UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

AVALIAÇÃO IMUNOGENÉTICA DOS GENES DOS RECEPTORES *TOLL-LIKE* 7, 8 E 9 EM PACIENTES COM LUPUS ERITEMATOSO SISTÊMICO

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SUMÁRIO

LISTA DE ABREVIATURAS

APC	Antigen Presenting Cell
BAFF	B-cell Activating Factor
BCR	B Cell Receptor
BDCA-2	Blood Dendritic Cell Antigen 2
BILAG	British Isles Lupus Assessment Group
CD	Cluster of Differentiation
CI	Confidence Interval
CRP	Proteína C Reativa
DC	Dendrític Cell
EBNA-1	Epsteion Barr Nuclear Antigen 1
EBV	Epstein Barr Vírus
Fc	Fração constante (cristalizável)
GM-CSF	Granulocyte/Macrophage Colony Stimulating Factor
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
IFN	Interferon
IFNAR	Type I Interferon receptor
Ig	Immuogloblin
IL	Interleukin
IRAK1	Interleukin-1 Receptor-Associated Kinase 1
IRF5	Interferon Regulatory Factor 5
ITGAM	Integrin Alpha M
mDC	Myeloid Dendritic Cell
MyD88	Myeloid Differentiation Primary Response gene 88
NF-kappaB	Nuclear Factor kappa B
NMDAR	N-metil-d-aspartate Receptor
ODN	Oligodesoxinucleotídeo
OR	Odds Ratio
PAMPs	Pathogen-Associated Molecular Patterns
PCR	Polymerase Chain Reaction
pDC	Plasmacytoid Dendritic Cell
PTPN22	Protein Tyrosine Phosphatase, non-receptor type 22

RBP	RNA-Binding Protein
RFLP	Restriction Fragment Lenght Polymorphism
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
SLICC	Systemic Lupus International Collaborating Clinic
SNC	Sistema Nervoso Central
STAT4	Signal Transducer and Activator of Transcription 4
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TNFAIP3	Tumor Necrosis Factor, Alpha-induced Protein 3
TREX1	Three Prime Repair Exonuclease 1
UTR	Untranslated Region

RESUMO

O Lupus Eritematoso Sistêmico (LES) é uma doença crônica auto-imune caracterizada pela alta produção de auto-anticorpos contra antígenos nucleares e pela formação de imunocomplexos que desencadeiam resposta citotóxica. As causas do LES são desconhecidas, porém se têm alguns indícios bem descritos que alterações genéticas, imunológicas e/ou ambientais podem desencadear processos autoimunes que levam ao LES. Esses fatores são, por exemplo, vírus e o mimetismo molecular causado por proteínas virais, ou genéticos que interfiram em rotas de processamento de imunocomplexos, produção de interferon (IFN) e transdução de sinal em linfócitos. Os receptores Toll-Like (TLR) são receptores de padrões moleculares de patógenos e estão envolvidos na produção de IFN, além de ser um elo importante entre a imunidade inata e a adquirida. Os TLR7/8/9 reconhecem ácidos nucléicos e são expressos em células dendríticas e células B, importantes na patogênese do LES. O objetivo do nosso trabalho foi avaliar a infuência dos polimorfismos genéticos potencialmente funcionais rs179008 no *TLR7*, rs3764880 no *TLR8*, rs5743836 e rs352140 no *TLR9* em uma amostra de 370 pacientes com LES e em uma amostra de 415 indivíduos saudáveis provenientes do sul do Brasil. O polimorfismo rs5743836 foi genotipado através da técnica de PCR alelo específico BIPASA enquanto que os demais foram genotipados por PCR-RFLP. As freqüências genotípicas e haplotípicas foram comparadas usando o teste de Qui-Quadrado e as freqüências alélicas usando o teste Exato de Fisher. As comparações foram realizadas subdividindo os indivíduos de acordo com a origem étnica e sexo. As freqüências genotípicas e alélicas diferiram para rs179008 (P=0,020 e P=0,003; OR para presença do alelo T: 1,74 CI 95% 1,12-2,70) e rs5743836 (P=0,045 e P=0,017; OR para presença do alelo C: 1,59 CI 95% 0,99-2,57) nas comparações entre mulheres eurodescendentes controles e pacientes. Houve uma tendência na presença do alelo C em pacientes com Anti-Ro/SS-A comparados com pacientes sem Anti-Ro/SS-A (P corrigido = 0,06). As análises com haplótipos ou genótipos combinados não apresentaram diferenças estatisticamente significativas. Nossos dados sugerem que os alelos T do rs179008 e o alelo C do rs5743836 estão envolvidos na suscetibilidade/patogênese do LES em mulheres Eurodescedentes do sul do Brasil.

Palavras-chave: reconhecimento de ácidos nucléicos, autoimunidade, happlótipos, Eurodescendentes, Afrodescendentes.

ABSTRACT

Systemic Lupus Erythematosus (SLE) is an autoimmune chronic disease characterized by high autoantibody production against nuclear antigens and by immunocomplexes formation that lead to citotoxicity. Causes of SLE are unknown, however there are some factors suggested to trigger autoimmune processes and result in SLE phenotype. These can be environmental, such as virus and molecular mimicry caused by their proteins, or genetic factors mainly related to immunocomplexes processing, interferon (IFN) production and the signal transduction pathway in lymphocytes. Toll-Like receptors (TLR) are pattern-recognition receptors and they are involved in IFN production, besides to be a link between the innate and acquired immune system. TLR7/8/9 recognize nucleic acids and are expressed mainly in dendritic and B cells. Our study aims to evaluate the prevalence of *TLR7* rs179008, *TLR8* rs3764880, *TLR9* rs5743836 and rs352140, potencially functional polymorphisms, in 370 SLE patients and 415 healthy blood-donnors from southern Brazil. Rs5743836 polymorphism was genotyped trough allele-specific PCR BIPASA and the rest were genotyped through PCR-RFLP. Genotypic and allelic frequencies were compared using chi-square and exact fisher test, respectively. OR was calculated and clinical characteristics were evaluated. All comparisons were carried out grouping individuals according ethnicity and gender. Genotypic and allelic frequencies were significantly different for rs179008 (P=0.020 and P=0.003, OR for T allele carriers: 1.74, CI 95% 1.12-2.70) and rs5743836 (P=0.045 and P=0.017, OR for C allele carriers: 1.59, CI 95% 0.99-2.57) comparing European-derived SLE and control women. A trend in Anti-Ro/SS-A presence was observed for rs5743836 C allele carriers (corrected P=0.06). There were no statistical differences when haplotypes and combined genotypes were analyzed. Our data suggest both *TLR7* rs179008 T allele and *TLR9* rs5743836 C allele involved in SLE susceptibility/pathogenesis in women European-derived.

Keywords: nucleic acid recognition, autoimmunity, haplotypes, European-derived, African-derived.

1. INTRODUÇÃO

1.1. Lúpus Eritematoso Sistêmico (LES)

O LES é uma doença inflamatória crônica auto-imune com envolvimento de múltiplos órgãos e sistemas. É caracterizada pela produção de anticorpos auto-reativos dirigidos especialmente contra antígenos nucleares e pela formação de imunocomplexos, os quais depositam-se, levando à intensa inflamação sistêmica e dano a múltiplos órgãos. Sua etiologia está sendo desvendada, sabendo-se da importante participação de fatores genéticos e ambientais para o desencadeamento desse desequilíbrio do sistema imunológico. Sua manifestação clínica é bastante variada e a evolução costuma ser crônica, com períodos de exacerbação e remissão. Devido a uma extensa heterogeneidade de sintomatologia clínica, podendo haver artrite, serosite, nefrite, vasculite, miosite, manifestações mucocutâneas, hemocitopenias imunológicas, diversos quadros neuropsiquiátricos, hiperatividade retículo-endotelial e pneumonite, entre outros, convencionou-se realizar seu diagnóstico através da associação de achados clínicos e laboratoriais, conforme os critérios de classificação propostos pelo *American College of Rheumatology* (ACR) em 1982 e revisados em 1997 (Tan, Cohen et al. 1982; Edworthy, Zatarain et al. 1988; Hochberg 1997). Os critérios de diagnóstico encontram-se detalhados no Anexo I e as informações referentes às características clínicas e laboratoriais dos pacientes no Anexo II.

1.1.1. Epidemiologia do LES

Estudos epidemiológicos de pacientes com LES são de difícil realização pela variabilidade de apresentações clínicas desta doença, pelo fato da definição do diagnóstico depender do acúmulo de determinados sinais e sintomas pré-estabelecidos pelos critérios de classificação e, também, pela sua baixa freqüência na população. Apesar de ser uma doença rara, o LES é registrado em todo o mundo e sua prevalência oscila de 15-50/100.000 habitantes. O LES não possui distribuição uniforme entre grupos raciais, gênero, idade, raça ou situação socioeconômica, podendo estes fatores ter influência na expressão da doença (Ward and Studenski, 1990a; Alarcon et al., 2002; Alarcon et al., 2004). Severidade, risco e expressão clínica variam de acordo com etnia, localização geográfica e sexo, com maior prevalência entre mulheres e algumas populações não européias (Uribe et al., 2004; Lau et al., 2006). A prevalência relatada de LES na

população norte-americana é de 40-50 casos para cada 100.000 pessoas, sendo que nos últimos 40 anos, devido provavelmente à detecção mais precoce da doença, houve um aumento dessa prevalência na ordem de até 3 vezes (Lawrence, Helmick et al.; Uramoto, Michet et al. 1999). Como citado anteriormente, a prevalência varia muito de acordo com a área geográfica e a população estudada. As estimativas de prevalência variam de 40 casos para cada 100.000 mulheres até 565 casos para cada 100.000 mulheres (Lahita, 1995). Taxas de incidência similares, variando de 3,3 a 4,8 casos para cada 100.000 pessoas por ano, foram observadas em coortes européias da Islândia, Inglaterra e Suécia (Hopkinson et al., 1994). Um estudo epidemiológico recente revelou uma variação de incidência de 1 a 32 casos para cada 100.000 pessoas por ano, englobando Estados Unidos, Ásia, Europa e Austrália. Dentre os países europeus, Espanha, Suécia e Islândia foram os que tiveram maior prevalência de LES (Danchenko et al., 2006). Um estudo brasileiro revelou uma incidência de LES de 8,7 casos para cada 100.000 pessoas por ano, sendo que para mulheres era de 14 casos para cada 100.000 pessoas por ano e para homens era de 2,2 casos para cada 100.000 por ano (Vilar and Sato, 2002).

O LES é mais comum entre mulheres, numa proporção de aproximadamente 9:1, principalmente comparando-se indivíduos em idade reprodutiva. Esse fato é atribuído a fatores hormonais e principalmente a efeitos do hormônio estrogênio (Cooper et al., 1998; Lahita, 1999). Nos Estados Unidos, mulheres de origem afro-americana apresentam maior prevalência de LES quando comparadas a mulheres de outras origens étnicas (Hochberg 1985; Hochberg 1990). Os primeiros sintomas começam a surgir principalmente na idade reprodutiva, geralmente entre a 2ª e 4ª décadas de vida, tendo o seu pico de incidência entre 35 e 39 anos, com incidência de 32,7 casos para cada 100.000 mulheres por ano (Vilar and Sato, 2002). O surgimento da doença ocorre entre 16-55 anos em 69% dos casos, abaixo dos 16 anos em 25% e acima dos 55 anos em 6% dos casos. (Dubois and Tuffanelli, 1964; Ballou et al., 1982; Schaller, 1982; Achour et al., 2011).

A sobrevida nos pacientes com LES tem melhorado muito nos últimos anos. Na década de 50 a sobrevida média em 5 anos após diagnóstico era de somente 50%, enquanto na última década a sobrevida média em 10 anos após o diagnóstico alcançou 80 a 90% (Hochberg, 1990; Gripenberg and Helve, 1991; Pistiner et al., 1991; Boumpas et al., 1995b; Tucker et al., 1995; Ward et al., 1995; Cervera et al., 2003; Kasitanon et al., 2006). O prognóstico do LES tende a ser menos favorável em afro-descententes quando comparado com euro-descendentes, assim como em populações com condições sócio-

econômicas desfavoráveis, em pessoas com baixo nível educacional e em crianças de um modo geral (Schaller 1982; Callahan and Pincus 1990; Ward and Studenski 1990). Nos homens, o diagnóstico é mais tardio e a mortalidade dentro do primeiro ano da doença é maior (Ward and Studenski, 1990b).

A mortalidade no LES segue um padrão bimodal (Urowitz et al., 1976). A mortalidade precoce se deve à atividade da doença, especialmente quando há acometimento renal e do sistema nervoso central e ao maior risco de infecções graves decorrentes da imunossupressão. Dados de um estudo brasileiro mostraram que até 58% das mortes nos paciente com LES resultaram de infecções (Iriya et al., 2001). A mortalidade em período mais tardio resulta de complicações da doença e do tratamento, sendo a doença cardiovascular um dos mais importantes fatores de morbidade e mortalidade nestes pacientes (Jonsson et al., 1989; Pistiner et al., 1991; Swaak et al., 1991; Esdaile et al., 1994; Boumpas et al., 1995a; Manzi et al., 1999).

1.1.2. Etiologia e patogênese do LES

Acredita-se que a participação de agentes infecciosos, drogas, radiação solar e fatores hormonais em um indivíduo geneticamente predisposto, proporcionariam a apresentação de auto-antígenos ao sistema imune e a perda da tolerância imunológica, que está associada à falha nos mecanismos supressores e de imunorregulação, com subseqüente ativação policlonal de linfócitos B e produção de auto-anticorpos. Auto-anticorpos dirigidos contra membranas celulares contribuem para as citopenias (anemia hemolítica, leucolinfopenia e trombocitopenia), além de outras manifestações detectadas na doença, como o envolvimento do sistema nervoso central. Auto-anticorpos dirigidos contra o complexo fosfolipídeo-beta-2-glicoproteína são responsáveis por eventos tromboembólicos venosos e arteriais observados em alguns pacientes com LES. A injúria celular promovida por auto-anticorpos e ativação do complemento com inflamação crônica predispõe ao surgimento de novos auto-antígenos que mantém a estimulação da resposta imunológica levando a uma perpetuação da resposta auto-imune.

Estudos de genética básica mostram alta taxa de concordância para a doença em gêmeos monozigóticos, que pode variar de 14 a 57%, enquanto que em gêmeos dizigóticos esta concordância é de 2 a 5% (Block et al., 1975; Deapen et al., 1992; Alarcon-Segovia et al., 2005). O encontro de outras doenças auto-imunes em famílias de pacientes com LES e

a associação de LES com outros distúrbios geneticamente determinados também reforçam a importância da predisposição genética para o desenvolvimento da doença.

Nos últimos anos tem-se utilizado varreduras genômicas capazes de identificar milhares de mutações de ponto independente de conhecimento prévio de genes ou regiões candidatas à suscetibilidade. Antes de 2007 nove loci para a suscetibilidade ao LES eram reconhecidos, hoje mais de 20 loci mostram associação com o LES (Harley et al., 2009).

As associações genéticas indicam muitas vias, processos e tipos de células diferentes envolvidos na geração do fenótipo do LES. A maioria desses genes está envolvida em 3 tipos de processos biológicos: (i) processamento de imunocomplexos; (ii) função de receptores Toll-Like (TLR) e produção de interferon (IFN) do tipo I; e (iii) transdução de sinal em linfócitos.

Processos mediados por células apresentadoras de antígenos (APC) como eliminação de células apoptóticas, processamento e apresentação aos linfócitos, têm sido implicados no desenvolvimento do LES. Variações em genes como *HLA-DR*, *CRP* e genes que codificam receptores Fc podem afetar o modo como essas proteínas reagem com imunocomplexos. Essa sugestão é sustentada pelo fato de baixos níveis de proteínas do sistema complemento serem encontradas na circulação de pacientes com LES ativo e pela associação do LES com a ausência de proteínas funcionais do sistema complemento como conseqüência da homozigose de alelos nulos para loci de membros da via clássica do complemento (Harley et al., 2009). *ITGAM*, que codifica uma cadeia alfa da integrina αMβ2 é uma molécula de adesão que não apenas se liga ao fragmento C3b, mas também a inúmeros outros ligantes que são relevantes ao LES. O polimorfismo H77R (rs1143679) parece explicar o efeito visto: essa variação parece causar mudanças estruturais significantes ao domínio de interação com o ligante da integrina αMβ2 (Nath et al., 2008). Além disso, aloanticorpos que reagem contra essa variante bloqueiam a adesão dependente de αMβ2 de neutrófilos ao endotélio (Sachs et al., 2004).

Outras associações genéticas bastante relacionadas ao LES vistas no processamento de imunocomplexos são em *CRP* e nos genes que codificam receptores Fc. A proteína C-Reativa, codificada pelo gene *CRP*, é uma proteína de fase aguda capaz de ativar o sistema complemento. Baixos níveis de proteína C-Reativa estão relacionados com o aumento na suscetibilidade a algumas infecções, eliminação deficiente de imunocomplexos e restos de células apoptóticas. Em um estudo com 586 famílias, Russell e colegas (2004) encontraram que níveis basais de proteína C-Reativa eram influenciados independentemente por 2 polimorfismos, rs1800947 e rs1205. Este último foi associado

com LES e com a produção de anticorpos antinucleares (Russell et al., 2004). Os receptores Fc são receptores de superfície, presentes em algumas células fagocíticas do sistema imune, que se ligam a anticorpos e promovem a fagocitose. Assim, os receptores Fc estão amplamente envolvidos na defesa de microrganismos, eliminação de restos de células apoptóticas e ativação celular. Em um estudo realizado por Kyogoku e colegas (2002), foi analizado o polimorfismo I232T (rs1050501) no gene *FCGR2B* em uma população japonesa com LES. Neste estudo os autores encontraram a freqüência do homozigoto TT aumentada em pacientes (Kyogoku et al., 2002). Do mesmo modo, Blank et al. (2005) seqüenciaram a região promotora do gene *FCG2B*, em um grupo de pacientes americanos euro-descendentes, e encontraram a freqüência de homozigotos para o alelo -343C (rs3219018) aumentada em pacientes quando comparada a controles. O alelo -343C foi associado à diminuição da transcrição e foi visto que linfócitos B ativados de pacientes com LES apresentavam redução significativa da expressão desse receptor na superfície. As rotas mediadas por opsoninas e Fc citadas acima foram recentemente revisadas (Kelley et al., 2010).

Os interferons têm sido implicados na patofisiologia do LES desde os anos 70 (Hooks et al., 1979). A observação de que alta atividade sérica de IFN-alfa parece ser um fator de risco herdável indica que o controle genético do sistema de IFN tipo I é importante no desenvolvimento de LES (Niewold et al., 2007). Vários estudos identificaram genes envolvidos na produção de IFN tipo I como fatores de suscetibilidade no LES, como por exemplo, *IRAK1, TREX1, IRF5* e *TNFAIP3* (Cunninghame Graham et al., 2007; Graham et al., 2007; Jacob et al., 2007; Koneru et al., 2007; Lee-Kirsch et al., 2007; Graham et al., 2008; Harley et al., 2008; Hom et al., 2008; Musone et al., 2008). A identificação de genes específicos na rota de produção de IFN promete complementar o entendimento da patologia do LES de dois modos: (i) promovendo evidências úteis na determinação de células e rotas que governam a produção de IFN, e (ii) auxiliando no desenvolvimento de terapias que reduzam a produção de IFN.

O gene *IRF5*, que codifica o fator de transcrição IRF5, é constitutivamente expresso em pDC (Izaguirre et al., 2003) e foi o primeiro gene identificado envolvido diretamente na produção de IFNα que foi associado ao aumento da suscetibilidade ao LES (Sigurdsson et al., 2005). As variantes alélicas com maior probabilidade de serem causais foram identificadas recentemente, e mostraram afetar *IFR5*, cuja expressão está aumentada em células mononucleares periféricas de pacientes (Sigurdsson et al., 2008a). Um haplótipo de risco no *IFR5* está associado ao aumento sérico de IFN-alfa em pacientes,

especialmente naqueles com anticorpos contra proteínas que se ligam a RNAs ou DNA fita dupla (Niewold et al., 2008). A cinase associada ao receptor de IL-1 (IRAK1), que está envolvida na sinalização de TLRs e na produção de IFN-alfa reforça a visão de que o controle genético da produção de IFN-alfa é um importante fator de risco para desenvolver LES (Jacob et al., 2009). Entre os genes envolvidos na resposta ao IFN-alfa, o produto codificado pelo gene *STAT4*, que interage com a parte citoplasmática do receptor de IFN do tipo I (IFNAR) (Tyler et al., 2007) está fortemente associado com LES (Remmers et al., 2007). Nos pacientes, há uma associação entre o genótipo de *STAT4*, aumento da sensibilidade IFN-alfa (Kariuki et al., 2009) e um fenótipo mais severo da doença, o qual inclui nefrite e a presença de anticorpos anti-DNA (Sigurdsson et al., 2008b; Taylor et al., 2008).

A transdução de sinais em células do sistema imune, especialmente linfócitos T e B, é uma outra rota que parece conter muitos genes de suscetibilidade ao LES. Por exemplo, *PTPN22* codifica uma fosfatase seletiva que modula a transdução de sinal em linfócitos T e possui 2 polimorfismos associados ao LES: R620W (rs2476601) e R263Q (rs33996649). O primeiro é um polimorfismo de ganho de função cuja capacidade catalítica da enzima é aumentada quando comparada à variante selvagem e, por isso, é considerado um supressor potente da sinalização de linfócitos T (Vang et al., 2005; Chung and Criswell, 2007; Harley et al., 2008). O outro polimorfismo é uma mutação de perda de função, que reduz a atividade da enzima e foi considerado um alelo de proteção ($p = 0.006$; OR95% 0.58, CI 0.38–0.86) (Orru et al., 2009).

De uma maneira geral, são considerados marcadores genéticos no LES: HLA-B8, HLA-DR2, HLA-DR3, DQW1, HLA-DMA*O401, deficiência de C2, deficiência de C1q, deficiência de C4 (especialmente C4A), baixos níveis de receptor do complemento-1 (CR1), determinados alelos do receptor Fc (especialmente o alelo nulo do FcRIIIB), polimorfismos de várias citocinas e receptores de citocinas, alelo nulo do gene para a enzima glutationa S-transferase M, proteinocinases, fosfatases, moléculas de sinalização intracelular, quimiocinas e opsoninas (Schur, 1995; Lazarus et al., 1997; Botto et al., 1998; Fraser et al., 2003; Morel et al., 2003; Nauta et al., 2003; Illei and Lipsky, 2004; Illei et al., 2004a; Illei et al., 2004b; Kyogoku et al., 2004; Tsao, 2004; Croker and Kimberly, 2005; Graham et al., 2006). Porém, no que se refere principalmente à associação de LES com haplótipos do HLA, tem-se verificado muita variação dessas associações nas diferentes populações estudadas, sendo alguns haplótipos vistos com maior frequência em alguns grupos populacionais, mas não em outros.

Nos pacientes com LES há numerosas anormalidades na regulação do sistema imune que podem ser secundárias à perda de autotolerância. Isto leva ao reconhecimento de auto-antígenos, ativação imunológica de linfócitos T e B com secreção de citocinas, principalmente interleucinas (IL) 4, 6 e 10, proliferação e diferenciação de linfócitos B e produção de anticorpos auto-reativos (Elkon, 1995). Várias das seguintes anormalidades têm sido descritas no LES: diminuição do número dos linfócitos T supressores e linfócitos T citotóxicos (Klinman and Steinberg, 1995), defeito na atividade citolítica dos linfócitos T (Stohl, 1995), aumento do número dos linfócitos T helper CD4+, caracterizando uma resposta imunológica predominantemente do tipo Th-2 (Tsokos, 1995), ativação policlonal precoce de linfócitos B, defeitos na tolerância de linfócitos B (Klinman and Steinberg 1995; Mohan and Datta 1995; Prodeus, Goerg et al. 1998; Yurasov, Wardemann et al. 2005), elevação dos níveis circulantes de IFN-alfa e aumento da transcrição do RNA indutor de IFN-alfa pelas células mononucleares (Baechler, Batliwalla et al. 2003; Bennett, Palucka et al. 2003; Kirou, Lee et al. 2004; Ronnblom, Eloranta et al. 2006). Recentemente, foi descrita relação dos TLRs com reconhecimento de auto-antígenos, produção de IFN e auto-anticorpos. No LES em atividade, foi encontrado aumento da proporção de células B de memória e células plasmáticas expressando TLR9, evidenciando uma possível contribuição deste fator na patogênese da doença (Papadimitraki et al., 2006). Defeitos na apoptose resultam na anormalidade da morte celular programada, com expressão de antígenos nucleares na superfície da célula. Isto, associado à eliminação ineficaz do material apoptótico, secundário a deficiências do complemento e alterações na função dos macrófagos, leva à estimulação continuada do sistema imune (Bijl et al., 2006). Conseqüentemente, ocorre quebra dos mecanismos de auto-tolerância e desencadeamento da produção de anticorpos auto-reativos.

Os pacientes com LES possuem anticorpos de múltiplas especificidades, como anti-fosfolipídeos, anti-Ro, anti-DNA. Foi identificado um grupo de anticorpos chamados R4A, anticorpos anti-DNA que se liga a um grande número de fagos, antígenos bacterianos e que exibe, também, patogenicidade renal e reação cruzada com o receptor de *N*-metil-d-aspartato (NMDAR). Esse fato apóia a hipótese de que anticorpos patogênicos podem ser produzidos por reatividade cruzada a antígenos que existem em patógenos (Gaynor et al., 1997; DeGiorgio et al., 2001). R4A se liga em regiões de domínios extracelulares das subunidades NR2A e NR2B do NMDAR. Além do mais, o anticorpo se liga ao NMDAR, provavelmente na sua forma nativa na membrana de neurônios em cultura. Essa observação foi de grande interesse, pois coincide com o aumento do

aparecimento de manifestações clínicas que acometiam o sistema nervoso central (SNC) em pacientes com LES (1999; Kozora et al., 2008). Esses sintomas que envolvem SNC, incluindo dificuldades cognitivas e desordens de humor, estão entre os sintomas mais freqüentes da neuropsiquiatria no LES. Ambas funções cognitivas e estabilidade de humor requerem função intacta do NMDAR (LeDoux, 2000). Como é estabelecido que nem todos os auto-anticorpos são patogênicos, há a sugestão de direcionar o tratamento apenas aos auto-anticorpos patogênicos para amenizar a atividade da doença e previnir danos em órgãos comumente afetados no LES, como os rins e o cérebro. Algumas terapias envolvendo esse grupo específico de auto-anticorpos como alvo foram propostas recentemente por Diamond e colegas (2010) (Diamond et al., 2010).

Muitas doenças caracterizadas como fenômenos auto-imunes podem ser, na verdade, de natureza infecciosa, principalmente causadas por vírus. Vários mecanismos para auto-imunidade induzida por vírus têm sido propostos, incluindo apresentação de complexos de proteínas virais/próprias para linfócitos auto-reativos e "ativação por testemunho" (*bystander activation*) (Dong et al., 1994; Christen and von Herrath, 2004b). Um importante evento no ciclo de muitos vírus é a interação das proteases virais com as proteínas da célula hospedeira. Isso pode resultar em sítios específicos de clivagem de moléculas que têm papel central no metabolismo da célula hospedeira, promovendo replicação viral e liberação de vírus. Além de inibir a transcrição e tradução da célula hospedeira, a clivagem proteolítica das proteínas do hospedeiro pode ter outra conseqüência: a geração de novos epítopos próprios que podem iniciar respostas auto-imunes. A ativação por testemunho consiste em um evento que induziria fortes respostas inflamatórias em vários órgãos e que pode, portanto, atrair uma gama de linfócitos auto-reativos ao sítio de inflamação.

As infecções combinam vários graus de efeito antígeno-específico e não-específico, danificam células-alvo ou órgãos diretamente, causando a liberação de auto-antígenos potenciais e aumento da apresentação de antígenos. Podem, também, ativar diretamente células autorreativas pela apresentação de antígenos que possuem reatividade cruzada, iniciando mimetismo molecular (*molecular mimicry*) (Christen and von Herrath, 2004a; Christen and von Herrath, 2004b). Infecções virais são associadas com uma variedade de condições auto-imunes, incluindo esclerose múltipla e diabetes tipo I. A explicação mais popular para essa associação clínica é a mimetismo molecular, definido como reatividade cruzada entre determinantes próprios e do patógeno reconhecido pelo sistema imune adaptativo (Christen and von Herrath, 2004a). Infecções pelo vírus Epstein-

Barr (EBV) têm sido associadas ao LES há muito tempo (James et al., 1997), entretanto a significância dessa associação não está inteiramente esclarecida. Anteriormente, James *et al* (1995) notaram similaridades entre a região do antígeno nuclear-1 (EBNA-1) e um epítopo do auto-antígeno Sm-BB. A imunização de coelhos com peptídeos derivados do Sm-BB, parecidos com um epítopo do EBNA-1, induziu a produção de auto-anticorpos contra outras regiões da proteína Sm-BB, bem como epítopos contra componentes do *spliceossome* (James et al., 1995). Mais recentemente, McClain *et al* (2005) analisaram amostras séricas coletadas de pacientes lúpicos antes do diagnóstico clínico. Esses autores determinaram que anticorpos direcionados contra o epítopo inicial do auto-antígeno humano Ro-60-kDa (Ro-60) reagem cruzadamente com EBNA-1. Interessantemente, esse epítopo compartilha na seqüência primária homologia com o epítopo linear de EBNA-1. Coelhos imunizados com o primeiro epítopo do Ro-60 ou o epítopo de reatividade cruzada do EBNA-1 desenvolvem auto-anticorpos direcionados contra epítopos de Ro e de auto-antígenos do *spliceossome* e eventualmente desenvolvem sintomas clínicos do LES (McClain et al., 2005). Juntas, essas observações fornecem um forte apoio à hipótese que anti-Ro e anti-Sm-BB no LES surgem por mimetismo molecular. Entretanto, esta hipótese só leva em conta um grupo específico de auto-anticorpos visto nos pacientes com LES (Sherer et al., 2004). Devem existir muito mais regiões com reatividade cruzada em EBNA-1 ou em proteínas diferentes de EBV que estão envolvidas no comprometimento com o LES. Além disso, EBV é extremamente prevalente: é presumido que mais de 90% da população mundial seja infectada (Macsween and Crawford, 2003). Outros vírus, como Citomegalovírus, Parvovírus B19 e alguns retrovírus, também já foram relacionados ao LES, porém as evidências não são tão claras quanto às relacionadas aos antígenos de EBV (Hession et al., 2010; Pavlovic et al., 2010; Perez-Mercado and Vila-Perez, 2010; Hachfi et al., 2011).

Outros fatores não virais, tais como tripanossomíase e micobacterioses podem induzir a produção de anticorpos contra DNA e sintomas semelhantes ao LES (Via and Handwerger 1993; Steinberg 1995). Raios ultravioletas podem estimular os queratinócitos a expressar antígenos nucleares em sua superfície e aumentar a secreção de citocinas que estimulariam linfócitos B à produção de anticorpos auto-reativos (Lehmann et al., 1990; Casciola-Rosen et al., 1994). Pó de silica e tabagismo podem aumentar o risco de desenvolvimento de LES (Cooper, Dooley et al. 1998; Ghaussy, Sibbitt et al. 2001; Parks, Cooper et al. 2002; Costenbader, Kim et al. 2004).

Um caso bem documentado de lúpus induzido por drogas é a exposição à procainamida, um fármaco utilizado na arritmia cardíaca. A injeção no timo de procainamida-hidroxilamina, um metabólito reativo da procainamida, resulta na indução de auto-tolerância e aparecimento de linfócitos T reativos à cromatina e produção de anticorpos contra cromatina. Os autores encontraram que procainamida-hidroxilamina não afeta a seleção negativa, mas previne o estabelecimento de não-responsividade a componentes próprios de baixa afinidade durante a seleção positiva (Kretz-Rommel and Rubin, 2000).

1.1.3. Terapias

A molécula CD20 é uma proteína transmembrana expressa na linhagem de células B começando no estágio pré-célula B tardio na medula óssea e é mantida durante a diferenciação e desenvolvimento da célula B na periferia. A expressão de CD20 na superfície é regulada negativamente em plasmoblastos que secretam anticorpos e é extinguida em plasmócitos (Tedder and Engel, 1994; Riley and Sliwkowski, 2000). CD20 é também expresso em níveis substancialmente menores em um pequeno subgrupo de células T basalmente ativadas que produzem constitutivamente IL1b e TNF-alfa e demonstram aumento da atividade apoptótica (Hultin et al., 1993; Wilk et al., 2009). Já que CD20 é expresso em células B normais e em situações de malignidade, o anticorpo monoclonal rituximab [Rituxan®⁄MabThera®; Genentech (São Francisco do Sul, CA, EUA)⁄F. Hoffman-LaRoche (Basel, Switzerland)⁄Biogen-Idec (Boston, MA, EUA)] tem sido um depletor efetivo de células CD20+ e é aprovado para tratamento de malignidades de células B e no tratamento de pacientes com atrite reumatóide que respondem inadequadamente a terapias anti-TNF (Smith, 2003; Cohen et al., 2006; Dorner et al., 2009).

Vários estudos envolvendo rituximab em LES reportam dados encorajadores na depleção efetiva de células B (Anolik et al., 2004; Looney et al., 2004; Leandro et al., 2005; Cambridge et al., 2006; Smith et al., 2006). Esses estudos indicam que a melhora clínica foi associada à depleção de células B circulantes, enquanto que a restauração da população dessas células B prevê recaída nos pacientes. Houve também redução nos títulos de anticorpos anti-DNA, mas não anti-RBP (proteínas que se ligam a RNA) e anticorpos antimicrobianos, sugerindo que os anticorpos anti-DNA são preferencialmente gerados por plasmoblastos de vida curta. Entretanto, resultados de ensaios clínicos placebo-controles fase II/III aleatórios duplo cego de rituximab em LES

não mostraram diferença significativa na resposta clínica da doença como determinado usando a medida de avaliação do *British Isles Lupus Assessment Group* (BILAG) (Merrill et al., 2010). Em contrapartida, consistente com os estudos anteriores, pacientes tratados com rituximab que eram positivos em níveis basais de auto-anticorpos mostraram redução nos níveis de auto-anticorpos anti-DNA e também de anti-cardiolipina depois do tratamento sem redução em outros auto-anticorpos, novamente apoiando a noção de diferente suscetibilidade de plasmócitos auto-reativos ao rituximab (Tew et al., 2010). Essas mudanças foram também associadas com a normalização de níveis séricos C3 e contagem de plaquetas.

Pelo fato de muitas observações indicarem um papel crucial do sistema de IFN tipo I na etiologia e patogênese do LES, várias companhias estão desenvolvendo terapias com o objetivo de inibir a produção ou efeitos desse sistema. Esse desenvolvimento tem sido estimulado pelo fato de modelos murinos de LES *knockout* para IFN tipo I apresentarem atividade reduzida da doença (Braun et al., 2003; Santiago-Raber et al., 2003). Resultados de ensaios clínicos de fase I usando uma única injeção de anticorpos monoclonais anti-IFN tipo I em pacientes têm sido reportados (Wallace, 2007; Yao et al., 2009). O tratamento anti-IFN-alfa causou uma inibição dose-dependente da expressão de genes induzíveis por IFN tipo I em ambos sangue periférico e biopsia de pele, bem como na redução da atividade clínica da doença. Além disso, a expressão dos genes de GM-CSF, TNF-alfa, IL-10 e IL-1-beta e BAFF foi diminuída a níveis menores em alguns pacientes (Yao et al., 2009), demonstrando a interação entre o sistema IFN tipo I e outras rotas pró-inflamatórias. A observação de que uma única injeção de anticorpos anti-IFN-alfa poderia neutralizar de maneira sustentada a assinatura de IFN é de interesse particular e apóia a visão de que a produção contínua de IFN no LES é pelo menos parcialmente o resultado de um círculo vicioso (Ronnblom and Alm, 2001a). O aumento da freqüência de infecções virais sérias, um potencial efeito colateral deste tratamento, não foi reportado até hoje entre pacientes tratados com anti-IFN-alfa. Isso pode ser devido ao fato que, além do IFN-alfa, existem outros IFN tipo I com forte atividade antiviral (Pestka et al., 2004). Se estes IFN tipo I serão suficientemente potentes na proteção de pacientes com LES tratados com anti-IFN-alfa com complicações sérias ainda não está bem estabelecido e pode somente ser verificado em ensaios clínicos maiores. Ainda existem outros alvos terapêuticos possíveis no sistema IFN tipo I, como IFNAR, antígeno BDCA-2 em pDC (Dzionek et al., 2001; Blomberg et al., 2003) ou oligodesoxirribonucleotídeos ou

oligorribonucleotídeos antagonistas de TLR (Barrat and Coffman, 2008). Nenhum desses alvos foi testado ainda em pacientes com LES.

O desenvolvimento de antagonistas para TLRs que se ligam a ácidos nucléicos tem se mostrado um processo fastidioso devido a similaridade entre os ácidos nucléicos eucarióticos e procarióticos. Apesar dessa dificuldade, já foi relatado o desenvolvimento de seqüências de DNA imunorregulatórias (IRS) que podem se ligar ao TLR9 e inibir sua ativação e efeitos posteriores à ligação ao receptor. Foi mostrado que esses oligonucleitídeos (ODN) podem amenizar a inflamação em múltiplos cenários. Camundongos injetados com seqüências imunoestimulatórias e D-galactosamina desenvolveram inflamação severa e morreram em poucos dias. Entretanto, quando co-injetados com IRS, a inflamação diminuiu e os camundongos sobreviveram por mais tempo (Barrat et al., 2005). Experimentos similares demonstraram o mesmo efeito sobre TLR7. Adicionalmente a esses estudos, o mesmo grupo de pesquisa desenvolveu um inibidor ambíguo para TLR7/9. Esses ODNs foram suficientes para a inibição da sinalização de ambos TLR7 e TLR9 e proteção contra inflamação. Essas seqüências IRS também inibiram a produção de IFN-alfa por pDC humanas, indicando a efetividade desses inibidores em células humanas (Duramad et al., 2005). Justamente por essa efetividade, Barrat e colegas (2007) também investigaram a capacidade de ODNs para tratar camundongos propensos ao LES (Barrat et al., 2007). Vários estudos estabeleceram previamente o papel do IFN-alfa na progressão da auto-imunidade na progênie de camundongos (NZB x NZW) (Rozzo et al., 2001; Santiago-Raber et al., 2003). Já que o IFN-alfa parece ter um papel central no LES humano (Crow, 2007), essa linhagem de camundongos tem sido selecionada como um modelo ideal para a aplicação de IRS. A injeção de IRS, duas vezes por semana, em camundongos F1 (NZB x NZW) resultou na diminuição dos níveis de anticorpos antinucleares, redução de glomerulonefrite em nove meses e aumento da taxa de sobrevivência (Barrat et al., 2007).

Além dos estudos de Barrat, outros dois grupos usaram diferentes ODNs inibitórios tendo como alvo TLRs em LES. Dong e colegas (2005) injetaram ODNs em camundongos F1 (NZB x NZW) que foram subseqüentemente analisados para a função renal e produção de auto-anticorpos característicos de LES. Os resultados sugeriram capacidade dos ODNs inibitórios em minimizar a glomerulonefrite e reduzir os níveis de auto-anticorpos dirigidos contra o DNA (Dong et al., 2005). Pawar e colegas (2007) usaram IRS em camundongos $MRL^{lpr/lpr}$ (linhagem de camundongos que desenvolve

LES severo caracterizado por forte produção de auto-anticorpos e linfoproliferação massiva) e observaram uma redução nos níveis de citocinas inflamatórias e títulos de auto-anticorpos, bem como diminuição do dano tecidual (Pawar et al., 2007). Esses experimentos usando IRS no tratamento de modelos murinos propensos a desenvolver LES sugerem forte potencial no tratamento do LES humano e de outras doenças auto-imunes tendo como alvo TLRs.

1.2 Receptores Toll-Like

Os TLRs são conservados desde *Caenorhabditis elegans* até mamíferos como proteínas transmembrana (Janeway and Medzhitov 2002; Hoffmann 2003; Oshiumi, Matsumoto et al. 2003; Akira and Takeda 2004; Beutler 2004). Toll, o primeiro membro descrito da família TLR foi inicialmente identificado como um produto essencial para o desenvolvimento da polaridade dorsoventral embrionária em *Drosophila*. Mais tarde, foi mostrado que essa proteína tem um papel crítico no sistema imunológico, participando na resposta contra fungos (Lemaitre, Nicolas et al. 1996). TLRs são receptores glicoprotéicos integrais de membrana, do tipo I, caracterizados por domínios extracelular contendo motivos repetidos rico em leucina (LRR) e um domínio de sinalização citoplasmática homólogo ao do receptor de interleucina do tipo I (IL-1R), denominado domínio de homologia Toll/IL-1R (TIR) (Bowie and O'Neill, 2000). O motivo LRR é composto por 19-25 LRR em *tandem*, cada um 24-29 aminoácidos de comprimento, contendo motivos XLXXLXLXX bem como outros resíduos de aminoácidos conservados (XØXXØXXXXFXXLX; Ø= resíduo hidrofóbico). Cada LRR consiste de uma folha beta pregueada e uma alfa hélice conectadas por alças. Baseado nas seqüências primárias, TLRs podem ser divididos em várias subfamílias, cada qual reconhece padrões moleculares associados a patógenos (PAMPs) relacionados: a subfamília composta por TLR1, TLR2 e TLR6 reconhece lipídios, enquanto que TLR3, TLR7, TLR8, TLR9 reconhecem ácidos nucléicos. Entretanto, alguns podem reconhecer várias estruturas não relacionadas, apresentando uma grande variedade de ligantes.

Os TLRs são expressos em várias células do sistema imunológico, incluindo macrófagos, DC, células B, subtipos específicos de células T e mesmo em células que não são dos sistema imunológico como fibroblastos e células epiteliais. A expressão de TLRs não é estática, e sim modulada rapidamente em resposta a patógenos, citocinas e estresses ambientais. Além do mais, TLRs são expressos extra ou intracelularmente. Enquanto

certos TLRs (TLR1, 2, 4, 5 e 6) são expressos na superfície, outros (TLR3, 7, 8 e 9) são encontrados quase que exclusivamente em compartimentos intracelulares tais como endossomos, e seus ligantes, principalmente ácidos nucléicos, requerem internalização no endossomo antes que a sinalização seja engatilhada.

Os TLRs ativam as mesmas moléculas de sinalização que são usadas para a sinalização do IL-1R (Akira and Takeda 2004). A estimulação de células com ligantes de TLRs recruta proteínas adaptadoras, tal como MyD88, à porção citoplasmática dos TLRs através de interações homofílicas dos seus domínios TIR. Isso resulta numa cascata de sinalização e produção de citocinas pró-inflamatórias e quimiocinas. As células que expressam TLRs são prioritariamente APCs tais como DCs e macrófagos, que fagocitam patógenos. APCs também ativam a resposta imune através da indução de migração do local de inflamação para a região do linfonodo, onde apresentam antígenos derivados de patógenos para células T $CD4^+$ virgens. Ao mesmo tempo, DCs ativados expressam moléculas coestimulatórias essenciais para a ativação de células T e podem induzir a diferenciação de células T $CD4^+$ virgens em células Th1 ou Th2. Células Th1 produzem principalmente IFN-tipo I e medeiam a resposta dirigida contra infecções bacterianas e virais, enquanto que células Th2, que produzem principalmente IL-4 e IL-13 estão envolvidas predominantemente na resposta contra infecções por helmintos. A estimulação de TLRs leva preferencialmente à diferenciação em Th1.

1.2.1. Envolvimento dos TLR 7, 8 e 9 na gênese do LES

A principal característica do LES é a alta produção de auto-anticorpos antinucleares. Uma hipótese que busca explicar esse evento leva em consideração a influência da apoptose incitando ou propagando a autoimunidade (Albert, 2004). A ocorrência de apoptose excessiva em pacientes com LES parece ser responsável pela liberação de ácidos nucléicos, histonas e outros antígenos intracelulares que promovem resposta imune (Rosen and Casciola-Rosen, 1999). Defeitos na eliminação desses resíduos apoptóticos podem promover a liberação de antígenos, que estariam normalmente seqüestrados, levando a uma resposta imune inadequada (Carroll, 2004). Esses resíduos se ligam a anticorpos, formando imunocomplexos, e esses anticorpos são reconhecidos por receptores específicos na superfície de fagócitos. Assim que são interiorizados num endossomo, os imunocomplexos são desfeitos e algumas moléculas são reconhecidas. Dentre elas, DNA é reconhecido por TLR9 e RNA por TLR7 e TLR8. Esse reconhecimento gera uma rota de sinalização que ativa fatores de transcrição pró-

inflamatórios (Marshak-Rothstein 2006; Baccala, Hoebe et al. 2007) e produção de citocinas pró-inflamatórias amplamente associadas à morbidade do LES (Ronnblom and Alm 2001; Ronnblom and Alm 2001; Pascual, Banchereau et al. 2003). Nos últimos tempos, um novo paradigma surgiu e fornece novas hipóteses no porque o sistema imune é tão especificadamente direcionado contra antígenos associados a RNA e DNA no LES. A base para esse paradigma pode estar nos TLRs, que por algum desvio de função ou expressão, podem engatilhar processos de auto-imunidade (Marshak-Rothstein, 2006).

As únicas células humanas que expressam constitutivamente TLR7 e TLR9 são as células dendríticas plasmocitóides (pDCs) (Hornung, Rothenfusser et al. 2002), mas outras células do sistema imune, se induzidas, também podem expressá-los. As pDCs constituem uma pequena porção das células mononucleares do sangue periférico, mas são responsáveis por grande parte da produção de IFN–alfa e IL-6 (Ronnblom and Alm, 2001b) em resposta a imunocomplexos (Marshak-Rothstein 2006; Baccala, Hoebe et al. 2007). Esse IFN-alfa estimula uma série de eventos que promovem a liberação de mais autoantígenos potenciais, ao mesmo tempo em que aumenta a sobrevivência e ativação de células pró-inflamatórias (Blanco, Pitard et al. 2005). O IFN-alfa e IL-6 também promovem ativação de linfócitos B em plasmócitos, células secretoras de anticorpos. Linfócitos B específicos para autoantígenos presentes nos resíduos apoptóticos podem ser preferencialmente ativados como resultado da estimulação através do receptor de célula B (BCR) e TLR7/9. Essa estimulação induz proliferação, diferenciação e troca de classes de imunoglobulina de linfócitos B, de maneira independente de linfócitos T (Marshak-Rothstein 2006; Baccala, Hoebe et al. 2007), gerando assim plasmócitos secretores de auto-anticorpos. Os auto-anticorpos resultantes perpetuam a formação de imunocomplexos e sustentam um ciclo vicioso de auto-imunidade (Kim et al., 2009).

Células T autorreativas que não são eliminadas no timo são controladas na periferia por DCs imaturas (tolerância periférica). No estado de equilíbrio (*steady state*), DCs imaturas capturam células apoptóticas e migram, sem amadurecer, para o linfonodo. No linfonodo, apresentam os peptídios próprios, na ausência de moléculas coestimulatórias, para células T virgens autorreativas, resultando em anergia ou em deleção. DCs imaturas podem também controlar a tolerância periférica através da indução e manutenção de células Treg. Tais mecanismos de tolerância previnem ou reduzem o desenvolvimento de auto-imunidade quando células apoptóticas são geradas ou processadas no momento da infecção. Banchereau e Pascual (2006) propõem que a ativação 'inapropriada' de DCs levaria a uma quebra da tolerância periférica (Banchereau and Pascual, 2006). Monócitos

representam a maior fonte de DCs sob condições inflamatórias e a combinação de IFN tipo I e GM-CSF os conduziria à diferenciação em DCs. De fato, monócitos de pacientes com LES se comportam como Células Dendríticas mielóides (mDC). A exposição de monócitos normais ao soro de pacientes com LES resulta na geração de DCs (Blanco et al., 2001), contribuindo para a produção de altas taxas de IFN tipo I. Assim, o sangue de um paciente com LES, pela alta quantidade de IFN tipo I, representa um ambiente indutor de DCs. A constante maturação de DCs poderia levar a uma ativação e expansão de células T autorreativas, explicando assim muitas características da doença. As células derivadas de monócitos expressam TLR8 e, na presença de IFN tipo I e do estímulo para esses TLR esses fatores se tornam indispensáveis para a maturação dessas células. As DCs derivadas de monócitos apresentam um papel muito importante no processamento/ eliminação de antígenos realizando o *crosspriming*, ou seja, apresentando autoantígenos e antígenos de células apoptóticas a células T CD8+, levando à produção de IL-6 e de IL-12p40, uma citocina crucial para o desenvolvimento de respostas Th1 (Wong et al., 2008).

Seguindo os raciocínios expostos acima, o papel fundamental dos TLRs, nas condições do LES, deve relacionar-se principalmente à ativação de células B e DC e subseqüente produção de citocinas pró-inflamatórias. Essas citocinas formam um ambiente propício para que haja um ciclo vicioso completado pela geração de autoantígenos e alta produção de auto-anticorpos patogênicos de alta afinidade (Banchereau and Pascual, 2006). Diferentes estudos estão em andamento com o objetivo de identificar antagonistas ou inibidores de TLR (Krieg and Vollmer 2007; Barrat and Coffman 2008; Bauer, Pigisch et al. 2008; Diebold 2008), visando reduzir a produção de IFN tipo I no LES (Blanco et al., 2001).

Devido ao LES ser caracterizado pela reatividade a ácidos nucléicos e a atividade dos TLRs citados acima [(i) os TLR7, TLR8 são específicos para reconhecimento de RNAs e TLR9 para o reconhecimento de DNA, (ii) eles direcionam o desenvolvimento de respostas pró-inflamatórias, através do IFN-alfa e (iii) fazem uma ligação complexa entre o sistema imune inato e adquirido], torna-se de vital importância analisar esses genes e suas variantes mais comuns no desenvolvimento, susceptibilidade e sintomatologia do LES.

1.2.2. Imunogenética dos *TLR7/8/9*

TLR7 e TLR8 são homólogos e têm o TLR9 como membro mais próximo evolutivamente (Chuang and Ulevitch 2000; Du, Poltorak et al. 2000). Ambos têm

afinidade por RNA e compartilham a cascata de sinalização celular, porém diferem no padrão de expressão tecidual (Chuang and Ulevitch 2000; Du, Poltorak et al. 2000; Diebold, Kaisho et al. 2004; Heil, Hemmi et al. 2004; Lund, Alexopoulou et al. 2004).

O gene *TLR7* localiza-se no cromossomo X, na região Xp22.3-p22.2 em humanos e possui 3 éxons. O polimorfismo rs179008 (Gln11Leu), localizado no éxon 3, corresponde a uma troca de uma adenina por uma timina e resulta na troca de um resíduo de Glutamina por um de Leucina na posição 11 da proteína. Uma análise *in silico* revelou que essa troca está localizada na seqüência peptídeo sinal, estendendo a região hidrofóbica e provavelmente afetando o processamento do TLR7 na membrana do Retículo Endoplasmático (Moller-Larsen et al., 2008). Oh e colegas (2009) viram que a variante 11Leu está relacionada com o aumento da carga viral, progressão acelerada da infecção por HIV, aumento da suscetibilidade a HIV-1 em mulheres e com diminuição na produção de IFN-alfa após estimulação de células de sangue periférico de controles saudáveis com o ligante de TLR7 imiquinod (Oh et al., 2009). Além disso, essa mesma variante foi associada com o aumento da suscetibilidade à infecção por HCV e com resposta não favorável à terapia baseada em IFN-alfa em mulheres infectadas por HCV (Schott et al., 2008). Um estudo recente, também com HCV, viu que o alelo 11Leu está associado com a presença de agregados linfóides no trato portal de pacientes infectados por HCV e com a menor expressão do mRNA de IL-29/IFN-lambda e de subunidades de IL-10R e IL-28R no fígado de mulheres homozigotas para 11Leu e homens 11Leu (Askar et al., 2010). Apesar dos poucos estudos existentes, esse polimorfismo já foi associado com fenótipos inflamatórios como a asma (Moller-Larsen et al., 2008) e degeneração macular relacionada à idade (Edwards, Chen et al. 2008), apesar de ter sido descrito como não associado com artrite reumatóide (Coenen et al., 2010). Dois estudos foram realizados com LES e *TLR7*. Um deles analisou o polimorfismo rs179008 e foi realizado em uma amostra de pacientes espanhóis caucasóides. Este estudo não encontrou associação nem com a suscetibilidade ao LES nem com a sintomatologia da doença (Sanchez, Callejas-Rubio et al. 2009). No outro, foram analisados vários polimorfismos em uma amostra de pacientes chineses e japoneses e foi visto que o polimorfismo rs3853839, localizado na região 3'UTR, está associado ao LES, tendo um efeito maior em homens (Shen et al., 2010)

O gene *TLR8* situa-se no cromossomo X, aproximadamente 16kb a jusante do gene do TLR7 e possui 3 éxons. Estudos de ligação analisando a região que os genes *TLR7* e *TLR8* estão localizados, revelou um grau de desequilíbrio de ligação muito baixo entre

eles, indicando que as associações observadas para quaisquer polimorfismos nestes genes devem representar sinais independentes (Edwards, Chen et al. 2008; Moller-Larsen, Nyegaard et al. 2008). A transcrição de *TLR8* dá origem a duas isoformas de TLR8, denominadas TLR8v1 e TLR8v2, com sítios de início de tradução alternativos (Chuang and Ulevitch, 2000; Du et al., 2000). Recentemente, Gantier e colegas (2010) analisaram o polimorfismo rs3764880 (Met1Val), cuja variante 1Val troca o códon de início traducional, e viram que não há alteração na taxa transcricional do gene. Foi visto que a variante 1Val controla a tradução fazendo com que o transcrito TLR8v1 seja mais traduzido que o TLR8v2, além da função dos transcritos não depender da porção N-terminal (Gantier et al., 2010). Um ensaio de superexpressão *in vitro* mostrou que a variante 1Val acarreta um diminuição na produção de NF-kappaB, resultando em um estado de menor ativação do sistema imune (Oh, Taube et al. 2008). Nesse mesmo estudo, analisando uma amostra de indivíduos infectados com HIV, essa variante foi associada a um efeito positivo no curso da infecção. Poucos estudos foram realizados com essa variante, dentre eles, destacam-se os realizados em pacientes com tuberculose (Davila, Hibberd et al. 2008), asma (Moller-Larsen et al., 2008), suscetibilidade à infecção pelo vírus da febre Crimean-Congo (Engin et al., 2010) e o estudo anteriormente citado, no curso da infecção com HIV (Oh, Taube et al. 2008).

O gene *TLR9* está localizado no cromossomo 3, numa região descrita como de susceptibilidade para o LES (Kelly et al., 2002) e tem somente 2 éxons, sendo o segundo responsável pela maior porção codificante (Du et al., 2000). Um estudo de exploração no *TLR9* conduzido por Lazarus (2003) encontrou que os polimorfismos rs5743836 (T-1237C) e a rs352140 (G2848A) distinguiam os quatro haplótipos mais freqüentes em amostras populacionais de diferentes etnias (Lazarus et al., 2003) e desde então esses polimorfismos são bastante estudados. Estudos acerca do polimorfismo rs5743836 mostraram que a variante -1237C insere um potencial sítio de ligação do fator de transcrição NF-kappaB no promotor do gene *TLR9* (Hamann et al., 2006). Mais tarde, um estudo conduzido por Novak e colegas (2007) mostrou que a sequência do alelo T leva a aumento da atividade do promotor em relação à do alelo C (P=0,018) (Novak et al., 2007). Mais tarde, Ng e colegas (2010) realizaram experimentos acerca da atividade do promotor e não acharam os mesmos resultados que o grupo de Novak. Eles não observaram diferença na atividade do promotor, porém o alelo -1237C era mais expresso em resposta a estímulos que resultam na ativação de NF-kappaB ($P \leq 0.001$) e esse aumento na atividade do promotor é devido à ligação de NF-kappaB à seqüência do alelo -1237C (Ng et al.,

2010). A variante -1237C já foi associada com doenças inflamatórias como a asma (Lazarus, Klimecki et al. 2003) e Doença de Crohn's (Torok et al., 2004). Quanto ao polimorfismo rs352140, ele consiste na troca de um G por um A na posição +2848 do éxon 2 e é uma variação silenciosa. O genótipo AA tem sido associado a maior expressão do gene e maior freqüência de células B positivas para IgM intracelular (Kikuchi et al., 2005), mas não há ainda estudos considerando a conseqüência funcional dessa variante. Alguns estudos avaliaram essas variantes em diferentes situações, como alergia (Noguchi, Nishimura et al. 2004; Berghofer, Frommer et al. 2005), suscetibilidade a infecções (Carvalho et al., 2008), doenças inflamatórias como asma (Lazarus, Klimecki et al. 2003; Noguchi, Nishimura et al. 2004; Lachheb, Dhifallah et al. 2008; Smit, Siroux et al. 2009) e arteriosclerose (Hamann et al., 2006). A respeito do LES, os resultados se mostraram contraditórios, provavelmente devido a diferentes *backgrounds* genéticos das populações. Estudos com amostras populacionais provenientes do leste asiático encontraram associação de variantes do *TLR9* (Hur, Shin et al. 2005; Demirci, Manzi et al. 2007; Tao, Fujii et al. 2007; Xu, Zhang et al. 2009) enquanto que estudos com amostras populacionais com ascendência Européia não encontraram associação com LES (De Jager et al., 2006; Demirci et al., 2007) . Nada ainda foi relatado a respeito da população brasileira com *TLR7/8/9*.

2. OBJETIVOS

Considerando a carência de estudos genéticos que buscam correlação entre as variantes dos genes *TLR7*, *TLR8* e *TLR9* em populações humanas, o presente projeto tem como objetivo estabelecer a freqüência das variantes rs179008 (Gln11Leu) do *TLR7*, rs3764880 (Met1Val) do *TLR8*, rs5743836 (T-1237C) e rs352140 (G2848A) do *TLR9* e dos haplótipos formados pelas duas últimas variantes citadas em um grupo de pacientes com LES do sul do Brasil.

Além disso, serão abordadas possíveis correlações entre essas variantes e haplótipos com a morbidade associada ao LES. O Laboratório de Imunogenética possui dados clínicos relevantes dos pacientes no que diz respeito ao período anterior e durante o tratamento, assim a associação dessas variantes com a sintomatologia clínica se torna muito importante.

CAPÍTULO I

3. ARTIGO CIENTÍFICO

Artigo em fase de preparação a ser enviado à revista científica Lupus

TLR7/8/9 polymorphisms and their associations in Systemic Lupus Erythematosus patients from Southern Brazil: a case control study and review of the literature

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Abstract

SLE is a chronic inflammatory autoimmune disease and can affect several organs and systems. It is characterized by high production of autoantibodies against nuclear compounds. TLR7/8/9 are responsible for nucleic acid recognition and they signalize to proinflamatory responses, through activation of NK-kappaB and Type I IFN production, making a bridge between the innate and the adaptative immune systems. We analyzed the frequency of *TLR7*,rs179008, *TLR8* rs3764880, *TLR9* rs5743836 and rs352140 in 370 SLE patients and 415 healthy controls from southern Brazil. All analyses were conducted according gender and ethnicity. Genotypic and allelic frequencies were different for *TLR7* rs179008 (0.253 vs. 0.163, P=0.020 and P=0.003, OR for T allele: 1.74 CI 95% 1.12-2.70) and *TLR9* rs5743836 (0.174 vs. 0.112, P=0.045 and P=0.017, OR for C allele: 1.59, CI 95% 0.99-2.57) between European-derived women groups. A higher frequency was observed to presence of Anti-SSa/Ro for *TRL9* rs5743836 C allele carriers (0.228 vs 0.126, Bonferroni corrected P=0.06). No statistical differences were found for *TLR9* haplotypic analyses. We suggest that *TLR7* rs179008 and *TLR9* rs5743836 can be considered SLE susceptibility factors for European-derived women in our population.

Keywords: nucleic acid recognition, autoimmunity, haplotypes, toll-like receptor, European-derived, African-derived.

Introduction

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease that affects many organs and systems. SLE is characterized by dysregulation in the production of antibodies, leading to high titres of autoantibodies, especially antinuclear antibodies such as anti-DNA, anti-RNA and anti-RNP. These antibodies lead to the formation and deposition of immunocomplexes, resulting in intense inflammatory response and tissue damage. Like other autoimmune diseases, SLE affects mainly women, in a 9:1 rate, probably due to hormonal effects (Cooper et al., 1998; Lahita, 1999). The causes of SLE are still unknown although genetic, immunological and environmental factors are certainly involved in its development.

One aspect largely related with SLE pathology is Type I Interferon (IFN) production (Block et al., 1975; Blanco et al., 2001; Banchereau and Pascual, 2006). Type I IFN is involved in typical immune responses against viruses and it can be released by various cell types, especially antigen presenting cells (APCs) (Blanco et al., 2001; Gilliet et al., 2008). One way to produce high amounts of Type I IFN is through APCs, when they recognize Toll-Like Receptors (TLRs) ligands (Bauer et al., 2008). TLRs are the best studied pattern-recognition receptors and, in SLE context, TLR7/8/9 stand out. TLR7 and TLR8 recognize RNA (Heil et al., 2004) and TLR9 recognizes DNA (Haas et al., 2008). TLR7 and TLR9 are expressed in both B and plasmacytoid Dendritic Cells (pDC), and are involved in 95% of all Type I IFN produced. TLR8 is expressed in monocyte-derived cells, such as macrophages and myeloid DC (mDC) (Jarrossay et al., 2001; Hornung et al., 2002).

It was suggested that the presence of high titres of autoantibodies against antinuclear antigens in SLE patients involves excessive apoptosis. This excessive apoptosis could release the nuclear autoantigens who leads to immunocomplexes formation (Rosen and

Casciola-Rosen, 1999). As soon as, the immunocomplexes are internalized by APCs, RNA can be recognized by TLR7 and TLR8 and DNA by TLR9, activating a signaling pathway that leads to the release of Type I IFN and other proinflamatory cytokines. Thus, in principle, TLR function or expression dysregulation could trigger an autoimmune process. As SLE is characterized by reactivity against nucleic acids and as TLR7/8/9 are responsible for their recognition, they are interesting study targets in SLE.

The *TLR7* gene is located on Xp22.3-p22.2. It harbors an interesting polymorphism, rs179008, who leads to the exchange of a Gln (A allele) to a Leu (T allele) at position 11 in the peptide, which according to a prediction made by Moller-Larsen and colleagues, shortens the TLR7 protein N region and extends the hydrophobic region within the signal sequence, indicating that it can affect TLR7 processing (Moller-Larsen et al., 2008). An elegant work carried out in HIV positive patients shown, *ex vivo,* that the presence of the Leu variant was associated to decreased IFNα but normal IL-6 production (Oh et al., 2009).

TLR8 is located on chromosome X, 16kb away from *TLR7*, with little linkage disequilibrium between them (Edwards et al., 2008; Moller-Larsen et al., 2008). This gene encodes two splice variants (TLR8v1 and TLR8v2) with alternative translation start sites (Chuang and Ulevitch, 2000; Du et al., 2000). Gantier and colleagues showed that the protein expression control is fine tuned by rs3764880, a polymorphism that leads to an 'A' to 'G' exchange at the first codon position, being the 'G' allele responsible for increasing TLR8v1 translation without changes on mRNA levels or protein function (Gantier et al., 2010). Interestingly, an earlier study showed, through overexpression assay, that the 'G' allele leads to a decreased NF-kappaB release and this can result in a lesser activation state of the immune system, leading to a slower clinical natural course of the disease in HIV

patients(Oh et al., 2008). ‘G’ allele was also related to protection against active tuberculosis damages (Davila et al., 2008).

TLR9 is located on 3p21.3. Lazarus et al scanned the gene and a total of 20 SNPs were identified, albeit two of them are enough to distinguish the four common *TLR9* haplotypes: rs5743836 and rs352140 (Lazarus et al., 2003). The former, is located in the promoter at the position -1237 and corresponds to a T to C exchange, creating a potential-binding site for NF-kappaB (Hamann et al., 2006). This polymorphism has been implicated in chronic inflammatory diseases including asthma (Lazarus et al., 2003) and Crohn’s Disease (Torok et al., 2004). The rs352140 polymorphism is located at position +2848 in the exon 2, corresponds to a G to A exchange, and does not alters the amino acid sequence. The AA genotype has already been associated with high TLR9 expression and intracellular IgM in B cells in patients with primary biliary cirrhosis (Kikuchi et al., 2005) but to our knowledge there is no studies reporting functional assays.

Considering the inflammatory status and the high levels of antinuclear autoantibodies in SLE patients and considering the role of TLR7/8 and 9 on the immune system activation, the present study aims to analyze the frequency of the polymorphisms rs179008 in *TLR7*, rs3764880 in *TLR8*, rs5743836 and rs352140 in *TLR9* among SLE patients and healthy controls from southern Brazil, looking for a possible association of these variants with clinical and laboratory expression of the disease. A possible participation of *TLR7* rs179008, *TLR9* rs5743836 and a bias of the latter with the presence of anti-SSa/Ro in women European-derived SLE patients will be presented and discussed here.

Materials and Methods

Study Population

The study population was comprised of 370 SLE patients: 342 (92.4%) women and 28 (7.6%) men; 282 (76.2%) identified as European-derived and 88 (23.8%) as African-derived. This classification was based on physical appearance, as judged by the researcher at the time of blood collection, and data about the ethnicity of parents/grandparents were reported by the participants. The issue arisen on the skin color-based classification criteria that is used in Brazil is well documented (Parra et al., 2003) and has been already assessed by our group in previous studies (Vargas et al., 2006; da Silva et al., 2010). Also, a recent study assessing individual interethnic admixture and population substructure by means of a panel composed of 48-insertion-deletion ancestry-informative markers has validated this classification in European-derived individuals from our region (Santos et al., 2010). The patients have received follow-up care at the Division of Rheumatology of the Hospital de Clínicas de Porto Alegre. All patients fulfilled the American College of Rheumatology revised criteria for the classification of SLE (Hochberg, 1997).

Clinical manifestations of SLE included the presence of photosensitivity, malar rash, discoid rash, oral or nasal ulcers, arthritis, serositis (pleuritis or pericarditis), nephritis and neurological diseases defined as seizures or psychosis. The laboratory evaluation included the presence of hematological disorders (hemolytic anemia, leukopenia, lymphopenia or thrombocytopenia), positive antinuclear antibody (ANA) (titer>1:100), or other autoantibodies such as anti-dsDNA, anti-Sm, anti-RNP, anti-Ro/SS-A, anti- La/SS-B, anticardiolipin, lupus anticoagulant and false positive VDRL. The patients were also evaluated in regard to secondary antiphospholipid syndrome and Sjogren's syndrome, according to the classification criteria for both disease (Vitali et al., 2002; Miyakis et al., 2006), SLEDAI (Bombardier et al., 1992) and SLICC damage index (Gladman et al., 1996) were applied to each patient as a measurement of disease activity and cumulative damage, respectively.

The control group was composed of 415 healthy people from the same urban center: 191 (46%) were women, 224 (54%) men; 309 (74.4%) individuals are European-derived and 106 (25.6%) African-derived. The study protocol was approved by the Ethics Committee of the Hospital de Clínicas de Porto Alegre and informed consent according to the Declaration of Helsinki was obtained from all patients.

Genotyping

DNA was isolated using salting out method (Lahiri and Nurnberger, 1991) and stored at -20°C. The polymorphisms *TLR7* rs179008, *TLR8* rs3764880 and *TLR9* rs352140 were amplified using protocol as described as Cheng and colleagues (Cheng et al., 2007). To genotype, it was used the restriction endonucleases *Apo*I, *Nla*III and *BstU*I, respectively. After treatment with restriction endonucleases, *TLR7* rs179008 and *TLR9* rs352140 cleavages were visualized in 6% polyacrylamide gel and *TLR8* rs3764880 was visualized in 8% polyacrylamide gel and all of them were stained with silver nitrate. About *TLR9* rs5743836, it was genotyped as described by Carvalho and colleagues (Carvalho et al., 2007) and visualized in 1.5% agarose gel stained with SYBR gold ®.

Statistical Analysis

A descriptive analysis of data through calculation of mean and standard deviation for quantitative variables was performed while the frequency and percentage were calculated for categorical data. We used the chi-square test or Fisher's exact test in the comparison between the presence and absence of polymorphic variants. Besides these tests, we calculated the odds ratio and confidence intervals. For the comparison of clinical and laboratory variables with the presence or absence of polymorphic variants, we used the chi-square test to compare qualitative variables and the Student's t test (or Mann-Whitney)

for quantitative variables, using the Bonferroni correction to the level of statistical significance. The Hardy-Weinberg equilibrium test was held in cases and controls using the chi-square test. Data were analyzed with SPSS 15.0 and WinPEPI version 11.1. Two-tailed value of $P<0.05$ was taken to indicate statistical significance.

Results

All analyses were performed with groups subdivided according to gender and ethnic origins since *TLR7* and *TLR8* genes are located at the X chromosome and since the literature already reports different allelic frequencies among European and African-derived populations for some of the analyzed variants. All control groups were in Hardy-Weinberg Equilibrium, although the *TLR7* and *TLR8* genotypic frequencies did not reach equilibrium among the European-derived SLE women.

Allelic and Genotypic Analyzes

Table 1 shows genotypic and allelic frequencies in European-derived individuals. When we compared women European-derived patients to controls, we noticed that *TLR7* rs179008 genotypic (P=0.020) and allelic frequencies were different (0.253 vs. 0.163, P=0.003). In the genotypic frequency analysis, the AA genotype was less represented while TT was overrepresented among patients compared to controls (P=0.013 and P=0.041, respectively). The overall Odds Ratio (OR) for T allele carriers was 1.74 with 95% Confidence Interval (CI) 1.12-2.70. For *TLR9* rs5743836, women European-derived patients and controls also showed different genotypic (P=0.045) and allelic frequencies (0.174 vs. 0.112, P=0.017). In the genotypic analysis, the CC genotype was overrepresented among patients when compared to controls (0.045 vs 0.007, P=0.041). The OR for C allele was 1.59 with 95% CI 0.99-2.57.

Table 2 shows genotypic and allelic frequencies in African-derived individuals. There were no statistical differences for both genotypic or allelic frequencies between groups.

Haplotype Analyzes

Since two polymorphisms were analyzed in *TLR9*, rs5743836 in promoter and rs352140 in exon 2, we estimated haplotypic frequencies (Table 3). Comparing patients and controls, no statistical differences were observed on the haplotypic frequencies, although a bias was observed between women European-derived patients and controls (P=0.082). Differences due to the ethnic origin of individuals become quite evident when European-derived and African-derived groups were compared. For instance, African-derived individuals have an overall CG haplotypic frequency higher than European-derived individuals.

Discussion

TLR7/8/9 are nucleic acid receptors involved in NF-kappaB activation and in the induction of Type I IFN. These receptors play an important role in activation and regulation of DC and B cells, which are responsible for pathogen clearance, antigen recognition and antibody production, critical findings in SLE.

TLR7

There are evidences indicating that TLR7 is involved on autoimmunity development. Studies in congenic mice bearing the Y-linked autoimmune accelerator (yaa) lupus susceptibility locus, have showed that differences in *Tlr7* expression as well as in environmental factors that induce TLR7 responses may result in increased B cell sensitivity to RNA-containing autoantigens (Pisitkun et al., 2006; Subramanian et al.,

2006). Also, transgenic mice with a two fold increased *TLR7* expression show increased production of RNA-related autoantibodies and spontaneous developed autoimmunity (Deane et al., 2007). Nevertheless, in humans, association studies with *TLR7* remain controversial: *TLR7* rs3853839 (3'UTR localization) was associated with SLE in Chinese and Japanese populations, with a stronger effect in men when compared to women (Shen et al., 2010). Recently, Kawasaki and colleagues evaluated Japanese SLE women and reproduced the previously association of rs3853839. They also observed associations of two other polymorphisms with SLE: rs179019 and rs179010, supporting the participation of *TLR7* in SLE among Asian populations (Kawasaki et al., 2011). In contrast, a study carried out in Spanish populations did not associated the *TLR7* rs179008 polymorphism with SLE susceptibility (Sanchez et al., 2009).

In the present study we investigated, a putative functional polymorphism in *TLR7* in SLE patients and ethnic-matched controls from the southernmost state of Brazil. Our results revealed an increased frequency of the T allele among European-derived women patients suggesting, for the first time, this variant as a susceptibility factor in SLE. As shown in Table 1, T allele was overrepresented in European-derived women (0.253 vs. 0.163; P=0.003), resulting in OR 1.74 (CI 95% 1.12-2.70) for T carriers, or OR 3.11 (CI 95% 1.19-9.42) for TT vs. CC carriers. The T allele was already associated to higher susceptibility to HCV infection and less response to an IFNα-based therapy in chronic HCV-infected German women (Schott et al., 2008). Furthermore, in a study performed by Oh and colleagues with HIV patients, the same variant was associated with higher viral loads, accelerated progression to advanced immune suppression, increased susceptibility to HIV-1 in women and decreased IFNα production after stimulation of peripheral blood mononuclear cells with imiquinod, a TLR7 ligand (Oh et al., 2009).

TLR8

It is believed that *TLR8* encodes two splice variants with alternative translation start sites (Chuang and Ulevitch, 2000; Du et al., 2000). A recent study showed that rs3764880 fine tunes translation of these two main isoforms in monocytes and that TLR8 biochemical function is independent of the N-terminus region (Gantier et al., 2010). Concerning its relationship with SLE, there are few studies assessing *TLR8* (Komatsuda et al., 2008; Cros et al., 2010) but none evaluates its polymorphisms in the SLE. Our study is the first to study *TLR8* within SLE and our results did not find an association between rs3764880 and SLE susceptibility or clinical symptoms. Nevertheless, these negative results do not discard *TLR8* as a candidate gene in SLE since it plays important roles in the regulation of monocyte-derived cells and it is not well elucidated the function and role of the translated TLR8 isoforms.

TLR9

It is important to remember that Hemmi et al showed that it recognizes bacterial DNA, through CpG motifs (Hemmi et al., 2000) and it remains as a truth until years later when Haas and colleagues showed that the DNA sugar backbone 2' deoxyribose represents a prime determinant for ssDNA-TLR9 interactions (Haas et al., 2008). It means that self DNA can act as a TLR9 ligand suggesting this molecule as potentailly relevant in SLE. We evaluated two polymorphisms in *TLR9*: rs5743836 in the promoter region, and rs352140 in exon 2. The former showed differences in genotypic and allelic frequencies between women European-derived patients and matched controls (P=0.045 and P=0.017, respectively), suggesting C allele as a susceptibility factor in SLE (OR 1.59 CI 95% 0.99-2.57 for C allele carriers). However, studies in SLE patients with European-derived or European genetic background did not observed this association (De Jager et al., 2006; Demirci et al., 2007), whereas studies with Japanese, Korean and Chinese samples did not perform any analysis because of low C allele frequency in

these populations (Hur et al., 2005; Ng et al., 2005; Tao et al., 2007). When clinical symptoms were analized, those patients who present Anti-SSa/Ro (n=92) showed increased frequency of CC genotype compared to those who do not present Anti-SSa/Ro (n=143) (P=0.005 for general comparison and P=0.003 revealing CC genotype increased in Anti-SSa/Ro positive). The overall OR for C allele carriers was 1.83 with CI 95% 1.03-3.22. Nevertheless, this finding lost statistical significance after Bonferroni correction (P=0.06), showing the need for a larger sample. For the exonic polymorphism rs352140, no associations were observed, neither between groups nor according clinical symptoms.

It has been discussed in the literature the functional consequences of rs5743836 C allele. Novak and colleagues showed a significantly higher promoter activity in TT allelic variant sequence compared to CC allelic variant sequence (P=0.018) (Novak et al., 2007). In this case, we can imagine that both variants: T for *TLR7* rs179008, and C for *TLR9* rs5743836 could play an important role in initial SLE susceptibility, where carriers could have increased susceptibility to virus infections, for example, which are largely related to SLE (Hession et al., 2010; Iskra et al., 2010; Pavlovic et al., 2010; Perez-Mercado and Vila-Perez, 2010; Quan et al., 2010; Younesi et al., 2010; Hachfi et al., 2011), and consequently become more prone to SLE. Nevertheless, results from Ng and colleagues are not in agreement with those from Novak et al (Ng et al., 2010). It had already known that C allele creates a potential binding site for NF-kappaB, and these authors show that the C allele has higher promoter activity in response to activators of the NF-kappaB pathway ($P \leq 0.001$). Therefore, we can hypothesize that, when activated, NF-kappaB can bind to *TLR9* promoter enhancing its expression and leaving more TLR9 available in endosomes. Thus, in SLE individuals, less host DNA or other ligand would be necessary to activate pDC and B cells in a TLR9 manner.

We analyzed *TLR9* haplotypes according to gender and ethnicity (Table 3) and no significant frequency deviations between patients and controls were observed. Nevetheless, differences among individuals from distinct ethnic origins become evident. For instance, African-derived individuals have an overall CG haplotypic frequency higher than European-derived individuals. Indeed, a previous study had already suggested a higher frequency of the CG haplotype among African-derived individuals even considering a small sample (n=24) (Lazarus et al., 2003). The haplotypic frequencies of rs5743836 and rs352140 from different human populations can be seen in Table 4. These data reinforces the fact that gene/disease association studies should take into account the genetic/ethnic background of the patients.

In conclusion, we analyzed *TLR7/8/9* polymorphisms in SLE patients from southern Brazil. Genotypic and allelic frequencies were significantly different for *TLR7* rs179008 (P=0.020 and P=0.003, OR for T allele: 1.74 CI 95% 1.12-2.70) and *TLR9* rs5743836 (P=0.045 and P=0.017, OR for C allele: 1.59 CI 95% 0.99-2.57) comparing European-derived SLE and control women groups from Southern Brazil. Therefore, we suggest that these variants can be involved in SLE susceptibility in European-derived women.

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References

1. Cooper GS, Dooley MA, Treadwell EL, St Clair EW, Parks CG, Gilkeson GS. Hormonal, environmental, and infectious risk factors for developing systemic lupus erythematosus. Arthritis Rheum 1998;41(10):1714-24.
2. Lahita RG. The role of sex hormones in systemic lupus erythematosus. Curr Opin Rheumatol 1999;11(5):352-6.
3. Block SR, Winfield JB, Lockshin MD, D'Angelo WA, Weksler ME, Fotino M, et al. Proceedings: Twin studies in systemic lupus erythematosus (SLE). Arthritis Rheum 1975;18(3):285.
4. Blanco P, Palucka AK, Gill M, Pascual V, Banchereau J. Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. Science 2001;294(5546):1540-3.
5. Banchereau J, Pascual V. Type I interferon in systemic lupus erythematosus and other autoimmune diseases. Immunity 2006;25(3):383-92.
6. Gilliet M, Cao W, Liu YJ. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. Nat Rev Immunol 2008;8(8):594-606.
7. Bauer S, Pigisch S, Hangel D, Kaufmann A, Hamm S. Recognition of nucleic acid and nucleic acid analogs by Toll-like receptors 7, 8 and 9. Immunobiology 2008;213(3-4):315-28.
8. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 2004;303(5663):1526-9.
9. Haas T, Metzger J, Schmitz F, Heit A, Muller T, Latz E, et al. The DNA sugar backbone 2' deoxyribose determines toll-like receptor 9 activation. Immunity 2008;28(3):315-23.

10. Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdorfer B, Giese T, et al. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol 2002;168(9):4531-7.

11. Jarrossay D, Napolitani G, Colonna M, Sallusto F, Lanzavecchia A. Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. Eur J Immunol 2001;31(11):3388-93.

12. Rosen A, Casciola-Rosen L. Autoantigens as substrates for apoptotic proteases: implications for the pathogenesis of systemic autoimmune disease. Cell Death Differ 1999;6(1):6-12.

13. Moller-Larsen S, Nyegaard M, Haagerup A, Vestbo J, Kruse TA, Borglum AD. Association analysis identifies TLR7 and TLR8 as novel risk genes in asthma and related disorders. Thorax 2008;63(12):1064-9.

14. Oh DY, Baumann K, Hamouda O, Eckert JK, Neumann K, Kucherer C, et al. A frequent functional toll-like receptor 7 polymorphism is associated with accelerated HIV-1 disease progression. AIDS 2009;23(3):297-307.

15. Edwards AO, Chen D, Fridley BL, James KM, Wu Y, Abecasis G, et al. Toll-like receptor polymorphisms and age-related macular degeneration. Invest Ophthalmol Vis Sci 2008;49(4):1652-9.

16. Chuang TH, Ulevitch RJ. Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. Eur Cytokine Netw 2000;11(3):372-8.

17. Du X, Poltorak A, Wei Y, Beutler B. Three novel mammalian toll-like receptors: gene structure, expression, and evolution. Eur Cytokine Netw 2000;11(3):362-71.

18. Gantier MP, Irving AT, Kaparakis-Liaskos M, Xu D, Evans VA, Cameron PU, et al. Genetic modulation of TLR8 response following bacterial phagocytosis. Hum Mutat 2010;31(9):1069-79.

19. Oh DY, Taube S, Hamouda O, Kucherer C, Poggensee G, Jessen H, et al. A functional toll-like receptor 8 variant is associated with HIV disease restriction. J Infect Dis 2008;198(5):701-9.

20. Davila S, Hibberd ML, Hari Dass R, Wong HE, Sahiratmadja E, Bonnard C, et al. Genetic association and expression studies indicate a role of toll-like receptor 8 in pulmonary tuberculosis. PLoS Genet 2008;4(10):e1000218.

21. Lazarus R, Klimecki WT, Raby BA, Vercelli D, Palmer LJ, Kwiatkowski DJ, et al. Single-nucleotide polymorphisms in the Toll-like receptor 9 gene (TLR9): frequencies, pairwise linkage disequilibrium, and haplotypes in three U.S. ethnic groups and exploratory case-control disease association studies. Genomics 2003;81(1):85-91.

22. Hamann L, Glaeser C, Hamprecht A, Gross M, Gomma A, Schumann RR. Toll-like receptor (TLR)-9 promotor polymorphisms and atherosclerosis. Clin Chim Acta 2006;364(1-2):303-7.

23. Torok HP, Glas J, Tonenchi L, Bruennler G, Folwaczny M, Folwaczny C. Crohn's disease is associated with a toll-like receptor-9 polymorphism. Gastroenterology 2004;127(1):365-6.

24. Kikuchi K, Lian ZX, Kimura Y, Selmi C, Yang GX, Gordon SC, et al. Genetic polymorphisms of toll-like receptor 9 influence the immune response to CpG

and contribute to hyper-IgM in primary biliary cirrhosis. J Autoimmun 2005;24(4):347-52.

25. Parra FC, Amado RC, Lambertucci JR, Rocha J, Antunes CM, Pena SD. Color and genomic ancestry in Brazilians. Proc Natl Acad Sci U S A 2003;100(1):177-82.

26. Vargas AE, Marrero AR, Salzano FM, Bortolini MC, Chies JA. Frequency of CCR5delta32 in Brazilian populations. Braz J Med Biol Res 2006;39(3):321-5.

27. da Silva GK, Guimaraes R, Mattevi VS, Lazzaretti RK, Sprinz E, Kuhmmer R, et al. The role of mannose-binding lectin gene polymorphisms in susceptibility to HIV-1 infection in Southern Brazilian patients. AIDS 2010.

28. Santos NP, Ribeiro-Rodrigues EM, Ribeiro-Dos-Santos AK, Pereira R, Gusmao L, Amorim A, et al. Assessing individual interethnic admixture and population substructure using a 48-insertion-deletion (INSEL) ancestry-informative marker (AIM) panel. Hum Mutat 2010;31(2):184-90.

29. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1997;40(9):1725.

30. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, et al. Classification criteria for Sjogren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis 2002;61(6):554-8.

31. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost 2006;4(2):295-306.

32. Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH. Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE. Arthritis Rheum 1992;35(6):630-40.

33. Gladman D, Ginzler E, Goldsmith C, Fortin P, Liang M, Urowitz M, et al. The development and initial validation of the Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index for systemic lupus erythematosus. Arthritis Rheum 1996;39(3):363-9.

34. Lahiri DK, Nurnberger JI, Jr. A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. Nucleic Acids Res 1991;19(19):5444.

35. Cheng PL, Eng HL, Chou MH, You HL, Lin TM. Genetic polymorphisms of viral infection-associated Toll-like receptors in Chinese population. Transl Res 2007;150(5):311-8.

36. Carvalho A, Marques A, Maciel P, Rodrigues F. Study of disease-relevant polymorphisms in the TLR4 and TLR9 genes: a novel method applied to the analysis of the Portuguese population. Mol Cell Probes 2007;21(4):316-20.

37. Pisitkun P, Deane JA, Difilippantonio MJ, Tarasenko T, Satterthwaite AB, Bolland S. Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication. Science 2006;312(5780):1669-72.

38. Subramanian S, Tus K, Li QZ, Wang A, Tian XH, Zhou J, et al. A Tlr7 translocation accelerates systemic autoimmunity in murine lupus. Proc Natl Acad Sci U S A 2006;103(26):9970-5.

39. Deane JA, Pisitkun P, Barrett RS, Feigenbaum L, Town T, Ward JM, et al. Control of toll-like receptor 7 expression is essential to restrict autoimmunity and dendritic cell proliferation. Immunity 2007;27(5):801-10.

40. Shen N, Fu Q, Deng Y, Qian X, Zhao J, Kaufman KM, et al. Sex-specific association of X-linked Toll-like receptor 7 (TLR7) with male systemic lupus erythematosus. Proc Natl Acad Sci U S A 2010;107(36):15838-43.

41. Kawasaki A, Furukawa H, Kondo Y, Ito S, Hayashi T, Kusaoi M, et al. TLR7 single nucleotide polymorphisms in the 3' untranslated region and intron independently contribute to systemic lupus erythematosus in Japanese women: a case-control association study. Arthritis Res Ther 2011;13(2):R41.

42. Sanchez E, Callejas-Rubio JL, Sabio JM, Gonzalez-Gay MA, Jimenez-Alonso J, Mico L, et al. Investigation of TLR5 and TLR7 as candidate genes for susceptibility to systemic lupus erythematosus. Clin Exp Rheumatol 2009;27(2):267-71.

43. Schott E, Witt H, Neumann K, Bergk A, Halangk J, Weich V, et al. Association of TLR7 single nucleotide polymorphisms with chronic HCV-infection and response to interferon-a-based therapy. J Viral Hepat 2008;15(1):71-8.

44. Komatsuda A, Wakui H, Iwamoto K, Ozawa M, Togashi M, Masai R, et al. Up-regulated expression of Toll-like receptors mRNAs in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. Clin Exp Immunol 2008;152(3):482-7.

45. Cros J, Cagnard N, Woollard K, Patey N, Zhang SY, Senechal B, et al. Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. Immunity 2010;33(3):375-86.

46. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000;408(6813):740-5.

47. Demirci FY, Manzi S, Ramsey-Goldman R, Kenney M, Shaw PS, Dunlop-Thomas CM, et al. Association study of Toll-like receptor 5 (TLR5) and Toll-

like receptor 9 (TLR9) polymorphisms in systemic lupus erythematosus. J Rheumatol 2007;34(8):1708-11.

48. De Jager PL, Richardson A, Vyse TJ, Rioux JD. Genetic variation in toll-like receptor 9 and susceptibility to systemic lupus erythematosus. Arthritis Rheum 2006;54(4):1279-82.

49. Tao K, Fujii M, Tsukumo S, Maekawa Y, Kishihara K, Kimoto Y, et al. Genetic variations of Toll-like receptor 9 predispose to systemic lupus erythematosus in Japanese population. Ann Rheum Dis 2007;66(7):905-9.

50. Hur JW, Shin HD, Park BL, Kim LH, Kim SY, Bae SC. Association study of Toll-like receptor 9 gene polymorphism in Korean patients with systemic lupus erythematosus. Tissue Antigens 2005;65(3):266-70.

51. Ng MW, Lau CS, Chan TM, Wong WH, Lau YL. Polymorphisms of the toll-like receptor 9 (TLR9) gene with systemic lupus erythematosus in Chinese. Rheumatology (Oxford) 2005;44(11):1456-7.

52. Novak N, Yu CF, Bussmann C, Maintz L, Peng WM, Hart J, et al. Putative association of a TLR9 promoter polymorphism with atopic eczema. Allergy 2007;62(7):766-72.

53. Quan TE, Roman RM, Rudenga BJ, Holers VM, Craft JE. Epstein-Barr virus promotes interferon-alpha production by plasmacytoid dendritic cells. Arthritis Rheum 2010;62(6):1693-701.

54. Younesi V, Nikzamir H, Yousefi M, Khoshnoodi J, Arjmand M, Rabbani H, et al. Epstein Barr virus inhibits the stimulatory effect of TLR7/8 and TLR9 agonists but not CD40 ligand in human B lymphocytes. Microbiol Immunol 2010;54(9):534-41.

55. Iskra S, Kalla M, Delecluse HJ, Hammerschmidt W, Moosmann A. Toll-like receptor agonists synergistically increase proliferation and activation of B cells by epstein-barr virus. J Virol 2010;84(7):3612-23.

56. Hachfi W, Laurichesse JJ, Chauveheid MP, Houhou N, Bonnet D, Longuet P, et al. Acute cytomegalovirus infection revealing systemic lupus erythematosus. Rev Med Interne 2011;32(1):e6-8.

57. Perez-Mercado AE, Vila-Perez S. Cytomegalovirus as a trigger for systemic lupus erythematosus. J Clin Rheumatol 2010;16(7):335-7.

58. Hession MT, Au SC, Gottlieb AB. Parvovirus B19-associated systemic lupus erythematosus: clinical mimicry or autoimmune induction? J Rheumatol 2010;37(11):2430-2.

59. Pavlovic M, Kats A, Cavallo M, Shoenfeld Y. Clinical and molecular evidence for association of SLE with parvovirus B19. Lupus 2010;19(7):783-92.

60. Ng MT, Van't Hof R, Crockett JC, Hope ME, Berry S, Thomson J, et al. Increase in NF-kappaB binding affinity of the variant C allele of the toll-like receptor 9 -1237T/C polymorphism is associated with Helicobacter pylori-induced gastric disease. Infect Immun 2010;78(3):1345-52.

61. Lammers KM, Ouburg S, Morre SA, Crusius JB, Gionchett P, Rizzello F, et al. Combined carriership of TLR9-1237C and CD14-260T alleles enhances the risk of developing chronic relapsing pouchitis. World J Gastroenterol 2005;11(46):7323-9.

62. Ito A, Ota M, Katsuyama Y, Inoko H, Ohno S, Mizuki N. Lack of association of Toll-like receptor 9 gene polymorphism with Behcet's disease in Japanese patients. Tissue Antigens 2007;70(5):423-6.

63. Berghofer B, Frommer T, Konig IR, Ziegler A, Chakraborty T, Bein G, et al. Common human Toll-like receptor 9 polymorphisms and haplotypes: association with atopy and functional relevance. Clin Exp Allergy 2005;35(9):1147-54.

Tables

Table 1. Genotypic and allelic frequencies in European-derived patients and controls.

	Genotype			Allele
TLR7	**AA**	**AT**	**TT**	**T**
♀ Patients (n=259)	**151 [0.583][a]**	**85 [0.328][a]**	**23 [0.089][a]**	**0.253[b]**
♀ Controls (n=144)	**102 [0.708][c]**	**37 [0.257][c]**	**5 [0.035][c]**	**0.163[d]**
	A		**T**	
♂ Patients (n=23)	18 [0.783]		5 [0.217]	0.217
♂ Controls (n=159)	136 [0.855]		23 [0.145]	0.145
TLR8	**AA**	**AG**	**GG**	**G**
♀ Patients (n=257)	107 [0.416]	100 [0.389]	50 [0.195]	0.389
♀ Controls (n=146)	69 [0.473]	56 [0.384]	21 [0.144]	0.336
	A		**G**	
♂ Patients (n=23)	16 [0.696]		7 [0.304]	0.304
♂ Controls (n=149)	97 [0.651]		57 [0.349]	0.349
***TLR9* T-1237C**	**TT**	**TC**	**CC**	**C**
♀ Patients (n=258)	**179 [0.694][e]**	**68 [0.264][e]**	**11 [0.043][e]**	**0.174[f]**
♀ Controls (n=147)	**115 [0.782][g]**	**31 [0.211][g]**	**1 [0.007][g]**	**0.112[h]**
♂ Patients (n=23)	16 [0.696]	6 [0.261]	1 [0.043]	0.174
♂ Controls (n=162)	112 [0.691]	45 [0.278]	5 [0.031]	0.170
***TLR9* G2848A**	**GG**	**GA**	**AA**	**A**
♀ Patients (n=257)	67 [0.261]	115 [0.447]	75 [0.292]	0.516
♀ Controls (n=145)	38 [0.262]	76 [0.524]	31 [0.214]	0.476
♂ Patients (n=23)	3 [0.130]	13 [0.565]	7 [0.304]	0.587
♂ Controls (n=162)	36 [0.222]	75 [0.463]	51 [0.315]	0.546

Absolute frequency and [relative frequency] are shown for genotypes

a x c (χ^2): P=0.020, b x d (Fisher): P=0.003

e x g (χ^2): P=0.045, f x h (Fisher): P=0.017

Table 2. Genotypic and allelic frequencies in African-derived patients and controls

	Genotype			Allele
TLR7	**AA**	**AT**	**TT**	**T**
♀ Patients (n=83)	59 [0.711]	19 [0.229]	5 [0.060]	0.175
♀ Controls (n=43)	29 [0.674]	10 [0.233]	4 [0.093]	0.326
	A		**T**	
♂ Patients (n=5)	5 [1.000]		0 [0.000]	0.000
♂ Controls (n=61)	52 [0.852]		9 [0.148]	0.148
TLR8	**AA**	**AG**	**GG**	**G**
♀ Patients (n=83)	38 [0.458]	33 [0.398]	12 [0.145]	0.343
♀ Controls (n=44)	21 [0.477]	17 [0.386]	6 [0.136]	0.330
	A		**G**	
♂ Patients (n=5)	4 [0.800]		1 [0.200]	0.200
♂ Controls (n=59)	43 [0.729]		16 [0.271]	0.271
***TLR9* T-1237C**	**TT**	**TC**	**CC**	**C**
♀ Patients (n=83)	46 [0.554]	29 [0.349]	8 [0.096]	0.271
♀ Controls (n=43)	25 [0.581]	18 [0.419]	0 [0.000]	0.209
♂ Patients (n=5)	4 [0.800]	1 [0.200]	0 [0.000]	0.100
♂ Controls (n=57)	22 [0.386]	30 [0.526]	5 [0.088]	0.351
***TLR9* G2848A**	**GG**	**GA**	**AA**	**A**
♀ Patients (n=83)	33 [0.398]	37 [0.446]	13 [0.157]	0.380
♀ Controls (n=40)	16 [0.400]	17 [0.425]	7 [0.175]	0.388
♂ Patients (n=5)	1 [0.200]	3 [0.600]	1 [0.200]	0.500
♂ Controls (n=62)	26 [0.419]	30 [0.484]	6 [0.097]	0.339

Absolute frequency and [relative frequency] are shown for genotypes.

Table 3. Haplotype estimated frequencies and number of chromosomes (n) in European- and African-Derived Individuals.

	Haplotype		European-derived		African-derived	
	T-1237C	**G2848A**	freq	n	freq	n
♀ Patients	T	G	0.450801	234[a]	0.472895	79[e]
(n=518/166)	T	A	0.375198	194[a]	0.256021	42[e]
	C	G	0.032013	17[a]	0.147587	24[e]
	C	A	0.141988	73[a]	0.123497	21[e]
♀ Controls	T	G	0.511698	150[b]	0.485093	42[f]
(n=294/86)	T	A	0.376057	111[b]	0.305605	26[f]
	C	G	0.013151	4[b]	0.127476	11[f]
	C	A	0.099094	29[b]	0.081826	7[f]
♂ Patients	T	G	0.413043	19[c]	0.500000	5[g]
(n=46/10)	T	A	0.413044	19[c]	0.400000	4[g]
	C	G	0.000000	0[c]	0.000000	0[g]
	C	A	0.173913	8[c]	0.100000	1[g]
♂ Controls	T	G	0.424313	138[d]	0.463610	59[h]
(n=326/128)	T	A	0.403908	132[d]	0.185173	24[h]
	C	G	0.026607	9[d]	0.196942	25[h]
	C	A	0.145172	47[d]	0.154275	20[h]

a x b (χ^2): P=0.082

c x d (Fisher): P=0.870

e x f (χ^2): P=0.641

a x e (χ^2): P=0.00000054

residual: TA: P=0.004, CG: P=0.00000012

b x f (χ^2): P=0.000034

residual: CG: P=0.0000017

d x h (χ^2): P=0.00000000027

residual: TA: P=0.000011, CG: P=0.000000001

Table 4. *TLR9* rs5743836 and rs352140 haplotype frequencies worldwide.

Haplotype		Lazarus et al., 2003			Lammers et al., 2005	Ito et al., 2007	Berghofer et al., 2005	Our Data	
rs5743836	rs352140	African american	European american	Hispanic american	Italian	Japanese	German	Euro-derived	Afro-derived
T	G	0.444	0.435	0.396	0.400	0.5196	0.4400	0.4651	0.4670
T	A	0.408	0.413	0.562	0.440	0.4657	0.4221	0.3914	0.2369
C	G	0.056	0.000	0.000	0.010	0.0147	0.0049	0.0207	0.1761
C	A	0.092	0.152	0.042	0.150	0.0000	0.1332	0.1228	0.1200
Total of individuals		24	23	24	224	102	102	310	110

4. DISCUSSÃO DA DISSERTAÇÃO

Decidimos estudar os *TLR7/8/9* pois são candidatos interessantes à suscetibilidade ao LES dado seus perfis de expressão, rotas de sinalização envolvidas e seus papéis potenciais coestimulatórios. Podemos imaginar que uma variação genética que afeta a função ou regulação da expressão desses receptores poderia afetar o limiar de ativação de células B e DC, e assim engatilhar ou perpetuar o fenômeno da auto-imunidade. Vários mecanismos poderiam ser implicados nesse processo: (i) ativação inapropriada de TLRs por ligantes endógenos ou exógenos; (ii) mimetismo molecular e o aumento da apresentação de auto-antígenos e (iii) regulação defeituosa mediada por TLRs em células regulatórias (Papadimitraki et al., 2007). Estudos com modelos murinos de LES salientam a importância desses receptores e incentivam mais estudos buscando esclarecer o conhecimento do LES humano (Fischer and Ehlers, 2008; Conti et al., 2010)

No nosso trabalho, avaliamos variantes polimórficas com potencial efeito funcional nos genes *TLR7/8/9* em pacientes com LES e em um grupo de controles saudáveis, ambas amostras provenientes do sul do Brasil. As freqüências alélicas foram similares às encontradas em populações com ascendência semelhante. Diferentemente de outros trabalhos, encontramos associação dos polimorfismos rs179008 no *TLR7* e rs5743836 no *TLR9* em mulheres com LES. Ambas variantes apresentaram maior freqüência no grupo de pacientes (Tabela 1 do artigo). A primeira já foi relacionada à baixa produção de IFN e assim, aumento na suscetibilidade de infecção a HCV e HIV (Schott et al., 2008; Oh et al., 2009; Askar et al., 2010), enquanto que a segunda já foi associada a fenótipos inflamatórios como asma e Doença de Crohn (Lazarus et al., 2003; Torok et al., 2004). Nossos resultados, juntamente com os dados da literatura, sugerem que o alelo T do polimorfismo rs179008 pode estar envolvido no aumento da suscetibilidade a infecções de origem viral. Como dito anteriormente, o LES está fortemente associado a infecções por vírus relativamente comuns na população, como EBV, parvovírus B19 e citomegalovírus. Já o alelo C do polimorfismo rs5743836 não possui uma conseqüência funcional muito bem definida, tendo sido associado à baixa atividade do promotor (Novak et al., 2007) e assim também estando relacionado ao aumento da suscetibilidade a vírus. Em contrapartida, o alelo C também foi relacionado com o aumento da expressão do promotor em resposta a ativadores de NF-kappaB (Ng et al., 2010), já que essa variante insere um

sítio de ligação potencial do NF-kappaB (Hamann et al., 2006). Já foi visto que o limiar para a ativação da sinalização de TLR7-IFN tipo I é maior que TLR7-NF-kappaB (Wang et al., 2006). Se esse limiar apresenta o mesmo padrão independente da origem de ativação, ao ser ativado, NF-kappaB pode promover maior expressão de *TLR9* tornando seu transcrito mais numeroso nos endossomos. Conseqüentemente, isso diminuiria o limiar para que fosse engatilhada a sinalização, ou seja, menos ligante seria necessário para ativar o sinal através de TLR9, e o IFN tipo I produzido seria, em grande parte, proveniente da ativação por TLR9. Se essa hipótese for verdadeira, o alelo C seria um fator que modificaria a regulação de células B e DC, facilitando a ativação celular. No ambiente do LES, DC ativadas, que expressam moléculas de coestimulação, apresentariam auto-antígenos para células T auto-reativas que, com os estímulos agora suficientes, se ativariam. Além disso, DC produziriam IFNs e IL-6, que promovem a ativação de células B. Essas células B, em presença de ligante de TLR9, teriam os estímulos necessários para serem ativadas e diferenciadas em plasmócitos, independente de células T.

Para afro-descendentes, não houve diferenças significativas nas comparações genotípicas e alélicas. Esse resultado negativo pode ser devido ao baixo numero amostral. No entanto, a comparação genotípica envolvendo o polimorfismo rs5743836 em mulheres resultou em um valor P=0,09. Quando analisamos os resíduos, vemos que o genótipo CC está super-representado em pacientes (P=0,035). Se essa tendência se mantiver com o aumento do número amostral, podemos sugerir o alelo C é como um fator de risco para o desenvolvimento de LES em mulheres independente da etnia. Ao compararmos a sintomatologia clínica dos pacientes, vimos que mulheres euro-descendentes homozigotas para o alelo C (rs5743836) apresentam maior freqüência na produção de Anti-SSa/Ro (P=0,005 na comparação geral por genótipos; P=0,003 para o aumento de CC; OR 1,83 CI 95% 1,03-3,22 para portadores do alelo C). Entretanto, esse achado se mostrou como tendência após correção de Bonferroni (P corrigido=0,06).

No trabalho realizado por Lazarus e colaboradores (2003) se viu que os haplótipos formados entre os polimorfismos rs5743836 e rs352140 poderiam distinguir os quatro haplótipos mais comuns (95,1%) (Lazarus et al., 2003) do gene *TLR9*. Quando estimamos as freqüências dos haplótipos pelo programa MLocus não observamos diferença estatisticamente significante entre a freqüência de casos e controles (tabela 3 do artigo). Porém, notamos o quão heterogêneos geneticamente são os dois grupos étnicos analisados.

O haplótipo CG, que em controles euro-descendentes é o mais raro, entre os controles afro-descendentes possui freqüência maior: é o segundo mais representativo em homens e terceiro mais representativo em mulheres. Estudos com dados históricos e com possíveis pressões ambientais de populações euro-descendentes e afro-descendentes poderiam revelar as causas destas diferenças, se realmente ocorre por pressões evolutivas ou se é um fenômeno decorrente do tempo e local de origem dessas variações genéticas.

Já que os alelos T do polimorfismo rs179008 no *TLR7* e o C do polimorfismo rs5743836 no *TLR9* foram associados ao LES e ambos possuem um perfil de expressão muito similar, principalmente em pDC e células B, analisamos os genótipos combinados de mulheres pacientes versus controles. As análises genotípicas não revelaram diferenças significativas por qui-quadrado de Pearson ou de tendência (dados não mostrados). O *odds ratio* para portadores de pelo menos um alelo de suscetibilidade de cada gene foi 1,91 CI 95% 0,93-4,20. Em conclusão, nosso trabalho sugere a participação dos alelos T do polimorfismo rs179008 no *TLR7* e o C do polimorfismo rs5743836 no *TLR9* e seus respectivos homozigotos como fatores de suscetibilidade ao LES.

Durante o período de execução deste trabalho, houve participação no desenvolvimento de outros trabalhos desenvolvidos pelo nosso grupo de pesquisa. Esses trabalhos estão detalhados nos anexos III e IV.

5. REFERÊNCIAS BIBLIOGRÁFICAS

(1999). The American College of Rheumatology nomenclature and case definitions for neuropsychiatric lupus syndromes. Arthritis Rheum 42 4: 599-608.

Achour A, Mankai A, Thabet Y, Sakly W, Braham F, Kechrid C, Bahri F, Bouajina E, Chouchene S, Haddad O, et al. (2011). Systemic lupus erythematosus in the elderly. Rheumatol Int

Alarcon-Segovia D, Alarcon-Riquelme ME, Cardiel MH, Caeiro F, Massardo L, Villa AR and Pons-Estel BA (2005). Familial aggregation of systemic lupus erythematosus, rheumatoid arthritis, and other autoimmune diseases in 1,177 lupus patients from the GLADEL cohort. Arthritis Rheum 52 4: 1138-1147.

Alarcon GS, Cianfrini L, Bradley LA, Sanchez ML, Brooks K, Friedman AW, Baethge BA, Fessler BJ, Bastian HM, Roseman JM, et al. (2002). Systemic lupus erythematosus in three ethnic groups. X. Measuring cognitive impairment with the cognitive symptoms inventory. Arthritis Rheum 47 3: 310-319.

Alarcon GS, McGwin G, Jr., Roseman JM, Uribe A, Fessler BJ, Bastian HM, Friedman AW, Baethge B, Vila LM and Reveille JD (2004). Systemic lupus erythematosus in three ethnic groups. XIX. Natural history of the accrual of the American College of Rheumatology criteria prior to the occurrence of criteria diagnosis. Arthritis Rheum 51 4: 609-615.

Albert ML (2004). Death-defying immunity: do apoptotic cells influence antigen processing and presentation? Nat Rev Immunol 4 3: 223-231.

Anolik JH, Barnard J, Cappione A, Pugh-Bernard AE, Felgar RE, Looney RJ and Sanz I (2004). Rituximab improves peripheral B cell abnormalities in human systemic lupus erythematosus. Arthritis Rheum 50 11: 3580-3590.

Askar E, Ramadori G and Mihm S (2010). Toll-like receptor 7 rs179008/Gln11Leu gene variants in chronic hepatitis C virus infection. J Med Virol 82 11: 1859-1868.

Ballou SP, Khan MA and Kushner I (1982). Clinical features of systemic lupus erythematosus: differences related to race and age of onset. Arthritis Rheum 25 1: 55-60.

Banchereau J and Pascual V (2006). Type I interferon in systemic lupus erythematosus and other autoimmune diseases. Immunity 25 3: 383-392.

Barrat FJ and Coffman RL (2008). Development of TLR inhibitors for the treatment of autoimmune diseases. Immunol Rev 223 271-283.

Barrat FJ, Meeker T, Chan JH, Guiducci C and Coffman RL (2007). Treatment of lupus-prone mice with a dual inhibitor of TLR7 and TLR9 leads to reduction of autoantibody production and amelioration of disease symptoms. Eur J Immunol 37 12: 3582-3586.

Barrat FJ, Meeker T, Gregorio J, Chan JH, Uematsu S, Akira S, Chang B, Duramad O and Coffman RL (2005). Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. J Exp Med 202 8: 1131-1139.

Bauer S, Pigisch S, Hangel D, Kaufmann A and Hamm S (2008). Recognition of nucleic acid and nucleic acid analogs by Toll-like receptors 7, 8 and 9. Immunobiology 213 3-4: 315-328.

Berghofer B, Frommer T, Konig IR, Ziegler A, Chakraborty T, Bein G and Hackstein H (2005). Common human Toll-like receptor 9 polymorphisms and haplotypes: association with atopy and functional relevance. Clin Exp Allergy 35 9: 1147-1154.

Bijl M, Reefman E, Horst G, Limburg PC and Kallenberg CG (2006). Reduced uptake of apoptotic cells by macrophages in systemic lupus erythematosus: correlates with decreased serum levels of complement. Ann Rheum Dis 65 1: 57-63.

Blanco P, Palucka AK, Gill M, Pascual V and Banchereau J (2001). Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. Science 294 5546: 1540-1543.

Block SR, Winfield JB, Lockshin MD, D'Angelo WA, Weksler ME, Fotino M and Christian CL (1975). Proceedings: Twin studies in systemic lupus erythematosus (SLE). Arthritis Rheum 18 3: 285.

Blomberg S, Eloranta ML, Magnusson M, Alm GV and Ronnblom L (2003). Expression of the markers BDCA-2 and BDCA-4 and production of interferon-alpha by plasmacytoid dendritic cells in systemic lupus erythematosus. Arthritis Rheum 48 9: 2524-2532.

Bombardier C, Gladman DD, Urowitz MB, Caron D and Chang CH (1992). Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE. Arthritis Rheum 35 6: 630-640.

Botto M, Dell'Agnola C, Bygrave AE, Thompson EM, Cook HT, Petry F, Loos M, Pandolfi PP and Walport MJ (1998). Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. Nat Genet 19 1: 56-59.

Boumpas DT, Austin HA, 3rd, Fessler BJ, Balow JE, Klippel JH and Lockshin MD (1995a). Systemic lupus erythematosus: emerging concepts. Part 1: Renal, neuropsychiatric, cardiovascular, pulmonary, and hematologic disease. Ann Intern Med 122 12: 940-950.

Boumpas DT, Fessler BJ, Austin HA, 3rd, Balow JE, Klippel JH and Lockshin MD (1995b). Systemic lupus erythematosus: emerging concepts. Part 2: Dermatologic and joint disease, the antiphospholipid antibody syndrome, pregnancy and hormonal therapy, morbidity and mortality, and pathogenesis. Ann Intern Med 123 1: 42-53.

Bowie A and O'Neill LA (2000). The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. J Leukoc Biol 67 4: 508-514.

Braun D, Geraldes P and Demengeot J (2003). Type I Interferon controls the onset and severity of autoimmune manifestations in lpr mice. J Autoimmun 20 1: 15-25.

Cambridge G, Leandro MJ, Teodorescu M, Manson J, Rahman A, Isenberg DA and Edwards JC (2006). B cell depletion therapy in systemic lupus erythematosus: effect on autoantibody and antimicrobial antibody profiles. Arthritis Rheum 54 11: 3612-3622.

Carroll MC (2004). A protective role for innate immunity in systemic lupus erythematosus. Nat Rev Immunol 4 10: 825-831.

Carvalho A, Marques A, Maciel P and Rodrigues F (2007). Study of disease-relevant polymorphisms in the TLR4 and TLR9 genes: a novel method applied to the analysis of the Portuguese population. Mol Cell Probes 21 4: 316-320.

Carvalho A, Pasqualotto AC, Pitzurra L, Romani L, Denning DW and Rodrigues F (2008). Polymorphisms in toll-like receptor genes and susceptibility to pulmonary aspergillosis. J Infect Dis 197 4: 618-621.

Casciola-Rosen LA, Anhalt G and Rosen A (1994). Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. J Exp Med 179 4: 1317-1330.

Cervera R, Khamashta MA, Font J, Sebastiani GD, Gil A, Lavilla P, Mejia JC, Aydintug AO, Chwalinska-Sadowska H, de Ramon E, et al. (2003). Morbidity and

mortality in systemic lupus erythematosus during a 10-year period: a comparison of early and late manifestations in a cohort of 1,000 patients. Medicine (Baltimore) 82 5: 299-308.

Cheng PL, Eng HL, Chou MH, You HL and Lin TM (2007). Genetic polymorphisms of viral infection-associated Toll-like receptors in Chinese population. Transl Res 150 5: 311-318.

Christen U and von Herrath MG (2004a). Induction, acceleration or prevention of autoimmunity by molecular mimicry. Mol Immunol 40 14-15: 1113-1120.

Christen U and von Herrath MG (2004b). Initiation of autoimmunity. Curr Opin Immunol 16 6: 759-767.

Chuang TH and Ulevitch RJ (2000). Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. Eur Cytokine Netw 11 3: 372-378.

Chung SA and Criswell LA (2007). PTPN22: its role in SLE and autoimmunity. Autoimmunity 40 8: 582-590.

Coenen MJ, Enevold C, Barrera P, Schijvenaars MM, Toonen EJ, Scheffer H, Padyukov L, Kastbom A, Klareskog L, Barton A, et al. (2010). Genetic variants in toll-like receptors are not associated with rheumatoid arthritis susceptibility or anti-tumour necrosis factor treatment outcome. PLoS One 5 12: e14326.

Cohen SB, Emery P, Greenwald MW, Dougados M, Furie RA, Genovese MC, Keystone EC, Loveless JE, Burmester GR, Cravets MW, et al. (2006). Rituximab for rheumatoid arthritis refractory to anti-tumor necrosis factor therapy: Results of a multicenter, randomized, double-blind, placebo-controlled, phase III trial evaluating primary efficacy and safety at twenty-four weeks. Arthritis Rheum 54 9: 2793-2806.

Conti F, Spinelli FR, Alessandri C and Valesini G (2010). Toll-like Receptors and Lupus Nephritis. Clin Rev Allergy Immunol

Cooper GS, Dooley MA, Treadwell EL, St Clair EW, Parks CG and Gilkeson GS (1998). Hormonal, environmental, and infectious risk factors for developing systemic lupus erythematosus. Arthritis Rheum 41 10: 1714-1724.

Croker JA and Kimberly RP (2005). Genetics of susceptibility and severity in systemic lupus erythematosus. Curr Opin Rheumatol 17 5: 529-537.

Cros J, Cagnard N, Woollard K, Patey N, Zhang SY, Senechal B, Puel A, Biswas SK, Moshous D, Picard C, et al. (2010). Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. Immunity 33 3: 375-386.

Crow MK (2007). Type I interferon in systemic lupus erythematosus. Curr Top Microbiol Immunol 316 359-386.

Cunninghame Graham DS, Manku H, Wagner S, Reid J, Timms K, Gutin A, Lanchbury JS and Vyse TJ (2007). Association of IRF5 in UK SLE families identifies a variant involved in polyadenylation. Hum Mol Genet 16 6: 579-591.

da Silva GK, Guimaraes R, Mattevi VS, Lazzaretti RK, Sprinz E, Kuhmmer R, Brandao L, Crovella S and Chies JA (2010). The role of mannose-binding lectin gene polymorphisms in susceptibility to HIV-1 infection in Southern Brazilian patients. AIDS

Danchenko N, Satia JA and Anthony MS (2006). Epidemiology of systemic lupus erythematosus: a comparison of worldwide disease burden. Lupus 15 5: 308-318.

Davila S, Hibberd ML, Hari Dass R, Wong HE, Sahiratmadja E, Bonnard C, Alisjahbana B, Szeszko JS, Balabanova Y, Drobniewski F, et al. (2008). Genetic association and expression studies indicate a role of toll-like receptor 8 in pulmonary tuberculosis. PLoS Genet 4 10: e1000218.

De Jager PL, Richardson A, Vyse TJ and Rioux JD (2006). Genetic variation in toll-like receptor 9 and susceptibility to systemic lupus erythematosus. Arthritis Rheum 54 4: 1279-1282.

Deane JA, Pisitkun P, Barrett RS, Feigenbaum L, Town T, Ward JM, Flavell RA and Bolland S (2007). Control of toll-like receptor 7 expression is essential to restrict autoimmunity and dendritic cell proliferation. Immunity 27 5: 801-810.

Deapen D, Escalante A, Weinrib L, Horwitz D, Bachman B, Roy-Burman P, Walker A and Mack TM (1992). A revised estimate of twin concordance in systemic lupus erythematosus. Arthritis Rheum 35 3: 311-318.

DeGiorgio LA, Konstantinov KN, Lee SC, Hardin JA, Volpe BT and Diamond B (2001). A subset of lupus anti-DNA antibodies cross-reacts with the NR2 glutamate receptor in systemic lupus erythematosus. Nat Med 7 11: 1189-1193.

Demirci FY, Manzi S, Ramsey-Goldman R, Kenney M, Shaw PS, Dunlop-Thomas CM, Kao AH, Rhew EY, Bontempo F, Kammerer C, et al. (2007). Association study of Toll-like receptor 5 (TLR5) and Toll-like receptor 9 (TLR9) polymorphisms in systemic lupus erythematosus. J Rheumatol 34 8: 1708-1711.

Diamond B, Bloom O, Al Abed Y, Kowal C, Huerta PT and Volpe BT (2010). Moving towards a cure: blocking pathogenic antibodies in systemic lupus erythematosus. J Intern Med 269 1: 36-44.

Dong L, Ito S, Ishii KJ and Klinman DM (2005). Suppressive oligodeoxynucleotides delay the onset of glomerulonephritis and prolong survival in lupus-prone NZB x NZW mice. Arthritis Rheum 52 2: 651-658.

Dong X, Hamilton KJ, Satoh M, Wang J and Reeves WH (1994). Initiation of autoimmunity to the p53 tumor suppressor protein by complexes of p53 and SV40 large T antigen. J Exp Med 179 4: 1243-1252.

Dorner T, Radbruch A and Burmester GR (2009). B-cell-directed therapies for autoimmune disease. Nat Rev Rheumatol 5 8: 433-441.

Du X, Poltorak A, Wei Y and Beutler B (2000). Three novel mammalian toll-like receptors: gene structure, expression, and evolution. Eur Cytokine Netw 11 3: 362-371.

Dubois EL and Tuffanelli DL (1964). Clinical Manifestations Of Systemic Lupus Erythematosus. Computer Analysis Of 520 Cases. Jama 190 104-111.

Duramad O, Fearon KL, Chang B, Chan JH, Gregorio J, Coffman RL and Barrat FJ (2005). Inhibitors of TLR-9 act on multiple cell subsets in mouse and man in vitro and prevent death in vivo from systemic inflammation. J Immunol 174 9: 5193-5200.

Dzionek A, Sohma Y, Nagafune J, Cella M, Colonna M, Facchetti F, Gunther G, Johnston I, Lanzavecchia A, Nagasaka T, et al. (2001). BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. J Exp Med 194 12: 1823-1834.

Edwards AO, Chen D, Fridley BL, James KM, Wu Y, Abecasis G, Swaroop A, Othman M, Branham K, Iyengar SK, et al. (2008). Toll-like receptor polymorphisms and age-related macular degeneration. Invest Ophthalmol Vis Sci 49 4: 1652-1659.

Elkon K (1995). Autoantibodies in systemic lupus erythematosus. Curr Opin Rheumatol 7 5: 384-388.

Engin A, Arslan S, Kizildag S, Ozturk H, Elaldi N, Dokmetas I and Bakir M (2010). Toll-like receptor 8 and 9 polymorphisms in Crimean-Congo hemorrhagic fever. Microbes Infect 12 12-13: 1071-1078.

Esdaile JM, Abrahamowicz M, MacKenzie T, Hayslett JP and Kashgarian M (1994). The time-dependence of long-term prediction in lupus nephritis. Arthritis Rheum 37 3: 359-368.

Fischer M and Ehlers M (2008). Toll-like receptors in autoimmunity. Ann N Y Acad Sci 1143 21-34.

Fraser PA, Ding WZ, Mohseni M, Treadwell EL, Dooley MA, St Clair EW, Gilkeson GS and Cooper GS (2003). Glutathione S-transferase M null homozygosity and risk of systemic lupus erythematosus associated with sun exposure: a possible gene-environment interaction for autoimmunity. J Rheumatol 30 2: 276-282.

Gantier MP, Irving AT, Kaparakis-Liaskos M, Xu D, Evans VA, Cameron PU, Bourne JA, Ferrero RL, John M, Behlke MA, et al. (2010). Genetic modulation of TLR8 response following bacterial phagocytosis. Hum Mutat 31 9: 1069-1079.

Gaynor B, Putterman C, Valadon P, Spatz L, Scharff MD and Diamond B (1997). Peptide inhibition of glomerular deposition of an anti-DNA antibody. Proc Natl Acad Sci U S A 94 5: 1955-1960.

Gilliet M, Cao W and Liu YJ (2008). Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. Nat Rev Immunol 8 8: 594-606.

Gladman D, Ginzler E, Goldsmith C, Fortin P, Liang M, Urowitz M, Bacon P, Bombardieri S, Hanly J, Hay E, et al. (1996). The development and initial validation of the Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index for systemic lupus erythematosus. Arthritis Rheum 39 3: 363-369.

Graham RR, Cotsapas C, Davies L, Hackett R, Lessard CJ, Leon JM, Burtt NP, Guiducci C, Parkin M, Gates C, et al. (2008). Genetic variants near TNFAIP3 on 6q23 are associated with systemic lupus erythematosus. Nat Genet 40 9: 1059-1061.

Graham RR, Kozyrev SV, Baechler EC, Reddy MV, Plenge RM, Bauer JW, Ortmann WA, Koeuth T, Gonzalez Escribano MF, Pons-Estel B, et al. (2006). A common haplotype of interferon regulatory factor 5 (IRF5) regulates splicing and expression and is associated with increased risk of systemic lupus erythematosus. Nat Genet 38 5: 550-555.

Graham RR, Kyogoku C, Sigurdsson S, Vlasova IA, Davies LR, Baechler EC, Plenge RM, Koeuth T, Ortmann WA, Hom G, et al. (2007). Three functional variants of IFN regulatory factor 5 (IRF5) define risk and protective haplotypes for human lupus. Proc Natl Acad Sci U S A 104 16: 6758-6763.

Gripenberg M and Helve T (1991). Outcome of systemic lupus erythematosus. A study of 66 patients over 7 years with special reference to the predictive value of anti-DNA antibody determinations. Scand J Rheumatol 20 2: 104-109.

Haas T, Metzger J, Schmitz F, Heit A, Muller T, Latz E and Wagner H (2008). The DNA sugar backbone 2' deoxyribose determines toll-like receptor 9 activation. Immunity 28 3: 315-323.

Hachfi W, Laurichesse JJ, Chauveheid MP, Houhou N, Bonnet D, Longuet P and Leport C (2011). Acute cytomegalovirus infection revealing systemic lupus erythematosus. Rev Med Interne 32 1: e6-8.

Hamann L, Glaeser C, Hamprecht A, Gross M, Gomma A and Schumann RR (2006). Toll-like receptor (TLR)-9 promotor polymorphisms and atherosclerosis. Clin Chim Acta 364 1-2: 303-307.

Harley IT, Kaufman KM, Langefeld CD, Harley JB and Kelly JA (2009). Genetic susceptibility to SLE: new insights from fine mapping and genome-wide association studies. Nat Rev Genet 10 5: 285-290.

Harley JB, Alarcon-Riquelme ME, Criswell LA, Jacob CO, Kimberly RP, Moser KL, Tsao BP, Vyse TJ, Langefeld CD, Nath SK, et al. (2008). Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci. Nat Genet 40 2: 204-210.

Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H and Bauer S (2004). Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303 5663: 1526-1529.

Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, et al. (2000). A Toll-like receptor recognizes bacterial DNA. Nature 408 6813: 740-745.

Hession MT, Au SC and Gottlieb AB (2010). Parvovirus B19-associated systemic lupus erythematosus: clinical mimicry or autoimmune induction? J Rheumatol 37 11: 2430-2432.

Hochberg MC (1990). Systemic lupus erythematosus. Rheum Dis Clin North Am 16 3: 617-639.

Hochberg MC (1997). Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 40 9: 1725.

Hom G, Graham RR, Modrek B, Taylor KE, Ortmann W, Garnier S, Lee AT, Chung SA, Ferreira RC, Pant PV, et al. (2008). Association of systemic lupus erythematosus with C8orf13-BLK and ITGAM-ITGAX. N Engl J Med 358 9: 900-909.

Hooks JJ, Moutsopoulos HM, Geis SA, Stahl NI, Decker JL and Notkins AL (1979). Immune interferon in the circulation of patients with autoimmune disease. N Engl J Med 301 1: 5-8.

Hopkinson ND, Doherty M and Powell RJ (1994). Clinical features and race-specific incidence/prevalence rates of systemic lupus erythematosus in a geographically complete cohort of patients. Ann Rheum Dis 53 10: 675-680.

Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdorfer B, Giese T, Endres S and Hartmann G (2002). Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol 168 9: 4531-4537.

Hultin LE, Hausner MA, Hultin PM and Giorgi JV (1993). CD20 (pan-B cell) antigen is expressed at a low level on a subpopulation of human T lymphocytes. Cytometry 14 2: 196-204.

Hur JW, Shin HD, Park BL, Kim LH, Kim SY and Bae SC (2005). Association study of Toll-like receptor 9 gene polymorphism in Korean patients with systemic lupus erythematosus. Tissue Antigens 65 3: 266-270.

Illei GG and Lipsky PE (2004). Biomarkers in systemic lupus erythematosus. Curr Rheumatol Rep 6 5: 382-390.

Illei GG, Tackey E, Lapteva L and Lipsky PE (2004a). Biomarkers in systemic lupus erythematosus. I. General overview of biomarkers and their applicability. Arthritis Rheum 50 6: 1709-1720.

Illei GG, Tackey E, Lapteva L and Lipsky PE (2004b). Biomarkers in systemic lupus erythematosus: II. Markers of disease activity. Arthritis Rheum 50 7: 2048-2065.

Iriya SM, Capelozzi VL, Calich I, Martins MA and Lichtenstein A (2001). Causes of death in patients with systemic lupus erythematosus in Sao Paulo, Brazil: a study of 113 autopsies. Arch Intern Med 161 12: 1557.

Iskra S, Kalla M, Delecluse HJ, Hammerschmidt W and Moosmann A (2010). Toll-like receptor agonists synergistically increase proliferation and activation of B cells by epstein-barr virus. J Virol 84 7: 3612-3623.

Ito A, Ota M, Katsuyama Y, Inoko H, Ohno S and Mizuki N (2007). Lack of association of Toll-like receptor 9 gene polymorphism with Behcet's disease in Japanese patients. Tissue Antigens 70 5: 423-426.

Izaguirre A, Barnes BJ, Amrute S, Yeow WS, Megjugorac N, Dai J, Feng D, Chung E, Pitha PM and Fitzgerald-Bocarsly P (2003). Comparative analysis of IRF and IFN-alpha expression in human plasmacytoid and monocyte-derived dendritic cells. J Leukoc Biol 74 6: 1125-1138.

Jacob CO, Reiff A, Armstrong DL, Myones BL, Silverman E, Klein-Gitelman M, McCurdy D, Wagner-Weiner L, Nocton JJ, Solomon A, et al. (2007). Identification of novel susceptibility genes in childhood-onset systemic lupus erythematosus using a uniquely designed candidate gene pathway platform. Arthritis Rheum 56 12: 4164-4173.

Jacob CO, Zhu J, Armstrong DL, Yan M, Han J, Zhou XJ, Thomas JA, Reiff A, Myones BL, Ojwang JO, et al. (2009). Identification of IRAK1 as a risk gene with critical role in the pathogenesis of systemic lupus erythematosus. Proc Natl Acad Sci U S A 106 15: 6256-6261.

James JA, Gross T, Scofield RH and Harley JB (1995). Immunoglobulin epitope spreading and autoimmune disease after peptide immunization: Sm B/B'-derived PPPGMRPP and PPPGIRGP induce spliceosome autoimmunity. J Exp Med 181 2: 453-461.

James JA, Kaufman KM, Farris AD, Taylor-Albert E, Lehman TJ and Harley JB (1997). An increased prevalence of Epstein-Barr virus infection in young patients suggests a possible etiology for systemic lupus erythematosus. J Clin Invest 100 12: 3019-3026.

Jarrossay D, Napolitani G, Colonna M, Sallusto F and Lanzavecchia A (2001). Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. Eur J Immunol 31 11: 3388-3393.

Jonsson H, Nived O and Sturfelt G (1989). Outcome in systemic lupus erythematosus: a prospective study of patients from a defined population. Medicine (Baltimore) 68 3: 141-150.

Kariuki SN, Kirou KA, MacDermott EJ, Barillas-Arias L, Crow MK and Niewold TB (2009). Cutting edge: autoimmune disease risk variant of STAT4 confers increased sensitivity to IFN-alpha in lupus patients in vivo. J Immunol 182 1: 34-38.

Kasitanon N, Magder LS and Petri M (2006). Predictors of survival in systemic lupus erythematosus. Medicine (Baltimore) 85 3: 147-156.

Kawasaki A, Furukawa H, Kondo Y, Ito S, Hayashi T, Kusaoi M, Matsumoto I, Tohma S, Takasaki Y, Hashimoto H, et al. (2011). TLR7 single nucleotide polymorphisms in the 3' untranslated region and intron independently contribute to systemic lupus erythematosus in Japanese women: a case-control association study. Arthritis Res Ther 13 2: R41.

Kelley JM, Edberg JC and Kimberly RP (2010). Pathways: Strategies for susceptibility genes in SLE. Autoimmun Rev 9 7: 473-476.

Kelly JA, Moser KL and Harley JB (2002). The genetics of systemic lupus erythematosus: putting the pieces together. Genes Immun 3 Suppl 1 S71-85.

Kikuchi K, Lian ZX, Kimura Y, Selmi C, Yang GX, Gordon SC, Invernizzi P, Podda M, Coppel RL, Ansari AA, et al. (2005). Genetic polymorphisms of toll-like receptor 9

influence the immune response to CpG and contribute to hyper-IgM in primary biliary cirrhosis. J Autoimmun 24 4: 347-352.

Kim WU, Sreih A and Bucala R (2009). Toll-like receptors in systemic lupus erythematosus; prospects for therapeutic intervention. Autoimmun Rev 8 3: 204-208.

Klinman DM and Steinberg AD (1995). Inquiry into murine and human lupus. Immunol Rev 144 157-193.

Komatsuda A, Wakui H, Iwamoto K, Ozawa M, Togashi M, Masai R, Maki N, Hatakeyama T and Sawada K (2008). Up-regulated expression of Toll-like receptors mRNAs in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. Clin Exp Immunol 152 3: 482-487.

Koneru S, Shishov M, Ware A, Farhey Y, Mongey AB, Graham TB, Passo MH, Houk JL, Higgins GC and Brunner HI (2007). Effectively measuring adherence to medications for systemic lupus erythematosus in a clinical setting. Arthritis Rheum 57 6: 1000-1006.

Kozora E, Hanly JG, Lapteva L and Filley CM (2008). Cognitive dysfunction in systemic lupus erythematosus: past, present, and future. Arthritis Rheum 58 11: 3286-3298.

Kretz-Rommel A and Rubin RL (2000). Disruption of positive selection of thymocytes causes autoimmunity. Nat Med 6 3: 298-305.

Kyogoku C, Dijstelbloem HM, Tsuchiya N, Hatta Y, Kato H, Yamaguchi A, Fukazawa T, Jansen MD, Hashimoto H, van de Winkel JG, et al. (2002). Fcgamma receptor gene polymorphisms in Japanese patients with systemic lupus erythematosus: contribution of FCGR2B to genetic susceptibility. Arthritis Rheum 46 5: 1242-1254.

Kyogoku C, Tsuchiya N, Wu H, Tsao BP and Tokunaga K (2004). Association of Fcgamma receptor IIA, but not IIB and IIIA, polymorphisms with systemic lupus erythematosus: A family-based association study in Caucasians. Arthritis Rheum 50 2: 671-673.

Lahiri DK and Nurnberger JI, Jr. (1991). A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. Nucleic Acids Res 19 19: 5444.

Lahita RG (1995). Special report: adjusted lupus prevalence. Results of a marketing study by the Lupus Foundation of America. Lupus 4 6: 450-453.

Lahita RG (1999). The role of sex hormones in systemic lupus erythematosus. Curr Opin Rheumatol 11 5: 352-356.

Lammers KM, Ouburg S, Morre SA, Crusius JB, Gionchett P, Rizzello F, Morselli C, Caramelli E, Conte R, Poggioli G, et al. (2005). Combined carriership of TLR9-1237C and CD14-260T alleles enhances the risk of developing chronic relapsing pouchitis. World J Gastroenterol 11 46: 7323-7329.

Lau CS, Yin G and Mok MY (2006). Ethnic and geographical differences in systemic lupus erythematosus: an overview. Lupus 15 11: 715-719.

Lazarus M, Hajeer AH, Turner D, Sinnott P, Worthington J, Ollier WE and Hutchinson IV (1997). Genetic variation in the interleukin 10 gene promoter and systemic lupus erythematosus. J Rheumatol 24 12: 2314-2317.

Lazarus R, Klimecki WT, Raby BA, Vercelli D, Palmer LJ, Kwiatkowski DJ, Silverman EK, Martinez F and Weiss ST (2003). Single-nucleotide polymorphisms in the Toll-like receptor 9 gene (TLR9): frequencies, pairwise linkage disequilibrium, and haplotypes in three U.S. ethnic groups and exploratory case-control disease association studies. Genomics 81 1: 85-91.

Leandro MJ, Cambridge G, Edwards JC, Ehrenstein MR and Isenberg DA (2005). B-cell depletion in the treatment of patients with systemic lupus erythematosus: a longitudinal analysis of 24 patients. Rheumatology (Oxford) 44 12: 1542-1545.

LeDoux JE (2000). Emotion circuits in the brain. Annu Rev Neurosci 23 155-184.

Lee-Kirsch MA, Gong M, Chowdhury D, Senenko L, Engel K, Lee YA, de Silva U, Bailey SL, Witte T, Vyse TJ, et al. (2007). Mutations in the gene encoding the 3'-5' DNA exonuclease TREX1 are associated with systemic lupus erythematosus. Nat Genet 39 9: 1065-1067.

Lehmann P, Holzle E, Kind P, Goerz G and Plewig G (1990). Experimental reproduction of skin lesions in lupus erythematosus by UVA and UVB radiation. J Am Acad Dermatol 22 2 Pt 1: 181-187.

Looney RJ, Anolik JH, Campbell D, Felgar RE, Young F, Arend LJ, Sloand JA, Rosenblatt J and Sanz I (2004). B cell depletion as a novel treatment for systemic lupus erythematosus: a phase I/II dose-escalation trial of rituximab. Arthritis Rheum 50 8: 2580-2589.

Macsween KF and Crawford DH (2003). Epstein-Barr virus-recent advances. Lancet Infect Dis 3 3: 131-140.

Manzi S, Selzer F, Sutton-Tyrrell K, Fitzgerald SG, Rairie JE, Tracy RP and Kuller LH (1999). Prevalence and risk factors of carotid plaque in women with systemic lupus erythematosus. Arthritis Rheum 42 1: 51-60.

Marshak-Rothstein A (2006). Toll-like receptors in systemic autoimmune disease. Nat Rev Immunol 6 11: 823-835.

McClain MT, Heinlen LD, Dennis GJ, Roebuck J, Harley JB and James JA (2005). Early events in lupus humoral autoimmunity suggest initiation through molecular mimicry. Nat Med 11 1: 85-89.

Merrill JT, Neuwelt CM, Wallace DJ, Shanahan JC, Latinis KM, Oates JC, Utset TO, Gordon C, Isenberg DA, Hsieh HJ, et al. (2010). Efficacy and safety of rituximab in moderately-to-severely active systemic lupus erythematosus: the randomized, double-blind, phase II/III systemic lupus erythematosus evaluation of rituximab trial. Arthritis Rheum 62 1: 222-233.

Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, Derksen RH, PG DEG, Koike T, Meroni PL, et al. (2006). International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost 4 2: 295-306.

Moller-Larsen S, Nyegaard M, Haagerup A, Vestbo J, Kruse TA and Borglum AD (2008). Association analysis identifies TLR7 and TLR8 as novel risk genes in asthma and related disorders. Thorax 63 12: 1064-1069.

Morel J, Simoes Cda S, Avinens O, Sany J, Combe B and Eliaou JF (2003). Polymorphism of HLA-DMA and DMB alleles in patients with systemic lupus erythematosus. J Rheumatol 30 7: 1485-1490.

Musone SL, Taylor KE, Lu TT, Nititham J, Ferreira RC, Ortmann W, Shifrin N, Petri MA, Kamboh MI, Manzi S, et al. (2008). Multiple polymorphisms in the TNFAIP3 region are independently associated with systemic lupus erythematosus. Nat Genet 40 9: 1062-1064.

Nath SK, Han S, Kim-Howard X, Kelly JA, Viswanathan P, Gilkeson GS, Chen W, Zhu C, McEver RP, Kimberly RP, et al. (2008). A nonsynonymous functional variant in integrin-alpha(M) (encoded by ITGAM) is associated with systemic lupus erythematosus. Nat Genet 40 2: 152-154.

Nauta AJ, Daha MR, van Kooten C and Roos A (2003). Recognition and clearance of apoptotic cells: a role for complement and pentraxins. Trends Immunol 24 3: 148-154.

Ng MT, Van't Hof R, Crockett JC, Hope ME, Berry S, Thomson J, McLean MH, McColl KE, El-Omar EM and Hold GL (2010). Increase in NF-kappaB binding affinity of the variant C allele of the toll-like receptor 9 -1237T/C polymorphism is associated with Helicobacter pylori-induced gastric disease. Infect Immun 78 3: 1345-1352.

Ng MW, Lau CS, Chan TM, Wong WH and Lau YL (2005). Polymorphisms of the toll-like receptor 9 (TLR9) gene with systemic lupus erythematosus in Chinese. Rheumatology (Oxford) 44 11: 1456-1457.

Niewold TB, Hua J, Lehman TJ, Harley JB and Crow MK (2007). High serum IFN-alpha activity is a heritable risk factor for systemic lupus erythematosus. Genes Immun 8 6: 492-502.

Niewold TB, Kelly JA, Flesch MH, Espinoza LR, Harley JB and Crow MK (2008). Association of the IRF5 risk haplotype with high serum interferon-alpha activity in systemic lupus erythematosus patients. Arthritis Rheum 58 8: 2481-2487.

Novak N, Yu CF, Bussmann C, Maintz L, Peng WM, Hart J, Hagemann T, Diaz-Lacava A, Baurecht HJ, Klopp N, et al. (2007). Putative association of a TLR9 promoter polymorphism with atopic eczema. Allergy 62 7: 766-772.

Oh DY, Baumann K, Hamouda O, Eckert JK, Neumann K, Kucherer C, Bartmeyer B, Poggensee G, Oh N, Pruss A, et al. (2009). A frequent functional toll-like receptor 7 polymorphism is associated with accelerated HIV-1 disease progression. AIDS 23 3: 297-307.

Oh DY, Taube S, Hamouda O, Kucherer C, Poggensee G, Jessen H, Eckert JK, Neumann K, Storek A, Pouliot M, et al. (2008). A functional toll-like receptor 8 variant is associated with HIV disease restriction. J Infect Dis 198 5: 701-709.

Orru V, Tsai SJ, Rueda B, Fiorillo E, Stanford SM, Dasgupta J, Hartiala J, Zhao L, Ortego-Centeno N, D'Alfonso S, et al. (2009). A loss-of-function variant of PTPN22 is associated with reduced risk of systemic lupus erythematosus. Hum Mol Genet 18 3: 569-579.

Papadimitraki ED, Bertsias GK and Boumpas DT (2007). Toll like receptors and autoimmunity: a critical appraisal. J Autoimmun 29 4: 310-318.

Papadimitraki ED, Choulaki C, Koutala E, Bertsias G, Tsatsanis C, Gergianaki I, Raptopoulou A, Kritikos HD, Mamalaki C, Sidiropoulos P, et al. (2006). Expansion of toll-like receptor 9-expressing B cells in active systemic lupus erythematosus: implications for the induction and maintenance of the autoimmune process. Arthritis Rheum 54 11: 3601-3611.

Parra FC, Amado RC, Lambertucci JR, Rocha J, Antunes CM and Pena SD (2003). Color and genomic ancestry in Brazilians. Proc Natl Acad Sci U S A 100 1: 177-182.

Pavlovic M, Kats A, Cavallo M and Shoenfeld Y (2010). Clinical and molecular evidence for association of SLE with parvovirus B19. Lupus 19 7: 783-792.

Pawar RD, Ramanjaneyulu A, Kulkarni OP, Lech M, Segerer S and Anders HJ (2007). Inhibition of Toll-like receptor-7 (TLR-7) or TLR-7 plus TLR-9 attenuates glomerulonephritis and lung injury in experimental lupus. J Am Soc Nephrol 18 6: 1721-1731.

Perez-Mercado AE and Vila-Perez S (2010). Cytomegalovirus as a trigger for systemic lupus erythematosus. J Clin Rheumatol 16 7: 335-337.

Pestka S, Krause CD and Walter MR (2004). Interferons, interferon-like cytokines, and their receptors. Immunol Rev 202 8-32.

Pisitkun P, Deane JA, Difilippantonio MJ, Tarasenko T, Satterthwaite AB and Bolland S (2006). Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication. Science 312 5780: 1669-1672.

Pistiner M, Wallace DJ, Nessim S, Metzger AL and Klinenberg JR (1991). Lupus erythematosus in the 1980s: a survey of 570 patients. Semin Arthritis Rheum 21 1: 55-64.

Quan TE, Roman RM, Rudenga BJ, Holers VM and Craft JE (2010). Epstein-Barr virus promotes interferon-alpha production by plasmacytoid dendritic cells. Arthritis Rheum 62 6: 1693-1701.

Remmers EF, Plenge RM, Lee AT, Graham RR, Hom G, Behrens TW, de Bakker PI, Le JM, Lee HS, Batliwalla F, et al. (2007). STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. N Engl J Med 357 10: 977-986.

Riley JK and Sliwkowski MX (2000). CD20: a gene in search of a function. Semin Oncol 27 6 Suppl 12: 17-24.

Ronnblom L and Alm GV (2001a). An etiopathogenic role for the type I IFN system in SLE. Trends Immunol 22 8: 427-431.

Ronnblom L and Alm GV (2001b). A pivotal role for the natural interferon alpha-producing cells (plasmacytoid dendritic cells) in the pathogenesis of lupus. J Exp Med 194 12: F59-63.

Rosen A and Casciola-Rosen L (1999). Autoantigens as substrates for apoptotic proteases: implications for the pathogenesis of systemic autoimmune disease. Cell Death Differ 6 1: 6-12.

Rozzo SJ, Allard JD, Choubey D, Vyse TJ, Izui S, Peltz G and Kotzin BL (2001). Evidence for an interferon-inducible gene, Ifi202, in the susceptibility to systemic lupus. Immunity 15 3: 435-443.

Russell AI, Cunninghame Graham DS, Shepherd C, Roberton CA, Whittaker J, Meeks J, Powell RJ, Isenberg DA, Walport MJ and Vyse TJ (2004). Polymorphism at the C-reactive protein locus influences gene expression and predisposes to systemic lupus erythematosus. Hum Mol Genet 13 1: 137-147.

Sachs UJ, Chavakis T, Fung L, Lohrenz A, Bux J, Reil A, Ruf A and Santoso S (2004). Human alloantibody anti-Mart interferes with Mac-1-dependent leukocyte adhesion. Blood 104 3: 727-734.

Sanchez E, Callejas-Rubio JL, Sabio JM, Gonzalez-Gay MA, Jimenez-Alonso J, Mico L, de Ramon E, Camps M, Suarez A, Gutierrez C, et al. (2009). Investigation of TLR5 and TLR7 as candidate genes for susceptibility to systemic lupus erythematosus. Clin Exp Rheumatol 27 2: 267-271.

Santiago-Raber ML, Baccala R, Haraldsson KM, Choubey D, Stewart TA, Kono DH and Theofilopoulos AN (2003). Type-I interferon receptor deficiency reduces lupus-like disease in NZB mice. J Exp Med 197 6: 777-788.

Santos NP, Ribeiro-Rodrigues EM, Ribeiro-Dos-Santos AK, Pereira R, Gusmao L, Amorim A, Guerreiro JF, Zago MA, Matte C, Hutz MH, et al. (2010). Assessing individual interethnic admixture and population substructure using a 48-insertion-deletion (INSEL) ancestry-informative marker (AIM) panel. Hum Mutat 31 2: 184-190.

Schaller J (1982). Lupus in childhood. Clin Rheum Dis 8 1: 219-228.

Schott E, Witt H, Neumann K, Bergk A, Halangk J, Weich V, Muller T, Puhl G, Wiedenmann B and Berg T (2008). Association of TLR7 single nucleotide polymorphisms with chronic HCV-infection and response to interferon-a-based therapy. J Viral Hepat 15 1: 71-78.

Schur PH (1995). Genetics of systemic lupus erythematosus. Lupus 4 6: 425-437.

Shen N, Fu Q, Deng Y, Qian X, Zhao J, Kaufman KM, Wu YL, Yu CY, Tang Y, Chen JY, et al. (2010). Sex-specific association of X-linked Toll-like receptor 7 (TLR7) with male systemic lupus erythematosus. Proc Natl Acad Sci U S A 107 36: 15838-15843.

Sherer Y, Gorstein A, Fritzler MJ and Shoenfeld Y (2004). Autoantibody explosion in systemic lupus erythematosus: more than 100 different antibodies found in SLE patients. Semin Arthritis Rheum 34 2: 501-537.

Sigurdsson S, Goring HH, Kristjansdottir G, Milani L, Nordmark G, Sandling JK, Eloranta ML, Feng D, Sangster-Guity N, Gunnarsson I, et al. (2008a). Comprehensive evaluation of the genetic variants of interferon regulatory factor 5 (IRF5) reveals a novel 5 bp length polymorphism as strong risk factor for systemic lupus erythematosus. Hum Mol Genet 17 6: 872-881.

Sigurdsson S, Nordmark G, Garnier S, Grundberg E, Kwan T, Nilsson O, Eloranta ML, Gunnarsson I, Svenungsson E, Sturfelt G, et al. (2008b). A risk haplotype of STAT4 for systemic lupus erythematosus is over-expressed, correlates with anti-dsDNA and shows additive effects with two risk alleles of IRF5. Hum Mol Genet 17 18: 2868-2876.

Sigurdsson S, Nordmark G, Goring HH, Lindroos K, Wiman AC, Sturfelt G, Jonsen A, Rantapaa-Dahlqvist S, Moller B, Kere J, et al. (2005). Polymorphisms in the tyrosine kinase 2 and interferon regulatory factor 5 genes are associated with systemic lupus erythematosus. Am J Hum Genet 76 3: 528-537.

Smith KG, Jones RB, Burns SM and Jayne DR (2006). Long-term comparison of rituximab treatment for refractory systemic lupus erythematosus and vasculitis: Remission, relapse, and re-treatment. Arthritis Rheum 54 9: 2970-2982.

Smith MR (2003). Rituximab (monoclonal anti-CD20 antibody): mechanisms of action and resistance. Oncogene 22 47: 7359-7368.

Stohl W (1995). Impaired polyclonal T cell cytolytic activity. A possible risk factor for systemic lupus erythematosus. Arthritis Rheum 38 4: 506-516.

Subramanian S, Tus K, Li QZ, Wang A, Tian XH, Zhou J, Liang C, Bartov G, McDaniel LD, Zhou XJ, et al. (2006). A Tlr7 translocation accelerates systemic autoimmunity in murine lupus. Proc Natl Acad Sci U S A 103 26: 9970-9975.

Swaak AJ, Nossent JC and Smeenk RJ (1991). Prognostic factors in systemic lupus erythematosus. Rheumatol Int 11 3: 127-132.

Tao K, Fujii M, Tsukumo S, Maekawa Y, Kishihara K, Kimoto Y, Horiuchi T, Hisaeda H, Akira S, Kagami S, et al. (2007). Genetic variations of Toll-like receptor 9 predispose to systemic lupus erythematosus in Japanese population. Ann Rheum Dis 66 7: 905-909.

Taylor KE, Remmers EF, Lee AT, Ortmann WA, Plenge RM, Tian C, Chung SA, Nititham J, Hom G, Kao AH, et al. (2008). Specificity of the STAT4 genetic association for severe disease manifestations of systemic lupus erythematosus. PLoS Genet 4 5: e1000084.

Tedder TF and Engel P (1994). CD20: a regulator of cell-cycle progression of B lymphocytes. Immunol Today 15 9: 450-454.

Tew GW, Rabbee N, Wolslegel K, Hsieh HJ, Monroe JG, Behrens TW, Brunetta PG and Keir ME (2010). Baseline autoantibody profiles predict normalization of complement and anti-dsDNA autoantibody levels following rituximab treatment in systemic lupus erythematosus. Lupus 19 2: 146-157.

Torok HP, Glas J, Tonenchi L, Bruennler G, Folwaczny M and Folwaczny C (2004). Crohn's disease is associated with a toll-like receptor-9 polymorphism. Gastroenterology 127 1: 365-366.

Tsao BP (2004). Update on human systemic lupus erythematosus genetics. Curr Opin Rheumatol 16 5: 513-521.

Tsokos GC (1995). Lymphocytes, cytokines, inflammation, and immune trafficking. Curr Opin Rheumatol 7 5: 376-383.

Tucker LB, Menon S, Schaller JG and Isenberg DA (1995). Adult- and childhood-onset systemic lupus erythematosus: a comparison of onset, clinical features, serology, and outcome. Br J Rheumatol 34 9: 866-872.

Tyler DR, Persky ME, Matthews LA, Chan S and Farrar JD (2007). Pre-assembly of STAT4 with the human IFN-alpha/beta receptor-2 subunit is mediated by the STAT4 N-domain. Mol Immunol 44 8: 1864-1872.

Uribe AG, McGwin G, Jr., Reveille JD and Alarcon GS (2004). What have we learned from a 10-year experience with the LUMINA (Lupus in Minorities; Nature vs. nurture) cohort? Where are we heading? Autoimmun Rev 3 4: 321-329.

Urowitz MB, Bookman AA, Koehler BE, Gordon DA, Smythe HA and Ogryzlo MA (1976). The bimodal mortality pattern of systemic lupus erythematosus. Am J Med 60 2: 221-225.

Vang T, Congia M, Macis MD, Musumeci L, Orru V, Zavattari P, Nika K, Tautz L, Tasken K, Cucca F, et al. (2005). Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant. Nat Genet 37 12: 1317-1319.

Vargas AE, Marrero AR, Salzano FM, Bortolini MC and Chies JA (2006). Frequency of CCR5delta32 in Brazilian populations. Braz J Med Biol Res 39 3: 321-325.

Vilar MJ and Sato EI (2002). Estimating the incidence of systemic lupus erythematosus in a tropical region (Natal, Brazil). Lupus 11 8: 528-532.

Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, Daniels TE, Fox PC, Fox RI, Kassan SS, et al. (2002). Classification criteria for Sjogren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis 61 6: 554-558.

Wallace DP, M; Olsen, N; Kirou, K; Dennis, G; Yao, Y; Jallal, B; Coyle, A; Zeng, L; Lupus Interferon Skin Activity (LISA) Investigators; White, B. (2007). MEDI-545, an anti-interferon alpha monoclonal antibody, shows evidence of clinical activity in systemic lupus erythematosus. Arthritis Rheum 56 S562-S563.

Wang JP, Liu P, Latz E, Golenbock DT, Finberg RW and Libraty DH (2006). Flavivirus activation of plasmacytoid dendritic cells delineates key elements of TLR7 signaling beyond endosomal recognition. J Immunol 177 10: 7114-7121.

Ward MM, Pyun E and Studenski S (1995). Long-term survival in systemic lupus erythematosus. Patient characteristics associated with poorer outcomes. Arthritis Rheum 38 2: 274-283.

Ward MM and Studenski S (1990a). Clinical manifestations of systemic lupus erythematosus. Identification of racial and socioeconomic influences. Arch Intern Med 150 4: 849-853.

Ward MM and Studenski S (1990b). Systemic lupus erythematosus in men: a multivariate analysis of gender differences in clinical manifestations. J Rheumatol 17 2: 220-224.

Wilk E, Witte T, Marquardt N, Horvath T, Kalippke K, Scholz K, Wilke N, Schmidt RE and Jacobs R (2009). Depletion of functionally active CD20+ T cells by rituximab treatment. Arthritis Rheum 60 12: 3563-3571.

Wong KL, Lew FC, MacAry PA and Kemeny DM (2008). CD40L-expressing CD8 T cells prime CD8alpha(+) DC for IL-12p70 production. Eur J Immunol 38 8: 2251-2262.

Yao Y, Richman L, Higgs BW, Morehouse CA, de los Reyes M, Brohawn P, Zhang J, White B, Coyle AJ, Kiener PA, et al. (2009). Neutralization of interferon-alpha/beta-inducible genes and downstream effect in a phase I trial of an anti-interferon-alpha monoclonal antibody in systemic lupus erythematosus. Arthritis Rheum 60 6: 1785-1796.

Younesi V, Nikzamir H, Yousefi M, Khoshnoodi J, Arjmand M, Rabbani H and Shokri F (2010). Epstein Barr virus inhibits the stimulatory effect of TLR7/8 and TLR9 agonists but not CD40 ligand in human B lymphocytes. Microbiol Immunol 54 9: 534-541.

ANEXOS

ANEXO I

CRITÉRIOS DIAGNÓSTICOS DO LÚPUS ERITEMATOSO SISTÊMICO (Hochberg, 1997):

1. Rash malar

Eritema fixo, plano ou elevado, nas eminências malares, tendendo a poupar a região nasolabial

2. Rash Discóide

Placas eritematosas elevadas, ocorrendo cicatrização atrófica nas lesões antigas

3. Fotossensibilidade

Rash cutâneo resultante de reação incomum ao sol, por história do paciente ou observação do médico

4. Úlcera oral

Ulceração oral ou nasofaríngea, geralmente não dolorosa, observada pelo médico

5. Artrite

Artrite não – erosiva, envolvendo 2 ou mais articulações periféricas caracterizada por dor à palpação, edema ou derrame

6. Serosite

(a) pleurite – história convincente de dor pleurítica ou atrito auscultado pelo médico ou evidência de derrame pleural

ou

(b) pericardite – documentada por ECG ou atrito ou evidência de derrame pericárdico

7. Alteração renal

(a) proteinúria persistente > 0,5 g por dia ou > 3 + se não quantificada

ou

(b) cilindros celulares: podem ser hematológico, granular, tubular ou misto

8. Alteração neurológica

(a) convulsão – na ausência de drogas implicadas ou alterações metabólicas conhecidas (ex.uremia, cetoacidose, distúrbios hidroeletrolíticos)

Ou

(b) psicose - na ausência de drogas implicadas ou alterações metabólicas conhecidas (ex.uremia, cetoacidose, distúrbios hidroeletrolíticos)

9. Alteração hematológica

(a) anemia hemolítica – com reticulocitose

Ou

(b) leucopenia - < 4000/mm3 total em 2 ou mais ocasiões

Ou

(c) linfopenia - < 1500/mm3 em 2 ou mais ocasiões

Ou

(d) trombocitopenia - < 100 000/mm3 na ausência de drogas causadoras

10. Alteração imunológica

(a) anti-DNA – anticorpo ao DNA nativo em títulos anormais

Ou

(b) Anti-Sm – presença do anticorpo ao antígeno nuclear Sm

Ou

(c) Achados positivos de anticorpos antifosfolipideos baseados em (1) concentração sérica anormal de anticardiolipina IgG ou IgM, (2) teste positivo para anticoagulante lúpico usando teste-padrão ou (3) VDRL falso positivo por pelo menos 6 meses e confirmado por FTA-Abs

11. Anticorpo antinuclear (FAN)

Título anormal do FAN por imunofluorescência ou método equivalente em qualquer momento, na ausência de drogas sabidamente associadas ao lúpus induzido por drogas

Para fins de classificação de doença, o (a) paciente deve apresentar ao menos 4 dos 11 critérios.

ANEXO II

PROTOCOLO DE AVALIAÇÃO CLÍNICA E LABORATORIAL DO AMBULATÓRIO DE LÚPUS ERITEMATOSO SISTÊMICO

IDENTIFICAÇÃO: **n°_____**

Nome:_____Registro:_____

_____ Sexo: ☐ F ☐ M Raça: ☐ Branco ☐ Não branco

Data de nascimento:___/___/_____

Profissão:_____Estado

civil:_____

Naturalidade/Procedência: -

_____

Endereço:_____

_____

Cidade:_____ CEP_____-___

Telefones:_____

DATA DO INÍCIO DOS SINTOMAS: ___/___/_____

DATA DO DIAGNÓSTICO: ___/___/_____

MANIFESTAÇÕES INICIAIS NO

DIAGNÓSTICO:_____

INÍCIO DO ACOMPANHAMENTO NO HCPA:___/___/_____

ÓBITO: ☐ S ☐ N DATA:___/___/_____

CAUSA:_____

_____

CRITÉRIOS PARA CLASSIFICAÇÃO PARA LES (ACR 1997)

☐ Rash malar

☐ Rash discóide

☐ Fotossensibilidade

☐ Úlceras orais/nasais

☐ Artrite)

☐ Serosite: ☐ Pleurite ☐ Pericardite

☐ Doença renal: Classe:_____(data: ___/___/___) ☐ sem biópsia

Índice de atividade ____/____ Ìndice de cronicidade:____/____

☐ Doença neurológica: ☐ Psicose

☐ Convulsão

☐ Hematológico: ☐ Anemia hemolítica

☐ Leucopenia / linfopenia

☐ Plaquetopenia

☐ FAN: Titulação:_____ Padrão: _____

☐ Imunológico: ☐ anti DNA ☐ anti Sm

☐ aCL: IgG:_____ IgM:_____ ☐ AL ☐ VDRL

ALTERAÇÔES CLÍNICAS E LABORATORIAIS ASSOCIADAS

☐ Hipertensão ☐ Diabetes ☐ Obesidade (IMC:___)

☐ Dislipidemia

☐ SAAF

☐ Síndrome de Sjögren

☐ Eventos tromboembólicos (☐ AVC, ☐ IAM, ☐ TVP, ☐ outros:_____)

História obstétrica: G:__/P:__/C:__/A:__

obs.:_____

☐ Tabagismo ☐ Ex-tabagismo ☐ Etilismo

Outras doenças autoimunes

associadas:_____

☐ ENA

_____

_

☐ Lupus band

test:_____

TRATAMENTO REALIZADO

☐ Corticoterapia	☐ Pulsoterapia	☐ Ciclofosfamida
☐ Azatioprina	☐ Cloroquina / Hidroxicloroquina	☐ Metotrexate
☐ Micofenolato mofetil	☐ Dapsona	☐ Ciclosporina
☐ Rituximabe	☐ AAS	☐Anticoagulante
☐ ACO/TRH	☐ CaCo3/D3	☐ Bisfosfonados
☐ Estatina	☐ Danazol	☐Anti-hipertensivos

ANEXO III

Mannose-binding lectin gene polymorphisms in Brazilian patients with systemic lupus erythematosus. Lupus. 2010;19(3):280-7. Epub 2009 Dec 18.

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PAPER

Mannose-binding lectin gene polymorphisms in Brazilian patients with systemic lupus erythematosus

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The mannose-binding lectin gene (*MBL-2*) has emerged as a candidate for systemic lupus erythematosus susceptibility, but studies in Brazilian population have not been conducted. To examine potential associations of mannose-binding lectin alleles G57E, G54D, IVSnt5, R52C and R52H with susceptibility to and clinical expression of systemic lupus erythematosus in southern Brazilian patients, we conducted a case–control study with 327 consecutive patients with diagnosis of systemic lupus erythematosus and 345 healthy controls. Genotyping was performed by restriction fragment length polymorphism–polymerase chain reaction assay. A statistically significant difference in the frequencies of allele R52C was observed in European-derived systemic lupus erythematosus patients when compared with controls (9.6% vs. 3.3%, $p < 0.001$, odds ratio: 3.15, 95% confidence interval: 1.76–5.62, $p < 0.05$). The frequencies of alleles G54D and G57E were not different between European-derived systemic lupus erythematosus patients and controls. There were no differences between clinical and laboratory features in systemic lupus erythematosus patients according to the presence or absence of mannose-binding lectin allelic variants. These results support an increased risk of systemic lupus erythematosus in European-derived individuals carrying allele R52C. Patients carrying this allele have an approximately three-fold higher odds ratio of developing systemic lupus erythematosus when compared with controls. Our data do not support associations between the mannose-binding lectin allelic variants studied and clinical expression of systemic lupus erythematosus. *Lupus* (2010) **19,** 280–287.

Key words: complement; genetics and immunology; mannose-binding lectin; risk factors; systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease that involves many organs and systems. It is characterized by autoantibody production mainly directed against nuclear antigens and immune complex formation and deposition, which lead to intense inflammatory response and tissue damage. Its multiple clinical and laboratory features make its diagnosis challenging.

The mannose-binding lectin gene (*MBL-2*), a single 4-exon gene located on chromosome 10, has emerged as a candidate for SLE susceptibility due to the MBL role in innate immunity and a possible association between its deficiency and autoimmune disease.[1] MBL is an acute-phase protein synthesized by the liver that can bind to apoptotic cell debris. It participates in the phagocytosis of apoptotic cells by macrophages.

Some polymorphic variants, mainly in the coding region of *MBL-2* gene, are associated with MBL deficiency. Three functional single-nucleotide polymorphisms (SNPs) have been described, giving rise to three structural variant alleles: at codons 54 (allele G54D or B), 57 (allele G57E or C), and 52 (allele R52C or D).[2–4] All variants (B, C and D) are independent and in complete linkage disequilibrium with each other. Altogether, the presence of any B, C or D allele has been collectively labeled as O, whereas the absence of variants at these three codons has been called allele A, the wild-type allele.

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In addition to polymorphic variants found in exon 1, SNPs were identified at promoter regions –550 (alleles H/L), –221 (alleles X/Y) and +4 (alleles P/Q).[5] Subsequently, four common haplotypes were described for these promoter variants: LXP, LYP, LYQ and HYP, where HYP is associated with medium to high serum levels of MBL, and LXP is associated with low levels of this protein.[6] These promoter haplotypes are in strong linkage disequilibrium with SNPs at exon 1, resulting in seven common extended haplotypes: HYPA, LYPA, LYQA, LXPA, HYPD, LYPB and LYQC.[5]

Several studies have observed a high occurrence of MBL polymorphic variants in SLE patients.[7] Furthermore, different studies observed the association of some allelic variants with specific clinical features in SLE patients, although the variety of clinical features and human populations studied made data interpretation very difficult.[1] Other two uncommon polymorphisms have already been described: at codon 52 (allele R52H) and in the first intron, at position 5 (allele IVSnt5). Both of them do not have well established relations with MBL phenotype and they have never been studied in SLE patients.[8,9]

Considering the inconsistent reports concerning the role of MBL in SLE and the lack of studies in Brazil, this study aims at investigating the associations between MBL alleles G57E, G54D, R52C, IVSnt5 and R52H and susceptibility and clinical expression of SLE in patients from southern Brazil.

Patients and methods

Study population

The study population was composed of 327 SLE patients; 249 (76.1%) of them were identified as European derived and 78 (23.9%) as African derived. This classification was based on physical appearance, as judged by the researcher at the time of blood collection, and ethnicity data of parents/grandparents reported by the participants. The skin color-based classification criteria used in Brazil are well documented [10] and have been already assessed by our group in previous studies.[11,12] The patients received follow-up assistance at the Division of Rheumatology of the Hospital de Clínicas de Porto Alegre. The medical records were reviewed for documentation of demographic, clinical and laboratory data (Table 1). All patients fulfilled the revised criteria of the American College of Rheumatology for the SLE classification.[13]

The clinical manifestations evaluated were: photosensitivity, malar rash, discoid rash, oral or nasal ulcers, arthritis, serositis (pleuritis or pericarditis), nephritis and neurological disease, defined as seizures or psychosis. The laboratory evaluation included the presence of hematological disorders (hemolytic anemia, leukopenia, lymphopenia or thrombocytopenia), positive antinuclear antibodies (ANAs) (titer > 1:100), or other autoantibodies, such as anti-double-stranded-DNA (anti-dsDNA), anti-Sm, anti-RNP, anti-Ro/SS-A, anti La/SS-B, anticardiolipin, lupus anticoagulant and false-positive VDRL. The patients were also evaluated in terms of secondary antiphospholipid syndrome and Sjögren's syndrome, according to the classification criteria for both diseases.[14,15] SLEDAI disease activity index[16] and SLICC damage index[17] were applied to each patient as a measurement of disease activity and cumulative damage, respectively.

The control group was composed of 244 European-derived and 101 African-derived healthy individuals from the same urban center, when compared with the SLE patients. The study protocol was approved by the Ethics Committee of the Hospital de Clínicas de Porto Alegre, and informed consent according to the Declaration of Helsinki was obtained from all patients.

DNA extraction and genotyping

DNA was isolated from peripheral blood cells using a salting-out method.[18] DNA samples were stored at −20°C. Genotyping was performed as previously described.[3,8] Polymerase chain reaction (PCR) amplification of exon 1 of the *MBL-2* gene was performed with sequence-specific primers: A (5′-ACCCAGATTGTAGGACAGAG-3′) and B (5′CCTTCCAGAGGAAACTGCCTGGGGATA T3′) for the determination of G57E, G54D, IVSnt5 and R52H polymorphisms, and with primers B and R52C (5′-CATCAACGGCTTCCCAGGCAAA GACGCG-3′) for R52C polymorphism. The amplifications were carried out in reactions containing PCR buffer, $MgCl_2$, dNTP, specific primers and Taq DNA polymerase (Invitrogen Corporation, San Diego, CA, USA) and were submitted to 35 cycles of 94°C for 30 s, 54°C for 30 s (56°C for the R52C polymorphism) and 72°C for 30 s; preceded by a 5-min denaturation stage at 94°C and finalized with a 5-min extension phase at 72°C. Amplified PCR products were cleaved by specific restriction enzymes in ideal conditions, according to the manufacturer's recommendations: *Mbo*II to G57E, *Ban*I to G54D, *Nla*III to IVSnt5 and R52H, and

Table 1 Demographic, clinical, and laboratorial features of SLE patients

Patients' features	*Whole (n = 327)*	*European-derived (n = 249)*	*African-derived (n = 78)*	*p-value*[a]
Females	91.7% (327)	91.2% (227)	93.6% (78)	0.658
Age (years)	42.2 ± 14.3 (327)	42.8 ± 14.7 (249)	40.3 ± 12.9 (78)	0.578
Age at diagnosis (years)	32.7 ± 13.6 (323)	32.6 ± 13.6 (246)	33.2 ± 12.8 (77)	0.503
Malar rash	53.5% (325)	54.8% (248)	49.4% (77)	0.476
Discoid rash	14.5% (325)	14.9% (248)	13.0% (77)	0.814
Photosensitivity	73.8% (325)	78.6% (248)	58.4 % (77)	0.001
Oral ulcers	36.3% (325)	37.5% (248)	32.5% (77)	0.505
Arthritis	83.1% (325)	82.7% (248)	84.4% (77)	0.853
Serositis	31.8% (324)	29.6% (247)	39.0% (77)	0.159
Nephritis	43.1% (325)	41.9% (248)	46.8% (77)	0.539
Neurologic disorders	11.7% (325)	12.1% (248)	10.4% (77)	0.838
Hematologic disorders	77.8 % (325)	75.4 % (248)	85.7% (77)	0.081
Hemolytic anemia	30.8% (325)	31.5% (248)	28.6% (77)	0.736
Leukopenia/lymphopenia	61.2% (325)	58.1% (248)	71.4% (77)	0.049
Thrombocytopenia	19.1% (325)	18.5% (248)	20.8% (77)	0.788
Immunologic disorders	65.5% (322)	64.9% (245)	67.5% (77)	0.774
Anti-DNA	47.2% (322)	46.1% (245)	50.6% (77)	0.573
Anti-Sm	19.6% (322)	18.8% (245)	22.1% (77)	0.637
Anticardiolipin	26.2% (321)	25.4% (245)	28.6% (77)	0.688
Lupus anticoagulant	5.3% (321)	6.1% (244)	2.6% (77)	0.380
False-positive VDRL	2.5% (321)	2.9% (244)	1.3% (77)	0.685
ANA	98.8% (323)	98.8% (246)	98.7% (77)	1.000
Anti-Ro/SS-A	44.2% (276)	37.4% (206)	64.3% (70)	<0.001
Anti-La/SS-B	14.1% (276)	11.2% (206)	22.9% (70)	0.026
Anti-RNP	30.8% (276)	31.1% (206)	30.0% (70)	0.986
Sjögren	10.9% (312)	10.9% (238)	10.8% (74)	1.000
APS	6.4% (311)	7.2% (237)	4.1% (74)	0.426
SLEDAI	1 (0–4) (263)	1 (0–4) (194)	1 (0–4) (69)	0.974
SLICC	1 (0–2) (304)	1 (0–2) (230)	1 (0–2) (74)	0.910

ANA: antinuclear antibody; APS: antiphospholipid syndrome; SLEDAI: systemic lupus erythematosus disease activity index; SLICC: systemic lupus international collaborating clinics. VDRL: venereal disease research laboratory test.
[a]Chi-squared test for qualitative variables and Mann–Whitney test for quantitative variables.

*Hha*I and *Mlu*I to R52C. Digested fragments were visualized in 6% polyacrylamide gel stained with ethydium bromide under ultraviolet light.

The summary nomenclature used for MBL genotypes was: A/A indicates homozygosis for the wild type allele, A/O indicates heterozygosis with the presence of one allelic variant mutant (allele B, allele C or allele D) and O/O indicates homozygosity for any variant or double heterozygosis of any two variants (B, C or D).

Statistical analysis

The descriptive data analysis used the calculation of mean and standard deviation values for quantitative variables, while frequency and percentage were calculated for the categorical variables. We used the chi-squared test or Fisher's exact test to compare the frequencies of polymorphic variants. The odds ratio and the confidence interval were also calculated. For the comparison of clinical and laboratory variables with the frequencies of polymorphic variants, we used the chi-squared test for qualitative variables and the Student's *t*-test (or Mann–Whitney test) for quantitative variables, using the Bonferroni correction to the level of statistical significance. The Hardy–Weinberg equilibrium test was performed in cases and controls using the chi-squared test. Data were analyzed with SPSS software version 13.0 and two-tailed value of $p < 0.05$ was obtained to indicate statistical significance.

Results

Three hundred and twenty seven SLE patients were included: 249 (76.1%) Euro-derived and 78 (23.9%) African-derived. Three hundred (91.7%) were female and 27 (8.3%) were male. The patients' mean age was 42.2 ± 14.3 years and the mean disease diagnostic age was 32.7 ± 13.6 years. Table 1 shows the frequencies of clinical and laboratory features. Secondary antiphospholipid syndrome was found in 6.4% (20/311) and Sjögren's

syndrome in 10.9% (34/312). The median for SLEDAI and SLICC was 1 (25–75th percentile). The European-derived group presented a higher proportion of individuals with photosensitivity (78.6% vs. 58.4%, $p = 0.001$) and a lower proportion of individuals presenting leukopenia or lymphopenia (58.1% vs. 71.4%, $p = 0.049$). The presence of anti-Ro/SS-A and anti-La/SS-B was significantly higher in the African-derived group (64.3% and 22.9% vs. 37.4% and 11.2%, $p < 0.001$ and $p = 0.026$, respectively). We observed that male patients had a higher frequency of nephritis (70.4% against 40.6%, $p = 0.005$), although the number of individuals in this group was relatively small (data not shown). No other statistically significant differences were found between genders.

The frequency of exon 1 *MBL-2* gene polymorphisms was studied in patients with SLE and healthy controls (Table 2). Variant alleles R52H and IVSnt5 were not found in the studied population. The genotypic distribution of all other three polymorphisms was in Hardy–Weinberg equilibrium in both cases and controls. Among the 249 European-derived patients with SLE, 129 (51.8%) were A/A, 95 (38.2%) were A/O and 25 (10%) were O/O. When compared with the European-derived healthy controls [150 (61.5%), 81 (33.2%) and 13 (5.3%), respectively], there was a statistically significant higher frequency of genotypes containing mutant allele O in SLE patients ($p = 0.034$). The African-derived patients did not present any statistically significant difference for genotypic distribution.

In the analysis of MBL allelic frequencies in SLE patients and healthy controls, considering alleles B, C and D individually, a statistically significant higher prevalence of allele D was found in the European-derived patient group [48 (9.6%) vs. 16 (3.3%), $p < 0.001$]. For the other two allelic variants (alleles B and C), no statistically significant differences were observed (Table 3). Consequently, allele A had a higher frequency in control group [381 (78.0%) vs. 353 (70.9%), $p = 0.01$]. The overall odds ratio (OR) of allele D was 3.15 [95% confidence interval (CI) 1.76–5.62, $p < 0.05$] in the comparison of European-derived patients to controls. The African-derived patients did not present any statistically significant difference for allelic distribution.

Table 4 shows the summarized differences observed in disease features in patients with SLE categorized by MBL genotypes. A higher frequency of leukopenia and lymphopenia and a higher frequency of lupus anticoagulant were observed in O/O European-derived SLE patients, when compared with A/O and A/A patients [76% vs. 50% and 60.6%, $p = 0.045$ (data not shown) and 16% vs. 7.5% and 3.2%, $p = 0.042$, respectively]. However, after applying the Bonferroni correction, these differences did not reach statistical significance.

Table 2 MBL genotypic frequency in SLE patients and health controls

	European-derived		*African-derived*	
Genotype	*Patients (%) n = 249*	*Controls (%) n = 244*	*Patients (%) n = 78*	*Controls (%) n = 101*
AA	129 (51.8)	150 (61.5)	45 (57.7)	58 (57.4)
AO	95 (38.2)	81 (33.2)	29 (37.2)	38 (37.6)
OO	25 (10.0)	13 (5.3)	4 (5.1)	5 (5.0)
p-value[a]	0.034		0.997	

A: wild type allele; O: allele B or C or D.
[a]Chi-squared test.

The prevalence of clinical features was compared considering the presence or absence of every variant allele. Table 5 shows the frequencies in the presence of allele B, C or D. Some interesting findings were obtained, such as: higher prevalence of hematological disorders (88.9% vs. 72.4%, $p = 0.033$), thrombocytopenia (33.3% vs. 15.3%, $p = 0.009$), lupus anticoagulant (13.3% vs. 4.5%, $p = 0.038$), psychosis (13.3% vs. 4.9%, $p = 0.049$) and lower frequency of anti-Ro/SS-A (21.4% vs. 41.5%, $p = 0.027$), with the presence of allele D in European-derived SLE patients. In African-derived patients, leukopenia and lymphopenia were more frequent in patients carrying allele B (94.1% vs. 65.0%, $p = 0.009$) and nephritis and anti-Ro/SS-A were less frequent in patients carrying alleles B and C, respectively (17.6% vs. 55.0%, $p = 0.014$ and 30.8% vs. 71.9%, $p = 0.009$, respectively). However, after applying the Bonferroni correction, no statistical significance was maintained.

Discussion

When comparing the results of our study with those of literature, we observed that our population presented a higher proportion of hemolytic anemia and photosensitivity.[19] The European-derived patients, when compared with the African-derived patients, presented a higher proportion of photosensitivity, but lower frequencies of anti-Ro/SS-A and anti-La/SS-B autoantibodies, which are frequently related to cutaneous manifestations. This result could be explained by the inherent difficulty in detecting this manifestation in dark-skinned patients.

Table 3 MBL allelic frequency in SLE patients and health controls

	European-derived			*African-derived*		
Alleles	*Patients (%) 2n = 498*	*Controls (%) 2n = 488*	*p-value*[a]	*Patients (%) 2n = 156*	*Controls (%) 2n = 202*	*p-value*[a]
Allele A	353 (70.9)	381 (78.0)	0.010	119 (76.3)	154 (76.2)	0.999
Allele B	79 (15.9)	77 (15.8)	0.971	19 (12.2)	16 (7.9)	0.210
Allele C	18 (3.6)	14 (2.9)	0.509	15 (9.6)	27 (13.4)	0.055
Allele D	48 (9.6)	16 (3.3)	<0.001	3 (1.9)	5 (2.5)	0.997

[a]Chi-squared test.

Table 4 Clinical and laboratorial characteristics in SLE patients categorized by different MBL genotype

	European-derived (n = 249)			*African-derived (n = 78)*		
Patients' features[a]	*A/A (%) n = 129*	*A/O (%) n = 95*	*O/O (%) n = 25*	*A/A (%) n = 45*	*A/O (%) n = 29*	*O/O (%) n = 4*
Malar rash	56.3	53.7	52.0	52.3	41.4	75.0
Discoid rash	12.5	20.0	8.0	13.6	13.8	0.0
Photosensitivity	82.8	74.7	72.0	65.9	48.3	50.0
Oral ulcers	36.7	42.1	24.0	29.5	37.9	25.0
Arthritis	79.7	88.4	76.0	81.8	89.7	75.0
Serositis	25.8	35.1	28.0	36.4	41.4	50.0
Nephritis	43.8	42.1	32.0	52.3	44.8	0.0
Neurologic disorders	8.6	14.7	20.0	11.4	10.3	0.0
Hematologic disorders	73.4	74.7	88.0	86.4	82.8	100.0
Immunologic disorders	61.4	69.9	64.0	70.5	65.5	50.0
Anti-DNA	47.2	45.2	44.0	59.1	37.9	50.0
Anti-Sm	18.9	20.4	12.0	20.5	27.6	0.0
Anticardiolipin	20.6	29.0	36.0	29.5	27.6	25.0
Lupus anticoagulant	3.2	7.5	16.0	4.5	0.0	0.0
False-positive VDRL	1.6	4.3	4.0	2.3	0.0	0.0
ANA	97.6	100.0	100.0	100.0	96.6	100.0
Anti-Ro	42.2	36.1	19.0	66.7	59.3	75.0
Anti-La	13.7	9.6	4.8	20.5	22.2	50.0
Anti-RNP	30.4	36.1	14.3	30.8	33.3	0.0
Sjögren	12.2	9.9	8.3	7.1	14.3	25.0
APS	6.5	7.8	8.3	4.8	3.6	0.0
SLEDAI[b]	2 (0–36)	1 (0–10)	0 (0–4)	1 (0–16)	2 (0–16)	1 (0–8)
SLICC[b]	1 (0–5)	0 (0–3)	2 (0–5)	1 (0–7)	1 (0–8)	1 (0–4)

A: wild type allele; O: allele B, C or D; ANA: antinuclear antibody; VDRL: venereal disease research laboratory test; APS: antiphospholipid syndrome; SLEDAI: systemic lupus erythematosus disease activity index; SLICC: systemic lupus international collaborating clinics; *n*: number of patients.
[a]Frequencies according to the presence of genotype. [b]Median (minimum–maximum). Statistical significance was considered with p-value < 0.0024 with Bonferroni correction.

The African-derived patients presented higher proportion of leukopenia and lymphopenia, when compared with the European-derived group. We observed that male patients had a higher prevalence of nephritis, when compared with female patients; however, this result should be considered with caution due to the relatively low number of male patients. The prevalence of secondary antiphospholipid syndrome and Sjögren's syndrome was 6.4% and 10.9%, respectively, considering all patients. Both frequencies were in accordance with previous reports.[20,21] Medians for SLICC and SLEDAI were relatively low, which indicate that our SLE patients had low activity and damage indexes, although the patients were diagnosed approximately ten years before the evaluation. Thus, these low indexes could reflect a higher number of patients with mild to moderate disease in our population or could be secondary to an efficient treatment.

Several studies have shown that *MBL-2* gene polymorphisms influence susceptibility to SLE and could be associated with some clinical and laboratory features, disease progression, cardiovascular disease and increased risk of infections.[1]

Table 5 Clinical and laboratory characteristics of SLE patients categorized by the presence of MBL variant allele

	European-derived (N = 249)			*African-derived (N = 26)*		
Patients' features[a]	*B (%) n = 79*	*C (%) n = 18*	*D (%) n = 48*	*B (%) n = 19*	*C (%) n = 15*	*D (%) n = 3*
Malar rash	55.6	50.0	46.7	58.8	33.3	33.3
Discoid rash	16.7	5.6	20.0	5.9	20.0	0.0
Photosensitivity	73.6	72.2	77.8	47.1	46.7	33.3
Oral ulcers	34.7	33.3	40.0	35.3	33.3	33.3
Arthritis	80.6	94.4	84.4	94.1	80.0	100.0
Serositis	32.4	44.4	31.1	35.3	46.7	100.0
Nephritis	45.8	38.9	31.1	17.6	53.3	66.7
Neurologic disorders	15.3	16.7	20.0	0.0	20.0	0.0
Hematologic disorders	73.6	77.8	88.9	94.1	80.0	66.7
Immunologic disorders	65.7	83.3	64.4	70.6	46.7	100.0
Anti-DNA	40.0	55.6	46.7	35.3	33.3	100.0
Anti-Sm	15.7	22.2	15.6	29.4	13.3	33.3
Anticardiolipin	31.4	38.9	33.3	23.5	33.3	33.3
Lupus anticoagulant	8.6	11.1	13.3	0.0	0.0	0.0
False-positive VDRL	4.3	5.6	4.4	0.0	0.0	0.0
ANA	100.0	100.0	100.0	100.0	93.3	100.0
Anti-Ro	34.5	41.2	21.4	76.5	30.8	100.0
Anti-La	12.1	5.9	4.8	29.4	15.4	66.7
Anti-RNP	31.0	29.4	23.8	35.3	15.4	33.3
Sjögren	10.3	5.9	11.4	5.9	0.0	0.0
APS	7.4	12.5	9.1	17.6	14.3	0.0
SLEDAI[b]	0 (0–16)	3 (0–10)	2 (0–12)	0 (0–4)	2 (0–10)	2 (0–4)
SLICC[b]	1 (0–5)	2 (0–8)	1 (0–4)	0 (0–5)	1 (0–3)	3 (0–5)

B: allele B; C: allele C; D: allele D; ANA: antinuclear antibody; VDRL: venereal disease research laboratory test; APS: antiphospholipid syndrome; SLEDAI: systemic lupus erythematosus disease activity index; SLICC: systemic lupus international collaborating clinics; *N*: number of patients; *n*: number of alleles.
[a]Frequencies according to the presence of mutant allele. [b]Median (minimum–maximum). Statistical significance was considered with p-value < 0.0024 with Bonferroni correction.

However, the true role of MBL levels in SLE onset and progression remains unclear, as data from different studies are controversial. There are two possible explanations for these controversial data: (a) the different ethnic backgrounds of the studied populations and b) the limited number of patients in the studies, with poor case/control matching. *MBL-2* polymorphisms on populations with specific ethnic backgrounds can determine different relative risks according to their interaction with distinct factors, which could involve other genetic markers, environmental exposure, immunological alterations and/or hormonal variations. It should be noted that, due to the high variability of *MBL-2* variant frequencies in different populations, attempts to increase the statistical power by grouping data from different studies and populations are very difficult.

A meta-analysis involving studies with three ethnic populations: European-derived, African-derived and Asian-derived concluded that allele B and allelic variants at the promoter region of *MBL-2* gene, specifically those found at positions –550 and –221, were risk factors for SLE development, with a relative risk of 1.40, 1.48 and 1.22, respectively.[7] In our study, no statistically significant associations were found between alleles B and C and SLE patients. However, we observed a significant difference concerning the presence of allele D in European-derived SLE patients when compared with healthy controls, determining an OR of 3.15, which contrasts with data from other studies.[22,23]

The frequency of mutant alleles varies greatly, depending on the population studied. Allele B has a prevalence of ~26% in European-derived populations and allele C is found at a frequency of 50–60% in African-derived populations.[24] For allele D, the prevalence among different studied populations is very low.[25] In Brazil, significant differences are found in the distribution of the *MBL-2* gene variants, considering geographically and ethnically distinct populations.[26] A study with European-derived healthy individuals in southern Brazil observed frequencies of 15.2%, 1.5% and 5.4% for alleles B, C and D, respectively.[27] Another study focused on ethnically mixed healthy individuals in northern Brazil observed frequencies

of 13.6% and 16.2% for alleles B and D, respectively.[28] According to Garred *et al.*, allele D did not reduce protein levels as much as alleles B and C.[29] This is an interesting observation, as our study failed to indicate any influence of alleles B and C, although allele D was detected as an important risk factor in European-derived patients. Perhaps other unknown genetic factors related to the presence of allele D, rather than MBL plasmatic level itself, might be associated with SLE development in these patients. In addition, we found no differences among African-derived SLE patients and the ethnically matched controls, reinforcing the assumption that the influence of MBL on the ethiopathogenesis of SLE is dependent on the genetic ancestry.

Another interesting fact is the high number of studies attempting to establish associations between clinical and laboratory features and specific MBL alleles, such as: a higher frequency of infections and decreased levels of C_3 and CH50 were observed in SLE patients homozygous to allele B[30]; SLE patients with low MBL levels presented a relative risk of 3.1 times to cardiovascular diseases[31]; a significant correlation was observed between some MBL allelic variants and increased risk of arterial thrombosis[32]; an association was made between *MBL-2* allelic variants and cerebrovascular disease in SLE European-derived patients[33]; an association was made of MBL deficiency with low frequency of autoantibodies and delayed development of the disease[34]; carriers of *MBL-2* gene variants in North-American patients with SLE presented low levels of serositis, renal involvement and antiphospholipid antibodies, but higher prevalence of leucopenia, when compared with controls[35]; the significantly higher prevalence of anti-Sm antibody was associated with genotypes A/B and A/C in a North-American group of SLE patients and they suggested that the MBL variants act as disease-modifying factors, particularly considering renal involvement[22]; the low serum MBL level predisposes Chinese SLE patients to infections, particularly bacterial infections.[36] It should be noted that not all studies indicate a strict correlation between functional MBL and the development of infections in SLE patients, as no associations were observed between functional MBL serum levels or MBL activity and major infections in SLE patients.[37] A study with Chinese patients observed that the frequency of juvenile-onset SLE ($\leq$20 years) was particularly high among XA/XA homozygotes (17.4%), when compared with the remaining patients (5.6%) and that XA/XA carriers presented a significantly higher risk of developing cutaneous manifestations $p = 0.003$) and pleuritis/pericarditis ($p = 0.013$), when compared with the remaining patients.[38] Associations between MBL polymorphisms and pediatric-onset SLE in a cohort of Chinese children did not find significant relations between allele B and SLE or its clinical manifestations. Interestingly, alleles C and D were not observed in these subjects, emphasizing that studies are required in populations with different ethnic backgrounds.[39] In our population, no statistically significant associations were found between clinical and laboratory characteristics and the frequency of variant alleles.

In this study, patients and controls were also genotyped for two minor *MBL-2* polymorphisms. The first one was a polymorphism involving a G to A nucleotide substitution at position five of the first intron (IVSnt5). This variant was first described in African-derived patients with sickle cell anemia. This polymorphism is associated with LY haplotype and lower MBL plasma levels, when compared with HY haplotype.[9] The second one was a G to A nucleotide substitution at codon 52, different from classical allele D. This polymorphism was first described in an African-derived population and its relationship with MBL phenotype has not been well established.[8] Both polymorphisms have never been studied in lupus patients. They were not observed in our sample, probably due to their low frequency.

In conclusion, our data indicated that the frequency of allele D was increased in SLE patients, when compared with controls from the same ethnic background, suggesting that this allele is an important risk factor for SLE development in the southern Brazilian population. However, *MBL-2* allelic variants do not seem to have a direct effect on clinical and/or laboratorial features of SLE in our population. Our data reinforce the idea that *MBL-2* alleles are risk factors for SLE development and that these risk factors vary according to the genetic background of the studied population. Therefore, further studies are needed to clarify the true role of MBL in SLE.

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References

1 Monticielo OA, Mucenic T, Xavier RM, Brenol JC, Chies JA. The role of mannose-binding lectin in systemic lupus erythematosus. *Clin Rheumatol* 2008; 27: 413–419.
2 Lipscombe RJ, Sumiya M, Hill AV, *et al*. High frequencies in African and non-African populations of independent mutations in the mannose binding protein gene. *Hum Mol Genet* 1992; 1: 709–715.
3 Madsen HO, Garred P, Kurtzhals JA, *et al*. A new frequent allele is the missing link in the structural polymorphism of the human mannan-binding protein. *Immunogenetics* 1994; 4037–4044.
4 Sumiya M, Super M, Tabona P, *et al*. Molecular basis of opsonic defect in immunodeficient children. *Lancet* 1991; 337: 1569–1570.
5 Madsen HO, Garred P, Thiel S, *et al*. Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. *J Immunol* 1995; 155: 3013–3020.
6 Madsen HO, Satz ML, Hogh B, Svejgaard A, Garred P. Different molecular events result in low protein levels of mannan-binding lectin in populations from southeast Africa and South America. *J Immunol* 1998; 161: 3169–3175.
7 Lee YH, Witte T, Momot T, *et al*. The mannose-binding lectin gene polymorphisms and systemic lupus erythematosus: two case–control studies and a meta-analysis. *Arthritis Rheum* 2005; 52: 3966–3974.
8 Mombo LE, Lu CY, Ossari S, *et al*. Mannose-binding lectin alleles in sub-Saharan Africans and relation with susceptibility to infections. *Genes Immun* 2003; 4: 362–367.
9 Neonato MG, Lu CY, Guilloud-Bataille M, *et al*. Genetic polymorphism of the mannose-binding protein gene in children with sickle cell disease: identification of three new variant alleles and relationship to infections. *Eur J Hum Genet* 1999; 7: 679–686.
10 Parra FC, Amado RC, Lambertucci JR, Rocha J, Antunes CM, Pena SD. Color and genomic ancestry in Brazilians. *Proc Natl Acad Sci USA* 2003; 100: 177–182.
11 Veit T, Cordero E, Mucenic T, *et al*. Association of the HLA-G 14 bp polymorphism with systemic lupus erythematosus. *Lupus* 2009; 18: 424–430.
12 Vargas AE, Marrero AR, Salzano FM, Bortolini MC, Chies JA. Frequency of CCR5delta32 in Brazilian populations. *Braz J Med Biol Res* 2006; 39: 321–325.
13 Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997; 40: 1725.
14 Vitali C, Bombardieri S, Jonsson R, *et al*. Classification criteria for Sjogren's syndrome: a revised version of the European criteria proposed by the American–European Consensus Group. *Ann Rheum Dis* 2002; 61: 554–558.
15 Miyakis S, Lockshin MD, Atsumi T, *et al*. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* 2006; 4: 295–306.
16 Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH. Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE. *Arthritis Rheum* 1992; 35: 630–640.
17 Gladman D, Ginzler E, Goldsmith C, *et al*. The development and initial validation of the Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index for systemic lupus erythematosus. *Arthritis Rheum* 1996; 39: 363–369.
18 Lahiri DK, Nurnberger JI, Jr. A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. *Nucleic Acids Res* 1991; 19: 5444.
19 Font J, Cervera R, Ramos-Casals M, *et al*. Clusters of clinical and immunologic features in systemic lupus erythematosus: analysis of 600 patients from a single center. *Semin Arthritis Rheum* 2004; 33: 217–230.
20 Manoussakis MN, Georgopoulou C, Zintzaras E, *et al*. Sjogren's syndrome associated with systemic lupus erythematosus: clinical and laboratory profiles and comparison with primary Sjogren's syndrome. *Arthritis Rheum* 2004; 50: 882–891.
21 Tarr T, Lakos G, Bhattoa HP, *et al*. Clinical thrombotic manifestations in SLE patients with and without antiphospholipid antibodies: a 5-year follow-up. *Clin Rev Allergy Immunol* 2007; 32: 131–137.
22 Piao W, Liu CC, Kao AH, *et al*. Mannose-binding lectin is a disease-modifying factor in North American patients with systemic lupus erythematosus. *J Rheumatol* 2007; 34: 1506–1513.
23 Villarreal J, Crosdale D, Ollier W, *et al*. Mannose binding lectin and FcγRIIa (CD32) polymorphism in Spanish systemic lupus erythematosus patients. *Rheumatology (Oxford)* 2001; 40: 1009–1012.
24 Lipscombe RJ, Beatty DW, Ganczakowski M, *et al*. Mutations in the human mannose-binding protein gene: frequencies in several population groups. *Eur J Hum Genet* 1996; 4: 13–19.
25 Turner MW. The role of mannose-binding lectin in health and disease. *Mol Immunol* 2003; 40: 423–429.
26 Boldt AB, Culpi L, Tsuneto LT, de Souza IR, Kun JF, Petzl-Erler ML. Diversity of the MBL2 gene in various Brazilian populations and the case of selection at the mannose-binding lectin locus. *Hum Immunol* 2006; 67: 722–734.
27 Alves Pedroso ML, Boldt AB, Pereira-Ferrari L, *et al*. Mannan-binding lectin MBL2 gene polymorphism in chronic hepatitis C: association with the severity of liver fibrosis and response to interferon therapy. *Clin Exp Immunol* 2008; 152: 258–264.
28 Hermes RB, Vallinoto ACR. Avaliação do polimorfismo no gene MBL (mannose-binding lectin) em pacientes infectados pelo vírus da hepatite C (VHC); in Revista Científica da UFPA - XVII Seminário de Iniciação Científica da UFPA, 2006.
29 Garred P, Larsen F, Madsen HO, Koch C. Mannose-binding lectin deficiency—revisited. *Mol Immunol* 2003; 40: 73–84.
30 Takahashi R, Tsutsumi A, Ohtani K, *et al*. Association of mannose binding lectin (*MBL*) gene polymorphism and serum MBL concentration with characteristics and progression of systemic lupus erythematosus. *Ann Rheum Dis* 2005; 64: 311–314.
31 Font J, Ramos-Casals M, Brito-Zeron P, *et al*. Association of mannose-binding lectin gene polymorphisms with antiphospholipid syndrome, cardiovascular disease and chronic damage in patients with systemic lupus erythematosus. *Rheumatology (Oxford)* 2006 Jun 26.
32 Ohlenschlaeger T, Garred P, Madsen HO, Jacobsen S. Mannose-binding lectin variant alleles and the risk of arterial thrombosis in systemic lupus erythematosus. *N Engl J Med* 2004; 351: 260–267.
33 Calvo-Alen J, Alarcon GS, Tew MB, *et al*. Systemic lupus erythematosus in a multiethnic US cohort: XXXIV. Deficient mannose-binding lectin exon 1 polymorphisms are associated with cerebrovascular but not with other arterial thrombotic events. *Arthritis Rheum* 2006; 54: 1940–1945.
34 Garcia-Laorden MI, Rua-Figueroa I, Perez-Aciego P, *et al*. Mannose binding lectin polymorphisms as a disease-modulating factor in women with systemic lupus erythematosus from Canary Islands, Spain. *J Rheumatol* 2003; 30: 740–746.
35 Bertoli AM, Fernandez M, McGwin G, Jr, *et al*. Systemic lupus erythematosus in a multiethnic US cohort: XXXVI. Influence of mannose-binding lectin exon 1 polymorphisms in disease manifestations, course, and outcome. *Arthritis Rheum* 2006; 54: 1703–1704.
36 Mok MY, Ip WK, Lau CS, Lo Y, Wong WH, Lau YL. Mannose-binding lectin and susceptibility to infection in Chinese patients with systemic lupus erythematosus. *J Rheumatol* 2007; 34: 1270–1276.
37 Bultink IE, Hamann D, Seelen MA, *et al*. Deficiency of functional mannose-binding lectin is not associated with infections in patients with systemic lupus erythematosus. *Arthritis Res Ther* 2006; 8: R183.
38 Jakab L, Laki J, Sallai K, *et al*. Association between early onset and organ manifestations of systemic lupus erythematosus (SLE) and a down-regulating promoter polymorphism in the MBL2 gene. *Clin Immunol* 2007; 125: 230–236.
39 Tsai Y, Yao T, Kuo M, Cheng T, Huang J. Lack of association of mannose-binding lectin gene polymorphisms with development and clinical manifestations of systemic lupus erythematosus in Chinese children. *Lupus* 2009; 18: 372–376.

ANEXO IV

Association Between Mannose-Binding Lectin Gene Polymorphisms and Pre-eclampsia in Brazilian Women. Am J Reprod Immunol. 2010 Nov;64(5):359-74.

Association Between Mannose-Binding Lectin Gene Polymorphisms and Pre-eclampsia in Brazilian Women

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Problem
Mannose-binding lectin (MBL) is involved in the maintenance of an inflammatory environment in uterus. High MBL levels have been associated with successful pregnancies whereas low levels are involved in pre-eclampsia (PE) development. Here, we evaluated *MBL2* gene polymorphisms in the structural and promoter regions addressing their association with PE.

Method of study
DNA samples from 162 control pregnant women and 157 pregnant PE women were genotyped and data compared with demographic and clinical characteristics.

Results
High frequency of C and D alleles (related to low MBL levels) was observed in PE women when compared to controls (C: 0.08 versus 0.03, $P = 0.006$; D: 0.10 versus 0.05, $P = 0.009$). Grouping the MBL genotypes according to phenotype, a higher frequency of OO genotype was observed in PE women when compared to control women (0.15 versus 0.04, $P = 0.007$).

Conclusion
Our data suggest that women with genotypes associated with low MBL levels could be potential PE developers.

Introduction

An immunological balance is required to allow the fetus growth during pregnancy. Inflammation plays a major role in the maintenance of pregnancy. Inflammatory responses are stimulated by different forms of tissue injury.[1] A TH2 profile has been associated with successful pregnancies[2–4] but some inflammatory environment in the uterus is of pivotal importance for appropriate placentation. In addition, an excessively inflammatory profile during pregnancy has been associated with disorders such as pre-eclampsia (PE), short gestation period, and miscarriages.[5,6] Inflammatory cells and immunological mediators, such as components of the complement system, are present in the uterine environment. The development of PE, a disorder that leads to high maternal and fetal mortality, involves inflammatory events and a spectrum of clinical presentations, including an incorrect placentation.[1,7–10] PE is characterized by abnormal vascular response to placentation, increased systemic vascular resistance,

enhanced platelet aggregation, activation of the coagulation cascade, endothelial cell dysfunction, and a systemic activation of maternal inflammatory cell responses.[1,11–14] The etiology and pathogenesis of PE may include maternal–fetal genetic and immunological factors. Genetic studies on PE development have suggested that NK cell activation during early pregnancy is beneficial for the placentation.[15] We had previously demonstrated that PE development is associated with polymorphisms of the HLA-G gene, largely studied in the regulation of pregnancy.[16]

The mannose-binding lectin (MBL) plays a fundamental role in inflammation and is also important for the maintenance of pregnancy. MBL is secreted by the liver and is present in the serum under oligo- or polymeric association activating the lectin pathway of complement through binding to specific bacterial motifs and promoting phagocytosis. MBL is a pro-inflammatory protein, modulating the inflammation and further inducing apoptosis.[17] Expression of paternal antigens on fetal cells can induce activation of the maternal complement cascade,[18,19] and activation of the complement by MBL contributes to the destruction of trophoblast cells at the maternal–fetal interface, increasing the possibility of an insufficient vascularization at time of implantation.[20] However, there are contradictory results regarding the role of MBL during pregnancy.[21] Indeed, previous studies have suggested that higher maternal MBL levels have been found associated with successful pregnancies and MBL levels were increased in the first trimester of pregnancy, suggesting a role of MBL in nidation, placentation, and maintenance of pregnancy.[22] Although a rise in MBL levels during pregnancy is related to the maternal genotype, a higher deposition of complement components have been identified in placentas from PE women.[19,23] MBL serum concentrations are influenced by single nucleotide polymorphisms (SNPs) located at both the structural and the promoter regions of the *MBL2* gene.[24] Some of these polymorphisms are associated with MBL protein deficiency and the development of several diseases and healthy complications.[25–28]

Three functional SNPs in *MBL2* gene were located at exon 1: at codons 54 (allele B, rs1800450), 57 (allele C, rs1800451), and 52 (allele D, rs5030737). At codon 54, a G to A substitution alters an aspartic acid to a glycine at the protein level. At codon 57 there is a G to A substitution (glycine to glutamic acid) and at codon 52, a C to T substitution leads to a change from arginine to cysteine. All three variants inhibit the correct oligomerization of MBL chains into the basic trimer structure, reducing the amount of functional MBL subunits in heterozygous individuals.[29] Concerning the mutations in exon 1, the wild-type allele is referred as A and the variants B, C, and D are collectively called as O. The AA wild-type genotype produces the highest MBL serum concentration and the OO genotype the lowest, disrupting multimer formation, and resulting in impaired function.[24,30] The variants in the first exon were never found together in the same chromosome and have been found in strong linkage disequilibrium with the variants in the promoter region of *MBL2* gene (H/L and X/Y). Therefore, considering that mutations in the promoter region prevent the binding of transcriptions factors, generating defectives protein forms and low secreted levels,[31,32] it is also important to analyze the polymorphisms of the promoter region of the *MBL2* gene. Low MBL levels have been associated with pregnancy complications,[33–38] and altered MBL levels could be involved in PE development.[20,39] Here, we analyzed the polymorphisms in the structural and promoter *MBL2* gene regions and investigated their association with the pathogenesis of PE.

Material and methods

Patients

The patients were recruited at the Maternity Unit of at public hospital in southern Brazil (Hospital Nossa Senhora da Conceição, Porto Alegre). We selected 162 control pregnant women and 157 pregnant women with PE. Their clinical characteristics are described in Table I. The inclusion criteria for controls were as follows: no rise in blood pressure, no hypertension or proteinuria, similar age in comparison with patients with PE (control women 28.08 years ± 7.37 and PE women 30.32 years ± 7.46), no biological relationship and a delivery date as close as possible to the delivery date of the matched patient. Controls were followed up for at least three months after delivery. If hypertension was observed during this follow-up period, the individual was excluded from the control group. Pre-eclampsia was defined by the presence of hypertension and proteinuria. Hypertension was characterized by blood pressure of at least 140 mmHg (systolic) or at least 90 mmHg (diastolic), on at least two occasions and 4–6 h apart following the 20th

Table I Demographic and clinical characteristics of the study group

Characteristics	PE women (*n* = 157)	Control Women (*n* = 162)
Maternal age at delivery (years)	30.32 ± 7.46	28.08 ± 7.37
Maternal body mass index (BMI)	32.95 ± 6.11	28.19 ± 4.61
Maternal smoking (*n* per day)	2.48 ± 7.17	3.31 ± 7.16
Race/ethnicity (*n*; % Caucasian)	107 (67.7)	128 (77.6)
Gestational age (weeks)	35.4 ± 3.72	38.3 ± 3.34
Birth weight of child (g)	2682 ± 76.7	3061 ± 57.5
Low birth weight (<2500 g) (%)	29 (18.4)	1 (0.6)
Sex of child (male/female)	62/73	70/71
Cesarean delivery (%)	84/125 (67)	57/141 (40)
Vaginal delivery (%)	41/125 (33)	84/141 (60)
Primiparous women (%)	43 (54.4)	36 (45.6)
Number of previous miscarriages		
0	121 (77%)	119 (73%)
1	25 (16%)	34 (20%)
≥2	8 (5%)	8 (5%)

Data are represented as mean ± S.D. or *n* (%).

week of gestation in women known to be normotensive beforehand.[40,41] Proteinuria was defined by the excretion of 300 mg or more of protein every 24 h. If 24-h urine samples were not available, proteinuria was defined by the protein concentration of 300 mg/L or more (≥1 + on dipstick) in at least two random urine samples taken at least 4–6 h apart following the 20th week of gestation.[41] The PE was classified as severe when blood pressure was ≥160/110 mmHg; or urinary protein excretion ≥5 g per 24 h; a platelet count of <100 000 mm^{-3} in at least two samples; the combination of hemolysis, abnormal liver enzymes associated with persistent epigastric or upper right quadrant pain; persistent and severe symptoms as altered mental status, headaches, blurred vision, or blindness; presence of multiorgan involvement such as pulmonary edema, oliguria (<500 mL per day).[14] Women who had chronic hypertension, renal disease, collagen vascular diseases, cancer, or thrombosis were not included in the study. All patients participating in this study gave their written informed consent, and the protocol was approved by the ethics committee of the Conceição Hospital (Porto Alegre, Brazil) and by the National Research Ethics Committee.

Genomic DNA

DNA was isolated from whole blood using a salting-out procedure according to Lahiri and Nurnberger.[42]

Polymerase Chain Reaction PCR–RFLP

A Polymerase Chain Reaction (PCR) – Restriction Fragment Length Polymorphism (RFLP) analysis was performed to identify SNPs in exon 1 (*codon 54* and *57*) of *MBL2* gene, as previously described.[43] The B *(*codon 54*)* and C (codon 57) alleles were detected respectively by *Ban*I (New England – BioLabs) and *Mbo*II (Fermentas, Life Sciences) restriction enzymes digestions of the product amplified by the MBL exon 1 PCR primers, followed by a 3% agarose gel electrophoresis. Controls with known genotypes were included in all experiments.

Polymerase Chain Reaction–Site-Directed Mutagenesis (SDM)

A SDM–PCR was employed to evaluate the SNPs in the exon 1 (codon 52) of *MBL2* gene as previously described.[22] Briefly, the restriction enzyme *Hha*l (New England – BioLabs) cleaves the A, B, and C alleles while *Mlu*I (New England – BioLabs) cleaves the amplified 125 bp product of allele D (codon 57) into two fragments (100 and 25 bp).

Variants in the Promoter Region of the MBL Gene

The detection of the promoter variants (-550) H/L and (-221) X/Y was performed using sequence-specific primers (PCR-SSP) as previously described.[24]

Inference of MBL Levels

The SNPs of the exon 1 of the MBL gene are in linkage disequilibrium with the promoter polymorphisms, resulting in six more frequent haplotypes: HYA, HYD, LYA, LYB, LYC, and LXA.[24] Moreover, the individuals were categorized into three haplotype groups based upon the genotypes and MBL levels associated with these haplotypes: group 1 was defined by high MBL serum levels associated with haplotypes (H/L)YA/(H/L)YA and (H/L)YA/LXA genotypes; group 2 was defined by intermediate MBL serum levels associated with LXA/LXA and (H/L)YA/O genotypes; and group 3 was defined by low MBL serum levels, resulting in MBL deficiency, associated with LXA/O and O/O genotypes.[44]

Statistical Analysis

MBL genotypic distribution was determined by direct counting. The genotypic frequencies were compared to Hardy–Weinberg expectations using Chi-Square tests. MBL allelic frequencies were compared using the Chi-square-test or Fisher exact test if appropriate. The significance level was set at $\alpha = 0.05$ (two-tailed). All statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Maternal MBL Allele Frequencies and Genotype Distribution

The genotype distribution of all analyzed SNPs in both PE and control women were in Hardy–Weinberg equilibrium (*data not shown*). The MBL allelic/genotypic distribution and frequencies of the SNPs in exon 1 are shown in Table II. Higher frequencies of the C and D alleles were observed in patients with PE when compared to controls (allele C: 0.08 versus 0.03, $P = 0.006$ and allele D: 0.10 versus 0.05 $P = 0.009$, respectively). We also observed higher frequencies of the AD genotype in patients with PE when compared to controls (0.138 versus 0.068; $P = 0.05$). Interestingly, higher frequencies of the AB genotype in control women were found when compared to PE women (0.28 versus 0.13; $P = 0.01$). Regarding the genotypic frequencies, we observed a trend for a higher frequency of the OO genotype (lower MBL producers) in patients with PE when compared to controls (0.11 and 0.04, respectively, $P = 0.07$).

Table II MBL allelic/genotypic overall distribution and frequencies of the SNPs in exon 1 between control and pre-eclamptic women

	Pre-eclamptic women [frequency(*n*)]	Control women [frequency(*n*)]
Genotypes		
A/A	0.519 (83)[a]	0.546 (89)[a]
A/B	0.131 (21)[b]	0.288 (47)[b]
A/C	0.100 (16)	0.049 (8)
A/D	0.138 (22)[c]	0.068 (11)[c]
B/C	0.038 (6)	0.018 (3)
B/D	0.044 (7)	0.012 (2)
C/D	0.018 (3)	0.000 (0)
B/B	0.006 (1)	0.012 (2)
C/C	0.006 (1)	0.000 (0)
D/D	0.000 (0)	0.006 (1)
Alleles		
A	0.70 (225)[d]	0.75 (244)[d]
O	0.29 (95)	0.25 (82)
B	0.11 (36)	0.17 (56)
C	0.08 (27)[e]	0.03 (11)[e]
D	0.10 (32)[f]	0.05(15)[f]
Genotypes		
AA	0.52 (83)[g]	0.54 (89)[g]
AO	0.37 (59)	0.42 (66)
OO	0.11 (18)[h]	0.04 (8)[h]

CI, confidence interval; OR, odds ratio; df, degrees of freedom.
Allele O = alleles B + C + D; Allele A = wild type.
Genotype AA: high serum MBL levels; Genotype AO: intermediate serum MBL levels; Genotype OO: lower serum MBL levels.
[a,b]$P = 0.01$ (OR = 1.7 CI: 1.09 – 2.67).
[a,c]$P = 0.05$ (OR = 2.14 CI: 0.98 – 4.69).
[d,e]$P = 0.006$ (OR = 2.48 CI: 1.25 – 4.89).
[d,f]$P = 0.009$ (OR = 1.63 CI: 1.06 – 2.49).
[g,h]$\chi^2 = 5.03$; df = 2; $P = 0.07$.

Haplotype and Genotype Groups According to MBL Polymorphisms

We sought to investigate whether any specific MBL genotype/haplotype (or potential phenotype) was related to PE (Table III). As the SNPs in the structural gene of MBL are in linkage disequilibrium with the two polymorphisms in promoter region, we grouped the individuals according to the haplotypes previously described in literature (see Material and Methods).[24]

No statistically significant association was observed in haplotype group frequencies between patients with PE and controls (Table III, χ^2 0.84, $P = 0.65$).

Table III MBL haplotype (promoter + exon 1 genotypes) distribution between control and pre-eclamptic women

	Pre-eclamptic women [frequency(*n*)]	Control women [frequency(*n*)]
Haplotype		
1*	0.53 (77)[a]	0.50 (81)[b]
2**	0.37 (54)[a]	0.40 (64)[b]
3***	0.09 (14)[a]	0.11 (17)[b]

Haplotypes: Group 1* high serum MBL levels (LYA/A, HYA/A); Group 2** intermediate serum MBL levels (LXA/LXA, LYA/O, HYA/O); Group 3*** lower serum MBL levels (LXA/O, O/O).
[a,b]χ^2 0.84; df = 2; P = 0.65.

Also, no associations between allelic and genotypic frequencies in the *MBL2* promoter region were observed comparing PE and control pregnant women (data not shown).

Association of Genotype/Haplotype Groups and the Disease Severity

Considering that PE can be presented in either mild or severe forms, we re-analyzed the data according to disease severity and subgrouped by the haplotype and/or genotype frequencies (Table IV). No association was observed between the haplotype/genotype distribution and PE severity. Interestingly, we observed a higher frequency of the OO genotype in severe PE when compared to mild PE. Also, a higher frequency of the OO genotype was observed among severe PE women when compared to control women (0.15 and 0.04, respectively $P = 0.07$), suggesting that the OO genotype is a risk factor for PE development. However, this only approached statistical significance. In addition, we have subgrouped the genotypes (Table V) according to MBL serum levels (i.e. AA and AO as high and intermediate MBL producers; OO as low MBL producers). Interestingly, adjusted odds ratios and confidence intervals (95% CI) indicated that women with genotypes associated with low MBL levels had more chance to develop severe PE in comparison with control women (OR = 3.95; CI: 1.36–11.47, $P = 0.007$). Patients were also characterized by clinical and socioeconomic parameters including ethnic origin, number of abortions, number of gestations, and weight. No significant differences were observed in genotypic/haplotypic frequencies between the control group and patients with PE subgrouped according to the aforementioned characteristics (Table S1).

Discussion

PE is a disease of multifactorial etiology that involves several immunological and genetic factors. The study of polymorphisms in genes of immunological interest is of great value in the understanding of the disease pathology. Here, we analyzed polymorphisms in the structural and promoter *MBL2* gene regions as well as their relationships with the pathogenesis of PE.

Several studies have observed low maternal MBL levels (or genotypes associated with low MBL levels) in association with adverse pregnancy outcome,

Table IV Haplotypes of promoter/exon regions and genotypes frequencies in control and pre-eclamptic women subgrouped by the development of mild or severe PE

	Haplotypes [frequency(*n*)]			Genotypes [frequency(*n*)]		
	1*	2**	3***	AA	AO	OO
Control pregnant women	0.51 (81)[a]	0.40 (64)[a]	0.09 (17)[a]	0.54 (89)[e]	0.42 (66)[e]	0.04 (8)[e]
Total PE	0.52 (77)[b]	0.37 (54)[b]	0.11(14)[b]	0.52 (83)[f]	0.37(59)[f]	0.11 (18)[f]
Mild PE	0.60 (32)[c]	0.33 (18)[c]	0.07 (4)[c]	0.61 (35)[g]	0.32 (18)[g]	0.07 (4)[g]
Severe PE	0.48 (23)[d]	0.37 (18)[d]	0.15 (7)[d]	0.45 (24)[h]	0.40 (22)[h]	0.15 (8)[h]

Haplotypes: Group **1*** high serum MBL levels (LYA/A, HYA/A); Group **2**** intermediate serum MBL levels (LXA/LXA, LYA/O, HYA/O); Group **3***** lower serum MBL levels (LXA/O, O/O).
Allele O = alleles B+C+D; Allele A = wild type.
[a,b]χ^2 P = 0.65 df = 2; [a,c]χ^2 P = 0.57 df = 2; [a,d]χ^2 P = 0.27 df = 2.
[e,f]χ^2 P = 0.07 df = 2; [e,g] χ^2 P = 0.30 df = 2; [e,h]χ^2 P = 0.07 df = 2.

Table V MBL aggregated genotypes (exon 1) overall distribution and frequencies between control and pre-eclamptic (PE) women

	MBL genotypes **AA** and **AO**	MBL genotype **OO**
Control pregnant women (frequency) [(*n*)]	0.96 (155)[a]	0.04 (8)[a]
Total PE women (frequency) [(*n*)]	0.89 (142)	0.11 (18)
Mild PE women (frequency) [(*n*)]	0.93 (53)[b]	0.07 (4)[b]
Severe PE women (frequency) [(*n*)]	0.85 (46)[c]	0.15 (8)[c]

CI, confidence interval; OR, odds ratio; df, degrees of freedom.
[a,b] $\chi^2 = 0.64$; df = 1; OR = 1.61; CI = [0.48-5.09] $P = 0.40$.
[a,c] $\chi^2 = 7.20$; df = 1; OR = 3.95; CI = [1.36-11.47] $P = 0.007$.

including recurrent miscarriages, PE, risk for chorioamnionitis and preterm delivery.[33–36,38] Conversely, other studies suggested that reduced MBL serum levels could decrease the capacity of MBL-mediated complement activation and also limit the complement-mediated destruction of semi-allogeneic fetal cells during pregnancy.[20]

In this study, we observed high frequencies of the AB genotype (codon 54) in controls when compared to patients with PE. This genotype has been correlated with intermediate MBL levels. This finding seems to corroborate with a previous work describing higher frequencies of the B allele and AB genotype among controls when compared to patients with PE.[20] However, it should be kept in mind that the simultaneously analysis of SNPs located at both the structural (codons 52, 54 and 57) and the promoter regions of the *MBL2* gene may provide more information for understanding the MBL role in PE development. Considering together alleles B, C, and D (therefore indicated as allele O), we have found a high frequency of the OO genotype among patients with PE. Importantly, disease severity was associated with a particular MBL genotype: women with severe forms of PE had the highest frequency of the MBL OO genotype.

The OO genotype has been associated with low MBL serum levels because of altered multimerization and impaired MBL protein function.[24,30] Although MBL is a key factor in enhancing inflammation, its role in pregnancy is largely unknown. While some authors associated high MBL levels with a successful pregnancy,[33,37] others found MBL levels correlated with pregnancy complications.[20,21,45] Certainly, other risk factors not evaluated in the present work may also contribute and interact with the MBL genotype in the pregnancy outcome.

Here, low MBL levels (inferred from the OO genotype) were found associated with severe form of PE, suggesting that genotypes associated with low MBL production should be considered as a risk factor for PE development. In another study, Kilpatrick et al. found an association between low MBL levels and the presence of PE.[36] Our data further suggest that the OO genotype is over represented in severe form of PE. In addition, low MBL levels could also change the inflammatory profile, leading to impaired angiogenesis in the maternal–fetal interface. Indeed, low MBL levels have been associated with complicated pregnancy outcomes.[33–38] Nevertheless, we should point out that some studies did not report associations between MBL haplotypes and PE development,[39] and conflicting data have been generated.[20,45] In addition, Than et al. have found elevated maternal plasma MBL in patients with PE when compared with normal pregnant women.[45] However, they did not evaluate *MBL2* genotypes.

In conclusion, considering that high MBL levels have been found essential in the first trimester of a successful pregnancy, our data suggest that women with genotypes associated with low levels of MBL could be potential PE developers. However, further studies should be performed to better understand the complex relationships between PE and genetic variations of MBL.

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References

1 Redman CW, Sacks GP, Sargent IL: Preeclampsia: an excessive maternal inflammatory response to pregnancy. *Am J Obstet Gynecol* 1999; 180:499–506.
2 Marzi M, Vigano A, Trabattoni D, Villa ML, Salvaggio A, Clerici E, Clerici M: Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy. *Clin Exp Immunol* 1996; 106:127–133.

3 Raghupathy R: Th1-type immunity is incompatible with successful pregnancy. *Immunol Today* 1997; 18:478–482.
4 Reinhard G, Noll A, Schlebusch H, Mallmann P, Ruecker AV: Shifts in the TH1/TH2 balance during human pregnancy correlate with apoptotic changes. *Biochem Biophys Res Commun* 1998; 245:933–938.
5 de Man YA, Dolhain RJ, van de Geijn FE, Willemsen SP, Hazes JM: Disease activity of rheumatoid arthritis during pregnancy: results from a nationwide prospective study. *Arthritis Rheum* 2008; 59: 1241–1248.
6 Lin D, Moss K, Beck JD, Hefti A, Offenbacher S: Persistently high levels of periodontal pathogens associated with preterm pregnancy outcome. *J Periodontol* 2007; 78:833–841.
7 Duckitt K, Harrington D: Risk factors for pre-eclampsia at antenatal booking: systematic review of controlled studies. *BMJ* 2005; 330:565.
8 Fekete A, Ver A, Bogi K, Treszl A, Rigo J Jr: Is preeclampsia associated with higher frequency of HSP70 gene polymorphisms? *Eur J Obstet Gynecol Reprod Biol* 2006; 126:197–200.
9 Venkatesha S, Toporsian M, Lam C, Hanai J, Mammoto T, Kim YM, Bdolah Y, Lim KH, Yuan HT, Libermann TA, Stillman IE, Roberts D, D'Amore PA, Epstein FH, Sellke FW, Romero R, Sukhatme VP, Letarte M, Karumanchi SA: Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nat Med* 2006; 12:642–649.
10 Redman CW, Sargent IL: Latest advances in understanding preeclampsia. *Science* 2005; 308: 1592–1594.
11 Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy. *Am J Obstet Gynecol* 2000;183:S1–S22.
12 Gu Y, Lewis DF, Deere K, Groome LJ, Wang Y: Elevated maternal IL-16 levels, enhanced IL-16 expressions in endothelium and leukocytes, and increased IL-16 production by placental trophoblasts in women with preeclampsia. *J Immunol* 2008; 181:4418–4422.
13 Rusterholz C, Hahn S, Holzgreve W: Role of placentally produced inflammatory and regulatory cytokines in pregnancy and the etiology of preeclampsia. *Semin Immunopathol* 2007; 29:151–162.
14 Sibai B, Dekker G, Kupferminc M: Pre-eclampsia. *Lancet* 2005; 365:785–799.
15 Hiby SE, Walker JJ, O'Shaughnessy K M, Redman CW, Carrington M, Trowsdale J, Moffett A: Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *J Exp Med* 2004; 200:957–965.
16 Vianna P, Dalmaz CA, Veit TD, Tedoldi C, Roisenberg I, Chies JA: Immunogenetics of pregnancy: role of a 14-bp deletion in the maternal HLA-G gene in primiparous pre-eclamptic Brazilian women. *Hum Immunol* 2007; 68:668–674.
17 Turner MW: The role of mannose-binding lectin in health and disease. *Mol Immunol* 2003; 40:423–429.
18 Faulk WP, Jarret R, Keane M, Johnson PM, Boackle RJ: Immunological studies of human placentae: complement components in immature and mature chorionic villi. *Clin Exp Immunol* 1980; 40:299–305.
19 Tedesco F, Radillo O, Candussi G, Nazzaro A, Mollnes TE, Pecorari D: Immunohistochemical detection of terminal complement complex and S protein in normal and pre-eclamptic placentae. *Clin Exp Immunol* 1990; 80:236–240.
20 Sziller I, Babula O, Hupuczi P, Nagy B, Rigo B, Szabo G, Papp Z, Linhares IM, Witkin SS: Mannose-binding lectin (MBL) codon 54 gene polymorphism protects against development of pre-eclampsia, HELLP syndrome and pre-eclampsia-associated intrauterine growth restriction. *Mol Hum Reprod* 2007; 13:281–285.
21 van de Geijn FE, Dolhain RJ, van Rijs W, Willemsen SP, Hazes JM, de Groot CJ: Mannose-binding lectin genotypes are associated with shorter gestational age. An evolutionary advantage of low MBL production genotypes?. *Mol Immunol* 2008; 45:1514–1518.
22 van de Geijn FE, Roos A, de Man YA, Laman JD, de Groot CJ, Daha MR, Hazes JM, Dolhain RJ: Mannose-binding lectin levels during pregnancy: a longitudinal study. *Hum Reprod* 2007; 22:362–371.
23 Sinha D, Wells M, Faulk WP: Immunological studies of human placentae: complement components in pre-eclamptic chorionic villi. *Clin Exp Immunol* 1984; 56:175–184.
24 Madsen HO, Garred P, Thiel S, Kurtzhals JA, Lamm LU, Ryder LP, Svejgaard A: Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. *J Immunol* 1995; 155:3013–3020.
25 Garred P, Madsen HO, Halberg P, Petersen J, Kronborg G, Svejgaard A, Andersen V, Jacobsen S: Mannose-binding lectin polymorphisms and susceptibility to infection in systemic lupus erythematosus. *Arthritis Rheum* 1999; 42:2145–2152.
26 Kilpatrick DC: Mannan-binding lectin and its role in innate immunity. *Transfus Med* 2002; 12:335–352.
27 Madsen HO, Videm V, Svejgaard A, Svennevig JL, Garred P: Association of mannose-binding-lectin deficiency with severe atherosclerosis. *Lancet* 1998; 352:959–960.
28 Tsutsumi A, Ikegami H, Takahashi R, Murata H, Goto D, Matsumoto I, Fujisawa T, Sumida T: Mannose

binding lectin gene polymorphism in patients with type I diabetes. *Hum Immunol* 2003; 64:621–624.
29 Monticielo OA, Mucenic T, Xavier RM, Brenol JC, Chies JA: The role of mannose-binding lectin in systemic lupus erythematosus. *Clin Rheumatol* 2008; 27:413–419.
30 Roos A, Bouwman LH, Munoz J, Zuiverloon T, Faber-Krol MC, Fallaux-van den Houten FC, Klar-Mohamad N, Hack CE, Tilanus MG, Daha MR: Functional characterization of the lectin pathway of complement in human serum. *Mol Immunol* 2003; 39:655–668.
31 Larsen F, Madsen HO, Sim RB, Koch C, Garred P: Disease-associated mutations in human mannose-binding lectin compromise oligomerization and activity of the final protein. *J Biol Chem* 2004; 279:21302–21311.
32 Terai I, Kobayashi K, Matsushita M, Miyakawa H, Mafune N, Kikuta H: Relationship between gene polymorphisms of mannose-binding lectin (MBL) and two molecular forms of MBL. *Eur J Immunol* 2003; 33:2755–2763.
33 Annells MF, Hart PH, Mullighan CG, Heatley SL, Robinson JS, Bardy P, McDonald HM: Interleukins-1, -4, -6, -10, tumor necrosis factor, transforming growth factor-beta, FAS, and mannose-binding protein C gene polymorphisms in Australian women: risk of preterm birth. *Am J Obstet Gynecol* 2004; 191:2056–2067.
34 Annells MF, Hart PH, Mullighan CG, Heatley SL, Robinson JS, McDonald HM: Polymorphisms in immunoregulatory genes and the risk of histologic chorioamnionitis in Caucasoid women: a case control study. *BMC Pregnancy Childbirth* 2005; 5:4.
35 Christiansen OB, Kilpatrick DC, Souter V, Varming K, Thiel S, Jensenius JC: Mannan-binding lectin deficiency is associated with unexplained recurrent miscarriage. *Scand J Immunol* 1999; 49:193–196.
36 Kilpatrick DC: Human mannan binding protein in pre-eclampsia. *Immunol Lett* 1996; 49:175–177.
37 Kilpatrick DC, Starrs L, Moore S, Souter V, Liston WA: Mannan binding lectin concentration and risk of miscarriage. *Hum Reprod* 1999; 14:2379–2380.
38 Kruse C, Rosgaard A, Steffensen R, Varming K, Jensenius JC, Christiansen OB: Low serum level of mannan-binding lectin is a determinant for pregnancy outcome in women with recurrent spontaneous abortion. *Am J Obstet Gynecol* 2002; 187:1313–1320.
39 van de Geijn FE, Dolhain RJ, van Rijs W, Hazes JM, de Groot CJ: Mannose-binding lectin genotypes and pre-eclampsia: a case-control study. *Hum Immunol* 2007; 68:888–893.
40 Brown MA, Hague WM, Higgins J, Lowe S, McCowan L, Oats J, Peek MJ, Rowan JA, Walters BN: The detection, investigation and management of hypertension in pregnancy: executive summary. *Aust N Z J Obstet Gynaecol* 2000; 40:133–138.
41 Sibai BM: Diagnosis and management of gestational hypertension and preeclampsia. *Obstet Gynecol* 2003; 102:181–192.
42 Lahiri DK, Nurnberger JI Jr: A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. *Nucleic Acids Res* 1991; 19:5444.
43 Madsen HO, Satz ML, Hogh B, Svejgaard A, Garred P: Different molecular events result in low protein levels of mannan-binding lectin in populations from southeast Africa and South America. *J Immunol* 1998; 161:3169–3175.
44 Frakking FN, Brouwer N, Zweers D, Merkus MP, Kuijpers TW, Offringa M, Dolman KM: High prevalence of mannose-binding lectin (MBL) deficiency in premature neonates. *Clin Exp Immunol* 2006; 145:5–12.
45 Than NG, Romero R, Erez O, Kusanovic JP, Tarca AL, Edwin SS, Kim JS, Hassan SS, Espinoza J, Mittal P, Mazaki-Tovi S, Friel L, Gotsch F, Vaisbuch E, Camacho N, Papp Z: A role for mannose-binding lectin, a component of the innate immune system in preeclampsia. *Am J Reprod Immunol* 2008; 60:333–345.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Haplotypes of promoter-exon regions and genotypes frequencies in both control and pre-eclamptic women classified accordingly ethnic origin.

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