ALFREDO DAMASCENO

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"AVALIAÇÃO LONGITUDINAL DA PATOLOGIA CEREBRAL POR RESSONÂNCIA MAGNÉTICA E DE SUA RELAÇÃO COM FATORES CLÍNICOS E IMUNOLÓGICOS EM PACIENTES COM ESCLEROSE MÚLTIPLA"

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Orientador: Prof. Dr. Fernando Cendes
Co-orientadora: Profa. Dra. Leonilda Maria Barbosa dos Santos

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“O mundo é o que você pensa que ele é"

Ike

Resumo

A esclerose múltipla (EM) é uma doença inflamatória e desmielinizante do sistema nervoso central que afeta cerca de 2,5 milhões de pessoas em todo o mundo e implica em um importante impacto social e econômico para o estado, resultante de incapacidades funcionais sensitivo-motoras e cognitivas.

Nas últimas décadas, o estudo e o entendimento da EM se beneficiaram dos avanços das técnicas de neuroimagem. A Ressonância Magnética (RM) tem sido usada para estudar tanto a história natural da doença quanto para monitorar a eficácia de tratamentos, mas a correlação dos achados da RM convencional com os dados clínicos ainda é insatisfatória. Com isso, tem surgido o interesse em outras técnicas de RM, entre elas a avaliação da substância cinzenta cerebral. Entretanto, apesar dos avanços em neuroimunologia e neuroimagem, ainda existem poucos dados que possam predizer a incapacidade em longo prazo.

Com isso, nosso objetivo foi identificar fatores clínicos e de RM relacionados a uma pior evolução clínica em pacientes com EM. Inicialmente nós realizamos um levantamento dos dados de 197 pacientes acompanhados no ambulatório de EM do HC-UNICAMP, levando em conta informações clínicas e epidemiológicas e o tempo que cada paciente levou para atingir escores específicos de incapacidade. Nós observamos que o grupo levou 25,8 anos para atingir o EDSS de 6,0, mas que pacientes do sexo masculino, e principalmente aqueles com surtos frequentes nos primeiros anos e com envolvimento do tronco cerebral ou cerebelo apresentaram uma evolução pior.

Posteriormente, estabelecemos um subgrupo menor de pacientes a fim de estudar o comportamento longitudinal da patologia cerebral e sua relação com a incapacidade clínica e cognitiva. Foram acompanhados, durante um período de 24 meses, 43 pacientes com EM forma remitente-recorrente e 29 indivíduos controles,

submetidos a exame neurológico, neuropsicológico e RM cerebral. O desempenho nos testes clínicos e neuropsicológicos foi pior no grupo dos pacientes, e 44,2% deles foram classificados como tendo disfunção cognitiva. Um pior desempenho cognitivo estava associado à presença de atividade subclínica da doença na RM, com uma alta carga lesional cortical e com a atrofia do corpo caloso. Além disso, uma maior incapacidade clínica também estava relacionada com estas lesões corticais, tanto cerebrais quanto aquelas presentes no córtex cerebelar. Como a presença de atividade subclínica foi um indicador importante de disfunção cognitiva, foi avaliado em um subgrupo de 15 pacientes a produção de citocinas pró-inflamatórias comparando com os dados de RM. Aqueles pacientes com lesões ativas na RM apresentaram uma produção significativamente maior de citocinas pró-inflamatórias, 10 vezes maior de INF-γ e 22 vezes maior de TNF-α.

O grupo de 43 pacientes foi acompanhado longitudinalmente e no final de 24 meses a atrofia cortical foi de 2,57% e da substância cinzenta subcortical de 3,8%, ambos significativamente maiores que no grupo controle. A presença de atrofia do tálamo no início estava relacionada a um maior risco de disfunção cognitiva após dois anos. Além disso, a presença de uma alta carga de lesões corticais no início do estudo estava relacionada a um risco 5,14 vezes maior de incapacidade clínica após 24 meses. Pode-se concluir que a substância cinzenta, cortical e subcortical, está difusamente afetada nos pacientes com EM, e que este dano progride consideravelmente em um período de dois anos, com importante impacto clínico e cognitivo.

Abstract

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system that affects about 2.5 million people worldwide. MS entails a significant economic impact due to both motor and cognitive functional impairments.

In recent decades, the study and understanding of MS have benefited from advances in neuroimaging techniques. Magnetic resonance imaging (MRI) has been used to study both the natural history and to monitor the effectiveness of treatments, but the correlation of conventional MRI findings with clinical data is not yet fully satisfactory. Thus, there has been great interest in other MRI techniques, including the assessment of grey matter. Nevertheless, despite advances in neuroimmunology and neuroimaging, there are few data that can predict the long-term disability in MS patients.

Therefore, our goal was to identify clinical and MRI factors related to a worse clinical outcome in patients with MS. Initially, we surveyed the data of 197 patients followed in the outpatient clinic of the MS center at UNICAMP University Hospital, gathering clinical and epidemiologic information and the time to achieve specific scores on EDSS disability scale. The median time from onset to the assignment of a disability score of 6 was 25.8 years, but male patients, especially those with frequent relapses in the first years of disease, and with involvement of the brainstem or cerebellum showed a worse outcome.

Subsequently, we established a smaller subgroup of patients in order to study the longitudinal behavior of brain pathology as seen by MRI and its relationship to clinical and cognitive disability. We followed for a period of 24 months, 43 patients with relapsing-remitting MS and 29 healthy subjects, who underwent neurological examination, neuropsychological testing and brain MRI. At baseline, performance on clinical and neuropsychological tests was worse in the patients group, and 44.2% were classified as having cognitive dysfunction. Worse performance on neuropsychological battery was

associated with the presence of subclinical MRI activity, with a high burden of cortical lesions and atrophy of the corpus callosum. In addition, worse clinical disability was also associated with these cortical lesions, both those in the brain as those present in the cerebellar cortex. As the presence of MRI subclinical disease activity was an important indicator of cognitive impairment, coupled with the fact that there are no strong biological markers so far, we assessed the production of proinflammatory cytokines in a subgroup of 15 patients and compared with MRI data. We found that patients with subclinical active MRI lesions had significantly higher production of proinflammatory cytokines, 10-fold greater in IFN-γ and 22-fold in TNF-α.

The group of 43 patients was followed longitudinally and after 24 months grey-matter atrophy was 2.57% in the cortex and 3.8% in subcortical structures, both rates significantly higher than in the control group. The presence of thalamus atrophy at the baseline was associated with an increased risk of cognitive dysfunction after 2 years. Furthermore, the presence of a high load of cortical lesions at baseline was related to a 5.14 fold increased risk of clinical disability after 24 months. It can be concluded that both cortical and subcortical grey matter are diffusely affected in MS patients, and that this damage progresses considerably over a period of two years, with important clinical and cognitive impact.

9HPT - teste dos noves pinos nos buracos

BOC – bandas oligoclonais

BRB-N - bateria de testes neuropsicológicos de Rao

DIR - seqüência de ressonância magnética com dupla inversão

EDSS – escala de incapacidade funcional de Kurzke

EM – esclerose múltipla

EMRR – esclerose múltipla forma remitente- recorrente

IFN - interferon

LCR – líquido celaforraqueano

PASAT - teste auditivo compassado de adição seriada

RM – ressonância magnética

SNC – sistema nervoso central

T25FW - teste de caminhada cronometrada de 25 pés

Th – linfócitos T auxiliadores

TNF - Fator de necrose tumoral

Sumário

Introdução

Introdução

A esclerose múltipla (EM) é uma doença inflamatória desmielinizante que afeta o sistema nervoso central (SNC), resultante da interação entre fatores genéticos e ambientais (1). Considerada como um exemplo típico de doença auto-imune, órgão – especifica e Th1-mediada, a EM é potencialmente a causa mais comum de incapacidade neurológica em adultos jovens, especialmente se excluirmos as causas traumáticas (2).

Atualmente, estima-se que a doença afete cerca de 2,5 milhões de pessoas em todo o mundo e estudos epidemiológicos mostram um aumento contínuo de sua incidência nas últimas décadas, principalmente em países desenvolvidos. Na cidade de São Paulo, a prevalência estimada em 1997 era de 15/100.000 habitantes (3). A EM implica em um importante impacto econômico para o estado, tanto pelos custos diretos e/ou indiretos (4), e este custo tem aumentado nos últimos anos (5). Além disso, muitos pacientes se afastam de suas atividades laborais e/ou se aposentam precocemente, algumas vezes no início da idade economicamente ativa. Este impacto social e econômico está relacionado principalmente com os eventos agudos da doença (surtos) e sua progressão insidiosa (5), os quais podem gerar incapacidades funcionais significativas para os pacientes, tanto da esfera sensitivo-motora, quanto cognitiva.

A disfunção cognitiva na EM tem sido bem estudada nos últimos anos e manifesta-se principalmente na capacidade de manter a atenção (concentração), velocidade de processamento de informações, memória (operacional e de longo prazo) e função executiva (fluência verbal e solução de problemas) (6,7). Entretanto, a escala de incapacidade mais utilizada na prática clínica, chamada “Expanded Disability Status Scale” (EDSS) (8), não mede adequadamente estes déficits. Outras escalas foram propostas como, por exemplo, a *Multiple Sclerosis Functional Composite* (MSFC) (9), compreendendo o teste de caminhada cronometrada de 25 pés (T25FW, avaliando a função dos membros inferiores), teste dos nove pinos nos buracos (9HPT, avaliando a função dos membros superiores) e o teste auditivo compassado de adição seriada (PASAT, avaliando

as funções cognitivas de velocidade de processamento de informações e memória operacional). No entanto, para uma avaliação cognitiva mais abrangente em pacientes com esclerose múltipla, Rao et al. (6) desenvolveram a Brief Repeatable Battery of Neuropsychological Tests (BRB-N), compreendendo testes específicos para detectar os déficits acima mencionados: (1) teste de memória verbal imediata e tardia (*Selective Reminding Test,* consistindo na memorização de lista de 12 palavras); (2) teste de memória espacial imediata e tardia (*10/36 Spatial Recall Test*, consistindo na memorização de 10 locais (quadrículas) diferentes em um tabuleiro de xadrez composto de 36 quadrículas); (3) teste de modalidade símbolo-dígito (*Symbol Digit Modality Test, SDMT,* versão oral, para avaliação da atenção seletiva); (4) teste de adição seriada de dígitos apresentados oralmente pelo computador a cada 3 segundos (*Paced Auditory Serial Addition Task, PASAT*, para avaliação da atenção mantida e memória operacional); e (5) teste de Geração de Lista de Palavras (*Word List Generation,* para avaliação da fluência verbal). Esta bateria tem se mostrado bastante útil para o diagnóstico e seguimento da disfunção cognitiva em pacientes com EM (6,10).

Nas últimas décadas, o estudo e o entendimento da EM também se beneficiaram dos avanços das técnicas de neuroimagem, e os próprios critérios diagnósticos sofreram recentemente uma importante revisão, incorporando a ressonância magnética (RM) como uma ferramenta chave para o diagnóstico e acompanhamento (11, 12). Atualmente, diferentes técnicas de RM são usadas para estudar tanto a história natural quanto para monitorar a eficácia de tratamentos, mas a correlação destes achados com os dados clínicos ainda não é totalmente satisfatória (13). Entre estas técnicas, a RM com captação de gadolínio é considerada como um bom marcador de atividade da doença em curto prazo. Por exemplo, a presença de lesões captantes de gadolínio está relacionada a um pior desempenho cognitivo em pacientes com EM (14). Entretanto, ainda não existe um consenso da definição de atividade de doença e das alterações imunobiológicas subjacentes a estas lesões subclínicas captantes de gadolínio (15). Na EM, normalmente existe um aumento da atividade de células Th1 e Th17, secretoras de citocinas pró-inflamatórias, assim como de células efetoras CD8+,

com uma relativa perda da atividade de linfócitos T reguladores. Além disso, existe evidência para uma atividade anormal de linfócitos B, células dendríticas e macrófagos (15). A imunopatogênese da EM pode diferir de paciente a paciente e também de acordo com o curso da doença em um mesmo paciente, tornando difícil, portanto, a identificação de biomarcadores seguros de atividade de doença. Entretanto, a descoberta dos mesmos poderá ter importantes aplicações clínicas, como auxiliar na avaliação da resposta terapêutica e identificar pacientes com risco de pior prognóstico (15).

Se por um lado a RM com captação de gadolínio sugere atividade subclínica de doença, por outro lado a carga lesional em seqüências convencionais ponderadas em T2 tem fraca correlação com o status clínico. Por este motivo, mudanças anuais nas imagens ponderadas em T2 dos pacientes são usadas apenas como desfechos (*end-points*) secundários nos grandes estudos clínicos de fase III (13). Com isso, tem surgido nos últimos anos o interesse por outras técnicas de RM, tais como as medidas de atrofia do corpo caloso (16) e da substância cinzenta cerebral (17), além de novas aquisições para detecção de lesões intracorticais (18). Um achado comum em pacientes com EM, encontrado a partir de estudos de autópsia e RM, é a atrofia do corpo caloso, observada tanto em pacientes com doença avançada quanto naqueles com incapacidade mínima, a qual que progride consideravelmente com o tempo independentemente do curso da doença (16, 19). Em parte essa atrofia pode ser explicada pelas lesões desmielinizantes tanto no próprio corpo caloso quanto em regiões da substância branca circunvizinha (pericalosa) (16).

Mais recentemente o foco das pesquisas tem se direcionado para a patologia da substância cinzenta, tanto cortical quanto subcortical. Lesões intracorticais são bem evidentes com técnicas imuno-histoquímicas em tecidos post-mortem (20), mas a RM convencional é capaz de detectar apenas a minoria delas, principalmente devido ao baixo contraste entre substância cinzenta, substância branca e LCR (21). Outro método não convencional para aquisição de imagens da RM mostrou uma sensibilidade muito maior na detecção de lesões intracorticais (18). Este método, chamado de *Double inversion recovery* (DIR), tem

sido usado tanto na EM quanto em outras doenças neurológicas (18, 21). Lesões intracorticais detectadas por DIR são encontradas em mais da metade dos pacientes com EM e são mais numerosas e frequentes em estágios mais avançados da doença, apesar de poderem ser detectadas mesmo nas fases iniciais. O volume destas lesões pode ser medido com auxílio de softwares semi-automáticos como o *Medical Image Processing, Analysis and Visualization* (MIPAV), desenvolvido no *National Institutes of Health* (22). Estas lesões parecem ser relevantes para a incapacidade clínica e medidas quantitativas volumétricas podem auxiliar ainda mais no estudo da relação entre a carga lesional cortical com a incapacidade clinica (18, 23).

Interessantemente, o risco de apresentar estas lesões se mostrou maior em pacientes do sexo masculino e naqueles com bandas oligoclonais presentes no LCR, o que pode estar relacionado com o fato de a maioria destas lesões serem de morfologia subpial (24). De fato, estruturas semelhantes a folículos linfóides são encontrados nas leptomeninges de pacientes com EM, e é possível que os linfócitos B e a imunidade humoral exerçam um papel importante no processo imunopatológico que ocorre no córtex (25, 26).

Outra abordagem da patologia da substância cinzenta tornou-se possível nos últimos anos com o desenvolvimento de técnicas computacionais, as quais, aliadas ao imageamento por meio da RM, permitem detectar com bastante acurácia a atrofia cerebral. Estudos de RM seriada mostraram que a perda volumétrica cerebral ocorre numa taxa de 0,5% a 1% por ano em pacientes com EM (27), em comparação com a taxa de 0,1% a 0,3% por ano em indivíduos normais (28), podendo esta perda volumétrica estar presente já nos estágios mais iniciais da doença ou até mesmo em pacientes com síndrome clínica isolada (*clinically isolated syndrome,* CIS) (27). Esta perda de substância cinzenta pode ser observada no decorrer de curtos períodos, de 1,5 a 2 anos, ou até mesmo em períodos menores que um ano (29-31), e parece mostrar correlação com o declínio cognitivo dos pacientes (32, 33).

Atualmente, existem diversos métodos para se avaliar a atrofia da substância cinzenta, entre eles o programa computacional *FreeSurfer*,

desenvolvido nos últimos anos com a capacidade de medir a espessura cortical (34-36). Este programa é um conjunto de ferramentas que segmenta a calota craniana e constrói modelos do limite entre substancia branca e cinzenta cortical, assim como a superfície pial. Uma vez que estas superfícies são conhecidas, uma série de medidas anatômicas torna-se possível, incluindo a espessura cortical, que é avaliada vértice a vértice como a menor distância entre o limite da substancia branca/cinzenta cortical e o limite entre substancia cinzenta cortical/superfície pial. Este procedimento tem permitido medidas de alta acurácia da espessura cortical não apenas do cérebro como um todo, mas também de regiões específicas (37). Entretanto, até agora o *FreeSurfer* tem sido pouco utilizado para investigar a atrofia cortical na EM (37).

Embora tenha havido grandes avanços na neuroimunologia e neuroimagem, a prática diária ainda é confrontada com a incerteza na previsão do curso e da incapacidade da EM em longo prazo. Os pacientes estão frequentemente preocupados com o seu status clínico no futuro, mas existem poucas medidas capazes de predizer a incapacidade de forma confiável, principalmente depois de dez ou mais anos de doença. Em termos de grupo, fatores clínicos como surtos frequentes nos primeiros anos de doença ou sexo masculino têm sido associados com um curso pior da doença, embora a maioria das informações venha da América do Norte e países europeus (39, 40). Fatores genéticos e ambientais também podem desempenhar um papel importante na heterogeneidade observada na evolução da doença, além de influenciar na resposta ao tratamento (41). Com o conhecimento destes fatores, novas terapias com alto impacto nas exacerbações da doença, mas às vezes com efeitos colaterais significativos (41), poderiam ser adaptadas para pacientes com maior risco de pior prognóstico.

Desse modo, nosso objetivo foi identificar fatores clínicos e de RM relacionados com uma pior evolução clínica em pacientes com EM.

Objetivos

Objetivos gerais:

Identificar fatores clínicos e de RM relacionados com uma pior evolução clínica e cognitiva em pacientes com EM.

Objetivos específicos:

1. Analisar fatores clínicos e demográficos relacionados com um pior curso da EM numa amostra de pacientes do HC-UNICAMP, comparando com dados publicados em outros países.

2. Avaliar o impacto clínico e cognitivo da patologia da substância cinzenta cerebelar, medida por RM, em pacientes com EMRR.

3. Avaliar características da patologia cerebral da EM (atrofia da substância cinzenta cortical e subcortical, lesões corticais e de substância branca, atrofia do corpo caloso e lesões ativas na RM), que estão mais relacionadas com disfunção clínica e cognitiva em pacientes com EMRR.

4. Investigar a associação entre lesões ativas na RM e a produção de citocinas pró-inflamatórias por células mononucleares do sangue periférico em pacientes com EMRR na fase de remissão.

5. Analisar de forma longitudinal a evolução dos parâmetros de RM e principalmente da substância cinzenta cerebral, e sua relação com a progressão clínica e cognitiva em um grupo de pacientes com EMRR.

Capítulos

Capítulo 1

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Prognostic indicators for long-term disability in multiple sclerosis patients

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ABSTRACT

Background: Daily practice is still faced with uncertainty in predicting the long-term disability of multiple sclerosis (MS). Most information comes from northern hemisphere cohorts, but in South America this information is scarce, and race, genetic and environmental factors could play an important role in the heterogeneity observed in disease outcomes.

Methods: We evaluated 197 patients attending our MS Center gathering clinical and demographic information. Outcome measures analyzed were time from first clinical symptom to EDSS of 6, 7 and 8. For survival analysis we employed Cox regression models and the Kaplan–Meier method.

Results: Time to EDSS 6 was 25.83 years (95% CI 15.36–36.31), and 36.25 years (95% CI 20.72–51.78) for EDSS 7. Male sex was associated with a 4.63 and 4.69 fold increased risk to EDSS 6 and 7, respectively ($p<0.001$ and $p=0.006$). Motor and brainstem symptoms at onset were also associated with an 8.1 and 13.1 fold increased risk to EDSS 6, respectively ($p=0.04$ and $p=0.01$). The number of relapses in five and ten years of disease onset was associated with a slightly increased risk to EDSS 8 (1.28 and 1.19, respectively; $p=0.032$ and $p=0.015$).

Conclusions: Male patients presenting with frequent relapses, especially those with motor and brainstem involvement, deserve close observation and should be cautiously monitored to early signs of treatment failure.

1. Introduction

Evolving clinical and neuroimaging criteria have allowed an earlier and more accurate diagnosis of multiple sclerosis (MS) in clinical practice [1]. The concepts of disease dissemination in space and time can now be apprehended in a single MRI. However, despite major advances in neuroimmunology and neuroimaging, daily practice is still faced with uncertainty in predicting the long-term course and disability of MS at the individual level. Patients are often concerned with their clinical status in the long-run, but few measures correlate reliably with disability after ten or more years of disease. At the group level, frequent relapses in the first years of disease, male sex or a short interval between the first and the second attack have been associated with a worse disease course, although most information come from large North America and European cohorts and some clinical predictors remain controversial [2,3]. In South America this information is scarce, and race, genetic and environmental factors could also play an important role in the heterogeneity observed in disease outcomes, besides influencing treatment response [4,5]. Therefore, emerging therapies with high short-term impact in disease exacerbations, yet sometimes with undesirable side effects, may be tailored to patients at higher risk for worse prognosis [6].

In this setting, we analyzed clinical and demographical factors related to shorter times to disability milestones in a Brazilian sample of multiple sclerosis patients, addressing similarities and differences to other cohorts reported in the literature.

2. Methods

2.1. Patient selection

We evaluated all patients attending our outpatient clinic of the MS center at UNICAMP University Hospital, Campinas, Brazil. MS was diagnosed according to 2005 revised Macdonald criteria [7]. Patients are followed every 3 or 4 months, and the majority lives in Campinas metropolitan area or nearby cities. All patients were seen since the first visit on the center by one of the authors (B.P.D.). We included all patients with a relapsing–remitting and secondary progressive course attending our MS center since 1984. Patients with a primary progressive course or those who fulfilled diagnostic criteria for neuromyelitis optica were excluded. The study was approved by the ethics committee of the Faculty of Medical Sciences of University of Campinas and patients provided written informed consent.

2.2. Clinical factors and outcome measures

We reviewed medical records gathering clinical and demographic information regarding sex, educational level (assigned as fundamental, intermediate, and superior level) and self-reported skin color

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(assigned as white, black or brown). In Brazil, fundamental school extends over eight grades and is compulsory from seven to 14 years of age, and required for entering intermediate school. Intermediate schooling spreads over at least three annual grades; superior education is taught at colleges or universities. Data on disease features including age at onset (i.e. first relapse), type of symptoms at onset, interval between first and second relapse, and number of relapses in two, five and ten years after disease onset were also collected, as well as the presence of oligoclonal bands (OCB) in the cerebrospinal fluid (CSF). We also searched for information about time from onset to first visit in our center and time from onset to treatment with disease-modifying drugs (DMDs) (i.e. interferon-beta, glatiramer acetate or natalizumab).

Overall mean annualized relapse rate (ARR) was calculated taking into account all relapses (including the first) from an individual. For example, for the ARR in the first two years we included only patients who completed at least two years of disease duration and those relapses that occurred during this period. Those presenting at the center with a relapse but with less than 2 years of disease duration were not included.

The majority of all patients (72.6%) could precisely recall month of onset at the first visit on the clinic and most of the other 27% could recall the month of onset within a range of 2 to 3 months.

Outcome measures analyzed were time to sustained Expanded Disability Status Scale (EDSS) scores of 6, 7 and 8. A sustained or irreversible EDSS was considered when the score persisted for ≥6 months, excluding any transient worsening of disability related to relapse.

Data was entered into a computerized database in January 2008 and updated annually since then — last update in November 2010.

2.3. Statistical analysis

Data was entered in Statistical Analysis System for Windows (SAS Institute Inc., 2002–2003, Cary, NC, USA). To test the association between two categorical variables we employed the chi-square or Fisher exact test. For continuous variables, Mann–Whitney (two groups) or Kruskal–Wallis (3 or more groups) tests were selected, due to a non-normal distribution of data. For survival analysis we employed Cox regression models, univariate and multivariate with stepwise selection of variables, and also the Kaplan–Meier method and log-rank test. Cox multivariate analyses included categorical (sex, educational level, skin color, and type of symptoms at onset) and continuous variables (age at onset, interval between first and second relapse, number of relapses in two, five and ten years after disease onset, and time from onset to treatment DMDs). The level of significance was $p<0.05$.

3. Results

3.1. Clinical features

We included 197 patients with a female/male ratio of 3.2:1 (Table 1). Mean follow-up period was 7.48 years (range 0.42–25.67), with 62% of patients with more than 5 years on follow-up (Table 2). The median proportion of retrospective time/data analyzed was 26.9% (i.e. from onset to initial visit at the center).

Overall mean annualized relapse rate (ARR) was 1.04 in the first two years of disease, decreasing to 0.39 between years 3 and 5, and to 0.2 between years 5 and 10. There was a similar trend in ARR considering only those patients without DMDs since disease onset: 0.86 in two years (n = 128), 0.25 between years 3 and 5 (n = 79), and 0.27 between years 5 and 10 (n = 34).

Patients whose initial visit to the center was more than 3 years after disease onset had a longer delay to treatment with DMDs (119 vs. 36.8 months, $p<0.00001$), and fewer relapses in two (1.65 vs. 2.51, $p<0.00001$) and five years of disease onset (2.76 vs. 3.76, $p=0.001$).

Table 1
Clinical and demographic data.

Demographic features	
Sex: no. (%)	
Females	151 (76.6)
Males	46 (23.4)
Educational level: no. (%)	
Fundamental	13 (14.3)
Intermediate	36 (39.6)
Superior	42 (46.1)
Skin color: no. (%)	
Black	6 (3.1)
Brown	24 (12.6)
White	161 (84.3)
Disease features	
Type of disease: no. (%)	
PPMS[a]	12 (5.7)
RRMS	128 (61.2)
SPMS	69 (33.1)
Presence of CSF oligoclonal bands: no. (%)	
Negative	21 (21.4)
Positive	77 (78.6)
Mean age at disease onset: years (range/median)	29.8 (7–61/29)
Type of symptom at disease onset: no. (%)	
Motor	68 (34.7)
Sensory	41 (20.9)
Brainstem	40 (20.4)
Optic neuritis	38 (19.4)
Multifocal	9 (4.6)
Mean interval between first and second relapse: months (range/median)	30.6 (1–252/13)
Mean number of relapses in	
Two years (SD)	2.09 (± 1.11)
Five years (SD)	3.26 (± 1.92)
Ten years (SD)	4.28 (± 2.62)
Mean time from onset to initial visit at the center: months (range/median)	57.7 (0–426 /29.5)
Mean time from onset to treatment with DMDs: months (range/median)	73.8 (0–473/51)
Mean disease duration: years (range/median)	12.4 (0.8–43.4/11.3)

CSF: cerebrospinal fluid; DMDs: disease-modifying drugs; PPMS: primary progressive multiple sclerosis; RRMS: relapsing–remitting multiple sclerosis; SD: standard deviation; SPMS: secondary progressive multiple sclerosis.
[a] PPMS patients were not included in the study.

Male patients had more brainstem symptoms at onset (37% vs. 15.3%) and fewer sensory symptoms (13% vs. 23.3%) or optic neuritis (8.7% vs. 22.7%) than female patients (chi-square = 15.67; df = 4; $p<0.01$), but both were similar regarding educational level, skin color, age at onset, interval between first and second relapse, number of relapses, and time from onset to treatment with DMDs.

Brainstem symptoms at onset were associated with higher number of relapses in the first two years of disease ($p=0.046$).

3.2. Outcome measures

3.2.1. Time to EDSS 6

Kaplan–Meier estimate of the median time from disease onset to EDSS 6 was 25.83 years (95% CI 15.36–36.31) (Fig. 1A).

Male patients had a shorter time to EDSS 6 than females (7.25 vs. 12.75 years, $p=0.022$), while educational level, skin color, and other disease features were not related to this outcome.

Univariate Cox regression analysis showed male gender with a 4.39 fold increased risk (*hazard ratio*) to EDSS 6 vs. females ($p<0.001$, 95% CI 2.13–9.03). Additionally, motor and brainstem symptoms at onset were also associated with an 8.1 and 13.1 fold increased risk to EDSS 6, respectively ($p=0.04$, 95% CI 1.06–61.97, and $p=0.01$, 95% CI 1.71–100.99). An older age at disease onset had a weak 1.04 increased risk ($p=0.038$, 95% CI 1.002–1.082). A longer delay from onset to treatment with DMDs was associated with a slight 0.992 decreased risk ($p=0.019$, 95% CI 0.985–0.999).

Table 2
Clinical and demographic characteristics stratified by follow-up times.

	Time on follow-up (years)		
	0–5	5–10	>10
Sex: no. (%)			
Male	15 (23.1)	16 (26.2)	11 (24.4)
Female	50 (76.9)	45 (73.8)	34 (75.6)
Age at onset: years (mean ± SD)	31.1 ± 10.2	28.8 ± 8.6	31.0 ± 10.6
Disease duration: years (mean ± SD)[a]	7.2 ± 5.3	12.7 ± 6.9	18.9 ± 7.4
Number of relapses in			
Two years (mean ± SD)	2.2 ± 1.1	2.2 ± 1.2	2.0 ± 0.9
Five years (mean ± SD)	2.9 ± 1.6	3.9 ± 2.2	3.1 ± 1.7
Ten years (mean ± SD)	NA	5.3 ± 2.9	4.3 ± 2.7
Interval between first and second relapse: months (mean ± SD)	25.9 ± 35.9	30.6 ± 40.7	31.6 ± 37.8
Time from onset to initial visit at the center: months (mean ± SD)	52.4 ± 61.2	61.5 ± 82.5	60.22 ± 81.4
Time from onset to treatment with DMDs: months (mean ± SD)[a]	53.5 ± 58.6	69.8 ± 77.6	110.5 ± 110.5
Patients that reached sustained EDSS 6: no. (%)[a]	9 (14.5)	23 (38.3)	23 (51.1)

DMDs: disease-modifying drugs; EDSS: Expanded Disability Status Scale; SD: standard deviation.
[a] $p<0.05$ (Kruskal–Wallis ANOVA).

Multivariate analysis confirmed the increased risk of male patients vs. females to EDSS 6 (*hazard ratio* = 4.63, $p<0.001$, 95% CI 2.15–9.96) (Fig. 2A) and also a slight 1.057 fold increased risk per year for older disease onset ($p=0.007$, 95% CI 1.015–1.101).

3.2.2. Time to EDSS 7

Kaplan–Meier estimate of the median time from disease onset to EDSS 7 was 36.25 years (95% CI 20.72–51.78) (Fig. 1B).

Univariate Cox regression showed again that male sex was associated with 3.29 fold increased risk to EDSS 7 ($p=0.016$, 95% CI 1.25–8.69), which rose to a 4.69 fold risk on multivariate analysis ($p=0.006$, 95% CI 1.57–14.00) (Fig. 2B). Univariate analysis also showed that a longer delay from onset to treatment with DMDs was associated with a weak 0.991 decreased risk to this outcome ($p=0.031$, 95% CI 0.984–0.999).

3.2.3. Time to EDSS 8

Kaplan–Meier estimate of the median time from disease onset to EDSS 8 was 33.75 years (95% CI 33.75–33.75) (Fig. 1C). Some patients reaching this outcome lacked information on time to EDSS 7.

In Cox regression, the number of relapses in five and ten years of disease onset was associated with a slightly increased risk to EDSS 8 (1.28 and 1.19, respectively; $p=0.032$, 95% CI 1.022–1.602 and $p=0.015$, 95% CI 1.035–1.373), and a longer delay from onset to treatment with DMDs was associated with a slight 0.990 decreased risk ($p=0.013$, 95% CI 0.982–0.998), but these associations were not present on multivariate analysis.

3.2.4. 'Benign' MS

From 112 patients fulfilling 10 years of disease duration, 26.8% had EDSS ≤ 3.0, and from 55 with at least 15 years of disease, only 21.8% had EDSS ≤ 3.0. These patients were not different to others regarding demographic or other disease features. Comprehensive neuropsychological evaluation was not performed in these patients.

4. Discussion

Several cohort studies have described the natural course of MS, including the accumulation of disability over time. However, there is some variability regarding disease duration up to irreversible disability scores. For example, from the London, Ontario, Canada cohort, the median time from onset to the assignment of an irreversible disability score of 6 was 15 years, while it was 20.1 years in the Lyon, France

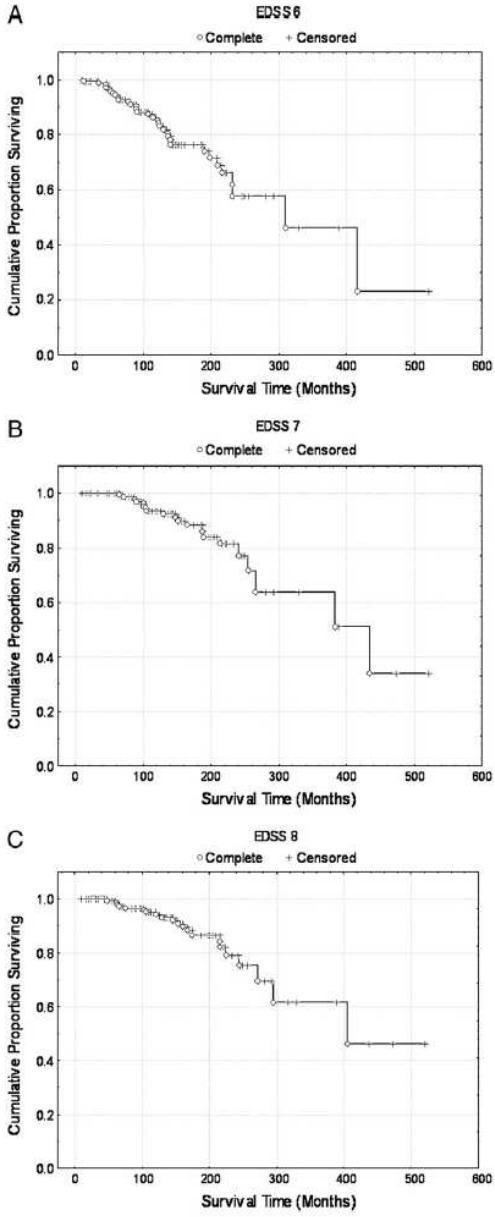

Fig. 1. Kaplan–Meier survival curves of time from disease onset to EDSS 6 (A), 7 (B) and 8 (C). The median time was 25.8, 36.25 and 33.75 years respectively. The estimated median time to EDSS 8 was shorter than EDSS7, probably due to the limited number of patients reaching this outcome and to a small amount for whom the only information available was time to EDSS 8.

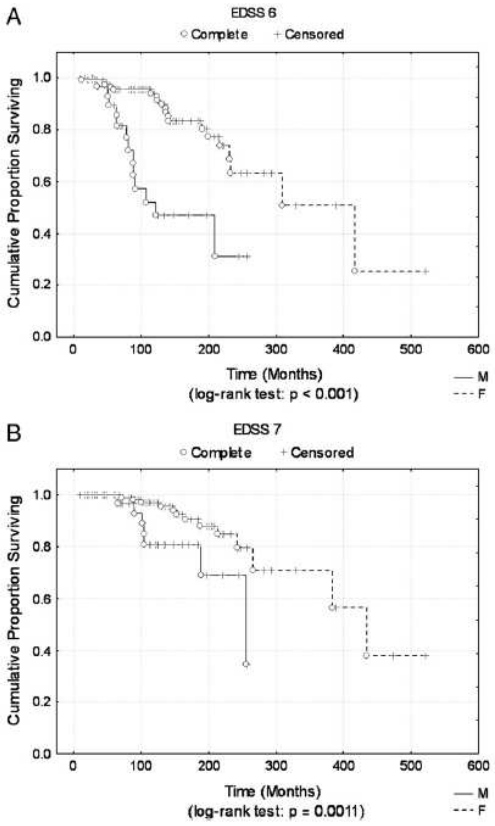

Fig. 2. Kaplan–Meier survival curves of time from disease onset to EDSS 6 (A) and 7 (B) separated by gender. The median time was significantly shorter in males than females for both outcomes (*hazard ratio* of 4.63 fold for EDSS 6 and 4.69 for EDSS 7).

cohort and 18 years in the Gothenburg, Sweden cohort [2,8,9]. In our sample it was 25.8 years, slightly longer than other studies previously mentioned. Race, genetic and environmental factors can play an important role in the heterogeneity observed in disease outcomes of our series and the abovementioned studies from Europe or North America [4,5]. Our study took place in Campinas City, situated in the southeast region of Brazil, where MS prevalence was around 15/100,000 inhabitants in one study. In this region, genomic ancestry has been estimated to be for the most part European, reaching 86.1% in whites and 67.5% in those self-categorized as having a brown skin, while African ancestry was present in 7.4% and 23.8% and Amerindian in 6.5% and 8.7% respectively [10,11]. Despite considering genetic and other environmental factors in the heterogeneity observed, it is also of note that our sample is relatively small compared to those large cohorts, and that we included patients under DMD treatment, what could have potentially influenced the results. Nevertheless, a longer delay to treatment with DMDs was related with a longer time to irreversible disability in this sample, what can be partially due to more severe cases seeking medical care and having started DMDs earlier than milder or benign ones.

Long-term prognosis of MS has been subject to extensive discussion in the literature, and several specific prognostic factors have been proposed to confer higher risk for a worse outcome, yet the results are not always consistent across studies [3,12]. The populations studied come from diverse countries and with different sampling techniques and again, race and environmental factors could explain some of the discrepancies found [4,5]. The influence of sex has been assessed in numerous studies and the overall impression is that male sex confers an increased risk for worse prognosis [3,12–14]. Accordingly, we found male sex to be the most consistent risk factor analyzed, reaching a 4.63 and 4.69 fold *hazard ratio* to EDSS 6 and 7, respectively, on *Cox* multivariate analyses. A younger age at onset has also been associated with a longer time for reaching disability landmarks in most studies yet with different strengths of influence [3,9,12,13]. In the multivariate analysis, we found a slight 1.057 fold increased risk per year for older disease onset; however, despite a longer time to reach disability landmarks, younger individuals at onset still reach these landmarks at a younger age than individuals with first symptoms at an older age, and therefore, are disabled for a longer part of their life, leading to an impression that a younger age at onset might not be a good prognostic factor [13–15]. Other disease features at onset, such as first symptoms, have also been subject to exploration. We found that motor and brainstem/cerebellar involvement at presentation implicated in an 8.1 and 13.1 fold increased risk, respectively, for EDSS 6 on *Cox* univariate analysis, but these associations were not found in the multivariate analyses. Indeed, in the literature there is an overall weak effect for motor, brainstem or sphincter involvement at presentation specially when taking other clinical information into account [3,16].

A higher relapse frequency during the first years of MS has been generally associated with a higher risk of disability, but this effect was considered only modestly predictive in a recent systematic review and type of relapse may be more important [3]. Moreover, once a certain threshold of irreversible disability has been reached, these variables are no longer predictive of the time course of the subsequent disability progression [13]. Indeed, we found that a higher relapse frequency was associated with a slightly increased risk of time to EDSS 8.

Other paraclinical investigations performed at onset, such as the search for OCB in the cerebrospinal fluid, have led to contradictory results regarding prognosis value [17]. Some studies found that patients with OCB had a higher risk for long-term disability than patients without them; however, this was not reproduced in our sample using survival analyses.

As previously mentioned, our sample is relatively small compared to some large cohort studies available from the northern hemisphere, where prevalence of MS is also considerably higher [2,8,9]. Our sample also comes from a tertiary university hospital, and hence is open to sampling/selection bias toward over-representation of more severe cases. However, our MS center attracts the great majority of patients within our region, and previous epidemiology studies in southeastern Brazil have shown similar demographic and clinical characteristics such as age at onset, male/female ratio, high predominance of white skin patients and similar symptoms at onset [18]. It is also of note that our cohort study included patients with different follow-up times and disease duration at presentation and about 40% of the patients have less than 5 years of follow-up. Moreover, being retrospective, it was open to information bias as the temporal relationship was more difficult to assess. It may have led to suboptimal accuracy for time of first relapse or between initial relapses, however, if we had included only those patients recalling exactly the month of onset we could have biased the results toward a more severe scenario. Future re-assessments can provide more robust information on prognosis.

In conclusion, male patients presenting with frequent relapses, especially those with motor and brainstem involvement, deserve close observation and should be cautiously monitored to early signs of treatment failure.

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Conflicts of interest

The authors report no conflict of interest.

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Capítulo 2

Artigo em submissão

THE CLINICAL AND COGNITIVE IMPACT OF CEREBELLAR GREY MATTER PATHOLOGY IN MULTIPLE SCLEROSIS

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Dr. Alfredo Damasceno participated in drafting/revising the manuscript, study concept/design and analysis/interpretation of data.
Dr. Benito Pereira Damasceno participated in drafting/revising the manuscript and analysis/interpretation of data.
Dr. Fernando Cendes participated in drafting/revising the manuscript and analysis/interpretation of data.

Disclosure: The authors report no conflict of interest.

ABSTRACT

Objective: Although modern neuropathological research propose the cerebellum as an important site for cortical demyelination in multiple sclerosis (MS), the functional significance of this finding is not completely clear. We aimed to evaluate the clinical and cognitive impact of cerebellar grey-matter pathology in a group of MS patients.

Methods: We prospectively enrolled 43 patients with relapsing-remitting MS and 30 healthy subjects. Clinical assessment included leg (T25FW) and arm (9HPT) function, overall disability (EDSS), and cognitive evaluation (PASAT and SDMT). MRI scans (3T scanner) included: FLAIR, DIR, and a volumetric T1. Brain white-matter lesion load and cerebellar intracortical and leukocortical lesion volumes were quantified on FLAIR and DIR images, respectively. Cerebellar white-matter and grey-matter volumes evaluation were performed on volumetric T1.

Results: Cerebellar intracortical lesions were observed in 42.5% and leukocortical in 32.5% of the patients. In multivariate regression analysis cerebellar intracortical lesions and white-matter volume were independent predictors for the EDSS (β = 6.93, $p < 0.001$; β = -0.085, $p = 0.006$) and 9HPT (β = 19.85, $p = 0.039$; β = -0.42, $p = 0.044$). Only intracortical lesions remained as an independent predictor for the T25FW (β = 5.93, $p < 0.001$), while leukocortical lesions (β = -35.38, $p = 0.048$), white-matter lesion load (β = -0.53, $p = 0.009$) and cerebellar grey-matter volume (β = 0.28, $p = 0.036$) were independent predictors for PASAT.

Conclusions: Cerebellar grey-matter pathology is widely present and significantly contributes to clinical and cognitive dysfunction in relapsing-remitting MS patients.

INTRODUCTION

In recent years, several neuroimaging studies have shown diffuse grey matter (GM) damage in multiple sclerosis (MS) patients, involving both cortical and subcortical structures, such as the spinal cord and cerebellum. Indeed, modern neuropathological research confirmed these findings and proposed the cerebellum as a major predilection site for cortical demyelination in MS, particularly in those with primary or secondary progressive disease.[1-3] Similar types of cortical lesions, as described in the forebrain, are also seen in the cerebellum, such as intracortical, leukocortical and subpial, and these lesions are also characterized by complete demyelination with relative preservation of neurons and axons.[1,3] However, the functional significance of these lesions is not completely clear. Moreover, besides its key role in motor function, increasing evidence supports an important function of the cerebellum in cognition, dependent upon the existence of different anatomical connections between high-level cortical regions, which may also be involved in MS lesions.[3-5] Although there are some studies reporting association between MRI atrophy measures and clinical performance, very few evaluated the clinical impact of these cortical lesions, but with limited clinical measures and no cognitive assessment.[6-8] Therefore, in this study, we evaluated the clinical and cognitive impact of cerebellar grey matter pathology, as measured by MRI, in a group of patients with relapsing remitting MS, addressing relative contributions of cerebellar cortical and white-matter atrophy, and also cerebellar leukocortical and intracortical lesions.

METHODS

Subjects

We prospectively and consecutively enrolled 43 patients with a relapsing-remitting MS diagnosis according to revised 2005 McDonald criteria,[9] and 30 age-, gender-, and educational level-matched healthy subjects for

comparison as a control group. All individuals were evaluated at the MS Center of UNICAMP University Hospital, Campinas, Brazil. All patients were clinically stable and on treatment with disease-modifying drugs. Exclusion criteria were: progressive course, fulfillment of diagnostic criteria for neuromyelitis optica, EDSS >5.0, any pre-existing condition known to be associated with brain atrophy or any relapse or steroid therapy within three months preceding the clinical and MRI evaluation. The study was approved by the ethics committee of the faculty of medical sciences of University of Campinas and all patients provided written informed consent.

Clinical assessment/Outcome measures

Neurological clinical examination included: assessment of leg function by means of the timed twenty-five foot walk (T25FW) and arm function with the nine-hole peg test (9HPT) for all participants. We also measured overall disability with the Expanded Disability Status Scale (EDSS) for all patients. Cognitive evaluation included the Paced Auditory Serial Addition Test (PASAT) and Symbol Digit Modalities Test (SDMT) for all individuals. Each of these tests was considered different outcome measures.

Magnetic resonance imaging

MRI scans for all subjects were acquired on a 3T scanner (Phillips Achieva-Intera). The study protocol consisted of: fluid attenuated inversion recovery (FLAIR) – acquired in the axial plane with 3mm slice thickness (TR 11000 ms, TI 2800 ms, TE 125 ms, matrix 328 x 210, gap 0, FOV 23 x 18 cm, flip angle 90°; in-plane resolution 0.7 mm x 0.85 mm); double inversion recovery (DIR) - acquired in the axial plane with 3mm slice thickness (TR 11000ms, TI 3400 ms, TE 50ms, delay 325 ms, matrix 328 x 210, gap 3, FOV 23 x 18 cm, flip angle 90°; in-plane resolution 0.7 mm x 0.85 mm) and a volumetric (three-dimensional) T1 gradient echo images - acquired in the sagittal plane with 1 mm slice thickness (TR

7.0 ms, TE 3.2 ms, matrix 240 × 240, FOV 24 × 24 cm, flip angle 8°; in-plane resolution 1.0 mm x 1.0 mm).

Image Analysis

Brain white-matter lesions

Brain white-matter lesion load (WML) was quantified on FLAIR sequences, blinded to clinical data, using the freely available Medical Image Processing, Analysis, and Visualization (MIPAV) software package developed at the Center for Information Technology, National Institutes of Health.[10] The intrarater reliability between WML quantification was assessed using intra-class correlation (ICC = 0.96).

Cerebellar cortical lesions

Cerebellar cortical lesions were identified and scored on DIR sequences, blinded to clinical data, in accordance to consensus recommendation,[11] and accurately controlled for artifacts. Lesion volume was quantified using the MIPAV software. Lesions were classified as purely cortical/intracortical (CoL) when lesion borders remained completely within the cortex or mixed white-matter/grey-matter lesions (MixL) when lesions occurred at the leukocortical junction, demyelinating white matter and cortex (figure 1). The intrarater reliability between each lesion quantification was assessed using intra-class correlation (ICC = 0.94 for both).

Cerebellar volumes

Cerebellar WM and GM volumes evaluation were performed on volumetric T1 gradient echo images by means of the FreeSurfer v5.1 image analysis suite, available online (http://surfer.nmr.mgh.harvard.edu/), as described

elsewhere.[12-14] All images were thoroughly controlled for errors and artifacts.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, Version 20.0., SPSS Inc, Chicago, Illinois).

Group comparisons on clinical and cognitive tests were performed with Mann-Whitney *U* tests. Comparisons on cerebellar volumes were analyzed by the General Linear Model univariate analyses of variance procedure, with gender and total intracranial volume as covariates.

A stepwise multivariate linear regression analysis was performed initially to assess a possible relative contribution of clinical factors (age, disease duration and gender) in clinical (EDSS, 9HPT and T25FW) and cognitive outcomes (PASAT and SDMT) and then, the same procedure was used to assess the relative contribution of MRI factors (CoL, MixL, WML, cerebellar WM and GM volumes) in the same clinical and cognitive outcomes. We also assessed the relative contribution of the same clinical factors and some MRI metrics (CoL, MixL and WML) to cerebellar GM and WM volume. Backward stepwise analyses were conducted (Wald statistic) with a p value for entry of 0.05 and a p value for removal of 0.1. Scatter plots were generated with Statistica software (Stat Soft, Inc., Tulsa, OC). The level of significance was $p < 0.05$.

RESULTS

Clinical and MRI characteristics

Clinical and MRI features are shown in Table. Patients and controls were similar regarding age, gender, and educational level distribution ($p > 0.25$). The T25FW time was higher in the patients group, and so was the 9HPT ($p < 0.001$). Patients also performed worse in SDMT ($p < 0.001$) and PASAT ($p = 0.001$) cognitive tests.

After correction for gender and total intracranial volume, the cerebellar GM and WM volume were higher in healthy subjects than patients (Table). Cerebellar CoLs were observed in 42.5% and MixLs in 32.5% of the patients.

Table. Clinical and MRI data.

	RRMS patients	Controls	Between-Subjects Comparisons *
Sex: no. (%) Female Male	 33 (76.7) 10 (23.3)	 23 (76.7) 7 (23.3)	p = 0.58
Age: years	30.79 ± 6.76	29.57 ± 7.40	p = 0.29
Disease duration: years	6.52 ± 4.90	NA	NA
EDSS score	2.12 ± 0.91	NA	NA
9HPT: seconds	22.80 ± 4.45	17.83 ± 1.49	p < 0.001
T25FW: seconds	4.78 ± 0.85	4.05 ± 0.67	p < 0.001
PASAT: raw score	33.33 ± 13.00	43.66 ± 9.44	p = 0.001
SDMT: raw score	50.12 ± 13.27	65.14 ± 11.37	p < 0.001
Cerebellar GM volume: cm3	92.48 ± 12.91	94.37 ± 11.43	F = 15.26, p < 0.001
Cerebellar WM volume: cm3	27.29 ± 4.68	29.03 ± 3.52	F = 3.53, p = 0.019
Cerebellar CoL volume: cm3	0.05 ± 0.08	NA	NA
Cerebellar MixL volume: cm3	0.05 ± 0.10	NA	NA
Brain WML volume: cm3	6.05 ± 9.12	NA	NA

Expressed are mean values and standard deviation.

9HPT: nine-hole peg test; CoL: cortical lesions; EDSS: Expanded Disability Status Scale; MixL: mixed white-matter/grey-matter lesions; NA: not applicable; PASAT: Paced Auditory Serial Addition Test; RRMS: relapsing-remitting multiple sclerosis; SDMT: Symbol Digit Modalities Test; T25FW: timed twenty-five foot walk test; WML: white-matter lesion load.

*Group comparisons were performed with Mann-Whitney *U* tests, except for gender distribution, where Fisher's exact test was used and brain volumes, where the General Linear Model was employed, with gender and total intracranial volume as covariates

Outcome measures

Clinical

After multivariate analysis, clinical factors (age, disease duration and gender) were not related to clinical outcomes, except for the T25FW test, where gender and age were independent predictors (β = 0.71, 95% CI 0.19 to 1.23, p = 0.009 and β = 0.63, 95% CI 0.03 to 0.09, p = 0.001, respectively).

For the EDSS, CoL (β = 6.93, 95% CI 4.21 to 9.65, p < 0.001) (figure 2A) and cerebellar WM volume (β = -0.085, 95% CI -0.14 to -0.02, p = 0.006) were independent predictors.

In the same way, CoL (β = 19.85, 95% CI 1.07 to 38.63, p = 0.039) and cerebellar WM volume (β = -0.42, 95% CI -0.83 to -0.01, p = 0.044) predicted the 9HPT time.

For the T25FW time, only CoL remained as an independent predictor (β = 5.93, 95% CI 3.02 to 8.83, p < 0.001). When those clinical factors found to be independent predictors of this test (age and gender) were included in the model, only CoL and age remained as predictors (β = 4.83, 95% CI 2.15 to 7.51, p = 0.001 and β = 0.044, 95% CI 0.008 to 0.079, p = 0.018, respectively).

Cognitive

In multivariate analysis, clinical factors (age, disease duration and gender) were not related to cognitive outcomes.

For the PASAT score, MixL (β = -35.38, 95% CI -70.52 to -0.24, p = 0.048), WML (β = -0.53, 95% CI -0.92 to -0.14, p = 0.009) and cerebellar GM volume (β = 0.28, 95% CI 0.02 to 0.55, p = 0.036) (figure 2B) were independent predictors.

The only predictor for the SDMT score was the WML (β = -0.49, 95% CI -0.93 to -0.04, p = 0.034).

Cerebellar atrophy

For both GM and WM volume, gender was an independent predictor when clinical factors were analyzed (β = -15.98, 95% CI -24.07 to -7.90, p < 0.001; and β = -3.87, 95% CI -7.09 to -0.64, p = 0.020). When MRI metrics were considered, there was a tendency to CoL predict GM volume (β = -49.35, 95% CI -103.3 to 4.6, p = 0.072), which disappeared when gender was included in the analysis (p = 0.23). Conversely, MixL was an independent predictor to WM volume (β = -17.1, 95% CI -31.97 to -2.22, p = 0.025) even when gender was included in the analysis (β = -16.35, 95% CI -30.42 to -2.29, p = 0.024).

DISCUSSION

This study confirms a major role of cerebellar cortical pathology in clinical and cognitive disability of MS patients. Cerebellar GM involvement in this disease has been clearly demonstrated in a number of neuropathological studies, concerning both cortical lesions and atrophy.[3] Alike the forebrain, similar types of cortical lesions are seen in the cerebellum, and the vast majority occurs in a band-like manner, affecting multiple folia.[1] However, pathological studies so far yielded little information about the clinical significance of cerebellar GM pathology and one

study found no association between cortical demyelination and clinical factors (i.e. age, gender and disease duration).[1-3]

MRI in-vivo visualization of cortical pathology provides a better opportunity to assess such clinical significance but lesions in the GM are mostly undetectable with traditional MRI sequences. However, GM atrophy measurements can be done with 3D MRI acquisitions using different types of software. Therefore, this approach has been used by several studies which documented a progressive loss of brain parenchyma, starting at the earliest stages and continuing throughout the long course of the disease.[3] In particular for the cerebellum, using voxel-based morphometry, significant correlations were found between cerebellar volume estimates and clinical metrics as measured by 9HPT and EDSS cerebellar functional score.[6] On the other hand, Anderson *et al*, on a comprehensive evaluation of cerebellar damage using diffusion tractography and volumetric analysis, found that cerebellar WM volume was associated with 9HPT score in patients with primary progressive MS, independently of cerebellar GM volume.[15] We also found a significant contribution of cerebellar WM volume to arm function as measured by the 9HPT and also the total EDSS score. Additionally, we found an independent contribution of cerebellar GM volume to cognitive function as measured by the PASAT test, supporting findings from recent research stressing the cerebellar role in cognitive functions and notably sequencing abilities.[4,5]

Cerebellar CoL are mostly undetected by conventional MRI and only recently more sensitive acquisition techniques have become available.[3] DIR is one such technique, and some studies have found significant association between brain CoL and cognitive dysfunction using this sequence.[16] However, very few evaluated the clinical impact of cerebellar GM lesions. Calabrese *et al* evaluated the relative contribution of cerebellar CoL in multivariate analysis and found them to be independent predictors of cerebellar disability. They also found cerebellar GM volume to be independent predictor of both cerebellar disability and EDSS score.[8] Despite being pioneer in assessing clinical significance of cerebellar CoL, this study had limited clinical outcome measures and no cognitive assessment. In accordance with that study, we found that cerebellar CoL are independent

predictors for both arm and leg dysfunction, and also for overall disability as measured by the total EDSS score. For example, a 1cm3 increase in cerebellar CoL increased T25FW time in almost 6 seconds. Furthermore, we aimed to discriminate the role of purely cortical and leukocortical lesions. While CoL contributed to clinical dysfunction, we found cerebellar MixL to be independent predictors of cognitive dysfunction as measured by the PASAT test. Although in the cerebellum purely CoL represent around 50% of GM plaques,[2] some demyelinated plaques in the cortex are also found in continuity with demyelination in the subcortical white matter and sometimes associated with large white matter plaques,[1] what may explain the contribution of MixL to cerebellar WM atrophy found in our study. Moreover, involvement of both GM and WM may disrupt important cerebello-cortical loops known to be involved in attention and other cognitive functions.[4,5] Indeed, a recent functional MRI study in MS has shown important cerebellar activation when PASAT test was used as paradigm.[17] Conversely, the SDMT relies more on integrity of the white matter fiber tracts in the occipito-parietal cortex thus being more related to brain WML,[15,18] as shown in our study.

Although DIR may improve detection of cortical lesions up to five times when compared with a conventional T2-weighted sequence, the vast majority is still missed by this technique, especially subpial lesions which may also have important contribution to clinical and cognitive dysfunction in MS patients.[3] Another disadvantage is its low signal-to-noise ratio, resulting in low agreement between observers. Therefore, some lesions considered as purely cortical may in fact be leukocortical and vice-versa. Future studies associating greater MRI field strengths and novel/improved sequences, may overcome this gap.[19]

In conclusion, cerebellar GM is widely affected in relapsing-remitting MS patients, and not only in those with primary or secondary progressive disease. Cerebellar GM pathology significantly contributes to clinical and cognitive dysfunction in MS patients, and can be monitored in-vivo with MRI. Further work is required to better characterize GM plaques and to assess its contribution to long term disability.

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Figure 1. Cerebellar cortical and mixed white-matter/grey-matter lesions lesions. Axial double inversion recovery images from relapsing-remitting MS patients showing purely cortical (long arrows, A and B) and mixed white-matter/grey-matter lesions lesions (short arrows, C and D).

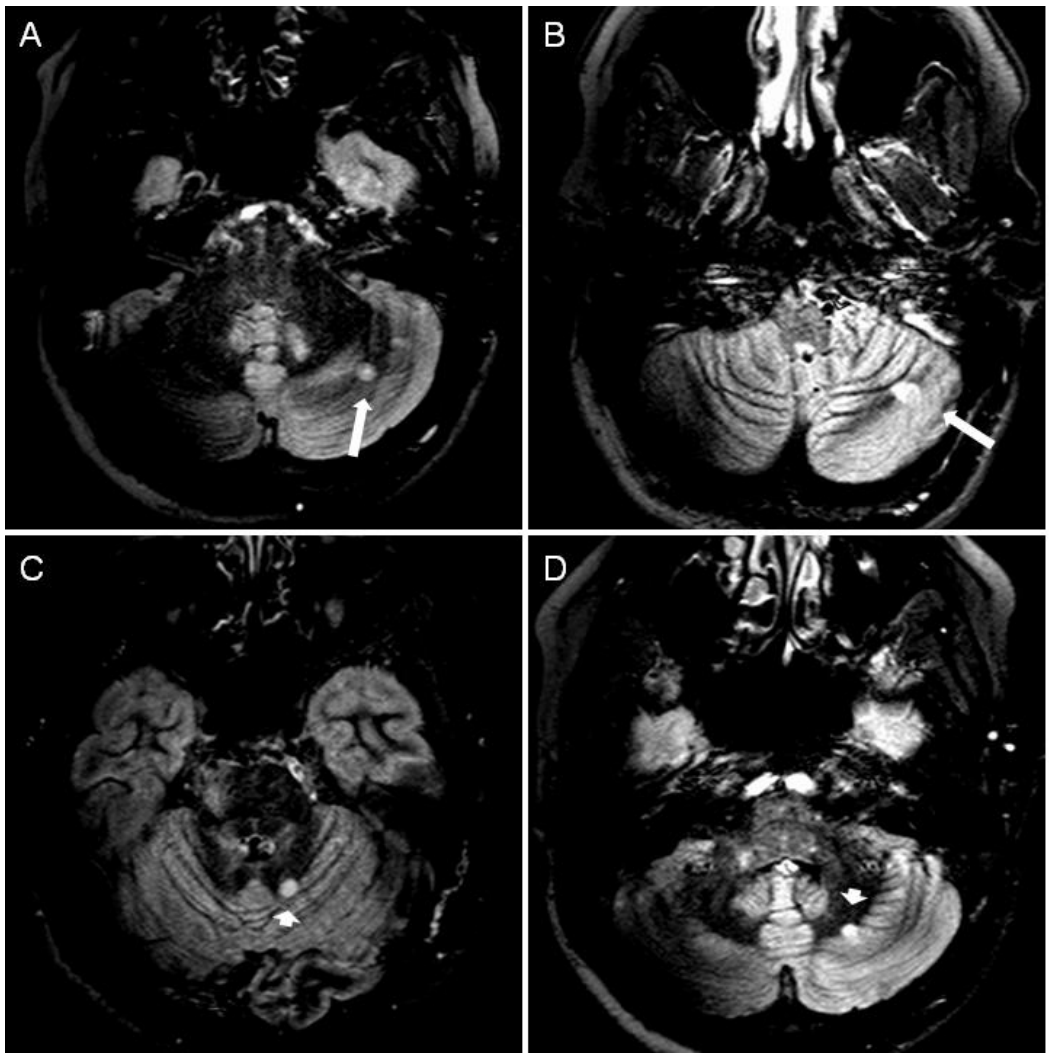
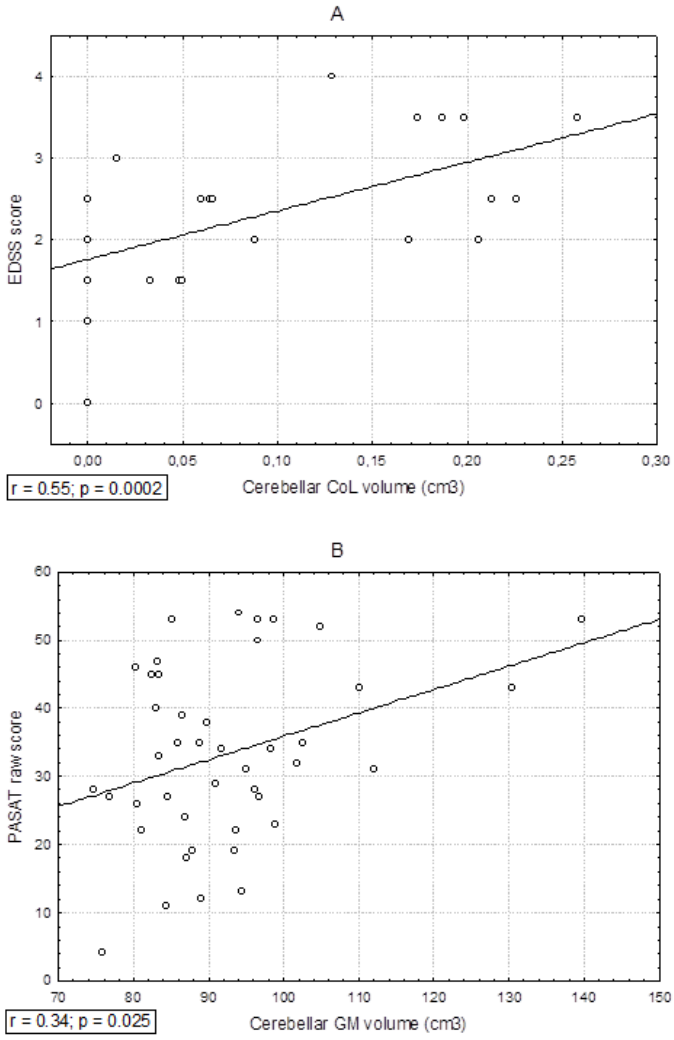


Figure 2. Scatter plots showing Spearman correlations between EDSS and cerebellar CoL volume (A) and between PASAT score and cerebellar GM volume (B).



Capítulo 3

Artigo em submissão

PREDICTING CLINICAL AND COGNITIVE DISABILITY IN MULTIPLE SCLEROSIS

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ABSTRACT

Introduction: Cognitive dysfunction is found in up to two thirds of the multiple sclerosis (MS) patients. Several studies have focused on understanding the pathophysiology underlying this dysfunction but, despite several MRI biomarkers have been suggested, the majority of available studies evaluated few MRI measures.

Objective: To evaluate the relative contributions of grey-matter cortical and subcortical volumes, cortical lesions, corpus callosum (CC) atrophy and gadolinium-enhancing lesions to cognitive dysfunction in a group of relapsing-remitting MS (RRMS) patients using multiple regression analysis.

Methods: We enrolled 43 patients with RRMS and 30 healthy subjects. Outcome variables included the EDSS score, 9HPT, T25FW, PASAT, SDMT score and total number of deficits in the Brief Repeatable Battery of Neuropsychological Tests (BRB-N). All individuals underwent MRI in a 3T scanner, including FLAIR, DIR, volumetric T1, and a T1-weighted scan prior to and after injection of gadolinium. Brain white-matter lesion load (WML) and cortical lesions were quantified on FLAIR and DIR sequences, respectively. Volume evaluation was performed on volumetric T1 images by means of the FreeSurfer. A stepwise multivariate linear regression analysis was performed to assess the relative contribution of MRI metrics to clinical and cognitive outcomes.

Results: Nineteen patients (44.2%) had cognitive impairment. MS patients had lower volume of CC and all grey-matter cortical and subcortical structures. Cognitively impaired patients had higher burden of cortical lesions and lower volume of CC and all grey-matter cortical and subcortical structures, when compared to those non-cognitively impaired. On multivariate analysis high burden of lesions was associated with a 0.58 increase in the EDSS score ($p = 0.042$). Cortical and striatum volume were independent predictors for the 9HPT ($p = 0.007$ and $p = 0.01$) and striatum volume was also the only independent predictor for the leg function T25FW test (0.040).The independent predictors for overall number of deficits in BRB-N were high burden of cortical lesions, presence of active lesions and CC volume ($p = 0.007$, $p = 0.012$, and $p = 0.036$, respectively).

Conclusions: Clinical and cognitive dysfunction is frequent in RRMS patients and significantly related to both grey-matter and callosal pathology. High burden of cortical lesions, callosal atrophy and presence of asymptomatic gadolinium-enhancing lesions were the best predictors of cognitive disability and they can be monitored in-vivo with MRI.

INTRODUCTION

Neuropsychological studies in multiple sclerosis (MS) have become increasingly available in the last decades, showing cognitive dysfunction in up to two thirds of the patients or even more in some series (1). This cognitive impairment, generally affecting information processing speed and episodic memory, has a substantial contribution to disability in these patients, impairing daily living and work capacity. A growing body of literature has focused on understanding the pathophysiology underlying cognitive dysfunction in MS and magnetic resonance imaging (MRI) has become particularly valuable to this aim (2). Current information suggest that focal white-matter lesions do play a role, but with limited overall effect, and focal cortical demyelinating lesions can have a greater impact on cognition (3- 5). Besides focal demyelinating lesions, irreversible tissue loss and axonal damage can disrupt important networks and are more robustly associated with cognitive deficits (2,6,7). Some studies have recently added that even deep grey matter structures such as the thalamus and putamen also have important contributions to cognitive impairment along with neocortical atrophy (8,9). Moreover, besides grey matter involvement, corpus callosum (CC) atrophy also progresses over time in MS and has been implicated in underlying cognitive dysfunction likely through a disconnection mechanism (10-12). Although several candidates have been suggested as potential MRI biomarkers of cognitive disability in MS, the majority of available studies evaluated each of those previously mentioned changes alone or in conjunction with few other MRI measures, yielding limited information on independent predictors (1,2). A few more comprehensive studies have incorporated additional MRI variables suggesting that brain atrophy is a strong predictor of cognitive impairment, but lacked assessment of cortical lesions or volume (7). Thus, in the present study, we evaluated the relative contributions of grey-matter volumes, cortical and subcortical, cortical lesions, and CC atrophy to cognitive dysfunction in a group of relapsing-remitting MS patients using multiple regression analysis. Furthermore, since the presence of gadolinium-enhancing lesions have been recently related to poor cognitive

performance, active lesions were also considered in the analyses (13).

METHODS

Subjects

We prospectively and consecutively enrolled 43 patients with a relapsing-remitting MS diagnosis according to revised 2005 McDonald criteria (14) and 30 age-, gender-, and educational level-matched healthy subjects for comparison as a control group. All individuals were evaluated at the MS Center of UNICAMP University Hospital, Campinas, Brazil. All patients were clinically stable and on treatment with disease-modifying drugs. Exclusion criteria were: progressive course, fulfillment of diagnostic criteria for neuromyelitis optica, EDSS >5.0, any pre-existing condition known to be associated with brain atrophy or any relapse or steroid therapy within three months preceding the clinical and MRI evaluation. The study was approved by the ethics committee of the faculty of medical sciences of University of Campinas and all individuals provided written informed consent.

Clinical and cognitive evaluation

Neurological clinical examination included the Expanded Disability Status Scale (EDSS) for all patients and Multiple Sclerosis Functional Composite (MSFC) for all individuals. Additionally, all participants filled the Fatigue Severity Scale (FSS) and were considered fatigued if scored 4 or higher. They were also assessed for depressive symptoms with the Zung Self-Rating Depression Scale (ZDS) and were considered depressed if scored 40 or higher. The neuropsychological assessment was performed in all individuals using the version A of the Brief Repeatable Battery (BRB-N) (15). This battery includes: verbal memory assessment with the Selective Reminding Test (SRT, with 3 subtests: SRT-LTS, long-term storage; SRT-CLTR, consistent long-term retrieval; SRT-DR,

delayed recall); visuospatial memory with the 10/36 Spatial Recall Test (SpRT, with 2 subtests: short-term and long-term recall); sustained attention and information processing speed with the Symbol Digit Modalities Test(SDMT); working memory and IPS with the Paced Auditory Serial Addition Test (PASAT) 3-second version; and verbal fluency with the Word List Generation test (WLG).

Outcome variables

Outcome variables were derived from clinical and cognitive evaluation and included the EDSS score, arm function (9HPT time), leg function (T25FW time), PASAT score, SDMT score and total number of deficits in the BRB-N (deficit defined as scores below 2SD of the control group mean on each subtest). Patients who had deficits in two or more BRB-N subtests were considered to have cognitive dysfunction (7).

Magnetic resonance imaging

MRI scans for all subjects were acquired on a 3T scanner (Phillips Achieva-Intera). The study protocol consisted of: fluid attenuated inversion recovery (FLAIR) – acquired in the axial plane with 3mm slice thickness (TR 11000 ms, TI 2800 ms, TE 125 ms, matrix 328 x 210, gap 0, FOV 23 x 18 cm, flip angle 90°; in-plane resolution 0.7 mm x 0.85 mm); double inversion recovery (DIR) - acquired in the axial plane with 3mm slice thickness (TR 11000ms, TI 3400 ms, TE 50ms, delay 325 ms, matrix 328 x 210, gap 3, FOV 23 x 18 cm, flip angle 90°; in-plane resolution 0.7 mm x 0.85 mm) and a volumetric (three-dimensional) T1 gradient echo images - acquired in the sagittal plane with 1 mm slice thickness (TR 7.0 ms, TE 3.2 ms, matrix 240 × 240, FOV 24 × 24 cm, flip angle 8°; in-plane resolution 1.0 mm x 1.0 mm). Additionally, for 40 patients, a T1-weighted spin-echo with 3mm slice thickness scan was acquired prior to and approximately eight minutes after intravenous injection of gadolinium (0.1 mmol /kg) (TR 548 ms, TE 10ms, matrix 256 x 183, gap 0, flip angle 90°, in plane-resolution 0.45 mm x 0.45

mm). Active lesions (Gad+) were considered as gadolinium-enhancing lesions.

Image Analysis

Brain white-matter lesions

Brain white-matter lesion load (WML) was quantified on FLAIR sequences, blinded to clinical data, using the freely available Medical Image Processing, Analysis, and Visualization (MIPAV) software package developed at the Center for Information Technology, National Institutes of Health (16). The intrarater reliability between WML quantification was assessed using intra-class correlation (ICC = 0.96).

Brain cortical lesions

Brain cortical lesions were identified and scored on DIR sequences, blinded to clinical data, in accordance to consensus recommendation, (17) and accurately controlled for artifacts. Lesion volume was quantified using the MIPAV software (Figure). The intrarater reliability between lesion quantification was assessed using intra-class correlation (ICC = 0.85).

Brain cortical/subcortical grey-matter and CC volumes

All volumes evaluation were performed on volumetric T1 gradient echo images by means of the FreeSurfer v5.1 image analysis suite, available online (http://surfer.nmr.mgh.harvard.edu/), as described elsewhere (18-20). All images were thoroughly controlled for errors and artifacts. We selected brain cortical grey-matter volume, caudate, putamem, thalamus, amygdala, hipoccampus and CC subareas (posterior, mid-posterior, central, mid-anterior and anterior) volumes as variables of interest (left + right hemispheres volumes). Caudate and putamen volumes were summed up to form the striatum volume. Similarly, amygdala and

hippocampus volumes were summed up to form the medial temporal lobe structures (MTL) volume. The CC volume was the sum of all CC subareas.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, Version 20.0., SPSS Inc, Chicago, Illinois). Group comparisons on clinical and cognitive tests were performed with Mann-Whitney *U* tests, except for percentage distribution, where Fisher's exact test was used. Comparisons on all brain volumes were analyzed by the General Linear Model univariate analyses of variance procedure, with gender and total intracranial volume as covariates.

Spearman correlation analyses were performed to test the associations between disease duration, age, FSS and ZDS and clinical/cognitive outcomes (EDSS, 9HPT, T25FW, PASAT, SDMT, and BRB-N deficits) as well as between MRI metrics and the same clinical variables and outcomes. Correlation analyses were also employed to test the association between MS lesions (cortical and WML) and grey-matter and CC volume.

A stepwise multivariate linear regression analysis was performed to assess the relative contribution of MRI metrics (CC, cortical lesions, cortical grey-matter volume, MTL, striatum, thalamus and WML volumes and also presence of gadolinium-enhancing lesions) to clinical and cognitive outcomes (EDSS, 9HPT, T25FW, SDMT, PASAT and total number of deficits in BRB-N). Given a non normal distribution of cortical lesions and WML volumes, these two variables were dichotomized to low/high burden of lesions (equal or higher than 1cm3 and 3cm3, respectively). A stepwise multivariate logistic regression analysis was also performed to assess which MRI factor better predicted cognitive impairment and Hosmer-Lemeshow test was used to assess how well the model accounts for outcome. Backward stepwise analyses were conducted (Wald statistic) with a p value for entry of 0.05 and a p value for removal of 0.1. The level of significance was $p < 0.05$.

RESULTS

Clinical and cognitive data

Basic clinical and cognitive information are shown in Table 1. Patients and controls were similar regarding age, gender and educational level, but the patients group performed worse on all clinical and cognitive tests (Table 1).

Table 1. Clinical and MRI data.

	RRMS patients	Controls	Between-Subjects Comparisons **
Sex: no. (%) Female Male	 33 (76.7) 10 (23.3)	 23 (76.7) 7 (23.3)	p = 0.58
Age: years	30.79 ± 6.76	29.52 ± 7.53	p = 0.29
Disease duration: years	6.42 ± 4.90	NA	NA
FSS score	3.49 ± 1.72	2.66 ± 0.89	p = 0.08
ZDS score	36.33 ± 8.51	28.10 ± 4.37	p < 0.001
EDSS score	2.15 ± 0.85	NA	NA
9HPT: seconds	22.80 ± 4.45	17.83 ± 1.49	p < 0.001
T25FW: seconds	4.78 ± 0.85	4.05 ± 0.67	p < 0.001
PASAT score	33.33 ± 13.00	43.66 ± 9.44	p = 0.001
SDMT score	50.12 ± 13.27	65.14 ± 11.37	p < 0.001
BRB-N deficits: no.	2.0 ± 2.05	0.28 ± 0.75	p < 0.001
Brain WML volume: cm3	6.05 ± 9.12	NA	NA
Brain CoL volume: cm3	0.82 ± 0.87	NA	NA
Brain cortical volume: cm3	432.05 ± 41.04	440.83 ± 35.14	F = 9.33; p < 0.001
CC volume: cm3	2.65 ± 0.66	3.16 ± 0.41	F = 4.46; p = 0.006
Thalamus volume: cm3	12.49 ± 1.62	13.76 ± 1.18	F = 9.30; p < 0.001
Striatum volume: cm3	17.37 ± 2.73	19.86 ± 1.86	F = 7.72; p < 0.001
MTL volume: cm3	11.61 ± 1.41	12.21 ± 1.22	F = 4.17; p = 0.009
Subjects with Gad+ lesions: no. (%)	6 (15%) *	NA	NA

Expressed are mean values and standard deviation.

9HPT: nine-hole peg test; BRB-N: Brief Repeatable Battery of Neuropsychological tests; CC: corpus callosum; CoL: cortical lesions; EDSS: Expanded Disability Status Scale; FSS: Fatigue Severity Scale; Gad+: Gadolinium-enhancing lesions; MTL: medial temporal lobe structures (Amygdala + Hippocampus); NA: not applicable; PASAT: *Paced Auditory Serial Addition Test; RRMS: relapsing-remitting multiple sclerosis; SDMT: Symbol Digit Modalities Test; T25FW: timed twenty-five foot walk test; WML: white-matter lesion load; ZDS:* Zung Self-Rating Depression Scale.

* Presence of gadolinium-enhancing lesions was assessed in 40 patients.

** Group comparisons were performed with Mann-Whitney *U* tests, except for gender distribution, where Fisher's exact test was used and brain volumes, where the General Linear Model was employed, with gender and total intracranial volume as covariates

Fatigue was present in 19 patients (44.2%) and depression in 12 (27.9%).

On correlation analyses disease duration was not related to outcomes and age was only related to the T25FW time ($R = 0.455$, $p = 0.002$).

The FSS score was strongly related to the EDSS score ($R = 577$, $p < 0.001$) and in a lesser degree to 9HPT and T25FW time ($R = 0.304$, $p = 0.047$; and $R = 0.413$, $p = 0.006$, respectively). The ZDS score was modestly related to the EDSS, SDMT and BRB-N deficits ($R = 0.306$, $p = 0.046$; $R = -0.320$, $p = 0.036$; and $R = 0.351$, $p = 0.021$, respectively).

Nineteen patients (44.2%) had cognitive impairment. These patients had similar age, disease duration, gender distribution, FSS and ZDS score to those without cognitive dysfunction ($p > 0.05$), but performed worse on clinical and cognitive outcomes (Table 2).

Table 2. Patients with and without cognitive impairment

	CI patients (n = 19)	Non-CI patients (n = 24)	Between-Subjects Comparisons**
Sex: no. (%) Female Male	 12 (63.2) 7 (36.8)	 21 (87.5) 3 (12.5)	p = 0.079
Age: years	29.79 ± 6.00	31.58 ± 7.32	p = 0.58
Disease duration: years	6.16 ± 5.32	6.63 ± 4.61	p = 0.65
FSS score	3.99 ± 1.72	3.10 ± 1.67	p = 0.112
ZDS score	39.16 ± 9.88	34.08 ± 6.65	p = 0.093
EDSS score	2.63 ± 0.76	1.77 ± 0.72	p = 0.001
9HPT: seconds	24.74 ± 5.35	21.27 ± 2.88	p = 0.011
T25FW: seconds	5.02 ± 0.81	4.59 ± 0.85	p = 0.087
PASAT score	25.21 ± 11.67	39.75 ± 10.23	p < 0.001
SDMT score	43.47 ± 9.71	55.37 ± 13.51	p = 0.001
BRB-N deficits: no.	3.89 ± 1.63	0.50 ± 0.51	p < 0.001
Brain WML volume: cm3	8.72 ± 12.39	3.87 ± 4.38	p = 0.077
Brain CoL volume: cm3	1.21 ± 1.05	0.54 ± 0.60	p = 0.034
Brain cortical volume: cm3	429.53 ± 40.62	434.04 ± 42.12	F = 7.2; p = 0.001
CC volume: cm3	2.33 ± 0.71	2.89 ± 0.52	F = 3.07; p = 0.039
Thalamus volume: cm3	11.98 ± 1.56	12.89 ± 1.58	F = 4.95; p = 0.005
Striatum volume: cm3	16.62 ± 2.60	17.96 ± 2.73	F = 2.96; p = 0.044
MTL volume: cm3	11.34 ± 1.44	11.81 ± 1.38	F = 3.54; p = 0.023
Subjects with Gad+ lesions: no. (%)*	5 (27.8)	1 (4.5)	p = 0.073

Expressed are mean values and standard deviation.

9HPT: nine-hole peg test; BRB-N: Brief Repeatable Battery of Neuropsychological tests; CC: corpus callosum; CoL: cortical lesions; EDSS: Expanded Disability Status Scale; FSS: Fatigue Severity Scale; Gad+: Gadolinium-enhancing lesions; MTL: medial temporal lobe structures (Amygdala + Hippocampus); NA: not applicable; PASAT: *Paced Auditory Serial Addition Test; RRMS: relapsing-remitting multiple sclerosis; SDMT: Symbol Digit Modalities Test; T25FW: timed twenty-five foot walk test; WML: white-matter lesion load; ZDS:* Zung Self-Rating Depression Scale.

* Presence of gadolinium-enhancing lesions was assessed in 40 patients.

**Group comparisons were performed with Mann-Whitney *U* tests, except for except for gender and subjects with Gad+ lesions distribution, where Fisher's exact test was used and brain volumes, where the General Linear Model was employed, with gender and total intracranial volume as covariates.

Non-cognitively impaired patients (55.8%) had similar age and gender distribution to controls ($p > 0.2$) but also performed worse on SDMT test ($p = 0.002$) and had more deficits in BRB-N ($p = 0.016$). They performed worse on 9HPT ($p < 0.001$) and T25FW ($p = 0.021$) as well.

MRI data

After correction for gender and intracranial volume, MS patients had lower volume of CC and all GM cortical and subcortical structures (Table 1).

On correlation analyses, disease duration was moderately related to WML and striatum volume ($R = 0.369$, $p = 0.019$; $R = -0.357$, $p = 0.019$, respectively). Age, FSS and ZDS score were not related to MRI variables.

Cognitively impaired patients had higher burden of cortical lesions and lower volume of CC and all grey-matter cortical and subcortical structures, after correction for gender and intracranial volume (Table 2).

When compared to controls, non-cognitively impaired patients also had

lower volumes of all grey-matter cortical and subcortical structures ($p < 0.01$, except for thalamus: $p = 0.046$), but with similar CC volume ($p = 0.23$).

High burden of MS lesions, and especially WML, were related to atrophy of all grey-matter structures and CC, except for cortical volume (supplemental table). Striatum volume was correlated with all clinical and cognitive outcomes while CC, MTL, cortical lesions and WML volume were related only to cognitive outcomes (supplemental table).

Multivariate analysis

Clinical outcomes

For the EDSS, only cortical lesions remained in the model as an independent predictor. High burden of lesions was associated with a 0.58 increase in the EDSS score ($p = 0.042$; 95% CI 0.022 – 1.138; R square = 0.110). When evaluating 9HPT as outcome, cortical and striatum volume remained as statistically significant independent predictors (Table 3). Striatum volume was also the only independent predictor for the leg function T25FW test (Table 3).

Table 3. Multivariate regression analyses.

		MRI variables	Unstandardized Beta	Standardized Beta	p	95% CI		R square
						Lower bound	Upper bound	
MSFC	T25FW	Striatum	-0.09	-0.33	0.040	-0.19	-0.01	0.112
	9HPT	Cortical volume Thalamus Striatum	0.07 -0.85 -0.85	0.64 -0.32 -0.55	0.007 0.090 0.010	0.02 -1.85 -1.49	0.12 0.14 -0.21	0.260
	PASAT	WML MTL	-16.39 2.17	-0.62 0.25	<0.001 0.064	- 23.27 -0.13	-9.50 4.48	0.627
BRB-N	overall deficits	Cortical lesions Gad+ CC	1.58 1.76 -0.89	0.39 0.33 -0.31	0.007 0.012 0.036	0.46 0.42 -1.72	2.71 3.10 -0.06	0.535

9HPT: nine-hole peg test; BRB-N: Brief Repeatable Battery of Neuropsychological tests; CC: corpus callosum; Gad+: Gadolinium-enhancing lesions; MTL: medial temporal lobe structures (Amygdala + Hippocampus); PASAT: *Paced Auditory Serial Addition Test; T25FW: timed twenty-five foot walk test; WML: white-matter lesion load.*

Cognitive outcomes

When evaluating information processing speed tests as outcome, high burden of WML was the only statistically significant predictor for the PASAT test (Table 2), while high burden of cortical lesions and presence of Gad+ lesions remained as independent predictors for the SDMT score (β = -11.922, p = 0.005, 95% CI -20.071 to -3.773 and β = -9.833, p = 0.068, 95% CI -20.435 to 0.770, respectively, R square = 0.260), but only cortical lesions was statistically significant (Figure).

On multivariate linear regression, the independent predictors for overall number of deficits in BRB-N were high burden of cortical lesions, presence of Gad+ lesions and CC volume (Table 3).

When performing multivariate logistic regression with cognitive dysfunction as outcome, CC atrophy and presence of Gad+ lesions remained in the model (odds ratio = 7.277, p = 0.019, 95% CI 1.380 – 38.374; and odds ratio = 8.431, p = 0.094, 95% CI 0.694 – 102.373, respectively; Hosmer and Lemeshow test chi-square = 5.851, p = 0.664), but only CC atrophy was statistically significant.

DISCUSSION

We found cognitive dysfunction in 44.2% of this group of early RRMS patients (mean of 6.2 years of disease duration), what is in accordance with previous studies showing cognitive dysfunction in up to two thirds of patients with MS depending on sample composition. Modern neuroimaging studies have proposed several pathological substrates underlying cognitive deficits in MS such as brain cortical lesions and atrophy, and subcortical grey matter structures, including the thalamus and hippocampal region (1, 2). Each of these structures does have a contribution to cognition in MS, but most available studies evaluated them in conjunction with few other MRI measures. We aimed to incorporate several of these structures and found that CC atrophy, high burden of cortical lesions and presence of subclinical disease activity as measured by Gad+ lesions were the best predictors of cognitive dysfunction, explaining 53.5% of the variance of deficits in BRB-N.

In-vivo visualization of cortical pathology with MRI has become a valuable mean to assess its clinical significance and several studies revealed that cortical atrophy predicts clinical and cognitive disability in MS (3, 4, 6, 8, 21-23). In our study, although cortical volume was correlated to the PASAT score, in multivariate analyses it did not remain in the model, what may be partially related to the early stage MS of our sample, with less cortical atrophy and shorter disease duration than previous studies. On the other hand, cortical lesions were more predictive of both clinical (EDSS) and cognitive dysfunction. Current pathological studies have shown that demyelinating lesions in the cortex are more common than previously appreciated and steadily progresses over time (3, 24). Accordingly, novel MRI studies have supported these findings and also suggested these lesions play a critical role in MS cognitive dysfunction (4, 5).

Subcortical grey-matter atrophy has also been proposed to influence cognitive dysfunction in MS patients, specially the thalamus (3, 9). Batista *et al*, have found that both thalamus and putamen were independent predictors of deficits in information processing speed (as measured by SDMT and PASAT tests)

in MS patients, after controlling for neocortical atrophy (8). We also included the thalamus and incorporated the striatum volume (putamen plus caudate) in our analysis, but even though these structures were related to both information processing speed and total deficits in BRB-N on correlation analyses, they did not remain in the model after multivariate statistics. On the other hand, the striatum volume was an independent predictor for clinical disability as measured by the 9HPT and T25FW tests. Another grey-matter rich structure recently shown to be associated with cognitive deficits in MS is the hippocampus (25, 26). A study by Benedict *et al*, for example, has revealed that atrophy of mesial temporal lobe structures (amygdala plus hippocampus) is strongly related to recognition memory tests (26). We have also included amygdala plus hippocampus in our analysis, and indeed, we observed a tendency to predict information processing speed deficits as measured by the PASAT ($p = 0.064$). However, when other MRI metrics were included, BRB-N overall deficits were better predicted by these other variables.

Besides grey-matter degeneration, other features of MS pathology contribute to cognitive dysfunction. Pathological studies have long associated callosal thinning with cognitive dysfunction in MS patients (27), a finding that was later confirmed by MRI. A study by Barkhof *et al* more than a decade ago showed that CC atrophy was associated with cerebral disconnection (28). This functional impairment of interhemispheric transfer was also shown to progress over time and in relation to continuous callosal atrophy (10). More recently, Llufriu *et al*, using a multimodal approach, also supported these findings, suggesting that CC atrophy contributes do cognitive dysfunction likely through a disconnection mechanism (12). We did not assess interhemispheric transfer time in our study but we found that CC atrophy was one of the best predictors of overall cognitive deficits.

If on the one hand the contribution of callosal atrophy to cognitive impairment has long been appreciated, on the other hand the influence of subclinical disease activity has been scarcely studied so far. There are few previous studies reporting a transient worsening of cognitive function during acute clinical relapses (29) and interestingly, Bellmann-Strobl *et al* have recently shown that presence of active lesions in MRI alone is related to poor information

processing speed performance (13). Based on this data, we also included Gad+ lesions in the multivariate analyses and indeed they remained as one of the best predictors. Subclinical disease activity on MRI likely reflects a diffuse inflammation process with negative impact on cognition and thus should ideally be monitored and controlled for in MS cognitive studies (13).

Despite aiming to incorporate important and novel MRI metrics in our study, we could not evaluate other significant measures such as magnetization transfer ratio and diffusion tensor imaging. These techniques have also been found to correlate with overall cognitive performance when analyzing changes in whole brain measures, cortical regions and CC (2, 7, 12, 23, 30). We have enrolled a limited number of subjects, and the inclusion of more variables would have a negative impact on effect size. Another limitation is related to the MRI detection of cortical lesions. Although DIR may improve detection of these lesions up to five times when compared with a conventional T2-weighted sequence, the vast majority are still missed, especially subpial lesions which may also have important contribution to clinical and cognitive dysfunction in MS patients (3). Novel sequences and greater MRI field strengths may overcome this gap. Additionally, our analyses were performed on a single time point and future longitudinal assessments can yield more information about predictors in the long-run.

In conclusion, clinical and cognitive dysfunction is frequent early in MS patients and significantly related to both grey-matter and callosal pathology. High burden of cortical lesions, callosal atrophy and presence of asymptomatic gadolinium-enhancing lesions were the best predictors of cognitive disability and they can be monitored in-vivo with MRI. Cognitive studies in MS should look over subclinical disease activity as a potential contributor to cognitive impairment.

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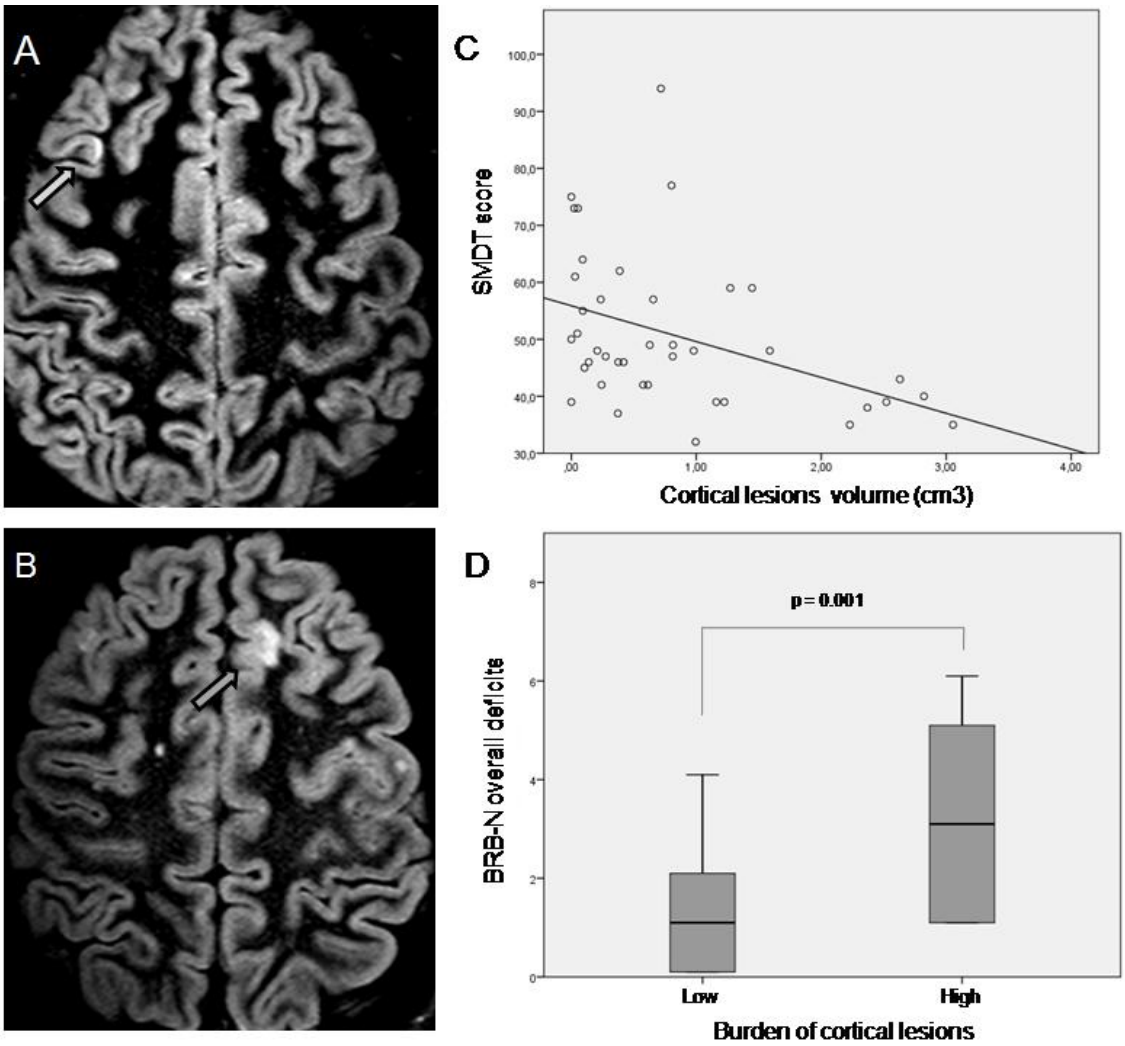
Figure. Cortical lesions and clinical/cognitive disability.

DIR visible cortical lesions can be either intracortical (A) or a mixed white matter-grey matter (B) (arrows). Cortical lesion volume was related to the SDMT test score (C) and patients with high burden of these lesions had more deficits on BRB-N tests (D).



Supplemental Table. Correlations between MS lesions and clinical/cognitive outcomes and grey-matter and CC volumes.

		Cortical volume	Thalamus volume	Striatum volume	MTL volume	CC volume	Cortical lesions	WML
Cortical lesions	Coefficient	-0.286	-0.342	-0.388	-0.452	-0.430	1.000	0.587
	Significance	0.074	0.031	0.013	0.003	0.006	.	< 0.001
WML	Coefficient	-0.234	-0.365	-0.657	-0.439	-0.706	0.587	1.000
	Significance	0.146	0.021	<0.001	0.005	<0.001	< 0.001	.
EDSS	Coefficient	-0.135	-0.137	-0.352	-0.194	-0.111	0.282	0.249
	Significance	0.388	0.382	0.020	0.212	0.477	0.078	0.121
T25FW	Coefficient	-0.293	-0.113	-0.328	-0.251	0.036	0.093	0.064
	Significance	0.057	0.472	0.032	0.105	0.820	0.568	0.695
9HPT	Coefficient	-0.023	-0.059	-0.367	-0.196	-0.279	0.242	0.231
	Significance	0.883	0.707	0.015	0.208	0.070	0.133	0.151
PASAT	Coefficient	0.418	0.468	0.630	0.623	0.470	-0.596	-0.667
	Significance	0.005	0.002	< 0.001	< 0.001	0.001	< 0.001	< 0.001
SDMT	Coefficient	0.109	0.214	0.375	0.369	0.445	-0.450	-0.373
	Significance	0.486	0.169	0.013	0.015	0.003	0.004	0.018
BRB-N deficits	Coefficient	-0.172	-0.351	-0.429	-0.334	-0.508	0.490	0.420
	Significance	0.270	0.021	0.004	0.028	0.001	0.001	0.007

9HPT: nine-hole peg test; CC: corpus callosum; EDSS: Expanded Disability Status Scale; MTL: medial temporal lobe structures (amygdala + hippocampus); PASAT: *Paced Auditory Serial Addition Test; SDMT: Symbol Digit Modalities Test; T25FW: timed twenty-five foot walk test; WML: white-matter lesion load.*

Capítulo 4

Artigo em submissão

SUBCLINICAL MRI DISEASE ACTIVITY IN MULTIPLE SCLEROSIS IS RELATED TO INCREASED PRODUCTION OF PROINFLAMMATORY CYTOKINES.

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ABSTRACT

Introduction: One important focus in multiple sclerosis (MS) research has been to develop reliable markers for disease activity, severity and efficacy of treatment, especially as therapeutic options for MS broaden. Nevertheless, no candidate biomarker has the validated reliability necessary for widespread clinical use so far.

Objective: We aimed to investigate the associations between cytokine production in stimulated peripheral blood mononuclear cells (PBMC), serum brain-derived neurotrophic factor (BDNF), and MRI disease activity in a sample of relapsing-remitting MS (RRMS) patients during remission phase.

Methods: We enrolled 15 IFNβ treated patients with RRMS and eight healthy subjects. MRI scans were acquired on a 3T scanner, with FLAIR sequences and T1-weighted spin-echo scan prior to and after intravenous injection of gadolinium. Brain white-matter lesion load (WML) and active lesions were quantified blinded to clinical data. Serum concentration of BDNF was determined using ELISA. Peripheral blood mononuclear cells (PBMC) were obtained and cultured. Cell free supernatants were then collected and evaluated for cytokine levels by ELISA (IL-10, IL-6, TNF-α and IFN-γ).

Results: The IFN-γ ($p = 0.019$), TNF-α ($p = 0.023$), and IL-10 ($p = 0.023$) production was higher in patients compared to controls. Cytokine production was neither correlated to the number of previous relapses nor to EDSS scores. Gadolinium enhancing lesions were observed in 13.3% of the patients. These patients had higher production of IFN-γ ($p = 0.019$), TNF-α ($p = 0.019$) and IL-6 ($p = 0.038$) when compared to patients without active lesions. IL-10 production was similar in both groups. The volume of enhancing lesions significantly correlated with production levels of IFN-γ, TNF-α and IL-6, but WML was not related to cytokines production. Serum concentration of BDNF was similar in patients and controls and neither related to clinical parameters nor to MRI lesions.

Conclusions: Although further studies are necessary for understanding the immunopathogenesis in subclinical MS, determination of both pro- and antiinflammatory cytokine secretion in PBMC suggest that a subclinical activity could be monitored to assist evaluating treatment response to different drugs.

INTRODUCTION

In the last years, one important focus in multiple sclerosis (MS) research has been to develop reliable markers for disease activity, disease severity and efficacy of treatment, especially as therapeutic options for MS broaden. These biomarkers would assist in several aspects such as monitoring response to treatments and detecting subclinical disease activity (1). Although hitherto the mainstay tool for assessing disease activity is magnetic resonance imaging (MRI) with gadolinium enhancement, it is expensive and may also under represent the overall extent of blood-brain barrier disruption. Novel biological markers could be easier to obtain and to measure in MS patients. Nevertheless, numerous candidate biomarkers in serum and cerebrospinal fluid have been described but none so far have the validated reliability necessary for widespread clinical use (1). MS is characterized by a complex system of interactions between proinflammatory and antiinflammatory cytokines in its course (2). Active MS lesions are characterized by T-cell and macrophage infiltration and the presence of immune mediators, including adhesion molecules, chemokines, and cytokines. However, serum evaluation of these inflammatory parameters have so far yielded few or no correlation with MRI markers of disease activity (3,4), and direct assessment of cytokine production in stimulated blood lymphocytes may produce better results (5-7). Moreover, several studies report correlations during clinical/relapse activity but few attempted to investigate biomarkers of subclinical disease activity (1, 6, 8).

In this setting, the present study investigated associations between cytokine production in stimulated peripheral blood mononuclear cells (PBMC), serum brain-derived neurotrophic factor (BDNF), and MRI disease activity in a sample of relapsing-remitting MS (RRMS) patients during remission phase.

METHODS

Subjects

We prospectively enrolled 15 patients with a RRMS diagnosis according to revised 2005 McDonald criteria (9), and eight age- and gender-matched healthy subjects for comparison as a control group. Neurological examination included assessment of overall disability with the Expanded Disability Status Scale (EDSS) for all patients. All individuals were evaluated at the MS Center of UNICAMP University Hospital, Department of Neurology, University of Campinas. All patients were clinically stable and on treatment with interferon beta (IFNβ). Exclusion criteria were: progressive course, fulfillment of diagnostic criteria for neuromyelitis optica, EDSS >5.0, or any relapse or steroid therapy within three months preceding the laboratory and MRI evaluation. The study was approved by the ethics committee of the faculty of medical sciences of University of Campinas and all patients provided written informed consent.

Laboratory analysis

Heparinized blood and serum samples were obtained from all patients and controls. Serum samples were stored at -80°C until use. Blood samples were analyzed in the Neuroimmunology Unit, Department of Genetics, Evolution and Bioagents, University of Campinas (UNICAMP). Serum concentration of BDNF was determined in duplicate using commercially available ELISA kits according to the protocol provided by the manufacturer (Abcam, USA). Peripheral blood mononuclear cells (PBMC) were obtained by separation on Ficoll-Hypaque gradients. One million PBMC per ml of complete RPMI media were cultured for 40 hours in 24Well plates in the presence of phytohaemagglutinin (10µg/mL) (6,7). Cell free supernatants were then collected and evaluated for cytokine levels by ELISA. Human ELISA Sets (BD Biosciences) for IL-10, IL-6, TNF-α and IFN-γ were used according to the protocol provided by the manufacturer. For cytokines

analyses, samples were diluted with assay diluent (1:5 for IL-10, TNF-α and IFN-γ, and 1:25 for IL-6).

Magnetic resonance imaging

MRI scans for all subjects were acquired on a 3T scanner (Phillips Achieva-Intera). Brain white-matter lesion load (WML) was quantified on fluid attenuated inversion recovery sequences, blinded to clinical data, using the freely available MIPAV software package developed at the National Institutes of Health (10). A T1-weighted spin-echo scan was acquired prior to and approximately 10 minutes after intravenous injection of gadolinium (0.1 mmol /kg). Active lesions were defined and outlined on T1-contrast enhanced scans. Gadolinium enhanced lesion volume was also quantified using MIPAV software, blinded to clinical data.

Statistical analysis

Data were entered into STATISTICA software (StatSoft, Inc., Tulsa, OC). Basic demographic, laboratorial and neuroimaging profile of patients were represented by descriptive statistics. Comparison of means was performed with Mann-Whitney *U* tests. All correlations were made with Spearman coefficient. The level of significance was established as $p<0.05$.

RESULTS

Clinical data

We enrolled 10 female and 5 male patients, and 8 age- and gender-matched controls (Table).

Table. Clinical data

	RRMS patients	Controls
Sex: no. (%)		
Female	10 (66.7)	5 (62.5)
Male	5 (33.3)	3 (37.5)
Age: years	30.07 ± 6.26	29.71 ± 8.17
Disease duration: years	5.57 ± 5.42	NA
EDSS score	1.9 ± 1.0	NA
Relapses in previous 12 months	1.0 ± 0.5	NA
Relapses in previous 24 months	1.9 ± 1.4	NA

Expressed are mean values and standard deviation.

EDSS: Expanded Disability Status Scale; NA: not applicable; RRMS: relapsing-remitting multiple sclerosis.

Cytokines

The IFN-γ (mean 1967 vs. 1012 pg/ml, p = 0.019), TNF-α (mean 440 vs. 181 pg/ml, p = 0.023), and IL-10 (mean 2299 vs. 312 pg/ml, p = 0.023) production in stimulated PBMC was higher in patients compared to controls but production of IL-6 was similar in both groups (mean 7726 vs. 6450 pg/ml, p = 0.68) (Figure 1). Cytokine production was neither correlated to the number of relapses in previous 12 or 24 months (P > 0.1) nor to EDSS scores (p > 0.5).

Gadolinium enhancing lesions were observed in 13.3% of the patients. These patients had significantly higher production of IFN-γ (mean 9256 vs. 845 pg/ml, p = 0.019), TNF-α (mean 2569 vs. 112 pg/ml, p = 0.019) and IL-6 (mean 15278 vs. 6564 pg/ml, p = 0.038) when compared to patients without active lesions (Figure 2). IL-10 production was similar in both groups (mean 1485 vs. 2423 pg/ml, p = 0.93). Furthermore, the volume of enhancing lesions significantly correlated with production levels of IFN-γ (R = 0.58, p = 0.021), TNF-α (R = 0.59, p = 0.020), and IL-6 (R = 0.54, p = 0.038).

WNL was not related to cytokines production in stimulated PBMC (p > 0.4).

BDNF

Serum concentration of BDNF was similar in patients and controls (p > 0.8). BDNF levels were neither related to clinical parameters (number of relapses in previous 12 and 24 months or EDSS score) nor to MRI lesions (presence/volume of active lesions or WML) (p > 0.05).

BDNF levels were not related to cytokine production levels in stimulated PBMC (p> 0.25).

DISCUSSION

The present study investigated associations between cytokines production, serum BDNF, and MRI in patients with relapsing-remitting MS treated with IFN-β. MRI disease activity during remission phase in RRMS patients is variable in frequency but seemingly related with both short- and medium-term prognosis. Therefore, assessment of ongoing subclinical disease activity may predict the risk of clinical relapse and/or disease course and assist in evaluating response to different treatment options (1).

There is a considerable number of studies investigating potential

biomarkers candidates in MS such as chemokines, immune cell subsets, co-stimulatory molecules, and cytokines (1, 6, 7, 11-13). Regarding disease activity, some previous studies reported association between Th1 proinflammatory cytokines and clinical relapses (1, 6, 7, 11), but few evaluated subclinical MRI activity. Here, we found that patients treated with IFN-β presented a subclinical disease activity. This group of patients was separated in those that presented or not active lesions on MRI analysis. MS patients as a group presented a significant increase in the production of proinflammatory cytokines such as IFN-γ, TNF-α and IL-10, which is in agreement with the observations that the inflammatory response is always present in MS. Patients with subclinical active MRI lesions had a further significantly increased production of proinflammatory cytokines such as IFN-γ (10-fold greater), TNF-α (22-fold greater) and IL-6 (2-fold greater). Indeed, TNF-α have been shown to induce specific changes in the morphology and permeability of CNS-derived endothelial cells (14), likely related to the blood-brain barrier disruption as seen by gadolinium enhanced MRI. Moreover, a longitudinal study demonstrated an increase of both IFN-γ and TNF-α production before clinical exacerbations, preceding clinical symptoms by up to 2 weeks (11). The production of IL-6 is also significantly increased in MS patients with MRI active lesions. IL-6 is a pleiotropic cytokine that is an important mediator of many inflammatory processes, including the increase of the level of this cytokine might enhance susceptibility to MS (15, 16). Our data is in agreement with previous studies that demonstrated increased levels of IL-6 in mononuclear cells in the blood and cerebrospinal fluid (CSF) (17, 18) and in brain tissue of patients with MS (17). Furthermore, studies from human MS patients suggest that IL-6 levels may correlate with disease severity (18).

It is noteworthy that MS patients as a group presented a significantly increase in the IL-10 production, whereas patients with active lesions did not. A protective role of IL-10 has been observed in the last decade in multiple sclerosis (19). This regulatory cytokine plays an important role in maintaining the antiinflammatory environment within the CNS (20). We previously demonstrated a significant decrease of IL-10 in patients with untreated and active MS (6).

Moreover, a significant increase of IL-10 production by the PBMC of MS patients was observed during the treatment with IFN-β (7), which may explain, at least in part, the beneficial effect of this treatment. Although the production of proinflammatory cytokines is increased in the treated patients, probably the increased levels of IL-10 are contributing to control the inflammatory process within CNS. All these findings suggest the importance of the production of cytokines with pro and antiinflammatory activity in controlling the inflammatory process in patients with subclinical MS. Regarding the association of these mediators with the MRI, our data partially agree with previous report that demonstrated association between low IL-10 production and higher WML in secondary progressive MS patients (5). We studied patients with a relapsing-remitting form of MS. Moreover, in our study, cytokine production levels were not related to the number of previous clinical relapses occurring in more than three months, suggesting that circulating immune cells secretion profile likely reflects immunological changes within a shorter time frame.

Serum levels of BDNF have also yielded conflicting results in MS (21, 22, 23). Some studies have shown similar BDNF levels in the serum of healthy controls, interferon beta treated and untreated MS patients (21). In agreement with them, we also found no difference between patients and controls and serum BDNF was neither correlated to clinical parameters (number of previous relapses or EDSS score) nor to MRI lesions (active or chronic). However, we did not assess platelet release of BDNF, what may potentially confound our results. Moreover, assessment of BDNF production by immune cells in MS may also provide more consistent results. Accordingly, some studies have revealed that BDNF production by PBMC is higher during relapse and significantly associated with contrast-enhanced lesion volumes (23).

Although we included T1 weighted gadolinium enhanced imaging in a 3T scanner to define active lesions, there is no consensus of what should be the “gold standard” to detect subclinical disease activity, and higher field strengths or newer contrast agents may detect a greater number and volume of active lesions (1, 24). Moreover, considering the exploratory nature of our study, we evaluated a

small number of individuals and relatively few cytokines, and indentifying an accurate and reproducible biomarker warrants further studies with larger number of patients and other candidate biomarkers.

In conclusion, although further studies are necessary for understanding the immunopathogenesis in subclinical MS, the determination of both proinflammatory and antiinflammatory cytokines secretion in PBMC of patients with RRMS suggest that a subclinical activity could possibly be monitored to assist evaluating treatment response to different drug options.

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Figure 1. Box-plots of cytokine production levels in RRMS patients and controls. Levels of IFN-γ (A), TNF-α (B), and IL-10 (C) where significantly higher in patients than controls ($p < 0.025$). IL-6 production (D) was similar in both groups ($p = 0.68$).

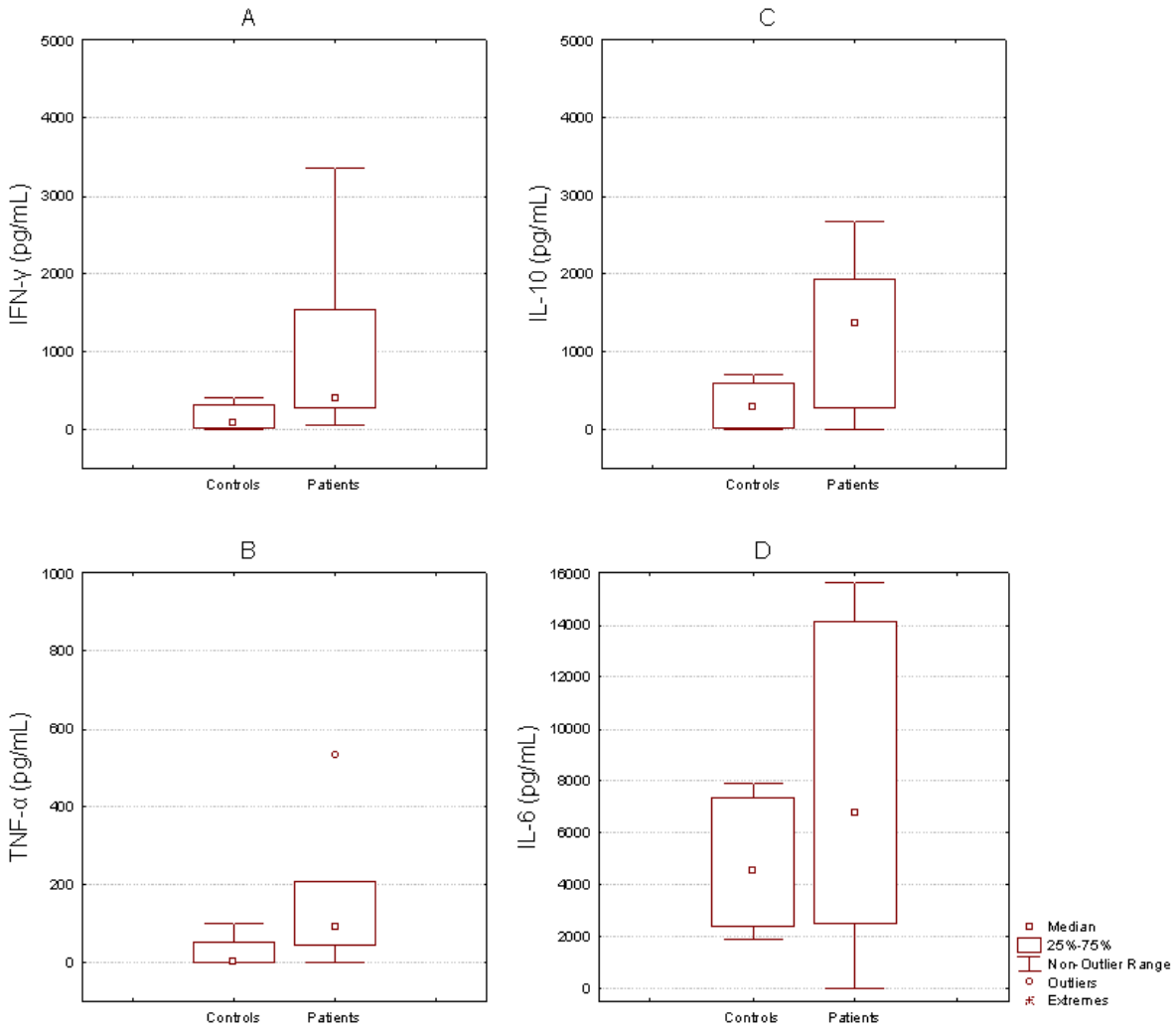
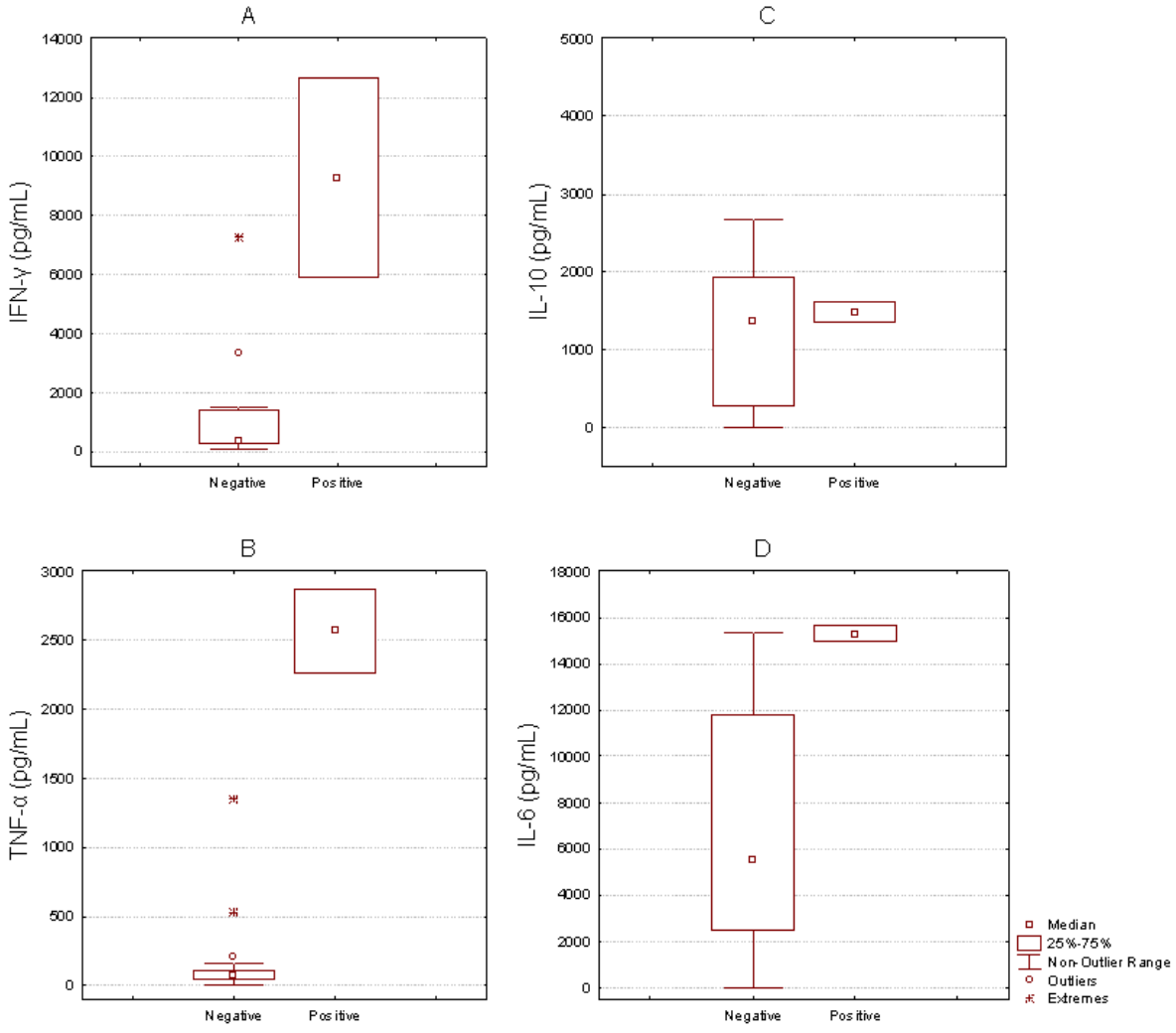


Figure 2. Box-plots of cytokine production levels in RRMS with (positive) and without (negative) gadolinium enhancing lesions on MRI. In patients with active lesions, mean levels of IFN-γ(A) were 10 times higher; TNF-α (B) 22 times higher; and IL-6 (C) 2 times higher than patients without gadolinium enhancing lesions. IL-10 (D) production was similar in both groups.



Capítulo 5

Artigo em submissão

A LONGITUDINAL EVALUATION OF MRI PREDICTORS OF COGNITIVE AND CLINICAL IMPAIRMENT IN MULTIPLE SCLEROSIS

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ABSTRACT

Introduction: Pathological and neuroimaging studies suggest that grey-matter (GM) pathology is better related to disability measures in multiple sclerosis (MS). However, longitudinal studies are necessary to determine how early stage MRI parameters predict clinical and cognitive impairment (CI) over time.

Objective: To perform a longitudinal evaluation of MRI metrics to assess their relative contributions to CI in a group of relapsing-remitting MS (RRMS) patients.

Methods: We enrolled 43 patients with RRMS and 30 healthy subjects. From the original cohort, 41 patients performed a second and 40 a third evaluation, after 12 and 24 months respectively. Outcome variables included the MSFC score and subtests (9HPT, T25FW, and PASAT), SDMT score and total number of deficits in the Brief Repeatable Battery of Neuropsychological Tests (BRB-N). All individuals underwent MRI in a 3T scanner, including FLAIR, DIR and a volumetric T1 images on each evaluation. White-matter lesion load (WML) and cortical lesions were quantified on FLAIR and DIR sequences, respectively. GM volume evaluation was performed on T1 images by means of the FreeSurfer. Multivariate regression analysis was performed to assess the relative contribution of MRI metrics to clinical and cognitive outcomes.

Results: Cognitive deterioration in the 24-month period was found in 52.3% of the patients. Over the 24-month interval, patients showed a 2.57% decrease in cortical GM volume, higher than the controls ($p = 0.036$). Subcortical GM volume also showed a higher atrophy rate in the patients group (3.80% vs. 1.29%, $p = 0.003$). There was an increase of at least 0.5cm3 in cortical lesions in 34.1% of the patients. Patients with any gadolinium-enhancing lesion during the 24-month follow-up had worse progression of T25FW z-score ($p = 0.029$) and also a tendency to worse progression of MSFC z-score ($p = 0.073$). At multivariate linear analysis, high burden of cortical lesions at baseline was associated with a 1.88 decrease in MSFC z-score over two years ($p = 0.002$). Logistic regression also showed that absence of thalamic atrophy in baseline was associated with a decreased risk of having cognitive impairment and/or decline at 2 years ($p = 0.012$). Decline of MSFC z-score was also associated with high increase of both

cortical lesions and WML ($p = 0.017$ and $p = 0.043$).

Conclusions: GM damage is extensively present in early RRMS patients and shows progression over time. Cognitive and clinical deterioration over time were significantly associated with thalamic atrophy and high burden of cortical lesions at baseline. Presence of subclinical disease activity, either reflected by new cortical lesions, new WML or Gad+ lesions were also associated with clinical and cognitive worsening.

INTRODUCTION

Multiple sclerosis (MS) has long been regarded as a predominantly demyelinating disease but pathological and neuroimaging studies suggest that focal white-matter (WM) lesions have a limited overall effect on clinical disability (1). Recently, several studies have focused on understanding the pathophysiology underlying clinical and cognitive impairment (CI) in MS and magnetic resonance imaging (MRI) has become specifically valuable to this aim (2). Grey-matter (GM) has been shown to be diffusely damaged and better related to disability measures than WM lesion load (1, 2). Atrophy measurements can be done with 3D MRI acquisitions using different types of software. Therefore, this approach has been used by several studies which documented a progressive loss of brain parenchyma, starting at the earliest stages and continuing throughout the long course of the disease, with important contributions to clinical impairment (1). Recent studies have also shown that deep GM structures such as the thalamus and putamen also have significant contributions to disability along with neocortical atrophy (3, 4). A number of candidates have been suggested as biomarkers of CI in MS but most available studies evaluated few MRI measures and/or did it on a single time point, yielding limited information on independent predictors on the long-term (2, 5). Despite major advances in neuroimmunology and neuroimaging, daily practice is still faced with uncertainty in predicting the long-term course and disability of MS at the individual level (6). Longitudinal in-vivo studies are necessary to investigate this and to determine the effect of early stage MRI parameters and their importance to predict CI over time.

Therefore, we performed a longitudinal evaluation of MRI metrics, including grey-matter volumes, both cortical and subcortical and also cortical lesions to assess the relative contributions to CI in a group of relapsing-remitting MS patients.

METHODS

Subjects

We prospectively and consecutively enrolled 43 patients with a relapsing-remitting MS diagnosis according to revised 2005 McDonald criteria (7) and 30 age-, gender-, and educational level-matched healthy subjects for comparison as a control group. All individuals were evaluated at the MS Center of UNICAMP University Hospital, Campinas, Brazil. All patients were clinically stable at the time of enrollment and on treatment with disease-modifying drugs. Exclusion criteria were: progressive course, fulfillment of diagnostic criteria for neuromyelitis optica, EDSS >5.0, any pre-existing condition known to be associated with brain atrophy or any relapse or steroid therapy within three months preceding the clinical and MRI evaluation. From the original cohort, 40 (93.0%) patients performed a second and third evaluation, after 12 and 24 months respectively. One patient was excluded from the study due to reconsideration of the MS diagnosis. Twenty-three (76.67 %) and twenty (66.67 %) healthy subjects from the original cohort were available for the second and third evaluation after 12 and 24 months, respectively. The study was approved by the ethics committee of the faculty of medical sciences of University of Campinas and all individuals provided written informed consent.

Clinical and cognitive evaluation

Neurological clinical examination included the Multiple Sclerosis Functional Composite (MSFC) for all individuals, comprising a leg-function test (timed twenty-five foot walk, T25FW), an arm-function test (nine-hole peg test, 9HPT) and a cognitive test (paced auditory serial addition test, PASAT). Additionally, all participants filled the Fatigue Severity Scale (FSS) and were considered fatigued if scored 4 or higher. They were also assessed for depressive symptoms with the Zung Self-Rating Depression Scale (ZDS) and were considered

depressed if scored 40 or higher. The neuropsychological assessment was performed in all individuals using alternate versions (A and B) of the Brief Repeatable Battery (BRB-N) (8). This battery includes: verbal memory assessment with the Selective Reminding Test (SRT, with 3 subtests: SRT-LTS, long-term storage; SRT-CLTR, consistent long-term retrieval; SRT-DR, delayed recall); visuospatial memory with the 10/36 Spatial Recall Test (SpRT, with 2 subtests: short-term and long-term recall); sustained attention and information processing speed with the Symbol Digit Modalities Test(SDMT); working memory and IPS with the PASAT 3-second version; and verbal fluency with the Word List Generation test (WLG).

Outcome variables

Outcome variables derived from clinical and cognitive evaluation. All clinical and cognitive subtests were converted to z-scores, which were obtained by subtracting the mean of the controls from the test result and then dividing by the standard deviation of the controls. Each component of the MSFC was averaged to create an overall composite score known as the MSFC score, according to published norms (9). The progression of each subtest z-score over time was also evaluated and those individuals who evolved 1SD below the mean of the controls were considered to have a decline on each subtest. Therefore, outcomes were the arm function (9HPT), leg function (T25FW), PASAT, overall MSFC and SDMT z-scores, and their progression over time. Total number of deficits in the BRB-N (deficit defined as scores below 2SD of the control group mean on each subtest) were also evaluated. Patients who had deficits in two or more BRB-N subtests were considered to have CI. Worsening in two or more BRB-N subtests was considered as cognitive decline.

Magnetic resonance imaging

MRI scans for all subjects were acquired on a 3T scanner (Phillips Achieva-Intera) at baseline and at 12 and 24 months intervals. The study protocol consisted of: fluid attenuated inversion recovery (FLAIR) – acquired in the axial plane with 3mm slice thickness (TR 11000 ms, TI 2800 ms, TE 125 ms, matrix 328 x 210, gap 0, FOV 23 x 18 cm, flip angle 90°; in-plane resolution 0.7 mm x 0.85 mm); double inversion recovery (DIR) - acquired in the axial plane with 3mm slice thickness (TR 11000ms, TI 3400 ms, TE 50ms, delay 325 ms, matrix 328 x 210, gap 3, FOV 23 x 18 cm, flip angle 90°; in-plane resolution 0.7 mm x 0.85 mm) and a volumetric (three-dimensional) T1 gradient echo images - acquired in the sagittal plane with 1 mm slice thickness (TR 7.0 ms, TE 3.2 ms, matrix 240 × 240, FOV 24 × 24 cm, flip angle 8°; in-plane resolution 1.0 mm x 1.0 mm). Additionally a T1-weighted spin-echo with 3mm slice thickness scan was acquired prior to and approximately eight minutes after intravenous injection of gadolinium (0.1 mmol /kg) (TR 548 ms, TE 10ms, matrix 256 x 183, gap 0, flip angle 90°, in plane-resolution 0.45 mm x 0.45 mm). Active lesions (Gad+) were considered as gadolinium-enhancing lesions.

Image Analysis

Brain white-matter lesions

Brain white-matter lesion load (WML) was quantified on FLAIR sequences, blinded to clinical data, using the freely available Medical Image Processing, Analysis, and Visualization (MIPAV) software package developed at the Center for Information Technology, National Institutes of Health (10). The intrarater reliability between WML quantification was assessed using intra-class correlation (ICC = 0.96). The progression of WML over time was calculated for each patient.

Brain cortical lesions

Brain cortical lesions were identified and scored on DIR sequences, blinded to clinical data, in accordance to consensus recommendation (11), and accurately controlled for artifacts. Lesion volume was quantified using the MIPAV software. The intrarater reliability between lesion quantification was assessed using intra-class correlation (ICC = 0.85). The progression of cortical lesions volume over time was calculated for each patient.

Brain cortical/subcortical grey-matter volumes

All volumes evaluation were performed on volumetric T1 gradient echo images by means of the FreeSurfer v5.1 image analysis suite, available online (http://surfer.nmr.mgh.harvard.edu/), as described elsewhere (12-14). All images were thoroughly controlled for errors and artifacts. We selected brain cortical grey-matter volume, caudate, putamem, thalamus, and total subcortical GM volume as variables of interest (left + right hemispheres volumes). Caudate and putamen volumes were summed up to form the striatum volume. Atrophy rates over time for each of these structures were calculated and those individuals with rates 1SD below the mean of the controls were considered to have high atrophy rate on each structure.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, Version 20.0., SPSS Inc, Chicago, Illinois). Group comparisons on clinical and cognitive tests were performed with Mann-Whitney *U* tests, except for percentage distribution, where Fisher's exact test was used. Within-group comparisons of test performances on different time points were carried out with the Wilcoxon test. Comparisons on all brain volumes were analyzed by the General Linear Model univariate analyses of variance procedure,

with gender and total intracranial volume as covariates.

A stepwise multivariate linear regression analysis was performed to assess the relative contribution of baseline MRI metrics (cortical lesions, cortical grey-matter volume, striatum, thalamus and WML volumes and also presence of gadolinium-enhancing lesions) in progression of clinical and cognitive outcomes (9HPT, T25FW, SDMT, PASAT and total number of BRB-N subtests with decline). Given a non normal distribution of cortical lesions and WML volumes, these variables were dichotomized to low/high burden of lesions (equal or higher than 1cm3 and 3cm3, respectively). For the same reason, increase of cortical lesions and WML volumes were dichotomized to low/high based on patients median (higher than 0.5cm3 and 1cm3, respectively). Linear regression was also employed to asses which MRI metrics progression better predicted the same clinical and cognitive progression. A stepwise multivariate logistic regression analysis was also performed to assess which baseline MRI factor better predicted presence of deficit and/or deterioration on MSFC and BRB-N at the 24-month follow-up. Hosmer-Lemeshow test was used to assess how well the model accounts for outcome. Backward stepwise analyses were conducted (Wald statistic) with a p value for entry of 0.05 and a p value for removal of 0.15. The level of significance was $p < 0.05$.

RESULTS

Clinical data

Clinical information on all time points is shown in Table.

Table. Clinical and MRI data of RRMS patients

	Baseline	1 year	2 years
Sex: no. (%)			
Female	33 (76.7)	30 (75)	31 (77.5)
Male	10 (23.3)	10 (25)	9 (22.5)
FSS score	3.49 ± 1.72	3.62 ± 1.81	3.35 ± 1.71
ZDS score	36.33 ± 8.51	34.62 ± 9.01	34.2 ± 8.56
9HPT: seconds	22.80 ± 4.45	22.07 ± 4.87	22.12 ± 5.35
T25FW: seconds	4.78 ± 0.85	4.84 ± 0.84	4.85 ± 1.09
PASAT score	33.33 ± 13.00	37.22 ± 12.74	37.32 ± 12.35
SDMT score	50.12 ± 13.27	50.02 ± 11.65	50.42 ± 11.59
BRB-N deficits: no.	2.0 ± 2.05	2.0 ± 1.97	1.75 ± 1.88
WML: cm3	6.05 ± 9.12	6.64 ± 10.66	8.00 ± 11.33
Cortical lesion volume: cm3	0.82 ± 0.87	1.12 ± 1.34	1.69 ± 2.00
Cortical GM volume: cm3	432.05 ± 41.04	424.91 ± 41.09	418.95 ± 39.98
Subcortical GM volume: cm3	163.99 ± 19.89	158.49 ± 18.68	156.15 ± 15.59
Subjects with Gad+ lesions: no. (%)*	6 (15)	9 (21.43)	9 (23.07)

Expressed are mean values and standard deviation.

9HPT: nine-hole peg test; BRB-N: Brief Repeatable Battery of Neuropsychological tests; FSS: Fatigue Severity Scale; Gad+: Gadolinium-enhancing lesions; PASAT: *Paced Auditory Serial Addition Test; RRMS: relapsing-remitting multiple sclerosis;*

SDMT: Symbol Digit Modalities Test; T25FW: timed twenty-five foot walk test; WML: white-matter lesion load; ZDS: Zung Self-Rating Depression Scale.

* Presence of gadolinium-enhancing lesions was assessed in 40, 40 and 39 patients on the first, second and third evaluation respectively.

The FSS and ZDRS scores were stable over time (p>0.1). Presence of fatigue was observed in 44.2%, 45%, and 37.5% of the patients on the first, second and third evaluation, respectively. In these evaluations, depression was observed in 27.9%, 25% and 30% of the patients.

On the patients group there was an improvement on 9HPT z-cores from the first to the second evaluation (p = 0.003), which became stable subsequently (p = 0.5). On the control group, despite a marginal improvement from the first to the second analysis (p = 0.1) a statistically significant difference was seen only between the second and the third evaluations (p = 0.003).

The T25FW z-score was stable over time in the patients group (p>0.3), but also showed an improvement between the second and third evaluation in the controls (p = 0.009).

The MSFC z-score improved initially on the patients group (p = 0.0006) but remained stable thereafter (p = 0.8), while in the controls, despite an initial worsening, it had a subsequent improvement (p = 0.002) (Figure1A).

There was no difference regarding gender on clinical progression over 2 years (p >0.3).

Cognitive performance

Cognitive information on all time points is shown in Table. The percentage of patients with CI was stable over time: 44.2%, 50% and 45% of the patients on the first, second and third evaluation, respectively. The number of failed BRB-N subtests also remained stable in each 12-month period (p>0.3). However, 23 (52.3%) patients had cognitive decline in the 24-month period.

The SDMT z-score was stable over time in the patients group (p>0.25) but had a tendency to improve between the first and the third evaluation (p = 0.056). For the controls, it was also stable in each 12-month period, but with a significant improvement between the first and the third evaluation (p = 0.013) (Figure1B). Moreover, the amount of improvement over the 24-month interval was higher in the controls when compared to the patients group (p = 0.033).

On the patients group, the PASAT z-score improved between the first and second evaluation (p = 0.001) and remained stable thereafter (p = 0.17). For the controls, the mean z-score slightly improved in each 12-month period, but this change did not reach statistical significance (p = 0.051 and p = 0.71). However, there was a significant improvement between the first and the third evaluation (p = 0.0056).

There was no difference regarding gender on cognitive progression over 2 years (p >0.15).

MRI data

Over the 24-month interval, the control group had a 0.0978% increase in cortical GM volume while it decreased 2.57% in the patients group (p = 0.036). Similarly, striatum volume had a 0.18% increase in the controls while it decreased 2.85% in the patients group (p = 0.084). Mean atrophy rate of thalamus was also slightly higher in the patients group (5.48% vs. 1.80%, p = 0.078). When considered altogether, subcortical GM volume showed a significantly higher atrophy rate in the patients group when compared to controls (3.80% vs. 1.29%, p = 0.003).

Mean increase in brain cortical lesions was 0.91 ± 1.57 cm3 in the 24-month period, while it was 1.90 ± 2.55 cm3 for WML (Figure 2), and 34.1% of the patients exhibited an increase in cortical lesions higher than 0.5 cm3, while 45.5% showed at least a 1.0 cm3 increase in WML.

Patients with any subclinical Gad+ lesion during the 24-month follow-up

had worse progression of T25FW z-score (0.57 vs. -0.21, p = 0.029) (Figure 3) and also a tendency to worse progression of MSFC z-score (0.35 vs. 1.38, p = 0.073). These patients also had a higher accumulation of WML (3.00 vs. 1.09 cm3, p = 0.015). The mean increase of cortical lesions was also slightly higher, but did not reach statistical significance (1.52 vs. 0.44 cm3, p = 0.125).

There was no difference regarding gender on MRI progression over 2 years (p > 0.18).

Multivariate analysis

Baseline MRI and clinical/cognitive progression in 24-month interval

When considering progression of T25FW z-score, both high burden of cortical lesions and presence of Gad+ lesions at baseline were associated with increasing (worse) z-score (β = 1.13, 95% CI 0.41 to 1.85, p = 0.003, and β = 0.79, 95% CI 0.02 to 1.57, p = 0.043, respectively; R square = 0.36)

Decline of 9HPT z-score was also predicted by a high burden of cortical lesions at baseline (β = -0.79, 95% CI -1.38 to -0.21, p = 0.009; R square = 0.18).

High burden of cortical lesions at baseline was associated with a 1.88 decrease in MSFC z-score over two years (p = 0.002, 95% CI -3.05 to -0.72; R square = 0.25). High WML also remained in the model but was not statistically significant (p = 0.1). Logistic regression also showed that high burden of cortical lesions was associated with a 5.14 increased risk of having MSFC z-score deficit and/or decline at the 24-month evaluation (p = 0.030; 95% CI 1.18 to 22.48; Hosmer and Lemeshow test chi-square 8.08, p = 0.32).

SDMT z-score progression was associated with initial thalamus volume (β = 0.150, 95% CI 0.02 to 0.28, p = 0.022; R square = 0.33), but no baseline MRI metric was associated with PASAT progression.

The number of BRB-N subtests with decline over the period was also

slightly associated with thalamus volume at baseline (β = -0.224, 95% CI -0.47 to 0.02, p = 0.07; R square = 0.09). Logistic regression also showed that for every 1cm3 increase in thalamus volume in baseline, the risk of having cognitive impairment and/or decline at the 24-month evaluation decreased 0.35 (p = 0.012, 95% CI 0.15 to 0.79; Hosmer and Lemeshow test chi-square 4.64, p = 0.70).

MRI progression and clinical/cognitive progression over 24-month interval

A high increase in cortical lesions was associated with a 0.93 worsening in T25FW z-score (p = 0.007, 95% CI 0.28 to 1.59; R square = 0.31). Presence of any Gad+ lesion in the period also remained in the model but was not statistically significant (p = 0.11).

High accumulation of WML and a high rate of striatum atrophy were associated with a decrease in 9HPT z-score (β = -0.63, 95% CI -1.12 to -0.04, p = 0.036, and β = -0.59, 95% CI -1.18 to -0.01, p = 0.045 respectively; R square = 0.38). A high rate of thalamic atrophy also had a tendency to predict 9HPT decrease (β = -0.50, 95% CI -1.08 to 0.08, p = 0.087).

Decline of MSFC z-score was also associated with high increase of both cortical lesions and WML (β = -1.22, 95% CI -2.21 to -0.23, p = 0.017 and β = -1.02, 95% CI -1.99 to -0.03, p = 0.043; R square = 0.30) (Figure). Logistic regression also showed that a high increase in WML was associated with a 7.81 increased risk of having MSFC z-score deficit and/or decline at the 24-month evaluation (p = 0.019; 95% CI 1.39 to 43.78; Hosmer and Lemeshow test chi-square 3.67, p = 0.59). High increase in cortical lesions and thalamus atrophy also remained in the model but were not statistically significant (p = 0.113 and p = 0.129, respectively).

No MRI metric progression was associated with SDMT or PASAT decline, but overall worsening on BRB-N subtests was associated with a high rate of striatum atrophy (β = 0.96, 95% CI 0.10 to 1.82, p = 0.029; R square = 0.18). A

high rate of cortical atrophy also remained in the model but was not statistically significant (p = 0.085).

No MRI metric progression was associated with the risk of having cognitive impairment and/or decline at the 24-month evaluation on logistic regression.

DISCUSSION

We found that GM damage is widely present in MS patients and shows progression over time. This damage had a significant influence on clinical and cognitive disability after a 24-month period. Accordingly, a number of recent studies have investigated the pathophysiology subjacent to CI in MS, and GM has been shown better related to disability measures than WM lesion load (1, 2).

In our study we could not find an increase in the percentage of CI in MS patients over two years. However, we did find that 52.3% of the patients had a decline in at least two of the BRB-N subtests. Both patients and control group showed a learning effect with repeated neuropsychological assessment, which is in accordance with previous studies (15). However, the learning effect on the patients group was less pronounced on the third evaluation, especially when compared to controls.

Pathological studies have shown that focal demyelinating lesions in the cortex are more frequent than appreciated in the past and gradually progresses over time (1, 16). Accordingly, MRI studies applying DIR sequences have supported these findings and also suggested a critical role of these lesions in MS cognitive dysfunction (17, 18). However, few studies evaluated accumulation of cortical lesions over time and their relation to both clinical and cognitive impairment (18, 19). We found that 34.1% of our RRMS patients showed an increase of at least 0.5 cm3 in cortical lesion volume, which is in accordance with previous findings (19). Moreover, high burden of cortical lesions at baseline was associated with a 5.14 increased risk for clinical disability at two years follow-up. Overall, presence of subclinical disease activity, as represented by either new cortical

lesions, new WML or Gad+ lesions was associated with worsening clinical and cognitive dysfunction.

The influence of cortical and subcortical GM atrophies on clinical and cognitive dysfunction of MS patients has also been studied recently (1, 3, 4). We found that cortical GM volume decreased at a rate of 2.57% over 24 months, significantly higher than that of age- and gender-matched controls, which is in accordance with previous studies (20). Similarly MS patients exhibited a higher atrophy rate of subcortical GM (3.8% vs. 1.3%). Moreover, we found that absence of thalamus atrophy at baseline was associated with a decreased risk of CI at follow-up. Thalamic involvement in MS has been the focus of both MRI and neuropathological investigation in the last few years. Accordingly, a study by Batista *et al* has found that both thalamus and putamen were independent predictors for information processing speed deficits in MS patients, after controlling for neocortical atrophy (3). Furthermore, serial measurement of thalamic volume has been recently recommended as a biomarker in MS clinical trials (4). Indeed, the thalamus plays a relevant role in semantic memory and other higher cognitive functions (as those evaluated by BRB-N) by a likely mechanism of thalamo-cortical interactions and synchronous co-activation of different cognitive operations and spatially widespread cortical regions by means of its 30 Hz gamma rhythm (21, 22). This role seems to be highly vulnerable to the widespread and diffuse MS brain lesions, particularly in tasks requiring speeded information processing and simultaneous mental associations.

Although we analyzed volumetric measurements of cortical atrophy, we did not assess cortical thickness measurements, also obtained by FreeSurfer software. For example, a recent study has shown a widespread cortical thinning in MS patients, which is related to cognitive impairment (23). Moreover, we were not able to evaluate other significant measures such as magnetization transfer ratio and diffusion tensor imaging, which have also been found to correlate with overall cognitive performance (2, 24-26). Given the limited number of subjects enrolled in our study, including more variables could have a negative impact on effect size. The interpretation of DIR findings also requires caution. This sequence has the

disadvantage of a low signal-to-noise ratio, which results in low agreement between observers (11). Although we used current consensus recommendation, volume quantification may not have been highly precise. Therefore, despite a 0.85 ICC, we dichotomized cortical lesions into high/low burden to mitigate smaller variations due to inaccuracy. Additionally, our analyses were performed within limited time points and future longitudinal assessments can provide more robust information in the long-term, such as an increasing percentage of cognitively impaired patients.

In conclusion, GM damage is extensively present in early RRMS patients and shows progression over time. Although a considerable percentage of patients with CI remained stable in the 24-month follow-up, a higher proportion showed cognitive and clinical deterioration over time, which is significantly associated with thalamic atrophy and high burden of cortical lesions. Presence of subclinical disease activity, as reflected in either new lesions, both cortical and in the white-matter or Gad+ lesions was also associated with clinical and cognitive worsening. All these measures should be routinely assessed in MS patients. Future longitudinal evaluations can provide more information about the long-term predictors.

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Figure 1. Longitudinal progression of clinical and cognitive scores.

(A)The MSFC z-score showed a mild decline in the control group in the first 12-month interval, but with significant subsequent improvement. In the patients group, even though there was a mild improvement in the first interval, it remained stable thereafter. (B) The overall improvement in the SDMT z-score in the 24-month interval was also statistically higher in the control group.

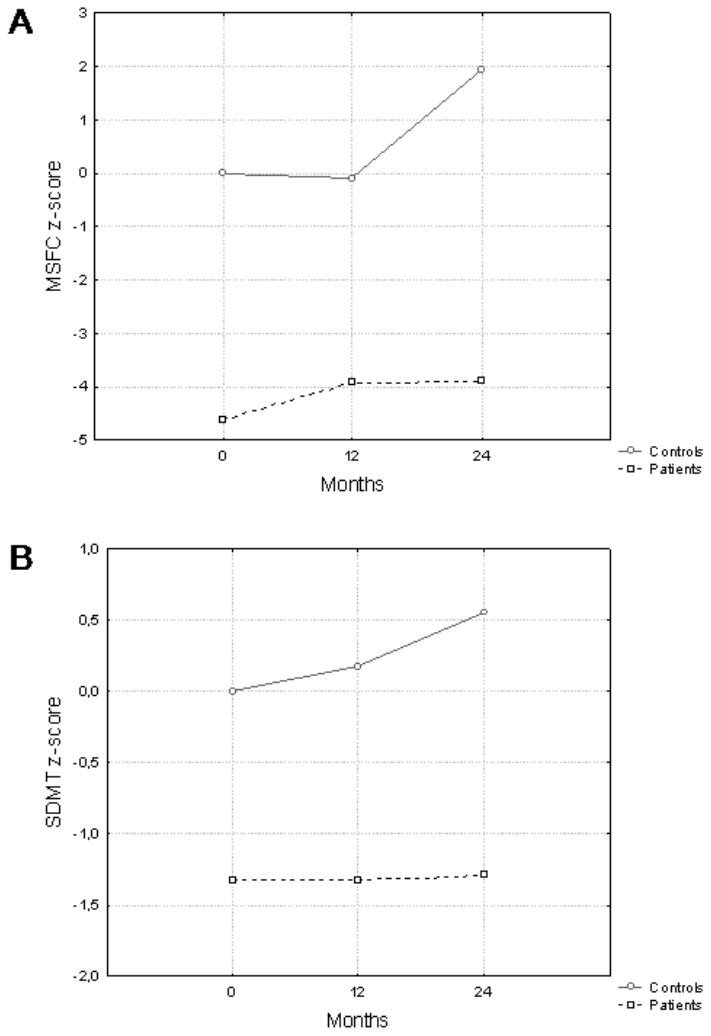
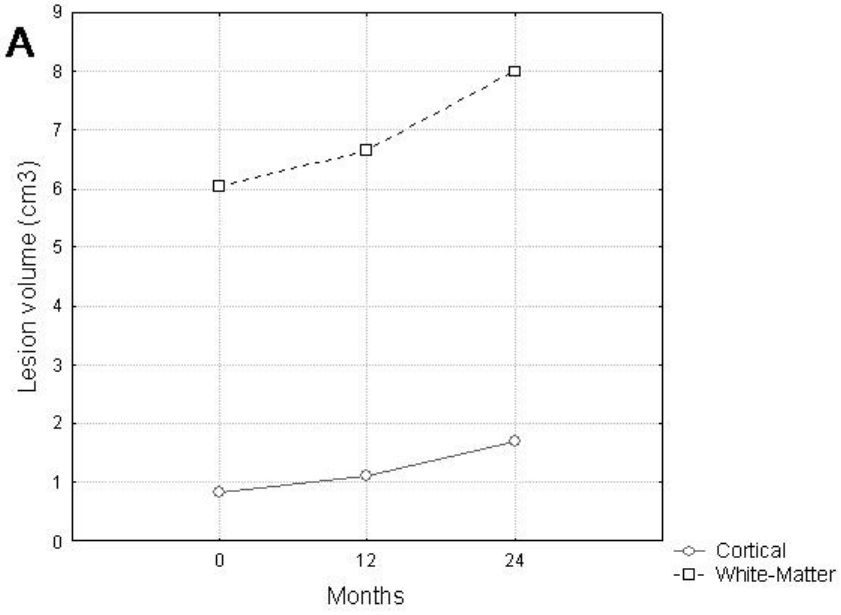


Figure 2. Lesion accumulation over time.

(A)There was a significant increase in white-matter lesions in each of the 12-month intervals ($p = 0.001$ and $p < 0.001$, respectively). There was also a noteworthy accumulation of cortical lesions over the same period ($p = 0.003$ and $p < 0.001$, respectively). (B and C) Axial double inversion recovery images obtained from a 30-years old female RRMS patient in the 12-month (B) and at the 24-month evaluation (C), showing a new lesion in the left frontal cortex (long arrow) and persistence of a parasagittal leukocortical lesion and an occipital cortical lesion (short arrows). High accumulation of cortical lesions over time was associated with clinical deterioration.



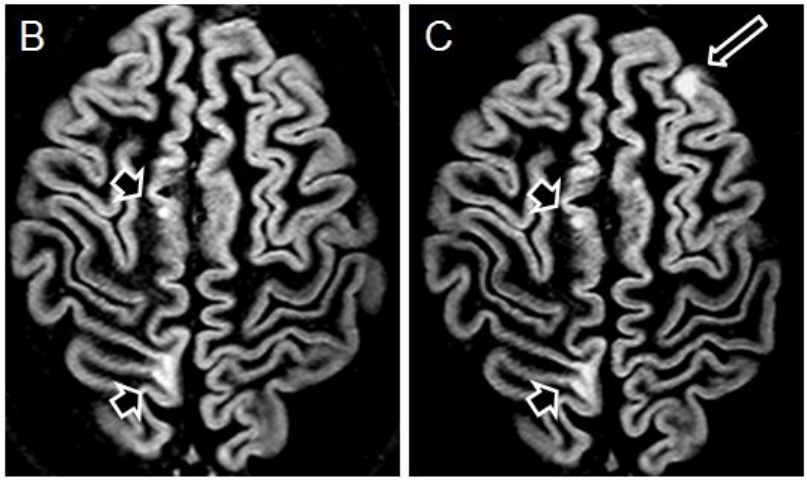
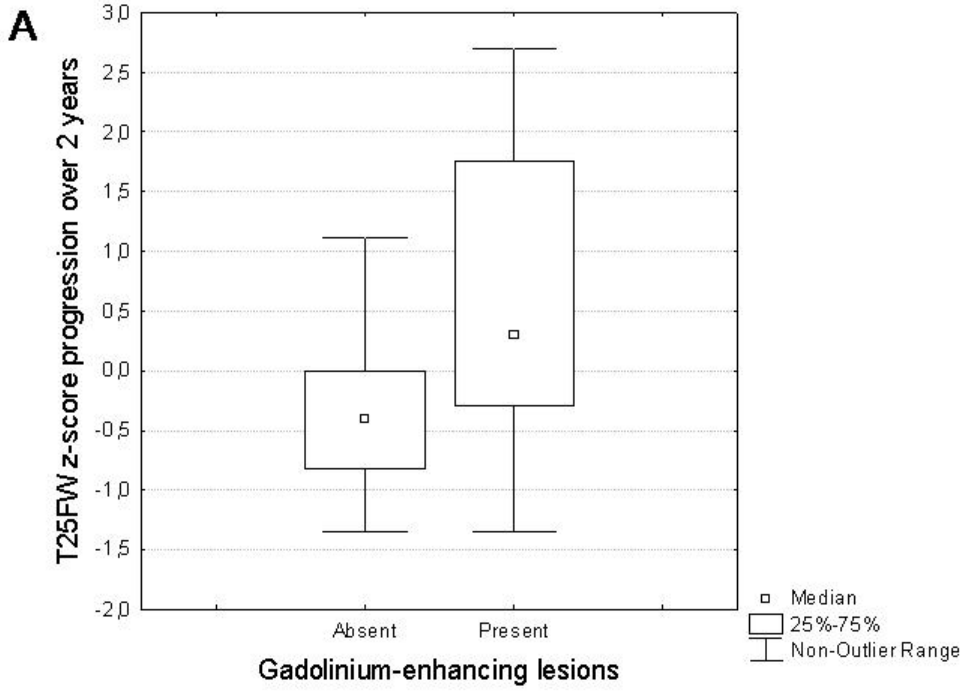
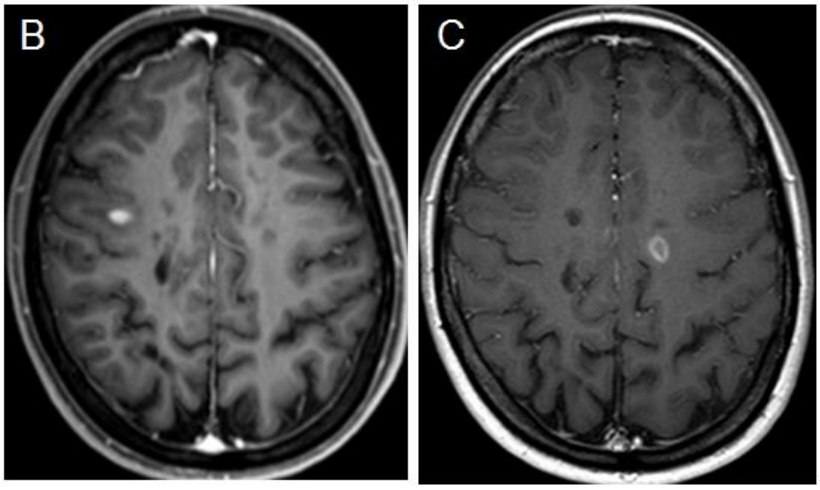


Figure 3. Subclinical disease activity.

(A)Box-whisker plots showing a higher decline in the T25FW z-score in those patients with any subclinical gadolinium-enhancing lesion over the 24-month follow-up. (B and C) Axial post-contrast T1-weighted scans obtained from a 30-years old female RRMS patient in the baseline (B) and at the 24-month evaluation (C) showing persistence of subclinical disease activity. The presence of any subclinical active lesions during the 24-month follow-up was also associated with higher accumulation of white-matter lesions.





Discussão

Conforme descrito no capítulo 1, para identificar fatores clínicos relacionados com uma pior evolução da doença em pacientes com EM, nós realizamos um levantamento dos dados de 197 pacientes acompanhados no ambulatório de EM do HC-UNICAMP, levando em conta informações clínicas e epidemiológicas e o tempo que cada paciente levou para atingir pontos específicos de incapacidade. Nós observamos que o grupo levou 25,8 anos para atingir o EDSS de 6,0. Alguns estudos de coorte já descreveram o curso natural da EM, incluindo a evolução da incapacidade ao longo do tempo (39, 43). No entanto, existe alguma variabilidade em relação a duração da doença até se atingir níveis altos de incapacidade. Por exemplo, Weinshenker *et al.* mostraram que o tempo médio entre o início da doença até a atribuição de uma pontuação de 6,0 foi de 15 anos no Canadá (43), enquanto que em Lyon, França, este tempo foi de 20,1 anos (38). Fatores genéticos e ambientais podem desempenhar um papel importante na heterogeneidade observada na evolução da EM e os estudos mencionados acima são de países da Europa ou América do Norte. Nosso estudo foi realizado na cidade de Campinas, situado na região sudeste do Brasil, onde a prevalência de EM é cerca de 15/100.000 habitantes (3). Apesar de considerar os fatores ambientais e genéticos, também é digno de nota que a nossa amostra é relativamente pequena e que incluimos pacientes em tratamento com drogas imunomoduladoras, o que poderia potencialmente ter influenciado os resultados. A influência do sexo tem sido bastante avaliada e a impressão geral é que o sexo masculino confere um risco aumentado de pior prognóstico (40). Nós também observamos que o sexo masculino foi um importante fator de risco, conferindo um risco 4,63 e 4,69 vezes maior para EDSS 6 e 7, respectivamente, em análise multivariada de Cox. Outras características da doença, como os primeiros sintomas, também têm sido alvo de estudo. Nós observamos que o envolvimento motor e de tronco cerebral/cerebelo no início estava relacionado com um risco aumentado em 8,1 e 13,1 vezes, respectivamente, para EDSS 6 na análise univariada de Cox, mas estas associações não foram encontradas nas análises

multivariadas. Uma frequência maior de surtos durante os primeiros anos de EM também tem sido associada com um maior risco de incapacidade, mas em uma revisão recente esse efeito foi considerado apenas modestamente preditivo, e o tipo de surto pode ser mais importante (40). De fato, nós observamos que uma frequência mais alta de surtos estava associada com um risco ligeiramente aumentado para EDSS 8.

Como mencionado anteriormente, a nossa amostra é relativamente pequena e provém de um hospital universitário terciário, e, portanto, aberta a viés de seleção, tendendo a um maior número de casos mais graves. No entanto, o nosso centro de EM atrai a grande maioria dos pacientes da população de Campinas e de municípios circunvizinhos (com mais de 5 milhões de habitantes), e estudos epidemiológicos prévios no sudeste do Brasil mostraram características demográficas e clínicas semelhantes (3, 44). Reavaliações futuras desta amostra podem fornecer informações mais robustas quanto ao prognóstico em longo prazo.

Posteriormente, nos capítulos seguintes, estabelecemos um subgrupo menor de 43 pacientes a fim de estudar melhor o comportamento longitudinal da patologia cerebral e sua relação com a incapacidade clínica e cognitiva na EM.

No capítulo segundo avaliamos o impacto clínico e cognitivo da patologia da substância cinzenta cerebelar neste grupo. Nós observamos que lesões corticais cerebelares são preditores independentes para disfunção dos membros superiores (teste 9HPT) e inferiores (teste T25FW), e também para a incapacidade geral, medida pelo escore EDSS (45). Por exemplo, um aumento de 1 cm3 no volume das lesões corticais cerebelares estava relacionado com um aumento de 6 segundos no teste T25FW. Calabrese *et al* mostrou dados semelhantes, mas não incluiu avaliação cognitiva (38). Nós observamos que as lesões mistas (leucocorticais) cerebelares foram preditores independentes de disfunção cognitiva, medida pelo teste PASAT. De fato, Sastre-Garriga *et al.* avaliaram a ativação cerebral com RM funcional em pacientes com EM e mostraram que a ativação cerebelar foi significativa quando se utilizou o teste PASAT como paradigma (46). Entretanto, devido a relação sinal/ruido ainda

insatisfatória da técnica DIR, algumas lesões consideradas como puramente corticais podem representar lesões leucocorticais e vice-versa. Pesquisas futuras associando RM de maior campo magnético e novas sequencias de aquisição podem superar esta insuficiência da técnica.

No capítulo 3 avaliamos, por meio de RM, a contribuição da patologia cerebral para a disfunção clínica e cognitiva neste grupo de pacientes com EMRR. Nós encontramos disfunção cognitiva em 44,2% deste grupo, o que está de acordo com a literatura que mostra esta disfunção em até dois terços dos pacientes com EM (47). Diversos fatores têm sido propostos como substrato patológico subjacente aos déficits cognitivos na EM, tais como lesões corticais cerebrais e atrofia da substância cinzenta cortical e subcortical, incluindo o tálamo e região hipocampal (47-49). Em nosso estudo, apesar de o volume cortical estar correlacionado com a pontuação no teste PASAT, nas análises multivariadas ele não permaneceu no modelo, o que pode estar parcialmente relacionada com o fato de nossa amostra ter sido de pacientes com EM inicial, com menos atrofia cortical e menor duração da doença do que nos trabalhos anteriores. Por outro lado, as lesões corticais foram mais preditivas de disfunção clínica cognitiva, concordando com os dados da literatura (38, 49).

Além da atrofia da substância cinzenta, outras características da patologia da EM parecem contribuir para a disfunção cognitiva. Há bastante tempo, estudos neuropatológicos têm mostrado que esta disfunção está também relacionada com a atrofia do corpo caloso (50). Mais recentemente, a neuroimagem ajudou a confirmar esses achados, sugerindo que a atrofia do corpo caloso contribui para a disfunção cognitiva provavelmente através de um mecanismo de desconexão cerebral (51). Nós não avaliamos o tempo de transferência inter-hemisférica, mas descobrimos que atrofia do corpo caloso foi um dos melhores preditores de déficits cognitivos em geral.

Se por um lado a contribuição da atrofia do corpo caloso no comprometimento cognitivo tem sido observada há muito tempo, por outro lado, a influência da atividade subclínica da doença tem sido pouco investigada até agora. Foong *et al.* descreveu há alguns anos uma piora transitória da função cognitiva

durante surtos clínicos agudos (52) e, interessantemente, Bellmann-Strobl *et al.* mostraram recentemente que a presença de lesões ativas na RM estava relacionada à deficits em testes de velocidade de processamento de informação (14). Com base nesses dados, nós também incluímos os dados de lesões ativas na RM nas análises multivariadas e, de fato, eles permaneceram como um dos melhores preditores. Esta atividade subclínica da EM provavelmente reflete um processo inflamatório difuso, com impacto negativo sobre a cognição e, portanto, deveria idealmente ser monitorada e controlada em estudos cognitivos nesta doença (14).

Como a presença de atividade subclínica da doença foi um indicador importante de disfunção cognitiva, aliado ao fato de ainda não existirem marcadores biológicos seguros, no capítulo 4, foi avaliada a produção de citocinas pró-inflamatórias e comparado com os dados de RM em um subgrupo de 15 pacientes. A presença de lesões ativas na EMRR durante a fase de remissão tem uma frequência variável, mas aparentemente está relacionada com o prognóstico a curto e médio prazo da doença (15). Nós observamos que a produção de citocinas como IFN-γ, TNF-α e IL-10 estava aumentada no grupo de pacientes como um todo (53). Entretanto, aqueles pacientes com lesões ativas na RM apresentaram uma produção ainda maior de citocinas pró-inflamatórias, com média de 10 vezes maior de IFN-γ e 22 vezes maior de TNF-α. A citocina TNF-α pode, de fato, induzir mudanças na morfologia e permeabilidade das células endoteliais do SNC, provavelmente relacionadas com a alteração da barreira hemato-encefálica, como vista na RM (54). Além disso, Beck *et al.* observou de maneira longitudinal que um aumento da produção de citocinas pró-inflamatórias precedia exacerbações clínicas da doença em até duas semanas (55). Embora mais estudos sejam necessários para o entendimento da imunopatogênese da fase de remissão da EM, a determinação da secreção de citocinas pró- e anti-inflamatórias pode contribuir para monitorar a atividade subclínica da doença, auxiliando na avaliação da resposta a diferentes agentes terapêuticos.

No Capítulo 5, nós analisamos de forma longitudinal a evolução dos parâmetros de RM, e principalmente da substância cinzenta cerebral, e suas

relações com a progressão clínica e cognitiva no grupo de 43 pacientes com EMRR, comparando com indivíduos sadios. Nós observamos que a substância cinzenta está difusamente afetada em pacientes com EMRR e que este dano progride consideravelmente com o tempo. Além disso, este dano tem influência na incapacidade clínica e cognitiva avaliada após um período de 24 meses. De fato, alguns estudos têm avaliado recentemente a fisiopatologia subjacente à disfunção cognitiva na EM, e a substância cinzenta parece mostrar melhor correlação com medidas de incapacidade do que as lesões de substância branca (56). Apesar de não encontrar um aumento da porcentagem de pacientes classificados como tendo disfunção cognitiva ao longo destes dois anos, nós observamos que 52,3% deles tiveram uma deterioração cognitiva, medida pela piora no desempenho de dois ou mais subtestes da bateria BRB-N. Como grupo, tantos os pacientes quanto os controles mostraram um efeito de aprendizado com a repetição destes testes, como observado previamente (57), mas este efeito foi menos pronunciado a partir do segundo ano de acompanhamento.

A influência da atrofia da substância cinzenta na incapacidade clínica e cognitiva tem sido mais investigada recentemente (56, 58). Nós verificamos que o volume cortical dos pacientes diminuiu numa taxa de 2,57% no período de dois anos, significativamente maior do que no grupo de controles. De fato, desde a avaliação inicial descrita no capítulo 3, foi observada uma atrofia significativa de diversas estruturas de substância cinzenta no cérebro, corticais e subcorticais, como o tálamo e o corpo estriado. Esta atrofia do tálamo, por exemplo, presente no início do estudo, foi um fator preditivo independente da disfunção cognitiva após dois anos de acompanhamento. Esta estrutura tem sido bastante implicada na patologia da disfunção cognitiva da EM estando, por exemplo, relacionada ao desempenho em testes de velocidade de processamento de informação (58-60).

Estudos de patologia também têm mostrado que lesões desmielinizantes focais no córtex cerebral são mais frequentes do que se achava previamente (56, 61). Entretanto, existe pouca informação em relação à progressão longitudinal destas lesões (38). Nós observamos que 34,1% de nossos pacientes tiveram um amento de pelo menos 0,5 cm3 no volume total destas

lesões corticais, de acordo com o que foi descrito recentemente por Calabrese *et al* (38). Além disso, nós observamos que uma alta carga de lesões corticais no início do acompanhamento estava associada a um aumento de 5,14 vezes no risco de incapacidade clínica após dois anos de acompanhamento. A despeito das características presentes no início do estudo, pacientes que desenvolveram um grande volume de lesões novas ao longo do acompanhamento, seja na substância branca ou no córtex, ou lesões captantes de gadolínio, também apresentaram um risco maior de incapacidade clínica após o período de 24 meses.

Embora o volume cortical tenha sido incluído em nossa análise, nós não avaliamos os dados de espessura cortical, também obtidos através do software *FreeSurfer.* Calabrese *et al.* mostrou recentemente uma redução difusa da espessura cortical em pacientes com EM, a qual está relacionada com a disfunção cognitiva dos mesmos (62). Nós também não incluímos ferramentas importantes de neuroimagem como o tensor de difusão e a taxa de transferência de magnetização (47). Dado o número limitado de pacientes incluídos no nosso estudo, acrescentar mais variáveis poderia ter uma influência negativa nos resultados. Além disso, nossas análises foram realizadas em um espaço de tempo relativamente curto, e análises futuras com um maior intervalo poderão fornecer informações mais robustas.

Conclusão

Conclusão Geral

Nossas observações permitem concluir que:

1. Pacientes do sexo masculino apresentando-se com surtos frequentes, e principalmente com envolvimento motor e de tronco cerebral, têm um risco aumentado para um pior prognóstico clínico e devem ser cuidadosamente monitorados.

2. A substância cinzenta cerebelar está difusamente afetada em pacientes com EMRR. Este dano tem uma contribuição significativa para a disfunção clínica e cognitiva observada nestes pacientes e pode ser monitorada *in-vivo* com RM.

3. A disfunção clínica e cognitiva está presente mesmo na fase inicial da EMRR, e significativamente relacionada com a patologia do corpo caloso e da substância cinzenta cerebral. Os melhores parâmetros de RM para predizer disfunção cognitiva foram uma alta carga de lesões corticais, atrofia do corpo caloso e presença de lesões subclínicas ativas na RM.

4. A presença destas lesões subclínicas ativas na RM está associada a um amento da atividade pró-inflamatória Th1, com aumento da produção de IFN-γ e TNF-α por células mononucleares do sangue periférico. Uma atividade subclínica da doença na fase de remissão poderia ser monitorada por meio destes parâmetros, auxiliando na avaliação da resposta a diferentes tratamentos.

5. A substância cinzenta cerebral está amplamente afetada em pacientes com EMRR e este dano progride consideravelmente com o tempo. Em médio prazo, uma proporção considerável de pacientes com EM mostra deterioração cognitiva, a qual está relacionada com a atrofia da substância cinzenta e com lesões desmielinizantes corticais. Presença de atividade subclínica da doença, seja por novas lesões corticais ou de substância branca, ou por lesões ativas na RM, também está relacionada com uma piora clínica e cognitiva em médio prazo.

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Anexos

Anexo 1: Parecer do Comitê de Ética em Pesquisa aprovando o trabalho.

FACULDADE DE CIÊNCIAS MÉDICAS
COMITÊ DE ÉTICA EM PESQUISA

www.fcm.unicamp.br/pesquisa/etica/index.html

CEP, 24/11/09.
(Grupo III)

PARECER CEP: N° 1057/2009 (Este n° deve ser citado nas correspondências referente a este projeto)
CAAE: 0819.0.146.000-09

I - IDENTIFICAÇÃO:

PROJETO: **"AVALIAÇÃO LONGITUDINAL DA ATROFIA CEREBRAL E ESPESSURA CORTICAL E SUA RELAÇÃO COM FATORES CLÍNICOS E IMUNOLÓGICOS EM PACIENTES COM ESCLEROSE MÚLTIPLA".**
PESQUISADOR RESPONSÁVEL: Alfredo Damasceno
INSTITUIÇÃO: Hospital das Clínicas/UNICAMP
APRESENTAÇÃO AO CEP: 10/11/2009
APRESENTAR RELATÓRIO EM: 24/11/10 (O formulário encontra-se no *site* acima)

II - OBJETIVOS

Estudar a taxa de atrofia cortical e redução da espessura cortical e suas relações com fatores clínicos e imunológicos em pacientes com EMRR.

III - SUMÁRIO

Serão estudados pacientes com diagnóstico definitivo de esclerose múltipla pelos critérios de McDonald revisados (McDonald et al, 2001; Polman et al, 2005), acompanhados no ambulatório de Esclerose Multipla do Departamento de Neurologia da Faculdade de Ciências Médicas da Universidade Estadual de Campinas (Hospital das Clínicas da UNICAMP). Serão incluídos pelo menos 20 pacientes com doença em estágio inicial (na fase de surto-remissão da doença, ou seja, EMRR), com escala de incapacidades EDSS e que estejam ou não recebendo tratamento com imunomodulador (interferon-beta ou acetato de glatiramer). Serão excluídos pacientes que apresentem a forma progressiva primária (EMPP) ou secundária (EMSP) da doença. Um grupo controle de pelo menos dez individuos normais será selecionado para comparação da variação dos níveis de melatonina e da resposta imunológica durante o ano, assim como para comparação da evolução da atrofia cerebral. A avaliação consistirá em exames neurológicos e neuropsicológicos seriados. Além disso, serão realizadas coletas de sangue venoso periférico e de urina de maneira trimestral e coleta de liquido cefalorraquidiano (LCR) por punção lombar no inicio da pesquisa, para aqueles que ainda não tiverem feito. Estes procedimentos fazem parte da rotina de atendimento dos pacientes no ambulatório. Será realizado também exame de ressonância magnética no inicio da pesquisa e após 12 e 24 meses, e costuma ser feito independentemente da pesquisa.

IV - COMENTÁRIOS DOS RELATORES

Trata-se de projeto de Doutorado a ser desenvolvido com pacientes do Ambulatório de Neurologia do HC-Unicamp, bem estruturado em seus aspectos éticos e metodológicos. Os pacientes serão submetidos a exames que fazem parte da rotina investigativa do grupo, nos dias da consulta programada não acarretando desconforto aos sujeitos da pesquisa. Será solicitado a Fapesp o

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13083-887 Campinas – SP

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cep@fcm.unicamp.br

financiamento do projeto. O Termo de Consentimento Livre e Esclarecido está bem redigido, claro, permitindo aos participantes do estudo boa compreensão dos objetivos da pesquisa.

V - PARECER DO CEP

O Comitê de Ética em Pesquisa da Faculdade de Ciências Médicas da UNICAMP, após acatar os pareceres dos membros-relatores previamente designados para o presente caso e atendendo todos os dispositivos das Resoluções 196/96 e complementares, resolve aprovar sem restrições o Protocolo de Pesquisa, o Termo do Consentimento Livre e Esclarecido, bem como todos os anexos incluídos na pesquisa supracitada.

O conteúdo e as conclusões aqui apresentados são de responsabilidade exclusiva do CEP/FCM/UNICAMP e não representam a opinião da Universidade Estadual de Campinas nem a comprometem.

VI - INFORMAÇÕES COMPLEMENTARES

O sujeito da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado (Res. CNS 196/96 – Item IV.1.f) e deve receber uma cópia do Termo de Consentimento Livre e Esclarecido, na íntegra, por ele assinado (Item IV.2.d).

Pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado e descontinuar o estudo somente após análise das razões da descontinuidade pelo CEP que o aprovou (Res. CNS Item III.1.z), exceto quando perceber risco ou dano não previsto ao sujeito participante ou quando constatar a superioridade do regime oferecido a um dos grupos de pesquisa (Item V.3.).

O CEP deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (Res. CNS Item V.4.). É papel do pesquisador assegurar medidas imediatas adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e enviar notificação ao CEP e à Agência Nacional de Vigilância Sanitária – ANVISA – junto com seu posicionamento.

Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas. Em caso de projeto do Grupo I ou II apresentados anteriormente à ANVISA, o pesquisador ou patrocinador deve enviá-las também à mesma junto com o parecer aprovatório do CEP, para serem juntadas ao protocolo inicial (Res. 251/97, Item III.2.e)

Relatórios parciais e final devem ser apresentados ao CEP, de acordo com os prazos estabelecidos na Resolução CNS-MS 196/96.

VII – DATA DA REUNIÃO

Homologado na XI Reunião Ordinária do CEP/FCM, em 24 de novembro de 2009.

Profa. Dra. Carmen Silvia Bertuzzo
VICE-PRESIDENTE do COMITÊ DE ÉTICA EM PESQUISA
FCM / UNICAMP

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Anexo 2- Termo de Consentimento Livre e Esclarecido (TCLE), conforme resolução 196/96.

Termo de Consentimento Livre e Esclarecido

Título do Projeto:

"Avaliação longitudinal da atrofia cerebral e espessura cortical e sua relação com fatores clínicos e imunológicos em pacientes com esclerose múltipla"

Pesquisador Responsável: Dr Alfredo Damasceno
Orientador Responsável: Professor Dr Fernando Cendes
Co-Orientador Responsável: Professora Dra Leonilda Santos

Nome do (a) Paciente	
Prontuário HC-UNICAMP	
RG do (a) Paciente	
Endereço	

Eu entendo que fui convidado(a) a participar de um estudo para avaliar pacientes com Esclerose Múltipla.

O objetivo geral do estudo é avaliar a atrofia do cérebro e a relação do grau de atrofia com fatores clínicos do paciente (por exemplo, o numero de surtos, déficit de memória, incapacidade funcional, etc), com fatores hormonais (dosagens da substância melatonina no sangue) e com fatores imunológicos (exame das células de defesa presentes no sangue e no líquido cérebro-espinhal). Todas essas avaliações vão nos ajudar a conhecer melhor a doença em sua fase inicial.

Se eu concordar em participar, e meu (minha) responsável também aceitar, farei testes para avaliar minha memória, exames de sangue, exames de urina, exame de líquido cérebro-espinhal obtido mediante punção da coluna lombar, bem como exames de ressonância magnética do cérebro. As consultas para

participação serão realizadas no Hospital das Clínicas – UNICAMP por neurologistas do Departamento de Neurologia. Se acontecer algum problema causado pelos exames realizados ou pelo tratamento, serei atendido(a) no Hospital das Clínicas – UNICAMP.

A pesquisa terá no máximo três anos de duração. Devo comparecer para consulta com o Dr Alfredo Damasceno no ambulatório de Neurologia conforme agendamento. Não terei nenhuma vantagem ou melhora da minha doença por participar do estudo. Só estarei colaborando para um melhor conhecimento dos problemas de pacientes com Esclerose Múltipla, como é o meu caso.

CONFIDENCIALIDADE DOS DADOS

Eu entendo que todas as informações desta pesquisa ficarão no meu prontuário, isto é, na minha pasta do hospital, e serão submetidas às normas do Hospital das Clínicas – UNICAMP relacionadas ao sigilo médico. Quando os resultados do estudo forem apresentados em aulas ou em publicações científicas, meu nome ou os nomes dos pacientes participantes não serão mostrados.

INFORMAÇÕES ADICIONAIS

Eu entendo que posso pedir informações sobre o estudo quando precisar. O Dr Alfredo Damasceno vai me atender durante as consultas; ele e o Professor Dr Fernando Cendes estarão disponíveis para aliviar minhas preocupações pelos telefones (+19) 3521-7754 e 3521-7372. Em caso de dúvidas ou reclamações, poderei entrar em contato com o Comitê de Ética em Pesquisa da Faculdade de Ciências Médicas – UNICAMP através do telefone (+19) 3521-8936. Rua Tessália Vieira de Camargo, 126; Caixa Postal 6111. CEP 13083-887. Campinas, SP. e-mail: cep@fcm.unicamp.br

VOLUNTARIEDADE DE PARTICIPAÇÃO

Eu entendo que a minha participação é voluntária, isto é, só participo se tiver vontade de participar. Não sou obrigado(a) a participar.. Se eu não aceitar participar, ou se após começar o estudo eu decidir sair dele, não terei nenhum problema e continuarei a ser atendido(a) normalmente no Hospital das Clínicas – UNICAMP. Eu compreendo também que o Dr Alfredo Damasceno poderá parar a minha participação neste estudo em qualquer momento que achar necessário.

CONFIRMAÇÃO DE CONCORDÂNCIA DO (A) PACIENTE

Eu, _____, confirmo que o Dr Alfredo Damasceno me explicou o objetivo de seu estudo, bem como os procedimentos aos quais eu serei submetido(a) e o fato de não existir possibilidade de riscos causados pelas avaliações, a não ser pelas complicações que podem surgir por causa dos exames complementares a que serei submetido(a). Se houver suspeita de riscos, estes exames não serão realizados. Compreendo que os exames e o tratamento empregado deverão aumentar os conhecimentos sobre as doenças neurológicas. Eu li e compreendi este Termo de Consentimento Livre e Esclarecido, e concordo por minha vontade em participar deste estudo.

____/____/20__

_____

ASSINATURA DO (A) PACIENTE

RESPONSABILIDADE DO PESQUISADOR

Eu expliquei a _____ e a seu (sua) responsável _____ o objetivo do estudo, os procedimentos necessários e o fato de não haver riscos advindos das consultas clínicas. Explicitei também as eventuais complicações que correm o risco de surgir em decorrência dos exames complementares que serão solicitados. Os exames de diagnóstico complementar realizados terão sempre por objetivo a investigação clínica para o enriquecimento científico com os resultados da pesquisa, de modo que eventuais riscos sejam sempre suplantados pelos benefícios provenientes de sua execução, utilizando sempre da melhor forma possível o meu conhecimento para a tomada deste tipo de decisão. Eu me comprometo a fornecer uma cópia deste Termo de Consentimento Livre e Esclarecido ao (à) participante, e outra cópia a seu (sua) responsável.

____/____/20__

_____

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