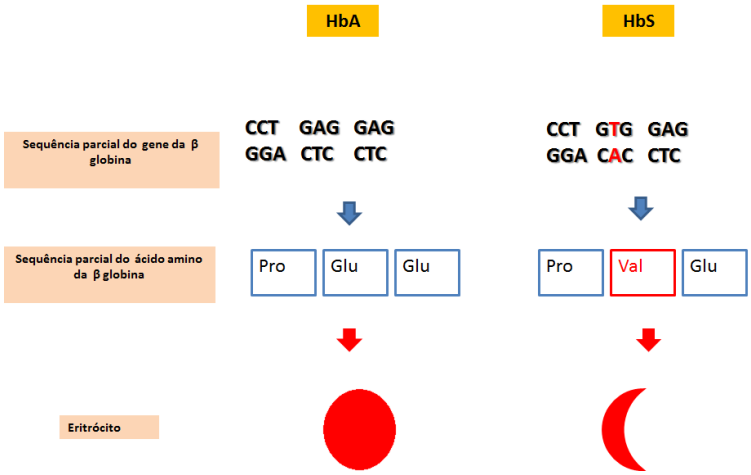

FIGURA 1. Mutação de ponto, com formação do alelo beta S (βS), no gene da globina beta (*HBB*). A HbS resulta da mutação de ponto que leva a substituição de adenina portimina (GAG > GTG), no sexto códon do alelo βS, com substituição do ácido glutâmico porvalina, na posição 6 da cadeia da globina beta.

2. REVISÃO DE LITERATURA

2.1. DISTRIBUIÇÃO E EPIDEMIOLOGIA DO ALELO β^S

A presença do alelo β^S, em quase todos os continentes, está associada a dois fatores: 1) a pressão genética ocasionada pelo *Plamodium sp* (microorganismo responsável pela malária) e 2) as migrações populacionais que ocorreram através da história da humanidade. Considerando os mapas, observa-se a coincidência entre as áreas do alelo β^S e as regiões que são ou eram endêmicas para a malária (**FIGURA 2**) (STUART e NAGEL, 2004; REES *et al.,* 2010; PIEL *et al*., 2010.

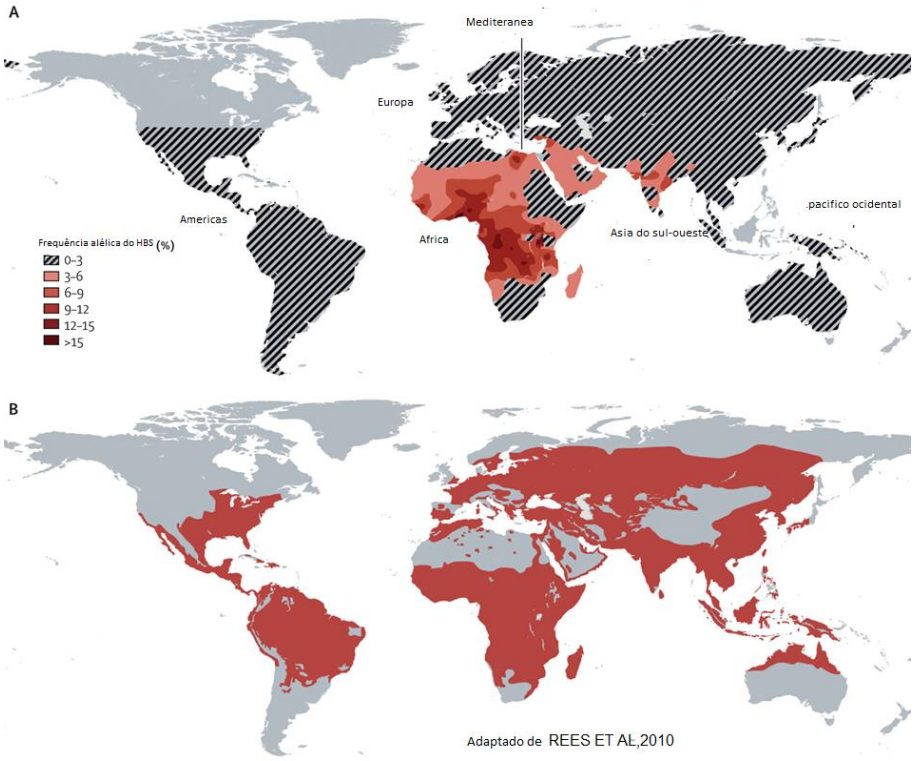

FIGURA 2.**Comparação das áreas de distribuição da malária e do alelo beta S no mundo (A)** o mapa monstra a distribuição do alelo beta S. No mapa o continente africano possui as maiores frequencias do alelo beta S(0 até <15%),enquanto na Europa e nas Américas, as frequências variam de 0-3%. (B) o mapa representa as regioes endêmicas para a malária e hemoglobina S (HbS) (adaptado de REES *et al.,*2010).

Independentemente da correlação geográfica entre a frequência do alelo β^S em populações africanas e a incidência histórica de malária (FIGURA 2), existem várias

outras evidências, especialmente aquelas obtidas em ensaios *in vitro,* sobre a resistência apresentada pelos portadores da hemoglobina (HbAS) (REES, WILLIAMS, e GLADWIN, 2010). Porém, o mecanismo exato dessa proteção ainda não está totalmente esclarecido (BILLO et al., 2012), alguns autores sugeriram que a HbS induz a expressão da heme oxigenase, que por sua vez produz monóxido de carbono. que confere tolerância à malária (FERREIRA et al., 2011). As migrações populacionais favoreceram a dispersão do alelo β^S para várias partes do mundo como o indica a sua presença nas Américas, especialmente no Caribe, Estados Unidos da América, México, Colômbia e Brasil, onde milhões de pessoas oriundas do continente africano foram deportadas para trabalharem plantações de açúcar, café ou algodão (FONG *et al.,* 2013; MAGAÑA *et al.*, 2005; CANÇADO e JESUS, 2007). As migrações de escravos de ascendência africana e seus destinos nas Índias Ocidentais e Américas ocasionaram a miscigenação racial da população atual. Na Europa Ocidental a ocorrência do alelo mutante β^S é decorrente de fluxo migratório relativamente recente (REES, WILLIAMS, e GLADWIN., 2010).

Atualmente, o alelo β^S tem frequência de 0-15% na população africana. Na Europa e na America do Norte ele está presente em cerca de 0-3% (REES, WILLIAMS, e GLADWIN, 2010;PIEL, STEINBERG, e REES., 2017). No Brasil, o grau de miscigenação racial é elevado porconta das migrações que ocorreram no país. Por exemplo, as regiões Norte e Nordeste apresentam taxa elevada de afrodescedência (PARRA *et al.,* 2003). Nessa população, são encontradas frequências mais elevadas de heterozigotos AS (HbAS), correspondendo a 6% até 10%, enquanto as demais regiões brasileiras apresentam menos de 5% (CANÇADO e JESUS, 2007).De acordo com os dados do programa nacional de triagem neonatal realizada pela Associação de Pais e Amigos dos Excepcionais (APAE) – BA sabe-se que a incidência e prevalência da DF na Bahia é uma das maiores do Brasil, acometendo um a cada 645 nascidos-vivos (SILVA *et al*., 2006).

2.2. ESTRUTURA DA HEMOGLOBINA S (HbS)

A hemoglobina (Hb) é a proteína responsável pelo transporte do oxigênio dos pulmões para os tecidos, é composta poruma parte proteíca, a globina, que possui dois grupos

de cadeias conhecidas como cadeias de globina que podem serdo tipo alfa (α) e não-alfa, com 141 e 146 aminoácidos, respectivamente (MARENGO-ROWE, 2006; SCHWARZE et al., 2014). A parte não protéica da molécula de Hb é denominada heme e apresenta estrutura tetracíclica, composta pela protoporfirina IX, que está ligada a um átomo de ferro ferroso ou Fe^{2+}.O oxigênio liga-se reversivelmente a estes átomos de ferro, sendo transportado através do sangue; o íon ferroso do heme está ligado ao azoto (N) de uma histidina **(FIGURA 3).**

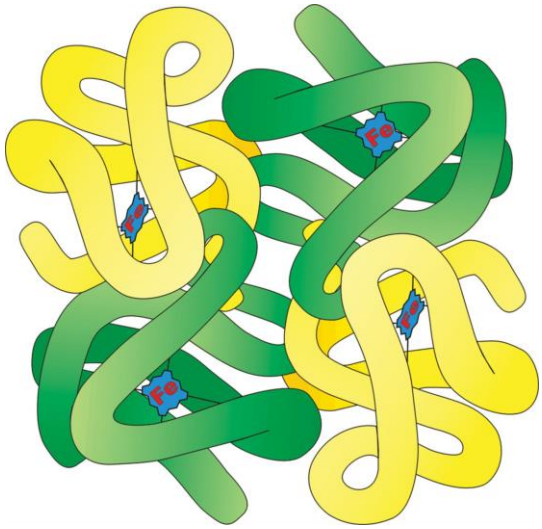

FIGURA 3. Molécula de hemoglobina e um grupo heme da hemoglobina . A hemoglobina é uma metaloproteína globular, que contém 4 cadeias duas alfa e duas beta. E possui um grupo heme ligado a cada uma das cadeias de globina. O ferro se liga ao grupo heme. Na figura as cadeias α estão em verde e as cadeias β em amarelo, o complexo heme-ferro está em azul (THOMAS; LUMB, 2012).

Todas as hemoglobinas humanas embrionárias e adultas tem 2 cadeias tipo α- e 2 tipo β, com alternância coordenada dos genes tipo α e β durante os estágios de desenvolvimento embrionário, fetal e adulto: Hb Gower-1 (ζ2ε2), Gower-2 (α2ε2), Portland-1 (ζ2γ2) e Portland-2 (ζ2β2), Hb F (α2γ2), Hb A (α2β2) e HbA2 ($\alpha_2\delta_2$) (HE; RUSSELL, 2001; MARENGO-ROWE, 2006).

Em certas condições existem deleções e mutações nos genes responsáveis pela síntese das cadeias globínicas, resultando na redução ou ausência total de uma ou mais cadeias, sendo denominadas talassemia (KOHNE, 2011; THEIN, 2013).Também existem outras hemoglobinopatias, nas quais as mutações, que podem estar associadas a substituições, inserções ou deleções de nucleotídeos, afetam as regiões codificantes dos genes e levam à formação de Hbs variantes. As variantes mais

comuns e clinicamente relevantes são: HbS (β6 Glu → Val), HbC (β6 Glu → Lis), HbD (β121 Glu → Gln) e HbE (β26 Glu → Lis) (KATO *et al*., 2018; THÉBERGE *et al*., 2015; THEIN, 2013). A denominação DF é um termo que envolve toda hemoglobinopatia na qual ocorre a associação de um alelo β^S com outra hemoglobina variante, tais como HbC, D, E, ou talassemia (STUART e NAGEL, 2004; REES, WILLIAMS, e GLADWIN., 2010). Existem também os casos onde a HbA está associada a hemoglobinas variantes, a mais comun é a HbS, com formação do chamado traço falciforme ou heterozigoto AS (HbAS) (FONG *et al*., 2013; JOHN, 2010).

2.3. O FENÔMENO DE POLIMERIZAÇÃO DA HbS

A homozigose para o alelo β^S (HbSS), também referida como AF, tem expressão clínica bem mais grave que em outros tipos da DF. Isto é devido, em grande parte, à variação celular da concentração de HbS que tem propensão a polimerização (THEIN, 2013). A polimerização da HbS leva a falcização da hémacia, que é reduzida na presença de concentrações elevadas de HbF (MARENGO-ROWE, 2006; SCHECHTER;2008; THEIN, 2011). *In útero*, a HbF é o principal constituinte das hemacias do feto e ao nascimento, sendo que a produção de γ-globina é normalmente reduzida, mas uma fração significativa de HbF aind está associada a Hb da vida adulta ou HbA (SCHECHTER, 2008). A HbF inhibe a polimerização da HbS porque ela se hibrida a esta, formando um complexo estável, diminuindo assim a polimerização da HbS (STUART e NAGEL 2004; THEIN, 2013). Os eritrócitos falcizados são encontrados na microcirculação quando expostos a concentrações reduzidas de oxigênio, desidratação e acidose. A presença de hipóxia dá origem a moléculas de desoxi HbS, que têm estrutura diferente da HbS oxigenada (oxyHbS) (MARENGO-ROWE, 2006; ILESANMI, 2010). A substituição do ácido glutâmico (glu), um aminoácido polar, pela valina (val), que porsua vez é apolar, leva ao aumento na formação de agregados de HbS entres eles, onde a fenilalanina (β-85) de uma HbS se liga a leucina (β-88) da HbS adjacente (ADACHI et al., 1994) no interiordo eritrócito.A HbS desoxigenada forma polímeros no interiordo eritrócito, modificando o seu citoesqueleto e diminuindo a sua deformabilidade celular, tornando o eritrócito rígido e em formato de foice (ALEXY *et al.*, 2010). A polimerização é um fenômeno cíclico e reversível, sendo que o eritrócito falcizado readquire o formato bicôncavo quando alcança um ambiente altamente

oxigenado, como os pulmões. A alternância destes ciclos acaba causando danos permanentes ao citoesqueleto e a membrana eritocitária que expõem a banda-3, espectrina, fosfatidilserina e glicoproteína IV (CD36) (LIU *et al*., 1996; YASIN *et al*., 2003). Por consequência, as hemácias tornam-se irreversivelmente falcizadas, estado que altera a capacidade do eritrócito em atravessaros vasos na circulação, sendo mais propicio a lise (PRESLEY *et al*., 2010; TARASEV *et al.*, 2017).A falcização dos eritrocitos reduz em cerca de 16 a 20 dias a sua sobrevida, quando comparados aos eritrócitos normais, os quais possuem em média 80 a 120 dias de vida (SERJEANT ,1997). Essa sobrevivência reduzida da hemácia falcizada está associada a sua destruição no baço ou no fígado, por intermédio das células do sistema fagocitário mononuclear que reconhecem as proteínas expostas. Deste modo, a hemólise mecânica mediada pelo baço (hemólise extravascular) e a hemólise intravascular que libera grande quantidade de arginase e produtos da lise eritrocitária nos vasos, hemoglobina/heme e ferro, explicam essa meia vida reduzida da hemácia falcizada (BROUSSE; BUFFET; REES, 2014; MISZTAL; TOMASIAK, 2011). Outra consequência associada a lise dos eritrócitos é o aumento da produção medulare da liberação de reticulócitos na corrente sanguínea (KAUSHAL et al., 2016).

2.4. FISIOPATOLOGIA DA ANEMIA FALCIFORME

A Anemia Falciforme possui grande diversidade fenotípica, mesmo em indivíduos com o mesmo genótipo. Essa diversidade clínica vem sendo atribuída a fatores socioeconômicos, ambientais, e fatores genéticos, tais como os haplótipos ligados aos genes da globina β, presença de talassemia alfa (Tal α), concentrações de Hb Fetal (HbF) e pela presença de mutações ou polimorfismos em diferentes genes (GONÇALVES *et al.,* 2003; THEIN, 2011;pIEL, STEINBERG, e REES, 2017).

Os indivíduos com AF são mais vulneráveis a infecções, principalmente as bacterianas, que se constituem em uma das maiores causas de internações; de fato, as crianças com AF são suceptíveis a desenvolver infecções provocadas pelo *Streptococcus pneunomiae e Hemophilus influenzae* devido a perda frequente da função esplênica. Além da sucetibilidade a infecções baterianas, os indivíduos com AF podem apresentar retardo de crescimento e várias complicações associadas à morbidade e mortalidade infantil são frequentemente observadas em crianças com a doença (GASTON *et al*.,

1986; RANKINE-MULLINGS e OWUSU-OFORI, 2017;PIEL, STEINBERG, e REES 2017).

A patogênese da Anemia Falciforme está associada as várias modificações fisico quimicas nas hemácias falcizadas, tais como danos na membrana eritrocitária e aumento na expressão de moléculas de adesão que vão desencadear eventos de vaso-oclusão e anemia hemolítica crônica associada a processos de isquemia-reperfusão, disfunção endotelial, hipercoabilidade, extresse oxidativo e inflamação que poderão induzir alterações em diferentes orgãos (BALLAS *et al*., 2010;PIEL, STEINBERG e REES, 2017). A anemia crônica observada na AF pode ser resultado de três causas que são a hiperhemólise, sequestro esplênico e crises aplásticas. A hiperhemolise é causada pela destruição exacerbada das hemácias devido ao sequestro esplênico ou hepático, podendo estar associada ao aumento da produção de reticulócitos (BALLAS *et al*., 2010). O sequestro esplênico (SE) é considerado como uma das causas mais importantes de óbito em criancas com AF, ele pode ocorrer já nas primeiras 8 semanas de vida e pode estar associado a infecções latentes. Durante o SE, occore o sequestro de hemácias (falcilizadas ou não) e de outros componentes do sangue pelo baço, o que ocasiona a queda no nível de Hb, podendo estar associada a trombocitopenia relativa e hipovolemia (BALLAS *et al*., 2010). As crises aplásticas, que são responsáveis por anemia crônica, podem ser explicadas pela supressão total ou parcial da eritropoese, o que pode evoluir rapidamente para anemia grave. A crise aplástica pode ser decorrente de infecções ou de inflamação, que são fenômenos bem comuns na AF (BALLAS *et al.,* 2010). As crises álgicas são uma das caracteristicas principais da AF, pois elas ocorrem bem precocemente, podendo ser provocadas pelos fenômenos vaso-oclusivos. No fenômeno de vaso-oclusão, a oclusão de vasos sanguíneos causa lesão tecidual e inflamação no sítio da oclusão (STUART e NAGEL, 2004; BALLAS *et al.,* 2010). A inflamação pode favorecer a hipercoagulabilidade e vasculopatia, enquanto a oclusão dos vasos pelas hemácias falcizadas pode levara danos e isquemias teciduais. A interação entre as hemácias e o endotélio vascular pode levar a ativação endotelial e a adesão de hemácias, leucócitos e plaquetas à parede vascular, reduzindo a microcirculação vascular (STUART e NAGEL, 2004; BALLAS *et al*., 2010; SEGEL, HALTERMAN e LICHTMAN, 2011).

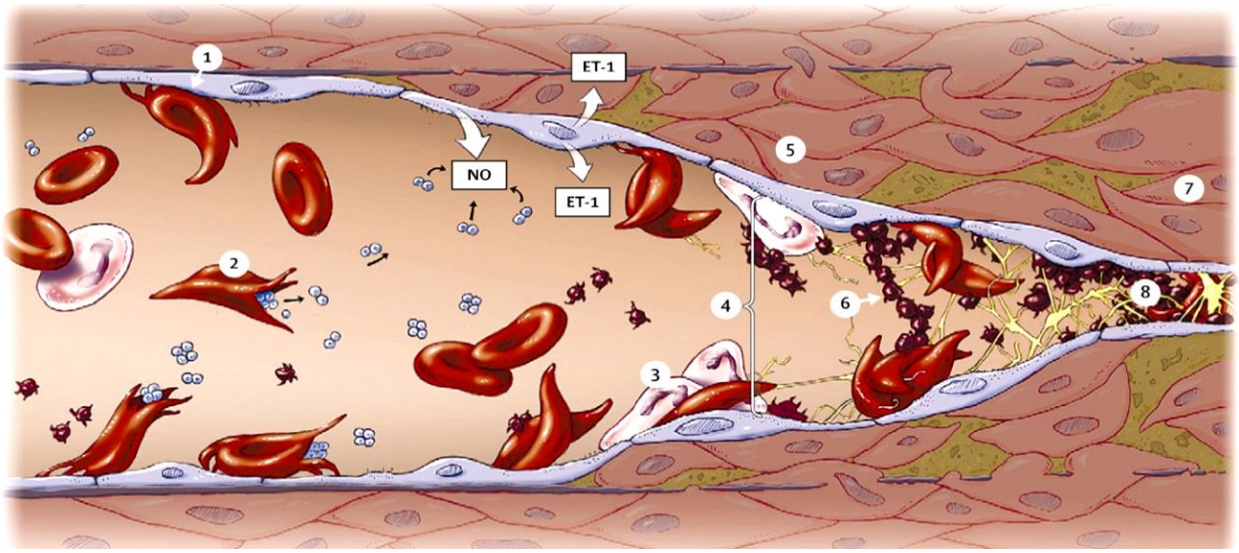

FIGURA 4. Vasculopatia e acidente vascularcerebral na doença falciforme. O eritrócito falciforme é proeminente no desenvolvimento de doença cerebrovascular como resultado da adesão anormal ao endotélio vascular(1) e hemólise (2). Esses fatores resultam no estado pró-inflamatório manifestado, em parte, pela adesão de leucócitos (3) e agregação plaquetária (6). A secreção aumentada de endotelina (ET-1) e a eliminação de óxido nítrico (NO) pordímeros de hemoglobina livres resultam no aumento do tônus vasomotor(4). O estreitamento luminal ocorre secundariamente à proliferação de células musculares lisas e fibroblastos dentro da camada íntima (5). O resultado final é vasculopatia (7) e oclusão (8) (Adaptado de SWITZERet al., 2006).

Quanto ao processo de vaso-oclusão, ele consiste na oclusão de microvasos em vários orgãos e leva a dor, danos teciduais e inflamação local. As hemácias falcizadas expressam moléculas de adesão que vão aumentara sua aderência à parede vascular; nesse meio, occore a ligação de reticulócitos pró-adesivos ao endotélio com ligações secundárias de hemácias falcizadas e também de leucócitos e plaquetas, que formam agregados com as hemácias diminuindo o fluxo sanguíneo (**FIGURA 4**) (FRENETTE e ATWEH, 2007; BALLAS *et al.*, 2010; SEGEL, HALTERMAN, e LICHTMAN, 2011).

O estresse oxidativo também é considerado modulador da AF pois influencia a vaso-oclusão em decorrência do aumento da adesão dos eritrócitos, leucócitos e plaquetas ao endotélio vascular (NURERFAN *et al*., 2011; CHIRICO e PIALOUX, 2012; SILVA *et al.,* 2013).

Também influenciada pelo estresse oxidatívo, a hemólise intravascular é considerada como um dos maiores eventos que ocore na AF, contribuindo para o aumento das concentrações de Hb livre no plasma bem como das espécies reativas de oxigênio (EROs) e peroxidação lipídica (NURERFAN *et al.*, 2011; SILVA *et al*. 2013).

2.5. ÓXIDO NÍTRICO

As EROs reduzem o NO, que é um gás que participa de vários eventos fisiológicos, atuando tanto como vasodilatadore neurotransmissor, quanto na ativação das plaquetas e expressão de moléculas de adesão no endotélio vascular. O NO é uma molécula extremamente reativa devido a presença de um radical livre que ele possui. O NO tende a se ligar preferencialmente ao heme e, por consequente, à hemoglobina (SNYDER; BREDT, 1992).

O NO é gerado a partirda L- arginina na presença do oxigênio, pela catálise da enzima óxido nítrico sintase (NOs). Os metabólitos do NO, $NO3^-$ e $NO2^-$ já foram considerados produtos finais inertes devido à sua oxidação, mas estudos recentes demonstraram que eles são novamente reciclados a NO (LUNDBERG 2005; YUYUN, et al., 2018). O NO estimula a enzima guanilato ciclase e tem a formação de monofosfato cíclico de guanosina (cGMP)(NATHAN; XIE, 1994) e, assim, diminui a proliferação do músculo liso e da parede endotelial, limitando as lesões de isquemia-reperfusão e modulando a proliferação endotelial e a inflamação (MORRIS 2008; YUYUN, et al 2018). A redução de NO em doenças vasculares reduz a perfusão tecidual e promove a formação de trombo, enquanto o aumento da produção de NO contribui para a vasodilatação pronunciada. Concomitamente, associada a redução da atividade plaquetária, a homeostase encontra-se desequilibrada (SCHMIDT et al., 1991).

O NO pode modificardiferentes proteínas, lipídios e ácidos nucléicos e, também, pode reagircom metais de transição (SHEN et al., 1995). Em caso de crises vaso-oclusivas, a redução dos níveis plasmáticos de NO é um fator de risco associado ao desenvolvimento da hipertensão pulmonar, priapismo, úlceras de membros inferiores e, possivelmente, de acidente vascular encefálico isquêmico (MORRIS 2008; KATO *et al*. 2009; KATO, STEINBERG, e GLADWIN 2017) .

2.6. O ACIDENTE VASCULAR CEREBRAL

O AVC é caracterizado pela perda rápida de função do cérebro devido a uma perturbação no fornecimento sanguíneo, que pode ser causada por obstrução ou rompimento de vaso. O AVC é a segunda causa de óbito e a terceira causa de perda da

função motora em todo o mundo (OMS, 2016). O AVC isquêmico é o tipo de AVC mais comum, no qual há obstrução decorrente de coagulação em vaso sanguíneo cerebral, causando dificuldade de entrada do sangue arterial no cérebro. Na população geral as principais causas são trombose e oclusão das artérias, mas também ocorre oclusão de pequenas artérias e veias. A anemia grave e a hiperviscosidade encontrada em pacientes com AF são citadas como possíveis causas de AVC (MENAA 2013; KATO, STEINBERG e GLADWIN 2017).

O AVC hemorrágico é o mais raro, sendo caracterizado pela ruptura de um vaso sanguíneo intracraniano ou por estrutura vascular anormal, porém está associado ao aumento de mortalidade maior que o AVC isquêmico na população geral (OHENE-FREMPONG *et al.*, 1998; MENAA, 2013;PIEL, STEINBERG e REES 2017)

Quanto ao AVC silencioso, ele afeta de 17 a 27% da população pediátrica com AF (BERNAUDIN *et al.*, 2011; DEBAUN e KIRKHAM, 2016). Os pacientes que sofrem de AVC silencioso não apresentam sintomas perceptíveis, mas podem apresentar danos cerebrais e sinais de lesão neurológica detectáveis nos achados radiológicos. O AVC silencioso pode contribuir para a deficiência cognitiva e afetar a capacidade intelectual e, consequentemente, o rendimento acadêmico.

Além disso, também representa fatorde risco para o AVC sintomático (MILLER*et al*., 2001; BERNAUDIN *et al.*, 2011; DEBAUN *et al*., 2012; MENAA, 2013; DEBAUN e KIRKHAM, 2016).O AVC isquêmico transitório possui sinais neurológicos com distribuição vascular, sendo que os sintomas podem serresolvidos dentro de 24 horas (MENAA, 2013).O AVC isquêmico é uma das principais complicações vasculares da AF e afeta cerca de 11% dos indivíduos com idade inferiora vinte anos (OHENE-FREMPONG *et al*., 1998; MENAA, 2013; DEBAUN *e* KIRKHAM, 2016). Ocorre geralmente na primeira década de vida, com incidência de 1% a 2% ao ano entre os dois e cinco anos de idade, com queda da incidência na segunda década, sendo 0,41% porano até atingir 24% aos 45 anos. O risco é menorem indivíduos com idade abaixo de 2 anos, provavelmente, devido à proteção conferida pela HbF (OHENE-FREMPONG *et al*., 1998; MENAA, 2013; DEBAUN e KIRKHAM, 2016). O AVC hemorrágico tem incidência menorem crianças com AF, com 3% dos casos nessa faixa etária. Porém, torna-se mais prevalente após os 29 anos.

Foram relatados índices reduzidos de mortalidade pós AVC isquêmico, ao contrário do que ocorre com o AVC hemorrágico, que apresenta mortalidade de 25% nos primeiros dias após o evento (OHENE-FREMPONG *et al*., 1998; SWITZER*et al.,* 2006). Porém, sem tratamento, o AVC isquêmico apresenta 66% de recorrência durante os 2 ou 3 anos após o primeiro AVC (DeBAUN *et al*., 2012; MENAA, 2013). A vasculopatia com estenose dos grandes vasos cerebrais, do Polígono de Willis, apresentada na parte distal da artéria carótida interna (ACI) e das regiões proximais das artérias cerebrais médias (ACM) e anteriores (ACA), é responsável pela maioria dos eventos agudos (SWITZER*et al*,. 2006), confirmando as observações já descritas nos exames histológicos que mostram hiperplasia intimal, fibroblastos e proliferação suave do músculo, com formação de trombos em vasos cerebrais (FASANO, MEIER, e HULBERT, 2015).

Os fatores de risco do AVC na AF incluem episódios isquêmicos prévios, concentrações diminuídas de Hb (inferiora 7g/dl), leucocitose, concentrações diminuídas de HbF e STA (MENAA, 2013; KATO, STEINBERG, e GLADWIN, 2017). A observação em irmãos sugere que fatores genéticos podem estar envolvidos, como foi demonstrado para a co-herança da talassemia alfa, que tem efeito protetor, uma vez que aumenta a concentração da hemoglobina e a viscosidade sanguínea (KATO, STEINBERG, e GLADWIN, 2017;PIEL, STEINBERG, e REES, 2017).Também foi associado o papel dos neutrófilos, que são recrutados para contera inflamação devido a hemólise e aderência das hemácias falcizadas que estão obstruindo os vasos (SEGEL, HALTERMAN, e LICHTMAN, 2011).

Embora os pacientes possam se recuperar completamente do AVC, estes podem também levara ocorrência de danos cerebrais permanentes. As consequências do AVC em pacientes com AF levam à necessidade de se realizar exames que consigam estimar o grau de prognóstico da doença, de maneira a identificar quais destas crianças apresentam risco maior de desenvolver esses eventos cerebrovasculares, com objetivo de evitaro primeiro acidente ou de prevenir aqueles que venham a ocorrer subsequentemente (BERNAUDIN *et al*., 2011; MENAA, 2013; FASANO, MEIER, e HULBERT, 2015; KATO, STEINBERG, e GLADWIN, 2017; KATO *et al*., 2018).

2.7. DIAGNÓSTICO E ASPECTOS TERAPÊUTICOS DO AVC NA ANEMIA FALCIFORME

2.7.1. **Doppler transcraniano**

O AVC é uma das complicações mais graves da DF, sendo que o melhor fator preditivo até o momento é a detecção do aumento da velocidade do fluxo sanguíneo cerebral pelo DopplerTranscraniano (DTC). O estudo intitulado *Stroke Prevention Trial in Sickle Cell Anemia* (*STOP*) *I* comprovou que a prevenção primária do AVC com transfusões sanguíneas crônicas é a conduta mais adequada para os pacientes com risco elevado para ocorrência do evento. O estudo *STOP II* mostrou que essas transfusões devem sermantidas indefinidamente, pois sua suspensão leva, frequentemente, ao aumento da velocidade do fluxo sanguineo cerebral para níveis de risco, sendo que se houvera suspensão das transfusões, independente do tempo transfusional, a taxa de recorrência volta a serde 70%. (ADAMS *et al*., 1988; ADAMS *et al.,* 1992; ADAMS *et al*., 1997; ADAMS, 2001, 2005). Atualmente, somente o DTC é recomendado para seleção de pacientes para tratamento preventivo do AVC primário.

Outros exames que avaliam as condições cerebrais, como a ressonância ou angiorressonância magnética cerebral (RM ou ARM), podem fornecer dados adicionais sobre o risco futuro de AVC (ADAMS, 2005; ZÉTOLA, 2012). A associação de infartos silenciosos detectados pela RM e risco de AVC clínico foi demonstrada pelo estudo cooperativo da DF, *Cooperative Study of Sickle Cell Disease* (*CSSCD*) (GASTON *et al.*, 1987; MILLER*et al.*, 2001). A ARM foi empregada para avaliação de riscos, porém não se pode estratificá-los em dados prospectivos (ADAMS, 2005). No Brasil, o DTC é o método escolhido para a prevenção primária do AVC em pessoas com DF e deve serrealizado e interpretado de acordo com os parâmetros estabelecidos pelo estudo *STOP* (LOBO et al., 2011).

O princípio do Doppler foi descrito por Christan Johann Doppler em 1843, mas em 1982, Rune e cols. descreveram o método não invasivo para se obteras velocidades médias do fluxo sanguíneo cerebral usando a ultra-sonografia pulsada com sonda de 2 MHz através das áreas específicas da calota craniana que apresentam menor espessura para ter acesso à circulação arterial intracraniana e que oferecem informações dinâmicas da circulação cerebral (KASSAB *et al*., 2007).O DTC utiliza

áreas específicas do crânio que apresentam menor espessura para acessarà circulação arterial intracraniana. São quatros regiões, chamadas de janelas acústica, nas quais penetra a onda ultrassonográfica, a temporal, orbital,a suboccipital, e a submandibular(KASSAB *et al*., 2007; BATHALA, MEHNDIRATTA, e SHARMA, 2013). A janela transtemporal é situada no osso da têmpora em cima do arco zigomático, sendo sub dividida na posterior, média e anterior, que são porsua vez irrigados pelos seguintes vasos: a porção distal da artéria carótida interna, artéria cerebral anterior, artéria cerebral média, artéria cerebral posterior(ACP), artéria comunicante anterior(ACoA) e artéria comunicante posterior(AcoP) (KASSAB *et al.,* 2007; BATHALA, MEHNDIRATTA, e SHARMA, 2013) **(FIGURE 5).**

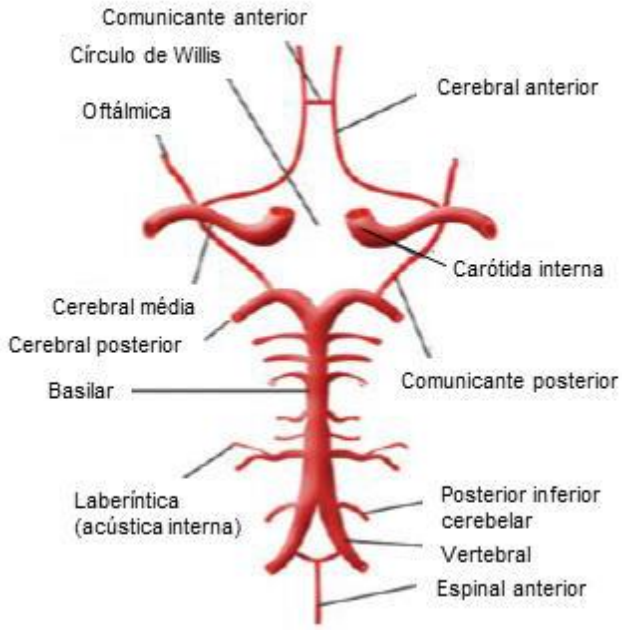

FIGURA 5. **O poligono de Willis.** Adaptado (D'ANDREA et al., 2016)

A velocidade do fluxo sanguíneo arterial acima de 200 centímetros/s está associada ao risco elevado de AVC. De fato, crianças com valores anormais de DTC VMMAX (≥200 cm/s) apresentam aproximadamente 44 vezes risco maiorde desenvolver AVC primário do que aquelas com velocidades normais (DTC VMMAX <170 cm/s) (ADAMS *et al.*,1998; LOBO *et al*., 2011).

No estudo *STOP*, realizado porAdams e cols., foram estabelecidos os primeiros critérios para detecção de estenose nas grandes artérias cerebrais de crianças com AF através do DTC. O método DTC apresenta boa segurança e é bem tolerado pelas

crianças, pois não é invasivo. Além disso, ele tem custo reduzido e, devido a sua portabilidade, pode ser realizado em ambulatório, clínicas ou em trabalho de campo. É importante notificar que o DTC apresenta grande sensibililidade em comparação a angiografia cerebral. Segundo Adams e cols., o DTC apresenta sensibilidade de 90% e especificidade de 100% (ADAMS *et al.*, 1992). De fato, o *National Institute of health* (NIH) recomendou o uso do DTC como teste de rastreamento para as crianças com AF. Os pacientes em sua grande maioria são obrigados a se deslocar para poder se beneficiar do exame nos poucos centros que o disponibilizam. No Brasil, segundo o ministerio da saude para poder fazer um monitoramento adequado dos pacientes desenvolveu se um planejamento com repetições conforme a tabela abaixo (LOBO et al., 2011) (tabela 1).

TABELA 1. Recomendações de periodicidade do dopplertranscraniano segundo consenso de DTC Brasil

Dopplertranscraniano (DTC)	**VFSC (cm/s)**	**Periodicidade do Exame**
Ausência de janela	---	Utilizaroutro recurso de imagem para analisaro evento cerebrovascular.
Dificuldade técnica porfalta de cooperação	---	Repetira cada 3 meses. Recomenda-se avaliação poroutro examinador.
Baixa VFSC	< 70	Repetirapós 1 mês.
Normal	< 170	Repetiruma vez porano.
Condicional baixo*	170-184	Repetira cada 3 meses. No caso de resultados subsequentes normais, deve-se adotara conduta do grupo normal.
Condicional alto*	185-199	Repetirapós 1 mês. Em casos de exames inalterados, recomenda-se repetira cada 3 meses. Em casos de dois exames alterados, recomenda-se discutirrisco de AVC e considerarregime transfusional crônico.
Anormal	Maiorou igual a 200 – 219	Repetirapós 1 mês. Caso o valorse mantenha ³ >= 200, recomenda-se discutiro risco de AVC e considerarregime transfusional crônico. Caso o resultado diminua para 170-199, recomenda-se repetição em 1 mês, se condicional alto (entre 185 e 199); ou em 6 meses, se condicional baixo (entre 170 e 184). Caso o resultado se normalize (< 170), recomenda-se repetição em 1 ano.

VFSC (cm/s): Velocidade do fluxo sanguíneo cerebral. Adaptado de http://bvsms.saude.gov.br/bvs/saudelegis/sas/2013/prt0473_26_04_2013.html consultado o 04/08/2016 (BRASIL,2009).

2.72**. Medidas terapêuticas para o AVC na anemia falciforme**

2.7.2.1 Transfusão

Estudos demonstraram que transfusões regulares de hemocomponentes constituem-se em medida de prevenção do AVC em pacientes com DF, com redução significativa das concentrações de Hb livre e de marcadores associados à hemólise, como a desidrogenase lática (LDH) e alanina aminotransferase (AST), em comparação a indivíduos controles não-submetidos à transfusão, ou que receberam transfusão esporadicamente (KATO *et al.*, 2009; WARE *et al.*, 2016). A transfusão tem por objetivo reduzira concentração de HbS para menos que 30%, com aumento consequente da oxigenação (MIRRE *et al*., 2010; MENAA, 2013; KATO, STEINBERG, *e* GLADWIN, 2017). O uso da transfusão também permite a diminuição dos níveis plasmáticos de Hb livre reduzindo os efeitos tóxicos dos produtos da degradação da Hb e a disfunção endotelial, com aumento da biodisponibilidade do NO (ADAMS *et al.*, 1998; AYGUN *et al.*, 2012). A tranfusão de hemoderivados é necessária em crianças vítimas de AVC ou em prevenção depois de um DTC alterado ou de uma ARM (ADAMS, 2005; AYGUN *et al*., 2012). Entretanto, a sobrecarga de ferro é o efeito associado ao uso frequente de transfusão necessitando do uso de terapia quelante de ferro (ADAMKIEWICZ *et al.,* 2009; RAGHUPATHY, MANWANI, e LITTLE, 2010; WARE *et al.,* 2016).

2.7.2.2 Hidroxiureia

A observação de que recém-nascidos AF são assintomáticos nos primeiros meses de vida, assim como os indivíduos com AF que possuem associação com a Persistência Hereditária de HbF(PHHF), explica o tratamento com a hidroxiureia (HU),o qual que estimula a produção “de novo” da HbF cujo aumento está associado a melhorias no quadro clinico dos pacientes(WARE *et al.* 2016; ABDELGADIR, ABDELSALAM, e MUDDATHIR, 2017).

A HU é uma terapia oral, sendo a primeira droga aprovada pelo *Food and Drug Administration* (FDA) para o tratamento da AF (PLATT, 2008); a HU é um inibidorda ribonucleotídeo redutase, um citostático que influencia na eritropese e favorece a saída dos progenitores mais imaturos que são os mais ricos em HbF.

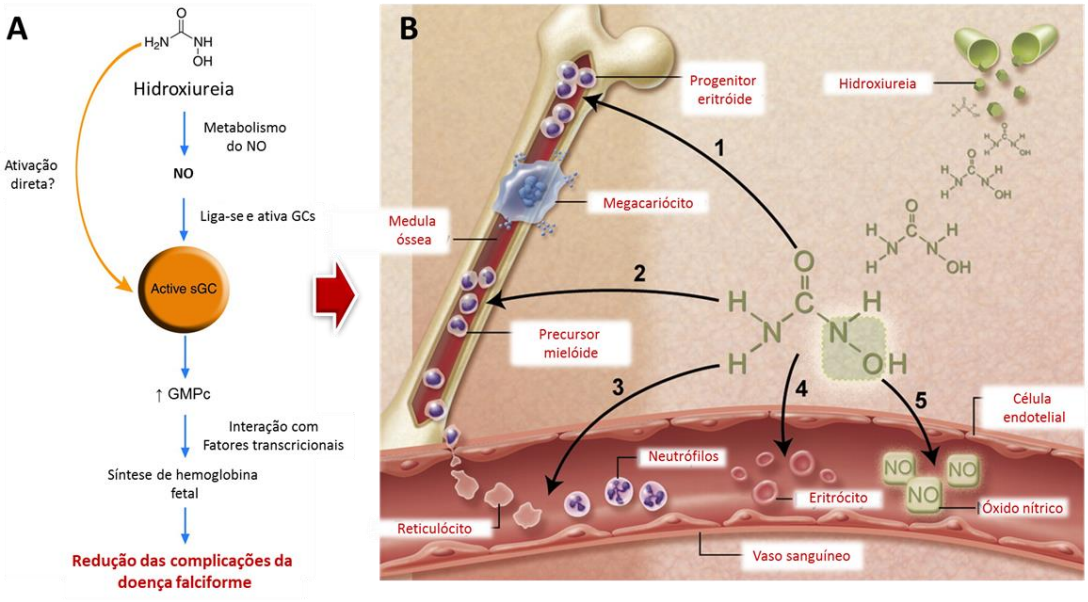

FIGURA 6. Os múltiplos benefícios da hidroxiureia no manejo terapêutico da doença falciforme. (A) Mecanismo de ação da hidroxiureia (Adaptado de King 2003). (B) Efeitos primários e secundários da HU, segundo Ware (2010).

A HU leva a diminuição da hemólise e melhoria da anemia associada a diminuição de leucócitos e plaquetas, sendo benéfica para a reologia do sangue e, por consequência, diminui a incidência de episódios dolorosos e vaso-oclusivos graves (STEINBERG, 2009b; WARE *et al*., 2016; KATO, STEINBERG, e GLADWIN, 2017). O aumento da HbF é altamente heterogêneo entre os pacientes, os mais sensiveis a droga podem atingir níveis elevados de HbF, enquanto aqueles menos sensíveis possuem aumentos discretos e outros, apesar de raros, não apresentam resposta ao tratamento (SCLAFANI *et al.*, 2016).

2.7.2.3 L –glutamina

Outra droga recentemente aprovada pelo FDA para o uso em indivíduos com AF a partirde 5 anos, é o *Endari*, que é uma terapia oral a base de L-glutamina. A L glutamina, desempenha vários e fundamentais papéis no metabolismo celular, incluindo síntese proteíca, produção de energia e formação de antioxidantes. A L-glutamina é o aminoácido mais abundante no corpo, a glutamina é produzida naturalmente (em

grande parte nos pulmões) e, geralmente, em quantidades suficientes para as necessidades do corpo.Pórem, é frequentemente usado como suplemento para combaterinfecções, lesões e estresse.O uso do Endari como terapia na DF está relacionado a diminuição das crises algicas e a neutralização do estresse oxidativo pela geração de moléculas anti-oxidantes (NAD+ /NADH). A L-glutamina pode melhorar o potencial redox do NAD em eritrócitos falciformes aumentando a disponibilidade de glutationa reduzida como um produto da degradação da glutamina que tem como consequência, ajudar na neutralização do estresse oxidativo presente nas hemácias falciformes que são mais suscetíveis a danos oxidativos que as normais, permitindo-lhes recuperara flexibilidade necessária para atravessar os vasos sanguíneos e capilares. Essa melhora tem como ação a redução da hemolise crônica e eventos vasos-oclusivos (NIIHARA *et al.*, 2014).

2.7.2.4 Transplante de células tronco hematopoiéticas

O uso de células tronco hematopoéticas é um tratamento potencialmente curativo voltado para a substituição das células hematopoiéticas dos indivíduos com a DF pelas do doador. Para que o processo ocorra é necessário um doador da família, HLA idêntico, que seja HbAA ou HbAS. A medula óssea é coletada por punção, sob anestesia. O uso do procedimento permite uma grande melhoria de vários sintomas da AF, tais como a prevenção do AVC e desaparecimento total das crises algicas e STA.Este tratamento requer internação e acompanhamento a longo prazo e tem 85% de taxa de sucesso, apesarde apresentar10% de risco relacionado a doença enxerto versus hospedeiro, em inglês *graft versus host disease*, (GVHD) e risco geral de rejeição de 5% (SHENOY, 2013; HOSOYA *et al.,* 2018). O tratamento preparatório para o procedimento tem como inconveniente o risco de esterilização. Como medida de prevenção desse risco, é altamente recomendado a criopreservação de óvulos antes do inicio do tratamento (BERNAUDIN *et al*., 2007; HOSOYA *et al*., 2018). Os transplantes baseados em sangue de cordão são aqueles que apresentam menor risco, especialmente, o risco de GVHD e o risco de óbito. Eles também possibilitam a cura para pacientes que não possuem doador intrafamiliar(HOSOYA *et al.* 2018).

2.7.2.5 Terapia gênica

A terapia genética suscitou uma grande esperança em pacientes com AF depois do sucesso obtido em um paciente francês tratado com vetorlentiviral de um gene anti-falcizante da globina β em células-tronco hematopoiéticas autólogas.O paciente não teve recorrência de crises de falcização e observou-se o desaparecimento de características biológicas da doença, devido ao nível da β-globina anti-falcizante terapêutica que permaneceu elevada 15 meses após o tratamento (RIBEIL *et al.*, 2017).

2.8. MODULAÇÃO GENÉTICA DO AVC NA ANEMIA FALCIFORME

A heterogeneidade fenotípica observada em indivíduos com AF tem sido atribuída a modificadores genéticos, como a presença de polimorfismos genéticos, geralmente single-nucleotide polymorphisms (SNPs). A observação referente ao aumento de cerebrovasculopatia entre irmãos e gêmeos (DRISCOLL et al., 2003) sugere que a patogênese do AVC na AF envolve, provavelmente, genes relacionados ao processo de hemólise, inflamação, resposta imune, coagulação, adesão celular, metabolismo de lipídios, regulação da pressão sanguínea e, hipóxia, entre outros. Vários estudos estão em andamento para verificar se polimorfismos genéticos, como aqueles implicados na adesão celular endotelial e na inflamação, quando presentes em fenótipos com doença cerebrovasculare outras complicações, estão associados a ocorrência ou proteção do AVC (GONÇALVES et al., 2003; STEINBERG; SEBASTIANI, 2012 SWITZER*et al*., 2006; MENAA, 2013;pIEL, STEINBERG e REES, 2017).Entre vários marcadores propostos e que estão sujeitos a controvérsia, podemos citar a proteção conferida pela alfa talassemia ao desenvolvimento de doença cerebrovascular, mas também todas as mutações ligadas ao perfil da HbF, tais como os haplótipos ligados ao grupo de genes da globina β e também a mutação no *BCL11A* e no gene *OR51B6* (SOLOVIEFF *et al.,* 2010;pIEL, STEINBERG, e REES 2017; SARAF *et al.,* 2017). A ocorrência de sintomas leves ou graves pode estarassociada a presença de alfa talassemia ou de concentrações elevadas ou diminuídas de HbF. Além disso, o histórico e a origem geográfica dos pacientes estão relacionados com a presença de haplótipos específicos ligados ao genes da globina β^S (GONÇALVES et al., 2003; STEINBERG; SEBASTIANI,

2012), tais como a presença de haplótipos, como haplótipo Benin (BEN), o haplótipo Bantu (CAR) ou haplótipo Senegal (SEN) e a região controladora do locus da globina β (*Locus Control Region* LCR) (ONER et al., 1992; THEIN, 2011). As mutações associadas a persistência hereditária de HbF (e a presença da talassemia α) estão associadas à prevalência diminuída de AVC (THEIN 2008, 2011; STEINBERG e SEBASTIANI, 2012; MENAA, 2013;pIEL, STEINBERG, e REES, 2017).

2.8.1. **Efeito modulatório da talassemia alfa**

A alfa talassemia é caracterizada pela diminução ou falta de produção da globina alfa. Cerca de um terço (1/3) dos afrodescendentes com AF apresentam também a co-existência da alfa talassemia (THEIN, 2011), que leva a redução na Concentração da Hemoglobina Corpuscular Média (CHCM), e no Volume Corpuscular Médio (VCM) com redução também da polimerização da HbS.Em adição, foi demonstrado também que a co-herança com a alfa talassemia reduz a taxa de hemólise e a contagem de reticulócitos, assim como os níveis de LDH (EMBURY *et al*., 1982; BALLAS, 2001; THEIN, 2011), demonstrando efeito protetorsobre as complicações ligadas a hemólise exacerbarda,assim como a hipertensão pulmonar, úlceras de membros inferiores, priapismo e albuminuria (STEINBERG, 2009; THEIN, 2011). Alguns estudos mostraram que a coexistencia da talassemia -α na AF otimiza a resposta a terapia com HU, mas esse aumento não estaria associdado ao aumento nas concentrações de HbF nem de VCM, que se constituem em parâmetros associados a resposta ao uso de HU (VASAVDA *et al*., 2008; THEIN, 2011). Desta forma, existe a hipótese de que a presença da alfa talassemia leve a uma resposta de canais bloqueadores (*e.g.,* senicapoc) que têm como objetivo preservara hidratação da hemácia e reduzira hemólise (ATAGA *et al*., 2008; THEIN, 2011). Alguns estudos revelaram o efeito epistatico negativo entre a AF e a alfa talassemia explicando o fato de que em locais nos quais a alfa talassemia é prevalente ocorre a redução da prevalência da AF (PENMAN *et al.,* 2009). Em resumo, a alfa talassemia beneficia a AF em relação a manutenção da membrana e em relação as alterações ocorridas no fenômeno da falcização, que induz a perda de cátions e perda de hidratação celular. Por consequência, concentrações reduzidas de CHCM levam a melhor hidratação das

hemácias e retardo na polimerização da desoxihemoglobina S (STEINBERG e EMBURY, 1986).

2.8.2. **Haplótipos ligados ao grupo de genes da globina beta S**

O efeito epistático e os haplótipos ligados ao grupo de genes da globina beta foram identificados como moduladores da expressão da HbF que foi reconhecida pelo efeito benéfico na clínica da AF (PIEL; STEINBERG e REES, 2017). A descoberta das enzimas de restrição permitiu aos perquisadores a investigação mais pormenorizada do gene *HBB*, usando a técnica de *Restriction Fragment Length Polymorphism* (*RFLP*), do *português* análise do comprimento de fragmentos de restrição. As pesquisas também revelaram que a mutação que deu origem ao alelo β^S é relativamente recente, ela teria occorido há 3000 a 10000 anos. Duas hipóteses tentam explicara origem da mutação que originou o alelo β^S. A mais conhecida é a “hipótese de origens independentes”, na qual a mutação responsável pela origem do alelo β^S surgiu pelo menos 4 vezes na África e propagou-se localmente em várias ocasiões (FLINT *et al.,* 1998) originando quatro haplótipos na África Sub-Sahariana (Bantu, Benin, Camarões e Senegal); um quinto tipo foi descoberto no subcontinente indiano que se propagou progessivemente até a Península Arábica Oriental (FIGURE 7), e a outra hipótese sugere uma única origem (FLINT *et al.*, 1998; BITOUNGUI *et al.*, 2015) .

Alguns haplótipos da globina beta foram associados a forma clinica grave da AF, como o Bantu e o Benin que foram associados a concentrações diminuídas de HbF e ao quadro clínico mais grave; enquanto os haplótipos Senegal e Arab-India foram associados a concentrações mais elevadas de HbF com 17%, 12,4 %, respectivamente e a um quadro clinico menos grave (STUART e NAGEL, 2004; MENAA 2013;pIEL, STEINBERG, e REES, 2017), ressaltando a relevância clínica importante da HbF. No entanto, dentro de cada grupo de haplótipos, o intervalo de níveis de HbF é muito variável, essa heterogeneidade dentro do grupo seria devido a moduladores da HbF (*Xmn1-HBG2*; *HMIP-2* e *BCL11A*) (STUART e NAGEL, 2004; THEIN, 2011; *BAUERet al*. 2013; ABDELGADIR, ABDELSALAM, e MUDDATHIR, 2017).

2.8.3. **Gene *BCL11A* e risco de AVC**

O gene *B-cell lymphoma/leukemia 11A* (*BCL11A*) é um repressor transcriptional (proteína de dedo de Zinco) expresso mais em hemácias e inibe a expressão de HbF, a cadeia γ da globina. e também estimula a proliferação de linfócitos e células dendríticas. O *knockout* do gene em camundungos levou ao aumento da expressão da HbF (BAUER*et al.,* 2013). O mecanismo de funcionamento do *BCL11A* ainda não está bem esclarecido, mas vários polimorfismos foram associados a melhora ou piora dos sintomas ou complicações do AVC e da vaso-oclusão (WONKAM *et al*. 2014; SARAF *et al.,* 2017). O gene *BCL11A* está também associado a regulação da HbA2, fenômeno atribuído como independente da regulação da HbF (GRIFFIN *et al.*, 2014) sendo considerado como um alvo na terapia de reativação da HbF (SANKARAN *et al.,* 2008; XU *et al.* 2010; BAUER*et al.,* 2013) usando a edição de genoma e o sistema, *Clustered Regular Interspaced Short Palindromic Repeats/ CRISPR associated protein 9* (CRISPR/Cas9) (BAUER e ORKIN, 2015).

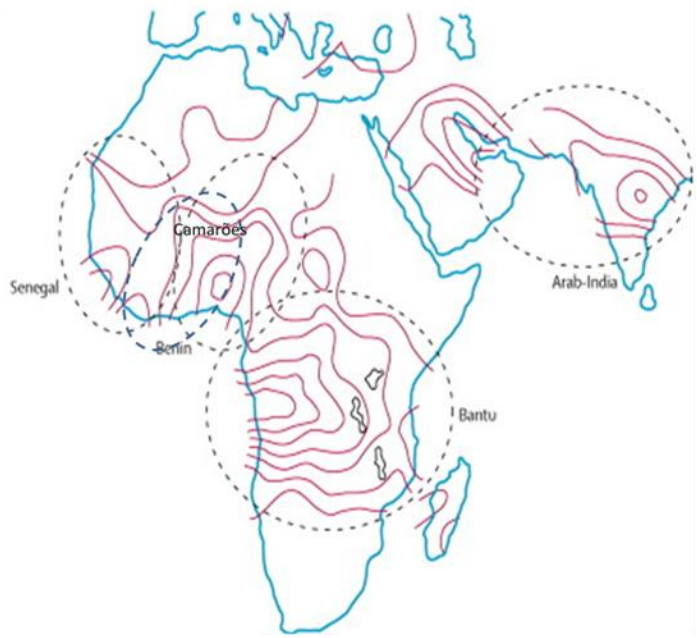

FIGURA 7. Distribuição mundial dos haplótipos associados ao grupo de genes da globina beta (Adaptado de Stuart e Nagel , 2004).

2.8.4. **Gene *APOB* e risco de AVC**

Os pacientes com AF são conhecidos por apresentarem metabolismo lipídico alterado, sendo que estudos realizados pelo nosso grupo sugeriram o fenótipo dislipidêmico em nossa população de pacientes (SEIXAS *et al*., 2010; ALELUIA *et al.,* 2017). A apolipoproteina B (*APOB)* é a forma majoritária das apolipoproteínas dos quilomícroms[1], very low density lipoprotein (VLDL) e Low density lipoprotein (LDL). A *APOB* é responsável pelo transporte dos lipídios, que é degradado no figado. O aumento no soro da apoB esteve associado aos níveis aumentados de LDL e diminuídos de high density lipoprotein (HDL) e correlacionados ao risco aumentado de doença cardiovascular, incluindo AVC isquêmico e doença coronariana. (KATHIRESAN *et al*., 2008). O gene *APOB* está envolvido no metabolismo dos lipídios e lipoproteínas, assim, uma mutação no gene tem sido associada a hipobetalipoproteinemia. Polimorfismos no gene *APOB* têm sido investigados quanto ao risco ou a proteção de ocorrência de AVC isquêmico (BENN *et al.,* 2007). Os pacientes com AF são conhecidos porterem o perfil dislipidêmico e o gene *APOB* é um gene com provável implicação nas alterações do metabolismo de lipídios (HU *et al.,* 2009; ALELUIA *et al*., 2017).

2.8.5. **Gene *MTHFR* e risco de AVC**

A metilenotetrahidrofolato redutase (MTHFR) é uma enzima que atua no metabolismo da homocisteina para metionina (FROSST et al., 1995) existem vários polimorfismos no gene *MTHFR*; sendo os mais comuns o são *MTHFR*677 C>T (FROSST et al., 1995) e o *MTHFR* 1298 A>C (VAN DERPUT et al., 1998). As mutações no gene *MTHFR* acarretam em deficiência de folato e estão presentes na forma homozigota mutante em cerca de 10% da população brasileira (ARRUDA et al., 1998). Alguns estudos estableceram que os homozigotos mutantes relacionados ao polimorfismo *MTHFR* 677C>T apresentam risco maior para ocorrência de AVC, apesar de necessitar de

[1] Quilomicróns: grandes partículas produzidas pelas células intestinais compostas de cerca de 85 a 95% de triglicerídeos de origem da dieta (exógeno), pequena quantidade de colesterol livre e fosfolipídios e 1 a 2% de proteínas

confirmação (KELLY et al., 2002; KLUIJTMANS et al., 1996; MORITA et al., 1997; SCHWARTZ et al., 1997; VAN BOCKXMEERet al., 1997).

2.8.6. **Gene *OR51B6* e risco de AVC**

O gene *OR51B6* (*Olfactory ReceptorFamily 51 Subfamily B Member6*) está localizado no cromossomo 11 e faz parte do gene do receptordo cluster do gene olfativo, cuja família é a maior do genoma. Os receptores olfativos compartilham uma estrutura de domínio 7-transmembrana com muitos receptores de neurotransmissores e hormônios e são responsáveis pelo reconhecimento e pela transdução mediada por proteína G dos sinais de odor. O *OR51B6* desempenha papel na regulação do gene gama da globina, influenciando de certa forma os níveis de HbF. Alguns polimorfismos foram associados a variação nos níveis de HbF, esses últimos, como biomarcadores do risco de AVC ou de desfecho clínico grave em pacientes com AF (SOLOVIEFF *et al*., 2010; WONKAM *et al.,* 2014).

2.8.7. **Gene *CYP4F2* e risco de AVC**

O gene *CYP4F2* (C*ytochrome P450 family 4 subfamily F member 2)* está localizado no cromossomo 19, codifica um membro da superfamília de enzimas do citocromo P450. As proteínas do citocromo P450 catalisam muitas reações envolvidas no metabolismo de drogas e na síntese de colesterol, esteróides e outros lipídios. O gene expressa uma enzima que regula o metabolismo do leucotriene B4 que é um importante mediador do 20-HETE (acido 20-hydroxyeicosatetraenoico), mediador de inflamação e responsável pela regulação da função vascular no cérebro agindo como um constritor das artérias do cérebro (STEC *et al.*, 2007). Pessoas com dois alelos TT variantes do *CYP4F2* irão requerer aproximadamente 1 mg / dia a mais de varfarina do que aqueles que possuem dois alelos do tipo selvagem (TAKEUCHI *et al*., 2009).

2.8.8. **Gene *SLCOB1* e risco de AVC**

O gene *SLCOB1* (*Solute CarrierOrganic anion transporterfamily member1B1*) é um gene localizado no cromossomo 12. Ele codifica o transportador de influxo do polipeptídio transportador de ânions orgânicos 1B1 (*OATP1B1*). Esta proteína é encontrada na membrana sinusoidal dos hepatócitos e transporta a bilirrubina. Ela está também envolvida na limpeza (varredura) de ácidos biliares (bilirubina), hormônios e toxinas e ânions orgânicos do fígado (MATARIN *et al*., 2010). O SnP 521T>C (rs4149056) foi associado a alteração da função transportadora (OSHIRO *et al*., 2010; ROMAINE *et a*l., 2010) e estudos revelaram que pode influenciar o tratamento com statina.

3. JUSTIFICATIVA

Segundo a Organização Mundial da Saùde (OMS), 330.000 crianças nascem a cada ano com hemoglobinopatias (83% com AF e 17% com talassemia), que são responsáveis por 3,4% dos óbitos em crianças com menos de 5 anos de idade (WEATHERALL, 2008) No Brasil, nascem por ano, cerca de 3.500 crianças com DF (FELIX; SOUZA; RIBEIRO, 2010), sendo a Bahia o estado com maior incidência da doença no Brasil, devido à miscigenação de sua população (1:645 nascimentos, segundo dados de triagem neonatal realizados pela APAE–Salvador). No estado homozigoto, ocorre risco elevado de complicações fatais, como a hipertensão pulmonar, priapismo, AVC, ulcerações nas pernas, episódios de doraguda, STA, e necrose vascular dos ossos, que podem contribuir para o óbito prematuro (LOBO et al., 2011; OHENE-FREMPONG et al., 1998; SEBASTIANI et al., 2007). Aproximadamente 10% dos pacientes com AF apresentam risco de desenvolver AVC antes de completar 20 anos de idade (BRAMBILLA; MILLER; ADAMS, 2007; OHENE-FREMPONG et al., 1998). Atualmente, a ferramenta disponível para a prevenção primária ou recorrência do AVC é o rastreamento periódico das velocidades médias máximas (VMMAX) de fluxo sanguíneo das artérias carótidas internas distais e artérias cerebrais médias usando o DTC (FLANAGAN et al., 2011). Valores acima do padrão de velocidade estão associados ao risco maior de AVC (BRAMBILLA; MILLER; ADAMS, 2007; MENAA, 2013). No entanto, o DTC apresenta limitações para identificar precisamente todos os pacientes com AF que irão desenvolver complicações vasculares cerebrais, expondo a necessidade de um painel mais sensível e específico de biomarcadores de predição do AVC (FLANAGAN et al., 2011).

Mais de cem diferents biomarcadores de sangue e urina foram descritos na AF e quase todos estão associados ao estado estável e tornam-se mais evidentes durante as complicações. Apesar de absolutamente necessários, atualmente não existem biomarcadores de prognóstico confiáveis, rápidos e específicos para o AVC na DF (MENAA, 2013; REES; WILLIAMS; GLADWIN, 2010). A identificação precisa das crianças com risco elevado de AVC permitiria intervenções precoces e evitaria o seu desenvolvimento e as consequências clínicas graves.

4. OBJETIVOS

4.1. OBJETIVO GERAL

Identificaro perfil de indivíduos com AF, com a investigação de biomarcadores laboratorias (bioquímicos, hematológicos, e genéticos) que possam estarassociados a ocorrência de AVC.

4.2. OBJETIVOS ESPECÍFICOS

4.2.1. Objetivo 1

Avaliar as correlações entre os marcadores laboratoriais e as VMMAX do DTC e a influência da HU nesses parâmetros laboratoriais;

4.2.2. Objetivo 2

Avaliar os níveis sistêmicos de NO em pacientes com anemia falciforme em uso ou não de HU e correlacionar com velocidades do DTC;

4.2.3. Objetivo 3

Avaliar o perfil lipídico dos pacientes com risco de AVC e associar aos polimorfismos no gene *APOB*;

4.2.4. Objetivo 4

Avaliar os polimorfismos nos genes *OR51B6*, *MTHFR* C766T e *CYP4F2* e sua associação ao risco de AVC e investigaras concentrações de HbF, a presença de haplótipos ligados ao grupo de genes da globina beta e, a talassemia alfa em pacientes com AF com risco de AVC.

5. RESULTADOS

O presente estudo foi subdividido em quatro capitulos apresentados na forma de manuscritos, descritos a seguir:

MANUSCRITO 1. *Transcranial DopplerVelocities and Laboratory Markers of Sickle cell Anemia severity.*

MANUSCRITO 2. *Systemic levels of nitric oxide in plasma: a possible biomarkerof stroke in sickle cell anemia.*

MANUSCRITO 3. *Lipids profiles associated with Transcranial Dopplervelocity of Sickle cell anemia patients. Evaluation of APOB gene influence.*

MANUSCRITO 4. *Evaluation of laboratorial markers and polymorphism of MTHFR, OR51B6, CYP4F2, SLCOB1, and APOB among sickle cell anemia patients with different Transcranial Doppler velocities.*

5.1. MANUSCRITO 1 – **Transcranial DopplerVelocities and Laboratory Markers of Sickle cell Anemia severity**

Principais resultados:

Nesse manuscrito encontramos correlações significativas entre DTC-VMMAX e marcadores laboratoriais como reticulócitos e leucócitos, que são marcadores conhecidos da gravidade da anemia falciforme. Também mostramos que o tratamento com HU diminui as contagens de leucócitos e linfócitos nos diferentes grupos de DTC aqui avaliados. Nós especulamos que a associação desses marcadores com o DTC deve ser mais estudada e cuidadosamente analisada, juntamente com as velocidades do DTC.

Situação: A ser submetido.

Title: Transcranial DopplerVelocities and Laboratory Markers of Sickle cell Anemia severity

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Abstract

Introduction. Stroke is one of the highest complications of Sickle Cell Anemia (SCA). Hydroxyurea (HU) is currently used to treat SCA complications and also to prevent stroke events. In the present work, we evaluated the correlation between laboratory markers and Transcranial Doppler(TCD) velocities and we also studied the influence of HU in groups stratified by stroke risks.

Procedure. The study included a total of 152 pediatric patients with SCA, without stoke, submit to TCD velocity screening, and the time-averaged maximum mean velocity (TAMMV) was determined in the middle cerebral artery (MCA), anterior cerebral artery (ACA), and distal intracranial internal carotid artery (ICA). We compare cerebral blood flow in patients stratified by: TCD1 - defined as normal, with TAMMV inferior to 170 cm/s; TCD2 – conditional, with TAMMV above 170cm/s, but less than 199cm/s; TCD3 – high Risk, with TAMMV greater than or equal to 200 cm/s.

Results. We observed negative correlations (with Spearman r< -0.2 and p<0.05) between left and right TAMMV and red blood cell (RBC), hemoglobin and hematocrit. There were positive correlations (Spearman r> 0.2 and p<0.05) with mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red cell distribution width (RDW), white blood cell (WBC), lymphocytes, reticulocytes, and aspartate aminotransferase (AST). Patients treated with HU showed decreased WBC counts in all TCD stratified groups, but only in a significant way comparing between TCD3 group (high risk) treated and those untreated. In the same line, the lymphocyte counts were significantly decreased by HU in TCD1 and TCD3. The Latent Class analysis (LCA) was conducted based on the laboratory markers and we modeled the profiles of inflammatory and hemolysis class. Sixty-two percent (62 %) of our population was found in the more inflammatory subclass. **Conclusions**. The current study shows significant correlations between TCD-TAMMV and laboratory markers like reticulocyte and white blood cell, which are known markers of SCA severity. We also show that HU treatment decrease WBC and lymphocyte counts in the different TCD groups evaluated here. We speculate that the association of these markers with TCD should be furtherstudied and carefully analyzed along with TCD velocities.

Key words: TAMMV; TCD, HU, WBC STROKE, Sickle Cell Anemia, LCA

Introduction

Sickle cell anemia (SCA) is a disease of genetic origin that is caused by a single point mutation of the beta globin gene (*HBB*), which manifests as a polymerization of hemoglobin S (HbS) in erythrocytes. SCA patients may present a heterogenic panel of various clinical aspects orchronic complications, the most common being acute painful episodes resulting in severe consequences on patients' quality of life. SCA patients are also exposed to risk of early childhood mortality ormorbidity. Stroke, one of the main outcomes of SCA, occurs in about 10 percent of affected individuals priorto age 20[1]. In children with SCA, the risk of stroke can be estimated using transcranial Doppler ultrasonography (TCD), which measures the time-averaged mean of the maximum velocity (TAMMV) in the internal carotid artery and the middle cerebral arteries. TAMMVs above 200 centimeters per second (cm/s) have been associated with an elevated risk of stroke[2,3]. Hydroxyurea (HU), a pharmaceutical drug known to stimulate the production of fetal hemoglobin (HbF) has been proven to prevent complications arising from SCA, and helps to improve the quality of life

of patients by reducing the incidence of acute painful crises. Research has indicated that HU is effective in the prevention of first stroke episode, although the exact mechanism of action remains unelucidated[4,5].The present study attempted to evaluate correlations between TAMMV and hematologic and biochemical laboratory markers, as well as investigate the influence of HU use on the markers studied. We also used latent class analysis (LCA) to analyze the specific hemolytic and inflammatory laboratorial profiles in ourpatients with risk of stroke.

Material and methods

A cross-sectional study including 152 SCA pediatric patients with an average age of 2-18 years was conducted at the Professor Hosannah de Oliveira Pediatric Hospital (HUPES/UFBA) and at the Sickle Cell Disease Reference Center, located in Salvadorand in Itabuna respectively, both cities in northeastern Brazil. The risk of stroke was assessed by Transcranial Doppler(TCD) in all subjects, which was consistently administered by a single trained professional using the same equipment. Time-averaged maximum mean velocity (TAMMV) was assessed using a 2 MHz probe connected to a Doppler-Box^{TM}X (Compumedics Germany GmbH, Singen, Hohentwiel, Germany). Three TAMMV outcomes were considered: TCD1, considered Normal (TAMMV <170 cm/s), TCD2, considered as Conditional (TAMMV 170-199cm/s) and TCD3, which represents an elevated risk of stroke (TAMMV> 200 cm/s). None of the patient had documented stroke event before. Parents or legal guardians signed a term of informed consent for each participating child. The present study received approval from the Institutional Review Board of the Professor Edgard Santos Hospital Complex, Federal University of Bahia (HUPES-UFBA), and was conducted in accordance with the guidelines of the Brazilian Government, the Declaration of Helsinki and its subsequent revisions.

After12 h of fasting and on the same day that TCD was performed, 10 mL of blood were collected by venous blood puncture for hematological profiling in Ethylenediaminetetraacetic acid (EDTA) tubes and biochemical analysis in dry tubes without anticoagulant.Serum nitrite levels (NOx) were evaluated using a colorimetric Griess assay with a NaNO3 standard curve, with spectrophotometry readings taken at a wavelength of 560 nm using a SpectraMax 190 Microplate Reader (MolecularDevices Corporation, Sunnyvale, California, USA). Results are expressed as micromolar concentrations of nitrite. Hemoglobin profiles were confirmed by high-performance liquid chromatography (HPLC) using the Variant™ II Hemoglobin Testing System (Bio-Rad, Hercules, California, USA). Hematological analyses were carried out using an electronic cell counter(Abbott Diagnostics, Lake Forest, Illinois, USA). Biochemical and inflammatory analyses were performed by immunochemical assay, and serum was analyzed by immunoassay using an A25 random access automatic analyzer(Biosystems SA, Barcelona, Catalunya, Spain), Access 2 Immunochemistry System (Beckman Coulter, Inc., Fullerton, CA, USA), and an Immage Immunochemistry System (Beckman Coulter, Inc., Fullerton, CA, USA).

Data were computed using Excel and Statistical analyses were performed using Graphpad prism v6.0 software. The software Mplus version 5.21, foradjusting LCA. The distribution of quantitative variables was analyzed using the Shapiro-Wilk test. The mean values of quantitative variables between three or more groups were compared using One-way Anova/Kruskall-Wallis respectively for parametric and non-parametric data. Mann-Whitney t-tests were utilized to compare between two groups. Correlation

analyses were performed between variables using Spearman's coefficient (r). LCA is a modeling approach that reveals the relationships between unseen groups (latents classes) within the population.

Results

The average age of the patient is 7.51 ± 4.14 and 54.90 % are male. Twenty-five percent (25 %) of them are taking HU. We evaluate the correlation between increases TAMMV, hematology and biochemical parameters and afterthis we compare the media value in each group of laboratorial parameters according to HU use and evaluate the LCA between the hemolytic and inflammatory profiles according to the figure1.

Correlation between TAMMV, hematology and biochemical parameters. As expected, elevated TAMMV was associated negatively with anemia parameters such RBC, hemoglobin and hematocrit. We observed negative correlations between increases in TAMMV and red blood cell (RBC) (r=-0.3016;p=0.0003), hemoglobin (r=-0.2524;p=0.0024) and hematocrit (r=-0.2530;p=0.0024) (figure1). Positive correlations were observed between TAMMV and mean corpuscularvolume (MCV) (r=0.3282;p<0.0001), mean corpuscular hemoglobin (MCH) (r=0.2910;p=0.004), red cell distribution width (RDW) (r=0.2310;p=0.0057), WBC (r=0.2325;p=0.0055), lymphocytes (r=0.2707;p=0.0012), reticulocytes (r=0.33381;p<0.0001), aspartate aminotransferase (AST) (r=0.2730;p=0.0010) and ferritin (r=0.2905;p=0.0007) (Figures 2, 3).

Comparing the mean value in each group of laboratorial parameters according to HU. When comparing the three TCD groups, and mean platelet volumes (MPV) (p=0.0134) were observed to vary between groups, with lower values observed in the TCD2 and TCD3 categories (figure 4). WBC counts progressively increased according to TCD grouping (TCD1<2<3), albeit not significantly (p=0.1905). Lymphocyte counts (p=0.006) were higher in the TCD3 group compared to the other two groups (figure 5). Ferritin mean values were also higher(p=0.0075) in the TCD2 and TCD3 groups (figure 6). MPV values were lower in the TCD3 group than in the other two categories, both for HU-treated and untreated patients (p=0.032). WBC counts differed significantly among the TCD groups of untreated patients (p=0.009), but this was not the case in patients treated with HU. Patients on HU showed decreased WBC counts in all TCD groups, yet this was only significant with regard to TCD3 (p=0.002) In addition, lymphocyte counts were found to be significantly decreased according to HU use in TCD 1 and TCD3 (r=0.04 and p=0.006), but not for TCD2 (figure 4, 5 and 6).

Latent Class Analysis.

We choose the variables and grouped them by hemolytic and inflammatory profile (Figure1). There were variables categorized by the median of each variable: one (1) is superior to the median and 0 is inferior to the median.

-The hemolytic profile was defined by direct bilirubin (0/1), total bilirubin (0/1), indirect bilirubin (0/1), and LDH (0/1) variables.

-The inflammatory profile was defined by variables lymphocytes (0/1), ferritin (0/1), platelet (0/1), and WBC (0/1).

The entropy indicates the good separation of the latent classes. The hemolytic profile present entropy equal to 1 and the inflammatory profiles is equal to 0.845 (Tab1).

In the hemolytic profile we notice an equal repartition in the more hemolytic latent class and in the less hemolytic latent class. Instead, we notice that 62.8 % of ourpatients are found, predominantly, in the inflammatory latent class, suggesting that our population seem to present an inflammatory profile more exacerbated, and an equal hemolytic profile (Figure 8 and 9).

Discussion.

Underthe WHO's definition for anemia, a patient is anemic when she/he presents less than 12g/L or13 g/L of hemoglobin, for women or men respectively. This critical symptom is associated with a variety of poor outcomes in many diseases, such as comorbidity in malaria, especially in children and pregnant women, in which anemia is cited as the principal cause of death[6]. In addition, anemia in the context of cardiovasculardisease is associated with the occurrence of cerebrovascularevents[7-9]. However**,** the literature is controversial with regard to the influence of anemia on stroke risk, except for patients with SCA[9]. Some authors, including Abramson et al have suggested that the risk of stroke in anemic patients increases in association with other comorbidities, e.g. kidney disease[10]. In anemia, it is clear that brain oxygenation is reduced as a result of decreased blood flow. While more study is needed to elucidate the true mechanism underlying anemia and stroke risk, anemia is a proven predictorof poor stroke outcome[11]. All patients in our study presented low levels of hematocrit (<30 %), which has been recognized as a predictor of poor stroke outcome[11,12], similarly to the worsened outcomes for anemia-related stroke as defined by the WHO. Chan and Ganasekaran[13] explained that anemia can cause alterations in cerebral blood vessels, and consequently, change oxygenation in the brain, thereby increasing the risk of stroke[13]. The present findings indicate a negative correlation between TAMMV and RBC count, as well as levels of hemoglobin and hematocrit; i.e. higherTCD velocities are correlated with low levels of RBC, hemoglobin and hematocrit [12,14]. Other markers of anemia, such as RDW, are also considered as predictors of stroke outcome. Increased RDW and WBC counts are known to be predictor of poorstroke outcome[15,16]. Increased WBC counts have been associated with both a risk of coronary heart disease and stroke incidence and mortality from cardiovascular disease in African American patients[17]. Our study population seems to support this notion, as we observed an increase in WBC counts in the TCD3 group that had the highest blood velocities and, consequently, higher risk of stroke. However, the First National Health and Nutrition Examination Survey (NHANES I) Epidemiologic Follow-up Study found no significant influence of WBC counts on stroke incidence [18]. Balkaran et al [19] found that WBC count were higher in patients who suffered stroke than in controls [19]. It is possible that the increased WBC counts seen in ourTCD3 group may be attributable to the recruitment of the inflammatory cells that become activated in case of stroke [15,20]. In addition, increased reticulocyte count have also been associated high WBC count, which were considered as a marker of stroke risk in a Brazilian cohort [21]. These author's data is consistent with the findings of ourstudy. It is not known whether inflammation occurs during orbefore the occurrence of stroke, but an inflammatory state is evidenced by increases in liver function biomarkers, such as AST, which were positively correlated with higher TAMMV in our study. Some authors have suggested that inflammation is a preexisting condition prior to a stroke event[22]. Serum ferritin levels is considered as a biomarker of inflammation, also have been described as a risk factor for a poordiagnosis of stroke in post-menopausal women[23,24]. The analysis of

latent class is gained popularity in the research world because of this capacity to overcome the limitation of the others model of statistic[25]. In ourstudy, the use of a LCA model permit us to see the general profile of our population according to the variables own characteristic. The analyses reveal that our subject can be regrouped in two latent class that are the hemolytic and the inflammatory profile. The inflammatory profile was found more abundant in our population. This observation suggests the important role of the inflammatory profile in SCA, especially, in regard to stroke risk prediction in ourpopulation in comparison to the hemolytic markers. Many studies have suggested that inflammation plays an important role in the pathogenesis of ischemic stroke[26,27]. The reduction of inflammation is an important therapeutic target in the ischemic stroke[27]. As reported, our HU-treated patients evidenced a lower count of WBC and lymphocytes than the other groups. The decreased incidence of stroke in SCA patients treated with HU reported in some studies may be a result of the anti-inflammatory properties of this drug [5,28]. Although the anti-inflammatory effect of HU may be indirect, the correlation between inflammatory markers and TCD velocities in SCA patients reported herein seem to provide support for this hypothesis.

Conclusions.

The current results show significant correlations between TAMMV and laboratory markers commonly used to monitor pediatric SCA patients. While reticulocyte and WBC counts are known markers of SCA severity, correlations with TAMMV have not been previously investigated. After an analysis of latent class (LCA), we conclude that SCA patients present an equal repartition of hemolytic latent class and a higher proportion of inflammatory latent class. Here we provide evidence that HU treatment impacted a variety of laboratory parameters in different TCD groups, including MPV, lymphocyte and leukocyte counts. Association between these markers and stroke risk in SCA patients should be further investigated and carefully analyzed, along with TCD velocities.

Acknowledgments.

The authors would like to thank the children with SCA and their families, whose participation was fundamental to the present study. The authors would also like to thank the staff at the ProfessorEdgard Santos University Hospital, as well as the Pharmacy School of the Federal University of Bahia, for their support. This present study was supported by grants from the Fundação de Amparo a Pesquisa do Estado da Bahia (FAPESB) (SUS0034/2013 and 8133/2014) and from The Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (COSAU 2012. N º400005/2013-1APQ) The authors would like to thank Andris K. Walter for his critical analysis and English language.

Conflict of interest: None conflict of interest to declare

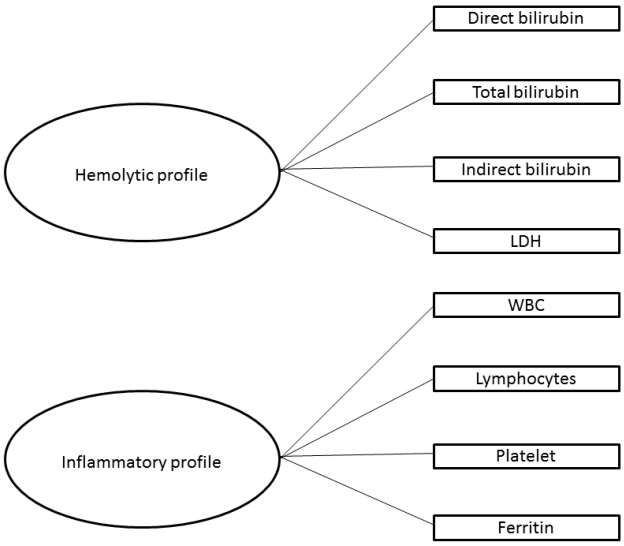

Figure 1. Theoretic Model of Latent Class Analysis on Laboratorial profile.

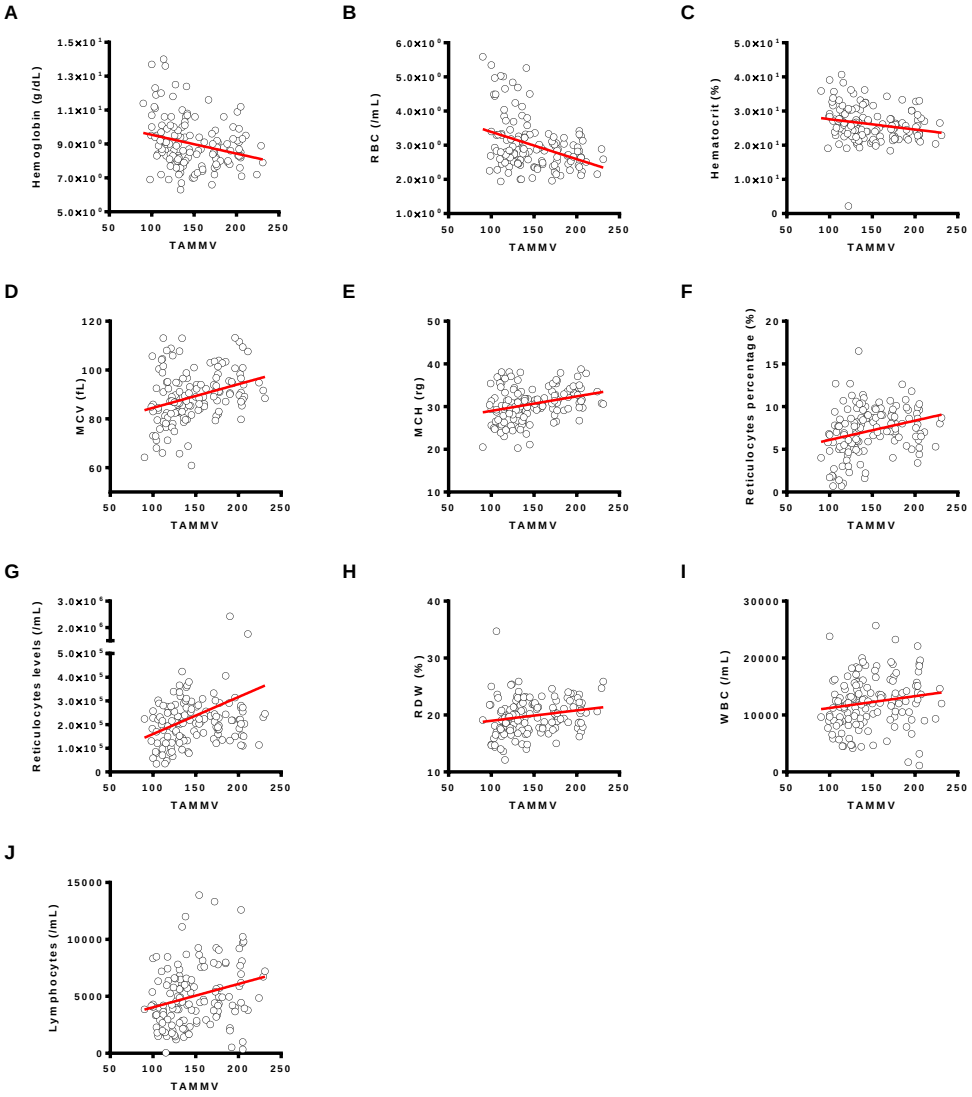

Figure 2. Correlation between TAMMV and hematological parameters. A. Hemoglobin (p=0.0024; r=-0.2524); B. Red blood cels (p=0.0003; r=-0.3016); C. Hematocrit (p=0.0024; r=-0.2530) had positive correlation between TAMMV and hematological parameters in SCA patients. D. Mean corpuscularvolume levels (p< 0.0001; r=0.3282); E. Mean corpuscularhemoglobin (p=0.0004; r=0.2910); F. reticulocyte percentage (p< 0.0001; r=0.3381); G. reticulocyte levels (p=0.002882; r=0.1889); H. RDW (%) (p=0.0057; r=0.2310); I. White blood Cells levels (p=0.0055; r=0.2325); J. Lymphocyte levels (p=0.0012; r=0.2707).

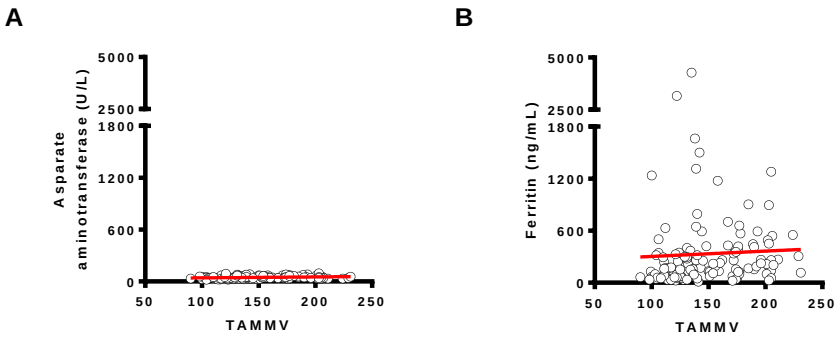

Figure 3. Correlations between TAMMV and biochemical parameters. **A** AST (p=0.0010; r=0.2730); **B** ferritin (p=0.0007; r=0.2905).

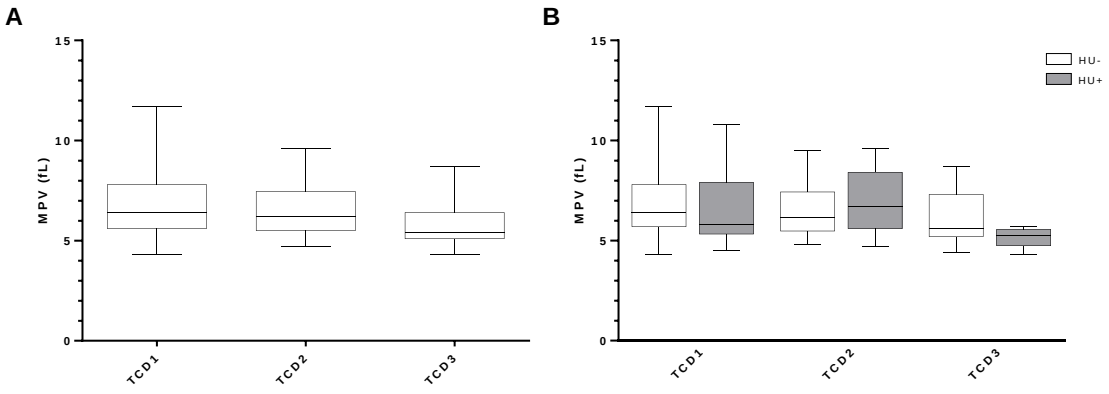

Figure 4. Mean values of Mean Platelet Volume were quantified in whole blood. **A** among TCD groups (p=0.0134; **B** between "HU-treated" (HU+) or" HU-untreated"(HU-) SCA patients (p=0.0320).

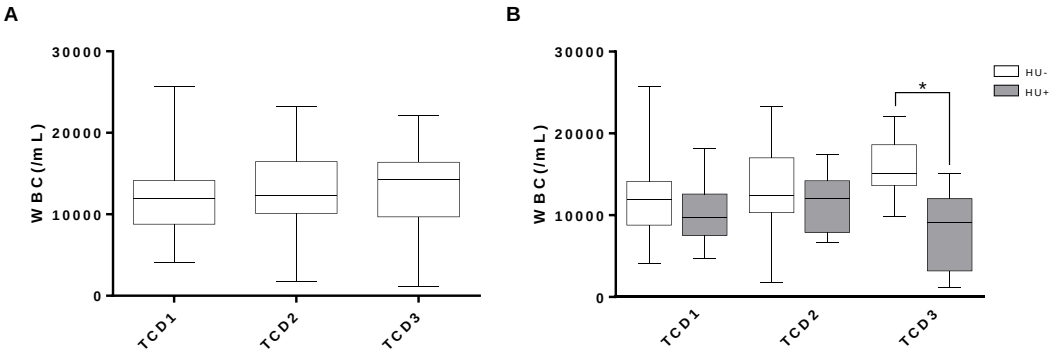

Figure 5. Mean White Blood Cell (WBC) counts were quantified in whole blood. **A** among TCD groups (p=0.1905; one way ANOVA Test); **B** Between "HU-treated" (HU+) or"HU-untreated" (HU-) SCA patients (p=0.007; one way ANOVA Test); TCD 3 HU- vs TCD3 HU+ (p=0.0008; independent t-test).

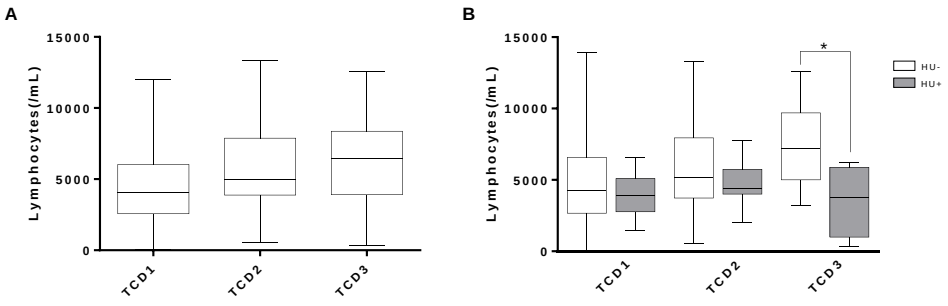

Figure 6. Mean value of lymphocytes were quantified in whole blood of SCA patients. **A** among TCD groups (p=0.0067); **B** between "HU-treated" (HU+) or" HU-untreated" (HU-) SCA patients (p=0.0020); between the HU- (p=0.0014); between the HU+ (p=0.4661); TCD1 HU- vs TCD3 HU- (p=0.0040); TCD 3 HU- vs TCD1 HU+ (p< 0.0001); TCD3 HU- vs TCD3 HU+ (p=0.0038).

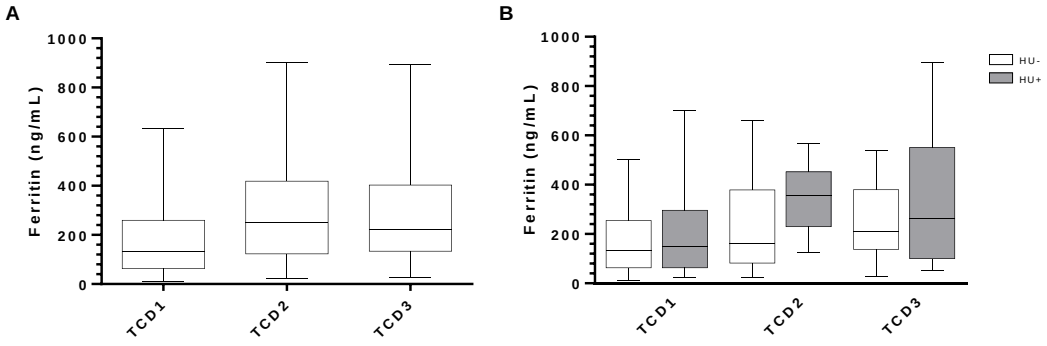

Figure 7. Mean values of ferritin levels: were quantified in serum of SCA patients. **A** Among TCD groups p=0.0075; **B** between "HU-treated" (HU+) or" HU-untreated" (HU-) SCA patients (p=0.0379); between the HU- (p=0.0858); between the HU+ (p=0.2902).

Table 1. Estimated parameters forAnalysis of latent classes for2 constructors related to ourstudy population

Constructors/indicators	**Entropy**	**Latent Class**	
Hemolytic profiles	1.000	50% More hemolytic	50% Less hemolytic
Direct bilirubin		0.724	0.289
Total bilirubin		1.000	0.000
Indirect bilirubin		0.934	0.079
LDH		0.658	0.355
Inflammatory profile	0.845	62.8% more inflammatory	37.2% less inflammatory
WBC		0.818	0.00
Lymphocytes		0.813	0.026
Platelet		0.652	0.279
Ferritin		0.679	0.357

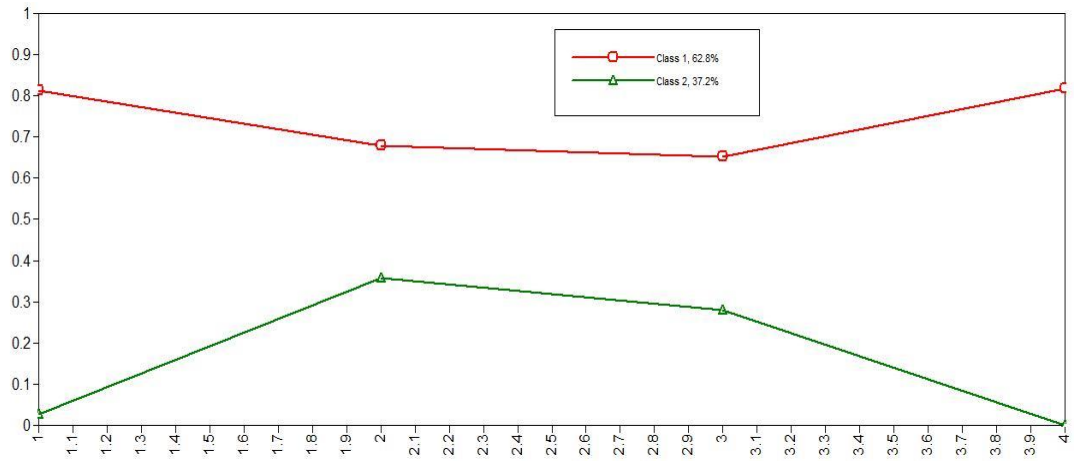

Figure 8. Latent Class representation of the inflammatory profile. Class1 = more inflammatory latent class (62.8%). Class2 = less inflammatory latent class (37.2%).

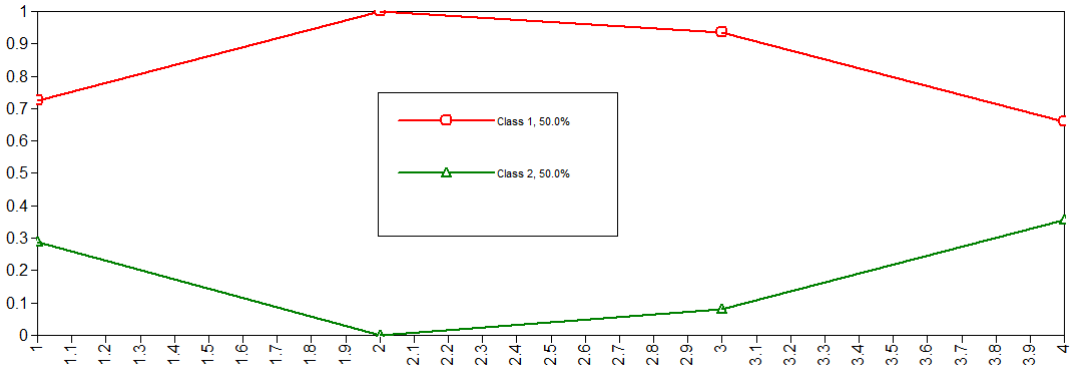

Figure 9. Latent Class representation of hemolytic profile. Class1 = more hemolytic latent class (62.8%). Class2 = less hemolytic latent class (37.2%).

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5.2. MANUSCRITO 2 – **Systemic levels of nitric oxide in plasma: a possible biomarkerof stroke in sickle cell anemia**

Principais resultados:

Observamos correlação positiva (com Spearman r≤0,6 e p <0,05) entre os metabolitos de NO (NOx) e VLDL (Lipoproteína de Baixa Densidade) e triglicerídeos (TG) nos grupos de DTC e com aspartato aminotransferase (AST) Após a investigação do uso da HU, notamos que a correlação positiva forte persiste em todos os grupos nos mesmos parâmetros. Sugere-se que os NOx, consequentemente o NO, pode ser considerado como um biomarcador interessante no que se refere à fisiopatologia do acidente vascular cerebral (AVC) e que o perfil lipídico dessa população específica de AVC deve ser mais estudado.

Situação: submetido a Annals of Hematology

Title: Systemic levels of nitric oxide in plasma: a possible biomarkerof stroke in sickle cell anemia

Authors:

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Abstract

Stroke is a severe clinical complication associated with SCA and many studies have called attention to the endothelial dysfunction associated with intravascularhemolysis as important events in its pathophysiology, also characterized by decreased nitric oxide (NO) bioavailability. By using the transcranial Doppler(TCD) it is possible to evaluate the patients that have more risk to stroke. Hydroxyurea (HU), one of the drugs approved forSCA treatment was shown to decrease the stroke risk and it is also known as a NO donor. In the present work, we define 4 outcomes based on the categorization of TCD velocities: TCD1 as normal group, TCD2 as conditional TCD3 as altered group and CVA as stoke group. The biochemical and laboratorial parameters of the SCA patients and correlated them with to NOx (Nitric Oxide metabolites) levels according to HU use. We observed positive correlation (with Spearman $r \leq 0.6$ and $p<0.05$) between NOx and (Very Low Density Lipoprotein) VLDL and triglyceride (TG) in the TCD2, TCD3 and CVA groups, and with aspartate aminotransferase (AST) and Nox in the TCD3 group and (Lactate dehydrogenase) LDH in the CVA groups. After investigation of the HU use, we notice that strong a positive correlation persist in all the groups in the same parameters. It suggest that NOx, consequently NO can be considered like an interesting biomarker with regard of stroke physiopathology and the lipid profile of this specific population of SCA should be furtherstudy.

Keywords: Nitric oxide, Sickle cell anemia, stroke, TCD, Hydroxyurea,

Introduction

Sickle cell disease (SCD) is one of the most commonly occurring hemoglobinopathies in the world, characterized by presence of abnormal S-type hemoglobin (HbS) [1]. The HbS is formed as a consequence of a point mutation in the beta (β) globin gene (*HBB*) (rs334), which replaces a glutamic acid in the sixth position of the β globin chain [2]. The homozygosis forthe β^S allele (HbSS), is referred as sickle cell anemia (SCA), while the association of β^S with wild-type β globin (heterozygosis) or other types of mutated in β globin gene is called SCD [2]. Sickle cell disease has a very complex range of clinical manifestations that dependent of many biological and genetic factors, and some are not well understood. The intravascular hemolysis and chronic anemia occurring in SCA patients play an important role in the disease pathophysiology and clinical variability. Stroke is a severe complication associated with SCA that can affect nearly 11% of patients under20 years of age [3]. Many studies have called attention to the endothelial dysfunction associated with intravascular hemolysis as an important event in the pathophysiology of stroke in SCA[2]. This intravascular hemolysis is characterized by decreased nitric oxide (NO) bioavailability, leading to vasomotor instability which ultimately results in a proliferative vasculopathy [4] Hydroxyurea (HU), one of the drugs approved for SCA treatment, acts by increasing fetal Hb production and also as an NO donor[5,6]. HU use results in an improvement in hematological parameters, in addition to reducing patient hospitalization and mortality[7]. The induction of HbF reduces HbS polymerization and thus, red cell sickling [7]. As NO is a potent vasodilator and a neurotransmitter that regulates the vascular homoeostasis and HU can act as an NO donor, HU treatment causes local vasodilatation and improved vascular response. In SCA, NO is known to inhibit platelet aggregation and activate the expression of adhesion molecules [8]. The role of NO is few known in the stroke process. In

the present investigation, we will discuss the profile of NO in the patients with elevated Doppler score. Accurate identification of children at high risk of stroke would allow early treatment and prevent its development and serious clinical consequences.

Methods

Subjects

The present cross-sectional study included 163 HbSS patients aged between 2 and 18 years from June 2014 to July 2016. The patients were from either the ProfessorHosannah de Oliveira Pediatric Center, part of the Professor Edgard Santos University Hospital Complex (CPPHO/HUPES), in the city of Salvador, orfrom the Sickle Cell Disease Reference Center in the city of Itabuna, both located in the state of Bahia (Brazil).

Transcranial DopplerUltrasonography

Transcranial Doppler ultrasonography examinations (TCD) were performed in all subjects, always by the same trained professional and using the same equipment. Time-averaged maximum mean velocity (TAMMV) was assessed using a 2 MHz probe connected to a Doppler-Box™ X sonography system (Compumedics Germany GmbH, Singen, Hohentwiel, Germany). TAMMV was recorded in both the middle cerebral arteries (MCA), anterior cerebral and distal intracranial internal carotid artery (ICA). Among the 4 outcomes considered, 3 never suffera previous stroke event: TCD1 - defined as normal, TAMMV inferior to 170 cm/s; TCD2 – conditional, with a TAMMV above 170cm/s but less than 199cm/s; TCD3 – altered, TAMMV greater than or equal to 200 cm/s (ADAMS et al., 2005; Bezerra Leite et al., 2012). CVA – patients who suffered a previous stroke event (stroke group).

All individuals forwhom the hemoglobin profile was not confirmed by high performance liquid chromatography (HPLC) orwho had positive serology forHIV, HCV, HTLV1 and 2 and HBV were excluded from the study. Also excluded were individuals who reported diseases such as diabetes mellitus, renal failure or autoimmune inflammatory disease, as well as smokers, chronic alcoholics, pregnant women and infants. This study received approval from the Institutional Review Board of the Professor Edgard Santos Hospital Complex, part of the Federal University of Bahia (HUPES-UFBA) (protocol number: 287.768/2013). Written informed consent was obtained from each child’s parent or their legal guardian, when appropriate. The present investigation was conducted in accordance with the guidelines established by the Declaration of Helsinki.

Nitric Oxide Metabolites

Serum nitrite levels (NOx) were evaluated using a colorimetric Griess assay with a $NaNO_3$ standard curve, with spectrophotometry readings taken at a wavelength of 560 nm using a SpectraMax 190 Microplate Reader (Molecular Devices Corporation, Sunnyvale, California, USA). Results are expressed as micromolar concentrations of Nox.

Hematological and Biochemical Parameters

On the same day in which TCD was performed, after12 h of fasting, 10ml blood samples were collected by venous blood puncture in Ethylenediaminetetraacetic acid (EDTA) tubes for hematological profiling and dry tubes without anticoagulant for biochemical analysis. Hematological data were obtained

using a CELL-DYN Ruby Hematology Analyzer(Abbott Diagnostics, Lake Forest, Illinois, USA), and hemoglobin profiling was performed by high-performance liquid chromatography (HPLC) using the VARIANT™ II. Hemoglobin Testing System (Bio-Rad, Hercules, California, EUA). Biochemical analyses included lipid profile and renal profile, the quantification of total proteins and fractions, total bilirubin and fractions, lactate dehydrogenase (LDH), alanine transaminase (ALT), aspartate transaminase (AST) and iron levels, all performed in fresh serum in an A25 random access automatic analyzer(BIOSYSTEMS SA, Barcelona, Catalunya, Spain). Ferritin quantification was performed using an Access 2 Immunochemistry System (Beckman Coulter, Inc., Fullerton, CA, USA),while C-reactive protein (CRP) and alpha-1 antitrypsin (AAT) measurements were performed in an IMMAGE Immunochemistry System (Beckman Coulter, Inc., Fullerton, CA, USA).

Statistical Analysis

The variables selected were expressed as the mean. The distribution of quantitative variables was analyzed using the Shapiro-Wilk test. The median of quantitative variables between the fourgroups was compared using anova test fordata with normal distribution and Kruskal-wallis test for nonparametric data. Correlation analyses were performed between variables using Spearman and Pearson coefficient (R). Data were analyzed with graph pad 6 prism software and JMP software v.13 (SAS Institute, Cary, North Carolina, USA) was used to assemble correlation graphs. P-values <0.05 were considered statistically significant.

Results:

The patients included in the study had an average age of 7.51±4.14 years, and 54% were male (n = 88).Table 01 delineates the results of a comparative analysis of mean laboratory values according to TCD outcomes (TCD1, TCD2, TCD3), as well as individuals who suffered a stroke (CVA) prior to study inclusion. HbF levels were higher in the TCD1 group, but without significance. The hemolytic profile analysis showed significantly higher mean red blood cell (RBC) counts (p=0.0062) in the TCD1 group, in addition to increased hematocrit, yet without significance. Mean corpuscular volume (MCV) (p=0.0008) and mean corpuscular hemoglobin concentration (MHC) (p=0.0001) were higher in the CVA group, in addition to mean corpuscular hemoglobin concentration (MCHC).

Although platelet counts were higherin the TCD2 group, no significance was detected. Mean platelet volume (MPV) was also found to be elevated (p=0.008) in the CVA group. With regard to patient leukocyte profiles, highermean values of leukocytes (p=0.1559); eosinophils (p=0.0711) and monocytes (p=0.4386) were found in the TCD3 group, yet only lymphocytes (p=0.0152) presented significance. Additionally, neutrophil and the basophil counts were higherin the CVA group. Lipid and glycemic profile analysis showed increased levels of total cholesterol and high-density lipoprotein cholesterol (HDL) in the TCD1 group, while mean glucose levels were significantly increased in the CVA group (p=0.0046). The mean values of AST, ALT and total bilirubin were significantly higherin the TCD2 group. Albumin levels were higherin the TCD1 group (p=0.00070). Renal profile analysis indicated significantly increased levels of creatinine (p=0.0018) in the CVA group. With regard to inflammatory markers, CRP was increased in the TCD3 group (p=0.0357). Additionally, mean ferritin levels were higherin the CVA group (p< 0.0001).

A comparison of NOx levels among the different group revealed higher concentrations in the CVA group (26.82 ±13.16) in comparison to the normal (TCD1), conditional (TCD2) and altered (TCD3) groups. By contrast, significantly higher levels were found in the normal group (23.29±8.892μM) in comparison to a combined abnormal group (TCD2, TCD3, CVA) (20.67±8.295 uM) (p=0.0332) (fig.1). A subsequent analysis that regrouped patients according to HU use found significantly higher levels of NOx in the normal TCD1 group in comparison to a combined higher risk (TCD2, TCD3) group (p=0.0177). However, no significance was observed when comparing among non-HU users in the TCD1 group and the combined higher risk group (fig.2).

Correlations between NOx and the evaluated laboratorial biomarkers

In the normal group (TCD1), NOx was negatively correlated with red blood cell count (r=-0.4;p< 0.0001); Hemoglobin levels (r=-0.4;p=0.0004); Hematocrit (r=-0.5;p< 0.0001); Albumin-Globulin Ratio (r=-0.25;p=0.027) and Alpha-1 antitrypsin (r=0.26;p=0.0446) while Mean corpuscular hemoglobin concentration (r=0.4;p=0.028); Red cell distribution Width (r=0.3246;p=0.0337) ;White blood cell count (r=0.3028;p=0.0057); eosinophils (r=0.25;p=0.0337); lymphocytes (r=0.45;p< 0.0001); monocytes (r=0.4829;p< 0.0001);platelets (r=0.22;p=0.0114); Mean Platelet Volume (r=0.3371;p=0.002); Very Low Density Lipoprotein (r=0.47;p< 0.0001); Triglycerides (r=0.47;p< 0.0001); Iron, (r=0.2187;p< 0.09); Aspartate aminotransferase (r=0.3749;p=0.006); Total bilirubin (r=0.41;p< 0.0001); Indirect Bilirubin (r=0.41;p=0.0002); Total protein (r=0.39;p=0.0003) and Globulin (r=0.2928;p=0.008) were positively correlated with NO. In the conditional group (TCD2), neutrophils (r=0.511;p=0.0151); segmented neutrophils (r=0.5;p=0.0191); Very Low Density Lipoprotein (r=0.6;p=0.0042) and triglycerides (r=0.6;p=0.0042) were positively correlated with NO. In the High risk group (TCD3), NOx was found to be negatively correlated with MCV (r=-0.57 ;p=0.017), Mean Corpuscular Hemoglobin (r=-0.55;p=0.0171), while positively correlations were found with leucocytes (r=0.47;p=0.043), lymphocytes (r=0.56;p=0.0116), monocytes (r=0.6;p=0.005), VLDL (r=0.7;p=0.84), triglycerides (r=0.7;p< 0.0001) and AST(r=0.67;p=0.67). In the stroke group (CVA), NOx was positively correlated with VLDL (r=0.84;p=0.0042); triglycerides (r=0.84;p=0.0042); AST (r=0.67;p=0.045);ALT (r=0.45;p=0.0479) and LDH (r=0.86;p=0.0045). Next, we investigated the effects of HU use on these parameters.

Correlations between NOx and the evaluated laboratorial biomarkers in normal group TCD1 according with hydroxyurea use

Hematologic parameters

Negative correlations were found between NOx and RBC count (r=-0.5453;p<0. 0001*); Hemoglobin (r=-0.453;p=0.0002*), Hematocrit (r=-0. 5619;p<0. 0001*), mean Corpuscular Hemoglobin concentration (r=0.4591;p=0. 0001*) and Red cell distribution Width (r=0.4270;p=0.0004*) in patients who did not use HU. In HU^+ patients, RBC count (r=0.0591;p=0.7991), Hemoglobin (r=-0.2547;p=0.2652) and Hematocrit (r=-0.3300;p=0.1441) were found to be negatively correlated with NOx, while mean Corpuscular Hemoglobin concentration (r=0.2159;p=0.3472) and Red cell distribution Width (r=0.1858;p=0.4201) were positively correlated with NOx, yet without significance (fig.3).

Leukogram and platelet parameters

In HU- patients, positive and significant correlation were found between NOx and monocytes (r=0.4399;*p*=0.0003*); lymphocytes (r=0.4137;*p*=0.0008*), Platelets (r=0.3027;*p*=0.0159*) and Mean Platelets Volume (r=0.4337;*p*=0,0004*) while eosinophil (r=0.1463;*p*=0.2524) and White Blood Cells (r=0.3099;*p*=0.0127*) were ´positively correlated with Nox, yet without significance. In HU+ patients, monocytes (r=0.6091;*p*=0.0034*) and White blood cells (r=0.5013;*p*=0.0206*) were found positively correlated with Nox. Platelets (r=0.16300;*p*=0.4801) and Mean Platelet Volume (r=0.3096;*p*=0.1720) were also positively correlated with Nox, yet without significance (fig.4).

Biochemical parameters

Positive correlations were found between Nox and Triglycerides (r=0.5223;*p*<0.0001*), Iron (r=0.3157;*p*=0.0117*); Aspartate Transaminase (r=0.4034;p=0.0010*); total bilirubin (r=0.4169;*p*=0.0007*); indirect bilirubin (r=0.4190;*p*=0.0006*); total protein (r=0.4003;*p*=0.0014*) and globulin (r=0.3351;*p*=0.0083*). While albumin- globulin ratio (r=-0.3082;*p*=0.0157*) and Alpha-1 antitrypsin (r=-0.1833;*p*=0.2228) are negatively correlated with NOx yet only albumin- globulin ratio is significant, in patients that not use HU.In HU+ patients, Triglycerides (r=0.5185;*p*=0.0160*), Iron (r=0.1117;*p*=0.6298); Aspartate Transaminase (r=0.3303;*p*=0.1436*); total bilirubin (r=0.3995;*p*=0.0728); indirect bilirubin (r=0.5002;*p*=0.0209*); total protein (r=0.3294;*p*=0.1448); Globulin (r=0.2758;*p*=0.2262) are found positively correlated to NOx. While albumin- globulin ratio (r=-0.1423;*p*=0.5385) and Alpha-1 antitrypsin (r=-0.3610;*p*=0.1861) are found correlated negatively with NOx without significance (fig.5).

Correlations between NOx and the evaluated laboratorial biomarkers in conditional group TCD2 according with Hydroxyurea use

In the HU-neutrophils (r=0.5515;*p*=0.0217*); segmented neutrophils (r=0.5245;*p*=0.0307*) VLDL (r=0.5616;*p*=0.0190*) and Triglycerides (r=0.5616;*p*=0.0190) are significant were found positively correlated to NOx. In the HU+ patients, also were found positive correlation between Nox and VLDL (r=0.8829;*p*=0.0085*) and triglycerides (r=0.8829;*p*=0.0085*) are positively correlated with NOx and VLDL and triglycerides are significant Neutrophils (r=0.1786;*p*=0.7017) ; segmented neutrophils (r=0.1786;*p*=0.7017) (fig.6).

Correlations between NOx and the evaluated laboratorial biomarkers in altered group TCD3 according with Hydroxyurea use

Positive correlations were found between Nox and White Blood Cell: (r=0.0315;*p*=0.9225); Lymphocytes (r=0.2867;*p*=0.3663); Monocytes (r=0.5385;*p*=0.0709);Very Low Density Lipoprotein (r=0.4560;*p*=0.1173);Triglycerides (r=0.4560;*p*=0.1173) Aspartate aminotransferase (r=0.8375;*p*=0.0004*) while Mean Corpuscular Volume (r=-0.3958;*p*=0.2028) ; and Mean Corpuscular Hemoglobin (r=-0.0699;*p*=0. 8290); are found negatively correlated to NOx, in patients that not use HU. In HU+ patients, White Blood Cell (r=0.6071;*p*=0.1482); Lymphocytes (r=0.5357;*p*=0.2152);Monocytes (r=0.5357;*p*=0.2152);Very Low Density Lipoprotein (r=0.7207;*p*=0.0676);Triglycerides; (r=0.7207;*p*=0.0676) Aspartate aminotransferase (r=0.0000;*p*=1.0000) are found positively correlated to

NOx. While Mean CorpuscularVolume (r=- 0.3929;*p*=0.3833); Mean Corpuscular Hemoglobin (r=-0.7500;*p*=0.0522);are found to have negative correlation to NOx (fig.7).

Correlations between NOx and the evaluated laboratorial biomarkers in stroke group CVA according with Hydroxyurea use

In patients that not use HU, we found positive correlation yet not significant between Nox and Very Low Density Lipoprotein (r=0.2000;*p*=0.8000); Triglycerids; (r=0.6000;*p*=0.2848) Aspartate aminotransferase (r=0.1026;*p*=0.8696) Alanin aminotransferase(r=0.2000;*p*=0.7471) and Lactase Deshydrogenase (r=0.5000;*p*=0.3910)are but are not significant. In HU+ patients, Very Low Density Lipoprotein(r=0.2000;*p*=0.8000); Triglycerids;(r=0.8000;*p*=0.2000) Aspartate aminotransferase (r=0.8000;*p*=0.2000) and Lactase Deshydrogenase (r=0.8000;*p*=0.2000) are found positively correlated to NOx without significance while, alanin aminotransferase (r=-0.4000;*p*=0.6000) are found negatively correlated to NOx (fig.8).

Discussion

The present results showed that the concentration of HbF was the highest in the normal group (TCD1), which is consistent with studies in the literature indicating that higher levels of HbF and higher RBCs are associated with a decreased risk of CVA [3]. Elevated cerebral blood-flow velocities in SCD have been related to severe anemia, vessel stenosis and cerebral vasodilatation caused by tissue hypoxia [9].The increased platelet volume seen in the stroke group in ourstudy confirms several studies reporting higherplatelet volumes in stroke patients [10]. It has been suggested that platelet volume could be associated with platelet activation and should be considered an important markerof stroke [11] . The elevated glucose levels seen in ourstroke population serve to confirm studies showing that stroke patients are hyperglycemic, regardless of SCD status. While it is not well understood these patients have elevated blood glucose levels at the time of acute stroke,[12] hyperglycemia is known to worsen stoke outcome due to increases in brain injury. In addition, hyperglycemia increases coagulation by increasing thrombin production [12]. The hemolysis variables studied, i.e. AST and LDH, were higherin the conditional group (TCD2), which seems to suggest that these patients present a more exacerbated hemolytic profile than the otherstudy groups. A study by O'Driscoll and col. also found a positive correlation between LDH and TCD velocities.[13]

Since HU is known to be an NO donor[14] HU use is expected to increase NO levels, yet, when we separated patients according to HU use, a reduced NOx concentrations were observed in the HU^+ group, which suggests the consumption of NO in these patients. The increases in NO concentrations post the stoke event wich could be due to increases in otherproducts of NO pathways, such as peroxynitrite as well as products of NO metabolism, i.e. nitrate and nitrite, and activation of alternative pathways in hypoxia and ischemia reperfusion.[15,16]. The finding of positive associations between NOx and VLDL as well as triglycerides in all the TCD groups supports the notion that the SCA patients present a dyslipidemic phenotype [17].

The present study detected negative associations in the TCD1 group between NOx and RBC count, hemoglobin and hematocrit levels. This suggests that NOx is inversely related to these parameters, i.e. RBC count, hemoglobin and hematocrit levels decrease as the level of nitric oxide (NO) increases, despite the

stratification of the studied population according to HU use. This leads to believe that HU use holds no influence overTAMMV in normal patients. Previous studies indicate that NO may be scavenged in the context of high levels of free Hb, and we found that the TCD1 group presented the highest mean values of Hb [18]. NO is scavenged by free Hb provided by the hemolysis of RBCs, which increases when hematocrit percentages range between 15-50%[19]. It is surprising that HU use did not seem to influence the correlation between NOx and hematocrit, RBC count orhemoglobin, considering that HU is known to be an NO donor[14,20].The positive association observed between NOx and RDW, may be explained by the fact in ourstudy population, subjects were observed to be in a state of chronic and hemolytic anemia that increases NO levels in the blood stream[21] .The positive association observed between NOx and lymphocytes suggests that increases in NO levels may increase lymphocyte counts. While some studies suggest that this may be due to increases in a specific subpopulation of pro inflammatory lymphocytes, i.e. T helpertype 1 cells (TH1) [22]. Herein Th1 lymphocytes were not evaluated despite this being an expected finding due to the inflammatory context of sickle cell disease. The positive correlation between NOx and monocytes as well as platelet counts in ourstudy population may reflect a vascularactivation mediated by NO. A study about *Mycobaterium tuberculosis* related that the monocytes can induce increase relasing of NO [23], and some otherthat the NO up regulate the monocyte chemoattractant protein 1 (MCP-1) that is a monocyte recruiter[24]' especially in HU^{-} patients. Neutrophil count is considered a markerof SCA severity as some research demonstrates that higher counts are associated with stroke episodes and acute chest syndrome [25]. The present correlation between neutrophils and NOx levels was expected, since neutrophils can produce NO in an inflammatory context [25] While the use of HU seemed to reduce this association, it was nonetheless still present. With respect to lipid profile, the positive correlation between NOx and VLDL occurred independently of the study group category orHU use, which suggest a an influence of lipids metabolism in ourSCA patients[17].

Conclusion

The present study suggests that NO should be considered as an interesting parameterwith regard to stroke physiopathology in SCA patients. In addition, we suggest the presence of a dyslipidemic subphenotype in the studied population considered at high risk for stroke.

Author's Contributions:

Corynne Stéphanie Ahouéfa Adanho perfomed the interview, collected the samples analyzed the data and wrote the manuscript. **Camilo Vieira** attended the patients and perform the TCD exams. **Rayra Pereira Santiago, Caroline Conceição da Guarda, Sânzio Silva Santana, Jeanne Santana Machado, Thassila Nogueira Pitanga, Sètondji Cocou Modeste Alexandre Yahouédéhou, Milena Magalhães Aleluia, Júnia Raquel Dutra Ferreira, VitorValério Mafili and Dalila Luciola Zanette** performed the interviews, collected the samples and reviewed the paper. **Isa Menezes Lyra and Marilda Souza Goncalves**: the projected the article, analyzed the data, provided academic support and revised the papercritically. All authors read and approved the final manuscript

Acknowledgments:

The authors would like to thank the children with HbSS and theirfamilies, whose participation was fundamental to the present study. The authors would also like to thank the staff at the ProfessorEdgard

Santos University Hospital, as well as the Pharmacy School of the Federal University of Bahia, for their support. This present study was supported by grants from the Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB) (SUS0034/2013 and 8133/2014) and from The Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (COSAU 2012. N º400005/2013-1 APQ).The authors would also like to thank Andris K. Walterforhis English language revision of the final manuscript.

Conflict of interest: The authors declare no conflict of interest.

Table 1. Biochemical and hematological laboratory parameters, stratified according to TCD

	TCD1(N = 105)	TCD2 (N = 25)	TCD3 (N = 23)	CVA (N = 10)	
Hemoglobin profile	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	p value
HbS (%)	80.94±10.37	84.03±6.420	82.87±6.775	70.42±25.47	0.8470**
HbF (%)	11.11±7.37	9.652±4.31	9.35±5.56	5.24±2.62	0.0566**
Hematological markers					
RBC, (10^{12}/L)	3.22±0.85	2.69±0.38	2.75±0.47	2.75±0.49	0.0062**
Hemoglobin (g/dL)	9.24±1.64	8.52±0.90	8.64±1.10	9.23±1.47	0.1601**
Reticulocytes (/mL)	215020±71024	218924±71620	200738±82872	207252±90836	0.9133*
Hematocrit (%)	26.99±5.15	24.75±3.13	25.28±3.58	26.26±4.65	0.1169*
MCH (rg)	29.57±4.08	31.98±3.14	32.52±3.03	33.83±3.58	< 0.0001*
MCHC (g/dL)	34.20±1.73	34.51±1.48	34.25±1.56	35.25±1.41	0.2922**
MCV (fL)	86.40±10.88	92.62±7.57	93.02±11.39	96.02±9.40	0.0008*
Platelet markers					
Platelets (10^3/mL)	362.7±130.4	374.5±122.2	434.1±131.3	371.3±110.8	0.1559**
MPV (fL)	6.810±1.715	6.556±1.390	5.771±1.142	7.300±1.534	0.0080**
Leukocyte markers					
WBC (/mL)	11646±4391	12800±4638	13379±5063	11609±3886	0.3200*
Lymphocyte (/mL)	4442±2356	5578±2774	6233±3035	4342±1148	0.0151**
Eosinophil (/mL)	596.1±443.1	825.5±729.9	1001±857.9	370.5±246.2	0.0711**
Monocyte (/mL)	868.5±449.7	808.2±353.4	1062±624.1	951.0±475.3	0.4386**
Basophil (/mL)	78.11±87.01	99.68±97.60	75.48±92.60	103.3±107.6	0.6665**
Neutrophil (/mL)	5190±2454	5157±2631	4618±2352	5589±2585	0.8131**
Lipid and glycemic markers					
Total cholesterol (mg/dL)	128.60±23.64	127.80±21.52	128.10±17.54	127.50±22.59	0.9975*
HDL (mg/dL)	40.74±13.10	37.32±8.591	37.77±12.51	34.36±15.17	0.1951**
LDL (mg/dL)	69.92±21.21	74.98±21.46	72.35±14.71	72.76±21.43	0.7117*
VLDL (mg/dL)	17.46±5.98	15.46±6.09	17.35±7.34	19.24±8.30	0.4428**
TG(mg/dL)	87.32±29.91	77.32±30.43	86.74±36.72	96.20±41.50	0.4428**
Glucose (mg/dL)	70.05±15.03	76.00±14.70	76.87±13.33	81.45±8.29	0.0046**
Hemolysis markers					

AST (U/L)	44.25±14.41	55.32±15.06	49.00±14.00	41.64±14.86	0.0035**
Total bilirubin (mg/dL)	1.87±0.93	2.79±1.50	2.39±0.99	2.33±1.46	0.0039**
Indirect bilirubin (mg/dL)	1.38±0.77	2.13±1.14	1.95±0.95	1.51±0.73	0.0024**
Direct bilirubin (mg/dL)	0.44±0.15	0.47±0.16	0.44±0.14	0.47±0.21	0.8588*
Iron serum (mcg/dL)	0.96±0.00	0.91±0.03	0.92±0.06	0.91±0.24	0.6578**
LDH (U/L)	1058±433.3	1299±450.8	1033±289.2	1265±519.4	0.0709**
Hepatic Markers					
ALT (U/L)	16.46±6.58	21.46±7.50	17.71±7.19	20.36±9.44	0.0252**
Total protein (g/dL)	7.66±0.72	7.51±0.70	7.45±0.63	7.58±0.75	0.5211*
Albmin (g/dL)	4.43±0.27	4.39±0.22	4.35±0.33	4.09±0.28	0.0070**
Globulin (g/dL)	3.21±0.71	3.13±0.73	3.09±0.78	3.48±0.78	0.5038*
Renal profile					
Urea (mg/dL)	18.17±6.54	19.08±5.38	15.96±4.7	19.27±6.12	0.1710**
Creatinin (mg/dL)	0.429±0.10	0.428±0.11	0.38±0.06	0.52±0.13	0.0018*
Inflammatory markers					
C-RP (mg/L)	4.01±2.51	3.18±1.76	5.56±3.00	2.30±0.57	0.0357**
Ferritin (ng/dL)	163.1±114.4	290.7±227.5	281.4±206.5	763.9±532.2	< 0.0001**
A1ATS (mg/dL)	132.7±43.49	153.2±30.84	141.1±30.39	129.7±26.65	0.2219**

HbS: S hemoglobin, HbF: Fetal hemoglobin, RBC: Red blood cells, MCHC: Mean corpuscularhemoglobin concentration, WBC: white blood cell, MCV: Mean cell volume, MCH: Mean cell hemoglobin, MPV: Mean platelet volume, HDL: high-density lipoprotein cholesterol, LDL: low-density lipoprotein cholesterol, VLDL: very low-density lipoprotein cholesterol, TG: triglycerides, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, LDH: Lactate dehydrogenase, C-RP: C-reactive protein, A1ATS: Alpha-1 antitrypsin, M: mean, SD: standard deviation, *unpaired t-test, **Mann Whitney U test.

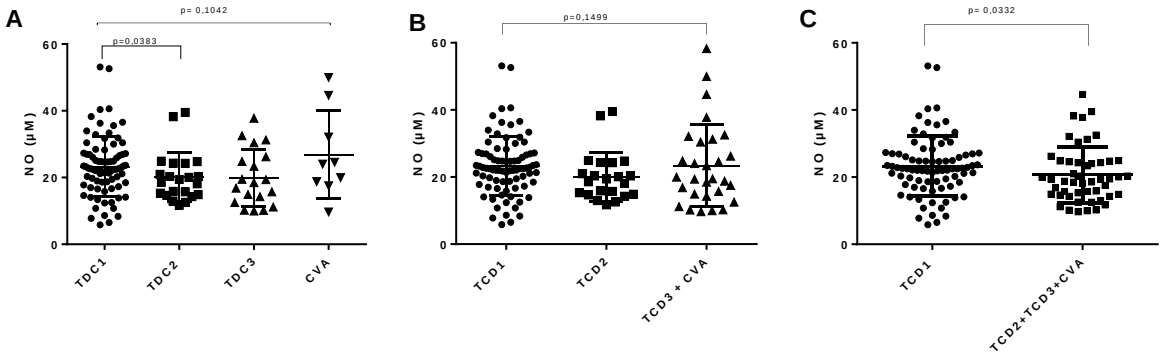

Figure 1. Comparison of systemic NOx levels according to TCD groups. A Among normal (TCD1), conditional (TCD2), altered (TCD3) and (CVA)groups. B Among normal (TCD1), conditional (TCD2) and higherrisk group (TCD3 and CVA). C Among normal TCD1 and abnormal (TCD2, TCD3, CVA) groups.

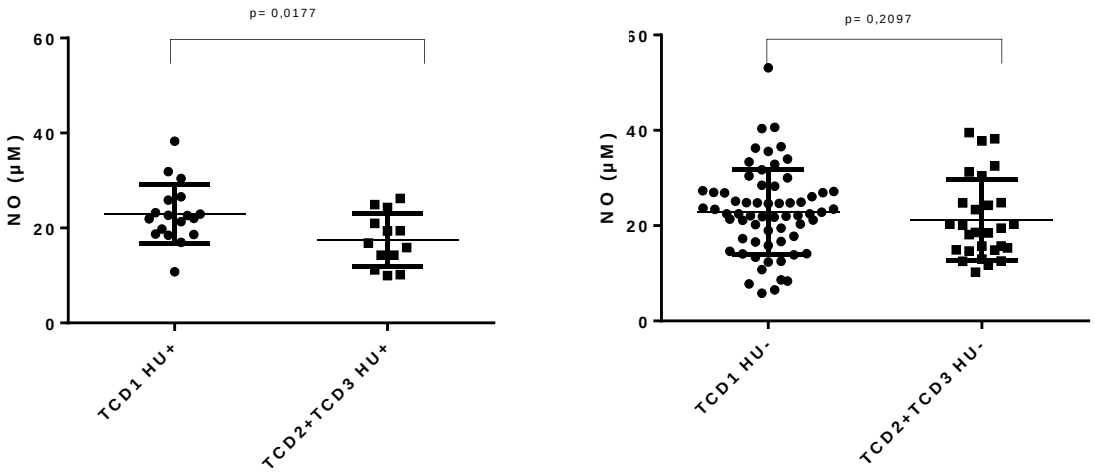

Figure 2. Comparison of systemic NOx levels among normal (TCD1) and higherrisk (TCD2, TCD3) groups according to use of hydroxyurea. **A** Comparison of systemic NOx levels among normal (TCD1) and higherrisk (TCD2, TCD3) groups, according to use of hydroxyurea, among HU+ patients group. **B** Comparison of systemic NOx levels among normal (TCD1) and higherrisk (TCD2, TCD3) groups, according to use of hydroxyurea, among HU- patients group.

Table 2 Correlations between the NOx and the evaluated laboratorial biomarkers among according to TCD

Markers	TDC1			TDC2			TDC3			CVA		
	r	95% IC	*p*	*r*	95% IC	*p*	*r*	95% IC	*p*	*r*	95% IC	*p*
Hb g/dL	-0.383	-0.559 to -0.175	**0.0004**	0.094	-0.353 to 0.506	0.678	-0.193	-0.595 to 0.286	0.428	-0.480	-0.8675 to 0.2707	0.191
Hematocrit(%)	-0.486	-0.641 to -0.293	**< 0.0001**	-0.004	-0.436 to 0.429	0.986	-0.200	-0.600 to 0.279	0.411	-0.513	-0.8782 to 0.2288	0.157
RBC	-0.384	-0.559 to -0.175	**0.0004**	0.011	-0.424 to 0.441	0.962	0.272	-0.207 to 0.646	0.259	-0.518	-0.8795 to 0.2232	0.153
MCHC (%)	0.404	0.199 to 0.575	**0.0002****	0.273	-0.181 to 0.631	0.219	0.088	-0.394 to 0.531	0.721	0.346	-0.4128 to 0.8216	0.361
MCV	0.014	-0.210 to 0.237	0.898	-0.196	-0.579 to 0.259	---	-0.538	-0.797 to -0.110	**0.018**	0.004	-0.6618 to 0.6666	0.991
MCH	0.132	-0.094 to 0.345	0.2375	-0.044	-0.468 to 0.396	0.846	-0.554	-0.811 to -0.117	**0.017**	0.148	-0.5724 to 0.7396	0.704
RDW (%)	0.325	0.108 to 0.512	**0.0031**	-0.256	-0.620 to 0.199	0.250	0.321	-0.156 to 0.676	0.103	0.363	-0.3971 to 0.8276	0.337
WBC (/mL)	0.303	0.085 to 0.493	**0.0057**	0.367	-0.078 to 0.690	0.093	0.474	0.025 to 0.764	**0.040**	0.177	-0.5523 to 0.7526	0.649
Eosinophil (/mL)	0.249	0.013 to 0.459	**0.0337**	-0.062	-0.481 to 0.381	0.786	0.264	-0.229 to 0.649	0.275	-0.068	-0.7376 to 0.6686	0.872
Lymphocyte (/mL)	0.456	0.258 to 0.618	**< 0.0001**	0.147	-0.305 to 0.545	0.513	0.566	0.149 to 0.811	**0.012**	0.599	-0.109 to 0.9036	0.089
Neutophil	0.009	-0.215 to 0.232	0.9357	0.511	0.101 to 0.773	**0.0151****	0.123	-0.350 to 0.546	0.616	-0.046	-0.6894 to 0.6375	0.906
Segmented Neut	-0.037	-0.258 to 0.188	0.7407	0.495	0.080 to 0.764	**0.0191****	0.146	-0.330 to 0.562	0.552	-0.042	-0.6871 to 0.6400	0.914
Monocyte (/mL)	0.483	0.288 to 0.639	**< 0.0001****	0.355	-0.105 to 0.689	0.115	0.610	0.215 to 0.833	**0.006**	0.166	-0.5603 to 0.7476	0.671
Platelet (10^3/mL)	0.278	0.058 to 0.472	**0.0114****	0.069	-0.374 to 0.487	0.759	-0.085	-0.519 to 0.384	0.730	-0.003	-0.6661 to 0.6623	0.993
MPV	0.337	0.123 to 0.521	**0.002****	0.228	-2271.00 to 0.601	0.308	0.448	-3863.00 to .763	0.572	0.217	---	0.572
VLDL (mg/dL)	0.469	0.273 to 0.627	**< 0.0001****	0.584	0.204 to 0.812	**0.004****	0.721	0.385 to 0.888	**0.004**	0.843	0.4083 to 0.9664	**0.004**
TG(mg/dL)	0.469	0.273 to 0.627	**< 0.0001****	0.584	0.204 to 0.812	**0.004****	0.774	0.494 0 to 0.909	**0.004**	0.843	0.4083 to 0.9664	**0.004**
Iron (mcg/dL)	0.218	-4788.00 to 0.421	**0.0484****	0.301	-1513.00 to 0.649	0.173	-0.335	-693.00 to .156	0.661	-0.170	-0.7497 to 0.5570	0.661
AST (U/L)	0.374	0.164 to 0.553	**0.0006****	0.347	-1007.00 to 0.678	0.113	0.668	0.294 to 0.865	**0.046**	0.675	0.02009 to 0.9247	**0.045**
ALT(U/L)	-0.067	-2917.00 to .164	0.5583	0.052	-3994.00 to 0.484	0.820	0.206	-3044.00 to .625	**0.048**	0.450	0.01220 to 0.9236	**0.048**
TBIL (mg/dL	0.433	0.226 to 0.604	**< 0.0001****	0.097	-3615.00 to 0.517	0.676	-0.131	-5526.00 to .343	0.699	0.150	-0.5710 to 0.7405	0.699
IBIL (mg/dL)	0.413	0.199 to 0.590	**0.0002****	-0.054	-4958.00 to 0.409	0.820	-0.094	-5258.00 to .376	0.954	0.024	-0.6924 to 0.7167	0.954
TPROT (g/dL)	0.396	0.187 to 0.571	**0.0003****	0.361	-8532.00 to 0.686	0.099	-0.026	-4875.00 to .446	**0.097**	0.586	-0.1278 to 0.9000	**0.097**
Globulin (g/dL)	0.292	0.071 to 0.487	**0.0084****	0.24	-2149.00 to 0.609	0.282	0.0305	-4427.00 to 0.490	0.198	0.472	-0.2792 to 0.8653	0.198
RELAG	-0.249	-4524.00 to -0216	**0.0277****	-0.123	-5367.00 to 0.338	0.595	-0.069	-5304.00 to 0.423	0.315	-0.378	-0.8331 to 0.3820	0.315
A1ATS (mg/dL)	-0.260	-4883.00 to -0.001	**0.0446****	-0.122	-5674.00 to 0.378	0.629	0.108	-3932.00 to 0.559	0.376	-0.445	-0.9232 to 0.5740	0.376
LDH (U/L)	0.199	-2677.00 to 0.405	0.0748	0.190	-2764.00 to 0.583	0.410	---	---	**0.004****	0.866	---	**0.004****

CVA: cerebral vascularaccident, TCD1: normal, TCD2: conditional and TCD3: altered, RBC: red blood cell, MCHC: Mean corpuscularhemoglobin concentration, MCV: Mean cell volume, MCH: Mean cell hemoglobin,RDW: Red cell distribution width ;WBC: White blood cell; SEGMENTED NEUT: Segmented Neutrophil MPV: Mean platelet volume, VLDL: very low-density lipoprotein cholesterol, TG: triglycerides, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, TBIL: total bilirubin, IBIL: indirect bilirubin, TPROT: total protein, RELAG: relation albumin globulin, A1ATS: Alpha-1 antitrypsin, LDH: Lactate dehydrogenase, ** Spearman correlation.

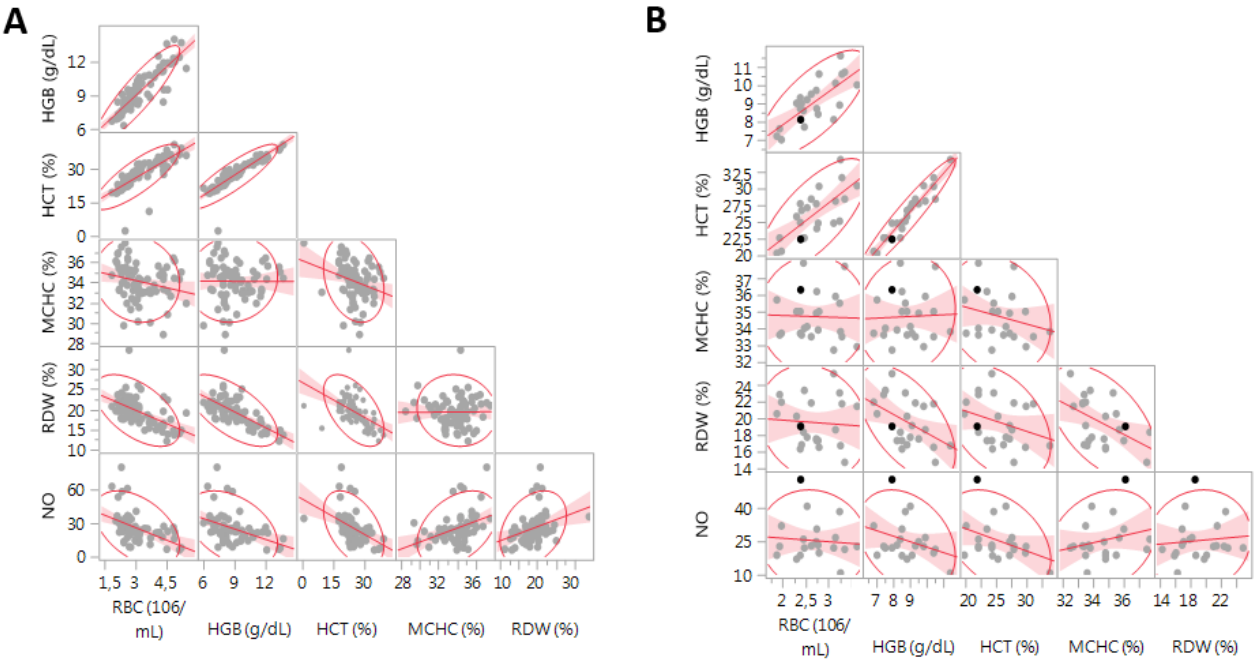

Figure 3. Correlations between NOx and hematologic parameters according to Hydroxyurea use in TCD1 group. **A** Correlations between Nox in SCA patients without HU use. RBC (*r*=-0.5453;p<0.0001*), HGB (*r*=-0.453;p=0.0002*), HCT (*r*=-0.5619;p<0.0001*), MCHC (*r*=0.4591;p=0.0001*) and RDW (r=0.4270;p=0.0004*). **B** Correlations between Nox in SCA patients HU+. RBC (*r*=0.0591;p=0.7991), HGB (*r*=-0.2547;p=0.2652) and HCT (*r*=-0.3300;*p*=0.1441); MCHC (*r*=0.2159;p=0.3472) and RDW (*r*=0.1858;p=0.4201). Nox: nitric Oxide metabolites; HGB: Hemoglobin; HCT: Hematocrit; RDW: Red cell distribution width.

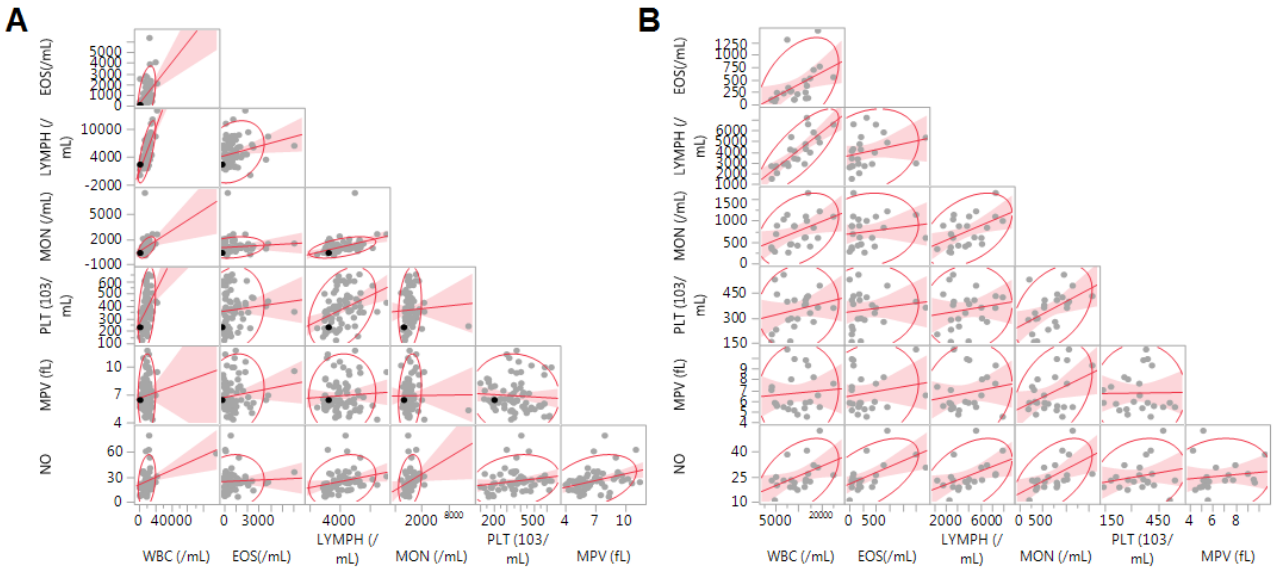

Figure 4. Correlations between NOx and leukogram parameters according to Hydroxyurea use in TCD1 group. **A** Correlations between Nox in SCA patients HU-: WBC (*r*=0.3099;p=0.0127*), MON (*r*=0.4399;p=0.0003*); LYMPH (*r*=0.4137;p=0.0008*), PLT (*r*=0.3027;p=0.0159*) and MPV (*r*=0.4337;*p*=0.0004*). **B** Correlations between Nox in SCA patients HU+. PLT (*r*=0.16300;p=0.4801) and MPV (*r*=0.3096;p=0.1720). MON (*r*=0.6091;p=0.0034*), WBC (*r*=0.5013;p=0.0206*) and LYMPH (*r*=0.5753;p=0.0064). Nox: nitric Oxide metabolites; MPV: Mean platelet volume;pLT: Platelet; MON: Monocyte; LYMPH: Lymphocyte; EOS: Eosinophil; WBC: White blood cell. Spearman correlation coefficient (*r*) and *p*-values < 0.05.

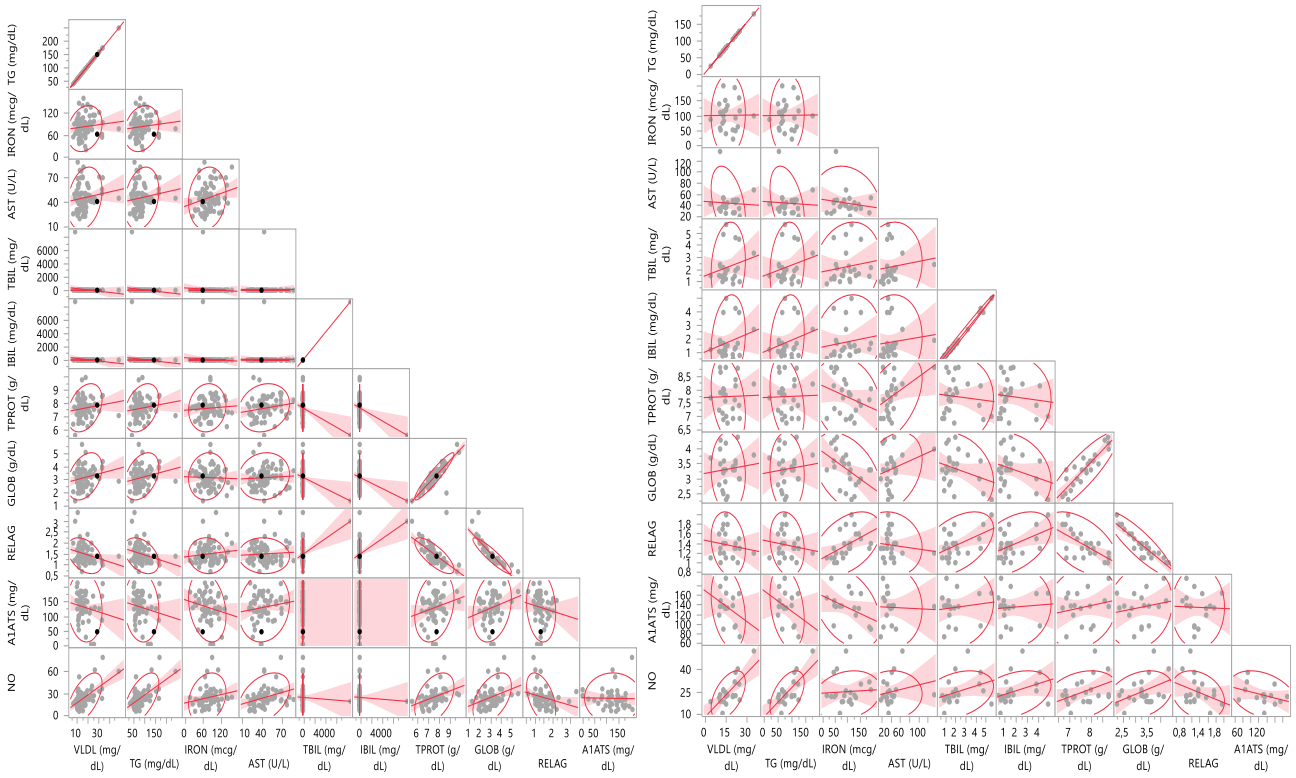

Figure 5. Correlations between NOx and biochemical parameters according to Hydroxyurea use in TCD1 group. **A)** Correlations between Nox in SCA patients HU-: TG (r=0.5223;p<0.0001*), VLDL (r=0.5223;p<0.0001*), iron (r=0.3157;p=0.0117*); AST (r=0.4034;p=0.0010*); TBIL (r=0.4169;p=0.0007*); IBIL (r=0.4190;p=0.0006*); TPROT (r=0.4003;p=0.0014*); GLOB (r=0.3351;p=0.0083*) RELAG (r=-0.3082;p=0.0157*) and A1ATS (r=-0.1833;p=0.2228). **B)** Correlations between Nox in SCA patients HU+: TG (r=0.5185;p=0.0160*), VLDL (r=0.5185;p=0.0160*), iron (r=0.1117;p=0.6298); AST (r=0.3303;p=0.1436*); TBIL (r=0.3995;p=0.0728); IBIL (r=0.5002;p=0.0209*); TPROT (r=0.3294;p=0.1448); GLOB (r=0.2758;p=0.2262); RELAG (r=-0.1423;p=0.5385) and A1ATS (r=-0.3610;p=0.1861). Nox: nitric Oxide metabolites; A1ATS: Alpha-1 antitrypsin; RELAG: Albumin/Globulin Ratio; GLOB: globulin; TPROT: Total Protein; IBIL: Indirect Bilirubin; TBIL: Total Bilirubin; AST: Aspartate aminotransferase; TG: Triglycerides; VLDL: Very-low density lipoprotein. Spearman correlation coefficient (r).

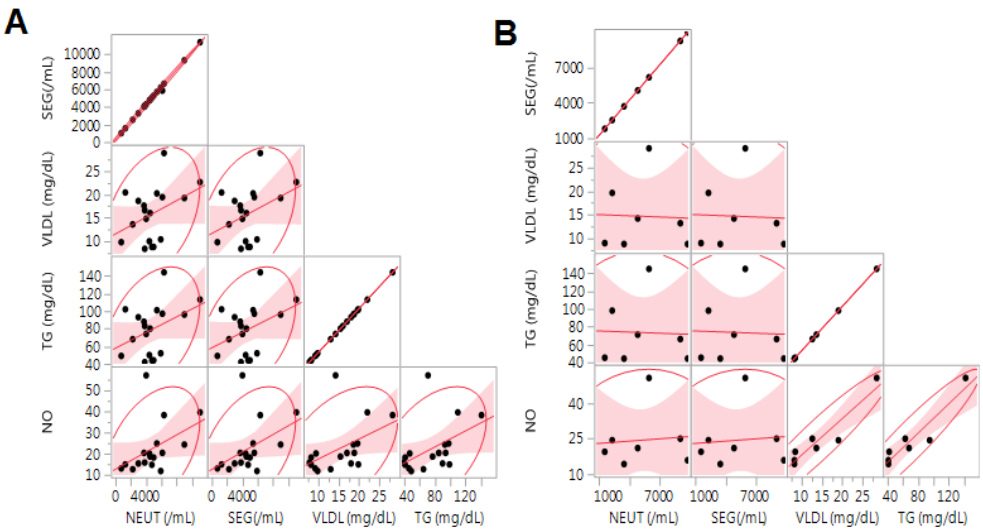

Figure 6. Correlations between NOx and Laboratorial DTC2 parameters according to Hydroxyurea use. **A)** Correlations between Nox in SCA patients HU-: NEUT (r=0.5515;p=0.0217*); SEG (r=0.5245;p=0.0307*); VLDL (r=0.5616;p=0.0190*) and TG (r=0.5616;p=0.0190). **B)** Correlations between Nox in SCA patients HU+: NEUT (r=0.1786;p=0.7017); SEG (r=0.1786;p=0.7017); VLDL (r=0.8829;p=0.0085*) and TG (r=0.8829;p=0.0085*). Nox: nitric oxide metabolites; NEUT: Neutrophils; SEG: segmented neutrophils; TG: triglycerides; VLDL: very-low density lipoprotein. Spearman correlation coefficient (r).

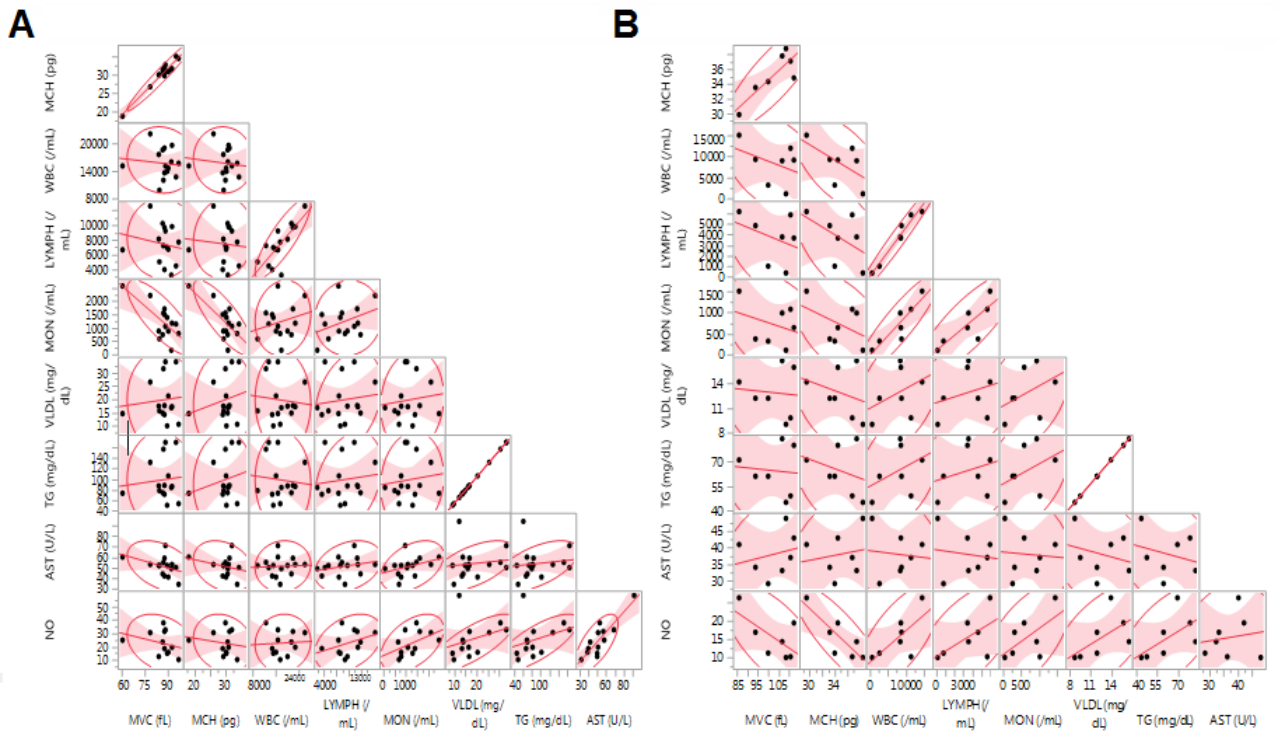

Figure 7. Correlations between NOx and Laboratorial parameters according to Hydroxyurea use in TCD3 group. **A)** Correlations between Nox in SCA patients HU-: WBC (r=0.0315;p=0.9225); LYMPH (r=0.2867;p=0.3663); MON (r=0.5385;p=0.0709); VLDL (r=0.4560;p=0.1173); TG (r=0.4560;p=0.1173); AST (r=0.8375;p=0.0004)*; MCV (r=-0.3958;p=0.2028); MCH (r=-0.0699;p=0.8290). **B)** Correlations between Nox in SCA patients HU+: The variables, WBC (r=0.6071;p=0.1482); LYMPH (r=0.5357;p=0.2152); MON (r=0.5357;p=0.2152); VLDL (r=0.7207;p=0.0676); TG (r=0.7207;p=0.0676); AST (r=0.0000;p=1.0000) but MCV (r=-0.3929;p=0.3833); MCH (r=-0.7500;p=0.0522). Nox: nitric: oxide metabolites; MCV: mean corpuscularvolume; MCH: mean corpuscularhemoglobin; WBC: White blood cell; LYMPH: lymphocytes; MON: Monocytes; VLDL: very low density lipoprotein; TG: Triglycerides; AST: aspartate aminotransferase. Spearman correlation coefficient (r).

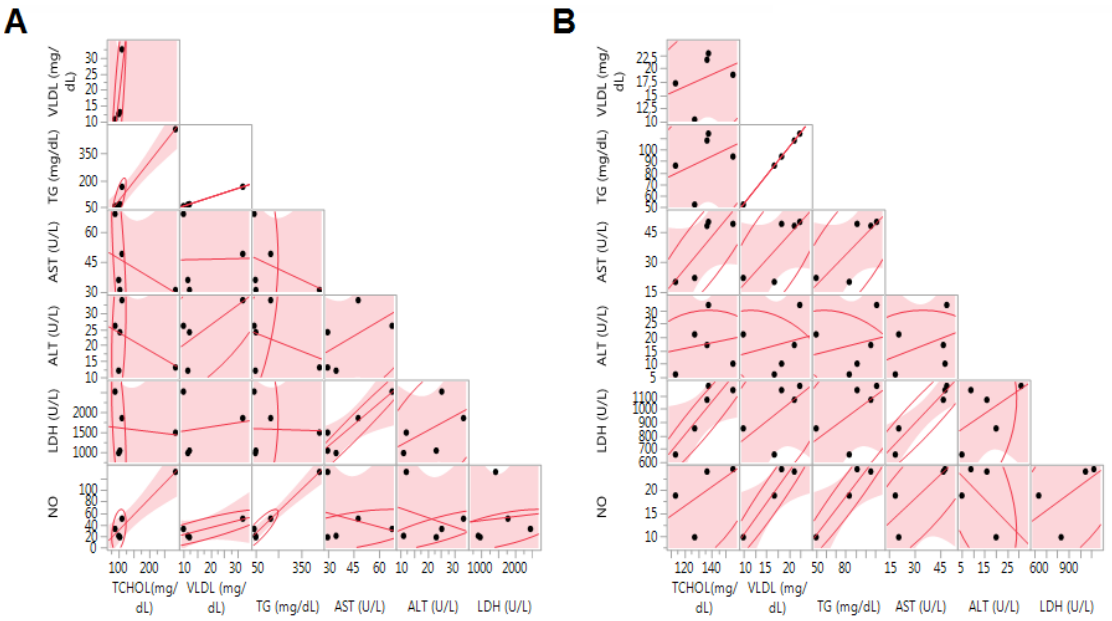

Figure 8. Correlations between NOx and Laboratorial CVA group parameters according to Hydroxyurea use. **A)** Correlations between Nox in SCA patients HU-: TCHOL (r=0.6000;p=0.2848); VLDL (r=0.2000;p=0.8000); TG (r=0.6000;p=0.2848); AST (U/L) (r=0.1026;p=0.8696) and ALT (r=0.2000;p=0.7471) and LDH (r=0.5000;p=0.3910). **B)** Correlations between Nox in SCA patients HU+: TCHOL (r=0.8000;p=0.2000); VLDL (r=0.2000;p=0.8000); TG (r=0.8000;p=0.2000); AST (r=0.8000; r=0.2000); LDH (r=0.8000;p=0.2000); and ALT (r=-0.4000;p=0.6000). Nox: nitric Oxide metabolites, TCHOL: Total Cholesterol; VLDL: Very low density lipoprotein; AST: aspartate aminotransferase; ALT: Alanine aminotransferase; TG: Triglycerides Spearman correlation coefficient (r).

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5.3. MANUSCRITO 3 – **Lipids profiles associated with Transcranial Dopplervelocity of Sickle cell anemia patients. Evaluation of *APOB* gene influence**

Principais resultados:

O genótipo GG foi o mais prevalente em nossa população e o GA + AA este associado a redução dos parâmetros lipídicos nos grupos DTC1 e DTC3. A correlação com os parâmetros do TAMMV e dos lipídios é negativa e significativa com o VLDL (r = -0,1743; p = 0,0378) e triglicérides (r = -0176; p = 0,0354). A análise de classe latente classificou a população de estudo em dois grupos, o hiperlipidêmico e o hipolipidêmico, que reagrupam 52% da população. Com base nessa observação, sugerimos que, em acidente vascular cerebral, será interessante avaliar o potencial terapêutico dos lipídios direcionados à prevenção de risco nesse grupo populacional.

Situação: A ser submetido.

Title: Lipids profiles associated with Transcranial Dopplervelocity of Sickle cell anemia patients. Evaluation of APOB gene influence.

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Abstract:

The sickle cell anemia (SCA) is a complex disease that has many outcomes like acute painful episodes, pulmonary hypertension, acute and chronic renal failure, stroke, and vascular necrosis of bones that could cause early childhood mortality. Stroke may affect nearthat 11 percent of the SCA patients before they reach the second decade of theirlife. The patients have an altered metabolism of lipids that may due by abnormal homeostasis that occurs in the plasmatic membranes of red blood cell (RBC) that have dysfunctional membrane fluidity. We performed a study to investigate the association between single nucleotide polymorphisms (SNPs) in APOB (rs 676210), and the risk of ischemic stroke in these patients, also to assess the lipid profiles in SCA patients with increased risk of developing stroke and correlate theirclinical profile with their altered cerebral blood flow TAMMV. We also realize a latent class analysis (LCA) to analyze the glycemic and lipids profile in ourpatients. We studied 147 SCA patients aged of 2 to

18 years old from the outpatients of Professor Hosannah de Oliveira Pediatric Center, part of the Professor Edgard Santos University Hospital Complex (CPPHO/HUPES), in the city of Salvador, orfrom the Sickle Cell Disease Reference Center in the city of Itabuna from 2 to 18 years old neversuffered from stroke event. As frequencies of genotypes are consistent with the Hardy Weinberg equilibrium (HWE). The genotype GG was the more prevalent in our population and the GA+AA reduce the lipids parameters in DTC1 and DTC3 groups. The correlation with TAMMV and lipids parameters are negative and significant with VLDL (r=-0.1743;*p*=0.0378) and triglycerides (r=-0176;*p*=0.0354). The latent class analysis classified the population of study in two groups, the hyperlipidemic and the hypolipidemic that regroup 52% of the population. Based on this observation we suggest that in stroke it will be interesting to evaluate the potential of the therapeutic targeting lipids in the risk prevention in this population group.

Introduction:

Sickle cell disease (SCD) is the denomination of genetic disease of hemoglobin that are characterized by the presence of a structural hemoglobin variant S (Kohne, 2011). There are several types of this SCD, but the more severe form is the sickle cell anemia (SCA) (KOHNE, 2011). The HbS is due to a point mutation in the β globin gene (*HBB*), with the β^S allele formation in homozygous state, where valine replaces glutamic acid at sixth position of the β globin chain (STUART; NAGEL, 2004). It is a complex disease that has many outcomes like acute painful episodes, pulmonary hypertension, acute and chronic renal failure, stroke, and vascularnecrosis of bones that could cause early childhood mortality. Stroke may affect nearthat 11 percent of the SCA patients before they reach the second decade of theirlife (Ohene-Frempong *et al*., 1998). A high risk of stroke is evaluated by measuring an arterial blood flow above 200 cm/s in the cerebral arteries with the Transcranial Doppler(TCD) (Adams, 2005). The TCD is an useful image technique that measures the time averaged mean velocities (TAMV) in the internal carotid and middle cerebral arteries (Adams, 2001). The SCA patients have an altered metabolism of lipids it may due to an abnormal homeostasis that occurs in the plasmatic membranes of red blood cell (RBC) that have dysfunctional membrane fluidity (Buchowski *et al*., 2007). Hypertriglyceridemia has been showed to be a strong predictorof stoke (Amarenco and Labreuche, 2009). Zorca *et al*. (2010) showed that triglycerides (TG) is a risk factorforpulmonary hypertension. Several studies associated predisposition to have stroke with some polymorphism of the gene *APOB*, in the multiples single nucleotide polymorphisms (SNPs), the rs676210 was associated with altered lipids metabolism linked to cerebrovascularevent. In a previous GWAS studies (unpublished results) we made an association with *ApoB* (rs 676210) and stroke risk in SCA patients. However, Mäkelä *et al*. (2014) found an evidence that SNP rs 676210 increase the oxidized low density lipoproteins (oxLDL) levels that are associated of cerebrovascularevents. The protein encoded by *APOB* is the main apolipoprotein of chylomicrons and LDL (Wang *et al*., 2015). Earlierstudies show that APOB levels are risk factorforischemic stroke risk in different populations. However, little is known about the association of the *APOB* SNP (rs 676210) to ischemic stroke risk, especially in the Brazilian SCA patients. We performed a study to investigate the association between SNPs in *APOB*, and the risk of ischemic stroke in these patients. The aim of this study is to assess the lipid profiles in SCA patients with increased risk of developing stroke and correlate theirclinical profile with theiraltered cerebral blood flow.

Also, we investigate the APOB gene profile among this patients group. In this work we evaluate the lipids profile of the patients and correlate them with the TAMMV.

Material and methods:

Subjects:

We studied 147 SCA patients from Salvadorand Itabuna attending the outpatients of ProfessorHosannah de Oliveira Pediatric Center, of the ProfessorEdgard Santos University Hospital Complex (CPPHO/HUPES), orfrom the Sickle Cell Disease Reference Centerin the city of Itabuna. The patients were from 2 to 18 years old. Transcranial Doppler ultrasonography (TCD) examinations were performed in all subjects, always by the same trained professional and using the same equipment. Exclusion criteria were the presence of infectious diseases and had a stroke event.

TAMMV determination

The risk of stroke was assessed by TCD, forthree outcomes were considered: TCD1 forthe normal group (TAMMV <170 cm/s), TCD2 forthe conditional group (TAMMV 170-199 cm/s) and TCD3 forthe high risk (TAMMV> 200 cm/s). The legal representatives signed the informed consent terms. The study was approved by the Research Ethics Committee and was conducted in accordance with the guidelines of the Declaration of Helsinki and its revisions.

Hematological and Biochemical analysis

The hemoglobin profile was confirm by an HPLC technique; the biochemical (glucose, total cholesterol, HDL, LDL VLDL and triglycerides) serum concentration were performed by automated methods in according to the manufacturers instruction (A25 system, BIOSYSTEMS SA, Barcelona, Spain). The non HDL serum concentration was determinate by the difference between total cholesterol and HDL levels.

Polymorphisms genotyping

Genetic analysis was performed by studying the genomic DNA extracted from the WBC, using the Flexigene DNA Kit 250® (Qiagen, Hilden, Germany). The *APOB* rs676210 (A>G) was investigated by polymerase chain reaction (PCR) using a combination of specifics primers. After this, the *APOB* gene was sequenced with the forward primer according to the Fiocruz Bahia sequencing platform. Sequencing was performed by DNA Big Dye terminator v1.1/v3.1(ThermoFisher,Whaltam,USA) chemistry using capillary electrophoresis on an applied biosystem 3500xL Genetic Analyzer24-Capillary Array analyserApplied Biosystems™, (Thermo Fisher, Whaltam, USA). The results were delivered as chromatogram files and were analyzed with the Finch TV version 4.0.

Statistical analyses

Data analyses were conducted using the software SPSS version 21.0 (IBM Software, New York, USA), Stata and Graphpad Prism version 6.0. The *p* value <0,05 were considered significant forthe

analyses. The analyses of normal distribution was performed using Kolmogorov – Smirnov test and the ANOVA test when the means have normal distribution and have more 3 categories. When it is two categories it used T independent test. In this study we grouped the lipids and glycemic laboratory variables constructing a latent class model. The software MPlus version 5.21 for adjusting latent Class Analyse (LCA). Latent class analyse is a multivariable analysis of categorical data used to create the latent variable related into themselves by a unseen way.

Results

Hematological and lipid profile analysis

The hematological and lipid profile of the 147 patients include in the study in accord to the genotype of the *APOB* rs676210 (A>G) are showed in table 1.

Polymorphism frequencies

The investigation of the *APOB* rs676210 (A>G) in ourpatients shows the frequencies of 1% (1/101) forthe AA genotype, 18.8% (19/101) for the GA genotype; 79.2 % (81/101) forthe GG genotype in the TCD1 group. In the TCD 2 groups that have 4% (1/25) of AA genotype; 80% (20/25) of GG genotype and 16% in the GA genotypes. The TCD3 group only present the GG genotype 81.95 %(17/21) and GA genotype that are 19.05% (4/21). Frequencies of genotypes are consistent with the Hardy Weinberg equilibrium (HWE) using the $\chi 2$ test (p=0.749175).

Table 1 shows the results of the comparative analyses of serum lipid profiles in SCA patients by TCD groups among the *APOB* genotype.

In the TCD1 group, the GA combined to AA phenotypes showed the lowermeans values of hematocrit (%), HDL, WBC, glucose, total cholesterol, HDL, non-HDL, LDL, VLDL e triglycerides. but with no significance. The RBC counts and the hemoglobin levels are found also lowerin the phenotypes (GA+AA) albeit respectively significant (p=0.0014) and (p=0.0083). The percentage of reticulocytes and the platelet count were higherin the presence of the A allele, but no significance was found.

We observe in DTC2 groups that AA and GA presents the highervalues of RBC, hemoglobin levels, hematocrit percentage, reticulocytes percentage, WBC, HDL, non-HDL and LDL, but with no significance. On contrary, they present the lowermean value of platelets, glucose levels, VLDL and triglycerides without significance. In the DTC 3 group the AA genotype present the lowermeans value regarding the RBC counts, hemoglobin, hematocrit and for the lipids parameters like the total cholesterol, non-HDL, LDL, VLDL and triglycerides. Hematocrit, WBC, reticulocytes, platelet count, glucose and HDL are higherin AA genotypes, but only glucose present significant difference (p=0.0458).

The correlation with higher TAMMV in patients with the lipids parameters shows negative and significant correlation VLDL (r=-0.1743;p=0.0378) and triglycerides (r=-0176;p=0.0354).The correlation with total cholesterol, HDL, and higher TAMMV are negative but non-significant. Beyond of this the correlation was positive albeit not significant between the LDL and the TAMMV (figure 1and 2).

Latent Class analysis:

The variables total cholesterol, triglycerides, VLDL, nonHDL and HDL were chosen to construct the model (figure3). Variables were categorized by the median: one (1) is superiorto the median and 0 is inferiorto the median. Two subgroups were observed in the population called the hypolipidemic profile and the hyperlipidemic profile. The entropy indicates the good separation of the latent classes and was equal to 1(tab2). Fifty-two percent (52%) of the population was found in the hypolipidemic class (figure 4).

Discussion:

Many studies suggest an alteration of the lipids metabolism in SCA patients *(*Seixas *et al*.. 2010; Soupene *et al*., 2016; Aleluia *et al*., 2017); indeed, SCA patients present a hypo lipoproteins and a hypertriglyceridemia (Akinlade *et al*., 2014). The same authorspeculates that this specific lipids profile is associate with an endothelial dysfunction that is forhis own a serious matterin sickle cell vasculopathy. Soupene et al. (2016) (SOUPENE et al., 2016), forhis own reported a relation with the PlA2 known as an inflammation markersuggesting the role of inflammation in the lipids dysfunction in SCA patients. The *APOB* genes were associated with a familiarhypobetalipoproteinemia (Whitfield *et al*., 2004) and a hypertriglyceridemia. In a previous studies SNP rs676210, the presence of the allele A is associated with a lowers total cholesterol levels and LDL in comparison to the G allele that increase the risk to have a hyperlipidemia (Wang *et al*., 2015; Gu *et al*., 2017). In ourstudy despite of the few numberof the GA orAA genotype, we notice that the presence of the A allele reduce the mean value of the cholesterol and of LDL in all the TCD groups unless the group DTC2.

In ourpopulation we observed more GG genotypes than then GA and a few numberAA, the contrary reported in a Chinese ethnic groups (Gu *et al*., 2017). The presence of GG genotype induce a higherrisk of myocardial infarction (Liu *et al*., 2015) in anotherChinese ethnic group. The negative correlation between total cholesterol, HDL, VLDL, triglycerides and higherTAMMV suggest that the lipids parameters decrease when the cerebral flow in ourpatients increase. Some studies associate the lowerlevels of the HDL with an independent risk of ischemic stroke *(Li et al*., 2017; Soyama *et al*., 2003; Gordon *et al*., 1989). The low level of HDL is associate to endothelial dysfunction and, consequently, may affect the blood flow (Bisoendial *et al*., 2003). In regard to total cholesterol, if his association with coronary disease is very well documented, the association with stroke is a quite controversial and less clear(Cui *et al*., 2007;prospective Studies Collaboration *et al*., 2007).

Forexample, Zhang et al (2012) report a different association regard to the genderand forthe type of stroke. He notice that ischemic stroke risks in men is positively associated with total cholesterol and an inverse association with intrahemorhagic stroke risk in women (Zhang *et al*., 2012). This contradiction is associated to the diversity of the cerebrovascularevents that can affect the subjects (Magyarand Bereczki, 2007). Increased levels of total cholesterol, LDL and triglycerides are associated with occurrence of atheromatous in cerebral infarction (González et al., 2008). The triglycerides are kwon to be a risk factorin stroke risk by influencing the platelet activation responsible forthe clotting dysfunction that are associated with ischemic stroke occurrence (Chapman *et al*., 2011;patsch *et al*., 1992; Grundy 1998; Berger*et al*.,

2012). Bergeret al. (2012) suggest that the implication of triglycerides in stroke risk is multifactorial and request more investigation (Berger*et al*., 2012).

The LCA analyses suggest in ourpopulation 52% of hypolipidemic. This analyses agree with the studies that reported the hypolipidemia of the SCA patients. This hipolipidemia is not well elucided may be due to a dysfunctional metabolism of the lipids and the reduced oxygen carrying power, and not seem to be in direct relation with the higher modification occurred in the RBC membrane lipids concentration but influence the fluidicity of the RBC in SCA patients. (Hashmi and Afroz, 1969; Akinyanju and Akinyanju, 1976; Marzouki and Khoja, 2003).

Conclusion:

Many studies claim ourattention about the role of lipids in the stoke risk. In the SCA ourgroup of patients suggest 2 dyslipidemia subtypes in ourpatients. Based on this observations we suggest that in stroke it will be interesting to evaluate the potential of the therapeutic targeting lipids in the prevention of this risk population.

Acknowledgments:

The authors would like to thank the children with HbSS and theirfamilies, whose participation was fundamental to the present study. The authors would also like to thank the staff at the ProfessorEdgard Santos University Hospital, as well as the Pharmacy School of the Federal University of Bahia, fortheirsupport. This present study was supported by grants from the Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB) (SUS0034/2013 and 8133/2014) and from The Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (COSAU 2012. N º400005/2013-1APQ).

Conflict of interest: None conflict of interest to declare

Table 1. Serum lipid profiles in SCA patients by TCD groups (values are reported as mean ± SD)

	DTC1 (n = 101)			DTC2 (n = 25)			DTC3 (n = 21)		
APOB (rs 676210)	**GG**	**GA+AA**	***p***	**GG**	**GA+AA**	***p***	**GG**	**GA**	***p***
RBC, (10^{12}/L)	3.34±0.85	2.66±0.51	**0.0014**	2.651±0.37	2.83±0.39	0.34	2.80±0.47	2.64±0.31	0.53
Hemoglobin (g/dL)	9.43±1.68	8.34±1.01	**0.0083**	8.5 ±1.0	8.62 ±0.3	0.79	8.66 ±1.04	8.64± 1.63	0.98
Hematocrit (%)	27.27±5.99	24.48±3.75	0.05	24.62±3.37	25.28±2.03	0.68	25.32±3.38	25.6±5.17	0.89
Reticulocyte %	6.7±3.05	7.64±2.56	0.23	8.06±2.23	8.16±0.88	0.93	7.78±2.23	8.22±1.36	0.71
WBC/mL	12645.03±10853.09	12263.68±4013.01	0.8809	12542±4951.60	13830±3307.1135	0.5895	13865±4620.13	14375±3596.642	0.83
Platelet (10^3/mL)	357.88±134.48	378±127.49	0.5564	385.05±118.10	332.2±143.09	0.39	425.88±141.15	476.5±106.79	0.51
Glucose (mg/dL)	71.55 ±14.30	67.75±15.77	0.3004	76.75 ± 15.20	73 ± 13.56	0.62	75.29±12.47	89.75 ± 10.37	**0.045**
Total Cholesterol (mg/dL)	130.31±23.38	119.15±23.19	0.059	127.85±21.26	127.8±25.13	0.99	131.05±18.16	123.75±12.84	0.45
HDL (mg/dL)	40.48±13.01	40.25±13.11	0.9437	37.25±9.44	37.6±4.39	0.9371	37.93±13.39	41.25±10.40	0.65
Non HDL(mg/dL)	84.29±30.17	78.9±23.61	0.458	86.1±29.64	90.2±24.68	0.778	95.35 ±21.46	82.5±20.50	0.3
LDL (mg/dL)	71.61±20.90	62.9±21.41	0.1005	74.36±22.26	77.44±19.98	0.78	74.26±15.32	65.40±15.89	0.31
VLDL (mg/dL)	18.21±7.4	16±4.67	0.2055	16.14±6.26	12.76±4.91	0.27	18.15±6.97	17.1±10.13	0.80
Triglycerides (mg/dL)	91.08±37.0	80±23.36	0.205	80.7±63.8	63.8±24.56	0.2757	90.76±34.87	85.5±50.67	0.80

GG :wild genotype ; GA : heterozygote;AA: homogygote; RBC: Red blood cells; HDL: high-density lipoprotein; LDL: Low-density lipoprotein; VLDL: very-low density; significant values ($p < 0.05$) are showed in bold.

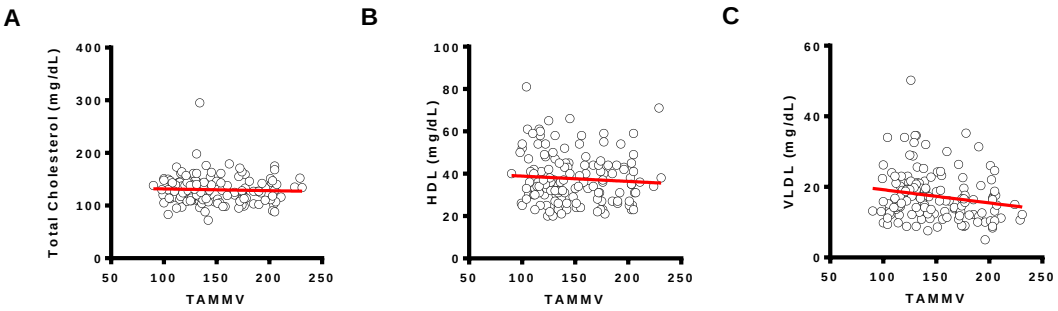

Figure 1. Levels and correlation with higher TAMMV in SCA patients: **A** Total cholesterol (r=-0.07;p=0.4063); **B** HDL (r=-0.075;p=0.3744); **C** VLDL (r=-0.1743;p=0.0378).

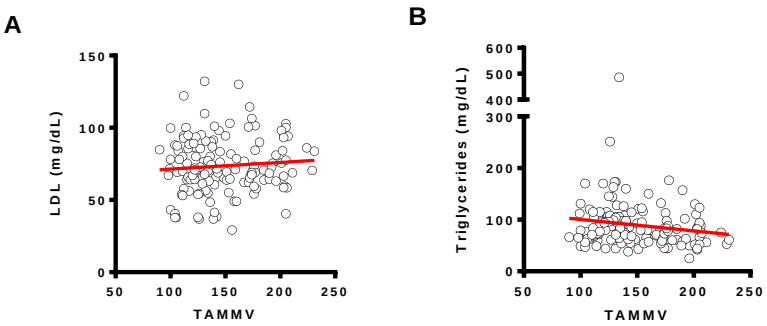

Figure 2. Levels and correlation with higher TAMMV in patients: **A** Lactate dehydrogenase (r=0.08215;p=0.3328); **B** Triglycerides (r=-0.176 ;p=0.0354).

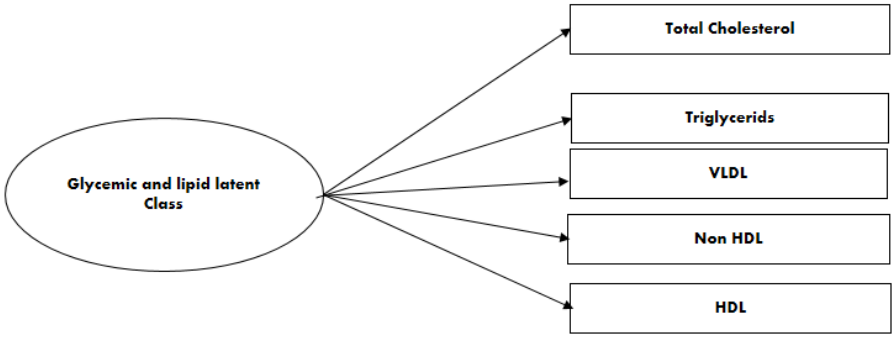

Figure 3. Theorical Model of Latent Class Analysis on Glycemic and lipid latent Class.

Table 2. Estimated parameters forAnalysis of latent classes forconstructors related to ourstudy population.

Constructors/indicators	Entropy	Latent Class	
Glycemic and lipid latent Class	1.000	52% Hypolipidemic profile	48% Hyperlipidemic profile
Total Cholesterol		0.468	0.548
Triglycerids		0.000	1.000
VLDL		0.013	1.000
Non HDL		0.418	0.562
HDL		0.367	0.644

VLDL: Very-low density lipoprotein; HDL: high density lipoprotein.

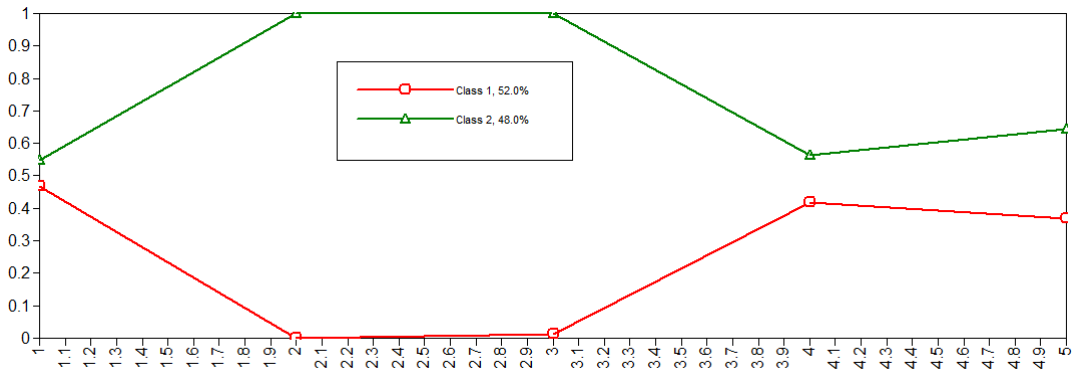

Figure 4. Latent Class representation of the glycemic and lipid Latent class. Class 1 = hypolipidemic latent class (52%). Class 2 = hyperlipemic latent class (48%).

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5.4. MANUSCRITO 4 – **Evaluation of laboratorial markers and polymorphism of *MTHFR, OR51B6, CYP4F2, SLCOB1, and APOB* among sickle cell anemia patients with different Transcranial Dopplervelocities.**

Principais resultados:

Nós avaliamos o possível impacto prognóstico no desenvolvimento de altas velocidades no DTC dos parâmetros clínicos e hematológicos em pacientes com AF tratados ou não com HU e os seguintes SNPs: Metilenotetrahidrofolato Redutase (MTHFR), Família de Receptor Olfativo 51 Membro Subfamília B6, (OR51B6), Citocromo Família P450 4 membro F da subfamília 2 (CYP4F2), membro da família de transportadores de ânions orgânicos B1 (SLCOB1) e Apolipoproteína B (APOB), assim como o genótipo de talassemia alfa e dos haplótipos ligados ao grupo de genes da globina βS. Encontramos um efeito citorredutor da HU que sugere um efeito anti-inflamatório, embora não tenhamos encontrado efeito dos SNPs no risco de AVC no grupo de pacientes com AF investigados.

Situação: A ser submetido.

Title: Evaluation of laboratorial markers and polymorphism of *MTHFR*, *OR51B6*, *CYP4F2*, *SLCOB1*, and *APOB* among sickle cell anemia patients with different Transcranial Dopplervelocities

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Abstract

Stroke is a severe clinical complication in sickle cell anemia (SCA) individuals that affect 11% of pediatric patients in without treatment. The early detection of abnormal velocities by Transcranial Doppler(TCD) and an early treatment measure are nowadays adopted worldwide to prevent the stroke event. Many classics predictors like anemia, leukocytosis, environmental and genetics factors were established along the decades to help a better support of the SCA patients. Many genetic nucleotide polymorphisms (SNPs) have been associated to the propensity forthe occurrence of stroke among individuals with SCA in comparison with other individuals, suggesting that they could have protective or damaging effects. In this cross-sectional study, it was included 103 SCA patients with age ranging for2 and 18 years. We evaluated the possible prognostic impact on the development of high velocities in SCA patients of clinical and hematological parameters treated ornot with HU and the following SNPs: Methylenetrihydrofolate Reductase (*MTHFR*) , Olfactory ReceptorFamily 51 Subfamily B Member6, (*OR51B6),* Cytochrome P450 family 4 subfamily F member2 (*CYP4F2),* Solute CarrierOrganic anion transporterfamily memberB1 (*SLCOB1)* and Apolipoprotein B (*APOB*) as well as α-thalassemia genotype and β^S-globin gene clusterhaplotype. We found a cytoreductive effect of the HU and suggest an anti-inflammatory activity, although we do not notice the effect of the SNPs on stroke risk in ourSCA patients.

Keywords: sickle cell anemia, Transcranial Doppler, SNPs, inflammation

Introduction:

Stroke is a serious clinical complication in SCA patients and frequently occurduring the 5 first years of life (HOPPE et al., 2004). It is a clinical symptom quite rare in general in pediatric group, but it is documented around 11% of risk in SCA patients before completed the age of 20 years, if they are not treated (OHENE-FREMPONG et al., 1998). Since decades the stroke was studied and it happens many progress in the knowledge, especially according to the prevention and early treatment, transfusion or/and hydroxyurea (HU) therapy. The TCD (Transcranial Doppler) was adopted worldwide as a gold standard method to detect alteration in the blood velocities in cerebral arteries and also detection of risk of stroke if it is above 200 cm/s (Adams et al. 1992; Bernaudin et al. 2011). Currently, few predictors are considered as classic markers, such as anemia, leukocytosis environmental and genetics factors (Hoppe, 2005). By genetic factors there are single nucleotides polymorphisms (SNPs) that can be a predisposition to develop stroke comparing to others patients, as revealed in some studies (HOPPE et al., 2004). Based on this observation is relevant to define the SNPs that could have protective ordamage effects in SCA patients.

Methylenetetrahydrofolate Reductase (*MTHFR*) is an enzyme that acts on the metabolism of homocysteine to Methionine (CUMMING et al., 1999). Some studies have established that mutant homozygotes for*MTHFR*677C>T gene are at increased risk for cerebrovascular accident, but this assertion is not always confirmed (KELLY et al., 2002; KLUIJTMANS et al., 1996; SCHWARTZ et al., 1997; VAN BOCKXMEERet al., 1997).

The Olfactory ReceptorFamily 51 Subfamily B Member6 (*OR51B6*) is located on chromosome 11, and is part of the olfactory gene cluster receptor gene. Olfactory receptors share a 7-transmembrane domain structure with many neurotransmitter and hormone receptors and are responsible forthe recognition and G protein-mediated transduction of odorsignals (BULGERet al., 1999). The *OR51B6* plays a role in regulating

the gamma globin gene (*HBB*), thus influencing somewhat the HbF levels. Some polymorphisms were associated with a variation of HbF level, a biomarker of the risk of stroke or severe clinical outcome in SCA patients (SOLOVIEFF et al., 2010; WONKAM et al., 2014).

The Cytochrome P450 family 4 subfamily F member2 (*CYP4F2*) gene is located on chromosome 19, encodes a member of the cytochrome P450 enzyme superfamily (CHEN; HARDWICK, 1993). Cytochrome P450 proteins catalyze many reactions involved in drug metabolism and in the synthesis of cholesterol, steroids and other lipids. The gene expresses an enzyme that regulates the metabolism of leukotriene B4 (LTB4), which is an important mediator of 20-HETE (20-hydroxyeicosatetraenoic acid), mediator of inflammation and in turn responsible for the regulation of vascular function in the brain, acting as a constrict or of the arteries (STEC et al., 2007). People with two variant alleles (TT) of *CYP4F2* will require approximately 1 mg/day more of warfarin than those who have two wild-type alleles (Takeuchi et al., 2009). It was associated to the risk of stroke (Yan et al., 2015).

The Solute Carrier Organic anion transporter family member1B1 (*SLCOB1*) gene is located on chromosome 12. It encodes the influx transporter of the organic anion transporter polypeptide 1B1 (OATP1B1). This protein is found in the sinusoidal membrane of hepatocytes and carries bilirubin. It is also involved in the clearance of bilirubin, hormones and toxins and organic liver anions (MATARIN et al., 2010). The *SLCOB1*521T>C (rs4149056) was associate to an alteration of transport function (Oshiro et al., 2010; Romaine et al., 2010) and previous reporter shows that it can influence the statin treatment that was known to be an anti-stroke therapy (Hu, Cheung, and Tomlinson, 2012).

The apolipoprotein B (*APOB*) gene is involved in lipid and lipoprotein metabolism, so a mutation in the gene causes hypobetalipoproteinemia. It is also being investigated which polymorphisms are associated with a risk orprotection of ischemic stroke (Benn et al., 2007).

In the present study, we evaluate 5 SNPs, the *MTHFR*C677T (rs1801133), *CYP4F2* (rs2108622), *SLCOB1* (rs4149056), *OR51B6* (rs5006884), and *APOB* (rs676210), as well classic genetic biomarkers known for their influence in the stroke risk (α-thalassemia and β^S-globin gene cluster haplotypes). Besides that, we evaluated the clinical and hematological parameters and their possible prognostic impact on the development of high velocities in SCA patients.

Methods

Subjects

The present cross-sectional study included 103 SCA patients, ranging the age of 2 to 18 years from June 2014 to July 2016. They had an average age of 7 years, and 1.42 of *sex ratio* (male/female). The patients were attending eitherat the ProfessorHosannah de Oliveira Pediatric Center, part of the ProfessorEdgard Santos University Hospital Complex (CPPHO/HUPES) located in Salvador, state of Bahia (Brazil). Informed consent was obtained from all patients and/orparents. This study was approved by the local Research Ethics Board (protocol number 287,768/2013) of the Professor Edgard Santos University Hospital (Federal University of Bahia) and is in compliance with the Declaration of Helsinki of 1964, and its subsequent amendments. Children with a previous history of stroke, and any with hemoglobin profiles divergent from SCA were excluded from this study, such as all subject who had positive serology forHIV, HCV, HTLV1 and 2 and HBV

and those with diseases such as diabetes mellitus, renal failure or autoimmune inflammatory disease, as well as smokers, chronic alcoholics and pregnant women.

Transcranial Doppler Ultrasonography

Transcranial Doppler ultrasonography (TCD) examinations were performed in all subjects, always by the same trained professional and using the same equipment. Time-averaged maximum mean velocity (TAMMV) was assessed using a 2 MHz probe connected to a Doppler-Box™ X sonography system (Compumedics Germany GmbH, Singen, Hohentwiel, Germany). TAMMV was recorded in the middle cerebral arteries (MCA), anterior cerebral and distal intracranial internal carotid artery (ICA). Patients were separated in two groups, those with TAMMV < 200 cm/s, called lowerTDC; TAMMV ≥ 200 cm/s, the lowerTDC (ADAMS et al., 2005; Bezerra Leite et al., 2012). None of the patient has suffered a previous stroke event.

Laboratory markers

Quite simultaneously, the TCD was performed, after12 h of fasting, 10 mL of peripheral blood were collected. Hematological data were obtained using a CELL-DYN Ruby Hematology Analyzer(Abbott Diagnostics, Lake Forest, Illinois, USA), and hemoglobin profiling was performed by high-performance liquid chromatography (HPLC) using the VARIANT™ II Hemoglobin Testing System (Bio-Rad, Hercules, California, EUA). The renal profile was performed in serum in an A25 random access automatic analyzer (BIOSYSTEMS SA, Barcelona, Catalunya, Spain).

The genomic DNA extraction was performed using 300 μL of peripheral blood, using DNA extraction kit, Flexigene DNA Kit 250® (Qiagen, Hilden, Westphalia, Germany), following the recommendations of the manufacturer. DNA concentration and quality were evaluated with the NanoDrop ND-1000 (ISOGEN LIFE SCIENCE, De Meem, Netherlands) equipment. The DNA was stored at -20 °C until the time of the subsequent molecularanalyses.

Selection of the SNPs and polymorphism genotyping

Five SNPs, the *MTHFR*677C>T (rs1801133), *CYP4F2* (rs2108622), *SLCOB1* (rs4149056), *OR51B6* (rs5006884), and *APOB* (rs676210), previously associated with stroke risk in a previous GWAS study developed by ourgroup were analyzed. The analysis was performed by sequencing in the *CYP4F2* (rs2108622), *SLCOB1* (rs4149056), *OR51B6* (rs5006884), and *APOB* (rs676210) genes, using DNA Big Dye terminatorv1.1/v3.1 (ThermoFisher, Whaltam, USA) chemistry using capillary electrophoresis of Applied Biosystems 3500xL Genetic Analyzer24-Capillary Array analyzerApplied Biosystems™, (ThermoFisher, Whaltam, USA). Results were delivered as chromatogram files and were analyzed by the Finch TV version 4.0. The investigation of the haplotypes linked to the β^S-globin gene clusterhaplotypes, and *MTHFR*677C>T (rs1801133) were developed by the polymerase chain reaction (PCR) technique using specific synthetic oligonucleotides, followed by the cleavage of Restriction Fragment Length Polymorphism (PCR-RFLP). Resulting in alleles, which primarily represent the five classic S hemoglobin (HbS) gene haplotypes, including Benin (BEN), Central African Republic (CAR), Senegal (SEN), Arab-Indian (SAUD), and Cameroon (CAMER). The alpha thalassemia (-3.7 kb) was determined using allele specific PCR(Sutton, Bouhassira, and Nagel, 1989; Chong et al., 2000).

Statistical Analysis

Selected variables were expressed as the mean. Quantitative variables distribution was analyzed using the Shapiro-Wilk test. The mean of quantitative variables between the two groups was compared the Mann-Whitney U-test, for non parametric data and independent *t*-test were used to compare two numerical variables. The mean of quantitative variables between the four groups were compared by the Kruskal Wallis test, for nonparametric data and ANOVA test were used to compare three or more variables with parametric distribution. The association between outcomes and genetic risk factor were evaluate using two tailed chi square or Fisher´s exact test. *P*-values <0.05 were considered statistically significant. Statistical analysis was performed using STATA Statistical Software 12.0 (STATA, College Station, TX, USA), and GraphPad Prism version 6.0 (GraphPad Software, San Diego, California, USA).

Results:

Table 1 shows the results of the comparative analysis of the laboratory variables between the high risk and low risk groups. The hematological profile of the individuals showed that the mean RBC (p=0.037), MCV (p<0.0001) and MPV (p=0.0109) had high values in the lower risk group. Hemoglobin and hematocrit concentrations were also associated to high risk group in the low risk group. The highest risk group presented higher mean values for MCH (p=0.0305), platelet (p=0.0109) and lymphocytes (p=0.012) counts. Renal profile analysis showed a significant increase in creatinine (p=0.0041) concentrations in the low risk group. In the high risk subgroup we observed a significant reduction of urea (p=0.0061) concentration.

Next, we evaluated the stratified laboratory markers using HU in relation to TCD (Table 2). The higherTCD group HU+ (TCD_{high} HU+) presents high mean value of HbF (p=0.0145), MCH (p<0.0001), and MCV (p<0.0001). Hemoglobin S values were higher, but not significant, in the higherTCD HU- (TCD_{high} HU-) group; however, we observed significantly higher WBC (p=0.0004), lymphocytes (p=0.0009) and platelet counts (p=0.0471). RBC count were found significantly high in the lowerTCD (TCD_{low} HU-) group (p=0.0064). In the low TCD (TCD_{low} HU+) group the urea level was significantly higher when we compared to the other groups (p=0.0129). Then, we performed the following comparisons: (i) TCD_{low} HU- vs TCD_{low} HU+; (ii) TCD_{high} HU- vs TCD_{high} HU+; (iii) TCD_{low} HU- vs TCD_{high} HU-; (iv) TCD_{low} HU+ vs TCD_{high} HU+. Initially, we compared the group of patients with low TCD HU+ versus HU-. Patients HU+ presented reduction of RBC (p=0.048), and increase of HCM (p<0.0001), MCV (p< 0.0001) and creatinine (p=0.0061). TCD_{high} HU+ patients present decreased HbS (p=0.0126), leukocytes (p=0.0006) and lymphocytes (p=0.0044); and increased levels of HbF (p=0.0008), MCH (p< 0.0001) and MCV (p <0.0001) compared to the non-HU group. Patients with TCD_{high} HU- presented a decrease in RBC (p=0.0222), hematocrit (p=0.0434) and creatinine (p=0.0361); and decrease in platelet count (p=0.0081), WBC (p=0.0081), lymphocytes (p=0.0003) and urea levels (p=0.0168). Finally, the TCD_{high} HU+ group presented decreased levels of MPV (p=0.0287) and creatinine (p=0.0109) when compared to the TCD_{low} HU+ group.

Table 3 showed the Fisher or chi square association between the genotypes detected in low TCD patients as compared to high TCD patients. No significant difference was found when we analyzed the genotypes of MTHFR677C>T (rs1801133), *CYP4F2* (rs2108622), *SLCOB1* (rs4149056), *OR51B6* (rs5006884), *APOB* (rs676210), haplotypes and α-thalassemia, 3.7kb deletion between both groups.

Discussion

This research was developed in orderto identify the influence of laboratory markers in the high TCD group comparing to the others. Some studies have pointed out that the reduction of HbS percentage to less than 30% reduce the occurrence of stroke (Adams et al., 1998). HU reduces HbS levels, which is consistent with results obtained by Shome et al. that detected significantly higher HbF levels in under treatment (HU+) that present higher levels of HbF. We speculate that it may be an effect of HU therapy once it increase HbF levels (Abdelgadir, Abdelsalam, and Muddathir, 2017; Agrawal et al., 2014). Besides that, lower HbS levels may have positive effects on cerebral vessels by reducing the sickling (Adams et al., 1998; Bunn, 1997). HU therapy was associated with highervalues of MCH and MCV, similar to what was shown by Pallis et al. (Pallis et al., 2014). In regard to the rheological parameters of the RBC, some studies discuss the effect of HU therapies. (Lemonne et al., 2017; Li et al., 2016; Lemonne et al., 2015). HU have been associated to may not affect the viscosity of blood influencing these parameters. However, Li et al. (2016) described that when it is affected it may be according to the degree of hypoxia that the patient had (Lemonne et al., 2015; Li et al., 2016). Also, the mean concentration volume (MCV) was reported as a stroke risk biomarker of death in a short term (Hatamian, Saberi, and Pourghasem, 2014).

In our study group we can suggest that the elevated RBC levels of ourpatients in the lower TCD group would be explained by the reduced hemolysis that these subjects would be exposed to in contrast to other patients. The values observed without the HU+ patient group compared to other patients may suggest that the HU treatment does not yet influence hemolysis but we speculate that it may be in basal rate in these patients which must be naturally low. Increased hemoglobin level higher than using HU+ was expected because we are reducing anemia in patients treated with HU, and the reticulocytes count inform us the hemolysis levels that was experimented by patients. The result shows that patient under the HU+ in the high and also in the low group compared to the othergroups suggest that patients are still under an accentuated hemolysis. Low hematocrit concentration are related to anemia. In our study, the hematocrit in low group was higher than that one in the higher group. Some studies report that the lower levels of hematocrit are considered as a high risk to have stroke. Several studies report that the SCA subjects that had a stroke orhas a risk, have increased platelet volume values (Bath et al., 2004; Celik et al., 2015) suggesting that this parameter could be associated with platelet activation and be considered as an important marker of stroke (Majumdar et al., 2013).

We observed an increase in WBC and lymphocytes counts in the higher risk group. However, many studies found that leukocytes were higher in patients who suffered stroke when compared with control. It is possible that the high count seen in this group is attributable to the recruitment of the inflammatory cells that become activated in case of stroke (Furlan et al., 2014; Jin, Yang, and Li, 2010; Belisário et al., 2016). The present study found that the patients under HU therapy present significant reductions in leukocyte, and lymphocytes counts, suggesting its cytoreductive effect as well as a possible anti-inflammatory effect (Lanaro et al., 2006; Giammarco et al., 2017; Yarbro, 1992). A Japanese study demonstrate that a high BUN (blood urea nitrogen) was associated with traditional coronary risk factors such as diabetes mellitus, dyslipidemia, and hypertension and affect the metabolism (Kawabe et al., 2014). Our patient group that was using HU improves the effect of HU treatment in the urea values. Creatinine, also is known to be an risk marker of stroke, and stroke poor outcome (Wannamethee, Shaper, and Perry, 1997; Saeed et al., 2009; Ibrahim, Rayyis, and

Almekhlafi, 2017; Snarska et al., 2016). Several studies have reported an association between the BEN/CAR and CAR/CAR haplotypes and the risk of stroke. This observation is confirmed in our population where the non-CAR hapotypes had the greatest frequencies in the group that have less risk of AVC. Moreover, the CAR and BEN are associated with lower HbF values associated with a more severe clinical profile (Menaa, 2013). In our study population, the highest proportion of mutants per gene found in the group with higher stroke risk corroborate the studies that associate the presence of alpha thalassemia as a protective factor in stroke patients (Menaa, 2013). Zhou et al. (2014) found that subjects with the *MTFHR C677T* rs1801133 TT genotype had significant increased risk of ischemic stroke; however, we don't find such as observation. Liao et al. (2016) and Deng et al. (2010) observed that the GG genotype, significantly increases the risk of Ischemic Stroke and contributed to elevate the 20-HETE level in individuals carrierof the gene polymorphism *CYP4F2 V433M* (rs2108622). In our study, the TT genotype of the *CYP4F2* 1347C>T variant was associated to the increase of the inflammation in accord to Sakiene et al. (2016).Very few work make the link of the *OR51B5/6* rs5006884 and the occurrence of stroke (Wonkam et al., 2014). The *SLCO1B1 C*-allele has been associated to Russian than the Chinese and Brazilian population (PASANEN; NEUVONEN; NIEMI, 2008). The carriers of at least one mutant C allele *SLCO1B15* has been associated with an increased risk of myopathy during treatment with statins, and indirectly with the lipid metabolism related to cardiovascularrisk (CARRet al., 2013); however, there is not report directly associated to the presence of this polymorphism and stroke risk. The presence of GG genotype of the *APOB* gene induces a higherrisk of myocardial infarction (LIU et al., 2015) in another Chinese ethnic group, and also this genotype did not present direct link with the stroke risk.

Conclusion:

This study find a benefic effect of the HU therapy in the high TCD group illustrated by the lowercounts of leukocytes, lymphocytes and platelets comparing with the others groups, confirming the cytoreductive effect of this therapy and suggesting an indirect anti-inflammatory performance. In our study we did not find the effect of the SNPs analyzed that can be due to the low number patients in the high risk group. More investigation with a larger number of patients are necessary to understand the influence of these genes and the stroke risk in SCA patients.

Acknowledgments:

The authors would like to thank the children with SCA and their families, whose participation was fundamental to the present study. The authors would also like to thank the staff at the ProfessorEdgard Santos University Hospital, as well as the Pharmacy School of the Federal University of Bahia, for their support. This present study was supported by grants from the Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB) (SUS0034/2013 and 8133/2014).

Conflict of interest: The authors declare no conflict of interest.

Table 1. Laboratory parameters, stratified according to TCD

Markers	LowerDTC Mean ± SD (N = 83)	HigherDTC Mean ± SD (N = 19)	*P*-Value
Hemoglobin profile			
HbS (%)	79.42 ± 13.73	82.87 ± 6.78	0.68**
HbF (%)	10.74 ± 6.74	9.35 ± 5.56	0.51**
Hematological			
RBC (10^6/mL)	3.051 ± 0.76	2.67 ± 0.37	**0.037****
Hemoglobin (g/dL)	9.073 ± 1.54	8.656 ± 1.11	0.316**
Reticulocytes (/L)	247.34 ± 260.10	298.62 ± 379.36	0.905**
Hematocrit (%)	26.63 ± 4.66	24.98 ± 3.63	0.165**
MCH (rg)	30.42 ± 3.96	32.61 ± 3.15	**0.0305***
MCV (fL)	98.56 ± 9.80	86.54 ± 7.85	**<0.0001***
Platelets (10^3/mL)	361.0 ± 131.20	450.4 ± 137.60	**0.0109***
MPV (fL)	6.33 ± 1.06	5.761 ± 1.22	**0.0480***
WBC (/mL)	12.076.00 ± 4.066.00	13,276.00 ± 5,465.00	0.3*
Lymphocyte (/mL)	4.720.00 ± 2.375.00	6,523.00 ± 3,218.00	**0.012***
Renal profile			
Urea (mg/dL)	19.25 ± 6.58	14.84 ± 3.92	**0.0061***
Creatinin (mg/dL)	0.46 ± 0.09	0.39 ± 0.06	**0.0041***

HbS: S hemoglobin; HbF: Fetal hemoglobin; RBC: Red blood cells; MCHC: Mean corpuscularhemoglobin concentration; WBC: white blood cell; MCV: Mean cell volume; MCH: Mean cell hemoglobin; MPV: Mean platelet volume. *Unpaired *t*-test, **Mann Whitney U-test.

Table 2. Laboratory markers stratified by HU according to TCD

Markers	TDC Low		TDC High		ANOVA	*Unpaired t-test* * orMann Whitney U-test** *p*-value			
	HU- Mean ± SD (N = 64)	HU+ Mean ± SD (N = 18)	HU- Mean ± SD (N = 14)	HU+ Mean ± SD (N = 5)	*P*-Value	*P1*	*P2*	*P3*	*P4*
Hemoglobin profile									
HbS (%)	78.90 ± 14.80	81.06 ± 9.54	84.82 ± 6.59	77.80 ± 4.47	0.21**	0.1844**	**0.0126****	0.2315**	0.1937**
HbF (%)	10.38 ± 6.937	12.05 ± 6.20	6.885 ± 2.878	15.76 ± 5.96	**0.0145****	0.1585**	**0.0008****	0.1228**	0.1467**
Hematological markers									
RBC (10^6/mL)	3.15 ± 0.78	2.617 ± 0.44	2.69 ± 0.32	2.598 ± 0.50	**0.0064****	**0.0048****	0.5285**	**0.0222****	>0.9999**
Hemoglobin (g/dL)	9.14 ± 1.62	8.82 ± 1.26	8.37 ± 0.67	9.40 ± 1.71	0.34**	0.4825**	0.2782**	0.0973**	0.4131**
Reticulocytes (/L)	229.22 ± 85.62	300.11 ± 53,485.00	221.14 ± 42.82	550.42 ± 806.00	0.247**	0.0553**	0.4336**	0.7789**	0.9665**
Hematocrit (%)	26.90 ± 4.84	25.60 ± 4.03	24.10 ± 2.23	27.28 ± 5.64	0.2242**	0.3300**	0.3490**	**0.0434****	0.4568**
MCH (pg)	29.53 ± 3.34	34.03 ± 3.59	31.19 ± 2.12	36.30 ± 2.29	**<0.0001***	**<0.0001***	**<0.0001***	**0.0892***	0.2642**
MCV (fL)	86.54 ± 7.85	98.56 ± 9.80	89.73 ± 4.85	104.90 ± 6.90	**<0.0001***	**<0.0001***	**<0.0001***	0.1259*	0.1989**
Platelets (10^3/mL)	362.8 ± 137.60	356.90 ± 122.80	475.5 ± 138.50	385.2 ± 124.70	**0.0471***	0.8679*	0.2546**	**0.0081***	0.6986**
MPV (fL)	6.39 ± 1.07	6.20 ± 1.03	5.992 ± 1.34	5.160 ± 0.57	**0.0846***	0.5133*	0.2027**	0.2501*	**0.0287****
WBC (/mL)	12,420.00 ± 4,067.00	11,043.00 ± 4,041.00	15,717.00 ± 3,483.00	6,931.00 ± 4,566.00	**0.0004***	0.2083*	**0.0006****	**0.0081***	0.1139**
Lymphocyte (/mL)	4,889.00 ± 2,539.00	4,255.0 ± 1,649.00	7,814.00 ± 2,496.00	3,168.00 ± 2,415.00	**0.0009***	0.3213*	**0.0044****	**0.0003***	0.4177**
Renal profile									
Urea (mg/dL)	18.01 ± 5.96	21.61 ± 8.35	14.57 ± 3.32	15.60 ± 5.68	**0.0129***	0.0893*	0.9858**	**0.0168***	0.1790**
Creatinin (mg/dL)	0.04 ± 0.09	0.51 ± 0.10	0.38 ± 0.07	0.39 ± 0.05	**0.0008***	**0.0061***	0.8372**	**0.0361***	**0.0109****

HbS: S hemoglobin; HbF: Fetal hemoglobin; RBC: Red blood cells; MCHC: Mean corpuscularhemoglobin concentration; WBC: white blood cell; MCV: Mean cell volume; MCH: Mean cell hemoglobin; MPV: Mean platelet volume. *P1*: Low TDC HU- vs Low TDC HU+;p*2*: High TDC HU- vs High TDC HU+;p3: Low TDC HU- vs High TDC HU-;p4: Low TDC HU+ vs High TDC HU+. *Unpaired *t*-test, **Mann Whitney U test;p-values < 0.05 (in bold) are considered significant.

Table 3. Allelic frequencies of haplotype and genotype frequencies of α-thalassemia$^{3.7kb}$, MTHFR677C>T, CYP4F2, SLCOB1, OR51B6 and APOB in SCA patients related to high risk of stroke

	Allele Frequency (%)		
	HigherTDC (≥ 200 cm/s)	LowerTDC (< 200 cm/s)	*P*-Value
Haplotype			
CAR	7 (6.68%)	20 (19.21%)	0.262
Non CAR	12 (11.76%)	63 (61.76%)	
Thalassemia$^{\alpha 3.7kb}$			
Absence of Tal$^{\alpha 3.7kb}$ deletion	15 (14.71%)	60 (58.82%)	0.774*
Presence of Tal$^{\alpha 3.7kb}$ deletion	4 (3.92%)	23 (22.55%)	
MC677T			
CC	9 (8.82%)	56 (54.90%)	0.100
CT+TT	10 (9.8%)	27 (26.47%)	
CYP4F2			
CC	12 (12.90%)	67 (72.04%)	0.252*
CT+TT	4 (4.30%)	10 (10.75%)	
OR51B5/6			
CC	18 (18%)	74 (74%)	1*
CT+TT	1 (1%)	7 (7%)	
SLCO1B1			
TT	18 (18.95%)	71 (74.74%)	0.591
TC+CC	0 (0%)	6 (6.32%)	
APOB			
GG	13 (13.13%)	67 (67.68%)	0.735
GA+AA	4 (4.04%)	15 (11.15%)	

CAR: Central African Republic haplotype; *MTHFR*: Methylenetrihydrofolate Reductase; *CYP4F2*: Cytochrome P450 family 4 subfamily F member2; *SLCOB1*: Solute CarrierOrganic anion transporterfamily member1B1; *OR51B6*: Olfactory ReceptorFamily 51 Subfamily B Member6; *APOB*: Apolipoprotein B.

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6. DISCUSSÂO

No nosso estudo, os valores de Hb encontrados corroboram com os valores médios descrito por (ADORNO et al., 2005), que encontraram a concentração média de 8,9g/dL. Considerando a definição da OMS para anemia, um paciente é considerado anêmico quando apresenta menos de 12g/dL ou 13g/dL de Hb, para mulheres ou homens, respectivamente. A presença de anemia é um sintoma crítico e está associado a vários desfechos desfavoráveis em muitas doenças. Como exemplo, a comorbidade na malária, especialmente em crianças e mulheres grávidas, na qual a anemia é citada como a principal causa de morte (WINTER; WAHLGREN, 2005).

A anemia tem sido também associada à ocorrência ou como preditor de gravidade de eventos cerebrovasculares (LASEK-BAL *et al*., 2015; NIKOLSKY *et al.,* 2004; SABATINE *et al*., 2005). No entanto, a literatura é controversa no que diz respeito à influência da anemia no risco de AVC, exceto em pacientes com AF (LASEK-BAL *et al*., 2015). Alguns autores, incluindo Abramson e cols., sugeriram que o risco de AVC em pacientes anêmicos aumenta em associação com outras comorbidades, porexemplo, na doença renal (ABRAMSON *et al.*, 2003).Todos os pacientes do nosso estudo apresentaram concentrações diminuídas de Ht (<30%), reconhecido como um preditor de desfecho desfavorável para o acidente vascular encefálico (TANNE *et al.*, 2010; SICO *et al*., 2013;).

Chan e Ganasekaran descreveram que a anemia pode causaralterações nos vasos sanguíneos cerebrais e, consequentemente, alterar a oxigenação cerebral, aumentando, assim, o risco de AVC (CHAN; GANASEKARAN, 2015). Nossos achados indicam uma correlação negativa entre VMMAX e contagem de hemácias, bem como de concentrações de Hb e Ht; isto é, velocidades mais altas de DTC estão correlacionadas com contagens diminuídas de eritrócitos e concentrações diminuídas de Hb e Ht (WHO;1968; TANNE *et al*., 2010). Outros marcadores de anemia, como o aumento do RDW e da contagem de leucócitos são conhecidos por serem preditores de desfecho desfavorável do AVC (FURLAN *et al.*, 2014; FENG *et al.*, 2017).

O aumento de leucócitos tem sido associado tanto ao risco de doença coronariana quanto à incidência de AVC e a mortalidade por doença cardiovascular em pacientes afro-americanos. Na nossa população de estudo observamos um número elevado na contagem de leucócitos no grupo DTC3 que apresentou as maiores velocidades sanguíneas cerebrais e, consequentemente, risco maior de AVC.

No entanto, o Primeiro Estudo Nacional de Saúde e Nutrição (NHANES I) não encontrou nenhuma influência significativa na contagem de leucócitos e a incidência de AVC (GILLUM; INGRAM e MAKUC, 1994).

Balkaran e cols. descobriram que a contagem de leucócitos é maior em pacientes com AVC que nos controles sem AVC (BALKARAN *et al.*, 1992). É possível que o número elevado de leucócitos observado em nosso grupo de estudo DTC3 seja atribuído ao recrutamento de células inflamatórias que seriam ativadas em caso de AVC (JIN; YANG; LI, 2010; FURLAN *et al.*, 2014). Associados a contagens elevadas de leucócitos, o aumento no número de reticulócitos mostrou ser um marcador de risco para AVC em uma coorte brasileira (BELISÁRIO *et al*., 2016). Esses dados foram consistentes com os achados em nosso estudo. Não se sabe se a inflamação ocorre durante ou antes a ocorrência do AVC, mas um estado inflamatório é evidenciado pelo aumento dos biomarcadores de função hepática, como a AST, que foram positivamente correlacionados com a VMMAX elevada em nosso estudo (MUSCARI *et al.*, 2014).

A análise de classe latente revelou que uma taxa maior de nossa população apresentou características acentuadas referentes ao perfil mais inflamatório. Alguns autores sugeriram que a inflamação é uma condição pré-existente a um evento de AVC (MUSCARI *et al.*, 2014; SCHREIBER, 2017). Os níveis séricos de ferritina, considerados como biomarcador de inflamação, também têm sido descritos como um fatorde risco para a graviidade e desfecho de AVC (ANRATHER; IADECOLA, 2016; ERDEMOGLU; OZBAKIR, 2002).A análise da classe latente ganhou popularidade no mundo da pesquisa devido a capacidade do método de superar os limites dos demais modelos de estatística (SCHREIBER, 2017). Em nosso estudo, o uso de um modelo de LCA permitiu visualizar o perfil geral de nossa população de acordo com as variáveis de características próprias. As análises revelaram que os indivíduos participantes da pesquisa podem ser agrupados em duas classes latentes, que são o perfil hemolítico e o perfil inflamatório. O perfil inflamatório foi encontrado mais abundante em nossa população de estudo sendo que essa observação sugere o papel importante desempenhado pelo perfil inflamatório na AF, especialmente em relação à predição de risco de AVC, quando comparados aos marcadores hemolíticos. Muitos estudos têm descrito a inflamação como um processo importante na patogênese do AVC isquêmico, sendo uma condição pré-existente ao evento

(MUSCARI et al., 2014), postulando-se a sua redução com alvo terapêutico no AVC isquêmico (LAKHAN; KIRCHGESSNER; e HOFER, 2009).

Conforme relatado, nossos pacientes tratados com HU apresentaram contagens mais baixas de leucócitos e linfócitos do que os outros grupos. A diminuição na incidência de AVC em pacientes com AF tratados com HU relatados em alguns estudos pode serresultado das propriedades anti-inflamatórias desta droga (OWUSU-ANSAH et al., 2016; WARE et al., 2016). Embora um efeito anti-inflamatório da HU seja indiretamente atribuído, as correlações entre marcadores inflamatórios e velocidades do DTC nos indivíduos com AF aqui investigados apoiam esta hipótese. Observamos que ao contrário do que se esperava, os indivíduos com AF tratados com HU apresentaram concentrações de Nox mais baixas, sugerindo o consumo de NO nesses indivíduos.

Nossos resultados mostraram que a concentração de HbF foi mais elevada no grupo normal (DTC1), sendo consistente com estudos na literatura, que sugerem que o aumento nas concentrações desta Hb está associado a diminuição do risco de AVC (WARE et al., 2017). As velocidades elevadas do fluxo sanguíneo cerebral na DF têm sido relacionadas à anemia grave, estenose e vasodilatação cerebral causadas por hipóxia tecidual (BERNAUDIN et al., 2011). O aumento do volume plaquetário observado no grupo com AVC em nosso estudo, confirma os resultados descritos associando plaquetas mais volumosas em indivíduos com DF, à ocorrência de AVC (CELIK et al., 2015). Foi sugerido que o volume plaquetário pode estar associado à ativação plaquetária e é considerado um marcador importante de AVC (MAJUMDAR*et al*., 2013). O LDH, esteve mais elevado no grupo de indivíduos com o DTC condicional (DTC2), assim como a AST e ALT. Um estudo de O'Driscoll e cols. descreveu a correlação positiva entre as velocidades de DTC e os valores de LDH (O'DRISCOLL *et al*., 2008).

O aumento nas concentrações de Nox observados após o evento de AVC poderia estarassociado a aumentos em outros produtos associados a via de NO, tais como peroxinitrito, bem como outros produtos do metabolismo do NO, tais com nitrato e nitrito, e a ativação de vias alternativas relacionadas a hipóxia e isquemia reperfusão(GLADWIN et al., 2000; LUNDBERG; WEITZBERG, 2005).

O presente estudo detectou correlações negativas no grupo DTC1 entre NOx e contagem de hemácias e as concentrações de Hb e Ht, sugerindo que as concentrações de NOx estão inversamente correlacionadas a esses parâmetros,

apesarda estratificação da população estudada estar de acordo com o uso de HU. Isso leva a crerque o uso de HU pode não estar exercendo influência sobre os indivíduos investigados no presente estudo, que apresentaram VMMAX normais. Estudos anteriores indicam que o NO pode ser eliminado em processos nos quais existam a liberação de concentrações elevadas de Hb livre (GLADWIN;pATEL, 2008). O NO é eliminado pela Hb livre liberada em decorrência da hemólise dos eritrócitos, que aumenta quando as concentrações de Ht sofrem variações (AZAROV et al., 2005), sendo que no grupo investigado, ela foi inferiora 30%. A associação positiva observada entre NOx e RDW pode ser explicada pelo fato dos indivíduos investigados apresentarem um quadro de anemia crônica e hemolítica que aumenta as concentrações de NO na corrente sanguínea (GLADWIN *et al.*, 2002). A correlação positiva observada entre o NOx e a contagem de linfócitos sugere que o aumento do NO pode estar aumentando populações específicas de linfócitos, especificamente a subpopulação de linfócitos pró-inflamatórios, ou seja, os linfócitos T helper tipo 1 (TH1)(CHOI *et al.*, 2002). Nosso estudo não chegou a avaliar populações especificas de linfócitos, apesarde ser um achado esperado devido ao conteúdo inflamatório presente na DF. A correlação positiva entre NOx e monócitos, bem como a contagem de plaquetas em nossa população de estudo, pode refletir a ativação mediada por NO. Um estudo sobre *Mycobaterium tuberculosis* relatou que os monócitos podem induziro aumento de NO (NIEDBALA; CAI; LIEW, 2006), sendo que outros sugerem que o NO regularia a proteína quimioatrativa de monócitos 1 (MCP-1), como recrutadores de monócitos (JAGANNATH; ACTOR;e HUNTER, 1998), especialmente em indivíduos que não estão em uso de HU e que devido a esse fato seriam mais expostos ao processo inflamatório que os sob tratamento (ZEIHER*et al*., 1995). A contagem de neutrófilos é considerada um marcador da gravidade da AF, pois alguns estudos demonstram que as contagens mais elevadas estão associadas a episódios de AVC e STA (ZHANG *et al.*, 2016). A presente correlação observada entre neutrófilos e níveis de NOx era esperada, uma vez que os neutrófilos podem produzirNO em ambiente inflamatório (ZHANG et al., 2016).

O uso de HU parece reduziressa associação, uma vez que os indivíduos com AF tratados apresentaram menos inflamação. As correlações positivas entre NOx e VLDL, bem como triglicérides foram descritas em todos os grupos de DTC e sugere a alteração no metabolismo de lipídios em nossos pacientes com AF

(ALELUIA et al., 2017). Muitos estudos sugerem a alteração do metabolismo lipídico em indivíduos com AF (ALELUIA *et al*., 2017; SEIXAS *et al*., 2010; SOUPENE *et al*., 2016); de fato, os indivíduos com AF apresentam hipolipoproteínia e hipertrigliceridemia (AKINLADE *et al.,* 2014).O mesmo autorespecula que essa alteração em lipídios está associada a disfunção endotelial que tem grande importância na vasculopatia da AF. Soupene e cols (2016) descreveram o papel da inflamação na disfunção lipídica em pacientes com AF (SOUPENE *et al.*, 2016).O gene *APOB* foi associado com a hipobetalipoproteinemia familiar(WHITFIELD *et al.*, 2004) e hipertrigliceridemia. Em estudos prévios do SNP rs676210, a presença do alelo A esteve associada a níveis diminuídos de colesterol total e LDL em comparação ao alelo G que esteve associado ao aumento no risco de hiperlipidemia (GU et al., 2017; WANG et al., 2015). Em nosso estudo, apesardo pequeno número de indivíduos com os genótipos GA ou AA, notamos que a presença do alelo A esteve associado a redução do valormédio do colesterol total e do LDL em todos os grupos de indivíduos que realizaram o DTC, mas não no grupo DTC2. Em nossa população, observamos mais genótipos GG do que GA e poucos AA, o contrário relatado em grupos étnicos chineses{Citation} (GU *et al.*, 2017). A presença do genótipo GG esteve associado ao aumento do risco de infarto do miocárdio em outro grupo étnico chinês (LIU *et a*l., 2015). A correlação negativa observada entre colesterol total, HDL, VLDL, triglicérides e a VMMAX elevada, sugere que os valores dos parâmetros lipídicos diminuem quando o fluxo cerebral de indivíduos com a AF aumenta. Alguns estudos consideram que as concentrações mais baixas do colesterol HDL constituem risco independente de AVC isquêmico (GORDON *et al*., 1989; LI *et al*., 2017; SOYAMA *et al*., 2003). As concentrações diminuídas de colesterol HDL estiveram associadas à disfunção endotelial e, consequentemente, podem afetar o fluxo sanguíneo (BISOENDIAL *et al*., 2003).

Em relação ao colesterol total, se sua associação com doenças coronarianas é muito bem documentada, a associação com AVC é ainda bastante controversa e menos clara (CUI *et al*., 2007;PROSPECTIVE STUDIES COLLABORATION *et al.*, 2007). Por exemplo, Zhang e cols. (2012) relataram a associação diferente segundo o gênero e ao tipo de AVC. Eles observaram que o risco de AVC isquêmico em homens é positivamente correlacionado ao colesterol total e inversa ao risco de AVC hemorrágico em mulheres (ZHANG *et al*., 2012).

Essa contradição é de vida a diversidade dos eventos cerebrovasculares que podem afetar indivíduos de diferentes sexos (MAGYAR e BERECZKI, 2007). Concentrações elevadas de colesterol total, LDL e triglicerídes estão associadas à ocorrência de ateroma no infarto cerebral (GONZÁLEZ *et al*., 2008). Os triglicerídeos são considerados como fator de risco para o AVC, uma vez que pode influenciar a ativação plaquetária responsável pela disfunção da coagulação associada à ocorrência do AVC isquêmico (BERGER et al., 2012; CHAPMAN et al., 2011; GRUNDY, 1998;PATSCH et al., 1992). Berger e cols (2012) sugerem que a implicação dos triglicérides no risco de AVC é multifatorial e requer investigações adicionais (BERGER*et al*., 2012). As análises da LCA (Classe latente) sugerem, em nossa população, que 52 % dos indivíduos investigados apresentam hipolipidemia. Esta análise concorda com os estudos que relataram a hipolipidemia em pacientes com AF. Essa hipolipidemia não está bem elucidada e pode ser atribuída ao metabolismo disfuncional dos lipídios e a redução na capacidade de transporte de oxigênio;porém, não parece estardiretamente relacionada com alterações na concentração de lipídios da membrana eritrocitária em pacientes com AF, mas influencia a fluidez da hemácia falcizada (AKINYANJU e AKINYANJU, 1976; HASHMI e AFROZ, 1969; MARZOUKI e KHOJA, 2003).

Vários estudos relataram uma associação entre os haplótipos *BEN / CARe CAR/ CAR*e o risco de AVC. Essa observação é confirmada em nossa população, onde os hapótipos “não CAR” apresentaram as maiores frequências no grupo que tem menorrisco de AVC (MENAA, 2013). Em nossa população de estudo, a maior proporção de mutantes por gene encontrada no grupo com maior risco de AVC corrobora os estudos que associam a presença da talassemia alfa como fator de proteção em pacientes com AVC (MENAA, 2013; ZHOU et al., 2014). Alguns estudos revelaram que os indivíduos com o genótipo rs1801133 TT tinham riscos aumentados significativos de AVC isquêmico. Não encontramos tal observação em nosso estudo (DENG et al., 2010; LIAO et al., 2016). No polimorfismo do gene *CYP4F2* V433M (rs2108622), foi observado que o GG aumenta significativamente o risco de do nível AVC e ter o nivél elevado de 20-HETE. Em nosso estudo, estudamos a variante *CYP4F2* (1347C> T) e o TT foi associado ao aumento da inflamação (SAKIENE et al., 2016). A ligação do SNP *OR51B5 / 6* rs5006884 e o AVC é ainda não é muito explorada, porém a ligação

com a expressão da HbF que está relacionada ao risco de AVC foi encontrada (WONKAM et al., 2014). Foi descrita que o alelo C *SLCO1B1* é mais comum na população russa, do que nos chineses e brasileiros (PASANEN; NEUVONEN; NIEMI, 2008) e a presença de pelo menos um alelo C mutante no *SLCO1B15* está associado ao risco aumentado de miopatia durante o tratamento com estatinas associadas ao metabolismo lipídico indiretamente relacionado ao risco cardiovascular (CARR *et al*., 2013). Entretanto, existe a associação direta com o risco de AVC. A presença do genótipo GG do gene *APOB* induz ao risco elevado de infarto do miocárdio (LIU *et al*., 2015) em outro grupo étnico chinês, também este genótipo não apresenta ligação direta com o risco de AVC.

7. CONCLUSÕES

Tendo como base os resultados apresentados nos diferentes manuscritos que compuseram o presente estudo, foi possível concluirmos:

Manuscrito 1

As associações e correlações significativas entre DTC- VMMAX e marcadores laboratoriais comumente utilizados para monitorar pacientes pediátricos com anemia falciforme, tais como percentual de reticulócitos, contagem de leucócitos e volume plaquetário médio, que são marcadores associados a gravidade da doença, sugerem que o estudo dos parâmetros laboratoriais, juntamente com a VMMAX, poderá compor o rol de procedimentos a serem incorporados no acompanhamento desses pacientes;

O uso de HU esteve associado a redução nas contagens de leucócitos e linfócitos e no aumento de ferritina nos indivíduos com anemia falciforme, que apresentaram DTC alterado e condicional, sugerindo que este grupo de marcadores pode ser importante no monitoramento deste tratamento e na avaliação da ocorrência de AVC;

A análise da Classe Latente (LCA), que analisou a população de estudo com base nos marcadores laboratoriais, detectou que sessenta e dois porcento (62%) da população avaliada pode ser classificada em um sub-fenótipo com características inflamatórias acentuadas, sugerindo a importância da inflamação nas repercussões clínicas da AF, incluindo as alterações no DTC- VMMAX.

Manuscrito 2

As associações e correlações significativas entre Nox e marcadores laboratoriais utilizados para monitorar os pacientes pediátricos com AF, tais como contagem de hemácias, dosagem de hemoglobina e hematócrito e valores de CHCM e RDW em pacientes que não se encontravam em uso de HU, no grupo DTC1; enquanto no grupo DTC2 ambos usando HU ou não, observamos correlação positiva significativa para o VLDL e TG. No grupo DTC3 e AVC não encontramos nenhuma correlação entre Nox e os parametros laboratoriais, após a investigação do uso da HU. O presente estudo sugere que o NO deve ser

considerado como parâmetro para avaliação da fisiopatologia do AVC em pacientes com AF; além disso, sugerimos que o subfenótipo dislipidêmico na população estudada parece ser um parâmetro importante na avaliação do grupo de pacientes avaliados.

Manuscrito 3

Quando avaliamos o perfil lipídico dos pacientes com risco de AVC e associamos aos polimorfismos no gene APOB, não foi descrito efeitos desse gene sobre os parâmetros lipidicos. A análise da Classe Latente (LCA), que avaliou o perfil da população em estudo com base nos marcadores laboratoriais, detectou que cinquenta e dois porcento (52%) da população avaliada pode ser classificada no sub-fenótipo hipolipidêmico, confirmando que o fenotipo sublipidêmico está associado a repercussões clínicas na AF, incluindo as alterações nas velocidades do Doppler. Sugerimos que os lipidios sejam utilizados como parâmetros de avaliação do potencial terapêutico na nossa população de pacientes com anemia falciforme.

Manuscrito 4

Avaliamos polimorfismos nos genes associados ao risco de AVC e investigamos as concentrações de HbF, em indivíduos com AF e AVC. Nesta avaliação não encontramos efeitos associados aos SNP OR51B6, MTHFR C766T e CYP4F2, bem como aos haplótipos ligados ao grupo de genes da globina beta e a talassemia alfa. Entretanto, o resultado alcançado pode ter sido devido ao número reduzido de indivíduos nos grupos de alto risco. Investigações adicionais, que incluam número maior de pacientes que estão sob risco de ocorrência de AVC, serão necessárias visando esclarecer a possível influência desses genes no risco de AVC em pacientes com AF.

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APÊNDICE I: QUESTIONARIO

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.

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 Falciforme: {ID} ( ) <6 m [0] ( ) 6m - 4anos [1] ( ) 5 - 9anos [2]

( ) 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 regularde 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 porum médico? {PRESMDOR} ( ) NÃO [0] ( ) SIM [1]

Assistido porespecialista 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]

Universidade Federal da Bahia
Hospital Universitário Prof. Edgard Santos
Centro Pediátrico Prof. Hosannah de Oliveira
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APÊNDICE II: TERMO DE ASSENTIMENTO LIVRE E ESCLARECIDO

Identificação de Pacientes Portadores de doença falciforme com risco aumentado para o desenvolvimento de acidente vascularcerebral através da realização do DopplerTranscraniano e perfil clínico associado.

Durante a leitura do documento abaixo, fui informado (a) que posso interromperpara fazerqualquerpergunta, com objetivo de tirardúvidas, para o meu melhoresclarecimento.

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, portadorde doença falciforme sob a minha inteira responsabilidade) foi selecionado porcomparecerpara a realização do dopplertranscraniano. Os médicos supracitados me explicaram que serão coletadas informações relacionadas à doença do (a) menorsob a minha responsabilidade, para tentaridentificaralguns 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 tentaridentificaralguns aspectos importantes da doença que poderão beneficiar, não somente o (a) menordo 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 colhero sangue na veia do braço varia de pessoa para pessoa e a criança pequena pode chorar. Pode havera 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 serresolvido colocando compressas com água gelada de quatro a seis vezes pordia.

Entendo também que eu tenho permissão para a qualquermomento revogaro meu consentimento e retiraro menordo estudo sem sofrernenhuma punição ou perda de direitos. Entretanto, exames adicionais poderão sersolicitados, caso o médico que o assiste julgue-os necessários para a saúde e bem estarda criança. Minha recusa em permitirque 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 formularperguntas 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 PARTICIPARDO ESTUDO, CONCORDEI COLOCARABAIXO A MINHA IMPRESSÃO DO DEDO POLEGAR.

Salvador, _____ de _____ de 201_

NOME: ..... Assinatura ⇒

Ou impressão digital ou datiloscópica

Test

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

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

Assinatura do Investigador: .....

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

APÊNDICE III- PCR-RFLP para Haplótipos

Reagentes

Reagente	Quantidade poramostra
Tampão	5,0 µL
$MgCl_2$ 50mM	2,5 µL
dNTP 2 mM	5,0 µL
Primers (3 ou 5 ou 6 ou 8 ou 10 ou 12) 25 pmol/µL	0,5 µL
Primers (4 ou 6 ou 7 ou 9 ou 11 ou 13) 25 pmol/µL	0,5 µL
Taq 5U/µl	0,25 µL
DNA	1,5 µL
H2O qsp 50µl	34,75 µL

Termociclagem

Desnaturação	94ºC,10 min.
35ciclos	94ºC, 45 seg. Temp. Variável de acordo com a Tab. 1 45 seg. 72ºC, 1min 30 seg.
Extensão	72ºC, 10 min 4ºC …

RFLP

Reagentes	Quantidade poramostra
Produto de PCR	20 µL
BSA*	0,3 µL
Tampão	3 µL
Enzima de restrição de acordo com tab. 1 Xmn I Hinc II Hind III ou Hinf I	 0,2 µL 0,1 µL 0,2 µL
H_2O	Qsp 30 µL

***** Verificaras reações que necessitam BSA na tab. 1

Tabela. Padrão de banda de PCRe corte

Gene	Primer	Fragmento (pb)	Após digestão	Temp. de pareamento	Enzima
$5'\gamma^G$	3 e 4	650	450+200	57ºC	Xmn I*
γ^G/γ^A	5 e 6	780	440+340	60ºC	Hind III
γ^G/γ^A	6 e 7	760	360+400	62ºC	Hind III
ψβ	8 e 9	700	360+340	60ºC	Hinc II
3' ψβ	10 e 11	590	470+120	57ºC	Hinc II
5'β	12 e 13	380	240+140	57ºC	Hinf I

*AcrescentarBSA

APÊNDICE IV- PCR PARA TALASSEMIA $\alpha^{2\ del\ 3.7kb}$

Reagentes

Reagente	Quantidade poramostra
Tampão 10X	1,25 μL
$MgCl_2$ 50mM;	0,5 μL
dNTP 10mM	0,25 μL
PrimerA (Comum)	0,125 μL
PrimerB ou C	0,125 μL
Q solution 5X	2,5 μL
Taq DNA Polimerase (5U/μL);	0,1 μL
DNA	Aprox. 1 μL (100ng/μL)
Água para PCR	qsp. 12 μL

Termociclagem

Abertura inicial	98ºC, 3 min 85ºC, 3 min
5 ciclos	98ºC, 30 seg 66ºC, 1min30seg 72ºC, 3 min
30 ciclos	96ºC, 30 seg 66ºC, 30 seg 72ºC, 3 min
Extensão	72ºC, 15 min 15ºC, infinito

Interpretação dos resultados

Tubo A+B	Tubo A+C	Paciente
+	-	Homozigoto para a talassemia $\alpha_2^{3,7Kb}$
+	+	Heterozigoto para a talassemia $\alpha_2^{3,7Kb}$
-	+	Portadorde genes α normais
-	-	A reação não funcionou

APÊNDICE V PCR para *OR51B6 /APOB/CYP4F2*

Reagentes

Reagente	Quantidade poramostra
Tampão 10X	2,5μL
$MgCl_2$ 50mM;	1,5 μL
dNTP 10mM	1μL
PrimerA	0,5 μL
PrimerB	0, 5 μL
Taq DNA Polimerase (5U/μL);	0,3 μL
DNA	Aprox. 1 μL (100ng/μL)
Água para PCR	qsp. 18,7 μL

Termociclagem

Desnaturação	94ºC,10 min.
35ciclos	94ºC, 45 seg. 60C 45 seg. 72ºC, 1min
Extensão	72ºC, 10 min 4ºC …

APÊNDICE VI PCR para *SLCO1B1*

Reagente	Quantidade poramostra
Tampão 10X	2,5μL
$MgCl_2$ 50mM;	1,5 μL
dNTP 10mM	1μL
PrimerA	0,3 μL
PrimerB	0, 3 μL
Taq DNA Polimerase (5U/μL);	0,3 μL
DMSO	1,25μL
Água para PCR	qsp. 18, μL
DNA	Aprox. 1 μL (100ng/μL)

Termociclagem

Desnaturação	94ºC,10 min.
35ciclos	94ºC, 45 seg. 58C 45 seg. 72ºC, 1min
Extensão	72ºC, 10 min 4ºC …