ANA PAULA KALLAUR

AVALIAÇÃO DO POLIMORFISMO GENÉTICO *Nco*I DO FATOR DE NECROSE TUMORAL BETA (TNF-β) E DOS NÍVEIS SÉRICOS DE CITOCINAS EM PACIENTES COM ESCLEROSE MÚLTIPLA DE LONDRINA E REGIÃO, PARANÁ

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Ciências da Saúde, Centro de Ciências da Saúde, Universidade Estadual de Londrina.

Orientador: Profa. Dra. Edna Maria Vissoci Reiche

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KALLAUR, Ana Paula**. Avaliação do polimorfismo genético *Nco*I do fator de necrose tumoral beta (TNF-β) e dos níveis séricos de citocinas em pacientes com esclerose múltipla de Londrina e região, Paraná.** 137 f. 2012. Dissertação (Mestrado em Ciências da Saúde) – Universidade Estadual de Londrina, 2012.

RESUMO

A esclerose múltipla (EM) é uma doença inflamatória crônica imuno-mediada do sistema nervoso central (SNC). Variações em genes que codificam a expressão e a regulação de citocinas desempenham um importante papel na susceptibilidade, atividade e progressão da doença. Os objetivos deste estudo foram: 1) determinar a frequência do polimorfismo genético *Nco*I do TNF-β em pacientes com EM e em indivíduos saudáveis da população sul-brasileira; 2) avaliar a associação entre o polimorfismo *Nco*I no gene do TNF-β e a susceptibilidade a EM, progressão e atividade da doença; 3) avaliar a associação entre o *Nco*I no gene do TNF-β e o polimorfismo do HLA-DRB1* em pacientes com EM; 4) avaliar o perfil de citocinas TNF-α, IL-1, IL-6 e IL-12, IFN-γ, IL-17, IL-4 e IL-10 em pacientes com EM recorrente-remitente (EM-RR) e controles saudáveis; e 5) avaliar a associação entre os níveis séricos das citocinas e a incapacidade clínica e atividade da doença nos pacientes com EM-RR. A incapacidade dos pacientes com EM foi avaliada pela Escala Expandida do Estado de Incapacidade (EDSS) e a atividade da doença foi avaliada por ressonância magnética nuclear (RMN). O DNA genômico foi extraído do *buffy coat* obtido de células do sangue periférico e um fragmento de 782 pares de base foi amplificado por meio da reação em cadeia da polimerase (PCR) utilizando *primers* específicos. O produto da PCR foi submetido à digestão pela enzima *Nco*I e o polimorfismo do comprimento dos fragmentos de restrição foram analisados em eletroforese em gel de agarose a 3%. Amostra de soro foi utilizada para determinação dos níveis das citocinas IL-1β, IL-4, IL-6, IL-10, IL-12, IL-17, IFN-γ e TNF-α pelo método de enzimaimunoensaio. Para a análise do polimorfismo genético foram inseridos 220 pacientes com EM e 278 indivíduos saudáveis, pareados quanto à idade, sexo e etnia. Entre os pacientes com EM, 106 (48,2%) apresentaram o genótipo TNFB2/B2, 96 (43,6%) apresentaram o genótipo TNFB1/B2 e 18 (8,2%) apresentaram o genótipo TNFB1/B1. Entre os controles saudáveis, 103 (37,1%) apresentaram o genótipo TNFB2/B2, 139 (50,0%) apresentaram o genótipo TNFB1/B2 e 36 (12,9%) apresentaram o genótipo TNFB1/B1. Diferenças significativas foram encontradas quando a frequência do genótipo TNFB2/B2 foi comparada com a do genótipo TNFB1/B2 em pacientes com EM e controles (p=0,0449; OR: 1,49; 95% IC= 1,099-2,855) e quando comparada com a frequência do genótipo TNFB1/B1 em pacientes com EM e controles (p=0,0225; OR: 2,058; 95% IC= 1,099-2,855). A frequência do alelo TNFB2 foi 0,700 (70,0%) nos pacientes com EM e 0,6205 (62,05%) nos controles, enquanto que a frequência do alelo TNFB1 foi de 0,300 (30,0%) nos pacientes com EM e 0,3795 (37,95%) nos controles (p=0,0089; OR: 1,427; 95% CI= 1,093-1,863). Não foi encontrado associação entre o alelos HLA-DRB1*01, 03, 04, 07, 08, 11, 13, e 15 e os alelos do polimorfismo genético *Nco*I do TNF-β (p>0.05). Os resultados sugerem que o polimorfismo genético *Nco*I do TNF-β não está em desequilíbrio de ligação com o polimorfismo do HLA-DRB1*. As variáveis como idade no diagnóstico, duração da doença, formas clínicas, EDSS basal, EDSS após cinco anos de acompanhamento, índice de progressão, progressão e local da lesão não estão associadas aos genótipos do TNF-β (p= 0,2884, p= 0,8201, p=0,4189, p=0,9889, p= 0,3913, p= 0,6002, p= 0,4509 e p= 0,8249, respectivamente). No entanto, foi encontrado uma tendência para a associação entre o polimorfismo genético *Nco*I do TNF-β e a atividade da doença (p=0,0722). Para o estudo do

perfil de citocinas, foram incluído 169 pacientes com EM-RR e 132 controles saudáveis, pareados quanto à idade, gênero, etnia, índice de massa corpórea e uso de corticoesteroides. Pacientes com EM-RR apresentaram níveis mais elevados de IL-6, IL-12, IFN-γ e IL-4 (p=0,0114, p=0,0297, p=0,0009 e p=0,0004, respectivamente) comparados aos controles. IL-1β mostrou uma tendência a estar mais elevada nos controles (p= 0.0992). TNF-α, IL-17 e IL-10 não apresentaram diferenças (p>0,05). Níveis diminuídos de TNF-α e aumentados de IFN-γ estavam independentemente associados à EM (p=0,0078 e p=0,0056, respectivamente). Pacientes com EM-RR e incapacidade leve apresentaram níveis séricos elevados de IL-4 comparados a pacientes com incapacidade moderada e grave (p = 0,0375). Os níveis séricos de TNF-α e IL-10 apresentaram-se elevados nos pacientes com lesão Gd negativo (p=0,0457 e p=0,0533, respectivamente) e os níveis séricos de IL-17 mostraram uma tendência, embora não significativa, a serem mais elevados em pacientes com a doença inativa (p=0,0631). Níveis diminuídos de TNF-α e aumentados de IFN-γ estavam independentemente associados à atividade da doença (p=0,0348 e p=0,0133, respectivamente). Os resultados sugerem que o polimorfismo genético *Nco*I do *TNF-β* contribui para susceptibilidade a EM independentemente dos alelos HLA-DRB1* na população sul-brasileira. No entanto, sozinhos, não contribuem para a atividade e gravidade da doença. Outros fatores genéticos devem interagir sinergicamente para contribuir com os mecanismos fisiopatológicos envolvidos na progressão e gravidade da EM. Os dados obtidos demonstram que mesmo em fase de remissão clínica da EM os pacientes apresentam alteração no balanço entre as citocinas pró-inflamatórias, Th1, Th17 e Th2. Os resultados contribuem para o melhor entendimento do papel do polimorfismo genético do TNF-β e das citocinas na EM e para o desenvolvimento de novas estratégias terapêuticas de imunomodulação e de neurorestauração.

Palavras-chaves: Polimorfismo genético *Nco*I. Esclerose múltipla. Fator de necrose tumoral alfa (TNF-α). Fator de necrose tumoral beta (TNF-β). EDSS.

KALLAUR, Ana Paula. **Evaluation of tumor necrosis factor beta (TNF-β) *Nco*I genetic polymorphism and cytokine serum level in patients with multiple sclerosis from Londrina and region, Paraná.** 137 f. 2012. Dissertation (Master's degree in Health Sciences) – Universidade Estadual de Londrina, Londrina, 2012.

ABSTRACT

Multiple Sclerosis (MS) is an immune-mediated chronic inflammatory disorder of the central nervous system (CNS). Variations in genes coding for the expression and regulation of cytokines may play a role in MS susceptibility, disease activity, and progression. The aims of this study were: 1) to determine the frequency of tumor necrosis factor beta (TNF-β) *Nco*I genetic polymorphism among the MS patients and healthy individuals from Southern Brazilian population; 2) to evaluate the association between TNF-β *Nco*I polymorphism with the susceptibility for MS, clinical progression, and activity of the disease; 3) to evaluate the association between TNF-β *Nco*I and HLA-DRB1* genetic polymorphisms; 4) to evaluate the serum TNF-α, IL-1β, IL-6, IL-12, IFN-γ, IL-17, IL-4, and IL-10 serum cytokine levels in relapsing-remitting MS (RR-MS) patients and healthy controls; 5) to evaluate the association between the cytokine profile with the clinical disability and disease activity of RR-MS patients. The disability of MS patients was evaluated using the Expanded Disability Status Scale (EDSS) and the disease activity was evaluated using magnetic resonance imaging (MRI). Genomic DNA was extracted from buffy coat of peripheral blood cells collected with EDTA as anticoagulant and a 782 base-pair fragment of the TNF-β gene was amplified using polymerase chain reaction (PCR) using specific primers. The PCR amplified product was subjected to *Nco*I restriction digestion and analyzed by restriction fragment length polymorphism in 3% agarose gel electrophoresis. The cytokines interleukin IL-1β, IL-4, IL-6, IL-10, IL-12, IL-17, IFN-γ, and TNF-α level was assessed using enzyme linked-immunosorbent assay. For the polymorphism analysis 220 MS patients and 278 blood donors were enrolled; age, gender, and ethnicity were controlled for the genotype analysis. Among the MS patients, 106 (48.2%) exhibited the TNFB2/B2 genotype, 96 (43.6%) the TNFB1/B2 genotype, and 18 (8.2%) the TNFB1/B1 genotype. Among the controls, 103 (37.1%) exhibited the TNFB2/B2, 139 (50.0%) exhibited the TNFB1/B2 and 36 (12.9%) were TNFB1/B1. The frequency of TNFB2/B2 genotype observed among the MS patients differed from the frequency observed for the TNFB1/B2 genotype in MS patients and controls (p=0.0449; OR: 1.49; 95% CI: 1.023-2.170) and when also compared with the TNFB1/B1 genotype in MS patients and controls (p= 0.0225; OR: 2.058; 95% CI: 1.099-2.855). The TNFB2 allele frequency was 0.700 (70.0%) among the MS patients and 0.6205 (62.05%) among the controls, and the TNFB1 allele frequency was 0.300 (30.0%) among the MS patients and 0.3795 (37.95%) among the controls (p=0.0089; OR: 1.427; 95% CI: 1.093-1.863). The association between HLA-DRB1*01, 03, 04, 07, 08, 11, 13, and 15 alleles and TNF-β *Nco*I alleles was not observed (p>0.05). These results could suggest that TNF-β *Nco*I polymorphism may not be in linkage disequilibrium within HLA-DRB1*. Variables such as age at diagnosis, disease duration, clinical forms, EDSS baseline, EDSS five-year follow-up, progression, progression index, and lesion location obtained by MRI, were not associated with TNF-β *Nco*I genotypes of MS patients (p= 0.2884, p= 0.8201, p= 0.4189, p= 0.9889, p= 0.3913, p= 0.6002, p= 0.4509, and p= 0.8249, respectively). The activity of MS showed a trend toward to be associated with the TNF- β genotypes (p=0.0722). The cytokine profile was evaluated in serum samples from 169 RR-MS patients and 132 healthy controls age-gender-ethnicity-body mass index-matched matched. The IL-6, IL-12, IFN-γ, and IL-4 levels

were higher among the RR-MS patients than controls (p=0.0114, p=0.0297, p=0.0009, and p=0.0004, respectively). IL-1β showed a trend towards higher levels in the controls than RR-MS patients (p= 0.0992). The logistic regression showed that low TNF-α and high IFN-γ were independently associated with MS (p=0.0078 and p=0.0056, respectively). The IL-4 level was higher in RR-MS patients with mild disability than those with moderate and severe disability (p=0.0375). The TNF-α and IL-10 levels were higher among the RR-MS patients with negative Gd than those with positive Gd (p=0.0457 and p=0.0533, respectively). The logistic regression showed that low TNF-α and high IFN-γ were independently associated with disease activity (p=0.0348 and p=0.0133, respectively). All together, the results suggest that TNF-β *Nco*I genetic polymorphism may contribute for the MS susceptibility in Southern Brazilian population independently of HLA-DRB1*. However, TNF-β genotypes were not associated activity and severity of the disease. Other genetic factors may interact and synergistically contribute to the pathophysiology process that affects the severity of MS. Even in the remission phase of the disease, the RR-MS patients exhibited an imbalance of the pro-inflammatory, Th1, Th17, and Th2 cytokines. These results may contribute to the better understanding the role of TNF-β genetic polymorphism and the cytokine profile in the MS and to the development of new therapeutic approach for immunomodulation and neurorestoration.

Keywords: *Nco*I genetic polymorphism. Multiple sclerosis, Tumor necrosis factor alpha (TNF-α). Tumor necrosis factor beta (TNF-β), EDSS.

LISTA DE ABREVIATURAS

AHC Ambulatório do Hospital de Clínicas

BHE Barreira hematoencefálica

CIS *Clinic isolated syndrome* - Síndrome clinicamente isolada

EDSS *Expanded Disability Status Scale*– Escala Expandida de Incapacidade

EDTA Ácido etilenodiaminotetracético

EM Esclerose Múltipla

EM-PP Esclerose Múltipla Primariamente Progressiva

EM-RR Esclerose Múltipla Remitente-Recorrente

EM-SP Esclerose Múltipla Secundariamente Progressiva

IFN Interferon

IL Interleucina

ICAM *Iintercellular adhesion molecule* - moléculas de adesão intercelular

PI *Progress index* – Índice de progressão

IMC Índice de massa corporal

LCR Líquido cefalorraquidiano

LFA1 *Lymphocyte function-associated antigen 1* - antígeno associado a função de linfócitos

MAG *Myelin associated glicoprotein* - Glicoproteína associada à mielina

MBP *Myelin basic protein* – Proteína básica da mielina

MHC *Major histocompatibility complex* – Complexo de histocompatibilidade principal

MOG *Myelin oligodendrocyte glycoprotein* – Glicoproteína oligodendrocítica da mielina

PLP *Proteolipidic protein* - proteína proteolipídica

SNC Sistema Nervoso Central

TNF-α *Tumor necrosis factor alpha* - Fator de necrose tumoral alfa

TNF-β *Tumor necrosis factor beta* - Fator de necrose tumoral beta

TGF-β Transforming growth factor – Fator de crescimento transformador

VLA4 *very late antigen 4* – antigen muito tardio-4

VCAM *vascular cellular adhesion molecule* - molécula da adesão celular vascular

SUMÁRIO

1 INTRODUÇÃO

A Esclerose Múltipla (EM) é uma doença crônica, imuno-mediada desmielinizante e neurodegenerativa com múltiplas áreas de inflamação, desmielinização e formação de cicatrizes gliais (esclerose) nas substâncias branca e cinzenta do sistema nervoso central (SNC). Clinicamente, se caracteriza por episódios de distúrbio focal dos nervos ópticos, tronco cerebelar, região periventricular, medula espinhal e cerebelo com remissão de magnitude variável e recidiva durante um período de muitos anos. As manifestações clínicas são determinadas pela localização e extensão variada dos focos desmielinizantes. As lesões apresentam predileção por determinadas áreas do SNC resultando em um conjunto sinais e sintomas que podem ser reconhecidos como características da EM (ADAMS e VICTOR, 1998; MILLER, 2002; KAIMEN-MACIEL et al., 2009).

O diagnóstico da EM sempre foi baseado em evidências clínicas; no entanto, vários testes como a ressonância magnética nuclear (RMN), exame do líquido cefalorraquidiano (LCR) e os potenciais evocados estão disponíveis para auxiliar o diagnóstico clínico. Rotineiramente, o clínico utiliza o histórico neurológico detalhado do paciente e realiza um exame neurológico para avaliar como o sistema nervoso foi afetado. Para estabelecer o diagnóstico de EM, o neurologista deve demonstrar a ocorrência de lesões no SNC disseminadas no tempo e no espaço e excluir qualquer outro diagnóstico. Critérios definidos são utilizados para o diagnóstico clínico com maior precisão, diminuindo a probabilidade de um diagnóstico incorreto. Em abril de 2001, o *National Multiple Sclerosis Society* (NMSS) *of America* recomendou o uso do critério de McDonald (2001) para o diagnóstico da EM em substituição ao critério de Poser (1991), pela facilidade do diagnóstico de pacientes que apresentam sinais e sintomas sugestivos da doença, incluindo a doença monossintomática (*clinically isolated syndrome* - CIS), esclerose múltipla remitente recorrente (EM–RR) e doença com progressão insidiosa sem surtos e remissões claras. O critério de McDonald foi revisado em 2005 para classificar exatamente o significado de surto, disseminação da doença no espaço e no tempo, RMN positiva e caracterizar a CIS. A forma CIS caracteriza-se por manifestações agudas monofásicas em localizações anatômicas específicas: neurites ópticas, mielites transversas, tronco cerebral e cerebelo, indicativas de alto risco para desenvolver a doença clinicamente definida, quando está associada à presença de lesões na RMN e às alterações imunológicas no LCR atribuídas à EM (POLMAN et al., 2005; KANTARCI et al., 2005; WHO, 2006). O Critério de McDonald foi revisado em 2010, para facilitar o diagnóstico de EM e diferenciar do diagnóstico de neuromielite óptica. No

entanto, esta nova revisão não invalida o Critério de McDonald original (2001) e o Critério de McDonald revisado (2005) (POLMAN et al., 2010).

A RMN é fundamental para a identificação de novas lesões que geralmente coincidem com distúrbios da barreira hematoencefálica (BHE) e são correlacionadas com a atividade da doença (ZHANG, 2012; SHINOHARA et al., 2012). A RMN é a peça central para o diagnóstico de EM. Atualmente, a presença de lesões positivas com o contraste gadolínio na RMN cerebral é um componente crítico para o diagnóstico da EM (POLMAN et al., 2007). A progressão da doença em pacientes com EM tem sido correlacionada com níveis elevados da citocina pró-inflamatória fator de necrose tumoral alfa (TNF- α) (DUDDY et al., 1999).

Lucchinete et al. (2000) realizaram um estudo detalhado em tecidos de pacientes com EM e mostraram que as lesões ativas poderiam ser classificadas em 4 subtipos patológicos distintos, de acordo com a natureza do infiltrado inflamatório e resposta das células gliais. As lesões com padrão I e II são caracterizadas por infiltrado de linfócitos e macrófagos, sobrevivência de oligodendrócitos e remielinização parcial. As lesões com padrão II também apresentam ativação das proteínas do sistema complemento e deposição de imunoglobulina. As lesões com padrão III e IV envolvem linfócitos T e macrófagos e existe falta de evidência sobre a deposição de proteínas do sistema complemento e remielinização; e ambas são caracterizadas por perda de oligodendrócitos.

Segundo a Organização Mundial da Saúde (WHO, 2006), a EM é classificada nas formas clínicas recorrente-remitente (EM-RR), secundária progressiva (EM-SP), primaria progressiva (EM-PP), síndrome clinicamente isolada (CIS) e benigna. Esta classificação utiliza os critérios de diagnóstico definidores de EM de Poser (1991), de Lublin e Reingnold (1996), de McDonald (2001) e revisados por Polman (2005). Koch et al. (2007) e Marques (2010) classificam a EM nas quatro formas de evolução clínica da doença: remitente-recorrente (EM-RR), secundariamente progressiva (EM-SP), primariamente progressiva (EM-PP) e progressiva recidivante (EM-PR).

A forma clínica mais comum é a EM-RR que representa 70,0% de todos os casos no início da doença. Esta forma clínica é caracterizada por episódios de surtos bem definidos no tempo, com alteração de uma ou mais funções neurológicas, com posterior recuperação que pode ser completa ou com sequelas. Aproximadamente, 80,0% dos pacientes irão apresentar inicialmente esta forma, com imprevisíveis surtos, exacerbação dos sintomas já existentes ou aparecimento de novos sintomas. Os quadros de surtos podem durar por períodos variáveis (dias a meses) com recuperação parcial ou total (remissão). A doença pode

apresentar-se clinicamente inativa durante meses ou anos, embora estudos de imagem de RMN mostrem, com frequência, atividade inflamatória assintomática carasterística da sindrome radiologicamente isolada (SRI). Com o passar do tempo, os sintomas podem se tornar graves com menor recuperação das funções após os ataques, possivelmente devida a perda axonal e gliose que, repetidamente, afeta as placas de EM (WHO, 2006).

A EM-SP é caracterizada por uma progressão que não está relacionada com a fase de surto. Aproximadamente 50,0% dos pacientes com a EM-RR irão desenvolver a forma EM-SP dentro de 10 anos e 80,0% irão desenvolver dentro de 20 anos após o início da doença. A EM-PP afeta cerca de 10,0 a 15,0% dos pacientes e é caracterizada pela ausência de ataques distintos e início insidioso com posterior piora dos sintomas. Ocorre um acúmulo de déficits e incapacidades que podem permanecer estáveis em algum momento ou progredir durante os anos (WHO, 2006). A EM-PP é similar à EM-SP, exceto pelo fato de que se apresenta desde o início da doença e que os surtos são mais frequentes (LUBLIN e REINGNOLD, 1996). O diagnóstico da EM benigna é retrospectivo, quando o acúmulo de incapacidades da EM-RR é leve ou ausente após longo período de tempo (considerado de 15 a 20 anos). Estudos longitudinais mostram que a maioria dos pacientes com esta forma clínica, eventualmente, terá incapacidade da fase secundária progressiva, sendo o termo benigno inadequado (WHO, 2006).

Os sinais e sintomas da EM indicam, em geral, mais de uma lesão e as manifestações clínicas podem ser transitórias. O paciente pode apresentar sensações incomuns que são difíceis de serem descritas e verificadas objetivamente. Segundo Miller (2002), os sinais e sintomas mais comuns na EM são: a) motor (fraqueza muscular, espasticidade, reflexos); b) sensitivo (distúrbio no sentido vibratório/ posicional, distúrbio da dor, temperatura ou tato, dor, sinal de Lhermitte); c) cerebelar (ataxia de membros/marcha/tronco, tremor, nistagmo, disartria); d) nervos cranianos/ tronco cerebral (visão afetada, distúrbios oculares excluindo nistagmo, nervos cranianos V, VII, VIII, sinais bulbares, vertigens), e) autonômico (disfunção vesical, intestinal e sexual, sudorese e anormalidades vasculares), f) psiquiátrico (depressão, euforia, anormalidades cognitivas) e g) outros (fadiga). Os sinais clínicos vistos raramente na EM são convulsões generalizadas, crise tônica, cefaléia, nevralgia do trigêmeo, disartia/ataxia paroxísticas, prurido paroxístico, coréia, atetose, mioclonias, hemiespasmos facial, mioquimia, torcicolo espasmódico, distonia focal, sinais do neurônio motor inferior (atrofia, hipotonia, arreflexia), pernas inquietas e histeria. (MILLER, 2002).

A gravidade da EM pode ser avaliada por marcadores de inflamação e desmielinização observados no exame de RMN que pode revelar uma lesão captante do

contraste gadolínio e pela Escala Expandida do Estado de Incapacidade (EDSS) de Kurtzke (1983). Na EDSS, os escores variam de 0,0 (nenhuma incapacidade) a 10,0 (morte devido à EM), dependendo do comprometimento dos sistemas funcionais apresentado pelo paciente.

Estudos epidemiológicos sugerem que a etiologia da EM seja multifatorial com uma complexa interação entre fatores genéticos e ambientais (SOSPEDRA e MARTIN, 2005). Outros fatores também podem estar envolvidos como fatores familiares, geográficos e climáticos (BUGEJA et al., 2006; SCHMIDT, WILLIAMSON e ASHLEY-KOCH, 2007). Vários estudos mostram que a influência da variabilidade genética não está apenas na susceptibilidade à EM, mas também no curso clínico e gravidade da doença (KANTARCI, 2001). Mecanismos autoimunes e infecções virais também podem ter um papel patogênico na EM (ADAMS e VICTOR, 1998; MILLER, 2002; WHO, 2006).

A idade de início da doença apresenta uma distribuição unimodal com pico máximo entre os 20 e 30 anos e os sintomas raramente se iniciam antes dos 10 ou após os 60 anos de idade. A incidência de EM é de, aproximadamente, 2 a 3 vezes mais elevada em mulheres do que em homens e, nos pacientes com início mais tardio, a razão tende a se igualar (MCDONALD e RON, 1999; DE GIROLAMI, ANTHONY e FROSCH, 2000; MILLER, 2002; SOSPEDRA e MARTIN, 2005; KAIMEN-MACIEL et al., 2007).

A alta prevalência de EM corresponde a > 50 casos/ 100.000 habitantes e ocorre no norte da Europa, norte dos Estados Unidos da América (EUA), Canadá, sul da Austrália e Nova Zelândia. A média prevalência corresponde a 30 – 50 casos/ 100.000 habitantes e ocorrem no sul da Europa, sul dos EUA e norte da Austrália. A baixa prevalência corresponde a < 30 casos/ 100.000 habitantes e ocorre na América do Sul e Ásia (KURTZKE, 1983; HOGANCAMP, RODRIGUEZ e WEINSHENKER, 1997; MARRIE, 2004). Estima-se que 2,5 milhões de pessoas vivam com EM no mundo (MILO e KAHANA, 2010). No Brasil, a taxa de prevalência é de, aproximadamente, 15 casos por cada 100.000 habitantes (CALLEGARO et al., 2001; KAIMEN-MACIEL e MEDEIROS, 2003; FRAGOSO e PERES, 2007).

Embora a latitude possa ser uma variável independente que atinge as taxas de prevalência da EM, as diferenças raciais podem explicar, em parte, a distribuição geográfica da doença. Ao se correlacionar as diferenças raciais às taxas de prevalência de EM no mundo, as populações brancas têm um risco maior e populações tanto asiáticas como negras têm um risco menor. Estudos epidemiológicos sugerem que a susceptibilidade à EM é hereditária e o risco de desenvolver EM é de 15 vezes mais alto quando a doença está presente em um parente de primeiro grau. Estudos de famílias e gêmeos proporcionam maior apoio à

susceptibilidade genética. Em regiões de prevalência elevada, o risco de desenvolvimento de EM por toda a vida é de 0,00125% na população em geral. Irmãos de pacientes com EM têm risco aproximado de 2,6%, pais têm risco de aproximadamente 1,8% e os filhos um risco aproximado de 1,5%. De modo geral, cerca de 15,0% dos pacientes com EM apresentam um familiar afetado. Dados de estudos em gêmeos indicam uma concordância de, aproximadamente, 25,0% em gêmeos monozigóticos e de apenas 2,4% para gêmeos dizigóticos do mesmo sexo (DE GIROLAMI, ANTHONY e FROSCH, 2000; MILLER, 2002; WHO, 2006). Estudos de populações migrantes fornecem evidências de alterações ambientais no risco a EM, ao mesmo tempo em que os fatores genéticos se mantêm constantes. O fator ambiental parece ter grande importância na patogênese da doença. Crianças nascidas em Israel, filhos de imigrantes de países tanto asiáticos como do norte da África, apresentam taxas de incidência relativamente mais altas, como as observadas em imigrantes europeus, em vez das baixas frequências características de seu país de origem. Estudos sugerem que a idade por ocasião da imigração tem um papel importante, ou seja, um imigrante que deixa o país de origem antes dos 15 anos de idade tem, praticamente, o mesmo risco de adquirir EM que um nativo de Israel ou da África do Sul. Já indivíduos que migram após os 15 anos de idade, apresentavam o risco do país de origem (DE GIROLAMI, ANTHONY, FROSCH, 2000; MILLER, 2002; WHO, 2006). Entre os fatores ambientais, os agentes infecciosos podem desencadear o processo de desmielinização. Alguns vírus como de sarampo, rubéola, *Herpesvírus Humano Tipo 1* (HHV-1), *Herpesvírus Humano Tipo 4* (HHV-4 ou *Vírus Epstein-Barr*), vírus da caxumba, coronavírus, parainfluenza, vaccinia e vírus linfotrópico de células T humanas tipo 1 (HTLV-1) já foram relatados como presentes em pacientes com EM. O *Herpesvírus Humano Tipo 6* (HHV-6) foi apontado como responsável pela atividade da doença e pesquisadores encontraram uma incidência maior da atividade viral em torno das placas de EM aguda, sugerindo que a atividade do vírus pode desencadear uma exacerbação dos sintomas (MILLER, 2002; WHO, 2006).

A susceptibilidade à EM é conferida, provavelmente, a múltiplos genes. Estudos mostram que a EM é uma doença com fatores independentes ou fatores epistáticos de vários genes, em que cada gene contribui com pequenos efeitos individuais do que poucos genes contribuindo com maior importância biológica, mostrando-se uma doença poligênica (EBERS et al., 1996). Dados de linhagens familiares com mais de um membro atingido são consistentes com a hipótese de que múltiplos genes não correlacionados predispõem à EM. O complexo de histocompatibilidade principal (*Major Histocompatibility Complex* ou MHC) localizado no braço curto do cromossomo 6 humano foi indicado como um determinante

genético na EM. A região MHC codifica as proteínas que compõem as moléculas MHC classe I e classe II ou do sistema de antígeno leucocitário humano (HLA) envolvidas na apresentação de antígenos para linfóticos T $CD8^+$ e T $CD4^+$, respectivamente. Das três classes de genes HLA, a associação mais forte com EM ocorre com os alelos de classe II. Inicialmente, foi encontrada associação com gene do HLA-Dw2 e –DR2 em europeus caucasianos e norte-americanos. No entanto, com o avanço das técnicas, foi encontrada uma associação positiva entre EM e os genótipos HLA-DRB1*1501, HLA-DRB5*0101 e HLA-DQA1*0602 em diferentes grupos étnicos no mundo (DE GIROLAMI, ANTHONY e FROSCH, 2000; MILLER, 2002; WHO, 2006; KALLAUR et al., 2011).

O clima, a não exposição à luz solar, a deficiência de vitamina D, o estresse e a poluição, são fatores determinantes para a prevalência da EM. A radiação ultravioleta, em particular, que está fortemente associada com a latitude, tem sido sugerida como um fator protetor devido às suas propriedades imunossupressoras, possivelmente mediadas pelo efeito do sol na síntese de melatonina ou vitamina D (SCHMIDT, WILLIAMSON e ASHLEY-KOCH, 2007). Traumas físicos já foram relatados como fator desencadeante ou agravante da doença e os efeitos da gravidez sobre a doença ainda são difíceis de avaliar. A EM é mais comum em mulheres em idade reprodutiva e as exacerbações parecem agrupar-se no período pós-parto e não durante a gravidez. Não foi esclarecido se este agrupamento está relacionado com alterações hormonais ou a outros fatores. A vacinação com vírus também é citada, com frequência, como um evento desencadeante, mas não existem evidências conclusivas (DE CARVALHO, PEREIRA e SHOENFEL, 2009). Cirurgias, anestesias e punção lombar também foram apontadas como fatores que contribuem para a EM, mas estudos controlados não conseguiram mostrar relação alguma. A EM é relatada como uma doença que ocorre mais frequentemente em indivíduos de classes socioeconômicas mais altas e que residem em áreas urbanas (MILLER, 2002).

Vários polimorfismos genéticos descritos nas diferentes populações mundiais têm sido associados com a susceptibilidade, resistência, curso clínico e gravidade da EM. Entretanto, resultados contraditórios são registrados na literatura, principalmente quando populações geneticamente mais homogêneas, como as do Hemisfério Norte, são avaliadas. Poucos estudos avaliam a frequência de polimorfismos genéticos associados à EM nas populações do Hemisfério Sul, incluindo a população brasileira, geneticamente mais heterogênea que as previamente estudadas.

1.1 IMUNOPATOLOGIA DA EM

A encefalomielite experimental autoimune (EAE) é um clássico modelo animal estudado para o entendimento das formas recidivantes e progressivas de desmielinização do SNC, apesar de modelos com ratos e primatas apresentarem importantes diferenças com os seres humanos (RIVER e SHWENTKER, 1935). Os linfócitos T $CD4^+$ Th1 e a alta expressão de integrina alfa 4 (α4) são os responsáveis pela EAE. Os linfócitos B e anticorpos antimielina, isoladamente, não são capazes de induzir a EAE. Anticorpos dirigidos contra a glicoproteína oligodendrocítica da mielina (*myelin oligodendrocyte glycoprotein* - MOG), uma pequena proteína encontrada na superfície da bainha de mielina, é fundamental para induzir a EAE em um modelo animal. Os anticorpos anti-MOG foram encontrados em macrófagos que digeriram a mielina, sugerindo que eles ajudariam na opsonização da mielina (GENAIN et al., 1999).

Dado que linfócitos T reativos dirigidos contra componentes da mielina podem induzir a EAE após transferência adotiva destas células a animais, a proteína básica da mielina (*myelin basic protein* – MBP) é considerada a mais importante. Assim, muitos esforços têm se concentrado em identificar e caracterizar as células T específicas para a MBP de pacientes com EM (ALLEGRETA et al., 1990).

Outra hipótese é de que a EM pode ser desencadeada por mimetismo molecular, em que os receptores das células T apresentam reação cruzada com epítopos expressos em agentes infecciosos, que imitam ou possuem epítopos semelhantes ao autoantígenos da mielina (GRAN et al., 1999). Existem peptídeos virais que contêm sequências que coincidem com várias regiões da MBP e que podem ativar clones células T específicas para MBP *in vitro* (VERGELLI et al., 1997). O vírus Epstein-Barr (EBV) estabelece uma imunogênica e persistente infecção dos linfócitos B, com uma expansão dos clones de linfócitos T citotóxicos em resposta ao vírus (ASCHEIRO et al., 2001). Estudos têm demonstrado que os LT podem fazer reação cruzada entre EBV e peptídeos da proteína básica da mielina, confirmando um possível mecanismo autoimune de mimetismo molecular (POHL, 2009; SALVETTI, GIOVANNONI and ALOISI, 2009).

Outra hipótese que tem sido estudada para avaliar a predisposição a EM é a perda da tolerância imunológica das células T autorreativas que circulam em baixos níveis pelo organismo. As células T regulatórias (Treg) desenvolvem um importante papel no controle das células T autorreativas *in vivo,* fazendo supressão ativa dessas células. A

diminuição das células T $CD4^+$ $CD25^+$ FOX $P3^+$leva ao início da doença autoimune em ratos (SAKAGUSHI, 2000; IRANI, 2005).

A extrapolação dos dados da EAE para a fisiopatologia da EM tem se centrado no papel dos linfócitos T $CD4^+$; no entanto, provavelmente os linfócitos T $CD8^+$, trazem contribuições significativas para a EM. O número de linfócitos T $CD8^+$ ultrapassa o número de linfócitos T $CD4^+$ nas lesões em todos os estágios da EM (LUCHINETTE et al., 2000). Estudos têm mostrado elevado número de linfócitos T $CD8^+$ na circulação de pacientes com EM quando comparado a controles e que essas células produzem citocinas funcionalmente distintas (CRAWFORD et al., 2004). Estudos em tecido cerebral de pacientes com EM mostraram a presença de expansão clonal de linfócitos T $CD8^+$ dentro e ao redor das lesões e ausência de linfócitos T $CD4^+$ (SKULINA et al., 2004; IRANI, 2005).

Doenças inflamatórias desmielinizantes do SNC apresentam importante deposição de imunoglobulina, principalmente da classe IgM, e proteínas do sistema complemento em lesões ativas. Anticorpos anti-MBP têm sido encontrados em soro de pacientes com doença precoce. Um estudo sugeriu que anticorpos anti-MOG e anti-MBP são preditores precisos de pacientes com alto risco converterem em EM definida (BERGER et al., 2003). Um acúmulo de evidências sugere que mecanismos humorais contribuem para a fisiopatologia da EM em alguns casos da doença. Achados como a presença de anticorpos antimielina, expansão clonal de células B nas lesões desmielinizantes (LUCHINETTI et al., 2000), a presença de mecanismos humorais em modelos animais como a EAE e estudos sorológicos realizados em pacientes com EM confirmam a presença de mecanismos humorais envolvidos da fisiopatologia da EM (BERGER et al., 2003).

A resposta inflamatória que ocorre na EM exige a migração de leucócitos para o SNC, mediada pela ação de inúmeras citocinas que agem em uma grande variedade de leucócitos na interação com os receptores transmembrana. As citocinas são importantes componentes do processo inflamatório e estão envolvidas na morte de oligodendrócitos e degeneração axonal (BJARTMAR et al., 2003) e na disfunção neuronal que são características da fisiopatologia da EM (LUCCHINET et al., 2000). As citocinas pró-inflamatórias são as mais investigadas na EM, especialmente o TNF-α e fator de necrose tumoral beta (TNF-β) MYCKO et al., 1998; BUGEJA et al., 2006).

Dada a importância de células inflamatórias dentro e ao redor das placas de EM, os mecanismos imunes que afetam a destruição da mielina têm sido objeto de muitas pesquisas. A EAE imita muitos aspectos da EM, na qual a desmielinização e inflamação ocorrem após a imunização de animais com mielina, proteínas da mielina ou certos peptídeos

dessa proteína (IMITOLA, CHTNIS e KHOURY, 2005). O distúrbio experimental pode ser passivamente transferido para outros animais por meio de células T que reconhecem esses componentes da mielina. O infiltrado nas placas e ao redor das regiões do cérebro consiste de células T $CD8^+$, T $CD4^+$ e macrófagos. Acredita-se que os macrófagos e células T induzam a lesão nos oligodendrócitos. Sugeriu-se que a lesão por células T $CD8^+$ citotóxicas ocorreria via Fas/Fas ligante em que os oligodendrócitos nas lesões expressam Fas, enquanto que o Fas ligante está presente nas células T infiltradas. No entanto, experiências demonstraram que o desenvolvimento de lesões semelhantes à EM requer outros fatores além da imunidade celular (DE GIROLAMI, ANTHONY e FROSCH, 2000).

A EM é considerada uma doença imuno-mediada por linfócitos T $CD4^+$ Th1, que quando ativados, reconhecem os principais autoantígenos da MBP, MOG, proteína proteolipídica (PLP) e glicoproteína associada à mielina (MAG), que se apresentam aumentadas em pacientes com EM (SOSPEDRA E MARTIN, 2005). Os linfócitos T $CD4^+$ Th1 produzem interleucina (IL)-2, IL-6, TNF-α e interferon gama (IFN-γ). Os linfócitos T $CD4^+$ Th2 produzem as citocinas IL-4, IL-5, IL-10 e IL-13 e os linfócitos $CD4^+$ Th17 produzem IL-17 (SUTTON et al., 2006). As atividades biológicas das citocinas e quimiocinas são mediadas pela interação com seus receptores e estão envolvidas na patogênese da resposta imune inflamatória no SNC, no controle da migração dos leucócitos através do endotélio cerebral, ativação e movimentação das células dentro do parênquima cerebral (SIMPSON et al., 2000).

Estudos mostram que existe um número desproporcional de células T $CD4^+$ ativadas no interior das lesões da EM e não está claro se estas células T desempenham um papel primário nos eventos iniciais que levam à destruição da mielina; tampouco foram estabelecidos os eventos desencadeantes que ocorrem dentro ou fora do SNC (SOSPEDRA E MARTIN, 2005).

Smith e McDonald (1999) sugeriram que as citocinas podem desempenhar um papel fundamental no bloqueio da condução dos neurônios, principalmente as citocinas pró-inflamatórias como o TNF-α e IFN-γ. O IFN-γ leva a ativação de células mononucleares, diferenciação de linfócitos T $CD4^+$ para o fenótipo Th1, indução da expressão de MHC classe I e classe II e apoptose de linfócitos T. É considerado o principal marcador da resposta Th1. IFN-γ é expresso pelo SNC no início da EAE e sua expressão aumenta durante os surtos da doença e diminui durante a remissão (BERGOLKA e MILLER, 1998). Uma expressão aumentada de IFN-γ no SNC de ratos leva a doença desmielinizante progressiva (RENNO et al., 1998). No entanto, estudos experimentais como EAE mostram que a ausência de IFN-γ

pode levar a maior suscetibilidade a doença, causar um grande infiltrado inflamatório no SNC e a uma forma progressiva da EAE (FERBER et al., 1996). Esse paradoxo de que a ausência de IFN-γ leva a um quadro clínico mais grave pode estar relacionado com as funções regulatórias de IFN-γ como citocina antiproliferativa (BADOVINAC, TVINNEREIM e HARTY, 2000) bem como de sua ação na indução de apoptose e finalização da resposta imune no SNC (CHU, WITTMWE e DAKTON, 2000). Pacientes com as formas clínicas EM-RR e EM-SP apresentaram níveis elevados de IFN-γ e IL-10; o que não ocorreu em pacientes com a forma EM-PP, sugerindo uma desregulação na sinalização de certos subtipos de EM (BALASHOV et al., 2000). Surtos clínicos estavam relacionados com aumento da produção de IFN-γ *in vitro* (BECK et al., 1988) e que a administração de IFN-γ em pacientes com EM precipitava os surtos (PANITCH et al., 1987).

TNF-α é produzido por células fagocíticas, células *natural killers* (NK), linfócitos B, linfócitos T ativados e micróglia no SNC. A produção de TNF-α está associada à resposta Th1, indução da ativação de uma grande variedade de células e expressão de moléculas de adesão, quimiocinas e citocinas. A expressão de TNF-α está relacionada com o curso da EAE (BEGOLKA e MILLER, 1998). Injeções de TNF-α levam a um significativo prolongamento da EAE e maior infiltrado de células inflamatórias na medula espinhal (KURODA e SHIMAMOTO, 1991). No SNC, a produção local de TNF-α mostrou induzir apoptose de oligodendrócitos (SEMAJ e RAINE, 1988). Estes resultados apontam para um papel inflamatório do TNF-α. No entanto, resultados contraditórios são encontrados na literatura. Ratos deficientes em TNF-α desenvolvem uma forma mais grave de EAE caracterizada por maior inflamação e desmielinização. Estes resultados podem ser explicados, em parte, por diferentes funções dos receptores de TNF (TNFR) na inflamação. Existem dois tipos de TNFR, o receptor de TNF-α I (TNFRI ou p55) e o receptor de TNF-α II (TNFRII ou p75) que podem existir em membrana de células e na forma solúvel. O TNFRI está relacionado com um curso mais grave da doença, inflamação e desmielinização, enquanto que o TNFRII está relacionado com um curso mais brando da doença e remielinização (BERGOLKA e MILLER, 1998; SUVANNAVEJH et al., 2000; EHLING et al., 2003; IMITOLA et al., 2005). Rieckmann et al. (1994) relataram que o TNFRI é encontrado em níveis mais elevados nos pacientes com EM em remissão e aumentam algumas semanas depois do início do surto. TNFRI é necessário para apoptose de células T e tem papel importante na regulação da resposta imune (BACHMANN et al., 1999). O TNF-α possui efeito direto na indução de apoptose de oligodendrócitos e demielinização e promove a proliferação e indução da morte celular de células progenitoras de oligodendrócitos

(ARNETT et al., 2001). Em humanos, a expressão de TNF-α aumenta as lesões na EM e também é expresso por macrófagos, micróglia e astrócitos em lesões crônicas ativas (CANELLA e RAINE, 1995). Vários estudos demonstram uma correlação positiva entre os níveis de TNF-α e o curso clínico de EM (ZIPP et al., 1995; ANDREWS et al., 1998; VAN OOSTEN et al., 1998). Outro estudo relatou um aumento da produção de TNF-α após estimulação *in vitro* que precede o início do surto (BECK et al., 1988). Níveis elevados de TNF-α obtidos no soro e no LCR têm sido relacionados com atividade da doença visualizada pela RMN (SPULER et al., 1996). Khoury et al. (1999) encontraram que os níveis de TNFRI e TNFRII estão aumentados em pacientes com doença crônica progressiva e esses altos níveis estariam relacionados com aumento dos ecores EDSS e aparecimento de novas lesões positivas impregnadas por gadolínio.

IL-12 é uma citocina pró-inflamatória produzida principalmente por monócitos, macrófagos e células dendríticas e é importante na diferenciação de linfócito T $CD4^+$ Th0 em células Th1. No modelo experimental EAE, o mRNA da IL-12 foi encontrado elevado imediatamente após o início da doença (ISSAZADEH et al., 1996). Em pacientes com EM, os níveis de IL-12 estavam elevados e expressos nas placas desmielinizantes (WINDHAGEN et al., 1995). O aumento da expressão de IL-12 no SNC leva ao aumento no processo inflamatório e no infiltrado de células (CAMPBELL et al., 1998).

IL-1 é produzida por monócitos, macrófagos, células endoteliais, linfócitos B e linfócitos T ativados e está presente em concentrações elevadas no SNC durante a indução da EAE (BAUER et al., 1992). Um dos efeitos mais importantes da IL-1β no cérebro é a indução de uma astrogliose reativa, com produção de IL-6 e ativação de astrócitos, efeito que é bastante aumentado na presença de TNF-α, IFN-γ, IL-6 e receptor solúvel de IL-6 (VAN WAGONER e BENVENSITE, 1999). A IL-1 tem se mostrado importante no dano neuronal e axonal no SNC em pacientes com EM (CARLSON et al., 1999).

IL-6 é produzida por fagócitos, células endoteliais, fibroblastos, células T ativadas, astrócitos e microglia. É uma citocina importante para o crescimento e diferenciação de células B. O aumento da expressão de IL-6 no SNC está relacionado com doenças neurodegenerativas (CAMPBELL et al., 1998). Na EM, foram identificados macrófagos e astrócitos que expressavam IL-6. Aproximadamente 10,0-17,0% dos astrócitos e 2,0% dos macrófagos presentes nas lesões expressam IL-6. No entanto, a maior parte de células que expressam IL-6 estavam presente nas lesões desmielinizantes inativas (SCHONROCK, GAWLOWSKI e BRUCK, 2000). Além disso, células T de pacientes com EM apresentam maior número de receptor de IL-6 (BONGIOANNI et al., 2000). No SNC, os astrócitos

produzem IL-6 em resposta ao TNF-α e à IL-1β, ambas citocinas que apresentam níveis elevados na presença de IL-17. IL-1β e TNF-α interagem sinergicamente para estimular a produção de IL-6 pelos astrócitos (VAN WAGONER e BENVENISTE, 1999).

IL-4 é produzida por células T $CD4^+$ Th2 e participa da diferenciação e crescimento de linfócitos B. *In vitro*, a IL-4 inibe a ativação de células Th1 levando a diminuição de IL-1 e TNF-α. Na EAE, a IL-4 atua como supressora da resposta inflamatória. O mRNA de IL-4 apresentou-se indetectável até a remissão da doença EAE em camundongos SLJ (BERKOLGA et al., 1998). Porém, uma pequena expressão de IL-4 no SNC tem sido encontrada em modelos experimentais (ISSAZADEH et al., 1996). A administração intraperitoneal de IL-4 após transferência adotiva de células reativas a MBP reduz a gravidade da EAE (CUA, HINTON e STOHLMAN, 1995). O aumento da expressão de IL-4 pode reduzir a gravidade da EAE, enquanto que a ausência não altera o curso da doença, possivelmente porque na sua ausência, outras citocinas do perfil Th2 possam substituir sua função e contribuir para o mecanismo de tolerância na EAE (IMITOLA et al., 2005). Na EM, níveis elevados de IL-4 são encontrados em lesões ativas agudas e crônicas (CANELLA e RAINE, 1995).

IL-10 é produzida por monócitos, macrófagos, células B e células Th2. *In vitro*, a IL-10 inibe a produção de várias citocinas, incluindo IL-1 e TNF-α, e a proliferação de células T. A sua principal função é inibir a produção de citocinas por macrófagos e reduzir a expressão de moléculas MHC II e co-estimulatórias. Camundongos que expressavam níveis elevados de IL-10 eram resistentes ao desenvolvimento de EAE (CUA et al., 1999). Camundongos deficientes de IL-10 foram mais suscetíveis e desenvolveram uma forma mais grave de EAE quando comparados a camundongos com produção deficiente de IL-4 (BETTELI et al., 1998). Em humanos, estudos mostram que os níveis de IL-10 estão diminuídos antes do início do surto em pacientes com EM-RR (RIECKMANN et al., 1994). Níveis de IL-10 apresentaram-se diminuídos quatro semanas antes da RMN demonstrar impregnação por contraste gadolínio e seis semanas antes do início do surto em pacientes com a forma EM-SP comparados com os que possuem a forma EM-RR (VAN BOXEL-DEZAIRE et al., 1999). Durante a remissão, clones de linfócitos Th2 isolados de pacientes com EM apresentaram produção aumentada de IL-10 e fator de crescimento transformador beta (TGF-β) quando comparados a controles (PELFREY et al., 2000).

A produção de IL-17 parece ter um importante papel na indução de EAE (HARRINGTON et al., 2005; PARK et al., 2005) e a transferência adotiva de células Th17 pode induzir a EAE (LANGRISH et al., 2005). Recentemente, estudos demonstraram que as

células Th17 constituem uma subpopulação diferente das células Th1 e Th2 e que sua diferenciação é inibida pelas citocinas Th1 e Th2. Células T naïve (Th0) se diferenciam nas células T produtoras de IL-17 na presença de TGF-β e IL-6 e ausência de citocinas Th1 e Th2 (IFN-γ e IL-4, respectivamente) (GRABER et al., 2008).

A produção de IL-23 por macrófagos e células dendríticas apresenta um importante papel na contínua estimulação e na sobrevivência das células Th17 (BETTELI et al., 2006). A IL-23 pode agir sobre células T $CD4^+$ de memória e células efetoras para induzir a proliferação de linfócitos T e secreção de IL-17 (AGGARWAL et al., 2003), além de promover a diferenciação das células T naïve em células Th17 (PARK et al., 2005). As células Th17 atuam induzindo a produção de IL-1β e TNF-α, presentes em condições inflamatórias em diversas doenças autoimunes. A citocina IL-12 possui propriedades antagonistas e inibe a expressão de IL-17 (HARRINGTON, MANGAN, WEAVER, 2006). Na EM também foi observado que a IL-17 estimula a produção de IL-6, TNF-α e IL-1β, mediadores implicados na patologia da doença (VAN WAGONER e BENVENISTE, 1999). A expressão de mRNA da IL-17 está aumentada nas lesões agudas da EM, no LCR e nas células mononucleares periféricas durante os surtos (LOCK et al., 2002).

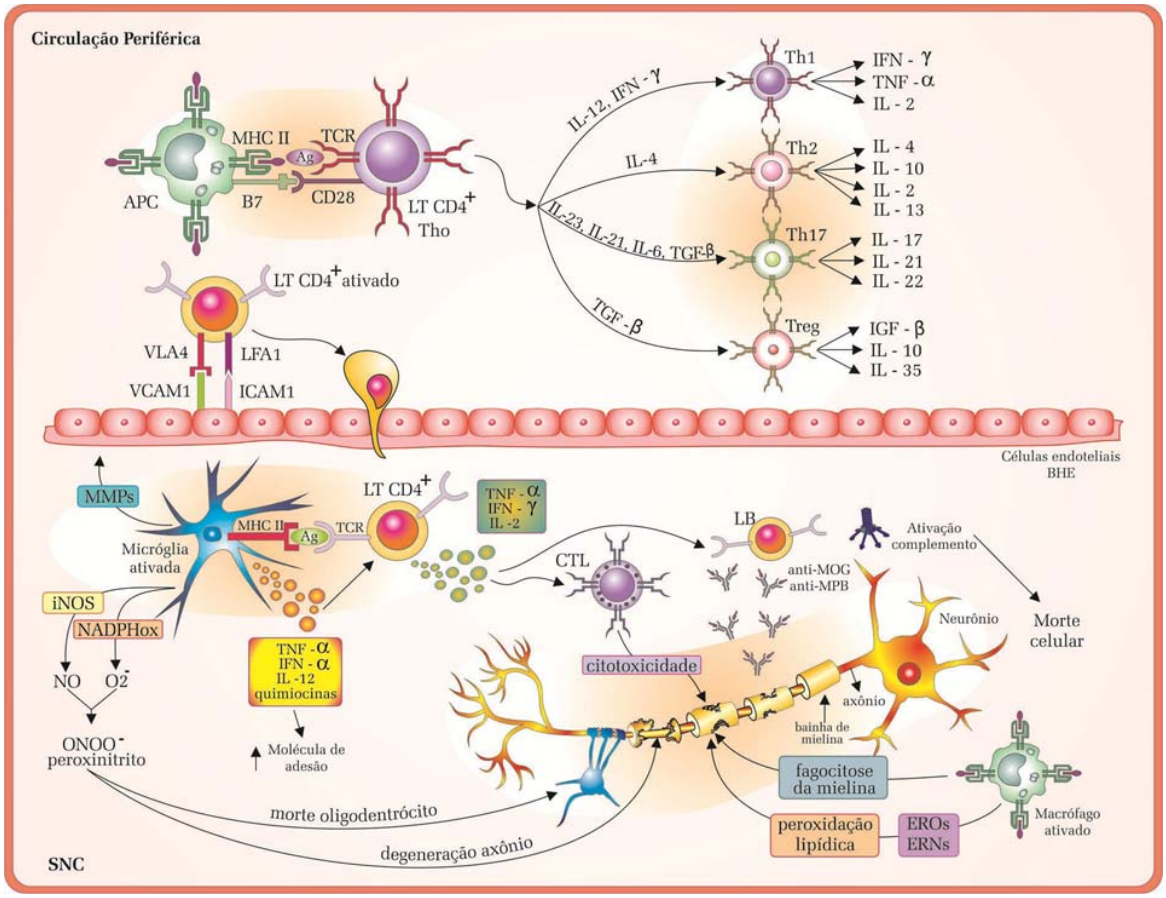
Nem todos os aspectos da inflamação são deletérios ao SNC, uma vez que vários componentes da resposta inflamatória possuem efeitos neuroprotetores. Estudos têm demonstrado que oligodendrócitos e micróglia expressam constitutivamente receptores para citocinas, principalmente do tipo Th2 (CANNELA e RAINE, 2004). A expressão de TGF-β e IL-10 está associada com recuperação da doença na EAE (BERGOLKA e MILLER et al., 1998) e a expressão de TGF-β está localizada nos neurônios (ISSAZADEH et al., 1998). *In vitro*, o TGF-β parece inibir a ativação da caspase-3 e a apoptose neuronal (DEIGNER, et al., 2000). Por outro lado, um aumento na expressão de TGF-β aumenta astrogliose e a infiltração de monócitos, resultando em uma EAE mais grave com início precoce quando comparado a controles (WYS-CORAY et al., 1997). A IL-10 parece diminuir a resposta imune na EAE e ter efeitos neuroprotetores (BETTELLI et al., 1998). Deste modo, citocinas Th2 parecem desempenhar um papel protetor na sobrevivência e diferenciação de neurônios e oligodendrócitos no SNC durante o processo de dano e desmielinização (MARCHIONNI et al., 1999).

Estudos têm demonstrado que, durante as condições inflamatórias e degenerativas, as células da glia no SNC são capazes de expressar as CC quimiocinas (ou β quimiocinas), tais como CCL2 (proteína quimioatraente de monócitos 1 ou MCP-1), CCL3 (proteína inflamatória de macrófagos 1α ou MIP-1α), CCL4 (proteína inflamatória dos

macrófagos 1 beta ou MIP-1β), CCL5 (proteínas reguladas sob ativação normalmente expressas e secretadas por células T normais ou RANTES), além dos receptores de CC quimiocinas, tais como CCR2, CCR3 e CCR5, e os receptores de CXC quimiocinas (CXCR) (SIMPSON et al., 2000). A expressão dos receptores CCR5 e CXCR3 ocorre, preferencialmente, na superfície dos linfócitos Th1 e a expressão de receptores CCR3 e CCR4 na superfície dos linfócitos Th2. O perfil de receptores nas células Th1 pode explicar, em parte, o recrutamento seletivo das subpopulações de células T nos locais de inflamação, incluindo a predominância de células Th1 que expressam CCR5 e CXCR3 nas lesões de EM. Em contraste, CCR2 não é expresso na superfície das células Th1 e, apesar disso, faz uma regulação positiva nas células T ativadas e também está presente na superfície dos fagócitos mononucleares (SIMPSON et al., 2000).

Um estudo realizado em Londres, Inglaterra, que avaliou 14 indivíduos com EM e 14 indivíduos sem EM, pareados pela idade e que foram a óbito, demonstrou níveis elevados de receptores de quimiocinas nos indivíduos com lesões ativas de EM. Este resultado está de acordo outros relatados na literatura, em que foram encontradas lesões com níveis elevados dos ligantes desses receptores. A maioria dos receptores CCR3 e CCR5 estava presente na membrana das células encontradas nas lesões, semelhante aos macrófagos. Células T perivasculares expressavam receptores CCR3 enquanto que células T $CD4^+$ expressavam CCR5, sugerindo infiltração de linfócitos com fenótipo Th1. O número de linfócitos T $CD4^+$ presente nos infiltrados crônicos de lesões da EM e que expressava CCR5 foi estatisticamente significativo ($p<0,001$) quando comparado com a expressão de CCR3. O receptor CCR2 foi identificado em infiltrados de células nos vasos das placas ativas (SIMPSON et al., 2000). A Figura 1 sumariza os principais eventos celulares e moleculares que ocorrem na fisiopatologia da EM.

Figura 1 – A esclerose múltipla (EM) é uma doença desmielinizante do sistema nervoso central (SNC). A ativação da resposta imunológica se inicia na periferia com as células apresentadoras de antígenos (APCs), como os macrófagos, apresentando antígenos (Ag) aos LT $CD4^+$ naïves (Th0) via MHC classe II e co-estimulador B7. O microambiente, por sua vez, produz uma série de citocinas e metaloproteinase que vão induzir a diferenciação dos LT $CD4^+$ Th0 em Th1, Th2, Th17 e Treg. A ativação do LT $CD4^+$ induz a expressão de moléculas de adesão em sua superfície com o antígeno muito tardio-4 (*very late antigen 4*- VLA4) e o antígeno associado à função de linfócitos 1 (*Lymphocyte function-associated antigen 1* - LFA1). A barreira hematoencefálica (BHE) passa a expressar moléculas de adesão celular vascular (*vascular cellular adhesion molecule* - VCAM1) e moléculas de adesão intercelular (*intercellular adhesion molecule* - ICAM 1). A interação do VLA4 com a VCAM1 e do LFA1 com a ICAM1 permite a entrada dos LT $CD4^+$ no SNC. No SNC, a micróglia irá apresentar Ag via MHC classe II a esses LT $CD4^+$. A micróglia, por usa vez, produz citocinas inflamatórias como fator de necrose tumoral alfa (TNF-α), interferon alfa (IFN-α), interleucina 12 (IL-12), quimiocinas e metaloproteinases (MMPs). As citocinas e quimiocinas irão aumentar a expressão de moléculas de adesão na BHE e ativar LT $CD4^+$, enquanto que as MMPs irão ajudar na degradação da BHE e assim, permitir a entrada de mais LT no SNC. Os LT $CD4^+$ ativados produzem citocinas como TNF-α, interferon gama (IFN- γ) e interleucina 2 (IL-2) que irão ativar a resposta citotóxica e humoral. Os LT $CD8^+$ citotóxicos irão fazer citotoxicidade direta na bainha de mielina, enquanto que a imunidade humoral irá produzir anticorpos contra componentes da mielina, que ativam as proteínas do sistema complemento, contribuíndo para a lesão da bainha de mielina.



1.2 POLIMORFISMOS NO GENE DO TNF E O SEU ENVOLVIMENTO NA EM

Os genes do TNF-α e TNF-β (também denominado de Linfotoxina alfa, LTa) estão inseridos na região do MHC classe III, no braço curto do cromossomo 6 humano. O TNF-α é a principal citocina pró-inflamatória secretada, principalmente, por macrófagos e linfócitos Th1. O aumento da expressão do TNF-α tem sido associado com a atividade clínica da EM-RR e com o desenvolvimento da EM-PP e EM-SP. O TNF-α aumenta a resposta imune por meio da apresentação de antígenos pelas moléculas MHC de classe II, pelo aumento da expressão de moléculas de adesão e pela disfunção da BHE (IMITOLA et al., 2005).

Polimorfismos nos genes do TNF-α e TNF-β estão relacionados com o aumento da susceptibilidade a doenças autoimunes (FUGGER et al., 1990; ROTH et al., 1994) e na fisiopatologia da EM (HUIZINGA et al., 1997; MYCKO et al., 1998; KAMALI-SAVERSTANI et al.; 2007). Um polimorfismo de um único nucleotídeo (SNP) na posição -308 na região promotora do gene do TNF-α, que consiste na troca de uma G (alelo TNF1) por uma A (alelo TNF2), têm sido associado com o aumento da expressão desta citocina após estimulação *in vitro* e ao aumento dos níveis de transcrição do gene do TNF-α, quando comparado com o tipo selvagem (alelo TNF1). Um estudo sobre o polimorfismo do TNF-α -308 realizado em um grupo de 87 pacientes com EM demonstrou que o alelo TNF2 estava presente em 26 (30,0%) pacientes e que este alelo não estava associado com o curso clínico da doença. Os autores concluíram que o alelo TNF2 como marcador genético, *per se*, não contribui significativamente para a gravidade da EM e que devem existir outros processos primários que afetem a atividade da EM não relacionados com as variações do gene do TNF-α. No entanto, foram demonstradas diferenças funcionais no polimorfismo -308 do TNF-α pelo aumento da expressão do mRNA do gene do TNF-α em indivíduos que apresentavam o alelo TNF2 (MÄURER et al., 1999).

Diferenças individuais na capacidade de produção do TNF-α podem ser causadas por diferenças na taxa de transcrição, de regulação da estabilidade do mRNA, na eficiente tradução ou processamento da proteína matura. Polimorfismos nos genes que codificam as proteínas que regulam esse processo podem causar diferenças na produção do TNF-α (HUIZINGA et al., 1997).

Outro estudo avaliou a distribuição dos polimorfismos nas posições -308, -238 e -376 na região promotora do gene do TNF-α em dois grupos diferentes de pacientes

com EM e demonstrou que o polimorfismo -238 G/A é menos comum em pacientes com danos neurológicos graves, sugerindo que o alelo -238 A exerceria um efeito protetor contra os danos causados pela EM. No entanto, não foi associado com diferenças na susceptibilidade a EM (HUIZINGA et al., 1997).

Estudos mostram que o TNF-α é um mediador crítico envolvido na imunopatogenicidade da EM e possui uma ampla variedade de respostas incluindo morte das células por apoptose, lesão isquêmica e citotóxica nos neurônios e também neuroproteção. A coexistência de efeitos neurotóxicos e neuroprotetores do TNF-α está relacionada à ligação a seus receptores TNFRI e TNFR II expressos na superfície das células ou na forma solúvel. Estudos *in vivo* têm demonstrado que TNFR I é o principal mediador da inflamação e desmielinização do SNC, enquanto que o TNFR II aumenta a remielinização. Concentrações da forma solúvel dos receptores têm sido encontradas e correlacionadas com a atividade clínica da EM. Pesquisadores investigaram as variações genéticas do TNFR II e a associação com susceptibilidade e progressão clínica da EM. EHLING et al. (2003) estudaram um SNP no exon 6, posição 783, com a troca de G por A que resultou na substituição não conservada do ácido glutâmico (códon GAA) por uma lisina (códon AAA). Também foram confirmados outros SNPs já conhecidos como no exon 6 localizados nas posições 676, 1668 e 1690. Somente o SNP da posição 1668 apresentou diferença significativa na frequência dos alelos e na distribuição dos genótipos observadas no grupo de pacientes com EM quando comparadas com um grupo controle (p=0,019).

Um SNP localizado na posição +252 do primeiro intron do gene do TNF-β consiste na troca de uma G (alelo selvagem) por uma A (alelo variante) foi detectado por meio do polimorfismo do comprimento do fragmento de restrição (RFLP) após a digestão com a enzima de restrição *Nco*I, sendo denominado de polimorfismo *Nco*I do gene do TNF-β (MESSER et al., 1991). Inicialmente foi denominado como polimorfismo +252 (G>A) e atualmente é reconhecido também como polimorfismo rs909253. Estudos têm demonstrado que este polimorfismo é potencialmente funcional e que está relacionado com aumento das condições inflamatórias por resultar em alterações na região transcricional do gene do TNF-α, com aumento na expressão deste gene e, consequentemente, em maior produção da citocina TNF-α, um importante fator na patogênese da EM (MESSER et al., 1991; EBERS e SANDOVNICK et al., 1994; SHARNA et al., 2006).

Kamali-Savertani e colaboradores (2007) avaliaram o polimorfismo *Nco*I no gene do TNF-β e o polimorfismo -308 no promotor do TNF-α na população iraniana com EM. A frequência alélica e genotípica encontrada em ambos os polimorfismos não diferiu

entre os pacientes com EM e controles, sugerindo que estes polimorfismos não estariam relacionados com a susceptibilidade à EM.

O polimorfismo na posição 26 do exon 3 no gene do TNF-β, com a substituição de um aminoácido aspartato por uma treonina, está correlacionado com a redução na produção de TNF-α. Já o polimorfismo *Nco*I no gene do TNF-β, localizado no intron 1, posição +252, tem sido descrito e correlacionado com o polimorfismo do exon 3 e afeta os níveis de expressão do TNF-β. A combinação dessas formas alélicas pode levar a níveis diferentes de produção de citocinas em resposta a vários estímulos fisiológicos ou patológicos que, por sua vez, pode aumentar a predisposição e desenvolvimento da EM ou explicar os diferentes cursos clínicos da doença (MYCKO, 1998). Em pacientes com EM-RR da população polonesa, foram avaliados os SNPs da região promotora do *TNF-α* (posição -308) e no exon 3 do *TNF-β* . A combinação dos alelos G+A (SNP do *TNF-α* -308) com os alelos C+C (SNP do TNF-β) estava presente seis vezes mais em indivíduos com EM do que nos controles, enquanto que as outras combinações alélicas não apresentaram diferenças significativas. Embora os dois SNPs foram previamente relacionados com aumento na produção das citocinas, os níveis séricos de TNF-α e TNF-β não apresentaram diferenças significativas (MYCKO, 1998).

1.3 TRATAMENTO DA EM

Resultados contraditórios têm sido encontrados na literatura sobre a eficácia dos medicamentos utilizados para o tratamento de pacientes com EM. Estudos mostram que o tratamento com IFN-β pode levar a uma elevação transitória nos níveis de TNF-α nos três primeiros meses de tratamento que não permanece após três meses de uso (DUDDY et al., 1999). Por outro lado, Furlan e colaboradores (2000) demonstraram que o tratamento com IFN-β causa uma profunda e constante diminuição nos níveis de IFN- γ e IL-4, sugerindo um amplo efeito imunomodulador. Rep e colaboradores (1999) sugerem que o tratamento com IFN-β leva a uma diminuição da resposta imune mediada por células Th1.

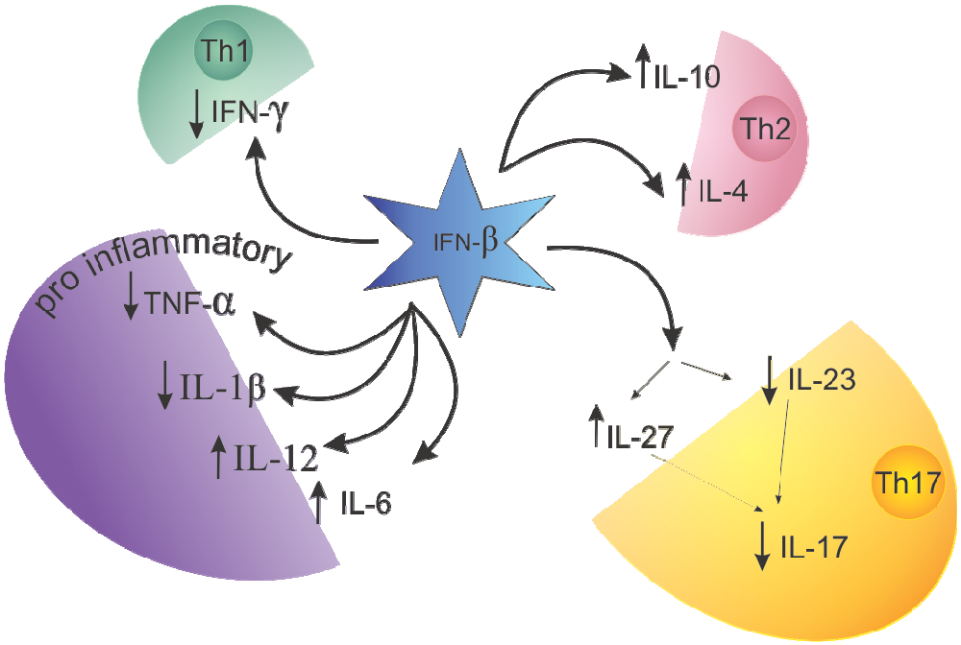
TNF-α e IFN-γ são potentes estimuladores da expressão da enzima óxido nítrico sintase induzível (iNOS) e esta enzima pode produzir, de forma contínua, níveis elevados de óxido nítrico (NO). De fato, a produção de NO está aumentada em pacientes com EM. Estudos (MOLLACE et al., 2001; GONSETE, 2008) têm demonstrado que baixas concentrações de NO podem levar ao bloqueio da condução em axônios normais e, em

particular, axônios afetados pela desmielinização. Existem evidências de que o NO seja produzido em altas concentrações nas lesões de EM e que alguns dos déficits clínicos estão associados com a inflamação mediada pelo NO. Assim, medidas que reduzem a produção de NO podem ser terapeuticamente efetivas e, neste contexto, é interessante mencionar que uma das ações do IFN-β é o bloqueio da iNOS.

A inflamação pode contribuir para o déficit neurológico por modificar as propriedades das células gliais, principalmente astrócitos e micróglia (HUA et al., 1998; IMITOLA et al., 2005; GONSETE, 2008;). Cucci e colaboradores (2010) compararam os níveis séricos de mRNA de citocinas envolvidas da imunopatogênese da EM em amostras de pacientes em tratamento com IFN-β 1b e demonstraram que o IFN- β diminui a expressão de citocinas pró-inflamatórias como IFN-γ e TNF-α e aumenta a expressão de citocinas da resposta Th2 como a IL-10. Sweeney e colaboradores (2011) relataram que a terapia com IFN-β inibe a produção de IL-27, e com isto, ocorre inibição das células $CD4^+$ Th17 diretamente ou por meio da inibição de citocinas que promovem o desenvolvimento destas células patogênicas. Sweeney e colaboradores (2011) também demonstraram, em ratos e em humanos, que o IFN-β inibe a produção de IL-1 e de IL-23, consideradas importantes citocinas para a estimulação da produção de IL-17 (Figura 2).

LANGRISH e colaboradores (2005) também verificaram que o tratamento com IFN-β inibe a síntese de IL-23, citocina importante na diferenciação das células Th17 e consequente produção de IL-17. IL-23 e IL-12 possuem propriedades antagonistas, ou seja, a IL-23 leva a produção de IL-17 enquanto que a IL-12 inibe sua diferenciação (HARRINGTON et al., 2006). A IL-17, por sua vez, aumenta a produção de IL-6, TNF-α e IL-1β por macrófagos e de IL-6 por astrócitos na EM, citocinas que tem sido implicado na patologia da doença (VAN WAGONER e BENVENISTE, 1999) (Figura 2).

Figura 2 - Mecanismos de ação da terapia com interferon-beta (IFN-β) utilizada para tratamento da esclerose múltipla. O IFN-β diminui a resposta Th1, reduz os níveis de interferon gamma (IFN-γ) e aumenta a resposta Th2 com consequente aumento de interleucina 4 (IL-4) e interleucina 10 (IL-10). O medicamento IFN-β tambem diminui os níveis de citocinas pró-inflamatórias como fator de necrose tumoral alfa (TNF-α) e interleucina 1 beta (IL-1β) enquanto aumenta os níveis de interleucina 6 (IL-6) e interleucina-12 (IL-12). A terapia com IFN-β também age sobre as células Th17 e diminui seus níveis séricos ao inibir a interleucina 23 (IL-23) e/ou ao aumentar a produção da interleucina-27 (IL-27).



O natalizumab é um anticorpo monoclonal dirigido contra a subunidade α4 do receptor *very late antigen* 4 (VLA 4), α4β1 e α4β7, localizado na superfície de linfócitos. A imunoglobulina IgG_4 humanizada se liga especificamente à superfície de células mononucleares circulantes que apresentam a subunidade α4 do VLA4 inibindo a ligação do VLA4 às moléculas de adesão molécula da adesão celular vascular (VCAM) presente no endotélio; inibindo, deste modo, a passagem das células através do endotélio. Na EM, os efeitos observados estão relacionados ao bloqueio do tráfego de células T através do endotélio e BHE (YEDNOCK et al., 1993; RAMOS-CEJUDO et al., 2011). Natalizumab é utilizado para o tratamento de pacientes com EM-RR. Estudos mostram que os níveis de IFN-γ e IL-12, assim como IL-4 e IL-10, permanecem elevados apenas algumas horas ou dias após a administração de natalizumab. Outras citocinas pró-inflamatórias, tais como IL-17, IL2 e IL-1β, apresentavam níveis aumentados após longo período de tratamento. Por outro lado, não foi encontrado nenhum efeito sobre as células Treg. Portanto, os resultados sugerem que

natalizumab apresenta outro mecanismo de ação além da interação e bloqueio do VLA4 e do extravasamento de células pela BHE (RAMOS-CEJUDO et al., 2011).

Outra estratégia terapêutica na EM é o acetato de glatiramer, um polímero composto por quatro aminoácidos que são encontrados na mielina. O medicamento funciona como uma falsa mielina e faz com as células de defesa ataquem o polímero ao invés da mielina. A administração de acetato de glatiramer leva a uma alteração do perfil pró-inflamatório Th1 para perfil anti-inflamatório Th2. Na EM, a citocina IL-1β está presente no SNC, sendo expressa principalmente nos infiltrados de macrófagos e células microglia. A atividade da IL-1β é inibida pela forma solúvel do antagonista do receptor de IL-1 (sIL-Ra). O acetato de glatiramer e o IFN-β induzem a expressão de sIL-1Ra pelos monócitos que exercem o efeito anti-inflamatório no SNC após atravessarem a BHE (CARPINTERO e BURGER, 2011).

A EM tem se mostrado uma doença poligênica, com o envolvimento de múltiplos genes, cada um contribuindo um pouco com a patogênese da doença. Desta forma, tornam-se necessário a realização de mais estudos na área de polimorfismos genéticos para um melhor entendimento da EM. Estudos prévios sobre polimorfismos genéticos, em especial do *TNF-β*, foram realizados em pacientes com EM de diferentes populações do Hemisfério Norte, como os Europeus e os Asiáticos (HUIZINGA et al., 1997; MYCKO et al., 1998; KAMALI-SARVESTANI, et al., 2007;). Os resultados obtidos são, por muitas vezes, contraditórios e de limitado poder de generalização para populações geneticamente mais heterogêneas como a brasileira. O perfil de citocinas inflamatórias e anti-inflamatórias tem sido muito pouco investigado em pacientes com EM da população brasileira. As citocinas estão envolvidas nos mecanismos de lesão e desmielinização na EM; no entanto, sua ação é de grande complexidade, pois elas agem como uma rede de moléculas com ações sinérgicas ou antagônicas. Além disto, estudos prévios avaliaram estes marcadores em um número limitado de pacientes, o que também limita o conhecimento do real papel do polimorfismo *Nco*I do gene do TNF-β e do perfil de citocinas na susceptibilidade e curso clínico da EM. Deste modo, este estudo tem como justificativa a investigação deste fator genético e do perfil de citocinas proinflamatórias e anti-inflamatórias em pacientes com EM da região sul do Brasil, na tentativa de identificar biomarcadores viáveis e que apresentem associação com a susceptibildiade e curso clínico da EM e, com isto, contribuam para o desenvolvimento de novos alvos terapêuticos para o tratamento de pacientes com EM.

2 OBJETIVOS

2.1 OBJETIVO GERAL

- ✓ Avaliar o polimorfismo genético *Nco*I do TNF-β e o perfil de citocinas em pacientes com EM da população sul brasileira.

2.2 OBJETIVOS ESPECÍFICOS

- ✓ Determinar a frequência do polimorfismo genético *Nco*I do TNF-β em pacientes com EM e em indivíduos saudáveis;
- ✓ Determinar os níveis séricos de citocinas inflamatórias (IL-1, IL-6, TNF-α, IFN-γ e IL-17) e anti-inflamtórias (IL-4 e IL-10) em pacientes com EM e em indivíduos saudáveis;
- ✓ Avaliar a associação entre o polimorfismo genético *Nco*I do TNF-β e o polimorfismo genético do HLA-DRB1*;
- ✓ Avaliar a associação entre o polimorfismo genético *Nco*I do TNF- β com a progressão da doença avaliada pelo EDSS;
- ✓ Avaliar a associação entre o polimorfismo genético *Nco*I do TNF- β com a atividade da doença avaliada pela RMN;
- ✓ Avaliar os níveis séricos das citocinas de acordo com a progressão da EM obtida pelo EDSS;
- ✓ Avaliar os níveis séricos de citocinas de acordo com atividade da doença obtida pela RMN.

3 METODOLOGIA

3.1 COMITÊ DE ÉTICA

O estudo foi aprovado pelo Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina (ANEXO A). Os indivíduos foram convidados a participar voluntariamente da pesquisa e um termo de consentimento livre e esclarecido (TCLE) foi obtido dos indivíduos envolvidos na pesquisa ou de seus responsáveis (ANEXO B).

3.2 DELINEAMENTO

Foi realizado um estudo observacional caso-controle.

3.3 CÁLCULO DO TAMANHO DA AMOSTRA

O cálculo do tamanho da amostra foi realizado no STATCALC do Programa Epi Info versão 6.04d. Para calcular o tamanho da amostra, foram considerados os dados obtidos em um estudo anterior (Kamali-Saverstani et al., 2007), em que a frequência do alelo TNFB2 foi de 70,0% em pacientes com EM e de 72,1% em controles saudáveis. De acordo com os cálculos, a estimativa do tamanho da amostra para o presente estudo foi de 215 indivíduos em cada grupo avaliado.

3.4 POPULAÇÃO

A população de casos foi constituída por pacientes com EM, ambos os sexos, atendidos no Ambulatório de Neurologia, do Ambulatório do Hospital de Clínicas (AHC) da Universidade Estadual de Londrina (UEL). A população controle foi constituída por doadores de sangue fidelizados, de ambos os sexos, do Hemocentro Regional de Londrina, em Londrina, Paraná.

3.5 AMOSTRA

A amostra de 220 pacientes com EM foi obtida por conveniência de tempo e local, de forma consecutiva. Os pacientes tiveram o diagnóstico de EM segundo os Critérios de McDonald (POLMAN et al., 2005) e classificados como EM-RR, EM-SP, EM-PP e CIS. A gravidade e progressão da doença foram avaliadas por meio da *Expanded Disability Status Scale* (EDSS), com escores que variam de 0,0 a 10,0 (KURTZKE, 1983). Os dados relativos ao diagnóstico e EDSS *baseline* foram coletados retrospectivamente de prontuários médicos. Em todos os pacientes, o ESS foi avaliado no período de remissão clínica, considerada como ausência de surtos nos últimos três meses. Os valores de EDSS foram categorizados em brando (0,0 – 4,0), moderado (4,5 – 5,5) e grave (6,0 – 10,0) (KAMALI-SAVERSTANI, et al.; 2007). Para avaliação da progressão da EM foram utilizados dois métodos de avaliação: a) o índice de progressão (*progress index*, PI) (MAÜRER et al., 1999) e b) progressão (KOCH et al. (2007). O PI é uma taxa de acúmulo de incapacidades calculado pelo EDSS/duração da doença em anos (MAÜRER et al., 1999). A progressão em pacientes com EDSS basal < 6,0 ocorre quando existe um aumento de um ponto no EDSS; e para pacientes com EDSS ≥ 6,0, a progressão da doença corresponde ao aumento de 0,5 ponto na EDSS (KOCH et al., 2007).

Os dados demográficos, clínicos e terapêuticos dos pacientes inseridos no estudo foram coletados pelo grupo de pesquisa por meio de um questionário padronizado (ANEXO C) e por consulta aos prontuários do Hospital Universitário da Universidade Estadual Londrina.

O grupo controle foi constituído por 278 indivíduos selecionados de forma consecutiva entre os doadores de sangue fidelizados do Hemocentro Regional de Londrina. Os dados demográficos e clínicos foram coletados por meio de um questionário padronizado (ANEXO C).

3.6 CRITÉRIOS DE INCLUSÃO E EXCLUSÃO

Para o grupo de pacientes com EM, foram incluídos indivíduos de ambos os sexos e que estavam em fase de remissão clínica da EM.

Para o grupo controle, foram incluídos doadores de sangue que não apresentaram reatividade em todos os testes sorológicos empregados na triagem sorológica para doadores de sangue, segundo as normas do Ministério da Saúde (Brasil, 2004). Os indivíduos também não apresentavam sinais e sintomas de processo inflamatório, infeccioso e

autoimune. Relataram não estar em uso de medicamentos que alterem a resposta imune como anti-inflamatórios ou imunomoduladores.

3.7 COLETA DE SANGUE

Amostras de sangue periférico dos indivíduos envolvidos na pesquisa foram coletadas com o sistema de coleta à vácuo em tubos com ácido etilenodiamino tetracético (EDTA) como anticoagulante e em tubos sem anticoagulante. Após a coleta, as amostras foram identificadas com número para garantir a confidencialidade. O material foi imediatamente centifugado a 3000 r.p.m e *buffy-coat* e soro foram aliquotados em tubos tipo *eppendorf* e armazenados em *freezer* -80°C para posterior análise.

3.8 POLIMORFISMO GENÉTICO *NCO*I DO TNF-β

O DNA genômico foi extraído com o kit de extração de DNA Biopur (Biometrix Diagnóstica, Curitiba, Paraná) de acordo com instruções do fabricante, com algumas modificações, como o volume de *buffy coat* utilizado (200μL) e a temperatura do tampão de eluição (70°C). A presença e integridade do DNA extraído foram avaliadas por meio de eletroforese em gel de agarose a 1%, corado com brometo de etídio e visualizado na presença de luz UV, comparando a um DNA padrão.

Um fragmento de 782 pares de base (pb) do gene do TNF- β foi amplificado utilizando a reação em cadeia da polimerase (*Polymerase Chain Reaction* - PCR) de acordo com dados descritos na literatura (MAJETSCHAK, et al., 1999; MAJETSCHAK et al., 2002; DELONGUI, et al., 2011) com algumas modificações. Os *primers* foram desenhados de acordo com *GenBank* número X02911 (MAJETSCHAK et al., 1999). O *Primer* 1 (TNFB1 *sense*) é formado pela sequência 5' CCG TGC TTC GTG CTT TGG GAC TA 3' e o *Primer 2* (TNFB2 *antisense*) é formado pela sequência 5' AGA GGG GTG GAT GCT TGG GTT TC 3' (Invitrogen™, Life Technologies, Carlsbad, CA, USA). A reação de PCR foi realizada em um volume final de 25μL, contendo 2,5mM de cada *primer*, 50 mM de $MgCl_2$, 1,25mM de dNTP *(*Invitrogen™, Life Technologies, Carlsbad, CA, USA*)*, 1,25U da enzima DNA polimerase recombinate diluída em seu tampão (Invitrogen™, Life Technologies, Carlsbad*, CA, USA)*, e *2*μL da amostra de DNA. A reação de amplificação foi realizada em termociclador PCR Sprint-Thermo Hybaid® (Biosystems, Barcelona, Espanha), com um ciclo

de desnaturação inicial de 5 minutos a 94ºC; seguido por 37 ciclos de 45 segundos a 94ºC para desnaturação, 45 segundos a 67°C para o anelamento e 45 segundos a 72°C para extensão; e 10 minutos a 72°C para a extensão final. Um controle negativo (sem amostra de DNA) e um positivo foi incluído em cada bateria de PCR. A amplificação do gene do TNF-β pela PCR foi avaliada por meio da eletroforese em gel de agarose a 1%, utilizando um marcador *ladder* de 100 pares de bases (Invitrogen™, Life Technologies, Carlsbad, CA, USA), corado com brometo de etídio e visualizado em presença de luz UV através do sistema *L-PIX HE* (Loccus Biotecnology, Cotia, Brasil).

O produto de PCR foi submetido à digestão enzimática pela enzima *Nco*I recombinante (Invitrogen, Life Technologies, Carlsbad, CA, USA). Dez microlitros do produto de PCR foi completamente digerido com 0,3μL de enzima *Nco*I 10U/μL por 4h a 37°C. Os fragmentos da digestão enzimática foram analisados pelo método de polimorfismo do comprimento dos fragmentos de restrição (*Restriction Fragment Length Polymorphism,* RFLP) em eletroforese com gel de agarose a 3% (70V, 70 minutos), após coloração com brometo de etídio, de acordo com dados descritos na literatura (MAJETSCHAK, et al., 1999; MAJETSCHAK et al., 2002; DELONGUI, et al., 2011). O alelo TNFB1 apresenta um fragmento de 196 pb e outro de 586 pb (inclui o sítio de restrição da enzima *Nco*I em ambos os alelos). O alelo TNFB2 resulta em um único fragmento de 782 pb (ausência do sítio de restrição em ambos os alelos). O genótipo heterozigoto TNFB1/TNFB2 resulta em 3 fragmentos, com 782 bp, 586 bp 196 bp. As imagens foram capturadas e armazenadas pelo sistema *L-PIX HE* (Loccus Biotecnology, Cotia, Brasil).

3.9 POLIMORFISMO GENÉTICO DO HLA-DRB1*

O polimorfismo genético do HLA-DRB1* também foi realizado a partir do DNA extraído de células do sangue periférico e fez parte de um estudo anterior na realizado na mesma população inserida no presente estudo (Kaimen-Maciel et al., 2009). Naquela ocasião, O DNA foi amplificado por PCR e hibridizado a sequências de primers de oligonucleotideos (PCR-SSO) utilizando tecnologia Limunex para genotipagem de HLA (One Lambda, Canoga Park, CA, USA). A leitura e interpretação foram realizadas por citometro de fluxo (One Lambda) de acordo com instruções do fabricante (KAIMEN-MACIEL et al., 2009).

3.10 CITOCINAS

As citocinas IL-1, IL-4, IL-6, IL-10, IL-12, IL-17, TNF-α e IFN-γ foram mensuradas em amostras de soro de pacientes com EM e de controles saudáveis pelo método de enzimaimunoensaio (ELISA) indireto com o emprego de conjunto de reagentes disponíveis comercialmente (eBioscience, San Diego, California, USA), de acordo com instruções do fabricante. Os resultados foram expressos em pg/mL.

3.11 ANÁLISE ESTATÍSTICA

Um banco de dados foi criado no Programa Microsoft Office Excell 2007 e a análise estatística foi realizada no Programa Graph Pad Prism 5. Variáveis categóricas foram analisadas pelo Teste de Qui-quadrado ou Exato de Fisher, quando apropriado, e a as variáveis contínuas foram analisadas pelo Teste de Mann Whitney. Para a análise de variáveis contínuas de dois ou mais grupos foi utilizado o Teste de Kruskal-Wallis com Pós-teste de Dunn. O Equilíbrio de Hardy-Weinberg foi calculado pelo teste de Chi-quadrado. Para determinar quais fatores estavam associados independentemente à EM, foi realizada uma análise multivariada de regressão logística, utilizando-se o programa GraphPad Instat versão 3.0 (GrahpPad Software, San Diego, CA). Valor de $p < 0,05$ foi considerado estatisticamente significativo.

4 RESULTADOS

Os resultados obtidos durante o desenvolvimento do projeto de pesquisa que fundamentou esta dissertação foram apresentados e discutidos em três artigos científicos descritos a seguir: O primeiro artigo foi de revisão da literatura sobre os polimorfismos genéticos envolvidos na EM com o título: **Genetic polymorphisms associated with the development and clinical course of multiple sclerosis**, publicado no periódico International Journal of Molecular Medicine, 4, 28, 467 – 479.

O segundo artigo apresenta os resultados sobre o papel do polimorfismo genético *Nco*I do TNF-β no desenvolvimento, progressão e atividade da EM com o título: **Tumor necrosis factor beta (TNF-β) *Nco*I genetic polymorphism is associated with multiple sclerosis independently of HLA-DRB1*,** submetido à publicação no periódico Cytokine. E o terceiro artigo, sobre os níveis séricos de citocinas obtidos nos pacientes com EM e a associação com a incapacidade e atividade da doença: **Cytokine profile in relapsing-remitting multiple sclerosis patients and the association with the progression and the activity of the disease,**também submetido à publicação no periódico Cytokine.

Genetic Polymorphisms Associated With the Development and Clinical Course of Multiple Sclerosis (Review)

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Abstract. Multiple sclerosis (MS) is an autoimmune disease characterized by areas of inflammation, demyelination and axonal damage. The etiology of MS is multifactorial with an interaction between genetic, environmental and geographical factors. The objective of this study was to review the physio-pathology and the genetic polymorphisms associated with the development and clinical course of MS. Studies carried out in populations worldwide showed that polymorphisms in the genes of the major histocompatibility complex (MHC) class IIand class IIIhave been associated with susceptibility, resistance and clinical forms of MS. Considerable attention has been focused on studies evaluating disease-modifying effects in MS that identified seven genes of probable importance such as the HLAclass II, ApoE, IL-1ra, IL-1β, TNF-α, TNF-β and CCR5 genes. However, the results described in the literature about genetic biomarkers in MS are not consistent in the worldwide population. The detection of a single nucleotide polymorphism involved in the etiology and physiopathology of MS is very difficult and, it is likely that, several genetic polymorphisms are involved, each with a small contribution to the susceptibility or resistance to MS. Taken together the results show the need for continued research in genetically heterogeneous populations to identify new biomarkers associated with MS that could be used as prognostic markers or as therapeutic targets to modulate the autoimmune response in MS patients. This information may contribute to a better understanding of the physiopathology and treatment of MS.

Key words: multiple sclerosis, genetic polymorphism, tumor necrosis factor α, tumor necrosis factor β, major histocompatibility complex, with the possibility of developing different therapeutic strategies according to the genetic profile of each individual.

1. Introduction

Multiple sclerosis (MS) is a chronic disorder of the central nervous system (CNS) characterized by an autoimmune response directed against myelin proteins and other unidentified antigens, resulting in demyelination and dense astrogliosis in the white substance

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of the CNS. Clinically, MS is characterized by episodes of focal disorders of the optic nerves, trunk, periventricular region, spinal cord and cerebellum, with remission of a variable magnitude and relapse in a period of many years. The clinical manifestations are vague, being determined by the location and extent of the demyelination (1-3).

Several clinical forms of MS have been identified, such as the relapsing-remitting (RR-MS), the secondary progressive (SP-MS), the primary progressive (PP-MS) and the benign forms (4-6). Approximately 80% of the patients will initially present the RR-MS form, in which there are unpredictable attacks (remission), appearance of new symptoms or increased severity existing symptoms. SP-MS is characterized by progres-sion that is not relapse-related. PP-MS affects around 10-15% of all MS patients and is characterized by a lack of distinct attacks, but with slow onset and then steadily worsening symptoms. There is an accumulation of deficits and disability, which may level off at some point or continue over the years.

The diagnosis of a benign form MS is retrospective when the accumulated disability from RR-MS is either mild or non-existent after a long period (usually considered to be 15-20 years) (7).

RR-MS and SP-MS may together be considered as about-onset disease, as ultimately the majority of patients with RR-MS develop a secondary progressive course (8). However, the interval between the onset of the disease and the beginning of the secondary progressive course may be influenced by genetic factors. Genetic markers predicting this interval could be pertinent to decisions about early institution of long-term immune modulation therapy protocols.

In addition to the broad clinical spectrum of the MS, there is also heterogeneity in the morphological alterations of the brain identified by magnetic resonance imaging (MRI) or by histopathological evaluation, as well as in the clinical presen-tation, e.g. in the response to therapy and in which CNS system and areas are primarily affected (9-11). The 2005 revisions to the McDonald diagnostic criteria for MS, with the use of imaging and cerebrospinal fluid (CSF) findings, introduced important changes, simplifying and speeding diagnosis, while maintaining adequate sensitivity and specificity (5,7,12). MS severity can be evaluated by assessing the levels of inflam-matory biomarkers and of demyelination observed in MRIwith gadolinium-enhancing imaging, and by the Expanded Disability Status Scale of Kurtzke (EDSS). Apositive MRIgadolinium-enhancement indicates an ongoing inflammation and activity of the MS process, whereas a gadolinium-negative imaging should be considered as a sign of stabilization of the MS process (13).

The age of onset has a unimodal distribution with a peak between 20 and 30 years and the symptoms are rarely initiated before 10 or after 60 years. The MS incidence is ~2 or 3-fold higher in women than men and in patients with late onset, the rate seems to equalize (1,14-17). Epidemiological studies suggest that MS etiology is multifactorial with a complex interaction between environmental factors, such as infectious agents, and genetic factors (14). Autoimmune mechanisms and viral infections may also have a pathogenic role in MS (1,2,7). Evidence is mounting that genetic variation influences not only the susceptibility to MS, but also the clinical course and severity (18).

Correlation studies of the racial differences in the prevalence rates of worldwide MS, reveals that the Caucasian population appears to have a high risk whereas the Asian population similarly to Blacks have a lower risk. Epidemiologic studies suggest that MS susceptibility is hereditary and the risk for developing the disease is 15-fold higher when MS is present in a first-degree relative. Studies with families and twins provide good support for the genetic susceptibility. In high prevalence areas, the risk of developing MS is 0.00125% in the general population. Siblings of patients with MS have a risk of ~2.6%, parents have a risk of 1.8% and sons have a risk of ~1.5%. Overall, about 15% of the patients with MS have an affected relative. Study data with twins indicate a concordance of 25% in monozygotic twins and only 2.4% in dizygotic twins with the same gender (1,7,16).

As a complex disease, MS susceptibility is probably conferred to multiple genes. Data from family lineages with more than one affected member are consistent with the hypothesis that multiple non-correlated genes are associated with susceptibility to MS. The major histocompatibility complex (MHC), located on the short arm of human chromosome 6, is considered as a genetic determinant in MS. At the MHCregion, the human leukocyte antigen (HLA) genes encode important molecules involved in the immune response. Of the three classes of HLAgenes, the strongest MS association occurs with class IIalleles (1,7,16).

Several genetic polymorphisms previously described in different populations worlwide have been associated with the susceptibility, resistance and outcome of MS. However, contradictory results are reported in the literature when a genetically different population is evaluated. The aim of this study was to conduct a review on polymorphisms in genes that encode molecules involved in the physiopathology, the develop-ment and the heterogeneity of the clinical course of MS in the worldwide population.

2. Study design

Information was selected and obtained from virtual database such as Medline and PubMed, including references, abstracts and full text of journal articles in Portugese and English, published between 1972 and 2011. Keywords and descriptors of health were used in association with MS, genetic poly-morphism, tumor necrosis factor, lymphotoxin-α, HLAalleles and MHCgenes.

3. The physiopathology of MS

MS is considered to be an autoimmune disorder mediated by activated CD4+ Thelper lymphocytes that recognize the major autoantigens, such as the myelin basic protein (MBP), proteo-lipidic protein (PLP), myelin oligodendrocytic protein (MOG) and the myelin-associated glycoprotein (MAG). Adisproportional number of activated CD4+ Tcells was found in MS lesions and it is not clear whether these cells play a primary role in the initial events and lead to the destruction of myelin or if they trigger the events that occur inside or outside of the CNS (14).

The activation of autoreactive CD4+ Tcells and their differentiation into a Th1 phenotype is an important event in the initial stages of the disease, and these cells are probably also important players in the long-term evolution of the disease. However, the damage of the target tissue, the CNS, is most likely mediated by other components of the immune system, such as antibodies, complement components, CD8+ Tcells, and factors produced by innate immune cells. Perturbations in immunomodulatory networks that include, mainly, Th2 cells, regulatory CD4+ Tcells, natural killer (NK) cells may in part be responsible for the relapsing-remitting or chronic progressive nature of the MS (14).

Myelin-specific CD4+ Tcells are thought to initiate and, in cooperation with B cells, perpetuate the immune processes of the disease. Both elimination of Tcells and damage to the CNS by immune cells are central pathogenic mechanisms of MS. It seems likely that apoptosis plays a central role in both of these processes (19). While elimination of Tcells by apoptosis is a physiological control mechanism, tissue damage in the CNS is a physiopathological feature. Tumor necrosis factor (TNF)-α, CD95 (Apo-1/Fas) ligand, and other ligands, such as the TNF-related apoptosis inducing ligand (TRAILor Apo-2 ligand) interact with their respective receptors to induce apoptotic cell death. Similar to the CD95 ligand (CD95L) and TNF-α, surface bound and soluble TRAILwere shown to rapidly induce

apoptosis in susceptible tumor cells upon trimerization of its receptors and subsequent activation of the caspases cascade, leading to fragmentation of DNA(20). TRAILis able to induce massive neuronal cell destruction in the human brain (21). Since TRAILreceptors, but not the ligand, are expressed in the normal human brain and human antigen-specific T cells upon activation up-regulate TRAIL, this system is expected to have a role in neuroinflammation. Blockade of TRAILexpressed in CD4+ myelin-specific Tcells reduces caspase-dependent neuronal cell death and markedly ameliorates clinical severity in experimental auto-immune encephalomyelitis, an animal model for MS (22).

MS demyelination leads to an axonal block. Some cyto-kines may play a role in the conduction block, particularly the pro-inflammatory cytokines TNF-α, and TNF-β, and interferon-γ (IFN-γ). Cytokines have indirect effects on neural function and direct effects on ion channels, but clear and direct effects on axonal conduction have been difficult to detect. On the other hand, TNF-α and IFN-γ are potent in stimulating the formation of the inducible form of the nitric oxide synthase (iNOS) enzyme and it can produce nitric oxide (NO) sustained and in high concentration. Theoretically, inflammation also contributes to neurological deficits by modifying the properties of glial cells, particularly, astrocytes and microglia (23,24).

The biological activities of chemokines are mediated by interactions with their corresponding chemokine receptors and they are involved in the pathogenesis of immune-mediated inflammation of the CNS, both in controlling leukocyte migration across brain endothelium and in the activation and movement of cells within the brain parenchyma. During a variety of inflammatory and degenerative conditions, glial cells within the CNS have the capacity to express chemokines, such as CCL2 (previously named monocyte chemoattractant protein-1 or MCP-1), CCL3 (previously named macrophage inflammatory protein-1α, MIP-1α), CCL4 (previously named MIP-1β), and CCL5 (previously named regulated upon activation protein normally T-cell expressed and secreted, RANTES). Glial cells also express the CCchemokines receptors (CCR) such as CCR2, CCR4, CCR5 and CXCreceptors (CXCR). The preferential expression of CCR5 and CXCR3 occurs in CD4+ Th1 cells, and the expression of CCR3 and CCR4 in CD4+ Th2 cells. The profile of chemokine receptors on CD4+ Th1 may, in part, explain the selective recruitment of the Tcell subpopulation to the sites of inflammation, including the predominance of CD4+ Th1 cells, expressing CCR5 and CXCR3 in MS lesions. By contrast, CCR2 is up-regulated in activated Tcells, and is also present in mononuclear phagocytes (25). Different pairs of chemokine receptors and their ligands seem to play a pathogenic role in MS, such as the receptor CXCR3 and its ligands

CXCL9 and CXCL10; CCR1 and CCL3, CCL4 and CCL5; CCR2 and CCL2; CCR5 and CCL3, CCL4 and CCL5. Interfering with the chemokine system may be an effective therapeutic approach in MS (26).

Four patterns of demyelination have been described in early active MS lesions suggesting that discrete pathways may lead to the common endpoint of myelin injury in MS (11). All four patterns share the infiltration of macrophages and T-cells. The most common patterns, types Iand II, are characterized by sharp lesion borders that surround blood vessels, oligo-dendrocyte survival and remyelination, and suggest a primary inflammation mechanism of myelin injury. Pattern IIis distinguished from pattern Iby immunoglobulin deposition and complement activation in regions of active myelin destruction. Patterns Iand IIresemble autoimmune experimental models of MS and suggest myelin as the target of injury. In contrast, patterns IIIand IV show very little remyelination due to depletion of oligodendrocytes. Pattern IIIis further distinguished by preferential loss of MAG, apoptotic oligo-dendrocytes, and features consisting of a distal, dying-back oligodendrogliopathy with degeneration of the inner glial loop. Pattern IV lesions demonstrate degeneration of oligodendrocytes in the periplaque white matter preceding demyelination. These patterns resemble toxic, viral and/or ischemic models of MS, and suggest that oligodendrocytes may be the targets of injury (27).

All four patterns co-express CCR1 and CCR5. In pattern IIlesions, the number of CCR1-expressing cells is decreased, while the number of CCR5-expressing cells is increased in late active vs. active regions. In contrast, CCR1+ and CCR5+ Tcells were equal in all regions of pattern IIIlesions. These suggest distinct inflammatory microenvironments in pattern II and IIIlesions and support MS pathological heterogeneity and that CCR5 expression could modulate patterns Iand IIbut not patterns IIIand IV pathology (27).

A significant increase in surface expression of CCR5 in CD4+, CD8+, CD19+ and CD14+ cells as well as an increased percentage of CXCR3 and CXCR4 in CD14+ cells was found in MS patients compared to controls. Increased levels of CXCL10 (IP-10) and CCL5 (RANTES) in CSFwere also observed in a subgroup of MS patients. These results support that chemokines and their receptors are involved in the patho-genesis of MS (28,29).

Arole for the CXCL12 (previously named stromal cell derived factor 1 or SDF1-α) in the pathogenesis of MS was also evaluated (30). This chemokine is constitutively expressed at low levels in the CNS and is essential in CNS development. It is also chemoattractant for resting and activated Tcells, as well as monocytes. Immunohistochemical analysis of chronic active and chronic silent MS lesions was performed to evaluate CXCL12 expression. In active

MS lesions, CXCL12 levels were high on astrocytes throughout lesion areas and on some monocytes/macrophages within vessels and perivascular cuffs, with low staining in endothelial cells. In silent MS lesions, CXCL12 stainning was lower than that observed in active MS lesions, and was also detected in endothelial cells and astrocytes, particularly hypertrophic astrocytes near the lesion edge. The endothelial cells expressed CXCR4, the receptor for CXCL12, suggesting that this chemokine may activate endothelial cells to produce other mediators involved in MS and may initiate and increase the inflammatory response during MS.

Due to the importance of inflammatory cells inside and around of MS plaques and the immune mechanisms that affect myelin distribution, these factors have been largely studied. In the allergic encephalomyelitis, an experimental model of MS, the inflammation and demyelination are seen after animal immunization with myelin, myelin protein and other peptides of this protein. The experimental disturbance can be adoptively transferred by encephalitogenic CD4+ Tcells into a naive animal. The plaques infiltrating around the brain contain CD8+ Tand CD4+ Tcells and macrophage. It is believed that macrophage and Tcells induce damage in the oligodendrocytes. It was suggested that CD8+ Tcytotoxic cells use Fas/FasLdue to that oligodendrocytes express Fas, whereas FasLis present in infiltrated T cells. However, a variety of studies demonstrated that factors other than cellular immunity are needed for the development of similar MS lesions (16,31).

4. Genetic polymorphisms and MS in the worldwide population

Cytokines and their receptor genes. Polymorphisms in genes that code cytokine and their receptors have been associated with the susceptibility or resistance to various infectious or autoimmune diseases, and to clinical disease variables, including MS. However, there is still no definitive evidence of such association (32).

Interleukin-1β (IL-1β) and IL-1 receptor antagonist (IL-1RA) genes. IL-1β and IL-1RAgenes, which exert opposing functions in the immune system and which are in linkage disequilibrium over an extraordinary distance of 360 kb (33), have been studied as disease modifying candidate genes in MS. Association of a single nucleotide polymorphism (SNP) in exon 5 (E5) of IL-1β with disease severity has been demonstrated in two studies utilizing different methods to correct for disease duration by assessing time-dependent disability (33,34). In both of these studies, allele 2 of the IL-1β E5, which is associated with higher expression of IL-1β, is found to be independently associated with a favorable prognosis in

MS. Studies on another SNP in the promoter region of the IL-1β gene (-511), have yielded conflicting results (35,36).

Avariable number of an 85-base pair tandem repeat (VNTR) in intron-4 of the IL-1ra gene has also been studied. The presence of allele 2 has been associated with higher expression of the protein and was found to be an independent and favorable prognostic factor in four studies (27). However, other studies did not confirm this result (33,34). The lack of association in these latter studies could be due to the fact that the population in these studies is ethnically distinct from that in the other studies. It may also be due the differences in the outcome analysis. Larger-scale studies and formal meta-analysis are needed to confirm the disease modifying effects observed for IL-1β and IL-1ra polymorphisms (27).

Interleukin-10 (IL-10) gene. IL-10 is an anti-inflammatory cytokine which may modulate disease expression in MS. The dimorphic functional polymorphisms within the IL-10 promoter region at positions -1082, -819 and -519 has been previously investigated for their possible influence on the susceptibility and outcome of MS. Specifically, a polymorphism at the IL-10 promoter gene located at position -1082 results in two alleles, named -1082Aand -1082G, that have been associated with low and high *in vitro* IL-10 production, respectively. When evaluated in healthy individuals compared to MS patients from a population with different genetic background and severity of disease, the results were contradictory. Several studies do not show any association with MS, nor with the progression related to patients' disability (37-39). The same polymorphism was evaluated in MS patients and healthy subjects from Finland in order to investigate the influence on the susceptibility and outcome of MS. Although the A→G substitution at position -1082 on the IL-10 gene proved to be protective against MS in all patients (OR, 0.32), the effect increased over the years (10 years, OR, 0.33; 15 years, OR, 0.21; 20 years, OR, 0.14) demonstrating that it is not associated with MS susceptibility. The authors suggest that differential production of IL-10 might be a factor in the severity of MS (40).

IL-10 expression is related to the polymorphisms -1082 (G→A), -819 (T→C) and -592 (A→C) in the promoter region of the IL-10 gene, which constitute three haplotypes, GCC, ATAand ACC. The ATA(a non-GCC) haplotype, which is associated with low IL-10 expression, was analyzed to determine whether it could influence the initial interferon (IFN) treatment response in RR-MS patients from Norway. During the first six months of treatment, MS patients with non-GCChaplotypes experienced fewer new MRIT1-contrast enhancing lesions than patients with the GCChaplotype. However, no differences were detected in the

clinical disease activity suggesting an influence of the IL-10 promoter polymorphism on the IFN treatment response in MS (41).

Interleukin 2 (IL-2) and the IL-2 receptor (IL-2R) genes. IL-2 and IL-2Rsignaling promotes proliferation and survival of activated CD4+ Tcells and has an essential non-redundant role in the production of regulatory Tcells (Treg). Arecent transversal study and meta-analysis of IL-2 and the IL-2Rantagonist (IL-2RA) were evaluated in an MS patient from Madrid (Spain) and the results confirmed the association of polymorphisms in the IL-2RAgene with susceptibility in this MS population (42).

TNF-a, TNF-β and the TNF receptor genes. Both TNF-α and TNF-β (also named Linfotoxin-α or LTa) genes are located on the HLAclass IIIregion. Studies of the TNFregion are of particular interest because TNF-α and TNF-β are tandemly located within the HLAcomplex, suggesting that these genes may be in linkage disequilibrium with the HLA-DRloci that are associated with MS (27). Previous allelic association studies in Northern Ireland using microsatellite markers (a, b, c and d) localized within a 20-kb region around the TNFgenes have shown significantly different allele distributions of the TNFa and b markers betwen MS patients and controls (43).

TNF-α is a pluripotent pro-inflammatory cytokine secreted mainly by macrophages and CD4+ Th1 cells. TNF-α modulates the immune response by induction of MHCclass IIantigens, adhesion molecule expression and dysfunction of the blood-brain barrier. The first step in the induction of many of the biological effects elicited by TNF-α is its binding to cell surface receptors. TNFreceptors (TNFR) have been detected in a variety of normal tissues and cell lines either sensitive or resistant to TNF. Two distinct receptors, a 55-60 kDa (TNFRI) and a 75-80 kDa (TNFRII), have been identified, and both are able to neutralize circulating TNF-α (44).

Several studies have indicated that TNF-α is a critical pro-inflammatory cytotoxic cytokine and immunopathogenetic mediator in MS. Increased expression of TNF-α has been shown to be associated with clinical activity in RR-MS and the development of chronic progressive disease. TNF-α induces a wide variety of responses including apoptotic cell death, enhanced ischemic and excitotoxic injury in neurons, but also neuroprotection (45). The co-existence of neurotoxic and neuroprotective effects of TNF-α may be partially explained by two different signaling pathways mediated by the two cell-surface receptors, TNFRIand TNFRII. *In vivo* studies have demonstrated that TNFRI mainly mediates CNS inflammation and demyelination, whereas TNFRIIenhances remyelination. Both receptors have also been shown to exist in soluble forms and are thereby able to neutralize circulating TNF-α. Serum and

CSFconcentration of the soluble forms of both receptors have been shown to correlate with the clinical activity in MS patients. These findings have encouraged researchers to investigate genetic variants of the TNFRIIgene and its association with the susceptibility to and clinical progression of MS (44).

The interindividual variation in the capacity to produce TNF-α may be caused by differences in either transcription rate, the regulation of mRNA stability, translation efficiency or processing of the mature protein. Polymorphisms in the genes encoding proteins that regulate this process may cause differences in TNF-α production. Different polymorphisms have been described in the promoter TNF-α gene, such as the SNP at position -308 of the TNF-α promoter, which consists of a substitution of a G (TNF1 allele, more common) to an A(TNF2 allele, less common) (46,47). The TNF2 allele has been associated with higher constitutive and inducible levels of TNFgene transcription as compared to the wild-type genotype. It has been also shown that this SNP is associated with increased TNF-α production in *in vitro* stimulation. Therefore, this genetic variation may result in altered TNF-α expression and thereby affects the susceptibility and clinical severity of inflammatory diseases (47). However, contradictory results have also been reported. In one study, the TNF2 allele did not correlate with the increased number of TNF-α expressing cells, indicating that the consistently observed increase of TNF-α production in MS by a variety of assays is better explained by the number of TNF-α expressing cells, than by a functionally relevant promoter polymorphism (48).

Astudy carried out in the Netherlands examined TNF-α levels and the distribution of the polymorphisms -308, -238 and -376 in the promoter of the TNF-α gene. Increased TNF-α levels in the CSFwere detected in patients with active MS compared to healthy individuals. In MS patients, the level of TNF-α produced by peripheral blood mononuclear cells was correlated with the extent of neurological deterioration and disability. Therefore, the distribution of -238 and -308 poly-morphisms were tested in two different groups of MS patients. In MS patients with severe neurological disability that needed hospitalization, the -238Aallele was not detected. In MS outpatients, the frequency of the -238G allele was higher, but not significantly different from the controls. The -308 genotypes were not differently distributed in patients compared to controls both in the first and the second group of MS patients, suggesting that the -238 G→Atransition polymorphism in the TNF-α promoter is less common in MS patients with neuro-logical damage. It may be possible that the -238Aallele is protective against damage in MS, while it is not associated with differences in susceptibility (46). Another study showed no significant differences in the TNF-α -238 and -

308 promoter polymorphisms evaluated in French MS patients and in healthy individuals (49).

The exon 3 variant of the TNF-β or LTa gene leads to a substitution at amino acid position 26 (aspartate to threonine), and correlates with a reduced level of TNF-β production. Another TNF-β gene polymorphism identified by restriction fragment length polymorphism (RFLP) using the restriction enzyme *Nco*I(named the NcoITNF-β polymorphism) resides within intron 1, has been described as being correlated with the exon 3 polymorphism and affects TNF-β gene expression levels (50). The combination of these allelic forms may lead to different levels of cytokine production in response to various physiological and pathological stimuli and in turn may result in a predisposition to the development of MS or a different clinical status of MS (23). The association of TNF-α and TNF-β allelic polymorphism frequency with the occurrence and clinical type and severity of MS was evaluated in a Polish Caucasian population. Both point mutations (-308 promoter of TNF-α gene and exon 3 TNF-β gene) seem to be inherited independently, since no difference was found between the number of the combined allelic types and the expected number calculated by the multiplication of the separated frequencies for TNF-α and TNF-β alleles in both control and MS patients. The frequency of the -308 G→Amutation in the TNF-α promoter region in normal controls was 15.0% and 24.0% in MS. For the TNF-β gene the exon 3 polymorphism allele Awas detected in 36.0% of controls and 34.0% of MS patients. In MS, the combined genotype TNF-α G and TNF-β C+Cwas present six times more frequently (12.0%) than in controls (2.0%), and the patients with this genotype showed the highest EDSS scores. Other combinations did not show a difference between the MS and non-MS groups. Both mutations are related with higher cytokine production; however, no statistical difference was observed between TNF-α and TNF-β levels (23).

In a different study the TNF-α -308 polymorphism was evaluated in 283 MS patients from Germany, in 72 patients with amyotrophic lateral sclerosis (ALS) and 66 patients with stroke, all of them from the same genetic background who served as controls. Disease severity was defined by the progres-sion index and by the EDSS. Of the 283 MS patients, 67.0% were homozygous for the TNF1 allele, 28.0% were hetero-zygous for the TNF2 allele, and 3.0% were homozygous for the TNF2 allele. No significant differences were found between the TNF2 allelic frequency in the group of patients with MS, ALS and stroke. In a subgroup of 87 MS patients with stable disease, 30.0% with the TNF2 allele and 70% without TNF2 allele, a quantitative polymerase chain reaction (PCR) was performed to determine TNF-α mRNAbaseline levels and the results did not show an association between the presence of the

TNF2 allele and the clinical course of MS. The investigators concluded that the TNF2 allele, as a genetic marker, itself, does not significantly contribute to the severity of MS. They suggested that primary factors other than variations in the TNF-α promoter region could contribute to MS susceptibility and disease progression. Nevertheless, functional differences in the -308 polymorphism were demon-strated because increased TNF-α mRNAlevels were detected in patients carrying the TNF2 allele. There is no doubt thagenotype distribution of SNPs in the *TNFRII* gene between MS patients and control, with the exception of one in exon 10, position 1668 with a T→G substitution polymorphism. In MS patients, 2.5% were positive for the 1668*G allele, whereas 5.5% were positive in the healthy control group (P=0.019). Genotype analysis revealed that the lower frequency of the 1668*G allele in MS patients was caused by an increased in the proportion of 1668*T/Thomozygous patients. However, the differences in the 1668* T→G polymorphism between patients and control may indicate that TNFRIIcontributes to overall MS susceptibility. When RR-MS and SP-MS patients were compared to those with PP-MS, no difference in genotype and phenotype frequencies were found. The data also showed that there was no association between genotype and carrier status of any polymorphism and gender, age, age at onset, EDSS, disease duration, and number of relapses in MS patients (56).

Chemokines and their receptors genes. Most chemokine genes are encoded in a cluster on chromosome 17q11.2-12, which has been identified in a number of genome-wide screens as being potentially associated with MS. The CCL5, CCL3 and CCL4 ligands use the same receptor CCR5 that has been implicated in the pathogenesis of MS. CCR5 is present mainly in cells of the immune system, such as macrophages and Tlymphocytes and plays a major role in the migration of these cells to sites of inflammation. The gene encoding CCR5 is located in the p21.3 region of the human chromosome 3 forming a cluster with other chemokine receptor genes (57). In previous reports, high levels of all of the ligands of CCR5 (CCL2, CCL3, CCL4 and CCL5), were detected in chronic active MS lesions (58), underscoring the role of CCR5 in the pathogenesis of MS. It thus appears that the CCR5 genetic polymorphisms are of particular interest in MS.

The deletion of 32 base pairs in the CCR5 gene, named CCR5-Δ32, results in a truncated protein and in failure to express the receptor on the cell surface. It was demonstrated that 15.0-20.0% of Caucasian individuals were heterozygous for the CCR5-Δ32 allele and 1.0% or less was homozygous for this variant allele (59). Further studies demonstrated that the CCR5-Δ32 allele was present in ~10.0% of the European descendants, was rarely observed in Asia and was virtually absent in the native population from sub-Saharan Africa (57,60-62). A study

of the allelic frequency of CCR5-Δ32 in 18 European populations revealed an interesting north-south gradient, with the highest frequencies of the variant allele being observed in the Finnish and Modrvinian populations (16.0%) and the lowest in Sardinia (4.0%) (63).

Several studies investigating the role of the CCR5-Δ32 polymorphism in MS have reported varied, often contradictory results. Whether the CCR5-Δ32 allele could influence the inflammatory response in MS was evaluated in 68 patients with possible onset symptoms of MS and 80 patients with an attack of RR-MS. CD4+ Tcell activation and commonly used measures of immune activation in MS such as intrathecal immunoglobulin synthesis, CSFleukocyte count, MMP-9 activity, and the CSFconcentration of neopterin were measured. In this study, the homozygosity frequency for the CCR5-Δ32 allele was comparable in MS patients and controls and the age, gender, EDSS score, disease duration, and other variables evaluated did not differ in patients carrying the CCR5-Δ32 and patients carrying the normal CCR5 alleles. However, data from a prospective 1-year follow-up study demonstrated that patients carrying the CCR5-Δ32 had a lower risk of recurrent disease activity than the patients with normal CCR5 alleles, and the onset of chronic progression was only observed in patients with wild-type CCR5 alleles, suggesting that treatment targeting CCR5 inhibitors may attenuate disease activity in MS (64).

The chromosome 3p21-24 region was examined in 125 MS families (322 total affected and 200 affected sib-pairs) and genetic analysis of CCR5 and CCR2B loci and two nearby markers (D3S1289 and D3S1300) was performed using both linkage-and association-based tests. The results showed no evidence of linkage to MS for any of the tested markers. Affected relative-pair and sib-pair analysis, and association testing for each locus were also not significant. However, age of onset was approximately three years later in patients carrying the CCR5-Δ32 deletion ($p=0.018$ after controlling for gender effects), suggesting that CCR5 expression may be associated with differential disease onset in a subset of MS patients, and may provide a therapeutic target to modulate inflammatory demyelination (65).

The association between the CCR5-Δ32 deletion and disease progression measured by clinical disability and MRIparameters was evaluated in a population-based patient sample ($n=70$). Patients with the CCR5-Δ32 allele showed a non-significant trend towards a small lesion burden (total lesion area/years duration), but the presence of this allele was not associated with a mild EDSS/year duration. The data support the previous hypothesis of a modulation of severity in MS by the CCR5-Δ32 genotype, which may result in less inflammation and tissue destruction (66). However, due the limited number of patients enrolled, weak associations between this polymorphism and disease variables cannot be

excluded. When 256 MS patients from Israel were evaluated, the progression to disability was prolonged in MS patients carrying the CCR5-Δ32 allele (homozygote and heterozygote genotypes for variant allele) compared with MS patients carrying the CCR5 wild-type genotype, suggesting that this variant allele may have favorable prognostic implications in MS (67).

Contradictory results have also been reported. Agroup of 120 unrelated Australian RR-MS patients and 168 unrelated control subjects were screened for the CCR5-Δ32 deletion. There was no significant difference in the allele frequency between the MS patients and the control group. The presence of two CCR5-Δ32 homozygotes in the MS patients may indicate that the absence of CCR5 is not protective against MS and that CCR5 is not an essential component in MS, though this may be due to a redundancy in the chemokine system with different chemokine receptors substituting for CCR5 when it is absent (68).

Astudy evaluating DNAisolated from postmortem brain tissue samples of 132 MS patients and from blood tissue samples of 163 gender and ethnicity-matched healthy controls was used to screen for the CCR5-Δ32 allele (69). An increased frequency of CCR5-Δ32 was found to be associated with early death and with a progressive reduction in the years of survival. The death hazard ratio of CCR5-Δ32 vs. the CCR5 allele was 2.12 (3.58 for female patients), suggesting that MS patients carrying the CCR5-Δ32 allele have twice the mortality rate of patients with wild-type genotype. Conflicting data with previous studies showing an association between CCR5-Δ32 carriage and a better prognosis of MS have been reported. No significant difference in the distribution of the CCR5-Δ32 allele was observed between 331 RR/SP-MS patients and controls, between the 108 PP-MS patients and controls or between the PP-MS and RR/SP-MS groups. Furthermore, no differences in the rate of disease progression were detected between carriers and non-carriers of the CCR5-Δ32 allele. In the population-based group of RR/SP-MS patients, carriage of the CCR5-Δ32 polymorphism was associated with a lower age at disease onset (mean age 26.562 vs. 31.065 years). However, significant differ-ences in the age of onset were also present in the PP-MS group or in a second RR-MS population, suggesting that the CCR5-Δ32 polymorphism is not a major determinant of susceptibility to MS (70).

In a study of the worldwide population, individuals who had the CCR5-Δ32 allelic variant presented lower MS activity and severity (32). The CCR5-Δ32 polymorphism was evaluated in 221 MS patients, and revealed that 75.6% of them had the wild-type genotype, 23.5% were heterozygous and 0.9% was homozygous for the CCR5-Δ32 allele. There was no association between the genotype or the presence of the CCR5-Δ32 allele and the disease and its severity,

age of onset of MS and gender. The frequency of genotypes observed in 94 patients with biopsy-derived, pathologically confirmed demyelinating disease was 81.9% wild-type, 16.0% heterozygous and 2.1% homozygous for the CCR5-Δ32 allele, similar to the frequency distribution in the population-based sample. No association between the genotype or carrier status for CCR5-Δ32 with the immuno-pathological classification of MS was observed. Although the results were not significant, the major differences in CCR5 expression in pattern IIcompared with pattern IIIMS lesion, may reflect differences in the pathogenic stimulus trigger and/or inflammatory microenvironment, rather than a genetic effect related to the CCR5 gene (27). This study suggests that CCR5 is unnecessary for the recruitment of CD4+ Tcells or macrophages to the CNS in MS, but CCR5-induced lymphocyte activation may be transduced by this receptor. Dependent CCR5 effector functions of mononuclear phagocytes may also be affected. This study has important pharmacogenomic implications considering that CCR5-Δ32 may influence the response to treatment by leading to low CCR5 levels.

The association of the CCR5-Δ32 allele with the risk of/or disease processes in Croatian and Slovenian MS patients was evaluated. Atotal of 325 MS patients and 356 healthy controls were genotyped by PCR and the results showed no significant differences in the distribution of the CCR5-Δ32 allele between the individuals, indicating that this allele does not influence susceptibility to MS. Furthermore, the study found that the CCR5-Δ32 carrier-status could not modulate age of disease onset or progression of the disease, suggesting that the CCR5-Δ32 is neither protective, nor a risk factor, for MS development (71).

Cytotoxic T lymphocyte associated-4 (CTLA-4) gene. CTLA-4 or CD152 gene is a strong candidate for involvement in auto-immune diseases because it plays an important role in the termination of Tcell activation (72). CTLA-4 gene is located on chromosome 2q33 region and studies carried out in different ethnic groups have demonstrated several SNPs on this gene that have been associated with susceptibility to MS. Some of these SNP are on exon 1 (+49A→G), on a microsatellite marker at position 642 of exon 4, and on the promoter regions -318C→Tand -651C→Tof the CTLA-4 gene (73). Interactions between CTLA-4 and HLA-DR2 genes in the development of MS were also reported (74,75). The SNPs on position +49A→G, analyzed and on promoter regions -318C→Tand -651C→Twere analyzed in 133 MS patients from the Japanese population and a statistical difference in the distribution of these SNPs between patients and control groups was not observed; however, the study suggested that the polymorphism may modulate the disease. Besides, an association between the CTLA-4 poly-morphisms and variables such as age, disease prognostic and HLA-

DRB1*1501 genotype was not observed. This study could not confirm that CTLA-4 gene polymorphisms are associated with susceptibility and clinical characteristics of MS (73).

Apolipoprotein E gene (ApoE). The protein apolipoprotein E(ApoE) has been the subject of considerable interest in MS (27) participating in repair processes within the CNS. Adifferent clinical course of MS was associated with the presence of specific alleles. The neuroprotective effect of ApoEis allele-specific (with ε2>ε3>ε4 allele) (76). Functional polymorphism of the ApoEgene would then be expected to affect the long-term outcome. The presence of the ApoE*ε4 allele or rather of the ε3/ε4 genotype (the ε4/ε4 genotype is rare) has been suggested as an unfavorable prognostic factor independent of age of onset, gender and duration of disease in different studies (77-83). However, other studies have not confirmed these findings (84-86). In most studies, individuals that carried the ε4 allele seemed to show faster progression of the disease, while others reported that there are neither changes in the distribution of ApoEalleles in patients with MS nor correlation between ApoEgenotypes and disease severity (87). The contradictory results may be explained, in part, by the fact that ApoEmay influence disease severity by modifying disease course, such as association with conversion to secondary progressive disease course, which may not reflect the overall accumulating disability when a small number of individuals is studied and PP-MS patients are also included. Data have shown that carriers of the ε4 allele of the ApoEgene had a significantly higher MRI lesion load than non-carriers (81). Although there was a trend for ε4 carriers to have worse clinical disease, the difference compared with non-carriers was not significant until the sample size was increased (from 83 to 374 individuals) (83). The association between the ApoEand MS progression measured by clinical disability and MRIparameters was evaluated in a population-based patient sample (n=70). Carriers of the ApoE-ε4 alleles did not show a more severe disease progression, neither by EDSS/years of duration nor by the total lesion area/years duration (66). Although the study was carried out on a genetically homogeneous Danish population, the limited number of MS patients enrolled could interfere with the results obtained and the association between this gene and disease variables could not be excluded.

Astudy carried out in Italy evaluating the -491 ApoEpolymorphism (A→Tsubstitution) in 236 individuals diagnosed with MS for at least three years showed no significant difference in the genotypic distribution (P=0.36) between MS and control group. Furthermore, no association was found between the ApoE-ε4 allele and the progression of the disease. The study showed a slight association between the ApoE-ε2 allele and a longer disease duration; patients with the ε3/ε2 the genotype showed longer disease duration when compared with

patients with the genotype ε3/ε3 (13.5±7.6 vs. 10.8±7.1 years, respectively) or ε3/ε4 (10.3±5.8 years) (87).

Genes associated with apoptosis. The Fas molecule, also named apolipoprotein-1 (Apo-1) or CD95, is a transmembrane glyco-protein apoptosis-signaling cell surface receptor belonging to the TNFreceptor family. The Fas molecule is expressed on the surface of many types of cells, including lymphocytes, epithelial cells, fibroblasts and certain endothelial cells. Both Fas and FasLare expressed in activated mature Tcells, and prolonged cell activation induces susceptibility to Fas-mediated apoptosis.

The Apo-1/Fas gene is located on chromosomal region 10q24.1 that shows linkage in MS genome screens, and studies indicate that there is an aberrant expression of Apo-1/Fas molecule in MS. The Mva1 polymorphism on the Apo-1/Fas promoter gene, which consists of an A→G substitution, was determined by PCR-RFLP from the DNAof 114 Japanese MS patients and of 121 healthy individuals. There was no statistical difference in the genotype and allelic frequencies among MS patients and controls showing no evidence that this polymorphism contributes to susceptibility to MS or that it is associated with the clinical course of the disease. Patients with the A/Agenotype seem to have a later onset than those with G/G or G/Agenotypes but this difference was not significant (88).

The TRAIL or Apo-2 ligand is a newly identified member of the TNF/nerve growth factor superfamily which has been mapped on human chromosome 3q26 (20) and is involved in cell death and some immunoregulatory mechanisms. The TRAIL gene is approximately 20 kb and is composed of five exons (89). Due to its implication in brain damage and the elevated expression in peripheral immune cells of MS patients, TRAILmay play a central role in the pathology of this disease. Ahighly polymorphic region in the TRAILpromoter with four SNPs within 11 base pairs was identified using single-strand conformation polymorphism analysis (90). The polymorphisms are located at positions -707 (C→T), -665 (T→C), -621 (C→T) and -597 (A→G) of the wild-type sequence. The SNP at position -707 was found within an AP-1 binding site. The variant haplotype frequency in 125 healthy individuals was 0.4% (-707 C→T), 17.6% (-597 A→G), 6.8% (-621 C→T), 4.0% (-665 T→C) and 71.2% (wild-type). In 123 RR-MS patients, the frequency of the SNPs detected was 7.7% (-621 C→T), 4.1% (-665 T→C), 16.2% (-597 A→G) and 72.0% (wild-type). The -707 SNP was not detected in RR-MS patients. When 15 PP-MS patients were genotyped, the frequency observed of these SNPs was 3.3% (-665 T→C), 20.0% (-597 A→G) and 76.6% (wild-type). The SNPs -707 and -621 were not observed in PP-MS patients. No significant differences in the distribution of the genotypes were observed as to mean age at onset of the disease, disease progression (EDSS

increase per year), and gender ratio. The authors suggested that these SNPs in the TRAILpromoter exert no impact on molecule expression, and are neither directly related to the increased risk of developing MS nor associated with a clinical course of this heterogeneous disease in German MS patients (90).

MHC classes I and II genes. The most important genetic factor of confirmed importance in MS has been identified in the HLAclass IIregion. Evidence suggests that MS patients differ from control subjects in their MHC. Initially association was observed between MS and MHCclass I(91,92) and later with MHCclass IIregions (93). An association between MS and HLA-A3, B7, Dw2 haplotype in a North European population and a Caucasian population from the USAwas observed (94); however, this association was not universal. Combination of HLA-DRB1*1501-DQA1*0102-DQB1*0602 (DR15) in northern Europeans (86,92,95,96) and -DRB1*0301-DQA1*0505-DQB1*0301 (DR3) in southern Europeans (97-99) was shown to increase the risk of MS 3-4 times. However, the data suggest that there may not be a single major susceptibility locus, and that genetic factors different than those related to the HLAregion may also be involved (27,93,100).

Susceptibility to MS was demonstrated, at first, with HLA-Dw2 and -DR2 specificities in Caucasian Europeans and North Americans. Further, with the use of genotype techniques, a positive association was demonstrated between MS and HLA-DRB1*1501, -DRB5*0101 and -DQA1*0602 genotypes in different ethnic groups worldwide (101-103). The relationship between HLAgenotypes and disease severity (measured by brain MRIquantitative markers of demyelinating and destructive pathology in patients with MS) was evaluated in 100 MS patients and 122 age-, gender-, ethnic- and residence-matched controls (104). HLA-DQB1*02 (OR, 19.9; 95% CI, 16.2-24.3), -DQB1*03 (OR, 16.8; 95% CI, 13.6-20.5), -DRB1*15 (OR, 4.6; 95% CI, 3.7-5.6), and -DFRB1*03 (OR, 3.9; 95% CI, 3.2-4.8) alleles were associated with MS. When evaluated by intermediary determination methods, some HLAalleles may predict the destructive pathological processes visible on an MRI; however, the severity of disease was not significantly different according to the carrier status of these alleles. When the genotyping was evaluated by a high deter-mination method, in a study carried out in Italian MS patients, the results demonstrated a strong association between the HLA-DRB1*1501, -DQB1*0301, -DQB1*0302, -DQB1*0602 and -DQB1*0603 alleles and severe neurodegenerative and inflammatory damages obtained by MRI (105).

Anegative association with HLA-DR1, HLA-DQ5 and -DRB1*01 was demonstrated in North and West European, Canary Island and Italian populations (98,106-108). HLA-DR2 was associated with susceptibility to MS in most of the Western hemisphere, while HLA-DR3

and -DR4 were associated with susceptibility to MS in Sardinia, Italy (109). Another study demonstrated that in the Sardinian population, MS was associated with HLA-DR4 (98) and, in individuals from Latin America (Mexico), was identified a relationship with HLA-DRw6 and the subtype DRw13 (110).

A significantly higher frequency of HLA-DRB1*1501 was observed in 133 Japanese MS patients (OR, 2.29; 95% CI, 1.32-3.98) than in healthy controls (73). An association between HLA-DRB1*1501, -DQA1*0102, -DQB1*0602 haplotypes with clinical form, disease duration and disability, evaluated with EDSS, was observed in Iranian MS patients (111). However, the authors discussed that patient enrollment from different parts of the country with varied ethnicities may be a limiting factor for the results.

T-cell receptor (TCR) gene. The HLA-DRB1* and TCRpoly-morphisms were evaluated in 358 clinically well-characterized MS patients from Germany, grouped according to the course of MS, and in 395 healthy subjects. The relative risk for MS in HLA-DRB1*15+ and -DRB1*03+ individuals were 3.6 and 1.4, respectively. In MS patients, certain TCRB gene polymorphisms were risk factors. In HLA-DRB1*03+ individuals, the relative risk was increased (>22.0) when a specific TCRBV6S3 allele was also inherited. Furthermore, distinct linkage disequilibria of TCRBV6S3 elements in patients and control subjects strongly suggest an additional risk factor in the TCRBV region for -DRB1*15+ individuals (112).

5. Genetic polymorphism and MS in the Brazilian population

Brazilians represent one of the most genetically heterogeneous population in the world, the result of five centuries of interethnic crosses between people from three continents including the European colonizers, represented mainly by Portuguese; African slaves (mainly from West-Central Africa); and the native Amerindians (113,114). The present Brazilian population includes Europeans from southern Europe (Portuguese and Italian) and Middle Easterners, Asians, Arabians, Africans and native Amerindians (115-117). This extensive ethnic miscegenation has suggested that other alleles may be involved in the autoimmune response and pathogenesis of MS and thus, influences the susceptibility and resistance to MS.

Cytokines and their receptor genes. Studies using tissue lesions obtained from MS patients showed an association between numerous cytokines with CNS inflammation. A change from the CD4+ Th1 phenotype to the CD4+ Th2 pattern response, which is observed during remission of the disease, may result in a decreased immune response with anti-inflammatory or regulatory cytokine production, such as IL-4 and IL-10. The IL-4 gene has mono and

biallelic expression and is located on 5q31-33 chromosome that codes cytokines from the Th2 pattern response. Polymorphism in the cytokine regulatory genes can influence the amount of cytokine produced. A SNP at the IL-4 gene, region +33 that results in a substitution of C→T, increases this cytokine production. The clinical symptoms that are found in MS can be correlated with the expression of IL-4 and the IL-4R. Aresearch carried out in Rio de Janeiro, Brazil, with 129 MS patients and 135 healthy individuals, showed no significant differences in the genotypic and allelic frequency of the IL-4 promoter gene polymorphism and for the Q551Rpolymorphism in the IL-4Rgene. Nevertheless, the SNP +33 genotyping showed a slight decrease in the TTgenotype (4.6% in MS patients vs. 6.7% in control subjects) and a slight increase in the CTgenotype in MS patients (41.1% in MS patients vs. 37.4% in control subjects). Likewise, MS patients showed a higher percentage (12.5%) of the CCallele of the Q551Rpolymorphism on the IL-4Rgene than controls (8.1%). When ethnicity was considered, Black MS patients had seven times higher chance to carry the TTallele of the IL-4 gene when compared to Caucasian patients (9.1 vs. 1.3%). In the control group, differences were not found. Similarly, Black MS patients showed a 3-fold greater chance to carry the CCallele of the IL-4Rgene when compared to Caucasian patients (20.0 vs. 6.8%); also no difference was found in the controls. These results suggest that IL-4 and IL-4Rgenotypes may influence the state of MS in the Brazilian population. The increased percentage of the TTallele of the IL-4 gene and the CCallele of IL-4Rgene in subjects African descent suggest an adaptation of African descendants to different antigenic and pathogenic challenges that influence cytokine equilibrium of the Th1 and Th2 responses. The data showed that both IL-4 and IL-4Rgenes are susceptibility factors to MS and may be able to modify the disease risk depending on the ethnicity and penetrance of susceptibility factors (118).

Chemokines and their receptor genes. The frequency of the CCR5-Δ32 allele was assessed in unrelated healthy individuals from different regions of Brazil. The frequencies of CCR5 homozygous (wild-type) and CCR5-Δ32 heterozygous indi-viduals were 93.0 and 7.0%, respectively. No homozygous genotype for the CCR5-Δ32 allele was detected, resulting in an allelic frequency of 0.035 (119). The prevalence of CCR5-Δ32 was investigated in a random sample of healthy individuals from Alegrete, in southern Brazil, where the population was basically established from a mixture of Spanish, Portuguese and African individuals and native Amerindians. No CCR5-Δ32 homozygous genotype was detected and the presence of the CCR5 wild-type genotype among whites, blacks and mulattos was 14.0, 8.0 and 13.0%, respectively, indicating a CCR5-Δ32 allele frequency of 0.068, 0.038 and 0.064, respectively (113).

The association between the CCR5-Δ32 allele with the clinical course and MRIwas analyzed in 124 unrelated MS outpatients (evaluated by the EDSS for the disease severity), and in 127 unrelated healthy blood donors from a similar geographical area, located in Londrina, southern Brazil (15). The frequency of the CCR5 wild-type and CCR5-Δ32 heterozygous genotypes was 90.4 and 9.6%, respectively, in MS patients. No homozygosity for the CCR5-Δ32 was found, resulting in an overall CCR5-Δ32 frequency of 0.0484 among the patients. The frequencies among healthy individuals was 90.5% for the CCR5 wild-type, 7.9% for the CCR5-Δ32 heterozygous and 1.6% form the CCR5-Δ32 homozygous genotypes, resulting in an overall frequency of CCR5-Δ32 of 0.0055 among healthy controls. No significant difference was observed in the distribution of genotypes between MS patients and healthy subjects. An association between the CCR5 gene polymorphism and the clinical course of MS was also not found. Despite the fact that the differences were not significant, the results suggest that the disease onset and progression to disability may be prolonged in MS carriers of the CCR5-Δ32 allele, when compared with individuals with fully functional CCR5, by 11.2 and 7.7 years respectively. MRIs identified lower positive gadolinium enhancing-imaging at brain lesions and lower brain atrophy n MS patients with the CCR5-Δ32 allele than in those with the wild-type genotype. Taken together, the results suggest that CCR5-Δ32 could be considered a favorable prognostic biomarker of MS in this population.

MHC class II genes. The evaluation of the HLA-DRB gene in a Brazilian population has a particular interest in showing the behavior of the HLA-DRB1 allele in a heterogeneous population placed in an environment of low prevalence of MS. Studies carried out in Brazilian patients with an African descent, showed a association between MS and HLA-DQB1*0602, in agreement to others studies, even without the presence of the -DRB1*1501 allele (120). Apositive association between the MS and -DQB1*0602, and -DQA1*0102 alleles was also found (121). The low frequency of the -DPA1*0301 allele observed indicated a protective role for MS. However, another study showed that the immune response seems not to be solely influenced by the expression of HLA-QB1*060 allele, because statistical significance was not observed between this allele and MS patients and control groups (122). As demonstrated from other researchers the HLA-DQA1*0201-0301 alleles were associated with the white Brazilian population; the -DRB1*1501 allele was present in white Brazilians and confers an ethnicity-dependent MS susceptibility in white MS patients; the -DQB1*0602 allele confers genetic susceptibility regardless of ethnicity (123). An association between the HLA-DRB1*1501 and -DRB1*1503 alleles and MS white and mulatto individuals from the Brazilian population was also observed (124).

The association between the HLA-DRB1*15 allele and MS was evaluated in a Brazilian Caucasian population sample from Londrina, southern Brazil (125). HLA-DRB1 alleles were analyzed by PCR with specific sequence oligonucleotide primers (PCR-SSOP) in 119 MS patients and in 305 healthy blood donors as controls. The HLA-DRB1*15 allele was identified as a significant susceptibility factor for MS development (OR, 2.53; 95% CI, 1.43-4.46) and the HLA-DRB1*11 allele with a trend significant to be a susceptibility factor for MS development (OR, 0.67; 95% CI, 0.44-1.03). It was observed that, for the -DRB1*11 allele, homozygocity may confer resistance in disease development. The homozygous for the -DRB1*15 allele did not present any effect in MS susceptibility, probably because of the limited number of homozygous patients for this allele evaluated in the study. In a study comparing the heterozygous pairs between controls and MS patients, represented by -DRB1*15/X, where X represents others alleles in combination with the 15 allele, a significant difference in the occurrence of heterozygous pairs -DRB1*15/03 and -DRB1*15/14, was verified, suggesting that this hetero-zygocity is associated with increased susceptibility to MS.

Astudy carried out among patients from Lagoa Hospital, Rio de Janeiro, identified a role for the HLA-DR2 haplotypes (HLA-DRB1*1501 and -DQB1*0602) in PP-MS patients. The disease was more severe in patients with the -DRB1*1501 haplotype, underscoring the importance of this genetic factor in the heterogeneity and clinical course of MS (126).

6. Final considerations

MS is a complex disease and its development and clinical course are not only related to individual genetic predisposition, but also dependent on a number of other factors, such as environmental, geographical and individual immune responses against certain infectious agents. The detection of a SNP involved in the etiology and physiopathology is very difficult and probably, several genetic polymorphisms are involved, each with a small contribution to the susceptibility or resistance to MS.

Considerable attention has been devoted to studies evaluating disease modifying effects in MS. Seven genes of probable importance have thus been identified: HLA class II, ApoE, IL-1RA, IL-1β, TNF-α, TNF-β (or LTa) and CCR5. The variant alleles may contribute to the heterogeneity in disease course by different mechanisms (27). However, the results described in the literature about genetic biomarkers of MS are not consistent in the worldwide population. All of the data reviewed in the present study have been obtained in population association studies. Although this design is the easiest to conduct, the results are confounded

by ethnic differences. Family-based association methods matching for ethnicity and sufficient confirmation in other data sets can circumvent this problem. All the results together show the need for continued research in the genetically heterogeneous populations. Collaborations between investigators are essential to identify new biomarkers associated with MS that could be used as therapeutic targets to modulate the autoimmune response in MS patients. This information may contribute to a better understanding of the physiopathology and treatment of MS, with the possibility of developing different therapeutic strategies according to the genetic profile of each individual.

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Tumor necrosis factor beta (TNF-β) *Nco*I genetic polymorphism is associated with multiple sclerosis independently of HLA-DRB1*

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Abstract: Multiple sclerosis (MS) is a progressive immune-mediated disorder caused by a demyelization process in the central nervous system with multifactorial etiology. Several studies emphasized the importance of chromosome 6 as a possible disease susceptibility locus, which includes human leukocyte antigen (HLA) classes I, II, and III genes, such as the tumor necrosis factor alpha (TNF-α), and tumor necrosis factor beta (TNF-β) genes. TNF-α and TNF-β have been implicated as the most important inflammatory cytokines in the pathophysiological mechanisms of MS. NcoI polymorphism in the TNF- β gene has been implicated with the susceptibility and clinical course of MS. However studies reported that this polymorphism may be inherited with the HLA polymorphism. The objective of this study was to investigate the association between TNF-β NcoI genetic polymorphism with the susceptibility for MS, the clinical progression, and the activity of the disease, and also to evaluate the association between TNF-β NcoI and HLA-DRB1* allelic polymorphisms. Demographic and clinical characteristics of 220 MS patients and 278 healthy individuals age-gender-ethnicity-matched were collected by a standard questionnaire and medical records between August 2010 to February 2012. The MS patients were evaluated using the Expanded Disability Status Scale (EDSS) and magnetic resonance imaging (MRI). Genomic DNA was extracted from peripheral blood cells of the individuals and a 782 base-pair fragment of the TNF-β gene was amplified using polymerase chain reaction (PCR). The PCR amplified products were subjected to NcoI restriction digestion and analyzed by restriction fragment length polymorphism. TNF-α serum level was determined using enzyme linked-immunosorbent assay. The frequency of TNFB2/B2 genotype differed from that of TNFB1/B2 and of TNFB1/B1 genotypes obtained among the MS patients and controls (p=0.0449, OR: 1.490 [IC: 1.023–2.170] and p=0.0225, OR: 2.058 [IC: 1.099–3.855], respectively. The TNFB2 allelic frequency also differed between MS patients and controls (p=0.0089, OR: 1.427 [1.093–1.863]). Although some HLA II haplotypes influence MS

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susceptibility, the association between HLA-DRB1*01, 03, 04, 07, 08, 11, 13, and 15 and TNF-β NcoI alleles was not observed (p>0.05). These results could suggest that TNF-β NcoI polymorphism may not be in linkage disequilibrium within HLA-DRB1* and may exerts a role in the MS susceptibility. TNF-β NcoI polymorphism was not associated with the age at diagnosis (p=0.2884), disease duration (p=0.8201), clinical forms (0.4189), EDSS baseline (p=0.9889), EDSS five-year follow-up (0.3913), progression index (p=0.6002), progression of the disease (p=0.4509), and lesions location (p=0.8169) observed at MRI. However, it was observed a tendency toward higher disease activity among the TNFB2/B2 genotype carriers (p=0.0722). All together, the results suggested that TNF-β NcoI polymorphism may contribute for MS susceptibility independently of HLA-DRB1* and could be used as a genetic biomarker for MS susceptibility in the Brazilian population.

Keywords: TNF-α. TNF-β. Genetic polymorphism. Multiple sclerosis. Susceptibility.

1. Introduction

Multiple sclerosis (MS) is a progressive immune-mediated chronic inflammatory disorder of the central nervous system (CNS) which the immune system attacks self molecules in the white matter and grey matter of the brain, spinal cord, and optic [1]. However, the pathogenesis of MS is incompletely understood, and involves complex interactions between genetic susceptibility and environmental triggers, as well as gene-environment interactions and epigenetic mechanisms [2]. Despite overwhelming evidence that the MS pathogenesis involves interplay between genetic and environmental effects, progress in identifying the relevant risk factors has been painfully slow and there is practically no understanding about how this might interact and result in disease.

Human leukocyte antigen (HLA) class II alleles were extensively studied in relation to MS disease. Susceptibility to MS was first associated with HLA-Dw2 and –DR2 alleles in Caucasian Europeans and North Americans. Positive associations between MS and HLA-DRB1*1501, -DRB5*0101, -DQA1*0102, and DQB1*0602 genotypes were demonstrated in Europeans [3]. Fernandez et al. (2009) [4] also found positive association between MS in patients from Biscay (Spain) and HLA-DRB1*1501 and -DQA1*0102; and particularly, with -DQB1*0602 allele, which showed the only consistent genetic association with MS. Research with Afro-Brazilian revealed association between MS and HLA-DQB1*0602 allele [5]. Kaimen-Maciel et al. (2009) [6] also found a positive association of HLA-DRB1*1501 allele and a negative association of HLA-DRB1*11 allele with Brazilian MS patients.

Variations in genes coding for the expression and regulation of cytokines and their receptors may also play a role in MS susceptibility and disease progression [7,8]. Several studies emphasized the importance of human chromosome 6 as a possible disease

susceptibility locus [1,9,10]. The pro-inflammatory cytokines are the most implicated, especially tumor necrosis factor alpha (TNF-α) and tumor necrosis factor beta (TNF-β) also named as Lymphotoxin-alpha (LTa) [10], which genes are tandemly located within the major histocompatibility complex class III (MHC III) on the short arm of human chromosome 6, suggesting that these genes may be in linkage disequilibrium with the HLD-DR loci that are associated with MS [11]. Since linkage disequilibrium is strongly within HLA complex, it has been also suggested that associations with HLA class I and II genes in autoimmunity disease may be secondary to the associations with the TNF genes [12].

A single nucleotide polymorphism (SNP) at position +252 located in the first intron of the TNF-β gene consists of a G in the wild type allele TNFB1 and of an A in the variant allele TNFB2 and is named as *Nco*I polymorphism [13]. It has been identified as a potentially influential locus in many inflammatory conditions. Studies have shown that TNF-β genetic polymorphism is especially interesting since variations in the region responsible for transcriptional regulation may have implications for the TNF-α gene expression and variability on TNF-α synthesis, which could be an important factor in the pathogenesis of MS [13-16]. The interindividual differences in the capacity to produce TNF-α may be resulted by differences in the transcription rate, the regulation of mRNA stability, translation efficiency or processing of the mature protein. Polymorphisms in the protein that regulates this process may cause differences in TNF-α production [17].

TNF-α regulates the induction of cell-surface HLA class II molecules and may have a pivotal role in the pathogenesis of the disordered immunoregulation of autoimmune diseases [18]. TNF-α can be produced by astrocytes which are the immunocompetent cells within the CNS. Since the lesions observed in MS are characterized by loci of demyelination with astrocytes proliferation, oligodendrocytes depletion, and infiltration of HLA class II positive cells and lymphocytes, the possibility of a role of TNF-α in the pathogenesis of MS has elicited much speculation [19]. Symptomatic attacks are related with onset of demyelinating lesions in the brain, optic nerve, and spinal cord [20]. Magnetic resonance imaging (MRI) is crucial in the identification of new lesions and disease activity. It is related with blood brain barrier (BBB) disrupt, entrance of T cells and increased inflammatory process in the CNS [20,21]. MRI is a critical component on the current diagnostic criteria for MS and can support, supplement, or even replace some clinical criteria [20,22]. The disease progression in MS patients has been correlated with high TNF-α level in cerebrospinal fluid (CSF) [23]. Elevated serum and CSF TNF-α levels have been associated with the onset of relapses [24].

TNF-β *Nco*I polymorphism was studied in several diseases including sarcoidosis [16], tuberculosis [11], sepsis [15,25-27], and psoriases [28]. TNF-β *Nco*I polymorphism was evaluated in very homogenous population including Iranian [1] and Polish [10] MS patients, which are genetically different from Brazilian population that is characterized by a great miscegenation, as reported elsewhere (29). Contradictory results are reported and the TNF-β *Nco*I polymorphism was not yet studied in Brazilian MS patients. To clarify this issue, the aim of the present study was to investigate the association between TNF-β *Nco*I polymorphism with MS susceptibility, progression, and disease activity; and toverified a possible association between HLA-DRB1* and TNF-β *Nco*I polymorphisms in Southern Brazilian MS patients.

2. Materials and methods

2.1 Study design

The protocol was approved by the Institutional Research Ethic Committee of the State University of Londrina, and a written consent form was obtained from all of the individuals. A total of 220 MS patients, diagnosed according to the revised McDonald Criteria [22,30], which correspond to the entire population with MS from the Neurology Outpatient Department of the Outpatient Clinical Hospital, State University of Londrina, Southern Brazil, were consecutively recruited. The control group was consisted of 278 healthy blood donors from the Blood Bank of Londrina, with similar demographic characteristics and from the same geographic area. All of the control individuals did not present either clinical symptoms or laboratory parameters of inflammation, infection [31], MS or other autoimmune disease. Parameters such as age, gender, ethnicity, and body mass index (BMI) were controlled.

2.2 Demographic and clinical data

The demographic and clinical data were assessed by a standard questionnaire, medical records, and the Blood Bank of Londrina database. MS clinical forms were determined according to the classification of Lublin and Reignold (1996) [32], as relapsing-remitting MS (RR-MS), primary progressive MS (PP-MS), secondary progressive MS (SP-MS), and clinical isolated syndrome (CIS). All of the MS patients were in the remission clinical phase,

defined as the period of recovery with no relapse episodes within the last three months prior to the time of enrollment in the study. The age at onset of the disease was based on retrospectives data from medical records [30]. The Expanded Disability Status Scale (EDSS) [33] was evaluated at baseline and five-year follow-up and was categorized as mild (EDSS 0.0 – 4.0), moderate (EDSS 4.5 – 5.5), and severe (EDSS 6.0 – 10.0) [1]. MRI revealed the lesion location (brain, spinal cord, and optic nerve) and the presence or absence of gadolinium (Gd+ or Gd-, respectively) contrast-enhancing lesions. The disease progression was evaluated by EDSS five-year follow-up less EDSS baseline and a relevant worsening of disability was defined as worsening of the EDSS score by at least one full point for patients with a baseline score of < 6.0, and a worsening by at least one-half point for patients with a baseline of 6.0 or higher [34]. The rate of accumulation of neurologic disability was expressed using the progression index: PI=EDSS/duration (years) [7]. The anthropometric measurements evaluated were body weight (measured to the nearest 0.1 kg using an electronic scale, with individuals wearing light clothing, but no shoes), and height (measured to the nearest 0.1 cm using a stadiometer). Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared.

2.3 *Nco*I genetic polymorphism

Peripheral blood samples were collected with EDTA as anticoagulant, centrifuged at 2,500 rpm for 15 min to obtain the buffy-coat that was stored at -80^{0}C freezer until used for the TNF-β genotyping. The genomic DNA was extract from the leukocytes using a commercial DNA extraction kit (Biometrix Diagnóstica, Curitiba, Brazil) according to the manufacturer's instructions. A 782 base-pair (bp) fragment of the TNF-β gene was amplified using polymerase chain reaction (PCR) as previously reported [15,25,26,]. The *primers* used were determined according to the GenBank number X02911 as follow: Primer 1 (TNF1 sense) 5' CCG TGC TTC GTG CTT TGG GAC TA 3'; Primer 2 (TNF2 antisense) 5' AGA GGG GTG GAT GCT TGG GTT TC 3' (Invitrogen™, Life Technologies, Carlsbad, CA, USA). PCR was performed with a final volume of 25μL, with 2.5mM of each primer, $MgCl_2$ 50 mM, dNTP 1.25mM (Invitrogen™, Life Technologies, Carlsbad, CA, USA), recombinant DNA polymerase 1.25 U in the buffer provided (Invitrogen™, Life Technologies, Carlsbad, CA, USA) and 200ng of the DNA sample. PCR conditions were performed in a thermocycler (PCR Sprint-Thermo Hybaid™, Biosystems, Barcelona, Spain) that comprised 5 min denaturation at 94^{o}C for initial denaturation; 37 cycles of 45 sec at 94^{o}C for denaturation, 45

sec at 67°C for the annealing and 45 sec at 72°C for the elongation; and 10 min at 72°C for final elongation. Ten microliters of PCR products were completely digested with 0.3μL of *Nco*I 10U/μL enzyme (Invitrogen, Life Technologies, Carlsbad, CA, USA) for 4h at 37°C. The *Nco*I genotypes were identified by restriction fragment length polymorphism (RFLP) analysis in a 3% agarose gel electrophoresis (70V, 70 min) and stained with ethidium bromide according to previous studies [15,25,26]. The TNFB1 allele includes a restriction site for *Nco*I which results in 196 bp and 586 bp fragments after digestion, and the TNFB2 allele (lacking the restriction site for *Nco*I) results a fragment with 782 bp. The heterozygous genotype TNFB1/B2 results 3 fragments (782 bp, 586 bp, and 196 bp).

2.4 HLA-DRB1* polymorphism

The results from the HLA-DRB1* genetic polymorphism were obtained from a previous research developed in the same cohort of MS patients and published elsewhere [6]. Briefly, the polymorphism was determined also from the buffy coat of peripheral blood samples and the target fragments of DNA were amplificated by PCR and hybridized by specific sequence oligonucleotide primers (PCR-SSO) using Luminex technology for HLA genotyping (One Lambda, Canoga Park, CA, USA). The reaction consisted of HLA locus-specific amplification with further hybridization by specifics probes in the fluorescent microsphere surface. Reading and interpretation of the results were undertaken using a flow cytometer (One Lambda) according to manufacture's instrucutions. Typefying resolutions ranged from low to intermediate [6].

2.5 Serum TNF-α level measurement

The blood samples of MS patients were also collected without anticoagulant and the serum was immediately separated, and storage at -80ºC freezer for posterior analyzes. The serum TNF-α level was determined by an enzyme-linked immunosorbent assay (ELISA) with commercially available reagents (Kit Human TNF-α *e*Bioscience™, San Diego, CA, USA), with detection limit of 2.0 ng/mL, according to manufacturer's instructions.

2.5 Statistical analysis

The sample size was calculated with the Statcalc Program from Epi Info version 6.04d, with 80.0% of power, based on genotype and allelic frequencies of TNF-β *Nco*I polymorphisms of previous study in Iranian population [1]. A database was created using the Excel Program of Microsoft Office and the data were evaluated by the statistical analysis programs Graph Pad Prism version 5.0 (GraphPad Software Inc., San Diego, CA). Categorical variables were analyzed using a chi-square test (or Fished Exact test when appropriate) and expressed as the absolute (n) and relative (%) values. Continuous variables were analyzed using Mann-Whitney test and expressed as median and interquartile range (IQR) 25%-75%. To compare continuous variables from more than two groups, Kruskal-Wallis test was used, with Dunn's post test. The odds ratio (OR) and 95% confidence interval (C.I.) were also analyzed. All of the results were considered significant when $p < 0.05$.

3. Results

As expected, the individuals did not differ in age, gender, and ethnicity ($p > 0.05$) (Table 1). The clinical and treatment data of MS patients are shown in Table 2. The most frequent clinical form was RR-MS, followed by SP-MS, PP-MS, and CIS. The median age at onset of the disease was 34.5 years (IQR 26.0 – 44.0) and disease duration median of 7.0 years (IQR 2.2 – 10.0). The most part of the patients presented mild disability at EDSS baseline and after five year follow-up, brain white matter lesions, Gd- contrast-enhancing lesions, and were treated with interferon beta (IFN-β).

TNF-β *Nco*I genotype frequencies observed among the MS patients and controls were in Hardy-Weinberg Equilibrium ($p>0.05$). Among the 220 MS patients, 106 (48.2%) were TNFB2/B2, 96 (43.6%) were TNFB1/B2, and 18 (8.2%) were TNFB1/B1. Among the controls, 103 (37.1%) were TNFB2/B2, 139 (50.0%) were TNFB1/B2, and 36 (12.9%) were TNFB1/B1. The genotype frequencies were different between MS patients and controls carrying TNFB2/B2 genotype and those carrying TNFB1/B2 genotype ($p=0.0449$); and also between MS patients and controls carrying TNFB2/B2 genotype and those carrying TNFB1/B1 genotype ($p=0.0225$). The TNFB2 allelic frequency was 0.700 (70.0%) among the MS patients and 0.6205 (62.05%) among the controls; the TNFB1 allelic frequency was 0.300 (30.0%) among the MS patients and 0.3795 (37.95%) among the controls ($p=0.0089$). MS patients carrying the TNFB2/B2 genotype exhibited approximately 1.5 more chances

(OR:1.49, 95% C.I.: 1.023 – 2.170) of developing MS than controls compared with the TNFB1/B2 genotype carriers, and approximately 2.0 more chances (OR: 2.058, 95% C.I.: 1.099 – 3.855) of developing MS compared to the TNFB1/B1 genotype carriers. Individuals with the TNFB2 allele exhibited 1.4 (OR: 1.427, 95% C.I.: 1.093 – 1.863) more chance of developing MS than the TNFB1 allele carriers (Table 3).

To elucidate the possible association between TNF-β *Nco*I polymorphism and HLA-DRB1*, the most frequent HLA-DRB1* alleles obtained previously in the same cohort of MS patients enrolled in the present study [6] were compared with the results of TNF-β *Nco*I allelic frequencies in these patients. Table 4 shows no association between the presence of HLA-DRB1*01, 03, 04, 07, 08, 11, 13, and 15 alleles and the TNF-β *Nco*I alleles ($p>0.05$), which could suggest that TNF-β *Nco*I polymorphism may not be in linkage disequilibrium within HLA-DRB1*15 and may exert a role in the MS susceptibility.

In the present cohort of MS patients, the TNF-β *Nco*I polymorphism was not associated with the age at diagnosis ($p=0.2884$), disease duration ($p=0.8201$), clinical forms (0.4189), EDSS baseline ($p=0.9889$), EDSS five-year follow-up (0.3913), PI ($p=0.6002$), progression ($p=0.4509$), and lesions location ($p=0.8169$) observed by MRI. However, the disease activity assessed using MRI Gd-enhanced lesions showed a trend towards to be associated with the TNFB2/B2 genotype [$p=0.0722$ (OR: 1.909; 95% C.I.: 0.9788-3.618)]. MS patients with the TNFB2/B2 genotype and with the TNFB1/B2+B1/B1 genotypes exhibited 30 (14.4%) and 19 (9.1%) Gd+ contrast-enhancing lesions, respectively; in contrast, 73 (34.9%) and 87 (41.6%) of them, exhibited Gd-contrast-enhancing lesions, respectively (data not shown).

The individuals were age-gender-ethnicity-BMI matched ($p>0.05$) for all the analysis involving TNF-α (data not shown). The serum TNF-α level was analyzed according to the TNF-β *Nco*I polymorphisms in MS patients and controls using a recessive model (Figure 1). Although MS patients carrying TNFB2/B2 genotype exhibited higher serum TNF-α levels than MS patients carrying TNFB1/B2+TNB1/B1 genotypes (median 2.0 mg/dL [IQR: 2.0 – 4.6] and median 2.0 mg/dL [IQR: 2.0 – 4.8], respectively), the difference was not significant ($p=0.3447$). Healthy controls carrying TNFB2/B2 genotype presented higher TNF-α levels than individuals carrying TNFB1/B2+TNB1/B1 genotypes (median 3.4 mg/dL [IQR: 2.0 – 7.4] and median 2.3 mg/dL [IQR: 2.0 – 7.1], respectively), but no difference was also found ($p=0.4054$). When the TNF-α levels obtained from MS patients and controls carrying TNFB2/B2 genotype were compared, the controls presented higher values than MS patients (median 3.3 mg/dL [IQR: 2.0 – 7.4] and median 2.0 mg/dL [IQR: 2.0 – 4.8], respectively)

(p=0.0287). Among the MS patients and controls carrying TNFB1/B2+TNFB1/B1 genotypes, the latter showed a trend towards higher TNF-α levels than MS patients (median 2.3 mg/dL [IQR: 2.0 – 7.1] and median 2.0 mg/dL [IQR: 2.0 – 3.8] (p=0.0750).

Although MS patients carrying TNFB2/B2 genotype and with severe EDSS exhibited higher TNF-α values than those with moderate and mild EDSS, no significant difference was found (p>0.05). The serum TNF-α level did not also differ according to the disease activity and TNF-β *Nco*I polymorphism (p>0.05).

4. Discussion

Although the TNF-α and TNF-β cytokines play an important role in the MS pathology, TNF-β genetic polymorphism that may alter the expression of TNF-α has been investigated only in two genetically homogeneous population such Polish and Iranian MS patients [1,10]. This host factor was not studied yet in a Brazilian MS population yet, that is considered one of the most heterogeneous population worldwide [29]. The main results obtained in this study were that TNF-β *Nco*I polymorphism may be associated with MS in Brazilian populations independently of the most frequent HLA-DRB1* alleles previously genotyped in this same cohort of MS patients. Other important result is that TNF-β *Nco*I polymorphism trend towards to be associated with MS disease activity when evaluated using MRI and may be associated with the increased serum TNF-α level in healthy subjects.

The demographic characteristics of MS patients evaluated in the present cohort are consistent with previous studies, and the predominance of female among MS patients reinforced that gender-related factors have an effect on the individual's susceptibility for MS; the occurrence of MS commonly in young adults and in Caucasians is also consistent with previous reports [1,2,15,35-37]. Clinical characteristics were also in agreement with previous studies [35,36,38]. The most of the MS patients exhibited low disability when evaluated using EDSS baseline and five-year follow-up. Some factor could explain this result as follow: a) all of the MS patients were in clinical remission, b) most of them presented the RR-MS clinical form which is characterized by lower severity and less disease progression than other clinical forms, c) most of them exhibited the inactive disease demonstrated by Gd-enhancing lesions, and d) most of them were treated with IFN-β (1a or 1b).

In the present study, MS patients showed higher frequencies of TNFB2/B2 genotype and TNFB2 allele than controls. Individuals with TNFB2/B2 genotype exhibited approximately 1.5 more chance of developing MS when compared with TNFB1/B2

individuals and approximately 2.0 more chance when compared with TNFB1/B1 individuals. In addition, the presence of the TNFB2 allele conferred almost 1.5 more chance of developing MS. The genotype and allelic frequencies in MS patients are consistent with those previously described [1, 10]; however these authors did not find association between this polymorphism and MS.

No association was found between HLA-DRB1* most frequent alleles and TNF-β *Nco*I polymorphism evaluated in this cohort of MS patients, which could suggest that the TNF-β *Nco*I polymorphism may not be in linkage disequilibrium within HLA-DRB1* alleles and may exert influence in MS susceptibility independently of HLA-DRB1*. Previous study [10] suggested that both TNF-β *Nco*I polymorphism and -308 TNF promoter polymorphisms are independently inherited. However, it was described an activation-dependent intrachromosomal interactions involved in the activation of TNF-α gene transcription in activated T cells (39). This finding leads to the hypothesis that the occurrence of the allelic forms of TNF-β gene could undergo intrachromosomal interactions that take place in close proximity to the TNF-α promoter in a configuration that potentially underlies selective activation of the TNF-α gene. This possible activation could increase the production of TNF-α contributing to the MS susceptibility, and this effect seems to be independently from the possible linkage disequilibrium with HLA-DRB1* alleles.

Although is expected that chronic inflammatory diseases show elevated TNF-α level, the present cohort of MS patients carrying with the TNFB2/B2 genotype presented lower levels of this cytokine compared to controls carrying the TNFB2/B2 genotype and this result can be explained by several factors, as follow: 1) TNF-α has short time half-lives, exerts both paracrine and autocrine pathways of action, and exhibits a long regulation loop [23]; 2) TNF-α is produced and consumed at sites of inflammation within the CNS of MS patients including lesions in white matter, spinal cord, and optic nerve; it have been detected in astrocytes of demyelinating plaques [40] and CSF fluid from MS patients with active disease [41]; 3) biological effects of the cytokines occur when they are binding with their receptors and almost all the cells express receptor for TNF-α (TNFRI is related with inflammation and severe course of the disease and TNFRII is related with remyelination and mild course of the disease) [42- 44]; and 4) both TNF-α and TNF-β are considered potent inflammatory cytokines that contribute to the white matter destruction at the onset of the disease [45], but not during repair, which is characterized by the predominance of Th2 lymphocyte cells [10].

There are inherent problems with the interpretation of the *in vivo* cytokines expression. First, the studies of cytokine in humans are limited to indirect measurements of cytokines in peripheral blood cells, in myelin-specific T cells clones generated from patients and controls, in CSF, or the local expression in *post mortem* brain and biopsy specimens [46]. Second, the cytokine profile in MS is confounded by the temporal dissemination of lesions, when the inflammation, demyelization, and remyelination processes and neuronal loss may occur simultaneously; and all of these processes contribute to the levels of any cytokine measured in CSF or blood [23]. Third, almost 70.0% of the MS patients enrolled in the present cohort were treated with IFN-β 1a or 1b and controversial results about its influence in cytokine profile were described [23,47-,49]. Previous study [23] also found higher level of TNF-α in controls than MS patients who were treated with IFN-β. Moreover, the patients enrolled in this study exhibited several years of diagnosis, were at clinical remission, and most of than were treated with IFN- β 1a or 1b therapy at the moment of blood collection.

Therefore, TNF-β *Nco*I polymorphism may influence the TNF-α production taken into account the highest TNF-α levels presented by the TNFB2/B2 controls. MS patients with TNFB2/B2 genotype also exhibited higher TNF-α level than TNFB1/B2+TNFB1/B1 carriers; however, probably due to the inflammatory process, the TNF-α was consumed at site of lesions or was linked to its receptors in cell membranes, when the cytokine are not free to be detectable by the sandwich ELISA.

TNF-β *Nco*I polymorphism does not influence the age at onset of the disease, disease duration, clinical form, severity, and progression of disability when evaluated using EDSS. Although TNFB2/B2 genotype carriers with severe EDSS presented higher serum TNF-α level than mild and moderate EDSS, no difference was found. Therefore, TNFB2/B2 genotype, itself, may not be sufficient to influence the disease severity and progression in Brazilian MS patients. However, TNFB2/B2 genotype showed a trend towards to be associated with disease activity using MRI, although no differences in TNF- α were found among disease activity and *Nco*I genotypes. Studies have shown that TNF-α level in serum and CSF are associated with disease activity using MRI [50]; expression of TNF-α in the CNS is up-regulated in MS lesions and is produced by macrophages, microglia, and astrocytes in active lesions [51]. Several studies have found positive correlation between TNF-α levels and clinical course of MS [52-54]. However, the present study did not find difference between the TNF-α level and the Gd+ or Gd- contrast enhanced lesions at MRI. Studies with Gd enhanced contrast have shown that MRI provides the most objective and sensitive tool for assessing disease progression and disease activity in MS because this imaging evaluation reveals

episodes of disease activity five to ten times more frequently than clinical measures [55]. Furthermore, there is accumulating evidences that MRI parameters are predictive of the course of disability in MS patients [56].

Genetic studies suggest that MS susceptibility depends on independents or epistatic effects of several genes with small individual effects rather than few genes of major biologic importance, consistent with a polygenic disease [14,44]. Several genetic polymorphisms potentially exert additive or synergistic influence with other factors, such as environmental agents and the immune response mediated by $CD4^+$ Th1 cells, the levels of other inflammatory cytokines and chemokynes in the CSF, the cytokine receptors expression [57-59], as well the HLA and non-HLA polymorphisms, as recently reviewed [8].

Taken together, our findings are consistent with the polygenic hypothesis for MS and suggest that TNF-β *Nco*I polymorphism may also have a role in the susceptibility for MS in Brazilian population independently of HLA-DRB1*15, and that the TNFB2 allele could be considered a genetic factor for the development of MS and may be involved with disease activity; however, by itself, may not be a major determinant for the disease progression in this population, suggesting that the TNF-α may not represent a new target for the treatment of MS patients.

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6. Conflict of Interest Statement

All the authors declare that there is no conflict of interest.

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Table 1 - Demographic characteristic of multiple sclerosis patients and healthy controls from Southern Brazilian population.

	MS patients (n = 220)	Healthy Controls (n = 278)	p value
Gender n (%)			
Female	155 (70.5)	186 (66.9)	0.2373[a]
Male	65 (29.5)	92 (33.1)	
Ethnicity n (%)			
Caucasian	179 (81.4)	223 (80.2)	0.3974 [a]
Non-Caucasian	41 (18.6)	55 (19.8)	
Age (years)			
Median	43.5	42.0	0.2373 [b]
IQR	21.3 – 53.0	34.0 – 49.0	

[a]Chi-Square test ($p<0.05$).
[b]Mann Whitney test ($p<0.05$)
IQR: interquartile range 25%-75%
n: absolute number; %: percentage.

Table 2 - Clinical characteristics of multiple sclerosis patients from Southern Brazil.

Clinical characteristic	MS patients
Clinical form (n = 220) n (%)	
RR-MS	162 (73.6)
SP-MS	23 (10.5)
PP-MS	9 (4.1)
CIS	9 (4.1)
Not defined	17 (7.7)
Age at onset of disease (years) (n = 220)	
Median	34.5
IQR	(26.0 – 44.0)
Disease duration (years) (n = 220)	
Median	7.0
IQR	(2.2 – 10.0)
EDSS baseline (n = 144) (%)	2.0 (0.0 – 3.5)
Mild (0.0 – 4.0) n	120 (83.3)
Moderate (4.5 – 5.5)	06 (4.2)
Severe (6.0 – 10.0)	18 (12.5)
EDSS five-year follow-up (n = 198) (%)	3.5 (1.0 – 4.5)
Mild (0.0 – 4.0)	141 (71.3)
Moderate (4.5 – 5.5)	29 (14.6)
Severe (6.0 – 10.0)	28 (14.1)
Lesion location by MRI (n = 209) (%)	
Brain white matter	171 (81.8)
Spinal cord	71 (34.0)
Optic nerve	22 (10.5)
Disease activity assessed by MRI (n=209) n (%)	
Positive gadolinium enhanced lesions	49 (23.4)
Negative gadolinium enhanced lesions	160 (76.6)
MS treatment (n=208) n (%)	
Interferon-β 1a	95 (45.7)
Interferon-β 1b	50 (24.0)

Glatiramer acetate	27 (13.0)
Natalizumab	4 (1.9)
Treatment naïve	13 (6.3)
No adhesion	19 (9.1)
Corticosteroids (n = 220) n (%)	25 (11.4)

n: absolute number; %: percentage.
IQR: interquartile range 25%-75%
MS: multiple sclerosis; RR: relapsing-remitting; SP: secondary progressive; PP: primary progressive; CIS: Clinically isolated syndrome; EDSS: Expanded Disability Status Scale; MRI: magnetic resonance imaging.
76 MS patients did not present EDSS baseline; 22 MS patients did not present EDSS five-year follow-up; 31 MS patients did not present MRI data; 12 MS patients did not present MS treatment data.

Table 3 - Frequency of tumor necrosis factor beta (TNF-β) *Nco*I polymorphism genotype and allele obtained from multiple sclerosis patients and healthy controls from Southern Brazilian population.

	TNF- β *Nco*I polymorphism			
	MS patients (n = 220) n (%)	Healthy controls (n= 278) n (%)	p value	Odds Ratio (95% CI)
B2/B2	106 (48.2)	103 (37.1)		Reference
B1/B2	96 (43.6)	139 (50.0)	0.0449[a]	1.490 (1.023 – 2.170)
B1/B1	18 (8.2)	36 (12.9)	0.0225[b]	2.058 (1.099 – 3.855)
B2 allele	0.700	0.6205	0.0089[c]	1.427 (1.093 – 1.863)
B1 allele	0.300	0.3795		

[a]Chi-square Test, p = 0.0449 (B2/B2 *vs* B1/B2)
[b]Chi-square Test, p = 0.0225 (B2/B2 *vs* B1/B1)
[c]Chi-square Test, p = 0.0089 (B2 allele *vs* B1 allele)
MS: multiple sclerosis; B1: allele with the restriction site of the *Nco*I; B2: variant allele without the restriction site of the *Nco*I; B1/B1: homozygous genotype for the allele B1; B1/B2: heterozygous genotype for the allele B1; B2/B2: homozygous genotype for the allele B2; TNF-β: tumor necrosis factor β; *Nco*I: *Nocardia corallis* I restriction enzyme; CI: confidence interval.

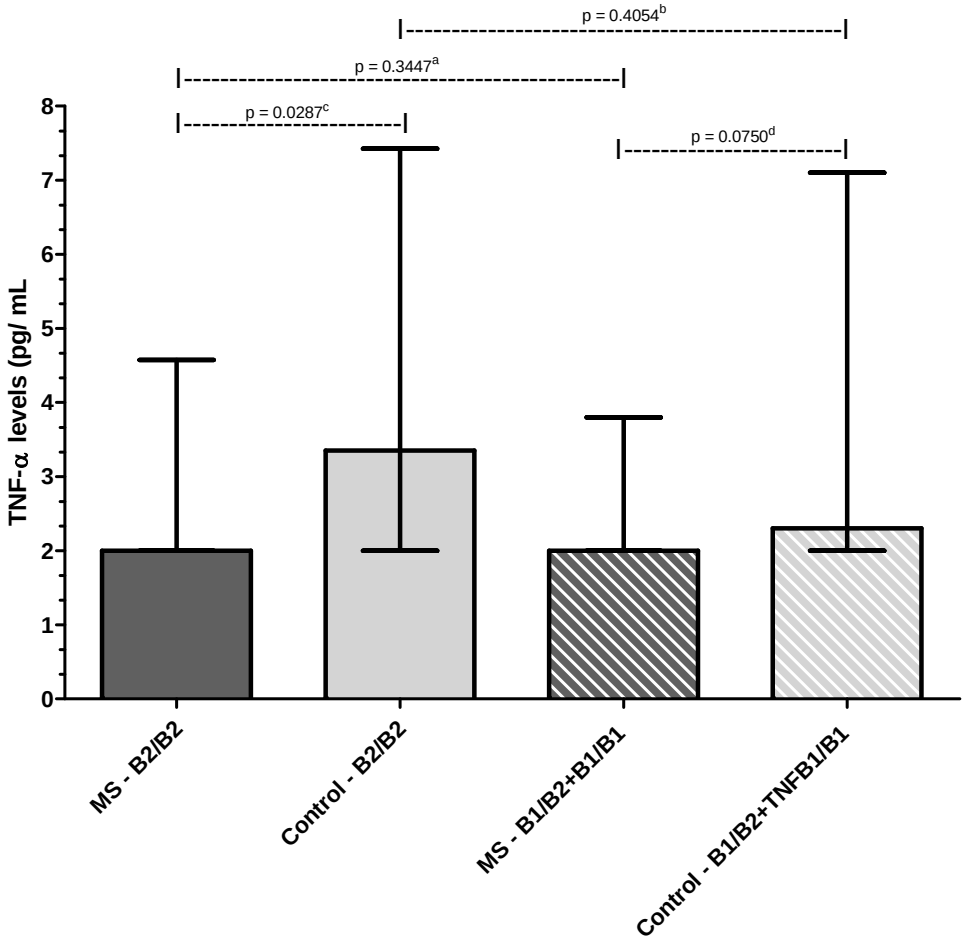
Table 4 - Frequency of TNF-β *Nco*I and HLA-DRB1* alleles in multiple sclerosis patients from Brazilian population.

HLA-DRB1 alleles[a]	TNF-β *Nco*I polymorphism		p value
	Allele B2 + (n=89) n (%)	Allele B2 – (n=10) n (%)	
01 +	19 (19.2)	04 (4.0)	0.2349
01 –	70 (70.7)	06 (6.1)	
03+	20 (20.2)	03 (3.0)	0.5939
03 –	69 (69.7)	07 (7.1)	
04 +	20 (20.2)	02 (2.0)	1.000
04 –	69 (69.7)	08 (8.1)	
07 +	18 (18.2)	02 (2.0)	1.000
07 –	71 (71.7)	08 (8.1)	
08 +	13 (13.1)	03 (3.0)	0.2025
08 –	76 (76.8)	07 (7.1)	
11 +	19 (19.2)	00 (0.0)	0.2014
11 –	70 (70.7)	10 (10.1)	

13+	15 (15.2)	02 (2.0)	0.6802
13 –	74 (74.7)	08 (8.1)	
15 +	17 (17.2)	00 (0.0)	0.2032
15 –	72 (72.7)	10 (10.1)	

Differences were assessed by Fisher Exact Test ($p<0.05$) and expressed as absolute number.
HLA: human leukocyte antigen; MS: multiple sclerosis; allele B2+: includes TNFB1/B2+TNFB2/B2; allele B2-: include TNFB1/B1; TNF-β: tumor necrosis factor β; *Nco*I: *Nocardia corallis* I recombinant restriction enzyme.
[a]The results from HLA-DRB1* genotyping were obtained previously from the same cohort of patients that were published elsewhere [6].

Figure 1 - Serum tumor necrosis factor-α level according to the TNF-β *Nco*I genotypes obtained in multiple sclerosis patients and healthy controls from Southern Brazil population.



[a]Mann Withney Test, p = 0.3447 (MS B2/B2 *vs* MS B1/B2+B1/B1)
[b]Mann Withney Test, p = 0.4054 (Control B2/B2 *vs* Control B1/B2+B1/B1)
[c]Mann Withney Test, p = 0.0287 (MS B2/B2 *vs* Control B2/B2)
[d]Mann Withney Test, p = 0.0750 (MS B1/B2+B1/B1 *vs* Control B1/B2+B1/B1)

Age-gender-ethnicity-body mass index matched ($p > 0.05$), (data not shown).
TNFB1/B1: homozygous genotype for the allele TNFB1; TNFB1/B2: heterozygous genotype for the allele TNFB1;TNFB2/B2: homozygous genotype for the allele TNFB2.

Cytokine profile in relapsing-remitting multiple sclerosis patients and the association with the progression and the activity of the disease

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ABSTRACT: Multiple sclerosis (MS) is a progressive immune-mediated disease caused by demyelination process of central nervous system (CNS). Cytokines and their receptors have an important role in the evolution of the lesions and have been correlated with changes in MS disease activity. The aims of this study were to evaluate the pro-inflammatory (TNF-α, IL-1β, IL-6, and IL-12), Th1 (IFN-γ), Th17 (IL-17), and anti-inflammatory Th2 (IL-4 and IL-10) cytokines serum levels in relapsing-remitting MS (RR-MS) patients; and to evaluate the association between cytokine profile with the clinical disability and disease activity of RR-MS patients. The serum cytokine level was assessed using enzyme linked-immunosorbent assay in 169 RR-MS patients in the remission clinical phase and in 132 healthy individuals age-gender-ethnicity--and body mass index matched. The disability of the RR-MS patients and the activity of the disease were evaluated using the Expanded Disability Status Scale (EDSS) and magnetic resonance imaging (MRI) with the gadolinium (Gd), respectively. IFN-γ, IL-6, IL-12, and IL-4 levels were higher in RR-MS patients than controls (p=0.0009, p=0.0114, p=0.0297, and p=0.0004, respectively) and the IL-1 levels trend towards higher in controls than RR-MS patients. IL-4 were higher in RR-MS patients with mild disability compared to those with moderate and severe disability (p=0.0375). TNF-α and IL-10 levels were higher in RR-MS patients with negative Gd-enhancing lesions than those with positive Gd-enhancing lesions presented at MRI. IL-17 levels trend towards higher in RR-MS patients with negative Gd (p=0.0631). Low TNF-α and high IFN-γ levels were independently associated with the RR-MS (p=0.0078 and p=0.0056, respectively) and also with the activity of the disease (p=0.0348 and p=0.0133, respectively). The results underscore that RR-MS patients, even in the remission clinical phase, exhibit a complex system of inflammatory and anti-inflammatory cytokines that may interact to modulate the progression and the activity of the disease.

Keywords: cytokines, multiple sclerosis, disease activity, disability, disease progression

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1. Introduction

Multiple sclerosis (MS) is a progressive immune-mediated disease caused by demyelination process of central nervous system (CNS), with perivascular infiltrates of auto reactive T-cells that recognize and react against auto antigens such as myelin basic proteins (MBP), proteolipid protein (PLP), myelin oligodendrocytic glycoprotein (MOG), and myelin associated glycoprotein (MAG) [1,2]. Cytokines and their receptors have an important role in the evolution of the lesions, and the pro-inflammatory and anti-inflammatory cytokine levels have been correlated with changes in MS disease activity [3].

MS is regarded as a T helper 1 (Th1) lymphocyte -mediated disorder, since the levels of Th1-related molecules are increased in MS, but the causes of the disease are still unclear. Th1 lymphocyte cells producing interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) have been found in the perivascular infiltrates of MS patients [4]; IFN-γ and interleukin-12 (IL-12) are increased in brain, cerebrospinal fluid (CSF) or peripheral blood of MS patients, especially during the acute exacerbations [5]. On the other hand, T helper 2 (Th2) lymphocyte-related molecules, such as interleukin-10 (IL-10) and interleukin-4 (IL-4), which can down regulate Th1-cells and exert anti-inflammatory functions, are increased during remission phases of MS [6]. The Th17, a subset of T-cells that secretes IL-17, has been associated with the pathogenesis of several inflammatory diseases including MS and encephalomyelitis autoimmune experimental (EAE) [7,8]. In the EAE, both Th1 and Th17 cells have shown to contribute to the disease pathogenesis, but no absolute requirement for either IL-17 or IFN-γ alone [9].

Cytokines have been identified as major regulators of the immune system and attempts have been made to correlate cytokine levels with MS disease activity assessed using magnetic resonance imaging (MRI). There is evidence that an acute dysregulation of the balance of cytokines is one of the key factors during an acute relapse leading to acute inflammatory lesions in MS patients. Elevated serum and CSF TNF-α and tumor necrosis factor beta (TNF-β) levels have been associated with the onset of MS relapses [10]. The disease progression in MS patients has also been correlated with high CSF TNF-α level [11].

The clinical disability of MS patients is evaluated using the Expanded Disability Status Scale (EDSS) [12] and the disease activity is evaluated using the MRI with gadolinium (Gd)-enhancing lesions which provide the most objective and sensitive tool for assessing the

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progression and the activity of the disease in MS patients. The MRI reveals episodes of disease activity five to ten times more frequently than clinical measurements [13]. Positive Gd-enhancing lesions are the earliest event in the formation of new MS lesions, and are predictive of the course of disability in MS patients [14].

The cytokine changes have been investigated in MS patients from different population worldwide and almost of the studies enrolled limited number of MS patients with different clinical forms of the disease, and during either the remission or the relapse phase [15-20]. The aim of the present study was to evaluate the serum cytokine profile in relapsing-remitting MS (RR-MS) patients from Southern Brazilian population, and to evaluate the association between the inflammatory and anti-inflammatory cytokine profiles with the clinical disability and disease activity of these patients.

2. Materials and methods

2.1 Study design and subjects

A case-controlled study was approved by the Institutional Research Ethic Committee of the State University of Londrina, and a voluntary written consent form was obtained from all the individuals before the start of the study. The study enrolled 169 consecutive RR-MS patients from the Neurology Outpatients Service of the Outpatient Clinic Hospital, State University of Londrina, Southern Brazil, during the year of 2011. The RR-MS patients were diagnosed according to the revised McDonald Criteria [21,22]. The RR-MS clinical form was determined according to the classification of Lublin and Reignold [23]. Demographic, clinical, and treatment data of the patients were recorded using a standard questionnaire and a research at medical records. The age at onset of the disease was based on retrospectives data from medical records [21]. All of the RR-MS patients were in the remission clinical phase, defined as the period of recovery with no relapse episodes within the last three months prior to the time of enrollment in the study. The clinical disability was evaluated using the EDSS [12] at the time of the enrollment of the patients and was categorized as mild (EDDS 0.0–4.0), moderate (EDSS 4.5–5.5), and severe (EDSS 6.0–10.0), according to previous study [24]. The lesion location (brain, spinal cord, and optic nerve) and the result of Gd contrast-enhancing lesions were obtained from the most recent MRI scan performed (± three months from the

enrollment) according described previously [12,25,26]. The control group consisted of 132 unrelated healthy blood donors from the Blood Bank of Londrina, with similar demographic characteristics and from the same geographical area of the patients. All of them presented negative reactivity for serologic screening tests for blood donation according to the government procedures [27]. The control subjects did not present either clinical or laboratory characteristics of autoimmune, infectious, or inflammatory diseases. In addition, they also reported that they were not using any anti-inflammatory drugs or other that could affect the immune system.

The RR-MS patients and control subjects were age-gender-ethnicity-and body mass index (BMI) controlled. Anthropometric measurements were obtained by body weight, measured to the nearest 0.1 kg by using an electronic scale, with individuals wearing light clothing, but no shoes; and height measured to the nearest 0.1 cm by using a stadiometer. BMI was calculated as weight (kg) divided by height (m) squared.

2.2 Cytokine measurements

Blood samples for cytokine analyzes were collected at the time of enrollment of the patients. The serum samples of MS patients were immediately separated, divided into aliquots, and stored at -80ºC freezer for posterior analyzes according to a consensus protocol [28]. Serum pro-inflammatory cytokine levels (IL-1β, IL-12p70, IL-6, and TNF-α), Th1 cytokine (IFN-γ), Th2 cytokines (IL-4 and IL-10), and Th17 cytokine (IL-17) were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) using a commercial immunoassay (eBioscience, San Diego, California, USA). The protocol was performed according to the manufacturer's instructions and the results were expressed in ng/mL. The limit of detection for these cytokines was defined according to the manufacturer's instructions (IL-1β: 2.0 ng/mL, IL-12p70: 2.0 ng/mL, IL-6: 1.0 ng/mL, TNF-α: 2.0 ng/mL, IFN-γ: 2.0 ng/mL, IL-4: 1.0 ng/mL, IL-10: 1.0 ng/mL, and IL-17: 2.0 ng/mL).

2.3 Statistical analysis

A database was created using the Excel Program of Microsoft Office and the statistical analysis was performed using Graph Pad Prism version 5.0 (Graph Pad Software, San Diego, CA, USA). Categorical variables were analyzed using a chi-square test (or Fished Exact test

when appropriate) and were expressed in absolute number and percentage (%). Continuous variables were analyzed using the Mann-Whitney test and were expressed as median, interquartile range (IQR) 25%-75%, and range. Continuous variables of two groups or more were compared using the Kruskal-Wallis test with Dunn's post test. All of the results were considered significant when $p<0.05$. To determine which factors were independently associated with RR-MS, the variables that presented $p<0.20$ in the univariate analyses and those values with supposed clinical relevance or previous data in the literature were included in the multivariate logistic regression model. The multivariate analyses were evaluated by the GraphPad Instat version 3.0 (Graph Pad Software, San Diego, CA, USA).

3. Results

The demographic and anthropometric characteristics of the RR-MS patients and healthy individuals are presented in Table 1. As expected, the RR-MS patients and controls did not differ in any of the controlled parameters ($p>0.05$). The clinical characteristics of RR-MS patients are shown in Table 2. The median age at onset of the disease was 33.0 years and median disease duration of 7 years. The most part of the patients presented mild EDSS and brain white matter lesions without activity. Data of MS therapy revealed that the most of the RR-MS patients (75.6%) were treated with interferon-beta (IFN-β) and only 18 (10.7%) of them were treated with corticosteroids.

The serum cytokine level was compared between RR-MS patients and controls (Table 3). Regard the pro-inflammatory cytokine profile, the IL-1 level trend toward higher values among the healthy controls than RR-MS patients ($p=0.0992$). The IL-6 and IL-12 levels were higher in RR-MS patients than those found in controls ($p=0.0114$ and $p=0.0297$, respectively). The univariate analysis showed that the serum TNF-α level observed among RR-MS patients did not differ from those observed among the controls (median: 2.0 pg/mL, IQR: 2.0 – 4.3 *vs* median: 2.0 pg/mL, IQR: 2.0 – 11.2, $p=0.1840$).

Regard the Th1 cytokine profile, the IFN-γ level was higher among the RR-MS patients compared with those obtained among the controls ($p=0.0009$). However, the Th17 cytokine IL-17 did not differ between the RR-MS and controls ($p=0.2298$).

The Th2 anti-inflammatory cytokine profile results revealed that the IL-4 level was higher in RR-MS patients than those observed among the controls ($p=0.0004$). Although IL-10 level were higher in RR-MS patients than controls, no difference was found ($p=0.1901$).

The logistic regression analysis between the results obtained from 169 RR-MS patients and 132 controls included the variables IL-1, IL-6, TNF-α, IFN-γ, IL-12, IL-4, and IL-10 showed that low TNF-α and high IFN-γ levels were independently associated with RR-MS (p=0.0078 and p=0.0056, respectively).

The serum cytokine level was also evaluated according to the disability of the RR-MS using the categorized EDSS scores in mild, moderate, and severe disability (table 4). Factors as gender (p=0.2357), ethnicity (p=0.2933), BMI (p=0.2998), and the use of corticosteroids (p=0.4333) were controlled. However, the age of RR-MS patients that exhibited severe disability was higher than those of RR-MS patients with mild and moderate disability (p=0.0034).

Serum IL-1 level did not differ according to the disability exhibited by the RR-MS patients (p=0.4422). Although IL-6, TNF-α, and IFN-γ levels were higher among the RR-MS patients with severe disability than those observed among the RR-MS patients with moderate and mild disability, the difference was not significant (p=0.7408, p=0.6896 and p=0.6032, respectively). No difference was also found in the IL-12 and IL-17 levels obtained among the RR-MS patients with mild, moderate, and severe disability (p=0.5083 and p=0.2359, respectively). Higher IL-4 level was observed among RR-MS patients with mild disability when compared with the values obtained from those with moderate and severe disability (p=0.0375). However, no difference was observed in the IL-10 level according to the disability of the patients (p=0.8680). After the logistic regression analysis, all of the variables presented no independent association with disease disability (p>0.05).

The serum cytokine level obtained from RR-MS patients were also evaluated according to the disease activity assessed by the Gd-enhancing lesions showed at MRI (Table 5). To this analysis, factors such as gender (p=0.5383), ethnicity (p=0.7656), BMI (p=0.6464), and the use of corticosteroids (p=0.9029) were also controlled. However, the age of RR-MS patients with positive Gd-enhancing lesions was lower compared with that presented by RR-MS patients with negative Gd-enhancing lesions (p=0.0032).

The IL-1, IL-6, IL-12, and IL-4 levels did not differ between RR-MS patients with positive Gd-enhancing lesions and negative Gd-enhancing lesions (p=0.4752, p=0.1129, p=0.9089, p=0.8980, respectively). Although the IFN-γ level was higher among the RR-MS patients presenting positive Gd-enhancing lesions than those obtained from RR-MS patients with negative Gd-enhancing, no difference was found in univariate analysis (p=0.1643). The TNF-α level was higher in RR-MS patients with negative Gd-enhancing lesions than patients with positive Gd-enhancing lesions (p=0.0572). The serum IL-17 level showed a trend toward

higher among the RR-MS patients with negative Gd-enhancing lesions than those among the RR-MS patients with positive Gd-enhancing lesions (p=0.0631). While the serum IL-10 level was higher among the RR-MS patients with negative Gd-enhancing lesions than those found among the RR-MS patients with positive Gd-enhancing lesions (p= 0.0533), the IL-4 levels did not differ between them (p=0.8980).

The logistic regression analysis between the results obtained from RR-MS patients and the disease activity including the variables age, IL-6, TNF-α, IFN-γ , IL-17, and IL-10 demonstrated that low TNF-α and high IFN-γ levels were independently associated with the activity of the disease (p=0.0348 and p=0.0133, respectively).

4. Discussion

This study evaluated the serum cytokine profile exhibited by RR-MS patients in a remission clinical phase from Brazilian population. Theoretically, CSF may better reflect the relevant inflammatory process due to its proximity to inflammatory lesion in the CNS. However, due to the flow pattern of CSF, it is unlikely that the CSF in the lumbar cistern accurately reflects the production of the inflammatory markers in the supratentorial region, where most of the MS-related inflammation occurs [29]. In addition, CSF collection is an invasive procedure, the intraparenchymal extracellular space may not necessary communicate with the free CSF space [30], and it remains unclear whether CSF markers possess definite advantages over markers collected from blood and/or urine [29]. These facts lead us to measure the cytokines profile in serum samples.

To obtain more homogeneity of the cohort, only patients with the RR-MS clinical form were included in this study. The main result obtained by this study was the elevated serum IL-6, IL-12, IFN-γ, and IL-4 levels in RR-MS patients compared to controls. MS patients with mild EDSS presented elevated IL-4 level. RR-MS patients with inactive disease exhibited elevated TNF-α and IL-10 levels and trend towards higher IL-17 levels. The logistic regression showed that low TNF-α and high IFN-γ levels were associated with both MS and the disease activity.

The demographic and clinical characteristics exhibited by the RR-MS patients are consistent with previous studies [18,24,31-34]. The most of the RR-MS patients exhibited mild clinical disability, predominance of lesions in bran white matter, and low disease activity. These are an expected result considering that all of the RR-MS patients were in

clinical remission, most of them were treated with IFN-β (1a or 1b) which has been shown to decrease clinical disability and disease activity [18,35-37].

RR-MS patients and controls were age-gender-ethnicity-BMI matched for the cytokine analyses. All of these parameters were controlled to minimize the influence of them on the serum cytokine level [38,39]. The IL-1 level trending towards higher among controls than among RR-MS patients could be explained by the fact that IFN-β therapy decreases the IL-1β expression by dendritic cells and monocytes of RR-MS patients [40]. This pro-inflammatory cytokine is considered an important mediator for the neuronal and axonal damage of the CNS in MS patients. One of the actions of IL-1β in the brain is the induction of astrogliosis with astrocytes activation and IL-6 production, an effect that is strongly augmented in the presence of TNF-α, IFN-γ, IL-6, and IL-6 receptor [41]. However, IL-1 has also been shown to induce the proliferation of nerve growth factor (NGF) *in vitro,* and may have some protective effects in MS patients [42].

The higher IL-6 levels among RR-MS patients than among controls are consistent with previous findings. One study showed that the number of IL-6 mRNA expressing mononuclear cells was enriched in CSF of MS patients [43]. Imamura et al. [44] obtained an increased IL-6 production by peripheral blood monocytes and macrophages stimulated with LPS from MS patients. IL-6 deficient mice were found to be resistant to the induction of EAE [17], and also showed a reduction of anti-MOG antibody titers, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) molecule expression, which may affect the entry of Th1 cells into CNS [45]. On the other hand, IL-6 also presented beneficial effects such as anti-inflammatory properties. IL-6 inhibits the IL-1β and TNF-α secretion *in vitro* [46], and induces circulating IL-1 receptor antagonist *in vivo* [47]. Our results are consistent with these previous studies considering that elevated IL-6 level was found in RR-MS patients, the IL-1 level trend towards higher values in controls, and no difference was observed on the TNF-α level.

Navikas et al. [48] demonstrated that MS patients present high number of MBP-reactive cells in their blood which after cultures with MBP and myelin proteolipid protein (PLP) respond to production and secretion of other cytokines including IFN-γ, IL-4, transforming growth factor beta (TGF-β), IL-10, and TNF-α. Navikas et al. [43] demonstrated that MS patients presented elevated MBP- and also PLP-reactive IL-6 mRNA expressing cells in peripheral blood. It is known that cytokine may modulate each other's production [49]. Therefore, the IL-6 acts with other cytokines within the cytokine network.

The TNF-α level was lower among the RR-MS patients than the controls, as showed by the multivariate analysis. This result is consistent with previous studies that also reported lower TNF-α level in MS patients than in controls [11,19]. Graber et al. [3] obtained decreased production of TNF-α by stimulated PBMC from MS patients when compared to controls.

The higher IFN-γ level obtained among MS patients than controls is also consistent with previous studies. Lymphocyte producing IFN-γ was found in the perivascular infiltrates of MS patients [4]. Cucci et al. [2] studied mRNA serum transcript in patients receiving different IFN-β 1b doses and confirmed that IFN-β decreases the expression of the Th1 inflammatory molecules, such as IFN-γ and TNF-α, and increases the Th2 related molecule IL-10.

Although the IL-17 expression has been shown to generate a highly pro-inflammatory environment and to induce severe pathological conditions [50], in the present study the IL-17 level did not differ between MS patients and controls. Brucklacher-Waldert et al. [51] showed that the proportion of Th17 cells in patients with relapses were significantly higher compared to patients during remission, in both clinically isolated syndrome (CIS) and RR-MS clinical forms of MS. Langrish et al. [52] also showed that IFN-β inhibits IL-23 which is implicated in the differentiation of Th0 (naïve) into Th17 cells and, consequently, the IL-17 production. In addition, Sweeney et al (2011) demonstrated that IFN-β therapy inhibits the IL-27 cytokine which, in turn, inhibits the Th17 cells both directly and through inhibition of IL-23 that promotes the development of these pathogenic T cells. IL-23 and IL-12 exert antagonistic effects: while IL-23 supports the IL-17 production, IL-12 inhibits it [53]. IL-17, in turn, stimulates astrocytes IL-6 production and macrophage production of IL-6, TNF-α, and IL-1β, cytokines that have been implicated in MS pathology [54].

The RR-MS patients enrolled in this study presented high levels of IL-12 which, in turn, could antagonize the IL-17 production and by this effect, could reduce the IL-1β and TNF-α induced production by IL-17. In addition to the consume of TNF-α in the CNS lesions, the reduced production could explained, in part, why the TNF-α level that did not differ among the individuals [54].

The higher IL-12 level exhibited by RR-MS patients compared with controls enrolled in the present study is also consistent with the previous finding that IL-12 level was up regulated in MS patients [55]. Over-expression of IL-12 in the CNS resulted in an increased inflammation and cellular infiltrates [56]. Although several studies have proposed a central pro-inflammatory role of IL-12 in the development of autoimmune responses, controversial

results have been shown regard the IL-12 level obtained from MS patients and the IL-12 level in MS patients treated with IFN-β. Ramgolam et al. [40] showed that the treatment with IFN-β 1a significantly induces the IL-12p35 gene expression in dendritic cells from RR-MS patients. On the other hand, Byrnes et al. [57] demonstrated that the treatment with IFN-β inhibits IL-12, decreases CXCL10, IL-18, IFN-γ, and TNF-α; however, this therapy increases the IL-10 and the TGF-β level [58].

In the Brazilian cohort enrolled in the present study, higher levels of IL-4 were found in MS patients than in controls, a consistent result with other previously reported [59,60]. Other study showed that IL-4 mRNA was undetectable until disease remission in SJL mice immunized with PLP [61]. IL-4 is a key cytokine produced by Th2 cells, and inhibits Th1 cells by decreasing IL-1 and TNF-α cytokines. Administration of IL-4 in EAE exerts an ameliorating and protective effect. Studies demonstrated that IL-4 up-regulation may reduce the severity of EAE, while its absence does not alter the course of the disease, possibly because in the absence of IL-4, other Th2 cytokine may substitute its functions and contribute to the tolerance of EAE [62].

Although IL-10 levels were higher in MS patients than controls, no statistically difference was found. Samoilova et al. [63] suggested that IL-10 plays an important role in recovery of EAE. In contrast with other Th2 cytokine (IL-4), the absence of IL-10 consistently results in a more severe EAE, suggesting that its function cannot be replaced by other Th2 cytokine.

The cytokine profile was also evaluated according to the EDSS of the RR-MS patients. Those with mild disability were younger than those with moderate and severe disability and this is an expected result because at the beginning of the disease, low EDSS scores are found among the RR-MS patients, and the disability increases during the disease progression [64]. However, the logistic regression analysis showed no influence of age at cytokine profile according to the EDSS. Although higher serum IL-6, TNF-α, IFN-γ and IL-12 levels were found in RR-MS patients with moderate and severe disability than those with mild disability, no significant differences were found. As observed, the results of TNF-α level are contradictory in different clinical disability. While some authors described low TNF-α levels in MS patients [11,19], other study reported that TNF-α is the principal mediator of inflammatory response and are increased among the MS patients suggesting that this cytokine is an important molecule in the pathogenesis and progression of MS [65].

The serum IL-17 and IL-10 did not also differ between EDSS categories. Several studies report that differential production of IL-10 might be a factor in the severity of MS

[31,63]. Regard the serum IL-4 level, the results showed a significant increase of this cytokine among the RR-MS patients suffering from mild disability than those with moderate or severe disability. This result confirms the important role of the IL-4 cytokine as an anti-inflammatory modulation of the MS lesive processes [6,19].

Regard the disease activity, the age of the RR-MS patients was not possible to be controlled. However, the logistic regression analysis showed that the age was not influencing the cytokine levels. There is evidence that an acute dysregulation in the balance of cytokines is one of the key factors during an acute relapse leading to acute inflammatory lesions. RR-MS patients with active disease presented lower TNF-α and higher IFN-γ levels than RR-MS patients without disease active, as showed by multivariate analysis. IFN-γ and IL-12 are increased in brain, CSF or peripheral blood from MS patients, especially during acute exacerbation phases [5]. Begolka and Miller [61] demonstrated that IFN-γ expression increases during the peak of the disease and decreases during the remission phase. Clinical attacks correlate with increased IFN-γ production [66] and patients experiencing relapses have significantly increased IFN-γ production by PBMCs after stimulation with PHA compared with patients in remission; however, this production was reduced after treatment with IFN-β [67]. IFN-γ is produced within MS lesions by infiltrating lymphocytes and induces apoptosis in human oligodendrocytes [68].

The cytokines effects only occur when they are binding to membrane receptors and TNF-α receptor are present in almost all of the cells [6]. In MS patients, TNF-α is produced and consumed at sites of inflammation, such as at the lesions in brain white matter, spinal cord, and optic nerve [69,70]; both TNF-α and TNF-β cytokines were detected in astrocytes from demyelinating plaques [70], contributing to white matter destruction at the onset of the MS [16]. Cytokines effects only occur when they are binding to fixed receptors, and almost all of the cells express TNF-α receptors [6,61,71]. The results obtained in the present study are consistent with these previous findings. Nevertheless, the serum TNF-α levels measured in the samples from RR-MS of the present study could be considered as the remaining of the expressed cytokine, that is neither acting at lesions on the CNS and nor binding to the transmembrane or soluble receptors. Other recent studies also showed lower serum TNF-α levels in the MS patients in remission than those in relapse clinical phase [19,20].

Although IL-6 level were higher in RR-MS patients with inactive disease, no differences were found. This result is in agreement with Schönrock et al. [72] that demonstrated that macrophage within the MS lesion expressed IL-6 and the highest number

of IL-6 expressing cell was found in inactive demyelination lesions. We should consider that IL-6 also has anti-inflammatory properties [46] that could influence in the disease activity.

Although studies have demonstrated that serum IL-12 level is correlated with active lesions [73], no difference was found in the serum IL-12 level obtained from the RR-MS patients with positive Gd- and negative Gd-enhancing lesions enrolled in the present study.

A trend toward higher levels of IL-17 was found in RR-MS patients with inactive disease. Th17 cell differentiation is orchestrated by multiple cytokines including IL-6, IL-1-β, TGF-β, IL-21, and IL-23 (stimulating) and IFN-γ, IL-4, IL-12, IL-10, and IL-27 (inhibiting) the differentiation of this cell. The Th17 cell represents a recently identified cell lineage that is proposed to play, in addition to the Th1 cells, a critical role in the development of the autoimmune response [74]. Studies from MS patients indicated that IL-17 mRNA is elevated in active MS brain lesions. In addition, IL-17 was also detected at lesions [75].

Serum IL-4 level did not differ between RR-MS patients with active and inactive disease, and this result is not consistent with previous studies that demonstrated high IL-4 levels in both acute and chronic lesions [62]. However, in the present study, the serum IL-10 level was statistically high among the RR-MS patients with inactive lesions. This result is consistent with the results of an increased production of IL-10 and TGF-β by isolated lymphocyte clones of MS patients in remission [76]. Decreased IL-10 level were found before the onset of an exacerbation in RR-MS patients [15]. IL-10 level was also significantly decreased in MS patients with the secondary progressive form (SP-MS) compared to RR-MS patients four weeks before the occurrence of MRI activity and six weeks before a clinical relapse [73].

The expression of the adhesion molecules, such as VCAM-1, ICAM-1, and their receptors on leukocytes very late activation antigen-4 (VLA-4) and lymphocyte function-associated antigen-1 (LFA-1), together with a selection of pro-inflammatory and immunomodulatory cytokines including IL-1, IL-2, IL-4, IL-10, TNF-α, TGF-β, and IFN-γ, play key roles in the lymphocytic infiltration and interactions during tissue inflammation and are in large part normally not expressed by CNS cells [62]. These authors demonstrated high levels of all of these molecules in MS patients, particularly in those with chronic active lesions and also found an increased expression of TNF-α and IL-4 in MS, that was statistically significant when compared with non-inflammatory neurological diseases.

All together, the results obtained in the present study underscore that RR-MS patients exhibit a complex system of cytokines that interacts to modulate the disability and the activity of the disease. Even in the remission phase of the disease and under immunomodulatory

therapy, the RR-MS patients present a imbalance between the pro-inflammatory, Th1, Th2, and Th17 cytokines, with a possible consume of TNF-α in the CNS lesions and the activation of cellular immune response mediated by high IFN-γ levels. These results contribute to the better understanding the role of cytokine profile in the RR-MS in an attempt to the development of new neuroprotective and neurorestorative therapeutic strategies.

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6. Conflict of Interest Statement

All the authors declare that there is no conflict of interest.

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Table 1 - Demographic characteristics of relapsing-remitting multiple sclerosis (RR-MS) patients and healthy controls from Southern Brazilian population

	RR-MS patients (n = 169)	Healthy Controls (n =132)	p value
Gender n (%)			
Female	123 (72.8)	104 (78.8)	0.2298 [a]
Male	46 (27.2)	26 (21.2)	
Ethnicity n (%)			
Caucasian	144 (85.2)	106 (80.3)	0.2604 [a]
Non-Caucasian	25 (14.8)	26 (19.7)	
Age (years)			
Median (IQR)	42.0 (30.0 – 51.5)	38.5 (32.0 – 46.7)	0.1090 [b]
Range	14.0 – 85.0	26.0 – 62.0	
BMI (Kg/m^2)			
Median (IQR)	23.5 (21.5 – 27.2)	24.8 (22.0 – 27.9)	0.1120 [b]
Range	16.9 – 44.0	16.4 – 40.2	

[a]Chi-Square test ($p<0.05$).
[b]Mann Whitney test ($p<0.05$).
IQR: interquartile range 25%-75%
n: absolute number
%: percentage
RR-MS: relapsing-remitting multiple sclerosis; BMI: body mass index.

Table 2 - Clinical characteristics of the relapsing-remitting multiple sclerosis patients (RR-MS) from Southern Brazilian population

Clinical characteristics	RR-MS patients
Age at onset (years) (n = 169)	
Median (IQR)	33.0 (24.5 – 44.0)
Range	10.0 – 68.0
Disease duration (years) (n = 169)	
Median (IQR)	7.0 (3.0 – 10.0)
Range	0.0 – 52.0
EDSS (n = 146) n (%)	
Mild (0.0 – 4.0)	113 (77.2)
Moderate (4.5 – 5.5)	20 (13.8)
Severe (6.0 – 10.0)	13 (9.0)
Lesion location at MRI (n = 161) n (%)	
Brain white matter	130 (80.8)
Spinal cord	54 (33.5)
Optic nerve	18 (11.2)
Disease activity at MRI (n = 161) n (%)	
Positive gadolinium enhanced lesions	35 (21.7)
Negative gadolinium enhanced lesions	126 (78.3)
RR-MS treatment (n = 156) n (%)	
Interferon-β 1a	37 (23.7)
Interferon-β 1b	81 (51.9)
Glatiramer acetate	21 (13.5)
Natalizumab	03 (1.9)
Treatment naïve	05 (3.2)

No adhesion	09 (5.8)
Corticosteroids (mg/ day)	18 (10.7)

n: absolute number
%: percentage
RR-MS: relapsing-remitting multiple sclerosis; BMI: body mass index; EDSS: Expanded Disability Status Scale (Kurtzke, 1983); MRI: magnetic resonance imaging. IQR: interquartile range 25%-75%.

Table 3 - Serum cytokine level obtained from relapsing-remitting multiple sclerosis (RR-MS) patients and healthy controls from Southern Brazil

Cytokine	RR-MS (n = 169)	Healthy controls (n =132)	p value[a]
IL-1β (pg/mL)			
Median (IQR)	2.0 (2.0 – 2.5)	2.0 (2.0 – 4.5)	0.0992
Range	2.0 – 40.7	2.0 – 63.2	
IL-6 (pg/mL)			
Median (IQR)	1.9 (1.0 – 3.9)	1.1 (1.0 – 3.4)	0.0114
Range	1.0 – 158.0	1.0 – 49.5	
TNF-α (pg/mL)			
Median (IQR)	2.0 (2.0 – 4.3)	2.0 (2.0 – 11.2)	0.1840
Range	2.0 – 68.4	2.0 – 384.5	
IL-12 (pg/mL)			
Median (IQR)	2.0 (2.0 – 2.0)	2.0 (2.0 – 2.0)	0.0297
Range	2.0 – 93.7	2.0 – 31.1	
IFN-γ (pg/mL)			
Median (IQR)	42.6 (23.4 – 74.9)	24.8 (11.7 – 80.8)	0.0009
Range	2.0 – 500.0	2.0 – 476.7	
IL-17 (pg/mL)			
Median (IQR)	2.0 (2.0 – 2.0)	2.0 (2.0 – 2.0)	0.2298
Range	2.0 – 53.2	2.0 – 88.2	
IL-4 (pg/mL)			
Median (IQR)	1.0 (1.0 – 4.0)	1.0 (1.0 – 1.0)	0.0004
Range	1.0 – 26.4	1.0 – 16.1	
IL-10 (pg/mL)			
Median (IQR)	4.7 (3.3 – 6.5)	4.0 (2.9 – 5.6)	0.1901
Range	1.0 – 104.9	1.0 – 28.2	

The results were expressed as median, interquartile range (IQR) 25%-75%), and range values. Differences were assessed by Mann Whitney test ($p<0.05$).

RR-MS: relapsing-remitting multiple sclerosis; IL-1β: interleukin 1β; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha; IL-12: imterleukin-12; IFN- γ: interferon gamma; IL-17: interleukin 17; IL-4: interleukin 4; IL-10: interleukin 10.

Table 4 - Serum cytokine level obtained from the relapsing-remitting multiple sclerosis (RR-MS) patients, according to the Expanded Disability Status Scale (EDSS

MS patients (n = 146)	Mild EDSS (0.0 – 4.0) (n = 113)	Moderate EDSS (4.5 – 5.5) (n = 20)	Severe EDSS (6.0 – 10.0) (n = 13)	p value
Gender n (%)				
Female	83 (56.9)	16 (11.0)	7 (4.8)	0.2357[a]
Male	30 (20.5)	4 (2.7)	6 (4.1)	
Ethnicity n (%)				
Caucasian	93(63.7)	19 (13.0)	10 (6.8)	0.2933 [a]
Non Caucasian	20 (13.7)	01 (0.7)	03 (2.1)	
Age (year)				
Median (IQR)	39.0 (28.0–48.0)	39.5 (34.3–56.0)	52.0 (39.5–57.0)	0.0034 [b]
Range	14.0 – 79.0	24.0 – 85.0	29.0 – 71.0	
BMI (Kg/m^2)				
Median (IQR)	23.4 (21.7–27.4)	24.8(20.5– 27.3)	20.9 (19.9–23.9)	0.2998 [b]
Range	17.5 – 44.0	16.9 – 33.6	19.8 – 24.6	
Corticosteroids (mg/day)[c]				
Median (IQR)	20.0 (20.0–20.0)	25.0 (20.0–30.0)	20.0 (20.0 -20.0)	0.3161 [b]
Range	2.0 – 40.0	20.0 – 30.0	20.0 – 20.0	
IL-1β (pg/mL)				
Median (IQR)	2.0 (2.0–2.0)	2.0 (2.0–5.0)	2.0 (2.0–2.0)	0.4422 [b]
Range	2.0 – 40.7	2.0 – 15.9	2.0 – 2.0	
IL-6 (pg/mL)				
Median (IQR)	1.8 (1.0–3.8)	2.1 (1.0–3.9)	3.9 (1.3–4.9)	0.7408 [b]
Range	1.0 – 122.3	1.0 – 158.0	1.0 – 5.6	
TNF-α (pg/mL)				
Median (IQR)	2.0 (2.0–5.0)	2.0 (2.0–3.3)	2.7 (2.0–28.8)	0.6896 [b]
Range	2.0 – 50.1	2.0 – 4.7	2.0 – 30.0	
IL-12 (pg/mL)				
Median (IQR)	2.0 (2.0–2.0)	2.0 (2.0–2.0)	2.0 (2.0–4.5)	0.5083 [b]
Range	2.0 – 93.7	2.0 – 7.5	2.0 – 11.8	
INF-γ (pg/mL)				
Median (IQR)	41.2 (19.6–77.7)	55.5 (27.1–36.3)	52.6(36.3–72.0)	0.6032 [b]
Range	5.0 – 500.0	9.8 – 500.0	27.0 – 500.0	
IL-17 (pg/mL)				
Median (IQR)	2.0 (2.0–2.0)	2.0 (2.0–2.0)	2.0 (2.0–5.5)	0.2359 [b]
Range	2.0 – 53.2	2.0 – 4.2	2.0 – 13.7	
IL-4 (pg/mL)				
Median (IQR)	1.0 (1.0–9.6)	1.0 (1.0–1.4)	1.0 (1.0–1.0)	0.0375 [b]
Range	1.0 – 26.4	1.0 – 17.2	1.0 – 1.0	
IL-10 (pg/mL)				
Median (IQR)	4.6 (3.2–6.6)	4.4 (3.4–6.8)	5.0 (3.0–5.2)	0.8680 [b]
Range	1.0 – 21.9	2.2 – 16.5	2.9 – 5.4	

[a]Categorical variables were assessed using chi-square Test ($p<0.05$).

[b]Differences were assessed using Kruskal-Wallis Test with Dunn's Post Test, for continuous variables ($p<0.05$).

Data were expressed as median, interquartile range (IQR) 25%-75%, and range values or absolute number (n), and percentage (%).

RR-MS: relapsing-remitting multiple sclerosis; EDSS: Expanded Disability Status Scale according to Kurtzke (1983); EDSS was categorized as mild, moderate or severe according to Kamali-Sarvestani et al. (2007); IL-1β: interleukin-1β; IL-6: interleukin-6; TNF-α: tumor necrosis factor-alpha; IFN- γ: interferon-gamma; IL-17: interleukin-17; IL-4: interleukin-4; IL-10: interleukin-10;
[c]18 RR-MS patients were treated with corticosteroid at the time of enrollment in the study

Table 5 - Serum cytokine level obtained from relapsing-remitting multiple sclerosis (RR-MS) Brazilian patients according to the gadolinium-enhancing lesions assessed by magnetic resonance imaging

RR-MS patients (n = 161)	Magnetic resonance imaging		p value
	Positive gadolinium (n = 35)	Negative gadolinium (n = 126)	
Gender n (%)			
Female	24 (14.9)	93 (57.8)	0.5383 [a]
Male	11(6.8)	33 (20.5)	
Ethnicity n (%)			
Caucasian	29 (18.0)	107 (66.5)	0.7656 [a]
Non Caucasian	06 (3.7)	19 (11.8)	
Age (years)			
Median (IQR)	32.0 (27.0 – 44.0)	44.0 (32.0 – 52.3)	0.0032 [b]
Range	14.0 – 79.0	15.0 – 85.0	
BMI (Kg/m^2)			
Median (IQR)	23.4 (22.1 – 27.7)	23.5 (21.1 – 27.1)	0.6464 [b]
Range	(19.3 – 35.5	16.9 – 44.0	
Corticosteroids (mg/day)[c]			0.9029 [b]
Median (IQR)	20.0 (20.0 – 20.0)	20.0 (20.0 – 22.5)	
Range	20.0 – 40.0	5.0 – 30.0	
IL-1β (pg/mL)			
Median (IQR)	2.0 (2.0 – 2.0)	2.0 (2.0 – 2.9)	0.4752 [b]
Range	2.0 – 18.0	2.0 – 40.7	
IL-6 (pg/mL)			
Median (IQR)	1.3 (1.0 – 3.2)	2.4 (1.0 – 4.0)	0.1129 [b]
Range	1.0 – 158.0	1.0 – 122.3	
TNF-α (pg/mL)			
Median (IQR)	2.0 (2.0 – 2.1)	2.0 (2.0 – 5.6)	0.0457 [b]
Range	2.0 – 17.5	2.0 – 68.4	
IL-12 (pg/mL)			
Median (IQR)	2.0 (2.0 – 2.0)	2.0 (2.0 – 2.0)	0.9089 [b]
Range	2.0 – 48.3	2.0 – 93.7	
IFN-γ (pg/mL)			
Median (IQR)	52.8 (25.0 – 131.4)	39.5 (23.0 – 70.7)	0.1643 [b]
Range	6.4 – 500.0	2.0 – 500.0	
IL-17 (pg/mL)			
Median (IQR)	2.0 (2.0 – 2.0)	2.0 (2.0 – 2.6)	0.0631 [b]
Range	2.0 – 15.6	2.0 – 53.2	
IL-4 (pg/mL)			
Median (IQR)	1.0 (1.0 – 14.9)	1.0 (1.0 – 4.1)	0.8980 [b]

Range	1.0 – 22.3	1.0 – 26.4	
IL-10 (pg/mL)			
Median (IQR)	3.4 (2.3 – 5.8)	4.9 (3.4 – 6.5)	0.0533 [b]
Range	1.0 – 15.9	1.0 – 104.9	

[a]Categorical variables were assessed using chi-square Test ($p<0.05$).
[b]Differences were assessed using Mann Whitney test for continuous variables ($p<0.05$). Data were expressed as median, interquartile range (IQR) 25%-75%, and range values or absolute number (n), and percentage (%).
RR-MS: relapsing-remitting multiple sclerosis; BMI: body mass index; IL-1β: interleukin-1β; IL-6: interleukin-6; TNF-α: tumor necrosis factor-alpha; IL-12: interleukin-12; IFN- γ: interferon-gamma; IL-17: interleukin-17; IL-4: interleukin-4; IL-10: interleukin-10.
[c]18 RR-MS patients were treated with corticosteroid at the time of enrollment in the study

5 CONCLUSÃO

O presente estudo possibilitou as seguintes conclusões:

1 - A frequência genotípica e alélica foi diferente nos pacientes com EM e controles; o genótipo TNFB2/B2 e o alelo B2 foi mais frequente nos pacientes com EM;

2- Os pacientes com EM apresentaram níveis séricos elevados de IL-6 e IL-12, IFN-γ e IL-4 comparados aos controles. A citocina IL-1 apresentou-se elevada nos controles; no entanto, apenas uma tendência à associação foi observada. Não foram encontradas diferenças significativas nos níveis séricos das citocinas IL-17 e IL-10 avaliadas entre os indivíduos;

3 - O polimorfismo genético *Nco*I do TNF-β está associado com a susceptibilidade e desenvolvimento da EM independentemente dos alelos HLA-DRB1*01, 03, 04, 07, 08, 11, 13, e 15;

4 - Não foi encontrada associação entre o polimorfismo genético *Nco*I do TNF-β e as variáveis idade no diagnóstico, duração da doença, forma clínica, EDSS basal, EDSS após cinco anos de acompanhamento, índice de progressão, progressão e local da lesão. No entanto, foi encontrada uma tendência à associação com a atividade da doença;

5 - Pacientes com a forma clínica EM-RR e incapacidade leve apresentaram níveis mais elevados de IL-4 quando comparados com pacientes com incapacidade moderada e grave. Os níveis de TNF-α, IL-6, IL-12, IFN-γ e IL-17 apresentaram-se mais elevados nos pacientes com incapacidade grave; no entanto, as diferenças não foram significativas;

6 - Pacientes EM-RR e doença inativa (lesões Gd negativas na RMI) apresentaram níveis aumentados de TNF-α e IL-10 comparados com pacientes com a doença em atividade (lesões Gd positivas na RMI). Os níveis de IL-17 mostraram uma tendência, embora não significativa, a serem mais elevados em pacientes com a doença inativa. Os níveis séricos de IFN-γ foram mais elevados nos pacientes com a doença em atividade; no entanto não foi encontrada diferença significativa;

7 - Níveis diminuídos de TNF-α e aumentados de IFN-γ estavam independentemente associados à EM e à atividade da doença.

6 PERSPECTIVAS FUTURAS

Os resultados encontrados em nosso trabalho estão de acordo com a hipótese de que a EM é uma doença poligênica e que o polimorfismo genético do TNF-β (*Nco*I) possui um importante papel na susceptibilidade para o desenvolvimento da doença. A presença do genótipo TNFB2/B2 e do alelo TNFB2 está associada com a susceptibilidade e o desenvolvimento da EM e estes biomarcadores genéticos podem estar envolvidos com a atividade da doença. No entanto, este polimorfismo não é o principal determinante para a progressão da doença nesta população, sugerindo que o TNF-α não seja um importante alvo terapêutico para o tratamento da EM.

Os resultados do perfil de citocinas mostram que a EM envolve um complexo sistema de citocinas que interagem entre si, com uma citocina modulando a ação e a produção da outra, participando do início e progressão das lesões desmielinizantes no SNC. Mesmo na fase de remissão clínica da doença, os pacientes com EM apresentam uma alteração no balanço entre as citocinas pró-inflamatórias (TNF-α, IL-1, IL-6 e IL-12), tipo Th1 (IFN-γ), tipo Th17 (IL-17) e anti-inflamatórias Th2 (IL-4 e IL-10). Os níveis séricos diminuídos de TNF-α e aumentados de IL-4 refletem a resposta imune que caracteriza a fase de remissão clínica da doença.

Apesar dos inúmeros estudos desenvolvidos na EM, a etiologia desta complexa doença ainda não está bem definida. As principais estratégias terapêuticas que irão dominar nos próximos anos serão a combinação de terapias imunomoduladoras e imunossupressoras já existentes e a busca por novos alvos terapêuticos. A terapia combinada apresenta uma vantagem sobre a monoterapia por se mostrar mais potente no tratamento da EM, no entanto, ela não causa um grande impacto na progressão da doença. Portanto, torna-se necessário o desenvolvimento de novas terapias direcionadas contra componentes do sistema imune envolvidos na fisiopatologia da EM. Desta forma, o conhecimento de fatores genéticos que estão envolvidos nos mecanismos imunológicos e fisiopatológicos da EM é de grande utilidade para o desenvolvimento de novas drogas que alterem o curso clínico desta doença.

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ANEXOS

ANEXO A

Parecer do Comitê de ética em pesquisa em seres humanos da UEL

Universidade Estadual de Londrina

COMITÊ DE ÉTICA EM PESQUISA

COMITÊ DE ÉTICA EM PESQUISA ENVOLVENDO SERES HUMANOS
Universidade Estadual de Londrina/ Hospital Universitário Regional Norte do Paraná
Registro CONEP 268

Parecer PF Nº. 074/09 **CAAE Nº. 0070.0.268.000-09** **FOLHA DE ROSTO Nº. 251304**	Londrina, 20 de abril de 2010.

PESQUISADORA: **EDNA MARIA VISSOCI REICHE**
CCS/PAC

Prezada Senhora:

O "**Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina/ Hospital Universitário Regional Norte do Paraná**" (Registro CONEP 268)– de acordo com as orientações da Resolução 196/96. do Conselho Nacional de Saúde/MS e Resoluções Complementares, avaliou o projeto:

"O PAPEL DO POLIMORFISMO GENÉTICO DO FATOR DE NECROSE TUMORAL BETA (TNF-BETA) E O DESENVOLVIMENTO E CURSO CLÍNICO DE ESCLEROSE MÚLTIPLA NA POPULAÇÃO BRASILEIRA"

Situação do Projeto: **APROVADO**

Informamos que deverá ser comunicada, por escrito, qualquer modificação que ocorra no desenvolvimento da pesquisa, bem como deverá apresentar ao CEP/UEL relatório final da pesquisa.

Atenciosamente,

Profª. Dra. Alexandrina Aparecida Maciel

Coordenadora
Comitê de Ética em Pesquisa-CEP/UEL

ANEXO B

Termos de Conscentimento Livre e Esclarecido (TCLE) para os pacientes com Esclerose Múltipla

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE 1)

Por favor, leia cuidadosamente este consentimento e não hesite em perguntar sobre qualquer dúvida que tenha.

O (A) senhor (a) está sendo convidado (a) a participar, voluntariamente, de um projeto de pesquisa com o título "O papel do polimorfismo genético do fator de necrose tumoral beta (TNF-β) e o desenvolvimento e curso clínico de esclerose múltipla, na população brasileira". O estudo será desenvolvido sob a coordenação da professora Dra. Edna Maria Vissoci Reiche, do departamento de Patologia, Análises Clínicas e Toxicológicas do Centro de Ciências da Saúde da UEL (telefone 43-3371-2321) com a colaboração do professor Dr. Damacio Ramon Kaimen-Maciel, do departamento de Clínica Médica do Centro de Ciências da Saúde (43-3371-2234) e demais professores da UEL. Cabe ao (a) senhor (a) decidir se pretende participar ou não. Caso não tenha condições de ler e/ou compreender as informações contidas neste termo, o mesmo poderá ser assinado e datado por um membro da sua família ou responsável legal.

O projeto de pesquisa tem como objetivo avaliar uma possível associação entre o gene do TNF-beta, uma molécula inflamatória, e a chance de desenvolvimento e curso clínico da esclerose múltipla. Neste momento em que o senhor (a) está sendo convidado (a) a participar do estudo, o diagnóstico de esclerose múltipla e as formas de tratamento já foram definidos pela equipe médica responsável pelo seu atendimento e a sua participação ou não no trabalho depende exclusivamente da sua vontade e não irá absolutamente modificar os rumos do seu tratamento, seja qual for a sua opção (participação ou não no estudo).

A sua participação no estudo implica na utilização de uma amostra do seu sangue, que foi coletada durante sua participação em dois projetos de pesquisa desenvolvidos anteriormente, aprovados pelo Comitê de Ética em Pesquisa em Seres Humanos da UEL, coordenados pelo professor Dr. Damacio Ramon Kaimen-Maciel e com a participação de outros pesquisadores. Os projetos estudaram o papel de outros fatores genéticos, como o sistema HLA e o CCR5, no desenvolvimento da esclerose múltipla. O primeiro projeto foi sobre "Genotipagem do sistema de antígeno leucocitário humano (HLA) classe II em pacientes com esclerose múltipla" e teve como objetivo avaliar uma possível associação entre os genes do sistema HLA e a chance de desenvolvimento e curso clínico da esclerose múltipla (Parecer n. 206/05 de 15 de setembro de 2005). Com a amostra de DNA extraído do sangue coletado, determinou-se os alelos dos genes do sistema HLA por métodos de biologia molecular. Os resultados obtidos demonstraram que existe uma associação do HLA com o risco de desenvolvimento e o curso clínico da esclerose múltipla e sugerem que o genótipo do HLA pode ser um biomarcador para a doença.

O segundo projeto que o (a) senhor (a) participou foi "Polimorfismo genético do receptor de quimiocina CCR5 e a susceptibilidade e curso clínico da esclerose múltipla" e teve como objetivo avaliar uma possível associação entre o gene do receptor de uma proteína envolvida na resposta inflamatória e a chance de desenvolvimento e curso clínico da esclerose múltipla (Parecer n.207/05, de 15 de setembro de 2005). Com a amostra de DNA extraído do sangue coletado, determinou-se os alelos do gene CCR5 por métodos de biologia molecular. Os resultados obtidos demonstraram que existe uma associação entre este gene e presença das lesões observadas na ressonância nuclear magnética dos pacientes com esclerose múltipla, sugerindo um papel deste fator genético no curso clínico da doença e que o genótipo do CCR5 pode ser um biomarcador para a doença.

A amostra de sangue coletada durante a sua participação nestes dois projetos de pesquisa foi armazenada no *freezer,* sob a responsabilidade da professora Dra. Edna Maria Vissoci Reiche, no Laboratório de Análises Clínicas do HU, e foi identificada com número, sem a possibilidade da identidade do (a) senhor (a) ser revelada a outras pessoas que não participam do estudo. Solicitamos sua autorização para que esta amostra possa ser utilizada no desenvolvimento de um novo projeto de pesquisa para o estudo de um outro gene chamado fator de necrose tumoral beta (TNF-beta) para avaliar se existe associação entre este gene e o desenvolvimento e progressão da esclerose múltipla. O mesmo estudo já foi realizado em outras populações mundiais mas não foi realizado na população brasileira, e os resultados poderão contribuir para o melhor conhecimento do desenvolvimento da doença assim como a possibilidade de se sugerir novas formas de tratamento da doença na nossa população.

Sua decisão para permitir a utilização da amostra armazenada é voluntária e em qualquer momento o (a) senhor (a) pode retirar o consentimento e deixar de participar do estudo, sem qualquer prejuízo à continuidade de seu tratamento na instituição. Não haverá necessidade de coletar amostras de sangue específicas para este projeto de pesquisa. **Neste documento, solicitamos também ao (a) senhor (a) a autorização para que o material armazenado possa, posteriormente, ser armazenado para uso futuro.** Comprometemos a submeter à análise do Comitê de Ética em Pesquisa os novos projetos de pesquisa.

Informamos que os dados pessoais fornecidos e os resultados do exame realizado serão mantidos sob sigilo e somente serão utilizados para fins de pesquisa. Durante todas as etapas do projeto, os participantes serão identificados por um número codificado que será utilizado nas análises posteriores para garantir a preservação da integridade do indivíduo, garantir o anonimato e evitar a quebra de confidencialidade. Ao final do projeto, os resultados serão divulgados em forma de artigos científicos e comunicações em eventos científicos, sempre mantendo o sigilo da identidade dos participantes.

Declara que está completamente esclarecido sobre a forma como a pesquisa será realizada, não tendo nenhuma dúvida sobre sua natureza e a ausência de riscos na sua participação. Declara também que está ciente de que sua participação é voluntária, de que será informado sobre os resultados da análise genética do gene TNF, objeto deste estudo, durante o seu atendimento, de não terá nenhum ônus e de que poderá se recusar ou abandonar a pesquisa em qualquer momento sem que haja penalização ou prejuízo algum para seu atendimento e tratamento. Está ciente também que o seu tratamento continuará sendo conduzido pelo seu médico e que nenhum pagamento ou benefício será feito ao participante ou aos familiares pela participação no presente estudo.

Eu estou disposto a participar dessa pesquisa e compreendi as condições acima descritas, concordo voluntariamente a participar desse estudo.

Assinaturas

Paciente ou representante legal (caso o paciente esteja impossibilitado de assinar ou compreender o conteúdo deste TCLE)

Nome:_____

Assinatura:_____**Local e data:** _____

Profissional que obteve o TCLE

Nome:_____

Assinatura:_____ **Local e data:** _____

Pesquisador responsável: Nome: Professora Dra. Edna Maria Vissoci Reiche
Endereço: Departamento de Patologia, Análises Clínicas do Centro de Ciências da Saúde, Hospital Universitário de Londrina. Av. Robert Koch, 60, Vila Operária, CEP 86038-440;
Fone: 43-3371-2321 (Imunologia), 43-3371-2670 (Diagnóstico Molecular)
Comitê de Ética em Pesquisa em Seres Humanos da Universidade Estadual de Londrina:
Fone: 43-3371-2490

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE 2)

Por favor, leia cuidadosamente este consentimento e não hesite em perguntar sobre qualquer dúvida que tenha.

O (A) senhor (a) está sendo convidado (a) a participar, voluntariamente, de um projeto de pesquisa com o título "O papel do polimorfismo genético do fator de necrose tumoral beta (TNF-β) e o desenvolvimento e curso clínico de esclerose múltipla, na população brasileira". O estudo será desenvolvido sob a coordenação da professora Dra. Edna Maria Vissoci Reiche do departamento de Patologia, Análises Clínicas e Toxicológicas do Centro de Ciências da Saúde da UEL (telefone 43-3371-2321) com a colaboração do professor Dr. Damacio Ramon Kaimen-Maciel, do departamento de Clínica Médica do Centro de Ciências da Saúde (43-3371-2234) e demais professores da UEL. Cabe ao (a) senhor (a) decidir se pretende participar ou não. Caso não tenha condições de ler e/ou compreender as informações contidas neste termo, o mesmo poderá ser assinado e datado por um membro da sua família ou responsável legal.

O projeto de pesquisa tem como objetivo avaliar uma possível associação entre o gene do TNF-beta e a chance de desenvolvimento e curso clínico da esclerose múltipla. Neste momento em que o senhor (a) está sendo convidado (a) a participar do estudo, o diagnóstico de esclerose múltipla e as formas de tratamento já foram definidos pela equipe médica responsável pelo seu atendimento e a sua participação ou não no trabalho depende exclusivamente da sua vontade e não irá absolutamente modificar os rumos do seu tratamento, seja qual for a sua opção (participação ou não no estudo).

A sua participação no estudo implica na utilização de uma amostra do seu sangue, que foi coletada durante sua participação em dois projetos de pesquisa desenvolvidos anteriormente, aprovados pelo Comitê de Ética em Pesquisa em Seres Humanos da UEL, coordenados pelo professor Dr. Damacio Ramon Kaimen-Maciel com a participação de outros pesquisadores. Os projetos estudaram o papel de outros fatores genéticos, como o HLA e o CCR5, no desenvolvimento da esclerose múltipla. O primeiro projeto foi sobre "Genotipagem do sistema de antígeno leucocitário humano (HLA) classe II em pacientes com esclerose múltipla" e teve como objetivo avaliar uma possível associação entre os genes do sistema HLA e a chance de desenvolvimento e curso clínico da esclerose múltipla (Parecer n. 206/05 de 15 de setembro de 2005). Com a amostra de DNA extraído do sangue coletado, determinou-se os alelos dos genes do sistema HLA por métodos de biologia molecular. Os resultados obtidos demonstraram que existe uma associação do HLA com o risco de

desenvolvimento e o curso clínico da esclerose múltipla e sugerem que o genótipo do HLA pode ser um biomarcador para a doença.

O segundo projeto que o (a) senhor (a) participou foi "Polimorfismo genético do receptor de quimiocina CCR5 e a susceptibilidade e curso clínico da esclerose múltipla" e teve como objetivo avaliar uma possível associação entre o gene do receptor de uma proteína envolvida na resposta inflamatória e a chance de desenvolvimento e curso clínico da esclerose múltipla (Parecer n.207/05, de 15 de setembro de 2005). Com a amostra de DNA extraído do sangue coletado, determinou-se os alelos do gene CCR5 por métodos de biologia molecular. Os resultados obtidos demonstraram que existe uma associação entre este gene e presença das lesões observadas na ressonância nuclear magnética dos pacientes com esclerose múltipla, sugerindo um papel deste fator genético no curso clínico da doença e que o genótipo do CCR5 pode ser um biomarcador para a doença.

A amostra de sangue coletada durante a sua participação nestes dois projetos de pesquisa foi armazenada no *freezer,* sob a responsabilidade da professora Dra. Edna Maria Vissoci Reiche, no Laboratório de Análises Clínicas do HU, e foi identificada com número, sem a possibilidade da identidade do (a) senhor (a) ser revelada a outras pessoas que não participam do estudo. Solicitamos sua autorização para que esta amostra possa ser utilizada no desenvolvimento de um novo projeto de pesquisa para o estudo de um outro gene chamado fator de necrose tumoral beta (TNF-beta) para avaliar se existe associação entre este gene e o desenvolvimento e progressão da esclerose múltipla. O mesmo estudo já foi realizado em outras populações mundiais mas não foi realizado na população brasileira, e os resultados poderão contribuir para o melhor conhecimento do desenvolvimento da doença assim como a possibilidade de se sugerir novas formas de tratamento da doença na nossa população.

Sua decisão para permitir a utilização da amostra armazenada é voluntária e em qualquer momento o (a) senhor (a) pode retirar o consentimento e deixar de participar do estudo, sem qualquer prejuízo à continuidade de seu tratamento na instituição. Haverá necessidade de coletar amostras de sangue específicas para este projeto de pesquisa. Neste documento, **solicitamos também ao (a) senhor (a) a autorização para que o material armazenado possa, posteriormente, ser armazenado para uso futuro**. Comprometemos a submeter à análise do Comitê de Ética em Pesquisa os novos projetos de pesquisa.

Informamos que os dados pessoais fornecidos e os resultados do exame realizado serão mantidos sob sigilo e somente serão utilizados para fins de pesquisa. Durante todas as etapas do projeto, os participantes serão identificados por um número codificado que será utilizado nas análises posteriores para garantir a preservação da integridade do indivíduo, garantir o anonimato e evitar a quebra de confidencialidade. Ao final do projeto, os resultados serão divulgados em forma de artigos científicos e comunicações em eventos científicos, sempre mantendo o sigilo da identidade dos participantes.

Declara que está completamente esclarecido sobre a forma como a pesquisa será realizada, não tendo nenhuma dúvida sobre sua natureza e a ausência de riscos na sua participação. Declara também que está ciente de que sua participação é voluntária, de não terá nenhum ônus e de que poderá se recusar ou abandonar a pesquisa em qualquer momento sem que haja penalização ou prejuízo algum para seu atendimento e tratamento. Está ciente também que o seu tratamento continuará sendo conduzido pelo seu médico e que nenhum pagamento ou benefício será feito ao participante ou aos familiares pela participação no presente estudo.

Eu estou disposto a participar dessa pesquisa e compreendi as condições acima descritas, concordo voluntariamente a participar desse estudo.

Assinaturas

Paciente ou representante legal (caso o paciente esteja impossibilitado de assinar ou compreender o conteúdo deste TCLE)

Nome:_____

Assinatura: _____**Local e data:** _____

Profissional que obteve o TCLE

Nome:_____

Assinatura: _____**Local e data:** _____

Pesquisador responsável: Nome: Professora Dra. Edna Maria Vissoci Reiche
Endereço: Departamento de Patologia, Análises Clínicas do Centro de Ciências da Saúde, Hospital Universitário de Londrina. Av. Robert Koch, 60, Vila Operária, CEP 86038-440;
Fone: 43-3371-2321 (Imunologia), 43-3371-2670 (Diagnóstico Molecular)
Comitê de Ética em Pesquisa em Seres Humanos da Universidade Estadual de Londrina:
Fone: 43-3371-2490

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE 3)

Por favor, leia cuidadosamente este consentimento e não hesite em perguntar sobre qualquer dúvida que tenha.

O (A) senhor (a) está sendo convidado (a) a participar, voluntariamente, de um projeto de pesquisa com o título "O papel do polimorfismo genético do fator de necrose tumoral beta (TNF-β) e o desenvolvimento e curso clínico de esclerose múltipla, na população brasileira". O estudo será desenvolvido sob a coordenação da professora Dra. Edna Maria Vissoci Reiche do departamento de Patologia, Análises Clínicas e Toxicológicas do Centro de Ciências da Saúde da UEL (telefone 43-3371-2321) com a colaboração do professor Dr. Damacio Ramon Kaimen-Maciel, do departamento de Clínica Médica do Centro de Ciências da Saúde (43-3371-2234) e demais professores da UEL. Cabe ao (a) senhor (a) decidir se pretende participar ou não. Caso não tenha condições de ler e/ou compreender as informações contidas neste termo, o mesmo poderá ser assinado e datado por um membro da sua família ou responsável legal.

O projeto de pesquisa tem como objetivo avaliar uma possível associação entre o gene do TNF-beta e a chance de desenvolvimento e curso clínico da esclerose múltipla. Neste momento em que o senhor (a) está sendo convidado (a) a participar do estudo, o diagnóstico de esclerose múltipla e as formas de tratamento já foram definidos pela equipe médica responsável pelo seu atendimento e a sua participação ou não no trabalho depende exclusivamente da sua vontade e não irá absolutamente modificar os rumos do seu tratamento, seja qual for a sua opção (participação ou não no estudo).

A sua participação no estudo implica na utilização de uma amostra do seu sangue, que foi coletada durante sua participação em dois projetos de pesquisa desenvolvidos anteriormente, aprovados pelo Comitê de Ética em Pesquisa em Seres Humanos da UEL, coordenados pelo professor Dr. Damacio Ramon Kaimen-Maciel e com a participação de outros pesquisadores. Os projetos estudaram o papel de outros fatores genéticos, como o HLA e o CCR5, no desenvolvimento da esclerose múltipla. Estes projetos. O primeiro projeto foi sobre "Genotipagem do sistema de antígeno leucocitário humano (HLA) classe II em pacientes com esclerose múltipla" e teve como objetivo avaliar uma possível associação entre os genes do sistema HLA e a chance de desenvolvimento e curso clínico da esclerose múltipla (Parecer n. 206/05 de 15 de setembro de 2005). Com a amostra de DNA extraído do sangue coletado, determinou-se os alelos dos genes do sistema HLA por métodos de biologia molecular. Os resultados obtidos demonstraram que existe uma associação do HLA com o risco de desenvolvimento e o curso clínico da esclerose múltipla e sugerem que o genótipo do HLA pode ser um biomarcador para a doença.

O segundo projeto que o (a) senhor (a) participou foi "Polimorfismo genético do receptor de quimiocina CCR5 e a susceptibilidade e curso clínico da esclerose múltipla" e teve como objetivo avaliar uma possível associação entre o gene do receptor de uma proteína envolvida na resposta inflamatória e a chance de desenvolvimento e curso clínico da esclerose múltipla (Parecer n.207/05, de 15 de setembro de 2005). Com a amostra de DNA extraído do sangue coletado, determinou-se os alelos do gene CCR5 por métodos de biologia molecular. Os resultados obtidos demonstraram que existe uma associação entre este gene e presença das lesões observadas na ressonância nuclear magnética dos pacientes com esclerose múltipla, sugerindo um papel deste fator genético no curso clínico da doença e que o genótipo do CCR5 pode ser um biomarcador para a doença.

A amostra de sangue coletada durante a sua participação nestes dois projetos de pesquisa foi armazenada no *freezer,* sob a responsabilidade da professora Dra. Edna Maria Vissoci Reiche, no Laboratório de Análises Clínicas do HU, e foi identificada com número, sem a possibilidade da identidade do (a) senhor (a) ser revelada a outras pessoas que não participam do estudo. Solicitamos sua autorização para que esta amostra possa ser utilizada no desenvolvimento de um novo projeto de pesquisa para o estudo de um outro gene chamado fator de necrose tumoral beta (TNF-beta) para avaliar se existe associação entre este gene e o desenvolvimento e progressão da esclerose múltipla. O mesmo estudo já foi realizado em outras populações mundiais mas não foi realizado na população brasileira, e os resultados poderão contribuir para o melhor conhecimento do desenvolvimento da doença assim como a possibilidade de se sugerir novas formas de tratamento da doença na nossa população.

Sua decisão para **permitir a utilização da amostra armazenada é voluntária** e em qualquer momento o senhor (a) pode retirar o consentimento e deixar de participar do estudo, sem qualquer prejuízo à continuidade de seu tratamento na instituição. Não haverá necessidade de coletar amostras de sangue específicas para este projeto de pesquisa. **Informamos ao senhor (a) que, após o encerramento deste projeto de pesquisa, o material NÃO será armazenado para uso futuro**.

Informamos que os dados pessoais fornecidos e os resultados do exame realizado serão mantidos sob sigilo e somente serão utilizados para fins de pesquisa. Durante todas as etapas do projeto, os participantes serão identificados por um número codificado que será utilizado nas análises posteriores para garantir a preservação da integridade do indivíduo, garantir o anonimato e evitar a quebra de confidencialidade. Ao final do projeto, os resultados serão divulgados em forma de artigos científicos e comunicações em eventos científicos, sempre mantendo o sigilo da identidade dos participantes.

Declara que está completamente esclarecido sobre a forma como a pesquisa será realizada, não tendo nenhuma dúvida sobre sua natureza e a ausência de riscos na sua participação. Declara também que está ciente de que sua participação é voluntária, de que será informado sobre os resultados da análise genética do gene TNF, **objeto deste estudo, durante o seu atendimento, de não terá nenhum ônus e de que poderá se recusar ou abandonar a pesquisa em qualquer momento sem que haja penalização ou prejuízo algum para seu atendimento e tratamento. Está ciente também que o seu tratamento continuará sendo conduzido pelo seu médico e que nenhum pagamento ou benefício será feito ao participante ou aos familiares pela participação no presente estudo.**

Eu estou disposto a participar dessa pesquisa e compreendi as condições acima descritas, concordo voluntariamente a participar desse estudo.

Assinaturas

Paciente ou representante legal (caso o paciente esteja impossibilitado de assinar ou compreender o conteúdo deste TCLE)

Nome:_____

Assinatura: _____Local e data: _____

Profissional que obteve o TCLE

Nome:_____

Assinatura: _____Local e data: _____

Pesquisador responsável: Nome: Professora Dra. Edna Maria Vissoci Reiche
Endereço: Departamento de Patologia, Análises Clínicas do Centro de Ciências da Saúde, Hospital Universitário de Londrina. Av. Robert Koch, 60, Vila Operária, CEP 86038-440; Fone: 43-3371-2321 (Imunologia), 43-3371-2670 (Diagnóstico Molecular)
Comitê de Ética em Pesquisa em Seres Humanos da Universidade Estadual de Londrina:
Fone: 43-3371-2490

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE 4)

Por favor, leia cuidadosamente este consentimento e não hesite em perguntar sobre qualquer dúvida que tenha.

O (A) senhor (a) está sendo convidado (a) a participar, voluntariamente, de um projeto de pesquisa com o título "O papel do polimorfismo genético do fator de necrose tumoral beta (TNF-β) e o desenvolvimento e curso clínico de esclerose múltipla, na população brasileira". O estudo será desenvolvido sob a coordenação da professora Dra. Edna Maria Vissoci Reiche do departamento de Patologia, Análises Clínicas e Toxicológicas do Centro de Ciências da Saúde da UEL (telefone 43-3371-2321) com a colaboração do professor Dr. Damacio Ramon Kaimen-Maciel, do departamento de Clínica Médica do Centro de Ciências da Saúde (43-3371-2234) e demais professores da UEL. Cabe ao (a) senhor (a) decidir se pretende participar ou não. Caso não tenha condições de ler e/ou compreender as informações contidas neste termo, o mesmo poderá ser assinado e datado por um membro da sua família ou responsável legal.

O projeto de pesquisa tem como objetivo avaliar uma possível associação entre o gene do TNF-beta e a chance de desenvolvimento e curso clínico da esclerose múltipla. Neste momento em que o senhor (a) está sendo convidado (a) a participar do estudo, o diagnóstico de esclerose múltipla e as formas de tratamento já foram definidos pela equipe médica responsável pelo seu atendimento e a sua participação ou não no trabalho depende exclusivamente da sua vontade e não irá absolutamente modificar os rumos do seu tratamento, seja qual for a sua opção (participação ou não no estudo).

A sua participação no estudo implica na utilização de uma amostra do seu sangue, que foi coletada durante sua participação em dois projetos de pesquisa desenvolvidos anteriormente, aprovados pelo Comitê de Ética em Pesquisa em Seres Humanos da UEL, coordenados pelo professor Dr. Damacio Ramon Kaimen-Maciel e com a participação de outros pesquisadores. Os projetos estudaram o papel de outros fatores genéticos, como o HLA e o CCR5, no desenvolvimento da esclerose múltipla. O primeiro projeto foi sobre "Genotipagem do sistema de antígeno leucocitário humano (HLA) classe II em pacientes com esclerose múltipla" e teve como objetivo avaliar uma possível associação entre os genes do sistema HLA e a chance de desenvolvimento e curso clínico da esclerose múltipla (Parecer n. 206/05 de 15 de setembro de 2005). Com a amostra de DNA extraído do sangue coletado, determinou-se os alelos dos genes do sistema HLA por métodos de biologia molecular. Os resultados obtidos demonstraram que existe uma associação do HLA com o risco de desenvolvimento e o curso clínico da esclerose múltipla e sugerem que o genótipo do HLA pode ser um biomarcador para a doença.

O segundo projeto que o (a) senhor (a) participou foi "Polimorfismo genético do receptor de quimiocina CCR5 e a susceptibilidade e curso clínico da esclerose múltipla" e teve como objetivo avaliar uma possível associação entre o gene do receptor de uma proteína envolvida na resposta inflamatória e a chance de desenvolvimento e curso clínico da esclerose múltipla (Parecer n.207/05, de 15 de setembro de 2005). Com a amostra de DNA extraído do sangue coletado, determinou-se os alelos do gene CCR5 por métodos de biologia

molecular. Os resultados obtidos demonstraram que existe uma associação entre este gene e presença das lesões observadas na ressonância nuclear magnética dos pacientes com esclerose múltipla, sugerindo um papel deste fator genético no curso clínico da doença e que o genótipo do CCR5 pode ser um biomarcador para a doença.

A amostra de sangue coletada durante a sua participação nestes dois projetos de pesquisa foi armazenada no *freezer,* sob a responsabilidade da professora Dra. Edna Maria Vissoci Reiche, no Laboratório de Análises Clínicas do HU, e foi identificada com número, sem a possibilidade da identidade do (a) senhor (a) ser revelada a outras pessoas que não participam do estudo. Solicitamos sua autorização para que esta amostra possa ser utilizada no desenvolvimento de um novo projeto de pesquisa para o estudo de um outro gene chamado fator de necrose tumoral beta (TNF-beta) para avaliar se existe associação entre este gene e o desenvolvimento e progressão da esclerose múltipla. O mesmo estudo já foi realizado em outras populações mundiais mas não foi realizado na população brasileira, e os resultados poderão contribuir para o melhor conhecimento do desenvolvimento da doença assim como a possibilidade de se sugerir novas formas de tratamento da doença na nossa população.

Sua decisão para permitir a utilização da amostra armazenada é voluntária e em qualquer momento o senhor (a) pode retirar o consentimento e deixar de participar do estudo, sem qualquer prejuízo à continuidade de seu tratamento na instituição. Não haverá necessidade de coletar amostras de sangue específicas para este projeto de pesquisa. Informamos ao (a) senhor (a) que, **após o encerramento deste projeto de pesquisa, o material NÃO será armazenado para uso futuro.**

Informamos que os dados pessoais fornecidos e os resultados do exame realizado serão mantidos sob sigilo e somente serão utilizados para fins de pesquisa. Durante todas as etapas do projeto, os participantes serão identificados por um número codificado que será utilizado nas análises posteriores para garantir a preservação da integridade do indivíduo, garantir o anonimato e evitar a quebra de confidencialidade. Ao final do projeto, os resultados serão divulgados em forma de artigos científicos e comunicações em eventos científicos, sempre mantendo o sigilo da identidade dos participantes.

Declara que está completamente esclarecido sobre a forma como a pesquisa será realizada, não tendo nenhuma dúvida sobre sua natureza e a ausência de riscos na sua participação. Declara também que está ciente de que sua participação é voluntária, de não terá nenhum ônus e de que poderá se recusar ou abandonar a pesquisa em qualquer momento sem que haja penalização ou prejuízo algum para seu atendimento e tratamento. Está ciente também que o seu tratamento continuará sendo conduzido pelo seu médico e que nenhum pagamento ou benefício será feito ao participante ou aos familiares pela participação no presente estudo.

Eu estou disposto a participar dessa pesquisa e compreendi as condições acima descritas, concordo voluntariamente a participar desse estudo.

Assinaturas

Paciente ou representante legal (caso o paciente esteja impossibilitado de assinar ou compreender o conteúdo deste TCLE)

Nome:_____

Assinatura: _____ Local e data: _____

Profissional que obteve o TCLE

Nome:_____

Assinatura: _____Local e data: _____

Pesquisador responsável: Nome: Professora Dra. Edna Maria Vissoci Reiche

Endereço: Departamento de Patologia, Análises Clínicas do Centro de Ciências da Saúde, Hospital Universitário de Londrina. Av. Robert Koch, 60, Vila Operária, CEP 86038-440; Fone: 43-3371-2321 (Imunologia), 43-3371-2670 (Diagnóstico Molecular). Comitê de Ética em Pesquisa em Seres Humanos da Universidade Estadual de Londrina: Fone: 43-3371-2490

ANEXO C

Questionário para coleta de dados demográficos, clínicos e terapeuticos dos indíviduos inseridos no estudo

FICHA DE AVALIAÇÃO – ESCLEROSE MÚLTIPLA

Nome:	Prontuário:
Data de nascimento: Idade:	Caucasiano ( ) Não Caucasiano ( )
Telefone casa: Celular:	
Medicamentos:	Dose diária:
Outras doenças:	

Data:	
Fumante:	
Peso:	
Altura:	
IMC:	
Pressão Arterial:	
Circunferência Abdominal:	
Tempo de diagnóstico:	
Atividade física:	( ) Sim Quantas vezes? ( ) Não