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Influência de variantes genéticas do sistema endocanabinoide sobre os subfenótipos de hemólise e de vasculopatia na anemia falciforme

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Dedico esse trabalho à minha amada famiília.

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“Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota.”

Madre Teresa de Calcutá

RESUMO

A anemia falciforme (AF) resulta da homozigose para a mutação no gene da beta-globina que leva a inúmeras complicações clínicas como resultado de mecanismos fisiopatológicos complexos. Os fenótipos clínicos da AF estão associados a modificadores genéticos e o sistema endocanabinoide (ECS) pode ser um candidato para modular as complicações da AF. O ECS está associado com a hematopoiese, na agregação plaquetária e nas respostas imunitárias. Sendo assim, o objetivo deste trabalho foi avaliar a associação de polimorfismos de base única (SNPs) relacionados ao ECS com os subfenótipos hemolíticos em pessoas com AF, bem como verificar a influência das variáveis bioquímicas de hemólise, concentração de hemoglobina (Hb) fetal (Hb F) e presença de alfa talassemia ($-\alpha^{-3,7\ kb}$). Os grupos estudados foram compostos por (1) 73 pessoas com acidente vascular encefálico (AVE), (2) 80 pessoas com priapismo e (3) 144 pessoas como grupo controle (para priapismo, 58). O fenótipo da AF foi diagnosticado por HPLC e confirmado por PCR-RE. A mutação da $\alpha^{-3,7kb}$ talassemia foi detectada por GAP-PCR e a genotipagem dos SNPs de *FAAH* rs324420, *MAGL* rs604300, *CNR1* rs7766029 e *CNR2* rs35761398 foi realizada com ensaios TaqMan. Os dados laboratoriais de hemoglobina (Hb) total, bilirrubina indireta (BI), contagem total de reticulócitos e lactato desidrogenase (LDH) foram obtidos a partir dos registos médicos. Níveis mais baixos de Hb F foram associados ao AVE ($P < 0,001$), e frequência mais baixa da mutação $\alpha^{-3,7kb}$-talassemia foi associada ao priapismo ($P < 0,001$). Em análises multivariadas com ajuste para $\alpha^{-3,7kb}$-talassemia, o *CNR2* rs35761398 foi associado a uma menor chance de desenvolver priapismo (OR = 0,386 [0,175-0,854], P = 0,019), independentemente da mutação $\alpha^{-3,7kb}$-talassemia (OR = 0,148 [0,059-0,375], $P < 0,001$) como covariável, e não houve associação estatística entre os SNPs e AVE. O priapismo isquêmico é um subfenótipo hemolítico da SCA que envolve vias desequilibradas de vasodilatação/vasoconstrição nos corpos cavernosos, como a diminuição da sinalização envolvida na via vasoconstritora das proteínas RhoA/Rho-kinase (ROCK), resultando no relaxamento dos corpos cavernosos e maior suscetibilidade ao priapismo. Uma vez que a ativação do receptor canabinoide tipo 2 (CB2) está envolvida na diminuição da ativação de RhoA, sugerimos, com base em nossos resultados, que o genótipo TT-CC para o SNP de *CNR2* analisado resulta no nível basal de ativação desse receptor, desempenhando

um papel na manutenção da via RhoA/ROCK normal e reduzindo as chances de desenvolver priapismo.

Palavras–chave: Priapismo isquêmico; *CNR2* rs35761398; Marcadores prognósticos; Receptor canabinoide tipo 2.

ABSTRACT

Sickle cell anemia (SCA) results from homozygosity for a mutation in the beta-globin gene that leads to numerous clinical complications due to complex pathophysiological mechanisms. The clinical phenotypes of SCA are associated with genetic modifiers, and the endocannabinoid system (ECS) may be a candidate for modulating the complications of SCA. The ECS is associated with several physiological pathways and impacts on hematopoiesis, platelet aggregation and immune responses. Therefore, this study aimed to evaluate the association of single base polymorphisms (SNPs) related to the ECS with hemolytic subphenotypes in patients with SCA, as well to verify the influence of the biochemical variables of hemolysis, fetal hemoglobin (Hb) concentration (Hb F) and the presence of alpha-thalassemia ($-\alpha^{-3.7\ kb}$) on the subphenotypes isolated or associated with the polymorphisms. The groups studied consisted of (1) 73 stroke patients, (2) 80 priapism patients and (3) 144 patients designated as the control group (for priapism, 58). The SCA phenotype was initially diagnosed by HPLC, and PCR-RE confirmed it. The $\alpha^{-3.7kb}$ thalassemia mutation was detected by GAP-PCR, and genotyping of the SNPs FAAH rs324420, MAGL rs604300, CNR1 rs7766029, and CNR2 rs35761398 was carried out using TaqMan genotyping assays. Laboratory data on total hemoglobin (Hb), indirect bilirubin (IB), total reticulocyte count, and lactate dehydrogenase (LDH) were obtained from the medical records. Lower Hb F levels were associated with stroke ($P < 0.001$), and a lower frequency of the $\alpha^{-3.7kb}$-thalassemia mutation was associated with priapism ($P < 0.001$). In multivariate analyses with adjustment for $\alpha^{-3.7kb}$-thalassemia, CNR2 rs35761398 was associated with a lower chance of developing priapism (OR = 0.386 [0.175-0.854], $P = 0.019$), independently of the $\alpha^{-3.7kb}$-thalassemia mutation (OR = 0.148 [0.059-0.375], $P < 0.001$) as a covariate, and there was no statistical association between SNPs and stroke. Ischemic priapism is a hemolytic subphenotype of SCA that involves unbalanced vasodilation/vasoconstriction pathways in the corpora cavernosa, such as decreased signaling involved in the RhoA/Rho-kinase (ROCK) protein vasoconstrictor pathway, resulting in relaxation of the corpora cavernosa and increased susceptibility to priapism. Since activation of the type 2 cannabinoid receptor (CB2) is involved in decreasing RhoA activation, we suggest, based on our results, that the TT-CC genotype for the CNR2 SNP analyzed results in the basal level of activation of this

receptor, playing a role in maintaining the normal RhoA/ROCK pathway and reducing the chances of developing priapism.

Keywords: Ischemic priapism; *CNR2* rs35761398; Prognostic markers; Cannabinoid receptor type 2.

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LISTA DE ABREVIATURAS E SIGLAS

Δ^9-THC - Delta^9-tetrahidrocanabinol

2-AG - 2-araquidonoilglicerol

7-TM - Sete domínios transmembranares, do inglês "*Seven-transmembrane domains*"

A – Adenina

AA – Ácido araquidônico

AC – Adenilato ciclase

AEA – Anandamida

AF – Anemia falciforme

Arg – Arginina

AVE – Acidente vascular encefálico

cAMP - Monofosfato cíclico de adenosina, do inglês "*cyclic adenosine monophosphate*"

CB1 – Receptor canabinoide tipo 1, do inglês "*cannabinoid receptor type 1*"

CB2 – Receptor canabinoide tipo 2, do inglês "*cannabinoid receptor type 2*"

CBD – Canabidiol

CBRs – receptores canabinoides, do inglês "*cannabinoid receptors*"

cGMP - Monofosfato cíclico de guanosina, do inglês "*cyclic gua monophosphate*"

CI – Intervalo de confiança, do inglês "*confidence interval*"

CNR1 – Gene do receptor canabinoide tipo 1

CNR2 – Gene do receptor canabinoide tipo 2

DAG – 1,2-diacil glicerol

DAGL - Diacil glicerol lipase

DAMPs – Padrões moleculares associados a danos, do inglês "*damage-associated molecular patterns*"

DF – Doença falciforme

ECs – Endocanabinoides

ECS – Sistema endocanabinoide, do inglês "*endocannabinoid system*"

EMT - Transportadores endocanabinoides de membrana, do inglês "*endocannabinoid membrane transporters*"

eNOS – Óxido nítrico sintase endotelial

ERO – Espécies reativas de oxigênio

FAAH - Amida hidrolase de ácidos graxos, do inglês "*fatty acid amide hydrolase*"

GABA - Ácido gama-aminobutírico, do inglês “*gamma-amino butyric acid*”

Gln – Glutamina

Glu – Ácido glutâmico

GPCRs - receptores acoplados à proteína G, do inglês “*G-protein-coupled receptors*”

Hb – Hemoglobina

HBB – Gene da beta globina, do inglês “*hemoglobin subunit beta*”

HPLC – Cromatografia líquida de alta performance, do inglês “*high performance liquid chromatography*”

HU – Hidroxiuréia, do inglês “*hydroxyurea*”

IB – Bilirrubina indireta, do inglês “*indirect bilirrubin*”

ICAM-1 – Molécula de adesão intercelular-1, do inglês “*intercellular adhesion molecule-1*”

IL – Interleucinas

LDH – Lactato desidrogenase, do inglês “*lactate dehydrogenase*”

MAGL - lipase de monoacilglicerol, do inglês “*monoacylglycerol lipase*”

MAPK - Proteína quinase ativada por mitógeno, do inglês “*mitogen-activated protein kinase*”

MCP-1 - Proteína quimiotática de monócitos 1, do inglês “monocyte chemoattractant protein-1”

NADA - Dopamina N-araquidonoil

NAEs - N-aciletanolaminas

NAPE-PLD - N-acil-fosfatidiletanolamina-específica fosfolipase D

NArPE - N-araquidonoil PE

NAT - N-acil transferase

NETs – Armadilhas extracelulares de neutrófilos, do inglês “*neutrophil extracellular traps*”

NF-κβ - Fator nuclear kappa beta, do inglês “*nuclear factor kappa beta*”

NO – óxido nítrico

OR – Razão de chances, do inglês “*odds ratio*”

PCR – Reação em cadeia da polimerase, do inglês “*polimerase chain reaction*”

PDE5 - Fosfodiesterase 5, do inglês “*phosphodiesterase type 5*”

PE – Fosfatidiletanolamina

PI3K - Fosfatidilinositol-3-quinase, do inglês “*phosphoinositide 3-kinase*”

PKA – Proteína quinase A, do inglês “*protein kinase A*”

PKB/Akt – Proteína quinase B, do inglês “*protein kinase B*”

PKG – Proteína quinase G, do inglês “protein kinase G”

PLC – fosfolipase C, do inglês “*phospholipase C*”

PPARs - Receptores ativados por proliferadores de peroxissoma, do inglês “*peroxisome proliferators-activated receptors*”

Pro – Prolina

Ret – Reticulócitos, do inglês “*retyculocytes*”

RhoA – Do inglês, “Ras homolog family member A”

ROCK – Rho quinase, do inglês “Rho-kinase”

SCA – Anemia falciforme, do inglês “*sickle cell anemia*”

SNC – Sistema nervoso central

SNP – Sistema nervoso periférico

SNPs – Polimorfismos de nucleotídeo único, do inglês “*single nucleotide polymorphisms*”

SNV – variantes de nucleotídeo único, do inglês “*single nucleotide variants*”

T – Timina

Thr – Treonina

TLR4 – Receptores do tipo toll 4, do inglês “*toll-like receptors 4*”

TNF – Fator de necrose tumoral, do inglês “*tumor necrosis factor*”

TRPV1 - Receptor de potencial transitório vaniloide tipo 1, do inglês “*transient receptor potential vanilloid 1*”

Val – Valina

VCAM-1 – Molécula de adesão vascular-1, do inglês “*vascular adhesion molecule-1*”

VGCC - Canais de cálcio dependentes de voltagem, do inglês “*voltage-gated calcium channels*”

VOC – vaso-oclusão

XO – Xantina oxidase

LISTA DE SÍMBOLOS

α - Alfa

β - Beta

γ – Gama

∆ - Delta

K - Kappa

SUMÁRIO

PREFÁCIO

A presente dissertação está sendo apresentada na forma de manuscrito a ser submetido em revista internacional indexada, intitulado “Association of single nucleotide variants (SNVs) of the endocannabinoid system with sickle cell anemia hemolytic subphenotypes”. O referido manuscrito será submetido na revista “*Annals of Hematology*”, com fator de impacto (2022) 3,5 e Qualis/CAPES – Ciências Biológicas I A3.

A introdução apresenta uma revisão da literatura sobre a fisiopatologia da anemia falciforme (AF), os componentes do sistema endocanabinoide (ECS) e seu modo de ação, e mecanismos fisiológicos relacionados à patologia da AF que podem ser modulados pelo ECS. Em sequência, apresentamos o objetivo geral e os objetivos específicos do trabalho. A metodologia, os resultados e as discussões dos dados são apresentados no manuscrito, em língua inglesa, seguindo as normas de formatação recomendadas pela revista. Por fim, descrevemos nossas conclusões.

1. INTRODUÇÃO

1.1. ANEMIA FALCIFORME

A doença falciforme (DF) é a afecção monogênica hematológica mais frequente no mundo, com incidência global em 2021 de 382 a cada 100 mil nascidos vivos e prevalência de 7,74 milhões de casos ao redor do mundo. Nas últimas duas décadas, o total global de mortes por DF apresentou um aumento de 43,4%, de 262 mil em 2000 para 376 mil em 2021 (Thomson *et al*., 2023). No Brasil, de 2015 a 2019 a incidência da DF foi estimada em 1.362 novos casos ao ano, sendo a prevalência de aproximadamente 60 mil casos e a taxa de mortalidade (somente considerando óbitos com DF como causa primária) de 0,25 a cada 100 mil óbitos (Cançado *el al*., 2023).

A DF é caracterizada pela presença de uma hemoglobina (Hb) com características bioquímicas diferentes da Hb normal, denominada Hb S. A alteração gênica é proveniente de uma troca de adenina (A) por timina (T) na posição 20 do gene da beta-globina (*HBB*), levando à troca de ácido glutâmico (Glu) por valina (Val) na sexta posição da cadeia beta(β)-globínica (*HBB*; β^S GAG→GTG; glu6val). A presença do alelo mutante (β^S) em homozigose resulta na anemia falciforme (AF), e a heterozigose composta com outras mutações no gene *HBB* resulta em diferentes fenótipos que juntos com a AF formam o grupo da DF (Kato *el al*., 2018; Ware *et al*., 2017).

A fisiopatologia da AF é complexa e envolve várias alterações moleculares, celulares e bioquímicas. A polimerização da Hb S é o principal evento que caracteriza a patogênese molecular da AF, dando início a uma série de mecanismos que se retroalimentam. Quando em condição de hipóxia, os tetrâmeros de Hb S expõem seus resíduos hidrofóbicos de valina e formam polímeros rígidos, deformando e danificando a membrana e o citoesqueleto dos eritrócitos. A polimerização é reversível, uma vez que as fibras se desfazem na presença de oxigênio e o eritrócito retorna ao formato discoide. No entanto, ciclos de falcização levam a danos repetidos na membrana, resultando em eritrócitos irreversivelmente falciformes e hemólise intra- e extravascular. A hemólise e a falcização eritrocitária geram consequências significativas, como a disfunção do endotélio vascular, a vasculopatia e a vaso-oclusão (Barabino; Platt; Kaul, 2010; Kato; Steinberg; Gladwin, 2017; Williams; Thein, 2018; Sundd; Gladwin; Novelli, 2019).

A hemólise intravascular resulta na liberação do conteúdo intraeritrocitário nos vasos sanguíneos, aumentando a concentração de Hb livre, grupo heme e de espécies reativas de oxigênio (ERO), como os radicais hidroxila (HO•) e superóxido (O_2•-), que depletam o óxido nítrico (NO) e formam outros oxidantes potentes, tais como o peroxinitrito (ONOO-) e dióxido de nitrogênio (NO_2) (Voskou *et al*., 2015). A hemólise também libera a arginase -1, que por sua vez, metaboliza o mesmo substrato que a enzima óxido nítrico sintase endotelial (eNOS) utiliza para a produção de NO, a L-arginina. A diminuição de NO e o aumento de ERO contribuem para a vasoconstrição, vasculopatia, ativação de moléculas de adesão endotelial e na produção de vasoconstritores potentes (Nader *et al*., 2021).

Os leucócitos, em resposta a esses eventos, aumentam a expressão de mediadores inflamatórios, tais como interleucina (IL) - 6, IL-1β, IL-8 e fator de necrose tumoral α (TNF-α), após ativação da via de sinalização do fator nuclear kappa beta (NF-kβ) por meio da interação do grupo heme e de outras moléculas de padrões moleculares associados a danos (DAMPs), que ativam o sistema imune inato via receptores do tipo toll 4 (TLR4) (Zhang *et al.*, 2016; Allali *et al*., 2020; Vona *et al*., 2021). Tais consequências promovem estado inflamatório, ativação endotelial com a exposição de proteínas de adesão (molécula de adesão intercelular-1 - ICAM-1, molécula de adesão vascular 1 - VCAM-1, P-selectina e E-selectina), recrutamento e adesão de plaquetas e outros leucócitos e disfunção endotelial (Nader *et al.*, 2021; Wang; Zennadi, 2021). A consequência dos eventos hemolíticos reflete em processos de interação dinâmica entre eritrócitos falciformes e o endotélio vascular aprisionando eritrócitos, leucócitos e plaquetas na microcirculação levando a obstrução vascular e isquemia tecidual, chamado de vaso-oclusão (VOC) (Figura 1) (Williams; Thein, 2018; Sundd; Gladwin; Novelli, 2019).

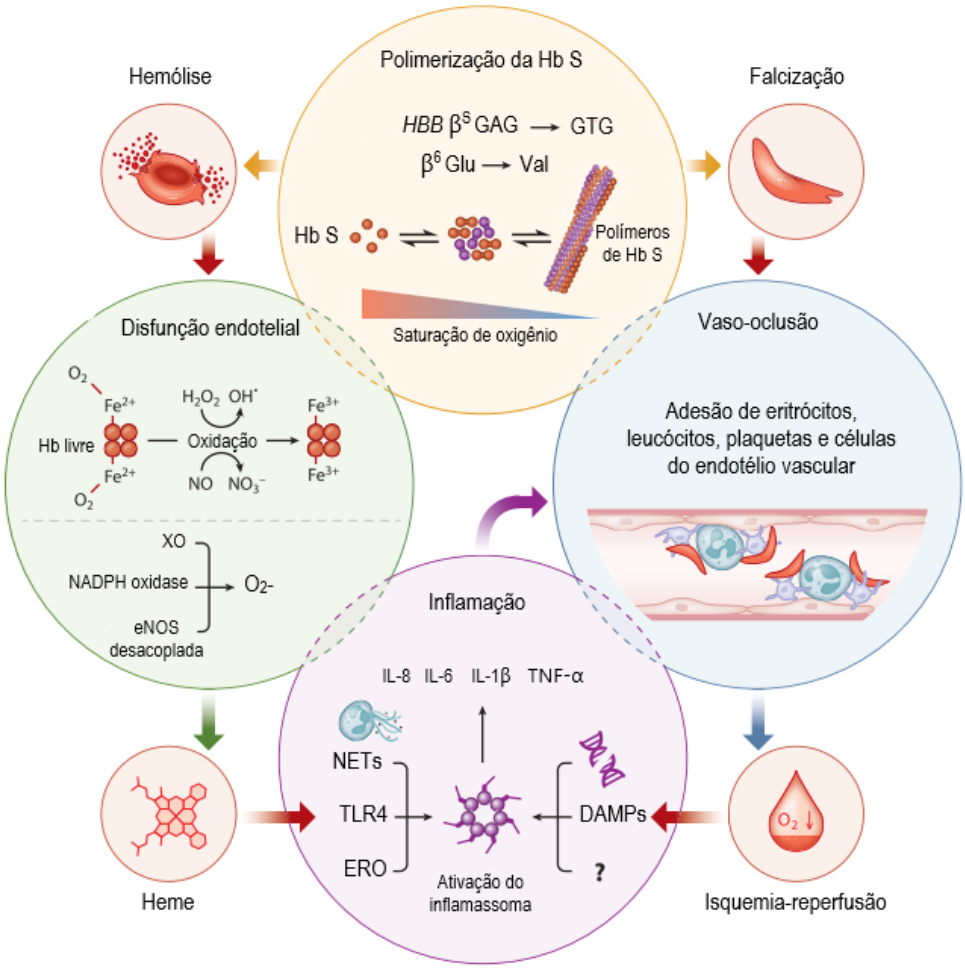

Figura 1. Fisiopatologia molecular da anemia falciforme (AF). A mutação no gene *HBB* ocasiona a substituição de um ácido glutâmico por uma valina na sexta posição da cadeia β globínica, resultando na formação da Hb S. Os rígidos polímeros de Hb S formados em hipóxia levam à falcização do eritrócito, com consequente formação de agregados de eritrócitos falcêmicos, leucócitos, plaquetas e células endoteliais, obstruindo o fluxo sanguíneo. Esse evento é referido como vaso-oclusão (VOC), o qual promove a lesão por isquemia-reperfusão. Os polímeros de Hb S também ocasionam a hemólise, a qual libera Hb na circulação sanguínea. A Hb livre promove disfunção endotelial por depletar o NO, formando EROs, e liberar o grupo heme, o qual juntamente com outras DAMPs ativam o inflamassoma. EROs são também produzidas pelas enzimas NADPH oxidase, XO e eNOS desacoplada. Por fim, o inflamassoma ativado por ERO, TLR4 e NETs (formadas por neutrófilos ativados), leva à produção de interleucinas e citocinas pró-inflamatórias. Os eventos são retroalimentados e constituem a complexa fisiopatologia da AF. DAMPs – padrões moleculares associados a dano; eNOS – óxido nítrico sintase endotelial; ERO – espécie reativa de oxigênio; Glu – ácido glutâmico; Hb – hemoglobina; *HBB* – gene da beta globina; IL – interleucina; NETs – armadilhas extracelulares de neutrófilos; NO – óxido nítrico; TLR4 – receptores tipo toll 4; TNF – fator de necrose tumoral; Val – valina; XO – xantina oxidase. Fonte: (Adaptado de Sundd; Gladwin; Novelli, 2019).

Como mencionado, a base molecular para a formação da Hb S é conhecida, mas somente esse mecanismo não é suficiente para explicar toda a diversidade fenotípica e a gravidade clínica variável encontrada nas pessoas com AF. Neste sentido, vários estudos têm sido propostos para a identificação de moduladores genéticos e bioquímicos a fim de relacioná-los com os mecanismos fisiopatológicos, estabelecer prognósticos mais precisos e, também, orientar o desenvolvimento de terapias mais específicas e eficazes (Belini-Júnior *et al.*, 2015; Habara; Steinberg, 2016; Pincez *et al*., 2022).

1.2. SISTEMA ENDOCANABINOIDE

Os componentes químicos da *Cannabis sativa* L. começaram a ser identidicados nas décadas de 50 e 60, sendo o canabidiol (CBD) e o Δ^9-tetrahidrocanabinol (Δ-^{9} THC) os primeiros a serem quimicamente caracterizados (Loewe, 1950; Mechoulam; Shvo, 1963; Gaoni; Mechoulam, 1964). Na década de 90, os receptores canabinoides tipo 1 e tipo 2 (CB1 e CB2) foram identificados como alvos do Δ-^{9} THC (Matsuda *et al*., 1990; Munro; Thomas; Abu-Shaar, 1993). A partir da identificação do CB1 e do CB2, foram isolados e caracterizados ligantes endógenos desses receptores - os quais foram denominados endocanabinoides (ECs) - e as principais enzimas responsáveis pela biossíntese e degradação desses compostos (Devane *et al*., 1992; Mechoulam *et al*., 1995; Sugiura *et al*., 1995; Di Marzo & Fontana; 1995; Okamoto *et al*., 2004). O sistema de sinalização composto pelos ECs, seus dois principais receptores e enzimas responsáveis pelo seu metabolismo foi denominado sistema endocanabinoide (ECS), o qual está envolvido em diversos aspectos – se não todos - da fisiologia de mamíferos (Ligresti; De Petrocellis; Di Marzo, 2016).

1.2.1. Endocanabinoides

Os ECs são lipídeos de sinalização endógenos, derivados de ácidos graxos e capazes de ativar os receptores canabinoides. Há ao menos 15 ECs com ligação ortostérica ou alostérica aos receptores CB1 e CB2, sendo exemplos ortostéricos o 2-araquidonoilglicerol (2-AG), a etanolamina N-aracnodoil (anandamida - AEA), a etanolamina O-araquidonoil (virandamida), a oleamida, a dopamina N-araquidonoil (NADA) e o éter glicerol 2-araquidonoil (noladina) (Pertwee, 2015); e exemplos alostéricos a lipoxina A4 (Pamplona *et al*., 2012) e o pepcan-12 (Petrucci *et al*., 2017).

Os ECs são considerados transmissores atípicos por não serem estocados em vesículas e por atuarem a partir de um mecanismo de sinalização retrógrado, sendo sintetizados sob demanda no neurônio pós-sináptico e liberados imediatamente após sua síntese, ligando-se a receptores do neurônio pré-sináptico (Giacobbe *et al*., 2021).

A síntese e a degradação dos ECs envolvem diferentes enzimas, sendo as vias do metabolismo da AEA e do 2-AG as mais estudadas. A AEA pertence ao grupo das N-aciletanolaminas (NAEs), e sua biossíntese inicia-se com a ligação de ácido araquidônico (AA) e fosfatidiletanolamina (PE), catalisada por uma N-acil transferase (NAT), formando o N-araquidonoil PE (NArPE). A enzima N-acil-fosfatidiletanolamina-específica fosfolipase D (NAPE-PLD) converte o NArPE em AEA (Biringer, 2021). A principal via de síntese do 2-AG, por sua vez, incia-se a partir da degradação de fosfolipídeos de membrana pela enzima fosfolipase C (PLC), formando o 1,2-diacil glicerol (DAG). O DAG é então convertido em 2-AG a partir da enzima diacil glicerol lipase (DAGL) (Murataeva; Straiker; Mackie, 2014; Rezende *et al*., 2023). Após serem liberados na fenda sináptica e interagirem com os receptores canabinoides (ou outros receptores), os ECs são reabsorvidos pelas células por um mecanismo envolvendo transportadores endocanabinoides de membrana (EMT), seguido por degradação intracelular. As principais enzimas envolvidas na hidrólise intracelular da AEA e do 2-AG são a amida hidrolase de ácidos graxos (FAAH) e a lipase de monoacilglicerol (MAGL), respectivamente (Rezende *et al*., 2023) (Figura 2).

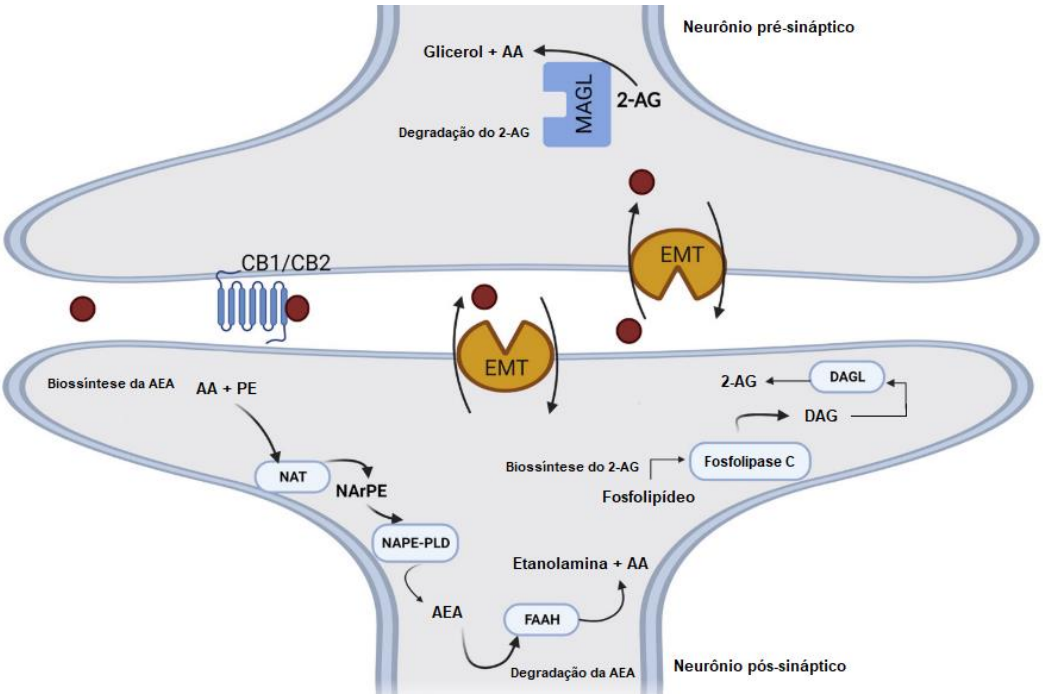

Figura 2. Principais vias de síntese e degradação da anandamida (AEA) e do 2-araquidonoilglicerol (2-AG). AA – ácido araquidônico; AEA – anandamida; 2-AG – 2-araquidonoilglicerol; DAG - 1,2-diacil glicerol; DAGL - diacil glicerol lipase; EMT – transportadores endocanabinoides de membrana; FAAH - amida hidrolase de ácidos graxos; MAGL - lipase de monoacilglicerol; NAPE-PLD - N-acil-fosfatidiletanolamina-específica fosfolipase D; NArPE - N-araquidonoil fosfatidiletanolamina; NAT - N-acil transferase; PE – fosfatidiletanolamina. Fonte: (Adaptado de Rezende *et al*., 2023).

1.2.2. Receptores endocanabinoides

Os dois receptores clássicos do ECS são o CB1 e o CB2, os quais compartilham 44% de homologia entre si (Munro, Thomas, Abu-Shaar, 1993). Ambos são receptores acoplados à proteína G (GPCRs) e membros da superfamília de receptores de 7 domínios transmembranares (7-TM), com um domínio amino-terminal extracelular, 7 domínios transmembranares hidrofóbicos, três *loops* exracelulares e três intracelulares e 1 domínio carboxi-terminal intracelular. (Sharma *et al*., 2021). O CB1 é altamente conservado entre humanos e outras espécies (97% de homologia com ratos e camundongos), e também encontrado em alguns invertebrados deuterostômios. O CB2, por sua vez, apresenta menor homologia; CB2 de humanos e camundongos, por exemplo, compartilham somente 82% da sequência de aminoácidos (Howlett; Abood, 2017).

O CB1 é, possivelmente, o GPCR mais abundante no cérebro de mamíferos e é amplamente encontrado no sistema nervoso central (SNC), com as maiores concentrações em regiões responsáveis por processos fisiológicos e mentais, como hipocampo, córtex cerebral, cerebelo, gânglio basal, hipotálamo e amígdala (Zou & Kumar, 2018; Sharma *et al*., 2021). Sua expressão em terminais pré-sinápticos sugere seu mecanismo de sinalização retógrado, inibindo a liberação de neurotransmissores como norepinefrina, dopamina, acelticolina, glutamato, e ácido γ-aminobutírico (GABA). O CB1 é também expresso por astrócitos, oligodendrócitos e em células da micróglia, ainda que em menor concentração em comparação a neurônios, e no sistema nervoso periférico (SNP) é altamente expresso por terminais nervosos simpáticos (Rezende *et al.*, 2023). Além dos SNC e SNP, o CB1 é encontrado (em menor concentração) no fígado, no trato gastrointestinal, no sistema cardiovascular, no endotélio vascular, na musculatura lisa e esquelética, no sistema imune e outros órgãos e tecidos (Zou & Kumar, 2018). O CB2 é principalmente expresso por órgãos

e células do sistema imune, como baço, tonsilas, timo, medula óssea, monócitos, macrófagos, mastócitos e linfócitos T e B. Nas células do sistema imune, o CB2 modula a ativação e migração das mesmas e a liberação de citocinas pró-inflamatórias (Ianotti; Di Marzo; Petrosino, 2016). Em concentrações bem menores em relação ao CB1, é encontrado também em neurônios do SNC e SNP e em células ativadas da micróglia (Di Marzo, 2018).

A ativação dos receptores canabinoides inicia uma via de sinalização que envolve, principalmente, a ligação do receptor às proteínas inibitórias Gi e Go (Gi/o), resultando na dissociação de seus heterodímeros e liberação das suas subunidades α e βγ (Lu *et al*., 2018). A subunidade Giα inibe a atividade da enzima adenilato ciclase (AC) e, consequentemente, diminui os níveis de monofosfato cíclico de adenosina (cAMP) (Lu *et al*., 2019; Sharma *et al*., 2021). A redução de cAMP afeta a ativação da proteína quinase A (PKA), a qual está associada a várias vias de sinalização relacionadas à proliferação, diferenciação e sobrevivência celular (Iannotti; Di Marzo; Petrosino, 2016; Rezende *et al*., 2023). A subunidade βγ estimula as vias da fosfatidilinositol-3-quinase (PI3K)/proteína quinase B (PKB/Akt) e da proteína quinase ativada por mitógeno (MAPK), envolvidas em processos de proliferação, sobrevivência e morte celular (Lu *et al*., 2019; Sharma *et al*., 2021). Além disso, a ativação dos receptores canabinoides é capaz de suprimir o influxo de cálcio (Ca^{2+}) para as células através da inibição de canais de cálcio dependentes de voltagem (VGCC) (Zou; Kumar, 2018) (Figura 3).

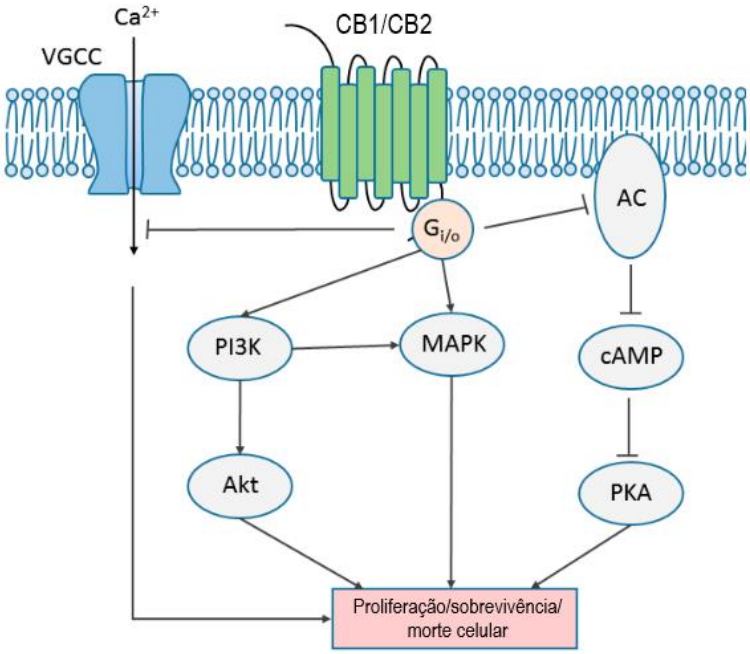

Figura 3. Principais vias de sinalização moduladas pela ativação dos receptores canabinoides. AC – adenilato ciclase; Akt – proteína quinase B; cAMP - monofosfato cíclico de adenosina; CB1 – receptor canabinoide 1; CB2 – receptor canabinoide 2; MAPK - proteína quinase ativada por mitógeno; PI3K - fosfatidilinositol-3-quinase; PKA – proteína quinase A; VGCC - canais de cálcio dependentes de voltagem. Fonte: (Adaptado de Zou; Kumar, 2018).

Além do CB1 e do CB2, outros GPCRs foram considerados possíveis receptores canabinoides; no entanto, até o momento somente os receptores GPR55 e GPR18 são alvos evidentes de uma ampla gama de ligantes canabinoides. O GPR55 apresenta baixa homologia de sequência com os receptores CB1 e CB2 (13% e 14%, respectivamente) e é amplamente expresso no cérebro e no SNP, coexistindo com esses receptores em vários tecidos. O GPR18 também apresenta baixa homologia com os receptores clássicos (13% com CB1 e 8% com CB2), sendo expresso principalmente em tecidos linfoides (Morales; Reggio, 2017). O receptor de potencial transitório vaniloide tipo 1 (TRPV1) e outros cinco da superfamília dos receptores de potencial transitório (TRP) são também modulados por canabinoides e constituem os chamados “receptores canabinoides ionotrópicos” (Di Marzo; De Petrocellis, 2010; Muller; Morales; Reggio, 2019). Por fim, os canabinoides são também capazes de ativar os receptores ativados por proliferadores de peroxissoma (PPARs), especialmente as isoformas α e γ, modulando respostas metabólicas, anti-inflamatórias e neuroprotetivas (Ianotti; Vitale, 2021). A capacidade de interagir com diversos receptores em diferentes tipos celulares confere aos canabinoides,

especialmente aos ECs, um efeito pleiotrópico, atuando em diversas vias fisiológicas e estados patológicos (Ligresti; De Petrocellis; Di Marzo, 2016).

1.2.3. Polimorfismos genéticos do sistema endocanabinoide

Polimorfismos de nucleotídeo único (SNPs) envolvidos em genes que codificam receptores canabinoides e enzimas que catabolizam os ECs podem alterar o padrão de sinalização e a metabolização dessas moléculas, possivelmente interferindo em diversas vias celulares e processos fisiopatológicos de diversas doenças. A função de SNPs relacionados ao gene *CNR1*, responsável por codificar o receptor CB1, bem como seus efeitos na expressão gênica e na atividade do receptor ainda não foram totalmente elucidados. Possivelmente, os SNPs envolvidos nesse gene, como o rs7766029, poderiam estar relacionados a uma diminuição na estabilidade do mRNA transcrito e, portanto, contribuiriam para uma redução da expressão de CB1 e sinalização de ECs que interagem com esse receptor (Domschke *et al*., 2008; Gonda *et al*., 2019). O SNP rs35761398 do gene *CNR2*, o qual codifica o receptor CB2, é responsável por uma substituição de uma arginina (Arg) por glutamina (Gln) na posição 63 da cadeia polipeptídica (Arg63>Gln) e afeta a resposta do CB2 aos ECs, interferindo na sinalização dessas moléculas e em processos associados, como a modulação do sistema imune (Carrasquer *et al*., 2010).

O SNP rs324420 do gene *FAAH*, que codifica a enzima responsável pela hidrólise da AEA, resulta em uma mutação *missense* na posição 385 do gene (385C>A) que converte um resíduo conservado de prolina (Pro) em uma treonina (Thr) (Pro129>Thr), produzindo uma enzima com propriedades catalíticas normais, porém mais suscetível à degradação proteolítica (Sipe *et al*., 2002). O genótipo A/A desse SNP é associado a um aumento da sinalização da AEA (Conzelmann *et al*., 2012) e a níveis reduzidos de FAAH quando comparado ao genótipo C/C (Chiang *et al*., 2004). O SNP rs604300, relacionado à MAGL, enzima responsável pela hidrólise do 2-AG, reside em uma região intrônica do gene *MAGL* próximo a uma sequência acentuadora da expressão do gene regulada por modificações epigenéticas relacionadas às histonas. Há evidências que a regulação epigenética nessa região pode exercer papel na transcrição do gene, produzindo níveis diferenciais de MAGL e influenciando na metabolização do 2-AG (Carey *et al*., 2015).

1.3. ANEMIA FALCIFORME E SISTEMA ENDOCANABINOIDE

Na busca de novas perspectivas sobre a fisiopatologia e terapias na AF, o ECS pode apresentar avanços promissores por ter demonstrado papel importante na atuação em várias vias bioquímicas e fisiológicas envolvidas na patologia da doença. Em cultura de células endoteliais da artéria coronária humana, agonistas sintéticos do CB2 diminuíram a expressão de proteína quimiotática de monócitos 1 (MCP-1) induzida por TNF-α, a ativação de NF-κB e a adesão e migração transendotelial de monócitos. No mesmo estudo, a expressão de ICAM-1 e VCAM-1 induzida por TNF-α foi atenuada, e em camundongos a adesão de monócitos ao endotélio aórtico foi mitigada (Rajesh *et al*., 2013). A diminuição da expressão de moléculas de adesão (ICAM-1, VCAM-1, P-selectina e E-selectina) mediada pela ativação de CB1/CB2 já havia sido demonstrada em modelo animal de aterosclerose (Zhao *et al*., 2010); assim como a quimiotaxia de macrófagos murinos para sítios de inflamação, modulada por CB2 (Raborn *et al*., 2008). O tratamento com um agonista de CB1/CB2 (CP55,940) reduziu a ativação de mastócitos e a liberação de citocinas pró-inflamatórias (IL-1α, IL-6, TNF-α e MCP-1), bem como preveniu a falcização dos eritrócitos induzida por hipóxia em camundongos falcêmicos (Vicent *et al*., 2016).

Em relação aos ECs, há evidências de que a AEA participa na estimulação da hematopoiese e tem papel na sobrevivência e fisiologia eritrocitária, além de diminuir a resposta inflamatória de macrófagos e células tronco mesenquimais (Bentzen; Lang, 2007; Gasperi *et al*., 2015; Ruhl *et al*., 2021). Tanto a AEA quanto o 2-AG são capazes de interagir com plaquetas, mas somente o 2-AG é um agonista verdadeiro de plaquetas humanas. Há evidências, no entanto, de que a AEA é capaz de prolongar o tempo de vida de plaquetas, reduzir sua agregação e de estimular a atividade da eNOS, aumentando a produção de NO e, consequentemente, a sobrevivência plaquetária (De Angelis *et al*., 2014; Gasperi *et al*., 2015). O 2-AG, em contrapartida, leva à ativação e à agregação plaquetária (Gasperi *et al*., 2015).

Portanto, a relação entre os mecanismos fisiopatológicos da AF e o ECS fundamenta a importância de estudos nesta área, contribuindo com a expansão da compreensão do impacto dos ECs em vias de interesse para potenciais terapias na AF, bem como no entendimento da influência de variantes genéticas desse complexo sistema em subfenótipos da doença.

2. OBJETIVOS

2.1. Objetivo Geral

Avaliar a associação de variantes genéticas nos genes envolvidos na sinalização e metabolização de canabinoides endógenos com os subfenótipos de características hemolíticas e de vasculopatia na anemia falciforme.

2.2. Objetivos Específicos

1) Rastrear os polimorfismos genéticos em genes (*FAAH*, *MAGL*, *CNR1* e *CNR2)* envolvidos na metabolização e sinalização dos endocanabinoides, em pessoas com anemia falciforme;

2) Avaliar a associação das variantes genéticas com os subfenótipos hemolíticos e de vasculopatia (acidente vascular encefálico vs. grupo controle) e (priapismo vs. grupo controle);

3) Verificar a influência das variáveis bioquímicas de hemólise, concentração de Hb F e presença de $\alpha^{-3,7kb}$-talassemia nos subfenótipos isoladamente ou associados com os polimorfismos.

3. ASSOCIATION OF SINGLE NUCLEOTIDE VARIANTS (SNVS) OF THE ENDOCANNABINOID SYSTEM WITH SICKLE CELL ANEMIA HEMOLYTIC SUBPHENOTYPES

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Abstract

Sickle cell anemia (SCA) results from a beta-globin gene mutation causing hemoglobin S production. The clinical SCA outcomes are associated with several genetic modifiers, and the endocannabinoid system (ECS) could be a candidate for modulating SCA complications. ECS is associated with several physiological pathways and impacts hematopoiesis, platelet aggregation, and immune responses. Here, we evaluated the association of ECS-related polymorphisms (*FAAH* rs324420, *MAGL* rs604300, *CNR1* rs7766029, and *CNR2* rs35761398) with hemolytic subphenotypes in 297 SCA patients. Phenotype SCA was first diagnosed by HPLC, and the Hb SS genotype was confirmed with PCR-RE. Alpha thalassemia ($-\alpha^{-3.7kb}$) mutation was detected with GAP-PCR, and genotyping was performed using TaqMan genotyping assays. Laboratory data were obtained from medical records. Overall, 73 developed

stroke (24.6%), 80 of the men presented priapism (46.8%), and 144 (48.5%) were assigned as the control group (for priapism, 58). Lower levels of Hb F were associated with stroke (P < 0.001), and a lower frequency of $\alpha^{-3.7kb}$-thalassemia mutation was associated with priapism (P < 0.001). In multivariate analyses with adjustment for $\alpha^{-3.7kb}$-thalassemia, the *CNR2* rs35761398 was associated with a lower chance of developing priapism (OR = 0.386 [0.175-0.854], P = 0.019), regardless of the $\alpha^{-3.7kb}$-thalassemia mutation (OR = 0.148 [0.059-0.375], P < 0.001) as a covariate. We did not find a statistical association between the SNPs and stroke. Priapism is a SCA hemolytic subphenotype involving imbalanced vasodilation/vasoconstriction pathways in corpora cavernosa. Based on our findings, we suggest a novel approach to the pathophysiology of priapism in SCA involving cannabinoide-receptor type 2 (CB2).
Keywords: Ischemic priapism, *CNR2*rs35761398, Prognostic markers, Cannabinoid-receptor 2.

Introduction

Sickle cell disease (SCD) is an inherited group of monogenic disorders characterized by a mutation in the beta-globin gene (*HBB*) that encodes hemoglobin (Hb) S, either in the homozygous state (sickle cell anemia, SCA) or compound heterozygous with another *HBB* mutation [1,2]. The primary determinant of SCA severity is the Hb S polymerization level, which generates rigid fibers of Hb S that distort and damage the membrane and cytoskeleton of erythrocytes, inducing sickling [1,3,4]. Erythrocyte injury results in both extra- and intravascular hemolysis, leading to endothelial dysfunction, vasculopathy, and the occlusion of small and large blood vessels. Consequently, this process gives rise to tissue ischemia/reperfusion injury and inflammation [5].

Since only the mutation is insufficient to explain the SCA subphenotypes, several studies have proposed the identification of genetic and biochemical modulators to relate them to the physiopathological mechanisms, establish more accurate prognoses, and guide the development of more specific and effective therapies [6,7]. In this context, the endocannabinoid system (ECS) emerges as a potential modulator of clinical complications in SCA.

Endocannabinoids (ECs) are endogenous signaling lipids involved in several molecular activities in cells, activating two G protein-coupled receptors (GPCRs) - cannabinoid receptors (CBRs) 1 and 2 [8]. The ECs, their specific receptors, and the enzymes responsible for their metabolism constitute the ECS [9]. Recent studies revealed that ECs interact with other GPCRs, peroxisome proliferator-activated nuclear receptors (PPARs), and selected ion channels, such as transient receptor potential vanilloid 1 (TRPV1) [10,11,12]. This pleiotropic effect gives a key role for ECS in many physiological pathways and pathological states [13].

In the SCA context, ECS participates in cellular and physiological processes of interest, as anandamide (AEA) can stimulate hematopoiesis, inhibit platelet aggregation and aggregates formation blood underflow, and play a role in erythrocytes survival and physiology [14,15,16]. Furthermore, activation of CB2 suppresses the expression of adhesive molecules and modulates leukocyte adhesion and macrophage chemotaxis to inflammation and injury sites [17,18]. Therefore, mutations in genes involved in the signaling and metabolism of ECs can contribute to the expression of specific subphenotypes in SCA. In this study, we aimed to evaluate the association of ECS single nucleotide variants (SNV) with SCA hemolytic subphenotypes in a northeast Brazilian cohort.

Methods

Patients

The observational, case-control, non-paired analytic study was performed with 297 SCA patients (median age 30 years, range 18 to 66 years, 171 males, 57.6%), enrolled and regularly followed in a reference center in Pernambuco, northeast Brazil. The following laboratory data were obtained from medical records: total hemoglobin (Hb), reticulocyte count, indirect bilirubin (IB), lactate dehydrogenase (LDH), and use of hydroxyurea (HU). For those submitted for HU treatment, data were obtained from the last time they were seen treatment-free.

Patients were divided into subgroups according to clinical complications related to hemolysis and vasculopathy phenotypes, which included 73 patients with stroke and 80 patients with priapism. The inclusion criteria for stroke were magnetic resonance and/or computed tomography data showing neurological injury and presenting evident

clinical manifestations and complaints resulting from strokes, such as cognitive impairment, paresthesia, and impairment of the senses, among others. For priapism, patients with current or previous records were included [19]. The control group was composed of 144 SCA patients ≥ 18 years who didn't present clinical complications (acute chest syndrome, stroke, leg ulcers, osteonecrosis, and priapism), following the definitions established in the literature [20,21].

This study was approved by the Federal University of Pernambuco and the Hemotherapy Foundation of Pernambuco (CAAE:58863316.6.3001.5195). Informed consent forms from all patients or their parents or legal guardians, when applicable, were obtained following the Declaration of Helsinki.

SCA confirmation and single nucleotide polymorphisms (SNPs) genotyping

Peripheral blood samples were collected from all patients, and genomic DNA was extracted using the phenol-chloroform protocol [22]. The Hb SS phenotype was diagnosed by high-performance liquid chromatography (HPLC). Polymerase Chain Reaction (PCR), followed by restriction analysis with *Dde*I, confirmed the genotype [23]. Alpha thalassemia ($-\alpha^{-3.7kb}$) mutation was detected with GAP-PCR [24]. *FAAH* (rs324420, C_1897306_10), *MAGL* (rs604300, C_2095673_20), *CNR1* (rs7766029, C_28979971_20) and *CNR2* (rs35761398, C_33555412_20) polymorphisms were genotyped using TaqMan SNPs assays following manufacturer's instructions.

Statistical analysis

We performed statistical analysis using SPSS Statistics 26.0 (IBM Corporation, Somers, NY, USA) and GraphPad Prism 9.0 (GraphPad Software, CA, USA). Continuous variables were expressed as medians and were tested for normal distribution using Lilliefor's test. Medians between groups were compared using the Mann-Whitney test. Fisher's exact and chi-square tests were applied for genotype frequency comparisons. Multiple binary logistic regressions were performed for SNP genotypes and potential prognostic factors for the complications, which included fetal hemoglobin (HbF) and $\alpha^{-3.7kb}$-thalassemia mutation. All *P-values* were two-sided with a significance level of 0.05.

Results

In general, 73 patients developed stroke (24.6%), 80 of the men presented priapism (46.8%), and 144 (48.5%) were assigned as the control group (for priapism, 58). Association analysis of genetics and laboratory data between groups is represented in Table 1. Lower levels of Hb F were associated with stroke (*P*<0.001), and a lower frequency of $\alpha^{-3.7kb}$-thalassemia mutation was associated with priapism (*P*<0.001). We did not observe a statistical difference between the other parameters.

Table 1 Association analysis of SCA hemolytic subphenotypes with genetics and laboratory variables.

Variables	All patients (n=297)	Stroke (n=73)	No Stroke (n=144)	*P* value	Priapism (n=80)	No priapism (n=58)	*P* value
Gender	**N (%)**	**N (%)**	**N (%)**		**N (%)**	**N (%)**	
Male	171 (57.6)	33 (45.2)	58 (40.3)	0.487	80 (100%)	58 (100%)	
Female	126 (42.4)	40 (54.8)	86 (59.7)		-	-	
Alpha thal.							
-α$^{3.7}$/αα	62 (21.4)	12 (16.7)	40 (28.6)	0.056	10 (12.8)	24 (41.4)	**0.000***
αα/αα	228 (78.6)	60 (83.3)	100 (71.4)		68 (87.2)	34 (58.6)	
Missing data	7	1	4		2		
	Median (Range)	**Median (Range)**	**Median (Range)**		**Median (Range)**	**Median (Range)**	
Hb F (%)	6.8 (0.8-24.2)	4.3 (1.0-15.8)	8.7 (0.8-23.5)	**0.000***	6.6 (0.8-24.2)	8.6 (0.8-23.5)	0.079
Hb (g/dL)	7.7 (4.8-11.5)	7.5 (4.8-9.9)	7.8 (4.9-11.5)	0.246	7.7 (4.9-9.8)	7.7 (5.9-11.5)	0.960
Ret. (%)	10.0 (0.2-119.0)	10.3 (2.5-119.0)	9.5 (0.2-29.2)	0.404	10.0 (3.0-28.0)	9.2 (2.4-29.2)	0.585
BI (mg/dL)	2.1 (0.04-13.6)	1.9 (0.14-8.6)	2.3 (0.04-13.6)	0.303	2.1 (0.5-9.3)	2.2 (0.3-13.6)	0.871
LDH (U/L)	811.0 (223-3542)	821.0 (235-3542)	738.5 (223-2381)	0.279	980.5 (244-3000)	750.0 (267-2381)	0.113

* indicates a statistically significant difference. Hb F, hemoglobin F; Hb, total hemoglobin; Ret, reticulocyte Count; BI, indirect bilirubin; LDH, lactate dehydrogenase. Quantitative data comparisons were made by Mann-Whitney test (non-parametric data). Categorical data comparisons were made by Pearson's Qui-square test

We analyzed all SNPs' co-dominant, dominant, recessive, and over-dominant inheritance models. Due to a better data fit, we considered the dominant model for *MAGL* rs604300 (*P*= 0.049) and the overdominant model for *FAAH* rs324420 (*P*= 0.049), *CNR1* rs7766029 (*P*= 0.025) and *CNR2* rs35761398(*P*= 0.038). The relative genotype frequencies for the SNPs in stroke and control groups (Fig. 1) and priapism and control groups (Fig. 2) are represented. We did not observe a statistical difference between the genotype frequencies in groups with clinical complications and the control group. We did not observe either a statistical difference in the impact of all possible inheritance models for the SNPs in our laboratory markers (total Hb, reticulocyte count, IB, and LDH), except for a lower level of total Hb related to the TT-CC genotype of *CNR2* (Supplementary Data).

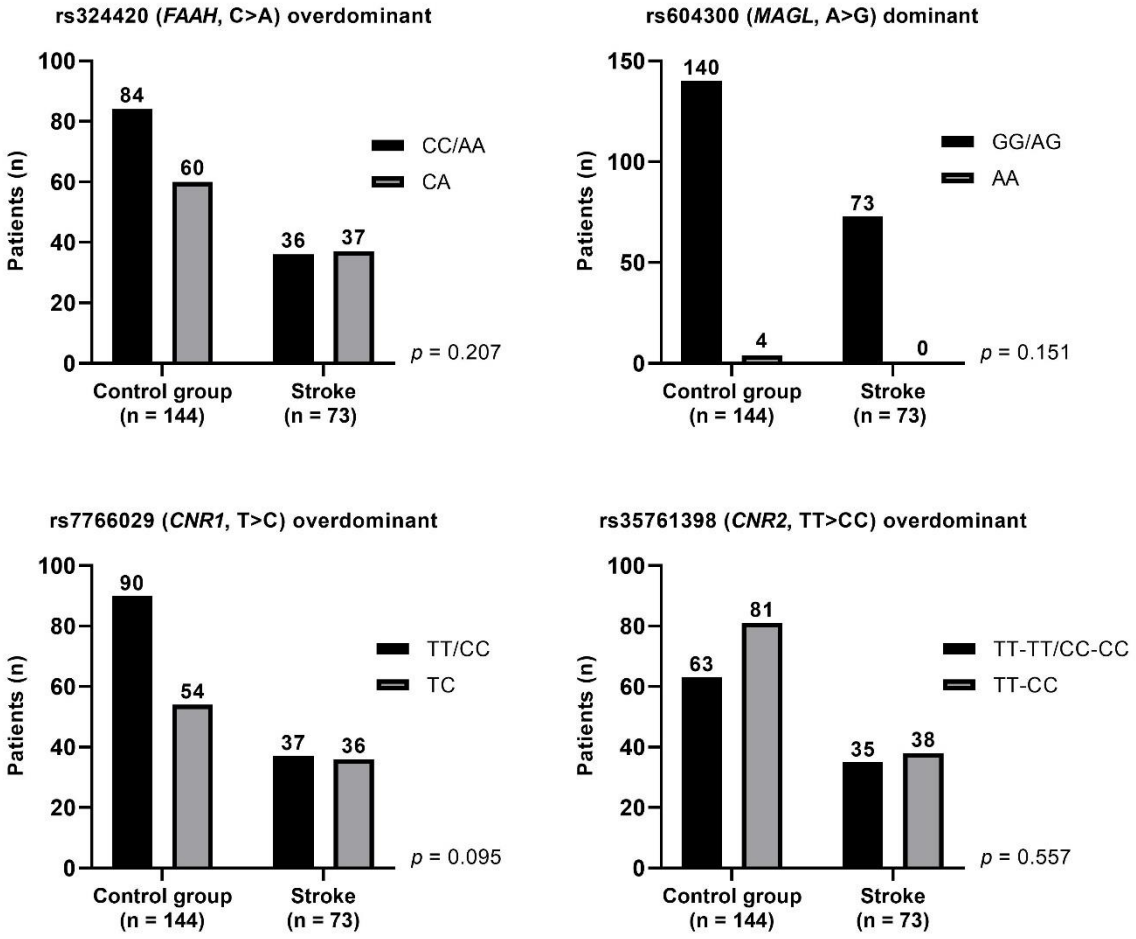

Fig. 1 Relative genotype frequencies in stroke and control groups for rs324420, rs604300, rs7766029, and rs35761398. Pearson's Qui-square test was performed for *FAAH*, *CNR1*, and *CNR2* frequencies comparisons and Fisher's exact test for *MAGL frequency* comparison. *FAAH*, fatty-acid amide hydrolase; *MAGL*, monoacylglycerol lipase; *CNR1*, cannabinoid receptor 1; *CNR2*, cannabinoid receptor 2

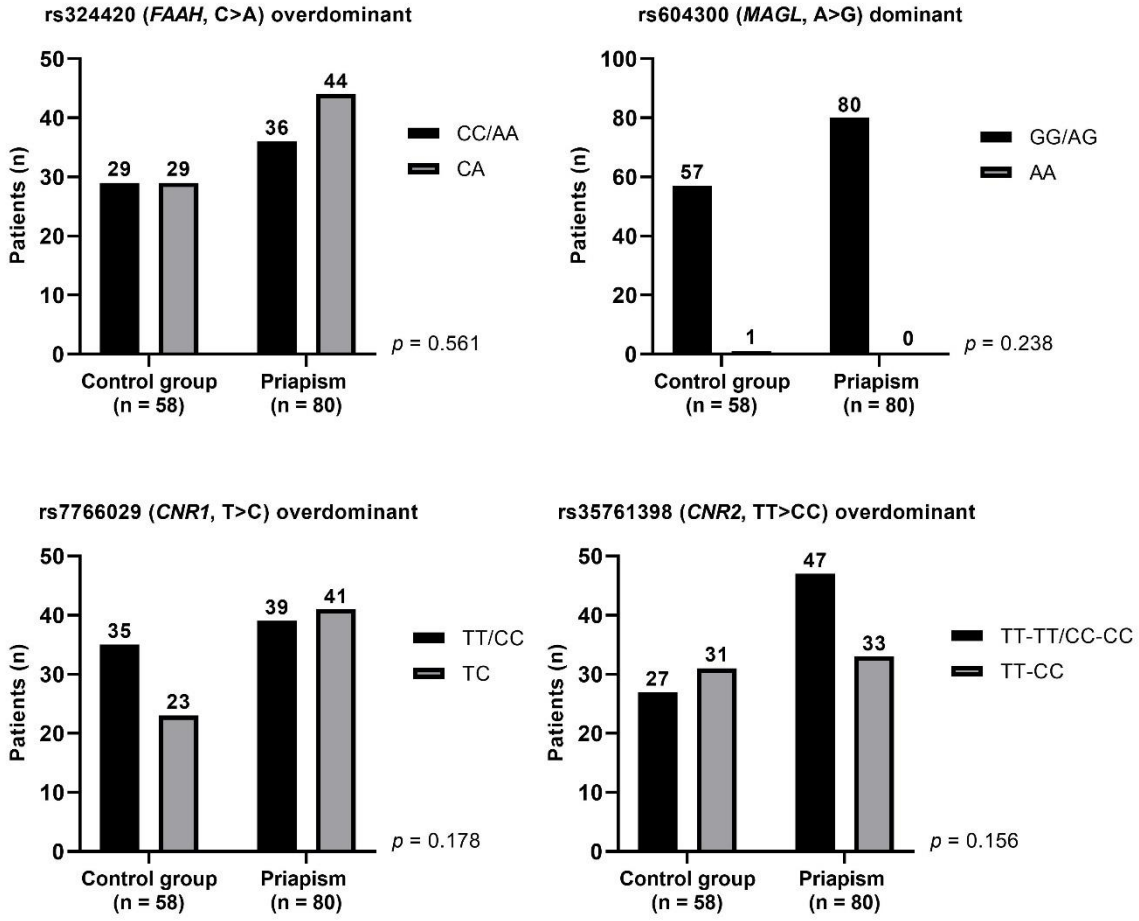

Fig. 2 Relative genotype frequencies in priapism and control groups for rs324420, rs604300, rs7766029, and rs35761398. Pearson's Qui-square test was performed for *FAAH*, *CNR1*, and *CNR2* frequencies comparisons and Fisher's exact test for *MAGL* frequencies comparison. *FAAH*, fatty-acid amide hydrolase; *MAGL*, monoacylglycerol lipase; *CNR1*, cannabinoid receptor 1; *CNR2*, cannabinoid receptor 2

We performed multivariate analyses adjusted by $\alpha^{-3.7kb}$-thalassemia mutation and Hb F as covariates for priapism and stroke, respectively (Table 1). We found that the TT-CC genotype for *CNR2* rs35761398 conferred a protective factor for priapism (OR = 0.386 [0.175-0.854], *P*= 0.019), regardless of the $\alpha^{-3.7kb}$-thalassemia mutation (OR = 0.148 [0.059-0.375], *P*< 0.001) (Fig. 3). We did not observe a statistical difference between the other three SNPs and priapism and between all SNPs and stroke, even with Hb F as a covariate (OR = 0.791 [0.718-0.872], *P* < 0.001).

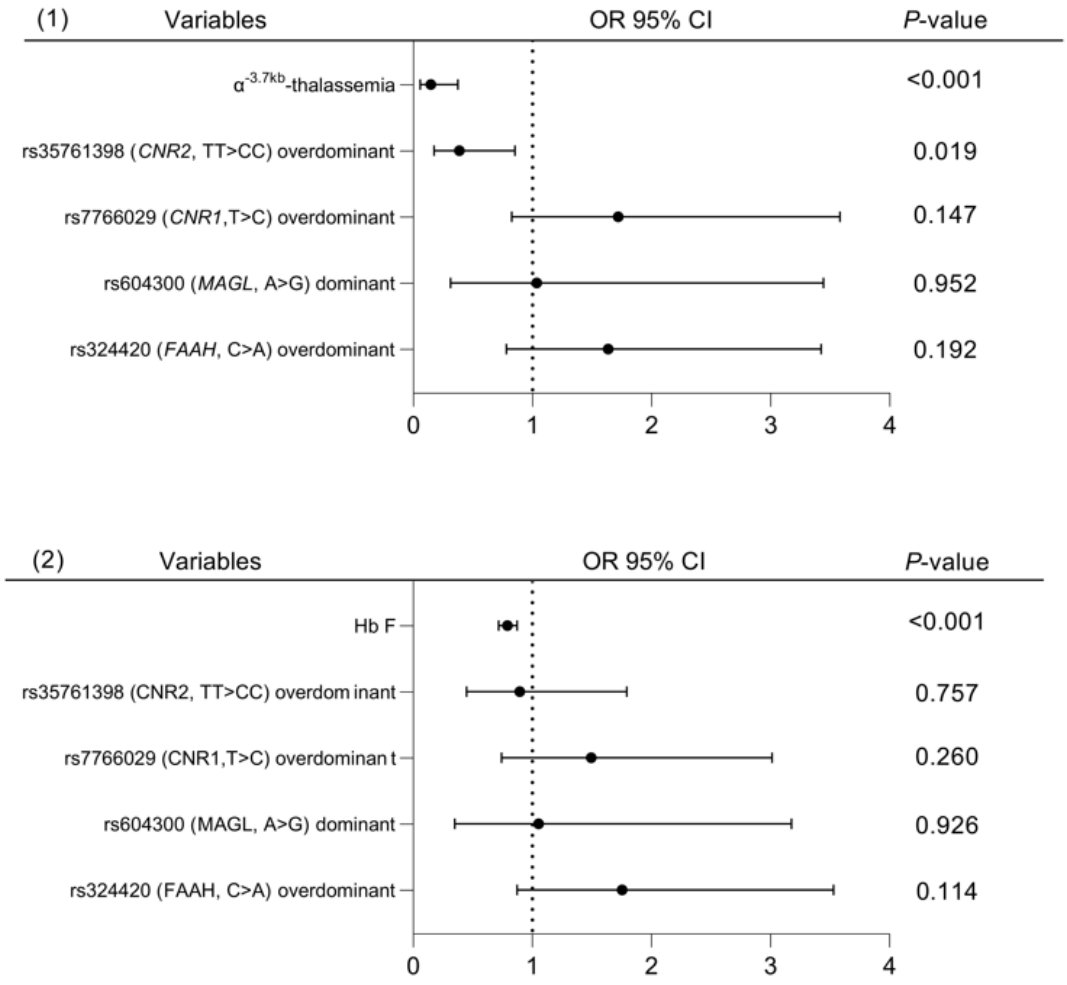

Fig. 3 Forest plot of multiple binary logistic regression analysis of selected SNPs and (1) priapism vs. control group and (2) stroke vs. control group. The horizontal lines correspond to the study-specific OR and 95% CI. Each of the SNPs was included separately in different models adjusted by (1) $\alpha^{-3.7kb}$-thalassemia mutation and (2) Hb F as covariates. *FAAH*, fatty-acid amide hydrolase; *MAGL*, monoacylglycerol lipase; *CNR1*, cannabinoid receptor 1; *CNR2*, cannabinoid receptor 2; Hb F, fetal hemoglobin; OR, odds ratio; CI, confidence interval

Discussion

Association studies with SCA clinical outcomes and genetic variants have the potential to help overcome several challenges that arise from this remarkable phenotypic heterogeneity [25]. Moreover, comprehending the causes of variability leads to identifying new pathological mechanisms and their association with well-known pathophysiology pathways, suggesting new therapeutic approaches [26]. Here, we investigated, for the first time to our knowledge, the association between ECS polymorphisms and SCA hemolytic subphenotypes.

Notably, HbF and alpha-thalassemia are the two main modulators of phenotype outcomes in SCA [5]. The HbF concentrations within the erythrocyte modified the rate and extent of HbS polymerization, delaying it [27,28]. HbF has more beneficial modulation in vaso-occlusive subphenotypes, perhaps due to insufficient HbF in some cells, allowing hemolysis. Only a few lysed sickle erythrocytes are needed in hemolytic subphenotypes to trigger the pathophysiology [27,29]. In this sense, the Hb F concentration in our study population was higher in patients without stroke; the results were consistent with other studies [30,31,32]. Alpha-thalassemia attenuates hemolysis by reducing mean cell Hb concentration and cell density, thereby reducing HbS polymerization rate, and co-inheritance with SCA is associated with lower chances of developing clinical complications [33,34,35]. Our data agrees with these findings since $\alpha^{-3.7kb}$-thalassemia mutation constituted a protective factor for priapism, giving an 85.2 percent less chance of developing this outcome.

Regarding the inheritance SNPs models and any hemolytic subphenotype (priapism and stroke), the dominant model for *MAGL* and the overdominant model for *FAAH*, *CNR1,* and *CNR2* were statistically significant. However, we did not find a statistical difference between the genotype frequencies in the control and priapism/stroke groups. When we analyzed the impact of these inheritance models in our laboratory markers, no statistical difference was found, except for a lower level of total Hb related to the CC-TT genotype of *CNR2*. Despite that, all patients are in a pathophysiological state marked by a Hb lower than normal levels, and this result could be related to other pathological mechanisms of SCA. Since no studies relate the ECS SNPs analyzed here to SCA, we can not compare our findings. Moreover, the inheritance model of a SNP in a disease can be a key for unraveling the genetic characteristics of complex diseases such as SCA and linking disease phenotypes to specific genotypes. Our study provides, for the first time, a model for ECS SNPs and SCA hemolytic subphenotypes.

SCA patients are in a constant state of intravascular and extravascular hemolysis, which releases free hemoglobin and arginase, initiating events that result in endothelial dysfunction and vascular injury [5, 26]. Patients with the highest hemolysis rates are more likely to develop SCA hemolytic subphenotypes, such as priapism and stroke [36,37].

Priapism is a pathological condition defined as a prolonged and painful penile erection not associated with sexual desire, and a late diagnosis can lead to penile fibrosis and erectile dysfunction [38,39]. Ischemic priapism, marked by penile pain and rigidity, is caused by blood entrapment in the corpora cavernosa sinusoids during erection. First, the mechanism underlying ischemic priapism in SCA was associated with obstruction of venous outflow from corporal bodies as a consequence of the interaction between sickle erythrocytes, endothelial cells, leukocytes, and platelets, leading to vaso-occlusion and impaired drainage [38,40]. Nevertheless, recent studies revealed a probable molecular basis of ischemic priapism involving nitric oxide (NO) depletion, resulting in persistent cavernosal tissue relaxation [41,42]. Although priapism has been previously related to marijuana and synthetic cannabis use, there is no well-established evidence of a molecular association between the ECS and this SCA outcome [43,44,45]. In our study, we demonstrated for the first time a protective association between the rs35761398 *CNR2* polymorphism and priapism, with an effect of 61.4 percent less chance of developing this outcome.

Normal erectile physiology is regulated by the NO/cyclic guanosine monophosphate (cGMP) pathway, involving both vascular and neurogenic pathways regulated by the nitric oxide synthase (NOS) [46]. Upon phosphorylation, endothelial NOS (eNOS) and neuronal NOS (nNOS) convert L-arginine in L-citrulline, releasing NO, which diffuses locally to smooth muscle cells and binds to guanylate cyclase. The activated guanylate cyclase converts guanosine-5’-triphosphate (GTP) to cGMP, which starts the cGMP-dependent protein kinase G (PKG), a downstream effector. This promotes the relaxation of corpora cavernosal smooth muscle and vasodilation of blood vessels, leading to penile erection [47]. The cGMP-specific type 5 phosphodiesterase (PDE5) inactivates cGMP, converting it to 5’-GMP, terminating erection [48].

The dysregulation of the NO/cGMP/PKG pathway is the primary molecular mechanism of ischemic priapism in SCA [49]. Hemolysis releases free hemoglobin and arginase, which scavenges NO and degrades L-arginine [50]. eNOS functional uncoupling, its decreased phosphorylation, and increased oxidative/nitrosative stress are also involved in the reduced activity of eNOS in SCA-related ischemic priapism [51,52,53,54,55]. The chronic decrease in endothelial NO results in reduced cGMP production and a compensatory reduction in PDE5 expression and activity. Thus, cGMP accumulates under erectogenic stimulation, resulting in priapism [42].

The dysregulated RhoA/Rho-kinase (ROCK) vasoconstrictor pathway also contributes to the pathophysiology of priapism [48]. In normal physiological conditions, RhoA agonist activation of ROCK mediates vasoconstriction of the penile vasculature and regulates eNOS expression and activation, maintaining the penis's flaccid state [56]. In sickle cell mice, both RhoA and total ROCK activities are significantly reduced, and further studies confirmed a dysregulated Rho signaling with reduced RhoA expression in human SCA penis [57,58]. This leads to reduced vasoconstriction in the penis in SCA, maintaining cavernous relaxation and increasing susceptibility to priapism.

In the ECS context, a study demonstrated that a CB2 agonist decreased TNF-α-induced RhoA activation in human coronary artery endothelial cells (HCAECs), and this effect was attenuated by a CB2 antagonist [59]. CB2 receptors are mainly expressed in immune cells, with the highest levels in macrophages, B cells, NK cells, monocytes, and polymorphonuclear neutrophils [60]. Endothelial cells and smooth muscle also express CB2 [61]. It potently modulates immune responses, positively or negatively, and its expression is highly induced by tissue injury or inflammation [62]. Recent studies demonstrated that CB2 is expressed in intracellular and extracellular forms, and the presence of GPCRs in different cell locations appears to be an essential feature of these receptors that confers functional heterogeneity [63,64,65,66]. According to our findings, we hypothesize that a basal level of CB2 activation in endothelial cells of penile vasculature, related to the TT-CC genotype for the *CNR2* SNP analyzed, plays a role in maintaining the normal RhoA/ROCK pathway. Therefore, it decreases the chances of developing priapism.

The rs35761398 SNP is a missense mutation of the second and third bases at codon 63 of the *CNR2* gene, leading to a glutamine (Q)/arginine (R) substitution, which causes a different polarization state of the protein [67]. The signal intensity caused by 63R activation seems weaker than the signal by 63Q activation, and a molecular docking study showed that the predicted structures of 63R could not bind to the G-protein at the correct position, decreasing signaling [68,69,70]. These findings suggest that it could have both 63Q/63R expressions in the TT-CC genotype. This expression may vary among different cell types or physiological states, explaining to some extent the protector effect in priapism and no relation to stroke.

Stroke is an SCA subphenotype related to hemolysis, endothelial dysfunction, and vascular injury [5,37]. Studies identify candidates' genetic variants involved in oxidative stress and inflammation that modulate stroke, and a recent genome-wide association study (GWAS) revealed 14 candidate genes associated with ischemic stroke occurring at earlier ages in SCD [71,72,73]. Unfortunately, our data revealed that the ECS SNPs analyzed are not good predictors for stroke. However, growing evidence exists for considering these genes as candidates in SCA modulation.

In conclusion, our data suggests a novel approach to the pathophysiology of priapism in SCA involving CB2. Nevertheless, the exact functional consequences of this SNP in CB2 activity still need to be well-established. So, our findings should be considered cautiously, and validations in independent cohorts could significantly strengthen our study.

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Authorship

A.C.M.B. conceived the study, performed experiments, analyzed and interpreted data, performed statistical analysis, and prepared the manuscript. V.S.R. reviewed the paper. G.S.A. and M.A.B.C. collected data. L.G. supported statistical analysis and reviewed the paper. D.G.H.S. and E.B.J. designed the study and reviewed the paper.

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Ethics approval and informed consent

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Federal University of Pernambuco Ethics Committee and Hemotherapy Foundation of Pernambuco (CAAE: 58863316.6.3001.5195).

Conflict of interest

The authors declare no competing interests.

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Supplementary data

Table S1 Association analysis of the SNPs overdominant models with laboratory variables

Laboratory variables	Polymorphisms										
	rs324420 *FAAH*		*P* value	rs604300 *MAGL*		*P* value	rs7766029 *CNR1*		*P* value	rs35761398 *CNR2*		*P* value
	CA	CC/AA		AG	AA/GG		TC	TT/CC		TT-CC	TT-TT/CC-CC	
	Median (Range)	Median (Range)		Median (Range)	Median (Range)		Median (Range)	Median (Range)		Median (Range)	Median (Range)	
Hb (g/dL)	7.8 (4.9-11.5)	7.6 (4.8-11.5)	0.391	7.7 (5.6-11.5)	7.7 (4.8-11.2)	0.255	7.8 (5.9-11.2)	7.6 (4.8-11.5)	0.310	7.5 (4.8-11.5)	7.9 (4.9-11.2)	**0.002***
Ret. (%)	10 (0.2-28.0)	9.6 (2.2-119.0)	0.638	10 (2.6-28.0)	9.8 (0.2-119.0)	0.764	10.2 (2.6-27.9)	9.6 (0.2-119.0)	0.259	9.4 (0.2-119.0)	10.1 (2.2-29.2)	0.431
BI (mg/dL)	2.10 (0.13-8.60)	2.09 (0.04-13.58)	0.943	1.60 (0.13-13.58)	2.16 (0.04-13.50)	0.151	2.00 (0.13-9.35)	2.28 (0.04-13.58)	0.622	2.14 (0.04-13.58)	2.08 (0.14-9.35)	0.447
LDH (U/L)	792.0 (230.0-2439.0)	825.0 (223.0-3542.0)	0.139	962.5 (235.0-3542.0)	798.0 (223.0-3000.0)	0.370	854.0 (223.0-3000.0)	792.0 (230.0-3542.0)	0.278	806.0 (223.0-3000.0)	811.0 (230.0-3542.0)	0.866

* indicates a statistically significant difference. Hb, total hemoglobin; Ret, reticulocyte Count; BI, indirect bilirubin; LDH, lactate dehydrogenase. Quantitate data comparisons were made by Mann-Whitney test (non-parametric data)

Table S2 Association analysis of the SNPs dominant models with laboratory variables

Laboratory variables	Polymorphisms										
	rs324420 *FAAH*		*P* value	rs604300 *MAGL*		*P* value	rs7766029 *CNR1*		*P* value	rs35761398 *CNR2*		*P* value
	CC	AA/CA		AA	GG/AG		TT	CC/TC		TT-TT	CC-CC/TT-CC	
	Median (Range)	Median (Range)		Median (Range)	Median (Range)		Median (Range)	Median (Range)		Median (Range)	Median (Range)	
Hb (g/dL)	7.6 (4.8-10.3)	7.8 (4.9-11.5)	0.209	7.4 (7.2-8.6)	7.7 (4.8-11.5)	0.754	7.7 (4.8-11.1)	7.7 (5.2-11.5)	0.771	8.2 (6.5-9.7)	7.7 (4.8-11.5)	**0.036***
Ret. (%)	9.9 (2.2-119.0)	9.9 (0.2-28)	0.547	9.0 (6.6-17.2)	10.0 (0.2-119.0)	0.914	9.6 (0.2-29.2)	10.0 (2.4-119.0)	0.490	11.0 (2.2-19.9)	9.8 (0.2-119.0)	0.432
BI (mg/dL)	2.24 (0.04-13.58).	2.08 (0.13-8.60)	0.728	2.31 (1.41-5.67)	2.10 (0.04-13.58)	0.647	2.23 (0.14-13.58)	2.05 (0.04-9.35)	0.982	2.42 (0.45-6.81)	2.08 (0.04-13.58)	0.363
LDH (U/L)	823.5 (223.0-3542.0)	798.0 (230.0-2439.0)	0.209	894.0 (723.0-996.0)	809.0 (223.0-3542.0)	0.736	822.0 (235.0-3542.0)	809.0 (223.0-3000.0)	0.840	814.0 (333.0-1416.0)	811.0 (223.0-3542.0)	0.962

* indicates a statistically significant difference. Hb, total hemoglobin; Ret, reticulocyte Count; BI, indirect bilirubin; LDH, lactate dehydrogenase. Quantitate data comparisons were made by Mann-Whitney test (non-parametric data)

Table S3 Association analysis of the SNPs recessive models with laboratory variables

Laboratory variables	Polymorphisms										
	rs324420 *FAAH*		*P* value	rs604300 *MAGL*		*P* value	rs7766029 *CNR1*		*P* value	rs35761398 *CNR2*		*P* value
	CC/CA Median (Range)	AA Median (Range)		AA/AG Median (Range)	GG Median (Range)		TT/TC Median (Range)	CC Median (Range)		TT-TT/TT-CC Median (Range)	CC-CC Median (Range)	
Hb (g/dL)	7.7 (4.8-11.5)	7.7 (6.3-11.2)	0.550	7.5 (5.6-11.5)	7.7 (4.8-11.2)	0.234	7.8 (4.8-11.2)	7.6 (5.2-11.5)	0.275	7.6 (4.8-11.5)	7.8 (4.9-11.2)	0.075
Ret. (%)	10.0 (0.2-119.0)	9.0 (2.6-27.0)	0.834	10.0 (2.6-28.0)	9.9 (0.2-119.0)	0.811	10.0 (0.2-29.2)	9.5 (2.4-119.0)	0.580	9.9 (0.2-119.0)	9.9 (2.6-29.2)	0.752
BI (mg/dL)	2.16 (0.04-13.58)	2.00 (0.50-6.92)	0.658	1.70 (0.13-13.58)	2.16 (0.04-13.50)	0.236	2.08 (0.13-13.58)	2.32 (0.04-8.72)	0.569	2.18 (0.04-13.58)	2.00 (0.14-9.35)	0.195
LDH (U/L)	809.0 (223.0-3542.0)	827.0 (387.0-1450.0)	0.688	962.0 (235.0-3542.0)	797.5 (223.0-3000.0)	0.331	823.5 (223.0-3542.0)	729.0 (230.0-2439.0)	0.297	810.5 (223.0-3000.0)	811.0 (230.0-3542.0)	0.841

* indicates a statistically significant difference. Hb, total hemoglobin; Ret, reticulocyte Count; BI, indirect bilirubin; LDH, lactate dehydrogenase. Quantitate data comparisons were made by Mann-Whitney test (non-parametric data)

Table S4 Association analysis of the SNPs codominant models with laboratory variables

Laboratory variables	Polymorphisms													
	rs324420 *FAAH*			*P* value	rs604300 *MAGL*			*P* value	rs7766029 *CNR1*			*P* value	rs35761398 *CNR2*			*P* value
	CC Median (Range)	AC Median (Range)	AA Median (Range)		AA Median (Range)	AG Median (Range)	GG Median (Range)		TT Median (Range)	TC Median (Range)	CC Median (Range)		TT-TT Median (Range)	TT-CC Median (Range)	CC-CC Median (Range)	
Hb (g/dL)	7.6 (4.8-10.3)	7.8 (4.9-11.5)	7.7 (6.3-11.2)	0.441	7.4 (7.2-8.6)	7.7 (5.6-11.5)	7.7 (4.8-11.2)	0.491	7.7 (4.8-11.1)	7.8 (5.9-11.2)	7.6 (5.2-11.5)	0.411	8.2 (6.5-9.7)	**7.5 (4.8-11.5)[a]**	7.8 (4.9-11.2)	**0.006***
Ret. (%)	9.9 (2.2-119.0)	10.0 (0.2-28.0)	9.0 (2.6-27.0)	0.834	9.0 (6.6-17.2)	10.0 (2.6-28.0)	9.9 (0.2-119.0)	0.952	9.6 (0.2-29.2)	10.2 (2.6-27.9)	9.5 (2.4-119.0)	0.528	11.0 (2.2-19.9)	9.4 (0.2-119.0)	9.9 (2.6-29.2)	0.629
BI (mg/dL)	2.24 (0.04-13.58)	2.10 (0.13-8.60)	2.00 (0.50-6.92)	0.883	2.31 (1.41-5.67)	1.60 (0.13-13.58)	2.16 (0.04-13.50)	0.329	2.23 (0.14-13.58)	2.00 (0.13-9.35)	2.32 (0.04-8.72)	0.820	2.42 (0.45-6.81)	2.14 (0.04-13.58)	2.00 (0.14-9.35)	0.360
LDH (U/L)	827.0 (223.0-3542.0)	792.0 (230.0-2439.0)	827.0 (387.0-1450.0)	0.246	894.0 (723.0-996.0)	962.5 (235.0-3542.0)	797.5 (223.0-3000.0)	0.623	822.0 (235.0-3542.0)	854.0 (223.0-3000.0)	729.0 (230.0-2439.0)	0.469	814.0 (333.0-1416.0)	806.0 (223.0-3000.0)	811.0 (230.0-3542.0)	0.980

* indicates a significant statistical difference

[a] indicates that the TT-CC group was statistically different from the TT-TT group ($P = 0.023$) and the CC-CC group ($P = 0.046$), compared with Dunn post hoc test. *P* values were adjusted with Bonferroni correction. Hb, total hemoglobin; Ret, reticulocyte Count; BI, indirect bilirubin; LDH, lactate dehydrogenase. Quantitate data comparisons were made by Kruskal-Wallis test (non-parametric data)

4. CONCLUSÕES

- O presente estudo forneceu, pela primeira vez, uma associação entre polimorfismos do sistema endocanabinoide (ECS) e a anemia falciforme (AF);

- Os polimorfismos analisados (*FAAH* rs324420, *MAGL* rs604300, *CNR1* rs7766029, e *CNR2* rs35761398) não foram bons preditores para o desenvolvimento de acidente vascular encefálico (AVE); no entanto, há evidências crescentes de que tais genes do ECS são bons candidatos à modulação da AF;

- O genótipo TT-CC do polimorfismo *CNR2* rs35761398 conferiu efeito protetor ao desenvolvimento de priapismo, e, portanto, sugerimos uma nova abordagem na fisiopatologia desse subfenótipo da AF envolvendo o receptor canabinoide tipo 2 (CB2);

- Nossos dados devem ser considerados com cautela, uma vez que consequências funcionais do polimorfismo *CNR2* rs35761398 na atividade do CB2 ainda precisam ser completamente estabelecidas.

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