**UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL**

**DISSERTAÇÃO DE MESTRADO**

# ENVOLVIMENTO DO POLIMORFISMO DE INSERÇÃO/DELEÇÃO DE 14 PB DA REGIÃO 3´UTR DO GENE HLA-G EM DOENÇAS REUMATOLÓGICAS

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**SUMÁRIO**

# ABREVIATURAS

ANOVA – *Analysis of Variance* (Análise de Variância)

bp – *base pairs* (pares de base)

CD – C*lusters of Differentiation* (Grupos de diferenciação) – Ex: "CD4"

CD4+ – positivo para a molécula de superfície CD4

HLA – *Human Leucocitary Antigen* (Antígeno Leucocitário Humano)

IL – *Interleukin* (Interleucina) – Ex: "IL-10"

Mb – mega pares de base (1.000.000 pb)

MHC – *Major Histocompatibility Complex* (Complexo Principal de Histocompatibilidade)

mRNA – *messenger RNA* (RNA mensageiro)

NK – *Natural Killer*

pb – pares de base

TCR – *T Cell Receptor* (Receptor de Célula T)

Th1 – *T-helper cell type 1* (célula T-auxiliar tipo 1)

UTR – *Untranslated Region* (região não-traduzida)

**RESUMO**

O antígeno leucocitário humano G (HLA-G) é um MHC de classe I não-clássico caracterizado por baixo nível de polimorfismo ao nível de DNA, limitada distribuição tecidual e pela expressão de isoformas tanto de membrana quanto solúveis. Estudos recentes demonstram que o HLA-G é induzido no curso de doenças inflamatórias e a sua expressão tem sido sugerida como um possível mecanismo de proteção tecidual contra respostas inflamatórias auto-imunes, agindo como um mecanismo de vigilância imunológica. É possível que variantes alélicas dentro do gene possam desempenhar um papel no risco e gravidade de doenças reumáticas. Assim, analizou-se a influência genética do polimorfismo de 14 pb do gene *HLA-G* em cinco doenças reumáticas: artrite reumatóide (AR), artrite idiopática juvenil (AIJ), lúpus eritematoso sistêmico (LES), síndrome de Sjögren (SS) e esclerose sistêmica (ES). Duzentos e sessenta e cinco pacientes para AR, 106 pacientes para AIJ, 127 pacientes para LES, 21 pacientes para SS e 111 pacientes para ES, bem como 356 controles saudáveis, foram genotipados por PCR para o polimorfismo de 14 pb. Pacientes com AIJ do sexo feminino apresentaram uma maior freqüência do alelo -14pb, comparado com controles do mesmo sexo, sugerindo que este alelo é provavelmente um fator de risco para AIJ, principalmente em indivíduos do sexo feminino. Não foram observadas diferenças estatísticas significativas nos outros grupos de pacientes em comparação aos controles, sugerindo que em AR, LES, SS e ES, esse polimorfismo não está relacionado ao risco ou à gravidade da doença. Nossos resultados encorajam trabalhos futuros que se concentrem na expressão de HLA-G nessas e em outras doenças reumáticas.

**ABSTRACT**

The Human Leukocyte Antigen G (HLA-G) is a non-classical class I MHC which is characterized by low polymorphism at DNA level, limited tissue distribution and expression of both membrane-bound and soluble isoforms. Recent works demonstrate that HLA-G is induced at the course of inflammatory pathologies and its expression has been suggested as a possible mechanism of tissue protection against autoimmune inflammatory responses, therefore acting as a mechanism of immune surveillance. It is likely that allelic variants within the gene might play a role in the risk and severity of rheumatic diseases. We analyzed the genetic influence of the 14 bp polymorphism of the *HLA-G* gene in five rheumatic diseases: Rheumatoid Arthritis (RA), Juvenile Idiopathic Arthritis (JIA), Systemic Lupus Erythematosus (SLE), Sjögren's Syndrome (SS) and Systemic Sclerosis (SSc). Two-hundred and sixty eight AR patients, 106 JIA patients, 127 SLE patients, 21 SS patients and 111 SSc patients were PCR-genotyped for the 14 bp polymorphism, as well as 356 healthy controls. Female JIA patients presented a higher frequency of the -14bp allele as compared to female controls suggesting that this allele is probably a risk factor for JIA, mainly in females. No statistic significant differences were observed for the other disease groups in comparison to controls, suggesting that, in RA, SLE, SS and SSc, this polymorphism is not related to disease risk or severity. Our data encourages future works which focus on HLA-G expression in these and other rheumatic diseases.

## 1. INTRODUÇÃO

O estudo sobre a função dos genes relacionados com o sistema imune em doenças reumáticas é uma área que há bastante tempo vêm ganhando a atenção dos pesquisadores. Durante muito tempo e até hoje, os genes do MHC de classe I e II, historicamente conhecidos como altamente polimórficos e como os principais responsáveis pelo processo de rejeição de transplantes alogênicos, captaram, por razões óbvias, a maior parte da atenção dos cientistas na busca da compreensão dos processos auto-imunes que caracterizam estas doenças. A descoberta de genes do MHC que não compartilhavam desse alto grau de polimorfismo levou os pesquisadores e se perguntarem se este tipo de molécula possuía alguma utilidade fisiológica (Hughes and Nei, 1989). Em 1990, entretanto, esta pergunta começou a ser respondida, com a identificação de uma dessas moléculas, o HLA-G, na interface feto-placentária, expresso na superfície de trofoblastos. Constatou-se que, além de o HLA-G ser responsável pela proteção do feto contra a resposta imune da mãe, essa molécula estava relacionada a processos de imunorregulação importantes em transplantes, em tumores e infecções virais, bem como em doenças inflamatórias, o que nos motivou a levar a cabo o presente trabalho. Na primeira parte dessa revisão bibliográfica será dada uma breve descrição da região do MHC, região esta em que está localizado o gene *HLA-G*, que será tratado logo a seguir. Por último, serão apresentadas brevemente as cinco doenças reumáticas objetos desse estudo.

## 1.1 O MHC

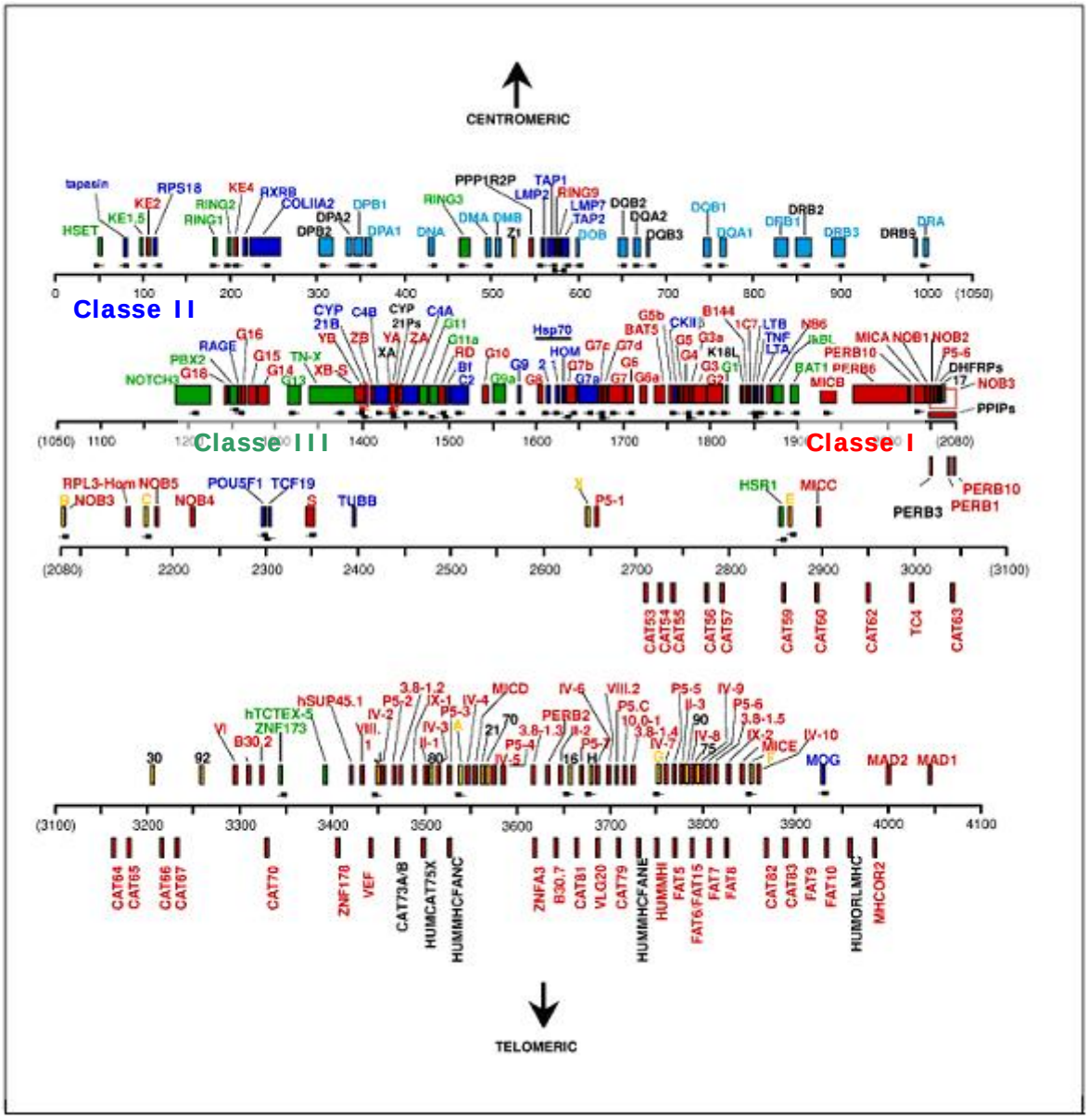
A família do Complexo Principal de Histocompatibilidade (MHC) caracteriza-se por uma coleção de genes situados no braço curto do cromossomo 6, na região 6p21.3, englobando mais de 4 Mb de DNA (Figura 1). O *locus* foi identificado e nomeado pelo seu papel na rejeição de tecidos em transplantes alogênicos e também pelo fato de muitos dos genes contidos nessa região (>10%) desempenharem importantes funções na biologia do sistema imune. Tanto a resposta mediada por células B (humoral) quanto a mediada por células T (celular) são iniciadas através da interação dessas células com os produtos dos genes do MHC, motivo pelo qual essa molécula representa a linha de frente da resposta imune adaptativa contra patógenos invasores. (Rhodes & Trowsdale 1999).

Os genes de MHC estão classificados principalmente em genes de classe I, II e III. Os genes da região do MHC de classe III codificam proteínas de diversas funções, entre as quais destacam-se proteínas do sistema de complemento (C2, C4 e fator-beta) e citocinas como o fator de necrose tumoral (TNF) (Milner & Campbell 2001). Os genes de classe II estão envolvidos na apresentação, em nível de membrana, de peptídeos derivados de proteínas extracelulares para linfócitos T *helper* e são expressos por células apresentadoras de antígenos profissionais. Como exemplos clássicos desta família destacam-se os *HLAs DP, DQ* e *DR* (Rhodes & Trowsdale 1999).

Os genes do MHC de classe I tiveram sua função originalmente descrita como a de apresentação de peptídeos antigênicos derivados de proteínas intracelulares para linfócitos T citotóxicos. De fato, esta função é desempenhada por alguns genes desta família de MHCs, como os *HLAs A, B* e *C*, chamados "clássicos" ou do grupo Ia. Tais genes têm a característica comum de serem altamente polimórficos, amplamente (mas não ubiquamente) expressos dentro do organismo humano e por direcionarem os linfócitos T citotóxicos para a eliminação de células infectadas por vírus ou outros patógenos intracelulares (Rhodes & Trowsdale 1999).

Em contrapartida, outros genes desta família parecem não desempenhar esta função, muito embora muitas das suas funções envolvam imunidade. São os chamados MHCs "não clássicos" ou do grupo Ib. Esses MHCs possuem poucos alelos (são oligomórficos) em comparação com os genes do grupo Ia e têm sua expressão limitada a poucos tipos de

**Figura 1**: Representação da região do MHC, região 6p21-3 (Rhodes & Trowsdale 1999).




células dentro do organismo. Estudos filogenéticos dos genes HLA de classe Ib indicam que eles são fracamente agrupados, sendo classificados em novos, de meia-idade e antigos, tendo-se como base a idade evolutiva aparente desses genes. Os genes de MHC classe Ib novos estão intimamente relacionados com os genes do grupo Ia. Entre estes genes incluem-se os genes *HLA-F* e *HLA-G*. Os genes antigos devem ter divergido dos genes do grupo Ia ainda no começo da evolução dos vertebrados (Rodgers & Cook 2005).

## 1.2 HLA-G

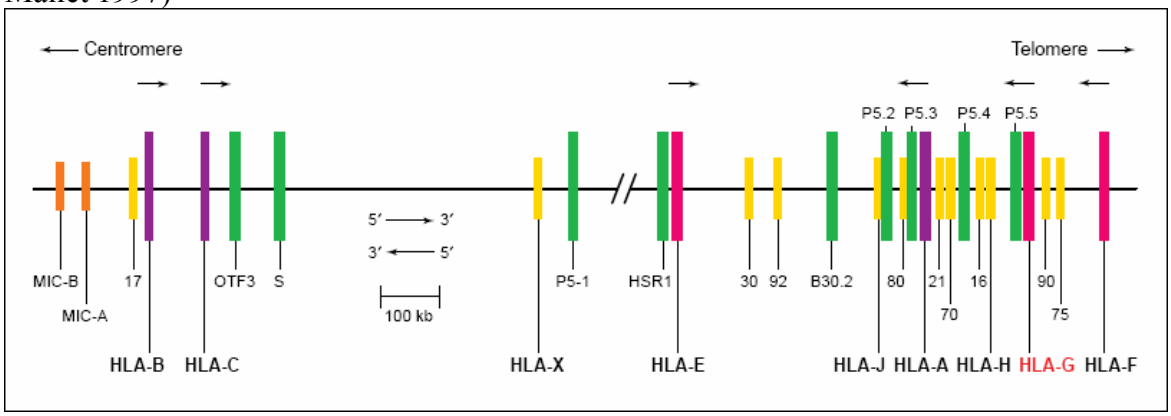
O Antígeno Leucocitário Humano (HLA)-G é um MHC classe I não clássico que tem se tornado objeto de curiosidade por parte dos pesquisadores por sua possível importância na manutenção da gravidez. Esta molécula parece estar envolvida na indução e manutenção da tolerância entre a mãe e o feto semi-alogênico, na interface feto-placentária, e parece ser importante inclusive na implantação do embrião. Uma expressão aberrante de HLA-G foi reportada nos casos de pré-eclâmpsia, abortos espontâneos recorrentes e em estudos envolvendo tratamentos de fertilização *in vitro* (Emmer *et al.* 2002; Fuzzi *et al.* 2002; Goldman-Whol *et al.* 2000; Hara *et al.* 1996; Jurisicova *et al.* 1996; Lim *et al.* 1997).

Além disso, vários estudos apontam para um papel imunorregulatório do HLA-G. Foi mostrado que a expressão de HLA-G é capaz de proteger células contra a ação lítica das células NK e influenciar a expressão de citocinas (Ponte *et al.* 1999; Rouas-Freiss *et al.* 1997). HLA-G pode reduzir a sensibilidade celular de vários tipos de tumores sólidos, células de desordens linfoproliferativas e transplantes alogênicos em relação à citólise promovida pelas células NK e linfócitos T citotóxicos, permitindo assim um escape da ação do sistema imune (Le Maoult *et al.* 2003; Aractingi *et al*., 2003; Rouas-Freiss *et al*., 2003; Le Maoult *et al.* 2004; Seliger *et al*., 2003; Urosevic *et al*., 2003; Nückel *et al* 2005, Amiot *et al*., 2003). Em um grupo determinado de pacientes transplantados do coração, rins ou fígado, a expressão de HLA-G mostrou-se associada a uma menor incidência de rejeição (Le Maoult *et al.* 2003; Creput *et al*., 2003; Lila *et al*., 2003).

Estudos recentes demonstram que a expressão de HLA-G é induzida no curso de doenças inflamatórias como lesões miosíticas, lesões psoriáticas na pele, em dermatite atópica e esclerose múltipla (Wiendl *et al*., 2000, Aractingi *et al*., 2001, Khosrotehrani *et al*., 2001, Wiendl *et al*., 2005). A expressão de HLA-G na superfície de células intestinais parece desempenhar um papel na supressão de citocinas pró-inflamatórias em colite ulcerativa (Torres *et al.* 2004). Em artrite reumatóide, mostrou-se que os níveis desta molécula em sangue periférico encontravam-se diminuídos nos pacientes, sugerindo que a atividade de células T e NK não se encontra eficientemente regulada por sHLA-G (HLA-G solúvel) nesta doença (Verbruggen *et al*., 2006). A expressão de HLA-G também parece desempenhar funções importantes em locais imunologicamente privilegiados como o timo,

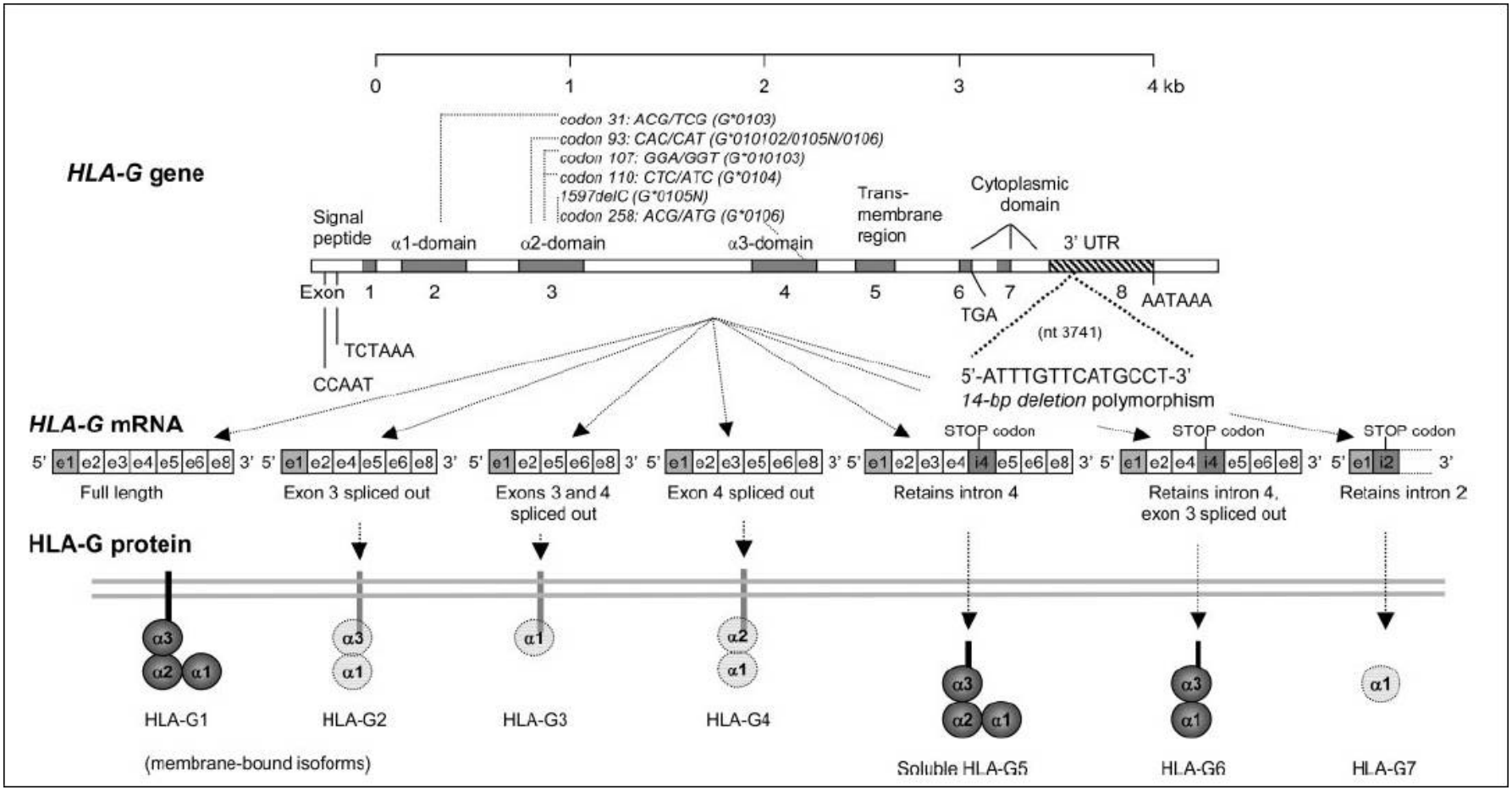
a córnea e o cérebro (Crisa *et al*., 1997; Mallet *et al*. 1999 , Le Discorde *et al.* 2003 , Wiendl *et al.* 2005). Muito recentemente, Feger *et al.* (2007) descreveram uma subpopulação de células T regulatórias que expressam e secretam HLA-G. Esse tipo celular foi encontrado em sangue periférico e sítios de inflamação em músculo (em miosite) e líquido cerebroespinhal (em esclerose múltipla), sugerindo um papel importante da molécula HLA-G na imunorregulação dessas doenças. Todos estas observações têm apontado o HLA-G como um dos mecanismos usados para proteger tecidos-alvos da ação inflamatória auto-imune e, assim sendo, servir como um mecanismo fundamental de controle imunológico.

**Figura 2**: Representação esquemática da região dos genes do MHC de classe I. O gene *HLA-G* encontra-se entre os genes *HLA-H* e *F*, próximos ao gene *HLA-A* (Le Boutteiller & Mallet 1997)




O *locus* HLA-G, descrito pela primeira vez por Geraghty *et al.* (1987), localiza-se na região telomérica da região cromossômica 6p21-3, proximalmente ao *locus* HLA-A (Figura 2). O gene exibe uma estrutura típica de um gene MHC de classe I, com uma organização exon/íntron muito parecida com a dos HLAs classe I clássicos (Le Boutteiller & Mallet 1997). HLA-G apresenta 7 diferentes isoformas, geradas por *splicing* alternativo dos transcritos de HLA-G, sendo quatro de membrana (G1, G2, G3 e G4) e três solúveis (G5, G6 e G7) (Figura 3). Esta molécula é caracterizada por um baixo polimorfismo ao nível de DNA. Dentre os polimorfismos descritos, destaca-se uma deleção/inserção de 14 pb na região 3´ não traduzida do gene, localizada no exon 8 (Figura 3). Foi mostrado que os transcritos que apresentam esta seqüência de 14 bp podem sofrer um processamento adicional que elimina os primeiros 92 pb do exon 8, o que torna este mRNA mais estável do que o mRNA que contém esta seqüência de 92 pb (Rousseau *et al.* 2003). O alelo +14

**Figura 3**: Representação do gene HLA-G e expressão gênica (Hviid 2006).



pb está também associado a níveis mais baixos de mRNA de HLA-G (tanto nas isoformas de membrana quanto solúveis) e, em alguma extensão, com níveis menores de sHLA-G (Hviid *et al.* 2003). O envolvimento deste polimorfismo específico na manutenção da gravidez, no sucesso da fertilização in vitro e em casos de pré-eclâmpsia já foi descrito (Triphati *et al.* 2004; Hviid *et al.* 2002; Hviid 2004; Hylenius *et al.* 2004, Vianna *et al.* 2007). Ainda, o polimorfismo de 14pb foi analisado em sarcoidose, apontando-se para o alelo +14pb como um possível fator de risco.

Interessantemente, foi mostrado que a IL-10, uma citocina de características antiinflamatórias, induz a expressão de HLA-G na superfície de monócitos e de células do trofoblasto (Moreau *et al.* 1999) e, por sua vez, sHLA-G parece estimular a expressão de IL-10 (e outras citocinas) em células mononucleares de sangue periférico (CMSP) (Kanai *et al.* 2001). Rizzo *et al.* (2005) investigaram os níveis de HLA-G e IL-10 em culturas de CMSP ativadas com LPS (lipopolissacarídeo) em relação ao genótipo da variante de 14 pb. Esse experimento demonstrou a existência de uma concentração de IL-10 significativamente aumentada no genótipo +14/+14 em comparação com o genótipo +14/-14 e com o genótipo -14/-14.

Reumatismo é um termo não-específico usado para descrever qualquer desordem dolorosa que afeta o sistema locomotor incluindo as articulações, os músculos, tecidos conjuntivos, tecidos suaves em volta das articulações e os ossos. As doenças reumáticas exercem um grande impacto nas sociedades e indivíduos, com custos econômicos para todos os países (Sangha 2000). A incapacidade funcional e laboral que geram tem um forte impacto sócio-econômico. Nos EUA, em 2001, a prevalência de artrites/sintomas articulares crônicos foi estimada em 33%, representando aproximadamente 69 milhões de pessoas (*Centers for Disease Control and Prevention (CDC*), 2002). Grande esforço tem sido feito nas últimas décadas no sentido da busca de novos tratamentos e da elucidação dos mecanismos de imunopatogênese e progressão desse grupo de doenças. No entanto, muitos desses mecanismos, incluindo aqueles associados à regulação da resposta imune, permanecem obscuros. A constatação que a molécula HLA-G está envolvida em diversos mecanismos de imunorregulação, considerando sua expressão em doenças inflamatórias, abre margem para a possibilidade da existência de relação entre as variantes alélicas deste gene e a suscetibilidade ou gravidade das manifestações clínicas em outras doenças reumáticas. Neste trabalho serão abordadas cinco doenças reumáticas, nas quais existe uma

forte contribuição do sistema imune: a artrite reumatóide, a artrite idiopática juvenil, o lúpus eritematoso, a síndrome de Sjögren e a esclerose sistêmica. A seguir, serão apresentadas sucintamente as diferentes doenças que serão objetos de nosso estudo.

### 1.3 Artrite Reumatóide

A artrite reumatóide (AR) é uma doença inflamatória de etiologia auto-imune e potencialmente debilitante. Caracteriza-se basicamente por sinovite crônica, simétrica e erosiva, preferencialmente de articulações periféricas e a maioria dos pacientes apresenta fator reumatóide positivo (Kelley *et al.* 1997). Sua prevalência no Brasil é de 1% da população (Marques Neto *et al.* 1993), similar à prevalência na população mundial (Wolfe *et al.* 1968). Afeta na sua maioria mulheres e tende a surgir a partir da quarta década de vida, com pico de incidência na quinta década (Alamanos *et al.* 2005).

O evento inicial da doença é o processo inflamatório iniciado na membrana sinovial com infiltrado de linfócitos e macrófagos, a qual pode adquirir uma estrutura similar ao de tecidos linfóides terciários com predomínio de linfócitos T CD4+. O quadro progride para a formação do *pannus* (membrana de tecido de granulação que cobre a superfície normal das cartilagens articulares), que atinge o osso subcondral e, em seguida, a cartilagem articular com destruição progressiva (Harris *et al.* 1997). A produção de citocinas com balanço predominante para as citocinas pró-inflamatórias tem papel fundamental na iniciação e perpetuação da inflamação crônica na membrana sinovial. A resposta do linfócito Th1 gera a produção de interferon-gama (IFN-γ) que estimula a liberação de TNF-α, interleucina-1 beta (IL-1β) e metaloproteinases pelos macrófagos e fibroblastos sinoviais (Klimiuk *et al.*1999).

Apesar de prováveis fatores ambientais deflagrarem o início da doença, a existência do componente genético na etiopatogênese da AR é clara a partir de observações de um maior risco de doença nos familiares de pacientes e de uma concordância maior em gêmeos monozigóticos em comparação com dizigóticos (Barton *et al.* 2002). A comparação entre as taxas de concordância em gêmeos monozigóticos e a prevalência na população revela que aproximadamente 50% na variação de ocorrência da doença é atribuída a fatores genéticos (van der Helm-van *et al.* 2005). Os genes do MHC são o fator genético identificado até o momento que contribui mais fortemente para o risco de

desenvolver AR. Diversos alelos de HLA-DRB1 vêm sendo associados à AR em diferentes populações: DRB1*0401, *0404, *0405, *0408, *0101, *0102, *1001, *1402 (2). Por estes alelos compartilharem uma seqüência de aminoácidos nas posições 70-74 da terceira região hipervariável da cadeia DRβ1, uma teoria foi proposta para unificar estes achados (Gregersen ate l, 1987), a "teoria do epitopo compartilhado". Outra teoria, proposta 12 anos depois, sugere que o fator de predisposição seria, na verdade, o HLA-DQ, com os alelos de DRB1 agindo como protetores ou permissivos (Zanelli *et al.* 1995). Nenhuma das teorias explica completamente todos os aspectos da susceptibilidade à AR. Assim, outros genes têm sido estudados nos últimos anos e diferentes associações com AR foram observadas, destacando-se os genes da proteína tirosina fosfatase N22 (*PTPN22*), do antígeno 4 dos linfócitos T citotóxicos (*CTLA-4*), do fator inibitório de migração de macrófagos (*MIF*) e peptidilarginina deaminase tipo 4 (*PADI4*) (Plenge *et al.* 2005, Worthington *et al.* 2005). Os "*genome-wide scans*" são abordagens poderosas que estão começando a ajudar na descoberta de novos genes candidatos. Um estudo envolvendo esta abordagem identificou a região genômica contendo os genes do fator associado ao receptor de TNF 1 (*TRAF1*) e do componente de complemento (*C5*) (Plenge *et al.* 2007). Até o momento, o fumo é o único fator ambiental convencional que mostrou-se consistentemente ligado ao risco do desenvolvimento de AR (Silman *et al.* 1996, Karlson *et al.* 1999, Stolt *et al.* 2003).

### 1.4 Artrite idiopática juvenil

A artrite idiopática juvenil (AIJ) compreende um grupo de doenças da infância que apresentam início, evolução e aspectos imunológicos distintos, tendo como fator comum a presença de sinovite persistente em crianças menores de 16 anos (Weldt *et al.* 2001). Desde a sua descrição, no final do século XIX, a classificação desse grupo de doenças tem se mostrado problemática. O termo e a classificação de "artrite idiopática juvenil" foi proposto pelos membros do comitê de Pediatria da Liga Internacional de Associações de Reumatologia (ILAR) em 1994 e adotado definitivamente em 1997 com o intuito de substituir os termos pré-existentes relativos à Artrite Crônica Juvenil, da Liga Européia Contra o Reumatismo (EULAR), e Artrite Reumatóide Juvenil (ARJ) do Colégio Americano de Reumatologia (ACR). A classificação atual engloba sete tipos diferentes de

artropatias da infância (Quadro 1) (Petty *et al.* 1998). O diagnóstico é dado após um período de seis meses de observação (Weiss & Ilowite, 2005).

Até o momento, não é conhecido um fator causal da AIJ e, em se tratando de um grupo de doenças distintas, a etiologia provavelmente deve ser diferente para cada subtipo. Uma das hipóteses é que AIJ ocorra em crianças geneticamente predispostas, a partir de um estímulo do tipo estresse, alteração hormonal, trauma articular, infecção viral ou bacteriana, que ative o sistema imune (Weiss & Ilowite, 2005). Sua de prevalência varia de 0,1 a 1 em cada 1000 crianças em países da América do Norte e da Europa. Tal como na AR, a manifestação mais importante em AIJ é o edema articular persistente, resultante do acúmulo de líquido sinovial e o espessamento sinovial, com um infiltrado de linfócitos e células plasmáticas. O passo seguinte na progressão da doença é a formação do *pannus.* A inflamação sinovial nas crianças com AIJ mostra densa infiltração de linfócitos T, que são vistos em aglomerados ao redor de células dendríticas (Silverman *et al.* 1993). O recrutamento continuado destas células T altamente diferenciadas pode contribuir para a persistência de uma sinovite destrutiva, mas os mecanismos desse recrutamento não estão completamente esclarecidos (Murray *et al.* 1997).

As evidências para um componente genético para a AIJ advêm de várias fontes, como estudos em gêmeos, estudos familiares e estudos de associação. A maioria dos trabalhos genéticos nas três últimas décadas concentrou-se nos genes do MHC (Thomson & Donn, 2002). Os genes *HLA-DR* parecem contribuir com 17% do risco para AIJ (chamada de artrite reumatóide juvenil à época do estudo) (Prahalad *et al.* 2000). "*Genome-wide scans*" com famílias de pacientes com AIJ confirmaram uma forte associação de *HLA-DRB1*, com uma transmissão aumentada do haplótipo *DR8* (Thompson *et al.* 2004). Genes fora do MHC também parecem desempenhar papéis importantes na patologia da AIJ. Além de estar associado à AR, o gene *CTLA4* também parece estar associado à AIJ. O gene do fator inibitório de migração (*MIF*) e da interleucina-6 (*IL-6*) parecem estar associados à AIJ sistêmica. O gene *CCR5* foi relacionado à AIJ sistêmica em um grupo de pacientes brasileiros (Scheibel *et al.* 2007), mas outros trabalhos não confirmam esta asociação (Prahalad *et al.* 2006, Lindner *et al.* 2007) Apesar de compartilhar características semelhantes com a AR, evidências crescentes, tanto em nível clínico quanto genético, indicam que a artrite nas crianças é diferente da artrite de adultos. (Woo & Wedderburn, 1998; Thomson & Donn, 2002; Glass & Giannini, 1999).

**Quadro 1:** A classificação dos subtipos de AIJ segundo a ILAR (1997)

1. Artrite Sistêmica (febre, *rash*, outros órgãos)
2. Oligoartrite (≤ 4 articulações afetadas)
   -persistente (≤ 4 articulações após 6 meses)
   -estendida (> 4 articulações após seis meses)
3. Poliartrite FR+ (> 4 articulações afetadas)
4. Poliatrite FR-
5. Artrite relacionada com entesite
6. Artrite Psoriásica
7. Não classificada ou outros
   -não preenche nenhuma categoria de 1 a 6
   -preenche mais de uma categoria de 1 a 6

FR-: fator reumatóide negativo; FR+: fator reumatóide positivo

### 1.5 Lúpus eritematoso sistêmico

O lúpus eritematoso sistêmico (LES) é uma doença auto-imune inflamatória que pode afetar virtualmente qualquer órgão do corpo e que se caracteriza pela formação de anticorpos dirigidos contra vários autoantígenos, especialmente DNA dupla fita e pequenas ribonucleoproteínas (Riemekasten & Hahn, 2005). Esta é uma doença inflamatória crônica do tecido conectivo, afetando a pele e vários órgãos internos. As características típicas da doença são a presença de uma mancha vermelha na face, afetando nariz e bochechas (eritema malar), artrite e dano progressivo nos rins devido à deposição de imunocomplexos. Seguidamente coração, pulmões e cérebro são também afetados por ataques progressivos de inflamação e fibrose. A Tabela 1 lista os critérios de diagnóstico propostos pelo *American College of Rheumatology* (ACR) em 1982, revisados por Sato *et al.*, (2002) e Petri (2005). A presença de quatro destes indicadores é suficiente para confirmar o diagnóstico de LES. Sua prevalência gira em torno 0,05% nos Estados Unidos,(Lawrence *et al.* 1998) e sua incidência é maior em mulheres (nove a dez casos em mulheres para cada caso diagnosticado em homens), especialmente na fase reprodutiva (Sato *et al.*, 2002).

A causa da doença permanece desconhecida, mas provavelmente envolve a interação entre fatores ambientais e genéticos. Cerca de 5 - 12% dos casos são familiais (Tsao *et al.* 2001). As razões de concordância entre pares de gêmeos monozigóticos estão entre as maiores encontradas em doenças auto-imunes, variando entre 24 e 58%, em comparação com 3 a 10% em gêmeos dizigóticos (Arnett Jr *et al.* 1997), o que evidencia o componente genético da doença. O papel da região do MHC no desenvolvimento desta doença tem sido extensivamente estudado nos últimos anos. Vários estudos têm apontado uma associação entre os alelos *DR3* (DRw17) e *DR2* (DRB1* 1501 e *1503) e doença, mas com um baixo risco relativo associado, o que sugere um papel limitado desses genes na patologia do lúpus (Arnett *et al.* 1997). Genes relacionados ao sistema complemento (*C2, C4 C1q*) parecem exibir uma forte relação com lúpus (Perdriger *et al.* 2002). Além dos genes do MHC, outros genes tiveram a atenção dos pesquisadores, como o da *MBP*

**Tabela 1.** Critérios para o diagnóstico de LES

| | |
|---|---|
| **1** | **Eritema malar:** lesão eritematosa fixa em região malar, plana ou em relevo. |
| **2** | **Lesão discóide:** lesão eritematosa, infiltrada, com escamas queratóticas aderidas e tampões foliculares, que evolui com cicatriz atrófica e discromia. |
| **3** | **Fotossensibilidade:** exantema cutâneo, como reação não usual à exposição à luz solar, de acordo com a história do paciente ou conforme observado pelo médico. |
| **4** | **Úlceras orais/nasais:** úlceras orais ou nasofaríngeas, usualmente indolores, observadas pelo médico. |
| **5** | **Artrite:** artrite não erosiva envolvendo duas ou mais articulações periféricas, caracterizadas por dor e edema ou derrame articular. |
| **6** | **Serosite:** pleuris (caracterizada por história convincente de dor pleurítica ou atrito auscultado pelo médico ou evidência de derrame pleural) ou pericardite (documentada por eletrocardiograma, atrito ou evidência de derrame pericárdico). |
| **7** | **Comprometimento renal:** proteinúria persistente ou cilindrúria anormal. |
| **8** | **Alterações neurológicas:** convulsão (na ausência de outra causa) ou psicose (na ausência de outra causa). |
| **9** | **Alterações hematológicas:** anemia hemolítica ou leucopenia (menos de 4.000 leucócitos/ml em duas ou mais ocasiões), linfopenia (menos de 1.500 linfócitos/ml em duas ou mais ocasiões) ou plaquetopenia (menos de 100.000 plaquetas/ml na ausência de outra causa). |
| **10** | **Alterações imunológicas:** anticorpo anti-DNA nativo ou anti-Sm, ou presença de anticorpo antifosfolípide baseado em: a) níveis anormais de IgG ou IgM anticardiolipina; b) teste positivo para anticoagulante lúpico ou teste falso-positivo para sífilis, por no mínimo seis meses. |
| **11** | **Anticorpos antinucleares:** título anormal de anticorpo anti-nuclear por imunofluorescência indireta ou método equivalente, em qualquer época, e na ausência de drogas conhecidas por estarem associadas à síndrome do lúpus induzido por drogas. |

(*mannose-binding protein*), *PARP* [*poly(ADP-ribose) polymerase*], genes de citocinas, genes do TCR, de proteínas de ativação de linfócito e ainda os genes de receptores Fc de imunoglobulinas (Perdriger *et al.* 2002).

### 1.6 Síndrome de Sjögren

A síndrome de Sjögren (SS) é uma doença auto-imune crônica caracterizada por uma infiltração progressiva de linfócitos e células plasmáticas nas glândulas salivares e lacrimais acompanhada da produção de autoanticorpos resultando em xerostomia (boca seca) e ceratoconjuntivite seca (Anaya & Talal, 1997). Sua prevalência pode variar de 0,5 a 2,7% na população e é mais comum em idosos (Anaya & Talal, 1997, Thomas *et al.* 1998). Esta variação pode ser atribuída à ausência de critérios diagnósticos uniformes. A doença afeta na sua maioria mulheres (razão mulher:homem de 9:1) na quarta e quinta década de vida. A SS pode ser primária ou ocorrer na presença de outras doenças do tecido conjuntivo, comumente em casos de artrite reumatóide, lúpus eritematoso sistêmico, esclerose sistêmica e polimiosite/dermatomiosite. Nestes casos, considera-se como SS secundária. O espectro da doença pode variar de uma desordem auto-imune órgão-específica (exocrinopatia auto-imune) a um processo sistêmico envolvendo os sistemas músculo-esquelético, pulmonar, gastrointestinal, hematológico, vascular, dermatológico, renal e nervoso. Vários pacientes apresentam fadiga e dor articular.

Um evento-chave no processo inicial da SS primária parece ser a apoptose aumentada das células epiteliais que progride para a infiltração linfocitária com a produção de autoanticorpos (Humphreys-Beher *et al.* 1999; Mitsias *et al.* 2006). A interação entre as células epiteliais, que apresentam alta expressão de HLA-DR, e as células T leva à produção de citocinas e à estimulação da proliferação e diferenciação de células B, contribuindo para o progresso da doença (Fox *et al.* 1986, Anaya *et al.* 2001). O diagnóstico da SS é baseado na combinação de sintomatologia e na presença de características auto-imunes, como a ativação de células T ou B. Nem todos os indivíduos que apresentam os sintomas de secura da boca e olhos têm SS, não existindo um teste único que seja sensível e específico o suficiente para diagnosticar esta doença. Apesar de não existir um consenso mundialmente aceito para o diagnóstico de SS, o critério de

classificação europeu modificado tem se tornado bastante popular (Vitali *et al.* 2002, tabela 3).

**Tabela 2.** Critérios revisados para o diagnóstico de sídrome de Sjögren primária (Vitali *et al.* 2002)

| | |
|---|---|
| **I** | **Sintomas oculares:** pelo menos uma resposta afirmativa das três questões abaixo:<br>a) Você tem, diária e persistentemente, problemas devido a olhos secos por mais de três meses?<br>b) Você tem sensação de areia nos olhos?<br>c) Você utiliza substituto de lágrima mais de três vezes ao dia? |
| **II** | **Sintomas orais:** pelo menos uma resposta afirmativa das três questões abaixo:<br>a) Você tem sentido a diariamente a boca seca por mais de trÊs meses?<br>b) Você tem edema persistente ou recorrente das glândulas salivares depois de adulto?<br>c) Você freqüentemente tem que adicionar líquidos para engolir comidas secas? |
| **III** | **Sinais oculares:** evidência objetiva de envolvimento ocular determinado com base no resultado positivo de pelo menos um dos dois testes abaixo:<br>a) Teste de Schirmer (<5mm/5 min)<br>b) Escore do teste de Rosa Bengala (4, de acordo com o sistema de escores de van Bijsterveld) |
| **IV** | **Características histopatológicas:** presença de um ou mais focos na biópsia de glândulas salivares (foco é definido como um aglomerado de 50 células mononucleares; o escore de foco é definido pelo número de focos numa superfície de 4mm$^2$ de superfície glandular) |
| **V** | **Envolvimento das glândulas salivares:** evidência objetiva do envolvimento de glândulas salivares com base no resultado positivo de pelo menos um dos três testes abaixo:<br>a) Cintilografia de glândulas salivares<br>b) Sialografia de parótidas<br>c) Medida do fluxo salivar sem estimulação (<1,5mL em 15min) |
| **VI** | **Auto-anticorpos:** presença dos auto-anticorpos séricos abaixo:<br>a) auto-anticorpos contra antígenos Ro/SS-A ou La/SS-B, ou ambos. |
| **Regras para classificação:** A presença de quatro dos seis itens (aceitando somente como padrão serológico anti-La e anti-Ro positivos) tem sensibilidade de 93,5% e especificidade de 94,0%, podendo ser utilizados para estabelecer o diagnóstico de SS primária definida. | |
| **Critérios de exclusão:** tratamento com radiação do pescoço ou da cabeça, infecção por hepatite C, pré-existência de linfoma, síndrome de imunodeficiência adquirida, sarcoidose, doença enxerto x hospedeiro, uso de fármacos anticolinérgicos. | |

Apesar de até o momento não existirem estudos com gêmeos em SS e de estudos com irmãos revelarem prevalências parecidas com as da população em geral (0,09% contra 0,1%, respectivamente) (Anaya *et al.* 2006, Bowman *et al.* 2004), a agregação observada

de doenças auto-imunes nas famílias de pacientes com SS, tais como LES, AR, doenças da tireóide e diabetes mellitus tipo 1, apóia a presença de um componente genético na etiologia da doença (Anaya *et al.* 2006b). Os esforços para desvendar o componente genético da SS têm se concentrado em estudos de associação com genes candidatos. Os fatores genéticos melhor identificados para SS primária são os genes *HLAs DR* e *DQ*, com os alelos de suscetibilidade variando de acordo com a origem étnica da população analisada (Mori *et al.* 2005). O gene do fator de necrose tumoral alfa (*TNF*$\alpha$) é outro gene relacionado ao sistema imune que parece estar associado. Entre os genes fora do MHC, destacam-se os da IL-10, o gene do antagonista do receptor de IL-1 (*IL1RN*) (revisado em Anaya *et al.* 2006b).

### 1.7 Esclerose Sistêmica

A esclerose sistêmica (ES) ou escleroderma é uma doença do tecido conjuntivo, de etiologia desconhecida, heterogênea, que afeta o tecido conjuntivo da pele, órgãos internos e as paredes de vasos sangüíneos. As características marcantes da doença são a auto-imunidade e inflamação, vasculopatia disseminada, afetando múltiplos leitos vasculares, e a presença de fibrose intersticial e perivascular progressiva (Abraham &Varga 2005). Os pacientes com ES são comumente classificados em dois grupos distintos, baseados no padrão de envolvimento cutâneo. ES cutânea difusa é dominada por fibrose de rápida progressão na pele, pulmões e outros órgãos internos. Em contrapartida, a ES cutânea limitada é dominada por manifestações vasculares, e por fibrose limitada e de progressão lenta. Apesar dos desfechos clínicos terem melhorado consideravelmente, a ES é ainda considerada incurável, e a forma cutânea difusa carrega a maior taxa de mortalidade entre as doenças do tecido conjuntivo, com 55% de sobrevivência em 10 anos de doença. Existe uma forte predominância de mulheres afetadas em relação aos homens e a principal idade de manifestação da doença é entre os 30 e 50 anos de idade. Cerca de 75 a 100 mil pessoas nos EUA têm ES, baseado nas taxas de incidência e de sobrevivência (Mayes *et al.* 2003).

A lesão e ativação vascular são os primeiros eventos perceptíveis na patogênese da ES (Kahaleh *et al.* 1979), evidenciada clinicamente no fenômeno de Raynaud, uma reação reversível caracterizada por vasoespasmo dos dedos da mão e do pé induzido pelo frio. A lesão causa a ativação e disfunção das células endoteliais, alteração da permeabilidade

vascular, à secreção alterada de mediadores vasoativos e à ativação das vias fibrolíticas e plaquetárias (Cerinic *et al.* 2003), fenômenos que levam ao remodelamento vascular, resultando no estreitamento progressivo e ao desaparecimento do lúmen vascular (Flavahan *et al.* 2003). A fibrose é a característica mais marcante da ES, constituindo-se da substituição progressiva da arquitetura tecidual por matriz extra-celular rica em colágeno, levando à perda de função dos órgãos afetados, sendo a maior responsável pela morbidade e mortalidade associada à ES (Denton *et al.* 2006). Os sistemas imunes inato e adaptativo têm participação na patogênese da ES (Abraham &Varga 2005). Pacientes com ES apresentam um balanço na produção de citocinas deslocado para o padrão Th2 (Tan *et al.* 2006, Mavalia *et al.* 1997, Atamas *et al.* 1999, Rottoli *et al.* 2005). Anticorpos altamente específicos para determinados autoantígenos são detectados em quase todos os pacientes com ES, correlacionando-se fortemente com o fenótipo da doença e seus níveis flutuam com a atividade da doença (Hu *et al.* 2003). Todavia, sua contribuição para as manifestações da doença permanece obscura.

Muitas evidências apontam para um fator genético para a ES. O risco de recorrência da doença encontra-se aumentado em irmãos e parentes em primeiro grau, comparados à população em geral (Arnett *et al.* 2001). Apesar de a história familiar ser o fator de risco mais forte identificado até hoje, o único estudo com gêmeos revelou uma concordância de 4,7% na expressão da doença. Apesar disso, a concordância na expressão de auto-anticorpos foi maior em gêmeos monozigóticos (90%) do que em dizigóticos (40%), sugerindo que a herança genética sozinha não é suficiente para causar a doença (Feghali-Bostwick *et al.* 2003). Os fatores ambientais envolvidos no risco e na gravidade da doença incluem sílica cristalina, solventes clorados, resinas epóxi e uma grande quantidade de solventes aromáticos. Dentre os estudos com genes candidatos, os resultados mais expressivos foram obtidos com os genes da fibrilina-1 (*FBN1*) e da osteonectina (*SPARC*), potencialmente envolvidos no processo de fibrose. Os genes do MHC de classe II mostraram estar altamente relacionados ao padrão de auto-anticorpos produzidos pelos pacientes. Um “genome-wide study” em uma população indígena com alta prevalência de ES revelou associações altamente significativas com a região do MHC, dos genes *SPARC*, *FBN1* e do gene da topoisomerase I (*TOPO1*) (revisado em Allanore *et al.* 2007).

## 2. OBJETIVOS

Através da determinação das freqüências alélicas e genotípicas do polimorfismo de inserção/deleção de 14pb da região 3´UTR do gene *HLA-G* nas doenças reumatológicas artrite reumatóide, artrite idiopática juvenil, lúpus eritematoso sistêmico, síndrome de Sjögren e esclerose sistêmica, investigar um possível papel regulador dessa molécula no processo inflamatório crônico presente nestas doenças.

**3. ASSOCIATION OF THE HLA-G 14 BP INSERTION/DELETION POLYMORPHISM WITH JUVENILE IDIOPATHIC ARTHRITIS AND RHEUMATOID ARTHRITIS** – ARTIGO COMPLETO SUBMETIDO À REVISTA *TISSUE ANTIGENS*

# ASSOCIATION OF THE HLA-G 14 BP INSERTION/DELETION POLYMORPHISM WITH JUVENILE IDIOPATHIC ARTHRITIS AND RHEUMATOID ARTHRITIS

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Short running title: HLA-G 14 bp polymorphism in JIA and RA

**ABSTRACT**

We tested the possible association of the 14 bp polymorphism of the HLA-G gene in the course of two inflammatory diseases, Rheumatoid Arthritis (RA) and Juvenile Idiopathic Arthritis (JIA). Patients and controls were genotyped for the 14 bp polymorphism by PCR with specific primers for the exon 8 of the HLA-G gene and the amplified fragment was visualized in a 6% polyacrylamide gel. 106 JIA patients, 265 RA patients and 356 healthy controls were genotyped for the 14 bp polymorphism. Female JIA patients presented a higher frequency of the -14bp allele as compared to female controls (0.743 and 0.576, corrected P = 0.0075). This increased frequency of the -14bp allele was observed in all JIA subtypes. In RA patients no differences in allelic and genotypic frequencies were observed between patients and controls. No correlations were observed among genotype and disease severity or clinical manifestations. Our data suggest that the HLA-G -14 bp allele is probably a risk factor for JIA, mainly in females. Considering the differences observed in relation to gender, we suggest that hormonal differences can interfere with the development of JIA. Considering the rheumatoid arthritis patients, our data agree with results from the literature and highlight the differences in the etiology of RA and JIA.

Key words: HLA-G, immunogenetics, Juvenile Idiopathic Arthritis, polymorphism, Rheumathoid Arthritis.

## INTRODUCTION

The Human Leukocyte Antigen G (HLA-G) is a non-classical class I MHC which is characterized by low polymorphism at DNA level, limited tissue distribution in non-pathological conditions and the expression of both membrane-bound and soluble isoforms by alternative splicing (1). This molecule has become object of interest for its possible role in pregnancy maintenance. HLA-G seems to be involved on the induction and maintenance of tolerance between the mother immune system and the semi-allogeneic fetus at the fetal-placentary interface and also seems to play an important role in embryo implantation (2-5).

Besides, several studies point out to a broader immunoregulatory role for this molecule. HLA-G seems to reduce cell sensitivity of several types of solid tumors, cells from lymphoproliferative disorders and allogeneic grafts to cytolysis mediated by NK cells and cytotoxic T cells, therefore acting as a mechanism of immune escape (1, 6-12). The expression of soluble HLA-G (sHLA-G) in blood and by heart and liver/kidney grafts has also been associated with significant better prognosis and fewer rejection episodes (1, 13-14).

Recent studies demonstrate that HLA-G is induced at the course of inflammatory pathologies such as myositic lesions, psoriatic lesions on skin, atopic dermatitis and multiple sclerosis (15-18). Furthermore, HLA-G expression on the surface of epithelial intestinal cells seems to play a role on suppression of pro-inflammatory cytokines in ulcerative colitis (19). HLA-G expression has been suggested as a possible mechanism of tissue protection against autoimmune inflammatory responses, therefore acting as a mechanism of immune surveillance (20-21). Recently, Verbruggen *et al*. 2006 observed that sHLA-G levels were lower in rheumatoid arthritis (RA) patients as compared to controls (22).

It was previously shown that a HLA-G gene polymorphism, a 14 bp insertion/deletion in exon 8 of the gene, might play a role in mRNA stability (23). The +14bp allele is associated with lower levels of HLA-G mRNA and, to some extent, with lower levels of sHLA-G (24-26). The involvement of this polymorphism in the maintenance of pregnancy, in success of *in-vitro* fertilization and in preeclampsia has been previously described (27-31).

It was previously described that IL-10, a cytokine known for its antiinflammatory and immunosuppressive roles, induces HLA-G expression in human trophoblasts and monocytes (32), and that HLA-G seems also to induce IL-10 expression in peripheral blood mononuclear cells (PBMCs) (33). Rizzo *et al.* (2005) (34) investigated HLA-G and IL-10 levels in PBMCs activated with lypopolyssacaride (LPS) in relation to the 14bp polymorphism. They observed a significant increase of IL-10 levels in PBMCs homozygous for the +14bp allele as compared to the other genotypes. This data, allied to the recent description of a subset of T cells which express and secrete HLA-G and that are present in normal physiological and inflammatory conditions (35), are elements that incite the study of the influence of polymorphic variants of HLA-G in inflammatory diseases.

Rheumatoid arthritis and juvenile idiopathic arthritis (JIA) are two inflammatory disorders that share some common features, such as a strong pro-inflammatory response directed to joints and tissue destruction. However, certain discrepancies concerning clinical (e.g. JIA presents a broad clinical heterogeneity, being classified in several subtypes and in RA, the vast majority of the patients are RF (rheumatoid factor) positive, while in JIA, there are few RF+ patients), molecular and genetic features suggest that they represent two quite different pathologies.

In the present work we assessed the frequency of the 14bp polymorphic variants in RA and JIA. Differences were observed in genotypic and allelic frequencies between these two disorders and a significant difference was observed in JIA patients subgrouped according to gender.

## MATERIALS AND METHODS

### Patients and controls:

The RA patient group was comprised of 265 European-derived individuals with diagnosis of RA, satisfying the American College of Rheumatology criteria (36), that were under the care of the Division of Rheumatology of the Hospital de Clínicas de Porto Alegre (HCPA), the capital of the southernmost state of Brazil. Among them, 23 lost to follow-up and 242 patients had their medical records reviewed for documentation of clinical, laboratory and radiographic data. Clinical data included pattern of joint involvement, atlantoaxial subluxation and extra-articular manifestations (rheumatoid nodules, amyloidosis, vasculitis, pneumonitis and episcleritis). Erosive disease was

characterized by the presence of erosions in any of the x-rays of the hands and feet since the beginning of follow-up in HCPA, otherwise patients were classified as having non-erosive disease. The disease activity score (DAS28) (37) and the health assessment questionnaire (HAQ) (38) were applied to each patient as a measure of disease activity and physical ability.

A total of 106 patients who fulfilled the criteria for juvenile rheumatoid arthritis as proposed by the American College of Rheumatology and redefined according to the International League of Associations for Rheumatology classification (39) were analyzed. The patients were recruited in three Medical Centers from Porto Alegre, and their relatives signed an informed consent. All patients were diagnosed before 16 years of age and presented inflammatory arthritis in, at least, one joint persistent for more than one year at the time of the research. This criterion was used to the exclusion of persistent reactive arthritis.

The control group was composed of 356 European-derived healthy individuals. The controls were from the urban population of Porto Alegre, the same geographic area of the patients, and share the same ethnic origins of the patients. All individuals included in the control group were adults (mean age 53.5±17.7 years). Previous history of arthritis or autoimmune diseases was an exclusion criterion. The control group was comprised by adults in order to avoid the inclusion of individuals (children) that eventually would later develop the disease. The study protocol was approved by the Ethics Committee of the Hospital de Clínicas de Porto Alegre and informed consent was obtained from all patients.

**Polymerase chain reaction (PCR) amplification of exon 8 of the HLA-G gene and genotyping:** DNA was isolated from peripheral blood cells using a salting out method (40). HLA-G genotyping was performed as previously described (28). Briefly, 100 ηg of genomic DNA was amplified in a 25µl reaction, with final concentrations as follows: PCR buffer 1X, dNTP 0.2 mM, $MgCl_2$ 1.5 mM, Taq DNA polymerase 0.75U, and 10 ρmol of each primer (GE14HLAG – 5´- GTGATGGGCTGTTTAAAGTGTCAC C, RHG4 – 5´-GGAAGGAATGCAGTTCAGCATGA). Thermocycling conditions: 94°C for 2 min; 35 cycles of 94°C for 30s, 64°C for 60s and 72°C for 60s; final extension of 72°C for 10 min. The amplified PCR products were visualized in 6% polyacrylamide gel stained with

ethydium bromide. The amplicon sizes for the 14bp polymorphism were: 224 pb for the +14bp allele and 210bp for the -14bp allele.

**Statistical analysis:** HLA-G genotypic frequencies were compared to Hardy–Weinberg expectations using Chi-Square tests. HLA-G allelic frequencies and HLA-G genotypes based upon the 14bp deletion polymorphism in exon 8 of controls, JIA and RA patients were compared using the $\chi^2$ test (with Yates correction when necessary) or Fisher's Exact Test. Bonferroni correction for multiple comparisons was applied when the p-value was significant. Relative risks were estimated by the odds ratio. Means for DAS28 and HAQ were analyzed by one-way ANOVA and Kruskall-Wallis test, respectively. The significance level was set at $\alpha = 0.05$ (two-tailed) and all statistical analyses were performed with SPSS 13.0 (SPSS Inc., Chicago, IL, USA) and winPEPI (41).

## RESULTS

### Juvenile Idiopathic Arthritis:

We analyzed 106 JIA patients (70 females and 36 males) for the 14bp insertion/deletion polymorphism. The mean disease age of onset was 4.8±3.1 years. (see further details on Table 1). Both control and JIA patient groups were in Hardy-Weinberg equilibrium (data not shown). We observed an increased frequency of the -14bp allele in the JIA group as compared to controls, which was significant, but not after Bonferroni correction was applied (Table 2). The genotypic frequencies differed significantly between gender inside the patients group: the -14bp/-14bp genotype was more frequent among JIA females (55.7% against 19.4%) while the +14/-14 e +14/+14 genotypes were more frequent among JIA males (66.7% against 37.1% and 13.9% against 7.1% respectively, see Table 3).

Hence, considering that our JIA sample was composed of 2/3 of female individuals and that the -14bp allele seems to be more frequent in this group we compared patients against controls subgrouping by gender (Table 3). Female patients presented a higher frequency of the -14bp allele as compared to female controls (0.7429 and 0.5755, respectively, corrected P = 0.0075). We observed an increased frequency of the -14bp/-14bp genotype among female patients as compared to female controls (corrected P = 0.0048), while in males this genotype was observed in a low frequency, though not

significant (OR=0.41, P=0.061). Females with the -14bp/-14bp genotype presented a higher chance of having JIA (OR= 2.82, CI 95%= 1.52~5.25).

We then observed the genotypic frequency distribution when subgrouping the patients according to JIA subtype. No significant differences were observed among subtypes when analyzing males and females together (data not shown). The genotypic frequency distributions were similar among the oligoarticular, polyarticular and systemic subtypes of JIA in females (p = 0.986) which were similar to the female JIA group as a whole. Similarly, the three male subtypes presented no differences in their genotypic frequency distributions (p = 0.908) which were similar to the male group as a whole. No influence of the HLA-G genotype on disease age onset was observed (data not shown).

**Rheumatoid Arthritis**

We analyzed 265 RA patients (218 women and 47 men) for the 14bp insertion/deletion polymorphism. The mean patient age was 58.1±12.7 years and the mean symptoms onset age was 41.6±14.2 years (see further details on Table 1). Both control and RA patient groups were in Hardy-Weinberg equilibrium (data not shown). No significant differences were observed in the allelic and genotypic frequencies between RA patients and controls (P = 0.727 and 0.463 respectively, see Table 2).

Next, we investigated whether the 14bp polymorphism had any relation with disease severity or with different RA associated clinical features. No patient presented pneumonitis, only one patient presented episcleritis (being heterozygous to the analyzed polymorphism), two patients had amyloidosis (both heterozygous) and four patients presented vasculitis (two heterozygous and two homozygous -14bp). The remaining results are presented in Tables 6 and 7. No differences were observed among the mean DAS28 or HAQ scores for each genotype (p-values 0.575 and 0.700 respectively). No differences on allelic frequencies were observed concerning the clinical features evaluated in the presented study (Table 4). No correlation between HLA-G genotype and disease onset was observed (data not shown).

**DISCUSSION**

In the present study, we assessed the frequency of the 14 bp insertion/deletion polymorphism in the HLA-G gene in rheumatoid arthritis and juvenile idiopathic arthritis. Although this polymorphism has been previously assessed in RA (42), this is the first case/control study in JIA. Since the HLA-G molecule seems to be involved in several immunoregulatory processes and, particularly in inflammatory disorders, HLA-G represents an excellent candidate gene for association to these two rheumatic diseases. The influence of the HLA-G molecule in rheumatoid arthritis was analyzed by two recent studies (22, 42), but there is no data concerning this molecule and JIA.

Our data suggest a gender-specific difference for the 14bp insertion/deletion polymorphism in JIA, with a significantly higher frequency of the -14bp allele among female patients, which seems to confer a higher disease susceptibility to the females that present this allele, particularly in homozygosis. In our sample, 56% of the JIA females were homozygous for this allele, as compared to 19% of the males. Another interesting fact is that this increased frequency was not limited to a given subtype of the disease; all the subtypes represented in our sample presented an increased frequency of the -14bp allele in the female subgroup (Table 3). However, due to the reduced sample size in some subtypes, this fact must be interpreted with caution. We can speculate that there might be an immunoregulatory mechanism, common to the different subtypes of the disease, that can be altered in the presence of the -14bp allele.

Our data suggest that the HLA-G -14 bp allele is a risk factor for JIA. Rizzo and cols., 2005 performed an *in vitro* study using PBMCs activated with LPS, observing that the IL-10 produced by those cells correlated directly with the HLA-G genotype, with +14bp/+14bp cells presenting the higher IL-10 levels (34). Considering IL-10 as a anti-inflammatory cytokine, if the -14bp allele is associated to lower levels of IL-10 *in vivo*, in situations of inflammation , such as in JIA, the presence of this allele would be, *per se*, harmful. Further studies that evaluate the correlation between serum and synovial levels of IL-10 and HLA-G allelic variants in juvenile idiopathic arthritis will be necessary to clarify this issue.

In disagreement with what was observed in JIA females, JIA males do not present a significantly different genotypic frequency distribution of the 14bp polymorphism as compared to control individuals of the same gender. The fact is intriguing since this is a childhood disease, in which physiological differences between gender, particularly hormonal levels, are not so pronounced. Still, a hypothesis for the differential involvement of the HLA-G molecule in JIA, according to gender, would be the possible regulation of the HLA-G gene by hormones. Indeed, Yie and cols. (43) demonstrated that HLA-G expression is regulated by progesterone through the presence of a progesterone response element at the 5' upstream region of the gene. However, no significant differences were observed on the progesterone levels between JIA patients and controls (44) or between healthy children subgrouped by gender (45) that could point to this possibility.

Interestingly, Khalkhali-Ellis *et al.* (1998) (44) observed an association between low androgen levels and disease in 20 JIA female patients. Testosterone serum levels are higher in boys, as compared to girls, from uterine life to the first year of life (46). In recent years, studies both in animal models as well as in humans have highlighted an imunossupressor role of testosterone (47-49). Therefore, it is possible that in situations where testosterone levels are present below a certain threshold (that would be attained/exceeded more frequently by boys), the HLA-G molecule would pass from a secondary role to an important factor in the regulation of inflammatory responses.

Concerning rheumatoid arthritis, our data is in accordance with the results obtained by Rizzo *et al* (2006) (42), where no differences between genotypic and allelic frequencies for this polymorphism were observed when RA patients were compared to control individuals. When DAS28 and HAQ scores, which were used to evaluate disease severity, were analyzed according to HLA-G genotype, no correlation was observed. This data suggest that, differently from JIA, in RA the 14bp polymorphism is not related to the disease physiopathology. However, we cannot rule out the possibility of involvement of other polymorphisms within the HLA-G gene with RA. The fact that RA patients present lower sHLA-G levels in serum (22) is indicative that HLA-G molecule might play a role in the physiopathology of RA.

Concluding, in this work we observed differences in the genotypic and allelic frequencies of the 14bp polymorphism between RA and JIA and between JIA patients subgrouped according to gender. Our results strengthen the argument that RA and JIA are

not etiopathologically related. We cannot exclude another polymorphic locus, even outside the HLA-G gene, in linkage-disequilibrium with the analyzed HLA-G variants, as a true responsible for the obtained results. However, the increasing evidences linking HLA-G and autoimmune diseases and inflammatory conditions, together with the recent description of a new regulatory HLA-G+ T cell subset (35), strongly suggest that this molecule plays essential roles in the regulation of immune responses.

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Table 1: Juvenile Idiopathic Arthritis patients characteristics and disease subtypes

| | N | % | Mean | Range |
|---|---|---|---|---|
| *JIA (total n = 106)* | | | | |
| Age (years) | | | 9.3 | 4-18 |
| Age at diagnosis | | | 4.8 | 1-13 |
| Disease time (years) | | | 4.6 | 1-16 |
| Oligoarticular | 50 | 47.2 | | |
| Polyarticular | | | | |
| RF- | 38 | 35.8 | | |
| RF+ | 3 | 2.8 | | |
| Systemic | 13 | 12.3 | | |
| unknown subtype | 2 | 1.9 | | |
| ANA positivity | 15 | 14.1 | | |
| | | | | Std. Dev. |
| *RA (total n = 265)* | | | | |
| Age (years) | | | 58.1 | 12.67 |
| Symptoms onset age | | | 41.6 | 14.17 |
| Age at diagnosis | | | 46.6 | 13.64 |
| Disease time (years) | | | 11.7 | 6.74 |
| DAS | | | 4.21 | 1,33 |
| HAQ | | | 1.41 | 0.78 |
| Erosions | 205[a] | 79.2 | | |
| EA Manifestations | 55[b] | 22.1 | | |
| Rheumatoid nodules | 42[b] | 16.9 | | |
| Sub-luxations | 10[c] | 4.2 | | |
| RF positivity | 220[d] | 89.1 | | |

[a] out of 259 [b] out of 249 [c] out of 239 [d] out of 247. RF – Rheumatoid factor; ANA – Anti-nuclear antibodies; DAS – Disease Activity Score; HAQ – Health Assessment Questionaire.

Table 2: Genotypic and allelic frequencies on Juvenile Idiopathic Arthritis and Rheumatoid Arthritis patients.

| | *JIA (%) n=106* | *RA (%) n=265* | *Controls (%) n=356* |
|---|---|---|---|
| Genotype | | | |
| +14bp/+14bp | 10 (9.4) | 49 (18.2) | 59 (16.6) |
| +14bp/-14bp | 50 (47.2) | 132 (49.1) | 175 (49.2) |
| -14bp/-14bp | 46 (43.4) | 84 (31.2) | 122 (34.3) |
| P-value [a] | 0.094 | 0.727 | NA |
| OR (CI 95%)[b] | 1.47 (0.92 – 2.34) | 0.89 (0.62-1.27) | NA |
| | | | |
| Allele | | | |
| +14 bp | 70 (0.330) | 230 (0.434) | 293 (0.412) |
| -14 bp | 142 (0.670) | 300 (0.566) | 419 (0.588) |
| P-value [c] | 0.041 | 0.463 | NA |
| Correction[d] | 0.164 | | |

[a] Frequencies of each group of patients are compared to those of the control group using $\chi^2$ test.
[b] Each patient subgroup is compared to its respective control group, according to gender, taking as a risk factor the -14bp/-14bp genotype.
[c] Same as [a] but using $\chi^2$ with Yates correction.
[d] Bonferroni correction was applied by multiplying P values by the number of tests (n = 4)

Table 3: Genotypic and allelic frequencies in Juvenile Idiopathic Arthritis (JIA) and Rheumatoid Arthritis (RA) Patients subgrouped by gender.

| | *Females* | | | *Males* | | |
|---|---|---|---|---|---|---|
| Genotype | JIA (%) | RA (%) | Controls (%) | JIA (%) | RA (%) | Controls (%) |
| -14bp/-14bp | 39 (55.7) | 74 (33.9) | 49 (30.8) | 7 (19.4) | 10 (21.3) | 73 (37.2) |
| +14bp/-14bp | 26 (37.1) | 105 (48.2) | 85 (53.5) | 24 (66.7) | 27 (57.4) | 89 (45.4) |
| +14bp/+14bp | 5 (7.1) | 39 (17.9) | 25 (15.7) | 5 (13.9) | 10 (21.3) | 34 (17.3) |
| OR (CI 95%)[a] | **2.82 (1.52 to 5.25)**[b] | 1.15 (0.73 to 1.83)[c] | NA | 0.41 (0.14 to 1.01)[d] | 0.46 (0.19 to 1.01)[e] | NA |
| Allele | | | | | | |
| -14 bp | 104 (74.3) | 253 (58.0) | 183 (57.5) | 38 (52.8) | 47 (50.0) | 235 (59.9) |
| +14 bp | 36 (25.7) | 183 (42.0) | 135 (42.5) | 34 (47.2) | 47 (50.0) | 157 (40.1) |
| P-value[f] | **0.0075** [g] | 0.954 | NA | NA[h] | 0.101 | NA |

[a] Each patient subgroup is compared to its respective control group, according to gender, taking as a risk factor the -14bp/-14bp genotype.
[b] P = 0.0048 after Bonferroni correction for multiple comparisons (n = 8) [c] P=0.597 [d] P=0.061 [e] P=0.057
[f] Each patient subgroup was compared to the respective control group, according to gender, using $\chi^2$ with Yates correction [g] After Bonferroni correction (n = 8) [h] The genotypic distribution on JIA males group was not in HW equilibrium.

Table 4: RA – Clinical manifestations in individual carrying the -14bp variant.

| *Feature* | | *% Allele -14bp (n)* | *n* | *P* |
|---|---|---|---|---|
| EA Manifestations | Yes | 57.3 (55) | 96 | 0.709 |
| | No | 54.5 (194) | 356 | |
| Rheumatoid nodules | Yes | 60.0 (42) | 70 | 0.443 |
| | No | 54.2 (207) | 382 | |
| Erosions | Yes | 53.7 (205) | 382 | 0.333 |
| | No | 60.0 (54) | 90 | |
| Sub-luxations | Yes | 45.5 (10) | 22 | 0.540 |
| | No | 54.5 (229) | 430 | |
| RF positivity | Yes | 54.5 (220) | 404 | 0.462 |
| | No | 48.2 (27) | 48 | |

EA – Extra articular; RF – Rheumatoid Factor

**4. LACK OF ASSOCIATION OF THE HLA-G 14 BP INSERTION/DELETION POLYMORPHISM WITH SYSTEMIC LUPUS ERYTHEMATOSUS, SYSTEMIC SCLEROSIS AND SJÖGREN'S SYNDROME –** ARTIGO SUBMETIDO À REVISTA *TISSUE ANTIGENS* COMO "BRIEF COMMUNICATION"

# LACK OF ASSOCIATION OF THE HLA-G 14 BP INSERTION/DELETION POLYMORPHISM WITH SYSTEMIC LUPUS ERYTHEMATOSUS, SYSTEMIC SCLEROSIS AND SJÖGREN'S SYNDROME

**Brief Communication**

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Short running title: HLA-G 14 bp polymorphism in autoimmune diseases

**ABSTRACT**

HLA-G is a non-classical HLA molecule which has been shown to be induced at the course of inflammatory pathologies and has been suggested as a possible mediator of tissue protection against autoimmune inflammatory responses. We investigated the influence of the *HLA-G* 14 bp polymorphism on disease susceptibility and severity of Systemic Lupus Erythematosus (SLE), Sjögren's Syndrome (SS) and Systemic Sclerosis (SSc). 127 SLE patients, 111 patients with SSc and 21 SS patients were PCR-genotyped for this polymorphism, as well as 356 control subjects. No statistic differences were detected between controls and patients for each disease. Our results do not indicate a significant role for the HLA-G 14 bp polymorphism as a modifier or susceptibility factor in these diseases.

The Human Leukocyte Antigen G (HLA-G) is a non-classical class I major histocompatibility complex (MHC) molecule which is characterized by low polymorphism at DNA level, limited tissue distribution in non-pathological conditions and expression of both membrane-bound and soluble isoforms by alternative splicing (1). Recent studies demonstrate that HLA-G is induced at the course of inflammatory pathologies such as myositic lesions, psoriatic lesions on skin and multiple sclerosis (2-4). Furthermore, HLA-G expression on the surface of epithelial intestinal cells seems to play a role on the suppression of pro-inflammatory cytokines in ulcerative colitis (5). HLA-G expression has been suggested as a possible mechanism of tissue protection against autoimmune inflammatory responses, therefore acting as a mechanism of immune surveillance (6-7). Recently, Verbruggen *et al.* 2006 observed that sHLA-G (soluble HLA-G) levels were lower in rheumatoid arthritis (RA) patients as compared to controls (8).

Expression of HLA-G may be influenced by genetic variations on the *HLA-G* gene. Particularly, it was previously shown that a HLA-G gene polymorphism, a 14 bp insertion/deletion in exon 8 of the gene, might play a role in mRNA stability (9) and is, to some extent, associated with alteration in the plasmatic levels of soluble forms of HLA-G (10, 11). The +14 bp allele of this polymorphism is associated with high IL-10 production by peripheral blood mononuclear cells (PBMC) *in vitro* (12). The involvement of this polymorphism in situations where immune regulation is required, such as in the maintenance of pregnancy (13-14), in the success of *in-vitro* fertilization (15), development of preeclampsia (16-17) and other pathological conditions such as sarcoidosis (18) has been also described. In our laboratory, we have found a positive association between the HLA-G -14bp/-14bp genotype and susceptibility to JIA (Juvenile Idiopathic Arthritis) in females, suggesting that HLA-G polymorphisms might have a role on the pathogenesis of rheumatic disorders (Veit *et al.* 2007, unpublished data).

Systemic Lupus Erythematosus (SLE), Sjögren's Syndrome (SS) and Systemic Sclerosis (SSc) are three rheumatic autoimmune diseases in which dysregulation of the immune response might play an important role. Although the role of classical MHC molecules for these three diseases has been intensively addressed during recent years, there are no information on the genetic contribution of nonclassical MHC molecules such as HLA-E, HLA-F and HLA-G in their pathogenesis. Since HLA-G is consistently associated with immunoregulatory responses, considering that its expression has been described in

several inflammatory disorders and since a possible genetic contribution of the HLA-G polymorphisms was suggested for JIA pathogenesis, it would be interesting to investigate the genetic influence of HLA-G polymorphisms in other rheumatic diseases. Therefore, we investigated the influence of the *HLA-G* gene 14bp polymorphism on disease susceptibility and severity of SLE, SS and SSc.

The SLE patients group was comprised of 127 European-derived individuals with diagnosis of RA, satisfying the American College of Rheumatology criteria (19), All patients had their medical records reviewed or underwent a medical interview for documentation of clinical and laboratory data. The disease activity index SLEDAI (20) and the SLICC/ACR damage index (21) were applied to each patient as a measure of disease activity cumulative damage. The evaluated clinical manifestations are presented on Table 1. The SSc group was composed by 111 European-derived patients which met the American College of Rheumatology (ACR) criteria for SSc (22) or the criteria suggested by LeRoy and Medsger for diagnosis of early forms of SSc (23). Among them, 107 patients had their medical records reviewed or underwent a medical interview for documentation of clinical, laboratory and radiographic data. Disease subtype was classified as follows: diffuse cutaneous SSc (truncal and acral skin tautness), limited cutaneous SSc (skin tautness restricted to extremities and/or face), and limited SSc (absence of skin tautness) (23). The SS group was comprised by 21 European-derived patients meeting the Diagnostic Criteria from the European Community for pSS, established in 1993 (24). A clinical assessment was performed by applying a clinical protocol previously standardized. The variables here analized were the presence of *Sicca Syndrome*, associated arthritis, positivity for systemic manifestations, and positivity for the autoantibodies ANA, RF, anti-Ro and anti-La. All patients were under the care of the Division of Rheumatology of the Hospital de Clínicas de Porto Alegre, the capital of the southernmost state of Brazil. The study protocol was approved by the Ethics Committee of the Hospital de Clínicas de Porto Alegre and informed consent was obtained from all patients.

The control group was composed by 356 European-derived healthy individuals. The controls were from the urban population of Porto Alegre or other cities from the same geographic area of the patients, and shared the same ethnic origins of the patients. An informed consent was obtained from all subjects.

DNA was isolated from peripheral blood cells using a salting out method (Lahiri and Nurnberger 1991) (25). HLA-G genotyping was performed as previously described in Hviid *et al.* 2002 (14). Briefly, 100 ηg of genomic DNA was amplified in a 25μl reaction, with final concentrations as follows: PCR buffer 1X, dNTP 0.2 mM, $MgCl_2$ 1.5 mM, Taq DNA polymerase 0.75U, and 10 ρmol of each primer (GE14HLAG – 5´- GTGATGGGCTGTTTAAAGTGTCAC C, RHG4 – 5´- GGAAGGAATGCAGTTCAG CATGA). Thermocycling conditions: 94°C for 2 min; 35 cycles of 94°C for 30s, 64°C for 60s and 72°C for 60s; final extension of 72°C for 10 min. The amplified PCR products were visualized in 6% polyacrylamide gel stained with ethydium bromide.

HLA-G genotypic frequencies were compared to Hardy–Weinberg expectations using Chi-Square tests. HLA-G allelic frequencies and HLA-G genotypes based upon the 14bp deletion polymorphism in exon 8 of controls, JIA and RA patients were compared using the $\chi^2$ test (with Yates correction when necessary) or Fisher's Exact Test. Means for continuous variables were analyzed by one-way ANOVA or Kruskall-Wallis test, conditions for ANOVA were not satisfied. The significance level was set at $\alpha = 0.05$ (two-tailed) and all statistical analyses were performed with SPSS 13.0 (SPSS Inc., Chicago, IL, USA) and winPEPI (26).

We analyzed 127 SLE patients (112 women and 15 men) for the 14 bp insertion/deletion polymorphism. The healthy control group presented an allelic frequency of 41.1%, which is similar to other Caucasian populations (27) and also similar to the frequency obtained by a previous study for a Brazilian population (28). The mean SLE patients age was 44.6±14.3 years and the mean disease diagnostic age was 29.8±14.0 years (see further details on Table 1). Both the patients and the control group were in Hardy-Weinberg equilibrium (data not shown). No statistically significant differences were observed in the allelic and genotypic frequencies between SLE patients and healthy controls (P = 0.844 and 0.425 respectively, see Table 2). We also investigated whether the insertion/deletion polymorphism was associated to disease severity or to different clinical features present in LES patients (all evaluated features are shown in table 1). No differences among genotypic and/or allelic frequencies or symptons associations were observed between affected and unaffected groups for all clinical characteristics evaluated in this study (data not shown). The mean SLEDAI and SLICC/ACR scores were also

similar when individuals were subgrouped according to genotye and a comparison was performed.

Considering the SSc patients, 111 individuals (97 females and 14 males) were analyzed for the 14 bp insertion/deletion polymorphism. The mean patients' age was 50.7±14.7 years and the mean disease diagnostic age was 37.3±15.4 years (see further details on Table 1). All patients presented positivity for the Raynaud's phenomenon. The observed genotype distribution among the patients follows the Hardy-Weinberg equilibrium. No significant differences were observed in the allelic and genotypic frequencies between SSc patients and healthy individuals (P = 0.636 and 0.774 respectively, see Table 2). In the same way, none of the evaluated clinical and molecular features evaluated in the study (Table 1) were significantly associated to the HLA-G genotype (data not shown).

We analyzed 21 SS patients for the 14 bp insertion/deletion polymorphism. All but one patient were females. The mean patients age was 54.5±8.3 years and the mean disease diagnostic age was 40.9±11.0 years (further details on Table 1). The patients group was in Hardy-Weinberg equilibrium (data not shown). No significant differences were observed in the allelic and genotypic frequencies between SLE patients and healthy controls (P = 0.401 and 0.595 respectively, see Table 2). None of the specific disease features analysed showed a statistically significant association with HLA-G genotype (data not shown).

We analyzed the role of the 14pb polymorphism of the HLA-G gene in SLE, SSc and SS, and assessed their possible relevance in modulating disease susceptibility, course, or severity in each of these three diseases. Recent works suggest that HLA-G plays a role on the imunoregulation of autoimmune diseases (15, 16, 18). Upregulation of HLA-G expression at sites of inflammation is supposed to limit organ damage and hence play a role in maintenance of tissue integrity (6, 7). Genetic variations of HLA-G, mainly the 14bp polymorphism, were shown to influence miscarriage rates and the risk of preeclampsia. Moreover, the 14bp polymorphism was suggested to play a role on the susceptibility to JIA, an inflammatory childhood disease (Veit *et al.* unpublished data). Analysis of our SLE, SSc and SS patients, in which several clinical features were taken into account, did not reveal evidence for a significant role of the HLA-G 14 bp polymorphism as a modifier or susceptibility factor in these diseases. These results, however, cannot exclude a possible role for this molecule on the immunoregulatory

mechanisms underlying these disorders. Kroner *et al.* (2006) were unable to find an association among HLA-G polymorphisms, including the 14bp polymorphism, and Multiple Sclerosis, even though the presence of immune cells expressing HLA-G in this disease is a well-established feature (29). Therefore, although our results indicate an absence of association between the HLA-G 14 bp polymorphism and the etiopathology of SLE, SSc and SS, further studies which focus on HLA-G expression might answer if this molecule plays a role on these and other rheumatic diseases.

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Table 1: Clinical features according to the disease.

| *Systemic Lupus Erythematosus (n=127)* | | *Systemic Sclerosis (n=111)* | | *Sjögren's Syndrome (n=21)* | |
|---|---|---|---|---|---|
| Age (years) | 44.6±14.6 (126) | Age (years) | 50.7±14.7 (106) | Age (years) | 54.5±8.3 (21) |
| Diagnostic age (years) | 29.8±14.0 (121) | Diagnostic age (years) | 37.3±15.4 (105) | Diagnostic age (years) | 40.9±11.0 (21) |
| Malar rash | 55.6% (126) | Skin score | 6.0 (2.0, 15.0)[1] (106) | Arthritis | 95.2% (21) |
| Discoid rash | 16.7% (126) | Type | (107) | *Sicca* syndrome | 95.2% (21) |
| Photosensitivity | 74.6% (126) | Diffuse cutaneous | 25.2% | ANA | 66.7% (21) |
| Oral ulcers | 37.3% (126) | Limited cutaneous | 61.7% | Rheumatoid factor | 61.9% (21) |
| Arthritis | 77.0% (126) | Limited | 13.1% | Anti-Ro antibody | 38.1% (21) |
| Serositis | 26.4% (125) | Digital pitting scars, amputations or loss of digital pad tissue | 67.9% (106) | Anti-La antibody | 33.3% (21) |
| Nephritis | 48.4% (126) | | | Systemic Manifestations | 52.4% (21) |
| Neurologic disorders | 11.1% (126) | | | | |
| Hematologic disorders | 74.0 %(126) | Calcinosis | 22.6% (106) | | |
| Hemolytic anemia | 33.3% (126) | ANA | 84.9% (106) | | |
| Leukopenia/ Lymphopenia | 56.3% (126) | Anticentromere antibody | 39.6% (106) | | |
| Thrombocytopenia | 19.0% (126) | Anti-Scl70 antibody | 23.6% (106) | | |
| Immunologic disorders | 67.8% (121) | Pulmonary Fibrosis | 54.9% (102) | | |
| Anti-DNA | 53.3% (120) | FVC (%) | 82.2±18.1 (98) | | |
| Anti-Sm | 12.5% (120) | DLCO (%) | 56.3±18.3 (96) | | |
| Anticardiolipin | 20.0% (120) | PAH (mmHg) | 33.8±12.8 (97) | | |
| Lupic Anticoagulant | 5.8% (120) | | | | |
| false positive VDRL | 2.5% (120) | | | | |
| ANA | 97.6% (123) | | | | |
| Secondary Sjögren S. | 7.7% (103) | | | | |
| SLEDAI | 2.0 (0.0, 4.0)[1] (76) | | | | |
| SLICC | 0.1 (0.0, 0.2)[1] (101) | | | | |

Numbers in parenthesis correspond to the number of patients for which the information was avaliable for analysis. [1] Median (percentiles 25, 75)
Abbreviations: ANA = Antinuclear antibody; VDRL = Venereal Disease Research Laboratory test; SLEDAI = Systemic Lupus Erythematosus Disease Activity Index; SLICC = Systemic Lupus International Collaborating Clinics; FVC = % of the predicted forced expiratory vital capacity (Normal range = 80 to 120%); DLCO = % of the predicted carbon monoxide pulmonary diffusing capacity (Normal range = 75 to 125%); PAH = pulmonary arterial hypertension (Normal range = 17 to 28 mmHg).

Table 2: Genotypic and allelic frequencies

| | ***Controls % (n)*** | ***SLE % (n)*** | ***SSc % (n)*** | ***SS % (n)*** |
|---|---|---|---|---|
| Genotype | | | | |
| -14bp/-14bp | 34.3 (122) | 29.9 (38) | 30.6 (34) | 42.9 (9) |
| +14bp/-14bp | 49.2 (175) | 55.9 (71) | 52.3 (58) | 47.6 (10) |
| +14bp/+14bp | 16.6 (59) | 14.2 (18) | 17.1 (19) | 9.5 (2) |
| Comparison with controls (P) | - | 0.425 | 0.774 | 0.595 |
| Allele | | | | |
| -14 bp | 0.588 (419) | 57.9 (147) | 56.8 (126) | 66.7 (28) |
| +14 bp | 0.412 (293) | 42.1 (107) | 43.2 (96) | 33.3 (14) |
| Comparison with controls (P)* | - | 0.844 | 0.636 | 0.401 |

* $\chi^2$ with Yates correction

## 5. DISCUSSÃO

O estudo da influência da molécula HLA-G em doenças reumáticas é ainda incipiente. Desde a primeira descrição da expressão de HLA-G em trofoblastos, Kovats *et al.* (1990), dez anos se passaram até que essa molécula fosse considerada como envolvida em uma doença inflamatória [Wiendl *et al.* (2000), em lesões miosíticas]. Desde então, outras doenças inflamatórias mereceram a atenção dos pesquisadores. Entretanto, ainda assim este grupo de doenças tem sido pouco abordado no que diz respeito à influência dessa molécula. Dessa maneira, propôs-se realizar um estudo que avaliasse a influência da molécula HLA-G através da comparação entre as freqüências genotípicas e alélicas da variante de 14 pb em pacientes e controles saudáveis em cinco doenças reumáticas, das quais apenas a artrite reumatóide havia merecido atenção por parte de outros grupos científicos.

Certamente o grande dado a ser destacado neste trabalho é a observação de que as freqüências genotípicas e alélicas do polimorfismo de 14pb diferiram significativamente entre os sexos nos pacientes com AIJ, o que se constitui na primeira evidência da influência de variantes alélicas de HLA-G na imunopatogênese de doenças reumáticas. Este dado torna-se mais intrigante se observarmos que AIJ é uma doença que acontece na infância, fase da vida em que, até os 10 anos de idade, poucas diferenças hormonais são observadas entre meninos e meninas (Elmlinger *et al.* 2002, Gassler *et al.* 2000). Ainda assim, é provável que a ação imunorregulatória mediada por HLA-G na prevenção dos processos inflamatórios presentes na AIJ, se realmente comprovada, ocorre em conjunto com outros fatores cujos níveis de expressão devem ser influenciados pelo sexo. A

testosterona, por exemplo, permanece em altos níveis em meninos até o primeiro ano de vida (Gässler *et al.* 2000) e é apontada, em diferentes trabalhos, como um agente imunossupressor (Liva *et al.* 2001, Yao *et al.* 2003, Page *et al.* 2006).

Nas meninas com AIJ foi observada uma maior freqüência do alelo de deleção de 14 pb. Este dado é consistente com os resultados de Rizzo *et al.* (2005), em que CMSP homozigotas para o alelo de deleção de 14 pb em cultura, ativadas com LPS, produziam menores níveis de IL-10 comparadas às células heterozigotas, que por sua vez produziam menos IL-10 que as homozigotas para o alelo de inserção de 14 pb. A IL-10 é uma citocina antiinflamatória relacionada a vários processos de imunorregulação (Grütz 2005). O envolvimento dessa molécula em AIJ já foi descrito. A capacidade de indução de IL-10 por células T foi previamente associada à remissão espontânea em AIJ (de Kleer *et al.* 2003). Ainda, variantes alélicas do gene IL-10 estão associadas à oligoartrite estendida, uma forma clínica mais grave, mas não à forma persistente, mais leve (Crawley *et al.* 2001). Assim, é possível que a indução de IL-10 por HLA-G seja um mecanismo importante para a prevenção de processos de desregulação do sistema imune que levam à artrite crônica em crianças.

Os resultados obtidos em AR, em oposição aos da AIJ, não revelaram quaisquer relações do polimorfismo estudado com a doença. Durante o decorrer do trabalho, após a genotipagem dos primeiros 111 pacientes, observou-se um padrão nas freqüências alélicas oposto àquele observado em AIJ: em AR, observava-se uma freqüência maior do alelo de inserção de 14 pb, ainda que não-significativa (P= 0,11), em relação aos controles, o que levou-nos a suspeitar que, nessa doença, esse alelo, e não o de deleção de 14 pb, constituir-se-ia no fator de risco para AR. Diante dessa perspectiva, e dispondo de material biológico para mais que dobrar o número amostral, decidiu-se genotipar mais 154 pacientes para verificar se essa tendência se confirmava. Ao final do processo de genotipagem, no entanto, essa tendência acabou não se confirmando. Esses resultados são corroborados por um trabalho anterior, que também descreveu a ausência de associação desse polimorfismo com artrite reumatóide, porém com um número menor de pacientes e controles (Rizzo *et al.* 2006). Os resultados apresentados nessa dissertação, além de contarem com um número amostral maior, ultrapassando inclusive os números prometidos no projeto de mestrado (265 pacientes genotipados contra 137 prometidos), envolveram a análise de um grande

conjunto de variáveis associadas à doença, permitindo avaliar não só a suscetibilidade, mas também a influência do polimorfismo na gravidade e curso da doença.

Em AR, os níveis plasmáticos das formas solúveis de HLA-G encontram-se mais baixos que em controles normais, o que poderia contribuir para um exacerbamento da resposta imune pró-inflamatória em AR. (Verbruggen *et al.* 2006). A razão para estes menores níveis pode advir de vários fatores. Já foi previamente descrito que HLA-G é expressa por linfócitos e monócitos, células do sistema imune normalmente presentes no infiltrado celular presente no líquido sinovial de pacientes AR (Feger *at al.* 2007, LeMaoult et al. 2003; Mitsdoerffer et al. 2005). É possível que a população celular responsável pela expressão de HLA-G encontre-se diminuída, ou que a expressão de HLA-G por parte desse conjunto de células esteja bloqueada por outros fatores inerentes à fisiopatologia da AR. Os resultados dessa dissertação, entretanto, não excluem a possibilidade da influência de outros polimorfismos dentro do gene HLA-G nos menores níveis séricos observados em pacientes AR.

A análise realizada em 127 pacientes com lúpus eritematoso sistêmico, 111 pacientes com esclerose sistêmica e 21 pacientes com síndrome de Sjögren, apresentada em forma de artigo, constituiu-se no primeiro estudo abordando a influência do HLA-G nessas doenças, o que reforça o campo aberto de pesquisa que essa molécula representa. Os números amostrais finais para essas doenças são ligeiramente inferiores àqueles propostos no início do trabalho por ter-se decidido excluir da análise os pacientes de origem diferente da européia, evitando assim confusões na análise dos resultados devido a diferentes etnias dentro da população. Com relação a LES e ES, os dados obtidos indicam a ausência de associação do polimorfismo de 14 pb com a suscetibilidade ou gravidade das manifestações clínicas nessas doenças. Com relação à SS, a ausência de associação é discutível, em termos do limitado número amostral atingido, fazendo-se necessário um aumento desse número a fim de obter resultados mais conclusivos. Apesar da ausência da associação do polimorfismo de 14 pb com HLA-G com essas três doenças, é impossível excluir um envolvimento desta molécula na fisiopatologia das doenças analisadas. Para averiguar esta questão, são necessários estudos direcionados para a expressão de HLA-G.

Muito ainda resta a ser descoberto sobre a influência de HLA-G em doenças reumáticas. Antes desse trabalho, apenas dois estudos haviam investigado a influência dessa molécula em AR (Verbruggen *et al.* 2006, Rizzo *et al.* 2006) e nenhum trabalho

havia se dedicado a estudar a ocorrência de HLA-G em AIJ, LES, ES ou SS. Entretanto, descobertas recentes relacionadas a células T regulatórias que expressam essa molécula prometem aumentar o interesse da comunidade científica para a potencial importância do HLA-G na regulação da resposta imune em doenças reumáticas.

Os estudos até hoje realizados com o HLA-G revelam que esta molécula pode exercer sua atividade reguladora através de vários mecanismos, como a inibição da atividade citotóxica mediada por células NK através de isoformas de membrana e solúveis, inibição de células T CD4+ através de HLA-G solúvel e inibição da maturação de células apresentadoras de antígenos, entre outros, revisados em LeMaoult et al, 2005. A indução de IL-10 aparece como um novo possível mecanismo de imunorregulação mediado por essa molécula (Rizzo et al, 2005) e, de acordo com os resultados exibidos no presente trabalho, pode ser o um mecanismo de regulação imunológica importante em AIJ. Os mecanismos pelos quais HLA-G desempenha sua função reguladora provavelmente dependerão do contexto imunológico (i.e. dos tipos celulares que expressam HLA-G e dos tipos celulares com os quais a molécula poderá interagir) em cada doença. Assim, é possível que a molécula de HLA-G, em AR e AIJ, esteja envolvido em rotas de imunorregulação distintas. Os trabalhos futuros motivados pelo presente trabalho constituem-se 1) na avaliação dos mecanismos de imunorregulação mediados por essa molécula em AR e AIJ; 2) na identificação dos tipos celulares envolvidos no processo de imunorregulação mediada por HLA-G nessas duas doenças, e 3) na investigação da expressão de HLA-G em LES, ES e SS.

Durante o período do mestrado também foi realizado um trabalho vinculado à determinação do genótipo HLA-G de mulheres que desenvolveram pré-eclâmpsia durante a gravidez (Vianna *et al.* 2007, ver Anexo 1). A pré eclâmpsia (PE) é um fenômeno inflamatório caracterizado por uma resposta vascular anormal ao fenômeno da placentação, tendo como principais manifestações clínicas hipertensão e proteinúria Alguns trabalhos sugerem que esse fenômeno seria o produto de um balanço imunológico em favor de uma resposta Th1, pró-inflamatória (Pregnancy WGRoHBPi 2000, Chaouat *et al.* 2004, Wegmann *et al.* 1993). Foi observado que, em mulheres primíparas com PE, o alelo -14pb encontrava-se em maior freqüência em comparação a mulheres primíparas sem PE, sugerindo uma associação mais ampla desse alelo a fenômenos pró-inflamatórios.

Os dados deste trabalho sugerem, portanto, que o polimorfismo de 14 pb do gene HLA-G está relacionado com a suscetibilidade genética ao desenvolvimento de AIJ. Os resultados obtidos apoiam o estudo da influência genética de variantes alélicas de HLA-G em doenças reumáticas. Estudos futuros voltados para a expressão de HLA-G e para os processos imunológicos mediados por essa molécula poderão clarificar os mecanismos de imunorregulação envolvidos na patogênese e na fisiopatologia das doenças apresentadas neste trabalho e em outras doenças reumáticas.

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## ANEXO I

**IMMUNOGENETICS OF PREGNANCY: ROLE OF A 14-BP DELETION IN THE MATERNAL HLA-G GENE IN PRIMIPAROUS PRE-ECLAMPTIC BRAZILIAN WOMEN** – ARTIGO PUBLICADO NA REVISTA *HUMAN IMMUNOLOGY*

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# Immunogenetics of pregnancy: Role of a 14-bp deletion in the maternal HLA-G gene in primiparous pre-eclamptic Brazilian women

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**KEYWORDS**
HLA-G polymorphism;
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**Summary** The etiology and pathogenesis of pre-eclampsia (PE) involve a combination of maternal-fetal genetic and immunologic factors. The immunologic maladaptation theory of PE predicts that the maternal immune system does not tolerate the semi-allogeneic fetus. Human leukocyte antigen-G (HLA-G) is expressed in some types of immune cells as well as in the fetal-maternal interface by trophoblasts, playing an immunoregulatory role. Here we have evaluated a 14-bp deletion polymorphism in the 3′-untranslated region of exon 8 of HLA-G gene in pregnant PE women and controls. HLA-G genotypes in both control and PE women were in Hardy-Weinberg equilibrium. The healthy pregnant and PE women had similar genotype frequencies ($p = 0.789$). This was similarly observed when PE women were subgrouped accordingly to severity of disease ($p = 0.646$). However, the primiparous PE women presented a tendency toward higher frequency of the 14-bp deletion allele (0.442) compared with the primiparous healthy women (0.286), $p = 0.09$. Our data suggest that the maternal 14-bp deletion of HLA-G is not associated with the risk for PE but that it could affect the development of PE in primiparous women.


## Introduction

Pre-eclampsia (PE) is a systemic disorder of unknown origin that is characterized by abnormal vascular response to placentation, increased systemic vascular resistance, enhanced platelet aggregation, activation of the coagulation cascade, and endothelial cell dysfunction [1]. The etiology and pathogenesis of PE involve a combination of maternal-fetal genetic and immunologic factors. The disorder is heterogeneous and pathogenesis can differ in women according to the presence of different risk factors. Pathogenesis of PE in pri-

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**ABBREVIATIONS**

| | |
|---|---|
| HLA-G | human leukocyte antigen-G |
| IL-10 | interleukin-10 |
| PE | pre-eclampsia |
| TH1 | T helper lymphocyte type 1 |
| TH2 | T helper lymphocyte type 2 |
| UTR | untranslated region |

miparous women may differ to that in women with pre-existing vascular disease, multifetal gestation, diabetes mellitus, or previous pre-eclampsia events [2,3]. Women with PE are usually diagnosed with hypertension and associated proteinuria. PE can be serious if severe hypertension is associated with proteinuria or if hypertension is associated with severe proteinuria (≥5 g per day) [4,5]. In general, maternal and perinatal outcomes are usually favorable in women with mild PE developing beyond of 36 weeks of gestation. In contrast, maternal and perinatal morbidities and mortalities are increased in women who develop the disorder before the 33$^{th}$ week of gestation [5–7]. PE is usually regarded as a disease of first pregnancy.

The immunologic maladaptation hypothesis of PE predicts that the maternal immune system does not tolerate the semiallogeneic fetus. During pregnancy, the maternal immune system is in close contact with cells and tissues of the semiallogeneic fetus. Therefore, there must be specific mechanisms engaged in modulating the maternal immune system to prevent the fetus rejection. Women with healthy pregnancies tend to present with a Th2 type of immune response, whereas a Th1 type response is incompatible with a successful pregnancy and plays a role in certain complications such as PE development [8–12]. Human leukocyte antigen-G (HLA-G) is a nonclassical HLA class Ib molecule that is predominantly expressed in the fetal-maternal interface and plays an important role during implantation and maternal acceptance of the fetus. HLA-G mRNA has been detected in many different tissues, whereas HLA-G protein expression is limited to a few specific cells such as trophoblasts in placenta, monocytes, lymphocytes, and thymus [13–15]. Some immunologic interactions can contribute to fetal maintenance. Expression of HLA-G by trophoblasts inhibits activation of maternal T cells, natural killer (NK) and antigen-specific CTL cytolysis via specific receptors [16–19], and IL-10 secreting cells may stimulate the HLA-G expression [20]. Interestingly, some effectors CD4+ and CD8+ T lymphocytes acquire immunosuppressive HLA-G1 from antigen-presenting cells (APCs) and reverse their function from effectors to regulatory cells [21]. Recently Fanchin *et al.* reviewed the possible relationship between HLA-G and human embryo implantation, exploring the HLA-G expression on human preimplantation embryos and in the endometrium, as well as its levels in embryo culture supernatants and circulating maternal blood [22]. HLA-G has few polymorphic alleles and shows a limited pattern of expression, contrasting to the highly polymorphic HLA class Ia and II antigens [15,23–32]. A very interesting variation of HLA-G involves a 14-bp deletion/insertion polymorphism in the 3′UTR of the HLA-G gene located at position 3741 at exon 8. The 14-bp sequence at the beginning of exon 8 is suggested to be responsible for the alternative splicing of the HLA-G transcript. This 14-bp polymorphism has been associated with HLA-G isoforms that lack 92 base sequences in the first part of exon 8 (3′UTR) [33]. During mRNA processing, this sequence functions as a cryptic branch point for mRNA splicing and is thus more stable in nature [34,35]. However, the 14-bp deletion causes the retention of 92 bases in the mature transcript, resulting in an unstable transcript. The 14-bp deletion/insertion polymorphism may influence both the HLA-G isoform splicing patterns and HLA-G mRNA stability. This may change the HLA-G function and could be of pivotal importance in certain pregnancy complications like PE [35,36]. The allele +14 bp has been associated with lower levels of soluble HLA-G and was implicated in the development of PE and recurrent abortions [35,37,38]. Several studies have been suggested the importance of the maternal HLA-G expression during cleavage embryo development and during the course of pregnancy. Yao *et al.*described a disparity between HLA-G mRNA isoforms and protein expression in embryos. They suggested that in some stage embryos, this difference might be caused by HLA-G protein remaining from maternal oocyte stores produced before embryonic genome activation. Thus HLA-G expressed at this stage may be more a marker of oocyte rather than embryonic quality [39]. In addition, Menezo *et al.* reported that the levels of secreted soluble HLA-G protein are higher than the capacity proposed for soluble HLA-G release by the embryo, so the signals secreted by the embryos are not in the order of magnitude of estimated HLA-G protein concentrations [40]. These findings shed some light on the contribution of the maternal HLA-G protein to a successful pregnancy. In this case-control study, we analyzed the maternal 14-bp HLA-G polymorphism, investigating both allelic and genotypic frequencies in Brazilian women who developed PE. The hypothesis of immune maladaptation in PE was studied here, evaluating the importance of maternal HLA-G genotype during a successful pregnancy.

## Subjects and methods

### Individuals

The patients were recruited at the Maternity Unit of at public hospital in Southern Brazil (Hospital Nossa Senhora Conceição, Porto Alegre). We identified 162 healthy pregnant women with uncomplicated pregnancies (controls) and 157 pregnant with PE. The inclusion criteria for selecting controls included: no rise in blood pressure, no hypertension or proteinuria, similar age (healthy women 28.08 years ± 7.37 years, and PE women 30.32 ± 7.46 years), no biologic relationship and a delivery date as close as possible to the delivery date(s) of the matched patient group. Controls were followed up for at least 3 months after delivery. If hypertension and/or proteinuria were observed during this follow-up period, this specific control individual was excluded. Pre-eclampsia was defined as the presence of hypertension and proteinuria. Hypertension is characterized by blood pressure of ≥140 mm Hg (systolic) or at least 90 mm Hg (diastolic), on at least two occasions and 4–6 hours apart after the 20$^{th}$ week of gestation in women known to be normotensive previously [5,41]. Proteinuria is defined as an excretion of ≥300 mg of protein every 24 hours. If 24-hour urine samples were not available, proteinuria was defined as a protein concentration of 300 mg/l or more (≥1 + on dipstick) in at least two random urine samples

**Table 1** Hardy-Weinberg equilibrium for HLA-G genotype between control and pre-eclamptic women

| HLA-G genotype (3′UTR polymorphism) | Healthy pregnant women ($n = 162$) [(freq)] | $\chi^2$ | Pre-eclamptic pregnant women M ($n = 157$) [(freq)] | $\chi^2$ |
|---|---|---|---|---|
| −14 bp/+14 bp | | | | |
| Observed count | 79.0 (0.491) | 0.01 | 81.0 (0.519) | 0.47 |
| Expected count | 82.3 | | 79.7 | |
| +14 bp/+14 bp | | | | |
| Observed count | 28.0 (0.172) | 0.001 | 23.0 (0.146) | 0.32 |
| Expected count | 25.9 | | 25.1 | |
| −14 bp/−14 bp | | | | |
| Observed count | 55.0 (0.337) | 0.002 | 53.0 (0.335) | 0.13 |
| Expected count | 54.8 | | 53.2 | |
| Total | | 0.013 | | 0.92 |

$\chi^2$ tabulated ($\alpha = 0.05$; df = 1) = 3.84 ($p > 0.05$).

taken at least 4–6 hours apart after the 20th week of gestation [5]. PE was classified as severe when blood pressure was ≥160/110 mm Hg; or urinary protein excretion ≥5 g per 24 hours; a platelet count of ≤100,000 $mm^{-3}$ in at least two samples; the combination of hemolysis and abnormal liver enzymes associated with persistent epigastric or upper right quadrant pain; persistent and severe symptoms such as altered mental status, headaches, blurred vision, or blindness; presence of multiorgan involvement such as pulmonary edema, oliguria (≤500 ml per day) [42]. 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 Hospitalar Conceição Group (Porto Alegre, Brazil) and by the National Research Committee of Ethics.

### Polymerase chain reaction amplification of exon 8 of the HLA-G gene

DNA was isolated from whole blood using a salting-out procedure [43]. The 14-bp polymorphism at exon 8 of HLA-G gene was detected through polymerase chain reaction (PCR) analysis: 200 ng of genomic DNA were added to a final volume of 25 μl containing 10 mol/l Tris-HCl (pH 8.8), 50 mol/l KCl, 0.08% (v/v) NonidetP40, 1.5 mol/l $MgCl_2$; 0.2 mol/l of each dNTPs; 10 pmol of each primer and 0.75 units of *Taq*-polymerase. Thermocycling conditions were as follows: 35 cycles of 94°C for 30 seconds, 64°C for 60 seconds, and 72°C for 120 seconds were preceded by an initial denaturation cycle of 94°C for 2 minutes and followed by a final extension step at 72°C for 10 minutes. The samples were genotyped by PCR using specific primers (5′GTGATGGGCTGTTTAAAGTGTCACC3′ and 5′GGAAGGAATGCAGTTCAGCATGA-3′) as described previously [26].

### Genotyping of 14-bp deletion polymorphism in exon 8 (3′-untranslated region) of HLA-G gene

The PCR products of exon 8 were analyzed by 6% polyacrylamide gel electrophoresis containing ethidium bromide and visualized under ultraviolet light. The amplicon sizes for the 14-bp polymorphism were 224 bp for the +14 bp allele and 210 bp for the allele.

### Statistical analysis

HLA-G genotypic frequencies were compared with Hardy–Weinberg expectations using $\chi^2$ tests. HLA-G allelic frequencies and HLA-G genotypes based on the 14-bp deletion polymorphism in exon 8 of control and PE women were compared using the $\chi^2$ test. The $p$ values were corrected for multiple comparisons when necessary. The significance level was set at $\alpha = 0.05$ (two-tailed). All statistical analyses were performed with SPSS 15.0 software (SPSS Inc, Chicago, IL).

## Results

### Maternal HLA-G allele frequencies and genotype distribution

The 14-bp polymorphism in HLA-G exon 8 is associated with different HLA-G mRNA isoforms that may change patterns and quantity of HLA-G mRNA expression and induce alternative splicing. In this way, maternal genotypic and allelic frequencies concerning the presence/absence of the 14-bp on exon 8 were compared among PE women and healthy controls. In all, 157 women who had experienced PE and 162 healthy control pregnant women were HLA-G genotyped. The maternal HLA-G genotype distribution in both PE and control women were in Hardy-Weinberg equilibrium for the 14-bp deletion polymorphism (Table 1). The HLA-G allele/genotype overall distribution and frequencies are shown in Table 2. No statistically significant differences were observed in allelic and genotypic

**Table 2** HLA-G allele/genotype overall distribution and frequencies in control and pre-eclamptic women

| | Healthy pregnant women ($n = 162$) [(freq)] | Pre-eclamptic pregnant women ($n = 157$) [(freq)] |
|---|---|---|
| HLA-G genotype (3′UTR polymorphism)[a] | | |
| −14 bp/+14 bp | 79 (0.491)[a] | 81 (0.519)[a] |
| +14 bp/+14 bp | 28 (0.172)[a] | 23 (0.146)[a] |
| −14 bp/−14 bp | 55 (0.337)[a] | 53 (0.335)[a] |
| HLA-G allele (3′UTR polymorphism)[b] | | |
| Minus 14 bp | 189 (0.583)[b] | 188 (0.599)[b] |
| Plus 14 bp | 135 (0.417)[b] | 126 (0.401)[b] |

[a] $\chi^2 = 0.474$; df = 2; $p = 0.789$.
[b] $\chi^2 = 0.156$; df = 1; $p = 0.693$.

**Table 3** HLA-G genotype frequencies according characteristics of development of the mild or severe pre-eclampsia

| HLA-G genotype (3′UTR polymorphism) | Healthy pregnant women ($n = 162$) [(freq)] | Mild pre-eclamptic pregnant women ($n = 57$) [(freq)] | Severe pre-eclamptic pregnant women ($n = 53$) [(freq)] |
|---|---|---|---|
| −14 bp/+14 bp | 79 (0.491)[a] | 31 (0.544)[b] | 25 (0.472)[c] |
| +14 bp/+14 bp | 28 (0.172)[a] | 7 (0.123)[b] | 10 (0.188)[c] |
| −14 bp/−14 bp | 55 (0.337)[a] | 19 (0.333)[b] | 18 (0.340)[c] |

CP = corrected p value for multiple comparisons.
[a,b] $\chi^2 = 8.74$; df = 2; $p = 0.646$/CP = 1.00.
[a,c] $\chi^2 = 0.96$; df = 2; $p = 0.953$/CP = 1.00.

frequencies between PE women and control pregnant women, however there was a tendency toward a lower frequency of homozygous individuals for the +14 bp allele (+14 bp/+14 bp) (0.146 and 0.172) when compared with the heterozygous (0.491 and 0.519) and homozygous (0.335 and 0.337) for the deletion genotype (Table 2). There were 75 women who developed gestational diabetes after the PE. However, all analyses performed excluding these subjects showed the same level of statistical significance (data not shown), indicating that the gestational diabetes did not interfere on the PE development.

## Association between specific maternal HLA-G polymorphism and different forms of pre-eclampsia

Pre-eclampsia is a multifaceted disorder that can present in either mild or severe form. We thus analyzed the patients subgrouped by the development of mild or severe PE. Patients who had previous hypertension and subsequent PE were excluded from this subgrouping ($n = 47$). No statistically significant differences were observed on genotypic frequencies between these two subgroups, as shown in Table 3 ($\chi^2$ test, healthy vs mild PE, $p = 0.646$; cp = 1.00 and healthy vs severe PE, $p = 0.953$; cp = 1.00). Patients were also characterized by clinical parameters including ethnic origin, number of abortions, number of gestations, and weight. No significant differences were observed in genotypic frequencies between the control group and PE women subgrouped accordingly the previously mentioned characteristics (Table 4). However, it is interesting to note that the primiparous PE women presented higher frequency of the −14/−14 bp genotype (n = 19; freq = 0.442) as compared with the primiparous healthy women (n = 15; freq = 0.283), although this only approached statistical significance ($p = 0.09$) (Table 4).

## Association between pre-eclampsia and dominating genotypes

To evaluate the role of maternal 14-bp deletion genotype (−14 bp/−14 bp) in PE development, we studied women who were heterozygous for the 14-bp deletion/insertion polymorphism (−14 bp/+14 bp) with those who were homozygous for the presence of the 14-bp segment (+14 bp/+14 bp), as shown in Table 5. In this analysis, no statistically significant differences were observed between the control women and the PE women with either mild or severe forms of PE (Table 5).

## Discussion

During pregnancy, the maternal immune system is in close contact with cells and tissues from the semiallogeneic fetus.

**Table 4** Genotype frequencies in both control and pre-eclamptic women classified accordingly clinical parameters

| | Control women *n* (freq) | | | Pre-eclamptic pregnant women *n* (freq) | | |
|---|---|---|---|---|---|---|
| Clinical parameters | HLA-G genotype (+14 bp/−14 bp) | HLA-G genotype (+14 bp/+14 bp) | HLA-G genotype (−14 bp/−14 bp) | HLA-G genotype (+14 bp/−14 bp) | HLA-G genotype (+14 bp/+14 bp) | HLA-G genotype (−14 bp/−14 bp) |
| Ethnic origin | | | | | | |
| Euro-derived | 63 (0.512)[a] | 22 (0.179)[a] | 38 (0.309)[a] | 55 (0.524)[a] | 15 (0.143)[a] | 35 (0.333)[a] |
| Afro-derived | 16 (0.444)[b] | 5 (0.139)[b] | 15 (0.417)[b] | 25 (0.500)[b] | 8 (0.160)[b] | 17 (0.340)[b] |
| Abortion | | | | | | |
| <2 | 75 (0.490)[c] | 26 (0.170)[c] | 52 (0.340)[c] | 75 (0.517)[c] | 23 (0.155)[c] | 50 (0.338)[c] |
| >2 | 5 (0.625)[d] | 1 (0.125)[d] | 2 (0.250)[d] | 4 (0.500)[d] | None | 4 (0.500)[d] |
| Weight | | | | | | |
| Normal | 24 (0.585)[e] | 3 (0.073)[e] | 14 (0.341)[e] | 14 (0.500)[e] | 7 (0.250)[e] | 7 (0.250)[e] |
| Obesity | 45 (0.450)[f] | 21 (0.210)[f] | 34 (0.340)[f] | 51 (0.515)[f] | 12 (0.121)[f] | 36 (0.364)[f] |
| Gestation | | | | | | |
| First | 26 (0.490)[g] | 12 (0.226)[g] | 15 (0.283)[g] | 15 (0.349)[g] | 9 (0.209)[g] | 19 (0.442)[g] |
| >1 | 61 (0.492)[h] | 20 (0.161)[h] | 43 (0.347)[h] | 65 (0.586)[h] | 14 (0.126)[h] | 32 (0.288)[h] |

**Table 5** HLA-G aggregated genotype overall distribution and frequencies in control and pre-eclamptic women

| | HLA-G genotypes (+14 bp/−14 bp) and (+14 bp/ +14 bp) | HLA-G genotypes (−14 bp/−14 bp) |
|---|---|---|
| Healthy pregnant women ($n$ = 162) [(%)] | 108 (0.663)[a] | 55 (0.337)[a] |
| Mild pre-eclamptic pregnant women ($n$ = 57) [(%)] | 38 (0.667)[b] | 19 (0.333)[b] |
| Severe pre-eclamptic pregnant women ($n$ = 53) [(%)] | 35 (0.660)[c] | 18 (0.340)[c] |
| Total pre-eclamptic pregnant women ($n$ = 157) [(%)] | 105 (0.665) | 52 (0.335) |

CP = corrected $p$ value for multiple comparisons.
[a,b] Fisher's exact test; $p$ = 0.546/CP = 1.00.
[a,c] Fisher's exact test; $p$ = 0.551/CP = 1.00.

This suggests that specific mechanisms must be operating to modulate and moderate the maternal immune system to prevent the fetus rejection, *i.e.*, promoting the acceptance of the semiallogeneic fetus. Several investigative groups have called a successful pregnancy a "Th2 phenomenon," characterized by a shifted Th2 cytokine profile. Indeed, certain complications during pregnancy, such as PE, have been associated with a Th1 response [12,44,45]. The etiology and pathogenesis of the PE involve a combination of maternal-fetal genetic and immunologic factors. One hypothesis for the pathogenesis of PE is the immunologic maladaptation theory, taking into consideration that the maternal immune system does not adapt to the semiallogeneic fetus. Considering the immunologic maladaptation hypothesis, candidate genes with immunologic functions should be sought to predispose for PE development. HLA-G genes code for important transmembrane proteins involved in the modulation of the maternal immune system during pregnancy and therefore in the maternal acceptance of the semiallogeneic fetus. It has been shown that IL-10 (an anti-inflammatory cytokine) is able to activate HLA-G expression [20,44]. In PE placentas, the HLA-G extravillous cytotrophoblast invasion is reduced, and this defect was associated with a lack of HLA-G expression [46–48]. Such defective HLA-G expression may contribute to the immune and vascular abnormalities associated with this pathology [31,46]. The strong HLA-G expression by invasive trophoblasts may, in part, explain maintenance of the semi-allogeneic fetus during pregnancy. It is thought that HLA-G inhibits the activation of maternal T cells and natural killer cells resident in deciduas favoring a Th2 type cytokine response [17,19,31]. Here, we aimed to investigate the underlying role of maternal 14-bp HLA-G gene polymorphism during successful pregnancy and PE.

It was observed that both the allelic and genotypic distributions of the 14-bp polymorphism were similarly represented between healthy and PE patients. Hviid *et al*. [26], in a study correlating the 14-bp HLA-G polymorphism and recurrent spontaneous abortions, did not observe significant differences in the frequencies of HLA-G alleles and/or genotypic distribution between control women and women with RSA. Hylenius *et al.*, in a family triads study correlating the risk of PE in women, did not find significant differences in the maternal 14-bp genotype distribution between PE and the control group. However differences regarding the maternal allele distribution suggested that the mother HLA-G genotype might confer risk for PE development in the primiparous women. Further analyses described an overrepresentation of the +14/+14 bp genotype in offspring from primiparous women with severe PE and suggested that the differences found in the distributions of the 14 bp polymorphism between PE cases and control could be related with a differential transmission of the paternal 14 bp polymorphism in exon 8 [49]. O'Brien *et al*. described low levels of HLA-G expression in placentas from primiparous pre-eclamptic women in comparison to normal placentas and observed differential distribution of HLA-G polymorphisms between normal and PE samples. They also observed an excess of the +14 bp allele in PE samples, suggesting a possible role of HLA-G in susceptibility to PE development [50]. Therefore, these previous studies have suggested the importance of differences in HLA-G mRNA alternative splicing and levels of HLA-G in complicated pregnancies, demonstrating the pivotal role of HLA-G in maintenance of a successful pregnancy.

When the samples were subgrouped accordingly to clinical parameters, no significant differences were observed in the maternal 14-bp polymorphism genotypic frequencies between control and PE women. However, it is interesting to note that the primiparous PE women showed a tendency toward higher frequencies of the 14-bp deletion allele as compared with the primiparous healthy women. This tendency can generate fewer and more unstable HLA-G molecules. The instability of the HLA-G molecule could lead to an increased pro-inflammatory profile and possible fetus rejection. In addition, PE is generally associated with the first pregnancy. Thus, in primiparous women, both the 14-bp deletion allele and a limited sperm exposure with the same partner before conception would induce an increased risk of PE [51]. Prolonged exposure to paternal sperm through sexual intercourse has been suggested to be protective again PE [52]. Women <20 years of age were at high risk for PE; thus the protective effects of a long-term sperm exposure with the same partner might explain this fact. A previous abortion (spontaneous or induced) or a healthy pregnancy with the same partner is associated with a reduced risk of PE, although this protective effect is lost by changing the partner [2,51,53,54]. Previous studies have produced conflicting results regarding the 14-bp HLA-G polymorphism and pregnancy complications. Hviid *et al*. [26,55] showed high levels of homozygous for the presence of the 14-bp sequence in women with reduced fertility and recurrent spontaneous abortion (RSA). In contrast, Tripathi *et al*. [35] demonstrated increasing number of hetererozygous for the 14-bp polymorphism in RSA women. HLA-G has previously been shown to be co-dominantly expressed [56]; however, HLA-G alleles containing the 14-bp sequence have been found to be associated with a lower HLA-G mRNA level for most isoforms in heterozygous (first trimester) trophoblast samples [24,26,33]. In this study, we did not find a dominancy of

HLA-G genotyped concerning the deletion of 14-bp in exon 8 of HLA-G gene as previously suggested.

It has been suggested that the presence of the 14-bp sequence in exon 8 of HLA-G gene is important for the maintenance of stable forms of mRNA and HLA-G molecules. In particular, our results showed a tendency of higher frequency of the maternal 14-bp deletion in primiparous PE women. The 14-bp deletion has been postulated to generate more unstable HLA-G transcripts, influencing both HLA-G isoform splicing patterns and HLA-G mRNA stability as well as changing HLA-G function leading to certain complications of pregnancy like PE [35,36]. Our data suggest that the maternal 14-bp polymorphism may be important in the outcome of pregnancy and address the possibility that the maternal HLA-G polymorphisms could influence functional parameters. However, further studies are necessary to clarify the molecular mechanisms and the specific cellular networks involved in HLA-G function.

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