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POLIMORFISMOS DE BASE ÚNICA (SNPs) DOS GENES *LIG4*, *RAD52*, *VDR* E *IFIH1* E A SUSCEPTIBILIDADE AO LÚPUS ERITEMATOSO SISTÊMICO E SUAS MANIFESTAÇÕES CLÍNICAS

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“Levanta, sacode a poeira e dá a volta por cima...”

Paulo Vanzolini

Resumo

O Lúpus Eritematoso Sistêmico (LES) é uma das mais relevantes desordens autoimunes no mundo com a prevalência variando entre 20 a 150 casos a cada 100.000 indivíduos. A formação de autoanticorpos e a deposição de imunocomplexos na circulação sanguínea é um dos principais mecanismos patogênicos da doença. Além disso, o LES é caracterizado por um diversificado quadro de manifestações clínicas que varia de paciente para paciente. A genética do LES é complexa e caracteriza-se pela participação de diversos genes atuando em conjunto na etiopatogênese da doença. Neste trabalho foi estudada a susceptibilidade dos polimorfismos nos genes *LIG4*, *RAD52, VDR* e *IFIH1* ao LES e suas manifestações clínicas. Os genes *LIG4* e *RAD52* são codificadores de enzimas de reparo do DNA e os danos ao DNA são potenciais ativadores da resposta imune. O *VDR* (receptor de vitamina D) atua como modulador da resposta imune através da ação da vitamina D. Uma vez que pacientes com LES apresentam com frequência alterações dos níveis séricos da vitamina D e o VDR está presente em importantes células do sistema imune, o *VDR* aparece como um candidato promissor à susceptibilidade ao LES. O gene *IFIH1* é capaz de induzir a ativação do IFN e, como esta citocina apresenta papel chave na patogênese do LES, a ação deste gene tem papel importante na resposta imune. Nos gene *LIG4 e RAD52* foram analisados quatro (rs10131, rs1805386, rs1805388 e rs3093740) e três (rs1051669, rs1106467 e rs3748522) SNPs respectivamente, em 158 pacientes e 212 controles da população do Sudeste Brasileiro. Os polimorfismos nos genes *LIG4* e *RAD52* não apresentaram associação ao LES nem às suas manifestações clínicas na população analisada. Os polimorfismos analisados no gene *VDR* (rs11168268, rs2248098, rs1540339, rs4760648 e rs3890733) não apresentaram associação com o LES, no entanto apresentaram associação com as seguintes manifestações clínicas: alterações cutâneas com genótipo G/G (rs11168269, OR=3,01e *p*=0,035), anticorpo anti ds-DNA com o genótipo C/T (rs4760648, OR=0,369 e *p*=0,03), alterações imunológicas com o genótipo G/G (rs2248098, OR=2,82 e *p*=0,04) e artrite com o genótipo T/T (rs3890733, OR=17,05 e *p*= 0,001). No gene *IFIH1* foram analisados dois polimorfismos (rs6432714 e rs10930046) e o genótipo C/C (rs10930046) foi associado com ao LES (*p*=0.032), no entanto, não foi encontrada associação com as manifestações clínicas. Os resultados obtidos neste estudo forneceram dados para o primeiro estudo de associação com LES e os genes de reparo *LIG4* e *RAD52*. Além disso, os genes *VDR* e *IFIH1* foram testados pela primeira vez na população brasileira, contribuindo como marcadores não somente na doença, mas nas manifestações clínicas do LES.

Palavras-chave: Lúpus Eritematoso Sistêmico, SNPs, *LIG4*, *RAD52*, *VDR*, *IFIH1*.

Abstract

Systemic lupus erythematosus (SLE) is one of the most important autoimmune disorders worldwide and the prevalence ranges from 20 to 50 cases per 100.000 individuals. The formation of autoantibodies and deposition of immune complexes throughout the body are the major pathogenic mechanisms in the disease. In addition, SLE is characterized by a heterogeneous clinical manifestations varying from patient to patient. The genetic component in SLE is characterized by the participation of several genes acting together in the etiopathogenesis of the disease. This work studied the genes *LIG4*, *RAD52*, *VDR* and *IFIH1* and the susceptibility to systemic lupus erythematosus and its clinical manifestations. The *LIG4* and *RAD52* genes products are DNA repair enzymes and DNA damage are potential activators of the immune response. The *VDR* acts as a modulator of the immune response through the action of vitamin D. Since SLE patients often have decreased serum levels of vitamin D and VDR is present in important immune system cells, *VDR* appears as a promising candidate to SLE susceptibility. The *IFIH1* gene is able to induce IFN activation and, since IFN is a key cytokine in the pathogenesis of SLE, the action of this gene plays an important role in immune response. In this thesis we analyzed SNPs in *LIG4*, *RAD52*, *VDR* and *IFIH1* genes and the susceptibility to SLE and its clinical manifestations. We analyzed four SNPs in *LIG4* gene (rs10131, rs1805386, rs1805388 and rs3093740) and three in *RAD52* (rs1051669, rs1106467 and rs3748522) in 158 SLE patients and 212 healthy controls in a Southeast Brazilian population. Polymorphisms in the *LIG4* and *RAD52* genes were not associated to SLE or to their clinical manifestations in the studied population. The analyzed polymorphisms in the *VDR* gene (rs11168268, rs2248098, rs1540339, rs4760648 and rs3890733) were not associated with SLE, but were associated to the following clinical manifestations: cutaneous alterations to genotype G/G (rs11168269, OR = 3.01 and p = 0.035), antibody anti dsDNA to genotype C/T (rs4760648, OR = 0.369 and p = 0.03), immunological alterations to genotype G/G (rs2248098, OR = 2.82, p = 0.04) and arthritis to genotype T/T (rs3890733, OR = 17.05 and p = 0.001). In *IFIH1* gene weanalyzed two polymorphisms (rs6432714 and rs10930046) and the genotype C/C at rs10930046 was associated to SLE (p = 0.032), however, no association was found with the clinical manifestations. The results obtained in this thesis provide data for the first association study with SLE and DNA repair genes *LIG4* and *RAD52*. In addition, *VDR* and *IFIH1* genes were tested for the first time in the Brazilian population, contributing not only as disease markers but as disease activity predictors.

Key words: Systemic Lupus Erythematosus, SNPs, *LIG4*, *RAD52*, *VDR*, *IFIH1*.

Lista de Ilustrações

Lista de Tabelas

Lista de Abreviaturas

Item	Definição
AITD	*Autoimmune thyroid disease* (Doença autoimmune da tireoide)
BANK	*B-cell scaffold protein with ankyrin repeats 1*(Proteína de andaime de célula B com repetições anquirina 1)
CTLA4	*Cytotoxic T-Lymphocyte Antigen -4* (Antígeno 4 de célula T citotóxica)
CD	Célula Dendrítica
Dcm1	*Meiotic recombination protein DMC1/LIM15 homolog* (Proteína homóloga de recombinação meiótica DMC1/LIM15)
DNA	Ácido desoxirribonucleico
DNA-PKcs	*DNA-protein kinase catalitc subunit* (DNA proteína quinase subunidade catalítica)
DSB	*Double Strand Break* (Quebra de dupla fita)
DM1	Diabetes Mellitus tipo I
FYB	*Fyn binding protein* (Proteína Ligante de Fyn)
FYN	*Fyntyrosine Kinase* (Quinase fyntirosina)
GWAS	*Genome wide association study* (Estudo de associação de genoma amplo)
HLA	*Human leukocyte antigen* (Antígeno leucocitário humano)
HR	*Homologous Recombination* (Recombinação Homóloga)
IFN	Interferon tipo 1
IFIH1	*Interferon induced with helicase C domain* (Interferon induzido com domínio helicase C)
IRF	*Interferon regulatory factor (*Fator de regulação do interferon)
LES	Lúpus Eritematoso Sistêmico
LIG 4	DNA Ligase 4
miRNA	Micro RNA
NF-KB	*Nuclear Factor Kappa B* (Fator nuclear Kappa B)
NHEJ	*Non Homologou End Joining* (Junção de extremidades não homólogas)

PCR	Reação em Cadeia de Polimerase
PTPN22	*Protein tyrosine phosphatase non-receptor type 22* (Proteína tirosina fosfatase não receptora tipo 22)
RAD52	RAD52 homólogo (S. cerevisiae)
RNA	Ácido Ribonucléico
SEM4	Semaforina 4a
SLC38A1	*Soluble carrier family 38, member 1*(Carreador solúvel da família 38, membro 1)
SNP	*Single Nucleotype Polymorphism* (Polimorfismo de Base Única)
STAT4	*Signal transducer and activator of transcription 4* (Transdutor de sinal e ativador de transcrição 4)
STK17A	Serine/threonine kinase 17a (Serina/treonina quinase 17A)
TCR	*T-cell receptor* (Receptor de célula T)
UTR	*Untranslated Region* (Região Não Traduzida)
UV	Ultra Violeta
VDR	*Vitamin D receptor* (Receptor da vitamina D)
XRCC4	*X-Ray Repair cross-complementing protein 4* (Proteína de reparo de raio-X de complemento cruzado 4)

Sumário

1. Introdução

O Lúpus Eritematoso Sistêmico (LES) é uma doença causada por uma resposta imune alterada e que apresenta manifestações clínicas heterogêneas, variando entre os indivíduos afetados. Diversos fatores possuem papel relevante no surgimento da doença, entre eles: genéticos, ambientais, hormonais e imunológicos. Em particular, os fatores genéticos representam um forte componente para o desenvolvimento do LES, sendo comumente o resultado de efeitos combinados de variações em um grande número de genes. Nos últimos anos, diferentes genes foram associados ao LES, como *IFR5, STAT4, PTPN22, CTLA-4, BANK1, IL-10,* etc. No entanto, apesar de promissores, os genes identificados representam apenas 15% da herdabilidade da doença.

Com a finalidade de ampliar o espectro de marcadores genéticos para o LES, estudos utilizando a tecnologia de microarranjos de DNA e estudos de associação de amplo genoma (GWAS, em inglês, *Genome-Wide Association Study*) tornaram-se cada vez mais frequentes. Sandrin-Garcia et al (2009), utilizaram a tecnologia de microarranjo de cDNA para identificar novos genes de susceptibilidade ao LES. Neste estudo, os genes *LIG4* e *RAD52* foram apontados como potenciais candidatos, devido à expressão diferenciada observada em pacientes na fase ativa da doença. Estes genes apresentam função de destaque em processos de reparo de quebras de dupla fita de DNA (DSB, em inglês, *Double-strand break*), representando assim o limite entre variabilidade genética e mutação. A expressão diferenciada desses genes pode indicar que pacientes com lúpus apresentam uma resposta inadequada ao reparo de DSBs. Desta forma, a identificação de polimorfismos que resultem na susceptibilidade à

doença poderá contribuir significativamente para o entendimento do processo de desenvolvimento do LES.

Além dos genes *LIG4* e *RAD52*, outros genes surgem como possíveis marcadores na patogênese do Lúpus. O gene receptor de vitamina D (*VDR*) atua como mediador da resposta imune, uma vez que modula a ação desta vitamina. A vitamina D é um hormônio capaz de induzir a resposta imune local, principalmente após infecção bacteriana com a produção de peptídeos antimicrobianos. Polimorfismos no gene *VDR* podem resultar em diminuição da atividade do receptor e consequente prejuízo para o papel da vitamina D (D3). Interessantemente, uma das características dos pacientes com Lúpus é a diminuição dos níveis séricos da vitamina D, o que pode levar a uma resposta imune alterada. Além disso, o *VDR* está localizado em diversos tecidos e células do sistema imune, incluindo as células B e T. Dessa forma, estudos de associação genética envolvendo o *VDR* e doenças autoimunes em geral vêm surgindo nos últimos anos, tornando este gene um promissor marcador genético para o LES.

Outro gene recentemente evolvido na susceptibilidade ao LES é o *IFIH1*, capaz de modular a via do interferon (IFN) após infecção viral. Os pacientes com lúpus apresentam o que se chama “assinatura do interferon”, que se caracteriza pela alteração dos níveis séricos desta citocina e dos genes envolvidos nesta via. Além disso, os níveis séricos do IFN nestes pacientes podem ser utilizados tanto como marcador quanto para prognóstico da doença. Assim os genes que modulam sua ação são de grande importância para aumentar o conhecimento da patogênese do LES.

Os polimorfismos de base única (SNPs) são a maior fonte de variações genéticas interindividuais, podendo ser utilizados para identificação da predisposição genética a doenças multifatoriais como o lúpus, funcionando como uma importante ferramenta na identificação e análise de marcadores genéticos principalmente por sua abundância no genoma. Desta forma, neste trabalho os polimorfismos nas sequências dos genes *LIG4, RAD52, VDR* e *IFIH1* foram avaliados com a susceptibilidade ao LES e suas manifestações clínicas em uma população do Sudeste Brasileiro.

2. Revisão da Literatura

2.1 Lúpus Eritematoso Sistêmico

2.1.1 Epidemiologia

A incidência mundial do lúpus eritematoso sistêmico (LES) pode variar entre 1 a 10 novos casos para cada 100.000 pessoas/ano, enquanto que a prevalência varia entre 20 a 150 casos a cada 100.000 indivíduos (Pons-Estel et al., 2010; Tsokos, 2011). A diferença significativa entre as taxas de prevalência e incidência nas diferentes regiões do mundo deve-se principalmente a variações nos fatores genéticos, populacionais e ambientais (Vilar & Sato, 2002). Essas diferenças podem ser observadas entre diferentes grupos étnicos onde os indivíduos com ascendência Africana, Hispânica ou Asiática, apresentam prevalência cerca de três vezes maior quando comparadas com indivíduos Caucasianos (Pons-Estel et al., 2010).

No Brasil dois estudos foram realizados abordando a epidemiologia do LES. No primeiro estudo realizado em Natal no Rio Grande do Norte, Vilar & Sato (2002) registraram uma incidência anual de 8,7 a cada 100.000 pessoas, com maior frequência em mulheres (14,1: 2,2). Entretanto, no estudo realizado em Cascavel no estado do Paraná, Nakashima et al. (2011) verificaram uma incidência de 4,8 casos por 100.000 habitantes/ano. A diferença de incidências encontradas entre as cidades de Natal e Cascavel podem ser explicadas pelas diferentes taxas de exposição aos raios UV encontradas entre as duas regiões (Vilar & Sato, 2002; Nakashima et al., 2011), assim como pela composição

étnica das cidades, uma vez que em Natal, o percentual de pardos e negros é superior à cidade de Cascavel (Nakashima et al., 2011).

Embora os estudos realizados apresentem divergências quanto à incidência e prevalência do LES em diferentes populações, o fato de que as mulheres, principalmente na idade reprodutiva, são mais afetadas do que os homens é um consenso. Esta diferença relacionada ao sexo pode variar entre 6-14 mulheres afetadas, para cada homem com LES. O LES também pode acometer crianças e idosos. Entretanto, nestes grupos, a taxa de mulheres e homens afetados não varia da mesma forma que em adultos, confirmando assim a influência dos fatores hormonais na doença (Kyttaris et al., 2010).

2.1.2 Imunopatologia

O LES é uma desordem autoimune, caracterizada pela produção patogênica de autoanticorpos contra componentes do núcleo celular, em associação com um diversificado conjunto de manifestações clínicas (Magalhães et al., 2003). A perda da autotolerância global com subsequente desregulação do sistema imune é consequência de fatores genéticos, ambientais e eventos estocásticos (Choi et al., 2012). As doenças autoimunes são caracterizadas por uma resposta inflamatória inapropriada e alterada, resultando no comprometimento de órgãos e tecidos, onde o sistema imune falha em diferenciar o próprio do não-próprio (Zenewicz et al., 2010). Essa alteração na resposta imune tem função significativa no desenvolvimento do LES, contribuindo com o dano aos tecidos, na via de liberação de citocinas inflamatórias, com a ativação descontrolada de células B e T, conduzindo no final a uma produção patogênica de autoanticorpos (Choi et al., 2012).

A ativação e a regulação das células T apresentam anormalidades complexas no LES e o processo desordenado de sinalização dessas células possui um papel importante na doença. Cada linfócito T possui um receptor (TCR, do inglês *T-cell receptor*) na membrana com a capacidade de interagir com um antígeno associado a uma molécula de MHC (*Major Histocompatibility Complex*) presente na superfície da célula apresentadora de antígeno (APC, do inglês *Antigen-Presenting Cell*) (Mendes, 2010). O complexo do receptor CD3 das células T, ao reconhecer e se ligar aos antígenos, envia sinais de ativação para o interior da célula através de uma cascata de sinalização, a qual envolve diversas estruturas e moléculas. Uma característica dos pacientes com Lúpus é a sinalização precoce e amplificada do TCR (Crispín et al., 2010; Kyttaris et al., 2010). Outra característica de indivíduos com LES é o aumento do número de linfócitos T que não possuem os co-receptores CD4 e CD8 (duplo negativos, DN) e que representam menos de 5% do total de linfócitos T nos indivíduos saudáveis. Este aumento de linfócitos T pode induzir a produção de autoanticorpos anti-DNA por células B autorreativas (Gualtierotti et al., 2010).

As células B imaturas, que em condições normais reconhecem e são ativadas contra autoantígenos durante seu processo de maturação, são submetidas à seleção negativa pelos mecanismos de tolerância do sistema imune, evitando a presença de linfócitos B autoreativos em órgãos do sistema imune periférico (Yurasov et al., 2006; Kyttaris et al., 2010). As células B funcionam como APCs na interação com células T, além de apresentar uma grande diversidade de funções regulatórias tanto na resposta imune quanto no LES. Apesar de mais comumente reconhecidas pela liberação de anticorpos as células B atuam na produção de citocinas e a interação direta com as células T

e células dendríticas (CD), com impacto significativo na modulação da resposta imune. As células B são eficientes na apresentação de antígenos, capazes de capturá-los através da superfície celular dos receptores Ig para subsequente internalização, processamento e apresentação via moléculas de MHC de classe I ou II. A apresentação de peptídeos autoantígenos é capaz de ativar diretamente células T autorreativas. As células B podem ainda modular as células T de memória e regular o desenvolvimento e ativação de CDs (Foster, 2008).

Os autoanticorpos são produzidos pelas células B autorreativas, as quais fazem parte do repertório normal de células dos indivíduos, entretanto, no LES, essas células desempenham um papel alterado, provavelmente resultante de um processo de maturação defeituoso (Kyttaris et al, 2010). Populações de células B "*naive*" em pacientes com LES apresentam mais de 50% das células autorreativas antes mesmo do primeiro encontro com antígenos. Esse dado sugere falha nos pontos de checagem durante a maturação desses linfócitos, as quais resultam em um maior número de células B autorreativas que produzem autoanticorpos (Yurasov et al., 2005). Em condições normais, a produção de autoanticorpos é importante para a remoção de autoantígenos, liberados tanto por células em apoptose como em necrose. A produção de autoanticorpos causa a formação de imunocomplexos, os quais se depositam nos tecidos e ativam o sistema complemento. Esses imunocomplexos, uma vez não removidos, podem desencadear a resposta inflamatória, causando o dano tecidual (Kyttaris et al., 2010). Essa teoria foi fortalecida pelo fato de que vários componentes do sistema complemento apresentam deficiência e possuem seus genes associados com a susceptibilidade ao LES. Indivíduos com

deficiência de C1q, C2 ou C4 mostram um risco maior de desenvolver o LES do que pessoas saudáveis (Manderson et al., 2004). Esses achados indicam o papel fundamental do sistema complemento, não apenas como primeira linha de defesa contra patógenos, mas também no processo de desenvolvimento da autoimunidade, uma vez que os componentes deste sistema opsonizam os imunocomplexos, facilitando a sua remoção e, consequentemente, prevenindo o desencadeamento de uma resposta inflamatória (Kyttaris et al., 2010).

As células dendríticas (CD) são outro grupo de células do sistema imune que também apresentam papel importante no desenvolvimento do LES, embora essa relação seja complicada pelo fato de existirem vários subconjuntos dessas células. As CDs podem influenciar no desenvolvimento do LES das seguintes maneiras: na apresentação de autoantígenos, secreção de citocinas pró-inflamatórias e induzindo a produção de autoanticorpos pelas células B, seja de forma direta ou indireta (Choi et al., 2012). As CDs são derivadas de monócitos e residem primariamente em tecidos epiteliais como a pele e a mucosa dos órgãos. Essa distribuição em vários tecidos permite a imunovigilância contra potenciais patógenos invasores. As duas principais funções das CDs são induzir a resposta imune contra patógenos e manter a autotolerância, posicionando essas células na interface da resposta imune inata e adaptativa (Seitz & Matsushima, 2010).

As citocinas são proteínas secretadas pelas células da imunidade inata e adaptativa e parecem ter um papel fundamental na modulação da resposta imune. Seus efeitos tanto podem ser estimuladores, como proliferação, ativação e quimiotaxia, como supressores, favorecendo a diminuição ou bloqueio de uma resposta imune (Apostolidis et al., 2011). Várias citocinas têm

sido associadas ao LES ao longo dos últimos anos. O Interferon tipo I (IFNα e IFNβ) apresenta diversas funções, como antiviral, antiproliferativa e na modulação da resposta imune. Devido a esses efeitos, o IFN I foi considerado como um dos possíveis responsáveis pelo processo patogênico de diversas doenças autoimunes, incluindo o LES (Apostolidis et al., 2011). Alguns estudos analisaram os níveis séricos de IFN I em pacientes com LES e observaram uma alta produção de IFNα em famílias com pessoas afetadas pela doença, tanto nos familiares saudáveis quanto nos pacientes (Niewold et al., 2007, 2008). Esses resultados indicam que o controle genético do IFN I é importante para a susceptibilidade à doença, mas que, para o desenvolvimento das manifestações clínicas, é necessário ainda a associação com outros fatores como os genéticos, ambientais e hormonais (Apostolidis et al, 2011). Além disso, mais da metade dos pacientes com LES apresentam uma desregulação na expressão de genes da via do IFN I, levando ao que se chama de "assinatura do interferon". O Interferon-α (IFN-α) apresenta-se elevado durante a fase ativa do LES e, a administração de IFN- α recombinante na terapia de certas neoplasias malignas ou de infecções virais crônicas pode levar à formação de anticorpos antinucleares (ANA's) e ocasionalmente ao desenvolvimento de sintomas característicos do lúpus (Borchers et al., 2012).

Barrat et al. (2005) mostraram que as CDs apresentam um papel importante na patogênese do LES através da secreção de citocinas pró-inflamatórias, como por exemplo, IFN tipo 1 (IFN-α). Estudos com camundongos mostraram que o IFN-α secretado por CDs ativadas podia estimular as células B a produzir anticorpos da subclasse IgG contra autoantígenos (Le Bon et al., 2006). Entretanto, é importante salientar que os

fatores que mediam a patogênese do LES envolvem todo o sistema imune e as alterações nas células B, T e dendríticas fazem parte de um mecanismo imune desregulado que alcança níveis sistêmicos com a participação de um amplo conjunto de células e proteínas, como mostrado na figura 1 (Apostolidis et al., 2011).

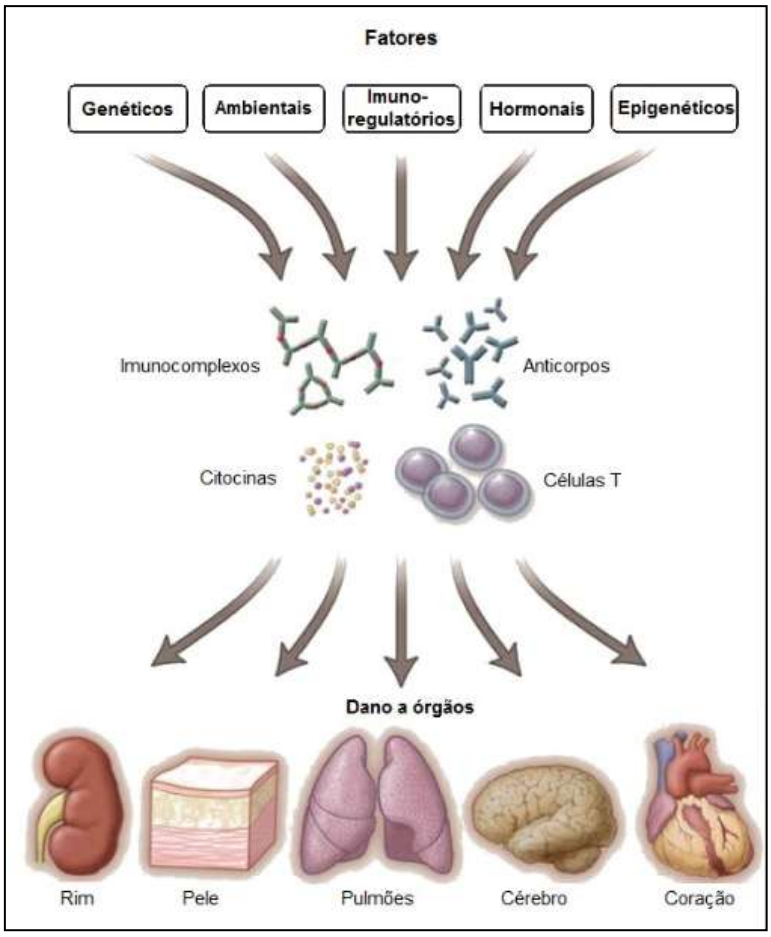


Figura 1. Visão geral da patogênese do Lúpus Eritematoso Sistêmico. O esquema ilustra a atuação dos fatores envolvidos na etiopatogênese do LES e a interação com os componentes do sistema imune, resultando no dano a órgãos e tecidos característica da doença. Adaptado de Tsokos, 2011**.**

2.1.3 Manifestações Clínicas

O diagnóstico oficial do LES é realizado através da presença de pelo menos quatro critérios clínicos dos onze estabelecidos pela Sociedade Americano de Reumatologia (ACR) (Hochberg, 1997), como mostrado na tabela 1.

Tabela 1. Critérios para o diagnóstico do Lúpus Eritematoso Sistêmico de acordo com o ACR. Adaptado de Tsokos (2011).

Critérios	Definição
Eritema Malar	Eritema fixo, plano ou elevado, sobre as eminências malares, tendendo a poupar sulco nasolabial;
Rash Discóide	Placas elevadas, eritematosas, com descamação ceratótica e crostículas;
Fotossensibilidade	Eritema cutâneo, às vezes maculopapular, como resultado de uma exposição solar;
Úlceras Orais	Ulceração oral ou nasofaringeana;
Artrite	Artrite não erosiva, envolvendo duas ou mais articulações periféricas;
Serosite	Pleurite ou pericardite;
Desordens Renais	Proteinúria e/ou desordens no sedimento urinário;
Distúrbios Neurológicos	Convulsões e psicose;
Desordens Hematológicas	Anemia, leucopenia, linfopenia e trombocitopenia;
Alterações Imunológicas	Presença dos anticorpos anti-dsDNA, anti-SM e antifosfolipídico;
Fator Anti-Nuclear	Presença de anticorpo antinuclear.

O LES é caracterizado por períodos de remissão, atividade e progressão da doença. No período de remissão, as manifestações podem durar semanas, meses ou até anos; no período de atividade geralmente é observado um aumento dos sintomas já estabelecidos enquanto que na progressão ocorre o aparecimento de novos sintomas. Fatores ambientais como estresse, infecções, exposição excessiva ao sol, causas emocionais ou interrupção do tratamento são possíveis motivos para o restabelecimento da atividade da doença (Robinson et al., 2011).

As manifestações clínicas do LES acometem órgãos vitais e tecidos como a pele, sistemas nervoso, renal, sanguíneo e imunológico como mostrados na figura 2.

Figura 2. Manifestações clínicas apresentadas pelos pacientes com LES, e o acometimento de diferentes órgãos e sistemas. (Fonte: http://semiologiamedica.blogspot.com.br/2011/10/semiologia-clinica-do-lupus-eritematoso.html)

Tais manifestações podem variar de indivíduo para indivíduo, com acometimentos simultâneos, aditivos ou sequênciais, cíclicos ou persistentes, agudos ou crônicos, com períodos de remissão e exacerbação relativos a órgãos e sistemas diferentes. Os sintomas iniciais do LES geralmente são inespecíficos, como mal-estar, fadiga, febre baixa, perda de peso e adenomegalia e costuma evoluir com manifestações cutâneas e articulares, alterações hematológicas e sorológicas (Assis & Baaklini, 2009). As alterações cutâneas, como a fotossensibilidade, rash malar e discoide e alopecia, atingem cerca de 85% dos pacientes. A fotossensibilidade ocasiona erupções cutâneas devido à exposição à luz ultravioleta e, diferente dos rash malar e discoide, podem acometer não somente a face, mas também os braços e mãos e persistir por mais de um dia, como mostrados nas figuras 3 e 4. (Uva et al., 2012).

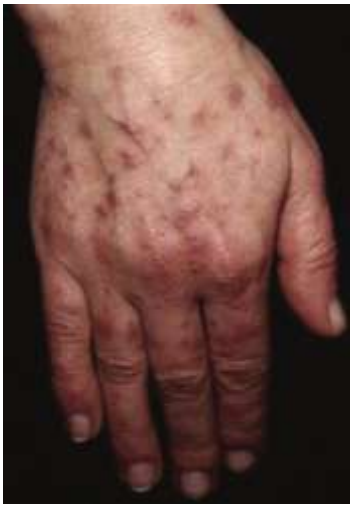


Figura 3. Lesões cutâneas ocasionadas por exposição à luz ultravioleta (UV) em pacientes com LES. Uva et al., 2012.

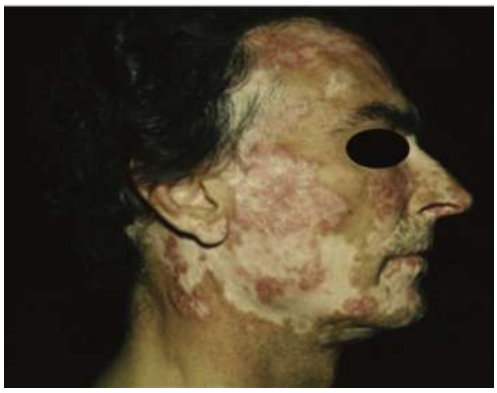
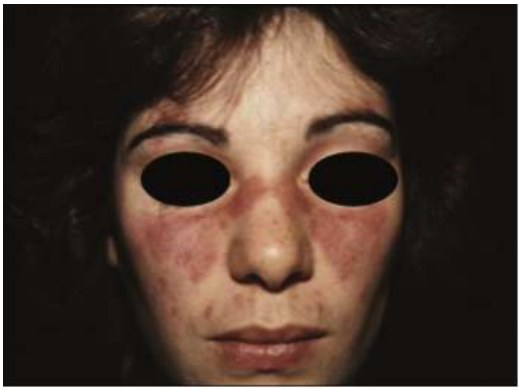
A  B 

Figura 4. A. Rash malar B. Eritema malar ou lesão em “asa de borboleta”, frequente nos pacientes com LES. Uva et al., 2012.

O comprometimento articular nos pacientes com LES atinge cerca de 90% dos indivíduos, com artralgia, artrite e osteonecrose como sendo as principais manifestações. Tais manifestações geralmente são não-erosivas, assimétricas, migratórias, moderadamente dolorosas e não causam deformações (Goh et al., 2012).

As desordens nefríticas são uma das manifestações mais perigosas na doença e causam substancial morbidade e mortalidade. A frequência de envolvimento renal no LES juvenil é alta e atinge cerca de 50-80% dos pacientes, sem diferir com etinicidade (Borchers et al., 2012). As alterações neurológicas relatadas acometem de 25% a 70% dos pacientes com LES e podem afetar qualquer parte do sistema nervoso (Cojocaru et al., 2011).

Os pacientes com LES podem apresentar manifestações vasculares, como por exemplo, vasculites cutâneas, as quais são observadas principalmente em vasos de pequeno calibre. A vasculite nos pacientes com LES é mediada tanto pela deposição de imunocomplexos como pela ligação direta de anticorpos aos componentes celulares e, consequentemente, pela ativação do sistema complemento e inflamatório (Uva et al., 2012). O fenômeno

de Raynaud é uma das manifestações vasculares dos pacientes com LES, e se caracteriza por uma resposta vascular exagerada a baixas temperaturas e ao estresse emocional. Esse fenômeno é caracterizado pela alteração da cor nas pontas dos dedos, resultado da vasoconstricção anormal das arteríolas das digitais, sendo esse um processo reversível (Uva et al., 2012).

O comprometimento do sistema cardiovascular pode atingir cerca de 65% dos pacientes com LES, sendo rara como primeira ocorrência da manifestação da doença (Goh et al., 2012). As alterações do sistema cardiovascular podem afetar o pericárdio, o miocárdio, as válvulas cardíacas e as artérias coronárias (Lalani et al., 2004). A pericardite é uma das manifestações cardíacas do LES mais comuns, sendo inclusive um dos critérios usados para o diagnóstico da doença na classificação ACR (Colégio Americano de Reumatologia) (Gladman et al. 1996; Hochberg, 1997). Autópsias em pacientes com LES revelam que em mais de 60% dos casos ocorre a pericardite, porém apenas 25% a manifestam clinicamente. A miocardite é uma manifestação menos comum, sendo muitas vezes clinicamente silenciosa, podendo estar associada com um processo inflamatório contra o músculo estriado, resultando em um dano ao miocárdio (Goh et al., 2012).

Manifestações neurológicas são relatadas em 25 a 70% dos pacientes com LES e podem afetar qualquer parte do sistema nervoso (Cojocaru et al., 2011). Pela classificação do ACR (Gladman et al. 1996; Hochberg, 1997), podem ocorrer comprometimentos diversos do sistema nervoso central como: estado confusional agudo, disfunção cognitiva, psicose, distúrbios do humor, distúrbios da ansiedade, cefaleia, doença cerebrovascular, mielopatia,

distúrbios do movimento, síndromes desmielinizantes, convulsões e meningite asséptica; assim como distúrbios do sistema nervoso periférico tais como: neuropatia cranial, polineuropatia, plexopatia, mononeuropatia simples ou múltipla, polirradiculoneuropatia aguda inflamatória desmielinizante (síndrome de Guillain-Barré), distúrbio autonômico e miastenia gravis (Assis & Baaklini, 2009). O envolvimento do sistema nervoso central é uma das maiores causas de mortalidade e morbidade em pacientes com LES (Goh et al., 2012), sendo as dores de cabeça e transtornos de humor as principais manifestações neurológicas relatadas (Cojocaru et al., 2011).

No curso da doença, mais da metade dos pacientes com LES desenvolvem manifestações hematológicas (Assis & Baaklini, 2009), entre elas, anormalidades dos elementos formadores do sangue, da coagulação e fatores fibrinolíticos, além de sistemas relacionados. As manifestações hematológicas mais relevantes do LES são anemia, leucopenia, trombocitopenia e a síndrome do anticorpo antifosfolipídico (APS) (Sasidharan et al., 2012).

Em relação às alterações imunológicas, uma das principais características do LES é a formação de autoanticorpos contra uma grande variedade de componentes celulares (Arbuckle et al., 2003), como antígenos nucleares, por exemplo, DNA nativo, DNA desnaturado e as proteínas histonas, Smith, U1-RNP, SSA, SSB e ribossomal (Cojocaru et al., 2011). Apesar da presença desses autoanticorpos ser uma característica constante na doença, a sua concentração e diversidade variam entre os pacientes (Nath et al., 2004).

2.1.4 Influência Ambiental

Embora os fatores que contribuem para a patogenia da doença não estejam completamente esclarecidos, alguns fatores ambientais são considerados importantes no desencadeamento do LES como exposição à radiação ultravioleta (UV), medicamentos que alteram a estrutura do DNA, infecções por alguns vírus e uso hormônios contraceptivos (D´Cruz et al., 2007).

A luz ultravioleta é considerada um dos principais fatores ambientais envolvidos com a exacerbação da doença, uma vez pode provocar quebras no DNA, aumentando sua imunogenicidade e levando a produção de novos autoantígenos e, consequentemente, contribuindo com a autoimunidade (Cooper et al., 2008). Além disso, a exposição à radiação UV é conhecida como um dos principais agravantes das alterações cutâneas como fotossensibilidade e eritema malar e discoide, tão comuns nos pacientes com lúpus (Uva et al., 2012).

O uso de drogas como, por exemplo, a hidralazina e a procainamida são outro fator ambiental considerado ativador do LES. Essas drogas podem inibir a metilação do DNA, causando a diminuição da produção de citocinas por células T $CD4^+$ e a hiperprodução de IgG por determinados tipos de linfócitos B, desordenando o sistema imune e, assim, induzindo o desenvolvimento do LES em um indivíduo geneticamente predisposto (Crispín et al., 2010). Pacientes com lúpus induzido por drogas podem apresentar alguns autoanticorpos específicos como o anti-fosfolipídico e o anti-histona. Esse tipo de lúpus normalmente permanece por 4 a 6 semanas após a interrupção do uso do medicamento, no entanto a detecção de autoanticorpo antinuclear

(ANA) pode permanecer positiva por 6 a 12 meses. Apesar dessas drogas serem capazes de induzir a doença, parecem não agravar o LES estabelecido previamente (Louis & Fernandes, 2001).

Os vírus apresentam propriedades de mimetismo molecular e suas proteínas podem levar à reação cruzada com antígenos próprios, desta forma as infecções virais têm sido consideradas possíveis desencadeadoras do LES. Como exemplo, há a proteína EBNA-1 do Epstein - Barr vírus (EBV), a qual apresenta reação cruzada com o autoantígeno Ro, um dos principais alvos da resposta imunológica na doença. A alta prevalência de EBV na população adulta mundial dificulta qualquer conclusão, no entanto, foi observado que alguns pacientes com LES apresentavam anticorpos para proteínas de EBV antes do desenvolvimento de autoanticorpos característicos do LES (Draborg et al., 2012).

Assim como na maioria das doenças autoimunes, os pacientes com LES são do sexo feminino, sugerindo a participação dos hormônios femininos no desencadeamento da doença (Mason & Isenberg, 2005) ou algum efeito dos genes do cromossomo X ou ainda um papel protetor dos hormônios masculinos contra o desenvolvimento de LES (Rahman & Isenberg, 2008). A administração oral de hormônios, como por exemplo, o uso de contraceptivos e reposição hormonal, mostrou ter influência no desenvolvimento do lúpus, apesar de apresentar resultados contraditórios (D´Cruz et al., 2007). Assim como os hormônios, foi demonstrado que os cromossomos sexuais também podem influenciar na expressão do LES. Em cobaias gonadectomizadas e manipuladas geneticamente para expressar XX ou XO, no caso das fêmeas, e

XY ou XXY, no caso dos machos, a presença de dois cromossomos X aumentou a severidade do LES (Smith-Bouvier et al., 2008).

2.1.5 Influência Genética

Os fatores genéticos conferem predisposição ao desenvolvimento do LES e na maioria dos pacientes a doença é resultado de um efeito combinado de variantes em um grande número de genes. Cada alelo contribui minimamente, e o efeito cumulativo de vários genes é necessário para aumentar de forma significativa o risco à doença. A maioria dos polimorfismos de base única (SNPs) associados ao lúpus está em regiões não codificadoras do DNA de genes relacionados à resposta imune (Tsokos, 2011).

As principais estratégias usadas até o momento na busca de genes marcadores do LES são os estudos de associação genômica em larga escala (GWAS) e os estudos de associação genética, normalmente realizados em estudos do tipo caso-controle. Qualquer uma das estratégias acima citadas apresenta limitações para o estabelecimento do risco genético, e trabalhos posteriores em diferentes populações são necessários para se confirmar os resultados encontrados (Sestak et al., 2007).

A primeira associação genética descrita no LES foi com a região cromossômica 6p21 a qual compreende o complexo de histocompatibilidade humano (HLA) e codifica mais de 200 genes, a maioria com funções imunológicas (Goldberg et al., 1976). A maioria dos genes do HLA estudados até hoje são de classe II, como o HLA-DR2 e o HLA-DR3, os quais mostraram consistentes associações com o LES em várias populações europeias com o dobro do risco de desenvolvimento da doença para cada alelo (Tsao et al.,

2003). Outros genes codificadores de proteínas com funções diversas no sistema imunológico, também mostraram ter associação com a susceptibilidade ao LES, como o *IFR5* (*Interferon Regulatory Factor 5*), *CTLA*-4 (*cytotoxic T lymphocyte antigen-4*) e o *PTPN22* (*Protein tyrosine phosphatase, non-receptor type 22),* STAT4 (*Signal transducer and activator of transcription 4*) e BANK1 (*B-cell scaffold protein with ankyrin repeats 1*) *(*Liu et al., 2001; Graham et al., 2006; Gregersen & Olsson, 2009; Tsokos, 2011). A figura 5 mostra os loci e genes associados ao LES, divididos em seis categorias de acordo com as funções dos genes.

O gene *IRF5*, codifica o principal fator de transcrição na via do IFN tipo I, regulando genes dependentes do IFN, citocinas inflamatórias e genes envolvidos na apoptose. Esse gene apresenta alguns polimorfismos que combinados formam diferentes haplótipos associados com o aumento ou o decréscimo do risco de desenvolvimento do LES (Graham et al., 2006; Graham et al., 2007; Sigurdsson et al., 2008). O gene que codifica a proteína STAT4 também tem sido associado com o LES em várias populações da Europa e da Ásia. O alelo presente no seu terceiro íntron mostrou-se associado com risco alterado ao desenvolvimento do lúpus e com um quadro mais severo da doença caracterizado pelo seu aparecimento precoce, alta frequência de nefrite e presença de anticorpos anti-dsDNA (Remmers et al., 2007; Taylor et al., 2008; Palomino-Morales et al., 2008; Kawasaki et al., 2008; Sigurdsson et al., 2008).

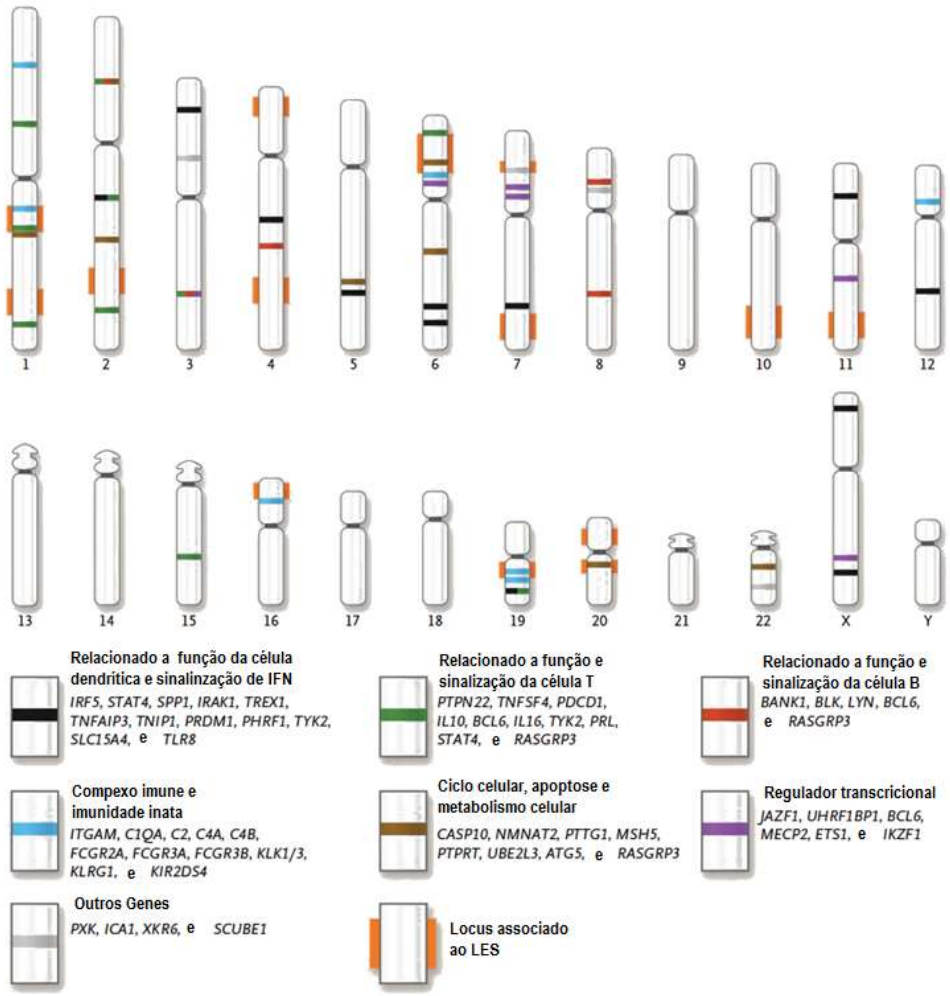


Figura 5. Distribuição dos loci e genes associados ao LES nos cromossomos humanos. Os genes estão separados em seis categorias de acordo com a função. Cada categoria é representada por uma cor diferente nos cromossomos. Uma categoria adicional (cinza) inclui genes que não pertencem aos outros grupos funcionais. Os loci com blocos em laranja dos dois lados indicam locus associado ao LES. Tsokos, 2011.

O gene PTPN22 codifica uma proteína que inibe a atividade de linfócitos T e apresenta um polimorfismo de base única não sinônimo (rs2476601) que está associado com a susceptibilidade a diversas doenças autoimunes, incluindo o LES (Gregersen & Olsson, 2009). Esse polimorfismo resulta na substituição de um aminoácido que aumenta a atividade da proteína, o que reduz a sinalização desencadeada pelos receptores de células T. Essa sinalização reduzida induz a autoimunidade através de alterações na seleção de linfócitos autoreativos e redução da atividade e do número de células Tregs (Vang et al., 2005; Rieck et al., 2007; Siggs et al., 2007).

Um dos mais importantes genes de susceptibilidade a doenças autoimunes é o *CTLA-4*. A proteína codificada por esse gene é expressa na superfície de linfócitos T ativados com uma função regulatória inibitória sobre essas células (Teft et al., 2006). Os polimorfismos do CTLA-4 mais estudados e considerados como associados ao LES em diferentes populações são T>C na posição -1722, C>T na posição -138 e A>G na posição 49 do éxon 1 (Liu et al., 2001; Hudson et al., 2002; Fernandez-Blanco et al., 2004; Barreto et al., 2004; Lee et al., 2005; Ulker et al., 2009; Taha Khalaf et al., 2011).

Os polimorfismos do gene *BANK1* surgiram como associados ao LES a partir de um estudo na população europeia. Esse gene codifica uma proteína adaptadora que faz parte da ativação de células B (Kozyrev et al., 2008). Os polimorfismos rs10516487 (G>A), rs17266594 (T>C) e rs3733197 (G>A) nesse gene podem contribuir com a manutenção da ativação dos receptores de células B e, consequentemente, com a hiperatividade dessas células, comumente observada nos pacientes com LES (Yokoyama et al., 2002).

Dessa forma, é importante salientar que o LES é uma doença complexa e a contribuição de vários fatores levam ao desencadeamento da doença, assim pequenas variações em diversos genes podem contribuir com a desregulação da resposta imune e consequentemente para o desenvolvimento do lúpus (Tsokos, 2011). Assim, o conhecimento de quais genes estão envolvidos na doença e quais variações são capazes de alterar o produto destes genes pode contribuir para o esclarecimento da etiopatogênese do LES.

2.1.6 Genes DNA Ligase 4 - LIG4 e Gene RAD52 homólogo

As células humanas acumulam cerca de 10.000 quebras no DNA todos os dias, sendo o reparo do DNA essencial para a manutenção da estabilidade genômica. A falha em reparar essas quebras pode levar a mutações, instabilidade genômica ou apoptose. Dentre os vários tipos de danos que podem ser expressos nas células, as quebras na dupla fita do DNA (DSBs) são as mais perigosas. DSBs são lesões espontâneas induzidas por eventos endógenos como radicais O_2 livres e a replicação do DNA, ou exógenos como a ação de agentes genotóxicos, radiação ionizante, ou mesmo estresse ambiental. As células danificadas podem seguir por duas vias de reparo de DSBs: recombinação homóloga (HR) e das extremidades não homólogas (NHEJ) (Tsail &Lieber, 2010;Neal & Meek, 2011).

A NHEJ é o processo menos complexo e o mais utilizado no reparo de DSBs por não exigir recombinação e nem um molde de DNA. É um processo que envolve várias etapas, requerendo assim o envolvimento de diversas proteínas. Basicamente o complexo protéico formado pelos heterodímeros Ku70 e Ku80 se ligam às quebras e recrutam o complexo Artemis e a proteína quinase DNA-dependente com subunidade catalítica – DNA-PKcs. Artemis e

DNA-PKcs possuem atividade de quinase e são capazes de atrair e de se ligarem a essas DSBs. Para que ocorra a finalização do processo de reparo, um último complexo é requerido: XRCC4 (do inglês X-Ray Repair cross-complementing protein 4) e a LIG4, que juntas promovem a ligação e finalização do processo (Neal & Meek, 2011).

O gene DNA ligase IV, ou *LIG4*, está localizado no cromossomo 13 (13q33–q34), apresenta 10,9 kb de tamanho e consiste de 2 éxons e 1 íntron (figura 6). O cDNA codifica 911 aminoácidos com peso molecular previsto de 96kDa (Chistiakov, Voronova, & Chistiakov, 2009). O gene *LIG4* codifica a proteína LIG4 que atua em associação com a proteína XRCC4 no reparo de recombinação de extremidade não-homólogas (NHEJ). Estudos em linhagens celulares animais, sem a presença do gene *LIG4* indicou uma elevada sensibilidade dessas células aos raios X e a agentes prejudiciais ao DNA, devido ao reparo ineficiente de DSBs por NHEJ. A ausência do gene *LIG4* em murinos é letal, causando morte embrionária e apoptose neural massiva, com bloqueio da linfogênese e múltiplos defeitos celulares (Frank, et al., 1998).

As quebras de dupla fita do DNA (DSBs) durante a recombinação V(D)J dos receptores de células B e T são reparadas via NHEJ. As proteínas Artemis e LIG4 são os únicos fatores V(D)J/NHEJ encontrados em desordens genéticas humanas. Mutações no *LIG4* são exclusivamente hipomórficas e só foram descritas em seis pacientes, dos quais quatro exibiam imunodeficiência associada à microcefalia e atraso no desenvolvimento, enquanto os outros dois eram pacientes com leucemia. Essas observações sugerem que o *LIG4* pode possuir papel adicional crítico na sobrevivência dos linfócitos durante a recombinação V(D)J (Buck, 2006).

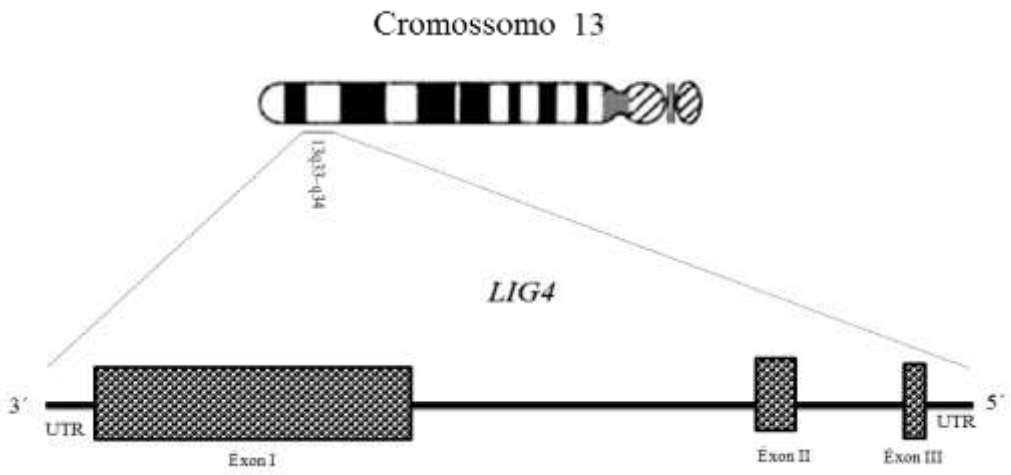


Figura 6. Organização esquemática do gene *LIG4*.

Polimorfismos no *LIG4* são capazes de prejudicar a atividade da proteína e podem originar a síndrome do LIG4, caracterizada por radiossensibilidade pronunciada, instabilidade genômica, anormalidades da medula óssea e imunodeficiência (Chistiakov, Voronova e Chistiakov, 2009). Uma vez funcionando incorretamente, o reparo de DSBs pelo mecanismo de NHEJ fica comprometido. Polimorfismos neste gene também foram associados à sobrevida e prognóstico de um tipo muito agressivo de glioma, o glioblastoma, com os índices mais baixos de sobrevida entre os pacientes com câncer (Liu et al. 2010). Ainda como um fator de risco, SNPs no *LIG4* foram associados ao câncer de pulmão na população Chinesa. No estudo foram avaliados os polimorfismos nos principais componentes da via NHEJ, incluindo *Ku70* e *80*, *LIG4* e a *XRCC4*. Os polimorfismos nos genes *XRCC4* e *LIG4* (rs1805388) indicaram uma associação significativa ao risco com câncer de pulmão de células não-pequenas (CPNPC) (Tseng et al. 2009).

Em pacientes com LES o reparo a DSBs apresenta deficiência em relação a indivíduos saudáveis, além disso, algumas enzimas de reparo da via NHEJ já foram associadas ao LES e a suas manifestações clínicas (Bassi et

al., 2008). Um estudo recente utilizando microarranjo de cDNA indicou que o gene *LIG4* apresentava repressão de 21 vezes nos pacientes com lúpus na fase ativa da doença. Outro gene envolvido na manutenção genômica também apresentou repressão no mesmo estudo, o serina/treonina quinase 17A (*STK17A)* (Bassi, et al., 2008; Sandrin-Garcia et al., 2009). No entanto, pouca atenção tem sido oferecida aos estudos envolvendo enzimas de reparo no LES, com literatura escassa no diz respeito aos estudos de associação desses genes e doenças autoimunes (Bassi et al., 2008).

O outro mecanismo de reparo utilizado pelas células para restituir as DSBs é a recombinação homóloga (HR). A HR é a forma mais complexa de reparo por exigir um molde de DNA (Rothenberg et al., 2008). De forma geral, o reparo por HR é iniciado por uma deterioração na dupla fita de DNA fornecendo assim uma cauda 3´de fita simples. Em seguida, as proteínas RAD51 e Dcm1 se ligam a essa região para iniciar o pareamento com uma sequência homóloga de DNA. A região 3´ fornecida pela degradação do DNA após a quebra é usada como iniciador na síntese da nova sequência. A proteína RAD51 é uma homóloga da recombinase RecA em bactérias e atua no pareamento inicial das fitas simples de DNA (Hiom, 1999).

A proteína RAD52 pertence a um grupo ubíquo de proteínas mediadoras de recombinação, cuja função é essencial para recombinação homóloga (HR), reparo direto do DNA e resgate de forquilhas de replicação em colapso, atuando com um papel chave na manutenção da integridade do DNA. O gene *RAD52 homólogo* está localizado no cromossomo 12 (12p13.33), apresenta 13 éxons e codifica uma proteína essencial nos estágios iniciais de reparo por HR (figura 7). A RAD52 interage com a proteína de recombinação do DNA RAD51,

mediando a sua função. A RAD52 humana atua também como uma proteína alternativa mediadora da ação de BRCA2 (*breast cancer type 2 susceptibility protein*) (Hiom, 1999).

Os estudos de associação envolvendo polimorfismos no gene *RAD52* humano até o momento, estão relacionados ao desenvolvimento de vários tipos de câncer. Os estudos com câncer de pulmão mostraram associação dos polimorfismos deste gene com a neoplasia. A RAD52 é uma das mais importantes proteínas que atuam na HR, e em células tumorais com mutações deletérias no RAD52 apresentam um mecanismo de HR completamente defeituoso (Danoy et al., 2008).

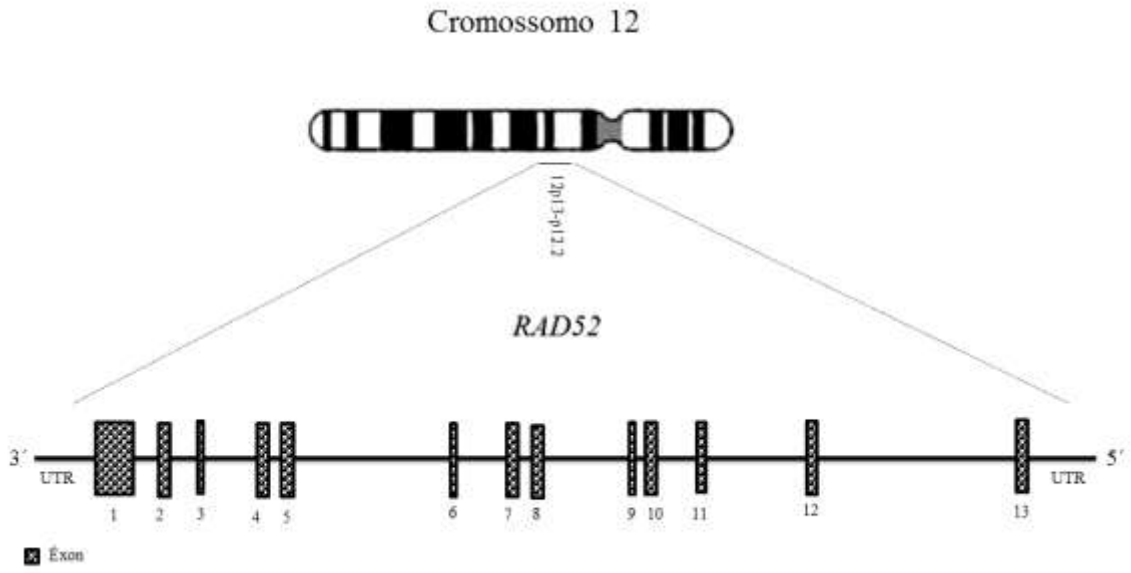


Figura 7. Organização esquemática do gene *RAD52*.

Estudos envolvendo polimorfismos em outros genes codificadores de proteínas de reparo da via de HR já foram associados ao LES. Dentre eles o *XRCC1*, que em o polimorfismo Arg399Gln foi associado tanto com a

susceptibilidade ao LES quanto com as alterações cutâneas relacionadas à doença. O estudo conduzido por Bassi et al. (2008), analisou o mesmo polimorfismo Arg399Gln do *XRCC1* em pacientes Brasileiros com LES e não encontrou associação com a doença, no entanto a análise combinada de três genes envolvidos nas principais vias de reparo, indicou associação com manifestações neuropsiquiátricas, síndrome antifosfolipídica e anticorpo anti-DNA.

Em 2009, Sandrin-Garcia et al., avaliaram o perfil de expressão gênica de 4500 genes comparando pacientes com LES nas fases ativa e inativa da doença em relação a indivíduos saudáveis. Um total de 156 genes mostrou-se diferencialmente expresso quando comparados os pacientes e controles. Desses 156 genes, um total de 8 genes apresentaram expressão diferencial em nas fases ativa e inativa do LES, dentre os quais o *LIG4* apresentou repressão de cerca de 21 vezes e o *RAD52* apresentou indução de cerca de 169 vezes, ambos na fase ativa a doença. Os genes *LIG4* e *RAD52* participam dos principais mecanismos de reparo de quebra de dupla fita de DNA (DSB). As DSBs quando não reparadas podem levar a autoimunidade por aumentar a imunogenicidade da molécula de DNA e a formação de corpos apoptóticos circulantes. Tais alterações são mecanismos chave na patogênese do LES (Bassi et al., 2008).

2.1.7 Receptor da vitamina D – VDR

A vitamina D apresenta duas formas principais: cholecalciferol (vitamina D3) e ergocalciferol (vitamina D2). Ambas as formas (D2 e D3) podem ser encontradas em alimentos e suplementos específicos, no entanto, somente a D3 é produzida pela pele. A pré-vitamina D3 é formada a partir do 7-

dehidrocolesterol, também chamada de pró-vitamina D3, após a exposição aos raios solares. Tal exposição resulta na forma inativa da vitamina D e seus componentes, o que atua como um mecanismo protetivo contra a ação tóxica da vitamina D (Kamen & Tangpricha, 2010). A D3 é um hormônio secoesteróide produzido na pela e metabolizado nos rins à sua forma ativa, apresenta efeito imunomodulatório e atua através do receptor de vitamina D, o VDR, que pode ser encontrado em mais de 50 tipos diferentes de células e tecidos (Wang et al, 2012). A D3 entra na célula alvo a partir da circulação e se liga ao VDR no citoplasma, o qual entra no núcleo e se heterodimeriza com o receptor de ácido retinóico (RXR). Esse heterodímero se liga a elementos de resposta a vitamina D (VDER) localizados no DNA, ativando assim a transcrição gênica (Kamen & Tangpricha, 2010).

A função clássica da vitamina D é de aumentar a absorção de cálcio pelo intestino, além disso, ela participa também de outras rotas metabólicas, como a da resposta imune, incluindo as células T, B e dendríticas (Haussler et al., 1998). Cada uma dessas células apresenta o VDR e produz a enzima 1α-hidroxilase e 24-hidroxilase sendo capazes de produzir a D3 localmente. As funções parácrinas e endócrinas da D3 estão sob uma delicada regulação do sistema imune e são dependentes da pré-vitamina D, fazendo com que a sua deficiência se torne crítica para o sistema imune (Kamen & Tangpricha, 2010). No sistema imune, a D3 ainda promove a diferenciação de monócitos, inibe a proliferação de células T e proliferação e secreção de citocinas, como a interleucina-2 (IL-2), interferon-γ (IFN-γ) e IL-12 (Uitterlinden et al., 2004).

O sistema imune inato age como a primeira linha de defesa contra a invasão microbial e a presença de alguns peptídeos antimicrobianos é

importante neste contexto. Em humanos, a catelicidina pode ser encontrada como um peptídeo antimicrobiano produzido por macrófagos, monócitos e queratinócitos, com alta eficiência contra bactérias, micobactérias, fungos e vírus (Nnoaham et al., 2008; Wejse et al., 2009). A vitamina D estimula a produção de catelicidinas por essas células; além disso, monócitos e macrófagos expostos a lipopolisacarídeos e ao *Mycobacterium tuberculosis* ativam o VDR e a 1-α-hidroxilase levando a um aumento local tanto da vitamina D quanto do seu receptor, o VDR, o que aumenta a produção de catelicidina (Adams et al., 2009).

Cada uma dessas células expressam o VDR e a 1α-hidroxilase e ainda podem produzir a vitamina D localmente. O efeito endócrino/parácrino da vitamina D depende da sua concentração, o que faz da deficiência desse hormônio um fator crucial no funcionamento do sistema imune (Merewood et al., 2009). Nos linfócitos pode se verificar diversas alterações relacionadas à vitamina D, dentre elas a supressão do receptor da célula T, alteração dos perfis de citocinas (diminuição de IFN-γ e IL-2 e aumento de TGF-β1, IL4, IL5, e IL10) e alterações no fenótipo de Th1 para Th2, com o aumento da tolerância imunológica. Além disso, ainda observa-se o estímulo das células T reguladoras em suprimir a proliferação de células T, a diferenciação das células B e o bloqueio da produção de imunoglobulinas (Monticielo et al., 2012).

O primeiro estudo sugerindo a associação da deficiência da vitamina D com o LES surgiu em 1979. O estudo avaliou 12 adolescentes com LES, fazendo utilização de glucocorticóides, que mostrou níveis baixos desse hormônio em quase 60% dos pacientes. Estudos subsequentes, em populações variadas apresentam resultados semelhantes (Muller et al., 1995;

Becker et al., 2001; Kamen et al, 2006). Quando a vitamina D se liga ao VDR ela induz a supressão de citocinas pró-inflamatórias, tais como IFN-γ e IL-12, que são elevadas no soro de pacientes com LES e em particular àqueles na fase ativa da doença, retardando assim o desenvolvimento dos processos patogênicos na doença (Monticielo et al., 2012).

O gene *VDR* apresenta mais de 100kb de comprimento e está localizado no cromossomo 12 (12q13.1). O *VDR* inclui oito éxons codificadores de proteínas (éxons 2-9) e seis éxons não traduzidos (1a-1f), que apresentam *splicing* alternativo, oito íntrons e duas regiões promotoras (Nejentsev et al., 2004). Os polimorfismos descritos no *VDR* estão principalmente nas regiões promotoras próximas aos sítios f e c do éxon 1, entre os éxons 2 e 9 e na região 3´UTR. Os polimorfismos no gene VDR mais descritos na literatura são: *Cdx2* (G>A), *FokI* (C>T), *BsmI* (A>G), *EcoRV* (G>A), *ApaI* (G>T) e *TaqI* (T>C) (Uitterlinden et al., 2004) (figura 8).

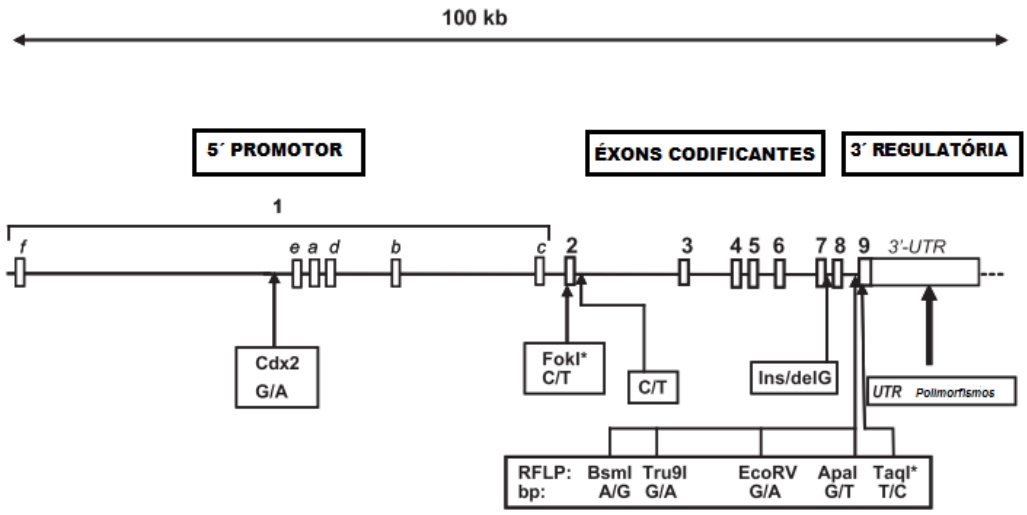


Figura 8. Estrutura do gene e posição dos polimorfismos mais estudados no gene VDR. Adaptado de Uitterlinden et al., 2004.

Os polimorfismos no gene *VDR* podem alterar tanto a função do gene quanto a sua expressão, levando assim a uma ação ineficiente da vitamina D. O polimorfismo *BsmI* está localizado no íntron 8 e resulta da substituição de uma adenina por uma guanina (A>G), já os *ApaI* e o *TaqI* estão localizados na região 3´do gene. No *ApaI* acontece uma substituição de timina por uma guanina (T>G) no íntron 8, enquanto o polimorfismo *TaqI*, no éxon 9, é definido por uma substituição de uma citocina por timina (C>T) resultando na mudança no códon ATC→ATT, mas mantendo o mesmo aminoácido (isoleucina). O papel funcional desses polimorfismos estão associados com o aumento da estabilidade do RNAm (Uitterlinden et al., 2004).

2.1.8 Gene Helicase interferon-induzinda 1 - IF1H1

Nos últimos anos tem sido investigado o papel dos marcadores genéticos para a atividade do LES, e dentre os candidatos a biomarcadores está o sistema do INF tipo 1, ou seja, genes relacionados ao IFN-α (Ahearn et al., 2012). O estudo realizado por Baechler et al (2002), utilizou a tecnologia de microarranjo de cDNA para analisar o perfil de expressão gênica em células mononucleares de sangue periférico (PBMC). Os autores observaram que o padrão de expressão dos genes induzidos pelo IFN estava super expresso nos pacientes com LES, originando o termo “assinatura do IFN”. No estudo ainda foi observado que a assinatura de IFN estava relacionada com a severidade da doença e com o envolvimento hematológico, cerebral e nefrítico (Beachler et al., 2002).

Estudos subsequentes confirmaram os resultados encontrados por Beachler et al. (2002), e ainda forneceram associações significantes com

genes e citocinas induzidos pelo IFN e a atividade aumentada da doença, hipocomplementemia (falta ou diminuição das proteínas do sistema complemento), e a presença de autoanticorpos específicos para dsDNA e Ro, U1-RNP e Sm, em pacientes adultos e crianças com LES (Ahearn et al., 2012).

O gene *IFIH1* (*Interferon-Induced Helicase 1*) está localizado no cromossomo 2 (2q24), possuindo 51,5 Kb de tamanho e 16 éxons, como mostrado na figura 9. Em sua região codificante, o gene *IFIH1* possui 3365 pb de comprimento, codificando uma proteína de 1025 aminoácidos e aproximadamente 116,7 KDa de peso molecular. O gene *IFIH1* codifica uma proteína citoplasmática com a capacidade de reconhecer dsRNA e ativar a sinalização do IFN-α. Ela também atua ativando a apoptose de células com a presença do dsRNA, iniciando a resposta de "limpeza" dos corpos apoptóticos da circulação sanguínea, além de ativar a transcrição de fatores como o IFR3, IFR7 e o NF-KB (Chistiakov, 2010).

A etiologia de diversas doenças autoimunes, como a artrite reumatóide (AR), esclerose múltipla (EM), psoríase, diabetes mellitus tipo 1 (DM1) e o LES, têm sido associadas a polimorfismos encontrados ao longo do gene *IFIH1* (Marinou et al., 2007). O estudo conduzido por Robinson et al. (2011) avaliou o polimorfismo C>T (rs1990760) e susceptibilidade ao LES da população Americana. Os autores identificaram associação do alelo T (rs1990760) e o aumento de genes induzidos pelo IFN em PBMCs em resposta ao estímulo por IFN-α em pacientes positivos para anticorpo anti ds-DNA. Desta forma, devido ao seu papel na imunidade e sua associação com desordens autoimunes, os autores concluíram que o *IFIH1* pode atuar de forma desregulada na tolerância humoral em pacientes com lúpus (Robinson et al., 2011).

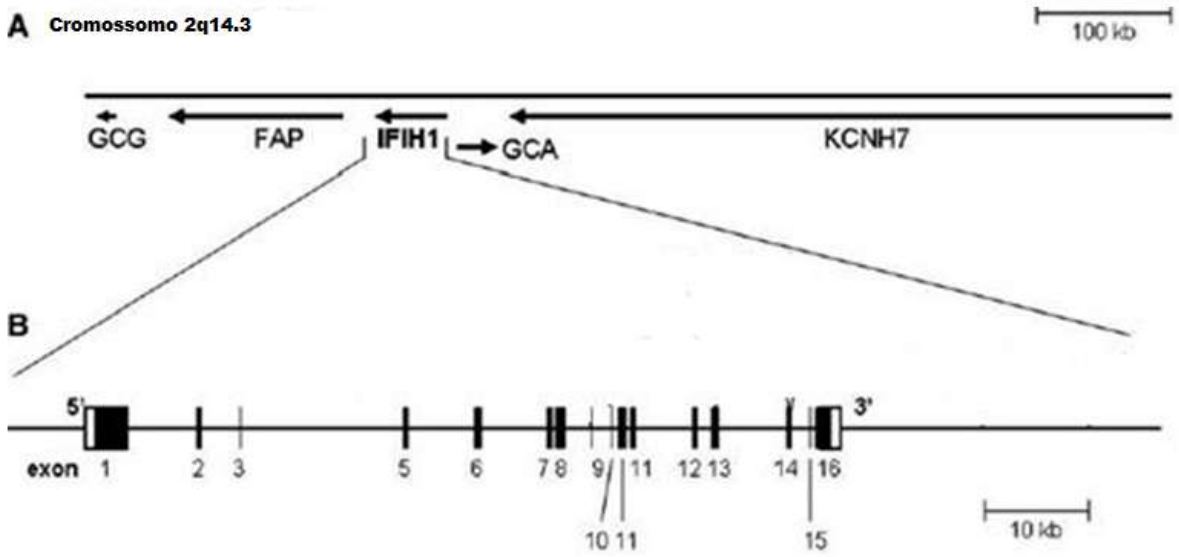


Figura 9. A. Posição do gene IFIH1 no cromossomo. B. Estrutura esquemática do gene**.** Chistiakov, 2010.

Dessa forma, sabendo-se que o LES é uma doença complexa, com a atuação de diversos genes envolvidos na sua etiopatogênese, neste estudo foram selecionados quatro genes que apresentam funções relevantes nas vias de desenvolvimento da doença. Os genes *LIG4* e *RAD52* são candidatos ao lúpus por suas atuações em mecanismos importantes de vias de reparo do DNA. Os genes *VDR* e *IFIH1* foram selecionados por sua ação na regulação da resposta imune. Assim, alterações nesses genes podem desencadear uma resposta imune desregulada e iniciar o processo patogênico de autoimunidade do LES.

3. Objetivos

3.1 Geral

O objetivo geral deste trabalho foi o de avaliar os polimorfismos de base única (SNPs) nos genes *LIG4*, *RAD52*, *VDR* e *IFIH1* na susceptibilidade ao lúpus eritematoso sistêmico e suas manifestações clínicas em uma população do Sudeste Brasileiro.

3.2 Específicos

- Determinar a frequência dos SNPs dos genes *LIG4*, *RAD52*, *VDR* e *IFIH1* em pacientes com Lúpus Eritematoso Sistêmico e indivíduos controle;
- Avaliar o grau de associação desses polimorfismos com a susceptibilidade ao LES;
- Verificar a formação de blocos haplotípicos entre os SNPs analisados em cada um dos genes *LIG4*, *RAD52*, *VDR* e *IFIH1*;
- Avaliar o grau de associação desses polimorfismos com as manifestações clínicas do Lúpus Eritematoso Sistêmico;
- Avaliar o grau de associação das manifestações clínicas em relação à etnia e sexo dos pacientes com Lúpus Eritematoso Sistêmico.

4. Capítulo I: Polymorphisms in *LIG4* and *RAD52* DNA repair genes and susceptibility to systemic lupus erythematosus and its clinical manifestations in Brazilian populations

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POLYMORPHISMS IN *LIG4* AND *RAD52* DNA REPAIR GENES AND SUSCEPTIBILITY TO SYSTEMIC LUPUS ERYTHEMATOSUS AND ITS CLINICAL MANIFESTATIONS IN BRAZILIAN POPULATION

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Running Head: *LIG4* and *RAD52* polymorphisms in SLE.

Key-words: SNPs, LIG4, RAD52 and SLE.

ABSTRACT

Systemic Lupus Erythematosus (SLE) is a complex autoimmune disorder with a strong genetic background. However, SLE might also be triggered due to environmental factors, such as UV light exposure. UV radiation can induce DNA double strand breaks (DSBs) which increases DNA immunogenicity. In SLE patients, DNA repair is diminished allowing the accumulation of DSBs and genomic instability. *LIG4* and *RAD52* genes play pivotal roles in DNA repair mechanisms and a recent microarray analysis showed their differential expression in active SLE patients. In this study we investigated a potential connection between *LIG4* and *RAD52* single nucleotide polymorphisms (SNPs) and SLE predisposition in a Southeast Brazilian population. We assessed 158 SLE patients and 212 healthy controls. We evaluated four Tag SNPs in *LIG4* and three in *RAD52* gene region, encompassing most of the gene sequence. We also performed SNPs analysis considering clinical manifestation, gender and ethnicity. Our data did not show association between *LIG4* and *RAD52* SNPs and SLE, its clinical manifestations or ethnicity in the tested population. To our knowledge, this is the first association study involving *LIG4* and *RAD52* genes and SLE predisposition. The lack of association from *LIG4* and *RAD52* polymorphisms in this study could exclude their role in SLE susceptibility in the genomic level, suggesting that the differential expression previously reported by *LIG4* and *RAD52* is not connected to SNPs found in these genes.

KEYWORDS: SNPs, LIG4, RAD52 and SLE.

INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a complex autoimmune disorder. The rising of autoantibodies against nuclear antigens is systemic, spares no organ or tissue and is the hallmark of the disease (1). The precise etiology of SLE is not completely clear. Even though genetic plays a major role in disease susceptibility, other factors can be important in triggering the disease, like hormonal factors, such as pregnancy, and environmental, such as ultraviolet (UV) radiation exposure (2). The heterogeneous clinical manifestations in SLE encompass vital organs and tissues and the most affected are women in childbearing ages (1). The incidence of the disease ranges from 1 to 10 cases in 100.000 person/years around the world, and Brazil has reported the highest worldwide incidence, presenting 8.7 cases per 100.000 person/years (3,4).

Genetic factors seem to play a pivotal role in SLE development. The genes associated to SLE are not the sole causative of the disease and most of them have been associated to several autoimmune disorders, such as *IFR5*, *STAT4* and *CTLA4* (1). Although various genes have already been described as associated to SLE, genome-wide association studies (GWAS) has provided a new and broader spectrum of candidate genes that might be connected to the disease. Sandrin-Garcia et al. (2009) evaluated the expression profile of 4500 genes in Southeast Brazilian patients with active and inactive SLE. A set of eight genes were considered candidates in SLE susceptibility, among them: DNA ligase 4 (*LIG4*) and RAD52 homolog (*RAD52*), both DNA repair genes (5).

The DNA contained in our cells is constantly exposed to a variety of potential agents capable of DNA damage. Among these lesions, the Double Strand Breaks (DSBs) are the most harmful to our genome (6). Although potentially hazardous, the DSBs may occur naturally, as an intermediate in meiosis and during the setting up of

the immune repertoire, i.e, V(D)J recombination (6,7). Therefore, DNA damage repair (DDR) is a decisive mechanism in maintaining the threshold between genetic variability and genomic instability (7).

The strategies used to solve DSB lesions are mainly the Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR). *LIG4* gene is located on chromosome 13q33–q34 and encompasses 10.9 kb of length, including 2 exons and one intron (8,9). During NHEJ, LIG4 protein is required in a complex with X-Ray Repair cross-complementing protein 4 (XRCC4), to provide a closure to the DSB repair (10). Homologous recombination is the most complex and efficient mechanism to solve the DSB problem. *RAD52* gene is located on chromosome 12 (12p13-p12.2) and plays a unique role in HR pathway by providing the annealing of complementary regions of single-stranded DNA and supporting *RAD51* in the initial pairing of homologous DNA molecules. (11,12).

Recent studies have shown defective DNA repair in SLE patients cell lines indicating that the genes involved in DNA repair pathways may not work properly in these patients. Moreover, DNA repair polymorphic sites were associated to predisposition of SLE clinical manifestations and antibody anti-DNA, reinforcing the role of DNA repair genes in autoimmunity (13,14). Hence DNA repair mechanisms are impaired in SLE patients and differential expression in active SLE were observed in *LIG4* and *RAD52* genes, we evaluated seven Tag SNPs along both genes to SLE susceptibility and its clinical manifestations. A tag SNP is described as a representative SNP in a particular genome region. So, it is possible to perform association studies without testing every single polymorphism within a gene (15). To our knowledge, this is the first association study involving *LIG4* and *RAD52* and susceptibility in SLE Brazilian population.

SUBJECTS AND METHODS

Patients and Controls

In this study, we performed a case-control study with 158 SLE patients (93% females and 7% males), mean age 37.8 years ±11.9 SD, enrolled from the Division of Clinical Immunology, University Hospital of the School of Medicine of Ribeirão Preto, University of São Paulo (SP, Brazil). The diagnosis of SLE was performed according to the criteria defined by the American College of Rheumatology (16). SLE patients were stratified according to clinical manifestations as depicted in Table 1. The patients group was classified as European or African-derived, 76% and 24% respectively, according to phenotypic characteristics of individuals and ethnicity data from parents/grandparents reported by the participants.

The control group consisted of 212 healthy unrelated individuals (47% females and 53% males, mean age 36.47 years ± 10.96 years), from Ribeirão Preto, São Paulo, Brazil. European and African-derived individuals represented 72.17% and 27.83%, respectively. As exclusion criteria for the control group, the presence of clinical manifestations and/or family history of autoimmunity were applied. Genotyping for HLA-A, B, C, DR and DQ were performed in order to exclude HLA risk haplotype in the control group. This study was previously approved by the local ethics committee (CEP/HCRP/FMRP #2234/2007).

SNPs Selection and Genotyping

Genomic DNA was isolated from whole blood using the Wizard genomic DNA purification kit (Promega, Madison, MA) and standard extraction protocol according to manufacturer's instructions. Polymorphisms were selected using the SNPBrowser

software 4.0 (Applied Biosystems, Foster City, CA) and HapMap database (http://hapmap.ncbi.nlm.nih.gov/). SNPs selection involved the following criteria: Tag SNPs and 10% Minimum Allele Frequency (MAF). Four Tag SNPs were selected at *LIG4* gene (rs10131, rs1805386, rs1805388 and rs3093740); and 3 at *RAD52* gene (rs1051669, rs11064607 and rs3748522).

Genotyping was performed using commercially available fluorogenic allele specific probes (Taqman Probes, Applied Biosystems, Foster City, CA) using the ABI7500 Real Time PCR platform (Applied Biosystems, Foster City, CA). Allelic discrimination followed as recommended by the manufacturer and analyzed using the SDS software 2.3 (Applied Biosystems, Foster City, CA).

Statistical Analysis

Genotypic and allelic frequencies and Hardy-Weinberg equilibrium were performed by using the SNPStats tool (http://bioinfo.iconcologia.net/SNPstats). In this study we considered for analysis: SLE clinical manifestations, sex bias and ethnicity, enabling the set up of subgroups. The combinations used in the analysis were: SNPs *versus* clinical manifestations, SNPs *versus* ethnicity, SNPs *versus* gender, clinical manifestation *versus* gender and clinical manifestation *versus* ethnicity. The Fisher's Exact Test was applied to determine the statistical significance of all comparisons. The Haploview Software, version 4.2, was used for haplotypes associations. P-values < 0.05 were considered statistically significant. Power analysis was performed using the *post hoc* test G*Power software (version 3.1.2) based on minor allele frequency and OR (Fisher's exact test) with an alpha error of 0.05 (17).

RESULTS

LIG4 gene polymorphisms

Four Tag SNPs at *LIG4* gene (rs10131, rs1805386, rs1805388 and rs3093740) were evaluated in this study, regarding their position and SNP coverage throughout the gene, as depicted in Figure 1a. A total of 158 SLE patients and 211 healthy controls (HC) were assessed, and allele and genotypic frequencies are shown in table 2. All allelic and genotypic frequencies of the tested polymorphisms were in Hardy-Weinberg equilibrium (H-W) in both groups, except for SNP rs10131, $p<0.05$ in the patient group. The allele and genotype frequencies did not significantly differ in SLE patients and HC, indicating no association between the tested polymorphisms and the SLE. We also performed analysis with genotypic and allelic frequencies considering gender and ethnicity (data not shown). No single genotype or allele was associated to SLE altered risk in none the above mentioned combined analysis. Haplotype analysis indicated a linkage disequilibrium between rs10131 and rs1805388 ($D' = 0.9$) as shown in Figure 1b, but no haplotype combination enclosing the tested SNPs show any association SLE (data not shown).

RAD52 gene polymorphisms

Three Tag SNPs in *RAD52* gene sequence were evaluated in this study (rs1051669, rs11064607 and rs3748522). Allelic and genotypic frequencies are shown in Table 3, and both groups were in Hardy-Weinberg equilibrium (H-W). As it was observed for *LIG4*, the allelic and genotypic frequencies for *RAD52* did not significantly differ in SLE patients and HC, once again indicating no association between the tested polymorphisms with SLE. The analysis regarding genotypic and allelic frequencies *versus* gender and *versus* ethnicity did not show association either

(data not shown). Haplotype analysis indicated a linkage disequilibrium between the 3 Tag SNPs (D´= 0.9), as shown in Figure 2b; however, no haplotype combination enclosing the tested SNPs showed association with SLE (data not shown).

LIG4 and *RAD52* gene polymorphisms and Clinical Manifestations

When assessing *LIG4* and *RAD52* polymorphisms in SLE clinical manifestations susceptibility, we did not find any statistically relevant results ($p > 0.05$) in neither genes. Clinical manifestations regarding gender and ethnicity described in our SLE group are show in table 4. European-derived SLE subgroup indicated altered risk for cutaneous and hematological alterations (OR = 2.73, CI = 1.21-6.35, *p*-value = 0.008 and OR = 2.76, CI= 1.22-6.51, *p*-value = 0.008, respectively) (Table 4). No association was found in the remaining SLE subgroups.

DISCUSSION

Several environmental stimuli have been associated to SLE susceptibility, particularly ultraviolet light exposure, which is able to produce DSBs and DNA oxidative damage (14). Even though DNA breaks are able to induce immune response, the underlying mechanism in the development of autoimmune diseases is not completely clear (18). In the present work we did not find genetic association to *LIG4* and *RAD52* and SLE susceptibility as well as its clinical manifestations. Our results provide evidences to exclude their participation at the genomic level in SLE development, at least regarding the studied Tag SNPs and those represented by them. Interestingly, both genes have shown differential expression when comparing active and inactive SLE patients and healthy subjects (5) from the same geographical area – Southeast Brazil, in which we performed our association study. In the cDNA microarray analysis conducted by Sandrin-Garcia et al. (2009), the transcription profile

of approximately 4500 genes was evaluated from peripheral blood lymphocytes obtained from active and inactive SLE patients and controls. A set of eight genes showed differential expression in comparison to controls. Among them *LIG4* showed repressed expression of -21 fold and *RAD52* an up regulation of +169 fold, both in active SLE phase (5). Moreover, the data presented by Davies et al. (2012) showed a defective response to DSB repair in juvenile SLE patient cells, even though all known core proteins in NHEJ were present in SLE cell lines (13).

In the present study, we assessed Tag SNPs covering most of the gene region, including UTRs, in both tested genes. Putting together our findings with the ones described by Sandrin-Garcia et al. (5), led us to hypothesize that the role of *LIG4* and *RAD52* genes in SLE susceptibility and clinical manifestations are not at the genomic level, but at transcriptional rates. However, it is strongly recommended replica studies in other populations and a complete sequencing of both genes, including the whole promoter regions, not covered by our Tag SNPs.

In the past few years, little attention has been devoted to DNA repair genes and chronic diseases, particularly SLE (14). Up until now, most of the studies are functional providing valuable data regarding efficiency and kinetics of DNA repair enzymes in SLE patients (13,14) . Bassi et al., 2008, evaluated the efficiency and association of DNA repair enzymes in SLE patients. The study indicated decreased efficiency in SLE leukocytes when compared to healthy individual cells. They also performed an association study, in which there was a lack of association from X*RCC1*, *XRCC3* and *XRCC4* gene variants and SLE altered risk (14), in agreement with ours results. The Tag SNPs in 3´UTR region from *LIG4* and *RAD52* (rs10131and rs1051669) are both within conservative regions described as binding sites for miRNA, known for its post-translational regulation (19). Hence, SNPs in linkage disequilibrium with the Tag SNPs

at rs10131 and rs1051669 may affect the sites of miRNA-495 and miRNA-210, known as target for *LIG4* and *RAD52* genes, respectively (19–21). So, the alterations on both genes, influencing SLE susceptibility might not be in its DNA sequence, becoming not deducible in an association study.

In this study, the association analysis with *LIG4* and *RAD52* polymorphisms and SLE clinical manifestations susceptibility did not provide statistical evidences for altered risk of any clinical feature from SLE patients, except a trend for cutaneous alterations. In the study conducted by Bassi et al. (2008), there was no evidence of association from the tested polymorphisms and SLE susceptibility, however they did find association to antiphospholipid syndrome –APS, a SLE clinical feature. Furthermore, Warchol at al., (2012), evaluated the role of *XRCC1* gene polymorphisms in SLE predisposition in a Polish population. The authors concluded that *XRCC1* Arg399Gln polymorphism was associated to SLE susceptibility and may increase cutaneous alterations (22).

Regarding clinical manifestation analysis, ethnicity and gender, the European-derived patients presented increased risk to cutaneous and hematological alterations than African-derived. Melanin acts as a natural optical strainer protecting the skin cells against UV radiation, and once European-derived presents less melanin than African –derived patients our association is justified (23). It is important to mention that the ethnic classification used in the present study was based on phenotypic characteristics of individuals and ethnicity data of parents/grandparents, often adopted in Brazil, even though, European-derived or African-derived groups can present a certain degree of admixture. A study by Santos et al (24) , assessing the individual interethnic admixture in Brazilian populations used a 48-insertion-deletion Ancestry-Informative Marker panel and a very high level of European contribution (94%) and fewer Native American

(5%) and African (1%) genes was identified in a sample of 81 European-derived individuals from southern Brazil (24). Hence, our classification in SLE patients and controls is in agreement to the ethnic/genetic background reported in South/Southeast Brazilian population. Our results are also in accordance to Sestak et al. (2008), which assessed different ethnical group of SLE patients (European, African and Hispanic) and found that African-derived patients had lower frequencies of cutaneous and immunological alterations and nephritic disorders, when compared to European and Hispanic-derived patients (25). Another study in a Northeast Brazilian population had similar results to ours, with cutaneous alterations as the most frequent clinical manifestation in SLE patients, specially photosensitivity (26). Since Brazil is a tropical country sun light incidence is relatively homogeneous along the year and SLE patients are constantly exposed to UV radiation and more susceptible to cutaneous alteration. So, our results are in accordance to others in Brazilian population (26,27).

CONCLUSION

Our study provided evidences for the lack of association of *LIG4* and *RAD52* DNA repair genes polymorphisms at genomic level to SLE susceptibility and its clinical features. Our hypothesis relies on their altered roles at a transcriptional level, not detected in this genetic association study. In this work, Caucasian-derived SLE patients are more susceptible to cutaneous and hematological alterations than African-derived patients.

ACKNOWLEDGMENT

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CONFLICT OF INTEREST

None of the authors has any potential financial conflict of interest related to this manuscript.

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Table 1. Demographical and Clinical profiles in 158 Brazilian SLE patients.

Table 2. Genotypic and allelic distribution of *LIG4* gene polymorphisms in SLE patients and HC.

Table 3. Genotypic and allelic distribution of RAD52 gene polymorphisms in SLE patients and HC **.**

Table 4. Clinical manifestations in SLE patients and stratification by ethnicity and gender.

Figure 1A. *LIG4* gene structure and Tag SNP distribution. **B.** Tag SNP position and linkage disequilibrium.

Figure 2A. *RAD52* gene structure and Tag SNP distribution. **B.** Tag SNP position and linkage disequilibrium

Table 1. Clinical manifestations in SLE patients and stratification by ethnicity and gender.

Clinical Manifestations	Ethnic group		Sex	
	European n= 120	African n=38	Female n= 147	Male n= 11
Cutaneous alterations	74 [1]	14	82	6
Photosensitivity	39	6	44	1
Arthritis	54	14	63	5
Serositis	33	7	37	3
Nephritic disorder	64	23	80	7
Neuropsychiatric disorder	26	4	28	2
Hematological alterations	71 [2]	13	78	6
Immunological alterations	68	19	83	4
Antinuclear Factor positive (FAN)	101	28	121	8
Antibody anti DNA (anti ds-DNA)	28	6	34	0
Antiohospholipid syndrome (APS)	40	12	47	5
Raynaud phenomenon	7	1	7	1

1. OR= 2.7394, CI= 1.21-6.35, *p*-value= 0.008, 2. OR =2.768 (1.22 - 6.511), *p*-value= 0.0089.

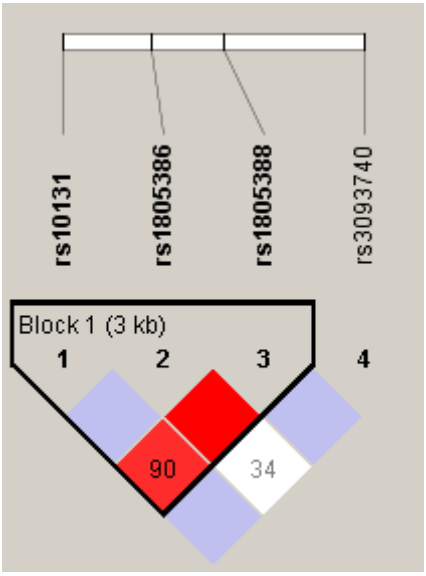
Table 2. Genotypic and allelic distribution of *LIG4* gene polymorphisms in SLE patients and HC.

Polymorphism	Patients n (%)	Controls n (%)	Odds Ratio (95% CI)	p-value
rs10131				
Allele	**272**	**392**		
C	248 (91.2%)	343 (87.5%)	1.00	
T	24 (8.8%)	49 (12.5%)	0.68 (0.39 - 1.16)	0.16
Genotype	**136**	**196**		
CC	115 (84.6%)	152 (77.6%)	1.00	
CT	18 (13.2%)	39 (19.9%)	1.63 (0.86 - 3.20)	0.13
TT	3 (2.2%)	5 (2.6%)	1.97 (0.22 - 24.04)	0.65
rs1805386				
Allele	**316**	**414**		
A	277 (87.7%)	350 (84.5%)	1.00	
G	39 (12.3%)	64 (15.5%)	0.77 (0.49 - 1.20)	0.24
Genotype	**158**	**207**		
AA	119 (75.3%)	144 (69.6%)	1.00	
AG	39 (24.7%)	62 (29.9%)	0.76 (0.46 - 1.25)	0.29
GG	0 (0%)	1 (0.5%)	0.00 (0.00 - 47.49)	1.00
rs1805388				
Allele	308	412		
G	256 (83.1%)	347 (84.2%)	1.00	
A	52 (16.9%)	65 (15.8%)	1.08 (0.71 - 1.64)	0.76
Genotype	**154**	**206**		
GG	105 (68.2%)	143 (69.4%)	1.00	
AG	46 (29.9%)	61 (29.9%)	1.02 (0.63 - 1.66)	0.90
AA	3 (1.9%)	2 (1.0%)	2.3 (0.22 - 24.79)	0.65
rs3093740				
Allele	**314**	**422**		
T	304 (96.8%)	411 (97.4%)	1.00	
G	10 (3.2%)	11 (2.6%)	1.22 (0.46 - 3.23)	0.66
Genotype	**157**	**211**		
TT	147 (93.6%)	200 (94.8%)	1.00	
GT	10 (6.4%)	11 (5.2%)	1.23 (0.45 - 3.30)	0.65

Table 3. Genotypic and allelic distribution of *RAD52* gene polymorphisms in SLE patients and HC.

Polymorphism	Patients N (%)	Controls N (%)	Odds Ratio (95% CI)	P-Value
rs1051669				
Allele	**316**	**420**		
C	234 (74.1%)	310 (73.8%)	1.00	
T	82 (25.9%)	110 (26.2%)	0.99 (0.69 - 1.39)	1.00
Genotype	**158**	**210**		
CC	86 (54.4%)	117 (55.7%)	1.00	
CT	62 (39.2%)	76 (36.2%)	1.11(0.70 - 1.75)	0.66
TT	10 (6.3%)	17 (8.1%)	0.80 (0.31 - 1.96)	0.68
rs11064607				
Allele	**316**	**410**		
A	170 (53.8%)	222 (54.1%)	1.00	
G	146 (46.2%)	188 (45.9%)	1.01 (0.74 - 1.37)	0.94
Genotype	**158**	**205**		
AA	42 (26.6%)	56 (27.3%)	1.00	
AG	86 (54.4%)	110 (53.7%)	1.04 (0.62 - 1.75)	0.90
GG	30 (19%)	39 (19%)	1.02 (0.52 - 1.99)	1.00
rs3748522				
Allele	**306**	**420**		
A	152 (49.7%)	212 (50.5%)	1.00	
G	154 (50.3%)	208 (49.5%)	1.03 (0.76 - 1.40)	0.88
Genotype	**153**	**210**		
AA	42 (27.5%)	55 (26.2%)	1.00	
AG	68 (44.4%)	102 (48.6%)	0.87 (0.51 - 1.49)	0.60
GG	43 (28.1%)	53 (25.2%)	1.06 (0.57 - 1.95)	0.88

A



B

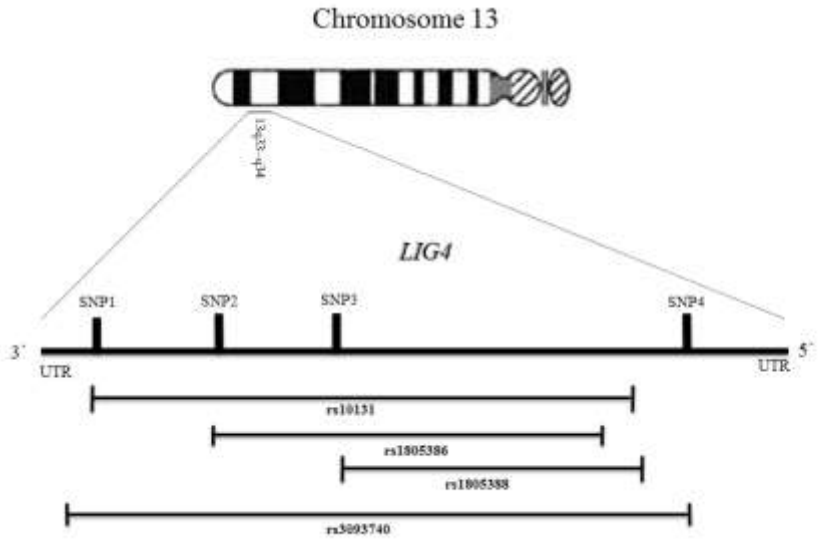


Figure 1. A Tag SNP position and linkage disequilibrium. **B** *LIG4* gene structure and Tag SNP distribution.

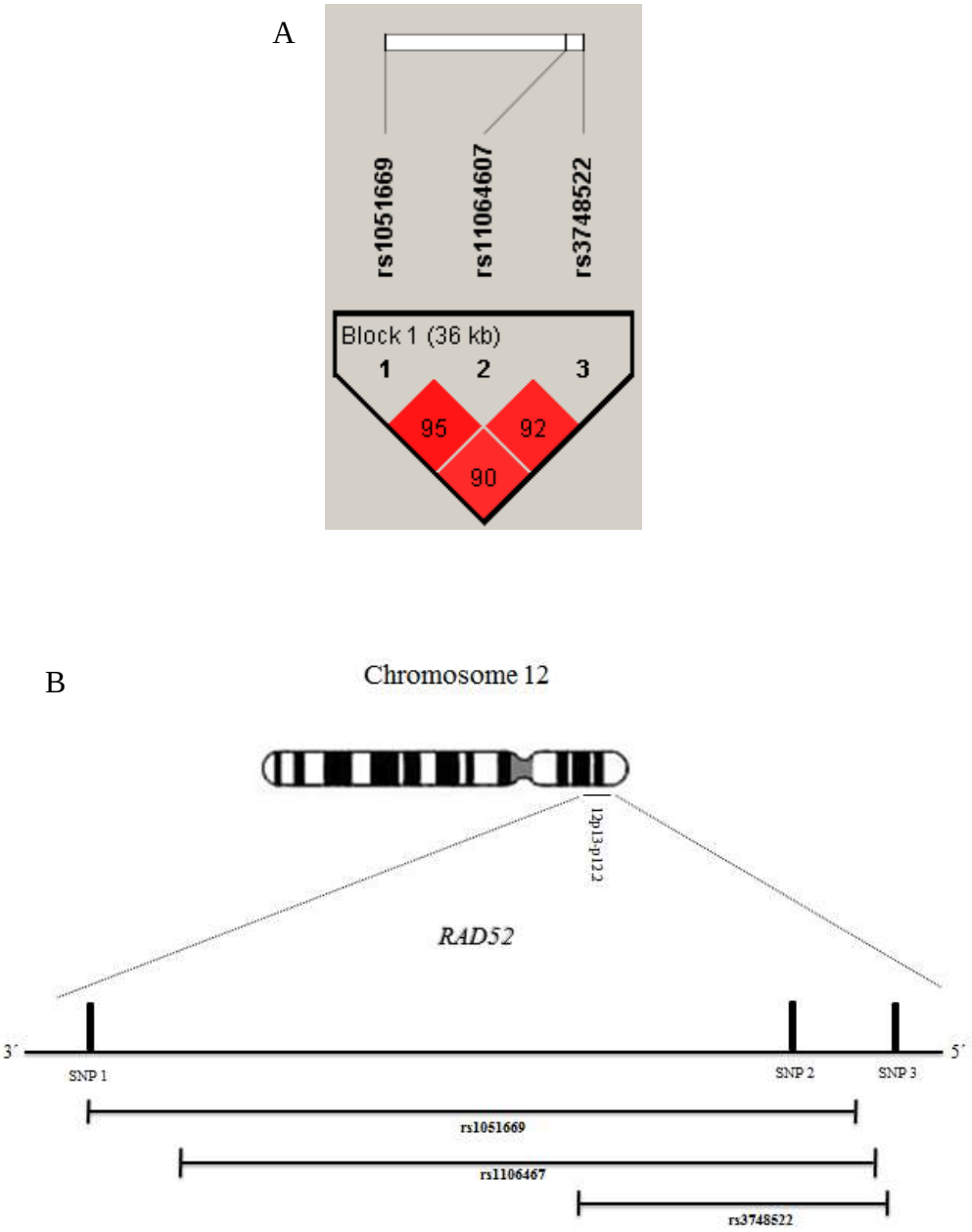


Figure 2. A Tag SNP position and linkage disequilibrium. **B** *RAD52* gene structure and Tag SNP distribution.

5. Capítulo II: Vitamin D receptor (VDR) polymorphisms and susceptibility to systemic lupus erythematosus and its clinical manifestations

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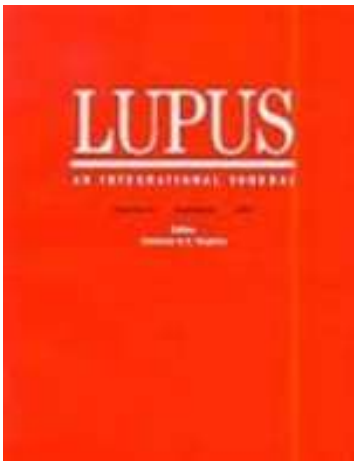

VITAMIN D RECEPTOR (VDR) POLYMORPHISMS AND SUSCEPTIBILITY SYSTEMIC LUPUS ERYTHEMATOSUS AND ITS CLINICAL MANIFESTATIONS

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Running Head: *VDR* polymorphisms in SLE.

Key-words: SNPs, VDR, SLE and SLE clinical manifestations.

ABSTRACT

Systemic Lupus Erythematosus (SLE) is an autoimmune disorder which has heterogeneous clinical manifestations and target tissue damage. SLE etiology and pathogenesis are complexes and encloses genetics, hormonal, immunological and environmental factors. Currently, several genes have been associated to SLE susceptibility, including Vitamin D Receptor (VDR), which is a mediator of immune responses through the action of vitamin D. Polymorphisms in *VDR* gene can impair vitamin D (D3) role and since SLE patients show deficient D3 blood levels, it leads to a possible connection to the disease´s onset. We performed an association study with *VDR* polymorphisms and SLE altered risk, as well as its clinical manifestations. This study included 158 SLE patients and 190 healthy controls from Southeast Brazilian who were genotyped for 5 Tag SNPs (Single Nucleotide Polymorphisms), which covered most of the VDR gene region. No association was reported between *VDR* polymorphisms and SLE susceptibility. However, we found association between *VDR* SNPs and SLE to the following clinical manifestations: Cutaneous alterations for G/G genotype (rs11168268) (OR=3.01, *p*=0.036) and C/T genotype (rs3890733) (OR=0.36, p= 0.003); Arthritis for T/T genotype (*rs3890733*) (OR=17.05, *p*= 0.001); Immunological alterations for G/G genotype (rs2248098) (OR= 2.82, *p*=0.040) and Antibody anti ds-DNA for C/T genotype (rs4760648) (OR=0.37, *p*=0.036). Our analysis also reported a trend of association to nephritic disorders (OR=0.37, *p*=0.041) and photosensitivity (OR= 0.33, *p*=0.046). In summary, our results show no association to *VDR* polymorphisms and SLE susceptibility; however in the present study we report association to SLE clinical manifestations in Brazilian population.

KEYWORDS: SNPs, VDR, SLE and SLE clinical manifestations.

INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a complex autoimmune disorder displaying heterogeneous clinical manifestations. The heterogeneity of SLE clinical characteristics is a confounding factor for diagnosis, therefore 4 of the 11 criteria defined by the American College of Rheumatology are simultaneously required for the formal diagnosis (1). SLE phenotype can range from different organ manifestations to autoantibody production (2). The most developed phenotypes are related to cutaneous alterations, affecting up to 85% of SLE patients (3). Lupus nephritis is a common clinical feature in SLE patients and the frequency of renal involvement is particularly high in juvenile-onset SLE, ranging from 50% to 80% in most cohorts described to date and biopsy from SLE patient´s kidney demonstrated some degree of renal involvement in almost all patients (4,5). Arthritis and arthralgia are another common manifestation in many systemic autoimmune diseases and affect 40-60% of SLE patients, usually nondeforming (4,6).

Vitamin D is a hormone produced in the skin and metabolized within the kidneys to 1α,25-dihydroxyvitamin D3 (calcitriol), the active form of vitamin D (7). Vitamin D classic function is to regulate calcium homeostasis and thus bone formation. However, D3 displays immunomodulatory effect, acting through the vitamin D receptor (VDR) (8). Since *VDR* was identified over three decades ago, more than 50 targets have been identified, providing a broader role for vitamin D function (9). The general immunologic effect of D3 includes: down regulation of Th1 immune responses, modulation of dendritic cells differentiation, depressing activated B cells proliferation,

up regulation of regulatory T cells and particularly, preserving immune response (10). Deficient levels of D3 are associated to SLE in several populations, as well as to its morbity and mortality in multiple chronic diseases (10,11).

VDR gene is highly polymorphic, with more than sixty described polymorphisms, including eight protein-encoding exons (exons 2-9) and six untraslated exons (exons 1a-1f), which are under alternative splicing, eight introns and two promoter regions (12,13). Polymorphisms in *VDR* sequence may change the gene function and so D3 action. Several single nucleotide polymorphisms (SNPs) in *VDR* gene have been described so far, but mainly four (namely *TaqI, BsmI, ApaI* and *FokI*), are intensively studied (13). Despite the fact that *BsmI, TaqI* and *ApaI* have no functional role, the reported association rely on linkage disequilibrium (14). Hence, the association studies with *VDR* polymorphisms and SLE susceptibility have been performed in different populations with controversial results (11,15,16).

We selected five Tag SNPs (rs11168268, rs2248098, rs1540339, rs4760648 and rs3890733), enclosing most of *VDR* gene sequence, and tagging some of the most studied polymorphisms (*TaqI, BsmI, ApaI)* for this association study. A Tag SNP is a representative polymorphism from a specific genome area with high linkage disequilibrium to other polymorphisms, so it is possible to perform an association study without the need of genotyping every SNP within a gene (17). In the present study we tested Tag SNPs in *VDR* gene and SLE susceptibility as well as its clinical manifestations in Southeast Brazilian population. To our knowledge, this is the first association study encompassing the whole *VDR* gene and SLE susceptibility and clinical features.

SUJECTS AND METHODS

Patients and Controls

In this study performed a case-control study with 158 SLE patients, 147 (93%) females and 11 (7%) males, mean age 37.8 years ±11.9 SD, recruited from the Division of Clinical Immunology, University Hospital of the School of Medicine of Ribeirão Preto, University of São Paulo (SP, Brazil). The diagnosis of SLE was performed according to the criteria defined by the American College of Rheumatology (1). SLE patients were stratified according clinical manifestations as showed in table 1. The patient group was classified as European 120 (76%) and African-derived 38 (24%), according phenotypic characteristics of individuals and ethnicity data from parents/grandparents reported by the participants.

The control group consisted of 190 healthy unrelated individuals 90 (47%) females and 100 (53%) males, mean age 36.47 years ± 10.96 years, from Ribeirão Preto, São Paulo, Brazil. European and African-derived individuals represented 72.17% (137) and 27.83% (53), respectively. As exclusion criteria for the control group, the presence of clinical manifestations and/or family history of autoimmunity were applied. Genotyping for HLA-A, B, C, DR and DQ were performed in order to exclude HLA risk haplotype in the control group. The present study was previous approved by the local ethics committee (CEP/HCRP/FMRP #2234/2007).

SNPs Selection and VDR genotyping

Genomic DNA was extracted from peripheral whole blood using a salting out procedure (18). Polymorphisms were selected using the SNPBrowser software 4.0 (Applied Biosystems, Foster City, CA) and HapMap database (http://hapmap.ncbi.nlm.nih.gov/). Tag SNPs (rs11168268, rs2248098, rs1540339,

rs4760648 and rs3890733) had least 10% Minimum Allele Frequency (MAF) (Figure 1a). Genotyping was performed with commercially available fluorogenic allele specific probes (Taqman Probes, Applied Biosystems, Foster City, CA) using the ABI7500 Real Time PCR platform (Applied Biosystems, Foster City, CA). Allelic discrimination followed as recommended by the manufacturer and analyzed using the SDS software 2.3 (Applied Biosystems, Foster City, CA).Statistical Analysis

Allelic and Genotypic frequencies and Hardy-Weinberg equilibrium were performed by using the SNPStats tool (http://bioinfo.iconcologia.net/SNPstats). The Exact Fisher Test was applied to determine the statistical significance of all comparisons. Haploview Software (version 4.2) was used for haplotype associations. "SNPassoc" R package (R software, version 2.12.2), developed for genetic studies, was used for evaluating the association between SNPs and SLE susceptibility (18). P-values < 0.05 were considered statistically significant.

RESULTS

The *VDR* allelic and genotypic frequencies were in Hardy–Weinberg equilibrium in SLE patients and healthy controls (HC). The frequencies distribution did not differ significantly between SLE patients and healthy controls (HC) as shown in Table 2. Haplotype analysis was performed to assess linkage disequilibrium among the tested Tag SNPs. No linkage disequilibrium for the *VDR* assessed polymorphisms were observed, except for Tag SNPs with rs1116828 and rs2248048 ($D' = 0.76$), suggesting combined genotype as shown in figure 1b. The haplotype combination did not provide any association neither to SLE or its clinical features susceptibility (data not shown) When assessing *VDR* polymorphisms and altered risk for SLE clinical manifestations and laboratorial profiles we report association to cutaneous and immunological

alterations, antibody anti-DNA, and arthritis. We also report a trend of association with *VDR* polymorphisms and nephritic disorders and photosensibility. The associations reported with *VDR* polymorphisms and clinical manifestations are depicted in table 3.

Regarding the SLE clinical manifestations stratification considering ethnicity and gender, we observed association in African-derived patients, indicating protection to cutaneous (OR = 0.36, CI = 0.16-0.82, p= 0.009) and hematological alterations (OR = 0.36, CI = 0.15-0.81, p= 0.009), when compared to European-derived patients, as demonstrated in table 4.

DISCUSSION

The first report suggesting that vitamin D deficiency was associated in SLE patients came over three decades ago and since then, several studies in different populations associated low vitamin D levels to the disease (19,20). Since VDR is the main regulator of vitamin D action, polymorphisms in the gene region may compromise its role (21). In the present work we performed an association study with 5 Tag SNPs, covering the whole the gene region, and SLE susceptibility, as well as its clinical features in Southeastern Brazilian population. We also considered for evaluation SLE clinical manifestations, gender and ethnicity.

Association studies with *VDR* polymorphisms and autoimmune disorders, including SLE susceptibility, have been performed in different populations with heterogeneous results. In the present work we did not provide evidence of association with *VDR* polymorphisms and SLE susceptibility in Southeast Brazilian population. Our results are in accordance to the ones from Thai, Iranian, Portuguese, Tunisian and Chilean populations where no association was reported (22–26). However, in some populations the association with *VDR* polymorphisms to several immune disorders, are reported, such as in Chinese, Japanese, Greek, German and Brazilian population

(15,27–30), evidencing the importance of replica studies in populations with diverse genetic background.

In the present study we reported association of *VDR* polymorphisms and the following clinical manifestations: cutaneous and immunological alterations (rs11168269, rs3890733 and rs2248098) antibody anti-DNA (rs4760648), and arthritis (rs3890733). We also found a trend of association with *VDR* polymorphisms and nephritic disorders (rs4760648) and photosensibility (rs4760648).

VDR is expressed in several types of cells and a recent review by Wang et al, 2012 (31), focused on tissue and the cell type-specific distribution of VDR throughout the body. VDR is selectively expressed in the skin epithelial cells and seems to be restricted to the nuclei (31) . A study performed by Hutchinson, et al. (32), assessed *VDR* variants and the risk to Malignant Melanoma (MM), a type of skin cancer. The study was based on the data showing that $1,25(OH)_2D3$ (the hormonal derivative of vitamin D3 and the ligand of the VDR) has antiproliferative and pro-differentiation effects in VDR-expressing cell types. Since MM cells express VDR they found association at *FokI* VDR polymorphic site with altered risk of MM (32). Skin alterations are involved in up to 85% of SLE cases and photosensitivity, one of the most common alterations, is a result of ultraviolet light exposure, causing a macular or erythematous rash (3). When the keratinocytes are exposed to ultraviolet light they become apoptotic due to DNA damage and release nuclear material, which is not efficiently cleared in SLE patients, causing immune response triggering (33). Moreover, vitamin D is able to reduce the UV-induced DNA damage and suppress cutaneous immunity, so vitamin D displays a key role in maintain cell integrity after UV light exposure (34).

Autoantibodies for double strand DNA (ds-DNA) is a widely known marker in SLE and the pathogenic potential is expressed as a correlation between the autoantibody titer and disease activity (remissions and exacerbations) (35). In the present study we report association of *VDR* polymorphisms at rs4760648 and anti-dsDNA. A recent study correlated vitamin D levels to SLE activity and anti-dsDNA titers (36). The authors reported that deficiency in D3 levels are related to increased SLE activity and anti-DNA titers, reinforcing the immune modulator role of D3 in chronic diseases (36). Besides vitamin D immunologic activity, it has a crucial role in calcium regulation and bone metabolism (37). Arthritis is a common feature in SLE patients and may occur in approximately 50% of patients, usually affecting the small joints of the hands, wrists, and knees, commonly asymmetric. Differently from rheumatoid arthritis (RA), arthritis in SLE does not induce severe bone damage and erosions. However, is often accompanied by pain and swelling (38). Most of the data with *VDR* polymorphisms regard rheumatoid arthritis and little attention has been devoted to SLE arthritis (37) . In the present study we report association to the *VDR* polymorphism at rs389073 and SLE arthritis increased risk (OR=17.05, p= 0.001). Nevertheless, the association to SLE arthritis should be interpreted with caution as the frequencies observed for this *VDR* polymorphism were not in Hardy-Weinberg equilibrium.

In the present study we also reported a association between *VDR* polymorphisms and nephritic disorders, one of the most frequent and damaging clinical feature in SLE (5). In 2002, Luo et al (15), investigated the association of the *VDR* polymorphism *BsmI* and SLE susceptibility and its clinical manifestations. The authors associated the *VDR BsmI* polymorphism B allele to the development of lupus nephritis and down-regulation of VDR mRNA expression in SLE patients (15). Li et al (2012) conducted a study assessing individual and clustered autoantibodies and their roles as disease´s

manifestations specific markers. The authors concluded that anti-dsDNA was associated with renal disorder and not to any other clinical manifestations. Noteworthy, the deposition of anti-dsDNA is the main pathogenic event in renal compromising in SLE(2). Regarding VDR haplotypes, we did not find association of genotype combinations neither to SLE nor to its clinical manifestations. Since the Tag SNPs assessed in this work did not show significant linkage disequilibrium among them no associations could be inferred regarding SLE and its clinical features. Even though, the formation of haplotypes blocks in *VDR* gene is widely studied with *BsmI, FokI* and *TaqI* most of the association studies are controversial and have not been proved yet (23,39–42).

In summary, despite the lack of association of *VDR* variants and SLE susceptibility in our study, we did find association to SLE clinical manifestations. This result may reinforce *VDR* as a potential marker in diseases activity; however, further studies with a large sample size and different ethnic background must be performed to support our data.

CONCLUSION

VDR gene polymorphisms are associated to particular SLE clinical manifestations in the Southeastern Brazilian population. Our results provide evidence for considering VDR as a potential marker in disease´s activity. However, further studies in populations with different ethnical background and larger number of subjects are necessary to reinforce our results.

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CONFLICT OF INTEREST

None of the authors has any potential financial conflict of interest related to this manuscript.

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Table 1. Demographical and Clinical/laboratory characteristics in 158 Brazilian SLE patients.

Demographic and clinical/laboratories characteristics	**SLE Patients**
	n (%)
Sex	
Male	11 (6.7%)
Female	147 (93.%)
Etnhic Group	
European derived	120 (75.9%)
African derived	38 (24.0%)
Clinical/Laboratories Characteristics	
Cutaneous alterations	88 (55.7%)
Photosensitivity	45 (28.5%)
Arthritis	68 (43%)
Serositis	40 (25.3%)
Nephritic disorder	87 (55.1%)
Neuropsychiatric disorder	30 (19%)
Hematological alterations	84 (53.2%)
Immunological alterations	87 (55.1%)
Antinuclear Factor positive (FAN)	129(81.64%)
Antibody anti DNA (anti ds-DNA)	34 (21.52%)
Antiohospholipid syndrome (APS)	52 (32.9%)
Raynaud phenomenon	8 (5.06%)

Table 02. Genotype and allele frequencies of *VDR* associated SNPs in SLE patients and healthy controls.

Polymorphism	**Patients n (%)**	**Controls n (%)**	Odds Ratio (95% CI)	***p-value***
rs11168268				
Allele	**158**	**187**		
A	193 (61.07%)	220 (58.51%)	1.00	
G	123 (38.93%)	154 (41.49%)	0.91 (0.66-1.25)	0.58
Genotype				
AA	62 (39.2%)	67 (35.8%)	1.00	
AG	69 (43.7%)	86 (46%)	0.87 (0.53-1.42)	0.63
GG	27 (17.1%)	34 (18.2%)	1.10 (0.47-1.58)	0.64
rs2248098				
Allele	**158**	**188**		
G	158 (50%)	199 (52.92%)	1.00	
A	158 (50%)	177 (47.08%)	1.12 (0.82-1.53)	0.45
Genotype				
GG	39 (24.7%)	55 (29.3%)	1.00	
AG	80 (50.6%)	89 (47.3%)	1.26 (0.74-2.18)	0.37
AA	39 (24.7%)	44 (23.4%)	1.25 (0.66-2.36)	0.54
rs1540339				
Allele	**158**	**188**		
C	221 (69.94%)	242 (64.36%)	1.00	
T	95 (30.06%)	134 (35.64%)	0.78 (0.56-1.08)	0.12
Genotype				
CC	81 (51.3%)	78 (41.5%)	1.00	
CT	59 (37.3%)	86 (45.7%)	0.66 (0.42-1.04)	0.08
TT	18 (11.4%)	24 (12.8%)	0.72 (0.36-1.43)	0.38
rs4760648				
Allele	**158**	**188**		
C	159 (50.32%)	189 (50.26%)	1.00	
T	157 (49.68%)	187(49.74%)	1.00 (0.73- 1.36)	1.00
Genotype				
CC	40 (25.3%)	51 (27.1%)	1	
CT	79 (50%)	87 (46.3%)	1.16 (0.69-1.94)	0.60
TT	39 (24.7%)	50 (26.6%)	0.99 (0.55-1.79)	1.00
rs3890733				
Allele	**158**	**189**		
C	219 (69.3%)	261 (69.41%)	1.00	
T	97 (30.7%)	117 (30.59%)	0.99 (0.70-1.38)	1.00
Genotype				
CC	71 (44.9%)	93 (49.2%)	1	
CT	77 (48.7%)	75 (39.7%)	1.34 (0.86-2,09)	0.21
TT	10 (6.3%)	21 (11.1%)	0.62 (0.28-1.44)	0.32

OR = odds ratio , CI = 95% confidence interval;

Table 3. SLE patients' clinical manifestation (%) accordingly to SNPs VDR gene associated genotype.

Clinical Manifestations	**SNPs**					
	rs11168268	rs2248097	rs4760647		rs3890733	
	G/G	G/G	C/T	T/T	C/T	T/T
	n=27 (%)	n=39(%)	n=79(%)	n=39(%)	n=77(%)	n=10(%)
Photosensitivity	11 (40.7)	14 (35.9)	22 (27.8)	**7 (18)[d]**	18 (23.4)	4 (40)
Serositis	7 (25.9)	12 (30.7)	19 (24)	10 (25.6)	17 (22.1)	1 (10)
Arthritis	11 (40.7)	17 (43.6)	24 (30.4)	14 (35.9)	35 (45.5)	**9 (90)[g]**
Cutaneous alterations	**20 (74.1)[a]**	21 (53.8)	45 (57)	22 (56.4)	**34 (44.2)[f]**	5 (50)
Neuropsychiatric disorder	6 (22.3)	8 (20.5)	16 (20.2)	7 (18)	12 (15.6)	3 (30)
Nephritic disorder	11 (40.7)	19 (48.7)	41 (51.9)	**18 (46.2)[e]**	43 (55.8)	6 (60)
Hematological alterations	18 (66.7)	17 (43.6)	42 (53.2)	18 (46.2)	42 (54.6)	5 (50)
Immunological alterations	11 (40.7)	**8 (20.5)[b]**	22 (27.8)	7 (18)	18 (23.4)	4 (40)
Antinuclear Factor positive (FAN)	21 (77.8)	33 (84.6)	65 (82.3)	19 (48.8)	59 (76.7)	9 (90)
Antibody anti DNA (anti ds-DNA)	7 (25.9)	5 (12.8)	**13 (16.5)[c]**	7 (18)	14 (18.2)	3 (30)
Antiphospholipid syndrome (APS)	5 (18.5)	11 (28.2)	19 (24)	9 (23.1)	15 (19.5)	2 (20)
Raynaud phenomenon	1 (3.7)	2 (5.1)	3 (3.8)	3 (7.7)	4 (5.2)	0 (0)

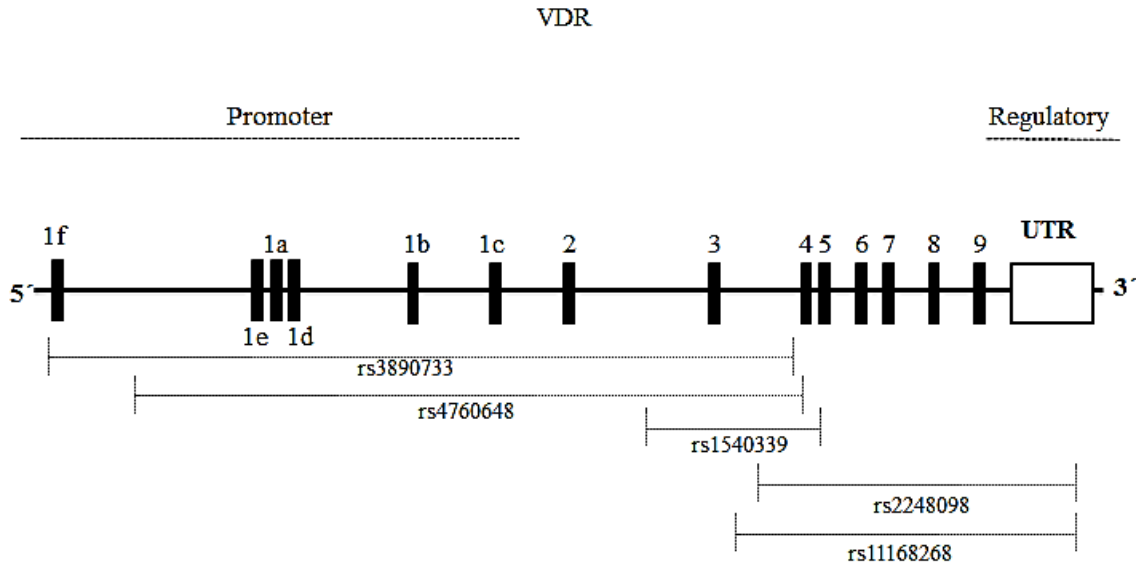
a. OR= 3.01, CI =1.04 – 9.70, p = 0.036; **b.** OR= 2.82, CI= 1.04-7.94, p = 0.040; **c .** OR=0.37, CI= 0.14-0.97, p = 0.036; **d.** OR = 0.33, CI 0.10 – 1.01, p = 0.046; **e.** OR= 0.37,CI= 0.13-1.02, p=0.041**; f.** OR = 0.36, CI= 0.17 – 0.74, p = 0.003; **g.** OR=17.05, p = 0.001, CI= 2.15-785.1.

Table 04. SLE patients' clinical manifestation (%) stratified accordingly to patients' ethnicity and gender.

Clinical Manifestations	**Ethnic group**		**Sex**	
	European n=120(%)	**African n=38(%)**	**Female n=147(%)**	**Male n=11(%)**
Photosensitivity	39(32.5)	6(15.8)	44(30)	1(9,1)
Serositis	33(27.5)	7(18.4)	37(25.2)	3(27.3)
Arthritis	54(45)	14(36.8)	63(42.9)	5(45.5)
Cutaneous alterations	74(61.7)	**14[1](36.8)**	82(55.8)	6(54.5)
Neuropsychiatric disorder	26(21.7)	4(10.5)	28(19)	2(18.2)
Nephritic disorder	64(53.4)	23(60.5)	80(54.4)	7(63.6)
Hematological alterations	71(59.2)	**13[2](34.2)**	78(53)	6(54.5)
Immunological alterations	68(56.7)	19(50)	83(56.5)	4(36.4)
Antinuclear Factor positive (FAN)	101(84.2)	28(73.7)	121(82.3)	8(72.7)
Antibody anti DNA (anti ds-DNA)	28(23.4)	6(15.8)	34(23.1)	0(0)
Antiphospholipid syndrome (APS)	23(19.2)	9(23.7)	31(21.1)	1(9.1)
Raynaud phenomenon	7(5.8)	1(2.6)	7(4.8)	1(9.1)

1. OR=0.36, CI= 0.16-0.82, *p*= 0.009; **2**. OR=0.36, CI =0.15-0.81, *p*= 0.009.

A



B

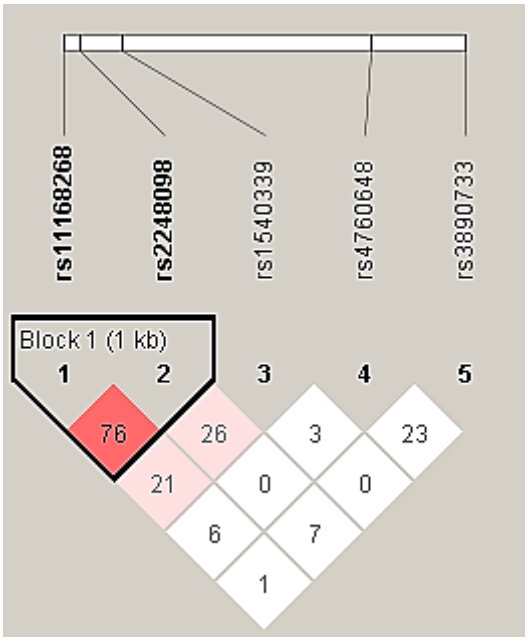


Figure 01: A *VDR* gene structure, distribution and coverage area of the tested Tag SNPs. **B.** Haplotype graphical representation showing the linkage disequilibrium.

6. Capítulo III: Interferon-Induced with helicase C domain (*IFIH1*) gene polymorphisms: association to systemic lupus erythematosus in Brazilian population

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INTERFERON-INDUCED WITH HELICASE C DOMAIN 1 (*IFH1*) GENE POLYMORPHISM: ASSOCIATION TO SYSTEMIC LUPUS ERYTHEMATOSUS IN BRAZILIAN POPULATION

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Running Head: *IFH1* and polymorphisms in SLE.

ABSTRACT

The formation of autoantibodies and the deposition of antibody-containing immune complexes in blood vessels throughout the body is the main pathogenic mechanism of the autoimmunity in Systemic Lupus Erythematosus (SLE). Type I Interferon (IFN) mediates innate immune response and increased levels IFN contributes to breakdown the peripheral tolerance. It is known that SLE patients present an "IFN-signature" due to overexpression of *IFN* responsive genes in peripheral blood. The Interferon-induced with helicase C domain 1 (*IFIH1*) gene, activate and modulate IFN response, pro-inflammatory cytokines and apoptotic processes through its CARD domain. Recent finding demonstrated an overexpression of IFIH1 protein in chronic discoid lupus erythematous, suggesting a possible role of *IFIH1* in SLE onset. The aim of this study was to analyze two single nucleotide polymorphisms (SNPs) in *IFIH1* gene able to modulate the protein response in SLE pathogenesis. We assessed the SNPs with rs6432714 and rs10930046, in 153 SLE Southeastern Brazilian patients and 188 healthy individuals as controls. The rs10930046 SNP indicated association to SLE protection (*p*-value= 0.032), but lacks any association regarding clinical manifestations. Our report describes the first association analysis of *IFIH1* polymorphisms and SLE in Brazilian population.

KEY-WORDS: SNPs; IFH1; SLE.

INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a chronic multifactorial autoimmune disorder characterized by the formation of pathogenic autoantibodies against nuclear antigens due to self-tolerance breakdown. SLE displays the most extensive and heterogeneous clinical manifestations varying according to ethnicity, geography and mainly, gender, with a strong sex bias[1]. Since SLE etiology remains unclear, genetic predisposition, environment and hormonal factors seem to play key roles in its pathogenesis. Until now, several genes have been associated to SLE, but most of them seems to be involved or related to Type 1 Interferon (IFN), leading to increased serum levels of IFN-inducible genes, known as "IFN-signature" [2,3].

Interferon-induced Helicase C domain 1 *(IFIH1)* gene, located at chromosome 2 (2q24), encodes a homonymous protein capable to recognize viral dsRNA on the cytoplasm of infected cells, thus, modulating type 1 IFN response, production of pro-inflammatory cytokines and apoptotic processes [6]. *IFIH1* has already been associated to type 1 diabetes (T1D) and Graves´ disease, both autoimmune disorders, and recently its role in SLE has been investigated [7]. The increased levels of IFIH1 in Lupus-prone mice may accelerate autoimmune processes and aggravates the disease by enhancing the levels of antinuclear antibodies [4]. Moreover, high levels of IFIH1 in tissue-specific regions of Chronic Discoid Lupus Erythematosus patients [5] suggest a key role of this protein on predisposition of SLE.

Since the association of *IFH1* and SLE is not well established, in this work, we propose to assess the role of two *IFIH1* polymorphisms with SLE pathogenesis, as well as with its clinical manifestations in Brazilian patients.

PATIENTS AND METHODS

We assessed 153 SLE patients (92.8% females and 7.2% males; mean age 37 years; ± 11.9 SD) from the Division of Clinical Immunology, University Hospital of the School of Medicine of Ribeirão Preto, University of São Paulo (SP, Brazil). The control group was set up with healthy Brazilian subjects (n = 188; 51% females and 49% males; mean age 36.9 years; ± 11.1SD). Detailed description of patients and controls enrolled in this study is described in a prior work [8]. This study was previously approved by the local ethics committee (CEP/HCRP/FMRP #2234/2007). The Clinical manifestations assessed in this study are described in Table 1.

We selected two SNPs of *IFH1*: rs6432714 and rs10930046. The Tag SNP rs6432714 is localized in intron 7, covering SNPs from intron 6 to intron 7 and the non-synonymous SNP rs10930046 located at exon 7. Genotyping procedures are detailed in the previous reference [5].

Hardy-Weinberg Equilibrium (HWE), association tests of the SNPs between genotypes were performed using likelihood ratio test. Association tests between alleles were used chi-squared test. The estimative of the best genetic model was made by Akaike's information criterion (AIC). All analysis was performed by SNPassoc package[9] from R software v.2.15.1. Linkage disequilibrium was calculated using Haploview software v. 4.2.[10]. Post-hoc statistical power tests were measured by using G*Power v.3.1.3 (http://www.psycho.uni-duesseldorf.de) for an α-probability of 0.025. P values lower than 0.025 were considered statistically significant after Bonferroni's correction.

RESULTS

The genotype and allele frequencies of the polymorphisms - rs6432714 and rs10930046 and the results of association tests are reported in Table 2. All groups were in Hardy-Weinberg equilibrium (HWE) except for the SNP rs10930046 in patient's group (p = 0.045). A significant difference between the C/C genotype frequencies from patient (0%) and control group (3.2%) was observed in the rs10930046 SNP analysis (p = 0.032) using the co-dominant model, adjusted by ethnicity and gender. However, this result could not be confirmed after Bonferroni's adjustment. No allele association was found in the both tested polymorphisms ($p > 0.05$).

Association tests for rs10930046 SNP were also applied to other genetic models and the recessive model was the most appropriated (AIC = 385.7) when compared to co-dominant, dominant, overdominant and log-additive models. The p-value for association using the recessive model (T/T + T/C versus C/C) was 0.011 indicating an association between the C/C genotype of the rs10930046 SNP and SLE.

No association was found between rs6432714 SNP and SLE and its clinical manifestations. No association with clinical manifestation was found when the SNP rs10930046 was analyzed.

DISCUSSION

IFN-1 plays an important role on SLE by enhancing autoimmune processes and disease´s complications [11]. The studies of IFN-induced genes are important in order to comprehend the regulatory mechanisms involved in type 1 IFN activity and the development of SLE. Here, we evaluated the association of *IFH1* gene with SLE predisposition and its clinical manifestations in a Brazilian population.

The first association study with *IFIH1* polymorphism was made by Gateva et al. (2009) which identified altered risk of A946T polymorphism (rs1990760) to SLE susceptibility in Sweden and US populations. Later, these results were replicated by Graham, *et al.* (2011) [7] confirming risk associated to SLE onset. The results provided by Graham et al. reinforced a previous genome-wide association study (GWAS), reporting an association trend between this *IFIH1* and SLE [12]. Finally, the *IFIH1* A946T polymorphism was associated to IFN-α increased levels from SLE patients with positivity for anti-dsDNA autoantibody [13].

The SNP rs10930046 consists in a non-synonymous substitution that changes a histidine for an arginine residue at codon 460 of the IFIH1 protein (H460R). This codon is in the helicase ATP binding-domain, a region important on recognizing ds-RNA and activates type I IFN expression. This same polymorphism was also associated with psoriasis but as a protective effect [14]. Despite our impairing in calculating the OR for the recessive genotypes between the groups (the percentage of C/C genotype was 0% in the patient group, our results pointed out to a protective role for C/C genotype in this SNP, and SLE onset. We found evidence of association between the SNP rs10930046 and the protection to SLE in the recessive genetic model for this mutation.

In this study the rs10930046 SNP was not consistent with HWE in the patient group. Significant deviations from HWE in the case group may be caused by association with the disease allele [15] . Therefore, we decided to keep the validations of the rs10930046 SNP tests for the sake of the argument.

The SNP rs6432714 was not associated with SLE in our population. However, this tagSNP presents a strong linkage disequilibrium with rs10930046 ($r^2 = 0.96$). The lack of association found in this analysis with the SNP rs6432714, might be addressed to a weaker statistical power (0.62) when comparing to SNP rs10930046 (0.84). We did not find association from both *IFIH1* polymorphisms tested to any clinical manifestations evaluated.

CONCLUSIONS

We reported association between the rs10930046 (H460R) polymorphism of *IFIH1* gene and SLE protection in the Brazilian population. The C/C genotype has a protective effect to SLE development. Moreover, no association between this SNP and clinical manifestations of the disease was found. Further analysis is required to understand the role of IFIH1 on modulation of type 1 interferon and apoptosis process in SLE patients.

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Table 1. Clinical manifestations in SLE patients subdivided by ethnicity and gender.

Clinical Manifestations	Ethnic group		Gender	
	European (117)	African (36)	Female (142)	Male (11)
Cutaneous alterations	71	14	79	6
Photosensitivity	38	5	42	1
Arthritis	53	12	60	5
Serositis	32	6	35	3
Nephritic disorder	63	23	79	7
Neuropsychiatric disorder	25	4	27	2
Hematological alterations	69	13	76	6
Immunological alterations	68	19	83	4
Antinuclear Factor positive (FAN)	99	26	117	8
Antibody anti DNA (anti ds-DNA)	28	5	33	0
Antiohospholipid syndrome (APS)	23	9	31	1
Raynaud phenomenon	7	1	7	1

Table 2. Genotypes and alleles frequencies of the single nucleotide polymorphisms (SNP) rs6432714 and rs10930046 of IFIH1 gene in healthy controls (HC) and Systemic Lupus Erithematosus patients (SLE). The odds ratio (OR), confidence intervals (CI) and P values from association tests are also shown.

SNP	Genotypes	SLE		HC		OR	CI (95%)	*P*
		N	%	N	%			
rs6432714	AA	110	71.9	135	71.8	1		0.229*
	AT	42	27.5	47	25	1.11	0.64 - 1.93	
	TT	1	0.7	6	3.2	0.19	0.02 - 1.76	
	A	262	85.62	317	84.31	1		
	T	44	14.38	59	15.69	0.9	0.59 - 1.38	0.634
rs10930046	TT	110	71.9	136	72.3	1		0.032*
	TC	43	28.1	46	24.5	1.18	0.68 - 2.04	
	CC	0	0	6	3.2	0	-	
	T	200	82.3	254	81.41	1		0.786
	C	43	17.7	58	18.59	0.94	0.61 - 1.46	

* *P* values adjusted by ethnicity and gender.

7. Discussão Geral

7.1 Polimorfismos nos genes *LIG4* e *RAD52* e a susceptibilidade ao LES e suas manifestações na população do Sudeste Brasileiro

Vários estímulos ambientais têm sido associados com a susceptibilidade ao LES, especialmente a exposição à luz ultravioleta, que é capaz de produzir DSBs e danos oxidativos do DNA (Bassi et al., 2008). No capítulo I foram avaliados quatro Tag SNPs no gene LIG4 e três no RAD52 para correlacionar com a susceptibilidade ao LES e/ou as suas manifestações clínicas. Além disso, foram considerados na análise das manifestações clínicas o sexo e a etnia dos pacientes com lúpus. O trabalho não encontrou associação genética com os genes LIG4 e RAD52 e susceptibilidade LES, bem como suas manifestações clínicas. Esses resultados fornecem evidências de que os polimorfismos na sequência desses genes não estão envolvidos com a susceptibilidade ao LES.

É importante notar que ambos os genes mostraram expressão diferencial nas fases ativa e inativa do LES quando comparados com indivíduos saudáveis da mesma área geográfica - Sudeste do Brasil, em que realizamos o nosso estudo de associação. Na análise de microarranjo de cDNA conduzida por Sandrin-Garcia et al. (2009), o gene LIG4 apresentou expressão reprimida de 21 vezes e o RAD52 uma indução 169 vezes, ambos na fase ativa da doença. Além disso, os dados apresentados por Davies et al. (2012) mostraram uma resposta deficiente ao reparo de DSBs em células de pacientes com LES juvenil, mesmo na presença de todas as proteínas envolvidas na NHEJ.

Em relação à análise de manifestações clínicas, etnia e gênero, os pacientes com ascendência Europeia apresentaram risco aumentado para alterações cutâneas e hematológicas em relação aos pacientes com ascendência Africana. A melanina

funciona como um filtro óptico natural protegendo as células específicas da epiderme contra os efeitos da radiação UV, e uma vez que os indivíduos com ascendência europeia apresentam menos melanina que os indivíduos com ascendência africana, pode-se justificar assim a associação encontrada (Böhm et al, 2005).

Neste capítulo, foram apresentados dados para o primeiro estudo de associação envolvendo os gene de reparo LIG4 e RAD52 com a susceptibilidade ao LES e suas manifestações clínicas.

7.2 Associação do gene *VDR* com as manifestações clínicas do LES na população Brasileira

No capítulo II foram avaliados cinco Tag SNPs cobrindo toda a sequência do gene VDR e a susceptibilidade ao LES e às suas manifestações clínicas na população do Sudeste brasileiro. Também foram considerados na análise o gênero e a etnia dos pacientes com lúpus. Não foi encontrada nenhuma associação do gene com a susceptibilidade ao LES, no entanto foram observadas associações com as seguintes manifestações clínicas da doença: alterações cutâneas, doenças imunológicas, anti-dsDNA e artrite. Além disso, foi observada uma tendência de associação à nefrite lúpica e fotossensibilidade.

O VDR é expresso em vários tipos de células, incluindo as do sistema imune, e a revisão de Wang et al (2012), descreve a distribuição deste receptor nas células do corpo. O VDR é expresso seletivamente em células epiteliais e sua localização parece ser restrita ao núcleo. Alterações da pele estão envolvidas em até 85% dos casos de LES e a fotossensibilidade, uma das alterações mais comuns, é devida a exposição à luz ultravioleta, causando eritemas na pele (Uva et al., 2010). Quando os queratinócitos, que possuem o VDR, quando expostos à luz ultravioleta podem se

induzir a formação de corpos apoptóticos que se não são eliminados corretamente nos pacientes com LES, contribuindo para a patogênese da doença (Lương& Nguyễn, 2012).

Outra associação importante encontrada no nosso trabalho foi com a artrite lúpica. A vitamina D atua de forma chave na homeostase do metabolismo do cálcio e também contribui na regulação do sistema imune (Snigh & Kamen, 2012). Em linhagens de murinos com artrite induzida, os agonistas do VDR foram associados com a prevenção da expressão e progressão da doença (Maruotti & Cantatore, 2010). Assim, as associações encontradas com as manifestações clínicas específicas no nosso estudo puderam ser corroboradas pela literatura.

No Brasil poucos estudos relacionaram os polimorfismos do *VDR* a doenças autoimunes e neste trabalho foram apresentados dados importantes de associação à doença e às suas manifestações clínicas. É importante notar que esse estudo associa os SNPs do *VDR* às manifestações clínicas relevantes do LES, sugerindo um possível papel marcador genético da atividade da doença.

7.3 Associação do gene IFIH1 com a susceptibilidade ao LES na população Brasileira

No Capítulo III, dois polimorfismos na sequência do gene *IFIH1* foram analisados com a susceptibilidade ao LES e as suas manifestações clínicas. Interferon tipo I (IFN) media a resposta imune inata e o aumento dos níveis de IFN contribuem para o colapso da tolerância periférica (Robinson eu al., 2007). Sabe-se que os pacientes com LES apresentam o que chama uma "assinatura IFN", que se caracteriza pela alteração não somente dos níveis séricos de IFN mas pela alteração da maioria dos genes envolvidos nesta via (Beachers et al., 2002). O gene *IFIH1* é capaz de ativar e modular a resposta ao IFN, a produção de citocinas pró-

inflamatórias e processos apoptóticos através do seu domínio CARD (Chistiakov, 2010). Dados recentes indicaram uma superexpressão da proteína IFIH1 no lúpus eritematoso discóide, sugerindo um possível papel deste gene na patogênese do LES. Neste trabalho foi observada associação de polimorfismo no gene *IFIH1* com o LES, entretanto não foi encontrada associação com as manifestações clínicas da doença, indicando que este gene pode estar envolvido na patogênese do LES na população Brasileira. Além disso, esse trabalho é o primeiro estudo de associação com o gene *IFIH1* e o LES no Brasil.

8. Conclusões Gerais

- Neste estudo não foi encontrada evidências estatísticas da associação dos polimorfismos nos genes *LIG4* e *RAD52* à susceptibilidade ao LES na população do Sudeste Brasileiro;
- Os pacientes com LES com ascendência Europeia apresentaram risco aumentado para desenvolvimento de alterações cutâneas e hematológicas;
- Não houve associação entre os polimorfismos do gene VDR e a susceptibilidade ao LES na população do Sudeste Brasileiro;
- Foi observada associação dos polimorfismos do *VDR* com a susceptibilidade às seguintes manifestações clínicas: alterações cutâneas (rs11168269), alterações imunológicas (rs2248098) e com artrite lúpica (rs3890733) na população do Sudeste Brasileiro;
- Foi observada associação dos polimorfismos do *VDR* com a proteção às seguintes manifestações clínicas: alterações cutâneas (rs3890733), anticorpo anti-dsDNA (rs4760648), fotossensibilidade (rs4760648) e nefrite lúpica (rs4760648) na população do Sudeste Brasileiro;
- O polimorfismo do gene *IFIH1* (rs10930046) está associado ao LES na população do Sudeste Brasileiro;
- Não foi encontrada associação entre os polimorfismos do gene *IFIH1* e as manifestações clínicas do LES;
- Nossos resultados podem contribuir com o aumento do número de genes associados com a etiopatogênese do lúpus.

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10. Anexos

10.1 Anexo I

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Autoimmunity

VITAMIN D RECEPTOR (VDR) GENE POLYMORPHISMS AND AGE ONSET IN TYPE 1 DIABETES MELLITUS IN BRAZILIAN POPULATION

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VITAMIN D RECEPTOR (VDR) GENE POLYMORPHISMS AND AGE ONSET IN TYPE 1 DIABETES MELLITUS IN BRAZILIAN POPULATION

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ABSTRACT

Vitamin D Receptor is a mediator of immune responses through the action of vitamin D, which is capable of regulate the insulin secretion by the pancreas. Since polymorphisms in the vitamin D receptor (VDR) gene might modulate vitamin D function, and thus immunologic response, VDR is possibly able to influence the predisposition to type 1 diabetes mellitus (T1DM). The aim of this work was to perform an association study among VDR polymorphisms and T1DM susceptibility, as well as the correlation with the disease onset. Two hundred and four T1DM patients and 217 controls, from Northeast Brazil, were genotyped for five tagSNPs, covering the whole VDR gene. Our results indicated an association between rs1540339 and rs4760648 SNPs (p=0.02 and p=0.03, respectively) and T1DM. No association was found with T1DM onset and age at diagnose. To our knowledge, this is the first association study in T1DM where the whole VDR gene was analyzed, and our results indicate that VDR polymorphisms could be important for T1DM susceptibility, but do not seem to be associated to age at disease onset.

INTRODUCTION

Vitamin D is a secosteroid hormone produced in the skin and metabolized within the kidneys to 1α,25-dihydroxyvitamin D3 (calcitriol), the active form of vitamin D [1]. D3 has immunomodulatory effect and acts through the vitamin D receptor (VDR) which can be found in over thirty different tissues, including islet cells of the pancreas and several cells of the immune system [2]. In animal models, administration of vitamin D3 is able to postpone the onset or prevent diseases such as arthritis, encephalomyelitis and types 1 and 2 diabetes [3–7].

Type 1 diabetes (T1DM) is a multifactorial autoimmune disorder where the genetic background is a key component. T1DM is a T-cell mediated immune disease in which β cells in the pancreas are mainly incapable of functioning properly, impairing the insulin secretion and leading to abnormal increased glucose levels in the blood [8]. VDRs are found in the pancreas and animal models have confirmed that vitamin D deficiency is able to inhibit pancreatic insulin secretion [1]. Vitamin D levels are inversely proportional to sunlight exposure; interestingly, the incidence of T1DM has likewise been described as correlated to geographical latitude and sunshine regularity, suggesting a seasonal pattern of the disease onset [9]. In Brazil, a tropical country, wherein the weather seasons are not well defined and the sunlight incidence is virtually even spread throughout the year, the incidence of T1DM was estimated at about 8 per 100,000 [10,11].

Vitamin D has been used in mouse models of T1DM to successfully prevent autoimmune insulitis and reduce the incidence of the disease [12,13]. In humans, studies in different populations have demonstrated that vitamin D supplementation during pregnancy and in early childhood decrease the incidence of T1DM. [2,5,14,15];

VDR gene spans over 100kb and is located on chromosome 12q (12q13.1) [16]. VDR is highly polymorphic, with more than sixty described polymorphisms [17]. VDR gene includes eight protein-encoding exons (exons 2-9) and six untraslated exons (exons 1a-1f) -which are

under alternative splicing-, eight introns and two promoter regions [18,19]. Polymorphisms in VDR sequence may change the gene function or its expression levels leading to an inefficient vitamin D action [17]. Several single nucleotide polymorphisms (SNPs) in VDR gene have been described so far, but four in particular (namely *TaqI*, *BsmI*, *ApaI* and *FokI*), have been intensively studied [18]. Despite the fact that *BsmI, TaqI* and *ApaI* have no functional role [19], the reported association rely on linkage disequilibrium [19]. Therefore, it is hypothesized that other polymorphisms, with unknown function, might be the real causal variant while *TaqI, BsmI* and *ApaI* became the genetic markers. [17-19]

Nevertheless, studies of VDR polymorphisms and association with T1DM are noticeably conflicting. In some cases, a clear correlation was reported [20-22] while in certain populations no association was observed [18,23]. This contradiction may be due to ethnic differences and environmental factors involved in T1DM pathogenesis.

In the present study, we analyzed five VDR tagSNPs in T1DM and their possible association to T1DM Brazilian patients, as well as their role in the disease age onset.

SUBJECTS AND METHODS

Patients and Controls

We performed a case–control study in T1DM patients from Pernambuco state, Northeast Brazil. We enrolled 204 T1DM patients, with 108 (53%) females and 96 (47%) males, children from 0-18 years old at diagnosis, and mean age at onset 7,3 years ± 4,06 SD. The patients were attended to three pediatric endocrinology services of public healthcare system in Recife, Brazil (Instituto Medicina Integral de Pernambuco Professor Fernando Figueira, Hospital da Restauração and Hospital das Clínicas) from February to July 2006. A Free Consent Term from all patients, or their responsible, enrolled in this study was obtained.

T1DM patients were diagnosed according to American Diabetes Association criteria and classified as T1DM regarding clinical presentation [24].

The control group consisted of 217 healthy unrelated volunteers, with no history of autoimmune or chronic diseases, with 144 females (66,4%) and 73 males (33,6%), from 16-72 years old and mean age 38,8 years ± 14,7 SD, from Pernambuco, Brazil. This study was carried out with advanced approval from the Local Ethics Committee (IMIP Number: 762/2006 and 1717/2010).

SNPs Selection and VDR genotyping

Genomic DNA was isolated from whole blood using the Wizard genomic DNA purification kit (Promega, Madison, MA) and standard extraction protocol according to manufacturer's instructions. Polymorphisms were selected using the SNPBrowser software 4.0 (Applied Biosystems, Foster City, CA) and HapMap database (http://hapmap.ncbi.nlm.nih.gov/). Five tagging SNPs (tagSNP): rs11168268, rs2248098, rs1540339, rs4760648 and rs3890733, each one with 10% Minimum Allele Frequency (MAF), were selected. Genotyping was performed using commercially available fluorogenic allele specific probes (Taqman Probes, Applied Biosystems, Foster City, CA) using the ABI7500 Real Time PCR platform (Applied Biosystems, Foster City, CA). Allelic discrimination followed as recommended by the manufacturer and analyzed using the SDS software 2.3 (Applied Biosystems, Foster City, CA).

Statistical Analysis

Genotypic and allelic frequencies and Hardy-Weinberg equilibrium were performed by using the SNPStats tool (http://bioinfo.iconcologia.net/SNPstats). The Exact Fisher Test was applied to determine the statistical significance of all comparisons. The Haploview

Software, version 4.2, was used for haplotypes associations. “SNPassoc” R package (R software, version 2.12.2), developed for genetic studies, was used for evaluating the association between SNPs and age onset [25]. P-values < 0.05 were considered statistically significant.

RESULTS

The frequencies of the five tagSNPs studied, which cover the whole VDR gene (figure 1a), in the 204 children with T1DM and 217 healthy individuals are reported on Table 1. Allelic and genotypic SNP frequencies for VDR polymorphisms were in Hardy-Weinberg equilibrium in both patients and controls groups.

Distribution of VDR genotype and allele frequencies was significantly different between patients with T1DM and controls for two tagSNP: rs1540339 and rs4760648. For the rs1540339 (C/T) SNP, the CT genotype was more frequent in the control group (47.7%) than in the patients (35.4%), (p = 0.03, OR = 0.63, 95%CI = 0.42-0.96) thus conferring protection for T1DM, but no differences in allele distribution could be evidenced between the assessed groups. For the rs4760648 (C/T) SNP, the T allele was more frequent in patients than in controls (51.5% *vs.* 44.1%, p=0.037), as well as the TT genotype (24.5% *vs* 15.6%, p = 0.026), suggesting an association with increased susceptibility to T1DM for both T allele and TT genotype (OR= 1.34, CI = 1.01-1.78; and OR = 2.00, CI = 1.11-3.60). The rs11168268 (A/G), rs2248098 (A/G) and rs3890733(C/T) showed no statistically significant differences in neither allele nor genotype frequencies comparisons.

Haplotype analysis showed no linkage disequilibrium for the VDR assessed polymorphisms, except for SNPS rs1116828 and rs2248048 (D´= 0.84), suggesting combined genotype as shown in figure 1b. In addition, the VDR polymorphisms did not influence the age of onset of T1DM as shown in figure 2.

DISCUSSION AND CONCLUSIONS

Since the first publication of an association study relating VDR polymorphisms with T1DM pathogenesis [20], other studies have been performed in different populations in order to replicate McDermott and co-workers findings. Studies confirming the VDR association with autoimmune diseases such as T1DM were able to pinpoint VDR gene as a potent modulator of the immune response [21,26,27]. The results we presented herein suggest evidence of association of VDR polymorphisms with T1DM susceptibility, but not with the age onset in a Brazilian population.

Supporting our findings with rs1540339 polymorphism, which were associated to protection in the Brazilian population, a recent study presented by Mory et al. [21] evaluated the prevalence of VDR polymorphisms *FokI* and *BsmI* in Brazilian individuals with T1DM. Even though they reported that the *BsmI* VDR SNP was more frequent in controls than in T1DM patients (bb+Bb = 79.1% *vs* 66.1%, respectively, p = 0.006), the frequencies were not in Hardy-Weinberg equilibrium. However, this was the first described relationship between VDR variants and β-cell autoimmunity in Brazilian T1DM patients [21]. In German population, a case-control study evaluated the role of VDR polymorphisms *ApaI, TaqI, BsmI* and *FokI* in a family set of T1DM, where VDR variants conferred protection against the disease, evidencing the role of vitamin D in immunoresponse [27]. Still in accordance to our findings, Panierakis [28], in a study assessing the Greek population, found association between VDR SNPs and T1DM; The *FokI* F allele and *BsmI* B allele were associated to protection in T1DM patients, while *ApaI* A and *TaqI* T to disease risk. In two different Spanish populations *FokI* analysis had similar results and influenced T1DM susceptibility in both populations [26].

In the present study, the rs4760648 polymorphism showed the T allele more frequently in patients than in controls, (*p* = 0.03), and the TT genotype conferred risk to T1DM in the assessed population (*p* = 0.02, OR = 2.00, CI = 1.11-3.60). The results presented by Panierakis [28] evidenced risk of VDR *ApaI* and *TaqI* polymorphisms in Greek population, supporting our findings. Another study in Turkish population presented *FokI* F allele as risk factor in T1DM pathogenesis [29].

Conversely, in other reports no association was found for VDR variants and T1DM susceptibility [2,23,30]. Recently, Lemos [23] assessed *FokI, ApaI, BsmI* and *TaqI*, SNPs as markers for T1DM susceptibility in a Portuguese population. The results showed a lack of association between these SNPs and T1DM susceptibility. Supporting these findings, a study in Chilean population, showed no association between VDR polymorphisms and T1DM etiology in a case-parent trios study [31]. Nejentsev and co-workers [17] performed a comprehensive association analysis including a large case-control group and 98 SNPs in VDR region. Although they did not show an association between *FokI, ApaI, BsmI* and *TaqI* VDR polymorphisms and T1DM, they did find evidence of association with the disease in four newly tested SNPs (rs4303288, rs11168275, rs12721366 and rs2544043) (*p* = 0.01 and *p* = 0.03) [2]. In the meta analysis by Guo [30], and in another study performed by Howson [32], little, if any, evidence was found among the VDR SNPs *FokI, BsmI, TaqI* and *ApaI* and T1DM etiology; however, is not reasonable to exclude VDR as a genetic marker in T1DM susceptibility, since new polymorphisms [2] have started to be studied and new associations, such as the ones provided by our study, are about to be described.

Haplotype analysis was performed to evaluate possible linkage disequilibrium among the tested polymorphisms. In our study we did not find any evidence to support the existence of haplotype blocks for the tested SNPs, although, a weak presence of linkage disequilibrium is suggested between SNPs rs11168268 and rs2248098. In most studies VDR gene haplotypes

are deduced from informative combinations of *FokI, ApaI, TaqI* and *BsmI* SNPs. The studies that relate VDR haplotype with T1DM susceptibility are, once again, inconsistent, although association reports can be found in different populations such as Indian-Asians, Croatian, Spanish and Germany [20,22,33,34]. Supporting our findings, no association could be found in Chilean, Portuguese and Finish population. [23,31,35].

In this study we also assessed the influence of VDR polymorphisms on the T1DM age of onset. Our results did not indicate association of VDR SNPs, however, age of onset of T1DM may vary due to several factors including environment and genetics. In the Japanese population, association of VDR gene polymorphisms to acute disease onset was described by Motohashi [36]. Their results showed association of VDR variant, *BsmI* B allele, and acute-onset of T1DM in the Japanese population [36]. In the VDR polymorphisms prevalence study presented by Mory [21], in which a Brazilian population was assessed, an association between *BsmI* B allele and later onset T1DM was evidenced as well. Although these results were not statistically significant this study was the first Brazilian report assessing the relationship between β-cell function and VDR polymorphisms [21]. Despite these findings, age onset analysis in T1DM and VDR polymorphisms are usually not taken into account [36].

Therefore, we consider that VDR polymorphism might be a relevant factor in customizing vitamin D intake therapies [37]. For instance, VDR function and vitamin D levels rely on several environmental factors, which might be specific to some T1DM patients and may change the risk to particular VDR polymorphisms [2,37]. Our results do not enclose the environmental factors that might enhance or inhibit VDR polymorphism and/or vitamin D status, which may be key points in understanding the relationship among gene, environment and T1DM.

In conclusion, our findings suggest an association between VDR polymorphisms and T1DM in the assessed population, in particular for the SNPs rs1540339 and rs4760648. This

may indicate new possible candidate polymorphisms for further studies with T1DM. The T1DM age onset analysis in this work did not provide any evidence of association with VDR polymorphisms.

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CONFLICTS OF INTEREST

None of the authors has any potential financial conflict of interest related to this manuscript.

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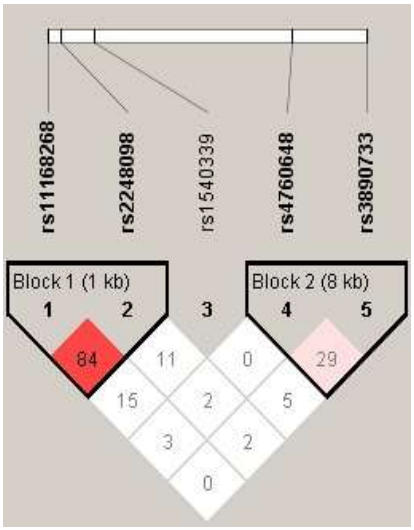
A



B

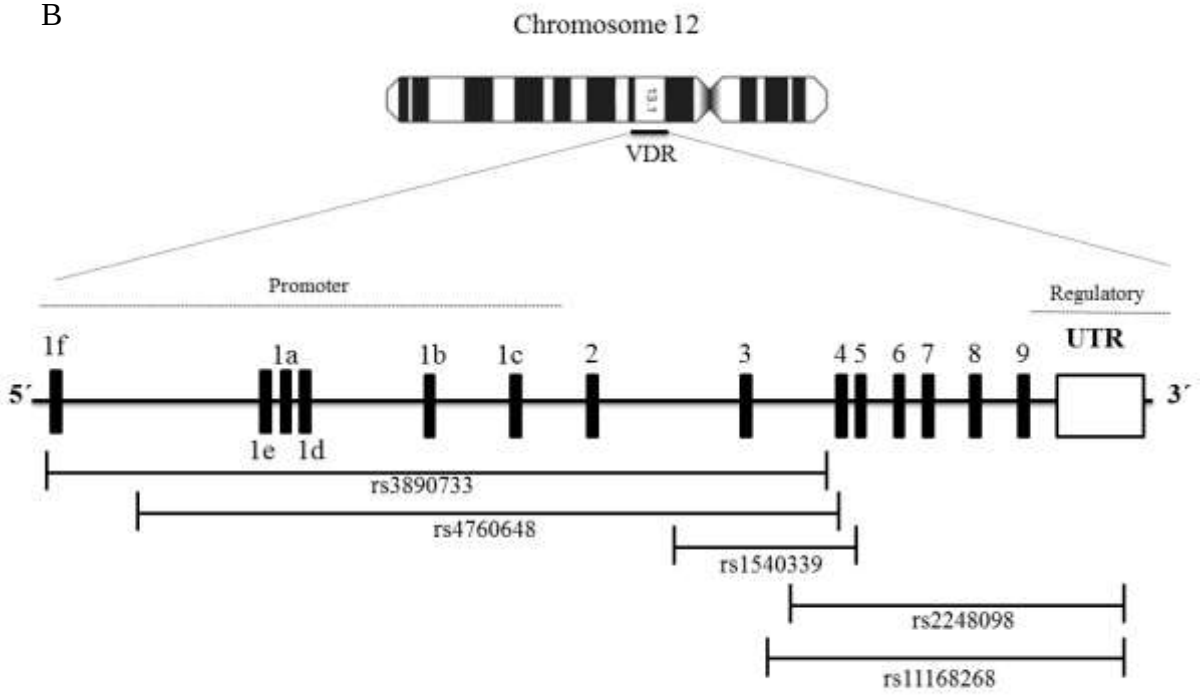


Figure 1. **A.** VDR chromosome structure and TaqSNPs distribution throughout the gene; **B**. Pairwise Linkage disequilibrium (LD) calculated by Haploview Software 4.2 showing the five selected SNPs in VDR gene. The intensity of the box color is proportional to the strength of the LD (D´) for the marker pair.

Table 1. Genotype and allelic frequencies in T1DM patients and controls.

SNP	Patients N (%)	Controls N (%)	Odds Ratio (95% CI)	P-Value
rs11168268				
Genotype	**191**	**199**		
AA	77 (40.3%)	76 (38.2%)	1.00	
AG	89 (46.6%)	93 (46.7%)	0.94 (0.61-1.45)	0.826
GG	25 (13.1%)	30 (15.1%)	0.82 (0.44- 1.53)	0.637
Allele				
A	243 (63.6%)	245 (61.6%)	1.00	
G	139 (36.4%)	153 (38.4%)	1.91 (0.80-1.47)	0.555
rs2248098				
Genotype	**199**	**210**		
GG	60 (30.1%)	55 (26.2%)	1.00	
AG	108 (54.3%)	107 (51%)	0.93 (0.59-1.46)	0.810
AA	31 (15.6%)	48 (22.9%)	0.59 (0.33-1.06)	0.081
Allele				
G	228 (57.3%)	217 (51.7%)	1.00	
A	170 (42.7%)	203 (48.33%)	0.79 (0.59-1.06)	0.122
rs1540339				
Genotype	**198**	**216**		
CC	105 (53%)	98 (45.4%)	1.00	
CT	70 (35.4%)	103 (47.7%)	0.63 (0.42-0.96)	**0.030**
TT	23 (11.6%)	15 (6.9%)	1.43 (0.71-2.90)	0.377
Allele				
C	280 (70.7%)	299 (69.21%)	1.00	
T	116 (29.3%)	133 (30.79%)	0.93 (0.68-1.26)	0.649
rs4760648				
Genotype	**204**	**212**		
CC	44 (21.6%)	58 (27.4%)	1.00	
CT	110 (53.9%)	121(57.1%)	1.20 (0.75 -1.92)	0.476
TT	50 (24.5%)	33(15.6%)	2.00 (1.11-3.60)	**0.026**
Allele				
C	198 (48.5%)	237 (55.9%)	1.00	
T	210 (51.5%)	187(44.1%)	1.34 (1.01- 1.78)	**0.037**
rs3890733				
Genotype	**200**	**217**		
CC	103 (51.5%)	97 (44.7%)	1.00	
CT	73 (36.5%)	94 (43.3%)	0.73 (0.48-1.11)	0.140
TT	24 (12%)	26 (12%)	0.87 (0.47-1.62)	0.752
Allele				
C	279 (69.7%)	288 (66.4%)	1.00	
T	121 (20.3%)	146 (33.6%)	0.85 (0.63-1.15)	0.290

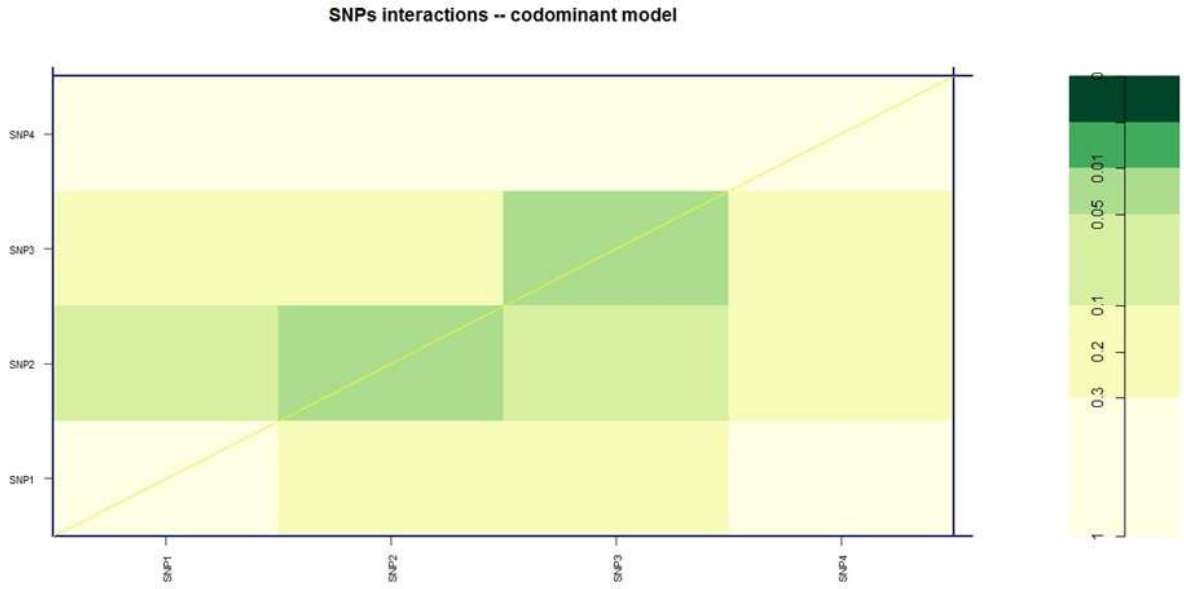


Figure 2. VDR SNPs interactions in the Codominant model. Interaction analysis of VDR polymorphisms and T1DM age onset from R sofware.

Supplementary Data Table S1: List of the assessed VDR TaqSNPs.

Supplementary Data

Table 1. List of the assessed VDR TaqSNPs.

rs4760648	**rs2248098**	**rs3890733**	**rs1540339**	**rs11168268**
rs2853564	rs9729	rs11168293	rs2239181	rs9729
rs2853559	rs3847987	rs2853564	rs2239179	rs3847987
rs4334089	rs739837	rs2853559	rs886441	rs739837
rs3890734	rs731236	rs4334089	**Total: 3**	rs731236
rs11168292	rs7975232	rs3890734		rs7975232
rs11168293	rs757343	rs11168292		rs1544410
Total: 6	rs1544410	**Total: 6**		rs2525044
	rs2525044			rs7962898
	rs7962898			rs7963776
	rs79637766			rs4760733
	rs4760733			rs7967152
	rs7967152			rs2239184
	rs22391846			rs7971418
	rs7971418			rs7975128
	rs7975128			rs7305032
	rs7305032			rs11168266
	rs11168266			**Total: 16**
	Total: 17			

10.2 Anexo II

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Prezado Senhor,

O trabalho intitulado **"ESTUDO DA NEUTROPENIA EM PACIENTES PORTADORES DE LÚPUS ERITEMATOSO SISTÊMICO"**, foi analisado pelo Comitê de Ética em Pesquisa, em sua 247ª Reunião Ordinária realizada em 21/05/2007, e enquadrado na categoria: **APROVADO, bem como o Termo de Consentimento Livre e Esclarecido,** de acordo com o Processo HCRP nº 2234/2007.

Atenciosamente.

PROF. DR. SÉRGIO PEREIRA DA CUNHA
Coordenador do Comitê de Ética em Pesquisa do HCRP e da FMRP-USP

Ilustríssimo Senhor
JOÃO ALEXANDRE TRÊS FANCOTO
PROF. DR. EDUARDO ANTONIO DONADI (Orientador)
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