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The rs3761548 *FOXP3* variant is associated with multiple sclerosis and transforming growth factor β1 levels in female patients

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Abstract

Objective The aim of this study was to evaluate the association between rs3761548 *FOXP3* (-3279 C > A) variant and multiple sclerosis (MS), disability, disability progression, as well as transforming growth factor (TGF)-β1 and interleukin (IL)-10 plasma levels in MS patients.

Methods and subjects The study included 170 MS patients and 182 controls. Disability was evaluated using Expanded Disability Status Scale (EDSS) and categorized as mild (EDSS ≤ 3) and moderate/high (EDSS > 3). Disability progression was evaluated using Multiple Sclerosis Severity Score (MSSS). The rs3761548 variant was determined with polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP). Plasma levels of TGF-β1 and IL-10 were determined using immunofluorimetric assay.

Results CA and AA genotypes were associated with MS [odds ratio (OR) 2.03, 95% confidence interval (CI) 1.66–3.53, $p = 0.012$; OR 8.19, 95% CI 3.04–22.07, $p < 0.001$, respectively). With the dominant model, the CA + AA genotypes were associated with MS (OR 2.57, 95% CI 1.50–4.37, $p < 0.001$). In the recessive model, the AA genotype was also associated with MS (OR 5.38, 95% CI 2.12–13.64, $p < 0.001$). After adjustment by age, ethnicity, BMI and smoking, all these results remained significant, as well as female patients carrying the CA + AA genotypes showed higher TGF-β1 than those carrying the CC genotype (OR 1.35, 95% CI 1.001–1.054, $p = 0.043$). No association was observed between the genotypes and disability, disability progression and IL-10 levels.

Conclusion These results suggest that the A allele of *FOXP3* -3279 C > A variant may exert a role in the T regulatory cell function, which could be one of the factors involved in the susceptibility for MS in females.

Keywords Multiple sclerosis · Disability · rs3761548 *FOXP3* variant · Transforming growth factor β1 · T regulatory cell

Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) characterized by demyelination and neurodegeneration mediated by a Th1 and Th17-immune response against white matter and grey matter of

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the brain, spinal cord, and optic nerve [1]. Its etiology is multifactorial with a complex interaction between genetic variants and environmental factors [2]. MS is a heterogeneous disease that affects women two to three times as often as men. While females are at higher risk for MS, males are more likely to display primary progressive disease and accumulate disability faster than female patients in relapse-onset MS [3].

Variations in genes coding for the expression and regulation of immune response may play a role in MS susceptibility and disease progression [4]. The *forkhead box protein 3* (*FOXP3*) gene, a member of transcription factor winged-helix family, is located on chromosome Xp11.23 within the area of autoimmune disease linkage [5, 6]. The *FOXP3* gene is primarily expressed in $CD4^+$ $CD25^+$ T regulatory (Treg) cells [7]. It encodes the FOXP3 protein that is a transcriptional factor for the Treg cell development and function, mainly in the downregulation of cytokine production by T helper (Th)-1 and Th17 effector cells [8].

Treg cells produce the pleiotropic cytokine transforming growth factor beta (TGF-β) and interleukin (IL)-10, which are dominantly viewed as critical mediators for tolerance and immunosuppression. TGF-β is a key regulator of the signaling pathways that initiate and maintain Foxp3 expression and suppressive function in $CD4^+$ $CD25^-$ precursors. In fact, in a cell type and environment-dependent fashion, Treg and TGF-β exert both negative and positive effects on the immune system. TGF-β suppresses immune responses through inhibiting the function of inflammatory cells and promoting the function of Treg cells. TGF-β suppresses the expression of important genes for T cell differentiation and function, such as GATA3, Tbet, signal transducer and activator of transcription 4 (STAT4), interferon (IFN)-γ, and granzyme-B. TGF-β also regulates the adaptive immunity components, such as T cells, as well as the innate immunity components, such as natural killer (NK) cells [9].

Different single-nucleotide variants (SNVs) in the promoter region of *FOXP3,* which can affect the expression of FOXP3 and impair the Treg differentiation and function, have been associated with the susceptibility and prognosis of autoimmune diseases, such as autoimmune thyroid diseases [10], psoriasis [11], systemic sclerosis [12], rheumatoid arthritis [13], and MS [14, 15].

The rs3761548 *FOXP3* -3279 C > A variant is located on the promoter region and the A allele is correlated with a reduction in FOXP3 expression [16].

Different genetic variants in the promoter region of *FOXP3* were evaluated in patients with MS. The rs3761548 (-3279 C > A) and the rs2232365 (-924 A > G) were associated with MS in Iran population [14, 15]. However, rs3761547 (-3499 A > G), rs3761548 (-3279 C > A) and rs3761549 (-2383 C > T) were evaluated in MS patients and controls from Polish and the results failed to confirm the association with MS [17]. In fact, it is well known that ethnicity influences the presence of variants in various genes, which may cause controversial results in different populations, that is why is important to survey this association in different ethnicities. Thus, the aim of this study was to evaluate the rs3761548 *FOXP3* (-3279 C > A) variant and its association with MS, disability, and disability progression, as well as with TGF-β1 and IL-10 levels in MS patients from Southern Brazilian population.

Materials and subjects

Subjects

The study included 170 MS patients, adults and both sexes, consecutively recruited from the Demyelinating Diseases Outpatient of the State University of Londrina, Londrina, Paraná, South Brazil. The MS diagnosis was established according to the McDonald criteria [18]. The patients were clinically evaluated for disability using the Expanded Disability Status Scale (EDSS) [19]. Based on their EDSS scores, patients were divided into two groups with EDSS less or equal to the median value of disability among them (minor disability) and more than this median (moderate/high disability) [20]. Disability progression was evaluated using the Multiple Sclerosis Severity Score (MSSS), as proposed elsewhere [21] and score ≥ 5.0 denoted higher than the average speed of disability accumulation [22].

As controls, 182 healthy individuals were selected among blood donors of the Regional Blood Bank of Londrina, from the same geographic region of the MS patients. None of the participants in the study presented clinical symptoms or laboratory biomarkers of heart, thyroid, kidney, hepatic, gastrointestinal, or oncologic diseases.

All MS patients were in the remission clinical phase, defined as the period of recovery with no relapse episodes within the last 3 months prior to the time of enrollment in the study. Demographic, epidemiological and anthropometric data (for patients and controls), as well as clinical history and the use of therapy for MS before the inclusion in this study (for patients) were obtained using a standard questionnaire at the admission of the individuals. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared and the ethnicity was self-reported as Caucasian and non-Caucasian [23].

The protocol was approved by the Institutional Research Ethics Committees of University of Londrina, Paraná, Brazil (CAAE: 22290913.9.0000.5231) and all of the individuals invited were informed in detail about the research and gave written informed consent.

Blood collection and immunological biomarkers

After fasting for 12 h, venous blood samples were obtained with anticoagulant ethylenediaminetetraacetic acid (EDTA), centrifuged at 3000 rpm for 15 min; further, plasma and buffy-coat were separated and divided into aliquots and stored at − 80 °C until use. TGF-β1 and IL-10 levels were determined using microspheres multiplex immunofluorimetric assay (Novex™, Life Technologies, Frederick, MD, USA) for Luminex platform (MAGPIX™, Luminex Corp., Austin, TX, USA), that was performed according to the manufacturer's instructions and their reference values.

Allelic variant rs3761548 (-3279 C > A) of FOXP3

Genomic DNA was extracted from a buffy-coat of peripheral blood cells using a resin column procedure (Biopur, Biometrix Diagnóstica, Curitiba, PR, Brazil). DNA was used to amplify a 155-base pair (bp) sequence of the *FOXP3* by polymerase chain reaction (PCR) as previously reported [24] with some modifications. Briefly, the primers used were determined according to the GenBank number NG_007392.1, 5′-GGCAGAGTTGAAATCCAAGC–3′ (forward) and 5′-CAACGTGTGAGAAGGCAGAA–3′ (reverse). PCR was performed with a final volume of 25 µL, with 0.25 mM of each primer, 1.50 mM $MgCl_2$, 0.10 mM dNTP, 1.25 units of Taq DNA polymerase (Invitrogen™, Life Technologies, Carlsbad, CA, USA). PCR conditions were performed in a thermocycler (Applied Biosystems Veriti™, 96-Well Thermal Cycler, Life Technologies, Foster City, CA, USA) and comprised of 4 min denaturation at 94 °C for initial denaturation; 35 cycles of 30 s at 94 °C for denaturation, 30 s at 65 °C for the annealing and 30 s at 72 °C for the elongation; and 10 min at 72 °C for final elongation. In all PCR analyses, a negative control (without a DNA sample) was included.

The PCR products (155 bp) were digested with *Pst*I restriction endonuclease (Anza™, Invitrogen, Life Technologies, Carlsbad, CA, USA) and analyzed with the restriction fragment length polymorphism (RFLP). The presence of two fragments (80 bp and 75 bp) corresponded to C allele, while the presence of one fragment (155 bp), corresponded to the A allele that did not undergo enzymatic cleavage. All PCR–RFLP products were analyzed on polyacrylamide gel (10%) stained with silver nitrate [24].

Statistical analysis

Analysis of contingency tables (χ^2 test) was employed to check the associations between categorical variables and diagnostic groups. The Kolmogorov–Smirnov test was used to assess the normality of distribution. TGF-β1 and IL-10 plasma levels were transformed into square root because the data were in right obliquity to ensure data normality. We assessed the differences in continuous variables between groups using the Mann–Whitney test. Categorical variables were expressed as absolute number (*n*) and percentage (%) and continuous variables were expressed as median and interquartile range 25–75% (IQR). The correlations between TGF-β1 and IL-10 were assessed using Spearman correlation coefficients. The Hardy–Weinberg equilibrium (HWE) was analyzed just in the females (patients and controls) because males carry only one copy of the X-chromosome. In male, the allele frequency was calculated.

The association between the *rs3761548 (-3279 C > A) of FOXP3* was analyzed in dominant, codominant, and recessive models according to the sex of the individuals. Multivariate general linear model (GLM) analysis was used to assess the effects of explanatory variables (including diagnosis) on dependent variables (TGF-β1 and IL-10) while controlling for age, sex, ethnicity, BMI, and smoking. The association between TGF-β1 levels and EDSS > 3, and the *FOXP3* variant according to sex was evaluated using automatic stepwise binary logistic regression analysis controlled for covariates that may confound the association of interest in three different models. The analysis also included the odds ratio (OR) and 95% confidence interval (CI) evaluated. All statistical analyses were performed using IBM SPSS windows version 24.

Results

Characteristics of the subjects

Table 1 shows the baseline data of MS patients and controls. As expected, MS patients did not differ in the variables age ($p = 0.251$), sex ($p = 0.958$), ethnicity ($p = 0.759$), BMI ($p = 0.102$), and smoking ($p = 0.326$) when compared to controls. The median age at MS diagnosis and disease duration was 32 years (26–43) and 6 years (3–12), respectively. According to the clinical form, 146 patients were classified with relapsing–remitting MS (RRMS) and 24 with progressive MS [5 with primary progressive MS (PPMS) and 19 with secondary progressive MS (SPMS)]. As the median disability in this cohort of patients was 3.0, this value was used as the cutoff to categorize the MS patients for this article, as wild disability (EDSS ≤ 3) and moderate/high disability (EDSS > 3).

Of the 162 patients with EDSS scores, 92 (56.8%) presented moderate/high disability (EDSS > 3) and 70 (43.2%) presented mild disability (EDSS ≤ 3). Eight patients did have the record of EDSS. Of the 144 MS patients with MSSS scores, 68 (47.2%) showed progression (MSSS ≥ 5) and 76 (52.8%) showed no progression (MSSS < 5). Twenty-six MS patients did have the

Table 1 Socio-demographic and clinical data in patients with multiple sclerosis and controls

	Control ($n=182$)	MS ($n=170$)	p value
Age (year)	40 (31–47)	39 (31–51)	0.251
Sex (female/male)	130 (71.4)/52(28.6)	121 (71.2)/49 (28.8)	0.958
Ethnicity			
Caucasian/non-Caucasian	141 (77.5)/41 (22.5)	134 (78.8)/36 (21.2)	0.759
Body mass index (kg/m^2)	25.41 (22.64–29.03)	24.40 (21.80–28.70)	0.102
Smoking (no/yes)	164(90.1)/18 (9.9)	144/22	0.326
Age at diagnosis (year)	–	32 (26–43)	
Duration of illness (year)	–	6 (3–12)	
Clinical forms			
Relapsing–remitting MS	–	146 (85.9)	
Secondary progressive MS	–	19 (11.2)	
Primary progressive MS	–	5 (2.9)	
EDSS range (median, IQR)[a]	–	0.0–9.0 (3.0, 1.0–4.5)	
EDSS > 3	–	92 (56.8)	
EDSS ≤ 3	–	70 (43.2)	
MSSS range (median, IQR)[b]		0.09–9.92 (4.63, 1.62–7.65)	
MSSS ≥ 5	–	68 (47.2)	
MSSS < 5	–	76 (52.8)	
MS treatment[c]			
Without treatment	–	16 (9.5)	
Interferon β	–	97 (57.7)	
Glatiramer acetate	–	42 (25.0)	
Natalizumab	–	12 (7.2)	
Fingolimod	–	1 (0.6)	

All results of Mann–Whitney test. χ^2: results of analyses of contingency tables. Continuous variables were expressed as median and interquartile range (25–75%) and categorical variables were expressed as absolute number (n) and percentage (%)

EDSS Expanded Disability Status Scale, *MSSS* Multiple Sclerosis Severity Score

[a]Eight patients did have the record of EDSS

[b]Twenty-six MS patients did have the record of MSSS

[c]Two patients did not have the MS treatment record

record of MSSS. According to the MS therapy, 16 (9.5%) patients were without treatment, 97 (57.7%) were treated with interferon β, 42 (25.0%) were treated with glatiramer acetate, 12 (7.2%) were using natalizumab and one (0.6%) was using fingolimod. Two patients did have the therapy recorded.

MS patients showed higher levels of TGF-β1 than controls, with median of 3015.3 pg/mL (IQR 1441.9–4675.6) versus 725.0 pg/mL (IQR 324.5–1555.7), $p < 0.001$; moreover, they showed higher IL-10, with median of 5.283 pg/mL (IQR 1.569–16.950) versus 0.010 pg/mL (IQR 0.010–0.957), $p < 0.001$, than controls (Fig. 1). It should be stressed that all these biomarker results were not adjusted for possible extraneous variables including age, sex, BMI, ethnicity, and smoking.

Associations with MS diagnosis

Table 2 shows the results of a multivariate GLM analysis with TGF-β1 and IL-10 as dependent variables and MS diagnosis as the explanatory variable while adjusting for age, sex, BMI, ethnicity, and smoking. We found that MS diagnosis, age and smoking had significant effects on the TGF-β1 and IL-10 levels, whereas sex, BMI, and ethnicity did not. Tests for between-subject effects showed that MS diagnosis was positively associated with TGF-β1 and IL-10, with a strong effect size of 40.3% and 32.5%, respectively. Smoking and age showed a modest effect on IL-10 levels. The GLM model showed the equivalence of covariance matrices ($p = 0.100$) and the dependent variables presented equal variances ($p > 0.05$).

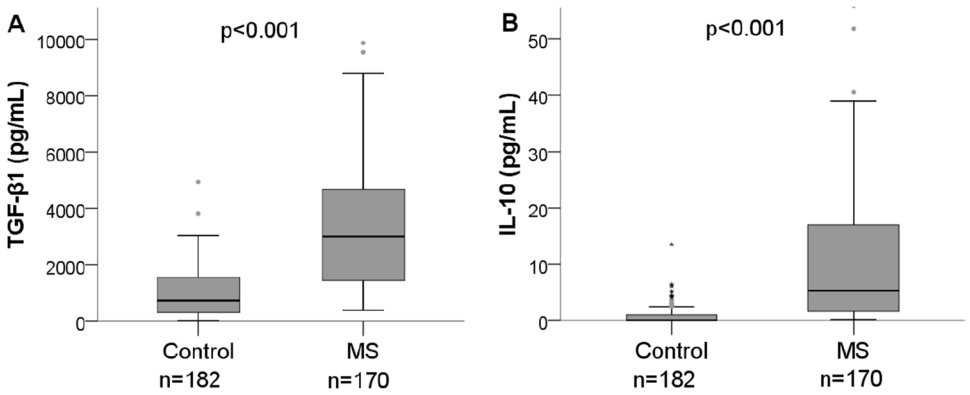

Fig. 1 Plasma levels of transforming growth factor beta 1 (TGF-β1) and interleukin 10 (IL-10) in patients with multiple sclerosis (MS) and controls. **a** Increased TGF-β1 levels in MS patients compared to controls ($p < 0.001$); **b** increased IL-10 levels in MS patients compared to controls ($p < 0.001$); results of Mann–Whitney test expressed as median and interquartile range (25–75%)

Table 2 Results of multivariate GLM analysis with transforming growing factor beta (TGF-β) and interleukin 10 (IL-10) as dependent variables and multiple sclerosis as independent variable

Model	Dependent variable	Independent variable	*F*	*df*	*p*	Partial eta square
Multivariable	TGF-β1	MS	112.20	2/192	**< 0.001**	0.549
	IL-10	Age	3.77	2/192	**0.025**	0.039
		Sex	1.24	2/192	0.292	0.013
		Ethnicity	0.39	2/192	0.674	0.004
		BMI	1.12	2/192	0.329	0.012
		Smoking	4.27	2/192	**0.015**	0.044
Univariate	TGF-β1	MS (+)	124.72	1/191	**< 0.001**	0.403
	IL-10	MS (+)	88.87	1/191	**< 0.001**	0.325
		Age (−)	6.20	1/191	**0.014**	0.032
		Non-smoking (+)	8.52	1/191	**0.004**	0.044

All the results of analyses of variance (*F* values)

df: degree of freedom, *MS* multiple sclerosis, *BMI* body mass index, *TGF-β1* transforming growing factor beta 1, *IL-10* interleukin 10

rs3761548 FOXP3 -3279 C > A genotypes

The HWE of rs3761548 *FOXP3* -3279 C > A was observed among the female MS patients and female controls and was consistent with the expected ($p > 0.05$).

Table 3 shows the rs3761548 *FOXP3* -3279 C > A allelic and genotype frequency in different genetic models, according to the sex of the individuals. In all genetic models tested, we observed an association between this genetic variant and MS susceptibility in female individuals. Among females, the allele model showed that the presence of A allele was associated with MS (OR 2.23, 95% CI 1.54–3.22, $p < 0.001$).

In a codominant model, the results demonstrated that the frequency of the CC, CA and AA genotypes in females differed between patients with MS and controls (25.6%, 53.7% and 20.7% versus 46.9%, 48.5%, and 4.6%, respectively, $p < 0.001$). The CA and AA genotypes were associated with MS (OR 2.03, 95% CI 1.66–3.53, $p = 0.012$; OR 8.19, 95% CI 3.04–22.07, $p < 0.001$, respectively). With the dominant model, the CA + AA genotypes were also associated with MS (74.4% versus 53.1%) with OR of 2.57 (95% CI 1.50–4.37, $p < 0.001$). Furthermore, in the recessive model, the AA genotype was also associated with MS (20.7% versus 4.6%) with OR of 5.38 (95% CI 2.12–13.64, $p < 0.001$). After adjustment by age, ethnicity, BMI and smoking, all these results remained significant. On the other hand, we have also observed that the frequency of A allele of the rs3761548 did not differ in male patients with MS in comparison to male controls (56.7% versus 46.2%, OR 0.72 and 95% CI 0.30–1.69, $p = 0.338$).

In Table 4, we evaluated whether the genotyping, using the dominant model (CC versus CA + AA) could interfere in disability, disability progression, and clinical forms of MS in both female and male MS patients. Female MS patients carrying the CA + AA genotypes were older and had a higher frequency of moderate/high disability (EDSS > 3, $p = 0.047$) than those carrying the CC genotype. However, there was no association between the genotypes and ethnicity ($p = 0.636$), BMI ($p = 0.859$), smoking ($p = 0.165$), IL-10 levels ($p = 0.785$), age of diagnosis ($p = 0.086$), disability progression ($p = 0.354$), MS clinical forms ($p = 0.262$), and MS treatment ($p = 0.074$). In male MS patients, no association was observed between allele frequency and age ($p = 0.329$), ethnicity ($p = 0.880$), BMI ($p = 0.553$), smoking ($p = 0.565$),

Table 3 Distribution of rs3761548 *FOXP3* -3279 C > A genotypes and allelic frequencies among Brazilian patients with multiple sclerosis (MS) and controls according to their sex

Sex	Controls ($n = 182$)	MS patients ($n = 170$)	OR (95% CI)	p value	Adjusted OR (95% CI)[a]	Adjusted p value[a]
Female						
Allele model						
C	185 (71.5)	127 (52.5)	Reference	< 0.001	Reference	< 0.001
A	75 (28.8)	115 (47.5)	2.23 (1.54–3.22)		2.21 (1.51–3.24)	
Co-dominant model						
CC	61 (46.9)	31 (25.6)	Reference	–	Reference	–
CA	63 (48.5)	65 (53.7)	2.03 (1.66–3.53)	0.012	2.04 (1.14–3.63)	0.015
AA	6 (4.6)	25 (20.7)	8.19 (3.04–22.07)	< 0.001	9.38 (3.19–27.59)	< 0.001
Dominant model						
CC	61 (46.9)	31 (25.6)	Reference	< 0.001	Reference	0.001
CA + AA	69 (53.1)	90 (74.4)	2.57 (1.50–4.37)		2.56 (1.46–2.47)	
Recessive model						
CC + CA	124 (95.4)	96 (79.3)	Reference	< 0.001	Reference	0.001
AA	6 (4.6)	25 (20.7)	5.38 (2.12–13.64)		6.01 (2.18–16.56)	
Male						
Allele model						
C	28 (53.8)	31 (63.3)	Reference	0.338	Reference	0.567
A	24 (46.2)	18 (56.7)	1.45 (0.66–3.27)		0.72 (0.30–1.69)	

[a]Adjusted by age, ethnicity, body mass index, and smoking

Table 4 Demographic and clinical data in relation to rs3761548 *FOXP3* -3279 C > A dominant model for multiple sclerosis (MS) according to sex of the patients

	Female		p value	Male		p value
	CC ($n = 31$)	CA + AA ($n = 80$)		C ($n = 31$)	A ($n = 18$)	
Age (year)	35.0 (28.0–47.0)	44.5 (31.0–54.0)	0.043	36.0 (31.0–51.0)	37.0 (29.0–44.0)	0.329
Ethnicity (C/NC)	26 (83.9)/5 (16.1)	72 (80.0)/18 (20.0)	0.636	23 (74.2)/8 (25.8)	13 (72.2)/5 (27.8)	0.880
BMI (kg/m^2)	23.7 (20.3–29.7)	24.20 (21.3–28.4)	0.859	25.4 (22.3–28.6)	26.2 (24.1–30.2)	0.553
Smoking (Yes)	6 (20.0)	9 (10.2)	0.165	4 (12.9)	3 (17.6)	0.565
IL-10 (pg/mL)	4.330 (1.844–12.342)	5.709 (1.328–16.224)	0.785	3.609 (1.904–26.788)	6.165 (1.316–28.050)	0.950
Age of diagnosis (year)	30.0 (24.0–37.0)	35.0 (26.0–43.0)	0.086	32.0 (23.0–41.0)	37.0 (29.0–44.0)	0.152
EDSS > 3[a]	9 (33.3)	48 (55.2)	0.047	14 (46.7)	7 (41.2)	0.716
MSSS ≥ 5[b]	10 (40.0)	39 (50.6)	0.354	13 (48.1)	6 (40.0)	0.611
RRMS	26 (83.9)	82 (91.1)	0.262	23 (74.2)	15 (83.3)	0.460
Progressive MS	5 (16.1)	8 (8.9)		8 (25.8)	3 (16.7)	
MS treatment[c]						
Without treatment	6 (19.4)	6 (6.8)	0.074	4 (12.9)	0 (0.0)	0.254
Interferon β	11 (35.5)	53 (60.2)		22 (71.0)	11 (64.0)	
Glatiramer acetate	11 (35.5)	24 (27.3)		3 (9.7)	4 (23.5)	
Natalizumab	3 (9.7)	5 (5.7)		2 (6.5)	2 (11.8)	

Results of Mann–Whitney test. χ^2: results of analyses of contingency tables. Continuous variables were expressed as median and interquartile range (25–75%) and categorical variables were expressed as absolute number (n) and percentage (%)

BMI body mass index, *EDSS* Expanded Disability Status Scale, *MSSS* Multiple Sclerosis Severity Score, *RRMS* relapsing–remitting multiple sclerosis

[a]Eight patients did have the record of EDSS

[b]Twenty-six MS patients did have the record of MSSS

[c]Two patients did have the therapy recorded

IL-10 levels ($p=0.950$), age of diagnosis ($p=0.152$), disability ($p=0.716$), disability progression ($p=0.611$), MS clinical forms ($p=0.460$), and MS treatment ($p=0.254$).

Figure 2 shows the plasma levels of TGF-β1 according to rs3761548 *FOXP3* genotypes and alleles and the sex of MS patients. In a codominant model, female MS patients carrying the AA and CA genotypes showed higher TGF-β1 levels than those carrying the CC genotype [median 2137.3 pg/mL (IQR 808.8–4251.2) for CC genotype; median 3509.0 pg/mL (IQR 2043.74–4694.28) for CA genotype, and median 3872.0 pg/mL (IQR 1589.7–5175.5), for AA genotype, $p=0.043$]. With the dominant model, female MS patients carrying the CA + AA genotypes showed higher TGF-β1 levels than those carrying the CC genotype [median 3524.4 pg/mL (IQR 2003.9–4793.9) versus median 2137.2 pg/mL (IQR 808.7–4251.2), $p=0.019$]. However, in the recessive model, female MS patients with AA genotype showed no difference in the TGF-β1 levels compared to those with CC + CA genotypes [median 3873.0 pg/mL (IQR 1589.7–5175.5) versus median 3142.8 pg/mL (IQR 1435.19–4581.0), $p=0.329$]. TGF-β1 did not differ in male MS patients according to allele frequency, with median 3254.3 pg/mL (IQR 2032.6–4955.3) among those with C allele versus median 1759.2 pg/mL (IQR 1326.3–3785.6) among those with the A allele ($p=0.080$).

To delineate whether TGF-β1 levels and moderate/high disability (EDSS > 3) were independently associated with the dominant model (CC versus CA + AA) of rs3761548 *FOXP3* variant in female MS patients, we carried out three different automatic stepwise regression analyses models with TGF-β1 levels, disability, age, BMI, smoking, age on diagnosis, and MS treatment as dependent variables.

Table 5, regression #1 shows that TGF-β1 levels (OR 1.026, 95% CI 1.001–1.053, $p=0.045$) and moderate/high disability (EDSS > 3) (OR 2.678, 95% CI 1.026–6.995, $p=0.044$) were positively associated with the CA + AA genotypes. Entering age, ethnicity, smoking, and age of diagnosis in the model #2, TGF-β levels and moderate/high disability remained associated with the CA + AA genotypes (OR 1.027, 95% CI 1.000–1.055, $p=0.050$; OR 2.842, 95% CI 1.042–7.750, $p=0.041$), respectively. After entering the MS treatment in the model #3, TGF-β1 levels remained positively associated with the CA + AA genotypes in female MS patients (OR 1.027, 95% CI 1.001–1.054, $p=0.043$); however, the moderate/high disability was no longer significantly associated with the presence of CA + AA genotypes (OR 0.480, 95% CI 0.159–1.450, $p=0.193$).

Discussion

The main finding of the present study is that, in all the genetic models, the rs3761548 *FOXP3* -3279 C > A variant was associated with MS diagnosis in female patients. In the allele, codominant, dominant, as well as recessive models, the presence of A allele in heterozygosity or in homozygosity increased the chance to have the MS diagnosis. Other important result is that IL-10 and TGF-β1 plasma

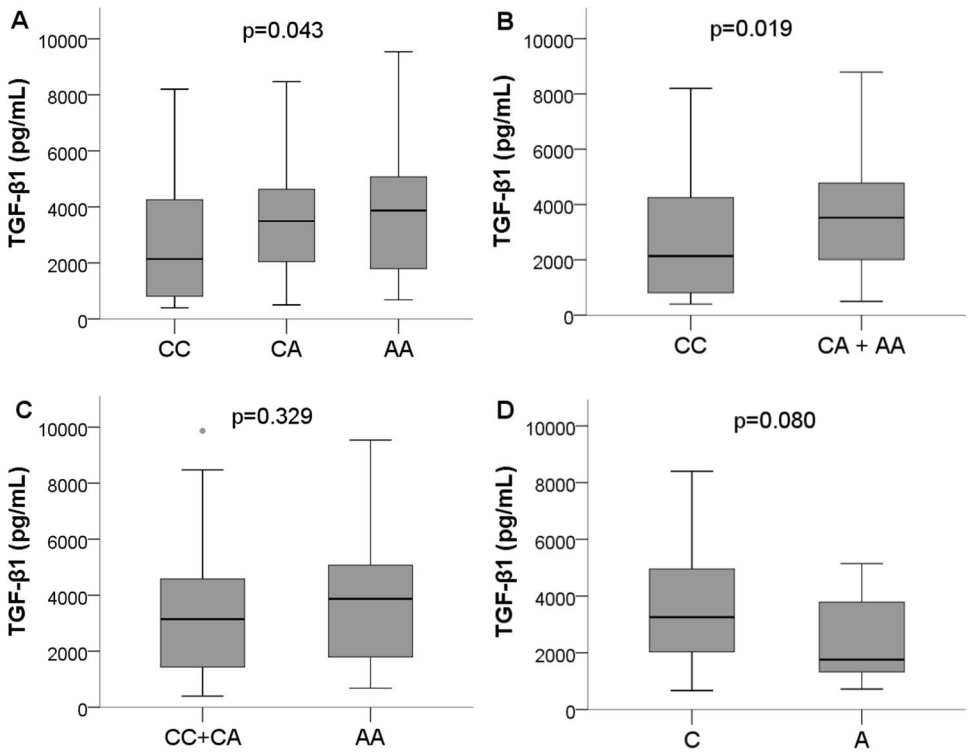

Fig. 2 Plasma levels of transforming growth factor beta 1 (TGF-β1) according to rs3761548 *FOXP3* -3279 C > A variant models and sex in multiple sclerosis (MS) patients. In female patients, TGF-β1 plasma levels were associated with MS according to: **a** codominant model (CC versus CA versus AA, $p=0.043$); **b** dominant model (CC versus CA + AA, $p=0.019$); **c** recessive model (CC + CA versus AA, $p=0.329$). **d** In male patients, TGF-β1 levels did not differ according to allele model (C versus A, $p=0.080$)

Table 5 Result of binary logistic regression analysis with the dominant model of *FOXP3* genetic variant in females MS patients as dependent variables

Sex	Model	Variable	Wald	*df*	*p* value	OR (CI 95%)
Female	#1	TGF-β1[a]	4.009	1	0.045	1.026 (1.001–1.053)
		EDSS > 3	4.046	1	0.044	2.678 (1.026–6.995)
	#2	TGF-β1[a]	3.838	1	0.050	1.027 (1.000–1.055)
		EDSS > 3	4.162	1	0.041	2.842 (1.042–7.750)
		Smoking	3.066	1	0.080	3.244 (0.869–12.109)
	#3	TGF-β1[a]	4.107	1	0.043	1.027 (1.001–1.054)
		EDSS > 3	1.692	1	0.193	0.480 (0.159–1.450)
		Age	3.548	1	0.060	1.35 (0.999–1.073)

MS multiple sclerosis, *TGF-β1* transforming growth factor β1

Model #1: TGF-β1 + EDSS

Model #2: TGF-β1 + EDSS + age + ethnicity + smoking + age of diagnosis

Model #3: TGF-β1 + EDSS + age + ethnicity + smoking + age of diagnosis + MS treatment

EDSS Expanded Disability Status Scale

[a]TGF-β1 plasma levels were transformed into square root because the data were in right obliquity

levels were higher in MS patients than controls, as well as female MS patients carrying the A allele in heterozygosity or homozygosity (CA + AA genotypes) showed higher levels of TGF-β1 than those female MS patients carrying the C allele in homozygosity (CC genotype). The presence of MS diagnosis was the explanatory variable that exerted the strongest effect in both TGF-β1 and IL-10 plasma levels (40.3% and 32.5%, respectively).

The higher IL-10 levels observed among the MS patients than controls are consistent with previous studies and underscored the role of IL-10 as anti-inflammatory cytokine produced by macrophages M2 and other cells to modulate the inflammatory autoimmune responses [9]. Huss et al. reported that IL-10 was being induced in a dose-dependent manner in Th1 cells by TGF-β and that this IL-10 expression in effector Th cells diminished the encephalitogenic capacity of myelin-specific Th1 cells [25]. Further, Huss et al. observed that the percentage of myelin-specific Th1 cells expressing IL-10, as well as the amount of IL-10, increased with each cycle of antigen activation, and that TGF-β1 induces IL-10 by the Th1 via Smad4 [26]. They also concluded that the encephalitogenicity could be restored by inhibiting IL-10. However, contradictory results are also reported [27]. According to these authors, MS patients have defects in peripheral immune regulation, including higher expression of costimulatory molecules on antigen-presenting cells (APCs), lower cytotoxic T lymphocyte antigen (CTLA)-4 levels, and lower IL-10 production [27].

The high levels of TGF-β1 observed in the present study are also in agreement with other studies carried out in MS patients [28–30], even in the cerebrospinal fluid (CSF) of MS patients during remissions [31] and in the stable phase of MS [32]. Both RRMS and progressive SPMS patients have more elevated blood levels of TGF-β1 compared with either healthy controls or patients with other neurological diseases, such as amyotrophic lateral sclerosis, multisystemic atrophy and lumbar discopathy [33]. However, other studies have contradicted these findings by reporting decreased TGF-β1 production in cultured peripheral blood mononuclear cells (PBMC) from patients with MS compared to healthy controls [34] and that T cell clones isolated from patients with MS during exacerbation were less likely to produce TGF-β1 upon proteolipid protein stimulation than those obtained during remission [35].

One study showed the disturbed cytokine levels in RRMS patients categorized as mild disability (EDSS 1.0–2.0) and moderate disability (EDSS 2.5–4.0) compared to controls. While IL-4 was increased in both RRMS patient groups, IL-10 was slightly increased in patients with minor neurological disability; however, TGF-β1 levels were decreased in both groups of RRMS patients compared to controls as well as did not differ between the both groups of RRMS patients [20].

Actually, TGF-β1 can have multifunctional roles and the net effect outcome appears to depend on the location, timing, and the nature of the cell that is targeted. In this respect, TGF-β1 has differential effects on virtually every cell type in the CNS [36–38]. It is also well established that in the presence of IL-6, TGF-β1 is critical for the induction of Th17 cells, while in the absence of IL-6, Tregs can be induced [39]. Some studies have demonstrated TGF-β1 as a strong immunoregulatory cytokine on antigen-presenting cells (APCs) both in vitro and in vivo [40]. These effects can be attributed to different major pathways including the control of IL-12 production by monocyte-derivate dendritic cells (moDCs) by the blockage of IL-12-induced tyrosine phosphorylation inhibiting the Jak–Stat pathway and differentiation of Th1 cell; subsequent inhibition of IFN-γ production by Th1 cells and the reactive oxygen species (ROS) production by

moDCs [40]. Moreover, TGF-β1, which is up-regulated in the remission phase of MS, induces microglia to secrete hepatocyte growth factor (HGF) to promote oligodendrocyte precursor cells (OPC) chemotaxis; with this effect, it may play pivotal role in myelin repair [37]. Yang et al. suggested that TGF-β1 negatively regulated the differentiation of encephalitogenic Th17 cells but that this can be overcome when myelin-specific T cells are reactivated in the absence of TGF-β1 [41]. TGF-β1 has also been shown to play a role in the differentiation of Th9 cells which produce robust amounts of IL-9 [42], a cytokine that negatively regulated Th17 cells.

Taken together, these data may suggest that enhancement of TGF-β1 signaling in T cells of MS patients may be beneficial. Although TGF-β1 has been implicated in some potentially inflammatory T cell populations, such as Th9 and Th17 cells, the data are not compelling in human CD4 T cells. In contrast, there is a consensus that TGF-β1 enhances Treg development and function, and negatively regulates encephalitogenic Th1 cells [43]. Therefore, we hypothesized that the increased TGF-β1 and IL-10 plasma levels observed among the MS patients in the present study, probably, could represent an endogenous anti-inflammatory response aimed at counteracting ongoing immunoinflammatory events in the MS.

The association between rs3761548 *FOXP3* variant with MS susceptibility among female patients in the present study in three genetic models, suggest a functional defect on Treg in patients with the AA and CA genotypes. The functional consequence of this variant in the promoter region of *FOXP3* is the loss of binding of some transcription factors, such as E47 and C-Myb, leading to defective transcription of *FOXP3* [16], and, therefore, might affect the function or quantity of Tregs [44]. Similarly with our results, the other authors found an association between rs3761548 *FOXP3* variant and MS in population of Iran [14, 15]. The frequencies of AA and CA genotypes were also higher in MS group as compared with healthy subjects [14]. On the other hand, other study failed to demonstrate this association [17].

The rs3761548 *FOXP3* genotypes have been associated with the function of Tregs in different conditions. While CC genotype was associated with the normal suppressor role of Tregs [45], the AA genotype was associated with impaired immune tolerance [46, 47]. Individuals with the rs3761548 *FOXP3* CC genotype showed better graft survival in kidney transplantation than those with the CA or AA genotype [45]. On the other hand, patients with the AA genotype showed a weaker Treg function and were more prone to allograft rejection in kidney transplantation than those with the CC genotype [46]. In addition, an association between the rs3761548 AA genotype and lower graft survival has been reported in Indian individuals [47]. Moreover, the rs3761548 *FOXP3* AA genotype has been associated with other autoimmune diseases, including psoriasis in Chinese individuals [11], and intractability of Graves' disease in Japanese individuals [10].

The high levels of TGF-β1 observed among the female MS patients carrying the CA + AA genotypes in the present study may be explained, in part, by the fact of many other cell types, such as macrophages M2, leukocytes and stromal cells, are able to generate TGF-β1 [9]. Considering that TGF-β1 is able to convert $CD4^+CD25^-$ non-Treg cells into $CD4^+CD25^-$ Treg cells, and this conversion was accompanied with increased Foxp3 expression [48, 49], one possible hypothesis is that the high levels of TGF-β1 observed in patients with the CA + AA genotypes, could be produced by other cells than Treg in an attempt to restore the number and function of Treg cells that may be decreased in these patients.

The inconsistent results observed on previous reported studies may be explained by the different autoimmune diseases included at them, the genetic heterogeneity presented by the genotyped individuals, the genotyping methods (RFPL versus non-RFLP), the assay for the cytokine measurement, as well as the sample size and the control population (hospital-based controls versus community-based controls) [50].

Some limitations of this study should be considered. This is a case–control design, which does not allow inferences on causal relationship. Moreover, the small number of male individuals included in the study, the single basal TGF-β1 and IL-10 measurement and one specific genetic variant analysis, which precludes the assessment of how other factors may impact on the complex relationship between *FOXP3* genotype, cytokine levels and MS. The study included patients with different clinical forms of MS, as well as treated with different MS therapies; however, all of them were in remission clinical phase of the disease and the results were adjusted by clinical forms and MS therapy. The statistical analysis with adjusting for many confounding variables including age, sex, ethnicity and smoking may be one of its strengths.

To our knowledge, this is the first study to investigate the relationship between rs3761547 *FOXP3* -3279 C > A variant in MS patients from Brazilian population. Taken together, our results demonstrated that rs3761548 *FOXP3* CA + AA genotypes were associated with MS diagnosis and higher levels of TGF-β1 among female MS patients than those with the CC genotype. However, the presence of the A allele was not associated with moderate/high disability as well as disability progression. These data suggest that the A allele of *FOXP3* -3279 C > A variant may be associated with the quantitative or functional alteration of Treg cells, which could be one of the factors involved in susceptibility to MS in females. The complex interaction between *FOXP3* genetic variants, TGF-β1 levels and MS deserves further investigation.

Author contributions Conception and research design: EMVR and ANCS; manuscript writing and discussion of results: EMVR, ANCS, and TF; data collection: DRK-M, WLCJP, TF, DFA, APK, and SRO, who contributed equally; laboratory analysis: TF, DFA, MABL, and KBO; statistical analysis: ANCS and DFA. All the authors have read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The protocol was approved by the Institutional Research Ethics Committees of University of Londrina, Paraná, Brazil (CAAE: 22290913.9.0000.5231) and all the individuals invited were informed in detail about the research and gave written Informed Consent.

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