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BIOQUÍMICA E FISIOLOGIA

TESE DE DOUTORADO

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Tese apresentada para o cumprimento parcial das exigências para obtenção do título de Doutor em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco.

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Chico Xavier

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RESUMO

Anemia Falciforme (AF) é uma doença monogênica grave e reflete a expressão clínica da homozigose do gene da hemoglobina falciforme (HbS), promovendo deformabilidadade na membrana do eritrócito, deixando-a em forma de foice. Estas alterações físicas da membrana podem estar relacionadas a modificações na sua composição lipídica. Pacientes com AF podem divergir quanto à sintomatologia e evolução da doença, caracterizando dois subfenótipos na expressão da doença falciforme: vaso-oclusivo (VO) e anemia hemolítica (AH). O objetivo deste estudo foi investigar alterações do metabolismo lipídico em pacientes com anemia falciforme relacionadas aos subfenótipos clínicos. Foram coletadas amostras de sangue de 13 indivíduos saudáveis e 51 pacientes com anemia falciforme, 23 com AH, 15 com VO e 13 com sobreposição (SP) de fenótipos. Foram determinados eletrólitos, glicose de jejum, perfil lipídico, funções renal e hepática. Colesterol de membrana eritrocitária foi avaliado por método enzimático. Fosfolipídios de plasma e de eritrócitos foram avaliados conforme Bartllet (1956), após terem sido isolados por cromatografia em camada delgada. LDL oxidada foi determinada através de ELISA e alelos da Apolipoproteína E (Apo E) foram detectados por PCR/RFLP. Tempo de hemólise foi avaliado conforme o teste de permeabilidade ao glicerol. Elasticidade do eritrócito e fragilidade osmótica também foram investigadas. O grupo com VO apresentou menores valores de colesterol e de lisofosfatidilcolina na membrana e menores níveis de fosfolipídios totais no plasma. Somente os indivíduos AH estiveram relacionados com diminuições das concentrações de fosfatidiletanolamina em eritrócitos. AF demonstrou promover um maior aumento no tempo de hemólise (SP > AH > VO), correlacionando-se positivamente com os níveis de colesterol da membrana. Pacientes com AF apresentaram menor elasticidade quando comparado aos controles, entretanto não foram observadas diferenças significativas entre os subfenótipos da AF. Pacientes com AF e portadores de alelo ε4 da Apo E apresentaram uma maior redução nos níveis de colesterol plasmático associada a uma elevação de suas concentrações na membrana eritrocitária. Alelo ε4 também esteve relacionado a menores valores plasmáticos das apolipoproteínas A-I e B, com aumento de LDL oxidada. Lisofosfatidilcolina, esfingomielina e fosfatidilserina tiveram suas concentrações elevadas em pacientes com AF na presença do alelo ε4. Fosfatidilserina correlacionou-se negativamente com os valores plasmáticos da Desidrogenase Láctica, em pacientes com alelo ε4, demonstrando uma maior participação deste fosfolipídio na influência sobre as propriedades físico-químicas desta enzima na presença do alelo ε4. Os resultados indicam que nesta população de pacientes estudados, a expressão de AF em seus subfenótipos está intrinsecamente relacionada a alterações divergentes no metabolismo lipídico, além de sofrer influência do polimorfismo da Apo E. Este é o primeiro estudo a demonstrar e investigar o subfenótipo sobreposição, assim como as alterações no metabolismo lipídico na anemia falciforme em pacientes portadores de subfenótipos VO ou AH, contribuindo para a caracterização detalhada da fisiopatologia dessa doença hematológica e das alterações metabólicas que as acompanham. O estudo permitiu conhecer maiores detalhes do metabolismo lipídico da AF, decifrando dados bioquímicos até então pouco explorados, que podem esclarecer alterações laboratoriais específicas com claras repercussões clínicas nos indivíduos acometidos.

Palavras-chave: Anemia Falciforme, Subfenótipo Vaso-oclusivo, Subfenótipo Hemolítico, Apolipoproteína E, Colesterol, Fosfolipídeos, Desidrogenase Láctica.

ABSTRACT

Sickle Cell Anemia (SCA) is a serious monogenic disease and reflects the clinical expression of homozygous sickle hemoglobin gene (HbS), promoting the deformability of the erythrocyte membrane, leaving the sickle-shaped. These physical modifications of the membrane may be related to changes in their lipid composition. Patients with AF may differ in terms of symptoms and disease progression, featuring two subphenotypes in the expression of the disease sickle cell vaso-occlusive (VO) and hemolytic anemia (AH). The aim of this study was to investigate whether changes in lipid metabolism in patients with SCA are related to different clinical subphenotypes. We collected blood samples from 13 healthy individuals and 51 patients with SCA, 23 with AH, 15 with VO and 13 with overlapping (SP) subphenotype. Electrolytes, fasting glucose, lipid profile, renal and liver functions were certain. Cholesterol erythrocyte membrane was assessed by enzymatic method. Plasma and erythrocytes phospholipids were evaluated according to Bartllet (1956), after having been isolated by thin layer chromatography. Oxidized LDL was determined by ELISA, and apolipoprotein E (Apo E) alleles were detected by PCR / RFLP. Time hemolysis test was evaluated as glycerol permeability and elasticity of the erythrocyte and fragility osmotic also were investigated. The VO group patients had lower levels of membrane cholesterol and lysophosphatidylcholine and lower levels of total plasma phospholipids. Only individuals with HA were associated with decreases in concentrations of phosphatidylethanolamine in erythrocytes. SCA patients showed a greater time of hemolysis (OL>HA>VO) and correlated positively with the levels of membrane cholesterol. Patients with SCA had lower elasticity when compared to controls, but no significant differences were observed between subphenotypes. SCA patients and carriers of the Apo E ε4 allele showed a greater reduction in plasma cholesterol levels associated with an increase in their levels in the erythrocyte membrane. ε4 allele was also associated with lower plasma levels of apolipoproteins A-I and B, an increase of oxidized LDL. Lysophosphatidylcholine, sphingomyelin and phosphatidylserine had their high concentrations in patients with SCA in the presence of the ε4 allele. Phosphatidylserine was negatively correlated with plasma levels of LDH in patients with the ε4 allele, demonstrating greater involvement of phospholipid in influencing physicochemical properties of this enzyme in the presence of the ε4 allele. The results indicate that in this population, the expression of subphenotypes SCA is intrinsically related to divergent alterations in lipid metabolism, and be influenced by the polymorphism of apolipoprotein E. This is the first study to demonstrate and investigate the subphenotype OL, as well as changes in lipid metabolism in SCA in patients with subphenotypes AH or VO, contributing to the detailed characterization of the pathophysiology of this disease hematological and the metabolic changes that accompany them. The study allowed us to walk towards deeper the knowledge of lipid metabolism in sickle cell anemia deciphering biochemical data hitherto little explored, which may account for specific laboratory abnormalities with clear clinical implications in affected individuals.

Keywords: Sickle Cell Anaemia, Subphenotype Vaso-occlusive, Subphenotype Hemolytic, Apolipoprotein E, Cholesterol, Phospholipids, Lactate Dehydrogenase.

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LISTA DE ABREVIATURAS

AF	Anemia falciforme
ANVISA	Agência de Vigilância Sanitária
AH	Subfenótipo com predomínio hemolítico da Anemia Falciforme
Apo A-I	Apolipoproteína A-I (Apolipoprotein A-I)
Apo B	Apolipoproteína B (Apolipoprotein B)
Apo E	Apolipoproteína E (Apolipoprotein E)
ATP	Adenosina trifosfato (Adenosine triphosphate)
AVEi	Acidente vascular encefálico isquêmico
cGMP	Guanina monofosfato cíclico (Cyclic guanine monophosphate)
Ca^{++}	Cálcio
CHCM	Hemoglobina corpuscular média (Mean corpuscular hemoglobina)
Cl^{-}	Cloro
CVO	Crise vaso-oclusiva
Desoxi-Hb	Hemoglobina desoxigenada (Deoxygenated hemoglobin)
DF	Doença falciforme
DHL	Desidrogenase láctica (Lactate dehydrogenase)
DTC	Doppler transcraniano
EUA	Estados Unidos da América
FVO	Subfenótipo com predomínio vaso-oclusivo
Glu	Ácido glutâmico
GTP	Guanosina trifosfato (Guanosine triphosphate)
H^{+}	Hidrogênio
Hb	Hemoglobina (Hemoglobin)
HbA	Hemoglobina A
HbAS	Traço falciforme
HbC	Hemoglogina C (hemoglobina variante)
HbF	Hemoglobina fetal
HbG	Hemoglobina G (hemoglobina variante)
HbS	Hemoglobina S
HbSC	Hemoglobinopatia SC
HbSS	Hemoglobinopatia SS (anemia falciforme)

HP	Hipertensão pulmonar
HU	Hidroxiuréia
IMC	Índice de massa corporal
K^+	Potássio
LCAT	Lecitina colesterol aciltransferase (Lecithin cholesterol acyltransferase)
LDL-c	Lipoproteína de baixa densidade – colesterol (Low-density lipoprotein – cholesterol)
HDL-c	Lipoproteína de alta densidade – colesterol (High-density lipoprotein – cholesterol)
Mg^{++}	Magnésio
Na^+	Sódio
NACF	Necrose avascular de cabeça de fêmur
NO	Óxido nítrico (Nitric oxide)
NOS	Óxido nitric sintetase (Nitric oxide synthase)
O_2	Oxigênio
oxLDL	Lipoproteína de baixa densidade oxidada
OL	Subfenótipo de superposição da anemia falciforme (Overlapping)
PA	Ácido fosfatídico (Phosphatidic acid)
PC	Fosfatidilcolina (Phosphatidylcholine)
PE	Fosfatidiletanolamina (Phosphatidylethanolamine)
PI	Fosfatidilinositol (Phosphatidylinositol)
OS	Fosfatidilserina (Phosphatidylserine)
PNTN	Programa Nacional de Triagem Neonatal.
ROS	Espécie reativa de oxigênio (Reactive oxygen species)
SHH	Síndrome Hemolítica Hereditária
SM	Esfingomielina (Sphingomyelin)
SP	Sufenótipo de superposição da anemia falciforme
STA	Síndrome torácica aguda
Steady-state	Período estacionário (fora de crise)
TG	Triglicerídeos
VAL	Valina
VLDL-c	Lipoproteína de muito baixa densidade (Very low density lipoprotein)

SUMÁRIO

I. INTRODUÇÃO

Hemoglobinopatia é o termo designado para as doenças hereditárias relacionadas às modificações da hemoglobina (Hb). A doença falciforme (DF) é representada pelo conjunto de patologias associadas à presença da hemoglobina S (HbS), hemoglobina variante de maior frequência. A DF está dispersa na população brasileira de forma heterogênea, com prevalência mais alta nos estados com maior concentração de afro descendentes. Em Pernambuco afeta 1:1.400 enquanto que o traço falciforme (HbAS) acomete 1:23 nascidos vivos (Bandeira *et al*, 2007; Loureiro & Rozenfeld, 2005; Ministério da Saúde, 2006). A hemoglobinopatia em homozigose para o gene da hemoglobina S (HbS), resultante de mutação no gene da β-globina, é conhecida com anemia falciforme (AF) (Ingraham, 1957), a qual representa uma condição grave da doença, e, é caracterizada por múltiplas manifestações clínicas. Os sintomas podem variar de forma individual, ainda que a doença seja oriunda do mesmo acometimento genético (Alexander*et al*, 2004; Martins *et al*, 2010; Steinberg, 2005).

O processo primário da fisiopatologia da AF é a polimeração da desoxi-hemoglobina (desoxi-Hb), ou seja, sob a redução do teor de oxigênio, vários tetrâmeros de HbS se unem e geram longos polímeros os quais modificam as propriedades físico-químicas dos eritrócitos, tais como alterações na fluidez e conformação da membrana celular, culminando em um evento conhecido como *falcização*, no qual a morfologia do eritrócito se apresenta em formato de *foice*. A cinética da polimerização é crítica para determinar o nível de gravidade clínica (Ikuta *et al,* 2011; Martins *et al*, 2010; Strypulkowski & Manfredini, 2010).

A taxa de difusão transversal dos lipídios é responsável pela deformabilidade da membrana, desta forma a reversão da deformação do eritrócito após passar nos pequenos vasos (área de menor concentração de oxigênio) é devido a movimentação lipídica de um lado a outro da membrana (Ataga & Key, 2007; Franck *et al*, 1985; Strypulkowski & Manfredini, 2009). As hemoglobinopatias têm efeito relevante na composição da bicamada lipídica da membrana eritrocitária, uma vez que a exposição da fosfatidilserina (PS - phosphatidilserine) na monocamada não citosólica contribui para efeitos protrombóticos e favorece aderência endotelial, permitindo o reconhecimento e a remoção das hemácias pelos macrófagos, caracterizando a hemólise (Kuypers, 2007). As vias bioquímicas que levam à exposição de PS são apenas, parcialmente, compreendidas, entretanto vários relatos indicam evidências de oxidação lipídica nas hemácias em foice, sugerindo que o reparo pelos fosfolipídios, apesar de

relevante, não é suficiente para manter a composição de espécies moleculares fisiológicas nessas células (Ataga & Key, 2007; Franck*et al*, 1985; Hannemann et al, 2011; Kuypers, 2007).

Alterações laboratoriais são evidenciadas na AF e abrangem o perfil lipídico. Hipocolesterolemia com baixos níveis de lipoproteína de baixa densidade (LDL), além de lipoproteína de alta densidade (HDL) têm sido descritos como potentes biomarcadores da gravidade clínica na AF (Daga *et al,* 2009; Naoum, 2005; Sasaki *et al,* 1983; Seixas *et al*, 2010; Zorca *et al,* 2010). Níveis séricos reduzidos de apolipoproteínas também têm sido encontrados em pacientes com AF quando em comparação à população em geral (Morris, 2008). Outras alterações observadas na AF estão relacionadas ao tempo de hemólise e elasticidade da membrana (Barabino, 2010; Uydu, 2012), assim como correlações das alterações lipídicas associadas às apolipoproteínas (Adams, 2003; Yuditskaya, 2009).

A DF é consequência de vários eventos e suscetibilidade genética que vão além da substituição de um único aminoácido da cadeia β da hemoglobina (Hb) (Morris, 2008). Embora tenha a mesma causa genética, os portadores de AF apresentam perfis clínicos distintos, como se houvesse dois tipos de "subfenótipos", um com características de vaso-oclusão e viscosidade e outro com predomínio hemolítico e disfunção endotelial (Alexander *et al*, 2004; Kato *et al*, 2007; Rees *et al*, 2010). Deste modo, muitos pacientes morrem por complicações dessa doença, devido a sua variabilidade clínica. Novos estudos são necessários (Bakanay *et al,* 2004) e o presente estudo, ao investigar alterações em plasma e eritrócitos de portadores dos diferentes subfenótipos da AF, poderá contribuir para elucidação de mecanismos de ação moduladores da gravidade clínica permitindo a instituição de novas abordagens terapêuticas individualizadas, evitando numerosas e desnecessárias intervenções, podendo, inclusive, melhorar a expectativa e /ou qualidade de vida desses pacientes.

II. SUBFENÓTIPOS DA ANEMIA FALCIFORME E ALTERAÇÕES LIPÍDICAS EM PLASMA E ERITRÓCITOS

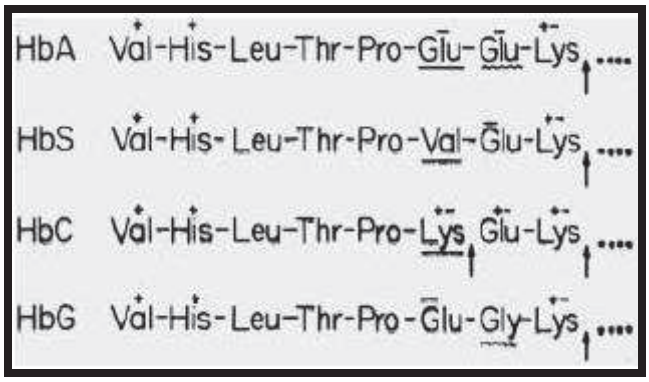
Em 1910, James Herrick reportou um caso clínico de anemia grave, em que pela primeira vez foi identificada a presença de glóbulos vermelhos alongados e em forma de foice. Alguns anos depois, Pauling *et al* (1949) verificaram, por eletroforese, que a Hb de pacientes com AF migrava mais lenta do que a do adulto normal e essa diferença era consequência de alterações moleculares. Essa descoberta tão relevante fez com que Linus Pauling recebesse o Prêmio Nobel de Química, em 1954. O estudo motiva pesquisadores em todo o mundo a caracterizar a modificação molecular acarretando na constatação da substituição do resíduo número 6 da cadeia β-globina, de ácido glutâmico por valina, verificada por Vernon Ingram (1957) pela técnica de "fingerprinting", análise simultânea de eletroforese e cromatografia (Herrick, 1910; Ingram, 1957; Pauling *et al*, 1949). Novos estudos vêm sendo realizados, favorecendo a melhor elucidação dos mecanismos envolvidos com as hemoglobinopatias. Alterações no metabolismo de lipídios e lipoproteínas, assim como modificações de fosfolipídios do plasma e de eritrócitos, em portadores de AF, vêm sendo estudados. No entanto, ainda não foram esclarecidos todos os mecanismos, como por exemplo, as características clínicas que podem ser tão distintas, apesar da AF ser uma doença genética, bem como se o polimorfismo de outros genes interferem no perfil clínico e laboratorial desses indivíduos. (Daga *et al,* 2009; Fredenrich & Bayer, 2003; Hannemann *et al*, 2011; Kuypers, 2008; Naoum, 2005; Sasaki *et al,* 1983; Seixas *et al*, 2010; Zorca *et al,* 2010).

II. 1. Anemia Falciforme

Hemoglobinopatias constituem um grupo de doenças genéticas, que se caracterizam por defeitos tanto estruturais, representadas pelas síndromes falciformes, quanto por alterações na síntese das cadeias da Hb, evidenciadas nas talassemias (Perutz & Mitchinson, 1950). Defeitos estruturais resultam na formação de moléculas de Hb com características bioquímicas alteradas em comparação com Hb normais (HbA, Hemoglobina A) e, por isso, são denominadas de Hb variantes (Powars, 1991; Steinberg, 2005). A Hb variante de maior frequência mundial é a HbS, causada pela substituição de adenina por timina (G<u>A</u>G > G<u>T</u>G) no códon 6 do gene β da globina, resultando na codificação de valina em vez de ácido

glutâmico (Figura 1). A DF agrupa um conjunto de fenótipos nos quais predomina a presença do gene da HbS (Hannemann et al, 2011; Martins *et al*, 2010).

Figura 1 – Codificação dos aminoácidos na Anemia Falciforme. No códon 6 da β-globina há substituição do nucleotídeo adenina por timina resultando na formação de valina (Val) em vez de ácido glutâmico (Glu), o que promove o surgimento da hemoglobina S (HbS) em detrimento da hemoglobina A (HbA). Outras hemoglobinas variantes (HbC e HbG) também são decorrentes de alterações na cadeia β-globina.



Fonte: Ingram, 1957.

Aproximadamente 7% da população mundial são acometidas por hemoglobinopatias (Loureiro & Rozenfeld, 2005). Em populações de origem africana, a AF é responsável por 70% das DF, sendo seguida pela hemoglobinopatia SC (HbSC) (Rees *et al*, 2010). No Brasil nascem cerca de 3.500 crianças por ano com a DF (1:1000) e 200.000 com traço falciforme (1:35) (Ministério da Saúde, 2006). Em Pernambuco, a DF afeta 1:1.400 enquanto que o traço falciforme acomete 1:23 nascidos vivos (Ministério da Saúde, 2006). A população negra é a que apresenta maior prevalência, cerca de 0,1% a 0,3%, e com tendência a atingir uma parcela cada vez maior de indivíduos, devido ao alto grau de miscigenação neste país. A DF está dispersa na população brasileira de forma heterogênea, com prevalência mais alta nos estados com maior concentração de afro descendentes. Todavia, estudos populacionais têm demonstrado a crescente presença de HbS também em indivíduos caucasóides (ANVISA, 2002; Bandeira *et al*, 2007; Loureiro & Rozenfeld, 2005).

Indivíduos com DF têm menor expectativa de vida, apresentando média de sobrevivência de 42 anos para homens e de 48 anos para mulheres (Araújo, 2010; Quinn *et al*, 2004). Estudo epidemiológico realizado nos EUA relatou estimativa de vida de 39 anos, com apenas 35% dos indivíduos portadores de DF ultrapassando 45 anos, enquanto que na Jamaica

50% sobrevivem mais de 55 anos (Hassell, 2010). No Brasil, 25% das crianças não alcançam 5 anos de vida se não tiverem acompanhamento médico adequado (Ministério da Saúde, 2006). A alta taxa de mortalidade infantil por causa da DF contribui para a redução do tempo de vida, apesar desse índice estar diminuindo e da sobrevidade com maior idade estar aumentando. Há menor proporção de mortes causadas por infecção (Quinn *et al*, 2004). Mesmo assim, as complicações estão presentes na maioria dos casos da AF, 82% dos pacientes são portadores da forma homozigótica e dois terços apresentaram crises dolorosas e necessitaram de tratamento hospitalar (Araújo, 2010; Martins *et al* 2010).

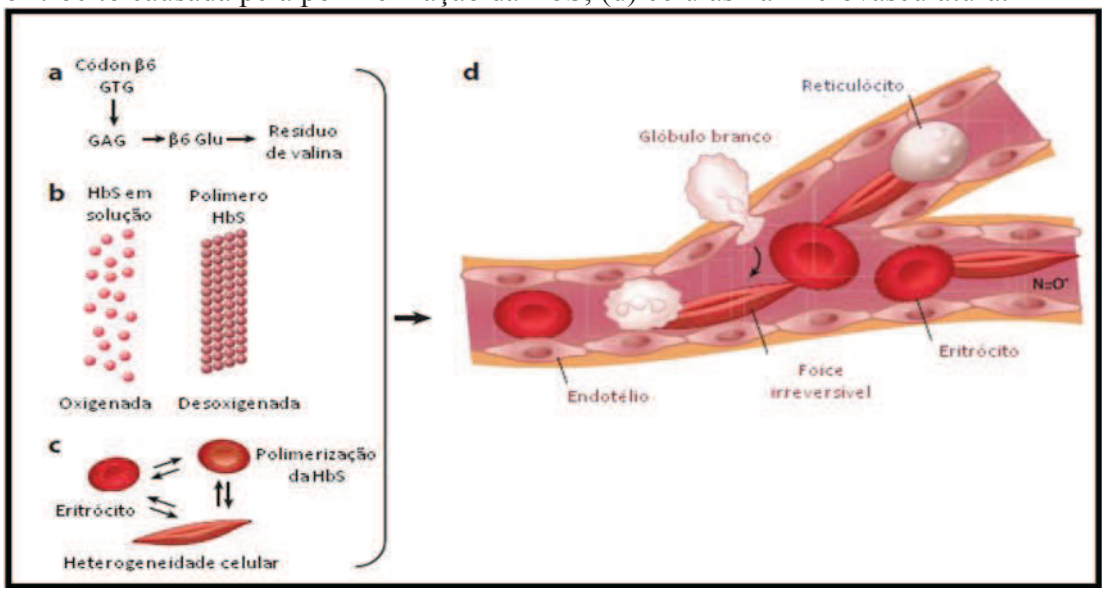
Cerca de 70% dos internamentos ocorrem em indivíduos abaixo dos 20 anos de idade. O motivo da admissão mais frequente é de caráter emergencial (90,8%) e a maior letalidade hospitalar acomete os adultos, principalmente HbSS, sendo a mediana do óbito entre 26,5 e 31,5 anos de idade (Loreiro & Rozenfeld, 2005). Associado a esses dados, também foi mostrado que o reconhecimento tardio de tais doenças pode levar à morte nos primeiros anos de vida. Outros estudos também relatam que tanto o maior número de internamentos quanto de alta mortalidade se dá em presença do genótipo SS (Hassell, 2010; Martins *et al*, 2010). A taxa de mortalidade entre os pacientes com DF que fazem uso da hidroxiuréia (HU) é reduzida em 40% quando comparada com aqueles que não fazem uso. O uso da HU foi a primeira intervenção terapêutica eficaz comprovada para melhorar ou prevenir complicações da AF (Bakanay *et al*, 2005). A elevada letalidade, que abrange especialmente jovens, reflete a gravidade da doença (Bandeira *et al*, 2007; Loureiro & Rozenfeld, 2005) tratando-se então de um problema de saúde pública (Ministério da Saúde, 2006; Paiva e Silva *et al*, 1993). Na tentativa de identificar precocemente novos casos, de poder proporcionar adequado tratamento de saúde e de minimizar complicações assim como reduzir a taxa de mortalidade desses indivíduos, as hemoglobinopatias foram inclusas no Programa Nacional de Triagem Neonatal (PNTN), o que permitiu a identificação precoce da AF e consequente abordagem terapêutica (Ministério da Saúde, 2006).

II. 1.1. Fisiopatologia e Manifestações Clínicas

A alteração das hemácias, tornando-os semelhantes a uma foice, deve-se à presença da HbS. O processo primário da falcização é a deficiência no transporte de oxigênio. Com a redução dos níveis de oxigênio, as globinas βS ficam mais próximas, favorecendo o contato entre as regiões da desoxi-Hb (hemoglobina desoxigenada) o que gera modificações das propriedades físico-químicas dos eritrócitos (Ikuta *et al,* 2011; Martins *et al*, 2010;

Strypulkowski & Manfredini, 2010). Essa polimerização determina o nível da gravidade clínica. Quando os eritrócitos falcizados ocluem a microvasculatura, incluindo capilares e pequenos vasos, inviabiliza a oxigenação tecidual gerando condições de hipóxia, papel central da fisiopatologia das DF (Ikuta *et al,* 2011). Este fenômeno é reversível com a oxigenação, desde que a membrana da célula não esteja definitivamente alterada (Hannemann *et al*, 2011; Martins *et al,* 2010) (Figura 2). No processo de falcização, as células irreversivelmente falcizadas representam entre 4 e 44% do total de eritrócitos de indivíduos homozigóticos. Eritrócitos irreversivelmente falcizados formam-se logo após sua liberação pela medula óssea e são rapidamente retirados da circulação, 1/3 por hemólise intravascular e 2/3 por fagocitose (Manfredini, 2008).

Figura 2 – Processo de Falcização. (a) mutação pontual da Anemia Falciforme; (b) polimerização da HbS quando há desoxigenação; (c) mudança da morfologia do eritrócito causada pela polimerização da HbS; (d) células na microvasculatura.

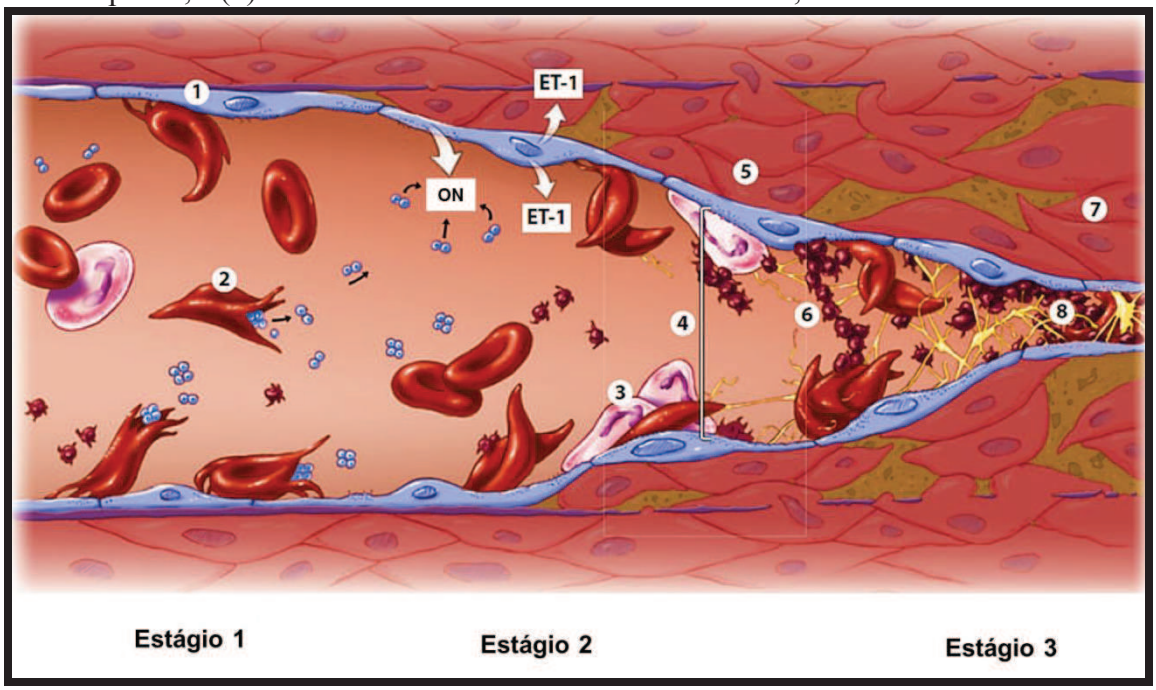


Fonte: Adaptado de Barabino, Platt & Kaul, 2010.

A obstrução vascular e a isquemia tecidual ocorrem pelo aprisionamento de hemácias e leucócitos na microcirculação, causando a dor aguda vaso-oclusiva (Figura 3). Embora esse processo requeira a polimerização da HbS, o evento é iniciado, na grande maioria das vezes, por processos inflamatórios. Juntamente com a inflamação, obstrução pré-capilar por rigidez, deformação do eritrócito com altos conteúdos de HbS também contribuem para a vaso-oclusão da microvasculatura. Esse evento é o resultado da interação dinâmica entre eritrócitos e endotélio vascular, resultando no episódio de oclusão microvascular e isquemia, seguida por restauração do fluxo sanguíneo, reperfusão, que promoverá ainda mais lesão tecidual. Esses ciclos de isquemia e reperfusão causam estresse oxidativo, com ativação de agentes oxidantes,

e estresse inflamatório aumentando a expressão de moléculas de adesão celular endotelial e a síntese de citocinas inflamatórias, podendo causar leucocitose. Infarto de medula óssea ocasionando embolização gordurosa também poderá contribuir para a oclusão vascular, em especial nos pulmões, desencadeando a STA (síndrome torácica aguda) (Ikuta *et al,* 2011; Rees *et al*, 2010).

Figura 3 – Desenvolvimento do fenômeno vaso-oclusivo em artérias. (1) Aderência do eritrócito falciforme ao endotélio; (2) Hemólise; (3) Adesão de leucócitos; (4) Aumento do tônus vascular; (5) Proliferação neo-íntima; (6) Agregação plaquetária; (7) Vasculopatias; (8) vaso-oclusão. ON – Óxido nítrico; ET-1 – Endotelina-1.



Fonte: Adaptado de Barabino *et al*, 2010.

O segundo processo fisiopatológico da DF é a anemia hemolítica, que também é impulsionada pela polimerização da HbS. Há muito tempo já se sabia que a hemólise pode levar à anemia, fadiga e colelitíase, mas somente agora é sabido que pode contribuir para o desenvolvimento de vasculopatias progressivas (Rees *et al,* 2010; Sebastiani *et al*, 2007).

Quando ocorre a hemólise intravascular, a Hb é liberada no plasma, onde interagem com as hemoglobinas coveiras: haptoglobina, CD163 e hemopexina. Dímeros da Hb liberada são capturados pela haptoglobina a fim de serem ligados ao CD163, na superfície dos macrófagos, para sofrerem endocitose e degradação. Enquanto outros dímeros sofrerão oxidação do heme férrico sendo ligado à hemopexina que irá conduzir até a superfície dos hepatócitos, local de degradação. Em vigência de hemólise excessiva, os sistemas de remoção de Hb não são suficientes para manter a homeostase, favorecendo o acúmulo de Hb e do heme

férrico plasmáticos. Esse acúmulo contribui para disfunção endotelial, visto que estimulam a proliferação e ações pró-inflamatórias e pró-oxidantes, bem como proliferação (Figura 4) (Rother *et al*, 2005).

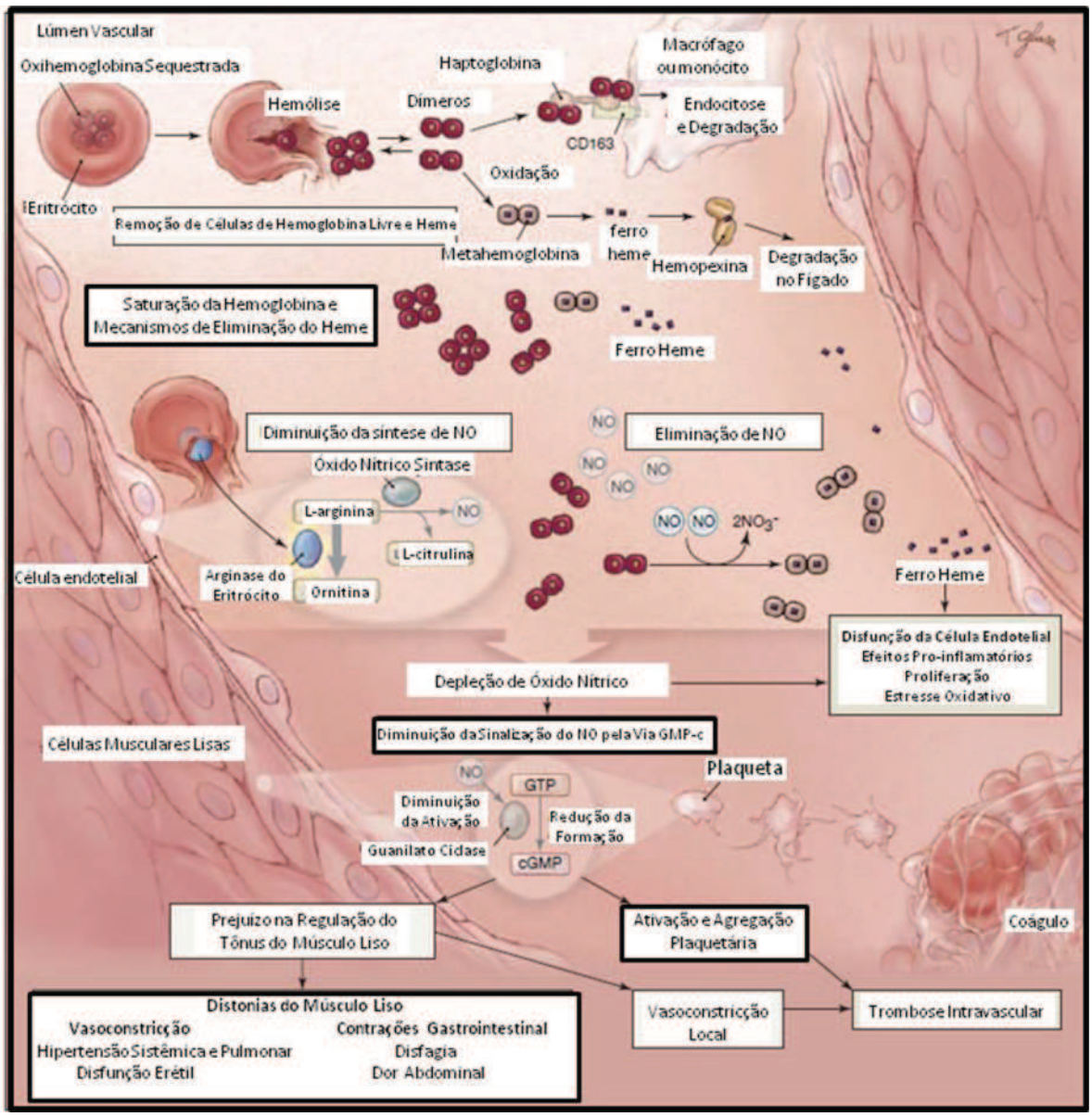
Outra descrição da fisiopatologia, associada às manifestações clínicas mais críticas, aborda o óxido nítrico (NO – nitric oxide) que é produzido nas células endoteliais a partir da L-arginina pela ação da enzima óxido nítrico sintetase (NOS – nitric oxide synthase). O NO mantém o relaxamento da musculatura lisa e inibe a agregação plaquetária, regulando o tônus vascular. Durante a hemólise intravascular, a disponibilidade de NO é limitada por causa da sua reação com o NO eliminado da oxihemoglobina e pela redução da L-arginase, mesmo diante das altas concentrações de NOS. A diminuição do NO reduz a ativação da enzima guanilato ciclase, responsável por gerar a guanina monofosfato cíclico (cGMP). Baixas concentrações de cGMP alteram o tônus vascular acarretando distonias, incluindo hipertensão arterial sistêmica, hipertensão pulmonar (HP), disfunção erétil, disfagia e dor abdominal. Além disso, também estimula a ativação e agregação plaquetária, promovendo a formação de coágulos (Rother *et al*, 2005).

Pacientes adultos com DF são fatores de risco para o desenvolvimento de vasculopatias. Estudos epidemiológicos sugerem que as complicações severas estão associadas com o aumento da taxa de hemólise e consequente redução da disponibilidade de NO. Muitas das manifestações clínicas já mencionadas, tais como hipertensão sistêmica e pulmonar, disfunção endotelial e mudanças na proliferação da íntima e da musculatura lisa dos vasos sanguíneos, além de colelitíase, úlceras cutâneas da perna e priapismo estão associados com baixas concentrações de Hb em *"steady-state"* e aumentam a taxa de hemólise intravascular (Rees *et al,* 2010; Sebastiani *et al*, 2007). Apesar de na DF 2/3 da hemólise ser extravascular, o 1/3 restante é capaz de liberar 10g por dia de Hb no plasma, o que acentua a gravidade hemolítica (Kato *et al,* 2006), favorecendo o surgimento de comorbidades como a hipertensão pulmonar, priapismo e úlceras em membros inferiores (Kato *et al*, 2006; Sebastini *et al*, 2007; Seixas *et al*, 2010).

Esses processos fisiopatológicos contextualizam os dois subfenótipos da hemoglobinopatia SS, um relacionado à hemólise, disfunção endotelial e vasculopatia proliferativa e outro relacionado à viscosidade, vaso-oclusão e falcização. De acordo com o predomínio do subfenótipo que o indivíduo apresente, as condições clínicas serão distintas (Figura 5) (Ikuta *et al,* 2011; Kato *et al*, 2007; Rees *et al*, 2010). A AF apresenta um complexo quadro de manifestações clínicas que variam desde um perfil quase assintomático até situações clínicas de grande gravidade (Ballas *et al*, 2010). A crise falcêmica pode ser

espontânea ou desencadeada por situações como o frio, alterações emocionais, esforço físico ou infecções. Há uma variação individual quanto à frequência e a duração das crises. Por tratar-se de uma patologia multiorgânica, várias podem ser as complicações (Tabela 1) (Stanley & Christian, 2013).

Figura 4 – Efeitos da Hemoglobina Livre Plasmática e Diminuição do Óxido Nítrico Durante a Hemólise Intravascular.



Fonte: Adaptado de Rother *et al*, 2005.

As crises vaso-oclusivas (CVO) podem ocorrer em diversos locais, entretanto são os leitos vasculares dos músculos profundos, do periósteo e da medula óssea os mais frequentemente afetados. É a complicação mais comum da Hemoglobinopatia SS (HbSS) e tem início ainda na infância. As durações das CVO bem como o surgimento e/ou a sua

recorrência apresentam variações de acordo com cada indivíduo. A maioria dos doentes apresenta valores laboratoriais distintos dos valores obtidos durante o "*steady-state*" (Ballas *et al*, 2010).

Figura 5 – Subfenótipos da Anemia Falciforme.

Fonte: Adaptado de Kato *et al*, 2007.

A CVO pode ocorrer na região torácica, promovendo o surgimento da síndrome torácica aguda (STA) que é a complicação mais frequente na AF, responsável por inúmeros internamentos e baixa sobrevida, visto que pode ter rápida progressão e evoluir para insuficiência respiratória aguda. A STA é uma lesão pulmonar aguda específica da HbSS e se caracteriza pelo surgimento de infiltrado pulmonar associado com hipóxia, tosse, febre, dispnéia e dor torácica. Recidivas da STA são comuns e podem acarretar em doença pulmonar crônica. Outras pneumopatias não relacionadas diretamente com a oclusão vascular, como por exemplo a pneumonia e asma, são capazes de piorar a oxigenação local ou sistêmica, visto que facilitam a polimerização da HbS (Ballas *et al*, 2010; Lamarre *et al*, 2012; Stanley & Christian, 2013).

A vaso-oclusão consequente da falcização dos eritrócitos na microcirculação óssea pode gerar infarto, trombose e necrose. A vulnerabilidade da medula óssea para essa oclusão ainda é incerta, entretanto pode justificar-se, parcialmente, pela hipercelularidade medular, que promove diminuição do fluxo sanguíneo e consequente hipóxia local. Osteonecrose na AF acomete principalmente a cabeça do fêmur e, muitas vezes, é bilateral. A necrose avascular de cabeça de fêmur (NACF) acomete cerca de 5% dos pacientes com HbSS,

geralmente adultos, e pode ser responsável por distúrbios da marcha. Dor artrítica, edema e derrame compõem essa condição clínica. Síndrome "mão-pé", também conhecida como dactilite, caracteriza-se por quadro doloroso nas superfícies dorsais de mãos e/ou pés, presença de edema, podem estar acompanhado por aumento de temperatura local e eritema. É causada por extensas áreas de infarto medular e, normalmente, tem duração de uma a duas semanas podendo ter recidivas, bastante frequente nos primeiros anos de vida (da Silva Junior *et al*, 2012). Estudos sugerem um fenótipo vaso-oclusivo composto por pacientes com níveis de Hb relativamente maiores, quando comparado ao período álgico, STA e NACF (Alexander *et al*, 2004; Ballas *et al*, 2010; Kato *et al*, 2007; Rees *et al*, 2010; Sebastiani *et al*, 2007).

Muitas vezes a colelitíase é confundida com infarto hepático por apresentar a mesma sintomatologia: dor no quadrante superior direito, febre, leucocitose, transaminases séricas e bilirrubinas elevadas. O tipo de lesão hepática na AF normalmente é colestática ou mista, ou seja, em associação com a vaso-oclusão hepática. A colestase, redução do fluxo biliar por diminuição ou interrupção, favorece o desenvolvimento de cálculos biliares pigmentados como consequência da hemólise crônica. Esses cálculos ocorrem em cerca de 50%-70% dos pacientes adultos (Ballas *et al*, 2010; Ebert *et al*, 2010).

A complicação mais devastadora da AF é o acidente vascular encefálico isquêmico (AVEi), que apresenta maior incidência na infância, tendo grande ocorrência por volta dos 6-9 anos, seguido de período de baixo risco, e um o segundo momento de risco de acometimento é após os 50 anos. Existem 50% a 70% de possibilidade de recorrência no prazo de 3 anos após o primeiro AVEi e o tratamento geralmente envolve transfusão de sangue a longo prazo. O AVEi ocorre mais frequentemente em virtude dos danos arteriais causados pela fisiopatologia da HbSS, levando a um infarto ou evento hemorrágico. Hemiparesia é característica de acidente hemorrágico e outras sequelas, como déficit cognitivo, podem surgir (Adams, 2001; Stanley & Christian, 2013). O uso de doppler transcraniano (DTC) para identificar pacientes portadores de HbSS em maior risco de AVEi foi introduzido no início dos anos 1990 e desde então vem sendo usado na prevenção de novos ictus (Ballas *et.al*., 2010). Histórias de AVEi costumam estar associadas com HP em pacientes com AF, ambas apresentam semelhanças quanto a epidemiologia, fisiopatologia e histopatologia, as quais parecem envolver a biodisponibilidade do NO (Kato *et al*, 2007).

Tabela 1 – Manifestações clínicas e comorbidades da anemia falciforme.

Sistema	Manifestação Clínica
Respiratório	Síndrome torácica aguda (STA), hipertensão pulmonar (HP), enfarto pulmonar, asma, hiperreatividade de vias aéreas.
Cardíaco	Débito cardíaco elevado, hipertrofia cardíaca (cardiomegalia), insuficiência cardíaca congestiva, disfunção cardíaca autonômica, vaso-oclusão das artérias coronárias.
Sistema Nervoso Central	Acidente vascular encefálico isquêmico (AVEi), ataque isquêmico transitório, enfarte cerebral silencioso.
Geniturinário	Insuficiência renal, pielonefrite, hematúria, acidose tubular renal, priapismo, enurese noturna.
Musculoesquelético	Dactilia, osteomielite, enfarto ósseo, osteopenia, osteoporose, necrose avascular.
Hematológico	Anemia hemolítica, anemia aplástica aguda.
Vascular	Úlceras de membros inferiores.
Imunológico	Predisposição à infecção devido à asplenia funcional e respostas deficientes dos neutrófilos, eritrócitos auto / aloimunização, reações transfusionais hemolíticas.
Hepatobiliar	Colecistite aguda / crônica, cálculos biliares, alterações hepáticas crônicas, sinusóides hepáticos distendidos, hepatomegalia, sequestro hepático.
Olhos	Retinopatia falciforme, glaucoma.
Baço	Esplenomegalia, sequestro esplênico, infarto esplênico levando à "autoesplenectomia".
Outros	Distúrbios do crescimento e desenvolvimento [baixo peso, baixo índice de massa corporal (IMC)], hemosiderose, síndromes álgicas.

Fonte: Adaptado de Ballas *et al* (2010) e Stanley & Christian (2013).

A hipertensão pulmonar corresponde a uma doença pulmonar crônica identificada pelo aumento da pressão da artéria pulmonar para valores acima de 25mmHg em repouso e atinge 20%-30% dos portadores de HbSS. Esses indivíduos apresentam taxa de mortalidade aumentada em comparação aos pacientes que não cursam com HP (Ballas *et al*, 2010; Machado & Gladwin, 2010). A trombose pulmonar é a complicação mais comum da HP e

pacientes com priapismo, que consiste em uma ereção peniana dolorosa e involuntária, de duração variável (Claudino & Fertrin, 2012; Kato, 2012; Stanley & Christian, 2013), têm cinco vezes maior risco em de evoluir com HP (Kato *et al*, 2007).

O priapismo pode apresentar início agudo, recorrente ou crônico. Acomete 45% dos pacientes com HbSS e pode levar à disfunção erétil em 30% dos casos (Claudino & Fertrin, 2012). Episódios de priapismo devem ser considerados emergência visto que um episódio com duração maior de 24 horas pode acarretar danos permanentes ao sistema vascular erétil, inclusive gerando a impotência sexual (Stanley & Christian, 2013). Fatores como baixos valores de Hb, altas concentrações da DHL, bilirrubinas, AST, reticulócitos, leucócitos e plaquetas, assim como HP e úlceras de perna, são fatores de riscos para a ocorrência do priapismo (Kato, 2012).

Úlceras de perna são relativamente comuns e podem ser incapacitantes. Aparecem espontaneamente ou em consequência de traumatismo. Apresentam uma pequena prevalência antes dos 10 anos de idade. As úlceras ocorrem em regiões com pouca gordura subcutânea, pele fina e fluxo sanguíneo reduzido, geralmente surgem próximas ao maléolo medial ou lateral e podem ocorrer bilateralmente. Quando não resolvida precocemente torna-se circunferencial e pode evoluir com quadro infeccioso. As úlceras são resistentes à cicatrização e sofrem recidivas e o tratamento é prolongado (Minniti *et al*, 2010).

Subfenótipo com predomínio hemolítico inclui manifestações clínicas como priapismo, AVEi, HP, colelitíase e úlceras maleolares. Embora todos os pacientes com AF apresentem o mesmo defeito molecular, existe considerável variabilidade fenotípica, havendo casos de extrema gravidade, com morte precoce na infância e casos com poucas complicações e perspectiva de vida próxima ao normal (Ballas *et al*, 2010; Kato *et al*, 2007; Rees *et al*, 2010; Sebastiani *et al*, 2007)

Vários marcadores estão associados com o prognóstico clínico da AF como, por exemplo, concentração de hemoglobina fetal (HbF), contagem de leucócitos e de reticulócitos e desidrogenase láctica (DHL) (Seixas *et al*, 2010). Células com concentração de Hb corpuscular média elevada, ou células densas, apresentam baixa afinidade pelo O_2, pouca deformabilidade, alta viscosidade, alta propensão à formação de polímeros de HbS na desoxigenação e menor sobreviva. Indivíduos com maior número de células densas têm maior grau de hemólise e de anemia, mas não necessariamente maior número de crise vaso-oclusiva (CVO) (Figueiredo, 2007). A DHL é considerada como marcador laboratorial de hemólise intravascular, visto que em casos de hemólise extravascular seus níveis séricos estão apenas

ligeiramente aumentados, diferente da hemólise intravascular quando estes níveis estão substancialmente elevados (Kato *et al,* 2006).

A AF vem sendo contextualizada em dois subfenótipos: (1) um que envolve hemólise, disfunção endotelial e vasculopatia proliferativa apresentando manifestações clínicas tais como HP, priapismo, colelitíase, AVEi e úlcera de perna, enquanto (2) o outro envolve viscosidade-vaso-oclusão e falcização, clinicamente sendo identificado com a presença de CVO, STA e osteonecrose (NACF). Alguns pacientes não conseguem ser classificados dentro desses subfenótipos, visto que apresentam características de ambos (Ikuta *et al,* 2011; Kato *et al*, 2007; Rees *et al*, 2010).

I. 1.2. Alterações do Metabolismo Lipídico

O metabolismo lipídico encontra-se alterado na AF (Rahimi *et al*, 2006). Tanto na AF quanto na talassemia maior, a disfunção hepática, que habitualmente está presente nessas situações, reduz a produção endógena de colesterol e promove alterações importantes nos seus perfis lipídicos (Daga *et al,* 2009; Naoum, 2005; Sasaki *et al,* 1983; Seixas *et al*, 2010; Zorca *et al,* 2010). O estresse oxidativo crônico gerado pelo estado hemolítico e sobrecarga de ferro, a exemplo do que decorre da terapia transfusional, torna a LDL mais susceptível à oxidação. A LDL oxidada (oxLDL) é removida do plasma com maior velocidade pelo sistema retículo-endotelial, contribuindo para a ocorrência de hipocolesterolemia. AF cursa com hiperplasia medular principalmente à custa da hiperproliferação dos precursores eritróides na medula óssea. O estado hiperproliferativo nessas doenças possivelmente causa redução do colesterol plasmático para atender à maior demanda deste elemento para síntese de novas membranas (Naoum, 2005).

A Lecitina Colesterol Aciltransferase (LCAT), enzima que esterifica o colesterol, é uma glicoproteína sintetizada pelo fígado e exerce papel crucial no metabolismo das lipoproteínas plasmáticas, especialmente na síntese e maturação das HDL circulantes (Jonas, 2000). A etapa inicial da reação envolve a transferência de colesterol das membranas celulares dos tecidos periféricos para pequenas subfrações de HDL-c, onde o colesterol é esterificado e armazenado no interior das lipoproteínas de alta densidade e levados ao fígado para serem degradados em ácidos biliares e excretados do organismo (Wang & Briggs, 2004; Vance & Den Bosch, 2000). O excesso desse colesterol esterificado gerado é transferido preferencialmente para as VLDL e LDL que seguem para o fígado onde são endocitadas mediante interação com receptores celulares (Guérin *et al*, 1994; Tall, 1998). Lipoproteínas de

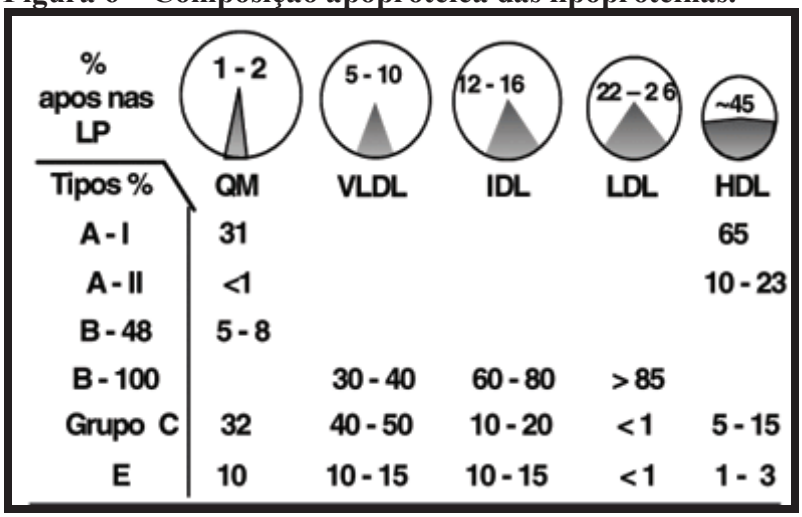
muito baixa densidade (VLDL-c) ricas em triglicerídeos (TG) podem representar um importante papel na oxidação lipídica. A hipertrigliceridemia provavelmente contribui para o aumento da produção hepática de VLDL-c, aumentando o número de receptores para LDL-c que é metabolizado rapidamente gerando redução dos seus níveis séricos (Fredenrich & Bayer, 2003; Seixas *et al*, 2010). Mutações do gene codificante da LCAT, como a Deficiência Familiar da LCAT, são responsáveis por apresentar deficiência de atividade enzimática ou por ausência total de enzima acarretando o surgimento de VLDL e LDL ricas em triglicerídeos (TG), anemia, proteinúria e doença renal (Lima *et al*, 2004).

As HDL são constituídas por 50% de apolipoproteínas (A-I em maior quantidade, A-II, C-I, C-II, C-III, E e J), 20% de colesterol livre e de colesterol esterificado, 15% de fosfolipídios e 5% de triglicerídeos (Figura 6) (Forti & Diament, 2007). Níveis séricos de apolipoproteínas em portadores com AF têm se apresentado menores quando comparado aos indivíduos hígidos (Morris, 2008). Através de mecanismos de transcrição e tradução dos alelos ε2, ε3 e ε4, seis fenótipos diferentes da apo E são produzidos, três homozigotos (E2/2, E3/3 e E4/4) e três heterozigotos (E3/2, E4/2 e E4/3) (Chan & Li, 1992). Desse modo surgem três isoformas e esta heterogeneidade resulta da substituição de um único aminoácido nas posições 112 e 158 da cadeia polipeptídica (Scott, 1993). Estudo utilizando ressonância magnética mostrou que tanto a presença do alelo ε3 quanto a presença de Apo C-III em portadores de AF podem estar associadas ao maior risco de AVEi (Hoppe *et al,* 2001). Portadores de AF com baixos níveis de Apo A-I têm prevalência elevada para o desenvolvimento de HP (Morris, 2008), bem como para desenvolverem CVO (Monnet *et al*, 1996; Tumblin *et al*, 2010). Diante desses achados torna-se claro que a DF é consequência de vários eventos e susceptibilidade genética que vão além da substituição de um único aminoácido da cadeia β da Hb (Morris, 2008).

Estudos bioquímicos e biofísicos vêm revelando um modelo detalhado da estrutura e composição das membranas biológicas. A organização dinâmica através da bicamada lipídica mostra assimetria dos lipídios e uma dimensão lateral com presença de domínios específicos. Apesar de milhares de diferentes tipos de lipídios já terem sido catalogados e as funções específicas de numerosos deles terem sido compreendidas, a definição completa da utilidade desse repertório ainda continua elusiva (van Meer *et al*, 2008). A rápida movimentação lipídica no plano da bicamada não é um movimento aleatório. Tanto a organização das moléculas de lipídios e quanto de proteínas levarão à formação de microdomínios ou "rafts" que estão envolvidos em processos fisiológicos específicos como transdução de sinal. Estes “rafts” são, em geral, enriquecidos em moléculas, tais como a esfingomielina,

glicerofosfolipídios saturados e colesterol. A diminuição do teor de colesterol da membrana afeta os "rafts", levando a alterações funcionais da membrana e consequentes alterações no citosol tais como um aumento do cálcio que pode levar à disseminação dessas vesículas enriquecidas em lipídios e proteínas específicas (Kuypers, 2007). Vale ressaltar que uma das funções mais relevantes do colesterol no metabolismo celular é o auxílio na síntese da membrana celular, que é composta por 52% de proteínas, 40% lipídios – sendo uma parte constituída por fosfolipídios e a outra parte por colesterol – e 8% de carboidratos (Naoum, 2005).

Figura 6 – Composição apoprotéica das lipoproteínas.



Tipos %	QM	VLDL	IDL	LDL	HDL
A - I	31				65
A - II	<1				10 - 23
B - 48	5 - 8				
B - 100		30 - 40	60 - 80	> 85	
Grupo C	32	40 - 50	10 - 20	< 1	5 - 15
E	10	10 - 15	10 - 15	< 1	1 - 3

Fonte: Forti & Diament, 2007.

A reversibilidade da deformação que um eritrócito sofre após passar pela microcirculação depende de um rápido fluxo de lipídios de um lado para outro da membrana eritrocitária, revelando que a deformabilidade da célula é influenciada pela taxa de difusão transversal de lipídios (Ataga & Key, 2007; Franck *et al*, 1985; Strypulkowski & Manfredini, 2009). O modelo estrutural da membrana dos glóbulos vermelhos é constituído por um mosaico bilipídico e fluídico que são formados por um complexo de mistura de lipídios e proteínas distribuídos de forma assimétrica. (Murador & Deffune, 2007)

Os repetidos episódios de falcização causam danos na membrana das hemácias desfavorecendo a reversibilidade do formato em foice dos eritrócitos mesmo quando a pressão de oxigênio é aumentada. Portanto, a vida útil da célula vermelha é reduzida, induzindo a anemia. Os glóbulos brancos e plaquetas são também afetados pela mutação do gene da β-globina (Kuypers, 2007).

O formato da célula é afetado por alterações na composição lipídica da membrana celular. Doenças associadas a alterações metabólicas como insuficiência renal e doença hepática, que podem ocorrer na AF, geram modificações na proporção de fosfolipídios e de colesterol da membrana eritrocitária e consequentemente afetam a fragilidade osmótica. Os eritrócitos maduros não são capazes de sintetizar lipídios pela ausência da enzima acetil CoA carboxilase, com isso, a membrana celular sofre alterações em sua composição lipídica de acordo com as alterações dos lipídios circulantes, o que promove modificações das funções básicas das membranas plasmáticas favorecendo processos patológicos (Barabino *et al*, 2010).

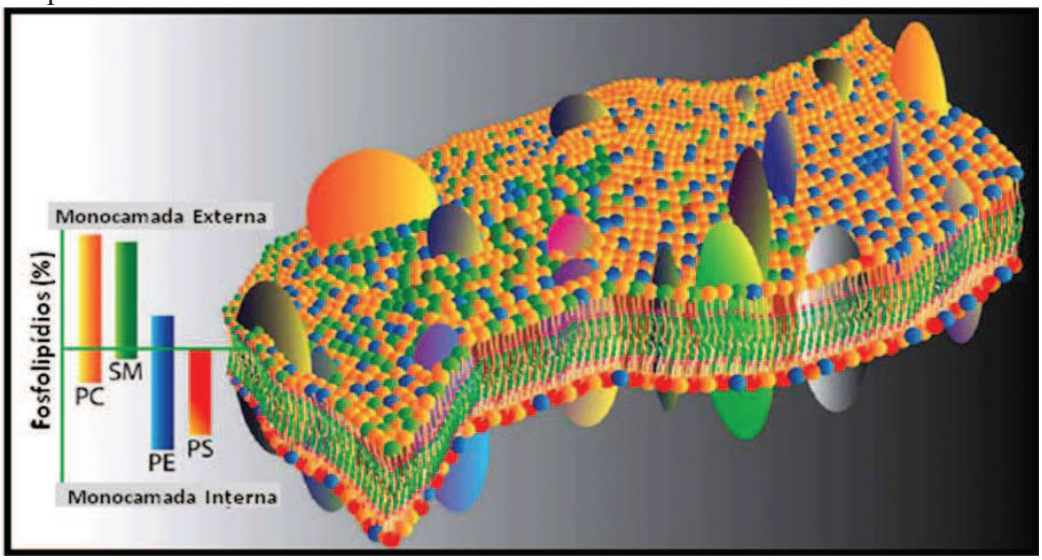
As membranas celulares de mamíferos contêm mais de mil tipos de fosfolipídios. As quantidades dos fosfolipídios sofrem variações entre os diferentes tipos de células e tecidos, definem a fluidez da membrana e, consequentemente, as funções das proteínas incorporadas. Fosfatidilcolina (PC) é o fosfolipídeo mais abundante, constitui cerca de 40%-50% dos fosfolipídios totais da membrana. O segundo mais abundante é a fosfatidiletanolamina (PE) que constitui 20%-50% dos fosfolipídios totais. A menor concentração corresponde a fosfatidilserina (PS) com 2%-10% dos fosfolipídios totais. Existem inter-relações metabólicas entre PS, PE e PC. Outros fosfolipídios, em menor quantidade nas membranas, incluem fosfatidilinositol (PI) e esfingomielina (SM) (Ataga & Key, 2007; Franck *et al,* 1985; Freikman *et al*, 2011; Kuypers, 2007; Strypulkowski & Manfredini, 2009; Vance, 2008; Zwaar *et al*, 1989).

Esses fosfolipídios organizam-se em sua bicamada lipídica com capacidade de mover-se rapidamente tanto no mesmo plano, bem como em toda bicamada, sendo essa dinâmica altamente organizada. O *flip-flop* através da membrana é organizado por proteínas localizadas na bicamada. Os fosfolipídios contendo colina, PC e SM, são encontrados principalmente na monocamada externa da membrana, enquanto os aminofosfolipídios são predominantemente PE ou exclusivamente PS localizados na monocamada interna (Figura 7) (Ataga & Key, 2007; Franck *et al,* 1985; Freikman *et al*, 2011; Kuypers, 2007; Strypulkowski & Manfredini, 2009).

Essa assimetria normalmente é mantida pela ação da aminofosfolipídeo translocase (flipase), ATP (adenosina trifosfato) dependente, que transporta PS e PE da superfície externa para a interna da membrana. Exposições anormais de PS (Figura 8) podem estar correlacionadas com a redução da atividade da flipase devido ao estresse oxidativo e modificações sulfidril causados pelos processos de polimerização e despolimerização da HbS (Ataga & Key, 2007; Franck *et al*, 1985; Kuypers, 2007). Tanto a composição quanto a organização são mantidas durante toda a vida da hemácia, e, alterações em qualquer dos lipídios e proteínas de membrana gera apoptose durante a eritropoiese ou morte precoce das

células circulantes. Dentre as alterações de membrana já evidenciadas na DF, têm-se os seguintes eventos: rearranjo das proteínas espectrina-actina, diminuição de glicoproteínas, geração de radicais livres, externalização da PS e aceleração da apoptose, em virtude do aumento da atividade citosólica de cálcio (Ca^{++}) (Ataga & Key, 2007; Kuypers, 2007; Strypulkowski & Manfredini, 2010).

Figura 7 – Composição Lipídica da Membrana de Eritrócitos. Os fosfolipídios contendo colina: fosfatidilcolina (PC) e esfingomielina (SM) estão localizados predominantemente na monocamada externa da membrana, enquanto os aminofosfolipídios fosfatidiletanolamina (PE) e fosfatidilserina (PS) são predominantemente ou exclusivamente localizados na monocamada interna, respectivamente.

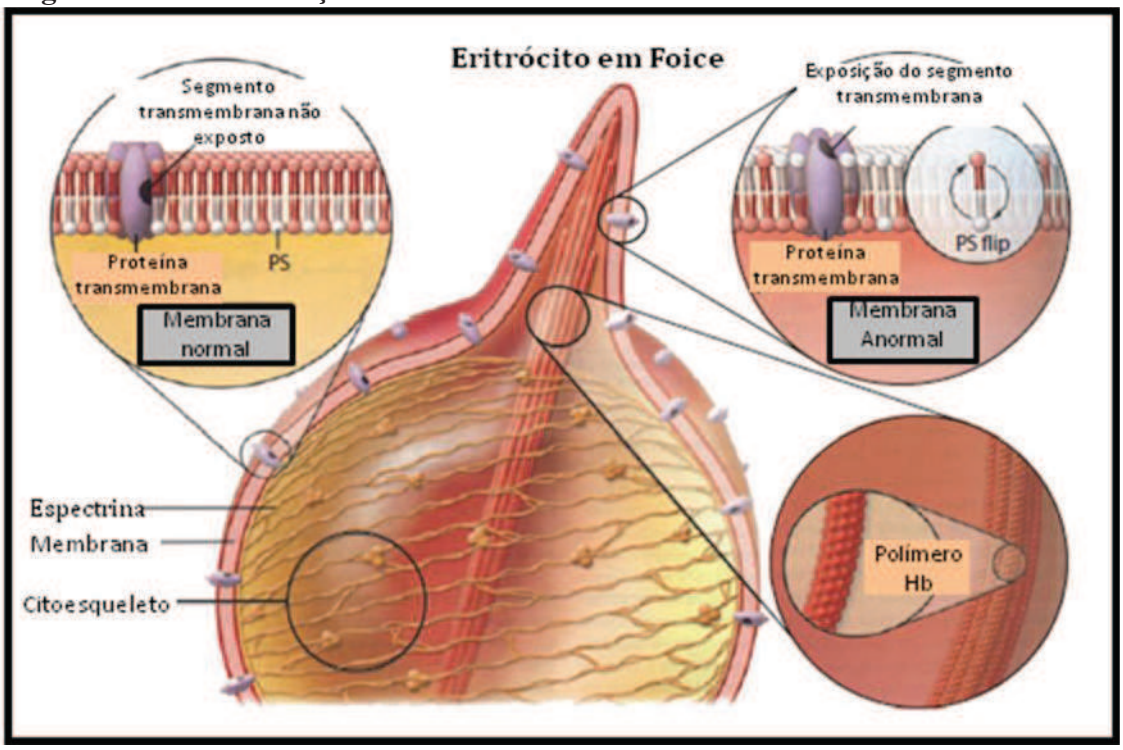


Fonte: Adaptado de Kuypers, 2008.

A exposição da PS na superfície celular também contribui para efeitos protrombóticos e aumento da aderência ao endotélio, o que permite o reconhecimento e a remoção das hemácias pelos macrófagos, caracterizando a hemólise (Kuypers, 2007; Strypulkowski & Manfredini, 2009). As vias bioquímicas que levam à exposição de PS são apenas, parcialmente, compreendidas, mas a perda de assimetria fosfolipídica pode ser desencadeada por uma série de condições e parecem envolver estresse oxidativo, Ca^{++} e atividade de proteínas cinases (Ataga & Key, 2007; Franck *et al*, 1985; Hannemann *et al*, 2011). A modificação do fluxo de Ca^{++} também participa dessa exteriorização (Freikman *et al,* 2011). Entretanto, ainda está sendo questionado se a ativação plaquetária e coagulação observada em portadores de AF contribuem para a fisiopatologia ou se são eventos secundários (Ataga & Key, 2007). A bilirrubina, que apresenta efeitos tóxicos levando à hemólise e esfoliação de lipídios, foi descrita como responsável por contribuir para a exposição de PS na membrana

eritrocitária (Brito *et al,* 2002). A taxa de hemólise e casos de esplenectomia (cirúrgica ou funcional) estão associados com danos na membrana dos eritrócitos pela exposição da PS que favorece a adesão no endotélio e trombose (Kato *et al*, 2007). Ao passo que as hemácias não possuem nova síntese de lipídios, os fosfolipídios sofrem transformações por processos de desacilação-reacilação que envolvem várias proteínas que podem ser afetados por fatores citosólicos e externos (Kuypers, 2008).

Figura 8 – Exteriorização da Fosfatidilserina no Eritrócito em Foice.



Fonte: Adaptado de Barabino, Platt & Kaul, 2010.

Vários relatos indicam evidências de oxidação lipídica nas hemácias de células falciformes, sugerindo que o reparo pelos fosfolipídios não é suficiente para manter adequada composição de espécies moleculares nessas células. Todas as hemácias são expostas à mesma fonte de substratos de ácidos graxos (no plasma), portanto a diferença está relacionada com atividade das enzimas que utilizam este conjunto para o reparo dos fosfolipídios. As duplas ligações das cadeias acila dos fosfolipídios são vulneráveis a modificações oxidativas, quando há adição de O_2 nas cadeias apolares, o local da embalagem da bicamada lipídica será alterado e sua funcionalidade será prejudicada. O reparo dessa violação é realizado pela desacilação de ROS (espécies reativas de oxigênio) nos fosfolipídios utilizando os ácidos graxos presentes no plasma. Em síntese, enquanto as células vermelhas não têm nova síntese de lipídios, os fosfolipídios sofrem rápidos processos de desacilação e reacilação envolvendo várias

proteínas, que podem ser afetadas por fatores citosólicos. A incorporação de ácidos graxos na membrana é alterada pelo estresse oxidativo, o que dificulta na manutenção da sua composição lipídica visto que o eritrócito também não é capaz de sintetizar novos lipídios (Kuypers, 2008).

O transporte de íons pela membrana eritrocitária é regulado por, pelo menos, três mecanismos, que também se encontram alterados nas hemoglobinopatias: (1) co-transporte de potássio (K^+) e cloreto (Cl^-) – ativo em hemácias jovens e desencadeado na presença de pH ácido ou meio hipotônico, quando ativado, K^+ e Cl^- deixam a célula e a água segue este fluxo, causando desidratação e consequente aumento da concentração de HbS; (2) canal de Gardos – canal de transporte de K^+ ativado pelo aumento do Ca^{++} intracelular resultante da desoxigenação e falcização. O efluxo de K^+ é acompanhado pelo de água, causando desidratação celular; (3) transporte de troca de sódio (Na^+) e hidrogênio (H^+) – capaz de desidratar células que foram previamente muito hidratadas. O transporte pela membrana também se encontra prejudicado em células HbSS, o que contribui para patogênese, uma vez que há influxo de Ca^{++}, efluxo de K^+ e Cl^- (Figueiredo, 2007; Hannemann *et al*, 2011) e perda relativa de magnésio (Mg^{++}) (Hannemann *et al*, 2011).

O aumento da permeabilidade da membrana gera perda de solutos por osmose levando à desidratação celular, que aumenta a concentração de hemoglobina corpuscular média (CHCM), facilitando a falcização, por aumentar a possibilidade de contato entre as moléculas de HbS. Por serem viscosos, os polímeros diminuem a deformabilidade dos eritrócitos, reduzindo o tempo de trânsito através da microcirculação. Esses fatores acarretam no encurtamento da vida média dos glóbulos vermelhos, fenômenos vaso-oclusivos, crises álgicas e lesão de órgãos (Hannemann *et al*, 2011; Martins *et al*, 2010). As células falciformes têm sobrevida muito curta, de 16 a 20 dias, quando comparadas aos 120 dias do eritrócito normal (Felix *et al*, 2010). Devido à redução da vida média das hemácias, pacientes com DF apresentam hemólise crônica que se manifesta por palidez, icterícia, elevação dos níveis de bilirrubina indireta, do urobilinogênio urinário e do número de reticulócitos (ANVISA, 2002). A DF representa um estado inflamatório, com o aumento crônico de citocinas, leucocitose, diminuição do tempo de meia vida dos leucócitos e ativação anormal de granulócitos, monócitos e endotélio. A estimulação de citocinas resultantes das células endoteliais aumenta a aderência com as hemácias falcizadas (Hannemann *et al*, 2011).

Pacientes com baixas concentrações de Hb e altas taxas hemolíticas parecem formar um subfenótipo de pacientes mais propensos a desenvolver doenças vasculares do que àqueles com maiores taxas de Hb, os quais aparentam ser mais susceptível a episódios de dor aguda

com provável síndrome torácica (Rees *et al*, 2010). Dessa forma, aparentemente, dois eventos interrelacionados e desencadeados pela polimerização da HbS parecem ser responsáveis pelas manifestações clínicas da AF: a vaso-oclusão e a anemia hemolítica. Como foram descritos acima, esses processos dependem não só de aspectos relacionados ao eritrócito, como o conteúdo de polímeros de HbS e grau de dano celular, bem como da interação com fatores externos, a exemplo do dano endotelial (Figueiredo, 2007; Steinberg, 2005). Apesar do fenômeno vaso-oclusivo ser importante em todos os pacientes, o mecanismo fisiopatológico da DF é controverso e foco de várias pesquisas (Rees *et al*, 2010). Esta divisão de fenótipos, um dependente de hemólise e outro da vaso-oclusão, indica que os tratamentos poderão ser dirigidos preferencialmente contra aspectos distintos da fisiopatologia e que combinações de tratamentos provavelmente serão superiores a uma única modalidade. Efeitos ambientais e nutricionais também podem ter papel fundamental na expressão fenotípica da AF (Figueiredo, 2007; Steinberg, 2005).

III. JUSTIFICATIVA

Estima-se o nascimento de 700-1.000 novos casos anuais de DF no país (ANVISA, 2002) e no Estado de Pernambuco a AF afeta 1/1.400 nascidos vivos. A Fundação de Hematologia e Hemoterapia de Pernambuco, Fundação HEMOPE, tem cadastrado atualmente 1.500 pacientes com DF. Embora tenha a mesma causa genética, a doença afeta o organismo de maneira diferente de pessoa para pessoa: enquanto alguns pacientes têm um quadro de grande gravidade e estão sujeitos a inúmeras complicações e frequentes hospitalizações, outros apresentam uma evolução mais benigna, em alguns casos quase assintomática (Rees *et al*, 2010). Diante dessa variabilidade clínica e do alto índice de mortalidade por complicações da AF (Bakanay *et al,* 2004), novos estudos são necessários visando melhora da sobrevida e qualidade de vida desses indivíduos. Afinal, apesar de se tratar de uma doença crônica e incurável, existe tratamento (Paiva e Silva *et al*, 1993; Quinn *et al,* 2004). Entretanto, o presente estudo, ao investigar alterações em plasma e eritrócitos entre os diferentes subfenótipos da AF, poderá contribuir como sendo um novo alvo para um tratamento individualizado, favorecendo uma melhor abordagem terapêutica, para um melhor desempenho funcional desses indivíduos, evitando numerosas e desnecessárias intervenções, aumentando, inclusive, a expectativa de vida desses pacientes.

IV. OBJETIVOS

IV. 1. Geral

Investigar possíveis alterações metabólicas em plasma e eritrócitos de pacientes portadores de HbSS nos seus diferentes subfenótipos com predomínio VO, AH ou superposição (SP).

IV. 2. Específicos

- Selecionar 3 grupos de pacientes com HbSS portadores de VO, AH e SP, e 1 grupo de indivíduos controle;
- Avaliar os parâmetros clínicos IMC, PAS, PAD dos portadores de HbSS de acordo com os subfenótipos;
- Investigar as alterações no metabolismo lipídico de pacientes com HbSS nos diferentes subfenótipos;
- Avaliar os níveis de colesterol e fosfolipídios plasmáticos e de membrana de pacientes com HbSS de acordo com os subfenótipos da doença;
- Analisar os índices hematimétricos, perfil hepático, glicose e marcadores de função renal dos portadores de AF e seus respectivos subfenótipos;
- Identificar níveis plasmáticos de DHL de pacientes com os diferentes subfenótipos da HbSS;
- Avaliar a fragilidade osmótica, permeabilidade ao glicerol e elasticidade de hemácias de pacientes com os diferentes subfenótipos da AF;
- Investigar a relação entre colesterol e fosfolipídios de membrana eritrocitária com a hemólise na AF;
- Identificar as frequências alélicas da ApoE na AF;
- Avaliar a influência dos alelos da ApoE sobre os lipídios do plasma e de membrana de eritrócitos de pacientes com AF;
- Investigar o papel dos alelos da ApoE sobre os níveis de LDL oxidada em pacientes com AF;
- Analisar uma possível correlação entre a concentração de fosfatidilserina de membrana de eritrócitos de pacientes com AF e os diferentes níveis plasmáticos de DHL.

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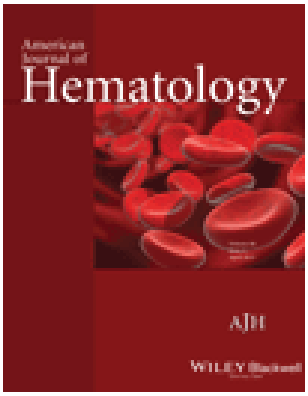
VI. ARTIGO 1 – *Lipid Composition of Plasma and RBC Membrane in Sickle Cell Anaemia. Are We Facing a Third and Intermediate Clinical Subphenotype?*



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Lipid Composition of Plasma and RBC Membrane in Sickle Cell Anaemia. Are We Facing a Third and Intermediate Clinical Subphenotype?

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ABSTRACT

Sickle cell anemia (SCA) is a genetic disorder caused by homozygosity for a single point mutation in the β-globin gene that formed HbS, changing the morphology of erythrocytes, leaving sickle shape. These physical modifications of the membrane may be related to changes in their lipid composition. Changes in various laboratory parameters can be observed in SCA, including lipid and apolipoprotein profiles. The globin mutations that lead to hemoglobinopathies as SCA have a profound effect on the red blood cells membranes (RBCm). Alterations in lipids of the membrane, like exposure phosphatidylserine (PS), will lead to apoptosis during erythropoiesis or early demise of the cell in the circulation. These altered lipid bilayer in RBCm leads anaemia and plays an important role in the pathology. Patients with SCA may differ in terms of symptoms and disease progression, there seems to be two subphenotypes in the expression of SCA: subphenotype associated with vaso-occlusion and viscosity (VO) and other with predominantly hemolysis and endothelial dysfunction (AH). The aim of this study was to investigate changes in lipid metabolism in patients with SCA are related to different clinical subphenotypes. The findings support the concept that there is a third clinical subphenotype in SCA, overlapping subphenotype, which attends most vulnerable in lipid metabolism suggesting a worse clinical severity.

INTRODUCTION

Sickle cell anaemia (SCA) is a monogenic disorder caused by homozygosity for a mutation in the β-globin gene. The HbS resulted from substitution of adenine for thymine (β^6GAG> GTG) encoding valine instead of glutamic acid (glu6val) (Ingram, 1957). The union of various Hb tetramers forms long polymers that precipitate in the cytoplasm, changing the morphology of erythrocytes to sickle shape, promoting change of rheology of red blood cells and plasma membrane (Ikuta *et al.*, 2011; Martins *et al.*, 2010; Strypulkowski & Manfredini, 2010). The sickling and unsickiling cycles lead to repeated damage to the red blood cells membranes (RBCm), eventually resulting in irreversibly sickle-shaped cells and hemolysis (Barabino, Platt & Kaul, 2010). Serum lactate dehydrogenase (LDH) is marker of intravascular hemolysis and was described as a biomarker of prognosis in sickle cell disease (SCD) (Kato *et al.*, 2006).

Hypocholesterolemia associated with low levels of low density lipoprotein (LDL-c) and high density lipoprotein (HDL-c) have also been described as potent biomarkers of clinical severity in patients with SCD (Dagger *et al.*, 2009; Seixas *et al*, 2010; Zorca *et al.*, 2010). Although mutations affect RBC, white blood cells (WBC) and platelets also undergo changes. SCD has been associated with multi-organ damage and risk mortality (Seixas *et al.*, 2010). By involving several organs, we can observe changes in various laboratory parameters (Clarke & Higgins, 2000; Minniti *et al.*, 2011; Seixas *et al.*, 2010).

The globin mutations that lead to hemoglobinopathies such as SCD have a profound effect on the RBCm (Kuypers, 2007, 2008).The RBCm is composed of a lipid bilayer with proteins embedded. Phospholipids are the main lipid constituents and are asymmetrically distributed across the lipid bilayer with choline containing phospholipids, phosphatidylcholine (PC) and sphingomyelin (SM) are located in the outer leaflet while amino phospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE), are found in the cytosolic side. These two surfaces, with distinctly different lipid and protein compositions, reflect the different physiological functions at the two sides of the membrane (Zwaar *et al.*, 1989). Alterations in either the lipids of the membrane, like exposure PS, will lead to apoptosis during erythropoiesis or early demise of the cell in the circulation (Kuypers, 2007).

The repeated sickling and unsickling damages the red cell membrane leading to irreversibly sickled red cell even when the oxygen pressure is increased thus reducing red cell life span as a result of membrane damage inducing anaemia (Akinbami *et al.*, 2012). The permanently deformed cells are subsequently removed from the circulation well before the usual 120-day life span of a healthy red cell, contributing to a chronic hemolytic anemia (Clarke & Higgins, 2000).

Although HbS polymerization and red cell sickling under deoxygenated conditions are primary event of this disease, evidence shows that there are two distincts pathophysiology which constitute subphenotypes of SCA with different clinical manifestations. Subphenotype associated with vaso-occlusion and viscosity (VO) including painful crisis, acute chest syndrome, hand-foot syndrome and avascular necrosis of bone, whereas hemolysis and endothelial dysfunction subphenotype (AH) has propensity to acquire pulmonary hypertension, leg ulcers, stroke, priapism and cholelithiasis, moreover the high serum lactate dehydrogenase (LDH) levels and reduces nitric oxide bioactivity (Alexander *et al.*, 2004; Kato *et al.*, 2007; Steinberg, 2005).

Despite having been discovered at the beginning of the last century, many issues related to the course of SCA still exist, for example, why individuals are affected differently if

the disease has the same genetic cause? There are only two subphenotypes in SCA? Patients who have no predominance of any of subphenotypes already known, where they fall? The aim of this study was to identify possible changes in biochemical parameters, including analysis of apolipoproteins A-I and B, the lipid composition of plasma and erythrocyte membrane between the different clinical subphenotypes of SCA, considering a third clinical subphenotype.

METHODS

Subject Selection

Our subjects consisted of 20 blood donors (HbAA) to group control and 60 patients with sickle cell anaemia (SCA - HbSS), both sex, aged 18 years and over who were studied at steady state at Fundação de Hematologia e Hemoterapia de Pernambuco (HEMOPE). Identification of homozygous for "A" or "S" was obtained by hemoglobin electrophoresis or HPLC. Serologic results for hepatitis B antivirus (anti-HBV), the hepatitis C antivirus (anti-HCV), human T-type antivirus lymph cells (anti-HTLV 1 and 2) and serological tests for syphilis (VDRL) were not reagents. Subjects were excluded if they had others hemoglobinopathies associated, smoking or alcohol consumption. The following events were considered to separate the subphenotypes: (1) subphenotype with blood viscosity and vaso-occlusion predominance (VO): vaso-occlusive pain crisis, acute chest syndrome, hand-foot syndrome, femoral head avascular necrosis and lactate dehydrogenase (LDH) values below 1,000 U/L; (2) leg ulcers, priapism, pulmonary hypertension, non-hemorrhagic stroke, cholelithiasis and LDH greater than 1,000 U/L were characteristics of the hemolytic and endothelial dysfunction subphenotype (HA) (Kato *et al.*, 2006; Kato, Gladwin & Steinberg, 2007; Sebastiani *et al.*, 2007). The individuals who showed two overlapping subphenotypes, however, not included exclusively in the VO or HA group were grouped in a third subphenotype of SCA called "overlapping" (OL). All subjects provided medical histories, using a standardized questionnaire, provided blood samples and underwent physical examination. Clinical data were accessed from their medical records. The Ethics Research Committee of the institution approved this protocol (nº.050/2011) and informed consent was obtained from each participant.

Blood Samples and Biochemical Analysis

Samples were obtained in individuals with fasting for 12 hours. Peripheral blood was collected into vacuum tubes (VACUETTE®) containing anticoagulant EDTA-$K3^+$ (1.8 mg/mL), serum clot activator tubes and sodium fluoride (NaF) with EDTA-K3 (3 mg and 1.8 mg/mL, respectively). Samples were used within 4h. The red cell indices include: red blood cell (RBC) number, hemoglobin concentration (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and red cell distribution width (RDW). Leukocyte count, platelet counts and reticulocyte count were included. The lactate dehydrogenase was used as a marker for intravascular hemolysis. Hepatic tests were also evaluated using hepatic enzymes: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyltransferase (GGT) and albumin levels were quantified by automated spectrophotometry (Cobas C501, Roche, Diamond Diagnostics, USA). Total, direct and indirect bilirubins were also studied. Renal function was assessed by dosing serum creatinine, urea and uric acid. Sodium (Na^{++}), potassium (K^+), chloride (Cl^-), calcium (Ca^{++}) and magnesium (Mg^{++}) were determined by the Flame Phgotometer analysis (Corning Instruments, England, UK).

Plasma levels of glucose, total cholesterol (TC), triglycerides (TG) and HDL-cholesterol (HDL-c) were determined by enzymatic spectrophotometry (Labtest Sistemas Diagnósticos Ltda.). LDL-cholesterol (LDL-c) and VLDL-cholesterol (VLDL-c) were determined by Friedewald equation [LDL-c = TC – HDL-c – VLDL-c; VLDL-c = TG/5]. Plasma apolipoprotein A-I (ApoA-I) and apolipoprotein B (ApoB) levels were mensured by immuneturbidimetric assay (Roche, USA) using Cobas c501.

Plasma and Red Blood Cell Membrane Phospholipids

The plasma was isolated after centrifugation (Sorvall, USA) at 1500 x g for 15 minutes. Erythrocytes were washed four times with cold isotonic saline buffer for removal of plasma lipoproteins and leukocytes (Nelson, 1967). The lipids were extracted from both erythrocyte membrane and plasma as described by Folch (1957), with chloroform: methanol (2:1, v/v). The main classes of phospholipids present in the plasma and erythrocytes were estimated following separation by one-dimensional and two-dimensional thin-layer chromatography (TLC), respectively. The TLC plates (20 x 20 cm) covered with 0.25 mm H silica gel 60 was used plasma (Merck, Brazil) while H silica gel with 2.5% magnesium acetate

was used to apply lipid extract erythrocytes. In detail, after activation of the TLC plates at 120º for 30 minutes, 50 µL of each reconstituted lipid extract was applied by means of Hamilton microsyringe as a 1cm long streak at the inferior the plate. TLC tanks lined with filter paper (Whatman filter paper (No 1)) were filled with the appropriate solvent mixture and allowed to settle for 30 min before the thinlayer plate was introduced. The solvent mixtures used for the first dimensional were chloroform-methanol- ammonia (65:35:5 v/v) and chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5 v/v) were used for second dimensional. Plasma TLC using the same solvent mixture for the second dimension erythrocytes. The solvent front was allowed to run 10 cm. The plate was then air-dried and transferred to a tank containing iodine crystals until the spots just became visible. The spots were outlined with a needle, the residual iodine was allowed to evaporate in air, and the spots were scraped off the plate and transferred to Pyrex test tubes. After, 0.3 mL of concentrated sulfuric acid was added to each tube. The tubes were heated to 180°C for 2 hours in an aluminum block. Add 2 drops of hydrogen peroxide and remained in the aluminum block for an additional 2 hours (Moore, 1968; Nelson, 1967). Inorganic phosphorus of the samples was quantified by the method of Bartlett (1959), based on a calibration curve prepared using inorganic phosphorus standard solution (2µg/mL) and total phospholipids (TPL) were measured from the lipid extract. Absorbance was measured in spectrophotometer at λ=735. Classes of phospholipids were analyzed plasma PC, PE, SM and lysophosphatidylcholine (LPC), whereas erythrocyte phospholipid classes studied PC, PE, SM, LPC, PS and phosphatidic acid (PA). All solvents were from Merck grade for analysis. Standards of phospholipids were from Sigma Chemical Company.

The determination of cholesterol content of the RBC membranes were determined by spectrophotometry using Colorimetric Method (Labtest Sistemas Diagnósticos Ltda.).

Statistical Analysis

Data were expressed as mean ± standart error of mean (SEM). Differences between all groups were analyzed for One-Way ANOVA followed by Fisher's Test. All analysis were adjusted for age, sex and BMI. P <0.05 implied statistical significance. All analyses were carried out using software StatView, version 4.5, Abacus Concept, Inc, Berkeley, CA

RESULTS

Blood pressure and body mass index in SCA

Patients had significantly lower diastolic blood pressure (DBP) compared to controls. The OL subphenotype did not differ from AH. Although, they were larger than VO. Sistolic blood pressure (SBP) was lower in VO compared to controls and others subphenotypes. The BMI was not significantly different between groups of SCA, but were lower when compared to controls.

Laboratory characteristics in subphenotypes of SCA

RBC number, Hb and Ht were lower in SCA, whereas MCV, HCM, MCHC and RDW were larger than compared to controls. About MCHC, only the VO group did not differ to control. OL and VO groups had similar characteristics and their values were larger than HA, except the values of RDW. OL subphenotype had larger values of RDW than VO and lowers than HA. No significant differences in MCV, HCM, MCHC and reticulocytes were found between subphenotypes. Reticulocytes were higher in SCA. The patients with SCA increased platelets and leukocytes number compared to controls. OL and HA subphenotypes had similar characteristics, the number of leukocytes was lower while the platelets were increased compared to VO group (**Table 1**).

The LDH was elevated in HbSS and the highest values were found in the AH group followed by the OL and VO subphenotypes. Patients with SCA had impaired liver function. They presented larger serum levels of AST, ALP, TB, DB and IB when compared to the levels obtained from the control group. Levels of AST in OL group were lower than AH, but increased if compared to VO, while the values of ALT levels were higher in OL relation to the HA, but compared to control did not difference. The OL group had the largest increase in values TB, DB and IB, following to AH and VO subphenotypes. Values of DB did not differ between OL and VO. ALP values were similar in subphenotypes of SCA. No significant differences in GGT were found.

Biochemical parameters that reflect renal function presented lower levels creatinine and urea compared to controls. Subjects with OL subphenotype had lower values creatinine than AH. Subphenotypes did not difference in urea. OL had more uric acid than VO group, but not when compared to control and AH group. The uric acid results were not different between OL and AH.

Magnesium and calcium were lower and potassium was highest in SCA compared to controls, but did not significantly difference between of subphenotypes. The OL group had

lower values of sodium than others subphenotypes and did not differ to controls. AH and VO did not show sodium alterations between them. Chlorine was lower when compared OL to AH groups. The AH subphenotype had significantly higher chlorine levels compared to VO and controls (**Table 2)**.

Lipid and apolipoprotein levels in subphenotypes of SCA

Changes in plasma lipids of patients with SCA were observed with reductions in the levels of TC, LDL-c and HDL-c. Plasma CT in OL group proved significantly reduced compared with other subphenotypes, without significant difference between HA and VO groups. No change was observed in LDL-c and HDL-c within the subphenotypes. Concentrations of VLDL-c and TG were decreased in the patients with OL when compared to control and HA. ApoA-I and ApoB were lower in SCA. All subphenotypes presented differences, except HA and VO that no showed differ between them. Patients with OL characteristics had the lowest values of apolipoproteins A and B when compared to all the others groups (**Figure 1**).

Plasma phospholipids in subphenotypes of SCA

SCA presented decrease values of TPL and PC, and increase levels of LPC without any changes between subphenotypes. The OL was reduced concentrations of PE and SM compared to others subphenotypes, but not compared to controls. VO and HA had the same characteristics about PE and SM relative to controls, both were higher (**Table 3**).

Total cholesterol of RBCm in subphenotypes of SCA

Patients with OL subphenotype showed highest levels of total cholesterol of RBCm (RBCm TC) compared to VO. The group HA had the same results like OL. However, no significant differences in SCA compared to controls were found (**Figure 2**).

RBCm phospholipids in subphenotypes of SCA

In general, there was no significant difference in values RBCm TPL between HbSS and HbAA. However, when analyzing the subphenotypes observed higher concentrations in the OL group than in the others. SCA had increased levels of RDCm PC, shown by the increase in OL and VO groups compared to controls. The OL subphenotypes presented higher values of PC. HA group did not differ compared to control, but had lower values of RBCm PC when compared to VO subphenotype. RBCm LPC values were also increased in SCA

compared to controls, except patients with VO characteristics, which had no difference with the controls. The only significant change between subphenotypes corresponded to higher values of RBCm LPC in the HA group when compared to the VO. The OL group had higher RBCm SM, while in group HA was found lower values of RBCm PE, both in relation to the controls. No differences in the amounts of RBCm PE and RBCm SM were found. All subphenotypes had higher RBCm PS concentrations compared to controls. In the case of RBCm PA, the OL group showed higher concentrations than the other groups. RBCm had PI values of OL and AH below the VO group, but no significant difference between subphenotypes and controls (**Table 4**).

DISCUSSION

This study supports the existence of distinct clinical subphenotypes in SCA in agreement with other authors. Studies classified SCA in two subphenotypes, one characterized by viscosity and vaso-occlusion (VO) and other with predominantly hemolytic and endothelial dysfunction (HA) (Alexander *et al*, 2004; Kato *et al*, 2007; Sebastiani *et al*, 2007; Steinberg, 2005). Our study found a third subphenotype composed of clinical criteria which do not meet the classification as VO nor HA groups, there may be overlapping features of subphenotypes. The third subphenotype of SCA (OL) showed significant differences in relation to both laboratory and blood pressure values even as the data of plasma and erythrocyte membrane phospholipids.

Studies show that children with SCD, especially in SCA, have delayed sexual and skeletal maturation, fat and muscle wasting, and impaired growth. Tissue hypoxemia caused by severe anemia, chronic and acute events of vaso-occlusion, endocrine dysfunction, low socioeconomic status and food intake contribute to low BMI (Barden *et al*, 2002; Oguanobi *et al*, 2010). Our patients had lower index, however no difference was observed between subphenotypes. Despite to BMI of subjects with leg ulcers was significantly lower than in patients without such history (Minniti *et al*, 2011).

Blood pressure is lower than normal in individual with SCA (Fourcan *et al*, 1999; Oguanobi *et al*, 2010; Pegelow *et al*, 1997). Our patients had lower DBP, but SBP only showed reduced in individuals of VO group compared to controls. OL and AH subphenotypes presented lager values of DBP than VO although were not difference from health subjects. These results may be due to the increased blood viscosity in VO. After all, viscosity limits the

flow of blood, providing the intrinsic resistance to blood flow in the presence of pressure, according to Poiseuille equation (Johnson, 2005; Swerdlow, 2006). Blood from an untransfused patient with SCA has a significantly higher viscosity than normal blood at the same hemoglobin level (Swerdlow, 2006). When clinical manifestations are taken into consideration, high values had increased risk of stroke and death (Oguanobi *et al*, 2010; Pegelow *et al*, 1997). Nevertheless, about SBP, neither OL nor HA had significant differences in relation to controls.

SCD is mostly a disorder of RBC, so there was already expected a reduction in the values of RBC number, Hb and Ht (Akinbami *et al*, 2012; Clarke & Higgins, 2000; Rahimi *et al*, 2006; Seixas *et al*, 2010) as found in all subphenotypes of SCA, especially in HA subphenotype. The lowest values were found in the HA group according to previous studies (Kato *et al*, 2007; Sebastani *et al*, 2007; Steinberg, 2005). Most patients have adapted to low RBC indices, there is therefore no clinical benefit to treat anaemia with blood transfusion (Akinbami *et al*, 2012). Values of MCV, HCM, MCHC and RDW were larger compared to controls. The significant difference in RDW between subphenotypes shows volumetric heterogeneity of erythroid populations. The OL subphenotype was more heterogeneous than VO, however anisocytosis in the HA is higher compared to other groups. This finding is one of the clues of the existence of the third subphenotype. There was an increase in the number of reticulocytes of patients compared to controls similar to the expected, but not between subphenotypes of SCA. Even the reticulocyte count is considered a biomarker of clinical severity in SCA as Rees & Gibson (2011) and Sebastiani *et al* (2010) reported.

Although, SCD is primarily a red cell disorders, the WBC and platelets are also affected by the mutation (Akinbami *et al*, 2012; Okpala *et al*, 2006; Paul *et al*, 2013; Shanley, Ebeling & Titus, 2011). Leukocytosis and thrombocytosis were observed in our patients, these results were expected considering the degree of chronic hemolysis, chronic painful crisis and risk of infection in sickle cell patients. OL and HA had similar characteristics, the number of leukocytes was lower while the platelets were increased compared to VO group. These findings may be the result of chronic hemolysis more pronounced in OL and HA groups. Furthermore, leukocytes contribute to SCD by adhering to blood vessel walls and obstructing the lumen, aggregating with other blood cells with more effective blockage of the lumen, stimulating the vascular endothelium to increase its expression of ligands for adhesion molecules on blood cells, and causing tissue damage and inflammatory reaction (Okpala, 2004). Akinbami *et al* (2012) showed that high absolute neutrophil count was statistically significant related with clinical severity of SCA, thus they conclued that leucocytosis is

associated with poor prognosis, while reducing neutrophil count is associated with good prognosis. Our findings for the VO group, with patients in steady state, corroborate with Paul *et al* (2013) that showed WBC count were independently associated with history of acute pulmonary events in hemoglobin SS patients.

Shanley, Ebeling & Titus (2011) related that patients with uncomplicated courses, discharge from emergency department without subsequent return, had larger platelet declines than those with complicated courses, several visits to emergency with episodes of acute chest syndrome. This may account for the higher values of platelets in the OL and HA compared to VO subphenotypes. Akinbami *et al*, 2012 said the negative feedback effect on erythropoietin production in subjects as a result of the anaemia could be responsible for the thrombocytosis. It is therefore, well recognized that thrombocytosis is associated with anaemia of chronic disease and several types of anaemia.

According to Kato *et al* (2004), LDH is a biomarker of intravascular hemolysis and indicates the prognosis of SCA. Despite to hemolysis consequent to the damaged RBCm could be intravascular or extravascular. (Akinbami *et al*, 2012). The link between LDH and a generalized state of endothelial activation is reflected by elevated blood plasma levels of soluble adhesion molecules, especially vascular cell adhesion molecule (VCAM-1). The highest values found in the DHL group OL suggest that this third subphenotype has a worse prognosis than the VO group, but not in relation to AH, which in this marker, continues to have the worst prognosis of all.

The lipid profile of our patients confirm the results found in several studies (Monnet *et al*, 1996; Rahimi *et al*, 2006; Sasaki *et al*, 1983; Seixas *et al*, 2010; Zorca *et al*, 2010), which report the low serum levels of CT, HDL-c and LDL-c in the SCA. Individuals in the OL group showed to be different from others in relation to the profile of lipids and apolipoproteins in plasma. It was the one subphenotype that showed a significant reduction in all analyzes compared to control, both the plasma lipid profile as the values of ApoA-I and ApoB. The OL subphenotype still had lower CT, VLDL-c, TG, ApoA-I and ApoB when compared to HA. These results demonstrate a greater lipid impairment from OL group, whereas plasma concentrations of VLDL-c and TG in HA and VO groups whether they had significant difference compared to the controls.

In 1995, el-Hazmi *et al* suggest that increased utilization or decreased production may account for the lower cholesterol level in severe anaemia, particularly those with SCA. Rahimi *et al* (2006) reported that the hemolytic stress can be associated with a significant reduction in the levels of lipids and lipoproteins by dilution of plasma constituents due to

increased plasma volume over the volume of red blood cells or by reduction of the enzyme β-hydroxy-methyl-glutaryl coenzima A reductase (HMG-CoA reductase), which participates in cholesterol biosynthesis. Although some authors (Monnet *et al*, 1996; Seixas *et al*, 2010; Zorca *et al*, 2010) agree that the SCA is hypertriglyceridemia, our study found a reduction in TG levels as well Shores *et al* (2003).

Patients with OL characteristics had the lowest values of apolipoproteins A and B when compared to all the others groups. This is in agreement with Monnet *et al* (1996) reported lower values of ApoA-I and ApoB in SCA. However, there are descriptions that apoB values are increased and associated with prevalence of HP (Yudistskaya *et al*, 2009). Morris *et al* (2008) said exist evidence that SCD patients with low apoA-I have endothelial dysfunction, a parallel finding to the general population. Some studies suggest that ApoA-I is further reduced during painful crisis (Monnet *et al*, 1996; Tumblin *et al*, 2010) while Yudistskaya *et al* (2009) have correlated with endothelial dysfunction and promoting HP in SCD. They explain that oxidative stress is a prominent feature of SCD and antioxidant activity linked to apoA-I might mediate part of its apparent protection against sickle vasculopathy and pulmonary hypertension. suggest a potential role for apoA-I in endothelium-regulated vasodilation in SCD. Yudistskaya *et al* (2009) suggest that apoA-I levels are significantly related to PAH, but not sensitive or specific enough to be a diagnostic test. The exact apolipoproteins mechanisms in SCA did not have clear, but the link between the reduced concentrations of apoA-I with painful episodes and incidence of HP may indicate that patients in the OL group compose one intermediary subphenotype.

The OL group decreased plasma concentrations of TPL and PC, as well as increased LPC similarly to the others subphenotypes compared to control. OL only had higher concentrations of TPL compared to HA. The reduction in values of plasma TPL in SCA has been well described (Sasaki *et al*, 1983; Kuypers, 2007, 2008; Okpala, 2006). Despite the evident reduction of TPL and the free glycerol content of the plasma was eight-fold higher than normal, Sasaki *et al* (1983) found no difference between the classes of phospholipids the distribution of plasma neither than fatty acid content, both were similar to normal values, which differs from our results that found differences in classes of phospholipids including differences between subphenotypes.

Ren *et al* (2005) revealed an imbalance between n-3 and n-6 long-chain polyunsaturated fatty acids (LCPUFA) in erythrocyte and plasma lipid moieties of the HbSS patients. Their study showed levels of the n-6 LCPUFA, arachidonic, adrenic and docosapentaenoic acids in erythrocyte choline and ethanolamine were higher in the patients

compared with the controls. In contrast, the proportions of eicosapentaenoic acid in choline and ethanolamine and docosahexaenoic acid and total n-3 metabolites in choline were lower in the patients. Okpala (2006) also showed reduced proportions of omega-3 fatty acids, occur in SCD. He described that these lipid abnormalities are more severe in patients with disease complications and in those with a greater degree of anaemia. Since lipid constitution of cell membranes affects surface expression of adhesion molecules, the above findings could account for earlier observations that omega-3 fatty acids reduce P-selectin expression and reduce the frequency of sickle cell crisis. Situations that promote reduction of these fatty acids involve oxidative damage and have been described as responsible for complications of SCA (Kuypers, 2007). Reen *et al* (2005) and Okpala (2006) indicate differences in the composition of fatty acids in the different classes of phospholipids, which may explain, in part, differences in the concentrations of plasma and RBCm as found in our study, more pronounced in subphenotype OL. We observed plasma concentrations of PE and SM were significantly reduced in HA and VO groups. Since the activated fatty acid is rapidly incorporated into a lysophospholipid in the RBC membrane (Kuypers, 2008).

This phospholipid turnover and repair can be expected to be higher in hemoglobinopathies due to the increased oxidant stress and damage to the lipids. Several reports indicate evidence of lipid oxidation in RBCs from sickle cell or thalassemia patients, suggesting that phospholipid repair is not efficient enough to maintain the proper molecular species composition in these cells (Kuypers, 2007).

OL and HA groups had higher concentrations of TC in RBCm than VO subphenotype. Although the OL and HA groups showed a tendency to increasing concentrations of TC in the control group, no significant differences were found. These results were similar to Ngogan *et al* (1989) which also showed no differences between patients with SCA and controls. Values of TC in RBCm had been contradictory (Kaplan & Simoni, 1985; Ngogan *et al* 1989; Sasaki *et al*, 1983). However, there is a consensus that the loss of membrane asymmetry affects cholesterol levels. Olsen *et al* (2009) proved that direct membrane perturbation by side-chain oxysterols is significant, and suggests that these membrane perturbations may play a role in the oxysterol regulation of cholesterol homeostasis. Furthermore, erythrocyte cholesterol is greatly influenced by exchange with plasma lipoproteins a disturbance of this exchange could modify the erythrocyte shape as well as the membrane fluidity (Ngogan et al, 1989).

One possible explanation for decreased exchange of cholesterol in irreversibly sickled cells is that their membranes have undergone cross-linking of aminophospholipids present in the inner bilayer of the erythrocyte membrane as a consequence of exposing to

malonyldialdehyde, an end product of lipid peroxidation. This, coupled with the decreased plasma lecithin-cholesterol acyltransferase (LCAT) activity limiting exogenous cholesterol uptake, may result in elevated cholesterol levels in irreversibly sickled cells. Elevated erythrocyte membrane cholesterol has been proposed to cause decreased membrane fluidity and changes in membrane permeability characteristics. Hence, altered mechanical and/or permeability properties of irreversibly sickled cell membranes may be related to the membrane cholesterol content of these unusual cells (Jain & Shohet, 1982). Lowering of the cholesterol content of the membrane tends to "dissolve" these rafts, leading to an altered function of the membrane, and changes in the citosol such as an increase in calcium can lead to shedding of vesicles enriched in specific lipids and proteins (Kuypers, 2007).

Saar et al (1998) noted a significant decrease of the PC rates, associated with significant accumulation of LPC into RBCm implied in hemolysis disorders. Saar et al (1998), conducted with sickle cell trait (HbAS), is different from our study, because we found a significant increase in PC as well as the LPC in RBCm, especially in the OL subphenotype of SCA. Diatta et al (2002) stated that the accumulation of LPC, by its cytolytic biomembrane perturbing properties, predispose the sickled red cell to the haemolysis phenomena and allow to forecast deeper disturbances among homozygous subjects. The highest values of LPC found in OL and AH groups are in accordance with the highest degree of hemolysis evidenced by RBC indices. The incorporation of fatty acids in PC was altered by oxidative stress (Kuypers, 2007).

The catalytic potential of the membrane surface depends increased exposure of negatively charged phosphatidylserine at the outer surface, plays an important physiological role in local blood clotting reactions (Vance & Steenberger, 2005; Zwaar et al, 1989). Due to the altered hydrophobicity of the oxidized acyl chain, phospholipases gain access to the exposed ester bond and will remove the oxidized fatty acid.The lysophospholipid that is generated by this process needs to be replaced. When RBCs lose their ability to maintain phospholipid asymmetry, PS is exposed, leading to RBC removal and these cells are not readily removed, they can induce pathophysiologic responses such as imbalanced hemostasis, and interactions with other blood cells and with endoendothelial cells of the vascular wall, as found in SCD and thalassemia (Kuypers, 2007).

Conditions leading to exposure of PS can be increased concentrations of the PA by the action of phospholipase D on PC. The LPA (lysophosphatidic acid) can also be formed and affect the integrity of the endothelium (Kuypers, 2008). The OL is the only group that significantly increases the PA compared to control and others subphenotypes. Although did

not significant difference, there was a tendency of increasing concentrations of PS compared to the others subphenotypes. Lipid breakdown products of PS exposing cells result in vascular dysfunction, including acute chest syndrome in sickle cell disease. Altered membrane lipids play an important role in the pathology of hemoglobinopathies and characterization of the proteins involved in lipid turnover will elucidate the pathways that maintain plasma membrane organization and cellular viability (Kuypers, 2007, 2008).

The PS can be synthesized from PC or PE. Phosphatidylserine can also synthesize PE. May be because it, the levels of PE in RBCm were tending to reduce OL and VO groups, but were significantly reduced compared to HA subphenotype. (Vance & Steenberger, 2005). The PE can be reduced in the SCA have been used as a substrate to form PS, whose concentrations are significantly increased, though no difference between subphenotypes.

Lubin *et al* (1989), after connecting information from their studies with sickle erythroctes, demonstrate a number of membrane lipid changes in these cells that include (1) changes in membrane phospholipid dynamics, (2) perturbation of the translocase protein that translocates aminophospholipids from the exterior leaflet to the interior leaflet, (3) perturbation of the interaction between membrane phospholipids and skeletal proteins, and (4) abnormal phospholipid molecular species compositions. They also reported that the mechanism underlying these alterations may involve several independent effects. Included in these will be the oxidative damage that occurs to these membranes and the dissociation of lipids and proteins that accompanies the sickling process. These informations agree with others authors (Diatta *et al*, 2002; Kuypers, 2007, 2008) contribute to the understanding of our findings. In summary, the inability of RBCs or RBC precursors to maintain phospholipid asymmetry therefore plays a role in the anemia (Kuypers, 2008).

By exposing these results, it is clear that there is a third clinical subphenotype of SCA, which we call overlapping. Differences in RBC indices, in particular the values of Hb and RDW, proved heterogeneous populations of erythrocytes between the three subphenotypes. This third subphenotype showed greater impairment of lipid profiles and apolipoprotein than others. Changes in plasma phospholipids and erythrocyte membrane were more prominent in subphenotype OL. Therefore, the most vulnerable in lipid metabolism OL group indicates the worst clinical severity.

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Table 1. Clinical and hematologic characteristics of subphenotypes in sickle cell anemia.

Parameters	Control	SCA Subphenotypes		
		VO	HA	OL
BMI	27.1 ± 0.4	21.8 ± 0.5*	20.8 ± 0.4*	21.9 ± 0.5*
SBP (mmHg)	118.1 ± 1.0	109.3 ± 1.8*	117.4 ± 2.1†	117.8 ± 1.6†
DBP (mmHg)	82.3 ± 0.6	66.1 ± 0.9*	70.0 ± 1.3*,†	72.3 ± 1.1*,†
Erythrocytes ($x10^6$)	4.9 ± 0.9	2.8 ± 0.9*	2.5 ± 0.5*,†	2.9 ± 1.3*,‡
Hb (g/dL)	14.2 ± 0.2	8.9 ± 0.2*	8.1 ± 0.1*,†	9.0 ± 1.2*,‡
Ht (%)	42.9 ± 0.5	26.3 ± 0.5*	23.8 ± 0.4*,†	26.6 ± 0.5*,‡
Reticulocytes (%)	1.33 ± 0.04	9.74 ± 0.78*	10.97 ± 0.67*	10.43 ± 0.65*
Platelets ($x10^3$)	270.4 ± 11.3	551.5 ± 48.5*	416.4 ± 18.6*,†	398.9 ± 16.7*,†
Leukocytes ($x10^3$)	6.4 ± 0.2	9.5 ± 0.4*	11.3 ± 4.7*,†	12.4 ± 6.2*,†
MCV fL	87.6 ± 0.8	95.1 ± 1.6*	97.1 ± 2.0*	95.3 ± 2.7*
MCH fL	28.9 ± 0.4	32.1 ± 0.7*	32.9 ± 0.7*	32.2 ± 0.9*
MCHC fL	33.0 ± 0.2	33.7 ± 0.2	33.9 ± 0.2*	33.8 ± 0.3*
RDW %	13.6 ± 0.2	18.9 ± 0.4*	23.1 ± 0.6*,†	20.7 ± 0.3*,†,‡

SCA – Sickle Cell Anaemia; VO – Vaso-occlusive; HA – Hemolytic Anaemia; OL – Overlapping; BMI – Body Mass Index; SBP – Systolic Blood Pressure; DBP – Diastolic Blood Pressure; Hb – Hemoglobin; Ht – Hematocrit; MCV – Mean Corpuscular Volume; MCH – Mean Corpuscular Hemoglobin ; MCHC - Mean Corpuscular Hemoglobin Concentration; RDW – Red Cell Distribution Width.

* $p<0.05$ for groups as compared to control;

† $p<0.05$ for groups as compared to VO group;

‡ $p<0.05$ for groups as compared to AH group.

Table 2. Biochemical characteristics of subphenotypes in sickle cell anemia.

Parameters	Control	SCA Subphenotypes		
		VO	HA	OL
LDH (U/L)	324.7 ± 7.60	646.4 ± 23.6*	1396.2 ± 67.7*,†	958.2 ± 73.2*,†,‡
AST (U/L)	23.9 ± 1.3	32.1 ± 1.5*	52.5 ± 2.1*,†	41.8 ± 2.4*,†,‡
ALT (U/L)	26.7 ± 1.4	25.9 ± 2.2	23.2 ± 1.6	29.2 ± 1.5‡
GGT (U/L)	53.8 ± 4.7	77.2 ± 13.4	73.0 ± 13.9	79.6 ± 9.0
TB (mg/dL)	0.51 ± 0.02	2.70 ± 0.22*	3.54 ± 0.19*,†	4.57 ± 0.63*,†,‡
DB (mg/dL)	0.15 ± 0.09	0.61 ± 0.04*	0.65 ± 0.02*	0.89 ± 0.11*,†,‡
IB (mg/dL)	0.37 ± 0.02	2.09 ± 0.22*	2.89 ± 0.18*,†	3.68 ± 0.58*,†,‡
ALP (U/L)	69.5 ± 2.8	84.5 ± 5.2*	91.1 ± 4.1*	96.1 ± 5.5*
Albumin (g/dL)	4.67 ± 0.04	4.81 ± 0.04*	4.62 ± 0.05†	4.72 ± 0.04
Urea (mg/dL)	29.0 ± 1.3	16.9 ± 0.8*	20.4 ± 1.9*	19.8 ± 0.9*
Creatinine (mg/dL)	0.83 ± 0.02	0.42 ± 0.01*	0.51 ± 0.04*,†	0.50 ± 0.02*
Uric Acid (mg/dL)	5.6 ± 0.2	4.8 ± 0.2*	6.5 ± 0.3*,†	6.1 ± 0.1†
Na^+ (mEq/L)	138.0 ± 0.1	138.8 ± 0.2*	138.7 ± 0.2*	137.8 ± 0.2†,‡
K^+ (mEq/L)	4.31 ± 0.04	4.74 ± 0.06*	4.86 ± 0.07*	4.78 ± 0.06*
Cl^- (mEq/L)	105.5 ± 0.2	105.4 ± 0.3	107.3 ± 0.3*,†	106.2 ± 0.3‡
Ca^{++} (mg/dL)	9.8 ± 0.8	9.5 ± 0.1*	9.4 ± 0.1*	9.3 ± 0.1*
Mg^{++} (mg/dL)	2.18 ± 0.18	2.01 ± 0.03*	1.98 ± 0.02*	1.99 ± 0.02*
Glucose (mg/dL)	93.1 ± 1.4	93.7 ± 1.9	92.8 ± 1.0	92.5 ± 1.9

SCA – Sickle Cell Anaemia; VO – Vaso-occlusive; HA – Hemolytic Anaemia; OL – Overlapping; LDH – lactate dehydrogenase; AST – aspartate aminotransferase; ALT – alanine aminotransferase; GGT – gamma glutamyl transferase; TB – total bilirubin; DB – direct bilirubin; IB- indirect bilirubin; ALP – alkaline phosphatase; Na^+ - sodium; K^+ - potassium; Cl^- - chlorine; Ca^{++} - calcium; Mg^{++} - magnesium.

* $p<0.05$ for groups as compared to control;

† $p<0.05$ for groups as compared to VO group;

‡ $p<0.05$ for groups as compared to AH group.

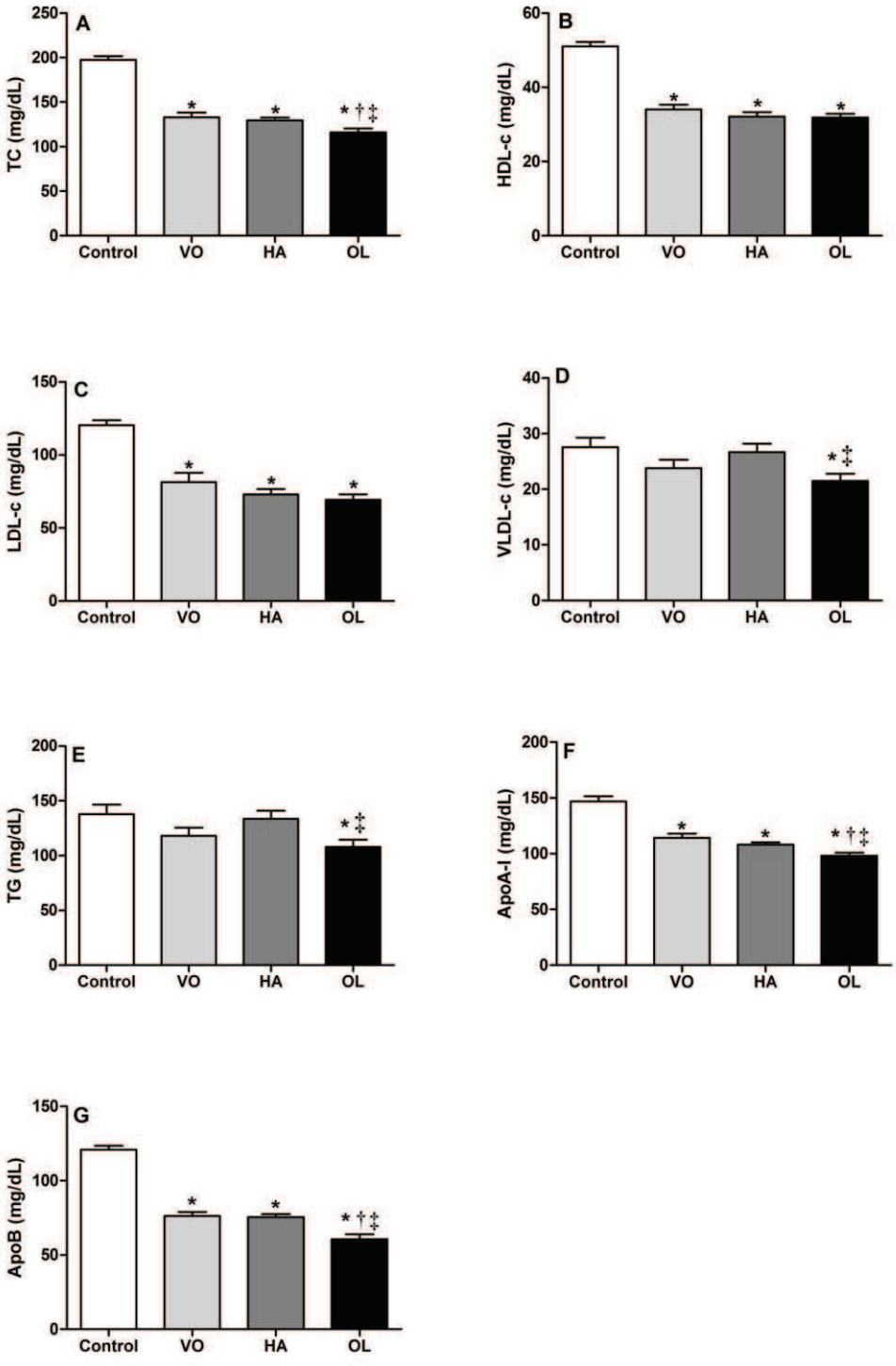


Figure 1. Lipid and apolipoproteic profile of subphenotypes in sickle cell anemia.
VO – Vaso-occlusive; HA – Hemolytic Anaemia; OL – Overlapping. (**A**) – Total Cholesterol (TC) levels; (**B**) – HDL-cholesterol (HDL-c) levels; (**C**) – LDL-cholesterol (LDL-c) levels; (**D**) – VLDL-cholesterol (VLDL-c) levels; (**E**) – Triglycerides levels; (**F**) – Apolipoprotein A-I (ApoA-I) levels; (**G**) – Apolipoprotein B (ApoB) levels.

* $p<0.05$ for groups as compared to control;

† $p<0.05$ for groups as compared to VO group;

‡ $p<0.05$ for groups as compared to AH group.

Table 3. Plasma phospholipids of subphenotypes in sickle cell anemia.

Plasma Phospholipids	Control	SCA Subphenotypes		
		VO	HA	OL
TPL	139.1 ± 3.5	92.1 ± 3.7*	100.1 ± 4.2*	108.0 ± 4.3*,†
PC	62.0 ± 2.2	49.1 ± 1.7*	47.0 ± 1.4*	45.3 ± 2.1*
LPC	7.7 ± 0.9	12.1 ± 1.1*	11.6 ± 1.0*	11.3 ± 1.3*
PE	8.5 ± 1.1	12.2 ± 0.9*	11.2 ± 0.8*	7.2 ± 0.7†,‡
SM	15.1 ± 0.8	19.9 ± 1.2*	20.3 ± 1.7*	13.9 ± 0.6†,‡

SCA – Sickle Cell Anaemia; VO – Vaso-occlusive; HA – Hemolytic Anaemia; OL – Overlapping; TPL – Total Plasma Phospholipids; PC – Phosphatidylcholine; LPC – Lysophosphatidylcholine; PE – Phosphatidylethanolamine; SM – Sphingomyelin.

* $p<0.05$ for groups as compared to control;

† $p<0.05$ for groups as compared to VO group;

‡ $p<0.05$ for groups as compared to AH group.

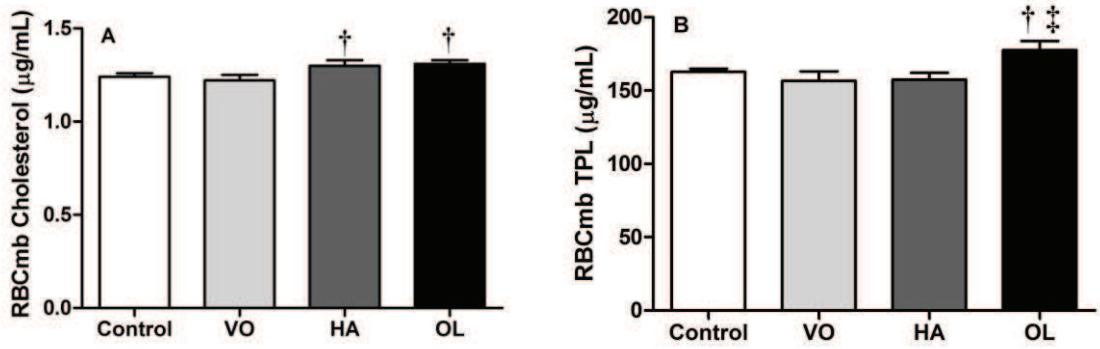


Figure 2. Cholesterol and phospholipids of red blood cell membrane of subphenotypes in sickle cell anemia.

VO – Vaso-occlusive; HA – Hemolytic Anaemia; OL – Overlapping. (**A**) Cholesterol of red blood cell membrane (RBCmb)–; (**B**) – Total Phospholipids (TPL) levels in RBCmb.

* $p<0.05$ for groups as compared to control;

† $p<0.05$ for groups as compared to VO group;

‡ $p<0.05$ for groups as compared to AH group.

Table 4. Membrane phospholipids of red blood cell membrane of subphenotypes in SCA.

RBCmb Phospholipids	Control	SCA Subphenotypes		
		VO	HA	OL
PC	26.0 ± 0.9	32.2 ± 1.3*	28.4 ± 1.3†	33.9 ± 2.0*,‡
LPC	2.1 ± 0.3	3.8 ± 0.5*	6.6 ± 1.1*,†	5.2 ± 0.7
PE	19.4 ± 1.2	17.2 ± 1.7	14.4 ± 1.5*	17.7 ± 1.8
SM	23.2 ± 1.3	26.7 ± 1.0	25.3 ± 1.7	28.9 ± 1.3*
PS	8.5 ± 0.8	14.4 ± 0.7*	12.4 ± 0.8*	14.6 ± 1.2*
PA	3.5 ± 0.7	3.4 ± 0.7	3.0 ± 0.4	5.7 ± 0.8*,†,‡
PI	4.3 ± 0.7	4.5 ± 0.7	2.9 ± 0.3†	2.4 ± 0.5†

VO – Vaso-occlusive; HA – Hemolytic Anaemia; OL – Overlapping; PC – Phosphatidylcholine; LPC – Lysophosphatidylcholine; PE – Phosphatidylethanolamine; SM – Sphingomyelin; PS – Phosphatidylserine; PA – Phosphatidylinositol; PA – Phosphatidic Acid; PI – Phosphatidylinosithol.

* $p<0.05$ for groups as compared to control;

† $p<0.05$ for groups as compared to VO group;

‡ $p<0.05$ for groups as compared to AH group.

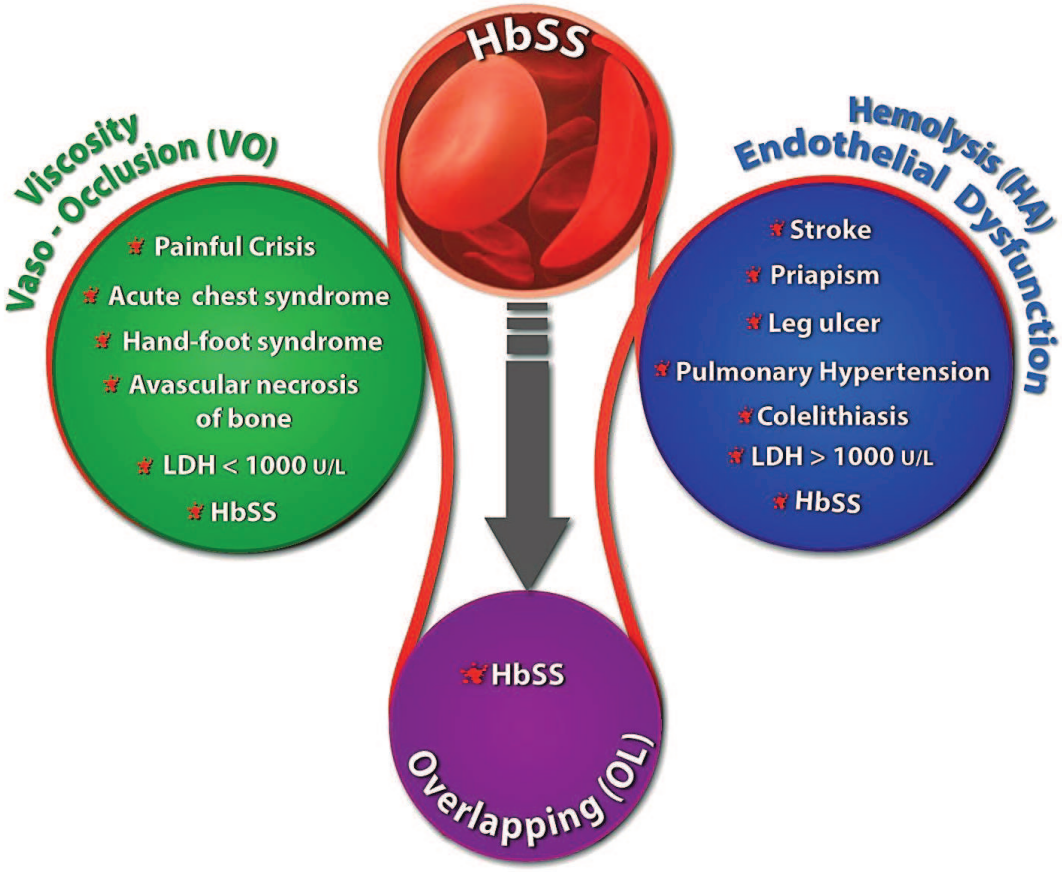
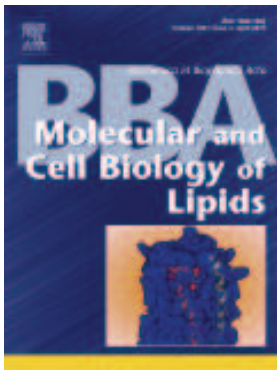


Figure 3. The third sickle cell anaemia subphenotype.

VII. ARTIGO 2 – *Altered Biophysical Properties of Red Blood Cells from Patients in Different Clinical Subphenotypes of Sickle Cell Anaemia*



Artigo a ser submetido ao periódico *Biochemica Et Biophysica Acta – Molecular and Cell Biology of Lipids* no formato *Original Research Article* (**FI:** 5.269; **QUALIS CB II:** A1).

Altered Biophysical Properties of Red Blood Cells from Patients in Different Clinical Subphenotypes of Sickle Cell Anaemia

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ABSTRACT

Sickle cell anaemia (SCA) is an inherited disease with a complex and diverse pathophysiology, it has two distinct clinical subphenotypes: vaso-occlusive (VO) and hemolytic anaemia (HA). The biophysical properties of the membranes of red blood cell (RBC) are related to lipid content and may differ among subphenotypes of SCA. This study aimed to study Biophysical Properties of Red Blood Cells from Patients in Different Clinical Subphenotypes of Sickle Cell Anaemia and their relation to membrane lipids. Blood samples were collected from 18 subjects with SCA participated in the study, 6 in each group VO, HA and overlapping (OL) and from 6 healthy individuals. Enzymatic methods and/or colorimetric were used for quantification of cholesterol and RBCmb phospholipids. The elasticity of the RBC was evaluated with the aid of optical tweezers. The fragility of the membrane across various concentrations of NaCl and glycerol permeability were also evaluated. One-Way ANOVA was used to analyze the data and values of $p<0.05$ represented significant differences. The elasticity of the RBC showed decreased in different subphenotypes, while the resistance to osmotic fragility was higher in these patients when compared to the control group. No significant differences were found between the SCA subphenotypes for elasticity and osmotic fragility. The time to 50% hemolysis was higher in individuals with SCA, vs. Control (Control> VO> HA> OL), and was significantly higher in individuals with HA and OL. The time of hemolysis was positively correlated to the cholesterol content of the RBCmb. Thus, the biophysical properties of elasticity, osmotic fragility and glycerol permeability are altered in sickle cell anaemia. The time for hemolysis differs between SCA subphenotypes and is directly related to the content of cholesterol, but not to the phospholipids, of the RBCmb of SCA patients.

Keywords: Sickle Cell Anaemia, Subphenotypes, Biophysical Properties and Membrane Cholesterol Composition.

HIGHLIGHTS

1. Biophysical properties of RBCmb were evaluated in different SCA subphenotypes;
2. Half maximum (50%) hemolysis time and RBCmb cholesterol composition were determined;
3. Elasticity and osmotic fragility were lower and did not differ between subphenotypes;
4. Glycerol permeability showed different 50% hemolysis time according to SCA subphenotypes;
5. 50% hemolysis time was positively correlated with RBCmb cholesterol content.

ABBREVIATIONS

EDTA – Ethylenediaminetetraacetic Acid; **HA** – Hemolytic Anaemia; ; **HbS** – Sickle Hemoglobin; **HCV** – Hepatitis C Virus; **HBV** – Hepatitis Virus B; **HPLC** – High-Performance Liquid Chromatography; **HTLV 1 and 2** – Human T Lymphotropic Virus Type 1 and 2; **HEMOPE** – Fundação de Hematologia e Hemoterapia de Pernambuco; **OL** – Overlapping; **RBC** – Red Blood Cell; **RBCmb** – Red Blood Cell membrane; **SCA** – Sickle Cell Anaemia; **VDRL** – Veneral Disease Research laboratory; **VO** – Vaso-occlusion.

1. INTRODUCTION

Sickle cell anaemia (SCA) is a systematic disorder caused by the homozygous occurrence of the mutation in the β-globin chain of hemoglobin known as sickle hemoglobin (HbS). HbS exists because of a single nucleotide substitution at the sixth position (replacement of glutamic acid to the less polar valine) on the hemoglobin. Sickling of the erythrocytes occurs by the aggregation of deoxy HbS into long straight fibers, which deform the cell. SCA is associated with many clinical manifestations as: acute episodes of pain, cerebral ischemic events, particularly overt stroke, priapism and acute chest syndrome and chronic organ damage, like renal failure, osteonecrosis and chronic hemolytic anaemia **[1-7]**.

The pathology of SCA is directly involved with the RBCmb structure, changes in resistance to hemolysis and the ability of elasticity have been reported during the SCA **[8,9]**. Since the structure and function of biological membranes are strongly influenced by the lipid components, the study of the relationship of clinical characteristics with the membrane molecules and its consequence on biophysical properties is fundamental to clarify the

mechanisms involved in the disease. The membrane lipids, particularly cholesterol and phospholipids, from sickle RBC are distributed asymmetrically and quantitatively altered **[10]**. Studies have reported the presence of abnormal exposures from phospholipids in sickle RBCmb. In this context, the externalization of phosphatidylserine appears to be related to the progression of SCA **[11,12]**.

In recent years, it has been observed that the SCA has two distinct subphenotypes: one with predominance of blood viscosity and vaso-occlusion, mainly characterized by vaso-occlusive pain crisis, acute chest syndrome, hand-foot syndrome, femoral head avascular necrosis; and another subphenotype that has been considered by the presence of hemolysis and endothelial dysfunction, which presents clinical events such as leg ulcers, priapism, pulmonary hypertension, non-hemorrhagic stroke and cholelithiasis. Still, the levels of lactate dehydrogenase have been the primary biochemical marker for the differentiation between these two subphenotypes **[13-15]**. Furthermore, some patients with SCA can show overlapping subphenotypes, but they are not included in any of these two classifications. These individuals can be included in a third SCA group named "overlapping".

The way in which these different classifications of subphenotypes and their clinical characteristics could be related to biophysical and chemical properties of RBC remains unknown. Above all, it is still unpublished the investigation of biophysical and chemical properties of RBCmb in individuals classified as "overlapping". The objective of this work was to study the biophysical properties of RBC from patients in different clinical subphenotypes of SCA and their relation with membrane lipids.

2. MATERIALS AND METHODS

2.1. Casuistics

Our subjects consisted of 06 blood donors (HbAA) to control group and 18 patients with sickle cell anaemia (HbSS), both sex, aged 18 years and over who were studied at steady state at Fundação de Hematologia e Hemoterapia de Pernambuco (HEMOPE). Identification of homozygous for “A” or “S” was obtained by hemoglobin electrophoresis or HPLC. Serological tests for hepatitis B antivirus (anti-HBV), the hepatitis C antivirus (anti-HCV), human T-type antivirus lymph cells (anti-HTLV 1 and 2) and syphilis (VDRL) were not reagents. Subjects were excluded if they had others hemoglobinopathies associated, smoking or alcohol consumption. The following events were considered to separate the subphenotypes:

(1) subphenotype with blood viscosity and vaso-occlusion predominance (VO): vaso-occlusive pain crisis, acute chest syndrome, hand-foot syndrome, femoral head avascular necrosis and lactate dehydrogenase (LDH) values below 1,000 U/L; (2) leg ulcers, priapism, pulmonary hypertension, non-hemorrhagic stroke, cholelithiasis and LDH greater than 1,000 U/L were characteristics of the hemolytic and endothelial dysfunction subphenotype (HA) **[13,14,15]**. The individuals who showed two overlapping subphenotypes simultaneously perform a third subphenotype of SCA named overlapping (OL). All subjects provided medical histories, using a standardized questionnaire, provided blood samples and underwent physical examination. Clinical data were accessed from their medical records. The Ethics Research Committee of the institution approved this protocol (nº.050/2011) and informed consent was obtained from each participant.

2.2. Blood Samples

Samples were obtained in individuals with fasting for 12 hours. Peripheral blood was collected into vacuum tubes (VACUETTE®) containing anticoagulant EDTA-$K3^+$ (1.8 mg/mL) and samples were used within 4h. Thus, the blood samples were centrifuged at 2500 xg for 15 minutes at 4 ° C (Sorvall RC6, NC, USA) for RBC separation. The pelleted RBC were washed with 4.5 mL of saline and then centrifuged at 2500 xg for 15 minutes at 4 ° C. This procedure was performed four times. Finally, the pelleted RBC were used for lipid extraction and to assess the biophysical properties.

2.3. RBC Membrane Lipid Composition

Lipids were extracted from erythrocyte membrane as described by Folch (1957)**[16]**, with chloroform: methanol (2:1, v/v). The determination of cholesterol content of the RBCmb were determined by spectrophotometry using Colorimetric Method (Labtest Sistemas Diagnósticos Ltda.). Inorganic phosphorus of the samples was quantified by the method of Bartlett (1959)**[17]**, based on a calibration curve prepared using inorganic phosphorus standard solution (2µg/mL) and total phospholipids (TPL) were measured from the lipid extract. Absorbance was measured in spectrophotometer at λ=735.

2.4. Glycerol Permeability of RBC

Glycerol permeability of erythrocytes were analyzed as described by Moore et al. (1968)**[18]**. Erythrocythes were obtained in the identical fashion as for lipid extraction. 0.2 mL of sedimented erythrocytes were diluted to 20 mL with buffered saline (pH 7.4). 3 mL of these resuspended cells were mixed with 45 mL of phosphate buffered saline; another 3 mL was transferred to an equal volume of phosphate-buffered 0.3 M glycerol and allowed to hemolyze completely. The O.D. of aliquots of both suspensions obtained at λ=675 nm. A linear plot of absorbance against time of hemolysis was obtained for every sample in the study.

Serial dilutions of hemolyzed RBC were prepared by mixing at 1 mL increments 1-10 mL of the unhemolyzed solution with 9-0 mL of the completely hemolyzed solution. Absorbance was immediately read at 675 nm. The absorbance values were translated into percentage of hemolysis by means of the standard curve. In this fashion a 50% hemolysis time was obtained for every different clinical subphenotype of SCA.

2.5. RBC Osmotic Fragility

The osmotic fragility of erythrocytes were analyzed as described by Moore et al. (1968)**[18]**, after some modifications. Sedimented erythrocytes (0.3 mL) were diluted to 4.5 mL (0.3% – 0.8%) with buffered saline (pH 7.4) and absorbance was at 675 nm after 60 seconds. The liner plot of absorbance against NaCl concentrations was obtained for all the different clinical subphenotypes of SCA.

2.6. Elasticity Measurments

RBC were added to a Neubauer chamber and captured by the optical tweezers (ζ = 1064 nm – IPG Photonics, EUA) and dragged against the blood serum with six constants velocities ranging from 140 μM/s to 290 μM/s by using the motorized stage **[19]**. When RBCs are dragged in blood serum they are deformed and two forces act upon the cells, a hydrodynamic force and an elastic force. Equilibrium occurs when elastic force cancels the drag force. At equilibrium,

Eq. (A1)

where m is the overall apparent elasticity **[20,21]** and $\Delta L = L - L_0$ is the cell length deformation (adopting L_0 as the cell length in the absence of any force) η is the viscosity measured using an Ostwald viscometer and n is the velocity. The cell is located at a distance Z_1 from the bottom of a Neubauer chamber and Z_2 from the cover slip, $1/Z_{eq} = 1/Z_1 + 1/Z_2$. Therefore, the measurement of the cell length as a function of the drag velocity can be used to extract a value for m, once the plasma viscosity g, the initial length L_0 and Z_{eq} are known. The cell movement at six velocities was registered by the optical tweezers camera using a video capture card in a computer. The L value was extract from video images with Image Pro-Plus software (Media Cybernetics, Silver Spring, MD). The depth Z_1 was measured by focusing the bottom of the Neubauer chamber and then lowering the chamber by the desired amount (in this case 50 mm) while keeping the cell fixed with the optical tweezers.

2.7. Statistical Analysis

Data was expressed as mean ± standard error of mean (SEM). Statistical significance was determined by one-way ANOVA. $P < 0.05$ implied significance. All analyses were carried out using software PRISMA (GraphPad Software, Inc., San Diego, CA, version 5.01).

3. THEORY

Studies show that despite the SCA have the same genetic origin, carriers of HbSS have distinct clinical features as if there were two subphenotypes a predominantly vaso-occlusive and another predominantly hemolytic associated with endothelial dysfunction. The degree of hemolysis has become a critical measure for the severity of the disease, which aids in the differential diagnosis of subphenotypes **[14]**. The presence of HbS alters the physical and chemical properties favoring the sickling of erythrocytes, and the reversibility of the deformation of the erythrocyte dependent of cross lipids rate **[22, 23]**. The lipids of cell membranes may confer rigidity characteristics, fluidity and permeability, among other biophysical properties to the membranes. Therefore, can changes in the biophysical properties of RBC be related to the different subphenotypes of SCA?

4. RESULTS

4.1 – Influence of Sickle Cell Disease Clinical Subphenotypes on Elastical Capability of RBC.

Overall, HbSS patients showed increased deformability independent of subphenotypes (Figure 1). The ability to maintain the original form was reduced in RBCs of patients as shown in Figure 2. It can be observed a wide variation of elasticity values among HbSS carriers, differently from healthy controls. In addition, some HbSS erythrocytes showed higher elasticity values than HbAA ones. However, there was a general tendency of lower elasticity.

4.2 – Osmotic Fragility

We observed increased RBC disruption resistance caused by osmolarity variation of the medium (Figure 3). There was a progressive increase of resistance among healthy, VO and HA individuals, respectively. Thus, HA patients showed significantly increased hemolysis resistance than the Control group. Interestingly, OL individuals, which showed clinical characteristics of both VO and HA, showed osmotic fragility intermediate between VO and HA.

4.3 – Glycerol Permeability

Sickle Cell Anaemia patients exhibited markedly increased resistance to hemolysis caused by presence of glycerol 0.3M (Figure 4A-B). The mean 50% hemolysis time increased progressively from control group to VO, HA and OL, respectively. Individuals of OL group presented values significantly higher even than sickle cell disease on VO subphenotype.

4.4 – Lipid Correlation with Hemolysis

In order to verify if the biophysical properties of erythrocyte membranes in SCD are related with their lipid composition we analyze cholesterol and phospholipids content and correlated them with mean 50% hemolysis time (Figure 5).
We observed a significant positive correlation between RBCmb cholesterol and hemolysis. Alternatively, there was no significant correlation between the RBCmb phospholipid content and hemolysis.

5. DISCUSSION

The report of biomechanical properties of sickle RBCs of different clinical subphenotypes brings markedly advances to the knowledge of how is the relation among clinical features at the molecular level. In order to retrieve the biophysical properties and its relation with RBCmb lipids, the dynamic structure of sickle RBCs were analyzed under different circumstances.

The use of optical tweezers gives the possibility to study the rheological properties of individual cells, providing an accurate assessment of sickle RBCs. Futhermore, the overall elasticity is very sensitive to several disturbances in the cell environment, such as osmotic pressure, changes because of molecules attached to the cell surface or hemoglobin defects **[24]**.

Some studies have shown variation in red cell deformability in diseases such as SCA **[25]** and malaria **[26]**. Our data suggest that the presence of hemoglobin S causes changes in the elasticity of the RBC in an expressive way, independent of the various clinical manifestations observed in patients.

In SCA patients, the RBC deoxygenation causes polymerization of Hb, which can be reversed by reoxygenation. However, during this process there is a structural breakdown of the key components of the red cell cytoskeleton called actin and spectrin, these proteins are directly involved in maintenance of the erythrocyte and their destabilization could be related to the loss of elastic ability observed in our study. In addition, Silva et al 2012**[19]** noted that erythrocytes of blood donors have decreased deformability with increasing storage time. Since the SCA erythrocytes have very short life expectancy, decline of elasticity observed could be in course due to the remotion of red blood cells from circulation before they become stiffened. Interestingly, sickle RBCs presented a wide variation in elasticity, with some sickle cells showing even higher values than healthy RBCs. Foretz et al, 2011**[27]** observed an increase of RBC distribution width related to decreased elasticity in SCA.

The resistance of RBC to hemolysis assessed by osmotic fragility was higher in the SCA, and this increase was significant only in the HA group. In this evaluation of biophysical properties, the RBC, when suspended in hypotonic medium, increases until it reaches a critical volume of hemolysis before being lysed, and according to some authors this resistance to hemolysis is influenced by factors such as the shape, size and volume of the erythrocyte, the type and amount of hemoglobin **[28]**. SCA is a disease characterized mainly by presenting

such changes in erythrocytes **[8]**. Thus, the increased resistance to hemolysis in individuals with HA subphenotype may be associated with the changes observed in the structure and content of the erythrocytes in this disease.

The content of cholesterol plays an important role in biological membranes, and changes in the amount of this lipid in the membrane RBC substantially reflects a change in serum lipid profile, since erythrocytes do not synthesize cholesterol because of the absence of the enzyme acetyl Coenzyme A carboxylase **[11,12,29]**. The enrichment of cholesterol in the membrane of erythrocytes leads to a decrease in the fluidity of these cells and this promotes alterations in level of hemolysis **[9]**.

Studies have reported that the permeability of the membrane to glycerol is closely related to the content of cholesterol and phospholipids of these membranes **[18]**. In our study, the time of hemolysis assessed by glycerol permeability increased in direct proportion to the content of cholesterol and phospholipids of erythrocyte membranes. Interestingly, cholesterol showed a stronger correlation with this finding. We observed that the time of hemolysis is higher in individuals with AH and OL. These subphenotypes hemolysis represents a pathological mechanism associated with the development of cardiovascular and renal diseases **[30]**.

Thus we believe that the change in permeability to glycerol in subphenotypes HA and OL may be associated with clinical manifestations that accompany these subphenotypes hemolytic. Studies have shown that chronic hemolysis, when associated with reduced levels of vascular nitric oxide, are mechanisms that contribute greatly to the development of pulmonary hypertension, a complication clinic that is responsible for a high rate of mortality in adults with sickle cell anemia. The priaprism and acute chest syndrome, other clinical manifestations present in individuals with SCA, are also a consequence of chronic hemolysis degree in these individuals **[31-35]**. Thus, we suppose that the differences in the RBCmb biophysical properties observed in this study may be important indicators of clinical manifestations in subphenotypes VO and HA and that the individuals who exhibit these overlapping phenotypes may have a greater predisposition to these clinical manifestations.

In conclusion, we observed that elasticity, osmotic fragility and glicerol permeability are altered in SCA patients. Particularly, half time hemolysis is positively correlated with membrane cholesterol content. These structural modifications are possibly related to clinical repercussions observed in the different SCA subphenotypes.

CONFLICT OF INTERESTS

The authors declare that are no conflicts of interest.

ACKNOWLEDGEMENTS

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APPENDICES

Eq. (A.1)

$$L = L_0 + \left(\frac{\eta L_0^2}{\mu Z_{eq}}\right) v$$

Fig. A1

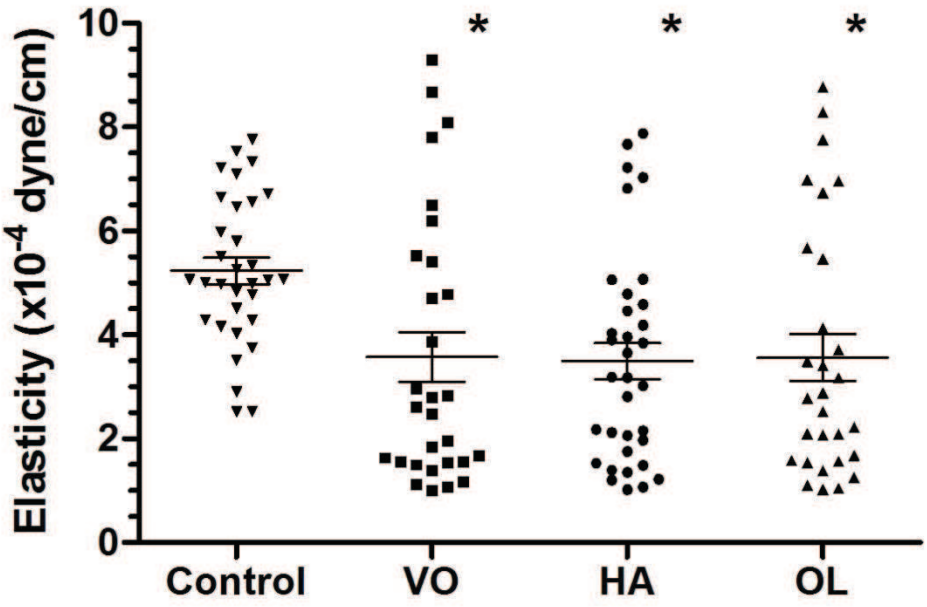

Figure 1. Dot plot of RBC elasticity measurements for control and sickle cell anaemia, according to vaso-occlusive (VO), Hemolytic Anaemia (HA) or Overlapping (OL) subphenotypes. *$p \leq 0.05$ vs Control.

Fig. A2

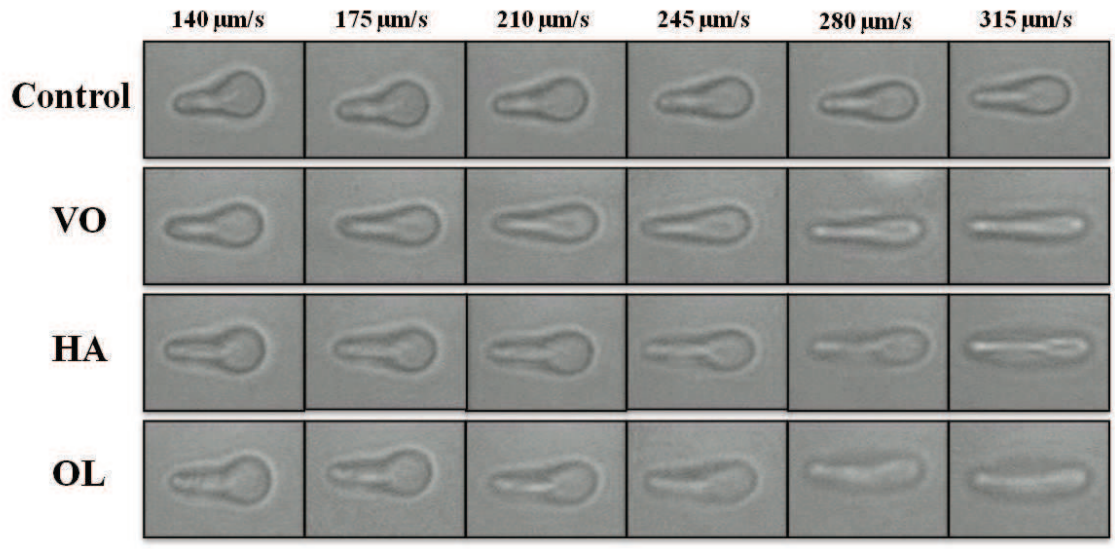

Figure 2. RBC elongation at the different velocities used for control and sickle cell anaemia, according to Vaso-occlusive (VO), Hemolytic Anaemia (HA) or Overlapping (OL) subphenotypes.

Fig. A3

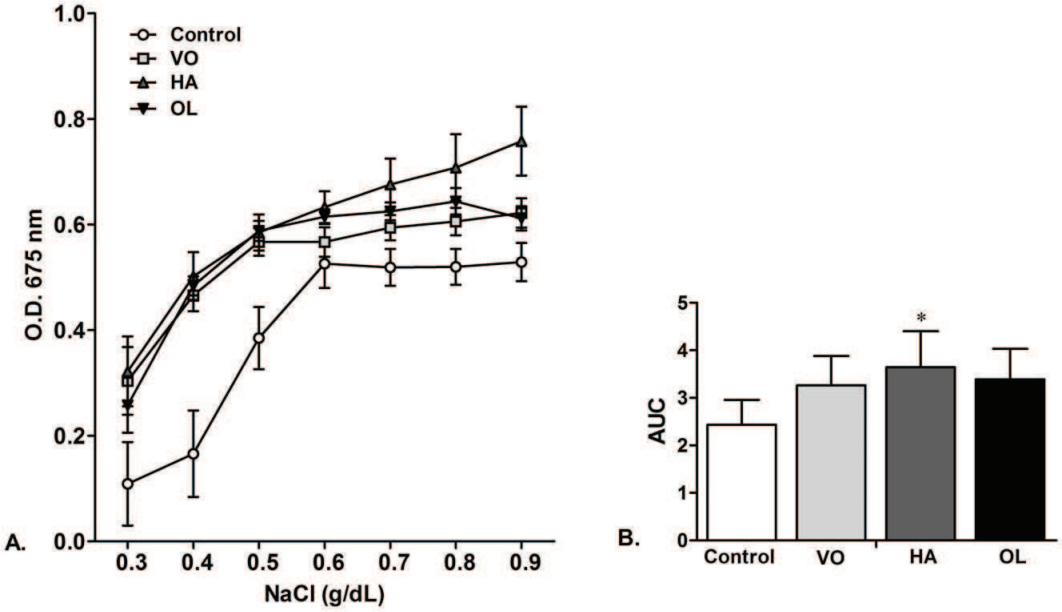

Figure 3. Hemolysis of patients with sickle cell disease and normal subjects erythrocytes in different concentrations of NaCl. (**A)** – NaCl hemolysis curve; (**B)** – AUC of NaCl hemolysis curve.

Data expressed as mean ± S.E.M.

*$p<0.05$ vs. control;

Fig. A4

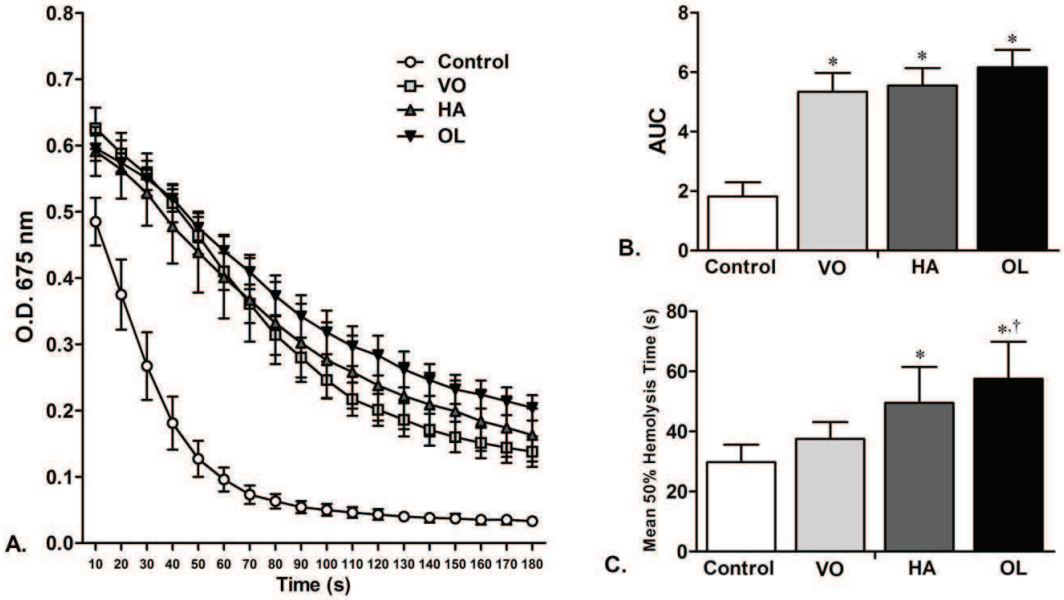

Figure 4. Hemolysis of patients with sickle cell disease and normal subjects erythrocytes in 0.3 M glycerol. (**A)** – 0.3 M glycerol hemolysis curve; (**B)** – AUC of 0.3 glycerol hemolysis curve; (**C)** – Mean 50% hemolysis time.

Data expressed as mean ± S.E.M.

*p<0.05 vs. control;

†p<0.05 vs.VO.

Fig. A5

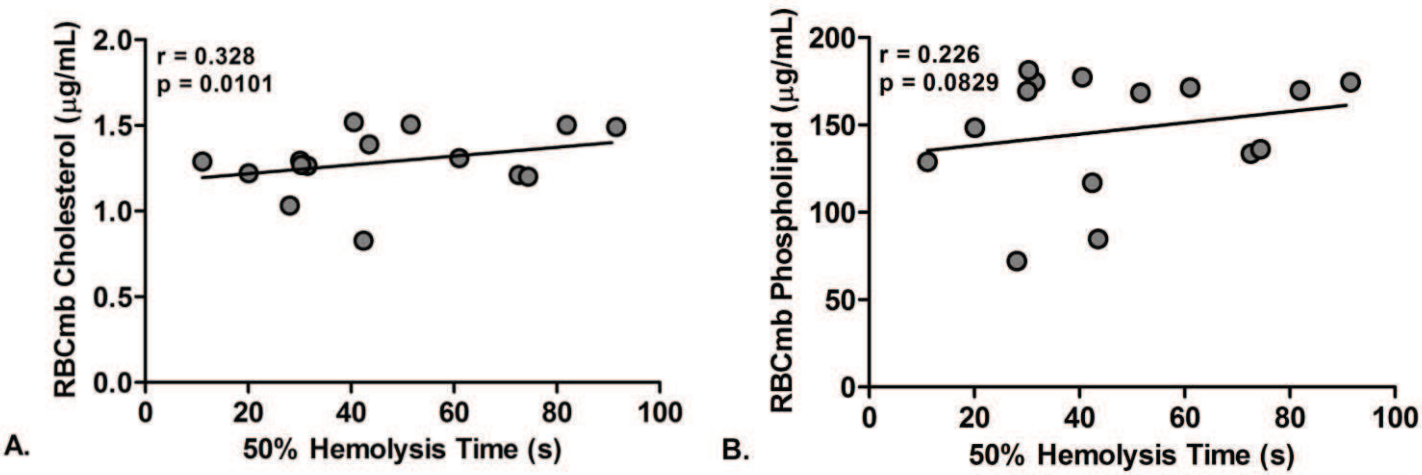

Figure 5. Correlation between lipid RBCmb composition and 50% hemolysis time in sickle cell anaemia. (**A**) – Correlation between RBCmb cholesterol and 50% hemolysis time; (**B**) – Correlation between RBCmb phospholipid and 50% hemolysis time.

VIII. ARTIGO 3 - *Relevant Role of Polymorphism of Apolipoprotein E on Lipids from Plasma and Red Blood Cell Membrane of Sickle Cell Anaemia Patients*

Artigo a ser submetido ao periódico *British Journal of Haematology* no formato *Original Research Article* (**FI:** 4.941; **QUALIS CB II:** A2).

Relevant Role of Polymorphism of Apolipoprotein E on Lipids from Plasma and Red Blood Cell Membrane of Sickle Cell Anaemia Patients

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Running Title: e4 allele of ApoE in plasma and RBCmb lipids of SCA patients

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SUMMARY

Sickle Cell Anaemia (SCA) is an important health problem, caused by a single point mutation, by substituting of glutamic acid for valine, in the 6th position of the β chain of the haemoglobin, which leads to sickle form of the Red Blood Cells (RBC). Plasma and membrane of RBC (RBCmb) suffer lipid alterations in their composition, and this may be interrelated to Apolipoprotein E (ApoE) alleles. This study aimed to investigate the possible influence of ApoE alleles on lipids of plasma and RBCmb from SCA patients, including levels of oxLDL and subclasses of phospholipids, beyond to investigate correlation between phosphatidylserine from RBCmb of SCA patients and plasma LDH, in the presence of the three ApoE alleles. 60 SCA patients were enrolled from *Fundação HEMOPE*, Brazil, and had their plasma and RBCmb levels of total cholesterol, total and phospholipid subclasses determined, and plasma levels of lipoproteins, Triglycerides, ApoA-I, ApoB, oxLDL, and LDH. Alleles e2, e3, and e4 of ApoE were identified by PCR/RFLP. e4 allele was related to lower values of plasma cholesterol, HDL-cholesterol, ApoA-I and ApoB; and to higher values of RBCmb cholesterol, and plasma oxLDL. Total phospholipids did not differ among alleles. However, e4 carriers presented the lowest values of plasma phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin. On the other hand, in the RBCmb, e4 was related to increase of lysophosphatidylcholine, sphingomyelin, and phosphatidylserine. A negative correlation between RBCmb levels of phosphatidylserine and plasma LDH was found in SCA patients with e4 allele. Therefore, this allele plays a key role on lipid composition of plasma and RBCmb in SCA.

Key-Words: Sickle Cell Anaemia, Red Blood Cell Membrane, Lipid Alterations, Alelles of Apolipoprotein E, Lactate Dehydrogenase.

INTRODUCTION

In 1910, Dr. James B. Herrick described the first case report of a patient with Sickle Cell Anaemia (SCA); and, in 1949, Linus Pauling et al. applied this term to a condition of severe chronic anaemia in which Red Blood Cells (RBC) are capable of sickling under decreased oxygen pressure (Pauling et al., 1949; Herrick, 2001). In 1957, Vernon Ingram described the chemical difference between normal (HbA) and sickle cell haemoglobin (HbS), and so SCA was the first genetic disorder that had its molecular abnormality precisely recognized (Ingram, 1957). A single point mutation in the normal codon GAG to GUG leads to codify the valine aminoacid in place of a glutamic acid residue, in the 6^{th} position of the β chain of the haemoglobin (Ingram, 1957; Alexy et al., 2010).

HbS suffers polymerization and becomes RBC distorted into elongated or spiculated shapes, that results in the sickle form. This hinders the crossing of the RBC through the small vessels, blocking circulation, maying to cause vaso-occlusion, pain crisis, acute chest syndrome, hemolytic anaemia, endothelial dysfunctions, leg ulcers, priapism, pulmonary hypertension, ischemic stroke, among other serious symptons. Therefore, despite advances in supportive therapies, the morbidity and the mortality of SCA remain high (Shores et al., 2003; Kato; Gladwin; Steinberg, 2007; Alexy et al., 2010).

Although the primary defect of SCA comes from the altered Hb molecule, the existing symptons in SCA may be associated to other abnormalities in the sickle RBC, especially those related to lipid composition of the membrane, as for example, the quantity of the phospholipids in the membrane RBC (RBCmb). Abnormal exposition of certain phospholipids, such as phosphatidylserine (PS), causes activation of coagulation proteins, platelet activation, increased expression of adhesion molecules of vascular endothelium, activation of the alternative complement pathway, and other biochemical events (Wang et al., 1993; Connor et al., 1997; Stypulkowski & Manfredini, 2010). Thus, lipid abnormalities are crucial points in SCA; and may be associated to the severity of the anaemia, as well as to the consistent presence of biomarkers for hemolysis, such as Lactate Dehydrogenase (LDH EC 1.1.1.27) (Zorca et al., 2010). This enzyme catalyzes the reaction of reduction and oxidation between piruvate and lactate and is involved in the glycolytic pathway. LDH interacts with phospholipid sites in the cells, and mays play a role in molecular mechanisms in pathological processes where the metabolism and internal environment of the cells are altered. It seems that PS is the most probable phospholipid to form membrane binding sites for LDH in normal and pathological conditions (Terlecki et al., 2006).

Reduction in plasma cholesterol levels, HDL-cholesterol and LDL-cholesterol in SCA patients have also been reported (Rahimi et al., 2006; Zorca et al., 2010). However, even in the presence of low concentrations of LDL-cholesterol, it is believed that plasma LDL from patients with SCA is more susceptible to oxidation (Belcher et al., 1999).

The lipid alterations in SCA are interrelated to apolipoprotein content too. Levels of apolipoproteins A-I (ApoA-I), and B (ApoB), are different of those found in normal individuals, and the symptons of SCA may be more aggressive depending on these levels (Yuditskaya et al., 2009). Apolipoprotein E (ApoE) plays an important role in the metabolism of lipids and lipoproteins. Three major isoforms of ApoE derive of three alleles (e2, e3, and e4) at a single gene locus on chromosome 19q13.2 (Rahimi; Vaisi-Raygani; Pourmotabbed, 2011). ApoE polymorphism mays interfere in the transport of cholesterol and other lipids between liver and peripheral tissues, and its allelic variation is associated to plasma lipid levels (Hixson & Vernier, 1990; Eichner et al., 2002; Yen et al., 2007). ApoE polymorphism may also be related to phenotypic diversity of SCA, and may act as a candidate to connect SCA, lipid alterations and associated pathologies, being a possible genetic factor that contributes to the different expression forms of this disease that varies of an asymptomatic presentation, mild, to a very severe form with multi-organ damage and high mortality (Adams et al., 2003).

However, studies that investigate the relationship between ApoE and lipids from plasma of SCA patients are very scarce; besides it is unheard the evaluation of the influence of ApoE alleles on lipids from RBCmb. Therefore, this study aimed to investigate the possible influence of ApoE alleles on lipids of plasma and RBCmb from SCA patients, including levels of oxLDL and subclasses of phospholipids. It was also objective of this study the investigation of a possible correlation between levels of PS from RBCmb of SCA patients and plasma LDH, in the presence of the three ApoE alleles.

METHODS

Patients

60 patients with SCA (HbSS), both sex, aged 18 years and over who were studied at steady state at Fundação de Hematologia e Hemoterapia de Pernambuco (HEMOPE). Identification of homozygous for “A” or “S” was obtained by hemoglobin electrophoresis or HPLC. Serologic results for hepatitis B antivirus (anti-HBV), the hepatitis C antivirus (anti-

HCV), human T-type antivirus lymph cells (anti-HTLV 1 and 2) and serological tests for syphilis (VDRL) were not reagents. Patients were excluded if they had others hemoglobinopathies associated, smoking or alcohol consumption. All subjects provided medical histories, using a standardized questionnaire, and provided blood samples. The Ethics Research Committee of the institution approved this protocol (nº.050/2011) and informed consent was obtained from each participant.

Blood Samples and Biochemical Analysis

Samples were obtained in patients fasting for 12 hours. Peripheral blood was collected into vacuum tubes (VACUETTE®) containing anticoagulant EDTA-$K3^+$ (1.8 mg/mL). Samples were used within 4h. LDH was used as a marker for intravascular hemolysis and its plasma levels were quantified by automated spectrophotometry (Cobas C501, Roche, Diamond Diagnostics, USA). Plasma levels of Total Cholesterol (TC), Triglycerides (TG) and HDL-cholesterol (HDL-c) were determined by enzymatic spectrophotometry (*Labtest Sistemas Diagnósticos Ltda.*). LDL-cholesterol (LDL-c) and VLDL-cholesterol (VLDL-c) were determined by Friedewald equation [LDL-c = TC – HDL-c – VLDL-c; VLDL-c = TG/5]. Plasma ApoA-I and ApoB levels were measured by immuneturbidimetric assay (Roche, USA) using Cobas c501. Plasma levels of oxLDL were assessed by Enzyme-Linked Immunosorbent Assay – ELISA – Mercodia (Sweden).

Plasma was isolated after centrifugation (Sorvall, USA) at 1500 x g for 15 minutes. RBC were washed four times with cold isotonic saline buffer for removal of plasma lipoproteins and leukocytes (Nelson, 1967). The lipids were extracted from both erythrocyte membrane and plasma as described by Folch (1957), with chloroform: methanol (2:1, v/v). The main classes of phospholipids present in the plasma and erythrocytes were estimated following separation by one-dimensional and two-dimensional thin-layer chromatography (TLC), respectively. The TLC plates (20 x 20 cm) covered with 0.25 mm H silica gel 60 was used plasma (Merck, Brazil) while H silica gel with 2.5% magnesium acetate was used to apply lipid extract erythrocytes. In detail, after activation of the TLC plates at 120º for 30 minutes, 50 µL of each reconstituted lipid extract was applied by means of Hamilton microsyringe as a 1cm long streak at the inferior the plate. TLC tanks lined with filter paper (Whatman filter paper (No 1)) were filled with the appropriate solvent mixture and allowed to settle for 30 min before the thinlayer plate was introduced. The solvent mixtures used for the first dimensional were chloroform-methanol- ammonia (65:35:5 v/v) and chloroform-acetone-

methanol-acetic acid-water (50:20:10:10:5 v/v) were used for second dimensional. Plasma TLC using the same solvent mixture for the second dimension erythrocytes. The solvent front was allowed to run 10 cm. The plate was then air-dried and transferred to a tank containing iodine crystals until the spots just became visible. The spots were outlined with a needle, the residual iodine was allowed to evaporate in air, and the spots were scraped off the plate and transferred to Pyrex test tubes. After, 0.3 mL of concentrated sulfuric acid was added to each tube. The tubes were heated to 180°C for 2 hours in an aluminum block. Add 2 drops of hydrogen peroxide and remained in the aluminum block for an additional 2 hours (Moore, 1968; Nelson, 1967). Inorganic phosphorus of the samples was quantified by the method of Bartlett (1959), based on a calibration curve prepared using inorganic phosphorus standard solution (2µg/mL) and total phospholipids (TPL) were measured from the lipid extract. Absorbance was measured in spectrophotometer at λ=735. Classes of phospholipids were analyzed plasma phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM) and lysophosphatidylcholine (LPC), whereas erythrocyte phospholipid classes studied PC, PE, SM, LPC, phosphatidylserine (PS) and phosphatidic acid (PA). All solvents were from Merck grade for analysis. Standards of phospholipids were from Sigma Chemical Company. The determination of cholesterol content of the RBC membranes was assessed by spectrophotometry using colorimetric method (*Labtest Sistemas Diagnósticos Ltda.*).

Alleles of Apolipoprotein E

Genomic DNA was extracted from leukocytes in sample of whole blood, following a standard salting-out technique (Miller, Dykes & Polesky, 1998). Genotypes for APOE polymorphisms (rs7412 and rs429358) were detected by polymerase chain reaction (PCR) (Kim et al, 2010). The amplified fragments were then digested with the enzyme HhaI (5 Units) for three hours and the restriction fragments were identified with 4% agarose gel electrophoresis and ethidium bromide staining (0.5 mg/L). Genotyping was performed with blinding to subject identity. Sequence-proven controls were run with each PCR. A random 1/24 of samples were genotyped again on another day; no discrepancies were observed.

Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM). Differences between all groups were analyzed for One-Way ANOVA followed by Fisher's Test. All

analysis were adjusted for age, sex and BMI. P <0.05 implied statistical significance. All analyses were carried out using software StatView, version 4.5, Abacus Concept, Inc, Berkeley, CA.

RESULTS

Allele Frequencies of ApoE in SCA Patients

The allele frequencies of ApoE were in Hardy-Weinberg equilibrium (p = 0.9385), as shown in Figure 1. The relative frequency (0.67) of e3 allele was the highest. e2 had a frequency relative of 0.17; and e4 of 0.16.

Influence of ApoE Alleles on Cholesterol Levels from Plasma and RBCmb of SCA Patients

Plasma TC was lower in SCA patients carrying e4 (116.9 ± 3.4 mg/dL) and e2 (119.7 ± 4.5 mg/dL) alleles than in those carrying e3 (128.4 ± 2.3 mg/dL; p = 0.0082 and p = 0.0390). On the other hand, e4 carriers presented the highest values of RBCmb cholesterol (1.33 ± 0.03 μg/mL), when compared to the presence of e2 (1.25 ± 0.03 μg/mL; p = 0.0299) or e3 (1.23 ± 0.02 μg/mL; p = 0.0005) alleles, as seen in Figure 2.

Association Between ApoE Alleles and Plasma Levels of Cholesterol from Lipoproteins, Triglycerides, and Apolipoproteins in SCA Patients

Plasma levels of HDL-c, LDL-c, VLDL-c, and TG are shown in Table 1. SCA patients with e4 allele presented lower levels of HDL-c than e2 carriers. In turn, patients with e2 allele had lower levels of LDL-c, VLDL-c, and TG than those found in the presence of e3 allele. Table 1 also shows the plasma concentrations of ApoA-I and ApoB; and e4 allele was related to the lowest values of these apolipoproteins. e2 carriers had higher concentrations of ApoA-I in comparison to the presence of e3 allele, however did not present differences in relation to ApoB levels when compared to e3 carriers.

Role of The ApoE Alleles on The Levels of ox-LDL in SCA Patients

The presence of e4 allele increases significantly the plasma levels of ox-LDL (81.9 ± 5.6 U/L) in comparison to the other alleles (e2 = 61.4 ± 4.3 U/L, and e3 = 67.0 ± 3.8 U/L; p = 0.0417 and p = 0.0347, respectively), as demonstrated in Figure 3. There was not statistical significance between levels of ox-LDL from e2 and e3 carriers (p = 0.4970).

Relationship Between ApoE Alleles and Plasma and RBCmb TPL and Their Respective Subclasses in SCA patients

Table 2 presents the plasma and RBCmb concentrations of TPL and subclasses. It was not observed a significant difference among the ApoE alleles in the concentrations of TPL from plasma, as well as in the concentrations of TPL from RBCmb. However, e4 allele carriers presented the lowest values of plasma PC, LPC, and SPH; besides e4 allele was related to increase of LPC and SPH in RBCmb from these patients in comparison to the values identified in RBCmb from e3 carriers. The presence of e2 allele was also related to increase of LPC and SPH from RBCmb of SCA patients. As for PE, it was not found any statistical difference in its plasma or RBCmb levels among the alleles of ApoE.

Correlation Between Levels of RBCmb Phosphatidylserine and Plasma Lactate Dehydrogenase in The ApoE Alleles of SCA patients

In relation to PS, e4 allele was associated to higher concentrations of PS in RBCmb, when compared to e3 or e2 allele, as shown in Figure 4. The SCA patients with presence of e4 allele had a significant negative correlation between levels of RBCmb PS and plasma levels of LDH. No correlation was found in the presence of other alleles, regarding to PS from RBCmb and plasma LDH.

DISCUSSION

The prevalence of e4 allele of ApoE is in agreement with that found in others admixed Brazilian population, as reported by Mendes-Lana et al. (2007) and De França et al. (2004). Rahimi et al. (2011) reported a higher prevalence of e4 allele of ApoE in SCA patients from Iran, with an odds ratio of 5.9 folds of the presence of HbSS in patients carriers of e4 allele when compared to the presence of e3 allele. On the other hand, the frequency of e2 allele was higher in our population than in others, since a prevalence of 16% was found in SCA patients

for this allele. In the general, the relative frequency observed for e2 allele is less than 10%. Gómez-Coronado et al. (1999) have reported a frequency of 8% for e2 allele in an European population, in addition to data in Brazilian subjects, as mentioned by Mendes-Lana et al. (2007) who found a same prevalence, equal to 8%, and De França et al. (2004) who detected a frequency of 6%. Economou-Petersen et al. (1998), studying β-thalassemia, observed prevalences of 6.4% and 12.8% for e2 and e4 alleles, respectively.

e3 was the allele most prevalent, being the precursor of the common isoform of ApoE, conforms Hixson & Vernier (1990), and is associated with a lower risk of cardiovascular disease in the general population, probably because of its considerable normal action in the metabolism of lipids. ApoE3 presents a normal affinity by its cellular receptor, differently of the other isoforms. ApoE2 presents reduced affinity and is associated to decreased levels of cholesterol and LDL; while ApoE4 has a greater affinity by apoB,E receptors, and, in population studies, this isoform is related to increased levels of total cholesterol and LDL, being linked to higher cardiovascular risk. However our results point lower values of plasma cholesterol in the presence of e4 allele in SCA patients, and not merely in the presence of e2 allele, when compared to the values found in SCA patients with e3 allele. These data did not corroborate with Rahimi et al. (2011), when studied the influence of alleles of ApoE on plasma lipids in Iranians. These researchers reported that the presence of non-ApoE e4 allele resulted in a significant decrease in the levels of total cholesterol and LDL-cholesterol. In our population, beyond the lower levels of plasma cholesterol, the values of LDL-cholesterol did not differ statistically between e4 allele and e3 allele, although the levels found in the presence of e4 allele have been lower than those found in SCA patients carriers of e3 allele.

Naoum (2005) has highlighted that, in SCA and in thalassemia major, there is a reduction of the endogenous synthesis of cholesterol by liver due to injury that this organ suffers in these hemoglobinopathies. Naoum (2005) has also reported that hypocholesterolemia and decrease in the levels of plasma LDL-cholesterol may also be associated to an increase in the removal of plasma LDL-cholesterol to supply a higher synthesis of new membranes. We found that e4 carriers presented the highest levels of cholesterol in their RBCmb. Besides, e4 SCA patients had the highest values of ox-LDL. Thus, SCA patients with e4 allele presented a higher chronic oxidative stress and their plasma LDL were more susceptible to oxidation, which may relate this allele to development of vasculopathy in SCA. Jofre-Monseny et al. (2008) reported that the impact of ApoE polymorphism on diseases risk is in large part due to the impact on oxidative status and found that e4 allele was also associated to higher oxidative stress and to a more pro-inflamatory

state. Morris (2008) described that the inflammatory state related to vasculopathy in SCA depends on the crucial role of the depletion of nitric oxide in endothelial dysfunction, increasing the expression of adhesion molecules in RBCmb and in endothelial cells, increasing plasma levels of cytokines; and Sacre et al. (2003) showed that e4 allele may be a enhancer to diminished production of nitric oxide by activity of the nitric oxide synthase.

In contrast, Hoppe et al. (2001) showed a trend towards increased stroke risk in the presence of e3 allele in SCA patients. However, the highest values of cholesterol in RBCmb and the highest plasma levels of ox-LDL in e4 allele SCA patients lead to reinforce the risk for the numerous pathologies present in SCA. These data also contribute to support the hypothesis that there is no evidence that ox-LDL induces cholesterol depletion in membranes, despite changes in endothelial biomechanics, being in accordance with Levitan & Shentu (2011). Inclusive, Kucuk et al. (1992) showed a greater sterol content in sickle RBCmb compared to normal RBCmb, and a synergism between oxysterol-cholesterol.

e2 allele ApoE carriers had lower levels of cholesterol from β-lipoproteins, lower levels of triglycerides, and higher plasma concentrations of apoA-I, linking this allele to an anti-atherogenic effect, as reported by De França et al. (2004), independently of ethnic group. On the other hand, the diminished levels of HDL-cholesterol found in e4 allele also reinforce the risk for a poor prognosis in SCA patients, as assessed and demonstrated by Seixas et al. (2010), which reported more prevalence of pneumonia, endothelial dysfunction, cardiac abnormalities, and even greater need for blood transfusions. It can be estimated that this decrease in levels of HDL-cholesterol corroborated with the increase in the plasma levels of ox-LDL, and this was more a point to the fact that e4 allele was related to oxidative stress, since, as reported by Kotosai et al. (2013), plasma HDL reduces hydroperoxides from ox-LDL; and as the levels of HDL-cholesterol were lower in the presence of e4 than in the presence of e2 allele, in SCA patients, the status of oxidative stress may be greater in e4 carriers patients. In parallel, e4 allele was related to the lowest values of apoA-I, which, in accordance to Yuditskaya et al. (2009), are associated with pulmonary hypertension, endothelial dysfunction and vasculopathy in SCA. Tumblin et al. (2010) reported a strong relationship between decrease in plasma levels of ApoA-I and acute painful episodes. Furthermore, our results showed diminished levels of ApoB in SCA patients with allele e4, and this in addition to decrease in levels of ApoA-I may of indirect form to represent a renal damage, since ApoA-I and ApoB have their plasma clearance trough these organs. Emokpae et al. (2011) described that individuals with SCA may develop glomerulopathy with proteinuria and progressive renal failure.

Oxidative stress is thought to change the phospholipid content of RBCmb, as showed by Freikman et al. (2011). Connor et al. (1997) hypothesized that sickle RBCmb might have a distorted lipid composition, and our results showed that e4 allele of ApoE was more related to the modifying in plasma and RBCmb levels of phospholipids. e4 carriers had the lowest values of PC, LPC, and SPH from plasma; and in contrast their levels of LPC, SPH, and PS from RBCmb suffered a significant increase, which shows that at least the increase in the content of LPC and SPH in RBCmb may be consequence of the decrease of these phospholipid subclasses in plasma, as reported by Kuypers (2008). Thus, although there was not differences between TPL levels and ApoE alleles, alterations on phospholipid subclasses were found depending on polymorphism of this apolipoprotein. Besides, it was reported by Freikman et al. (2011) that RBCmb from patients with sickle cell disease presented around 3-fold more cholesterol content than TPL, and in our results we saw levels much more of total cholesterol in RBCmb from patients with e4 allele and without differences in TPL content.

There is no data about the role of ApoE polymorphism on phospholipids from plasma or from RBCmb, as in normal RBCmb as in sickle RBCmb. PC is the major phospholipid subclasse in eukaryotic cell membranes, as mentioned by Testerink et al. (2009), and we did not find a significant alteration in its levels in RBCmb. PC can be acquired by diet and can also be produced through PE, as cited by Li & Vance (2008). In SCA, we did not find a significant difference among ApoE alleles on plasma and RBCmb levels of PE.

SPH is the second most present in plasma lipoproteins, and in the plasma membranes, SPH is colocalized with cholesterol, interacting strongly each other, as reported by Nilsson & Duan (2006). Increased levels of SPH from sickle RBCmb were found in our study in correspondence to e4 allele presence, and they can be associated to the increase of cholesterol in these sickle membranes. It was also seen that e2 allele was related to higher levels of SPH in RBCmb of SCA patients. These lipid changes may alter the structure and may decrease the half-life of the RBC in conjunction to the genetic and biochemical disorders of HbSS, contributing for a bad prognosis in SCA, as reported by Kuypers (2008).

LPC is a major plasma lipid recognized as an important cell signaling, as mentioned by Schmitz & Ruebsaamen (2010). These researchers also pointed LPC like the major phospholipid component of ox-LDL and hence with an important role in acute and chronic inflammation state. e4 and e2 alleles presented higher levels of LPC in RBCmb than e3. Watanabe et al. (2002) and Matsumoto et al. (2007) emphasized the link between vascular smooth muscle cell proliferation and ox-LDL through LPC. Lima et al. (1998), Owen et al. (1996) and Lima et al. (2004) reported that LPC is a lysophospholipid derivative of PC, when

free cholesterol is esterified with fatty acid that provides of PC under catalytic action of lecithin-cholesterol acyltransferase (LCAT). So the fall in the levels of PC from plasma, in patients with e4 allele, should be followed by an elevation in the plasma levels of LPC. However, plasma LPC also fell. This corroborated with the described by Kuypers (2008), hence it is possible that plasma be acting as a source of phospholipids to the RBCmb.

The content of PS in RBCmb is one of the most highlited in many studies, as evidenced by Freikman et al. (2008), Connor et al. (1997), Tokumasu et al. (2009), Kuypers (2008), Wang et al. (1993), among others. These studies reported the asymmetric distribution of the anionic phospholipids, like PS. Normally, PS is localized in inner leaflet of the membrane; but when the RBCmb is sickled, PS may be on the cell surface, in outer leaflet of the plasma membrane. PS exposure on the cell surface is a normal process in hemostasis, however our results showed a higher concentration of PS in RBCmb from SCA patients with e4 allele when compared to the other ApoE alleles. This alteration in the quantity of PS may be related to a consequent disbalance of coagulator activity more pronounced in SCA patients carriers of e4 allele, since membranes from sickled shape cells have an increase in the chance of acquire a procoagulant surface with the presence of PS. Procoagulant property of PS was reported by Zwaal et al. (1989). PS is reported as a phospholipid subclasse with other properties too. Wang et al. (1993) showed that exposure of PS in erythrocites from SCA patients activates alternative complement pathway.

PS is also required as a cofactor for several important enzymes, conforms mentioned by Vance & Steenbergen (2005). SCA patients with e4 allele presented a correlation negative between RBCmb levels of PS and plasma levels of LDH. This enzyme is considered a marker of haemolysis, according to Kato et al. (2007), Zorca et al. (2010), Ballas et al. (2013). Inclusive, Kato et al. (2007) reported that plasma levels of LDH may be used to define SCA in two subphenotypes, one presenting viscosity vaso-occlusion and correspondent symptoms and other with haemolysis-endothelial dysfunction. It was interesting to note that as higher the values of PS in RBCmb, lower the plasma levels of LDH in the presence of e4 allele. This may be an important protective factor to maintain the RBCmb with some kind type of resistance to haemolysis, hence cytosolic LDH has strong affinity for to bind to PS that should also be increased in inner leaflet and not only in outer leaflet of the RBCmb of SCA patients carriers of e4 allele, in comparison to other alleles, since there was no correlation between PS and LDH in the presence of e2 neither e3. Terlecki et al. (2006) showed this strong binding of LDH to PS, and reported that this is an essential mechanism to retaining high activity of this enzyme in hostile conditions, like those that occur in SCA, low pH values, hypoxia, among

others, which are the main conditions where have the need of the catalytic action of LDH. This corroborated with Ballas (2013), when not only reported LDH as a marker of haemolysis and placed the increase of LDH not always because haemolysis and yes due to tissue damages that occur during painful vaso-occlusive crisis. Thus our results suggest that even in front of so many factors that enhance haemolysis and, the increase of PS in e4 allele patients may be helpful to a lower risk of a worse prognosis of SCA and its associated pathologies, like a compensatory mechanism.

Therefore, it is notable the relevant role of polymorphism of ApoE on abnormalities lipid from plasma and from RBCmb that are enrolled in SCA. This study provides the firsts informations about phospholipid subclasses and ApoE alleles in SCA, pulling out this theme of obscurity into the light, and reinforcing this genetic link with the prognosis of SCA patients and bringing up many factors that may be involved with this disease and that depends on the polymorphism of ApoE.

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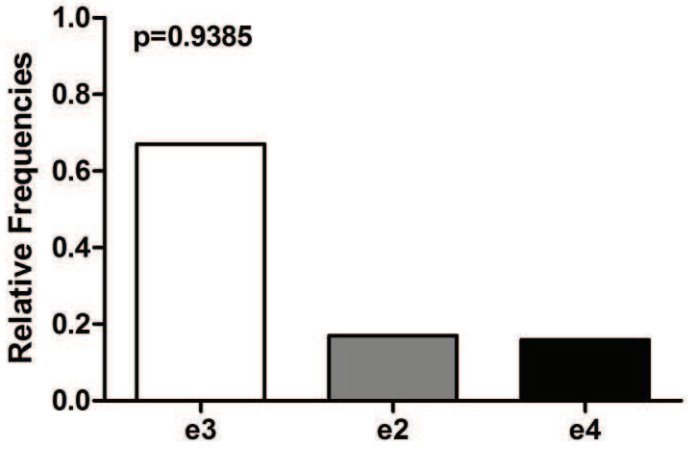

Figure 1. Allele relative frequencies (RF) distribution of apolipoprotein E in sickle cell anaemia patients.

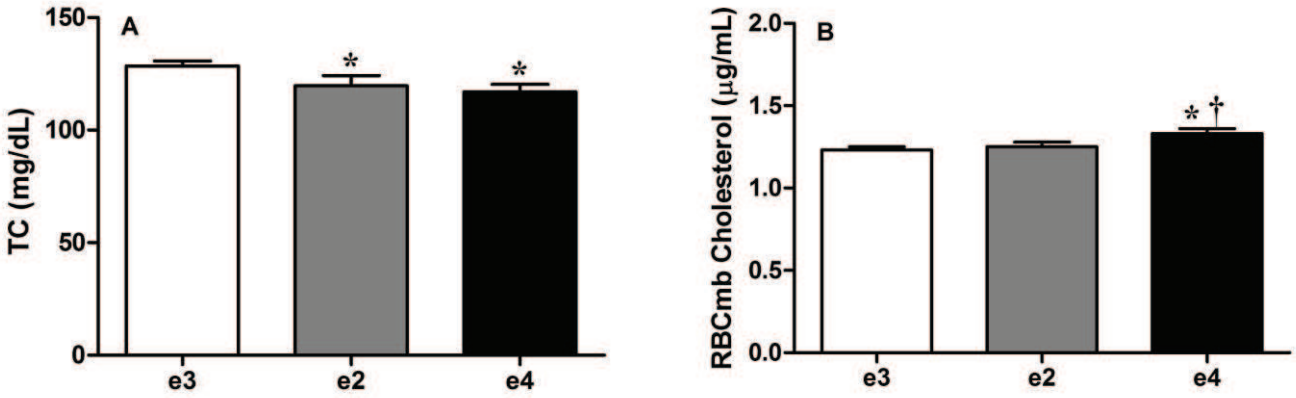

Figure 2. Influence of apolipoprotein E alleles on plasma and RBCmb cholesterol levels in sickle cell anaemia patients.

*$p<0.05$ for groups as compared with e3;

†$p<0.05$ for groups as compared with e2.

Table 1. Plasma levels of cholesterol from lipoproteins, triglycerides and apolipoproteinsA-I e B regarding to apolipoprotein E alleles in sickle cell anaemia patients.

Parameters	e3	e2	e4
HDL-c (mg/dL)	32.6 ± 0.7	34.4 ± 1.0	31.0 ± 1.6†
LDL-c (mg/dL)	73.8 ± 2.1	65.3 ± 3.6*	67.4 ± 4.1
VLDL-c (mg/dL)	24.1 ± 0.7	21.0 ± 1.3*	23.7 ± 1.8
TG (mg/dL)	119.3 ± 3.6	105.0 ± 6.6*	119.2 ± 8.9
ApoA-I (mg/dL)	108.2 ± 1.6	114.6 ± 3.4*	91.8 ± 2.5*,†
ApoB (mg/dL)	72.6 ± 1.4	73.6 ± 3.5	65.1 ± 2.4*,†

TG – triglycerides; ApoA-I – apolipoproteinA-I; ApoB- apolipoproteinB.

*$p<0.05$ for groups as compared with e3;

†$p<0.05$ for groups as compared with e2.

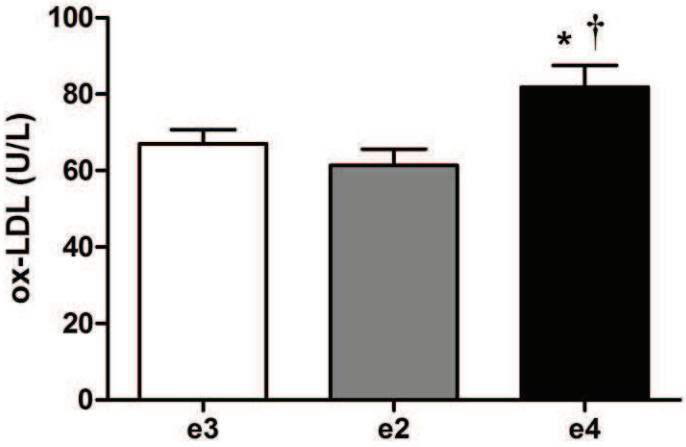

Figure 3. Role of the apolipoprotein E alleles on the levels of oxidized LDL in sickle cell anaemia patients.

*p<0.05 for groups as compared with e3;

†p<0.05 for groups as compared with e2.

Table 2. Plasma and red blood cell membrane levels of total phospholipids and subclasses according to apolipoprotein E alleles in sickle cell anaemia patients.

Parameters	e3	e2	e4
TPL (µg/mL)			
Plasma	99.3 ± 2.5	102.2 ± 3.7	93.2 ± 5.1
RBCmb	161.9 ± 3.1	165.5 ± 5.3	129.7 ± 1.7
PC (µg/mL)			
Plasma	47.2 ± 0.9	48.5 ± 1.5	41.4 ± 0.9*,†
RBCmb	29.7 ± 0.9	32.5 ± 1.9	32.2 ± 1.6
LPC (µg/mL)			
Plasma	12.5 ± 0.6	12.3 ± 0.9	9.9 ± 0.6*,†
RBCmb	4.6 ± 0.3	7.5 ± 1.2*	9.8 ±2.5*
SPH (µg/mL)			
Plasma	21.3 ± 1.1	18.8 ± 1.3	14.8 ± 0.8*,†
RBCmb	24.9 ± 0.7	27.6 ± 1.3*	29.7 ± 2.2*
PE (µg/mL)			
Plasma	11.0 ± 0.5	11.2 ± 0.9	9.9 ± 1.0
RBCmb	16.5 ± 0.9	13.8 ± 2.3	16.8 ± 1.2

*$p<0.05$ for groups as compared with e3;

†$p<0.05$ for groups as compared with e2.

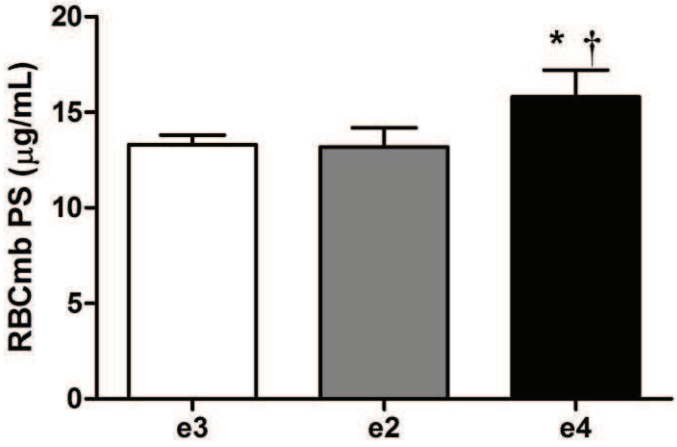

Figure 4. Relationship between apolipoprotein E alleles and red blood cell membrane phosphatidylserine levels in sickle cell anaemia patients.

*$p<0.05$ for groups as compared with e3;

†$p<0.05$ for groups as compared with e2.

Table 3. Correlation between the levels of red blood cell membrane phosphatodylserine and plasma lactate dehydrogenase in the apolipoprotein E alleles of sickle cell anaemia.

Parameters	r	95% CI	p
e3	0.088	0.081 – 0.253	0.3062
e2	0.119	-0.218 – 0.431	0.4919
e4	-0.530	-0.788 – 0.114	0.0149

IX. CONCLUSÕES

- Os resultados mostram a existência do terceiro subfenótipo clínico da anemia falciforme;
- As diferenças nos índices hematimétricos, em especial os valores de Hb e de RDW, mostram populações de eritrócitos heterogêneas entre os três subfenótipos;
- O subfenótipo SP apresentou maior comprometimento dos perfis lipídicos e apolipoprotéicos do que os demais grupos;
- As alterações nos fosfolipídios plasmáticos e de membrana eritrocitária foram mais proeminentes no subfenótipo SP;
- O metabolismo lipídico mais vulnerável no grupo SP indicam pior gravidade clínica;
- A anemia falciforme provoca diminuição de elasticidade de maneira independente dos subfenótipos clínicos;
- A anemia falciforme promove diminuição da fragilidade osmótica e permeabilidade ao glicerol de hemácias;
- O subfenótipo com predomínio hemolítico apresenta elevada resistência à hemólise entre os pacientes com anemia falciforme;
- Os níveis de colesterol, mas não os de fosfolipídios, de membrana eritrocitária estão diretamente relacionados à resistência ao rompimento da hemácia em pacientes com anemia falciforme;
- Os alelos da ApoE encontram-se distribuídos em pacientes com anemia falciforme dentro de uma variação esperada nas diversas populações;
- Pacientes com hemoglobinopatia SS possuem diferenças em seu metabolismo lipídico dependentes do alelo de ApoE;
- Alelo ε4 da ApoE associa-se com mudanças na composição lipídica da membrana de eritrócitos de pacientes com anemia falciforme, parecendo contribuir para uma maior incorporação de colesterol, além de uma maior quantidade de LPC, SM e PS;
- Pacientes com anemia falciforme portando o alelo ε4 da ApoE tiveram sobremaneira uma maior propensão a apresentarem LDL oxidada em seus plasmas;
- Alelo ε4 de ApoE influencia negativamente na liberação de DHL no plasma de pacientes com anemia falciforme, podendo contribuir para a modificação de suas características estruturais e funcionais, devido à elevação de PS na membrana dos eritrócitos.

X. APÊNDICES

APÊNDICE A - TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (para adultos)

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (para adultos)

Dados de Identificação

Título do Projeto: ***Investigação de Alterações do Metabolismo Lipídico em Subfenótipos com Predomínio Vaso-oclusivos ou Hemolíticos da Anemia Falciforme (HbSS)***

Pesquisador responsável: ***Profª Drª Vera Lúcia de Menezes Lima***

Instituição a que pertence: *Universidade Federal de Pernambuco (UFPE). Departamento de Bioquímica – Centro de Ciências Biológicas (CCB)*

Endereço da Instituição: *Av. Prof. Moraes Rego, s/n – Cidade Universitária. Prédio do Centro de Ciências da Saúde (CCS) – 2º andar. CEP: 50.670-420. Recife/PE.*

Telefone / Fax: (81) 2126-8541 / 2126-8540 ramal 202.

Eu, _____, aceito participar como voluntário (a) do estudo intitulado "Investigação de Alterações do Metabolismo Lipídico em Subfenótipos com Predomínio Vaso-oclusivo ou Hemolítico da Anemia Falciforme (HbSS)", recebi da Doutoranda em Bioquímica e Fisiologia, Priscila Pereira Passos, uma das responsáveis pela execução desta pesquisa, as seguintes informações que me fizeram entender sem dúvidas os seguintes aspectos:

1. O objetivo deste estudo é analisar o metabolismo lipídico de pacientes com diagnóstico de anemia falciforme que apresentam evolução benigna, quase assintomática, comparando com os pacientes que evoluem com quadro clínico de grande gravidade, sujeitos a inúmeras complicações e freqüentes internamentos.
2. Esta pesquisa poderá ou não trazer benefícios a minha pessoa no momento e, no entanto, ajudará futuros pacientes.
3. Esse estudo será realizado da seguinte maneira: *Primeiro*: será respondido um questionário, o qual demora cerca de 15 minutos. O questionário abrange informações sobre minhas condições sociais, econômicas, nutricionais e minha história / condição clínica para que possam ser correlacionados com os dados laboratoriais obtidos pela coleta de sangue; *Segundo*: Será verificada minha pressão arterial com esfigmomanômetro aneróide (aparelho mais comum usado para medir a pressão arterial) e estetoscópio (aparelho que também é usado para auscultar / escutar os batimentos cardíacos), serão medidos minha altura, cintura e quadril com fita métrica, e, meu peso corporal será verificado em balança destinada para esse fim; *Terceiro:* será realizada a coleta de 24 mL de sangue periférico de uma das veias do braço para que possam ser feitos os exames. A coleta de sangue será realizada por profissional capacitado e com todas as técnicas adequadas, dura no máximo 10 minutos e a quantidade de sangue que será retirada não fará falta alguma. Todo o material utilizado é estéril e descartável. Não há risco de contrair doenças. Entretanto, os riscos associados ao presente estudo são apenas os de uma coleta de sangue venoso, ou seja, raramente, pode acontecer o surgimento de uma mancha roxa no local da punção devido a um extravasamento de sangue, o qual será absorvido espontaneamente pelo próprio organismo. Em caso de dor após a punção, a mesma deverá passar com o uso de compressas geladas.
4. Meu prontuário será consultado para verificação de dados clínicos.
5. Sempre que eu desejar, a qualquer momento, terei esclarecimentos sobre cada uma das etapas do estudo, e que poderei recusar a continuar participando do estudo, bem

como retirar o meu consentimento, sem que isso traga qualquer prejuízo ao meu tratamento no HEMOPE.

6. As informações conseguidas através da minha participação não permitirão identificação da minha pessoa, exceto aos responsáveis pelo estudo, bem como a divulgação das informações mencionadas só será feita entre profissionais estudiosos do assunto, nos meios científicos.

Finalmente, após eu ter compreendido perfeitamente tudo o que me foi informado sobre a minha participação nesse estudo e estando consciente dos meus direitos, das minhas responsabilidades, dos benefícios que a minha participação implicam, concordo em dele participar e para isso ***DOU O MEU CONSENTIMENTO SEM QUE PARA ISSO EU TENHA SIDO FORÇADO OU OBRIGADO.***

ATENÇÃO: Para informar ocorrências irregulares ou danosas, dirija-se ao Comitê de Ética em Pesquisa do HEMOPE.
Localizado na Rua Joaquim Nabuco, nº 171. 2º andar. Fone: 3182-4771

Recife, ____ de _____ de 20____.

Assinatura ou Impressão Datiloscópica do (a) Voluntário (a)	Assinatura de um dos Responsáveis pelo Estudo

1ª Testemunha	2ª Testemunha

Demais pesquisadores inclusos nesse estudo:

Profº Dr. Aderson da Silva Araújo
Hematologista – Diretor de Hematologia
Rua Joaquim Nabuco, 171. Graças. Recife/PE. CEP: 52011-900.
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Profª Drª Bianka Santana dos Santos
Biomédica – Pós-doutoranda em Bioquímica e Fisiologia pela UFPE
Av. Prof. Moraes Rego, s/n – Cidade Universitária. Prédio do Centro de Ciências da Saúde (CCS) – 2º andar. CEP: 50.670-420. Recife/PE. Telefone / Fax: (81) 2126-8541 / 2126-8540 ramal 202.

Msc. Priscila Pereira Passos
Fisioterapeuta Respiratória – Doutoranda em Bioquímica e Fisiologia pela UFPE
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APÊNDICE B – FORMULÁRIO / QUESTIONÁRIO DE PESQUISA

FORMULÁRIO / QUESTIONÁRIO DE PESQUISA

Título do Projeto: ***Investigação de Alterações do Metabolismo Lipídico em Subfenótipos com Predomínio Vaso-oclusivos ou Hemolíticos da Anemia Falciforme (HbSS)***

Dados do (a) Voluntário (a):
Nome: _____ Registro: _____
Data de Nascimento: ___ / ___ / _____ Idade: _____ Sexo: M ( ) F ( )
Responsável: _____
Endereço: _____ Bairro: _____
Cidade/Estado: _____ CEP: _____ Fone: _____
Estado Civil: Solteiro ( ) Casado ( ) Outro ( )
Origem étnica: Branco ( ) Índio ( ) Negro ( ) Pardo ( )

Grau de Escolaridade: Ensino Fundamental: Incompleto ( ) Completo ( )
Ensino Médio: Incompleto ( ) Completo ( )
Ensino Superior: Incompleto ( ) Completo ( )

Renda Familiar (salários mínimos): Inferior a 1 salário ( ) 1 salário ( )
Valor de Referência: _____ 2 a 3 salários ( ) mais de 3 salários ( )

Aspectos nutricionais: Quantas refeições faz por dia ? 1 ( ); 2 ( ); 3 ( ); mais de 3 ( )
Toma algum tipo de suplemento ? Não ( ) Sim ( )

Hábitos de vida: É fumante ? Não ( ) Sim ( )
Faz uso de bebidas alcoólicas ? Não ( ) Sim ( )
Pratica atividade física ? Não ( ) Sim ( ). Qual? _____
Freqüência: 1x/sem ( ) 2x/sem ( ) 3x/sem ( )

Doença Falciforme: Idade do diagnóstico: _____
Critério: Eletroforese de Hb ( ) Teste de Falcização ( )
História familiar de DF: Não ( ) Sim ( ). Qual? _____
Parentesco: _____

Esquema Vacinal: Completo ( ) Incompleto ( )
Descrever: _____

Profilaxia Antibiótica: Não ( ) Sim ( )
Descrever: _____

Medicações em uso: Não ( ) Sim ( )
Qual(is)? _____

Transfusões de Sangue: Não ( ) Sim ( ) Quantas ? _____
Data da última transfusão: ___ / ___ / _____

Internamentos: Não ( ) Sim ( ) Quantos ? _____ Motivo: _____
Data do último internamento: ___ / ___ / _____

Cardiopatias associadas: Não ( ) Sim ( )
Qual(is)? _____

Hepatopatias associadas: Não ( ) Sim ( )

Qual(is)? _____

Diabetes: Não ( ) Sim ( )
Histórico familiar: Não ( ) Sim ( ) Grau de parentesco: _____

Dados Físicos: Peso: _____ Kg
Estatura: _____ cm; Cintura: _____ cm; Quadril: _____ cm
IMC: _____ Percentil: _____
PA: _____ mmHg

EVENTO	AUSENTE	PRESENTE
STA: Síndrome torácica aguda: dor torácica, infecção, infiltrado pulmonar respiratório recente no raio-X e febre.		Quantas vezes ? Data do último episódio: ___ /___ / _____
AVCi: Acidente vascular cerebral isquêmico: diagnosticado a partir de relato em prontuário médico confirmado por avaliação neurológica clínica ou por imagem;		
NACF: Necrose óssea ou asséptica de cabeça de fêmur: diagnosticada por exame de imagem ou parecer ortopédico;		
Priapismo: ereção dolorosa do pênis que pode ocorrer em episódios breves e recorrentes, ou episódios longos, podendo causar impotência sexual.		Quantas vezes ? Data do último episódio: ___ /___ / _____
SMP: Síndrome mão-pé: crise de dor, dactilite que ocorre nos pequenos ossos das mãos e pés, com edema destes membros.		Quantas vezes ? Data do último episódio: ___ /___ / _____
CVO: crise vaso-oclusiva consiste em admissão hospitalar por episódio doloroso na ausência de outra causa que não a doença de base, requerendo uso de medicação narcótica.		Quantas vezes ? Data do último episódio: ___ /___ / _____
Hipertensão Pulmonar: hipertrofia ventricular direita com refluxo de válvula tricúspide.		Quantas vezes ? Data do último episódio: ___ /___ / _____

APÊNDICE C – CARTA DE APROVAÇÃO DO COMITÊ DE ÉTICA

Fundação HEMOPE

1 - DADOS SOBRE O PROJETO **PARECER FINAL : Nº. 050/2011**

Título do Projeto: Investigação de alterações do metabolismo lipídico em subfenótipos com predomínio vaso-oclusivos ou hemolíticos da anemia falciforme (HbSS).
Instituição Solicitante: Universidade Federal de Pernambuco-UFPE e Fundação de Hematologia e Hemoterapia de Pernambuco -HEMOPE.
Pesquisadora: Vera Lucia de Menezes Lima
Identidade: 874590/SSP-PE **CPF:** 094.193.574-49 **Telefone:** 81- 2126-8541
Endereço: Av. Professor Moraes Rego, S/N – Cidade Universitária- Recife –PE
CEP: 50670-420
Orientada: Priscila Pereira Passos
Local de Desenvolvimento do Projeto: Departamento de Bioquímica do Centro de Ciências Biológicas e Diretoria de Hematologia do Hospital Hemope.
Finalidade: Pós-graduação em Bioquímica e Fisiologia- nível doutorado
Técnico responsável da Fundação Hemope: Dr. Aderson da Silva Araújo

2 - COMENTÁRIOS DOS RELATORES:
Objetivo Geral: Analisar o metabolismo lipídico de pacientes com diagnósticos de AF que apresentam evolução benigna, quase assintomática, comparando com os pacientes que evoluem com quadro clínico de grande gravidade, sujeitos a inúmeras complicações e frequentes internamentos.

3 - PARECER DO RELATOR: O Comitê de Ética em Pesquisa do Hemope (CEP), em cumprimento aos dispositivos da Resolução 196/96 e complementares, após acatar as considerações do relator, membro deste Comitê, relativamente às exigências apontadas no **Parecer nº. 050/2011**, considera **APROVADO** o protocolo de pesquisa supracitado, uma vez que este não colide, aparentemente com os princípios básicos da bioética – a não maleficência, a beneficência, a autonomia e a justiça, além do sigilo.

4 - INFORMAÇÕES COMPLEMENTARES:

- O sujeito da pesquisa tem a liberdade de recusar-se a participar ou retirar seu consentimento em qualquer fase da pesquisa, sem prejuízo ao seu cuidado (Res. 196/96 – Item IV.1.f), devendo receber uma cópia do Termo de Consentimento Livre e Esclarecido, por ele assinado (Item IV.2.d).
- O pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado e descontinuar o estudo somente após serem analisadas as razões da descontinuidade, pelo CEP, que o aprovou (Res. CND Item III. 1.z), exceto quando perceber risco ou dano não previsto ao sujeito participante ou, quando constatar a superioridade do regime oferecido a um dos grupos de pesquisa (Item V.3).
- O CEP deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (Res. CNS Item V.4). É papel do pesquisador assegurar medidas imediatas adequadas frente a evento adverso grave, ocorrido – mesmo que tenha sido em outro centro e enviar notificação ao CEP e ANVISA, junto com o seu posicionamento.

Fundação de Hematologia e Hemoterapia de Pernambuco – Comitê de Ética em Pesquisa
Rua Joaquim Nabuco, 171, Graças, Recife - PE - CEP: 52011-000
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Fax: (81) 3182-4660

PERNAMBUCO
GOVERNO DO ESTADO

Fundação HEMOPE

- Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas. Em caso de projetos do grupo I ou II apresentados anteriormente à ANVISA, o pesquisador ou patrocinador deve enviá-los também à ANVISA, junto com o parecer aprovatório do CEP, para serem juntadas ao protocolo inicial (Res. 251/97. Item III.2.e).
- **Relatórios parcial e final devem ser apresentados ao CEP, de acordo com os prazos estabelecidos na Resolução CNS-MS 196/96.**

Homologado na Reunião do CEP de 30/11/2011

Ana Lúcia de Sena
Coordenadora
Comitê de Ética em Pesquisa em Seres Humanos -HEMOPE

Fundação de Hematologia e Hemoterapia de Pernambuco – Comitê de Ética em Pesquisa
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PERNAMBUCO
GOVERNO DO ESTADO

APÊNDICE D – *Differential vulnerability of substantia nigra and corpus striatum to oxidative insult induced by reduced dietary levels of essential fatty acids.*

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ORIGINAL RESEARCH ARTICLE
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Differential vulnerability of substantia nigra and corpus striatum to oxidative insult induced by reduced dietary levels of essential fatty acids

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Oxidative stress (OS) has been implicated in the etiology of certain neurodegenerative disorders. Some of these disorders have been associated with unbalanced levels of essential fatty acids (EFA). The response of certain brain regions to OS, however, is not uniform and a selective vulnerability or resilience can occur. In our previous study on rat brains, we observed that a two-generation EFA dietary restriction reduced the number and size of dopaminergic neurons in the substantia nigra (SN) rostro-dorso-medial. To understand whether OS contributes to this effect, we assessed the status of lipid peroxidation (LP) and anti-oxidant markers in both SN and corpus striatum (CS) of rats submitted to this dietary treatment for one (F1) or two (F2) generations. Wistar rats were raised from conception on control or experimental diets containing adequate or reduced levels of linoleic and α-linolenic fatty acids, respectively. LP was measured using the thiobarbituric acid reaction method (TBARS) and the total superoxide dismutase (t-SOD) and catalase (CAT) enzymatic activities were assessed. The experimental diet significantly reduced the docosahexaenoic acid (DHA) levels of SN phospholipids in the F1 (∼28%) and F2 (∼50%) groups. In F1 adult animals of the experimental group there was no LP in both SN and CS. Consistently, there was a significant increase in the t-SOD activity ($p < 0.01$) in both regions. In EF2 young animals, degeneration in dopaminergic and non-dopaminergic neurons and a significant increase in LP ($p < 0.01$) and decrease in the CAT activity ($p < 0.001$) were detected in the SN, while no inter-group difference was found for these parameters in the CS. Conversely, a significant increase in t-SOD activity ($p < 0.05$) was detected in the CS of the experimental group compared to the control. The results show that unbalanced EFA dietary levels reduce the redox balance in the SN and reveal mechanisms of resilience in the CS under this stressful condition.

Keywords: substantia nigra, corpus striatum, oxidative stress, superoxide dismutase, catalase, lipid peroxidation, DHA, neurodegeneration

INTRODUCTION

Docosahexaenoic acid (DHA) and arachidonic acid (AA) are long chain polyunsaturated fatty acids (LC-PUFA) which play important roles as critical modulators of brain function under physiological or pathological conditions (Zhang et al., 2011). They are derived from the essential fatty acids (EFA) α-linolenic and linoleic acids, respectively, and can exert opposite effects on brain metabolism (Schmitz and Ecker, 2008). Imbalance in their levels, early in life, and especially DHA deficiency, can decrease anti-inflammatory responses that can induce neurodegeneration (Yavin, 2006; Schmitz and Ecker, 2008). Recent studies using microarray technology have shown that DHA is able to regulate the transcription of many genes related to oxidative stress (OS), cell signaling, and apoptosis (Kitajka et al., 2004; Lapillonne et al.,

2004; Yavin, 2006). Consistent with this evidence, it has been demonstrated that DHA protects against peroxidative damage of lipids and proteins in developing and adult brains in experimental models of ischemia-reperfusion (Glozman et al., 1998; Green et al., 2001; Pan et al., 2009; Mayurasakorn et al., 2011) or reduce OS-induced apoptosis of retina photoreceptors (Rotstein et al., 2003). Moreover, the DHA-derived docosanoid, named neuroprotectin D1, protects human retinal pigment epithelial cells from OS (Mukherjee et al., 2004) as well as inhibits brain ischemia-reperfusion-mediated leukocyte infiltration and pro-inflammatory gene expression (Marcheselli et al., 2003).

It is well established that OS is caused by the disequilibrium between the production and detoxification of highly reactive oxygen species (ROS), including singlet oxygen, superoxide anion, and hydrogen peroxide, which can disrupt the redox balance inside cells if not properly neutralized. The superoxide anion is known to induce protein and nucleic acid dysfunction and to initiate lipid peroxidation (LP) (Kohen and Nyska, 2002). Endogenous anti-oxidant mechanisms against superoxides include a series of linked enzyme reactions. The first of these enzymes is superoxide dismutase (SOD; EC1.15.1.1), that converts superoxide anion to hydrogen peroxide (H_2O_2), which can be removed by catalase (CAT; EC 1.11.1.6) and/or glutathione peroxidase (GPx; EC 1.11.1.9) (Kohen and Nyska, 2002; Melo et al., 2011).

Neuron response to OS is not uniform in the brain. This differential vulnerability depends on a number of factors including high intrinsic OS, high demand for ROS-based intracellular signaling, low ATP production, mitochondrial dysfunction, and high inflammatory response (Wang and Michaelis, 2010). Strong evidence indicates that OS may be one of the most important mechanisms involved in the etiology and evolution of a number of neurodegenerative diseases (Hashimoto and Hossain, 2011; Thomas and Beal, 2007; Melo et al., 2011). DHA is considered as a potential target for therapeutic intervention in some of these disorders, including Parkinson's Disease (PD), where the dopaminergic neurons of substantia nigra (SN) are especially affected by OS and mitochondrial dysfunction (Jenner et al., 1992; Sayre et al., 2001). In experimental models of PD, for example, it has been shown that the dietary supplementation of DHA may partially restore dopaminergic neurotransmission after 6-hydroxidopamine (6-OHDA)- or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced striatal lesions which produce OS (Bousquet et al., 2008; Cansev et al., 2008). Moreover, DHA supplementation is able to increase the SOD activity in the corpus striatum (CS) (Sarsilmaz et al., 2003) as well as significantly decrease cyclooxigenase-2 activity and prostaglandin E2 levels in the SN, decreasing MPTP-induced dopaminergic cell death (Ozsoy et al., 2011). Conversely, combination of successive parity and α-linolenic acid deficient maternal diet reduced the number of dopaminergic neurons in the rat SN pars compacta and ventral tegmental area of adult offspring (Ahmad et al., 2008).

Recent evidence from our laboratory, adopting a two generation model of EFA dietary restriction and stereological assessment, showed a differential vulnerability of two distinct SN dopaminergic cell populations to this type of nutritional insult. In addition to a reduction in the number of dopaminergic neurons in the SN rostro-dorso-medial region, this dietary treatment was able to change body and brain weights, TH protein levels, and the size of these neurons in young animals (Passos et al., 2012). The mechanisms involved in such effects are not yet completely understood. It is well established that under physiological conditions, the SN has unique biochemical features which provide a higher vulnerability to OS (Kidd, 2000) when compared to other brain regions, including the CS (Mythri et al., 2011). The present study was conducted to test the hypothesis that OS can be a potential mechanism involved in the neurodegeneration of SN dopaminergic cells induced by EFA dietary restriction. We tested whether this restriction for one or two generations could induce LP or modify the anti-oxidant activity of SOD or CAT in the SN and CS of rats.

MATERIALS AND METHODS

All procedures were approved by the Ethics Committee for Animal Research of Federal University of Pernambuco (protocol # 009428/200633), which complies with the "Principles of Laboratory Animal Care" (NIH, Bethesda, USA). Adult female Wistar rats weighing 200–250 g were fed from mating throughout pregnancy and lactation on a control or experimental diets, each containing approximately 400 Kcal 100 g and differing only in the lipid source. The diets were prepared according to Soares et al. (1995) and meet all current nutrient standards for rat pregnancy and growth (**Table 1**). The control diet contained 50 g/Kg of soybean oil with adequate amounts of saturated, monounsaturated, α-linolenic (6% of total fatty acids) and linoleic (56% of total fatty acids) acids. The experimental diet contained 50 g/Kg of coconut oil (from Babaçu, *Orbignia martiana*) with reduced levels of linoleic and α-linolenic acids and higher levels of saturated (2-fold) and monounsaturated (2.5-fold) fatty acids (**Table 2**).

Table 1 | Diet composition (grams/100g diet).

Ingredients	Control diet	Experimental diet
Casein	20.7	20.7
Cellulose	1.8	1.8
Corn starch	46.8	46.8
Sucrose	21.0	21.0
Soyabean oil	5.0	–
Coconut oil	–	5.0
Vitamin mix[a]	0.9	0.9
Mineral mix[b]	3.7	3.7
D.L-Cystine	0.1	0.1
Butyl hydroxytoluene	0.001	0.001
Kcal/100 g	399.1	400.5

[a] *Vitamin mixture (Rhoster Ind.Com. LTDA. SP. Brazil) containing (m%): folic acid (20); niacin (300); biotin (2); calcium pantothenate 160; pyridoxine (70); riboflavin (60); thiamine chloride (60); vitamin B12 (0.25); vitamin K1 (75). Additionally containing (UI%): vitamin A 40.000; vitamin D3 10.000; vitamin E (750).*

[b] *Mineral mixture (Rhoster Ind. Com. LTDA. SP. Brazil) containing (m%): CaHP04 (38); K2HP04 (24); CaCO3 (18.1); NaF (0.1); NaCl(7.0); MgO (2.0); MgS04 7H20 (9.0); FeS04 7H20 (0.7); ZnS04 H20 (0.5); MnSO+ H20 (0.5); CuS04 5H20 (0.1); Al2 (S04)3K2S04 24H20 (0.02); Na2SeO3 5H20 (0.001); KCl (0.008).*

Table 2 | Fatty acid composition of the diets (% of total fatty acids).

Fatty acids	Control diet	Experimental diet
8	0.02	3.27
10	0.03	3.95
11	nd	0.07
12	0.20	28.04
13	nd	0.06
14	0.19	19.56
15	0.02	0.02
16	9.27	11.32
17	nd	0.02
18	15.31	0.72
20	0.33	0.16
22	0.51	0.08
23	0.07	0.02
24	0.04	nd
Total saturated	**26.01**	**67.29**
16:1	2.72	0.06
18:1n9	9.36	23.51
20:1	0.24	0.16
Total monounsaturated	**12.32**	**23.73**
18:2n6	55.36	8.10
18:3n3	6.04	0.49
20:2	0.04	0.06
20:5n3	0.03	nd
22:2n	0.05	0.04
22:6n3	0.13	0.06
Total polyunsaturated	**61.65**	**8.75**
18:2n6 /18:3n3	9.17	16.39

nd, not detected. Bold values indicate $p < 0.001$.

Rat offspring ($n = 112$) were the object of the present study and only males were used for the experimental assays. Litters were culled to six pups on postnatal day 1 and weaned on postnatal day 21. Dams and pups were distributed into two main groups according to the nutritional condition: control (C) and experimental (E) rats. After weaning, pups were separated and fed *ad libitum* the same diet as their respective mothers. First generation (CF1 and EF1) male rats were weighed and evaluated for biochemical parameters related to LP and anti-oxidant markers at 90–110 days. The remaining males and females were allowed to mate to provide the second-generation groups (CF2 and EF2), which were analyzed at 30–42 days. In each group, animals were sampled randomly from different litters, housed three per cage in a room maintained at 22 ± 2°C with 67% relative air humidity and kept on a 12 h light/dark cycle (lights on 6:00 h).

Each experimental day, six animals per group were anesthetized with isofluorane and then decapitated. The regions containing the SN or CS were rapidly dissected in 0.9% (w/v) NaCl solution at 2°C. After weighing, the pooled tissue was homogenized in a 0.9% (w/v) NaCl solution (1:10) at 4°C and centrifuged for 10 min at 1000 g at 4°C for an analysis of LP for the determination of thiobarbituric acid-reactive substances (TBARS) level and for 10 min at 10,000 g at 4°C in order to assess either the total (Cu–Zn and Mn) superoxide dismutase (t-SOD) and catalase enzymatic activities. An aliquot of supernatant was analyzed for total protein content using a bicinchroninic acid protein kit (Sigma-Aldrich, St. Louis, MO).

LIPID PEROXIDATION

LP was measured by estimating malondialdehyde (MDA) using a thiobarbituric acid (TBA) reaction (TBARS method) according to Ohkawa et al. (1979). In the TBA test reaction, MDA or MDA-like substances and TBA react to produce a pink pigment with maximum absorption at 532 nm. The reaction was developed by the sequential addition of 0.2 mL of 8.1% sodium duodecil sulfate, 1.5 mL of 20% acetic acid (pH 3.5), and 1.5 mL of 0.8% TBA solutions in a boiling water-bath for 30 min to triplicates of supernatants. After tap water cooling, 1.5 mL of n-buthanol / pyridine (15:1 v/v) was added to the sample, centrifuged at 2500 g for 10 min and the organic phase was read at 532 nm using a plate reader. The results were expressed as nmol per mg of protein using a standard curve generated using different concentrations 1,1,3,3-tetramethoxypropane solution. The control SN and CS samples were incubated in a 30 μM sodium nitroprusside (SNP) solution for 45 min before the assay and used as positive controls for LP.

SUPEROXIDE DISMUTASE (SOD) ASSAY

Assessment of total SOD (t-SOD) enzymatic activity was performed according to Misra and Fridovich, (1972) at 25°C. Triplicates of SN or CS supernatants (100 μL) were previously incubated in a water bath at 37°C and then added to 880 μL of 0.05% sodium carbonate solution pH 10.2 in 0.1 mM EDTA. The reaction was developed by adding 20 μL of 30 mM epinephrine (in 0.05% acetic acid). The absorbance was measured at 480 nm for 4 min. One unit of t-SOD was defined as the enzyme amount causing 50% inhibition of epinephrine oxidation. Tissue t-SOD enzymatic activity was also expressed as units per milligram of protein (U/mg protein). Positive controls were obtained incubating control homogenate samples of SN and CS in a 30 μM SNP solution for 45 min before the enzymatic assay.

CATALASE (CAT) ASSAY

CAT activity was measured according to Aebi (1984). The rate constant k of H_2O_2 decomposition under our experimental conditions of temperature (∼20°C) and pH (7.0) was determined to be 4.6×10^7 by measuring the absorbance changes per minute, for 4 min. The enzymatic activity was expressed as the H_2O_2 consumed in nM/min/mg protein. Positive controls for catalase activity were obtained by incubation of SN and CS homogenates of the control group in increasing concentrations of H_2O_2 (3.156 to 100 μM) for 30 min at 37°C before the enzymatic assay.

STATISTICAL ANALYSIS OF OXIDATIVE STRESS PARAMETERS AND BODY WEIGHT

All biochemical experiments were carried out in triplicate and repeated at least twice. Six animals from three litters per group were used each time. A total of 38 and 58 animals were used in the F1 and F2 generations, respectively. Biochemical data of TBARS levels, t-SOD, and catalase enzymatic activity were plotted using GraphPad Prism 5.0 software and the statistical analysis

was performed using ANOVA followed by Tukey as the *post-hoc* test or Student's *t*-test in some cases. The analysis of body weight was carried out using unpaired Student's *t*-test. Differences were considered significant when $p < 0.05$.

FLUORO JADE C (FJC) ASSAY

Considering our recent evidence that a loss of SN dopaminergic cells is induced by EFA dietary restriction for two generations (Passos et al., 2012), FJC, a polyanionic fluorescein derivative, was applied to examine signals of neurodegeneration. It has been shown that this protocol specifically labels damaged neurons and not glial cells in the SN and CS (Bian et al., 2007; Ehara and Ueda, 2009) when these regions are submitted to certain types of insult, especially under conditions that induce OS (Ehara and Ueda, 2009; Li et al., 2009; Yang et al., 2011).

Animals from the F1 and F2 groups ($n = 6$/group) were anesthetized with a sodium pentobarbital solution (100 mg/kg, i.p. Sigma-Aldrich, St. Louis, MO), perfused with a 0.9% NaCl solution, followed by 4% paraformaldehyde in a phosphate buffered saline (PBS), pH 7.4. The brains were post-fixed in the same fixative for two hours, rinsed in a phosphate buffer (PB) and subsequently cryoprotected in solutions of 10, 20, and 30% sucrose in PB. Brain blocks were serially cut on a freezing microtome (Leitz Wetzlar) into 50 μm-thick sections in the parasagittal plane. All sections were collected serially in PB and arranged in six series. The Atlas of Paxinos and Watson (1986) was used to delimit cytoarchitectonic regions of interest. Sections of one series per animal were mounted on gelatin-coated slides, air-dried, and subjected to FJC staining according to Ehara and Ueda (2009). Slides were immersed in a 1% NaOH solution (in 80% ethanol) for 5 min, rinsed for 2 min in 70% ethanol, and for 2 min in distilled water, and then incubated in 0.06% potassium permanganate solution for 5 min. After water washing (2 min), the slides were immersed in a FJC solution (0.0001%) in 0.1% acetic acid for 10 min followed by washing in distilled water. The slides were air-dried on a slide warmer at 50°C for 30 min, cleared in xylene, cover slipped with Entellan (Merck). As a positive control for FJC labeling we used brain sections of rats previously treated with the mitochondrial toxin 3-Nitropropionic Acid (3-NP) which induces striatal neurodegeneration. The animals treated with 3-NP were from another study not related to the present work. As a better positive control for FJC labeling in the SN, we used also brain sections of animals which previously received intracerebral injections of pilocarpine in order to induce epilepticus status. The number of FJC-positive neurons was analyzed in the CS and SN in six animals of C and EF2 groups at the stereotaxic coordinate identified as corresponding approximately to lateral 1.9 mm (plate 81) according to Paxinos and Watson (1986).

Double fluorescence staining against FJC and tyrosine hydroxylase in brain sections of two EF2 animals was achieved by the method described by Ehara and Ueda (2009). Tissue sections were incubated first with blocking solution containing 1% BSA, 0.3% triton X-100 for 60 min and then with rabbit polyclonal anti-TH antibody (1:500; Millipore) for 24 h at 4°C. The sections were washed three times in phosphate buffer (PB) 0.1 M, pH 7.4, and incubated for 4 h with Rhodamine-conjugated 546-labeled anti-rabbit IgG (1:600; Jackson). After washing twice in PB, they were mounted onto gelatin coated slides and dried at 50°C for 30 min. The samples were rehydrated for 1 min, incubated in 0.06% potassium permanganate solution for 5 min, and then rinsed for 1 min in distilled water followed by FJC (0.0001% dissolved in 0.1% acetic acid) for 30 min. After rinsed in distilled water, the sections were dried at 50°C for 20 min, clearead in xylene for 1 min and coverslipped with Entellan. Fluoro-Jade C and TH in the CS and SN were analyzed using an epifluorescence microscope (Leica, DM LB).

FATTY ACID DETERMINATION IN THE CORPUS STRIATUM AND MIDBRAIN

The fatty acid profiles of CS and midbrain phospholipids were assessed in F1 groups at 95 days and F2 groups at 35 days of age. The pups ($n = 6$/group) were decapitated and the regions containing the CS or midbrain were rapidly dissected in an ice bath. The tissues were homogenized in a 50 mM Tris-HCl buffer (pH = 7.4) with EGTA and centrifuged for 30 min at 28,000 g at 4°C. The pellets were immediately re-suspended in 50 mM Tris-HCl buffer (pH = 7.4). The total lipids of CS or midbrain homogenates were extracted according to Folch et al. (1957). The phospholipids were then separated by means of a Sep-Pak procedure (Juaneda and Rocquelin, 1985) and transmethylated (Berry et al., 1965). These samples were analyzed using a Shimatzu GC apparatus equipped with a flame ionization detector and HP-inowax 20 M) capillary column (30 m × 0.32 mm × 0.3 μm). The column temperature was initially 40°C for 1 min, then increased to 150°C by 55°C/min, and finally increased to 220°C by 1.7°C/min. The injector and detector temperatures were 200 and 220°C, respectively. Hydrogen was used as the carrier gas at a flow rate of 1.0 mL/min; injection was in split-less mode and the injection volume was 1.0 μL of the sample isoctane extract. A standard fatty acid methyl ester mixture (Supelco™, 37 Component FAME mix, USA) was used to identify the fatty acid methyl esters by their retention time. Fatty acid data were expressed as percentage of total peak area. Data are expressed as the mean ± standard deviation (SD). Differences between the groups were analyzed by Student's *t*-test and considered significant at $P < 0.05$.

RESULTS

Data on body weights of F1 and F2 groups are presented in **Table 3**. Adult animals of the EF1 group and young animals of the EF2 group showed significantly lower body weights when compared to the control ($p < 0.05$).

Table 3 | Body weights of F1 and F2 animals.

Groups	Body Weight (g)	
CF1	402.54 ± 40.04	($n = 38$)
EF1	376.97 ± 36.92**	($n = 43$)
CF2	79.65 ± 14.87	($n = 33$)
EF2	71.91 ± 10.09*	($n = 43$)

Values are expressed as Mean ± SD.

**P < 0.05; **P < 0.01 Unpaired Student's t-test.*

CORPUS STRIATUM AND MIDBRAIN FATTY ACID PROFILE

Table 4 shows the midbrain fatty acid profile of F1 generation adult animals and **Table 5** combines data of midbrain and CS fatty acids of the F2 generation young animals raised under either control or experimental diets. As can be observed, the midbrain phospholipids from the EF1 and EF2 groups exhibit, respectively, 28 and 50% lower DHA levels (22:6n–3) as compared to their control groups. DHA levels were also lower in the EF2-CS phospholipids (∼50%) when compared to control. The reduced levels of DHA in both EF1 and EF2 groups was accompanied by a significant increase in the docosapentanoic fatty acid (DPA; 22:5n6) contents (2-tail *t*-test, $P < 0.001$). On the other hand, the values for AA (20:4n–6) did not differ between both groups of F1 or F2 generations. With respect to saturated and monounsaturated fatty acids, the presence of coconut oil in the maternal diet significantly increased the levels of palmitic (16:0), stearic (18:0), palmitoleic (16:1), and oleic (18:1n9) acids (2-tail *t*-test, $P < 0.01$) in the EF2 midbrain phospholipids.

Table 4 | Fatty acid composition (% of total) in midbrain phospholipids of F1 generation groups raised on Control or Experimental diets.

Fatty acid	Midbrain	
	Control diet	Experimental diet
C16	16.41 ± 1.9	15.85 ± 0.81
C16:1	0.96 ± 0.34	1.10 ± 0.28
C18	22.47 ± 1.63	23.99 ± 1.87
C18:1n9	24.55 ± 0.96	24.64 ± 2.00
C20	0.74 ± 0.13	0.91 ± 0.10
C20:1	2.04 ± 0.21	2.83 ± 0.12
C20:4n6	8.76 ± 0.32	8.73 ± 0.30
C20:3n6	0.45 ± 0.30	0.35 ± 0.24
C22	0.97 ± 0.53	0.80 ± 0.11
C23	3.11 ± 0.51	3.10 ± 0.52
C22:5n6	1.03 ± 0.74	3.16 ± 0.75***
C22:6n3	14.41 ± 1.81	11.25 ± 0.69**
C24:1n	2.24 ± 0.52	1.07 ± 0.18

Values are expressed as means ± SD.
***p < 0.01; ***p < 0.001.*

LIPID PEROXIDATION AND T-SOD ENZYME ACTIVITY IN ADULT ANIMALS OF F1 GENERATION

Biochemical results of the F1 groups are summarized in **Figure 1**. As expected, LP (measured as TBARS levels) was found to be significantly increased in SN (0.770 ± 0.136 nmol MDA/mg protein) and CS (0.834 ± 0.140 nmol MDA/mg protein) homogenates of CF1 group previously treated with 30 μM SNP, compared to the control condition (0.425 ± 0.105 and 0.532 ± 0.015 nmol MDA/mg protein for SN and CS, respectively; $P < 0.001$). However, TBARS levels in both regions were not modified in rats fed on the experimental diet (0.494 ± 0.089 and 0.570 ± 0.038 nmol MDA/mg protein for SN and CS, respectively) when compared to the control animals (**Figure 1A**). Consistent with these results, a significant increase in the t-SOD enzyme activity was observed in the EF1 group ($P < 0.01$) either in the SN (0.735 ± 0.020 U/mg protein) or CS (0.640 ± 0.192 U/mg protein) compared to the control condition not submitted to pre-treatment with SNP (0.606 ± 0.028 and 0.355 ± 0.034 U/mg protein for SN and CS, respectively). As can be observed, the SNP treatment used as a positive control, significantly increased SOD activity in the SN (1.241 ± 0.206 U/mg protein) and CS (1.832 ± 0.046 U/mg protein).

LIPID PEROXIDATION, T-SOD, AND CAT ENZYME ACTIVITIES IN YOUNG ANIMALS OF F2 GENERATION

In young animals of the F2 generation, distinct effects were induced by the experimental diet in the two regions analyzed. Evidence of LP, assessed by a significant increase in TBARS levels, was detected in the SN of EF2 group (0.564 ±

Table 5 | Fatty acid composition (% of total) in Corpus Striatum and Midbrain membrane phospholipids of F2 generation groups raised on Control or Experimental diets.

Fatty acid	Corpus Striatum		Midbrain	
	Control diet	Experimental diet	Control diet	Experimental diet
C16	17.99 ± 1.04	21.74 ± 0.74	16.51 ± 1.90	24.09 ± 0.27*
C16:1	0.73 ± 0.10	0.73 ± 0.12	0.64 ± 0.04	0.85 ± 0.03
C17	Nd	nd	0.16 ± 0.00	0.20 ± 0.02
C18	25.74 ± 0.25	25.20 ± 0.51	24.88 ± 0.67	29.09 ± 0.37*
C18:1n9	17.07 ± 0.34	15.18 ± 0.69	16.38 ± 0.25	19.21 ± 0.21*
C18:2n6t	nd	nd	0.74 ± 0.17	0.65 ± 0.22
C20:1	nd	nd	0.62 ± 0.10	0.60 ± 0.05
C20:4n6 (AA)	14.08 ± 0.27	13.12 ± 0.34	13.74 ± 0.83	14.93 ± 0.48
C23	3.88 ± 0.13	3.46 ± 0.18	3.98 ± 0.20	3.52 ± 0.19
C22:5n6	1.54 ± 0.06	9.60 ± 0.26**	1.49 ± 0.11	9.39 ± 0.31**
C22:6n3 (DHA)	19.23 ± 0.88	9.48 ± 0.84**	19.70 ± 0.69	8.70 ± 0.73**

Values are expressed as means ± SD.
**p < 0.01; **p < 0.001; nd, not detected.*

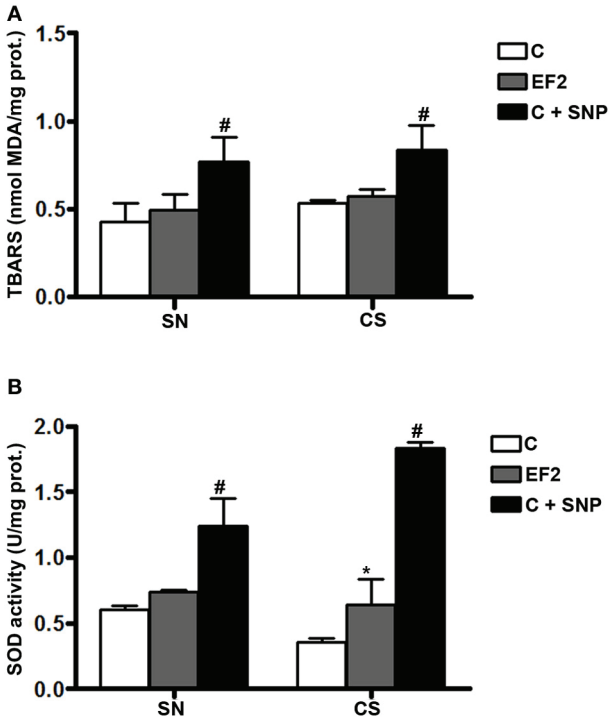

FIGURE 1 | Thiobarbituric acid-reactant substances (TBARS) levels (A) and total superoxide dismutase (t-SOD) activities (B) in the pool of Substantia Nigra and Corpus Striatum from first generation adult rats fed essential fatty acid restricted diet and controls ($n = 12$ per group). $^{*}P < 0.05$ compared to control group. Treatment of control homogenates with sodium nitroprusside (SNP) was used as positive control in all the experiments. #$P < 0.001$ compared to control or EF1 groups.

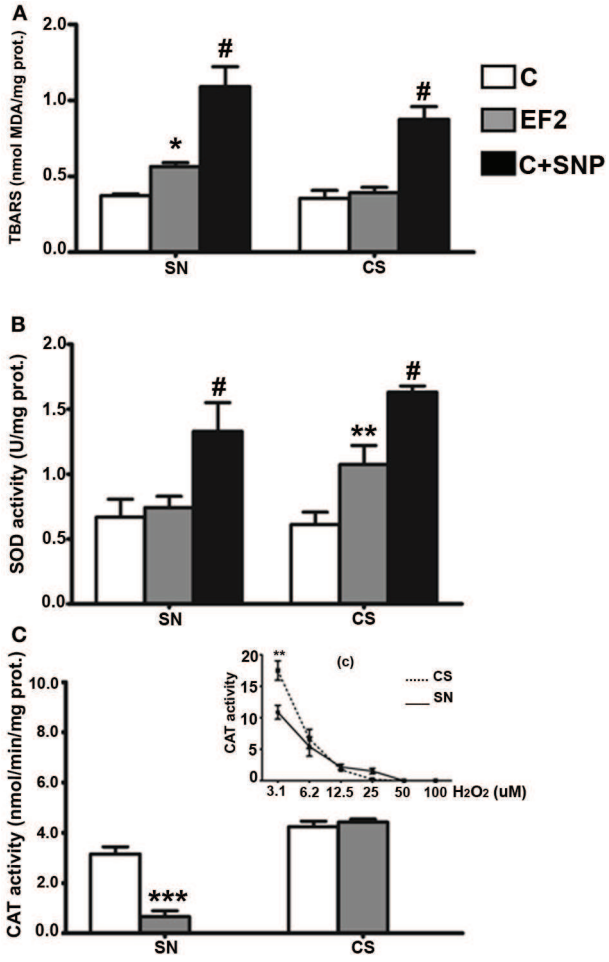

FIGURE 2 | Thiobarbituric acid-reactant substances (TBARS) levels (A), total superoxide dismutase (t-SOD) activities (B), and catalase (CAT) activities (C) in the pool of Substantia Nigra and Corpus Striatum from young rats fed an essential fatty acid restricted diet over two generations and respective controls ($n = 12$ per group). $^{*}P < 0.05$; $^{**}P < 0.001$ compared to control group. Treatment of control homogenates with sodium nitroprusside (SNP) was used as positive control for TBARS and t-SOD in all the experiments. The insert (c) in the panel **(C)** shows the H_2O_2 concentration-dependent manner of the CAT activity in the SN and CS control homogenates obtained as positive controls. #$P < 0.0001$ compared to control or EF2 groups.

0.02 nmol MDA/mg protein) in comparison with the control group (0.372 ± 0.01 nmol MDA/mg protein, $P < 0.05$). The magnitude of LP induced by the experimental condition in the SN is about 50% less than that obtained by using 30 μM SNP (1.330 ± 0.220 nmol MDA/mg protein). No difference between the EF2 (0.354 ± 0.005 nmol MDA/mg protein) and the C (0.391 ± 0.083 nmol MDA/mg protein) groups was found in the CS (**Figure 2A**). A significant increase in t-SOD enzyme activity was found in the CS of the EF2 group (1.074 ± 0.145 U/mg protein) compared to the control group in the absence of pretreatment with SNP (0.610 ± 0.096 U/mg protein, $P < 0.01$). Nevertheless, the increase in SOD activity in the EF2 group CS was smaller than that induced by 30 μM SNP in the C group (1.633 ± 0.046 U/mg protein). No difference between the groups was detected for t-SOD activity in the SN (0.741 ± 0.087 and 0.667 ± 0.138 U/mg protein for the EF2 and C groups, respectively) as shown in the **Figure 2B**. On the other hand, the CAT activity was significantly reduced in the SN of the EF2 group (0.652 ± 0.238 nmol/min/mg protein) compared to the control group (3.159 ± 0.279 nmol/min/mg protein in the control; $P < 0.001$). No difference between the groups was detected in CAT activity in the CS (4.339 ± 0.217 nmol/min/mg protein and 4.420 ± 0.125 nmol/min/mg protein for the EF2 and C groups, respectively) as shown in **Figure 2C**. The insert in the **Figure 2C** shows the H_2O_2 concentration-dependent manner of the CAT activity in the SN and CS control homogenates obtained as positive controls. As can be observed, at lower concentrations of H_2O_2, the CAT activity is significantly greater in the CS as compared to SN ($p < 0.05$) but this difference disappears at higher concentrations.

FLUORO JADE C AND TYROSINE HYDROXILASE LABELING

Fluoro-Jade C-positive cell bodies were not detected in the SN or CS in the groups (6 animals/group) of F1 generation (**Figure 3A**). In the SN of the EF2 group, several FJC-positive cells were seen either in the pars compacta or in the pars reticulata while no

labeling was detected in cell bodies of the CS in all animals ($n = 6$) analyzed (**Figure 3B**). In the EF2 group ($n = 6$), the number of FJC-positive cells distributed in the *pars compacta* and *pars reticulata* at the middle level of SN changed from 59 to 70 cells and the average number was estimated as 63.8 ± 6.4 cells.

Double fluorescence staining for FJC and TH of a representative EF2 animal is shown in the **Figure 4**. As can be seen, signals of degeneration were detected in SN dopaminergic and non-dopaminergic neurons either in the *pars reticulata* or in the *pars compacta*. Nevertheless, no staining for FJC was found in cell

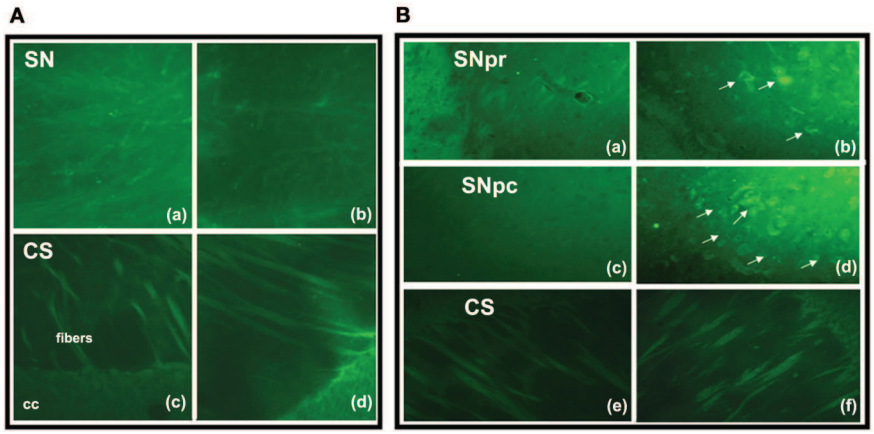

FIGURE 3 | Fluoro-Jade C staining in brain parasagittal sections of F1- (panel A) or F2- (panel B) generation groups at the level of Substantia nigra (SN) or Corpus Striatum (CS). No labeling was detected in cell bodies or processes of SN **(Aa, Ab)** and CS **(Ac, Ad)** in adult animals of F1 generation. However, FJC positive cell bodies and processes were detected in the SN pars reticulata **(Bb)** and pars compacta **(Bd)** of EF2 group while no labeling in these regions was seen in the controls **(Ba, Bc)**. No FJC labeling was detected in cell bodies of the CS in the EF2 **(Bf)** or control **(Be)** groups. A slight and non-specific labeling was seen in regions rich in myelin such as cerebral peduncle (cp), corpus callosum (cc), or myelinated fibers crossing the CS.

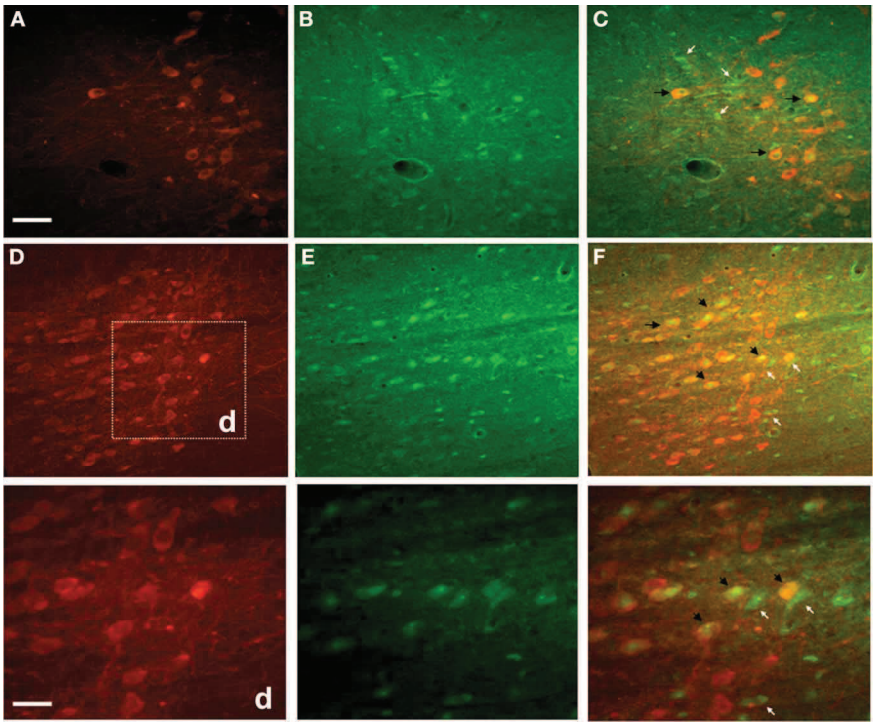

FIGURE 4 | Photographs of epifluorescence microscopy showing SN sections from a representative EF2 animal subjected to TH immunostaining followed by Fluoro-Jade C staining. Examples of single (FJC; yellow arrows) or double (TH + FJC; black arrows) labeled cells can be seen either in the SN *pars reticulata* (**A,B**, and **C**) or in the *pars compacta* (**D,E**, and **F**). High magnification of the region (d) is shown in the bottom panel. Scale bar of **A** = **B,C,D,E**, and **F** represents 60 μm while the scale bar of bottom panel represents 20 μm.

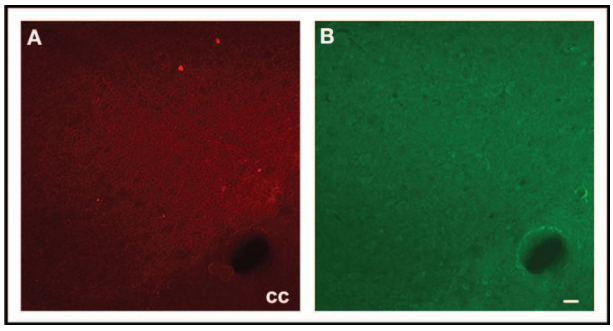

FIGURE 5 | Photographs of epifluorescence microscopy showing CS sections from a representative EF2 animal subjected to TH immunostaining (A) followed by Fluoro-Jade C staining (B). Note the absence of FJC-positive cells surrounded by TH-positive neuronal terminals. cc = corpus calosum, Scale bar = 40 μm.

bodies surrounded by TH-positive neuronal terminals in the CS, confirming data obtained using single labeling for FJC (**Figure 5**).

DISCUSSION

The current study investigated whether a dietary restriction of both linoleic and α-linolenic fatty acids for one or two generations could affect the redox balance in the SN and CS. We hypothesized that OS could be a potential mechanism involved in the loss of dopaminergic cells previously demonstrated (Passos et al., 2012). Our data showed signals of degeneration in SN dopaminergic and non-dopaminergic neurons and indicated a differential vulnerability of SN and CS to oxidative insult induced by two generations of EFA dietary restriction.

REPERCUSSION OF DIETARY TREATMENT ON BODY WEIGHT

The significant lower body weight gain of adult EF1 and young EF2 animals is in agreement with previous studies using coconut oil as the only source of dietary lipids (Deuel et al., 1954; Soares et al., 1995; Borba et al., 2010). Regarding this effect, this type of dietary treatment has been associated with dysfunction of growth hormone regulation (Soares et al., 1995). Moreover, it has been reported that coconut oil can reduce body weight due to high saturated medium chain fatty acids (8:0–14:0) turnover rates, which are predominant in its lipid profile. Such effect seems to be independent of essential fatty acid deficiency (Hargrave et al., 2005).

MIDBRAIN AND CORPUS STRIATUM FATTY ACID PROFILE

It has been demonstrated that a diet containing coconut oil as the only source of lipids depletes DHA in the brain more than a fat free diet, even for a short-term treatment, especially due to the diet's high content of saturated fatty acids (Ling et al., 2010). In the present study, the experimental diet based on coconut oil significantly reduced DHA levels about 28 and 50% in the midbrain phospholipids of the EF1 and EF2 groups, respectively, as compared to their controls. The DHA depletion was accompanied by a significant increase in DPA levels, which reinforces the condition of DHA deficiency. On the other hand, despite containing 8% linoleic acid (about 30% of recommended minimal dietary requirement for rodents (Bourre et al., 1990), the experimental diet did not modify the AA levels in either region of EF2 group. These results agree with other studies, indicating that AA is more tightly controlled than DHA in the central nervous system and that its brain concentrations are less vulnerable to limitations in the supply of precursor than other organs (Bourre et al., 1990; Brenna and Diau, 2007; Igarashi et al., 2009; Ling et al., 2010). In fact, recent evidence has indicated that even when using a diet containing 2.3% linoleic acid for 15 weeks, starting at weaning, the brain AA concentration is reduced by only 28%, while a 74% reduction has been observed in the liver of the same rats (Igarashi et al., 2009). Thus, in addition to DHA deficiency, our dietary treatment was able to increase AA/DHA ratio in the fatty acid profile of SN and CS phospholipids.

REPERCUSSION OF DIETARY TREATMENT ON LIPID PEROXIDATION AND ENZYMATIC ANTI-OXIDANT ACTIVITY

It has been established that an imbalance in the AA/DHA ratio and especially DHA deficiency can decrease anti-inflammatory and anti-oxidant responses and induce cellular damage in different classes of neurons (Yavin, 2006; Schmitz and Ecker, 2008). An inverse relation between the number of some brain neurons and increasing ratios of n–6/n–3 EFAs in the maternal diet has been also recently reported (Tian et al., 2011). In the present study, an increase in the t-SOD activity observed in the SN and CS of the EF1 group was able to protect these regions from membrane LP measured as TBARS levels. The absence of FJC labeling in neuronal cell bodies of both brain regions reinforces these results, considering the efficacy of this reagent in detecting signals of neurodegeneration induced by conditions of OS, such as ischemia (Yang et al., 2011), glutamate excitotoxicity (Ehara and Ueda, 2009) or dopaminergic lesions induced by 6-OHDA (Ehara and Ueda, 2009) or MPTP (Bian et al., 2007; Li et al., 2009).

EFA dietary restriction over two generations, which induced a more expressive DHA deficiency in midbrain phospholipids (∼50%) and AA/DHA ratio (∼2), was able to provoke LP and impaired the anti-oxidant responses at least in SOD and CAT enzymes in the SN of the EF2-group as compared to the control. Such results are consistent with recent evidence of the protective action of DHA dietary supplementation on SN cell populations under experimental conditions that induce OS, such as MPTP (Ozsoy et al., 2011). The lack of efficient t-SOD reactivity and the expressive reduction in the CAT activity observed in the EF2 group shows the vulnerability of SN to conditions that reduce DHA availability during the critical period of brain development. Studies on rats or human SN have indicated a progressive decrease in the activity of several anti-oxidant enzymes including SOD and CAT during physiological brain aging (Kolosova et al., 2003; Venkateshappa et al., 2012). The present findings in the EF2 young animals corroborate our initial hypothesis indicating that a decreased anti-oxidant function can be a potential mechanism by which long-term EFA dietary restriction induces loss of SN dopaminergic neurons (Passos et al., 2012). Thus, increased levels of OS in the young brain might act synergistically with other deleterious effects induced by DHA deficiency, accelerating the degenerative profile of SN. The FJC staining in the SN of EF2 animals reinforces these data, demonstrating the presence of

neuronal damage in several dopaminergic neurons either in the *pars compacta* or in the *pars reticulata*. Moreover, we also detected signals of degeneration in non-dopaminergic cells at the same regions of SN, suggesting that the oxidative insult induced by EFA dietary restriction affects neuronal populations with distinct neurochemical profile.

In contrast to the effects detected in the SN and despite a similar DHA deficiency, we did not observe LP or anti-oxidant dysfunction in the CS of the EF2 young rat brains, when compared to their respective controls. In support of this biochemical data, we did not find FJC-positive cell bodies in parasagittal or transversal sections of this nucleus. These findings reinforce some early and recent evidence in human and experimental animals that this region is more resistant than SN under physiological (Kolosova et al., 2003; Venkateshappa et al., 2012) or pathological conditions where SN dopaminergic neurons are affected (Floor and Wetzel, 1998; Mythri et al., 2011). The significant increase in the t-SOD activity in the CS of the EF2 animals indicates that this region has differential compensatory means which can be triggered from the insult induced by DHA deficiency. It is noteworthy that under normal conditions, dietary DHA supplementation, even for a short period (30 days), is able to increase the t-SOD activity in the CS of adult rats, which has been suggested as a potential regulatory action of this LC-PUFA on this enzyme (Sarsilmaz et al., 2003). If this is the case, our findings suggest that such action could be activated even under conditions of 50% DHA depletion in the CS phospholipids. A differential reactivity of CS under OS conditions was also recently reported: in animals injured with 6-OHDA, the dopamine turnover is significantly increased in this nucleus by fish oil supplementation (Delattre et al., 2010).

The increased t-SOD activity in the CS was not accompanied by a similar CAT reaction, which did not change its activity as compared to the control condition. These enzymes play complementary activities in the anti-oxidative defense system, considering that the H_2O_2 generated by SOD activity is the substrate for CAT. Thus, the absence of LP in the CS suggests that other anti-oxidant mechanisms involved in the degradation of H_2O_2 could be implicated in the relative resistance of this nucleus. An expressive increase in the total glutathione levels and in the glutathione peroxidase activity associated with glial cell proliferation has been found in the CS and frontal cortex of human postmortem PD brains (Mythri et al., 2011). Although future studies need to be carried out in order to address this issue in our experimental model, preliminary results of our group indicate that the glial cell reactivity might be also implicated in the lower vulnerability of CS to oxidative insult described herein.

Despite the resistance of CS to OS under the present experimental conditions, we cannot discard potential effects of EFA dietary restriction on other parameters involving neuroprotection in the nigrostriatal system. The anti-oxidant parameters here investigated under conditions of DHA deficiency probably are not working alone. A recent study demonstrated that a relatively short-term feeding of an α-linolenic acid-restricted diet was able to lower the DHA content and the brain derived neurotrophic factor (BDNF) levels in the mouse striatum, while two other brain regions were not affected (Miyazawa et al., 2010). Conversely, when DHA is supplemented in the diet, CS strongly reacts to OS induced by MPTP, increasing the synthesis of BDNF more than in control conditions (Bousquet et al., 2009). The disrupted relation between OS and neurotrophin availability could be involved in behavioral or neurochemical effects observed in animals or human beings submitted to EFA dietary restriction (Fedorova and Salem, 2006; Kuperstein et al., 2008).

CONCLUSION

The present data shows the importance of adequate dietary levels of EFA to maintain an effective redox balance in the SN. Our results demonstrate that LP associated with an impaired anti-oxidant response increases the vulnerability of SN dopaminergic and non-dopaminergic neurons to degeneration induced by long-term EFA dietary restriction. These results reinforce the hypothesis that this dietary treatment increases the risk of certain neurological disorders. The data also demonstrate that biological mechanisms of resilience can be activated in the CS even under a similar condition of DHA deficiency.

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APÊNDICE E – *Dopaminergic cell populations of the rat substantia nigra are differentially affected by essential fatty acid dietary restriction over two generations.*

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Dopaminergic cell populations of the rat substantia nigra are differentially affected by essential fatty acid dietary restriction over two generations

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ABSTRACT

Essential fatty acids play a crucial role in the activity of several neurotransmission systems, especially in the monoaminergic systems involved in cognitive and motor aspects of behavior. The present study investigated whether essential fatty acid dietary restriction over two generations could differentially affect dopaminergic cell populations located in the substantia nigra rostro-dorso-medial (SNrm) or caudo-ventro-lateral (SNcv) regions which display distinct neurochemical profile and vulnerability to lesions under selected pathological conditions. Wistar rats were raised from conception on control or experimental diets containing adequate or reduced levels of linoleic and α-linolenic fatty acids, respectively. Stereological methods were used to estimate both the number and soma size of tyrosine hydroxylase (TH)-immunoreactive neurons in the SNrm and SNcv. TH protein levels were assessed with Western blots. Long-term treatment with the experimental diet modified the fatty acid profile of midbrain phospholipids and significantly decreased TH protein levels in the ventral midbrain (3 fold), the number of TH-positive cells in the SNrm (~20%) and the soma size of these neurons in both SNrm (~20%) and SNcv (~10%). The results demonstrate for the first time a differential sensitivity of two substantia nigra dopaminergic cell populations to unbalanced levels of essential fatty acids, indicating a higher vulnerability of SNrm to the harmful effects induced by docosahexaenoic acid brain deficiency.

1. Introduction

The nervous system is predominantly composed of lipids containing saturated, monounsaturated and polyunsaturated fatty acids. It is well established that balanced dietary levels of these fatty acids are necessary for the structural, metabolic and developmental integrity of the brain (Uauy and Dangour, 2006; Innis, 2007). Long-chain polyunsaturated fatty acids (LC-PUFA) such as arachidonic acid (AA, 20:4*n*-6) and docosahexaenoic acid (DHA, 22:6*n*-3) are major components of neuronal membrane phospholipids and are synthesized by successive desaturation and elongation of the essential fatty acids (EFA) linoleic (LA, 18:2, *n*-6) and α-linolenic (LNA, 18:3, *n*-3) respectively, which must be provided by the diet (Schmitz and Ecker, 2008).

A growing body of evidence has indicated that several different fatty acids may act as neuroprotectors in the brain. For example, DHA has been implicated in reducing apoptosis and necrosis in different classes of neurons (Lang-Lazdunski et al., 2003; Kim et al., 2010) while AA can be a neurotrophic factor on sensory neurons (Robson et al., 2010). The saturated stearic acid (18:0) is able to protect cortical neurons against the effects of oxidative stress (Wang et al., 2007) and the monounsaturated oleic acid (18:1*n*9) can behave as a neurotrophic factor in primary cell cultures (Medina and Tabernero, 2002) or promote axonogenesis in the

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striatum during brain development (Polo-Hernández et al., 2010). LC-PUFA are functionally involved with several neurotransmission systems including the monoaminergic (Zimmer et al., 2000; Chalon, 2006; Vines et al., 2012) system. Decreased levels of DHA during brain development can affect serotoninergic and dopaminergic functions in cortical or subcortical regions, modifying several aspects of behavior (Levant et al., 2004; Yavin, 2006; Chalon, 2006; Kuperstein et al., 2008; Fedorova et al., 2009; Levant et al., 2010) and contributing to the etiology of some neurological diseases (McNamara and Carlson, 2006). Thus, the dietary supplementation of LC-PUFA, especially those of the *n*-3 family, has been indicated as a therapeutic strategy for a variety of these diseases (Heinrichs, 2010). In experimental models of Parkinson disease, for example, it has been shown that the dietary supplementation of DHA or fish oil may partially restore dopaminergic neurotransmission after 6-hydroxidopamine (6-OHDA) – or 1-metil-4-fenil-1,2,3,6-tetrahydropyridine (MPTP)-induced striatal lesions (Bousquet et al., 2008; Cansev et al., 2008). On the other hand, in a model that associated successive parity with a maternal diet containing deficient levels of α-linolenic acid, a reduced number of TH-immunoreactive neurons was detected in the offspring's substantia nigra pars compacta (SNpc) at postnatal day 70 (Ahmad et al., 2008). These data suggest that an adequate *n*-3 EFA dietary supply is necessary for the survival of dopaminergic neurons, at least from the SNpc. To our knowledge, no information is available on the vulnerability of other dopaminergic cell populations located in the substantia nigra (SN).

In the SN, dopaminergic cell populations are mainly distributed in two regions: the rostro-dorso-medial region of the SNpc (SNrm), and the caudo-ventro-lateral region (SNcv) including the SN pars reticulata and the ventrolateral region of pars compacta. Such populations differ in their ontogeny, morphological and neurochemical features (Bayer et al., 1995; González-Hernández et al., 2004; Duke et al., 2007) and their projections to corpus striatum can be segregated into distinct functional divisions (Joel and Weiner, 2000; Prensa and Parent, 2001). They also differ on their susceptibility to degeneration in Parkinson's disease in humans (Damier et al., 1999; Duke et al., 2007) and in rodent models (Rodríguez et al., 2001). According to these previous studies, dopaminergic neurons located in the SNcv are relatively more vulnerable to lesions and degenerate before the others. The distinct vulnerability of these dopaminergic populations has been ascribed to differences in their neurochemical profile, particularly the expression of calcium binding proteins, (Yamada et al., 1990; Gaspar et al., 1994), availability of the glial cell line-derived neurotrophic factor (GDNF) from the striatum (Barroso-Chinea et al., 2005), levels of plasma membrane dopamine transporter (Uhl et al., 1994; González-Hernández et al., 2004), increased expression of genes encoding pro-inflammatory cytokines and decreased expression of several glutathione-related genes (Duke et al., 2007).

Considering these neurochemical differences, the present study investigated whether the dopaminergic cells of the SNcv would be more vulnerable than those from the SNrm to the effects of a diet commonly used to induce EFA deficiency, due its reduced levels of both linoleic and α-linolenic acids and high contents of saturated fatty acids (Deuel et al., 1954; Hargrave et al., 2005; Ling et al., 2010). We also investigated whether this dietary treatment over two generations could affect the amount of the dopamine-synthesizing enzyme in the ventral midbrain.

2. Materials and methods

2.1. Animals and diets

Adult female Wistar rats weighing 200–250 g were fed from mating throughout pregnancy and lactation on a control or an experimental diet, both containing around 400 kcal/100 g and differing only in the lipid source. The composition of the diets and their fatty acids content are shown in Tables 1 and 2, respectively. The diets were prepared according to Soares et al. (1995), submitted to transesterification according to Hartman and Lago (1973) and analyzed using gas chromatography. The control diet contained 5% of soybean oil with adequate amounts of saturated, monounsaturated, α-linolenic and linoleic fatty acids. The experimental diet contained 5% coconut oil with reduced levels of linoleic acid (~7 times reduction, corresponding to about 30% of recommended minimal dietary requirement for rodents (Bourre et al., 1990)) and α-linolenic acid (~12 times reduction), and higher levels of saturated (2 fold) and monounsaturated (2.5 fold) fatty acids. The diets differed also regarding the linoleic/α-linolenic acids ratio which was 9:1 for the control and 16:1 for the experimental version. They were prepared at least twice a month and stored in a refrigerator at 4 ± 2 °C.

The offspring (*n* = 80 males) of both groups were the object of the present study. At parturition, litter size, total litter weight and mean birth weight of the pups was recorded. Litters were then reduced to 6 pups each, by keeping the median weighted animals (identified with distinct marks). Dams and male pups were divided into two main groups according to the nutritional condition: control (C) and experimental rats (E). After weaning, on postnatal day (P) 21, the rat pups were

Table 1
Diet composition (g/100 g diet).

Ingredients	Control diet	Experimental diet
Casein	20.7	20.7
Cellulose	1.8	1.8
Corn starch	46.8	46.8
Sucrose	21.0	21.0
Soyabean oil	5.0	–
Coconut oil	–	5.0
Vitamin mix[a]	0.9	0.9
Mineral mix[b]	3.7	3.7
D,L-Cystine	0.1	0.1
Butyl hydroxytoluene	0.001	0.001
kcal/100 g	399.1	400.5

[a] *Vitamin mixture* (Rhoster Ind.Com. LTDA, SP, Brazil) containing (*m*%): folic acid (20); niacin (300); biotin (2); calcium pantothenate 160; pyridoxine (70); riboflavin (60); thiamine chloride (60); vitamin B_{12} (0.25); vitamin K_1 (7.5). Additionally containing (UI%): vitamin A 40.000; vitamin D_3 10.000; vitamin E (750).
[b] *Mineral mixture* (Rhoster Ind. Com. LTDA, SP, Brazil) containing (*m*%): $CaHPO_4$ (38); K2HPO_4 (24); $CaCO_3$ (18.1); NaF (0.1); NaCl (7.0); MgO (2.0); $MgSO_4$ $7H_2O$ (9.0); $FeSO_4$ $7H_2O$ (0.7); $ZnSO_4$ H_2O (0.5); $MnSO^+$ H_2O (0.5); $CuSO_4$ $5H_2O$ (0.1); $Al_2(SO_4)_3K_2SO_4$ $24H_2O$ (0.02); Na_2SeO_3 $5H_2O$ (0.001); KCl (0.008).

Table 2
Fatty acid composition of the diets (% of total fatty acids).

Fatty acids	Control diet	Experimental diet
8	0.02	3.27
10	0.03	3.95
11	nd	0.07
12	0.20	28.04
13	nd	0.06
14	0.19	19.56
15	0.02	0.02
16	9.27	11.32
17	nd	0.02
18	15.31	0.72
20	0.33	0.16
22	0.51	0.08
23	0.07	0.02
24	0.04	nd
Total saturated	26.01	67.29
16:1	2.72	0.06
18:1*n*9	9.36	23.51
20:1	0.24	0.16
Total monounsaturated	12.32	23.73
18:2*n*6	55.36	8.10
18:3*n*3	6.04	0.49
20:2	0.04	0.06
20:5*n*3	0.03	nd
22:2*n*	0.05	0.04
22:6*n*3	0.13	0.06
Total polyunsaturated	61.65	8.75
18:2*n*6/18:3*n*3	9.17	16.39

nd = not detected.

separated and fed *ad libitum* the same diet as their respective mothers. They were the first generation (CF1 and EF1) and were allowed to mate at 90–110 days to provide the second generation groups (CF2 and EF2) which were analyzed at 30–42 days. In each group, animals were sampled randomly from different litters, housed three per cage in a room maintained at 22 ± 2 °C with 67% relative air humidity and kept on a 12 h light/dark cycle (lights on 6:00 h). All animal procedures were carried out in accordance with the norms of the Ethics Committee for Animal Research of Federal University of Pernambuco (CEEA) which specifically approved this study (protocol # 009428/200633), and complies with the "Principles of Laboratory Animal Care" (NIH, Bethesda, USA).

2.2. Fatty acid determination in the midbrain and cerebral cortex

The fatty acid profiles of midbrain and cerebral cortex phospholipids were assessed in F2 groups at 35 days of age. Pups were decapitated and the regions containing the midbrain or cerebral cortex were rapidly dissected in an ice bath. Tissues were homogenized in a 50 mM Tris–HCl buffer with EGTA and centrifuged for 30 min at 28,000 × *g* at 4 °C. Pellets were immediately resuspended in 50 mM Tris. Total lipids of brain homogenates were extracted according to Folch et al. (1957). Phospholipids were then separated by means of a Sep-Pak procedure (Juaneda and Rocquelin, 1985) and transmethylated (Berry et al., 1965). Samples were analyzed using a Thermo Trace Ultra GC apparatus equipped with a flame ionization detector and HP-20 (carbowax 20 M) capillary column (25 m × 0.32 mm × 0.3 μm). The column temperature was initially 40 °C for 1 min, then increased to 150 °C by 55 °C/min, and finally increased to 220 °C by 1.7 °C/min. The injector and detector temperatures were 200 and 220 °C, respectively. Nitrogen was used as the carrier gas at a flow rate of 1.0 ml/min; injection was in split mode (1:20) and the injection volume was 1.0 μl of sample isooctane extract. A standard fatty acid methyl ester mixture (Supelco, USA) was used to identify most of fatty acid methyl esters by their retention time. The docosapentanoic acid (DPA) of our samples was identified by using gas chromatography–mass spectrometry (GC-MS). Thus, the presence of DPA peak and its mass spectrum was compared with that obtained in the mass spectrum libraries of the equipment (Willey, Nist and Adams). Fatty acid data were expressed as % of total peak area. Data are expressed as the mean ± standard error of the mean (SEM). Differences between groups were analyzed by Student's *T* test. Differences were considered significant at $P < 0.05$.

2.3. Protein extraction and Western blotting analysis

Ventral midbrain homogenates containing samples of 6 animals per group were obtained at P35-42 from the CF2 and EF2 groups. Such samples were homogenized in freshly prepared 20 mM Tris/HCl buffer (pH 7.4) containing 10 mM $MgCl_2$, 0,6 mM $CaCl_2$, 0.5 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin and 0.05% Triton X-100. An aliquot was taken to determine protein content. An equal volume of sample buffer (62.5 mM Tris/HCl, pH 7.4, containing 4% SDS, 10% glycerol, 10% mercaptoethanol and 0.002% bromophenol blue) was added, and samples were boiled for approximately 3 min. Fractioning of protein samples was achieved using 10% polyacrylamide gels containing 0.1% SDS. After separation, the protein bands were transferred from the gel onto nitrocellulose paper, as described by Towbin et al. (1979). The nitrocellulose blots were incubated with mouse anti-TH polyclonal antibody (1:500; Chemicon, Temecula, CA, USA) or mouse anti-β-actin polyclonal antibody (1:5000; Chemicon, Temecula, CA, USA) and then incubated for 3 h at room temperature. They were subsequently exposed to mouse secondary antibodies conjugated to horseradish peroxidase and developed with a 0.16% solution of 3-amino-9-ethylcarbazole in 50 nM sodium acetate buffer (pH 5) containing 0.05% Tween-20 and 0.03% H_2O_2. Digital images of the blots were obtained and the integrated optical density was estimated by using Labworks software (UVP Products, CA). TH protein levels were normalized to that of the β-actin protein that was used as an internal standard.

2.4. Immunohistochemical procedures

Rats (seven animals per group) were anesthetized with sodium pentobarbital (60–80 mg/kg, i.p.) and perfused transcardially first with saline (0.9% NaCl; 50–100 ml) followed by 4% paraformaldeyde in 0.1 M phosphate buffer (PB), pH 7.4 (200 ml). Perfusion was always performed between 12:00 and 18:00, with a continuous infusion pump (Harvard equipment) through a cannula inserted into the left ventricle with a perfusion rate at 7.64 ml/min. After perfusion, the brains were dissected starting from the prefrontal cortex back to the inferior limit of the brainstem (the olfactory bulb and cochleas were excluded). They were then postfixed for 2 h in the same fixative, rinsed in PB and weighed (wet weight). Subsequently, the brains were cryoprotected in solutions of 10%, 20% and 30% sucrose in PB.

Brain blocks were serially cut on a freezing microtome (Leitz Wetzlar) into 50 μm-thick sections in the parasagittal plane, throughout the latero-medial extent of each hemi-brain. All sections were collected serially in PB and arranged in six series. The atlas of Paxinos and Watson (1986) was used to delimit the cytoarchitectonic regions. The series of sections used for immunohistochemistry was first treated with 0.01 M citrate buffer, pH 6.0, at 60 °C for 2 h. Thereafter, free-floating sections were rinsed in PB several times and incubated with rabbit anti-TH polyclonal antibody (Chemicon, USA) diluted 1:500 in PB containing 0.3% Triton X-100 (PBX) and 1% normal goat serum (NGS) for 24 h at 4 °C. Sections were then incubated for 1.5 h in secondary antiserum (biotinylated goat anti-rabbit IgG; Vector Labs, Burlingame, CA) diluted 1:200 in PBX, and processed for immunoperoxidase staining using the avidin biotin peroxidase complex (Standard ABC kit, Vector Labs). The binding of antibodies was revealed by the addition of diaminobenzidine tetrahydrochloride 0.05% (Sigma) and 0.01% H_2O_2 in PB, for 10 min. Subsequently, the free-floating sections were rinsed in PB and mounted on gelatin-coated slides. These procedures were carried out simultaneously in brain sections from both control and experimental animals. For the control of the staining specificity, some sections were subjected to an immunohistochemical procedure, omitting the primary antibody.

2.5. Stereological and morphometric analysis

Total estimates of the number of dopaminergic neurons in the SNrm and SNcv were obtained from seven brains per group processed for TH immunohistochemistry. In order to encompass the full latero-medial extent of the SN in the left side of the brain, six sections from a 1:6 series were analyzed for each brain. Optical fractionator sampling was carried out using a Nikon Eclipse 80 microscope equipped with an advanced scientific instrumentation motorized stage input into a high resolution plasma monitor and linked to a MBF CX 9000 color digital video camera. Sampling used to count stained cells was implemented using Stereo-investigator software (MicroBrightField Inc.; Williston, USA). Areas of interest containing dopaminergic populations were outlined with reference to an atlas of the rat brain (Paxinos and Watson, 1986). Boundaries separating the SNrm and SNcv were identified based on the location of anatomical landmarks and digitized directly from each section using a 4× objective. Every sixth section containing the regions of interest was selected from a random initial sort to ensure random overall sampling. The stereology was performed at high magnification with an 100×/1.4 aperture oil immersion lens which allows for clear visualization of the nucleus and precise definition of the cell walls.

Pilot studies were carried out to determine suitable counting frame and sampling grid dimensions prior to counting in each region of SN to allow approximately 25–30 and 70–85 sampling sites per section for SNrm and SNcv respectively. The level of acceptable error in the stereological estimations was defined by the ratio between the intrinsic error introduced by the methodology and the coefficient of the variation. The coefficient of error (CE) expresses the accuracy of the cell number estimates and a value of $CE \leq 0.10$ was deemed appropriate for the present study, because variance introduced by the estimation procedure contributes little to the observed group variance (Glaser and Wilson, 1998). The optical fractionator method determines the number of cells by multiplying the number of objects identified inside each counting box by the values of three ratios: (a) the ratio between the number of sections sampled and the total number of sections (section sampling fraction, ssf); (b) the ratio of the counting box and the area of the grid (area sampling fraction, asf); and (c) the ratio between the height of the counting frame and the section thickness after histological procedures (thickness sampling fraction, tsf) (West, 2002). The total number of TH-positive cells was obtained using the following equation: $N = \sum Q \times (1/ssf) \times (1/asf) \times (1/tsf)$ where N is the total number of cells and $\sum Q$ is the number of counted objects (West, 2002). The experimental parameters adopted for the stereological analysis are shown in Table 3.

For the soma size the area measurements were carried out using the Neurolucida System for Neuroanatomical Analysis (MicroBrightField Inc.; Williston, USA). A systematic random sampling of cells was implemented using high magnification images with 100×/1.3 aperture oil immersion lenses in those cases in which the cell nucleus could be clearly identified. This analysis was carried out in six animals per group. Ten cells per section were analyzed in each region, corresponding to a total of 60 cells per region. Thus, a total of 360 cells were analyzed per group in the SNrm or SNcv. The nonparametric Kruskal–Wallis ANOVA Ranks test, $\alpha < 0.05$, was used to analyze the effects of diets and regions on the measures of neuronal soma size and the Dunn's test, $\alpha < 0.05$ was further utilized to determine *post hoc* comparison among groups and regions using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com.

Fig. 1A illustrates the regional borders of SNrm and SNcv used for stereological quantification and Fig. 1B shows a random sampling of cells for analysis of soma area in six parasagittal sections of two representative control animals.

3. Results

3.1. Body and brain weights

Body and brain weights at 30–42 days were significantly smaller in the EF2 group as compared to the control. Nevertheless, the brain weight/body weight ratio did not differ between the groups (Table 4).

Table 3
Experimental parameters adopted for stereological estimation of total number of TH-immunoreactive neurons in the substantia nigra rostro-dorso-medial and caudo-ventro-lateral of control (CF2) and experimental (EF2) groups.

Regions and subjects	α (frame) (μm^2)	A (x,y step) (μm^2)	asf	tsf	ssf	Number of counting frames	Number of sections	Total markers counted
SNrm								
CF2 group								
S1	120 × 120	120 × 120	1	0.62	1/6	152	6	1018
S2	120 × 120	120 × 120	1	0.68	1/6	147	6	980
S3	120 × 120	120 × 120	1	0.71	1/6	192	6	956
S4	120 × 120	120 × 120	1	0.69	1/6	174	6	972
S5	120 × 120	120 × 120	1	0.52	1/6	150	6	1146
S6	120 × 120	120 × 120	1	0.67	1/6	151	6	972
S7	120 × 120	120 × 120	1	0.61	1/6	178	6	1118
EF2 group								
C1	120 × 120	120 × 120	1	0.70	1/6	184	6	841
C2	120 × 120	120 × 120	1	0.67	1/6	175	6	797
C3	120 × 120	120 × 120	1	0.68	1/6	153	6	867
C4	120 × 120	120 × 120	1	0.67	1/6	159	6	908
C5	120 × 120	120 × 120	1	0.71	1/6	163	6	997
C6	120 × 120	120 × 120	1	0.68	1/6	148	6	1100
C7	120 × 120	120 × 120	1	0.62	1/6	132	6	730
SNcv								
CF2 group								
S1	120 × 120	120 × 120	1	0.60	1/6	437	6	649
S2	120 × 120	120 × 120	1	0.70	1/6	487	6	458
S3	120 × 120	120 × 120	1	0.60	1/6	425	6	363
S4	120 × 120	120 × 120	1	0.83	1/6	502	6	370
S5	120 × 120	120 × 120	1	0.76	1/6	471	6	675
S6	120 × 120	120 × 120	1	0.68	1/6	402	6	525
S7	120 × 120	120 × 120	1	0.65	1/6	480	6	595
EF2 group								
C1	120 × 120	120 × 120	1	0.55	1/6	421	6	361
C2	120 × 120	120 × 120	1	0.59	1/6	452	6	313
C3	120 × 120	120 × 120	1	0.76	1/6	404	6	233
C4	120 × 120	120 × 120	1	0.63	1/6	429	6	290
C5	120 × 120	120 × 120	1	0.69	1/6	455	6	419
C6	120 × 120	120 × 120	1	0.82	1/6	401	6	483
C7	120 × 120	120 × 120	1	0.88	1/6	451	6	385

All evaluations were performed using a 100× objective lens (N.A. 1.4). α (frame), area of the optical dissector counting frame; A (x,y step), x and y step sizes; asf, area sampling fraction [α (frame)/A (x,y step)]; tsf, thickness sampling fraction, calculated by the height of optical dissector divided by section thickness; h, section thickness; ssf, section sampling fraction.

3.2. Midbrain and cerebral cortex phospholipid fatty acid composition

Table 5 shows the mean percentage of midbrain fatty acid in rats raised on either the control or experimental diets. As can be observed, rats from the EF2 group exhibited a 50% less DHA (22:6*n*-3) levels accompanied by a compensatory increase (~6 fold) in the DPA (22:5*n*-6) when compared to the control group. On the other hand, the values for AA (20:4*n*-6) did not differ between the groups. With respect to saturated and monounsaturated fatty acids, higher levels of palmitic (16:0), stearic (18:0), palmitoleic

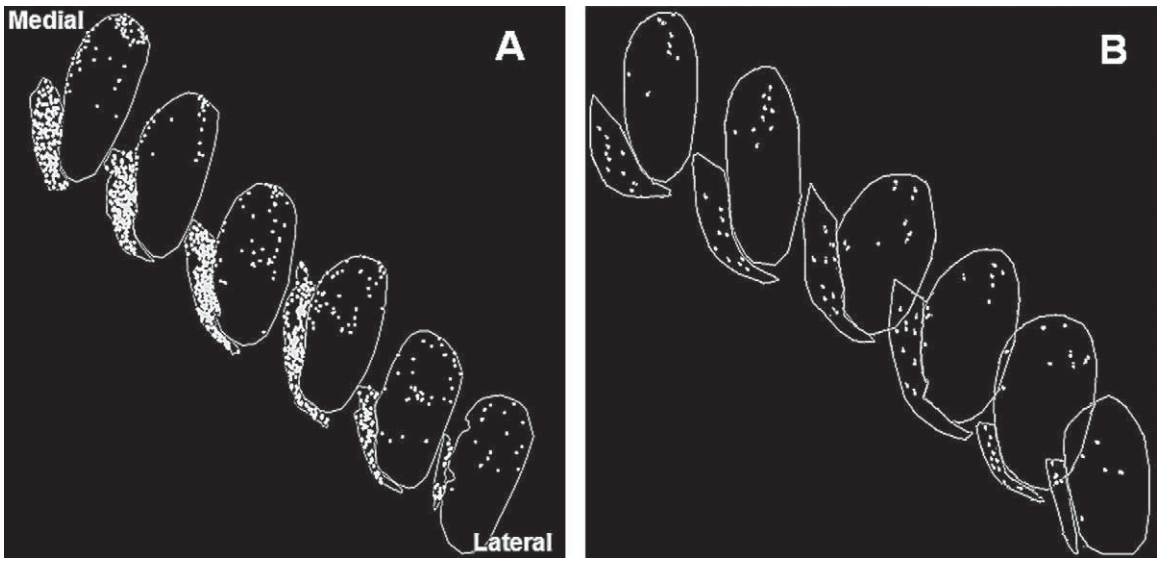

Fig. 1. Cytoarchitetonic limits for stereological analysis. Representative schematic drawings of six parasagittal sections along the latero-medial extension of substantia nigra (4× objective) showing how the cytoarchitetonic limits of SNrm and SNcv were identified for stereologic assessment of dopaminergic cell number (panel A) and soma size (panel B). Dots represent TH positive neurons counted in each section (panel A) and ten of these neurons randomly selected per region and per section (panel B).

Table 4
Body and brain weights.

Groups	Body weight	Brain weight	Brain weight/body weight ratio
CF2	85.67 ± 14.12 g (*n* = 18)	1.68 ± 0.06 g (*n* = 12)	0.0158 ± 0.003 g (*n* = 12)
EF2	74.41 ± 5.90 g* (*n* = 13)	1.56 ± 0.11 g* (*n* = 8)	0.0148 ± 0.002 g (*n* = 8)

Values are expressed as mean ± SD. *n*, number of animals; unpaired Student's *T*-test.
* $P < 0.05$.

Table 5
Fatty acid composition (% of total) in midbrain membrane phospholipids of F2 generation groups raised on control or experimental diets.

Fatty acid	Midbrain	
	Control diet	Experimental diet
C16	16.51 ± 1.90	24.09 ± 0.27*
C16:1	0.64 ± 0.04	0.85 ± 0.03
C17	0.16 ± 0.00	0.20 ± 0.02
C18	24.88 ± 0.67	29.09 ± 0.37*
C18:1*n*9	16.38 ± 0.25	19.21 ± 0.21*
C18:2*n*6t	0.74 ± 0.17	0.65 ± 0.22
C20:1	0.62 ± 0.10	0.60 ± 0.05
C20:4*n*6 (AA)	13.74 ± 0.83	14.93 ± 0.48
C23	3.98 ± 0.20	3.52 ± 0.19
C22:5*n*6	1.49 ± 0.11	9.39 ± 0.31**
C22:6*n*3 (DHA)	19.70 ± 0.69	8.70 ± 0.73**

Values are expressed as means ± SD. nd = not detected.
* $P < 0.01$.
** $P < 0.001$.

(16:1) and oleic (18:1*n*9) acids (2-tail *T*-test, $P < 0.01$) were detected in the EF2 group midbrain phospholipids in comparison to the control. Similar results were obtained in the cerebral cortex phospholipids where DHA levels in the EF2 group (9.99 ± 0.41%) were significantly lesser than that observed in the control (21.22 ± 0.41%). Increased levels of DPA were detected in the cerebral cortex of EF2 group (9.6 ± 0.26%) when compared to control (1.54 ± 0.06%) but no difference between the groups was detected in the AA levels (14.79 ± 0.35% and 14.93 ± 0.43% in the control and EF2 groups respectively).

3.3. Characterization and stereological analysis of SN dopaminergic cell populations

Fig. 2A and D depicts low magnification images of representative parasagittal sections through the SN in animals of CF2 and EF2 groups, respectively. The TH-immunoreactivity pattern shows the distribution of dopaminergic neurons in the SNrm and SNcv as well as in the retrorubral field (RRF). Fig. 2B, C, E, and F shows higher magnification images of SNrm (B and E) and SNcv (C and F) of both groups. As can be observed, neurons located in the SNrm or SNcv differ with respect to morphological features and soma size. TH-immunoreactivity is less intense in the EF2 animal when compared to the control.

Stereological analysis of TH-positive cells in the SN is presented in Table 6 which shows individual and average values for the seven subjects per group, including the planimetric volumes of SNrm or SNcv, coefficient of errors and biological variability. On average, the numbers of TH-immunoreactive cells in the SNrm and in the SNcv of the control group were respectively 9715 ± 1835 and 4570 ± 1510. Combining the data of these two regions, a total of 14,285 ± 2995 dopaminergic neurons in the left SN were estimated. A comparative analysis between the two groups showed that in the SNrm the number of TH-immunoreactive cells in rats fed the deficient diet was 20% lower than in those fed the control

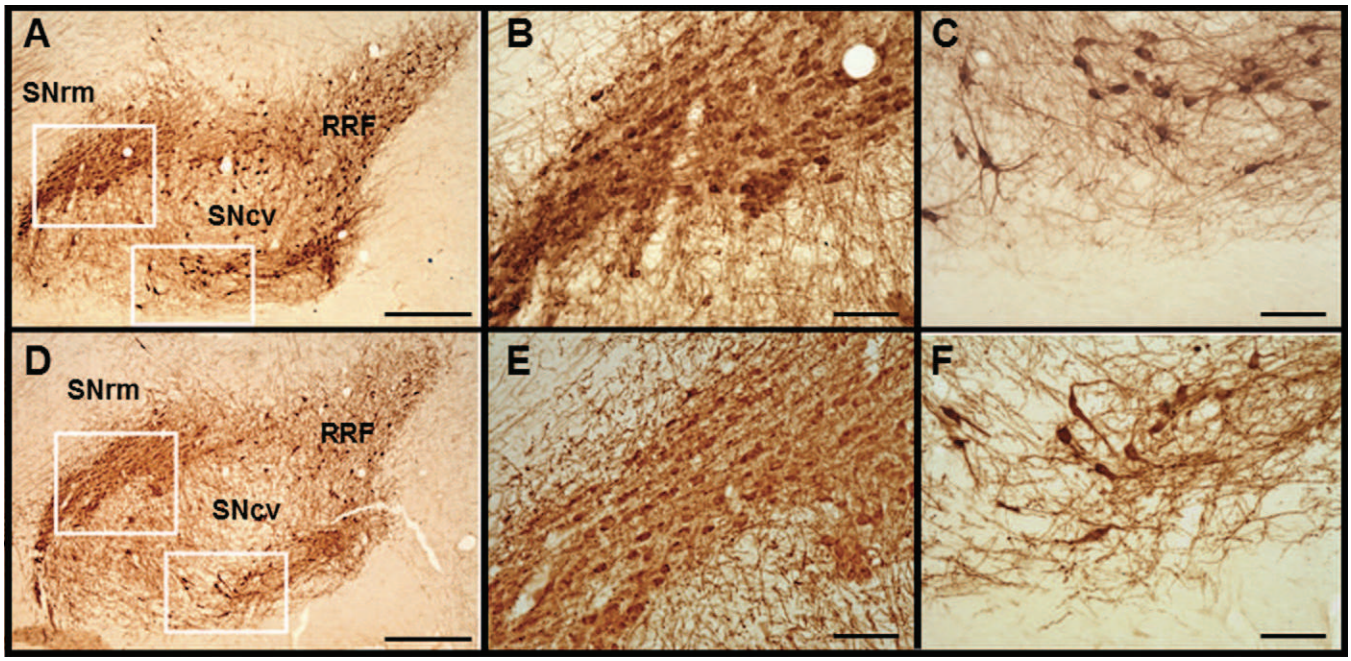

Fig. 2. Effect of EFA dietary restriction for two generations on TH-immunoreactivity in the substantia nigra. Representative photomicrographs of TH-immunoreactive parasagittal sections at the middle level of substantia nigra from rats fed control or experimental diet. Low magnification images of CF2 (A) and EF2 (D) animals showing cytoarchitetonic limits of substantia nigra rostro-dorso-medial (SNrm), caudo-ventro-lateral (SNcv) and the retrorubral field (RRF) (bar = 250 μm). High magnification images of (A) and (D) show dopaminergic cell features in the SNrm and SNcv from CF2 (B and C) and EF2 (E and F) animals (bar = 30 μm).

Table 6
Estimated individual unilateral planimetric volumes of SNcv and SNrm and correspondent unilateral number of their respective dopaminergic cells.

Subjects	SNcv					SNrm					SNcv + SNrm
	Thickness	Volume (mm^3)	CE	Total cells	CE	Thickness	Volume (mm^3)	CE	Total cells	CE	Total cells
CF2 group											
S1	13.4	1.48	0.04	6.720	0.08	12.8	0.33	0.06	9.884	0.07	16.604
S2	11.3	1.19	0.06	3.865	0.08	11.6	0.30	0.06	8.579	0.07	12.444
S3	13.2	1.07	0.05	3.617	0.08	11.2	0.24	0.11	8.180	0.07	11.797
S4	9.6	1.39	0.07	2.149	0.10	11.6	0.24	0.09	8.427	0.07	10.576
S5	10.4	1.29	0.06	5.369	0.08	15.1	0.27	0.13	13.138	0.07	18.507
S6	11.6	1.19	0.06	4.618	0.08	11.8	0.19	0.05	8.656	0.07	13.274
S7	12.4	1.32	0.05	5.650	0.07	12.9	0.36	0.05	11.141	0.06	16.791
Mean	11.7	1.28	0.055	4.570	0.081	12.4	0.276	0.060	9.715	0.068	14.285
SD	1.4	0.14	0.009	1.510	0.009	1.3	0.058	0.031	1.835	0.004	2.995
CV^2		0.012		0.109			0.044		0.035		
CE^2		0.003		0.006			0.006		0.005		
CE^2/CV^2		0.259		0.060			0.138		0.129		
CVB		0.010		0.102			0.038		0.031		
CVB^2 ($\%CV^2$)		74.02		93.98			86.36		87.07		
EF2 group											
C1	14.3	1.57	0.06	4433	0.09	11.4	0.33	0.08	7237	0.06	11,670
C2	13.4	1.62	0.04	3035	0.09	11.9	0.32	0.09	7122	0.07	10,157
C3	10.5	1.39	0.06	2304	0.09	11.6	0.31	0.07	7543	0.07	9847
C4	12.7	1.32	0.07	4143	0.10	11.9	0.35	0.07	8125	0.07	12,268
C5	11.6	1.44	0.06	4253	0.10	11.2	0.32	0.10	8416	0.07	12,669
C6	9.7	1.08	0.04	1722	0.09	11.7	0.33	0.08	9687	0.07	11,409
C7	9.1	1.62	0.08	2670	0.09	11.2	0.42	0.07	6158	0.07	8828
Mean	11.6	1.28	0.058	3222	0.093	11.6	0.344	0.080	7755[#]	0.068	10,978[#]
SD	1.9	0.14	0.015	1065	0.005	0.3	0.039	0.011	1123	0.004	1400
CV^2		0.009		0.109				0.113	0.021		
CE^2		0.003		0.008				0.006	0.005		
CE^2/CV^2		0.354		0.079				0.056	0.220		
CVB^2		0.006		0.100				0.106	0.016		
$CVB^2(\%CV^2)$		64.63		92.11				94.33	77.99		

CE, Scheaffer coefficient of error; CV, coefficient of variation; CVB, biological coefficient of variation; $CVB^2 = CV^2 - CE^2$; SD, standard deviation.
[#] $P < 0.05$ *versus* control.

diet (7755 ± 1123; 2-tail *T*-test, $P = 0.036$). On the other hand, in the SNcv no significant difference between the groups was detected (EF2 group = 3222 ± 1065; 2-tail *T*-test, $P = 0.116$). A total of 10,978 ± 1400 dopaminergic neurons was estimated in the left SN of the EF2 group which is ~23% lower than the value estimated in the respective control (2-tail *T*-test, $P = 0.028$).

3.4. Effects of the dietary treatment on dopaminergic cell soma size

In both the control and EF2 groups, the soma area of these cells ranged from 86 to 496 μm^2 in the SNrm and from 110 to 527 μm^2 in the SNcv. The soma size frequencies of these neurons were found to be distinguishable from a normal distribution (Shapiro Wilk's *W* test, $P < 0.05$) in both regions within each group. Fig. 3A and B illustrates comparative frequency histograms of soma size in the SNrm and SNcv of both groups. The predominant classes of soma size differed significantly between these two regions in each group ($P < 0.01$). The long term dietary treatment with the experimental diet resulted in lower dopaminergic cell size both in the SNrm (median = 195.89 μm^2 (84.25–514.79 μm^2)) and the SNcv (259.09 μm^2 (69.70–519.62 μm^2)) when compared to the respective controls (SNrm, median = 246.43 μm^2 (86.35–496.17 μm^2) and SNcv, median = 292.475 μm^2 (109.28–527.454 μm^2)) according to Kruskal–Wallis ANOVA Ranks test, followed by the Dunn's test, $P < 0.01$ for both regions (Fig. 3C).

3.5. Dietary EFA-deficiency over two generations decreases TH protein levels of ventral midbrain

Quantitative analysis of TH levels in homogenates of the ventral midbrain showed a pronounced decline in the expression of this enzyme in the EF2 group when compared to control. Fig. 4 (panel A) shows the single band of 62 kDa in both groups and Fig. 4 (panel B) shows the TH/actin ratio and reveals a threefold difference between the groups.

4. Discussion

In the present study we investigated whether a long term treatment with a maternal diet containing reduced levels of both linoleic and α-linolenic acids and high contents of saturated plus monounsaturated fatty acids could affect key components of midbrain dopaminergic system and two distinct dopaminergic cell populations of substantia nigra. The results demonstrated that this multigenerational model of EFA dietary restriction changed body and brain weights, TH protein levels in the ventral midbrain and the size of dopaminergic neurons. The number of these cells in the SNrm and SNcv were differentially affected.

4.1. Repercussion of dietary treatment on body and brain weights

The smaller body weight in the EF2 group is in agreement with previous studies using coconut oil as the only source of dietary lipids for one (Deuel et al., 1954; Soares et al., 1995) or two generations (Borba et al., 2010). Regarding this effect, it has been speculated that coconut oil can reduce body weight gain due to higher fatty acid turnover rates of the saturated medium chain fatty acids (8:0–14:0) which are oxidized more rapidly than long chain fatty acids (Hargrave et al., 2005). Moreover, this effect has also been associated with dysfunction of growth hormone regulation (Soares et al., 1995). A smaller EF2 group brain weight contrasts with previous studies adopting a dietary deficiency specific for α-linolenic fatty acid for two or three generations, where no difference between the groups was detected either at 21 days (Ahmad et al., 2002a,b) or at adulthood (Ahmad et al., 2002b). Although distinct factors may be involved in brain and body weight

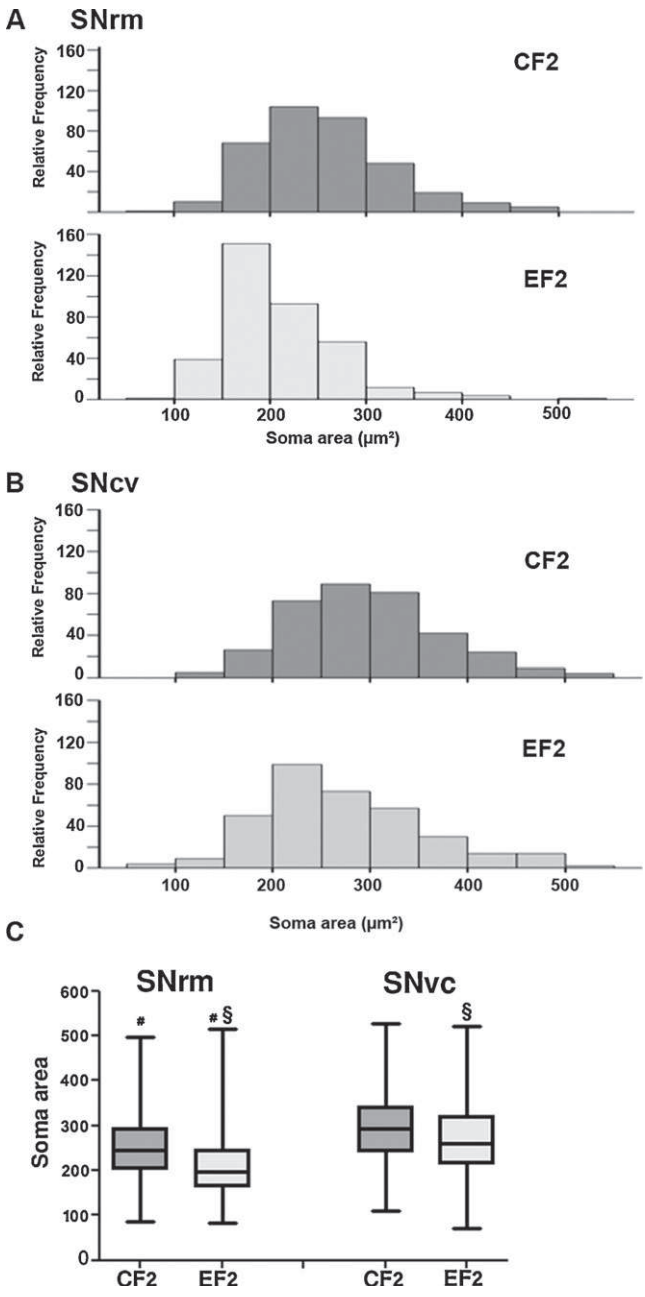

Fig. 3. EFA dietary restriction over two generations affects SN dopaminergic soma size. Comparative frequency histograms of dopaminergic cell soma size in the SNrm (A) and SNcv (B) of both groups show the predominant classes of soma size among the regions and groups ($N = 360$ cells per region). (C) The experimental condition resulted in lesser average soma size in both the SNrm (~20%) and SNcv (~10%) when compared to the control condition ($P < 0.01$, Kruskal–Wallis test followed by the Dunn's test). #, difference between regions; §, difference between groups.

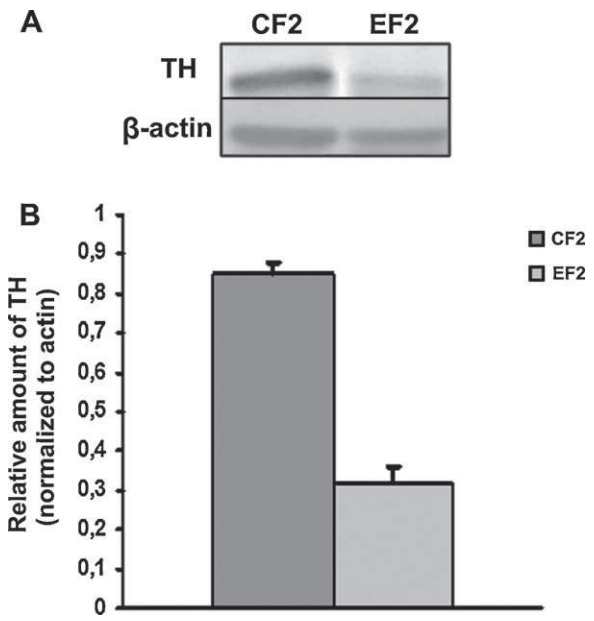

Fig. 4. Effect of EFA dietary restriction over two generations on TH protein levels in the ventral midbrain. Panel A shows the reduction in TH-immunoreactive band of 62 kDa as compared to β-actin (42 kDa). Panel B shows the TH/actin ratio and reveals a threefold difference between the groups. Values are expressed as arbitrary units normalized to β-actin and are average of six different animals of different dams.

gains in the EF2 group, the modifications herein detected were similar in magnitude and did not change the brain/body weight ratio when compared to the control group. Taken together, these data show the effectiveness of the experimental diet in inducing systemic and localized developmental effects on the rat somatic growth.

4.2. Midbrain fatty acid profile

Previous second generation studies using balanced levels of saturated but a high linoleic/α-linolenic acid ratio (~134) have shown distinct levels of DHA depletion among brain regions, with midbrain being less affected (~64 reduction) than the cerebral cortex and cerebellum (~71% reduction) (Levant et al., 2006; Xiao et al., 2005). Recent evidence has indicated that a diet containing coconut oil as the only source of lipids can increase DHA depletion in the brain when compared to a fat free diet, even for a short term treatment (Ling et al., 2010). In the present study, the diet based on coconut oil and containing a linoleic/α-linolenic acid ratio of 16 resulted in lesser DHA levels (~50%) and increased the contents of some saturated and monounsaturated fatty acids in the midbrain phospholipids. The DHA deficiency was also confirmed by the compensatory increase in the DPA levels (~6 fold) which is an specific marker of *n*-3 fatty acid deficiency. Similar modification on the fatty acid profile was also observed in the cerebral cortex which indicates a widespread dietary impact on other brain regions. On the other hand, AA levels did not differ between the two groups, increasing the AA/DHA ratio in both regions analyzed. We suspect that at least three factors may have contributed to these results. First, the liver has a special ability to retain AA; and sustained AA levels in the young rat brain are consistent with up-regulated conversion from linoleic acid in the maternal, fetal and newborn liver (Satomi and Matsuda, 1973; Rapoport et al., 2007). Second, potential modifications in the transport of AA across the placenta can occur (Haggarty, 2002) and third, dietary α-linolenic acid deficiency can upregulate the brain's expression of AA-selective metabolizing enzymes including cytosolic and secretory phospholipases A2 (Rao et al., 2007). Early and recent studies have also indicated that AA is more tightly controlled than DHA in the central nervous system and that its brain concentrations are less vulnerable to limitations in the supply of precursor than in other organs (Bourre et al., 1990; Brenna and Diau, 2007; Igarashi et al., 2009).

4.3. Repercussion of dietary treatment on the number of dopaminergic cell populations

Unbiased stereology demonstrated that the average total number of TH-positive cells in the SNrm of the EF2 young animals

was 20% lower than the controls. This reduction is less than that reported for the entire SN pars compacta (33%) of adult rats, associating successive parity and an α-linolenic acid dietary deficiency (Ahmad et al., 2008). Despite the choice of the EFA deficiency experimental model, altogether, these data agree with the evidence of a protective action of DHA on subsets of SN dopaminergic neurons (Bousquet et al., 2008; Cansev et al., 2008) and seems to be consistent with previous data showing modifications in terminal fields of midbrain dopamine neurotransmission or dopamine-related behavior in *n*-3 deficient rats (Delion et al., 1996; Levant et al., 2004, 2010). Moreover, the increased AA/DHA ratio (∼2 fold) in the midbrain phospholipids of EF2 group could also have an effect on the magnitude of the outcomes. An inverse relation between the number of some brain neurons and increasing ratios of *n*-6/*n*-3 EFAs in the maternal diet has been recently reported (Tian et al., 2011). Imbalance in EFA levels, early in life, and especially *n*-3 LC-PUFA deficiency can lead to changes in brain metabolism, decreasing anti-inflammatory or anti-oxidative responses which can induce cellular suffering in different classes of neurons (Schmitz and Ecker, 2008; Innis, 2007). The increased AA/DHA ratio in the EF2 group could enhance the effects of *n*-6 fatty acids *via* prostaglandin synthesis (Schmitz and Ecker, 2008; Rapoport et al., 2007). It has been demonstrated that the prostaglandin PGE2, a derivative of AA *via* cyclooxygenase-2 (COX-2), can induce neurotoxic effects on subpopulations of SN dopaminergic neurons in a dose and receptor-dependent manner (Carrasco et al., 2007). Furthermore, it has been also shown that dietary treatment with DHA caused a significant decrease in COX-2 activity and PGE2 levels in mice SN, reducing MPTP-induced dopaminergic cell death (Ozsoy et al., 2011).

A smaller average number of SNcv TH-immunoreactive cells in the EF2 group was observable, but no significant difference between the groups was detected, failing to confirm our initial hypothesis that such neurons could be more vulnerable than those of SNrm to the harmful effects of EFA dietary restriction. Although future studies will have to be carried out to determine mechanisms underlying the apparent resistance of these cells in our experimental model, one aspect is of immediate interest in the discussion of this data. Midbrain dopaminergic cell populations differ regarding the availability of some neurotrophins. For example, most dopaminergic neurons of ventral tegmental area (VTA) and SNrm, but not most those of SNcv, contain GDNF retrogradely transported from striatum (Barroso-Chinea et al., 2005). Recent evidence has indicated that DHA supplementation is able to increase GDNF and neurturin (NTN) levels in the SN, reduce dopaminergic cell death induced by MPTP (Tanriover et al., 2010) as well as increase brain-derived neurotrophic factor (BDNF) mRNA levels in the striatum (Bousquet et al., 2009). GDNF, NTN and BDNF in addition to other trophic factors can play differential and synergistic roles on developing SN dopaminergic neurons, maintaining their phenotypic profile and exerting cytoprotective effects (Akerud et al., 1999; Baquet et al., 2005; Stahl et al., 2011). However, dietary *n*-3 fatty acid deficiency for a short-term or over two generations is able to reduce mRNA and/or protein levels of BDNF in the rat cerebral cortex (Rapoport et al., 2007; Rao et al., 2007) and mouse striatum (Miyazawa et al., 2010). Considering that the repercussion of LC-PUFA deficiency on the levels of some neurotrophins can differ among brain regions (Miyazawa et al., 2010), thus leads us to speculate that the differential effects on dopaminergic cell number in SNrm and SNcv could involve a distinct sensitivity of these cell populations to potential modifications on neurotrophin-related mechanisms. With respect to this, *n*-3 EFA deficiency could impair compensatory mechanisms that usually confer less vulnerability to degeneration in some midbrain dopaminergic cells. Adopting a bidimensional analysis we also detected fewer dopaminergic neurons at the middle level of VTA in the EF2 group (Supplementary Fig. 1). Such results are qualitatively similar to that described by Ahmad et al. (2008) using stereology. Likewise the SNrm, the VTA region usually displays highest resistance to degeneration when compared to SNcv (Barroso-Chinea et al., 2005).

4.4. Repercussion of dietary treatment on dopaminergic cell size

The lower values of soma area observed in both SN dopaminergic cell populations of the EF2 group seems to be qualitatively similar to previous data on neurons of the hippocampus, hypothalamus, and both piriform and parietal cortices in multigenerational models of α-linolenic deficiency (Ahmad et al., 2002a,b). These previous studies did not identify the neurotransmitter phenotypes and showed that reduction in neuron size in the DHA-deficient diet group was greater in rats at weaning age (21 days) compared to older ones (68 days) in most brain regions, except in the piriform cortex (Ahmad et al., 2002a). In the CA1 region of hippocampus, for example, *n*-3 deficient diet induced lesser size of neurons without altering other parameters including total number of neurons (Ahmad et al., 2002b). The present data on SN dopaminergic cells of the EF2 group at 30–42 days differs from those reported in the SNpc of Long Evans rats at 70 days (Ahmad et al., 2008) where no difference on soma volume was detected between the control and *n*-3 deficient groups. Although methodological issues and age of animals may have contributed to the observed differences, the present findings reinforce previous evidence that DHA levels can affect the growth and development of neurons and suggest that this effect may not be specifically restricted to a particular neurochemical profile or brain region. With regard to this, it is noteworthy to consider that in the developing brain, DHA is a ligand for the retinoid X-receptor (Lengqvist et al., 2004), which plays a major role in the morphological differentiation of dopaminergic neurons (Castro et al., 2001).

4.5. TH protein levels in the ventral midbrain

Western blotting analysis in ventral midbrain of the EF2 group shows a lower TH protein levels when compared to the controls. Taking into account that our samples contain other TH-immunoreactive nuclei adjacent to SN, these results show a diffused effect of EFA dietary restriction on midbrain dopaminergic systems. They are also consistent with the reduced TH-immunoreactivity pattern and number of TH-immunoreactive neurons herein detected in the SN. A recent study using a diet containing ten times higher linoleic to α-linolenic acid ratio (∼178) than the ratio presented in our experimental diet, reported a time-dependent decline in the TH levels of SN in lactant pups of the first generation (Kuperstein et al., 2008). The present work using a two-generation dietary treatment reinforces the sensitivity of this rate-limiting enzyme in the biosynthesis of dopamine to unbalanced levels of fatty acids, indicating a long-lasting, non-compensated effect on the ventral midbrain.

5. Conclusion

Our results demonstrate for the first time a differential sensitivity of two functionally distinct SN dopaminergic cell populations to unbalanced dietary levels of EFA. Considering that under physiological conditions SNrm is usually more resistant than SNcv to other different kinds of insults, the present results suggest that EFA dietary restriction during brain development, could impair homeostatic mechanisms, modifying the degeneration profile of dopaminergic cells under a variety of pathological conditions. In this respect, the current findings seem to be in

agreement with a recent prospective study in humans, that has positively associated a high intake of PUFA, specifically *n*-3 PUFA, with a lower risk of developing Parkinson disease (de Lau et al., 2005).

Conflict of interest

There is no conflict of interest in the work reported in the present paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchemneu.2012.05.003.

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APÊNDICE F – *Essential fatty acid deficiency reduces cortical spreading depression propagation in rats: a two generation study.*

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Research article

Essential fatty acid deficiency reduces cortical spreading depression propagation in rats: a two-generation study

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Cortical spreading depression (CSD) propagation was investigated in rats under dietary essential fatty acid (EFA) deficiency over two generations (F1 and F2). Wistar rat dams received diets containing 5% fat either from coconut-oil (EFA-deficient) or soybean-oil (control). F1-pups received their dams' diets until the day of CSD recording (30–40 days or 90-100 days). F2-pups were kept on their F1 dams' diet until 30–40 days. Compared to the controls, the EFA-deficient group had reduced ($P < 0.05$) body weights in both F1 and F2 conditions. This effect was more conspicuous ($P < 0.001$) in the F2-animals where brain weight was also reduced ($P < 0.05$). All EFA-deficient groups displayed lower CSD velocities ($P < 0.001$) than the corresponding controls. Within the same dietary group and generation, F1 young rats showed higher CSD velocities ($P < 0.001$) than adults. Data show that EFA deficiency reduces CSD propagation, and this effect is long lasting as it persists up to the second generation.

Keywords: successive generations' effect, cortical spreading depression, essential fatty acids, brain development, polyunsaturated fatty acids

Introduction

Long-chain polyunsaturated fatty acids (LC-PUFAs) are synthesized in mammals from their respective dietary essential fatty acid (EFA) precursors, α-linoleic acid (18:2*n*-6) and α-linolenic acid (18:3*n*-3). They can also be obtained directly from dietary sources such as vegetable oils (corn, safflower, soyabean), eggs, breast-milk and fish. In the adult brain, approximately 35% of the total lipids are in the form of LC-PUFA, mainly arachidonic acid (AA, 20:4*n*-6) and docosahexaenoic acid (DHA, 22:6*n*-3).[1] Different organs present distinct proportions of these compounds and, in the central nervous system, the amount varies depending of the brain area. In general, linoleic, linolenic and eicosapentaenoic acids are in low concentrations while DHA and AA are found in high concentrations in neuronal membranes, mainly in the pre-frontal cortex.[2]

EFAs are important constituents of structural lipids in nervous cell membranes and, as such, are involved in many brain functions.[3] Recently, a variety of functions have been demonstrated for both α-linoleic and α-linolenic acids. Both types of fatty acids are precursors of signaling molecules with opposing effects, modulating membrane fluidity and gene expression.[4,5] In addition, they are involved in the synthesis and functions of brain neurotransmitters, and in the molecules of the immune system.[3] It has also been shown that α-linolenic acid

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deficiency possibly induces an enhanced vulnerability to stress that can affect behavioural, sensory and motor performance in rodents.[6] Although the modifications cannot be precisely related to a specific neurotransmission system, there is evidence suggesting the involvement of the mono-aminergic system.[7] Under physiological conditions, EFAs have also multiple effects on the glutamatergic system; some of these effects would be expected to favor hyperexcitability,[8,9] while others could contribute to decreased synaptic glutamate transmission and increased neuroprotection.[10–12]. Vreughdenhil *et al.*[10] showed that LC-PUFAs and their metabolites may diminish neuronal excitability by modulating ion channels, *i.e.* in the presence of DHA and eicosapentaenoic acid, both sodium and calcium currents were inactivated in the CA1 hippocampal isolated neurons. Pathological conditions in the brain (such as ischemia, trauma and seizure) are accompanied by increased levels of free n-6 and n-3 LC-PUFAs, mainly AA and DHA,[13,14] which are synthesized and released from astrocytes.[15,16] A neuroprotective role has been suggested for LC-PUFAs involving the opening of K^+ channels like TREK1 and TRAAK in the neurons whose activation would be expected to hyperpolarize synaptic terminals.[11] Moreover, it has been shown that AA and DHA have various effects on different pathways of Ca^{2+} intracellular regulation in astrocytes.[12] These LC-PUFAs inhibit store-operated Ca^{2+} entry, reduce the amplitudes of Ca^{2+} responses evoked by agonists of G protein-coupled receptors and suppress intracellular Ca^{2+} concentration oscillations. Prolonged exposure of astrocytes to AA and DHA drives the cells into a new steady state with moderately elevated intracellular Ca^{2+} concentrations, where cells become virtually insensitive to external stimuli.[12] Altogether, such mechanisms have been proposed as important neuroprotective actions of LC-PUFAs, because AA and DHA released by disturbed parts of the brain protect surrounding cells from pathological overstimulation.[12] Such data are compatible with the idea that increasing dietary consumption of LC-PUFAs could prevent epileptic discharges since these compounds easily cross the blood–brain barrier and, consequently, could decrease neuronal excitability.[10–12] The relationship between EFA dietary manipulation and neuronal excitability can be experimentally studied by using the electrophysiological phenomenon known as cortical spreading depression (CSD).

CSD is characterized by a reduction of spontaneous electrical brain activity evoked by mechanical, electrical or chemical cortical stimulation. During the initial phase of CSD, a burst of neuronal electrical activity, similar to that found in epileptic EEG, can occur. Following this, the spontaneous brain electrical activity is depressed and this EEG depression spreads slowly all over the brain cortical surface.[17] This phenomenon is reversible and is characterized by particular ionic, metabolic and hemodynamic changes.[18] It is dependent of neuron–glia interactions and can be affected by several conditions including nutritional[19,20] and pharmacological[21–23] manipulations. There are several reports showing that CSD seems to be involved in various pathophysiological events including ischemia,[24] migraine,[25] and epilepsy.[26]

In this study, we used CSD as a neurophysiological parameter to investigate, in the young and in the adult rat, the long-lasting effects over two generations (F1 and F2) fed a diet deficient in both α-linolenic and α-linoleic acid. The present two-generation study is based on the evidence from others that such long-lasting treatment seems to be required to induce the brain DHA and AA decline necessary (around 50–80%) to induce more severe changes in neural function.[27] In addition, we also evaluated CSD propagation at a young and an adult age (respectively, 30–40 days and 90–100 days of life) since age is related to regional changes in fatty acid composition of brain phospholipids.[2]

Materials and methods

Animals and diets

Female Wistar rats from the colony in our department received one of the two following diets at mating: (i) the experimental diet containing 5% fat as coconut oil (Rhoster®), which is specifically deficient in both α-linolenic and α-linoleic acid (EFA-deficient); or (ii) the standard diet (control) containing 5% fat as soybean oil, which provided normal amounts of the EFA. Both diets were prepared according to Soares *et al.*[28] and were balanced in all nutrients, except for the lipid source. After weaning, all the pups were raised on their respective dams' diets. They constituted the so-called first generation group of animals. The second generation group was formed by pups born from dams of the first generation. After weaning, the pups also received the diets of their respective dams.

The animals were maintained in a room at a temperature of 23 ± 2ºC with a 12-h light:12-h dark cycle (lights on at 6:00 am) with free access to food and water. All animal procedures utilized in this study were in accordance with the *Principles of Laboratory Animal Care* (NIH, Bethesda, MD, USA) and with the norms of the Ethics Committee for Animal Research, of the Universidade Federal de Pernambuco.

CSD recordings

After birth, all the pups were weighed on days 0, 7, 14, 21 (weaning) and 30 as well as on the day of CSD recording. The CSD was recorded when the rats were

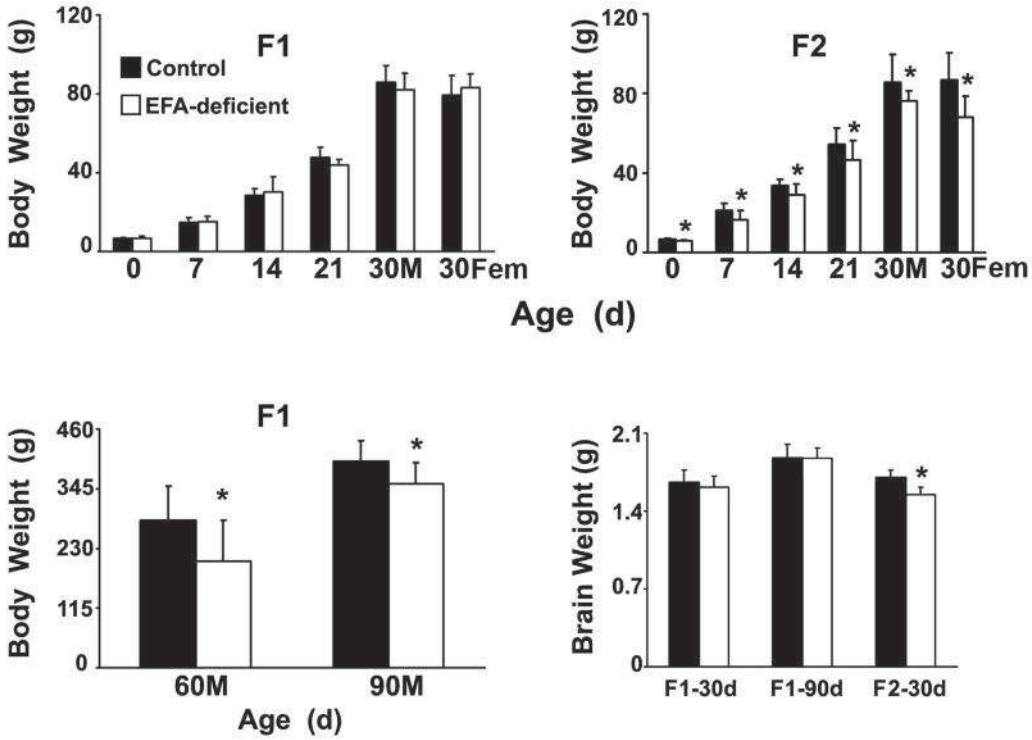

Figure 1 Body and brain weights (mean ± SD) of control and EFA-deficient rats, fed diets in which the lipid sources were, respectively, soya bean oil and coconut oil. The two upper panels compare the body weights of young animals (up to 30 days of age) in two consecutive generations (respectively, F1 and F2). The bottom-left panel shows F1 intergroup body weight differences in adult rats (60 and 90 days of age). The brain weights of young (30 days of age) F1 and F2 rats, as well as of adults (90 days) F1 animals are in the bottom-right panel. M and Fem denote, respectively, male and female animals. The asterisks indicate the EFA-deficient values that were significantly lower than the corresponding controls ($P < 0.05$; ANOVA plus Tukey test)

30–40 days-old (young rats) and at 90–100 days (adult rats) for the first generation. For the second generation, CSD was recorded only at 30–40 days of life (young rats). On the day of the electrophysiological experiment, the animals were intraperitoneally anesthetized with a mixture of 1 g/kg urethane plus 40 mg/kg chloralose. The trachea was opened and a tracheal cannula inserted, followed by the trepanation of three trephine holes on the right side of the skull. These holes were aligned in the anteroposterior direction and parallel to the midline. The first hole (2 mm diameter) was positioned on the frontal bone and was used to apply KCl to elicit CSD. The other two holes were drilled on the parietal bone (3–4 mm diameter) and were used to record the propagating CSD wave. During the recording time, rectal temperature was continuously monitored and maintained at 37 ± 1°C by means of a heating blanket.

CSD was elicited at 20-min intervals by 1-min application of a cotton ball (1–2 mm in diameter) soaked with 2% KCl solution (approximately 0.27 M), applied to the anterior hole drilled at the frontal region. Both the slow potential change and the spontaneous cortical electrical activity (ECoG) accompanying CSD were continuously recorded for 4 h, using two Ag–AgCl agar-Ringer electrodes (one in each hole) against a common reference electrode of the same type, placed on the nasal bones. The CSD velocity of propagation was calculated from the time required for a CSD wave to pass the distance between the two cortical electrodes. After the electrophysiological recordings, the brain was immediately removed and weighed.

Statistical analysis

Data were expressed as mean values ± SD in all groups. Body and brain weights, as well as CSD propagation rates, were compared between groups by using ANOVA, followed by Tukey test, where indicated. *P*-values of less than 0.05 were considered significant.

Results

Body and brain weights

In the first generation, animals receiving the EFA-deficient diet only presented a significantly reduced ($P < 0.05$) body weight at 60 days and 90 days (205.3 ± 79.0 g and 355.1 ±

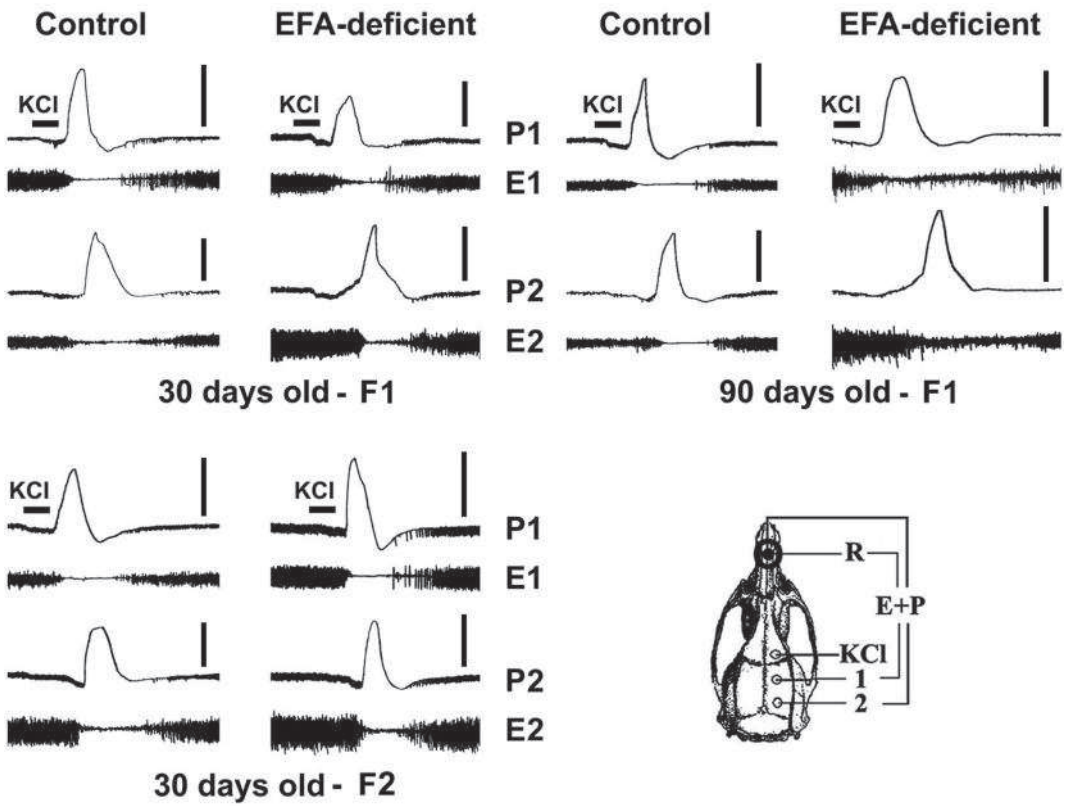

Figure 2 **Electrocorticogram (E) and slow potential change (P) recorded from two points of the right cortical parietal surface in 30-day-old (two left vertical columns) and 90-day-old (two right columns). The horizontal black bars in P1-traces indicate the period (1 min) in which stimulation with 2% KCl was applied to the frontal region to elicit CSD. The inset (bottom right) shows the recording positions 1 and 2, from which the traces marked with the same numbers were obtained. The position of the common reference electrode (R) and the application place of stimulus (KCl) are also shown. Vertical bars correspond to 10 mV in P and 1 mV in E (negative upwards)**

40.1 g, respectively), as compared with the control group (284.5 ± 65.6 g and 398.2 ± 39.5 g) as shown in Figure 1 (upper and lower left panels).

At the second generation, animals chronically fed an EFA-deficient diet showed a persistent body weight reduction ($P < 0.001$) from birth (5.69 ± 0.73 g) until 30 days of life (76.27 ± 5.01 g and 68.04 ± 10.34 g for males and females, respectively), as compared to the controls (from 6.54 ± 0.63 g at birth to 90.08 ± 10.73 g and 86.67 ± 13.61 g for 30-day-old males and females, respectively). Data are shown in Figure 1 (upper right panel).

Regarding the brain weight, rats submitted to an EFA-deficient diet for two generations presented a significant reduction at 30 days (from 1.701 ± 0.075 g to 1.548 ± 0.063 g for control and EFA-deficient, respectively). This effect was not seen at 30 days in the first generation group (1.660 ± 0.108 g and 1.616 ± 0.099 g; $P = 0.235$). Data are shown in Figure 1 (lower-right panel).

CSD velocity

Topical 2% KCl stimulation on a point in the frontal cortical surface elicited a single CSD wave. This wave propagated without interruption along the whole cortex and could be recorded (electrocorticogram and slow potential changes) by the two electrodes located at the parietal cortex. Within a few minutes after CSD had been recorded, the ECoG and the slow potential gradually returned to the pre-CSD pattern. Figure 2 presents examples of CSD recordings in control and EFA-deficient animals of both ages.

The effect of EFA-deficient diet on the CSD propagation can be seen in Figure 3, which shows the mean CSD-velocities of propagation for all groups during the 4 h of recording. F1–rats receiving the EFA-deficient diet displayed significantly lower ($P < 0.001$) CSD velocities (3.29 ± 0.10 mm/min and 3.03 ± 0.13 mm/min, for the young and adult groups, respectively) as compared to the corresponding control groups (3.73 ± 0.11 mm/min and 3.36 ± 0.09 mm/min). Furthermore, in the F1-rats, comparison of the two age-groups revealed that adult animals displayed lower CSD propagation velocities than the corresponding young animals ($P < 0.001$; two way ANOVA). The EFA-deficiency effect on CSD propagation persisted in the second generation young rats

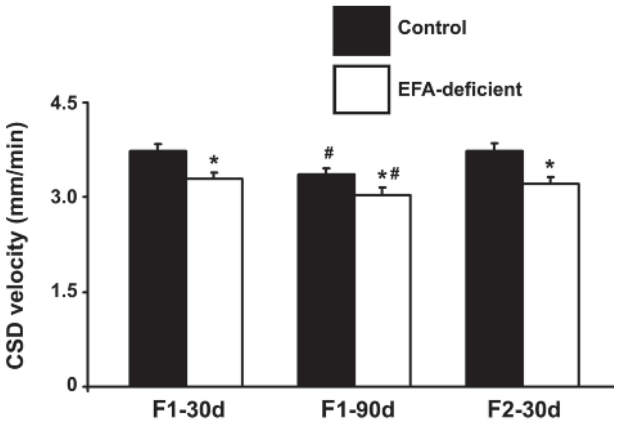

Figure 3 CSD velocities in F1 30- and 90-day-old rats, as well as in F2 30-day-old animals fed a control or an EFA-deficient diet in which the lipid sources were, respectively, soya bean oil and coconut oil. Values are mean ± SD. Asterisks indicate that all EFA-deficient rat values are significantly lower than the corresponding control values. The symbol # in the 90-day-old rats show that their velocities are significantly lower than those of the respective 30-day-old groups ($P < 0.05$; ANOVA plus Tukey test)

(control, 3.74 ± 0.12 mm/min; EFA-deficient 3.21 ± 0.10 mm/min; $P < 0.001$).

Discussion

The main finding of the present study was that chronic EFA-deficiency reduced cortical spreading depression (CSD) susceptibility, as indexed by its lower propagation velocities. This effect was seen in both rat generations investigated.

A number of experimental data obtained from our laboratory have shown that several clinically relevant conditions can interfere with CSD elicitation and/or propagation.[20] For example, improving the nutritional status lowers CSD propagation, whereas under protein deficiency CSD propagates with a faster velocity.[29–31] In our study, although both control and EFA-deficient diets were balanced in all other nutrients, the EFA-deficient diet reduced body and brain weights as well as the CSD propagation velocity, probably due to the specific absence of essential α-linolenic and α-linoleic acids. Xiao *et al.*[2] have shown that the second generation rat offspring raised on the omega-3 deficient diet have cortical phospholipid DHA contents 75% lower than those raised on the control diet. In the present experiments, we did not measure cortical phospholipid DHA and AA levels. However, considering that our dietary model for two generations included α-linolenic as well α-linoleic acid deficiency, we could speculate that similar levels of deficiency in cortical DHA and AA might be detected. This hypothesis needs to be confirmed in future experiments.

It is already documented that neurotransmitter systems, such as the serotoninergic[32] and dopaminergic[33,34] systems, can be affected by EFA-deficiency. Kodas *et al.*[32] proposed that chronic α-linolenic deficiency could increase the release of serotonin, and also decrease its re-uptake and degradation in the synaptic cleft. On the other hand, high levels in PUFAs from the omega-6 and omega-3 family can affect physiological properties of 5-HT receptors in the prefrontal cortex.[35] In agreement with these findings, our data support the possibility that serotoninergic neurotransmission could be increased in the cortex of EFA-deficient rats, since it has been shown that serotoninergic system activation exerts an antagonistic effect on the CSD phenomenon.[22,36] Therefore, an increased extracellular concentration of serotonin would explain our finding of reduced CSD propagation in the EFA-deficient animals.

In addition to the effect of serotonin on their specific receptors, an alternative mechanism that could explain the antagonistic role of serotonin on CSD would be via its action on the *N*-methyl-D-aspartate (NMDA) receptors. Experimental evidence shows that pharmacological stimulation of the serotoninergic system can structurally change NMDA receptor by affecting its pharmacological and physiological properties.[37] Furthermore, it seems that NMDA receptors play an important role in the initiation, propagation and duration of CSD since NMDA antagonists can impair those CSD features.[38–40] Electrophysiological recordings in acutely dissociated cortical cells also have indicated that, under physiological conditions, a direct excitatory effect of DHA and AA occurs on the NMDA receptor, increasing the excitability of cortical neurons.[8,9]

A number of studies in experimental animals have shown that plasma LC-PUFAs, either obtained directly from the diet or synthesized in the liver from their precursors, are the main source for the brain.[41,42] However, other studies show that both cerebral endothelium and astrocytes avidly elongate and desaturate precursors of the LC-PUFAs. AA and DHA are released from these cells supplying the neurons which are unable to carry out fatty acid desaturation.[15] Such release can be increased by activation of astroglial serotonin receptors and it has been suggested that the release of AA and DHA in response to serotonin may represent a mechanism through which astroglia provides these LC-PUFAs to neurons.[43]

It has been shown that the synthesis of DHA in the brain may be regulated by the availability of DHA or other LC–PUFAs in the brain tissue or cerebral circulation.[44,45] According to these authors, there is an inverse relationship between EFA levels in the diet and

DHA synthesis in the brain. Some pathophysiological conditions, such as ischemia and seizure where CSD seems to be involved, are accompanied by increased levels of free AA and DHA[13,14] released from astrocytes.[15,16] It has been speculated that such release could reduce the cortical excitability, especially via K^+ channel activation in neurons and suppressing intracellular Ca^{2+} concentration oscillations in astrocytes, as potential mechanisms of neuroprotection.[11,12] Although, in the present study, we did not analyze the amount of AA and DHA released in the brain, we can not discard the possibility that a fine balance in the neurochemical interactions between astrocytes and neurons, especially those involving serotonin, glutamate and ionic mobilization, could be involved in the effects induced by the EFA deficiency upon CSD propagation. Our data seem to be also consistent with potential modifications in the electrophysiological properties of plasma membranes that could be established since the growth spurt period of brain development in the young animals of F1. In accordance with this, it should be mentioned that a recent study described that relative power of fast activities in the EEG recorded from α–linolenic acid deficient rats was significantly lower than that in the rats receiving adequate DHA amounts during the lactation period of the F1 generation.[46]

Another possibility that could explain the effect of the EFA-deficient diet used in this study in reducing CSD velocity would be based on the impairment of the cerebral blood flow (CBF). The first description of CSD demonstrated dramatic changes in the tone of the cortical resistance vasculature, *i.e.* a transient dilation of pial arterioles was noticed.[47] The mechanisms responsible for regulating the cerebral circulation during CSD involve cerebral blood vessels, astroglia, neurons and perivascular nerves as functionally inter-related components of the neurovascular unit.[48] These components may, directly or secondarily, modulate cerebral blood flow through releasing neurotransmitters, neuronal and astroglial-derived factors (*e.g.* prostaglandins and thromboxane), nitric oxide, carbon monoxide, adenosine, hydrogen and potassium ions, lipoxygenase and cytochrome P-450 monooxygenase products; of note, such factors participate in the mechanisms involved in promoting and counteracting cerebral vasodilator responses consequent to CSD.[49] The role of PUFAs, particularly α-linolenic and docosahexanoic acid, in promoting vasodilation is well known[50] and this function is related to the fact that these PUFAs are potent protectors against focal and global ischemia of the brain.[11] This vasodilation seems to involve TREK-1 potassium channels, since the PUFA-mediated activation of these channels induced a robust vasodilation of the basilar arteries, where such channels are expressed.[50] Taken together, the above findings suggest that the reduction in CSD velocity could involve, at least in part, some of the effects induced by the chronic dietary PUFA deficiency in the hemodynamic changes that accompany and favor brain susceptibility to CSD.

Another important finding of the present study was the persistence of the EFA-deficiency effect on CSD propagation until the second generation offspring. The implication is that no additive mechanisms had been detected during the two-generation period where the EFA deficiency is more expressive; especially taking into account that such deficiency could induce a greater susceptibility of biological nervous membranes to stress.[51] Alternatively, if such additive effects occurred, considering that the brain weight was reduced in F2 animals, they were effective in maintaining an electrophysiological steady-state that could reduce, but did not block, the CSD propagation. On the other hand, the absence of compensatory mechanisms that could restore the CSD velocities to control levels re-inforces the importance of DHA and AA in brain development and function. The lasting CSD effect of EFA deficiency would be consistent with observations in humans showing that cognitive deficits associated with EFA deficiency occurring early-in-life persisted until high-school age.[52]

Conclusions

The data document, for the first time, an impairing effect of long-lasting EFA-deficiency on CSD propagation in the rat cortex, which persisted in the second generation EFA-deficient animals. Our data advance understanding of the mechanisms of EFA-deficiency and cerebral functional relationships. Therefore, they might be useful in shedding light on the changes in cortical excitability associated with fatty acid-dependent structural and functional neuron–glial changes involved in some neurological diseases.

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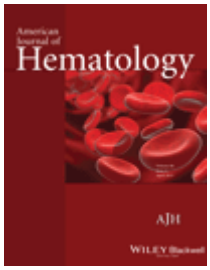

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ANEXO 2 - GUIA PARA AUTORES: *Biochemica Et Biophysica Acta – Molecular and Cell Biology of Lipids*

BBA - MOLECULAR AND CELL BIOLOGY OF LIPIDS

One of the nine topical journals of Biochimica et Biophysica Acta

AUTHOR INFORMATION PACK

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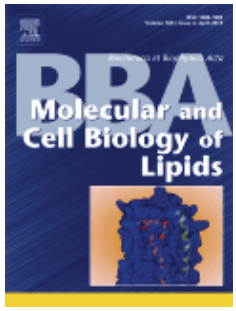

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