Universidade Estadual de Londrina

BRUNA MIGLIORANZA SCAVUZZI

AVALIAÇÃO DE ESTRESSE OXIDATIVO E NITROSATIVO NO LÚPUS ERITEMATOSO SISTÊMICO:

ASSOCIAÇÃO COM A ATIVIDADE DA DOENÇA, MARCADORES IMUNOLÓGICOS E MOLÉCULAS DE ADESÃO

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Estadual de Londrina, como requisito parcial para a obtenção do Título de Doutor.

Orientador: Prof. Dr. Isaias Dichi.
Co-orientadora: Prof. Dra Andréa Name Colado Simão.

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Londrina, 6 de dezembro de 2017.

Dedico este trabalho e todo meu amor à minha filha Júlia, ao meu marido Wagner, aos meus pais, Edison e Lucia e meus irmãos, Roberta e Leonardo.

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SCAVUZZI, Bruna Miglioranza. **Avaliação de estresse oxidativo e nitrosativo no lúpus eritematoso sistêmico: associação com a atividade da doença, marcadores imunológicos e moléculas de adesão.** 2017. 115 f. Tese (Doutorado em Ciências da Saúde) – Universidade Estadual de Londrina, Londrina, 2017.

RESUMO

Introdução: O lúpus eritematoso sistêmico (LES) é uma doença inflamatória autoimune crônica de origem multifatorial, incluindo fatores hormonais, genéticos e ambientais. O aumento exacerbado na produção de espécies reativas de oxigênio e nitrogênio (EROs e ERNs, respectivamente) pode produzir substratos que agravam a antigenicidade, desencadeando a produção característica de autoanticorpos que definem a doença. Além do estresse oxidativo e nitrosativo, níveis de antioxidantes diminuídos têm sido demonstrados em vários estudos com pacientes com LES, contribuindo ainda mais para o desequilíbrio redox. Assim, para analisar parâmetros relacionados ao estresse oxidativo e nitrosativo em pacientes com LES foram desenvolvidos um artigo de revisão e um artigo original na presente Tese de Doutorado.

Artigo 1: Pesquisas recentes indicam que as medidas de biomarcadores de dano oxidativo e nitrosativo em pacientes com LES podem se tornar ferramentas interessantes para ajudar a coletar informações sobre a patogênese do LES, monitorar a atividade da doença, identificar e prever quais pacientes estão em risco de complicações e danos específicos a órgãos devido ao LES. Assim, foi realizada uma revisão bibliográfica que reúne publicações recentes e/ou relevantes sobre a fisiopatologia do estresse oxidativo e nitrosativo no LES e autoimunidade. O artigo também analisa os principais biomarcadores do estresse oxidativo e os principais mecanismos envolvidos em sua participação. Finalmente, o artigo avalia os sistemas antioxidantes assim como as descobertas recentes de terapias antioxidantes em pacientes com LES.

Artigo 2: O objetivo do trabalho foi delinear mudanças em biomarcadores nitro-oxidativos e defesas antioxidantes e verificar suas associações com o score SLEDAI, autoimunidade, respostas imunes e moléculas de adesão. Este estudo caso-controle teve a participação de 204 pacientes com LES e 256 indivíduos controles. O LES foi diagnosticado utilizando os critérios revisados do *American College of Rheumatology,* 2013, e a atividade da doença foi determinada utilizando pontuação *Systemic Lupus Erythematosus Disease Activity Index* (SLEDAI). A peroxidação lipídica e o Parâmetro Antioxidante de Aprisionamento Total do Radical (TRAP) foram avaliados por quimioluminescência. Os produtos avançados de oxidação protéica (AOPP), os metabólitos de óxido nítrico (NOx) e os grupamentos sulfidrila (-SH) foram dosados por métodos espectroforométricos. Produtos de degradação oxidativa de DNA /RNA foram determinados pelo ensaio imunoenzimático (ELISA), bem como os níveis plasmáticos das citocinas IFN-γ, IL-4, IL-6, IL-12 e IL-17 e os anticorpos anti-DNA de dupla fita (dsDNA). As respostas T helper foram consideradas de acordo com as citocinas: Th1 (IL-12 + IFN-γ), Th2 (IL-4) e Th17(IL-17+ IL-6). Os níveis plasmáticos do inibidor do ativador do plasminogênio Tipo 1 (PAI-1) e das moléculas de adesão: molécula de adesão celular endotelial plaquetária (PECAM-1), molécula de adesão celular-vascular-1 (VCAM-1), molécula de adesão intercelular-1 (ICAM), E-selectina, P-selectina foram dosados pelo *Human Magnetic Adhesion 6-Plex Panel*. Os niveis séricos de proteína C reativa ultrasensível (usPCR) foram determinados por ensaio turbidimétrico. Os títulos de autoanticorpos contra antígenos nucleares (ANA) foram avalidos por imunofluorescência indireta. Foi observado que o LOOH ($p < 0,001$) e AOPP ($p < 0,001$) foram significativamente maiores, enquanto a TRAP foi significativamente menor ($p < 0,001$) em pacientes com LES que em controles. AOPP e LOOH foram associados significativamente e positivamente com os escores de SLEDAI, anticorpos antinucleares e níveis de anti-dsDNA, enquanto a TRAP foi significativamente e inversamente correlacionada com SLEDAI, ANA e dsDNA. Houve associações positivas significativas

entre AOPP e LOOH e marcadores imuno-inflamatórios, indicando aumento da resposta (Th17, Th17/Th2) e (Th1+ Th17, Th1+Th17/Th2), (p = 0,002 e p = 0,001, respectivamente). AOPP e LOOH (positivamente) e TRAP (inversamente) foram associados à expressão da molécula de adesão. Um modelo que indica associação entre marcadores e o LES foi calculado mostrando que, usando-se LOOH, AOPP, NOx, moléculas de adesão e índice de massa corporal, 94,2% dos pacientes foram corretamente classificados com uma especificidade de 91,5%. Concluiu-se que o aumento do estresse nitro-oxidativo faz parte da fisiopatologia (auto) imune do LES, afeta o escore SLEDAI, modula a expressão de moléculas de adesão, e estão fortemente relacionados ao diagnóstico do LES.

Palavras-chave: Lúpus eritematoso sistêmico. SLEDAI. Espécies reativas de oxigênio e nitrogênio. Citocinas. Antioxidante. Molécula de adesão. Fisiopatologia. Estresse oxidativo.

SCAVUZZI, Bruna Miglioranza. **Evaluation of oxidative and nitrosative stress in systemic lupus erythematosus: association with disease activity, immunological biomarkers and adhesion molecules.** 2017. 115 pp. Thesis (Doctorate in Health Sciences) – State University of Londrina, Londrina, 2017.

ABSTRACT

Introduction: Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease of multifactorial origin, including hormonal, genetic and environmental factors. An exacerbated increase in the production of reactive oxygen and nitrogen species (ROS and RNS, respectively) can produce substrates that aggravate the antigenicity, triggering the characteristic production of autoantibodies that define the disease. In addition to excessive oxidative stress, decreased antioxidant levels have been demonstrated in several studies with SLE patients, further contributing to redox imbalance.

Article 1: Recent findings have indicated that measurements of reactive oxygen and nitrogen species in SLE patients may become an interesting tool to help gather information on the pathogenesis of the disease, monitor disease progression, identify and predict which patients are at risk for specific organ damage and complications due to SLE. Thus, a review article was written to gather recent and/or relevant publications on the pathophysiology of ROS and RNS in SLE and autoimmunity. The main biomarkers of oxidative stress and the main mechanisms involved in its participation were also analyzed. At last, the review evaluates antioxidant systems as well as the recent findings on antioxidant therapies in SLE patients.

Article 2: The aim of this study was to delineate changes in nitro-oxidative biomarkers and antioxidant defenses and examine their associations with severity of SLE, autoimmunity, immune responses and adhesion molecules. 204 SLE patients and 256 controls participated in this case-control study. SLE was diagnosed using the revised American College of Rheumatology criteria and disease activity was determined using Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score. Lipid peroxidation and Total radical-trapping antioxidant parameter (TRAP) were determined by chemiluminescence. Advanced protein oxidation products (AOPP), nitric oxide (NOx) metabolites and sulfhydryl (-SH) groups were measured by spectrophotometric methods. Oxidative DNA/RNA degradation products, anti-double-stranded DNA antibodies (dsDNA) and plasma levels of the cytokines IFN-γ, IL-4, IL-6, IL-12 and IL-17 were determined by ELISA. T helper responses were estimated according to the following cytokines: Th1 (IL-12 + IFN-γ), Th2 (IL-4) and Th17 (IL-17+ IL-6). Plasma levels of Plasminogen activator inhibitor type-1 (PAI-1) and the adhesion molecules: platelet endothelial cell adhesion molecule 1 (PCAM-1), vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM), E-selectin, selectin, were measured by the Human Magnetic Adhesion 6-Plex Panel. Serum levels of ultrasensitive C-reactive protein (usCPR) was determined by turbidimetric assay. Antibody titers against nuclear antigens (ANA) were assessed by indirect immunofluorescence. LOOH ($p<0.001$) and AOPP ($p<0.001$) were significantly higher, while TRAP was significantly lower ($p<0.001$) in SLE patients than in controls. AOPP and LOOH were significantly and positively associated with SLEDAI score, antinuclear antibodies and anti-dsDNA levels, whilst TRAP was significantly and inversely correlated with SLEDAI, ANA, and dsDNA. There were significant positive associations between AOPP and LOOH and immune-inflammatory markers, indicating an increase in (Th17, Th17/Th2) and (Th1+ Th17, Th1+Th17/Th2 ($p=0.002$ and $p=0.001$ respectively). AOPP and LOOH (positively) and TRAP (inversely) were associated with adhesion molecule expression. A model predicting SLE was calculated showing that, using LOOH, AOPP, NOx, adhesion molecules and body mass index, 94.2% of the patients were correctly classified with a specificity of 91.5%. We concluded that increased nitro-oxidative stress takes part in the (auto) immune pathophysiology of SLE and modulates severity of illness and adhesion molecule expression. Thus, a model predicting SLE was

computed showing that, using LOOH, AOPP, NOx, adhesion molecules and body mass index, 94.2% of the patients were correctly classified with a specificity of 91.5%. Thus, increase of nitrooxidative stress is part of the (auto) immune pathophysiology of SLE, affects the SLEDAI score, modulates the expression of adhesion molecules, and is strongly related to the diagnosis of SLE.

Keywords: Systemic Lupus Erythematosus. SLEDAI. Reactive oxygen and nitrogen species. Cytokines. Antioxidant. Adhesion molecules. Physiopathology. Oxidative stress.

LISTA DE ABREVIATURAS, SIGLAS E FÓRMULAS MOLECULARES

3NTyr	3-nitrotirosina
4-HNE	4-hidroxinonenal
8-OHdG	Nucleósido 8-hidroxi-2'-desoxiguanosina
ACR	*American College of Rheumatology* (Colégio Americano de Reumatologia)
ANA	*Antinuclear antibodies* (anticorpos antinucleares)
Anti-dsDNA	*Anti-double-stranded DNA antibody* (anticorpos anti-DNA de cadeia dupla)
Anti-Sm	Antígeno anti-Smith
Anti-snRNP	Antígeno anti-ribonucleoproteínas
AOPP	*Advanced oxidation protein products (*Produtos avançados oxidação protéica)
AU	Ácido úrico
BMI	*Body mass index* (índice de massa corporal)
C3	*Complement component 3* (componente do complemento C3)
C4	*Complement component 4* (componente do complemento C4)
CA	Circunferência abdominal
CAT	Catalase
CRP	*C-reactive protein* (proteína C-reativa)
DCV	Doença cardiovascular
DNA	*Deoxyribonucleic acid* (ácido desoxirribonucléico)
EDTA	*Ethylenediamine tetraacetic acid* (ácido etileno diamino tetra-acético)
ELISA	*Enzyme-linked immunosorbent assay* (ensaio de imunoabsorção enzimática)
eNOS	Óxido nítrico sintase endotelial
EROs	Espécies reativas de oxigênio
ERNs	Espécies reativas de nitrogênio
F2	Isoprostanos
FRAP	*Ferric Reducing Antioxidant Power*, poder de redução do íon ferro
GLM	*Multivariate general linear model* (modelo de multivariada linear geral)
GPx	Glutationa peroxidases
GSSG	Glutationa dissulfeto
HDL	*High Density Lipoprotein* (lipoproteína de alta densidade)
H_2O_2	Peróxido de hidrogênio
ICAM	Molécula de adesão intercelular-1
iNOS	Óxido nítrico sintase induzível

IFN	*Interferon* (interferon)
IL	Interleucina
IMC	Índice de massa corporal
LDL	*Low Density Lipoprotein* (lipoproteína de baixa densidade)
LES	Lúpus eritematoso sistêmico
LOOH	Hidroperóxidos lipídicos
MDA	Malondialdeído
MetS	Síndrome Metabólica
nNOS	Óxido nítrico sintase neuronal
NF-kB	*Factor nuclear kappa B* (fator nuclear kappa B)
NO_2^-	Nitritos
NO_3^-	Nitratos
NOx	Metabólitos de óxido nítrico
NO	Óxido nítrico
NOS	Óxido nítrico sintase
$O_2^{\cdot -}$	Ânion superóxido
OH·	Radical hidroxila
ONOO-	Peroxinitrito
PAI-1	Inibidor do ativador do plasminogênio Tipo 1
PECAM-1	Molécula de adesão celular endotelial plaquetária
PCR	Proteína C reativa
RNA	*Ribonucleic acid* (ácido ribonucléico)
SLE	*Systemic Lupus Erythematosus* (Lúpus Eritematoso Sistêmico)
SLEDAI	*Systemic Lupus Erythematosus Disease Activity Index* (índice de atividade do lupus eritematoso sistêmico)
SOD	Superóxido dismutase
TAS	*Total Antioxidant Status* (Estado Antioxidante Total)
TEAC	*Trolox Equivalent antioxidant Capacity* (Capacidade Antioxidante Equivalente ao Trolox)
TG	Triglicerídeos
TRAP	*Trapping Antioxidant Parameter*, (Parâmetro Antioxidante de Aprisionamento Total do Radical)
Th	*T helper lymphocytes* (linfócitos T auxiliares)
Tregs	*Regulatory T cells* (Células T reguladoras)
UV	*Ultraviolet radiation* (radiação ultravioleta)
VCAM-1	Molécula de adesão celular-vascular-1

SUMÁRIO

1. INTRODUÇÃO

O lúpus eritematoso sistêmico (LES) é uma doença autoimune inflamatória crônica de origem multifatorial, incluindo fatores hormonais, genéticos e ambientais (OATES, 2010; TSOKOS, 2011). A doença é caracterizada por ativação e proliferação anormais de células B e T, assim como sinalização anormal para apoptose e necrose, levando a uma grande produção de autoanticorpos, dirigidos principalmente contra componentes nucleares, e formação de complexos imunes, causando inflamação crônica generalizada, destruição de tecido, acelerada aterosclerose e mortalidade prematura (M. MCMAHON, BH. HAHN, 2011; OATES, 2010; TSOKOS, 2011).

A taxa de incidência do LES varia de aproximadamente 0,3 a 10 novos casos por 100.000 habitantes/ano e a taxa de prevalência varia de 10 a 97 casos por 100.000 habitantes. Essas taxas são altamente variáveis e dependentes de diversos fatores sócio-demográficos, econômicos e genéticos, como o sexo, acometendo cerca de 9 mulheres a cada homem; faixa etária, sendo mais comum em mulheres em idade reprodutiva; etnia, sendo menos frequente em indivíduos caucasianos e particularmente mais frequente dentre indivíduos negros; região demográfica, sendo mais comum em indivíduos que habitam a zona rural (CARTER; BARR; CLARKE, 2016; DANCHENKO; SATIA; ANTHONY, 2006; NASONOV et al., 2014; PONS-ESTEL, G. J.; ALARCÓN, G. S.; SCOFIELD, L.; REINLIB, L.; COOPER, 2010; REES et al., 2014).

A principal característica sorológica da doença é a presença de autoanticorpos dirigidos contra componentes celulares, como proteínas ou complexos de proteínas nucleares, antígenos citoplasmáticos, antígenos de membrana celular, antígenos associados a fosfolipídios, células sanguíneas, células endoteliais e antígenos do sistema nervoso, proteínas plasmáticas, proteínas de matriz e antígenos diversos. O LES é a doença autoimune com o maior número de autoanticorpos detectáveis, já havendo sido descritos mais de cento e oitenta autoanticorpos relacionados à doença (COZZANI *et al.*, 2014; SHERER *et al.*, 2004; YANIV *et al.*, 2015).

Os principais autoanticorpos no LES são os direcionados a estruturas do núcleo, denominados anticorpos antinucleares (ANA). Os ANAs consistem em vários tipos de autoanticorpos caracterizados por diferentes especificidades antigênicas, incluindo DNA de cadeia simples, DNA de dupla fita (ex. anti-dsDNA), proteínas histonas, nucleossoma (complexo de histona-DNA), ribonucloproteínas, proteínas centroméricas e antígenos extraíveis de núcleo (anti-ENAs), como antígeno anti-Smith (anti-Sm), Ro (anti-SSA/Ro), La (anti- anti-SSB/La), ribonucleoproteínas (anti-snRNP), entre outros (COZZANI *et al.*, 2014;

OATES, 2010). Os ANAs estão presentes em cerca de 95% dos pacientes com LES com a forma ativa da doença (COZZANI *et al.*, 2014). A revisão atual do *American College of Rheumatology* (ACR) inclui quatro autoanticorpos nos critérios de diagnóstico para LES: presença de ANA, presença de anticorpo anti-Sm, níveis anormais de anticorpos anti-DNA de dupla fita (dsDNA), e presença de anticorpos antifosfolípides (HOCHBERG, 1997).

Os autoanticorpos se ligam aos seus antígenos, originando imunocomplexos autorreativos circulantes e/ou de deposição, responsáveis pelas principais manifestações clínicas observadas no LES (TSOKOS, 2011), levando a complicações como vasculite, nefrite, miosite, anemia grave (devido a autoanticorpos contra eritrócitos) e trombocitopenia (devido a autoanticorpos contra plaquetas) (FERNANDEZ; KIROU, 2016; MAK *et al.*, 2012). Além da presença de anticorpos específicos, o ACR considera para o diagnóstico do LES a presença de outros critérios, baseados nas características clínicas da doença, que afetam principalmente: a pele, causando eritema malar, lesão discoide, e/ou fotossensibilidade; as mucosas, provocando ulcerações na cavidade oral e/ou narisofaríngea; serosa, evidenciada pela presença de pleurite e/ou pericardite; as articulações, podendo causar artrite; os rins, ocasionando proteinúria e/ou cilindrúria; o sistema nervoso central, levando a alterações como convulsão e/ou psicose; desordens hematológicas, tais como anemia hemolítica, leucopenia, linfopenia e/ou trombocitopenia (HOCHBERG, 1997).

O curso do LES é geralmente crônico, recidivante e imprevisível e as manifestações clínicas da doença podem oscilar entre leve a grave, alternando entre períodos em que os sintomas são reduzidos ou reaparecem. A fase aguda da doença - também denominada *flare* - é o período em que ocorre um aumento mensurável na atividade da doença, ocorrendo piora nos sintomas clínicos e/ou laboratoriais. Já na fase de remissão, que pode durar anos, as manifestações clínicas ficam reduzidas. Durante os períodos de fase aguda da doença, podem se manifestar erupções cutâneas, feridas na cavidade oral, pleurite, atralgia, fadiga, febre entre outros. No entanto, os sintomas podem agravar-se e envolver manifestações específicas da doença, como envolvimento de órgãos e sistemas ou outras manifestações que requerem aumento da medicação ou hospitalização, ocorrendo até o risco de morte pelo acometimento de órgãos vitais (FERNANDEZ; KIROU, 2016; MAK *et al.*, 2012). A avaliação da atividade da doença é geralmente realizada através de escalas de escores, como o *Systemic Lupus Erythematosus Disease Activity Index* (SLEDAI), que consideram parâmetros clínicos e laboratoriais semelhantes aos listados para o diagnóstico (BOMBARDIER *et al.*, 1992), demonstrado no (Anexo 2).

Embora nas últimas cinco décadas as taxas de mortalidade aguda tenham diminuído drasticamente nos primeiros cinco anos de tratamento devido ao

diagnóstico mais precoce e a estratégias de tratamento mais adequadas, a aterosclerose prematura e acelerada é a principal comorbidade entre os pacientes com LES (M. MCMAHON, BH. HAHN, 2011). Pacientes com LES têm um risco significativamente aumentado de eventos cardiovasculares devido à aterosclerose, tendo sido observado que mulheres com LES na faixa de 35 a 44 anos possuem probabilidade 50 vezes maior de sofrerem um infarto agudo do miocárdio do que mulheres da mesma faixa sem a doença; risco esse que não é explicado apenas por fatores tradicionais de avaliação de risco cardíaco, como no estudo de Framingham (KARRAR; SEQUEIRA; BLOCK, 2001; MCMAHON; HAHN; SKAGGS, 2011). A etiologia do risco aumentado de aterosclerose tem sido atribuída a uma combinação de fatores predisponentes, como atividade e duração da doença, presença e grau de acometimento renal, presença de síndrome metabólica (MetS), terapia com glicocorticóides, inflamação crônica, resposta imunológica ativa e ao estresse oxidativo (MOK *et al.*, 2010; SHERER; ZINGER; SHOENFELD, 2010).

2. LES E ESTRESSE OXIDATIVO E NITROSATIVO

Espécies reativas de oxigênio (EROs) é um termo geral para espécies geradas a partir de uma redução incompleta do oxigênio. Inclui tanto os radicais de oxigênio (ex. ânion superóxido, $O_2^{\cdot -}$; o altamente reativo radical hidroxila, OH·) como também alguns derivados não-radicalares (ex. peróxido de hidrogênio, H_2O_2). O termo espécie reativa inclui também espécies reativas de nitrogênio (ERNs) (ex. óxido nítrico, NO·; peroxinitrito, ONOO-) (HALLIWELL, 2006). Já o termo radical livre refere-se a qualquer espécie que contenha um ou mais elétrons desemparelhados (HALLIWELL; GUTTERIDGE, 2006). Embora a etiologia do LES seja multifatorial, tem sido demonstrado que o aumento exacerbado da produção de EROs e ERNs pode produzir substratos oxidados que agravam a antigenicidade, desencadeando a produção característica de autoanticorpos que definem a doença (MORGAN, PHILIP E; STURGESS; DAVIES, 2009; OATES, 2010; PERL, 2013; PRASAD *et al.*, 2015; WALL *et al.*, 1980).

As EROs são produtos normais do metabolismo celular e são geradas principalmente pela cadeia respiratória mitocondrial. Em quantidades controladas, essas espécies reativas modulam vários aspectos fisiológicos importantes da função celular e são necessárias para vias de sinalização, como as envolvidas na ativação e proliferação de células T, apoptose de células anormais ou envelhecidas, fagocitose de células infectadas, dentre outras. (PHANIENDRA; JESTADI; PERIYASAMY, 2015). No entanto, quando há um desequilíbrio entre a produção dessas espécies reativas e sua eliminação por mecanismos antioxidantes, elas podem desempenhar um papel deletério para o organismo, conhecido

como estresse oxidativo (BONOMINI; RODELLA; REZZANI, 2015; SIMONE REUTER, 2011). O estresse oxidativo tem um papel importante no desenvolvimento e progressão de doenças crônicas e autoimunes, como esclerose múltipla, artrite reumatóide, doenças cardiovasculares e LES (DICHI *et al.*, 2014).

O óxido nítrico (NO) é uma molécula de sinalização que desempenha um importante papel em diversas funções fisiológicas, como a regulação do tônus vascular, regulação imune, regulação do potencial da membrana mitocondrial nas células T, transdução de sinal (por exemplo, a sinalização de Ca^{2+}) e a regulação da apoptose (NAGY, GYÖRGY *et al.*, 2010). No entanto, o NO pode tornar-se patológico quando produzido em excesso podendo então reagir com EROs e formar moléculas altamente reativas como o peroxinitrito, gerando neoepítopos com o potencial de levar à quebra da tolerância imunológica (OATES; GILKESON, 2006).

Existe um grande interesse clínico nos biomarcadores que avaliam EROs e ERN que, de maneira individual ou conjunta, estejam associados ao LES e sua atividade. O conhecimento sobre o perfil oxidativo, defesas antioxidantes e biomarcadores imunológicos ajudam na compreensão da fisiopatologia do LES e pode se tornar uma ferramenta interessante para ajudar a coletar informações sobre a patogênese da doença, monitorar sua progressão, identificar e prever quais pacientes estão em risco de complicações ou danos específicos a órgãos, além de oferecer opções para novos alvos terapêuticos direcionados e inovadores, ainda insuficientes no arsenal terapêutico para pacientes com LES.

2.1. FONTES DE ERO E ERN

A principal fonte endógena de EROs em pacientes com LES é sua produção excessiva pelas mitocôndrias das células T, devido a uma disfunção conhecida como hiperpolarização mitocondrial. Nessas condições, ocorre neutralização deficiente do peróxido de hidrogênio, originando espécies reativas danosas, como o oxigênio radicalar e o radical hidroxila. O oxigênio radicalar pode ser convertido em peróxido de hidrogênio através de superóxido dismutases, podendo ser subsequentemente convertido em água por ação da catalase. No entanto, o excesso de peróxido de hidrogênio, na presença de um íon metálico (ex. íon ferroso, Fe^{+2}), pode sofrer a reação de Fenton ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH$) desencadeada pela luz UV, formando o radical hidroxila, que não pode ser neutralizado e é altamente reativo, ocasionando modificações imediatas de biomoléculas celulares como proteínas, lipídios, ácido desoxirribonucleico (DNA) e ácido ribonucleico (RNA) que estejam em seu entorno (PERL, 2013). Os lipídios são os principais alvos oxidativos das espécies reativas (AHSAN, H.; ALI; ALI, 2003). Essa peroxidação lipídica produz aldeídos altamente

reativos como malondialdeído (MDA) e 4-hidroxinonenal (HNE), que se difundem rapidamente para outras organelas na célula e para a corrente sanguínea, propagando o dano oxidativo (PERL, 2013; WANG *et al.*, 2010) (Figura 1). Os aldeídos reativos, por sua vez, se ligam de forma covalente a proteínas, levando a modificações estruturais e de função biológica. Essas proteínas modificadas por aldeídos reativos são altamente imunogênicas, induzindo uma forte resposta de anticorpos T-dependentes (WANG *et al.*, 2010, 2016). Estas e outras alterações moleculares causadas pela oxidação de biomoléculas podem gerar neoepítopos com potencial para produzir um amplo espectro de autoanticorpos, levando à inflamação, danos a órgãos e tecidos e exacerbação da autoimunidade no LES (LOZOVOY *et al.*, 2013; MORGAN, P E; STURGESS; DAVIES, 2005; SHAH, DILIP *et al.*, 2013, 2014; ZHANG *et al.*, 2010).

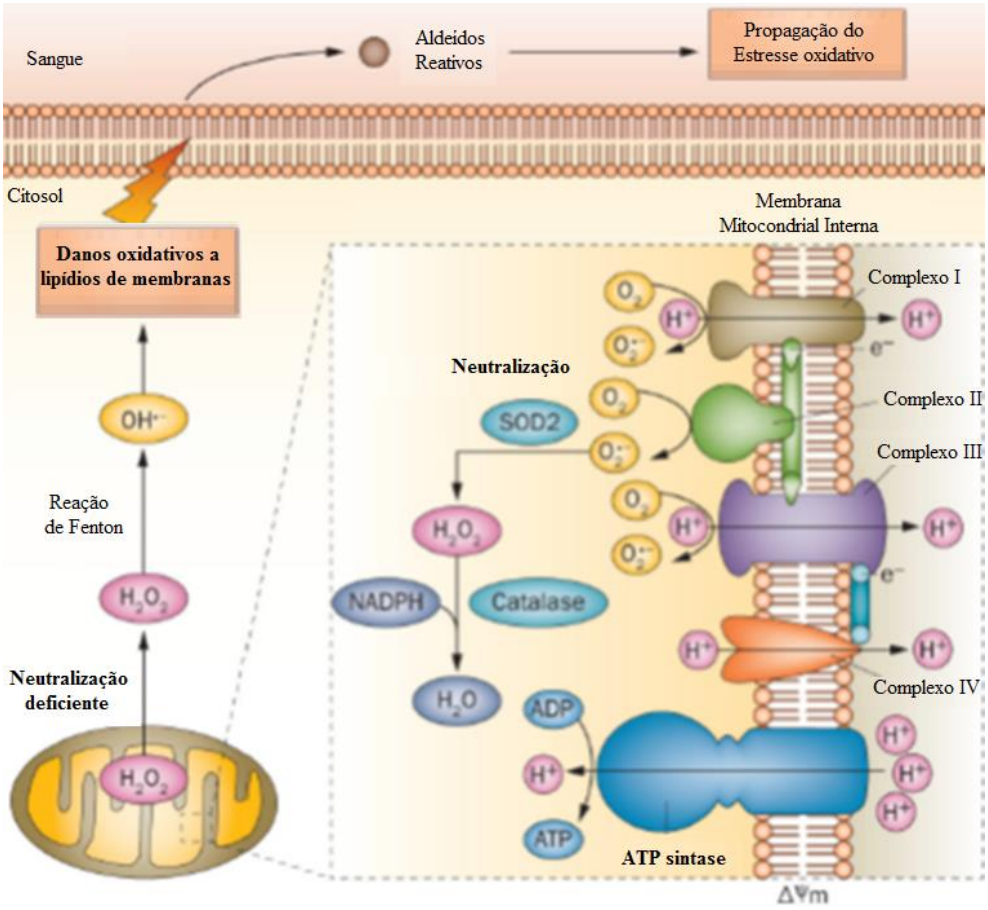


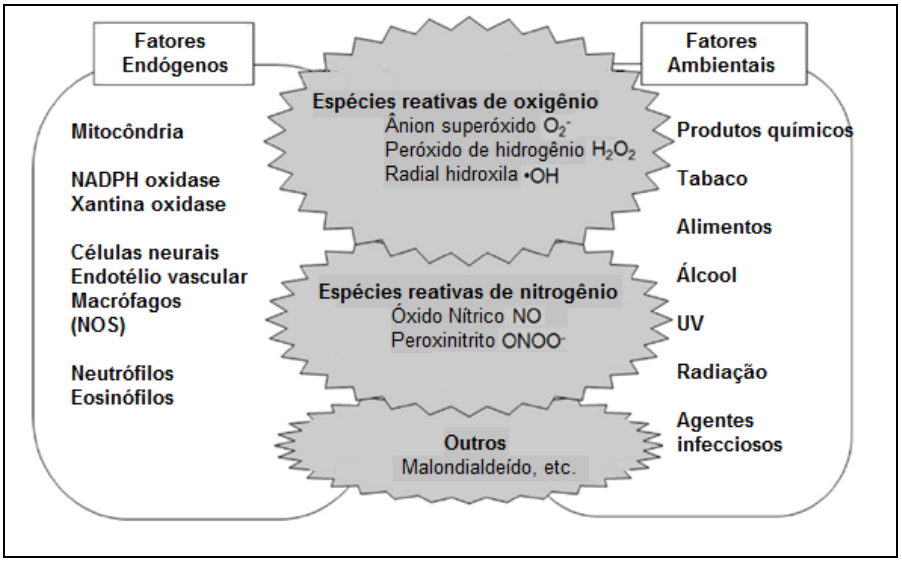
Figura 1 Geração mitocondrial de estresse oxidativo e sua propagação sistêmica, Adaptado de (PERL, 2013). A hiperpolarização mitocondrial, presente em células T de pacientes com LES, ocasiona aumento na produção de espécies reativas de oxigênio através da promoção da transferência de elétrons para o oxigênio molecular, gerando oxigênio radicalar ($O_2^{\bullet-}$). Esse último é convertido em peróxido de hidrogênio (H_2O_2) pela SOD2. O peróxido de hidrogênio é subsequentemente neutralizado, sendo convertido em água (H_2O) através da redução do NADPH, em reação mediada pela enzima catalase. O excesso de H_2O_2 é transformado em radical hidroxila ($^{\bullet}OH$) através da reação de Fenton. O radical hidroxila é fortemente reativo e provoca danos oxidativo em lipídios e outras macromoléculas no entorno. A peroxidação lipídica produz aldeídos altamente reativos, que se difundem rapidamente para outras organelas na célula e para a corrente sanguínea.

Outras fontes endógenas de intermediários reativos de oxigênio e nitrogênio são as metaloenzimas (ex. nicotinamida adenina dinucleótido fosfato oxidase e óxido nítrico sintase), peroxissomos, células polimorfonucleares (ex. neutrófilos), lisossomos e microssomos, macrófagos, células epiteliais, plaquetas e leucócitos (SHAH, DILIP; SAH; NATH, 2013a). Dentre as fontes exógenas de geração de EROs destacam-se a radiação ultravioleta, alguns medicamentos (ex. acetaminofeno), compostos químicos (ex. tabagismo, pesticidas) e infecções bacterianas ou virais (ex. Epstein-Barr) (COSTENBADER *et al.*, 2004; DAS *et al.*, 2010; JAMES *et al.*, 1997; LEHMANN *et al.*, 1990; PERL, 2013).

O NO é sintetizado por óxido nítrico sintase (NOSs) usando oxigênio e aminoácido arginina como substratos (NAGY, G *et al.*, 2010). NOS induzíveis (iNOS) são expressos principalmente em células imunes, como macrófagos em resposta a estímulos inflamatórios (OATES; GILKESON, 2006). NOS endoteliais (eNOS) e NOS neuronais (nNOS) são expressos em linfócitos no sangue periférico humano (OATES; GILKESON, 2006). iNOS produz quantidades mais elevadas de NO do que as NOS constitutivas e quando esta produção ocorre nas imediações de mitocôndrias com um estado oxidativo, ela pode reagir com o ânion superóxido para formar o peroxinitrito, que é altamente reativo. ERN têm demonstrado ter capacidade para induzir apoptose ou causar modificações a biomoléculas, como proteínas, lipídios e DNA, levando a uma quebra da tolerância imunológica (KHAN; ALI, 2006; OATES; GILKESON, 2006).

As principais fontes endógenas e exógenas de EROs e ERNs estão apresentadas na Figura 2.

Figura 2 Principais fontes endógenas e exógenas de espécies reativas de oxigênio e nitrogênio, segundo Thanan et al., 2014.



2.2. Principais Marcadores de Danos Oxidativos e Nitrosativos a Biomoléculas

Produtos de modificações oxidativas de lipídios, proteínas e DNA podem ser detectados em diversos fluidos biológicas (ex. como urina, soro, plasma) e células (ex. linfócitos, hemácias) e têm sido relacionados à atividade da doença em pacientes com LES (MANSOUR *et al.*, 2008; MORGAN, P E; STURGESS; DAVIES, 2005; SHAH, DILIP *et al.*, 2010, 2014; SHAH, DILIP; WANCHU; BHATNAGAR, 2011; WANG *et al.*, 2010, 2016).

2.2.1. Marcadores de Peroxidação Lipídica

Os lipídios são os principais alvos das espécies reativas devido à sua abundância nas membranas celulares. A peroxidação lipídica pode ser resumida como a oxidação - por EROs - de lipídios contendo ligações duplas carbono-carbono, como membranas de fosfolípidos de ácidos graxos poliinsaturados, ésteres de colesterol, glicolípidos e ácidos graxos livres, resultando em inúmeros produtos de oxidação (AHSAN, H.; ALI; ALI, 2003; AYALA; MUÑOZ; ARGÜELLES, 2014). Os principais produtos primários de peroxidação lipídica são hidroperóxidos lipídicos. Os principais produtos secundários de peroxidação lipídica incluem aldeídos altamente reativos, tais como malondialdeído (MDA), 4-hidroxinonenal (4-HNE) e hexanal, que se difundem rapidamente para outras organelas na célula e na corrente sanguínea, propagando danos oxidativos (AHSAN, H.; ALI; ALI, 2003; AYALA; MUÑOZ; ARGÜELLES, 2014; PERL, 2013; WANG *et al.*, 2010).

Os hidroperóxidos lipídicos (LOOH) são marcadores relativamente estáveis de peroxidação lipídica. Após sua formação, LOOH podem ser reduzido pelas enzimas glutationa peroxidase (GPx) e selenoproteína P (SeP) aos seus álcoois correspondentes ou continuar no processo de propagação / terminação da peroxidação lipídica, causando maior dano oxidativo (AYALA; MUÑOZ; ARGÜELLES, 2014).

O MDA é um marcador confiável de peroxidação lipídica e, após a sua formação, pode ser metabolizado em CO_2 e H_2O em uma série de reações enzimáticas ou pode se ligar covalentemente a proteínas e ácidos nucleicos, levando a modificações estruturais e danos à função biológica (AYALA; MUÑOZ; ARGÜELLES, 2014).

Assim como o MDA, o 4-HNE tem a capacidade de se ligar covalentemente a proteínas e ácidos nucleicos. Essas moléculas reativas modificadas com aldeídos são altamente imunogênicas, induzindo uma forte resposta de anticorpos dependentes de T (WANG *et al.*, 2010, 2016).

Os isoprostanos (F2) são liberados em formas livres por fosfolipases e podem estar circulantes no plasma ou serem excretados na urina (IULIANO *et al.*, 1997). A

quantificação de isoprostanos na urina e plasma vem sendo considerada biomarcador confiável na determinação de peroxidação lipídica *in vivo* e seus níveis plasmáticos possuem grande associação com doenças coronárias (DAVIES; ROBERTS, 2011).

O aumento da peroxidação lipídica tem sido relatado de forma consistente no LES, sendo associado com maiores valores de SLEDAI (SCAVUZZI *et al.*, 2017; SHAH, DILIP *et al.*, 2010; WANG *et al.*, 2010, 2016), maior atividade de doença autorrelatada, fadiga e menor qualidade de vida (AVALOS *et al.*, 2007).

2.2.2. Marcadores de Oxidação de Proteínas

As proteínas são alvos importantes de dano oxidativo devido à sua abundância na maioria dos tecidos humanos (MORGAN, PHILIP E; STURGESS; DAVIES, 2009). O dano oxidativo às proteínas pode ocorrer a partir do ataque direto de espécies reativas ou através de aldeídos reativos de oxidação lipídica (ex. MDA e 4-HNE), que se ligam covalentemente a proteínas, gerando neoepítopos que levam a respostas autoimunes (WANG *et al.*, 2010, 2016). Além disso, a oxidação de resíduos de aminoácidos de proteínas, também pode alterar a função enzimática e vias de sinalização, agravando ainda mais a fisiopatologia do LES (AFANAS'EV, 2015; KHAN; SIDDIQUI; ALI, 2006). Múltiplos marcadores de oxidação de proteínas foram associados ao aumento da atividade da doença no LES (MORGAN, P E; STURGESS; DAVIES, 2005). Morgan et al., 2009 demonstraram que os pacientes com LES apresentaram níveis elevados de oxidação de proteínas e encontraram evidências de que a oxidação de proteínas desempenha um papel importante na patogênese desta doença (MORGAN, PHILIP E; STURGESS; DAVIES, 2009).

O método "Produtos avançados da oxidação protéica", do inglês *advanced oxidation protein products* (AOPP), avalia o dano oxidativo decorrente da oxidação de resíduos do aminoácido tirosina das proteínas plasmáticas, como albumina, fibrinogênio e lipoproteínas e são quantificados por espectrofotometria (WITKO-SARSAT *et al.*, 1996).

O grupamento sulfidrila (-SH) das proteínas, também chamado de grupamento tiol, tem a capacidade de atuar como antioxidante e remover radicais peroxil do plasma e, assim, inibir a oxidação de proteínas (PÉREZ *et al.*, 2012; ZHANG *et al.*, 2010). Portanto, seus níveis *in vivo* geralmente são inversamente associados à oxidação protéica.

Os derivados de carbonílicos (aldeídos e cetonas) são produtos primários da oxidação dos resíduos de aminoácidos tais como lisina, treonina, prolina, arginina, e são relativamente estáveis (DALLE-DONNE *et al.*, 2003). Os derivados de proteína carbonílica

também podem surgir da reação secundária com aldeídos derivados de peroxidação lipídica (por exemplo MDA, 4-HNE) produzidos durante a peroxidação lipídica (DALLE-DONNE *et al.*, 2003).

2.2.3. Oxidação de ácidos nucleicos

O DNA liberado de células apoptóticas e necróticas também pode sofrer dano oxidativo, favorecendo seu reconhecimento pelo sistema imunológico (TSOKOS, 2011).

A interação do radical hidroxila com as nucleobases da cadeia de DNA como a guanina leva à formação de C8-hidroxiguanina ou 8-hidroxi-2'-desoxiguanosina (8-OHdG), que é amplamente usado como um biomarcador para o estresse oxidativo do ácido nucleico (VALAVANIDIS; VLACHOGIANNI; FIOTAKIS, 2009). Foi demonstrado que os níveis de 8-OHdG no plasma de pacientes com LES possuem associação positiva com a atividade da doença (SLEDAI) (LEE *et al.*, 2014), exposição solar de alta intensidade (MAESHIMA *et al.*, 2002) e exposição a produtos químicos como pesticidas (SIMONIELLO *et al.*, 2017).

Apesar da importância da oxidação de ácido nucleico na indução de produção de autoanticorpos, poucos estudos investigaram o dano oxidativo em ácidos nucleicos no LES humano.

A figura 3 resume os principais biomarcadores de estresse oxidativo e nitrosativo no LES.

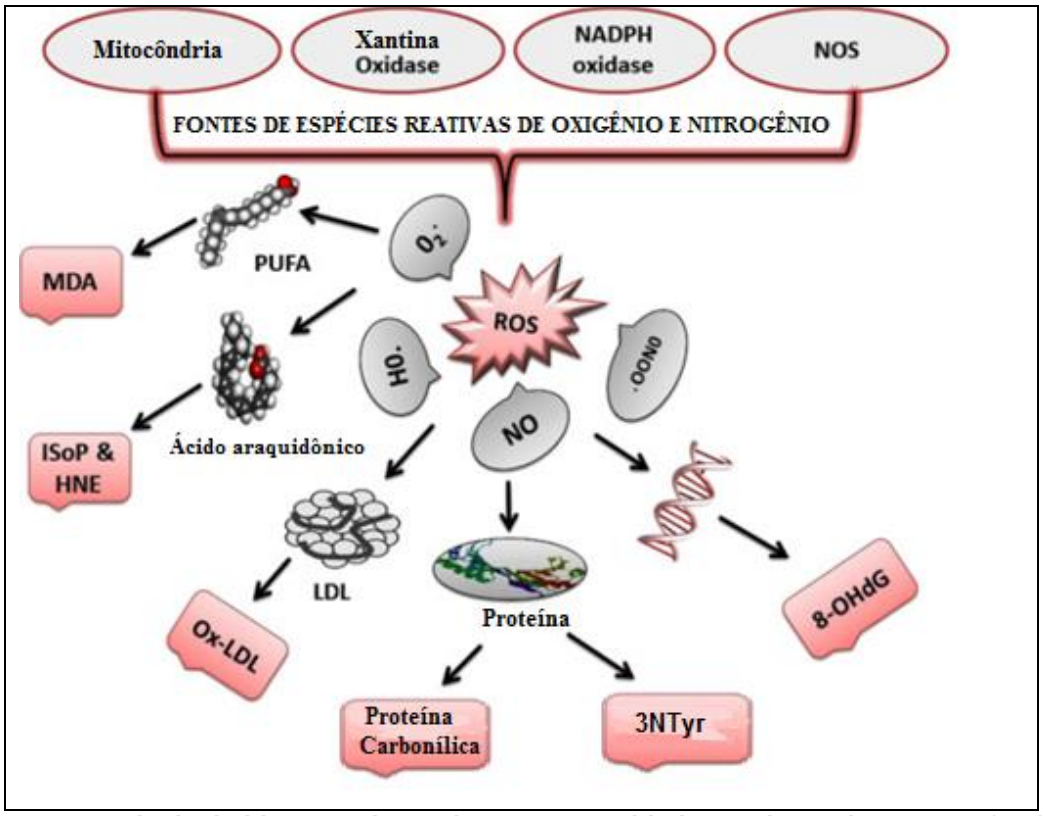


Figura 3 Principais biomarcadores de estresse oxidativo e nitrosativo, segundo Shah et al., 2014. Oxidação de biomoléculas e formação de biomarcadores de estresse oxidativo e nitrosativo. São biomarcadores de peroxidação lipídica: malondialdeído (MDA), F2-isoprostano (ISoP), hidroxinonenal (HNE), lipoproteínas de baixa densidade (Ox-LDL). Marcadores de oxidação de proteínas: proteína carbonílica e 3-nitrotirosina (3NTyr). Biomarcador de dano oxidativo ao DNA: 8-hidroxi-2'-desoxiguanosina (8-OHdG).

2.2.4. NITROSAÇÃO

Os resíduos de tirosina são geralmente expostos e, portanto, suscetíveis a ataques de ERN, resultando na formação de proteínas como a 3-nitrotirosina (3NTyr) (AHSAN, HASEEB, 2013). A 3NTyr é considerada um marcador bioquímico para nitração de proteínas devido à ação do peroxinitrito, dióxido de nitrogênio, ácido nitroso, cloreto de nitrilo e algumas peroxidases (AHSAN, HASEEB, 2013). As proteínas modificadas pelo peroxinitrito podem servir como neoantígenos, provocando uma resposta imunogênica (WANG *et al.*, 2010).

Além da 3NTyr, que mede os efeitos do peroxinitrito em proteínas contendo tirosina, a concentração de NO nas amostras pode ser estimada pela medição de seus metabólitos, como nitritos (NO_2^-) e nitratos (NO_3^-), pois o NO é uma molécula de curta duração (NAVARRO-GONZÁLVEZ; GARCÍA-BENAYAS; ARENAS, 1998).

No LES, embora alguns estudos tenham relatado elevação dos níveis dos metabólitos de óxido nítrico (NOx) e associações positivas com nefrite lúpica e

atividade da doença avaliada por SLEDAI (OATES *et al.*, 2008), outros não conseguiram encontrar essas associações (GONZALEZ-CRESPO *et al.*, 1998). Em uma pesquisa anterior, nosso grupo de estudo mostrou que os níveis de NOx foram inversamente e independentemente associados à atividade da doença avaliada pelo escore de SLEDAI (IRIYODA *et al.*, 2017). Portanto, concluímos naquele estudo que em condições de inflamação e aumento do estresse oxidativo, o NO é consumido em uma reação com o ânion superóxido, produzindo peroxinitrito e diminuindo assim a sua biodisponibilidade.

2.3. ANTIOXIDANTES

O corpo humano possui numerosos sistemas antioxidantes que agem para neutralizar as EROs e manter um equilíbrio redox adequado (ESRA BIRBEN *et al.*, 2012; WANG *et al.*, 2010). Os antioxidantes são geralmente classificados como enzimáticos (ex., superóxido dismutases, catalase e glutationa peroxidase) e não enzimáticos tais como vitaminas C e E, ácido úrico e glutationa (ESRA BIRBEN *et al.*, 2012). Os sistemas antioxidantes enzimáticos e a glutationa são predominantemente encontrados no meio intracelular, enquanto os antioxidantes não enzimáticos são principalmente encontrados no meio extracelular (VASCONCELOS *et al.*, 2007).

No LES, há evidências de que as defesas antioxidantes são incapazes de lidar com o estresse oxidativo e nitrosativo na doença. Há diversos estudos em que marcadores de capacidade antioxidantes que estão diminuídos, e essa redução está associada ao aumento da atividade da doença (AL-SHOBAILI *et al.*, 2013; WANG *et al.*, 2010). Essa relação inversa entre níveis de antioxidantes e atividade da doença é particularmente importante no caso da glutationa, que tem sido consistentemente associada ao estresse oxidativo no LES e predisposição celular à apoptose (SHAH, DILIP; SAH; NATH, 2013b). Embora já tenham sido relatados aumentos compensatórios de enzimas antioxidantes, foram insuficientes para prevenir danos oxidativos (SIMONIELLO *et al.*, 2017). Assim, além do estresse oxidativo, a diminuição dos níveis antioxidantes contribui ainda mais para o desequilíbrio redox no LES (MANSOUR *et al.*, 2008; MORGAN, PHILIP E; STURGESS; DAVIES, 2009; SHAH, DILIP; WANCHU; BHATNAGAR, 2011; WANG *et al.*, 2010).

2.3.1. Antioxidantes enzimáticos

A superóxido dismutase (SOD) é um grupo de três metaloenzimas que catalisa a conversão do superóxido em peróxido de hidrogênio eliminando o anion

superóxido ($O_2^{\bullet-}$), levando a sua conversão em peróxido de hidrogênio, conforme resumido na Figura 4. SOD1, SOD2 e SOD3 estão primariamente localizadas no citoplasma, mitocôndria e no meio extracelular, respectivamente. Nos seres humanos, essas enzimas são habitualmente ligadas aos íons metálicos de cobre, zinco e manganês (JOHNSON, FELICITY; GIULIVI, 2005). O peróxido de hidrogênio é posteriormente reduzido a água por ação da enzima catalase (CAT) ou glutationa peroxidases (GPx), como mostrado na Figura 4 (LEE *et al.*, 2014). As catalases são principalmente localizadas em peroxissomos, GPx-1 no citossol e GPx-4 na mitocôndria (CHELIKANI; FITA; LOEWEN, 2004; LEE *et al.*, 2014). A GPx cataliza a oxidação da glutationa (GSH) para a glutationa dissulfeto (GSSG). A GSSG pode ser novamente reduzida a GSH através da glutationa redutase (GR) (LEE *et al.*, 2014).

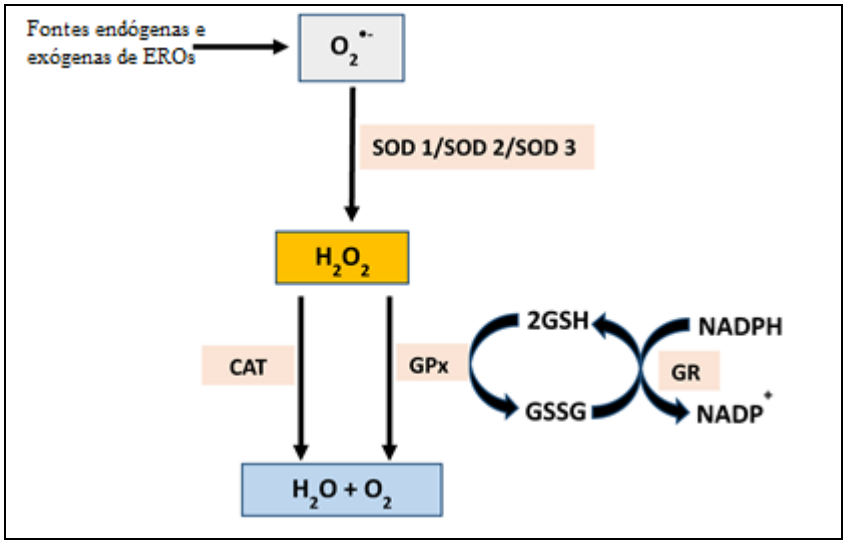


Figura 4 Principais defesas antioxidantes enzimáticas, adaptado de Ajuwon et al., 2015.

No LES foi relatado que existem autoanticorpos contra SOD e CAT modificados pelo MDA, demonstrando que essas enzimas podem se tornar alvos da resposta imune após modificação oxidativa (BEN MANSOUR *et al.*, 2010).

2.3.2. Antioxidantes não-enzimáticos

A capacidade antioxidante pode ser quantificada em diversos fluidos biológicos (por exemplo, soro, plasma) através de métodos que basicamente verificam o poder total antioxidante da amostra comparando-a a uma solução padrão, não sendo possível determinar diretamente qual o componente da amostra responsável pela ação antioxidante. Exemplos de metodologias são: poder de redução do íon ferro [*Ferric Reducing Antioxidant Power* (FRAP)]; Capacidade Antioxidante Equivalente ao Trolox [*Trolox*

Equivalent antioxidant Capacity (TEAC)]; Estado Antioxidante Total [*Total Antioxidant Status* (TAS)]; e Parâmetro Antioxidante de Aprisionamento Total do Radical, [*Trapping Antioxidant Parameter* (TRAP)] (SHAH, DILIP *et al.*, 2014; VASCONCELOS *et al.*, 2007). A maioria dos estudos mostra níveis diminuídos da capacidade antioxidante no LES comparado ao controle (IRIYODA *et al.*, 2017; LOZOVOY *et al.*, 2011; SCAVUZZI *et al.*, 2017; SINCER *et al.*, 2015; ZHANG *et al.*, 2010).

O TRAP, por exemplo, detecta antioxidantes plasmáticos hidrossolúveis e/ou lipossolúveis, medindo o tempo de inibição da quimioluminescência induzido por 2,2-azobis (2- aminopropano) (REPETTO *et al.*, 1996). O ácido úrico (AU), detectável pelo método TRAP, possui ação antioxidante e responsável por 60% da eliminação de radicais livres no plasma humano (VENTURINI *et al.*, 2012). Os níveis de AU são afetados pelo sexo e por muitas condições de saúde, como o índice de massa corporal (IMC) (STÖCKL *et al.*, 2012; VENTURINI *et al.*, 2012), e a insuficiência renal (JOHNSON, RJ *et al.*, 2013; YANG *et al.*, 2011). Assim, ao usar este método, o ácido úrico também deve ser quantificado para permitir as correções estatísticas apropriadas (SIMÃO *et al.*, 2008).

2.4. Moléculas de Adesão e LES

As moléculas de adesão celular (CAMs) controlam a migração transendotelial de leucócitos para os tecidos inflamados com o auxílio de quimiocinas e quimioatratores. Essas moléculas podem ser consideradas marcadores de ativação de disfunção endoteliais (SPRINGER, 1994). As CAMs estão aumentadas em pacientes com LES e já foram associadas positivamente com o aumento da atividade da doença e presença de manifestações clínicas como as erupções cutâneas e a nefrite lúpica (HEJAZI; WERTH, 2016; LALWANI *et al.*, 2015; LEWIS *et al.*, 2016; SANTOS *et al.*, 2017; SKEOCH *et al.*, 2013). Diversos fatores aumentam a expressão dessas moléculas, porém a inflamação crônica é um dos fatores mais importantes (BARTOLONI; SHOENFELD; GERLI, 2011; PRASAD *et al.*, 2015). Adicionalmente, estudos que demonstraram que EROs teriam a capacidade de aumentar a expressão de moléculas de adesão (RUI *et al.*, 2016) e que antioxidantes teriam a capacidade de diminuir a expressão das CAMs sugerem que (MARUI *et al.*, 1993) o estado redox também pode influenciar os níveis dessas moléculas.

2.5. Resposta Imune Th e LES

As células T helper (Th) são usualmente agrupadas em quatro classes Th1, Th2, Th17 e Tregulatórias (Tregs), baseado nas principais citocinas produzidas e seus

efeitos funcionais. A diferenciação das células Th depende dos estímulos inflamatórios do meio (SHAH, KAMINI *et al.*, 2010). As células Th desempenham um papel importante na fisiopatologia do LES e estudos têm demonstrado que no LES há um desequilíbrio entre as classes Th, favorecendo um perfil altamente pró-inflamatório, culminando no aumento da atividade da doença (GUIMARÃES *et al.*, 2017; MAK; KOW, 2014; SHAH, DILIP *et al.*, 2010).

Foi demonstrado que o estresse oxidativo pode ativar a proteína alvo da rapamicina em mamíferos (mTOR) (LAPLANTE; SABATINI, 2009) e potencial transmembranar mitocondrial de células T de pacientes com LES (MAK; KOW, 2014). O aumento da atividade mTOR resulta no aumento da expressão da IL-2 e IL-17, aumentado a polarização Th1 and Th17 e inibindo as células T CD8+ (MAK; KOW, 2014). Essa polarização foi associada a maior atividade da doença e lesão de órgãos (SHAH, KAMINI *et al.*, 2010).

3. JUSTIFICATIVA

Devido ao importante papel do estresse oxidativo e nitrosativo na fisiopatologia do LES e na atividade da doença, é de fundamental importância elucidar suas associações com biomarcadores imuno-inflamatórios e moléculas de adesão. Portanto, foi explorada a relação do estresse oxidativo e nitrosativo com esses biomarcadores em pacientes com LES, além de ter sido avaliado o efeito do uso de antioxidantes sobre esses marcadores e, assim, contribuir para compreensão da complexa etiopatogenia da doença.

4. OBJETIVOS

4.1. OBJETIVO GERAL

Avaliar biomarcadores de estresse oxidativo e nitrosativo e verificar uma possível associação com moléculas de adesão, perfil de citocinas, marcadores de autoimunidade e atividade da doença em pacientes com LES.

4.2. OBJETIVOS ESPECÍFICOS

- Avaliar os perfis oxidativo e nitrosativo, índices imuno-inflamatórios e moléculas de adesão em pacientes com LES e indivíduos controles;
- Avaliar a associação entre os biomarcadores de estresse oxidativo e nitrosativo e os dados sócio-demográficos IMC, sexo, presença de síndrome metabólica e idade;
- Avaliar o efeito do estresse oxidativo e nitrosativo sobre os índices imuno-inflamatórios, moléculas de adesão, SLEDAI, anticorpos antinucleares e anti-dsDNA;
- Propor modelos preditores do LES utilizando biomarcadores do estado redox, moléculas de adesão, índices imuno-inflamatórios e dados demográficos.

5. MATERIAIS E MÉTODOS

5.1. POPULAÇÃO, AMOSTRA E DELINEAMENTO

No total, 460 indivíduos foram selecionados para participar no estudo, entre pacientes atendidos no Ambulatório de Reumatologia do Ambulatório de Especialidades do Hospital Universitário (AEHU) da Universidade Estadual de Londrina (UEL) e voluntários do Hospital Universitário da UEL, Londrina, Paraná. Esse estudo caso-controle incluiu 204 pacientes com LES e 256 voluntários controles. O LES foi diagnosticado utilizando os critérios revisados do *American College of Rheumatology* (ACR) (HOCHBERG, 1997). A atividade da doença foi determinada utilizando pontuação SLEDAI (BOMBARDIER *et al.*, 1992).

5.2. CRITÉRIOS DE INCLUSÃO E EXCLUSÃO

Os critérios de inclusão dos pacientes com LES e indivíduos controles foram indivíduos de ambos os sexos, com idade entre 18 e 65 anos. Os critérios de exclusão foram a presença de doenças da tireóide, suprarrenais, renais, hepáticas, gastrointestinais, doenças infecciosas, oncológicas, outras doenças autoimunes, terapia de reposição hormonal e uso de suplementos antioxidantes. Fumantes também foram excluídos do estudo.

5.3. ASPECTOS ÉTICOS, CONSENTIMENTO, SAÚDE E SEGURANÇA

A pesquisa foi conduzida em conformidade com todas as normas estabelecidas de experimentação humana. Além disso, o Comitê de Ética em Pesquisa Envolvendo Seres Humanos da UEL aprovou todos os procedimentos envolvendo os participantes humanos, conforme aprovação de número CAAE: 0186512.0.0000.5231, Parecer CEP n. 210.328, de 04/03/2013 (Anexo 1) da UEL, e um termo de consentimento livre e esclarecido (TCLE) foi obtido de todos os indivíduos incluídos no estudo (Apêndices 1 e 2). Esta investigação clínica foi conduzida de acordo com os princípios expressos na Declaração de Helsinki e suas alterações posteriores. Todos os procedimentos obrigatórios de saúde e de segurança do laboratório foram cumpridos.

5.4. DADOS DEMOGRÁFICOS, EPIDEMIOLÓGICOS, MEDIDAS ANTROPOMÉTRICAS E MENSURAÇÕES DE PRESSÃO SANGUÍNEA

As informações sobre o histórico médico foram obtidas na avaliação clínica realizada por médico reumatologista. Informações sobre a duração da doença, como também a utilização de medicamentos, anti-inflamatórios não-esteroides, corticosteróides, antimaláricos, contraceptivos orais, medicamentos anti-hipertensivos e suplementos com vitaminas com propriedades antioxidantes conhecidas (ex. vitamina A, C, E e D) e também minerais que constituem sistemas antioxidantes importantes, como superóxido dismutase (zinco, cobre e magnésio) foram registradas para cada paciente. Os indivíduos de ambos os grupos relataram não fazer uso de bebidas alcoólicas regularmente (Apêndices 3 e 4).

As medidas antropométricas foram tomadas. O peso corporal foi medido com precisão de 0,1 kg no período da manhã, utilizando uma balança eletrônica, com os indivíduos vestindo roupas leves e sem sapatos; a altura foi medida com precisão de 0,1 cm usando um estadiômetro. O IMC foi calculado a partir do peso (kg) dividido pela altura (m) elevado ao quadrado. A circunferência abdominal (CA) foi medida com os pacientes em pé, na metade da distância entre a face inferior da última costela e a porção superior da crista ilíaca e expressa em cm. Após o paciente ter permanecido sentado, foram realizadas três aferições de pressão arterial no braço esquerdo usando um esfigmomanômetro calibrado com um intervalo de 1 minuto entre as mensurações. A média destas leituras foi utilizada na análise, expressas em mmHg.

A MetS foi definida seguindo o critério do Adult Treatment Panel III, que classifica o paciente como tendo MetS se três ou mais dos seguintes cinco critérios forem atendidos: 1) circunferência abdominal superior a 94 cm em homens e 80 cm em mulheres, 2) nível de triglicerídeos em jejum maior que ou igual a 1,7 mmol/L (150 mg/dL), 3) lipoproteína de alta densidade (HDL) inferior a 1,0 mmol/L (40 mg/dL) em homens ou 1,3 mmol/L (50 mg/dL) em mulheres; 4) pressão arterial superior a 130/85 mmHg (ou uso de medicação anti-hipertensiva) e 5) nível de glicose em jejum superior ou igual a 5,6 mmol/L (100 mg/dL) ou ao uso de medicação antidiabética (NATIONAL CHOLESTEROL EDUCATION PROGRAM (NCEP) EXPERT PANEL ON DETECTION EVALUATION AND TREATMENT OF HIGH BLOOD CHOLESTEROL IN ADULTS (ADULT TREATMENT PANEL III), 2002).

5.5. MARCADORES LABORATORIAIS

Após jejum de 12 horas, amostra de sangue venoso foi colhida usando tubos estéreis (BD Vacutainer® UltraTouch™, Franklin Lakes, NJ, EUA), sem anticoagulante ou contendo ácido etilenodiaminotetracético (EDTA). O sangue total foi deixado em repouso durante 30 minutos e centrifugado a 3000 força g durante 10 minutos. As amostras de plasma e de soro foram separadas e seguidamente distribuídas em alíquotas e armazenadas a -80°C para análises subsequentes.

O colesterol total, lipoproteína de alta densidade (HDL), lipoproteína de baixa densidade (LDL), triacilglicerol (TG), glicose e ácido úrico foram avaliados por um auto-analisador bioquímico (Dimension Dade AR Dade Behring, Deerfield, IL, EUA) usando kits Dade Behring®.

Os ANAs foram quantificados utilizando imunofluorescência indireta com células HEp2 como substrato (IFI-ANA-HEp2-IgG, VIRO-IMMUN LaborDiagnostika, GmbH, Oberursel, Alemanha) e foram consideradas significativas quando os títulos ≥1: 160; os anticorpos anti-DNA de dupla fita (anti-dsDNA) foram determinados utilizando imunoensaio de enzima de anticorpo (ELISA, anti-dsDNA, Orgentec Diagnostika, GmbH, Alemanha) e foram considerados significativos quando os títulos ≥ 20 UI / mL. Os níveis plasmáticos das citocinas IFN-γ, IL-4, IL-6, IL-12 e IL-17 foram determinados utilizando um ELISA (eBioscience, San Diego, CA, EUA). As respostas T helper foram consideradas de acordo com as citocinas: Th1 (IL-12 + IFN-γ), Th2 (IL-4) e Th17(IL-17+ IL-6). Os níveis séricos de proteína C-reativa (PCR) foram medidos usando um ensaio turbidimétrico (C8000, ABBOTT, Architect Abbott Laboratories, Abbott Park, IL, EUA).

Os hidroperóxidos lipídicos foram avaliados por quimioluminescência iniciada por terc-Butil hidroperóxido (CL-LOOH), conforme descrito anteriormente Gonzalez Flecha et al, 1991, e os resultados foram expressos em contagens por minuto (cpm) (GONZALEZ FLECHA; LLESUY; BOVERIS, 1991). Produtos avançados de oxidação protéica (AOPP) foram determinados no plasma usando o método descrito por Witko-Sarsat et al. 1996. As concentrações de AOPP foram expressas como micromoles por litro (µmol / L) de equivalentes de T de cloramina (WITKO-SARSAT *et al.*, 1996). A concentração de metabólitos de óxido nítrico na amostra foi estimada pela medição dos nitritos metabólicos (NO_2^-) e nitratos (NO_3^-) utilizando esferas de cadmio para redução de nitrato em nitrito. As concentrações destes metabólitos foram posteriormente determinadas de acordo com o método proposto por Navarro-Gonzáles et al. 1998. Os valores de NOx foram expressos em µM (NAVARRO-GONZÁLVEZ; GARCÍA-BENAYAS; ARENAS, 1998). Os grupos sulfidrila (-SH) de proteínas foram avaliados em amostras de plasma por um ensaio espectrofotométrico baseado em ácido 2,2-ditiobisnitrobenzóico

(DTNB), como relatado anteriormente por Hu, 1994 e os resultados são expressos em μM (HU, 1994). A capacidade antioxidante total foi determinada pelo método TRAP, por quimioluminescência conforme Repetto et al. 1996. Este método detecta antioxidantes plasmáticos hidrossolúveis e/ou lipossolúveis medindo o tempo de inibição da quimioluminescência induzido por 2,2-azobis (2- aminopropano). O sistema foi calibrado com o Trolox analógico de vitamina E, e os valores de TRAP foram expressos em equivalente de μM Trolox / mg UA (REPETTO *et al.*, 1996). As degradações oxidativas de DNA / RNA foram avaliadas por 8-hidroxi-2-desoxiguanosina (8-OHdG) - um produto de guanina de base de DNA oxidativamente modificada - usando um ensaio imunossorvente ligado a enzima em sanduíche (ELISA) (eBioscience, San Diego, CA, EUA).

Os níveis plasmáticos do Inibidor do ativador do plasminogênio Tipo 1 (PAI-1) e das moléculas de adesão: molécula de adesão celular endotelial plaquetária (PECAM-1), molécula de adesão celular-vascular-1 (VCAM-1), molécula de adesão intercelular-1 (ICAM), E-selectina, P-selectina foram dosados pelo *Human Magnetic Adhesion 6-Plex Panel* (Novex Life Technologies, Frederick, Estados Unidos da América) para a plataforma Luminex®.

5.6. ANÁLISE ESTATÍSTICA

O tamanho da amostra foi estimado estatisticamente usando GPower usando poder estatístico de 80% e nível de significância de $p < 0,05$, e com um efeito de 0,15 (com base na média e desvio padrão de alguns dos parâmetros previamente avaliados em outros estudos). A amostra em estudo para análise de covariância (2 grupos) deveria ser em torno de 351. Portanto, 204 pacientes e 256 controles foram incluídos. As análises de variância (ANOVAs) ou o teste não paramétrico de U de Mann-Whitney foram empregados para verificar diferenças entre os grupos em variáveis contínuas. As análises de tabelas de contingência (X^2-test) foram utilizadas para verificar as associações entre variáveis categóricas. As análises do modelo de análise multivariada linear geral (do inglês *multivariate general linear model* - GLM) foram utilizadas para avaliar os efeitos multivariados das variáveis explanatórias (incluindo o diagnóstico) sobre as variáveis dependentes (incluindo biomarcadores de estresse oxidativo e nitrosativo), controlando sexo, idade, IMC e SM. Foram utilizados “testes dos efeitos entre sujeitos” para avaliar os efeitos univariáveis das variáveis preditoras significativas nas variáveis dependentes. As médias marginais estimadas (± SE) foram calculadas para interpretar as diferenças inter-grupos entre as variáveis independentes categóricas e as estimativas de parâmetros foram utilizadas para interpretar a direção e o impacto de variáveis independentes contínuas. Foram utilizadas análises de regressão logística binária

automática para delinear as variáveis explanatórias mais significativas que predizem o LES (controles como grupo de referência) utilizando as seguintes variáveis explanatórias: biomarcadores de estresse oxidativo e nitrosativo, idade, presença de síndrome metabólica, IMC com ou sem biomarcadores imunes ou moléculas de adesão. Alguns biomarcadores de estresse oxidativo e nitrosativo foram transformados em Ln (LOOH, AOPP, NOx) visando ajustá-los a uma distribuição normal (avaliada usando o teste de Kolmogorow-Smirnov). Utilizamos também as pontuações transformadas z para os biomarcadores para mostrar as diferenças entre os grupos e para calcular as pontuações compostas ponderadas da unidade z, incluindo um índice de estresse oxidativo como valor z de AOPP (zAOPP) + zLOOH (zAOPP + LOOH); zNOx (diminuição de NOx indicando um consumo de NO para nitrosilação e formação de peroxinitrito) e zTRAP (TRAP reduzido sendo um antioxidante) como zTRAP + NOx e índice de estresse nitro-oxidativo como zAOPP + zLOOH- (zTRAP + zNOx) como zAOPP + LOOH / TRAP + NOx. Também usamos dados imunológicos, como apresentado anteriormente (GUIMARÃES *et al.*, 2017), ou seja, o índice de atividade Th17 (Th17), que é o valor z de IL-17 (zIL-17) + zIL-6; um índice de ativação imune Th17 em relação à atividade Th2 (Th17 / Th2), calculado como zIL-17 + zIL-6-zIL-4; um índice de ativação imune ou atividade Th1 + Th17 (Th1 + Th17), calculado como zIL12 + zIFNγ + zIL-6 + zIL-17; e um índice de ativação imune geral versus supressão (Th1 + Th17 / Th2), calculado como zIL12 + zIFNγ + zIL-6 + zIL-17 - zIL-4. Todas as análises estatísticas foram realizadas usando o IBM SPSS windows versão 22. Todos os resultados de regressão foram verificados para multicolinearidade usando VIP e tolerância. Resultados dos testes bicaudais a um nível alfa de 0,05 ($p<0,05$).

6. RESULTADOS

ARTIGOS CIENTÍFICOS

O presente estudo deu origem a dois trabalhos: o primeiro, um artigo de revisão intitulado ¨Targeting Oxidative and Nitrosative Stress in Systemic Lupus Erythematosus¨ a ser enviado para a revista *Expert Opinion on Therapeutic Targets*. O segundo, um artigo científico original intitulado ¨Increased lipid and protein oxidation and lowered antioxidant defenses in systemic lupus erythematosus are associated with severity of illness, autoimmunity, increased adhesion molecules and Th1 and Th17 immune shift¨, aceito para publicação na revista *Immunologic Research* com fator de impacto de 2,905.

6.1. ARTIGOS 1: TARGETING OXIDATIVE AND NITROSATIVE STRESS IN SYSTEMIC LUPUS ERYTHEMATOSUS

Targeting Oxidative and Nitrosative Stress in Systemic Lupus Erythematosus

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ABSTRACT

Introduction: Systemic Lupus Erythematosus (SLE) is a chronic inflammatory autoimmune disease characterized by autoantibody production especially directed against nuclear components, leading to chronic widespread inflammation and tissue destruction. Increased production of reactive oxygen and nitrogen species (RO&NS) can lead to modifications of cellular biomolecules such as proteins, lipids and deoxyribonucleic acid, generating neo-epitopes with the potential to produce an exacerbation of autoimmunity in SLE. Oxidative and nitrosative stress (O&NS) upregulates proinflammatory processes, which in turn can increase O&NS in a vicious cicle. Additionally, metabolic syndrome and obesity may amplify the inflammation process due to the production of proinflammatory cytokine by the visceral adipose tissue.

Areas covered: This review gathers recent and relevant publications on the pathophysiology of O&NS in SLE and autoimmunity and discusses the main mechanisms involved. This review also discusses the antioxidant system and the main antioxidant therapies investigated in SLE to date.

Expert opinion: Antioxidant therapy, and especially N-acetylcysteine, may confer numerous health benefits to patients with SLE due to modulation of O&NS. Further studies should consider antioxidants in addition to the standard therapy for management of SLE.

Keywords: Systemic Lupus Erythematosus; reactive oxygen species; pathophysiology; intervention; antioxidant; N-acetylcysteine*;* fish oil; vitamins.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease of multifactorial origin, including hormonal, genetic and environmental aspects [1,2]. The disease is characterized by abnormal B cell and T cell activation and proliferation, as well as impaired death signaling, leading to a large production of autoantibodies, directed mainly against nuclear components and formation of immune complexes, causing disseminated chronic inflammation, tissue destruction, accelerated atherosclerosis and premature mortality [1–3]. It has been consistently demonstrated that an exacerbated increase in the production of reactive intermediates (RI) can produce substrates that aggravate the antigenicity, triggering the characteristic production of autoantibodies that define the disease [1,4–7].

Reactive oxygen species (ROS) is a general term for reactive species arising from incomplete oxygen reduction, including oxygen radicals (e.g. superoxide anion $O_{2^{\cdot}}^{-}$ -; hydroxyl radical, OH·), non-radical derivatives (e.g. hydrogen peroxide, H_2O_2) and [8]. ROS are products of cellular metabolism and are generated primarily by the mitochondrial respiratory chain. In controlled quantities, ROS modulate several important physiological aspects of cell function and are required for signaling pathways, such as those involved in T cell activation, cytokine production and proliferation, apoptosis of abnormal or aged cells, phagocytosis of infected cells, among other important physiological processes [9,10]. However, when there is an imbalance between the production of these reactive species and their elimination by antioxidant mechanisms, they may become pathogenic, a condition known as O&NS [11,12]. O&NS plays an important role in the development of SLE and several other chronic and autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and cardiovascular diseases [13].

O&NS favors activation of a variety of transcription factors including nuclear factor-kappa B (NF-kB), leading to an upregulation of pro-inflammatory cytokines and chemokines [14]. Furthermore, the inflammatory process itself activates macrophages and neutrophils, increasing O&NS. Additionally, metabolic syndrome and obesity, conditions that are remarkably more prevalent within SLE patients, may amplify the inflammation process due to the production of proinflammatory cytokine by the visceral adipose tissue [15,16]. O&NS is so strongly associated with SLE that in a recent study, we were able to establish a model predicting SLE diagnosis using markers of lipid and protein oxidation, nitric oxide metabolites, platelet endothelial cell adhesion molecule 1 (PECAM-1) and body mass index (BMI). The algorithm correctly classified 93.4% of the subjects with a sensitivity of 94.2% and specificity of 91.5% [17]. Wang *et al*. [18] demonstrated an association between levels of markers of lipid peroxidation and circulating immune complexes (CICs)

suggesting a causal role of lipid-derived reactive aldehydes in the pathogenesis of SLE.

The term reactive intermediates also includes reactive nitrogen species (e.g. nitric oxide, NO; peroxynitrite, $ONOO^-$), known as reactive nitrogen species (RNS) [8]. Nitric oxide (NO) is a signaling molecule that plays a central role in numerous physiologic functions, such as regulation of blood vessel tone, immune regulation, regulation of mitochondrial membrane potential in T cells, signal transduction (e.g. Ca^{2+} signaling) and regulation of apoptosis [19]. However, NO may become pathological when overproduced and depending on the redox state of its immediate cellular milieu, where NO may react with ROS and form highly reactive molecules such as peroxynitrite, generating neo-epitopes with the potential to break immune tolerance [20].

Recent findings have indicated that measurements of reactive oxygen and nitrogen species (RO&NS) in SLE patients may become an interesting tool to help gather information on the pathogenesis of SLE, monitor disease activity, identify and predict which patients are at risk for specific organ damage and complications due to SLE. Thus, this review gathers recent and relevant publications available in the electronic databases PUBMED, Lilacs, Scientific Electronic Library Online (SCIELO), and Science Direct investigating the pathophysiology of ROS in SLE and autoimmunity. The main biomarkers of oxidative and nitrosative stress are reviewed as well as the main mechanisms involved. This review also gathers studies evaluating endogenous antioxidant systems and discusses the main antioxidant therapies tested in SLE patients.

Sources of Reactive Oxygen Species

Endogenous sources of ROS include free radicals eliciting from the electron transport chain (ETC) in the mitochondria; enzymatic systems such as nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), xanthine oxidase, uncoupled nitric oxide synthase, and cytochrome P450; peroxisomes, endoplasmic reticulum, polymorphonuclear cells (e.g. neutrophils), lysosomes and microsomes, macrophages, epithelial cells, platelets and leukocytes [7,21]. Among the exogenous sources of ROS are ultraviolet radiation, certain drugs (e.g. acetaminophen), chemical compounds (e.g. smoking, pesticides), and bacterial or viral infections (e.g. Epstein-Barr) [7,22–25].

The main endogenous source of ROS in patients with SLE is its overproduction by T-cell mitochondria due to a dysfunction known as mitochondrial hyperpolarization, which is persistent in SLE patients [10]. Under this condition, the cytochromes within the electron transport chain become more reduced, generating harmful reactive species, such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$) and hydroperoxyl ($HO^{\bullet}_2$) [26]. Under physiological conditions, as shown on Figure 1, superoxide anion can be converted to hydrogen peroxide (H_2O_2) through superoxide dismutases (SOD) (1), and can

subsequently be converted to water by the action of catalase (CAT) (2) [7]. However, when hydrogen peroxide is excessive and is in the presence of transition metals, such as ferrous ion (Fe^{+2}), it can undergo Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH$) triggered by UV light, forming the hydroxyl radical (${}^{\bullet}OH$), which cannot be neutralized and is highly reactive (3). The hydroxyl radical then causes immediate modifications of cellular biomolecules such as proteins, lipids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in the proximity [7]. In addition to ferrous ion (Fe^{+2}), other transition-metals ions from copper, nickel, cobalt, and vanadium can participate in the generation of hydroxyl radicals [27].

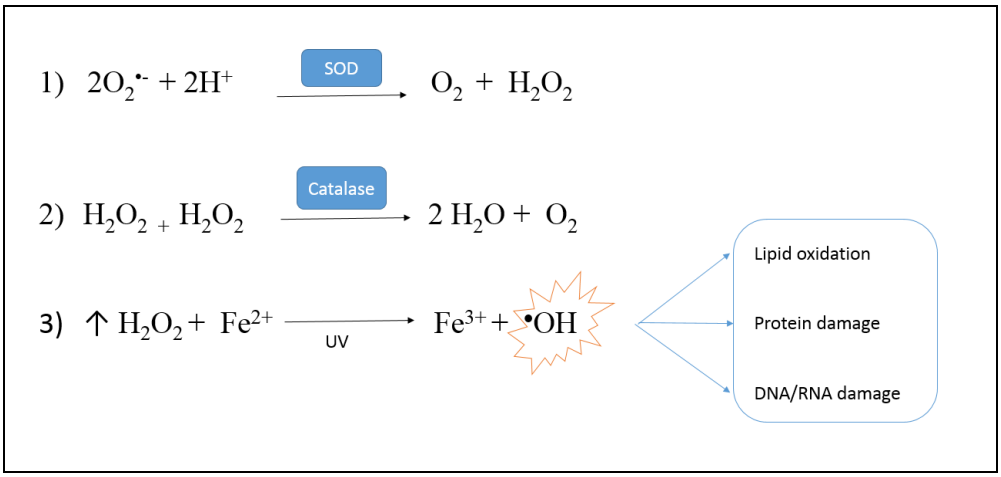

Figure 1: Main enzymatic reactions involved in the neutralization of reactive oxygen species (1,2) and Fenton reaction (3). 1) Superoxide anion is converted to hydrogen peroxide (H_2O_2) through superoxide dismutases. 2) H_2O_2 is converted to water and oxygen by catalase. 3) Excessive H_2O_2 in the presence of Fe^{+2} undergoes Fenton reaction triggered by UV light, forming the highly reactive hydroxyl radical (${}^{\bullet}OH$) (3), causing oxidative damages in lipids, proteins and nucleic acids.

These and other molecular changes caused by oxidation of biomolecules can generate neo-epitopes with the potential to produce a broad spectrum of autoantibodies, leading to inflammation, organ and tissue damage, and exacerbation of autoimmunity in SLE [28–32]. Products of oxidative modification of lipids, proteins and nucleic acids can be detected in various biological fluids (e.g., urine, serum, plasma) and cells (e.g. lymphocytes, red blood cells) though various quantification methods [18,28,32–36].

Sources of Reactive Nitrogen Species

NO is synthesized by nitric oxide synthases (NOSs) using oxygen and the amino acid arginine as substrates [37]. Inducible NOS (iNOS) is mainly expressed in immune cells such as macrophages in response to inflammatory stimuli [20]. Endothelial NOS (eNOS) and neuronal NOS (nNOS) are expressed in lymphocytes in human peripheral blood [20]. iNOS produces higher amounts of NO than the constitutively NOS and when this production occurs in the immediate vicinity of the mitochondria in a highly

oxidative state, it can react with superoxide anion to form the highly RNS peroxynitrite, which has been shown to have te ability to induce apoptosis or cause modifications of cellular biomolecules such as proteins, lipids and DNA, leading to a break in immune tolerance [20,38].

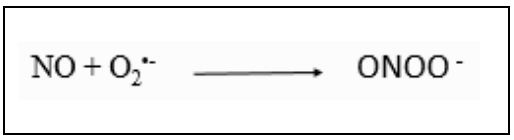

Figure 2: Nitric oxide (NO) reacts with superoxide anion to form highly reactive peroxynitrite.

Amongst the exogenous sources of nitric oxide metabolites, diet is a major confounder in measures of systemic NO production [39].

OXIDATIVE AND NITROSATIVE DAMAGE TO BIOMOLECULES

Lipid peroxidation

Lipid peroxidation can be summarized as the oxidation - by ROS - of lipids containing carbon-carbon double bonds such as polyunsaturated fatty acids (PUFAs) phospholipid membranes, cholesterol esters, glycolipids and free fatty acids, resulting in numerours oxidation products [27,40]. The main primary products of lipid peroxidation are lipid hydroperoxides. The main secondary products of lipid peroxidation include highly reactive aldehydes such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and hexanal, which diffuse rapidly to other organelles in the cell and into the bloodstream, propagating oxidative damage [7,27,33,40].

When ROS production occurs in physiological rates, lipid peroxidation is generally inhibited by antioxidant enzymes present in the mitochondrial, such as SODs, CAT, and by cellular glutathione [27,41]. However, when peroxidation rates are excessive, cells may suffer apoptosis or necrosis, leading to molecular damage, various pathological states and aging [27]. Furthermore, most biological membranes are composed of PUFAs, which are the main oxidative targets of ROS. When these membranes are oxidized by ROS, they suffer structural changes leading to alterations in fluidity, permeability and integrity [27,40]. Spengler *et al*. [42] found higher erythrocyte deformability and decreased membrane fluidity in red blood cells of SLE patients and demonstrated that it was associated with MDA levels.

The process of lipid peroxidation can be divided in three steps: 1) initiation, where ROS abstracts an hydrogen from an polyunsaturated chain and forms a lipid radical (L•); 2) propagation, where molecular oxygen (O_2) addition occurs to the carbon-centered radical (L•) generating a peroxyl radical (LOO•) and lipid hydroperoxide (LOOH); 3)

termination, where the radical molecule gains a hydrogen atom and forms nonradical products [21,27]. These events are shown in Figure 3.

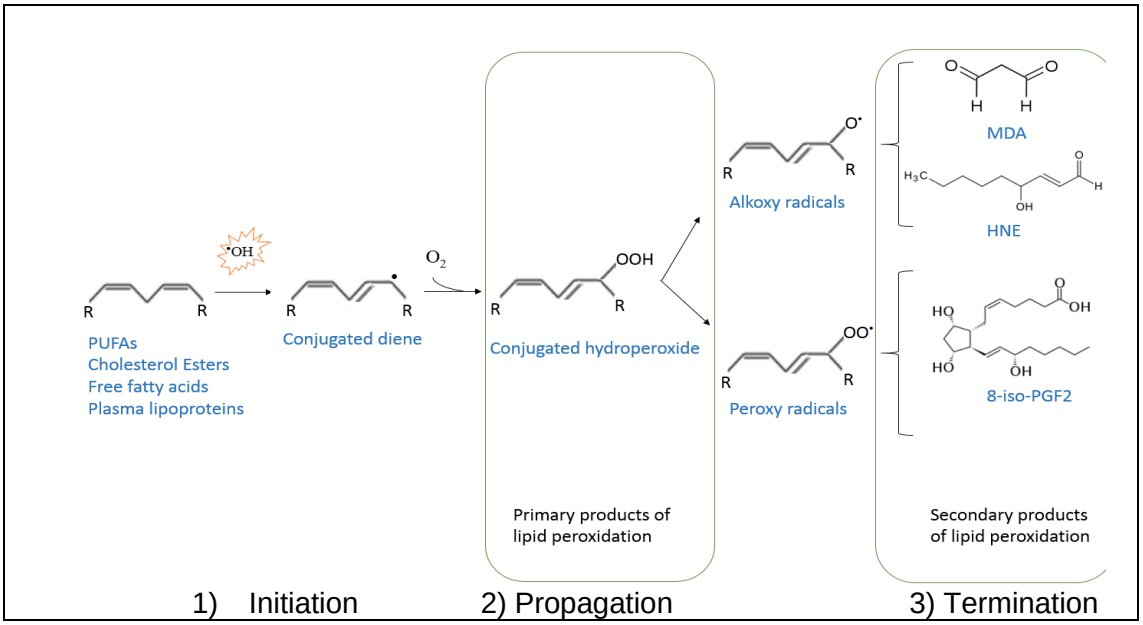

Figure 3: Lipid peroxidation process. Reactive oxygen species target mainly polyunsaturated fatty acids, producing numerous oxidation products in three steps: 1) initiation, where ROS abstracts an hydrogen from an polyunsaturated chain and forms a lipid radical (L•); 2) propagation, where molecular oxygen (O_2) is added to the carbon-centered radical (L•) generating a peroxyl radical (LOO•) and lipid hydroperoxide (LOOH); 3) termination, where the radical molecule gains a hydrogen atom and forms non radical products. Adapted from Lima & Abdalla, 2001 [43].

Lipid hydroperoxides are relatively stable markers of lipid peroxidation. Upon formation, LOOH can be reduced by the enzymes glutathione peroxidases (GPx) and selenoprotein P (SeP) to its corresponding alcohols using glutathione (GSH) as reductant or continue on the propagation/termination process of lipid peroxidation, causing further oxidative damage [27].

Although the lipid peroxidation process forms non radical secondary products, these substances are highly reactive. MDA is a reliable marker of lipid peroxidation and after its formation, it can be metabolized to CO_2 and H_2O in a series of enzymatic reactions or it can bind covalently to proteins and nucleic acids to form adducts, leading to structural modifications and damages to biological function [27]. MDA is easily quantifiable using a colorimetric assay, where MDA reacts with thiobarbituric acid in a method known as Thiobarbituric Acid Reacting Substances Test (TBARS). TBARS is a method that was widely used for quantification of MDA however; it has been shown to be nonspecific and has been discouraged for the analysis of *in vivo* samples. Thus, more specific techniques such as high performance liquid chromatography and immunoassays should be preferred [27].

In turn, 4-HNE has the ability to form adducts and may function as a signaling molecule and regulate several transcription factors, such as erythroid 2-related factor 2

(Nrf2), NF-kB, and peroxisome-proliferator-activated receptors (PPAR) [27]. These reactive aldehyde-modified molecules are highly immunogenic, inducing a strong response of T-dependent antibodies [18,33].

Upon formation, F_2 -isoprostanes are esterified on phospholipids, released in free forms by phospholipases, circulate in plasma and are excreted in urine [44]. Measurement of F_2-isoprostanes in urine and plasma have recently emerged as reliable biomarkers of lipid peroxidation in vivo and their levels have shown striking association with coronary heart diseases [45].

Increased lipid peroxidation has been consistently reported in SLE and it has also been associated with higher SLEDAI scores [17,18,33,34] and higher self-reported disease activity, fatigue and lower quality of life [46]. To study the hypothesis that autoimmunity may be mediated by increased formation of proteins modified by 4-HNE and MDA following excessive ROS generation and oxidative stress, Wang *et al*. [18] examined lipid-derived reactive aldehydes 4-HNE and MDA and immune complexes (ICs) specific to 4-HNE and MDA in SLE patients, which were divided into two groups based on their SLEDAI (<6 and ≥ 6) and compared to healthy controls. The authors found increased prevalence of both 4-HNE and MDA-protein adducts and their specific immune complexes in SLE patients compared to controls. Most importantly, the investigators also found that, in SLE patients, increased 4-HNE and MDA-specific ICs are positively associated with SLEDAI and elevated circulating immune complexes (CICs) suggesting a causal role of lipid-derived reactive aldehydes in the pathogenesis of SLE [18]. Furthermore, MDA has been associated with comorbidities in SLE, such as lupus nephritis [47].

The levels of these oxidation products may be measured through various techniques, such as chemiluminescence, HPLC (UV, EM, QL), CG, immunoassays [27]. Table 1 summarizes recent studies investigating biomarkers of lipid peroxidation in SLE patients.

Table 1. Studies and methodologies evaluating biomarkers of lipid peroxidation in SLE patients compared to healthy controls.

Authors	Lipid hydroperoxides	8-iso-PGF2	MDA	MDA-protein adducts	4-HNE-protein adducts
Iriyoda *et al*. [48]	↑C				
Scavuzzi *et al*. [17]	↑C				
Wang *et al*. [18]				↑I	↑I
Simoniello *et al*. [49]			↑T		
Spengler *et al*. [42]			↑T		
Lozovoy *et al*. [50]	↑C				
Shah *et al*. [29]			↑T		
Pérez *et al*. [51]			↑H		
Lozovoy *et al*. [52]	↑C				
Hassan *et al*. [47]			↑T		
Wang *et al*. [33]				↑I	↑I
Ben Mansour *et al*. [53]				↑T	
Avalos *et al*. [46]		↔G			
Turgay *et al*. [54]			↔T		
Abou-Raya *et al*. [55]		↑I			
Iuliano *et al*. [44]		↑G			

8-iso-PGF2α, 8-isoprostaglandin F2; MDA, malondialdehyde; 4-HNE, 4-hydroxynonenal.
↑: higher levels; ↓: lower levels; ↔ unchanged.
C, chemiluminescence; I, immunoassay; T, TBARS; H, High Performance Liquid Chromatography; G, gas chromatography/mass spectrometry.

Therefore, increased lipid peroxidation was reported in five sudies with MDA [29,42,47,49,51], four studies with lipid hydroperoxides [17,48,50,52], three studies with MDA-protein adducts [18,33,53] and two studies with 8-iso-PGF2 [44,55] and 4-HNE-protein adducts [18,33]. In contrast, one study with 8-iso-PGF2 [46] and one with MDA [54] found no differences between SLE patients and controls.

Protein oxidation and nitrosative stress

Proteins are important targets of oxidative damage because of their abundance in most human tissues [6]. Oxidative damage to proteins may occur from the direct attack of reactive oxygen species or through reactive lipid-oxidation derived aldehydes, which bind covalently to proteins forming adducts, generating neo-epitopes from self-proteins, leading to autoimmune responses [18,33]. Furthermore, oxidation of amino acid residues of proteins, can also alter enzyme function and oxidative signaling, and further aggravate the pathophysiology of SLE [56,57]. Multiple markers of protein oxidation have been associated

with increased disease activity in SLE [32]. Morgan *et al*. [6] demonstrated that SLE patients have sustained, elevated levels of protein oxidation and found evidence that protein oxidation plays an important role in the pathogenesis of this disease.

Tyrosine is a non-essential amino acid present in most proteins. These residues are usually surface-exposed and, thus, susceptible to attack by various factors, resulting in the formation of 3-nitrotyrosine (3NTyr) or tyrosine nitrated proteins [58]. Nitrotyrosine is considered a biochemical marker for nitration of proteins due to the action of peroxynitrite, nitrogen dioxide, nitrous acid, nitryl chloride, and certain peroxidases [58]. Proteins modified by peroxynitrite could serve as neoantigens, triggering an immunogenic response [33]. Advanced oxidation protein products (AOPP) is a method that also evaluates oxidative damage arising from the oxidation of tyrosine residues of the plasma proteins such as albumin, fibrinogen and lipoproteins and are assayed by spectrophotometry [59]. Wang *et al*. [33] demonstrated that serum nitrotyrosine formation was significantly increased in patients with SLE (both active and inactive) compared to healthy controls. Furthermore, the increases were much greater in patients with higher disease activity, indicating that the formation of nitrated proteins is associated with the pathophisiology of SLE [33].

The attack of ROS on proteins may lead to the formation of protein hydroperoxides [30]. Sulfhydryl groups of proteins, also called a thiols, have the ability to work as antioxidants and scavenge peroxyl radicals in plasma and thus, inhibit protein hydroperoxide formation [30,51]. Therefore, their levels *in vivo* are usually inversely associated to protein oxidation.

Carbonyl (aldehydes and ketones) derivatives are produced upon oxidation of amino acids residues such as prolyl, arginyl, lysyl, and threonyl [60]. Protein carbonyl derivatives can also arise from secondary reaction with lipid peroxidation-derived aldehydes (e.g. MDA, 4-HNE) produced during lipid peroxidation [60]. Protein carbonyls have a relatively early formation and are relatively stable, which allows detection and storage and can be detected by various means, such as spectrophotometric assay, ELISA, electrophoresis and Western blot immunoassay [60]. Table 2 summarizes recent studies investigating biomarkers of protein oxidation in SLE patients.

Table 2. Studies investigating biomarkers of protein oxidation in SLE patients compared to healthy controls.

Authors	AOPP	Nitrotyrosine	Protein carbonyl	Thiols
Iriyoda *et al*. [48]	↑^{S}			
Scavuzzi *et al*. [17]	↑^{S}			
Lozovoy *et al*. [50]	↑^{S}			
Shah *et al*. [29]				↓
Pérez *et al*. [51]				↓
Lozovoy *et al*. [52]	↑^{S}			
Wang *et al*. [33]		↑^{I}		
Ben Mansour *et al*. [53]				↓^{S}
Zhang *et al*. [30]		↑^{I}	↑^{S}	↓^{S}
Khan *et al*. [56]		↑^{I}		
Morgan *et al*. [32]		↑^{H}	↑^{I}	↓^{S}

AOPP, advanced oxidation protein products.
↑: higher levels; ↓: lower levels; ↔ unchanged.
S, spectrophotometry; I, immunoassay; H, High Performance Liquid Chromatography.

In summary, all studies investigating biomarkers of protein oxidation and nitration found evidence of significant oxidative damage to proteins. Increased AOPP [17,48,50,52] and nitrotyrosine [30,32,33,56] levels were reported in four studies [17,48,50,52] each, increases in protein carbonyl in two studies [30,32] and five reported decreases in thiols [29,30,32,51,53].

Nucleic Acid Oxidation

DNA released from apoptotic and necrotic cells may suffer oxidative damage, favoring its recognition by the immune system [2]. The interaction of the hydroxyl radical with the nucleobases of the DNA strand, such as guanine, leads to the formation of C8-hydroxyguanine or its nucleoside form 8-hydroxy-2′-deoxyguanosine (8-OHdG), which is widely used as a biomarker for nucleic acid O&NS [61], and may be measured through various techniques, such as HPLC with electrochemical detection, gas chromatography/mass spectrometry (GC-MS), and HPLC-MS and immunoassays [27,61].

Lee *et al*. [62] studied levels of 8-OHdG in plasma of SLE patients and found that those with more severe clinical symptoms and higher SLEDAI had increased plasma 8-OHdG levels. Maeshima *et al*. [63] found that periods of high-intensity sunlight exposure were associated with higher oxidative DNA damage measured by urinary 8-OHdG, whereas there was no difference between SLE patients and healthy controls in periods of low-

intensity sunlight exposure. These findings suggest that sunlight exposure may be an important contribuitor to oxidative DNA damage [63]. Simoniello *et al*. [49] found that SLE patients living in rural areas had 3.52 times more oxidative DNA damage than those living in cities, which was probably related to exposure to pesticide mixtures[49].

Despite the importance of nucleic acid oxidation in the induction of autoantibody production, only a small number of studies have examined oxidative damage on nucleic acids in human SLE. Lunec *et al*. [64] and Lee *et al*. [62] found that SLE patients had higher plasma 8-OHdG levels than healthy controls[62,64]. In a subsequent study, Lee *et al*. [65] found that polymorphism of human 8-oxoguanine glycosylase 1 (hOGG1) gene confers the susceptibility to lupus nephritis and increases plasma levels of 8-OHdG. More recently, Iriyoda *et al*. [48] investigated three oxidized guanine species: 1) 8-hydroxy-20 - deoxyguanosine from DNA; 2) 8-hydroxyguanosine from RNA; and 3) 8-hydroxyguanine from either DNA or RNA using enzyme linked immunoassay (ELISA) in inactive (SLEDAI < 6) and active (SLEDAI > 6) SLE. The authors found that serum levels of oxidized DNA/RNA were inversely associated with disease activity assessed by SLEDAI regardless of the corticosteroid use. The investigators argued that this could occur due to increased binding of anti-DNA antibodies to modified DNA, since DNA oxidation could increase the likelihood of interactions with circulating autoantibodies, promoting immune complex formation, thus, these molecules would not be available for serum detection [48].

Nitrosative Stress

Besides 3NTyr, which measures the effects of peroxynitrite on proteins containing tyrosine, NO concentration in samples may be estimated by measuring its metabolites, such as nitrites (NO_2^-) and nitrates (NO_3^-), since NO is a short-lived molecule [66].

In SLE, although some studies have reported higher levels of nitric oxide metabolites (NOx) and demonstrated associations with SLEDAI score and lupus nephritis [67], others have failed to find these associations [68]. In a previous investigation, our study group found that NOx levels were inversely and independently associated with disease activity evaluated by SLEDAI score [48]. We hypothesize that in conditions of inflammation and increased oxidative stress, NO is consumed in a reaction with superoxide anion, yielding peroxynitrite (Figure 2) an, thus, decreasing NO bioavailability.

ANTIOXIDANTS

Numerous endogenous antioxidant systems in the human body act to counterbalance ROS and maintain an adequate cellular redox equilibrium [14,33]. These antioxidants are generally categorized as enzymatic (e.g. superoxide dismutases, catalase and glutathione peroxidase) and nonenzymatic such as vitamins C and E, uric acid and glutathione (GSH), which is the major non-enzymatic anti-oxidant in human cells [14]. The enzymatic antioxidant systems and glutathione are predominantly found intracellularly, while non-enzymatic antioxidants are mainly found extracellularly [69].

Superoxide dismutases (SOD) is a group of three metal-containing enzymes that scavenge superoxide anion ($O_2^{\bullet-}$), catalyzing its conversion to hydrogen peroxide (H_2O_2), as summarized in Figure 1. SOD1, SOD2 and SOD3 are primaraly located in the cytoplasm, mitochondria, and extracellularly, respectively. In humans, these enzymes are usualy bonded to the metal ions copper, zinc, and manganese [70]. Hydrogen peroxide is further reduced to water by catalase or glutathione peroxidases (GPx), as shown on Figure 4 [62]. Catalases are mainly located in peroxisomes, GPx-1 in the cytosol and GPx-4 in the mitochondria [62,71]. Glutathione peroxidase catalizes the oxidation of glutathione (GSH) to glutathione disulphide (GSSG). GSSG can be reduced back to GSH by glutathione reductase (GR) [62].

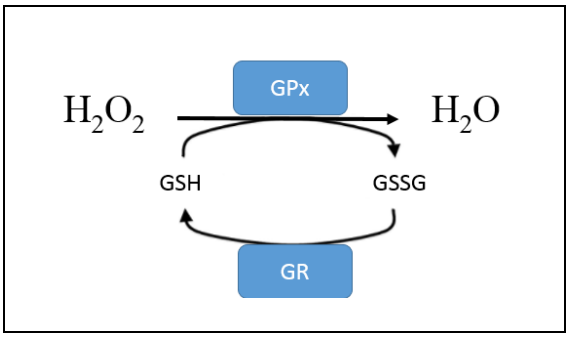

Figure 4: Hydrogen peroxide (H_2O_2) is converted to water (H_2O) through glutathione peroxidase, with the oxidation of glutathione (GSH) to glutathione disulphide (GSSG). GSSG can be reduced back to GSH by glutathione reductase (GR).

In SLE, there has been overwhelming evidence that the antioxidant defenses are unable to cope with excessive O&NS and numerous markers of impaired antioxidant capacity have been associated with increased disease activity [33,72]. This is particular evident with glutathione depletion, which has been consistently associated with O&NS in SLE and cell predisposition to apoptosis [73]. Although compensatory increases of antioxidant enzymes and/or activity have been reported, these were still insufficient to prevent oxidative damage [49]. Thus, in addition to O&NS, decreased antioxidant levels further contribute to redox imbalance in SLE [6,33,35,36].

Ben Mansour *et al*. [53] reported a higher reactivity of SLE autoantibodies against MDA-modified SOD and CAT, suggesting that these enzymes could become targets of immune response and elicit production of autoantibodies following oxidative modification.

The levels of individual antioxidants as well as antioxidant capacity can be quantified in various biological fluids (e.g., serum, plasma, erythrocytes, saliva) through various methods, such as Ferric Reducing Antioxidant Power (FRAP), Trolox Equivalent antioxidant Capacity (TEAC), Total Antioxidant Status (TAS) and Total Radical-Trapping Antioxidant Parameter (TRAP) [28,69]. Most studies show decreased levels of enzymatic and non-enzymatic antioxidants, as shown in Table 3.

TRAP detects hydrosoluble and liposoluble plasma antioxidants by measuring the chemiluminescence inhibition time induced by 2,2-azobis (2- aminopropane) [74]. Uric acid (UA) is an antioxidant in part responsible for 60% of the scavenging of free radicals in human plasma, and is detectable by TRAP method [75]. Levels of UA are affected by sex and numerous health conditions, such as body mass index BMI [75,76]. Furthermore, renal impairment, a common finding in SLE, can affect plasma levels of uric acid [77,78]. Thus, when using this method, uric acid should also be quantified to allow appropriate statistical corrections.

Table 3. Studies evaluating antioxidants and antioxidant capacity in SLE patients.

Authors	ANTIOXIDANTS						ANTIOXIDANT CAPACITY		
	SOD	CAT	GPx	GPx-1	GPx-4	GSH	TRAP	TAC	TAS
Iriyoda *et al*. [48]							↓		
Scavuzzi *et al*. [17]							↓		
Wang *et al*. [18]	↓	↓							
Simoniello *et al*. [49]	↑	↑							
Zaieni *et al*. [79]	↓	↓							
Sincer *et al*. [80]									↓
Lee *et al*. [62]		↓		↔	↓				
Lozovoy *et al*. [50]							↓		
Shah *et al*. [29]	↓	↓	↓			↓			
Lozovoy *et al*. [52]							↔		
Hassan *et al*. [47]						↓			
Wang *et al*. [33]	↓								
Zhang *et al*. [30]	↑	↓	↓					↑	
Tewthanom *et al*. [81]						↓			
Kurien *et al*. [82]	↓								

SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GPx-1, glutathione peroxidase-1; GPx-4, glutathione peroxidase-4; GSH, glutathione; TRAP, total radical-trapping antioxidant parameter; TAS, total antioxidant status.

↑: higher levels; ↓: lower levels; ↔ unchanged.

In summary, SOD levels were lower in most [18,29,33,79,82] but not all studies [30,49], where compensatory elevations were likely to have occurred. Lower levels of CAT were reported in all [18,29,30,62,79] but one [49] study. GPx were consistently decreased [29,30], as was GSH [29,47,81]. As for antioxidant capacities, TRAP was mostly decreased [17,48,50] but showed no significant changes in one study [52]. TAS was lower [80] and TAC was higher [30] in a single study.

IMPACT OF INCREASED OXIDATIVE AND NITROSATIVE STRESS IN SLE PATIENTS

Aberrant T-cell response

T helper (Th) cells are commonly grouped into subsets Th1, Th2, Th17 and regulatory-T cells (Tregs), based on the cytokines they primarily produce and their functional effects. Th-cell differentiation is dependent upon the local cytokine milieu and stimulation of antigen-presenting cells [83]. T cells are crucial to the pathogenesis of SLE and while the contributions of each T helper cell subset to the pathogenesis of SLE are still a matter of debate, recent findings have indicated that O&NS may disrupt the balance of these subsets, enhancing severity of SLE [34,84,85].

In a previous study, we demonstrated that increased lipid peroxidation and protein oxidation were associated with a Th1 and Th17 immune shift in SLE [17]. O&NS has been shown to activate the mammalian target of rapamycin (mTOR) - a signaling kinase that is a major regulator of cellular growth, proliferation, metabolism and survival [86] and mitochondrial transmembrane potential in T cells of SLE patients [85]. Increased in mTOR activity causes CD3 downregulation leading to higher calcium flux, which in turn activates cAMP response element modulator (CREM), inhibiting IL-2 and enhancing IL-17 expressions. These changes lead to Th1 and Th17 polarization and inhibition of CD8+ T cells [85]. This polarization towards Th1 and Th17 has been related to increased disease activity and organ damage [83]. Shah *et al*. [34] suggested that increased O&NS in SLE is associated with a shift towards Th1 cytokine production, which could worsen severity of illness. Furthermore, Th17 - a highly proinflammatory subset - has been associated with SLE and increased severity of illness [87].

Endothelial activation

Cell adhesion molecules (CAMs) facilitate leucocyte-endothelial cell interactions and the transmigration of inflammatory cells to sites of inflammation and may act as markers of endothelial activation and dysfunction [88]. An investigation using cultured human umbilical vein endothelial cells demonstrated that two antioxidants (pyrrolidine

dithiocarbamate and N-acetylcysteine) partially repressed gene transcription and expression of adhesion molecules [89]. Furthermore, another study with human endothelial cells demonstrated that ROS caused a significant and dose-dependent increase in expressions of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) [90]. Collectivelly, these studies indicate that oxidative stress may have a role in the induction of CAMs. ROS induces the oxidant-sensitive transcription factors such as NF-kB and activator protein 1 (AP-1) leading to increased expression of CAMs [91]. In a recent investigation, our study group was able to demonstrate a positive association between oxidative stress and increased levels of Plasminogen Activator Inhibitor Type-1 (PAI-1) and the CAMs: PECAM-1, VCAM-1, E-selectin and P-selectin in SLE patients [17]. Furthermore, elevated levels of CAMs have been associated with disease activity and clinical manifestations of the illness, such as cutaneous manifestations, neurological disorders and lupus nephritis [92–94].

Deregulation of apoptosis and necrosis

Excessive ROS and GSH depletion have been shown to cause increased apoptosis and delayed clearance of apoptotic bodies, generating autoimmunity caused by neo-epitopes [28,29]. Furthermore, ATP depletion caused by disruption of the mitochondrial membrane potential ($\Delta\Psi_m$) and excessive intracelular H_2O_2 may elicite necrosis of T cells in SLE, stimulating the release of several proinflammatory and chemotactic factors, leading to further inflammation, O&NS and tissue damage [10].

Increased inflammation

O&NS and inflammation are closely related and act in an interdependen manner to cause a progressive damage [95]. When O&NS is the primary disturb, a variety of transcription factors including nuclear factor (NF-kB) pathways are activated, upregulating proinflammatory cytokines and chemokines; the inflammation develops and leads to further O&NS in a vicious cycle. On the other hand, when inflammation is the primary disturb, it will lead to activation of macrophages and neutrophils, increasing O&NS, which propitiates inflammation aggravation [95]. Thus, it seems reasonable to speculate that the use antioxidants may be a beneficial therapy to be considered in addition to the standard immunomodulatory drug regimen in SLE. Figure 5 summarizes the involvement of oxidative and nitrosative stress in SLE.

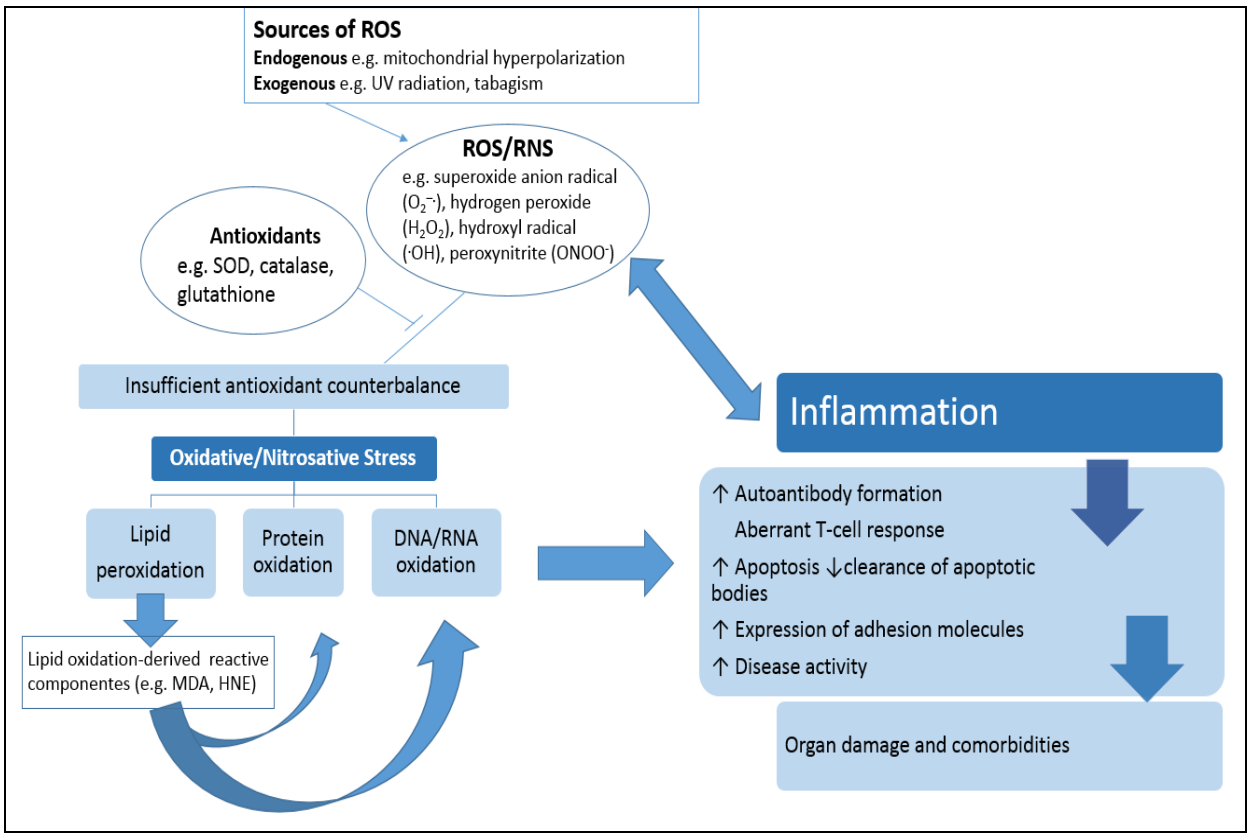

Figure 5: Overview of Oxidative and Nitrosative Stress in Systemic Lupus Erythematosus. ROS and RNS are generated from both endogenous and exogenous sources. Numerous antioxidant systems in the human body act to counterbalance ROS to maintain an adequate cellular redox balance. However, when there is an insufficient antioxidant counterbalance, oxidative stress occurs. Excessive and chronic production of ROS and RNS can lead to oxidation of cellular biomolecules such as proteins, lipids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Furthermore, lipid peroxidation produces highly reactive aldehydes such as malondialdehyde, which may bind covalently to proteins and nucleic acids, leading to further changes in molecular structures and biological functions. These oxidized molecules have the potential to produce a broad spectrum of autoantibodies, cause an aberrant T-cell response, increase expression of adhesion molecules and cause inflammation, in a vicious cycle where inflammation and oxidative stress can induce each other through activation of multiple pathways, leading to exacerbation of autoimmunity in SLE.

ANTIOXIDANT THERAPIES IN SLE

N-Acetylcysteine

The most significant findings with antioxidant therapy in SLE are related to interventions with N-Acetylcysteine (NAC), which is as a precursor of the antioxidant GSH [96]. As aforementioned, GSH is depleted in SLE [29,47]. This deficiency correlates with

baseline mitochondrial hyperpolarization and has been associated with a dysfunction of redox signaling and induction cell death by reactive oxygen intermediates [26]. Thus, supplementation with NAC would have the ability to reverse GSH depletion and improve oxidative damage in SLE.

Lai *et al*. [96] studied daily interventions of 1.2 g, 2.4 g, or 4.8 g of NAC in patients with SLE. Although the authors did not evaluate markers of oxidative and nitrosative stress, the doses of 2.4 g and 4.8 g were associated with a highly significant reduction in disease activity and mTOR activity. These concentrations also reversed expansion of CD4-CD8- T cells, stimulated FoxP3 expression in CD4+CD25+ T cells, and reduced anti-DNA production [96]. Furthermore, this supplementation resulted in clinical improvement in disease activity and reduced fatigue [96].

Tewthanom *et al*. [97] studied the effect of daily supplementation of NAC (1.8 g/6 months) in mild SLE in addition to the standard therapy. Although this intervention was insufficient to significantly increase GSH levels, it significantly reduced MDA levels and increased the number of patients who could tapper prednisolone dosage. Besides a reduction in CRP and MDA, Kudaravalli 2011 found evidence of improvement of endothelial function with NAC supplementation (1.8 g/day), marked by a decrease of reflection index [98]. In a recent case report, Li *et al*. [99] describe two cases of early-stage lupus nephritis that were treated with daily doses of NAC (1.2 g/ 3 months) and standard therapy (hydroxychloroquine and calcitriol). A significant increase in the antioxidant GSH and decrease in lipid peroxidation was reported, as well as significant improvements in routine blood counts, 24-h urine protein, ESR and disease activity index [99].

n-3 polyunsaturated fatty acids

Fish oil contains long chain n-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which have anti-inflammatory and antioxidant effects. These PUFAs have been proposed as arachidonic acid antagonists, and would have the ability to modulate several components of natural and acquired immunity, such as reduce the expression of proinflammatory cytokines (e.g. TNF-α, IL-1β and IL-6) [100], and thus affect Th-type response [101]. Furthermore, fish oil supplementation has been shown to up-regulate gene expression of antioxidant enzymes such as glutathione S-transferase (GST) and Mn-superoxide dismutase (Mn-SOD) and down-regulate hydroxysteroid sulfotransferase (SULT), contributing to decreases in ROS generation, due to decreased sulfation of bile acids and steroid hormones [100,102].

In animal models, autoimmune-prone NZB/W female mice treated with fish oil exhibited a significant increase in the activities of the antioxidant enzimes catalase, superoxide dismutase and glutathione peroxidase [103]. Furthermore, dietary n-3 fatty acids have been

shown to lower the production of ROS such as superoxide anions, hydrogen peroxide and nitrite radicals [104].

Although several studies have investigated the effects of omega-3 fatty acids in SLE [100], very few have investiged the effects of this intervention on biomarkers of oxidative stress in humans. Nakamura *et al*. [105] investigated the effect of fish oil supplementation and reported a significant reduction of urinary 8-isoprostane after 3 months of EPA treatment (1.8 g/day) in patients with lupus nephritis. Wright *et al*. [106] found improvements in endothelial function (flow-mediated dilation of the brachial artery) and oxidative stress (reduction of platelet 8-isoprostanes) after a 24-week intervention with omega-3 PUFAs. Additionally, fish oil has been shown to significantly decrease disease activity index (SLEDAI) and increase adiponectin levels, demonstrating the beneficial clinical potential of PUFAs on SLE [107]. These findings indicate that interventions with n-3 FA may modulate oxidant stress and decrease disease activity in SLE, contributing to improvements in the pathophysiology of the disease.

Vitamins

Human intervention studies with vitamins yielded limited improvements in SLE patients. Tam *et al*. [108] found significant reduction of only MDA after a 12-week intervention with vitamins C and E. Maeshima *et al*. [109] studied the effect of vitamin E on urinary 8-OHdG and levels of anti-ds DNA antibodies. The authors found that levels of anti-ds DNA antibodies decreased with the intervention, while urinary 8-OHdG remained unchanged.

Vitamin D deficiency has been associated with the onset of SLE and several studies have demonstrated that oral vitamin D (cholecalciferol) supplementation can have immunomodulatory effects, increasing expression of CD38 on B cells and a decreasing T-cell-dependent proinflammatory cytokines such as IFN-γ, TNFα and IL-17 [110,111], as well as have beneficial effect on Th response, by inhibiting Th1 and Th17 responses and enhancing Th2 and T-reg function [112]. Nevertheless, we are not aware of studies investigating the effect of vitamin D supplementation on markers of oxidative stress in SLE.

Table 4. Studies evaluating antioxidant interventions on markers of oxidative and nitrosative stress in patients with SLE.

Ref.	Study design	Population	Intervention	Conclusion
[99]	Case report of NAC intervention on early-stage lupus nephritis	2 female SLE patients (17 and 26 year old)	1.2 g of NAC/day for 3 months	↑GSH ↓ 8-iso-PGF2α
[97]	Randomized clinical trial investigating the effect of adjunctive NAC supplementation in mild SLE	40 male and female SLE patients (> 18 years)	1.8 g of NAC/day for 6 months	↔GSH ↓ MDA
[98]	Clinical trial investigating the effect of NAC and atorvastatin on endothelial dysfunction in patients with SLE	32 male and female SLE patients (between 17 and 65 years)	1.8 g of NAC/day for 2 weeks	↓ MDA
[106]	Randomised double-blind placebo-controlled parallel trial of the effect of n-3 PUFAs on disease activity and endothelial function	60 SLE patients	3 g of n-3 PUFAs for 24 weeks	↓8-isoprostane
[48]	Clinical trial investigating the effect of highly purified EPA on oxidative stress and plasma fatty acid composition in patients with lupus nephritis	6 male and female SLE patients (between 25 and 47 years)	1.8 g of EPA ethyl-ester for 3 months	↓8-isoprostane
[109]	Clinical trial to investigate the efficacy of vitamin E against both oxidative DNA damage and autoantibody production in SLE	36 women with SLE	150 to 300 mg/day	↓ 8-OHdG
[108]	Double blind, placebo controlled pilot study to asses the effects of longterm antioxidant vitamins on markers of oxidative stress, antioxidant defense and endothelial function in patients with SLE	39 male and female SLE patients	500 mg vitamin C and 800 IU vitamin E/ day for 12 weeks	↓ MDA ↔SOD ↔GPx ↔FRAP

Ref, reference. PUFA, polyunsaturated fatty acids. EPA, eicosapentaenoic acid. NAC, N-Acetylcysteine. GSH, glutathione. 8-iso-PGF2α, 8-isoprostaglandin F2. MDA, malondialdehyde. 8-OHdG, 8-hydroxy-2′-deoxyguanosine. SOD, superoxide dismutase. GPx, glutathione peroxidase. FRAP, plasma total antioxidant power.
↑: higher levels; ↓: lower levels; ↔ unchanged.

CONCLUSION

In SLE, the number of investigations reporting diminished antioxidant defenses and extensive oxidation of biomolecules resulting in a a wide range of oxidation products are overwhelming. Studies investigating O&NS should evaluate multiple biomarkers to shed more light in the pathophysiology of disease. Antioxidant therapy has yielded positive results and should be further investigated.

EXPERT OPINION

The investigations of several groups as well as our own studies have demonstrated a strong association between O&NS and SLE and have suggested a causal role of O&NS in the pathogenesis of the disease. The reports of diminished antioxidant defenses and extensive oxidation of biomolecules resulting in a a wide range of oxidation products in SLE are overwhelming. In spite of the potential of antioxidant therapies in SLE, few investigations have been conducted on the topic. It is important to note that antioxidant therapies should be regarded as adjunctive treatments, aimed to improve the response of standard primary treatment, since the main complications of the disease are caused by formation and deposition immune complexes, and O&NS is not the main cause of this formation, but can aggravate this condition.

The most significant findings with antioxidant therapy in SLE have come from interventions with NAC, which would have the potential to mitigate glutathione depletion and not only improve markers of oxidative damage in SLE, but also decrease disease activity, fatigue, markers of inflammation and endothelial function. Furthermore, this is a safe and inexpensive intervention, demonstrating the promissing potential for this supplementation in patients with SLE. Nevertheless, studies with longer durations and larger sample sizes are needed to investigate the long-term effects of the therapy on disease prognosis, and to asses if the supplementation is capable of reducing the frequency and/or severity of flares as well as the frequency of common complications, such as lupus nephritis and cardiovascular events. Interventions with fish oil, vitamins C and E have shown limited but positive results. Surprisingly, the effect of Vitamin D on O&NS has not been investigated yet.

When investigating the effect of antioxidant therapies in SLE, several markers of O&NS should preferably be tested in order to give a better indication of the effect of the therapy on the whole body oxidative damage. Furthermore, biomarkers of inflammation should also be tested to acess the ability of the intervention to regulate immune system responses, such as oxidant-sensitive transcription factor NF-kB, as well as to shed light into the possible mechanisms involved.

Article Highlights Box

- **Markers of lipid, protein and nucleic acid oxidation are increased in Systemic Lupus Erythematosus**
- **Endogenous antioxidant defenses are decreased in Systemic Lupus Erythematosus**

- **Oxidative and nitrosative stress is highly associated with Systemic Lupus Erythematosus and exacerbates immune responses**
- **Adjuntive antioxidant therapy with N-Acetylcysteine decreases not only markers of oxidative stress, but also disease activity, markers of inflammation and endothelial function**

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(*= of interest, **= of considerable interest)

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6.2. ARTIGO 2: INCREASED LIPID AND PROTEIN OXIDATION AND LOWERED ANTIOXIDANT DEFENSES IN SYSTEMIC LUPUS ERYTHEMATOSUS ARE ASSOCIATED WITH SEVERITY OF ILLNESS, AUTOIMMUNITY, INCREASED ADHESION MOLECULES AND TH1 AND TH17 IMMUNE SHIFT

Increased lipid and protein oxidation and lowered antioxidant defenses in systemic lupus erythematosus are associated with severity of illness, autoimmunity, increased adhesion molecules and Th1 and Th17 immune shift.

Lupus: Oxidative Stress

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ABSTRACT

This study investigated nitro-oxidative stress in patients with Systemic Lupus Erythematosus (SLE) in association with disease activity, immune-inflammatory biomarkers and adhesion molecules. 204 patients with SLE and 256 healthy volunteers were enrolled in this case-control study, which measured nitro-oxidative stress biomarkers, including lipid peroxides (LOOH), advanced oxidation protein products (AOPP), nitric oxide metabolites (NOx), sulfhydryl (-SH) groups, products of DNA/RNA oxidative degradation; and total radical-trapping antioxidant parameter (TRAP). Also measured were antinuclear antibodies (ANA), antibodies against double-stranded DNA (dsDNA), plasma levels of diverse cytokines, C-reactive protein and adhesion molecules.

LOOH ($p<0.001$) and AOPP ($p<0.001$) were significantly higher, while TRAP was significantly lower ($p<0.001$) in SLE patients than in controls. AOPP and LOOH were significantly and positively associated with SLEDAI scores, antinuclear antibodies and anti-dsDNA levels, whilst TRAP was significantly and inversely correlated with SLEDAI and ANA. There were significant positive associations between AOPP and LOOH and immune-inflammatory markers, indicating T helper (Th)-17 and Th1 bias and Th1+Th17/Th2 ratio ($p=.0.002$ and $p=0.001$ respectively). AOPP and LOOH (positively) and TRAP (inversely) were associated with adhesion molecule expression. A model predicting SLE was computed showing that, using LOOH, AOPP, NOx, adhesion molecules and body mass index, 94.2% of the patients were correctly classified with a specificity of 91.5%. Increased nitro-oxidative stress takes part in the (auto) immune pathophysiology of SLE and modulates severity of illness and adhesion molecule expression.

Keywords: Systemic Lupus Erythematosus; Reactive oxygen and nitrogen species; Cytokines; Antioxidant; Adhesion molecules.

INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a chronic inflammatory autoimmune disease characterized by autoantibody production especially directed against nuclear components, leading to chronic widespread inflammation, tissue destruction, accelerated atherosclerosis and premature mortality (1). Although the etiology of SLE is multifactorial, it has been suggested that the increased production of reactive oxygen and nitrogen species (ROS and RNS, respectively) could produce oxidized substrates that aggravate antigenicity, contributing to immune dysregulation, organ damage and the development of fatal comorbidities (1,2).

Reactive oxygen species are products of cell metabolism and are generated mainly by the mitochondrial respiratory chain. When there is an imbalance between the production of ROS and RNS and their scavenging by antioxidant mechanisms, an excessive accumulation occurs, leading to nitro-oxidative stress (O&NS)(3). Nitro-oxidative stress plays a major role in the development and progression of chronic and autoimmune diseases (4). Studies have suggested that nitro-oxidative stress is associated with chronic injuries in SLE patients and that the excessive and chronic production of ROS and RNS can lead to modifications of cellular biomolecules such as proteins, lipids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). These molecule alterations could generate neo-epitopes with the potential to produce a broad spectrum of autoantibodies, leading to inflammation, organ damage and exacerbation of autoimmunity in SLE (5,6). Excessive nitro-oxidative stress and decreased antioxidant levels have been demonstrated in SLE patients and have been associated with disease activity (7,8).

Oxidative stress may have a key role in the induction of cell adhesion molecules (CAMs) (9). CAMs facilitate leucocyte-endothelial cell interactions and the transmigration of inflammatory cells to sites of inflammation and may act as markers of endothelial activation and dysfunction (10). Studies have shown higher levels of adhesion molecules in patients with SLE when compared to healthy controls (11). These elevated levels of CAMs are associated with disease activity and clinical manifestations of the illness, such as cutaneous manifestations, neurological disorders and lupus nephritis (12–14).

T helper (Th) cells are commonly grouped into subsets Th1, Th2, Th17 and regulatory-T cells (Tregs), based on the cytokines they primarily produce and their functional effects. Th-cell differentiation is dependent upon the local cytokine milieu and stimulation of antigen-presenting cells (15). Upon activation, Th1 cells secrete pro-inflammatory cytokines, which stimulate macrophages to produce ROS and nitric oxide (NO) thereby mediating innate immunity (16). Th2 cells play an active role in the development of autoantibody-mediated autoimmunity in SLE, since several Th2 cytokines, such as interleukins (IL) IL-6 and IL-10, promote antibody production by B cells (17). Th17, a highly proinflammatory

subset, plays a major role in the initiation and development of SLE and is additionally significantly associated with SLE and severity of illness (18). The contributions of each T helper cell *subset* to the pathogenesis of SLE are still a matter of debate, however recent findings have indicated that oxidative stress may disrupt the balance of cytokine production by these subsets, enhancing severity of SLE (16)(19).

Given the important role of ROS and RNS in the pathophysiology of SLE and disease activity, the aim of the present study was to delineate changes in nitro-oxidative biomarkers and antioxidant defenses and examine their associations with severity of SLE, autoimmunity, immune responses and adhesion molecules. These associations could increase our understanding of physiopathology and could help identify new therapeutic targets in SLE.

MATERIALS AND METHODS

Subjects

A total of 460 individuals were selected from among rheumatology ambulatory patients and volunteers of the local University Hospital to participate in the study. The study included 204 patients with SLE and 256 healthy volunteers. SLE was diagnosed using the American College of Rheumatology (ACR) 2013 revised criteria (20). Disease activity was determined by using SLEDAI score (21). Inclusion criteria were patients (both genders) aged from 18 to 65 years. Exclusion criteria were thyroid, adrenal, renal, hepatic, gastrointestinal, infectious or oncological diseases, hormone replacement therapy and antioxidant supplements. Information on medical history was obtained at clinical evaluation. Disease duration, non-steroidal anti-inflammatory drugs, corticosteroids, antimalarial, oral contraceptives, and antihypertensive medications were recorded for each patient. The individuals of both groups did not drink alcohol regularly. Sample collection and analysis, as well as data evaluation were performed in a blinded fashion.

Ethics, consent, health and safety

All procedures performed in this study were approved by the institutional Ethics Committee and were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all the participants, who had acknowledgement that they would not be identified. All mandatory laboratory health and safety procedures have been complied.

Anthropometric and blood pressure measurements

Anthropometric measurements and laboratorial parameters were assessed. Body weight was measured to the nearest 0.1 kg in the morning by using an electronic scale, with individuals wearing light clothing and no shoes; height was measured to the nearest 0.1 cm

by using a stadiometer. BMI was calculated as weight (kg) divided by height (m) squared. Waist circumference was measured on standing subjects midway between the lowest rib and the iliac crest. Three blood pressure measurements using a calibrated sphygmomanometer were taken with a 1-min interval after the participant had been seated were recorded on the left arm. The mean of these measurements was used in the analysis.

MetS was defined following the Adult Treatment Panel III criteria, where MetS is present if three or more of the following five criteria are met: 1) waist circumference over 102 cm in men and 88 cm in women, 2) fasting triglyceride levels greater than or equal to 1.7 mmol/L (150 mg/dl), 3) high density lipoprotein (HDL) lower than 1.0 mmol/L (40 mg/dl) in men or 1.3 mmol/L (50 mg/dl) in women; 4) blood pressure over 130/85 mmHg (or antihypertensive medication use), and 5) fasting glucose levels greater than or equal to 5.6 mmol/L (100 mg/dl) or the use of antidiabetic medication.

Biochemical, immunological, and hematological biomarkers

After fasting for 12 hours, venous blood was withdrawn in Ethylene Diamine Tetra acetic acid (EDTA) and BD Vacutainer® UltraTouch™ serum sterile tubes. Whole blood was allowed to stand for 30 min and centrifuged at 1500 rpm for 10 min. Plasma and serum samples were separated and divided into aliquots and then stored at -80^{o}C for subsequent analysis.

Total cholesterol, HDL, low-density lipoprotein, triacylglycerol, glucose and uric acid were evaluated by a biochemical auto-analyzer (Dimension Dade AR Dade Behring, Deerfield, IL, USA) using Dade Behring® kits. Plasma insulin level was determined by chemiluminescence microparticule immunoassay (Architect, Abbott Laboratory, Abbott Park, IL, USA).

ANA were quantified using indirect immunofluorescence with HEp2 cells as substrate (IFI-ANA-HEp2-IgG, VIRO-IMMUN LaborDiagnostika, GmbH, Oberursel, Germany) and were considered significant when titers ≥1:160; antibodies against double-stranded DNA (anti-dsDNA) were quantified using enzyme-linked immunoassay (ELISA, anti-dsDNA, Orgentec Diagnostika, GmbH, Germany) and were considered significant when titers ≥20 IU/mL.

Plasma cytokines levels of IFN-γ, IL-4, IL-6, IL-12 and IL-17 were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) (eBioscience, San Diego, CA, USA) and serum CRP levels were measured using a turbidimetric assay (C8000, ABBOTT, Architect Abbott Laboratories, Abbott Park, IL, USA).

Oxidative and Nitrosative stress measurements

All nitro-oxidative stress measurements were performed in triplicate. Lipid hydroperoxides were evaluated by tert-butyl hydroperoxide-initiated chemiluminescence (CL-

LOOH), as previously described (22), and the results were expressed in counts per minute (cpm).

AOPP were determined in the plasma using the semi-automated method (23). AOPP concentrations were expressed as micromoles per liter (µmol/l) of chloramine T equivalents.

TRAP was determined as reported previously (24). This method detects hydrosoluble and/or liposoluble plasma antioxidants by measuring the chemiluminescence inhibition time induced by 2,2-azobis (2- aminopropane). The system was calibrated with the vitamin E analog Trolox, and the values of TRAP were expressed in equivalent of µM Trolox/mg UA(24).

It is known that TRAP levels are in part associated with BMI and uric acid (25) and therefore, we have adjusted our TRAP data statistically for possible effects of BMI (and MetS) and uric acid where needed.

NO concentration in sample was estimated by measuring the metabolites nitrites (NO2-) and nitrates (NO3-) using cadmium beads for the reduction of nitrate to nitrite. The concentrations of these metabolites were later determined according to the method proposed by (26). The values NOx values were expressed in µM.

SH groups of proteins were evaluated in plasma samples by a spectrophotometric assay based on 2,2-dithiobisnitrobenzoic acid (DTNB), as reported previously and the results are expressed in µM (27).

DNA/RNA oxidative degradations were assessed by 8-hydroxy-2-deoxyguanosine (8-OHdG) - a product of oxidatively modified DNA base guanine - using a sandwich enzyme-linked immunosorbent assay (ELISA) (eBioscience, San Diego, CA, USA).

Cell Adhesion Molecules and Plasminogen Activator Inhibitor Type-1 (PAI-1)

Levels of PECAM-1, VCAM-1, ICAM, E-selectin, P-selectin, and PAI-1 were determined by Human Magnetic Adhesion 6-Plex Panel (Novex Life Technologies, Frederick, United States of America) for Luminex® platform.

Statistics

The sample size was estimated statistically using GPower indicating that using a power of 0.80, at $\alpha<0.05$ level, and with an effect size of 0.15 (based on mean and standard deviation for some of the parameters previously evaluated in other studies), the study sample for analysis of covariance (2 groups) should be around 351. Therefore, 204 patients and 256 controls were included. Analyses of variance (ANOVAs) or the non-parametric Mann-Whitney U test were employed to check differences among groups in continuous variables. Analyses of contingency tables (X^2-test) were used to check associations between categorical variables. GLM analyses were used to assess the multivariate effects of explanatory

variables (including diagnosis) on dependent variables (including O&NS biomarkers), while controlling for sex, age, BMI and MetS. Tests for between-subject effects were employed to assess the univariate effects of significant predictor variables on the dependent variables. Estimated marginal means (±SE) were computed to interpret the inter-group differences between categorical independent variables and parameter estimates were used to interpret the direction and impact of continuous independent variables. Automatic stepwise binary logistic regression analyses were used to delineate the most significant explanatory variables predicting SLE (controls as reference group) using the following explanatory variables: O&NS biomarkers, age, MetS, BMI with or without immune biomarkers or CAMs. Some O&NS biomarkers were Ln transformed (LOOH, AOPP, NOx) to normalize their distribution (assessed using the Kolmogorow-Smirnov test). We also used the z transformed scores for the biomarkers to display the differences among groups and to compute z-unit weighted composite scores, including an index of oxidative stress as z value of AOPP (zAOPP) + zLOOH (zAOPP+LOOH); zNOx (lowered NOx indicating an increased usage of NO for nitrosylation and peroxynitrite formation) and zTRAP (lowered TRAP being an antioxidant) as zTRAP+NOx, and nitro-oxidative stress index as zAOPP+zLOOH-(zTRAP+zNOx) as zAOPP+LOOH/TRAP+NOx.

We also used immune data as presented previously (19), namely index of Th17 activity (Th17), that is z value of IL-17 (zIL-17) + zIL-6; an index of Th17 immune activation with respect to Th2 activity (Th17/Th2), computed as zIL-17 + zIL-6 – zIL-4; an index of immune activation or Th1+Th17 activity (Th1+Th17), computed as zIL12 + zIFNγ + zIL-6 + zIL-17; and an index of overall immune activation versus suppression (Th1+Th17/Th2), computed as zIL12 + zIFNγ + zIL-6 + zIL-17 – zIL-4.

All statistical analyses were performed using IBM SPSS windows version 22. All regression results were checked for multicollinearity using VIP and tolerance. Tests were 2-tailed and an alpha level of 0.05 indicated statistically significant results.

RESULTS

Descriptive statistics

Table 1 shows the socio-demographic, clinical and biomarker data of participants with SLE and controls. The authors did not use p-corrections to interpret the multiple results of univariate tests presented in Table 1 as these results (and the Pearson and point-biserial correlation matrices between the variables) were used to delineate the most significant predictor variables to be used as independent explanatory variables in the ultimate multivariate general linear model (GLM) and binary logistic regression analyses. SLE participants were somewhat older than healthy controls, while there were more women than men in the SLE group as compared to controls. Body mass index (BMI) was significantly

higher in SLE patients than in controls. There were differences in self-declared ethnicity among the groups, whilst there were more subjects with hypertension and metabolic syndrome (MetS) in the SLE group as compared with controls. Consequently, we have controlled our data for age, sex, BMI, MetS (and possible interactions, including SLE X sex), hypertension and ethnicity by entering these variables as additional explanatory variables in our multivariate analyses (Tables 2-6). Mann-Whitney-U tests showed that SLEDAI, antinuclear antibodies and antibodies against double-stranded DNA (dsDNA) were significantly increased in participants with SLE as compared to controls. Lipid peroxides (LOOH) and advanced oxidation protein products (AOPP) were significantly higher in SLE patients than in controls. There were no significant differences in deoxyguanosine, nitric oxide metabolites (NOx), uric acid and levels of sulfhydryl (SH) groups of proteins between both study groups, while TRAP was significantly lower in the patient group. C-reactive protein (CRP), all immune indexes, platelet endothelial cell adhesion molecule 1 (PECAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin, P-selectin and Plasminogen Activator Inhibitor Type-1 (PAI-1) were significantly higher in patients than in controls. There were no significant differences in ICAM between both study groups.

Aberrations in O&NS biomarkers between SLE and controls

Table 2 displays the results of GLM analysis with 7 O&NS data (AOPP, NOx, LOOH, TRAP and the 3 O&NS indexes) as dependent variables and diagnosis as explanatory variable, while controlling for age, sex, BMI and MetS. Self-declared ethnicity ($F=1.67$, $df=4/381$, $p=0.157$) and hypertension ($F=0.55$, $df=4/340$, $p=0.699$) did not have effects in this GLM analysis and, therefore, were not included in the GLM analysis. SLE diagnosis had a significant effect on the 7 O&NS data, while there were significant multivariate effects of sex, sex X diagnosis interaction, BMI and MetS, but not age. Tests for between-subject effects show significant sex X SLE diagnosis effects on AOPP, NOx production, zAOPP+LOOH and zTRAP+NOx (see **Figure 1** for effects of the interaction sex X diagnosis). Thus, AOPP, NOx, zAOPP+LOOH and zTRAP+NOx were significantly higher in male SLE patients than in female SLE patients, whereas in controls no such differences were found. Tests for between-subject effects and estimated marginal means (Table 2) show that SLE is accompanied by lower TRAP, increased LOOH, and increased zAOPP+LOOH/zTRAP+NOx ratio and that males have higher TRAP and LOOH concentrations than women. There were no significant effects of SLE on the other O&NS biomarkers, including –SH groups ($F=0.50$, $df=1/350$, $p=0.481$), deoxyguanosine ($F=2.72$, $df=1/218$, $p=0.100$) and uric acid ($F=0.51$, $df=1/379$, $p=0.474$).

Table 3 (regression #1) shows the outcome of an automatic stepwise regression analysis with SLE as dependent variable (and controls as reference group). Entered were all

O&NS variables (including the 3 indexes), age, sex, BMI and MetS. We found that zLOOH+AOPP and zTRAP+NOx together with sex, BMI and MetS were significantly associated with SLE (X^2=120.73, df=5, p<0.001, Nagelkerke=0.408; 76.1% of the subjects were correctly classified with a sensitivity of 63.6% and specificity of 85.1%).

Effects of confounding variables including the drug state

Table 2 shows that both BMI and MetS have significant effects on the O&NS biomarkers. Tests for between-subject tests showed that BMI was associated with TRAP, LOOH, zAOPP+LOOH and zAOPP+LOOH/zTRAP+NOx ratio (all positively), while MetS was accompanied by increased AOPP and zAOPP+LOOH levels. We have also examined the effects of drug state of the patients on the O&NS biomarkers by forced entry of the separate drug state variables in the multivariate GLM analysis shown in Table 2. There were no significant effects of prednisolone (F=1.27, df=4/358, p=0.283), antimalarial drugs (F=2.07, df=4/381, p=0.084), immunosuppressive drugs (F=0.88, df=4/381, p=0.475), mycophenolate (F=1.90, df=4/381, p=0.110), statins (F=0.76, df=4/381, p=0.551), hypoglycemic drugs (F=1.64, df=4/381, p=0.163), and vitamin D (F=1.54, df=4/351, p=0.191) on the O&NS biomarkers.

Positive associations between nitro-oxidative biomarkers, SLEDAI, and autoimmunity

Spearman's rank order correlation analyses showed that the SLEDAI was significantly associated with TRAP (r=-0.295, p<0.001, n=449), AOPP (r=0.160, p=0.001, n=447), NOx (r=-0.107, p=0.024, n=444), LOOH (r=0.195, p<0.001, n=439), zAOPP+LOOH (r=0.228, p<0.001, n=433) and zAOPP+LOOH/zTRAP+NOx ratio (r=0.318, p<0.001, n=415). Spearman's rank order correlation analyses showed that antinuclear antibodies (ANA) were significantly correlated with TRAP (r=-0.202, p<0.001, n=442), AOPP (r=0.117, p=0.014, n=440), LOOH (r=0.213, p<0.001, n=432), zAOPP+LOOH (r=0.217, p<0.001, n=426) and zAOPP+LOOH/zTRAP+NOx ratio (r=0.214, p<0.001, n=408). Spearman's rank order correlation analyses showed that anti-dsDNA antibodies were significantly correlated with TRAP (r=-0.136, p=0.004, n=449), AOPP (r=0.100, p=0.034, n=447), LOOH (r=0.125, p=0.009, n=439), zAOPP+LOOH (r=0.135, p=0.005, n=433) and zAOPP+LOOH/zTRAP+NOx ratio (r=0.177, p<0.001, n=415).

Table 4 shows the results of a multivariate GLM analysis with SLEDAI and ANA and anti-dsDNA antibodies as dependent variables and O&NS biomarkers as explanatory variables. We found significant effects of TRAP, AOPP, LOOH, sex, BMI, but not age, on SLEDAI and ANA and anti-dsDNA antibodies. SLEDAI was associated with TRAP, AOPP, and LOOH after controlling for sex and BMI. ANA titers were associated with TRAP, AOPP and female sex, while anti-ds DNA antibodies were associated with AOPP and LOOH (both

positively). There were no significant effects of NOx (F=1.75, df=3/380, p=0.0156), -SH groups (F=1.73, df=3/354, p=0.161) and uric acid (F=1.28, df=3/417, p=0.281) on SLEDAI and the autoimmune biomarkers. There was a significant multivariate effect of deoxyguanosine on the three dependent variables (F=3.42, df=3/218, p=0.018), whilst the tests for between-subject effects showed a significant effect (positive) on antinuclear antibodies only (F=6.37, df=1/220, p=0.012).

We have also examined the effects of zAOPP+LOOH and zAOPP+LOOH/zTRAP+NOx on the three dependent variables, after controlling for age, sex and BMI. zAOPP+LOOH had a significant effect on SLEDAI and ANA and anti-dsDNA antibodies (F=8.12, df=3/400, p<0.001), while tests of between-subject effects showed associations among this oxidative stress biomarker and SLEDAI (F=17.39, df=1/402, p<0.001), ANA antibodies (F=7.56, df=1/402, p=0.006) and anti-dsDNA antibodies (F=12.62, df=1/402, p<0.001). zAOPP+LOOH/zTRAP+NOx ratio had a significant effect on SLEDAI and ANA and anti-dsDNA antibodies (F=18.03, df=3/383, p<0.001), while tests for between-subject effects showed significant associations with SLEDAI (F=46.64, df=1/385, p<0.001), ANA antibodies (F=15.91, df=1/385, p<0.001) and anti-dsDNA antibodies (F=13.95, df=1/385, p<0.001).

Positive associations between nitro-oxidative and immune biomarkers

Table 5 shows the results of a multivariate GLM analysis with immune-inflammatory indexes (namely Th17, Th17/Th2, Th1+Th17, TH1+Th17/Th2 and CRP) as dependent variables and nitro-oxidative biomarkers as explanatory variables, while adjusting for sex, age and BMI. We found significant effects of AOPP, LOOH, sex, BMI but not age on the immune-inflammatory markers. We found significant between-subject effects of AOPP and LOOH (both positively) on Th17 and Th17/Th2 indexes, significant effects of LOOH on Th1+Th17 and Th1+Th17/Th2 indexes (all positive) and significant associations between AOPP and CRP (positive) after adjusting for sex and BMI. TRAP showed a significant effect on the immune-inflammatory variables (F=2.93, df=4/182, p=0.022), while there were no significant between-subject effects. We found no significant effects of –SH groups (F=2.36, df=4/172, p=0.055), NOx (F=2.02, df=4/177, p=0.094), desoxyguanosine (F=0.86, df=4/78, p=0.494) and uric acid (F=0.95, df=4/182, p=0.434). There was a significant effect of the oxidative index, zAOPP+LOOH, on the immune-inflammatory biomarkers, while tests of between-subject effects showed significant effects of this index on Th17 (F=11.21, df=1/189, p<0.001), Th17/Th2 (F=19.68, df=1/189, p<0.001), Th1+Th17 (F=6.02, df=1/189, p=0.015) and Th1+Th17/Th2 (F=11.02, df=1/189, p=0.001) indices. There was, however, no significant effect of zAOPP+LOOH/zTRAP+NOx on the immune-inflammatory variables (F=1.71, df=4/177, p=0.151).

Table 3, second regression analysis, examines the combined effects of nitro-oxidative and immune biomarkers on SLE and therefore we added the immune variables (Th17, Th1+Th17, Th17/Th2, Th1+Th17/Th2) as additional explanatory variables and found that zAOPP+LOOH, Th1+Th17/Th2, BMI, MetS and sex were strongly associated with SLE (X^2=97.63, df=5, p<0.001, Nagelkerke=0.605; 88.1% of the subjects were correctly classified with a sensitivity of 65.9% and specificity of 93.8%).

Nitro-oxidative biomarkers positively correlate with upregulation of adhesion markers

There were no significant associations between any of the nitro-oxidative stress biomarkers and ICAM. **Table 6** shows the results of a multivariate GLM analysis with 5 adhesion molecules as dependent variables and the nitro-oxidative biomarkers as explanatory variables, while adjusting for sex, age and BMI. We found significant effects of AOPP, LOOH, TRAP and NOx on the 5 adhesion molecules. Tests for between-subject effects showed that PECAM-1 was associated with TRAP and NOx (both negatively), E-selectins with AOPP (positively), and P-selectins and PAI-1 with LOOH (positively) and TRAP (negatively). There were no significant effects of –SH groups (F=0.71, df=5/130, p=0.621), deoxyguanosine (F=1.25, df=5/135, p=0.289) and uric acid (F=1.24, df=5/132, p=0.296) on the adhesion molecules. There was a significant effect of zAOPP+LOOH on the 5 adhesion molecules (F=4.09, df=5/144, p=0.002). Tests for between-subject effects showed significant effects on PECAM-1 (F=7.37, df=1/148, p=0.007), VCAM-1 (F=4.69, df=1/148, p=0.032), E-selectins (F=12.84, df=1/148, p<0.001). P-selectins (F=14.07, df=1/148, p<0.001) and PAI-1 (F=15.37, df=1/148, p<0.001). There was also a highly significant effect of zAOPP+LOOH/zTRAP+NOx on the 5 adhesion molecules (F=10.18, df=5/139, p<0.001). Tests for between-subject effects showed significant effects on PECAM-1 (F=36.08, df=1/143, p<0.001), VCAM-1 (F=10.02, df=1/143, p=0.002), E-selectins (F=19.68, df=1/143, p<0.001). P-selectins (F=31.27, df=1/143, p<0.001) and PAI-1 (F=21.07, df=1/143, p<0.001).

Table 2, regression analysis #3, examines the combined effects of nitro-oxidative biomarkers and adhesion molecules on SLE. Toward this end we entered the 5 adhesion molecules together with the nitro-oxidative biomarkers as explanatory variables and found that zAOPP+LOOH (positive), NOx (inverse), PECAM-1 and BMI were very strongly associated with SLE (X^2=128.52, df=4, p<0.001, Nagelkerke=0.806; 93.4% of the subjects were correctly classified with a sensitivity of 94.2% and specificity of 91.5%).

DISCUSSION

The major findings of this study are that the imbalances between ROS/RNS and the ensuing lipid and protein oxidation are strongly associated with SLE, and impact severity of illness, and the immune profiles, adhesion molecule levels and autoimmune responses in SLE. Moreover, this study established a model predicting increased risk towards SLE versus controls indicating that lipid and protein oxidation, lowered NOx, and increased PECAM-1 are important features of SLE.

Differences among SLE and controls

Patients with SLE showed significantly higher lipid and protein oxidation, hs-CRP levels, indices of immune activation and CAMs and lower antioxidant defenses as compared with controls. Previous reports found increased protein oxidation (8) and AOPP levels (28) in SLE as compared with controls. Furthermore, chronically elevated oxidation was reported in SLE patients with inactive and active disease, indicating that protein oxidation could play an important role in the pathogenesis of SLE (8). AOPP production results from oxidation of amino acid tyrosine residues on plasma proteins, including albumin, fibrinogen and lipoproteins (23). Modifications in the amino acid sequence or structure can generate neo-epitopes from self-proteins, leading to autoimmune responses (29). These amino acid modifications may also alter enzyme functions and oxidative signaling, thereby further aggravating SLE pathophysiology (30).

SLE patients show a significant increase in lipid peroxidation as determined by lipid hydroperoxides, which originate mostly from phospholipids, cholesterol esters and free fatty acid oxidation triggered by the action of free radicals. Lipids are the main oxidative target in cell membranes, causing damage in cell structure and functions (31). Prior studies reported increased lipid peroxidation in SLE (16). Patients with SLE show a significant decrease in plasma antioxidant capacity as measured by TRAP methodology. Our findings are in line with previous studies reporting impaired antioxidant status in SLE (32). It is important to note that uric acid has antioxidant properties, which are in part responsible for the free radical scavenging by TRAP. However, uric acid levels were not significantly altered in SLE.

SLE patients also showed significant increases in hs-CRP and indices of immune activation. An exacerbation of immune-inflammatory responses is known to initiate and sustain SLE disease activity (33). In agreement with previous studies, adhesion molecules were significantly higher in patients than in controls (12,13).

The nitro-oxidative biomarkers were significantly associated with increased BMI and the presence of MetS. MetS is generally defined as a complex condition represented by a combination of risk factors such as central obesity, dyslipidemia, hypertension, and disturbed glucose metabolism (34). Therefore, in accordance with other investigations, this study found

that both BMI and MetS contribute to increased inflammation and oxidative stress (35,36).

A newly computed oxidative stress index using the sum of z scores of LOOH and AOPP (reflecting lipid and protein oxidation), NOx and PECAM-1 showed a good diagnostic performance for SLE versus controls. ANA is highly sensitive (~95%), but lacks specificity, while other parameters such as anti-dsDNA, Anti-SSA/Ro and anti-SSB/La are specific but less sensitive for SLE (37,38). However, we underscore that our new algorithm cannot replace useful conventional markers of SLE. Indeed, elevations in oxidative stress and adhesion molecules also occur in other inflammatory and BMI-dependent diseases and are therefore not specific. Furthermore, any prediction obtained in a training set should be checked in a validation set.

To our knowledge, this is a first study to demonstrate significant diagnosis X sex interaction effects on nitro-oxidative biomarkers in SLE. Thus, these biomarkers were significantly higher in male than in female patients, while no such differences were detected in controls. This indicates a sexual dimorphism in the contributions of nitro-oxidative stress to the pathophysiology of SLE. Thus, in male SLE patients, lipid and protein oxidation may play a more important role than in females, while in the latter increased utilization of NO and lowered TRAP values may be more important. The increased oxidative processes in males may perhaps explain that male patients have a more severe course of illness (39). Nevertheless, other factors may explain sexual dimorphism in autoimmune diseases (40). In addition, male patients are usually less aware of SLE as a disease and therefore, may have their illness diagnosed later as compared to women leading to a greater impact of oxidative stress (39,41).

Postive associations between O&NS biomarkers, SLEDAI, and autoimmunity

Our results show that imbalances between pro-oxidant (LOOH and AOPP) and antioxidant (TRAP) biomarkers are associated with increased disease activity. Increased lipid peroxidation is associated with higher SLEDAI scores in several studies (7,16,42). Lipid peroxidation produces highly reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE), which may bind covalently to proteins, leading to changes in molecular structure and biological functions (7). These reactive aldehydes-modified proteins are immunogenic and may induce T-cell dependent antibody response that may have a causative role in SLE (42). Oxidation of amino acid residues of proteins, such as cysteine and methionine, also alter the molecular structure and biological functions of these proteins *(6).* Multiple markers of protein oxidation are associated with increased disease activity in SLE *(6).*

The human antioxidant defense system consists of enzymatic antioxidants such as SOD, CAT, glutathione-related enzymes, and non-enzymatic reactions (5). Biomarkers of

impaired antioxidant capacity have been associated with disease activity in SLE (7,43). In this study, we found a significant inverse association between disease activity and TRAP, but not NOx. These findings do not corroborate previous findings reporting a small but significant effect of NOx on SLEDAI (44).

In the present study, BMI had a significant effect on SLEDAI score. This has been previously described even in patients with inactive or mild disease (35). Visceral fat contributes to systemic inflammation and promotes oxidative stress *(36).* Our study also showed that the female sex impacted the SLEDAI score, whilst previous studies investigating gender disparities in SLE yielded mixed results (41,45).

Anti-dsDNA positivity was associated with increased lipid and protein oxidation. Morgan et al. (2005) reported that increased anti-dsDNA antibody positivity was associated with several markers of protein oxidation (6). Another study demonstrated an association between anti-dsDNA positivity and lipid peroxidation and suggested that oxidative stress may damage cell membranes and nucleus, increasing antigenicity thereby generating autoantibodies, including anti-dsDNA and ANA (46). Our findings that female sex was significantly associated with antinuclear antibodies corroborate those of previous studies (47).

Positive associations between nitro-oxidative and immune biomarkers

In our study, increased peroxides and protein oxidation were associated with a Th1 and Th17 immune shift. These findings extend previous reports indicating skewing towards Th17 with higher Th1/Th2 and Th17/Th2 ratios in SLE (15). Shah et al. suggested that increased oxidative stress in SLE is associated with a shift towards Th1 cytokine production (16). We also found an association between oxidized proteins and CRP in SLE, findings which corroborate a study reporting an *in vitro* association between oxidized phospholipids and CRP (48). Increased body weight was strongly associated with CRP, supporting the notion that this proinflammatory protein is produced by the visceral adipose tissue (35,36,49). We found that female sex was associated with higher CRP levels, which is in agreement with previous reports showing significant higher CRP levels in women than in men (50).

Positive associations between nitro-oxidative stress biomarkers and adhesion molecules

This is also a first study reporting that in SLE nitro-oxidative and antioxidant biomarkers are significantly associated with increased adehesion molecules levels, including PECAM, E-selectin, P-selectin and PAI. A study using cultured human umbilical vein endothelial cells demonstrated that two antioxidants (pyrrolidine dithiocarbamate and N-acetylcysteine) were able to partially repress gene transcription and expression of some

adhesion molecules (9). Another human endothelial cell study showed that ROS provoked a significant increase in the expression of ICAM-1 and VCAM-1 in a time- and dose-dependent manner (51). Altogether, these findings indicate that oxidative stress may play an role in CAM expression.

The main limitation of our study is that this is a case-control study, which does not allow inferences on causal relationships. A second limitation is that our study sample shows a relatively lower disease activity as indicated by a SLEDAI score of 3.7 and, therefore, our findings may not be applicable to patients with more severe phenotypes. Thirdly, we did not distinguish between different SLE presentations, including cutaneous SLE, lupus nephritis and central nervous and psychiatric presentations. Moreover, most SLE patients received medications with anti-inflammatory and/or immunomodulatory properties that could affect the results. Nevertheless, we found that treatments with these immunosuppressive drugs did not significantly interfere with the results of our study. Another limitation of this study was that subjects were not placed on a 24-hour low nitrate diet, which may have influenced variability in serum levels of nitrate and nitrite (52). Strengths of our study are that we used multivariate statistical analyses thereby adjusting for many confounding variables including BMI, MetS and sex. A second strength is that we recruited a large study sample and sampled blood in the same month of the year, thereby minimizing possible effects of seasonality (53).

CONCLUSION

Increased protein and lipid oxidation and lowered antioxidant defenses in systemic lupus erythematosus are associated with severity of illness, autoimmunity, increased adhesion molecules and Th1 and Th17 immune shift. A model predicting SLE diagnosis was proposed in which lipid and protein oxidation, nitric oxide metabolites, adhesion molecule PECAM-1 and BMI correctly classified SLE patients with high sensitivity and specificity. These results show that nitro-oxidative stress is a possible new drug target and that new drugs could be developed targeting nitro-oxidative stress and immune-inflammatory processes.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest: “The authors declare that they have no conflict of interest”.

Ethical approval: “All procedures performed in this study were in accordance with the ethical

standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards."

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

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Table 1 Socio-demographic and biomarker data in patients with systemic lupus erythematosus (SLE) and healthy controls.

	Variables	Controls (n= 256)	SLE (n=204)	F/X²	df	p
Clinical data	Age (years)	37.4 (11.7)	41.4 (13.2)	11.30	1	**0.001**
	Sex (F/M)	183 / 73	191 / 13	36.62	1	**<0.001**
	BMI (kg/m^2)	25.4 (4.4)	27.6 (5.8)	20.38	1	**<0.001**
	Ethnicity (Caucasian/not Caucasian)	206/50	134/70	12.87	1	**<0.001**
	Metabolic Syndrome (No/Yes)	201/45	101/102	51.57	1	**<0.001**
	Hypertension (No/Yes)	179/24	116/87	49.21	1	**<0.001**
	Uric Acid (mg/dL)	4.26 (1.41)	4.22 (1.27)	0.09	-	0.771
	SLEDAI	-	3.7 (3.9)	-	-	-
	ANA (titer)	-	1122 (2032)	-	-	-
	Anti-dsDNA (U/mL)	-	22.9 (105.7)	-	-	-
Markers of oxidative stress	Hydrogen peroxide (RBC)	16160 (13848)	22138 (17767)	23.68	1/343	**<0.001**
	AOPP (μmol/L of chloramine T equivalents)	147.5 (60.2)	176.4 (80.2)	16.47	1/442	**<0.001**
	NOx (μM)	33.6 (26.7)	30.5 (22.3)	0.51	1/447	0.474
	TRAP (μM Trolox/mg UA)	694.2 (151.6)	583.4 (161.0)	55.90	1/413	**<0.001**
	-SH groups (μM)	343.2 (96.6)	326.6 (86.7)	3.29	1/261	0.070
	DNA/RNA oxidative degradation	6120 (2299)	6694 (4838)	0.50	1/447	0.480
Immune parameters	CRP (mg/L)	2.89 (4.65)	6.02 (10.82)	21.72	1/443	**<0.001**
	Th17 (z score)	- 0.48 (1.36)	0.69 (1.37)	51.53	1/293	**<0.001**
	Th17 / Th2 (z score)	- 0.63 (1.39)	1.43 (1.79)	76.20	1/225	**<0.001**
	Th1 + Th17 (z score)	- 0.72 (2.13)	1.54 (2.79)	37.35	1/223	**<0.001**
	Th1 + Th17 / Th2 (z score)	- 0.87 (1.98)	2.13 (2.91)	66.43	1/216	**<0.001**
Adhesion molecules	PECAM-1 (pg/mL)	24.4 (5.8)	39.9 (11.1)	113.27	1/172	**<0.001**
	VCAM-1 (pg/mL)	635.2 (206.5)	958.9 (441.9)	36.08	1/172	**<0.001**
	E-Selectin (pg/mL)	9.6 (4.2)	19.7 (11.3)	58.58	1/172	**<0.001**
	P-Selectin (pg/mL)	87.7 (56.9)	165.1 (74.9)	69.50	1/172	**<0.001**
	PAI-1 (pg/mL)	44.9 (43.7)	119.7 (109.3)	45.88	1/172	**<0.001**
	ICAM (pg/mL)	641 (109)	895 (163)	3.02	1/172	0.084

Results are shown as mean (SD). F: results of analyses of variance; X^2: results of analyses of contingency table.
F, female; M, male; BMI, body mass index; SLEDAI, SLE Disease Activity Index; ANA, antinuclear antibodies; anti-dsDNA, anti–double-stranded DNA; AOPP, advanced oxidation protein products; NOx, nitric oxide metabolites; TRAP, total radical-trapping antioxidant parameter; -SH groups, sulfhydryl-groups of proteins; CRP, C reactive protein; PECAM-1: platelet endothelial cell adhesion molecule 1; VCAM-1: vascular cell adhesion molecule 1; PAI-1: plasminogen activator inhibitor type-1.
Th17: index for Th17 shift computed as z value of IL-17 (zIL-17) + zIL-6 ; Th17/Th2: index of Th17 activation with respect to Th2, computed as Th17 – zIL-4; Th1+Th17: index of Th1+Th17 immune activation, computed as zIFNγ+zIL-12+zIL-17+zIL-6; Th1+Th17/Th2: index of general immune activation computed as Th1+Th17 – zIL4

Table 2. Results of multivariate general linear model (GLM) analysis with oxidative and nitrosative stress biomarkers as dependent variables.

Type test	Dependent variable	Explanatory variable	F	df	P
Multivariate	TRAP, AOPP, NOx, LOOH, zAOPP+LOOH, zTRAP+NOx, zAOPP+LOOH/TRAP+NOx	SLE	11.70	4/382	**<0.001**
		Sex	6.16	4/382	**<0.001**
		MetS	6.19	4/382	**<0.001**
		SLE x Sex	4.84	4/382	**0.001**
		BMI	3.19	4/382	**0.014**
		Age	0.22	4/382	0.927
Between-subject effects	TRAP	SLE	10.78	1/385	**0.001**
		Sex	11.12	1/385	**0.001**
		BMI	4.38	1/385	**0.038**
	AOPP	SLE	10.90	1/385	**<0.001**
		Sex	6.74	1/385	**0.010**
		SLE x Sex	6.70	1/385	**0.010**
		MetS	21.93	1/385	**<0.001**
	NOx	SLE	4.40	1/385	**0.037**
		SLE x Sex	9.63	1/385	**0.002**
	LOOH	SLE	16.37	1/385	**<0.001**
		Sex	4.07	1/385	**0.044**
		BMI	6.86	1/385	**0.009**
	zAOPP+LOOH	SLE	27.41	1/385	**<0.001**
		Sex	10.88	1/385	**0.001**
		BMI	6.12	1/385	**0.014**
		SLE x Sex	5.13	1/385	**0.024**
		MetS	10.10	1/385	**0.002**
	zTRAP+NOx	SLE x Sex	10.24	1/385	**0.001**
	zAOPP+LOOH /zTRAP+NOx	SLE	15.99	1/385	**<0.001**
		BMI	8.93	1/385	**0.003**

Estimated marginal means (SE) (all values expressed as z scores)

Variables	Controls	SLE	Female	Male
TRAP (z score)	0.44 (0.07)	-0.09 (0.14)	-0.09 (0.06)	0.44 (0.15)
AOPP (z score)	-0.30 (0.07)	0.58 (0.15)	-0.09 (0.06)	0.52 (0.16)
NOx (z score)	-0.12 (0.07)	0.23 (0.15)	0.10 (0.06)	0.01 (0.16)
LOOH (z score)	-0.17 (0.07)	0.49 (0.15)	-0.01 (0.06)	0.32 (0.15)
zAOPP+LOOH (z score)	-0.14 (0.11)	1.07 (0.21)	-0.08 (0.08)	0.84 (0.22)
zTRAP+NOx (z score)	0.32 (0.12)	0.14 (0.23)	-0.01 (0.09)	0.45 (0.24)
zAOPP+LOOH/zTRAP+NOx (z score)	-0.46 (0.16)	0.92 (0.31)	-0.07 (0.12)	0.39 (0.33)

TRAP: total radical-trapping antioxidant parameter; AOPP: advanced oxidation protein products; NOx: nitric oxide metabolites; LOOH: Lipid hydroperoxides; zAOPP+LOOH: z value of AOPP (zAOPP) + zLOOH (oxidative stress index); zTRAP+zNOx (nitro-oxidative stress and antioxidant index); zAOPP+zLOOH-zTRAP-zNOx (overall nitro-oxidative stress index versus antioxidant defenses); MetS: Metabolic Syndrome; BMI: Body mass index.

Table 3. Results of automatic stepwise binary regression analyses with systemic lupus erythematosus as dependent variable.

Explanatory variables		OR	CI 95%	Wald	df	p
Regression #1	zAOPP+LOOH	1.63	1.33 – 1.99	22.26	1	<0.001
	zTRAP+Nox	0.71	0.60 – 0.83	18.25	1	<0.001
	MetS	4.79	2.59 – 8.86	24.99	1	<0.001
	BMI	1.06	1.00 – 1.12	4.11	1	0.043
	Sex (Male)	0.09	0.04 – 0.20	34.67	1	<0.001
Regression #2	zAOPP+LOOH	1.62	1.06 – 2.50	4.89	1	0.027
	Th1+Th17/Th2	1.84	1.50 – 2.26	34.19	1	<0.001
	MetS	5.66	1.70 – 18.87	7.95	1	0.005
	BMI	1.19	1.06 – 1.34	8.90	1	0.003
	Sex (Male)	0.07	0.01 – 0.48	7.50	1	0.006
Regression #3	zAOPP+LOOH	2.59	1.49 – 4.51	11.37	1	0.001
	Nox	0.009	0.03 – 0.28	16.97	1	0.001
	PECAM	21.56	5.26 – 85.97	18.38	1	<0.001
	BMI	1.20	1.04 – 1.38	6.20	1	0.013

AOPP: advanced oxidation protein products; LOOH: lipid hydroperoxides; NOx: nitric oxide metabolites; zAOPP+LOOH: z value of AOPP (zAOPP) + zLOOH (oxidative stress index); zTRAP+NOx: zTRAP + zNOx (nitro-oxidative and antioxidant index); Th1+Th17/Th2: zIL-6 + zIL-17 + zIL-12 + zIFNγ – zIL-4; MetS: metabolic syndrome; BMI: body mass index.

Table 4. Results of multivariate GLM analysis with systemic lupus erythematosus disease activity index (SLEDAI), antinuclear antibodies (ANA) and anti-double-stranded DNA (anti-dsDNA) as dependent variables.

Tests	Dependent variable	Explanatory variable	F	df	P
Multivariate	SLEDAI, ANA, Anti-dsDNA	TRAP	10.05	3/394	<0.001
		AOPP	3.74	3/394	0.011
		LOOH	3.14	3/394	0.025
		Sex	4.79	3/394	0.003
		BMI	7.06	3/394	<0.001
		Age	0.29	3/394	0.832
Between-subject effects	SLEDAI	TRAP (-)	26.83	1/396	<0.001
		AOPP (+)	6.02	1/396	0.015
		LOOH (+)	7.61	1/396	0.006
		Sex (F>M)	11.79	1/396	0.001
		BMI (+)	19.88	1/396	<0.001
	ANA	TRAP (-)	7.99	1/396	0.005
		AOPP (+)	4.77	1/396	0.030
		Sex (F>M)	4.70	1/396	0.031
	Anti-dsDNA	AOPP (+)	6.12	1/396	0.014
		LOOH (+)	4.87	1/396	0.028

SLEDAI, SLE disease activity index; ANA, antinuclear antibodies; anti-dsDNA, anti–double-stranded DNA; TRAP: total radical-trapping antioxidant parameter; AOPP: advanced oxidation protein products; LOOH: Lipid hydroperoxides; BMI: body mass index; F, female; M, male.

Table 5. Results of multivariate GLM analyses with the immune biomarkers as dependent variables.

Tests	Dependent variable	Explanatory variable	F	df	P
Multivariate	Th17, Th17/Th2	AOPP	4.32	4/185	0.002
	Th1+ Th17, Th1+Th17/Th2	LOOH	5.24	4/185	0.001
	CRP	Sex	4.73	4/185	0.001
		BMI	8.02	4/185	<0.001
		Age	1.61	4/185	0.173
	Th17	AOPP (+)	6.57	1/188	0.011
		LOOH (+)	5.15	1/188	0.024
	Th17/Th2	AOPP (+)	6.02	1/188	0.011
		LOOH (+)	16.31	1/188	<0.001
	Th1+Th17	LOOH (+)	5.82	1/188	0.017
	Th1+Th17/Th2	LOOH (+)	13.65	1/188	<0.001
	CRP	AOPP (+)	8.86	1/188	<0.001
		Sex (F>M)	17.98	1/188	<0.001
		BMI (+)	31.50	1/188	<0.001

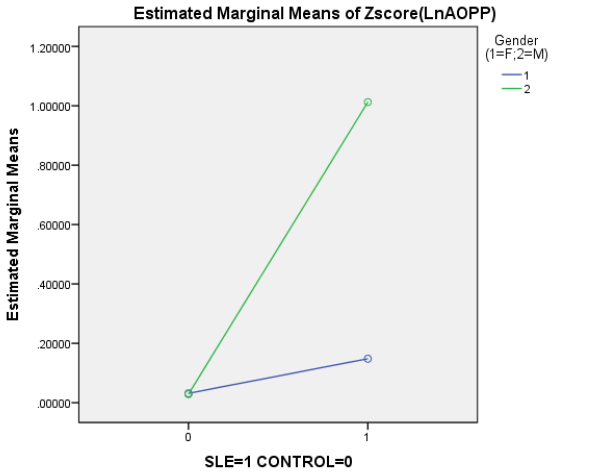
AOPP: advanced oxidation protein products; LOOH: lipid hydroperoxides; BMI: body mass index; Th17: z value of IL-6 (zIL-6) + zIL-17 (index of Th17 shift); Th17/Th2: Th17 – zIL-4 (Th17 / Th2 shift); Th1+Th17: Th17 + zIL-12 + zIFNγ (Th1 and Th17 activation); Th1+Th17/Th2: Th1+Th17 – zIL-4 (Th1 and Th17 / Th2 ratio); CRP: C reactive protein; F, female; M, male.

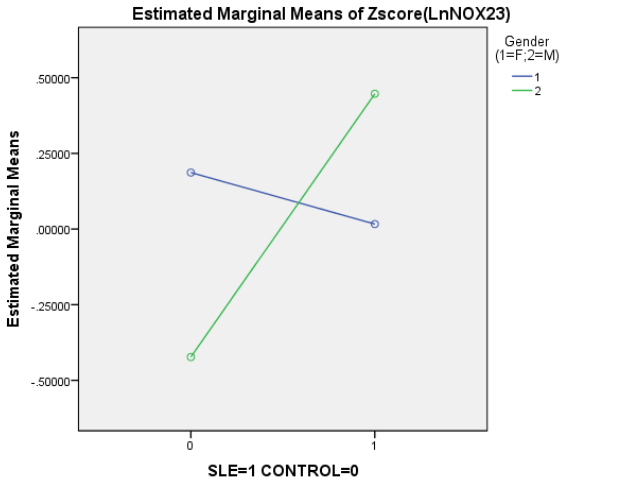
Table 6. Results of multivariate GLM analysis with adhesion molecules as dependent variables.

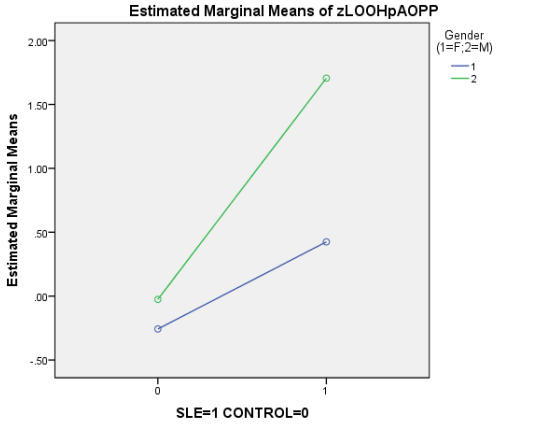
Tests	Dependent variables	Explanatory variables	F	df	P
Multivariate	PECAM, VCAM, E-Selectin, P-Selectin, PAI-1	AOPP	2.28	5/136	0.050
		LOOH	2.36	5/136	0.043
		TRAP	3.21	5/136	0.009
		NOx	3.70	5/136	0.004
		Sex	2.06	5/136	0.074
		BMI	4.99	5/136	<0.001
		Age	2.59	5/136	0.028
Between subject	PECAM	TRAP (-)	6.85	1/140	0.010
		NOx (-)	13.88	1/140	<0.001
		BMI (+)	5.52	1/140	0.020
	VCAM	Age (+)	6.37	1/140	0.013
	E-Selectin	AOPP (+)	10.48	1/140	0.002
		BMI (+)	16.64	1/140	<0.001
	P-Selectin	LOOH (+)	7.15	1/140	0.008
		TRAP (-)	8.41	1/140	0.004
		BMI (+)	6.74	1/140	0.010
	PAI-1	LOOH (+)	9.14	1/140	0.003
		TRAP (-)	9.22	1/140	0.003
		BMI (+)	13.37	1/140	<0.001

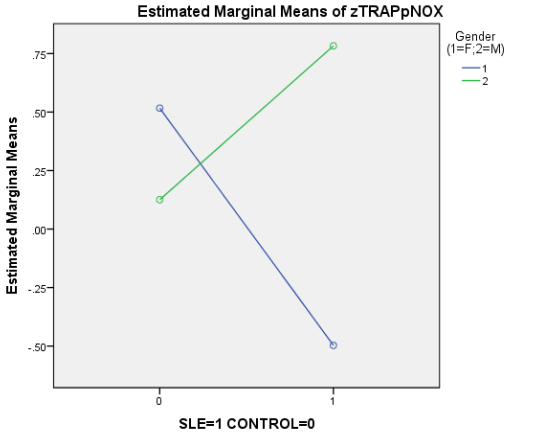
PECAM-1: platelet endothelial cell adhesion molecule 1; VCAM-1: vascular cell adhesion molecule 1; PAI-1: plasminogen activator inhibitor type-1; AOPP, advanced oxidation protein products; LOOH: Lipid hydroperoxides; TRAP, total radical-trapping antioxidant parameter; NOx, nitric oxide metabolites; BMI, body mass index.

Figure 1. Tests of between-subject effects of the interaction sex X diagnosis on oxidative and nitrosative stress markers.









7. CONCLUSÃO

Os resultados obtidos neste trabalho nos permitem concluir que:

- O LES está relacionado a um significativo desequilíbrio redox, caracterizado principalmente pelo aumento de peroxidação lipídica, oxidação protéica, diminuição das defesas antioxidantes, diminuição metabólitos de óxido nítrico indicando um consumo de NO para nitrosilação e formação de peroxinitrito.

- Aumento de peroxidação lipídica e aumento da oxidação de proteínas estão associados ao aumento da atividade da doença (SLEDAI), anticorpos antinucleares e anticorpos anti-dsDNA.

- Aumento de peroxidação lipídica e aumento da oxidação de proteínas estão relacionados ao aumento das respostas Th17 e Th1, além do aumento da razão Th1+Th17/Th2.

- Aumento de peroxidação lipídica, aumento da oxidação de proteínas e diminução de capacidade antioxidante estão relacionados ao aumento da expressão do PAI-1 e das moléculas de adesão: PECAM-1, VCAM-1, E-selectina, P-selectina nos pacientes com LES.

- O agorítimo que considera índice de estresse oxidativo dado por AOPP+LOOH (positivamente), metabólitos de óxido nítrico (inversamente), defesas antioxidantes totais diminuídas (TRAP), PECAM-1 e IMC classificam corretamente 94,2% de todos os indivíduos com LES, indicando forte associação desses marcadores com o a fisiopatologia da doença.

8. CONSIDERAÇÕES FINAIS

Com a avaliação de uma série de biomarcadores de dano oxidativo, como marcadores de peroxidação lipídica, oxidação de proteínas, DNA, capacidade antioxidante e metabólitos de óxido nítrico, os dados deste estudo foram relevantes para contribuir com o entendimento global da contribuição do estresse oxidativo para o LES, além de terem revisado o efeito do estresse oxidativo e nitrosativo sobre a atividade da doença, moléculas de adesão e diversos outros marcadores imuno-inflamatórios. De forma geral, os estudos disponíveis na literatura apresentam poucos biomarcadores de estresse oxidativo, não refletindo todo o perfil de dano oxidativo e seu papel na fisiopatologia do LES.

O estresse oxidativo leva ao desequilíbrio das respostas Th1, Th17, e essa polarização tem sido relacionada ao aumento da atividade da doença. Além disso, o estresse oxidativo está relacionado a um aumento dos níveis de moléculas de adesão. Finalmente, a obesidade pode amplificar o processo oxidativo e inflamatório devido à produção de citocinas pró-inflamatórias pelo tecido adiposo visceral, aumentando a atividade da doença.

Estes resultados indicam que o estresse nitro-oxidativo pode se tornar um novo alvo terapêutico para tratamento do LES, visando o aumento das defesas antioxidantes e diminuição do estresse nitro-oxidativo e com isso, processos imune-inflamatórios e a atividade da doença.

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ANEXO 1 – APROVAÇÃO DO CEP/UEL

UNIVERSIDADE ESTADUAL DE LONDRINA - UEL/ HOSPITAL REGIONAL DO NORTE DO

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Associação entre polimorfismos genéticos e a susceptibilidade ao Lúpus Eritematoso Sistêmico em pacientes atendidos no Ambulatório do Hospital de Clínicas da Universidade Estadual de Londrina, Londrina, Paraná

Pesquisador: Andréa Name Colado Simão

Área Temática:

Versão: 4

CAAE: 01865212.0.0000.5231

Instituição Proponente: Universidade Estadual de Londrina - UEL

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 210.328

Data da Relatoria: 19/12/2012

Apresentação do Projeto:

Estudos com famílias e gêmeos sugerem que os fatores genéticos desempenham um papel significativo na predisposição ao Lupus Eritematoso Sistêmico (LES). Assim, a hipótese levantada neste projeto é de que indivíduos que apresentam polimorfismo genético nos genes que codificam a Proteína C Reativa, o HLA e o TNF apresentam maior susceptibilidade ao desenvolvimento de LES e apresentam maior estresse oxidativo. Para isso, o sangue dos indivíduos selecionados será colhido para realização de investigação gênica e dosagem de Proteína C Reativa e TNF.

Objetivo da Pesquisa:

Este projeto objetiva determinar a associação de polimorfismos genéticos e a susceptibilidade ao LES e ao aumento do estresse oxidativo em pacientes atendidos no Ambulatório do Hospital de Clínicas (AHC) da Universidade Estadual de Londrina (UEL), Londrina, Paraná.

Avaliação dos Riscos e Benefícios:

O projeto não apresenta riscos ao paciente e a população poderá ser beneficiada com os resultados obtidos, caso a equipe de pesquisa determine fatores genéticos que possam estimar a chance de um indivíduo desenvolver a doença ou a chance de um indivíduo previamente com a

UNIVERSIDADE ESTADUAL DE LONDRINA - UEL/ HOSPITAL REGIONAL DO NORTE DO

doença em desenvolver quadros clínicos mais graves como a nefrite lúpica. Além disso, os resultados obtidos neste estudo poderão, também, indicar uma possível relevância da inclusão na rotina laboratorial de testes de genotipagem dos
genes indicados para indivíduos atendidos no AHC e no Hospital Universitário da UEL. Indivíduos que apresentarem um genótipo ou um conjunto de haplótipos associado ao LES poderão ser beneficiados com estratégias terapêuticas diferentes ou serem submetidos a um monitoramento clínico e laboratorial em intervalos menores de tempo, ou ambos procedimentos, o que poderá contribuir para uma melhor avaliação e monitorização clínica
destes indivíduos.

Comentários e Considerações sobre a Pesquisa:

O Projeto está bem estruturado e é relevante para o avanço das investigações sobre LES.

Considerações sobre os Termos de apresentação obrigatória:

Todas as pendências foram respondidas adequadamente.

Recomendações:

Encaminhar relatório ao final do estudo.

Conclusões ou Pendências e Lista de Inadequações:

Não há.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Prezada Pesquisadora,

Favor retirar seu parecer de aprovação junto ao CEP/UEL.

Endereço: AVENIDA ROBERT KOCH, 60
Bairro: VILA OPERÁRIA CEP: 86.038-440
UF: PR Município: LONDRINA
Telefone: (43)3371-2490 E-mail: cep258@uel.br

UNIVERSIDADE ESTADUAL DE LONDRINA - UEL/ HOSPITAL REGIONAL DO NORTE DO

LONDRINA, 04 de Março de 2013

Assinador por:
Alexandrina Aparecida Maciel Cardelli
(Coordenador)

Endereço: AVENIDA ROBERT KOCH, 60
Bairro: VILA OPERÁRIA **CEP:** 86.038-440
UF: PR **Município:** LONDRINA
Telefone: (43)3371-2490 **E-mail:** cep268@uel.br

ANEXO 2 - Systemic Lupus Erythematosus Disease Activity Index - SLEDAI

(Bombardier et al., 1992)

Entrar com o peso específico na coluna do Score do SLEDAI se a alteração estiver **presente na visita ou nos ùltimos 10 dias antes**

Fator de ponderação	**SLEDAI SCORE**	**DESCRIÇÃO**	**DEFINIÇÃO**
8		Convulsão	Instalação recente excluindo causa metabólica, infecciosa ou causada por drogas
8		Psicose	Alteração da função mental normal devido a alterações graves da percepção da realidade. Inclui alucinações, incoerência, associações livres, empobrecimento do conteúdo do pensamento, pensamento marcadamente ilógico, comportamento bizarro desorganizado ou catatónico. Excluir uremia e causas farmacológicas/drogas
8		Síndrome orgâno-cerebral	Função mental alterada com alteração da orientação, memória ou outra função intelectual com rápida instalação e flutuação dos achados clínicos incluindo obnubilação da consciência com diminuição da capacidade de concentração e incapacidade de manter a atenção ao ambiente envolvente, mais pelo menos duas das seguintes: distúrbios da percepção, discurso incoerente, insónia ou sonolência diurna, aumento ou decréscimo da actividade psicomotora Excluir causa metabólica, infecciosa ou causada por drogas
8		Distúrbios visuais	Alterações da retina ligados ao LES, incluindo corpos coroideus, hemorragias retinianas,exsudado seroso ou hemorragia no coroide ou nevrite óptica Excluir HTA ou causa infecciosa ou causada por drogas
8		Distúrbios nos pares cranianos	Instalação recente de neuropatia sensitiva ou motora atingindo os pares cranianos
8		Cefaleia lúpica	Cefaleia severa, persistente; pode ser tipo migranoso mas deve ser resistente à terapêutica narcótica
8		AVC	Instalação recente de AVC Excluir arteriosclerose
8		Vasculite	Ulceração, gangrena, nódulos digitais dolorosos, infarto periungeal,

			hemorragias sub-ungueais**,** ou biópsia ou angiograma compativeis com vasculite
4		Artrite	Mais de duas articulações com dor e sinais inflamatórios
4		Miosite	Dor/fraqueza muscular proximal, associado com elevação da CK/aldolase ou eletromiografia ou biopsia compatível com miosite
4		Cilindros urinários	Cilindros de Eritrócitos ou de granulosos
4		Hematúria	> 5 células por campo. Excluir litíase, infecção ou outra causa
4		Proteinúria	> 0.5g/24h. Instalação recente ou aumento > 0.5g/24h
4		Piúria	> 5 leucocitos por campo. Excluir, infecção
2		Novo rash	Instalação recente ou recorrência de rash tipo inflamatório
2		Alopécia	Instalação recente ou recorrência de perda anormal difusa ou localizada de cabelo
2		Ulcerações nasais	Instalação recente ou recorrência de ulcerações nasais
2		Pleurisia	Dor torácica pleurítica com atrito pleural derrame ou espessamento pleural
2		Pericardite	Dor pericárdica mais pelo menos um dos seguintes: atrito, derrame, ou confirmação eletrocardiográfica ou por ecocardiograma
2		Hipocomplementémia	C3, C4 ou CH50 abaixo dos valores de referência do laboratório
2		Aumento do DNA	>25 % no ligando pelo ensaio de Farr ou acima dos valores de referência do laboratório
1		Trombocitopénia	<100.000 plaquetas/mm^3
1		Leucopénia	<3.000 leucocitos/mm^3 Excluir causas farmacológicas
1		Febre	>38º C excluir causa infecciosa
		SCORE SLEDAI TOTAL (1-105)	

APÊNDICE 1 - Termo de Consentimento Livre e Esclarecido (para grupo de pacientes)

"Associação entre polimorfismos genéticos e a susceptibilidade ao Lúpus Eritematoso Sistêmico em pacientes atendidos no Ambulatório do Hospital de Clínicas da Universidade Estadual de Londrina, Londrina, Paraná"

Prezado(a) Senhor(a):

Gostaríamos de convidá-lo (a) a participar da pesquisa **"Associação entre polimorfismos genéticos e a susceptibilidade ao Lúpus Eritematoso Sistêmico (LES) em pacientes atendidos no Ambulatório do Hospital de Clínicas da Universidade Estadual de Londrina, Londrina, Paraná,"** realizada no "Hospital Universitário da Universidade Estadual de Londrina (HU/UEL), Londrina, Paraná". O objetivo da pesquisa é "determinar se existe associação entre fatores genéticos do indivíduo e a chance de desenvolver LES e se existe associação com o quadro clínico da doença". A sua participação é muito importante e ela se daria da seguinte forma: no momento da entrada no projeto de pesquisa, será realizada uma avaliação clínica e coleta de 20 mL de sangue periférico para realização de exames laboratoriais relacionados ao LES, e uma entrevista para você fornecer informações sobre estilos de vida como dieta e exercícios físicos. Gostaríamos de esclarecer que sua participação é totalmente voluntária, podendo você: recusar-se a participar ou mesmo desistir a qualquer momento sem que isto acarrete qualquer ônus ou prejuízo à sua pessoa. Informamos, ainda que as informações serão utilizadas somente para os fins desta pesquisa e serão tratadas com o mais absoluto sigilo e confidencialidade, de modo a preservar a sua identidade.

As amostras de sangue coletadas serão identificadas por códigos com letra e número garantindo o absoluto sigilo e confidencialidade dos resultados. Após sua utilização, as amostras serão armazenadas em *freezer* sob a responsabilidade do pesquisador responsável para outros estudos genéticos relacionados ao LES.

A participação no projeto não apresenta riscos ao (a) senhor (a) e a população poderá ser beneficiada com os resultados obtidos, caso a equipe de pesquisa determine fatores genéticos que possam estimar a chance de um indivíduo desenvolver a doença ou a chance de um indivíduo previamente com a doença em desenvolver quadros clínicos mais graves como a nefrite lúpica.

Informamos que o(a) senhor(a) não pagará nem será remunerado por sua participação. Garantimos, no entanto, que todas as despesas decorrentes da pesquisa serão

ressarcidas, quando devidas e decorrentes especificamente de sua participação na pesquisa.

Caso você tenha dúvidas ou necessite de maiores esclarecimentos pode nos contactar: **Professora Dra. Andrea Name Colado Simão, no Setor de Imunologia Clínica do Laboratório de Análises Clínicas do HU/UEL, fone 43-3371-2321, e-mail:** deianame@yahoo.com.br**,** ou procurar o Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina, na Avenida Robert Kock, nº 60, ou no telefone 33712490.

Este termo deverá ser preenchido em duas vias de igual teor, sendo uma delas, devidamente preenchida e assinada entregue a você.

Londrina, ___ de _____de 2012.

_____

Pesquisador Responsável: Profa. Dra. Andrea Name Colado Simão,
RG: 6226736-4.

_____ , tendo sido devidamente esclarecido sobre os procedimentos da pesquisa, concordo em participar **voluntariamente** da pesquisa descrita acima.

Assinatura (ou impressão dactiloscópica):_____

Data:_____

Obs: Caso o participante da pesquisa seja menor de idade, deve ser incluído o campo para assinatura do menor e do responsável.

APÊNDICE 2 - Termo de Consentimento Livre e Esclarecido (para grupo controle)

"Associação entre polimorfismos genéticos e a susceptibilidade ao Lúpus Eritematoso Sistêmico em pacientes atendidos no Ambulatório do Hospital de Clínicas da Universidade Estadual de Londrina, Londrina, Paraná"

Prezado(a) Senhor(a):

Gostaríamos de convidá-lo (a) a participar da pesquisa "Associação entre polimorfismos genéticos e a susceptibilidade ao Lúpus Eritematoso Sistêmico (LES) em pacientes atendidos no Ambulatório do Hospital de Clínicas da Universidade Estadual de Londrina, Londrina, Paraná, realizada no "Hospital Universitário da Universidade Estadual de Londrina (HU/UEL), Londrina, Paraná". O objetivo da pesquisa é "Determinar se existe associação entre fatores genéticos do indivíduo e a chance de desenvolver LES e se existe associação com o quadro clínico da doença". A sua participação é muito importante e ela se daria da seguinte forma (avaliação clínica, coleta de sangue periférico para realização de exames laboratoriais relacionados ao LES, fornecer informações sobre estilos de vida como dieta e exercícios físicos. Gostaríamos de esclarecer que sua participação é totalmente voluntária, podendo você: recusar-se a participar, ou mesmo desistir a qualquer momento sem que isto acarrete qualquer ônus ou prejuízo à sua pessoa. Informamos ainda que as informações serão utilizadas somente para os fins desta pesquisa e serão tratadas com o mais absoluto sigilo e confidencialidade, de modo a preservar a sua identidade.

Sua participação é importante para compor o grupo de indivíduos saudáveis que serão utilizados para a comparação dos resultados obtidos com o grupo de pacientes com a doença.

As amostras de sangue coletadas serão identificadas por códigos garantindo o absoluto sigilo e confidencialidade dos resultados. Após sua utilização, as amostras serão armazenadas em *freezer* sob a responsabilidade do pesquisador responsável par outros estudos genéticos relacionados ao LES.

A participação no projeto não apresenta riscos ao (a) senhor (a) e a população poderá ser beneficiada com os resultados obtidos, caso a equipe de pesquisa determine fatores genéticos que possam estimar a chance de um indivíduo desenvolver a doença ou a chance de um indivíduo com a doença em desenvolver quadros clínicos mais graves como a nefrite lúpica. Os resultados serão discutidos entre os pesquisadores da área e poderão contribuir para a implabntação de novos exames laboratoriais possam estimar a chance de um

indivíduo desenvolver a doença ou a chance de um indivíduo com a doença em desenvolver quadros clínicos mais graves como a nefrite lúpica.

Informamos que o(a) senhor(a) não pagará nem será remunerado por sua participação. Garantimos, no entanto, que todas as despesas decorrentes da pesquisa serão ressarcidas, quando devidas e decorrentes especificamente de sua participação na pesquisa.

Caso você tenha dúvidas ou necessite de maiores esclarecimentos pode nos contactar **(Professora Dra. Andrea Name Colado Simão, no Setor de Imunologia Clínica do Laboratório de Análises Clínicas do HU/UEL, fone 43-3371-2321, e-mail:** deianame@yahoo.com.br**, o**u procurar o Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina, na Avenida Robert Kock, nº 60, ou no telefone 33712490.

Este termo deverá ser preenchido em duas vias de igual teor, sendo uma delas, devidamente preenchida e assinada entregue a você.

Londrina, ___ de _____de 2012.

Pesquisador Responsável

RG::_____

_____ , tendo sido devidamente esclarecido sobre os procedimentos da pesquisa, concordo em participar **voluntariamente** da pesquisa descrita acima.

Assinatura (ou impressão dactiloscópica):_____

Data:_____

Obs: Caso o participante da pesquisa seja menor de idade, deve ser incluído o campo para assinatura do menor e do responsável.

APÊNDICE 3 - Questionário Controles

Dados demográficos			Nº do Controle:
Nome			
Endereço			
Telefone			
Data de nascimento			
Faz uso de algum Medicamento?	Quais? Qual dosagem?		
Tem alguma Doença?			
Etnia	( ) Caucasiano ( ) Negro ( ) Mulato ( ) Asiático		
Cor da pele	( ) Branca ( ) Negra ( ) Pardo ( ) Amarela		
Exposição solar diária	( ) Não se expõe ao sol diariamente ( ) Baixa exposição (≤ 20 min/dia) ( ) Exposição solar adequada (> 20 min/dia)		
Usa protetor solar?	( ) Sim ( ) Não Qual a frequência?		
Tabagismo	( ) Sim ( ) Não		
Consumo de álcool	( ) Sim ( ) Não		
Profissão			
Hábitos de dieta	( ) Suplementação alimentar ( ) Antioxidante ( ) Vitaminas ( ) Dieta específica		
	Obs.:		
Dados Clínicos			
IMC:	Peso:	Altura:	Circunferência:
Pressão Arterial:	Atividade física?	( ) Sim ( ) Não Quantas vezes?	
Teve Inflamação/ Infecção na última semana?	( ) Sim ( ) Não	Qual?	
Pós Menopausa	( ) Sim ( ) Não	Data última menstruação:	

APÊNDICE 4 - Ficha de avaliação pacientes - Projeto LES

NOME:	PRONTUÁRIO:
DATA NASC:	CAUCASIANO ( ) NAO CAUC ( )
END:	TEL:
MEDICAMENTOS PREDNISONA: HIDROXICLOROQUINA/CLOROQUINA: METOTREXATE: AZATIOPRINA: MICOFENOLATO MOFETIL: OUTROS IMUNOSSUPRESSORES: OUTROS:	
OUTRAS DOENÇAS: HAS SIM ( ) NÃO ( ) DIABETES SIM ( ) NÃO ( ) AVC/IAM SIM ( ) NÃO ( ) OUTROS:	
NEFRITE LÚPICA SIM ( ) NÃO ( ) OBS:	
TEMPO DE DOENÇA:	
ESCORE SLEDAI:	
TABAGISMO: SIM ( ) NÃO ( )	
ATIVIDADE FÍSICA: SIM ( ) NÃO ( )	

PESO	ALTURA	IMC	CIRC. ABDOMINAL	PRESSÃO ARTERIAL