UNIVERSIDADE ESTADUAL PAULISTA
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Andréia da Silva Souza

Análise do polimorfismo da região 3'não traduzida (3'NT) do gene *HLA-G* de pacientes com esclerose múltipla e neuromielite óptica da região noroeste do estado de São Paulo

Dissertação apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Mestra em Patologia.

Orientadora: Profa. Dra. Doralina Guimarães Brum Souza

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Botucatu, 22 de fevereiro de 2016

Dedicatória

Dedico este trabalho...

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ANEXO 69

Lista de Abreviaturas e Siglas

AIM Marcador informativo de ancestralidade (do inglês, *Ancestry Informative Markers*)

APC Célula apresentadora de antígeno (do inglês, *antigen presenting cell*)

AQP Aquaporina

AR Artrite reumatoide

CCR5 Receptor de quimiocinas CC tipo 5 (do inglês, *C-C chemokine receptor type 5*)

CPH Complexo principal de histocompatibilidade (MHC, do inglês, *major histocompatibility complex*)

EAE Encefalomielite experimental autoimune (EAE, do inglês, *experimental autoimmune encephalomyelitis*)

EDSS Escala de Incapacidade Funcional (do inglês, e*xpanded disability status scale*)

EM Esclerose múltipla

EMPP Esclerose múltipla primária-progressiva

EMRR Esclerose múltipla recorrente-remitente

EMSP Esclerose múltipla secundária-progressiva

HLA Antígeno leucocitário humano (do inglês, *human leukocyte antigens*)

IC Intervalo de confiança

IFN Interferon

IL Interleucina

LD Desequílibrio de ligação (*LD – Linkage disequilibrium*)

LES Lúpus eritematoso sistêmico

Mb Megabases (10^6 bases)

mL Mililitro (10^{-3} Litro)

MOG Glicoproteína da mielina do oligodendrócito (do inglês, myelin oligodendrocyte glycoprotein)

mRNA Ácido ribonucleico mensageiro ou RNA mensageiro

MTLE Mielite transversal longitudinal extensa

MTP Metilprednisolona

Ng Nanograma (10^{-9} gramas)

NK Célula assassina natural (do inglês, *natural killer*)

NMO Neuromielite óptica

NMOSD Desordens do espectro de NMO (do inglês, *neuromyelitis optica spectrum desorder*)

NO Neurite óptica

NT Não traduzida

Pb Pares de bases

PBM Proteína básica de mielina

PF Plasmaférese

PLP Proteína proteolipídica

RM Ressonância magnética

SNC Sistema nervoso central

SNP Polimorfismo de base única (do inglês, *single nucleotide polymorphism*)

TCR Receptor de célula T

Th Linfócito T auxiliar (do inglês, T *helper*)

TNF Fator de necrose tumoral

UTR Região não traduzida (do inglês, *untranslated region*)

RESUMO

A esclerose múltipla (EM) e as desordens do espectro de neuromielite óptica (NMOSD) são doenças inflamatórias autoimunes com predileção pelo sistema nervoso central, sendo que a EM ocorre mais frequentemente em populações caucasianas, enquanto a NMO é mais frequente em indivíduos não caucasianos. O gene *HLA-G* codifica uma molécula com um importante papel na tolerância imunológica. Essa molécula foi primeiramente descrita na interface materno-fetal, onde foi associada ao sucesso da gravidez. Além disso, a expressão da molécula de HLA-G tem sido relatada em condições patológicas como câncer, infecções virais e doenças autoimunes, incluindo EM. O nível de expressão de HLA-G depende principalmente de fatores que modulam sua expressão transcricional e pós-transcricionalmente, tais como a região *3'não traduzida (3'NT)* do gene através da manutenção da estabilidade do mRNA e da interação com microRNAs específicos. Alguns haplótipos de *3'NT* foram associados a maior expressão de HLA-G, o que seria benéfico em desordens autoimunes. O objetivo deste estudo foi analisar os sítios polimórficos da região *3'NT* do gene *HLA-G* de pacientes com EM e NMOSD. A região *3' NT* do gene *HLA-G* de 105 pacientes com EM, 54 pacientes NMOSD e 108 controles, foi sequenciada através do método Sanger. Foram identificados 10 sítios de variações, incluindo *14pb indel +3001C/T,+3003T/C, +3010G/C,+3027C/A, +3035C/T, +3142C/G, +3187G/A, +3196C/G, +3227G/A.* Não foi encontrada qualquer associação desses alelos à EM quando comparada a controles saudáveis ou a NMOSD. Porém, comparado a controles, o alelo *+3187A* apareceu em maior frequência no grupo NMOSD e consequentemente o alelo *+3187G* e *UTR-1*(*DelCTGCCC**G**CG*), único haplótipo que carrega esse alelo, foram sub-representados nesse grupo, particularmente no grupo de pacientes soropositivos para anticorpos anti-AQP4. Por outro lado, o grupo de pacientes NMOSD soronegativos para anti-AQP4 apresentou maior frequência de UTR-6 (*DelCTGCCC**A**CG*) quando comparado a controles saudáveis. Considerando que: i) o alelo *+3187A* está associado a menor expressão de HLA-G e ii) o alelo *+3187A* e o genótipo *+3187AA* são mais frequentes no grupo NMOSD, principalmente nos pacientes anti-AQP4 positivos, a baixa expressão de HLA-G poderia estar contribuindo para a quebra da tolerância imunológica na patogenia de NMOSD.

Palavras-chaves: *HLA-G*, *3'NT*, *UTR-1*, *+3187A*, esclerose múltipla, neuromielite óptica.

ABSTRACT

Multiple sclerosis (MS) and neuromyelitis optica spectrum disorders (NMOSD) are autoimmune inflammatory diseases with predilection for the central nervous system, and the MS occurs most frequently in Caucasian populations, while NMOSD is more frequent in non-Caucasian individuals. A key feature of MS and NMOSD is the breakdown of immunological tolerance. *Human leukocyte antigen G* (*HLA-G*) gene encodes a nonclassical HLA class I molecule that plays a pivotal role in immune tolerance, inhibiting different cell subsets involved in innate and adaptive immunity and autoimmune responses. Constitutive expression of HLA-G has been primarily observed in placenta, and ectopic expression been observed in cancer, allografts and autoimmune disorders. As a corollary, HLA-G expression is expected to be advantageous in autoimmune disorders. The regulation of the *HLA-G* gene may depend on transcriptional and posttranscriptional regulatory elements, such as *HLA-G* 3'untranslated region (UTR) by maintaining mRNA stability and interaction with specific microRNAs. The aim of this study was to verify polymorphic sites at *HLA-G* 3'UTR in MS and NMOSD patients. *HLA-G* 3'UTR of Brazilian patients with MS (*n*=105), NMO (*n*=54) and in healthy individuals (*n*=108) was typed using Sanger method. Ten variation sites were observed, including the *14bp indel +3001C/T,+3003T/C, +3010G/C,+3027C/A, +3035C/T, +3142C/G, +3187G/A, +3196C/G, +3227G/A. HLA-G* 3'UTR allele, genotype and haplotype frequencies showed no differences after comparison of MS with controls and MS with NMOSD were closely similar. Compared to controls; i) the *+3187A* allele was overrepresented and UTR-1(*DelCTGCCC**G**CG*) that contains *+3187G* was underrepresented in NMOSD patients; ii) *+3187A* and *+3187AA* were overrepresented, whereas UTR-1(*DelCTGCCC**G**CG*) was underrepresented in NMO AQP4+ patients, and iii) the UTR-6 (*DelCTGCCC**A**CG*) was overrepresented in NMO AQP4- patients. *HLA-G* 3'UTR polymorphic sites are differentially associated with neuromyelitis optica and multiple sclerosis further corroborating that NMO and MS are distinct diseases. Considering that: i) the *+3187A* allele has been associated with decreased expression of HLA-G, ii) the *+3187A* allele and *AA* genotype were overrepresented in NMOSD patients, particularly in those exhibiting AQP4 antibodies (AQP4 group), a low HLA-G expression may contribute to breakdown of immune tolerance in the pathogenesis of NMOSD.

Keywords: *HLA-G, 3'UTR, UTR-1, +3187A,* multiple sclerosis, neuromyelitis optica

Capítulo I - Revisão de Literatura

INTRODUÇÃO

As doenças desmielinizantes inflamatórias do sistema nervoso central (SNC) são as principais causas de incapacidade neurológica não traumática em adultos jovens em todo o mundo. Esclerose múltipla (EM) e neuromielite óptica (NMO) são as principais doenças inflamatórias desmielinizantes, autoimunes restritas ao SNC (1).

Esclerose múltipla

A EM é a doença desmielinizante mais comum do SNC. Estudos de prevalência mostram que EM afeta aproximadamente 2,3 milhões de jovens adultos em todo o mundo, é duas vezes mais frequente em mulheres e têm maior prevalência em populações caucasianas (2). Por afetar adultos jovens, com início entre 20 e 40 anos, e ser uma doença incapacitante, gerando déficits sensoriais, motores, autonômicos e de funções cognitivas, a EM tem uma relevante importância socioeconômica (3,4).

A prevalência difere conforme a região geográfica no globo, aumentando com o distanciamento da linha do equador em ambos os hemisférios (5). Europa e América do Norte têm as maiores prevalências (140 e 108 por 100.000, respectivamente), enquanto África Subsaariana e Ásia Oriental, as menores (2,1 e 2,2 por 100.000, respectivamente) (2). No Brasil, a prevalência de EM é igualmente variável, a menor frequência é encontrada no Nordeste brasileiro (1,36/100.000) e as maiores nas regiões Sudeste e Sul, com 15 a 18/100.000 e 27,2/100.000, respectivamente (6–9).

A doença pode apresentar diferentes formas clínicas. Aproximadamente 85% dos pacientes com EM apresentam a forma recorrente-remitente (EMRR), caracterizada por exacerbações clínicas com diminuição da capacidade funcional do paciente, seguidas por recuperação total ou parcial. Em torno de 50% dos pacientes com EMRR converte em secundária-progressiva (EMSP) após 10 anos de evolução, que é caracterizada por uma história de piora gradual com ou sem exacerbações agudas. O terceiro tipo, primária-progressiva (EMPP) é caracterizado por progressão contínua da incapacidade do paciente desde o início da doença com acúmulo progressivo de déficits neurológicos (10–12).

A incapacidade funcional resultante dos diversos surtos (piora neurológica) ou atividade inflamatória silenciosa é acumulada ao longo dos anos de evolução e mensurada pela Escala Expandida do Estado de Incapacidade de Kurtzke *(EDSS, do inglês, Expanded Disability Scale Score*). Essa varia de 0 a 10, sendo zero caracterizado pela ausência de sinais

e sintomas neurológicos; de 1,0 a 2,5, por incapacidade leve; de 3,0 a 4,5, por incapacidade moderada; ≥ 5,0 por incapacidade grave, particularmente pelo déficit de marcha; e 10, óbito pela doença (13).

O diagnóstico é essencialmente por exclusão de diagnóticos altenativos, sendo atualmente utilizados os critérios de McDonald e revisões, que estabelecem o diagnóstico com base na demonstração objetiva de disseminação de lesões no espaço e no tempo por motivos clínicos, isoladamente, ou por cuidadosa e padronizada integração de achados clínicos, de ressonância magnética (RM) e laboratorial (análise do líquor) (14–16).

A etiologia da doença não é conhecida. Porém, há evidências que fatores ambientais e genéticos podem contribuir para o seu desenvolvimento. Estudos familiares sistematizados evidenciam risco diferencial para consaguíneos não gêmeos (1,9%), gemêos dizigóticos (1,7-2,3%) e monozigóticos (15-25%) que enfatiza o papel genético na doença (17,18). Aproximadamente 100 genes já apresentaram associação com a EM, muitos deles relacionados à resposta imune, tais como, apresentação de antígenos, transdução de sinais, citocinas (IL-10 e IFN-γ) e receptores (recetor α de IL-4 e receptor β de IL-2), receptores de quimiocinas (CCR5), vitamina D e estrógenos (3,19). Porém, genes do complexo principal de histocompatibilidade (CPH ou MHC, do inglês *major histocompatility complex*), localizados no cromossomo 6, principalmente os antígenos leucocitários humanos (HLA, do inglês *human leucocytes antigens*), têm sido os mais robustamente associados a EM há mais de 40 anos. Os alelos classe II, *HLA-DRB1*15:01*, *HLA-DRB1**13:03, *HLA-DRB1**03:01, *HLA-DRB1**08:01 e *HLA-DQB1**03:02 têm sido associado à susceptibilidade, enquanto alguns alelos de *HLA* de classe I relacionados à proteção, entre eles, *HLA-A**02:01, *HLA-B**44:02, *HLA-B**38:01 e *HLA-B**55:01 (20).

A neuropatologia das lesões na EM caracteriza-se por inflamação, desmielinização, dano axonal e gliose (21). O principal mecanismo é a imunomediação celular por linfócitos T CD4+, mas linfócitos T CD8+, células NK (do inglês, *Natural Killler*), macrófagos, linfócitos B, anticorpos e moléculas do sistema complemento também contribuem para a patogenia da EM (3,12,22). A encefalomielite experimental autoimune (EAE, do inglês, *experimental autoimmune encephomyelitis*), modelo animal da EM, pode ser induzida em camundongos, ratos e cobaias pela inoculação de componentes da bainha de mielina do SNC, tais como proteína básica da mielina (PBM), proteína proteolipídica (PLP) e glicoproteína da mielina do oligodendrócito (MOG) (23) e se desenvolve em uma a duas semanas após a inoculação do antígeno (23). Ativação de células TCD4+ auto-reativas, expansão clonal, diferenciação em

células *Th*1 (linfócitos T auxiliares 1 – do inglês, *T helper*) e transmigração através da barreira hemotoencefálica são etapas cruciais para o mecanismo inicial da EAE (23).

Apesar da predominância do perfil de resposta celular (Th1 e Th17) apontado pela EAE (24–26), achados de biópsia e necropsia de pacientes com EM evidenciam heterogeneidade nos mecanismos da patogenia da doença, com quatro subtipos histopatológicos - I, II, III e IV (22). O mecanismo mediado por célula T, subtipo histopatológico I, é o predominante na EM. Porém, a deposição de imunocomplexos nas placas de lesões ativas, particularmente do padrão histológico tipo II, demonstra que a participação das células B é relevante (22). O papel das oligoclonais IgG no líquor de mais de 95% dos pacientes caucasianos não foi especificado (27) e ainda necessita de esclarecimentos. Adicionalmente, pessoas saudáveis apresentam anticorpo anti-PBM (28,29). Portanto, não há um biomarcador para a EM (12) e o modelo patogênico considerando T CD4+, isoladamente, não é suficiente para explicar a patogênia da doença (30).

Posto que a etiologia da EM é desconhecida e há evidências que o mecanismo patogênico é inflamatório, o tratamento baseia-se na redução da atividade inflamatória durante os surtos, com o uso dos corticóides, e na prevenção de novos surtos com o uso de imunomoduladores (31–33).

Neuromielite óptica

A NMO ou doença de Devic é uma doença inflamatória desmielinizante do SNC com uma particular predileção pelo nervo óptico e medula espinhal (34). Por mais de um século, essa doença foi considerada uma variante da EM devido as suas características clínicas em comum, como a manifestação de neurite óptica e mielite (35,36). Em 2004, a descoberta do anticorpo anti-AQP4 (NMO-IgG), que evidenciou 91% de especificidade para a NMO, foi um marco na diferenciação dessas duas doenças (37). Portanto, o anticorpo anti-AQP4 é o primeiro biomarcador nas doenças desmielinizantes.

Estudos de prevalência de NMO ainda são escassos, mas alguns estudos populacionais mostraram prevalência de 0,52 a 4,4 por 100.000 habitantes (38–41). Apesar da baixa prevalência, casos já foram relatados em diversas populações em todos os continentes (34,42–44). A NMO representa 15% a 48% das desordens inflamatórias desmielinizantes do SNC em populações não caucasianas, como asiáticas (20% a 48%) e brasileiros (15%), diferentemente da EM que tem sido considerada mais comum em populações caucasianas (45–50). A idade de início varia desde a infância até a idade adulta, incluindo idosos, e a idade média de início

da doença é superior à da EM (51–54). Adicionalmente, a NMO é 3 a 11 vezes mais frequente em mulheres (55).

A NMO pode ser monofásica ou apresentar curso recidivante. Inicialmente, os critérios diagnósticos da NMO consideravam aspectos clínicos e de RM (34). Os critérios diagnósticos revisados após a descoberta do anticorpo anti-AQP4 exigem: a ocorrência de mielite aguda, neurite óptica (NO) e duas das três características seguintes: i) RM do encéfalo não típica da esclerose múltipla; ii) RM da medula espinhal com mielite transversa longitudinal extensa (MTLE) correspondente a, pelo menos, três corpos vertebrais e/ou iii) NMO-IgG (anti-AQP4) reagente (34,56).

Episódios de MTLE com anti-AQP4+ são considerada síndromes de alto risco para conversão a NMO em um ano (57). A importância desse conceito é permitir o tratamento precoce desde o primeiro episódio. Assim, formas clínicas limitadas de NMO, como NO bilateral ou MTLE (eventos únicos ou recorrentes), com soropositividade para anti-AQP4 foram definidas como transtornos do espectro de NMO (NMOSD - NMO *spectrum disorder*) (46). No entanto, devido ao envolvimento mais restrito ou mais extenso do SNC e a um subgrupo de pacientes soronegativos para anti-AQP4, recentemente, uma revisão desses critérios estabeleceu o termo unificador, NMOSD estratificado pelo teste sorológico (NMOSD com ou sem AQP4-IgG) (57).

Pelo menos um terço dos pacientes soronegativos para anti-AQP4 podem apresentar positividade para anticorpos anti-MOG e estes parecem ter características clínicas mais favoráveis do que aqueles com anticorpos anti-AQP4 ou negativos para ambos os anticorpos (58).

A etiologia de NMO não é conhecida. Porém, a descoberta do anticorpo contra a molécula aquaporina 4 (AQP4), canal de água presente nos prolongamentos dos astrócitos (37,59), e a deposição periarteriolar de imunocomplexos nos espaços de Virchow-Robin são pilares da patogenia da doença (60) e respaldam o mecanismo autoimune humoral. Alguns estudos têm sugerido que células T específicas para AQP4 são necessárias para a produção de anti-AQP4 por linfócitos B no compartimento imunológico periférico, bem como para o desenvolvimento de lesões no SNC (61–63). O perfil de resposta T*h*2 é sugerido como o predominante (64,65), porém há ainda aumento de células T*h*1 e T*h*17 no sistema periférico de pacientes com NMO (66,67). Modelos animais da NMO não são ainda bem sucedidos (68).

A coexistência de outras doenças autoimunes – lúpus eritematoso sistêmico (LES) e síndrome de Sjoegren, em 20% a 30% dos pacientes com NMO ou apenas dos marcadores laboratoriais dessas doenças (40%), sugerem que os indivíduos acometidos podem ter uma

maior predisposição genética a autoimunidade (69,70). A associação da NMO com genes *HLA* (44,71–73) corroboram esse conceito. Não há estudos familiares metodologicamente adequados na NMO. Porém, análise de 12 famílias com mais de um membro acometido sugere que o fator genético pode ter um papel importante na doença (74).

Ainda não foram realizados estudos clínicos randomizados, controlados, duplo-cegos no tratamento de surto ou prevenção de novos surtos na NMO. Os poucos estudos são restritos à análise de série de casos ou coortes, retrospectivos ou observacionais utilizando metilprednisolona (MTP) e plasmaférese (PF), em surtos não responsivos à MTP. Terapia imunossupressora é prescrita para a prevenção de novos surtos da doença (75,76)**.**

Sumariando, a EM e NMO podem ser consideradas doenças distintas em termos de patogenia, epidemiologia, fatores de risco genéticos e ambientais, manifestações clínicas, laboratoriais, prognóstico e tratamento (77). Porém, ambas apresentam em comum a quebra da tolerância imunológica e associação com genes do CPH.

CPH e Tolerância imunológica

O CPH, localizado no braço curto do cromossomo seis (6p21.3), compreende uma região de aproximadamente 3,6 Mb e é dividido didaticamente em três regiões: as teloméricas classe I e III, e a centromérica classe II, sendo alguns dos genes de classe I e classe II os responsáveis por codificar as moléculas transmembrânicas que realizam a apresentação antigênica, moléculas HLA (78,79).

As moléculas HLA de classe I são expressas pela maior parte das células nucleadas, embora com níveis de expressão variáveis de acordo com o tecido, e atuam na apresentação de antígenos intracelulares às células T citotóxicas ou CD8 (79,80). Por sua vez, as moléculas de HLA de classe II são expressas em subgrupos de células imunológicas e apresentam peptídeos processados de proteínas exógenas pela interação com os receptores de células T auxiliares ou CD4 (79).

A interação entre moléculas de HLA associadas a peptídeos e os receptores dos linfócitos T (TCRs) é estabilizada por uma série de moléculas na superfície do linfócito, que sustenta a interação e realiza a transdução de sinal, permitindo ativação e expansão clonal do linfócito e sua ação contra antígenos, geralmente não próprios (81,82).

Além da sua importante contribuição para a resposta imunológica, as moléculas de HLA estão diretamente envolvidas na seleção do repertório de células T no timo, cuja falha é um dos principais fatores contribuintes para o desenvolvimento de doenças autoimunes (83). A seleção tímica é realizada de duas formas: na seleção positiva, cujo objetivo é gerar um

repertório de células T capazes de interagir com moléculas de HLA do indivíduo, são selecionados apenas linfócitos cujos TCRs ligam-se, fracamente, a moléculas de HLA; posteriormente, na seleção negativa, onde há presença de moléculas coestimulatórias, a forte afinidade entre HLA/peptídeo-próprio e TCR gera sinalização a apoptose do linfócito, dessa forma são eliminadas as células com capacidade de iniciar uma resposta autorreativa (79,83,84). Alguns linfócitos autorreativos que conseguem entrar em tecidos periféricos precisam ser inativados, suprimidos ou eliminados pela tolerância periférica para não desencadear autoimunidade (83,85).

Dado a importância das moléculas de HLA para a resposta e tolerância imunológica, diversos trabalhos tem mostrado a contribuição de múltiplas variantes destes *loci* em doenças autoimunes, tais como espondilite anquilosante, lúpus eritematoso sistêmico (LES), artrite reumatoide (AR), diabetes tipo 1 (DT1), colite ulcerativa, doença de Crohn e EM (78,86).

Na EM, alguns alelos de *HLA* classe II foram associados a risco, particularmente *HLA-DRB1*15* (20,87–89), destacando-se a maior frequência do alelo *HLA-DRB1*1501* em pacientes brancos e do alelo *HLA-DRB1*1503* em pacientes afrodescendentes (87,90). Enquanto isso, vários estudos têm associado o alelo HLA-*DRB1*03* à NMO (44,72,73).

Outra forma de olhar o cenário de tolerância imunológica tem sido o estudo de genes do MHC de classe I conhecidos como não clássicos ou Ib (*HLA-G*, *-E* e *-F*) que embora semelhantes estruturalmente aos clássicos (*HLA-A, -B* e *-C*) são menos polimórficos que estes, e estão envolvidos no processo modulação da resposta imunológica (91). As moléculas codificadas pelos genes HLA não clássicos são capazes de desempenhar funções tolerogênicas através de ligações a receptores inibitórios específicos em monócitos, células T, B e NK (92–94).

HLA-G

O gene *HLA-G* codifica a molécula de HLA classe Ib cuja as características, funções biológicas e distribuição tecidual estão melhores esclarecidas. Uma das características biológicas da molécula é que existem isoformas ligadas à superfície celular (HLA-G1, -G2, -G3 3 -G4) e solúveis (sHLA-G5, -G6 e -G7), geradas por edição alternativa (*splicing)* do transcrito primário do RNA mensageiro (mRNA). Adicionalmente, a isoforma HLA-G1 pode gerar uma forma solúvel através da clivagem por matriz metaloproteinase-2 (MMP-2), produzindo sHLA-G1 (92,95). As moléculas sHLA-G1 e sHLA-G5 possuem estruturas similares.

Ambas as isoformas, sHLA-G ou de membrana exercem funções imunomodulatórias: inibem a atividade citotóxica de células T CD8+ e NK induzindo-as a apoptose (96–98); inibem a proliferação de linfócitos T CD4+ ou os direcionam ao perfil imunossupressor (99,100); inibem células apresentadoras de antígenos (APCs) e ativação de linfócitos B (101,102); induzem células T regulatórias (103) e células dendríticas tolerogênicas (104,105); liberação de interleucina-10 (IL-10) e polarização do perfil de citocinas na resposta imune (106,107).

Apesar do potencial da molécula de HLA-G de induzir a resposta Th2, Kapasi e colaboradores (107) encontraram respostas bifásicas sobre a produção de citocinas TNF-α, IFN-γ (Th1) e IL-10 (Th2) por linfócitos T citotóxicos quando submetidos a variadas concentrações de HLA-G. Em concentrações de HLA-G de ≤50 ng / mL, a secreção de TNF-α e IFN-γ aumentou e a de IL-10 foi reduzida, porém, a uma concentração de HLA-G ≥100 ng / mL, os níveis dessas citocinas foram invertidos. Isso sugere que o nível de expressão de sHLA-G pode direcionar a resposta imunológica por efeito concentração dependente.

As funções da molécula de HLA-G no processo de imunomodulação são realizadas pela interação com receptores específicos presentes nas células do sistema imunológico, entre eles: ILT-2(CD85j/LILRB1), expresso em células NK, T, B e mielomonocíticas (102); ILT4 (CD85d/LILR2), expresso em monócitos, células dendríticas e neutrófilos (108–110); e KIR2DL4 (CD158d), presente em células NK (111). Interessantemente, a expressão desses receptores é aumentada na superfície de células expostas a HLA-G de membrana e solúvel, tornando estas células mais sensíveis à inibição e promovendo a tolerância imunológica (112,113).

A expressão de HLA-G foi primeiramente identificada na interface materno-fetal, em células do citotrofoblasto extraviloso, onde foi associada à proteção do feto semialogênico da citólise por células NK maternas (114–116). Essa molécula também parece ser primordial para a implantação do embrião, pois a interação da molécula com KIR2DL4 em células NK induz a secreção de citocinas e fatores angiogênicos fundamentais para o remodelamento das artérias espiraladas uterinas (117,118).

À parte do contexto gestacional, o *HLA-G* é transcrito em níveis basais na maioria das células e tecidos (115), no entanto a expressão proteica é restrita a alguns tipos celulares de tecidos do timo (119), da córnea (120), da matriz ungueal (121), do pâncreas (122) e precursores eritroides e endoteliais (123). Adicionalmente, a expressão de HLA-G pode ser induzida em condições não fisiológicas como após transplantes alogênicos (99,124,125), como mecanismo de escape à vigilância imunológica por células tumorais (126–128), como

escape de células infectadas em infecções virais (129,130) e em doenças autoimunes (131–133). A expressão de HLA-G nessas condições sugere que fatores do microambiente podem controlar a expressão da molécula (115).

Menor expressão de sHLA-G no soro foi relatada em doenças autoimunes como AR (134) e artrite juvenil idiopática (135), e apesar dos resultantes conflitantes encontrados em LES (136,137), a menor expressão de sHLA-G têm sido associada à quebra da tolerância imunológica nessas doenças. Por outro lado, a expressão em tecidos lesionados por desordens autoimunes tais como: pele, na psoríase (138), no pênfigo vulgar (139) e na esclerose sistêmica (140); e no parênquima cerebral, na EM (141); sugere implicação da molécula no controle da resposta imunológica, uma vez que esses tecidos não expressam moléculas de HLA-G em condições fisiológicas (115,141,142).

Na EM, foram detectados níveis significativamente diminuídos de RNAm do HLA-G e de sHLA-G no período pós-parto das pacientes quando comparados ao pós-parto de mulheres saudáveis (143). A menor expressão foi detectada principalmente nas pacientes com ativação da doença, apontando para uma possível relação entre baixos níveis da molécula nesse período e recidiva pós-parto (143). Por outro lado, a maior expressão de HLA-G no líquor de pacientes tem sido correlacionada à resolução da inflamação no SNC, evidenciada por imagem de RM de lesões inativas (144–147). Esses achados são sugestivos de que os níveis de HLA-G podem estar relacionados à atividade da doença e contribuir ou para recidiva ou para remissão da doença.

Em um panorama geral, a expressão da molécula HLA-G é vista como benéfica no contexto de transplantes, gravidez e desordens autoimunes, porém prejudicial quando a reposta imune deve ser mantida, como em infecções e câncer (92), por isso o conhecimento das regiões que controlam sua expressão é fundamental.

As regiões regulatórias do gene *HLA-G*, promotora e 3'não traduzida (3'NT ou 3'UTR – do inglês, 3'*untranslated region*), são responsáveis pelo controle dos níveis de expressão e, diferente da região codificadora do gene, são altamente polimórficas (92,148). A região promotora é responsável pelo controle transcricional do *HLA-G*, através da interação com fatores de transcrição para o início da síntese de mRNA, enquanto a região 3'NT do gene atua no controle pós-transcricional (92,148–150).

Região *3'NT* do gene *HLA-G*

A região 3'*NT* do gene *HLA-G*, localizada principalmente no éxon 8, é uma das responsáveis pelo controle dos níveis de expressão da molécula de *HLA-G* e sua variabilidade

influencia, principalmente, a estabilidade da molécula de mRNA e ligação a microRNAs (92,148–150). Os principais polimorfismos e haplótipos da região *3'NT* do *HLA-G* e suas frequências na população mundial estão representados na Figura 1.

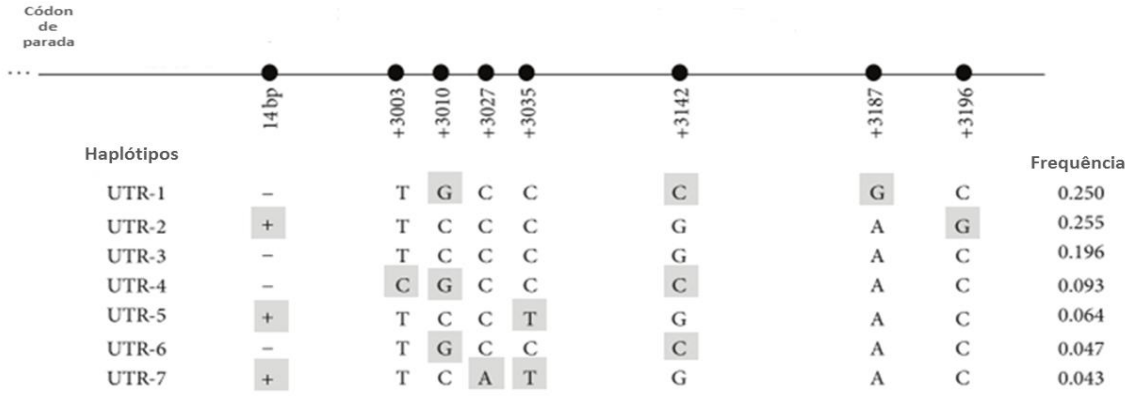

Figura 1 - Estrutura da região 3'não traduzida do gene *HLA-G* com seus principais polimorfismos e frequência dos haplótipos na população mundial.
Fonte: Adaptado de Castelli et al., 2014.

Pelo menos três dessas variações da região *3'NT* podem influenciar o nível de expressão de HLA-G: uma variação do tipo inserção/deleção (indel) de 14 pares de bases e duas variações de nucleotídeos únicos (SNP - do inglês *Single Nucleotide Polymorphism*) nas posições *+3142* e *+3187*, definidas considerando como nucleótideo +1 a adenina (A) do primeiro ATG traduzido (92,150).

Esses polimorfismos podem afetar a expressão da molécula por mecanismos diferentes. A presença dos 14pb no transcrito primário está relacionado à edição alternativa do RNA resultando em maior estabilidade do mRNA (151). Apesar disso, a inserção desses nucleotídeos foi associada a menor expressão de mRNA em amostras de trofoblastos e a abortos recorrentes em mulheres dimamarquesas (152,153). A presença de uma guanina na posição +3142 aumenta a afinidade com microRNAs específicos, tais como o *miR-148a*, *miR-148b* e *miR-152*, que leva ao aumento da degradação do mRNA e diminuição da produção de HLA-G (154). Por último, uma adenina na posição +3187 diminui a estabilidade do mRNA, pela sua proximidade a uma sequência rica em adenina-uracila (AU) que influencia a degradação do mRNA e está associada à pré-eclampsia (155).

Apesar de não estar bem esclarecida a influência das outras variações sobre a estabilidade do mRNA, análises *in silico* têm mostrado que diversos miRNAs humanos têm potencial para ligar ao mRNA do *HLA-G* e influenciar sua expressão, inclusive nas posições *+3003*, *+3010*, *+3027* e *+3035* (92,156,157).

Outro aspecto importante é que esses polimorfismos geralmente estão associados entre si. A inserção dos *14pb*, por exemplo, está associada, na maioria dos haplótipos de *3'NT*, aos alelos *+3142G* e *+3187A* (150,158,159), o que poderia justificar a menor produção de HLA-G relacionada aos *14pb* (92). Estas associações são decorrentes de um forte padrão de desequilíbrio de ligação (*LD*, do inglês *Linkage Disequilibrium*) encontrado entre quase todas as variantes da região *3'NT* (150,158).

Há evidências da correlação entre a variabilidade da 3`NT e o fenótipo produtor de sHLA-G (160–163). A influência conjunta desses SNPs em um haplótipo foi estudada por Martelli-Palomino e colaboradores (163) e seus resultados mostraram a produção diferencial de sHLA-G por cada *UTR*. A *UTR-1* está associada aos mais altos níveis de produção de sHLA-G, enquanto as *UTR-5* e *-7* aos níveis mais baixos. As *UTRs* (*-2, -3, -4* e *-6*) foram associadas a níveis intermediários (163). Dado a importância dos níveis de expressão de HLA-G para a modulação da resposta imunológica, esses polimorfismos e haplótipos podem estar associados ao desenvolvimento de diversas patologias. No entanto, não se pode descartar a influência de outros segmentos do gene *HLA-G* na regulação dos níveis de expressão e função da molécula, tais como a região promotora e codificadora, respectivamente. Vários trabalhos têm mostrado a variabilidade desses segmentos e um forte padrão de *LD* no gene *HLA-G* inteiro, fazendo que algumas *UTRs* estejam associadas a determinados haplótipos de promotora e codificadora com maior frequência (149,150,164). Assim, a associação de haplótipos de *3'NT* do *HLA-G* com algumas patologias pode ser causal ou um efeito do forte *LD* entre a região *3'NT* e os outros segmentos do gene.

Estudos de variabilidade da região *3'NT* do gene *HLA-G* têm associado os polimorfismos dessa região, individual ou conjuntamente, à uma série de contextos patológicos, tais como doenças autoimunes (158,165–170), neoplasias (171–173), aceite/rejeição de transplantes (174,175) e infecções (176–179).

Na EM, apenas três trabalhos exploraram variações em regiões regulatórias do gene *HLA-G*. O primeiro, realizado por Kroner e colaboradores (180), analisou três polimorfismos em regiões distintas do gene, na população alemã: o polimorfismo *-725C/G*, na região promotora; *G*0105 null*, éxon 3; e *14pb* na *3'NT* do *HLA-G*, e nenhuma das variações apresentou relação com a suscetibilidade a doença ou qualquer associação com idade de início, gravidade e progressão (*n*=698). O segundo analisou *-725C/G/T* (metodologia incluindo o alelo T) e *-716G/T*, na região promotora; e *14pb* na *3'NT*, em 227 pacientes poloneses com EM, onde Wiśniewski e colaboradores (181) encontrou maior frequência de *725G* em pacientes com EM e relação entre os *14pb Ins* e menor idade de início da doença.

Por último, Rizzo e colaboradores (182) analisaram apenas a produção de sHLA-G, em pacientes com EM (*n*=69), relacionada as variações dos *14pb indel* e *+3142 C/G*.

Na NMO, não há qualquer estudo que referencie o papel do *HLA-G* ou que tenha analisado variações do gene associadas à suscetibilidade ou proteção a doença.

É importante mencionar que muitos desses estudos de associação à doença foram realizados em populações diferentes, com tamanhos amostrais variáveis, assim como o número de variações analisadas. Estes fatores podem estar relacionados a diferentes resultados em estudos da mesma patologia.

Estudos que analisaram a diversidade genética da região *3'NT* do gene *HLA-G* em populações mundiais mostraram diferentes frequências alélicas e haplotípicas entre as populações estudadas, principalmente europeia e africana (159,164). De maneira similar, Lucena-silva e colaboradores (183) encontraram diferenças na frequência de alelos, genótipos e na diversidade de haplótipos entre as populações do Nordeste e Sudeste do Brasil.

O Brasil é um país com alta diversidade genética e sua composição populacional possui influência europeia, africana e ameríndia. Adicionalmente, informações sobre autoclassificação pela cor da pele não correlacionam com a contribuição genética ancestral individual (184). Portanto, o uso de marcadores informativos de ancestralidade (AIM) é relevante para evitar associações espúrias ou conflitantes em estudos genéticos em populações miscigenadas, como a brasileira.

Ao considerar que: a EM e a NMO são doenças com riscos étnicos e genéticos diferentes (46); o Brasil possui população estruturada com contribuição europeia, africana e ameríndia (185); a frequência dos haplótipos da região *3`NT* do *HLA-G* difere entre europeus e africanos (159,164), a distribuição e a diversidade dos haplótipos da região *3`NT* do *HLA-G* é diferente entre grupos populacionais brasileiros (158,183), o uso de AIMs é imprescindível para este estudo e em estudos multicêntricos futuros, particularmente com o gene *HLA-G*.

Uma investigação prévia sobre a contribuição ancestral europeia, africana e ameríndia em pacientes brasileiros com NMO e EM, avaliada por um conjunto de 12 AIM, mostrou que apesar da contribuição étnico-genética conhecida nessas doenças, a contribuição ancestral europeia foi predominante em todos os grupos analisados – NMO, MS e controles (186) e as medianas do índice de ancestralidade africana foram similares entre os três grupos (186). Assim, devido à homogeneidade quanto à contribuição ancestral nessas populações, revelada no estudo anterior, os mesmos grupos de pacientes e controles analisados para AIMs foram considerados adequados para a análise proposta por esse estudo.

Além disso, posto que: i) a expressão de HLA-G está desbalanceada na EM e outras doenças autoimunes e ii) estudos da região *3'NT* do *HLA-G* em pacientes com EM são escassos e ausentes na NMO; os estudos sobre o gene *HLA-G* na NMO e EM tornam-se relevantes para a melhor compreensão do aspecto imunogenético dessas doenças. O objetivo do estudo apresentado no II capítulo desse manuscrito foi verificar os sítios polimórficos da região *3`NT* do *HLA-G* em pacientes brasileiros com EM e NMOSD combinada com o uso de AIMs.

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Objetivos

1. Objetivo Geral

Analisar os polimorfismos da região *3'NT* do gene *HLA-G* de pacientes com esclerose múltipla e neuromielite óptica.

2. Objetivos específicos

1. Descrever a frequência das variações encontradas na região *3'NT* do gene *HLA-G* em pacientes com EM, NMO e controles saudáveis;
2. Avaliar as frequências alélicas, genotípicas de cada ponto;
3. Determinar os haplótipos de *3'NT* encontrados nas amostras populacionais estudadas;
4. Comparar a frequência dos alelos, genótipos e haplótipos da região *3'NT* do gene *HLA-G* entre pacientes com NMO e controles, EM e controles, e entre EM e NMO;
5. Utilizar a frequência dos marcadores informativos de ancestralidade para estimar a ancestralidade individual e dos grupos estudados.

Capítulo II - Artigo

Artigo escrito segundo as recomendações para a publicação no Jornal *Multiple Sclerosis* – Factor Impact: 4.82 (http://msj.sagepub.com)

TITLE: HLA-G 3' Untranslated Region polymorphic sites are associated with neuromyelitis optica but not with multiple sclerosis

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ABSTRACT

Background A key feature of multiple sclerosis (MS) and neuromyelitis optica spectrum disorders (NMOSD) is the breakdown of immunological tolerance. HLA-G molecule plays a pivotal role in immune tolerance, inhibiting different cell subsets involved in innate and adaptive immunity and autoimmune responses. Constitutive expression of HLA-G has been primarily observed in placenta, and ectopic expression been observed in cancer, allografts and autoimmune disorders. As a corollary, HLA-G expression is expected to be beneficial in autoimmune disorders. The regulation of the *HLA-G* gene may depend on transcriptional and posttranscriptional regulatory elements. **Objective:** The aim was to verify polymorphic sites at *HLA-G* 3'UTR in MS and NMOSD patients. **Methods**: *HLA-G* 3'UTR was typed using sequencing analyses in Brazilian patients with MS (*n* = 105), NMO (*n* = 54) and in healthy individuals (*n* = 108). **Results**: Ten variation sites were observed, including the *14pb indel +3001C/T,+3003T/C, +3010G/C,+3027C/A, +3035C/T, +3142C/G, +3187G/A, +3196C/G, +3227G/A*. *HLA-G* 3'UTR allele, genotype and haplotype frequencies showed no differences after comparison of MS with controls and MS with NMOSD were closely similar. Compared to controls; i) the *+3187A* allele was overrepresented and the *UTR-1* (*DelCTGCCC**G**CG*) that contains *+3187G* was underrepresented in NMOSD patients; ii) *+3187A* and *+3187AA* were overrepresented, whereas *UTR-1* (*DelCTGCCC**G**CG*) was underrepresented in NMOSD AQP4+ patients, and iii) the *UTR-6* (*DelCTGCCC**A**CG*) was overrepresented in NMOSD AQP4- patients. Compared to AQP4+ patients, the *+3003TT* genotype was underrepresented, whereas the *+3003TC* genotype was overrepresented in AQP4- patients.

Conclusion: Polymorphic sites at the 3' untranslated region of the immunoregulatory *HLA-G* gene are differentially associated with neuromyelitis optica and multiple sclerosis further corroborating that NMO and MS are distinct diseases. Considering that: i) the *+3187A* allele has been associated with decreased expression of HLA-G, ii) the *+3187A* allele and *AA* genotype were overrepresented in NMOSD patients, particularly in those exhibiting AQP4 antibodies (AQP4 group), it may contribute to breakdown of immune tolerance in the pathogenesis of NMOSD.

Keywords: *HLA-G*, *3'UTR*, *UTR-1*, *+3187*, multiple sclerosis, neuromyelitis optica, Brazilians

INTRODUCTION

Neuromyelitis optica (NMO) and multiple sclerosis (MS) are inflammatory demyelinating autoimmune diseases of the central nervous system (CNS), presenting overlapping clinical features, which have placed NMO as a worse prognostic variant of MS (1). A serum antibody that reacts with aquaporin-4 (NMO-IgG), specific for NMO was identified, thereby contributing to MS differential diagnosis (2). The major impact of the differential diagnosis was on treatment because the drugs used in MS do not have efficacy on NMO (3). Furthermore, the discovery of NMO biomarker corroborate the humoral mechanism of the disease (4), enabling expansion of clinical phenotypes, discriminating seropositive anti-AQP4 (AQP4ab+) and seronegative anti-AQP4 (AQP4ab-) groups, referred to as neuromyelitis optica disorders spectrum (NMOSD) (5). NMO exhibits a higher frequency in non-Caucasian individuals - Asians, Hispanic and Afrodescendents, whereas MS is relatively more frequent in Caucasians population (6).

Although the etiology of NMO and MS is unknown, genetic factors have been associated with both diseases (7,8). Almost one hundred genes have been associated with MS, however, the Human Leukocyte Antigens (HLA) have been the most robustly associated with MS (9). The *HLA-DRB1*15* allele group has been associated with MS, mainly *HLA-DRB1*15*01 allele in Caucasian and Afrodescendents populations (10–12) and *HLA-DRB1*1503* allele in Afrodescendents with MS (11). On the other hand, the *HLA DRB1*03* allele group was overrepresented in NMO patients compared with healthy controls (13,14). The pathogenesis of MS is predominantly mediated by lymphocytes (15), whereas NMO pathogenesis is mediated by antibody mechanisms (4). Despite of the distinct epidemiologic, clinical (6), genetic (13), laboratory (2) and pathogenic mechanisms (4,16), a key feature of MS and NMO is the breakdown of immunological tolerance, wherein the HLA-G molecule may influence (17).

HLA-G is a non-classical MHC class I gene that encodes a molecule presenting immunoregulatory and tolerogenic functions (17). These functions are performed by the interactions of HLA-G molecules with specific receptors present on cells of the immune system, including: ILT-2 (CD85j / LILRB1) expressed on NK, T, B cells and myelomonocytics (18); ILT4 (CD85d / LILRB2) expressed in monocytes, dendritic cells (19,20) and neutrophils (21); and KIR2DL4 (CD158d), present in NK cells (22). In physiological conditions, HLA-G expression is restricted to trophoblast cells (23,24),

medullary thymic epithelial cells (25), pancreatic islets (26), cornea (27), keratinocytes and melanocytes of the proximal nail matrix (28), and erythroid and endothelial precursors (29). However, the expression of HLA-G can be induced in non-physiological conditions, such as after allogeneic transplants (30–32), in autoimmune diseases (33–37) and as the mechanism of escape immune surveillance by tumor cells (38–40), or by infected cells in viral disorders (41,42).

The HLA-G molecule may be present as soluble (sHLA-G) forms and on cell surfaces (43). The quantity of HLA-G molecules produced primaly depend on factors that modulate gene expression by transcriptional and posttranscriptional regulatory mechanisms, through the promoter region and 3'untranslated region (3'UTR), respectively (43,44). The *HLA-G* gene has 8 exons and the 3'UTR is mainly localized in the exon 8. Studies indicated that *HLA-G* 3'UTR alleles and haplotypes correlated with sHLA-G level in the peripheral blood (45–48). Three major *HLA-G* variable sites have been described in association with HLA-G expression levels: i) the *14-bp deletion/insertion* (*rs371194629*) polymorphism, which contributes to mRNA stability (49); ii) a single-nucleotide polymorphism (SNP) C>G at the position *+3142 (rs1063320),* in which the G allele increased affinity for specific microRNAs (miRNAs), such as miR-*148a*, *miR-148b* and *miR-152* (50); and iii) the *+3187A/G SNP (rs9380142),* in which the presence of an Adenine contributes for increased *HLA-G* mRNA degradation by modifying an AU-rich motif (51). Although the influence of other *HLA-G 3'UTR* variations on the mRNA stability is unclear, *in silico* analyses have shown that several human miRNAs are potential ligands of the *HLA-G* mRNA, including the *+3003, +3010, +3027 and +3035* positions, and could influence HLA-G expression (52,53).

HLA-G 3'UTR variability studies have been performed on several autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), pemphigus vulgaris and sarcoidosis (54–60). In MS, only the *14-bp* variation at the *HLA-G* 3'UTR was analyzed and no association has been found with the disease (61,62), however, the presence of the *14-bp* was associated to younger disease onset in MS patients (62). Furthermore, the *14-bp Del* together with the *+3142C* allele were associated with increased sHLA-G levels in MS patients (45). Other studies demonstrated that the HLA-G is upregulated in CNS inflammation sites and in cerebrospinal fluid (CSF) in MS (63,64) and the higher HLA-G expression in CSF of patients has been correlated to the resolution of inflammation in the CNS as observed by magnetic resonance imaging (MRI) (65–68). In contrast, the lower expression of this molecule has been linked to postpartum relapses in MS patients (69). In

neuromyelitis there are no reports regarding the level of HLA-G expression, but diseases with similar humoral mechanisms, such as RA and SLE, present unbalanced sHLA-G levels (70–72).

Considering that: i) The distribution of *HLA-G 3'UTR* haplotype frequencies may be different among European and African populations (73,74); ii) the Brazilian population has contribution of African, European and Amerindian (75); iii) NMO and MS present differential ethnic distribution (6); iv) ancestry plays a major role on NMO and MS suscptibility (80); v) NMO and MS are autoimmune diseases (4,15); vi) *HLA-G 3'UTR* alleles and haplotypes are associated with different HLA-G expression levels; vi) there are many studies that reveal an influence of HLA-G on MS pathogenesis (63–69); a case-control association study of *HLA-G* in highly diverse Brazilian population is relevant.

OBJECTIVE: The aim of this study was to evaluate the *HLA-G* 3'UTR polymorphic sites and haplotypes in patients with MS or NMOSD taking into account ancestry revealed by ancestry informative markers (AIMs) to avoid spurious associations.

MATERIALS AND METHODS

Subjects

A total of 105 MS (67 women; 72 Relapsing–Remitting MS or RRMS and 33 Secondary-Progressive MS or SPMS) and 54 NMOSD patients (47 women), diagnosed using the revised McDonald and Wingerchuck criteria, respectively (1,76–79), were included in the study. NMOSD patients were stratified according to serological status for AQP4 antibodies, into seropositive anti-AQP4 (anti-AQP4ab+), seronegative anti-AQP4 (anti-AQP4ab-) and untested (n=26, n=20 and n=8, respectively) groups. Untested patients were included in analyses only as NMOSD. All patients were recruited at the Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo (USP), Brazil, as well as 108 healthy controls (42 female, 66 male). The study protocol was approved by the Ethics Research Committee at Faculdade de Medicina de Botucatu, Universidade Estadual Paulista, Botucatu, Brazil (#4205-2012) and each subject provided written informed consent.

The three groups were genotyped for a panel of ancestry informative markers (AIMs) with 12 highly informative marker loci required to characterize tri-hybrid populations that vary in substructure or the degree of admixture: FY-NULL, RB1, LPL, AT3, APOA, PV92, CKM, DRD2-BclI, MID-52, MID-575, MID-93 and SB19.3, in previous study (80). At least

half of the AIMs studied allowed the discrimination between African and European or Amerindian ancestry. African Ancestry Indexes (AfrAI) was estimated for each individual using the Bayesian clustering algorithm, implemented by the software STRUCTURE (81), showed that NMOSD, MS and Control groups from Ribeirão Preto (RP) were closely similar (P>0.05), according to a previous study (80). Therefore, these same groups were selected for the current analysis.

Typing of the *HLA-G 3'UTR*

Genomic DNA was extracted from peripheral blood by salting-out method (82). The 3'UTR of the *HLA-G* gene was PCR-amplified using the primers HLAG8F: 5'-TGTGAAACAGCTGCCCTGTGT-3' (83) and HLAGR1: 5'-TCTTCTGATAACACAGGA ACTTC-3'. Amplification was performed, following the method described previously (83), in a final volume of 25 µl containing 1× PCR buffer (0.2M Tris-HCl pH 8.5; 0.5M KCl), 0.2mM of each dNTP, 1.5mM of $MgCl_2$, 12.5 pmol of each primer, 1U of Platinum Taq DNA-polymerase (Invitrogen, Carlsbad, CA, USA) and 50ng of genomic DNA. The initial denaturation cycle was carried out at 94°C for 5min, followed by 30 cycles at 95°C for 45s, 57° for 45s and 72°C for 60s, and a final extension step at 72°C for 7 min.

The amplification product was evaluated by electrophoresis on 1% agarose gel stained with GelRed™ (Biotium, Hayward, CA). PCR products of 597bp (with the 14-bp deletion) and/or 611bp (with the 14-bp insertion) were directly sequenced in an ABI3100 Genetic Analyzer (Applied Biosystems®, Foster City, CA, USA) using the reverse primer HLAG1R. All polymorphic sites observed were individually annotated.

Statistical analysis

The allelic and genotypic frequencies were estimated by direct counting using the GENEPOP software version 4.1.2 (84) and the number of expected homozygotes or heterozygotes were estimated using the exact test with Levene's correction. Adherence to the Hardy-Weinberg equilibrium (HWE) expectations were tested by the Markov chain method described by Guo and Thompson (85) using the GENEPOP software version 4.1.2 (84). Linkage disequilibrium (LD) between 3'UTR polymorphic sites was evaluated by means of a likelihood ratio test of LD (86) using the Arlequin program version 3.5 (87) and the LD plot was generated by Haploview 4.2 (88).

Given the positive LD between polymorphic sites studied, but unknown gametic phase, the most likely haplotype pair for each sample was determined by two independent

computational methods, without considering any prior information: the Expectation-Maximization (EM) algorithm (89) and the PHASE method (90), implemented by the softwares Arlequin 3.5 (87) and PHASE v2 (90), respectively, as described previously (83). The results were compared. Only haplotypes inferred with probabilities between 0.98 and 1.0 and concordant according to both methods were considered. The exact test of population differentiation based on haplotype frequencies was performed by Arlequin software 3.5.

To compare the allelic, genotype and haplotype frequencies between patients and controls and between each selected group of patients, 2x2 contingency tables were constructed and two-sided Fisher's exact test with odds ratio (OR) and 95% confidence interval (95%CI) were calculated by using the GraphPad InStat 3.0 software for Windows (GraphPad Software, San Diego, CA, USA). For genotype and haplotype frequency analysis, 2x2 tables were constructed by fixing 1 genotype or haplotype and comparing it against the others.

Comparison of clinical and demographic data between patients groups was performed by the Fisher test, by GraphPad Stat 3.0 software, and Mann-Whitney or Kruskal-Wallis tests, using Sigma Stat 3.5 software (Systat Software Inc. - USA).

RESULTS

Demographical, clinical and laboratory findings of NMOSD and MS patients, and controls

The studied groups showed differences in sex distribution**.** NMOSD and MS patients exhibited higher frequency of females than the healthy controls ($P \leq 0.0001$). The comparison of median age of control group (35 years), MS (40 years) and NMO (45 years), showed significant differences between the control group and NMO ($P = 0.018$).

Regarding to skin color, the control is composed of 70% white people and 30% have dark skin, while the MS group can be stratified in 53.3% presenting white skin and 46.7% present dark skin color. In the NMOSD group, 100% have dark skin. All clinical and demographic factors were significantly different between MS and NMOSD groups. NMOSD patients presented higher frequency of females (P=0.004), older disease onset (P=0.035), higher EDSS at the first attack (P<0.001), shorter disease duration (P=0.022), and higher EDSS at the last follow up (P<0.001) when compared with MS patients (Table 1). NMOSD patients were stratified according to serological status for AQP4 antibodies, in positive and

negative anti-AQP4ab group, and both were different from MS in EDSS at the first attack and EDSS at the last follow up (P<0.001 for each comparison). Only NMOSD positive anti-AQP4ab group was significantly different from MS patients in age of disease onset and duration of disease, presenting older disease onset (P=0.011) and shorter duration of disease (P=0.025) (Table 2). Anti-AQP4ab+ NMOSD patients also presented older disease onset than anti-AQP4ab- NMOSD patients (Table 2).

HLA-G 3'UTR variability was associated with NMOSD

Ten variables sites were detected in the *HLA-G 3'UTR* of patients and healthy controls, including the *14-bp INS/DEL* (position:+2960; accession number: *rs371194629*), and the SNPs in the positions *+3001C/T*, *+3003T/C* (*rs1707*), *+3010G/C* (*rs1710*), *+3027A/C* (*rs17179101*), *+3035C/T* (*rs17179108*), *+3142C/G* (*rs1063320*), *+3187G/A* (*rs9380142*), *+3196C/G* (*rs1610696*) and*+3227G/A* (*rs1233331*), considering the Adenine of the first translated ATG as the position +1. These polymorphic sites were already described for the *HLA-G 3'UTR* (74,83).

Genotype frequencies were consistent with the Hardy-Weinberg assumptions (P>0.05) with the exception for *+3001*, for which the exact test of HWE was not performed by GENEPOP software due to the presence of a single copy of the T allele at each population sample. All variable sites presented allelic frequencies higher than 1%, except for the *+3001T* allele (Table 3).

No significant difference was found between the allelic frequencies of MS and control groups. However, the *+3187A* allele was significantly more frequent in NMOSD patients than in controls (P=0.037; OR=1.90, 95%CI: 1.06-3.43) and consequently, the *+3187G* allele less frequent for NMOSD (P=0.037; OR=0.53, 95%CI: 0.29-0.95). The *+3187AA* genotype was more frequent in patients with NMO than in controls (P=0.06; OR=2.0, 95%CI: 0.99-4.03) but, with marginal significance (Table 3).

The exact tests of LD indicated strong LD patterns among all variation sites (Figure 1), allowing the use of probabilistic models for haplotype inference. Almost all samples were inferred with maximum probability value (1.0) for both PHASE and EM algorithm. The lowest valid probability value was 0.986. Two samples of the MS group were excluded for not estimating a single pair of haplotypes with high probability (>0.90).

A total of nine *HLA-G 3'UTR* haplotypes were inferred, all of them already described and previously named as *UTR-1* to *UTR-7* (83), *UTR-17* and *UTR-18* (73,74). Most of the

UTR sequences were represented in all groups with frequencies higher than 1%, except for *UTR-17* (Table 4). The exact test of population differentiation based on haplotype frequencies revealed no significant differences among the studied groups (*P*=0.295 ± 0.025) – MS and NMOSD (*P*=0.451 ± 0.013), MS and healthy controls (*P*=0.281 ± 0.011), NMOSD and healthy controls (*P*=0.213 ± 0.008).

When compared with healthy controls, *UTR-1* (*DelCTGCCC**G**CG*) was underrepresented in NMOSD patients (*P*=0.028; OR=0.52, 95%CI: 0.24-0.94) while *UTR-6* (*DelCTGCCC**A**CG*) was overrepresented in these patients (*P*= 0.065; OR=4.18, 95%CI: 1.02-17.0), with marginal significance (Table 4). The UTR-5 was more frequent in NMOSD patients group than in MS patients group (*P*=0.088; OR=2.03, 95%CI: 0.91-4.55), also with marginal significance. Moreover, the frequency *UTR-1* (*DelCTGCCC**G**CG*) found in NMOSD group was similar to those already described for African populations (73,74).

HLA-G 3'UTR variability in anti-AQP4ab+ and anti-AQP4ab- NMOSD patients

Anti-AQP4ab+ patients showed significant differences from control group in allelic, genotype and haplotype frequencies. The *+3187A* allele and *+3187AA* genotype were overrepresented among AQP4ab+ patients (*P*=0.006; OR=3.55, 95%CI: 1.36-9.44 and *P* = 0.015; OR=3.55, 95%CI: 1.25-10.1, respectively). However the *+3187G* allele and consequently *UTR-1* (*DelCTGCCC**G**CG*), associated to this variable site, were underrepresented (*P*=0.006; OR=0.28, 95%CI: 0.11-0.74, for both comparisons) (Tables 5 and 6).

The anti-AQP4ab- patients group presented higher frequency of *UTR-6* (*DelCTGCCC**A**CG*) than healthy controls (*P*=0.003; OR=10.1, 95%CI: 2.32-44.4). The *+3003TT* genotype was underrepresented and *+3003TC* genotype overrepresented in AQP4ab+ patients group when compared to the AQP4ab- patients group (*P*=0.04; OR=0.17, 95%CI: 0.03-0.88 and *P*=0.04; OR=6.0, 95%CI: 1.13-31.8, respectively). The *+3003C* allele and consequently UTR-4 (*DelCCGCCC**A**CG*), uniquely associated with it, were more frequent in AQP4ab+ patients group than in anti-AQP4ab- group (*P*=0.06; OR=4.75, 95%CI: 0.98-23.1 and *P*=0.06; OR=4.52, 95%CI: 0.93-22.0, respectively), with marginal significance.

DISCUSSION

In this series, no association was observed for of *HLA-G* 3'UTR alleles, genotypes and haplotypes for MS patients, however, several polymorphic sites were associated with NMOSD.

The *+3003CT* genotype was more frequent in anti-AQP4ab+ NMOSD patients group due to increase of *+3003C* allele whereas *+3003TT* genotype was in lower frequency when compared to AQP4ab- NMOSD patients group. *In silico* analyzes have showed that some miRNAs may have differential affinity with the C or T allele in the *+3003* position HLA-G mRNA (52,53). For example, miR-4653-3p and miR-5787 appear to bind to HLA-G mRNA with more stability in the presence of a Cytosine in this position, while miR-6510-5p and miR-5703 seem to bind preferentially in the presence of a Thymine (53). Thus, the influence of this polymorphism on the HLA-G expression profile may depend on the microenvironment. Some miRNAs are differentially expressed in blood in NMOSD patients differentiating them from patients with clinically isolated syndrome (CIS)/relapsing-remitting MS (RRMS) and healthy controls (91). However, more studies are needed to differentiate miRNA expression profile between anti-AQP4ab+ and anti-AQP4ab- NMOSD patients**.**

The *+3187A* allele and *AA* genotype were overrepresented in NMOSD patients, particularly in those exhibiting anti-AQP4ab+ patients group, whereas the *+3187G* was underrepresented, when compared with healthy controls. Corroborating the last finding, the *UTR-1* (*DelCTGCCC**G**CG*), the unique haplotype that carries the *+3187G* allele, also appears with low frequency in the NMOSD group (*P*=0.028 OR=0.52 95%CI: 0.29-0.94), particularly in anti-AQP4ab+ group (*P*=0.006 OR=0.28 95%CI: 0.11-0.73). Considering that the *UTR-1* (*DelCTGCCC**G**CG*) has been associated with increased production of sHLA-G (46), the lower frequency of this haplotype in NMOSD patients was expected and may contribute with susceptibility to disease. The *UTR-6* (*DelCTGCCC**A**CG*) haplotype was overrepresented in anti-AQP4ab- group when compared to control group. These haplotypes differ only in the position *+3187*, *UTR-1* (*DelCTGCCC**G**CG*) carries the allele *+3187G* and *UTR-6* (*DelCTGCCC**A**CG*) carries allele *+3187A*.

Analysis of two Brazilian samples of SLE patients, from Northeastern and Southeastern regions, compared to healthy controls from same regions, showed a higher frequency of *+3187A* allele only in SLE patients from Northeastern and low frequency *UTR-1* haplotype when the two populations were analyzed together (56). The *+3187A* allele was also

associated with rheumatoid factor positivity in RA (57), and type II reactive leprosy reaction (92). The pathogenesis of NMO, SLE and RA have in common the predominance of humoral response with the presence of diagnostic or pathogenic antibodies in serum and deposition of immune complexes in vessel walls or injured tissues (4,93–96). Interestingly, an *in vitro* study demonstrated that the interaction between HLA-G molecule and ILT-2 receptor can inhibit the proliferation of B lymphocytes and its differentiation into plasma cells, inhibiting the production and secretion of antibodies (97). Moreover, the HLA-G molecule attenuates the expression of chemokine receptors (CXCR4 and CXCR5) in B lymphocytes, and thus can control B lymphocyte traffic into secondary lymphoid organs (97). These data suggest that polymorphic sites that unbalance HLA-G expression could contribute to disorders in which humoral mechanism predominates, such as NMOSD.

Although the variability of *HLA-G* 3'UTR has an important role in the regulation of HLA-G expression levels, we cannot rule out the influence of the promoter and coding regions polymorphisms regarding their influence in molecule structure and function, as well as its mRNA and protein levels (43,44). Furthermore, these segments of the *HLA-G* gene have a strong pattern of LD among them, due to this, extended haplotypes may have higher frequency in certain combinations of promoter, coding and *3'UTR* haplotypes (73,83,98). Thus, future studies are needed to assess whether the association of *HLA-G 3'UTR* haplotype with some diseases is causal or an effect of strong LD between *3'UTR* and other segments of the gene.

The *HLA-G 3'UTR* diversity analysis in worldwide populations using *1000 Genomes* project data (99) showed differences in haplotypes frequency between European and African population, including *UTR-1* and *UTR-6* frequencies (73,74) which were associated with NMO in this study. The lower frequency of *UTR-1* is relatively common in African populations, such as Senegal – 11.5% (74), Nigeria – 13% (73,74) and Democratic Republic of Congo – 14.6% (73), whereas in European populations the *UTR-1* frequency is notably higher (25% - 39%) (73,74). The opposite is observed for *UTR-6* frequency (2%-10% in African populations and 0.6%-2% in European populations) (73,74). The Brazilian population is highly admixed and has contributions of African, European and of native Americans, however the *UTR-1* frequencies found in a Southeastern Brazilian population (23.6% and 25.8%) (83,100) and in Northeastern Brazilian population (29,5%) (100) were similar to *UTR-1* frequencies found in the European populations. The *HLA-G 3'UTR* frequencies in MS and Control groups in this study, mainly *UTR-1* frequency (20.9% and 27.8% respectively), were

similar to those described in previous studies that also considered a Brazilian population sample (83,100). However, the frequency of *UTR-6* may have been overestimated, in previous studies, due to methodological limitation (e.g. the inability to distinguish between *UTR-6* and *UTR-18*).

Taken together, these observations point out the essential use of AIM in diseases association studies, especially with *HLA-G*, in Brazilian population. In addition epidemiological studies suggest possible ethnic and genetic risk differences in MS and NMOSD. Therefore, to avoid spurious associations, the groups of patients with MS, NMOSD and controls analyzed in this study were formed only by individuals previously genotyped for AIMs (80). Analyses of African Ancestry Indexes (AfrAI) for MS-RP, NMOSD-RP and healthy controls-RP revealed no difference among them ($P > 0.05$) (80). Moreover the genetic ancestry estimates in these groups revealed the predominance of European component in MS, NMOSD and healthy controls, supporting that these populations are homogeneous regarding the ancestry (80). Since these populations are comparable, the results here found for allelic and haplotype frequencies in NMOSD group may be genuinely associated with the breakdown of immunological tolerance in the disease.

Limitations in this study are mainly due to the size of MS and NMO population samples, even though being representative of the studied diseases. Since only the *HLA-G 3'UTR* was studied, we cannot rule out the influence of other segments of the *HLA-G* gene, particularly in MS, in which the influence of changes on HLA-G expression levels has been observed on the pathogenesis of MS (63,65–69).

CONCLUSION

Polymorphic sites at the *3'UTR* of immunoregulatory *HLA-G* gene are differentially associated with NMO but not with multiple sclerosis further corroborating that NMO and MS are distinct diseases. Considering that the expression of HLA-G molecule is pivotal for the immune tolerance mechanism and the allele *+3187G* is associated with more efficient production of HLA-G, the low frequency of allele *+3187G* and *UTR-1* haplotype in NMOSD group may contribute to the breakdown of immune tolerance in the pathogenesis of NMO.

TABLES AND FIGURES

Table 1. Demographic and clinical findings of neuromyelitis optica spectrum disorders (NMOSD) and multiple sclerosis (MS) Brazilian patients

	NMOSD 54	**MS** 105	***p-value***
Gender ratio (Female:Male)	6.7: 1	1.8: 1	0.004*†
Age of onset, years Median (IQ)	34.5 (23-51)	30 (23-38)	0.035*‡
EDSS first attack Median (IQ)	6.0 (6.0-7.0)	2.5 (2.0-3.5)	<0.001**‡
Duration of disease, years Median (IQ)	5.0 (2.5-7.0)	6.5 (4.0-10)	0.022*‡
EDSS last follow up Median (IQ)	6.0(3.0-8.0)	2.5 (2.0-5.5)	<0.001**‡

* *P*<0.05; ** *P*<0.001; † Fisher's exact test; ‡ Mann Whitney U test; IQ: Interquartile; EDSS: Expanded Disability Status Scale.

Table 2. Demographic and clinical findings of NMOSD stratified according to serological status for AQP4 antibodies and MS Brazilian patients

	NMOSD		**MS**	***p-value***
	AQP4ab+ (26)	**AQP4ab- (20)**	**(105)**	
Gender ratio (Female:Male)	4.2: 1	9: 1	1.8: 1	-
Age of onset Median (IQ)	46 (26-60)[a]	29.5 (16.5-44)[b]	30 (23-38)[b]	0.011‡
EDSS first attack Median (IQ)	6.25 (6.0-7.5)[a]	6.0 (5.25-6.75)[a]	2.5 (2.0-3.5)[b]	<0.001‡
Duration of disease, years Median (IQ)	4 (2.0-6.0)[b]	5(3.0-7.5)[ab]	6.5 (4.0-10)[a]	0.025‡
EDSS last follow up Median (IQ)	4.75(2.5-7.5)[a]	6.25 (2.75-7.25)[a]	2.5 (2.0-5.5)[b]	<0.001‡

‡ Kruskal-Wallis test; IQ: Interquartile; EDSS: Expanded Disability Status Scale; same letters in the same row means that the groups are similar.

Table 3. Comparison of the *HLA-G* 3'UTR allelic and genotype frequencies among NMOSD, MS patients and healthy control (CTL) from northwestern region of São Paulo, Brazil.

		MS	NMOSD	CTL	MS x CTL			NMOSD x CTL			NMOSD x MS		
Position[a]	***2n***	**(210)**	**(108)**	**(216)**	***p-value***[b]	**OR**	**IC 95%**	***p-value***[b]	**OR**	**IC 95%**	***p-value***[b]	**OR**	**IC 95%**
14bp[c]	**D**	0.533	0.482	0.556	0.697	0.91	0.62-1.34	0.238	0.74	0.47-1.18	0.408	0.81	0.51-1.29
	I	0.467	0.518	0.444		1.09	0.75-1.60		1.35	0.85-2.14		1.23	0.77-1.96
	DD	0.267	0.259	0.324	0.372	0.76	0.42-1.37	0.470	0.73	0.35-1.52	1.000	0.96	0.46-2.03
	ID	0.533	0.444	0.463	0.339	1.33	0.77-2.27	0.868	0.93	0.48-1.79	0.318	0.70	0.36-1.35
	II	0.200	0.296	0.213	0.867	0.92	0.48-1.80	0.249	1.56	0.74-3.28	0.234	1.68	0.79-3.58
	HWE	0.558	0.419	0.859									
+3001	**C**	0.995	1.000	0.995	-	-	-	-	-	-	-	-	-
	T	0.005	0.000	0.005									
	CC	0.991	1.000	0.991	-	-	-	-	-	-	-	-	-
	CT	0.009	0.000	0.009									
	TT	0.000	0.000	0.000									
	HWE	-	-	-									
+3003	**T**	0.847	0.887	0.880	0.388	0.76	0.43-1.33	1.000	1.07	0.52-2.22	0.387	1.42	0.69-2.90
	C	0.153	0.113	0.120		1.32	0.75-2.32		0.93	0.45-1.93		0.71	0.35-1.45
	TT	0.704	0.774	0.778	0.265	0.68	0.36-1.27	1.000	0.98	0.44-2.15	0.444	1.44	0.66-3.12
	TC	0.286	0.226	0.204	0.195	1.56	0.82-2.97	0.838	1.14	0.52-2.54	0.562	0.73	0.34-1.59
	CC	0.010	0.000	0.018	1.000	0.55	0.05-6.13	-	-	-	-	-	-
	HWE	0.454	1	0.695									
+3010	**C**	0.601	0.643	0.566	0.476	1.17	0.78-1.74	0.216	1.38	0.84-2.26	0.524	1.18	0.71-1.97
	G	0.399	0.357	0.434		0.86	0.57-1.28		0.72	0.44-1.19		0.85	0.51-1.39
	CC	0. 404	0. 449	0. 311	0.141	1.56	0.87-2.79	0.107	1.80	0.90-3.62	0.723	1.16	0.57-2.33
	CG	0.394	0.388	0.509	**0.086**	**0.59**	**0.34-1.04**	0.171	0.61	0.31-1.22	1.000	1.03	0.51-2.10
	GG	0.202	0.163	0.179	0.718	1.19	0.59-2.42	1.000	0.89	0.36-2.21	0.655	0.75	0.30-1.86
	HWE	0.087	0.348	0.859									
+3027	**C**	0.938	0.972	0.958	0.387	0.66	0.27-1.58	0.757	1.52	0.40-5.74	0.279	2.31	0.64-8.29
	A	0.062	0.028	0.042		1.52	0.63-3.63		0.66	0.17-2.48		0.43	0.12-1.55
	CC	0.886	0.944	0.926	0.354	0.62	0.24-1.59	0.753	1.36	0.35-5.35	0.268	2.19	0.59-8.14
	CA	0.105	0.056	0.065	0.333	1.69	0.63-4.54	1.000	0.85	0.21-3.42	0.385	0.50	0.13-1.88
	AA	0.009	0.000	0.009	1.000	1.03	0.06-16.7	-	-	-	-	-	-
	HWE	0.326	1	0.231									

(Continued)

Table 3. Continued.

		MS	NMOSD	CTL	MS x CTL			NMOSD x CTL			NMOSD x MS		
Position[a]	*2n*	(210)	(108)	(216)	*p-value*[b]	OR	IC 95%	*p-value*[b]	OR	IC 95%	*p-value*[b]	OR	IC 95%
+3035	C	0. 867	0. 852	0. 852	0.678	1.13	0.65-1.95	1.000	1.00	0.52-1.92	0.734	0.89	0.46-1.72
	T	0.133	0.148	0.148		0.88	0.51-1.53		1.00	0.52-192		1.13	0.58-2.19
	CC	0.752	0.741	0.731	0.756	1.12	0.60-2.06	1.000	1.05	0.50-2.20	1.000	0.94	0.44-2.00
	CT	0.229	0.222	0.241	0.873	0.93	0.50-1.76	0.846	0.90	0.41-1.96	1.000	0.96	0.44-2.12
	TT	0.019	0.037	0.028	1.000	0.68	0.11-4.15	1.000	1.35	0.22-8.31	0.605	1.98	0.27-14.5
	HWE	1	0.313	1									
+3142	G	0.564	0.623	0.565	1.000	1.00	0.67-1.48	0.338	1.27	0.79-2.05	0.394	1.27	0.79-2.07
	C	0.436	0.377	0.435		0.99	0.68-1.47		0.79	0.49-1.27		0.79	0.49-1.28
	GG	0.324	0.396	0.305	0.766	1.12	0.62-2.01	0.288	1.49	0.75-2.96	0.478	1.33	0.67-2.66
	GC	0.480	0.453	0.519	0.492	0.82	0.48-1.42	0.503	0.77	0.40-1.49	0.866	0.93	0.48-1.82
	CC	0.196	0.151	0.176	0.724	1.17	0.58-2.35	0.824	0.83	0.34-2.05	0.516	0.71	0.29-1.75
	HWE	0. 843	0. 778	0. 601									
+3187	A	0.791	0.833	0.724	0.115	1.44	0.92-2.25	**0.037***	**1.90**	**1.06-3.43**	0.455	1.33	0.72-2.43
	G	0.209	0.167	0.276		0.70	0.45-1.10		**0.53**	**0.29-0.95**		0.76	0.41-1.38
	AA	0.657	0.704	0.542	**0.094**	**1.62**	**0.93-2.82**	**0.061**	**2.01**	**0.99-4.03**	0.596	1.24	0.61-2.52
	AG	0.267	0.259	0.365	0.141	0.63	0.35-1.14	0.215	0.61	0.30-1.26	1.000	0.96	0.46-2.03
	GG	0.076	0.037	0.093	0.806	0.80	0.30-2.11	0.340	0.37	0.08-1.77	0.496	0.47	0.10-2.28
	HWE	0.070	0.624	0.263									
+3196	C	0.662	0.630	0.694	0.534	0.86	0.57-1.29	0.260	0.75	0.46-1.22	0.620	0.87	0.54-1.41
	G	0.338	0.444	0.306		1.16	0.77-1.74		1.34	0.82-2.17		1.15	0.71-1.87
	CC	0.457	0.407	0.528	0.338	0.75	0.44-1.29	0.183	0.62	0.32-1.19	0.614	0.82	0.42-1.59
	CG	0.410	0.444	0.333	0.260	1.39	0.79-2.42	0.173	1.60	0.82-3.13	0.736	1.15	0.59-2.24
	GG	0.133	0.148	0.139	1.000	0.95	0.44-2.09	1.000	1.08	0.43-2.73	0.812	1.13	0.44-2.89
	HWE	0.386	0.772	0.085									
+3227	G	0.976	0.926	0.977	1.000	0.97	0.28-3.41	0.490	0.62	0.16-2.34	0.495	0.63	0.17-2.41
	A	0.024	0.074	0.023		1.03	0.29-3.61		1.62	0.43-6.17		1.58	0.42-6.00
	GG	0.952	0.926	0.954	1.000	0.97	0.27-3.46	0.483	0.61	0.16-2.36	0.490	0.63	0.16-2.43
	GA	0.048	0.074	0.046	1.000	1.03	0.29-3.67	0.483	1.65	0.42-6.41	0.490	1.60	0.41-6.22
	AA	0.000	0.000	0.000	-	-	-	-	-	-	-	-	-
	HWE	1	1	1									

[a] Position considering +1 the A of the first translated ATG; [b]*p-value* were calculated using Fisher's exact test by fixing one allele against the other or each genotype against the others; [c] Polymorphism type insertion / deletion of 14 base pairs starting at the position +2960; HWE: Hardy-Weinberg equilibrium; * $p<0.05$; Bold values represent statistical significance or marginal significance.

Table 4. Comparison of the *HLA-G* 3'UTR haplotype frequencies among NMOSD, MS patients and healthy control from northwestern region of São Paulo, Brazil.

		MS		Control		NMOSD		MS *versus* Control			NMOSD *versus* Control			NMOSD *versus* MS		
Haplotype[a]	**14bp +3001 +3003 +3010 +3027 +3035 +3142 +3187 +3196 +3227**	***n***		**n**		***n***		***p-value***[b]	**OR**	**95%CI**	***p-value***[b]	**OR**	**95%CI**	***p-value***[b]	**OR**	**95%CI**
UTR1	Del C T G C C C G C G	43	0.209	60	0.278	18	0.167	0.113	0.69	0.44-1.08	**0.028***	**0.52**	**0.29-0.94**	0.453	0.76	0.41-1.39
UTR2	Ins C T C C C G A G G	71	0.345	66	0.305	40	0.370	0.407	1.20	0.80-1.80	0.260	1.34	0.82-2.17	0.709	1.12	0.69-1.82
UTR3	Del C T C C C G A C G	23	0.112	26	0.120	12	0.111	0.879	0.92	0.51-1.67	0.857	0.91	0.44-1.89	1.000	0.99	0.47-2.09
UTR4	Del C C G C C C A C G	31	0.150	26	0.120	12	0.111	0.395	1.30	0.74-2.27	0.857	0.91	0.44-1.89	0.390	0.71	0.35-1.44
UTR5	Ins C T C C T G A C G	13	0.063	20	0.093	13	0.120	0.281	0.66	0.32-1.37	0.441	1.34	0.64-2.81	**0.088**	**2.03**	**0.91-4.55**
UTR6	Del C T G C C C A C G	6	0.029	3	0.014	6	0.056	0.328	2.13	0.53-8.63	**0.065**	**4.18**	**1.02-17.0**	0.352	1.96	0.62-6.23
UTR7	Ins C T C A T G A C G	13	0.063	9	0.042	3	0.028	0.384	1.55	0.65-3.71	0.757	0.66	0.17-2.48	0.279	0.42	0.12-1.52
UTR17	Ins T T C C T G A C G	1	0.005	1	0.005	0	0.000	-	-	-	-	-	-	-	-	-
UTR18	Del C T G C C C A C A	5	0.024	5	0.023	4	0.037	1.000	1.05	0.29-3.68	0.488	1.62	0.43-6.17	0.500	1.55	0.41-5.88
Total		206	1.000	216	1.000	108	1.000	-	-	-	-	-	-	-	-	-

[a] Nomenclature described by Castelli et al. 2010 and Sabbagh et al. 2014; [b] *p-value* were calculated using Fisher's exact test by fixing each UTR against the others; *n*: number of chromosome; OR: *Odds ratio*; 95%CI: 95% Confidence Interval; * $p<0.05$; Bold values represent statistical significance or marginal significance.

Table 5. Comparison of the *HLA-G* 3'UTR allelic and genotypes frequencies among NMOSD patients stratified according to serological status for AQP4 antibodies and healthy control (CTL).

		AQP4ab+	AQP4ab-	CTL	AQP4ab+ x CTL			AQP4ab- x CTL			AQP4ab+ x AQP4ab-		
Position[a]	*2n*	(52)	(40)	(216)	*P*[b]	OR	95%CI	*P*[b]	OR	95%CI	*P*[b]	OR	95%CI
14bp[c]	D	0.500	0.500	0.556	0.536	0.80	0.44-1.47	0.605	0.80	0.41-1.57	1.000	1.00	0.44-2.28
	I	0.500	0.500	0.444		1.25	0.68-2.29		1.25	0.64-2.46		1.00	0.44-2.28
	DD	0.269	0.250	0.324	0.646	0.77	0.29-2.00	0.607	0.69	0.24-2.07	1.000	1.11	0.29-4.19
	ID	0.462	0.500	0.463	1.000	0.99	0.42-2.35	0.811	1.16	0.45-3.01	1.000	0.86	0.27-2.76
	II	0.269	0.250	0.213	0.602	1.36	0.51-3.63	0.770	1.23	0.40-3.75	1.000	1.11	0.29-4.19
+3001	C	1.000	1.000	0.995	-	-	-	-	-	-	-	-	-
	T	0.000	0.000	0.005									
	CC	1.000	1.000	0.991	-	-	-	-	-	-	-	-	-
	CT	0.000	0.000	0.009									
	TT	0.000	0.000	0.000									
+3003	T	0.800	0.950	0.880	0.167	0.55	0.25-1.23	0.272	2.60	0.59-11.4	**0.059**	**0.21**	**0.04-1.02**
	C	0.200	0.050	0.120		1.83	0.82-4.09		0.38	0.09-1.69		**4.75**	**0.98-23.1**
	TT	0.600	0.900	0.778	**0.078**	**0.43**	**0.17-1.08**	0.363	2.57	0.56-11.9	**0.040***	**0.17**	**0.03-0.88**
	TC	0.400	0.100	0.204	**0.066**	**2.61**	**1.03-6.59**	0.363	0.43	0.09-2.02	**0.040***	**6.00**	**1.13-31.8**
	CC	0.000	0.000	0.018	-	-	-	-	-	-	-	-	-
+3010	C	0.640	0.625	0.566	0.426	1.36	0.71-2.58	0.570	1.28	0.59-2.75	1.000	1.07	0.43-2.68
	G	0.360	0.375	0.434		0.73	0.39-1.39		0.78	0.36-1.68		0.94	0.37-2.35
	CC	0.440	0.375	0. 311	0.244	1.74	0.71-4.24	0.581	1.33	0.44-3.96	0.753	1.31	0.36-4.73
	CG	0.400	0.500	0.509	0.378	0.64	0.26-1.56	1.000	0.96	0.34-2.76	0.748	0.67	0.19-2.36
	GG	0.160	0.125	0.179	1.000	0.87	0.27-2.84	0.736	0.65	0.14-3.12	1.000	1.33	0.21-8.29
+3027	C	0.981	0.975	0.958	0.693	2.22	0.27-17.9	1.000	1.70	0.21-13.8	1.000	1.31	0.08-21.6
	A	0.019	0.025	0.042		0.45	0.06-3.64		0.59	0.07-4.79		0.76	0.04-12.6
	CC	0.962	0.950	0.926	1.000	2.00	0.24-16.7	1.000	1.52	0.18-12.9	1.000	1.32	0.08-22.4
	CA	0.038	0.050	0.065	1.000	0.58	0.07-4.91	1.000	0.76	0.09-6.54	1.000	0.76	0.04-12.9
	AA	0.000	0.000	0.009	-	-	-	-	-	-	-	-	-
+3035	C	0.865	0.800	0. 852	1.000	1.12	0.46-2.70	0.476	0.70	0.29-1.65	0.411	1.61	0.53-4.88
	T	0.135	0.200	0.148		0.89	0.37-2.16		1.44	0.61-3.40		0.62	0.21-1.89
	CC	0.731	0.700	0.731	1.000	0.99	0.38-2.62	0.790	0.86	0.30-2.44	1.000	1.16	0.32-4.23
	CT	0.269	0.200	0.241	0.802	1.16	0.44-3.07	0.782	0.79	0.24-2.57	0.732	1.47	0.36-5.96
	TT	0.000	0.100	0.028	-	-	-	0.174	3.90	0.61-24.9	-	-	-
+3142	G	0.620	0.600	0.565	0.528	1.26	0.67-2.36	0.730	1.16	0.58-2.30	1.000	1.10	0.46-2.55
	C	0.380	0.400	0.435		0.79	0.42-1.50		0.86	0.44-1.72		0.92	0.39-2.16
	GG	0.400	0.300	0.305	0.477	1.52	0.62-3.72	1.000	0.97	0.34-2.76	0.544	1.56	0.45-5.42
	GC	0.440	0.600	0.519	0.513	0.73	0.30-1.75	0.627	1.39	0.53-3.68	0.373	0.52	0.16-1.73
	CC	0.160	0.100	0.176	1.000	0.89	0.27-2.90	0.525	0.52	0.11-2.44	0.678	1.71	0.28-10.5
+3187	A	0.904	0.800	0.724	**0.006***	**3.58**	**1.36-9.44**	0.434	1.52	0.66-3.49	0.228	2.35	0.71-7.84
	G	0.096	0.200	0.276		**0.28**	**0.11-0.74**		0.66	0.29-1.51		0.43	0.13-1.42
	AA	0.808	0.600	0.542	**0.015***	**3.55**	**1.25-10.1**	0.807	1.27	0.48-3.35	0.187	2.80	0.75-10.5
	AG	0.192	0.400	0.365	0.109	0.42	0.15-1.19	0.804	1.16	0.44-3.09	0.187	0.36	0.09-1.34
	GG	0.000	0.000	0.093	-	-	-	-	-	-	-	-	-
+3196	C	0.635	0.700	0.694	0.411	0.76	0.41-1.44	1.000	1.03	0.49-2.14	0.657	0.74	0.31-1.80
	G	0.365	0.300	0.306		1.31	0.69-2.47		0.97	0.47-2.03		1.34	0.56-3.24
	CC	0.423	0.450	0.528	0.387	0.66	0.28-1.56	0.628	0.73	0.28-1.91	1.000	0.89	0.28-2.90
	CG	0.423	0.500	0.333	0.493	1.47	0.61-3.52	0.205	2.00	0.76-5.24	0.766	0.73	0.23-2.37
	GG	0.154	0.050	0.139	0.764	1.13	0.34-3.73	0.464	0.33	0.04-2.62	0.369	3.46	0.35-33.6
+3227	G	0.942	0.975	0.977	0.187	0.39	0.09-1.68	1.000	0.92	0.11-8.13	0.630	0.42	0.04-4.19
	A	0.058	0.050	0.023		2.59	0.59-11.2		1.08	0.12-9.52		2.39	0.24-23.9
	GG	0.885	0.950	0.954	0.185	0.37	0.09-1.67	1.000	0.92	0.10-8.35	0.622	0.40	0.04-4.21
	GA	0.115	0.050	0.046	0.185	2.69	0.60-12.1	1.000	1.08	0.12-9.81	0.622	2.48	0.24-25.8
	AA	0.000	0.000	0.000	-	-	-	-	-	-	-	-	-

[a] Position considering +1 the A of the first translated ATG; [b]*p-value* were calculated using Fisher's exact test by fixing one allele against the other or each genotype against the others; [c] Polymorphism type insertion / deletion of 14 base pairs starting at the position +2960; * $p<0.05$; Bold values represent statistical significance or marginal significance.

Table 6. Comparison of the HLA-G 3'UTR haplotype frequencies among NMOSD patients stratified according to serological status for AQP4 antibodies and healthy control

		AQP4ab+		AQP4ab-		Control		AQP4ab+ *versus* Control			AQP4ab- *versus* Control			AQP4ab+ *versus* AQP4ab-		
Haplotype[a]	**14bp +3001 +3003 +3010 +3027 +3035 +3142 +3187 +3196 +3227**	***n***		***n***		***n***		***p-value***[b]	**OR**	**95%CI**	***p-value***[b]	**OR**	**95%CI**	***p-value***[b]	**OR**	**95%CI**
UTR1	Del C T G C C C G C G	5	0.096	8	0.200	60	0.278	**0.006***	**0.28**	**0.11-0.73**	0.338	0.65	0.28-1.49	0.228	0.43	0.13-1.42
UTR2	Ins C T C C C G A G G	19	0.365	12	0.300	66	0.305	0.411	1.31	0.69-2.47	1.000	0.97	0.47-2.03	0.657	1.34	0.56-3.24
UTR3	Del C T C CC G A C G	7	0.135	4	0.100	26	0.120	0.815	1.14	0.46-2.78	1.000	0.81	0.27-2.47	0.751	1.40	0.38-5.16
UTR4	Del C C G C C C A C G	10	0.192	2	0.050	26	0.120	0.178	1.74	0.78-3.88	0.272	0.39	0.09-1.69	**0.061**	**4.52**	**0.93-22.0**
UTR5	Ins C T C C T G A C G	6	0.115	7	0.175	20	0.093	0.606	1.28	0.49-3.36	0.156	2.08	0.82-5.30	0.548	0.62	0.19-2.00
UTR6	Del C T G C C C A C G	1	0.019	5	0.125	3	0.014	0.580	1.39	0.14-13.7	**0.003***	**10.1**	**2.32-44.4**	**0.082**	**0.14**	**0.02-1.23**
UTR7	Ins C T C A T G A C G	1	0.019	1	0.025	9	0.042	0.693	0.45	0.06-3.64	1.000	0.59	0.07-4.79	1.000	0.77	0.05-12.6
UTR17	Ins T T C C T G A C G	0	0.000	0	0.000	1	0.005	-	-	-	-	-	-	-	-	-
UTR18	Del C T G C C C A C A	3	0.058	1	0.025	5	0.023	0.187	2.58	0.59-11.2	1.000	1.08	0.12-9.52	0.630	2.39	0.24-23.9
Total		52	0.999	40	1.000	216	1.000	-	-	-	-	-	-	-	-	-

[a]Nomenclature described by Castelli et al. 2010 and Sabbagh et al. 2014; [b] *p-value* were calculated using Fisher's exact test by fixing each UTR against the others; *n:* number of chromosome; OR: *Odds ratio*; 95%CI: 95% Confidence Interval; * $p<0.01$; Bold values represent statistical significance or marginal significance.

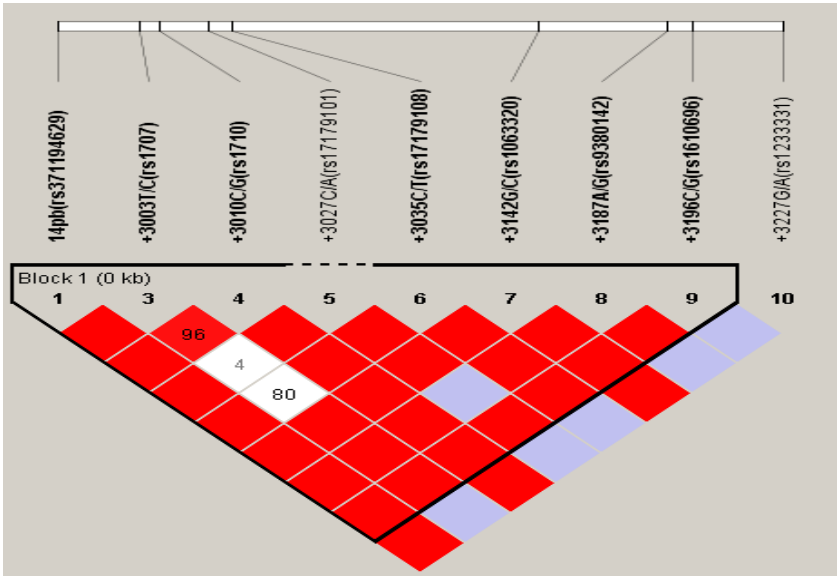

Figure 1. LD patterns for the 3'UTR region of *HLA-G* in the analyzed groups. Considering the variable sites with MAF>0.01 generated by Haploview 4.2. Areas in red indicate strong LD (LOD⩾2, *D'* = 1), blue boxes indicate weak LD (LOD<2, *D'* = 1) and white indicate very weak LD (LOD<2, *D'*<1).The haplotype block was defined by the confidence intervals method of Gabriel (99). D′ values different from 1.0 are represented in each diamond as percentages. MAF: minor allele frequency; LOD: *Log of the odds* – measure of confidence in the value of *D'*.

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ANEXO

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Ética

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Ilustríssima Senhora
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Com referência ao Projeto de Pesquisa: (Protocolo CEP 4205/2012) **"Polimorfismo da região 3' não traduzida (3'UTR) do gene HJA-G em Brasileiros com Esclerose Múltipla ou Neuromielite Óptica"** coordenado por Vossa Senhoria, com a colaboração de Anderson Kuntz Grezyec, Amilton Antunes Barreira, Eduardo Antonio Donadi, Fernando Coronetti Gomes da Rocha, Jeffersonn Becker, Soniza Vieira Alves Leon, Andréia da Silva Souza e Renata Brant de Souza, aprovado por este colegiado em **07/05/2012**, conta com os seguintes sub-projetos à saber:

Sub-Projeto I: (Protocolo CEP 4205/2012-A) **"Análise do polimorfismo da região 3' não traduzida (3'NT) do gene HLA-G de pacientes com esclerose múltipla e neuromielite óptica da Região Noroeste do Estado de São Paulo"**, que foi conduzido por Andréia da Silva Souza, (com objetivo de Dissertação de Mestrado), orientada pela Profª Drª Doralina Guimarães Brum Souza.

Sub-Projeto II: (Protocolo CEP 4205/2012-B) **"Análise de painel de genes informativos de ancestralidade e aspectos demográficos e clínicos em pacientes da Região Noroeste do Estado de São Paulo"**, que foi conduzido por Renata Brant de Souza, (com objetivo de Dissertação de Mestrado), orientada pela Profª Drª Doralina Guimarães Brum Souza.

Fica alterado o cronograma de execução do Projeto (Protocolo CEP 4205/2012) até o ano de **2020**.

O CEP solicita aos pesquisadores que ao final da execução do Projeto **4205/2012**, bem como de seus **Sub-Projetos I e II 4205/2012-A e B** respectivamente, sejam enviados de forma separada os respectivos "Relatórios Finais de Atividades"

Atenciosamente,

Profª Drª Silvana Andréa Molina Lima
Coordenadora do CEP-FMB-UNESP.