Article published online: 2023-08-21

https://doi.org/10.1590/0004-282X20190026

ARTICLE

Polymorphisms in the *CIITA* -168A/G (rs3087456) and *CIITA* +1614G/C (rs4774) may influence severity in multiple sclerosis patients

Os polimorfismos no gene *CIITA* -168A/G (rs3087456) e *CIITA* +1614G/C (rs4774) podem influenciar a gravidade em pacientes com esclerose múltipla

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ABSTRACT

It is currently unknown how genetic factors may influence the clinical course of multiple sclerosis (MS). **Objective:** We examined the impact of *CIITA* polymorphisms -168A/G (rs3087456) and +1614G/C (rs4774) on the risk of disability progression, severity and on responses to first-line immunomodulator treatments. **Methods:** Genomic DNA was extracted from blood samples. We used ABI3730xl and GeneMapper v.4.0 software to identify genotype variations. All patients were followed up and clinically reassessed at three-month intervals. Disability progression was measured by the Expanded Disability Status Scale and disease severity by the Multiple Sclerosis Spasticity Scale (MSSS). **Results:** We included 37 men and 80 women. We found no evidence regarding the influence of the single nucleotide polymorphisms studied in the Expanded Disability Status Scale or therapeutic response of the evaluated drugs. We performed a logistic regression analysis with the MSSS and found that a less severe MS course was associated with wild type *CIITA* -168AA and *CIITA* +1614GG, as the chance of the patient progressing to MSSS2 and MSSS3 decreased in 61% and 75% with *CIITA* -168AA and 66% and 75% with *CIITA* +1614GG, respectively ($p < 0.0001$). Although less significant, the *CIITA* +1614 GC also pointed to a less severe MS course and the chance of the patient progressing to MSSS3 decreased 79% ($p = 0.015$). We also observed that the *CIITA* -168GG genotype was more frequent in MSSS2 and MSSS3 and had 40% lower odds ratio to becoming more severe MS. **Conclusion:** These data suggest that *CIITA* -168AA, *CIITA* +1614GG and *CIITA* +1614 GC polymorphisms may be associated with a better MS clinical course. This knowledge may be useful for a better understanding of MS and its therapeutic management.

Keywords: Multiple sclerosis; Polymorphism, Genetic; Therapeutics; Disease progression.

RESUMO

Atualmente não se sabe como os fatores genéticos podem influenciar o curso clínico da esclerose múltipla (EM). **Objetivo:** Examinamos o impacto dos polimorfismos *CIITA* -168A/G (rs3087456) e *CIITA* +1614G/C (rs4774) no risco de progressão da incapacidade, gravidade e resposta aos tratamentos imunomoduladores de primeira linha. **Métodos:** O DNA genômico foi extraído de amostras de sangue. Utilizamos o software ABI3730xl e GeneMapper v.4.0 (Applied Biosystems) para identificar variações genotípicas. Todos os pacientes foram acompanhados e reavaliados clinicamente em intervalos de três meses. A progressão da incapacidade foi medida pela EDSS e a gravidade da doença pelo MSSS. **Resultados:** Incluímos 37 homens e 80 mulheres. Não encontramos evidências sobre a influência dos SNPs estudados no EDSS e na resposta terapêutica aos fármacos avaliados. Realizamos uma análise de regressão logística com o MSSS e observamos uma evolução menos grave da EM associada aos tipos selvagens *CIITA* -168AA e *CIITA* +1614GG, pois a chance do paciente atingir MSSS2 e MSSS3 diminuiu em 61%/75%, e 66/75% respectivamente ($p < 0,0001$). Embora menos significativo, o *CIITA* +1614GC também foi relacionado com evolução menos grave da EM e a chance do paciente atingir o MSSS3 diminuiu 79% ($p = 0,015$). Nós também observamos que o genótipo *CIITA* -168GG foi mais frequente no MSSS2 e MSSS3 e teve uma razão de chance 40% menor para atingir forma mais grave da EM. **Conclusão:** Estes dados sugerem que os polimorfismos *CIITA* -168AA, *CIITA* +1614GG e *CIITA* +1614GC podem estar associados a um melhor curso clínico da EM. Este conhecimento pode ser útil para uma melhor compreensão da EM e o seu manejo terapêutico.

Palavras-chave: Esclerose múltipla; Polimorfismo genético; Terapêutca; Progressão da doença.

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Conflict of interest: There is no conflict of interest to declare.

Support: This study was funded by support from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-Brazil).

Received 25 June 2018; Received in final form 22 October 2018; Accepted 29 November 2018.

Multiple sclerosis (MS) is an inflammatory and degenerative demyelinating disease of the central nervous system (CNS). It represents the most common inflammatory condition of the CNS and is the second leading cause of disability among young adults[1]. Although the mechanism that triggers the autoimmune disorder remains unclear, the disorder tends to be triggered by environmental factors in genetically susceptible individuals[2,3].

The natural history of relapsing-remitting MS includes periods of disease exacerbation followed by a progressive period[4]. Clinical phenotypes of bout-onset MS may widely vary: the Expanded Disability Status Scale (EDSS)[5] and the Multiple Sclerosis Severity Scale (MSSS)[6] are the most common methods used to quantify disability and the severity of disease progression over time, and the scores can be correlated with clinical, immunological and genetic phenotypes. Previous studies have recently associated various genetic markers with disability progression[7,8].

Several studies have shown that variations in human leukocyte antigen (HLA) genes, class I or class II, may be correlated with susceptibility to or severity of autoimmune disorders[9]. Associations with *HLA-DR15* haplotype (*DRB1**1501-*DQA1**0102-*DQB1**0602) in MS have repeatedly been demonstrated in multiple populations, such as in northern European, African American and African-Brazilian descendants[10,11]. The *HLA* expression is regulated by the assembly of a transcriptional complex consisting of several different molecules that interact with sequence elements in their promoters. The HLA class II transactivator gene (*CIITA* in humans and *C2TA* in mice), loci 16p13, encodes an important transcription factor that has been described as the master control factor of *HLA II* gene expression[12]. Thus, *CIITA* is an attractive candidate for genetic studies of autoimmune diseases for which *HLA* associations have been well established.

Strong evidence for an association of the *CIITA* +1614G/C (rs4774) missense single nucleotide polymorphism (SNP) with MS in the presence of DRB1*15:01, a well-established MS risk allele, has been reported[13,14]. The *CIITA* +1614C variant, located in exon 11, causes an amino acid substitution from glycine to alanine at amino acid position 500. Additionally, the *CIITA* -168A/G SNP (rs3087456), located in the gene promoter, is also associated with MS susceptibility[15]. In humans, *CIITA* +1614 C and *CIITA* -168 G homozygous polymorphic genotypes have been associated with abnormal gene expression in several autoimmune diseases[16,17,18,19,20,21]. The present study is the first to associate these variants with MS severity.

Therefore, the aim of this study was to investigate the association between the *CIITA* -168A/G (rs3087456) and *CIITA* +1614G/C (rs4774) SNPs in clinical disability, disease progression and to investigate the clinical responses to first-line treatment with glatiramer acetate (GA) or interferon beta (IFN-b).

METHODS

Study population and samples

This retrospective cohort study was conducted according to a predefined protocol. The included patients were followed up at the University Hospital Clementino Fraga Filho, which is a hospital that patients with MS are routinely referred to, for diagnosis and follow-up in the city of Rio de Janeiro. The included patients fulfilled the criteria outlined by McDonald et al., revised in 2010 by Polman et al.[22]. Patients with comorbidities, other autoimmune diseases and neuromyelitis optica were excluded. For each patient, neurological assessments were performed every three months. The database included the number of relapses, progression of disability and time intervals to reach EDSS scores of 3.0, 6.0, 7.0, 8.0 and 10. We also compared clinical scores with disease duration using the Global MSSS table. Patients with defined slow progressing patterns of MS progression (MSSS-1) were located in the first three deciles of the table with MSSS < 3; patients with mid-rate progression (MSSS-2) were located in the deciles > 4 to < 7; and patients with a rapidly progressing pattern (MSSS-3) were located in the last three deciles with MSSS > 7 (modified from Čierny et al.[23]). Several criteria defined by Río et al.[24] were used to classify treatment responses to GA or IFN-b. We defined a non-response to treatment as an increase of at least one EDSS step or any relapse within one year of follow-up[24,25], as these criteria best reflect the clinical evolution of patients. All patients signed an informed consent to undergo a genetic analysis, and the study procedures were reviewed and approved by the National Council for Ethics in Research (CONEP; #1265) on May 29, 2000.

Genotypic analysis

Genomic DNA was extracted from blood samples collected on filter paper (Flinders Technology Associates) according to Bereczky et al.[26], with some modifications. Briefly, each filter paper was transferred to a 1.5 ml tube containing 200 µl lysis buffer (10 mM Tris pH 8.0, 100 mM NaCl, 10 mM EDTA, pH 8.0, 0.5% SDS) and proteinase K (5 mg/ml), and the material was incubated at 56°C for two hours. Then, 500 µl phenol:chloroform:isoamyl alcohol (25:24:1) was added. After gently stirring the tube, the material was centrifuged for two minutes at 10,000 g, and the aqueous phase was transferred to a new tube, to which the same volume of absolute isopropanol was added. The sample was kept on ice for 30 minutes and then centrifuged at 10,000 g for 10 minutes. The supernatant was discarded, and the precipitate was washed with 70% ethanol and then stored in TE solution at pH 8.0 (10 mM Tris HCl; 1 mM EDTA). The DNA extracts were quantified by spectrophotometry at 260/280 nm.

The SNPs investigated in this study, *CIITA* -168A/G (rs3087456) and *CIITA* +1614G/C (rs4774), are detailed in Table 1 and the primer sequences were previously described by Patarroyo et al.[27]. Initially, alleles were identified by PCR amplification with sequence-specific primers using 100 ng of genomic DNA in 10 µl volume reaction using a One Lambda

Table 1. Polymorphisms analyzed in this study.

Polymorphism	Reference	fHt*	MAF*	Functional position
CIITA-168A/G	rs 3087456	0.46	0.37	promoter
CIITA+1614G/C	rs 4774	0.41	0.25	Not synonymous

fHt: heterozygosity frequency; MAF: minor allele frequency; *According 1,000 genomes.

Table 2. Allelic and genotypic frequencies of the studied SNPs.

Genotypes and alleles	n (%)
CIITA -168A/G (rs3087456)	
AA	96 (82)
AG	13 (11)
GG	8 (7)
G allele frequency	0.12
CIITA +1614G/C (rs4774)	
GG	90 (77)
GC	17 (14)
CC	10 (9)
C allele frequency	0.16

(Canoga Park, USA) kit, according to the manufacturer's recommendations, followed by capillary electrophoresis using an ABI PRISM® 3500 Genetic Analyzer (Applied Biosystems, Foster City, USA) and an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, USA). Sequencing data were used for alignment with the available reference sequences in the National Center of Biotechnology Information (NCBI, USA) database.

Statistical analysis

Allelic and genotypic frequencies were calculated by direct gene counting. Predictors of MS outcomes according to the time to reach EDSS 3.0 and EDSS 6.0 scores compared with genotypes were evaluated using Kaplan-Meier curves and log rank tests. Patients were allocated to one of three groups according to disease progression rate: MSSS1 was characterized as slow progression, MSSS2 as intermediate progression and MSSS3 as rapid progression, and analyzed by logistic regression. The patients were also classified as responders and nonresponders to drug treatments. The chi-square test or Fisher's test were used to test associations between polymorphisms and drug treatments; p-values < 0.05 were considered statistically significant, and all statistical analyses were conducted using SPSS software (version 24.0).

RESULTS

This study included a group of 117 patients (37 men and 80 women) with MS. The mean age was 46 (15–75) years (standard deviation [SD] ± 11.99 years), and the mean age of disease onset was 30 (12-58) years (SD ± 10.43). From the time of diagnosis, patients had a median disease duration of 15 years (SD ± 8.04 years) in 2017. The MS cohort comprised 99 relapsing-remitting, 13 secondary-progressive and 5 primary-progressive.

The distributions of allelic and genotypic frequencies of the SNPs *CIITA* -168A/G (rs3087456) and *CIITA* +1614G/C (rs4774) in our studied population are shown in Table 2. Frequencies of variant alleles were 0.12 for *CIITA* -168A/G and 0.16 for *CIITA* +1614G/C.

No association between the *CIITA* -168A/G and *CIITA* +1614G/C SNPs and possible progression to EDSS ≥ 3 or EDSS ≥ 6 was observed in the Kaplan-Meier survival analysis (Figure).

We applied the MSSS value as the most powerful indicator to estimate MS severity in individual patients. As shown in Table 3, a less severe MS course was associated with *CIITA* -168AA, because the chance of the patient progressing to MSSS2 and MSSS3 decreased by 61% and 75% respectively ($p < 0.0001$). Although the *CIITA* -168GG genotype did not reach statistical significance, it was possible to observe a trend for the more severe phenotype, as this genotype was more frequent in the MSSS2 and MSSS3 groups and had 40% lower odds ratio to reach a more severe MS course. Thus, the homozygous polymorphic genotype could be associated with MS severity, according to the MSSS.

The *CIITA* +1614GG genotype was also more frequent in the MSSS1 group and associated with a less severe MS course; the chance of the patient progressing to MSSS2 and MSSS3 decreased by 66% and 75% respectively ($p < 0.0001$). We could not determine a relationship of the variant allele with the severity of the disease. Although less significant, the *CIITA* +1614 GC was also associated with a less severe MS course and the chance of the patient progressing to MSSS3 decreased 79% ($p = 0.015$).

We analyzed treatment responses in 107 patients who received regular treatments with GA or IFN-b as a first-line immunomodulating drug for at least one year. Although this study included 117 patients with MS, 10 did not use an immunomodulating drug during the course of this study. According to the definition of responses to treatment, 72.7% of patients carrying the *CIITA* -168AG genotype and 75% carrying the GG genotype were nonresponders. For *CIITA* +1614G/C, only a homozygous polymorphic genotype (66.7% of patients carrying CC) had a worse response than patients carrying a normal genotype (Table 4). However, it was not possible to observe significant differences between the groups.

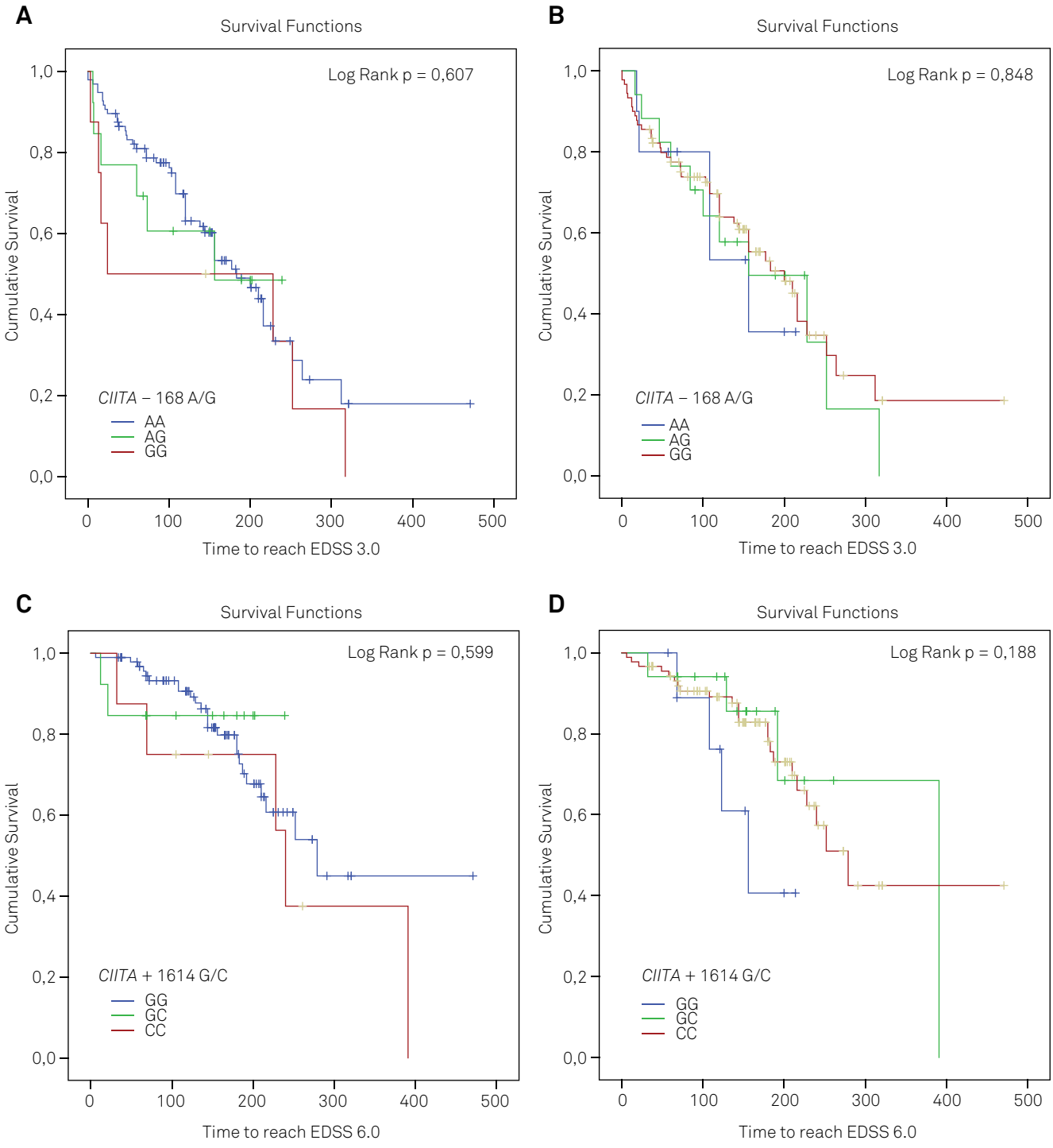

Figure. Survival curves in relation to the variants and the time to reach the two disability milestones (EDSS 3.0 and EDSS 6.0): A) *CIITA* rs3087456 (-168A/G); B) *CIITA* rs4774 (+1614G/C).

DISCUSSION

The characteristics of the MS patients included in this paper are representative of the neurological phenotypes reported by other MS cohort studies in the city of Rio de Janeiro, Brazil, such as female prevalence, highest frequency relapsing-remitting MS, low frequency of progressive course, and African-descendants representing one third of the patients[10]. In this study, we investigated the correlations among polymorphisms, disease course, disease morbidity and therapeutic responses.

We describe here, for the first time, the correlation between SNPs in *CIITA* and disease severity in a Brazilian cohort. We showed that MSSS values tended to be higher for patients who carried the *CIITA* -168 GG genotype. In fact, all genome-wide association studies have corroborated the

Table 3. Polymorphisms and MSSS.

Variable	n (%)	MSSS1 (%)	p-value (OR)	MSSS2 (%)	p-value (OR)	MSSS3 (%)	p-value (OR)
rs3087456 (-168A/G)							
AA	96 (82)	50 (42.8)	0.683 (1.09)	27 (23)	< 0.0001 (0.39)	19 (16.2)	< 0.0001 (0.25)
AG	13 (11.1)	7 (5.9)	0.782 (1.17)	3 (2.6)	0.067 (0.30)	3 (2.6)	0.067 (0.30)
GG	8 (6.9)	2 (1.7)	0.178 (0.33)	3 (2.6)	0.484 (0.60)	3 (2.6)	0.484 (0.60)
Overall	117 (100)	59 (50.4)		33 (28.2)		25 (21.4)	
rs4774 (+1614G/C)							
GG	90 (77)	49 (41.9)	0.400 (1.20)	23 (19.7)	< 0.0001 (0.34)	18 (15.4)	< 0.0001 (0.25)
GC	17 (14.5)	6 (5.1)	0.232 (0.55)	8 (6.8)	0.808 (0.89)	3 (2.6)	0.015 (0.21)
CC	10 (8.5)	4 (3.4)	0.530 (0.67)	2 (1.7)	0.079 (0.25)	4 (3.4)	0.530 (0.67)
Overall	117 (100)	59 (50.4)		33 (28.2)		25 (21.4)	

OR: odds ratio. Statistical analysis performed with respect to each genotype and frequency in MSSS1, MSSS2 and MSSS3 groups. Statistical significance ($p < 0.05$). Logistic regression.

Table 4. Responder and nonresponder status.

1st Treatment	Patients n (%)	R and NR	Polymorphism					
			rs3087456 (-168A/G)			rs4774 (+1614G/C)		
			n (%)			n (%)		
			AA	AG	GG	GG	GC	CC
GA or IFN-b	107	R	40 (45.5)	3 (27.3)	2 (25)	35 (43.2)	7 (41.2)	3 (33.3)
		NR	48 (54.5)	8 (72.7)	6 (75)	46 (56.8)	10 (58.8)	6 (66.7)
					p = 0.376			p = 0.943
Glatiramer acetate	25 (23.4)	R	5 (27.8)	1 (16.7)	0	5 (25)	1 (33.3)	0
		NR	13 (72.2)	5 (83.3)	1 (100)	15 (75)	2 (66.7)	2 (100)
Interferon-b	82 (76.6)	R	35 (50)	2 (40)	2 (28.6)	30 (49.2)	6 (42.9)	3 (42.9)
		NR	35 (50)	3 (60)	5 (71.4)	31 (50.8)	8 (57.1)	4 (57.1)

*Statistical significance ($p < 0.05$). Fisher's test (groups with $n < 5$) and X^2 test. R: responder; NR: nonresponder.

role of major histocompatibility complex (MHC) genes in MS susceptibility[28]. Moreover, CIITA is referred to as the "master control factor" for expression of the MHC class II gene[29] and restores expression of all MHC class II isotypes in mutant cells.[30] The primary function of CIITA is the complete or partial control of the expression of multiple genes involved in antigen presentation to T cells by MHC molecules, which is essential for an adaptive immune response[29,30]. George et al. analyzed 52 independent non-MHC genome-wide association study SNP variants in 7,125 MS patients and found no association with MSSS disease severity[8]. Based on this context, we hypothesized that *CIITA* could be a candidate gene for determining MS disease severity. Using a retrospective cohort of 117 patients with a 15-year mean disease duration, we found that the distributions of allelic and genotypic frequencies of the *CIITA* -168A/G and *CIITA* +1614G/C SNPs presented the highest frequency in wild type subjects. Although several case-control studies have evaluated the genetic association of SNPs in the *CIITA* gene in relation to MS development[13,14,31-35], none have evaluated whether these variants are related to clinical disability, progression or treatment responses. Considering the influence of CIITA protein in MHC class II expression, *CIITA* could potentially influence MS severity.

The demographic and clinical parameters showed that MS is typically diagnosed in the second or third decade of life, and the clinical course of MS (conversion to active disease, relapse or disability progression) was previously analyzed by Pan et al.[7]. Weinshenker showed that the median time for MS conversion to a progressive course was approximately ten years[36]. In the last years, the availability of disease-modifying drugs and escalation to higher potency therapies may have changed the natural history of disease and evolution to secondary-progressive MS has become substantially lower[37]. In this context, identifying predictive factors is critical for diagnosing the impact on the disease progression. Although not available for clinical use, biomarkers, such as serum neurofilament light, can monitor tissue damage and the effects of therapies in MS patients[38]. The association of SNPs with MS and markers of disease progression, such as the time to reach disability milestones (EDSS 3.0, 6.0, 7.0, 8.0 and 10) or the time to secondary progression in MS patients, has only been considered in a few genetic studies such as that reported by Pan et al.[7], who

identified three non-HLA SNPs that predicted MS conversion after an episode of clinical isolated syndrome and seven non-HLA SNPs that were correlated with faster progression based on the EDSS. Lin et al. investigated the MS clinical course and found SNPs that predicted 25-hydroxyvitamin D levels and disease relapses. They found significant associations of five SNPs with increased frequency of relapses but no association with disease disability risk associated with the clinical course[39].

We observed significant decreases in the frequency of a wild-type genotype for both *CIITA* +1614G/C and *CIITA* -168A/G in the MSSS2 and MSSS3 groups. Furthermore, we observed a trend of homozygous polymorphic genotype to progress to the MSSS2 or MSSS3 severity groups for the *CIITA* -168A/G SNP (Table 3). Considering these data, we believe that the polymorphic allele may be associated with MS severity measured by the MSSS.

The *CIITA* gene is controlled by several distinct promoters, two of which direct specific constitutive expression in dendritic cells (promoter I) and B lymphocytes (promoter III), while another mediates IFN-γ-induced expression (promoter IV)[33,40]. Masternak et al.[12] observed that cellular, temporal and functional diversity in MHC-II expression is not regulated at the level of MHC-II genes themselves but is ultimately under the control of several promoter sequences that differentially activate the same transactivator gene[40]. Swanberg et al.[17] and Martínez et al.[33] compared MHC molecules with other SNPs and found an association of the -168A/G polymorphism (rs3087456) with low expression of MHC molecules and susceptibility to MS and another disease with inflammatory components in Nordic countries. Thus, the polymorphic allele could be associated with MS severity based on the MSSS. Rasmussen et al.[31] studied 111 MS patients and 105 controls from the UK and identified an association of the -168A/G SNP with primary progressive MS ($p < 0.04$).

Among 107 patients receiving GA or IFN-b treatment in our study, we found that those who carried the *CIITA* -168AG or GG genotype and the *CIITA* +1614CC genotype had the most negative influence on the treatment, compared with patients carrying a normal genotype. An association of this polymorphism with a worse treatment response was observed, but without statistical significance, possibly due to the small cohort of patients. We cannot compare this result with other studies because treatment responses including *CIITA* SNPs have not previously been analyzed. Previous studies that have investigated the therapeutic responses to IFN-b for MS treatment did not find an association with class II and class I alleles. Fernández et al.[41] did not find an association between *HLA* class II alleles and therapeutic responses. Swanberg et al.[17] observed a lower expression of this gene in cells of individuals with the *CIITA*-168GG genotype than in cells of individuals with other genotypes, after stimulation with IFN-g. Additionally, Dominguez-Mozo et al.[42] found that patients with a polymorphic allele of *CIITA* +1614 C exhibited significantly higher expression of mRNA levels[42]. Thus, as these SNPs induce variations in gene expression compared with wild-type genotypes, we suggest that changes should be made in the therapy applied to individuals carrying at least one polymorphic allele.

In a study of 195 MS cases and 195 healthy controls from Spain, Garcia-Montojo et al.[35] analyzed the association of human herpes virus 6 (HHV-6) and two *CIITA* polymorphisms (rs3087456G and rs4774C). They found a strong association of the *CIITA* +1614C allele with HHV-6 positive MS patients. Upon analyzing the clinical response to IFN-b, only 13/61 (21.3%) MS patients with the *CIITA* +1614C allele were considered to be clinical responders, while 49/136 (36.1%) of MS patients with the *CIITA* +1614G allele were clinical responders ($p = 0.004$). In accordance with our study, (Table 4), 33.3% of patients carrying *CIITA*+1614CC genotype (homozygous polymorphic) were clinical responders, while 43.2% MS patients with the *CIITA* +1614GG genotype were clinical responders. However, Alvarez-Lafuente et al.[34] did not find any significant association for the *CIITA* -168 allele. Improving our knowledge of the stages and mechanisms associated with MS will enable the further development of therapeutic drugs that can target these disease-related mechanisms and allow us to establish a therapeutic window of opportunity for early intervention in nonresponders[43].

We did not find significant differences between genotypes and the time taken to reach EDSS 3.0 or EDSS 6.0. The frequency results and statistical analysis may be limited by the small sample size, although this was a pilot study that tested the potential association of these *CIITA* gene SNPs with clinical disability and treatment responses. Indeed, a future study incorporating a larger population may have greater statistical relevance.

In conclusion, our findings support an association between the *CIITA* -168AA genotype with a less severe MS clinical course, and *CIITA* -168GG genotype with a more severe MS clinical course. The *CIITA* +1614 GG and, less significantly, the *CIITA* +1614 GC were also associated with a less severe MS clinical course. Comprehensive studies conducted across different populations are required to validate these results. These findings may contribute to a better understanding of the clinical course of MS and lead to decreased morbidity and mortality through risk assessment and improvements in therapeutic interventions.

Acknowledgments

The authors thank the team at Hospital Universitário Clementino Fraga Filho (UFRJ) for help with the sample collection. All authors critically reviewed the manuscript and approved the final version for submission.

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