The Effects of the MTHFR 677C>T (rs1801133) Genetic Variant on Susceptibility and Disability in Multiple Sclerosis Patients are Mediated by Homocysteine but not Folate Levels

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Abstract

We investigated whether the *MTHFR* 677C>T (rs1801133) variant and plasma homocysteine and folate are associated with multiple sclerosis (MS), disability, disability progression, and inflammatory biomarkers. We included 163 MS patients categorized using the Expanded Disability Status Scale (EDSS) as mild (EDSS<3) and moderate/high (EDSS≥3) disability, and 226 healthy controls. Disability progression was evaluated using Multiple Sclerosis Severity Score (MSSS) and the *MTHFR* 677C>T was genotyped using real time polymerase chain reaction. The levels of some inflammatory biomarkers and inflammatory activity index (IAI) were determined. There was no association between the *MTHFR* 677 C>T genotypes and MS, EDSS, and MSSS ($p>0.05$). Plasma folate and homocysteine were higher and adiponectin was lower in MS patients than controls ($p<0.001$). Moreover, 21.8% of the EDSS variance was explained by age, IAI and C-reactive protein (CRP) (all positively associated); 10.9% of the MSSS variance was predicted by IAI and CRP (both positively) and vitamin D3 (negatively), whereas 54.4% of the MS-EDSS-MSSS score was explained by the regression on age, IAI, homocysteine, folate, and CRP (all positively) and adiponectin, body mass index, and vitamin D3 (all negatively), female sex and the *MTHFR* 677 TT genotype. In patients and controls, 16.6% of the variance in the homocysteine was explained by the *MTHFR* 677 TT genotype and age (both positively), folate (negatively) and male sex. In conclusion, the *MTHFR* 677C>T variant was not directly associated with MS, disability, and disability progression; however, the TT genotype showed indirect effects on MS susceptibility and disability mediated by homocysteine.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory illness caused by an autoimmune response. It is characterized by demyelination of the central nervous system (CNS), which is frequently followed by progressive and irreversible neurological impairment. Although the etiology of MS is not fully characterized, it has been recognized that interactions between genetic and environmental variables contribute to the autoimmune inflammatory process [1]. Vitamin D, folate, and B12 deficiency, as well as elevated homocysteine levels, are all significant environmental variables related with the pathogenesis of MS [2–8].

Hyperhomocysteinemia, or elevated homocysteine levels, may have harmful effects on neurons and blood vessels, including endothelial dysfunction and oxidative damage, contributing to the development of neurodegenerative disorders such as MS [9–12]. Homocysteine is a pro-inflammatory biomarker whose plasma levels are controlled by a variety of physiological and acquired factors and their interactions, including age, sex, medications, and *MTHFR* genetic variations [13–15]. The *MTHFR* gene encodes methylenetetrahydrofolate reductase (MTHFR), a critical enzyme involved in the metabolism of folate and homocysteine [16]. MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate, the predominant folate form in plasma and a carbon donor for homocysteine remethylation to methionine [17]. Numerous genetic variations in the *MTHFR* have been found, the most studied and clinically significant of which is the 677C>T (rs1801133) in exon 4, which results in the substitution of alanine for valine at position 222 (p.Ala222Val or A222V) of the protein [18, 19]. There have been reports of racial-ethnic disparities in the distribution of the *MTHFR* 677C>T variant [20, 21], as well as a relationship between the *MTHFR* 677C>T variant and elevated homocysteine levels [22–26]. Individuals carrying the TT genotype had decreased MTHFR enzyme activity and elevated plasma homocysteine levels [27, 28]. Previous research has revealed elevated plasma homocysteine levels in patients with MS when compared to healthy controls [8, 29, 30]. Homocysteine levels are significantly higher in MS patients than in controls, as well as in those with moderate/high disability compared to those with mild disability [8]. There have been observed discrepancies in the relationships between MS and plasma homocysteine, vitamin B12, and folate levels. While some studies found that MS patients had higher homocysteine levels and lower vitamin B12 and folate levels than controls [30, 31], others found no difference in homocysteine, vitamin B12, or folate levels between MS patients and controls [32–34]. Given the lack of knowledge regarding the relationship between *MTHFR* 677C>T (rs1801133), homocysteine, and folate in MS patients and their role in the clinical course of the disease, the purpose of this study was to determine whether the *MTHFR* 677C>T variant and plasma homocysteine and folate levels are associated with MS susceptibility, disability, disability progression, and inflammatory biomarkers.

Material And Methods

Subjects

The case-control study included 163 MS patients, adults and both sexes, consecutively recruited from the Demyelinating Diseases Outpatient of the State University of Londrina, Londrina, Paraná, South Brazil, one of the specialized reference centers for the diagnosis and treatment of MS at the Parana State, Southern Brazil. The MS diagnosis was established according to the McDonald criteria [35]. The patients were clinically evaluated for disability using the Expanded Disability Status Scale (EDSS) [36]. Based on their EDSS scores, the patients were divided into two groups: EDSS < 3 (mild disability) and EDSS ≥3 (moderate/high disability). Disability progression was evaluated using the Multiple Sclerosis Severity Score (MSSS), as proposed elsewhere [37] and score ≥ 5.0 denoted higher than the average speed of disability accumulation [38]. A single MS severity index was entered as a latent vector extracted from the MS diagnosis, EDSS and MSSS scores, named as MS-EDSS-MSSS.

All MS patients were in the remission clinical phase, defined as the period of recovery with no relapse episodes within the last three months prior to the time of enrollment in the study. As controls, 226 healthy individuals (HC) were selected among blood donors of the Regional Blood Bank of Londrina, from the same geographic region of the MS patients. None of the study's participants had clinical symptoms or laboratory biomarkers for

cardiovascular, thyroid, kidney, hepatic, gastrointestinal, or oncologic disorders, as well as other inflammatory and autoimmune diseases. Demographic, epidemiological and anthropometric data (for MS patients and HC), as well as clinical history and the use of therapy for MS before the inclusion in this study (for MS 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 classified according to individual's self-perception of skin color as Caucasian and non-Caucasian [39]. Other data were obtained including waist circumference, current smoking, systemic arterial hypertension (SAH), metabolic syndrome (MetS) and type 2 diabetes mellitus (T2DM). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured twice and the mean of these two measurements was used in the analysis. Moreover, use of antihypertensive medication was an indication of SAH [40]. T2DM was defined as a fasting serum glucose ≥ 126 mg/dL and/or use of hypoglycemic medication [41].

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 samples

Peripheral blood samples were drawn after 12 h of fasting using vacuo system tubes (Vacutainer System, Becton-Dickinson, New Jersey, U.S) with and without ethylenediamine tetra-acetic acid (EDTA) as anticoagulant. The specimens were centrifuged at 2,500 rpm for 10 minutes within 2 hours after sampling. The buffy-coat, plasma and sera were frozen at -80°C until analysis.

MTHFR 677C>G Genetic Variant Genotyping

Genomic DNA was extracted from the buffy-coat of peripheral blood cells using a resin column procedure (Biopur, Biometrix Diagnostika, Curitiba, Brazil) following the manufacturer's instructions. DNA concentration was measured using a spectrophotometer at 260 nm (NanoDrop 2000c™, ThermoScientific, Waltman, MA, USA) and the DNA purity was assessed by 260/280 nm ratio. The *MTHFR* 677C>T (rs1801133) variant was determined using TaqMan® allelic discrimination validated assay on real-time polymerase chain reaction (qPCR) system (StepOne, Applied Biosystems by Life Technologies, Carlsbad, CA, USA) with the allele-specific fluorogenic oligonucleotide probe (C__1202883_20). The reaction was performed using 5 ng of genomic DNA, 0.25 μL TaqMan SNP Genotyping Assay 40x (Applied Biosystems, Foster City, CA, USA) containing two sequence-specific primers and two allele-specific TaqMan® MGB probes with a reporter dye at its 5' end (VIC® or FAM™), 5 μL TaqMan Universal Genotyping Master Mix (Applied Biosystems, Foster City, CA, USA). Negative and positive controls were also included in the reactions.

Inflammatory Biomarkers

Plasma levels of homocysteine, folate, and vitamin D3 (measured as 1,25 dihydroxyvitamin D) were determined by chemiluminescence microparticle immunoassay (CMIA, Architect, Abbott Laboratory, Abbott Park, IL, USA). The reference values for homocysteine are up to 9.0 $\mu\text{mol/L}$ (male) and up to 7.0 $\mu\text{mol/L}$ (female); for the folate, the values are 3.1-20.5 ng/mL, as recommended by the manufacturers. Uric acid plasma levels were evaluated using a biochemical auto-analyzer (Dimension Dade AR Dade Behring, Deerfield, IL, USA). C-reactive protein (CRP) determined with high sensitivity assay (hsCRP) using turbidimetry (Architect C8000, Abbott Laboratory, Abbott Park, IL, USA), and plasma levels of interleukin (IL)-2, IL-4, IL-6, IL-10, interferon (IFN)- γ , tumor necrosis factor (TNF)- α , soluble TNF- α receptor (sTNFR)1 and sTNFR2 were determined using immunofluorimetric method with microspheres multiplex immunoassay (Novex Life Technologies, Frederick, USA) for Luminex platform in MAGPIX® instrument (Luminex Corp., TX, USA).

Two new composed inflammatory scores were proposed: the first, namely as immune activation index (IAI), was entered as a latent vector extracted from macrophage M1 cytokines (IL-6 and TNF- α) + T helper (Th)1 cytokines (IL-2 and IFN- γ) + Th17 cytokines (IL-6 and IL-17), and Th2+T regulatory (Treg) cytokines (IL-4 and IL-10); the second, namely as TNF- α and its receptors, was entered as a latent vector extracted from TNF- α + sTNFR1+sTNFR2.

Statistical analysis

Analysis of variance (ANOVA) was employed to assess differences in continuous variables between study groups, and analysis of contingency tables (chi-square test) to check associations between classifications. Univariate general linear models (GLM) analysis was performed to assess the differences in biomarkers between three subgroups (HC and two patient classes). Multiple pair-wise differences were assessed with protected Least Significant Difference (LSD) tests. Automatic multiple regression analysis was employed to predict dependent variables (e.g., the EDSS or MSSS scores) using explanatory variables (e.g., biomarkers, age, sex, and BMI). An automatic stepwise (step-up) method was used with a p-to-enter of 0.05 and p-to-remove 0.06 while checking R^2 changes, homoscedasticity (using White and modified Breusch-Pagan tests for homoscedasticity), multicollinearity (using tolerance and VIF), and multivariate normality (Cook's distance and leverage). Results of multiple comparisons were p-corrected for false discovery rate (FDR) [42]. Automatic binary logistic regression analysis was conducted with MS or MS subgroups as dependent variables and the biomarkers as input variables. Odds ratios (OR) with 95% confidence intervals (CI) and the accuracy of classification (with sensitivity and specificity) were computed and Nagelkerke's pseudo- R^2 values were used as effect size measurement. The results of these regression analyses were always bootstrapped using 5.000 bootstrap samples and the latter results are shown if the results are not concordant. All tests are two-tailed and a p-value of 0.05 was employed to determine statistical significance. Statistical analyses were carried out using IBM SPSS Windows version 25, 2017.

Partial Least Squares (SmartPLS) analysis [43] was used to measure the multi-step multiple mediation associations between biomarkers (input variables) and the MS-severity index. The latter was entered as a latent vector extracted from the EDSS and MSSS scores and the diagnosis MS. All biomarkers were entered as single indicators except IAI, which was entered as a latent vector extracted from different immune profiles. Both latent vectors were entered as reflective models. Consequently, complete PLS path analysis using 5.000 bootstrap samples was performed only when the outer and inner models complied with pre-specified quality data: a) all outer model loadings on both latent vectors are > 0.7 at $p < 0.001$ and all latent vectors show good construct validity as indicated by Cronbach's alpha (> 0.7), composite reliability (> 0.7), rho A (> 0.8), and average variance extracted (AVE) > 0.5 ; b) the overall fit of the model is adequate as indicated by Standardized Root Mean Squared Residual (SRMR) < 0.08 ; and c) Confirmatory Tetrad analysis indicates that both latent vectors models are not mis-specified as reflective models. PLS predict with 10-fold cross-validation was used to assess the predictive performance when analyzing new data. Predicted-Oriented Segmentation analysis, Multi-Group Analysis and Measurement Invariance Assessment were employed to examine compositional invariance.

Results

Sociodemographic and clinical data

Table 1 shows the sociodemographic and clinical data of the HC and MS patients divided into those with and without an increased EDSS score (≥ 3 as threshold value). This table presents the data on all HC and the selected group of MS patients who were not treated with folic acid ($n=141$) because all computations (except the genotypic associations) are calculated using this data set. Among these 141 MS patients, 58 (41.1%) presented mild disability (EDSS < 3) and 83 (58.9%) presented moderate/high disability (EDSS ≥ 3). Univariate analysis showed that MS patients with moderate/high disability were older and showed a higher frequency of MetS than those with mild disability and HC ($p < 0.001$). Patients with moderate/high disability showed a higher frequency of SAH and T2DM than HC ($p=0.011$ and $p=0.049$, respectively). There were no significant differences regarding sex, BMI, ethnicity and smoking between the three study groups. Patients with moderate/high disability showed higher duration of disease and disability progression than those with mild disability ($p < 0.001$). There were significantly more patients with progressive clinical forms (SPMS+PPMS) in the patient group with EDSS ≥ 3 as compared with the EDSS < 3 group.

Table 1

Socio-demographic and clinical data of patients with multiple sclerosis (MS), subdivided according to the EDSS threshold value of ≥ 3 and healthy controls (HC)

Variables	HC ^A (n= 226)	MS EDSS<3 ^B (n = 58)	MS EDSS ≥ 3 ^C (n = 83)	F/ χ^2	df	p value
Age (years)	36.3 (10.6) ^C	36.1 (10.3) ^C	46.4 (14.0) ^{A,B}	25.42	2/363	<0.001
Sex (Femae/Male)	155/71	38/20	58/25	0.311	2	0.856
BMI (Kg/m ²)	25.37 (4.47)	25.15 (4.63)	25.64 (5.15)	0.80	2/356	0.821
MetS (No/Yes)	182/39 ^C	51/7 ^C	48/28 ^{A,B}	15.86	2	<0.001
Duration of illness (years)	-	5.57 (4.55) ^C	8.24 (7.76) ^B	5.54	1/139	0.020
Clinical forms		58	64	-	-	<0.001
RRMS		0	14			
SPMS		0	05			
PPMS						
EDSS	-	1.01 (0.86) ^C	4.91 (1.61) ^B	282.86	1/139	<0.001
MSSS	-	1.89 (1.65) ^C	7.02 (2.20) ^B	204.33	1/126	<0.001
Ethnicity C/NC	179/47	47/11	58/25	3.54	2	0.170
SAH (No/Yes)	170/21 ^C	49/9	62/21 ^A	9.11	2	0.011
T2DM (No/Yes)	176/29 ^C	56/2	76/7 ^A	6.02	2	0.049
Smoking (No/Yes)	200/26	54/4	70/13	2.56	2	0.278
Continuous variables were expressed as mean (SD); the categorical variables were expressed as number (n); A: healthy controls; B: MS EDSS <3; Multiple sclerosis patients with EDSS < 3; C: MS EDSS ≥ 3 ; Multiple sclerosis patients with EDSS ≥ 3 ; df: degree of freedom; BMI: body mass index; MetS: metabolic syndrome; RRMS: relapsing-remitting multiple sclerosis; SPMS: secondary progressive multiple sclerosis; PPMS: primary progressive multiple sclerosis; EDSS: Expanded Disability Status Score; MSSS: Multiple Sclerosis Severity Score; C: Caucasian; NC: non-Caucasian; SAH: systemic arterial hypertension; DMT2: type 2 diabetes mellitus						

Biomarker data in MS subgroups

Table 2 shows the biomarker assessment in HC and MS patients divided into two subgroups using the EDSS score. Both the HC group ($\chi^2 = 0.01$, df=1, p=0.907) and total study group ($\chi^2 = 0.15$, df=1, p=0.698) were in Hardy-Weinberg equilibrium regarding the *MTHFR* allelic frequencies. The first part of the table (computed on the total sample) shows that there were no significant associations between these diagnostic groups and the *MTHFR* phenotypes using different genetic models. The second part of the table (calculated on the selected study groups) shows the measurements of the non-genetic biomarkers in the study groups. We found that folate and homocysteine were significantly higher in MS patients than in HC, whereas adiponectin was reduced in both patient groups. There were no significant differences in vitamin D3 and uric acid between the three groups. CRP was significantly higher in patients with an EDSS score ≥ 3 *versus* those with EDSS <3. The M1, Th1, Th17, and Th2 Treg cytokine values, named IAI, were significantly different between the three study groups and increased from HC to MS patients with EDSS<3 and to MS patients with EDSS ≥ 3 . The composite score of the levels of TNF- α and their soluble receptors sTNFR1 and sTNFR2 was significantly higher in MS patients with EDSS ≥ 3 than in the two other groups (EDSS<3 and HC). These differences remained significant after FDR p-correction.

Table 2
Genetic and biomarker data in patients with multiple sclerosis (MS) divided according to an EDSS threshold of ≥ 3 and controls

Variables	Controls ^A (n=226)	MS, EDSS<3 ^B (n=74)	MS, EDSS ≥ 3 ^C (n=89)	F/ χ^2	df	p value	R ²
<i>MTHFR</i> 677C>T model							
Codominant CC vs. CT vs. TT	106/97/23	35/31/8	41/47/10	0.94	4	0.919	-
Dominant CC vs. CT + TT	106/120	35/39	41/57	0.80	2	0.671	-
Recessive CC + CT vs. TT	203/23	66/8	88/10	0.03	2	0.987	-
Overdominant CC vs. TT	129/97	43/31	51/47	0.86	2	0.649	-
Folate (ng/mL)*	6.07 (0.40) ^{B,C}	10.37 (0.78) ^A	9.61 (0.65) ^A	18.37	2/253	<0.001	0.127
Hcy (μ mol/L)*	10.48(0.27) ^{B,C}	11.46(0.52) ^A	12.67(0.46) ^A	10.03	2/346	<0.001	0.055
Adiponectin (μ g/mL)*	7.34(0.25) ^{B,C}	2.74(0.42) ^A	2.97(0.39) ^A	127.59	2/292	<0.001	0.467
Vitamin D3 (ng/mL)*	30.11(0.94)	29.52(1.76)	30.32(1.56)	0.89	2/334	0.411	0.005
Uric acid (mg/dL)*	4.32 (0.08)	4.05(0.15)	4.05(0.13)	2.27	2/355	0.105	0.013
CRP (mg/L)*	3.29 (0.67)	2.18 (1.31) ^C	4.97 (1.17) ^B	2.53	2/349	0.081	0.014
M1	-0.562(0.098) ^{A,C}	0.189(0.192) ^{A,C}	1.014(0.169) ^{A,B}	32.89	2/360	<0.001	0.154
Th1	-.579(0.107) ^{B,C}	0.221(0.209) ^{A,C}	0.779(0.183) ^{A,B}	21.97	2/360	<0.001	0.109
Th17	-.790(0.104) ^{B,C}	0.474(0.203) ^{A,C}	1.166(0.178) ^{A,B}	49.37	2/360	<0.001	0.215
Th2 + Treg	-.041(0.103) ^{B,C}	0.802(0.202) ^{A,C}	1.420(0.177) ^{A,B}	85.95	2/359	<0.001	0.324
TNF- α + receptors	-0.178(0.063) ^C	-0.159(0.122) ^C	0.581(0.107) ^{A,B}	18.58	2/359	<0.001	0.094
*All results of univariate GLM adjusted for age, sex and Body mass index (BMI), and performed as log transformation. A: healthy controls; B: Multiple sclerosis patients with EDSS < 3; C: Multiple sclerosis patients with EDSS ≥ 3 ; df: degree of freedom; R ² : Nagelkerke's pseudo-R ² values were used as effect size measurement. <i>MTHFR</i> 677C>T methylenetetrahydrofolate reductase genetic variant with C as the major allele and T as the minor allele; Hcy: homocysteine; CRP: C-reactive protein. M1: as zIL-6 + zTNF α ; Th1: as zIL-2 + zIFN- γ ; Th17: as zIL-17 + zIL-6; Th2 + Treg: as zIL-4 + zIL-10; TNF- α + receptors: TNF- α + soluble TNF- α receptors: as zTNF- α + zsTNFR1 + zsTNFR2							

Prediction of MS and subgroups using biomarkers

Table 3, regression #1, shows the results of a binary logistic regression analysis with MS as dependent variable and HC as reference group and all biomarkers shown in Table 2 as explanatory variables while allowing for the effects of age, sex, BMI, MetS, SAH, and T2DM. We found that folate, homocysteine, IAI, and age (all positively), adiponectin (negatively) and female sex were significant predictors of MS ($\chi^2=327.38$, df=6, p<0.001) with a Nagelkerke pseudo-R² value of 0.788 and an accuracy of 89.0% (sensitivity=84.7% and specificity=92.2%). The *MTHFR* 677C>T genotypes were not significant in this model. Regression #2 shows that the disability (MS patients with EDSS ≥ 3 versus those with EDSS<3 as reference group) was significantly ($\chi^2=31.69$, df=3, p<0.001) associated with increased age, IAI, and CRP (all positively) with a Nagelkerke pseudo-R² value of 0.271 and an accuracy of 71.6% (sensitivity=74.7% and specificity=67.2%). The *MTHFR* genotypes were again not significant.

Table 3
Results of binary logistic regression analysis with multiple sclerosis patients with EDSS ≥ 3 as dependent variable

Dependent Variables	Explanatory variables	B	SE	Wald (df=1)	p value	OR	95% CI
MS vs. HC	Adiponectin	-2.718	0.381	53.62	<0.001	0.06	0.03 - 0.13
	Folate	1.535	0.304	25.44	<0.001	4.64	2.56 - 8.43
	Homocysteine	1.116	0.247	20.45	<0.001	3.05	1.88 - 4.95
	IAI	2.052	0.479	18.39	<0.001	7.79	3.05 - 19.90
	Sex (male)	-1.870	0.483	15.00	<0.001	0.15	0.06 - 0.40
	Age	0.056	0.017	11.40	0.001	1.06	1.02 - 1.09
EDSS ≥ 3	Age	0.062	0.016	14.30	<0.001	1.06	1.03 - 1.10
vs. EDSS <3	IAI	0.760	0.355	4.59	0.032	2.14	1.06 - 4.28
	CRP	0.444	0.208	4.56	0.033	1.56	1.04 - 2.34
OR: odds ratio; CI: confidence interval; MS: Multiple Sclerosis; HC: healthy controls.							
EDSS: Expanded Disability Status Score; IAI: inflammatory activity index, computed as first principal component extracted from M1, Th1, Th17, Th2Treg, TNF- α + TNFR1 + TNFR2; M1: zIL-6 + zTNF α ; Th1: zIL-2 + zIFN- γ ; Th17: zIL-17 + zIL-6; Th2 + Treg: zIL-4 + zIL-10; TNF- α + receptors: TNF- α + soluble TNF- α receptors: zTNF- α + zTNFR1 + zTNFR2; CRP: C-reactive protein							

Prediction of EDSS and MSSS scores by biomarkers

Table 4 shows the results of multiple regression analyses with the MS-EDSS-MSSS scores as dependent variables and all biomarkers as explanatory variables while allowing for the age, sex, BMI, SAH, and T2DM. We found that 21.8% of the variance in the EDSS score in MS patients was explained by age, IAI and CRP (all positively associated). IAI and CRP (both positively) and vitamin D3 (negatively) predicted 10.9% of the variance in the MSSS score. Not one of the genotypic *MTHFR* 677C>T models was significant in this regression model. The third multiple regression in Table 4 shows that 54.4% of the variance in the MS-EDSS-MSSS score was explained by the regression on age, IAI, homocysteine, folate, and CRP (all positively associated) and adiponectin, BMI, and vitamin D3 (all negatively associated), female sex and the *MTHFR* TT genotype.

Table 4

Results of multiple regression analysis of multiple sclerosis patients with EDSS and MSSS as dependent variables and biomarkers as explanatory variables

Dependent variables	Explanatory variables	β	f	p value	F	df	p value	R ²
EDSS in MS	Model #1				13.59	3/146	<0.001	0.218
	Age	0.329	4.34	<0.001				
	IAI	0.148	2.70	0.008				
	CRP	0.162	2.16	0.033				
MSSS in MS	Model #2				5.97	3/146	0.001	0.109
	IAI	0.199	2.55	0.012				
	CRP	0.184	2.35	0.020				
	Vitamin D3	-0.157	-2.01	0.046				
EDSS+MSSS + MS in all subjects	Model #3				43.39	10/363	<0.001	0.544
	Adiponectin	-0.427	-10.08	<0.001				
	Age	0.211	5.28	<0.001				
	IAI	0.250	6.54	<0.001				
	Homocysteine	0.239	6.06	<0.001				
	Folate	0.162	4.10	<0.001				
	Sex	-0.097	-2.48	0.014				
	CRP	0.129	3.22	0.001				
	BMI	-0.113	-2.86	0.004				
	<i>MTHFR 677C>T</i>	-0.086	-2.38	0.018				
	(recessive model)							
	Vitamin D3	-0.072	-1.98	0.049				

df: degree of freedom; R²: Nagelkerke's pseudo-R² values were used as effect size measurement. EDSS: Expanded Disability Status Score; MSSS: Multiple Sclerosis Severity Score; IAI: inflammatory activity index, computed as first principal component extracted from M1, Th1, Th17, Th2, Treg, TNF- α + TNFR1 + TNFR2; CRP: C-reactive protein; BMI: body mass index; *MTHFR 677C>T* methylenetetrahydrofolate reductase genetic variant with C as the major allele and T as the minor allele: recessive model: CC +CT vs. TT

Prediction of homocysteine and folate using *MTHFR 677C>T* genotypes

Table 5 shows the results of multiple regression analyses with homocysteine as dependent variable and folate and *MTHFR* genotypes as explanatory variables while allowing for the effects of age, sex, and BMI. In the total study group, we found that 16.6% of the variance in homocysteine was explained by the *MTHFR* TT genotype and age (both positively) and folate (negatively) and male sex. In the HC group, the same variables predicted 15.9% of the variance in homocysteine levels. In the total study group, 4.5% of the variance in folate levels was predicted by the *MTHFR* additive model (negatively) and age (positively). We found that 11.7% of the variance in the IAI was explained by the regression on adiponectin and age (both negatively) and folate (positively). The *MTHFR* genotypic models were not significant in this regression.

Table 5
Results of multiple regression analysis of multiple sclerosis patients with homocysteine and folate as dependent variables and biomarkers as explanatory variables

Dependent variables	Explanatory variables	β	F	p value	F	df	p value	R ²
Hcy in all subjects	Model #1				18.44	4/370	<0.001	0.166
	Age	0.301	6.23	<0.001				
	Folate	-0.173	-3.56	<0.001				
	Sex	0.178	3.74	<0.001				
	<i>MTHFR</i> 677C>T recessive model	0.129	2.69	0.007				
Hcy in HC	Model #2				10.37	4/220	<0.001	0.159
	Folate	-0.269	-4.25	<0.001				
	<i>MTHFR</i> 677C>T recessive model	0.187	3.01	0.003				
	Age	0.168	2.67	0.008				
	Sex	0.159	2.57	0.011				
Folate in all subjects	Model #1				8.78	2/372	<0.001	0.045
	Age	0.178	3.51	0.001				
	<i>MTHFR</i> 677C>T Additive model	-0.118	-2.33	0.020				
IAI in all subjects	Model #2				16.34	3/370	<0.001	0.117
	Adiponectin	-0.267	-5.28	<0.001				
	Folate	0.143	2.78	0.006				
	Age	-0.124	-2.51	0.013				

df: degree of freedom; R²: Nagelkerke's pseudo-R² values were used as effect size measurement.; Hcy: homocysteine; HC: healthy controls; IAI: inflammatory activity index computed as first principal component extracted from M1, Th1, Th17, Th2, Treg, TNF- α + sTNFR1 + sTNFR2; *MTHFR* 677C>T methylenetetrahydrofolate reductase genetic variant with C as the major allele and T as the minor allele: recessive model: CC + CT vs. TT; additive model: CC=-1; CT=0; TT=+1

Results of PLS analysis

The associations between the *MTHFR* genotypic models and homocysteine with folate as putative mediator (while allowing for the effects of age, sex, and BMI) were firstly examined in the HC group. Figure 1 shows the results of complete PLS path analysis conducted on 5.000 bootstrap samples. We found that 15.9% of the variance in homocysteine was explained by the TT genotype, age, and folate (all positively) and male sex. In addition, 5.7% of the variance in folate was predicted by the additive *MTHFR* genotype. Most importantly, the specific indirect effect of this additive model on homocysteine mediated by folate was not significant ($t=1.64$, $p=0.14$). Folate partly mediated the effects of age on homocysteine ($t=2.45$, $p=0.014$). All in all, there were significant direct effects of the TT genotype, but not any of the other genotypes, on homocysteine.

Consequently, we have examined the same associations in the total study group after entering the other biomarkers of MS, namely IAI (entered as a latent vector extracted from the M1, Th1, Th17 and Th2Treg cytokine profiles), adiponectin, uric acid, age, sex, CRP, BMI (all entered as single indicators). The final outcome variable, named MS-EDSS-MSSS, was a latent vector extracted from MS, EDSS, and MSSS, which thus is an index of MS and its severity. We used a multi-step multiple mediation model whereby the effects of the *MTHFR* genotypes (recessive and additive models), age, sex and BMI could be explained by the biomarkers. Figure 2 shows the PLS path model using complete PLS path analysis on 5.000 bootstrap samples and after feature selection, PLS predict analysis, prediction-oriented segmentation with multi-group analysis. This figure shows only the significant pathways. The overall fit of this model was adequate with SRMR=0.019. Moreover, the construct reliabilities of both latent vectors were adequate, namely Cronbach $\alpha > 0.9$, rho A and composite reliability > 0.936 , and AVE > 0.78 . The outer model loadings on both latent vectors were all > 0.82 with $p<0.0001$. Blindfolding showed that the construct cross-validated redundancies were adequate, namely the MS latent vector 0.099 and IAI 0.480. We found that 56.0% of the variance in MS-EDSS-MSSS latent vector was explained by the direct effects of adiponectin and uric acid (both negatively), homocysteine, folate, CRP, and age (all positively). Furthermore, 33.8% of the variance in uric acid was explained by age, sex and BMI, and 20.2% of the variance in CRP by sex and BMI.

Most importantly, there were significant indirect effects of the *MTHFR* TT genotype on MS mediated by homocysteine ($t=2.01$, $p=0.022$) and of *MTHFR* additive model which was mediated by folate ($t=-2.01$, $p=0.045$). There were no other significant indirect effects or direct effects of *MTHFR* genotypes on the MS-EDSS-MSSS index. Nevertheless, analyses of the total direct effects showed that only the *MTHFR* TT genotype ($t=2.22$, $p=0.027$), but not the additive model ($t=-1.87$, $p=0.062$) had a significant effect on MS-EDSS-MSSS. Predicted-Oriented Segmentation analysis coupled with Multi-Group Analysis and Measurement Invariance Assessment showed that full compositional invariance was obtained. The Q^2 Predict values of all endogenous construct indicators were positive indicating that they outperform the most naïve benchmark (the prediction error is smaller than the error of the most naïve benchmark).

Discussion

The primary finding of this work is that the *MTHFR* 677C>T variation influences MS susceptibility indirectly via the elevated homocysteine (but not folate) levels associated with the TT genotype in a recessive model. Indeed, homocysteine levels have a direct impact on the inflammatory response, as measured by the IAI index, and consequently on the course of MS disability. Another significant finding is that the *MTHFR* 677C>T genotypes had no direct effect on the disability or progression of disability in MS patients. Other biomarkers, such as age, IAI, and CRP, had an effect on the EDSS and MSSS scores. Together, these biomarkers had a significant effect on disability, as measured by the EDSS. Additionally, a panel of biomarkers for IAI, CRP, and vitamin D3 had a small effect on disability progression. On the other hand, the *MTHFR* 677C>T genotype was associated with a minor influence on MS diagnosis, disability, and progression of disability. In fact, other variables were also involved in these clinical biomarkers of the MS.

MTHFR C677T and multiple sclerosis and homocysteine

The relevance of the *MTHFR* 677C>T variation in the pathogenesis of MS has been investigated in a number of genetically distinct worldwide populations, with contradictory results. Our findings corroborate prior research that demonstrated no association between the *MTHFR* 677C>T variation and susceptibility to MS [44–47]. While three case-control studies established a relationship between the T allele and MS [48–50], others [44–47] did not. In an Australian population, MS patients were more likely to have the TT homozygous genotype than controls; however, the difference was not statistically significant, and the authors concluded that their findings did not support a significant role for this functional gene variation in MS susceptibility [47]. However, the T allele of the *MTHFR* 677C>T variant was associated with MS susceptibility [48], as was the CT genotype, for both recessive (TT vs. CT + CC) and codominant (CT vs. CC) inheritance patterns [49]. In another study, the frequency of the TT genotype was higher in MS patients than in controls [50]. These results point to the need for additional studies involving individuals from genetically different populations.

Several factors could account for these seemingly contradictory outcomes. First, the MS is heterogeneous in terms of clinical characteristics, as distinct subgroups of individuals may share a variety of hereditary variables that predispose them to the disease. Second, whereas some alleles of candidate genes may be highly related with disease in one population, they may be weak or absent in another due to the existence of additional genetic variables or genetic-environment interactions, such as smoking, nutrition, and lifestyle habits [51]. Third, MS development cannot be predicted only on the basis of genotype, as even the greatest major histocompatibility complex (MHC) class II-linked risk genes for MS are only partially penetrant [52]. These authors affirm that the incomplete penetrance of MS susceptibility alleles is most likely due to interactions with other genes, post-transcriptional regulatory mechanisms, and major nutritional and environmental impacts. As a result, changeable environmental exposures may play a role in determining whether individuals who inherit risk genes develops MS. Fourth, conflicting findings regarding the association between the *MTHFR* variant and MS may be explained by the study design, sample size, time points at which biomarkers were tested, and, technically, by the use of different laboratory methods for measuring homocysteine and folate, as well as for *MTHFR* genotyping analysis.

In the current investigation, we found that the TT genotype explained 16.6% of the variance in homocysteine in all participants, regardless of age, folate, or sex. As a result, patients with the TT genotype had higher homocysteine levels than those with the CC + TT genotypes (recessive model). The association between the *MTHFR* 677C>T variant and elevated homocysteine levels is well recognized in both the general population [53–55] and individuals with various disorders [56–58]. Individuals carrying the TT genotype have a 55–65% reduction in *MTHFR* enzyme activity, while those carrying the CT genotype have a 25% reduction in enzyme activity [28, 57].

Homocysteine is a sulfur-containing non-essential amino acid that acts as an intermediary in the methionine metabolism pathway. It can be metabolized by two different reactions: trans-sulfuration or remethylation. The reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate by the *MTHFR* enzyme is required for the methylation of homocysteine to methionine [16, 17]. This enzyme acts as a link between two essential metabolic pathways, regulating one-carbon metabolism, nucleotide synthesis, and the synthesis of the universal methyl donor S-adenosyl methionine (SAM). Patients with a *MTHFR* deficiency have elevated homocysteine levels [16].

In terms of folate levels and the genetic variant explored in this work, our findings indicated that folate levels, age, and the *MTHFR* C677T variant explained 4.5% of the variance in folate levels across all participants using a dominant model (CC vs. CT vs. TT). Individuals carrying the CT genotype exhibited an intermediate folate phenotype compared to those carrying the two homozygous genotypes (CC and TT), with the highest folate levels in CC carriers, intermediate levels in CT carriers, and the lowest levels in TT carriers. Our hypothesis that the *MTHFR* 677C>T variant

regulates homocysteine and folate levels (in a recessive model CC+CT vs. TT) may be proven. Additionally, in MS, this variant and the same explanatory variables account for 15.9% of the variance in homocysteine levels.

Homocysteine and folate levels in multiple sclerosis

Our results of the present study indicated that MS patients had significantly higher homocysteine and folate levels than controls. Numerous researches have examined the possible involvement of homocysteine, vitamin B12, and folate in the neurodegenerative process [59]. Previous investigations have found that MS patients had higher plasma homocysteine levels than controls [8, 29, 30]. We previously demonstrated that MS patients had elevated homocysteine levels and that hyperhomocysteinemia is related with disease progression as measured by the MSSS [8]. Li et al. found that individuals with MS had higher homocysteine levels than controls, but no significant changes in vitamin B12 or folate levels between MS and controls. When clinical manifestations of MS were investigated, patients with relapsing-remitting MS (RRMS) had higher homocysteine levels than controls; however, there was no difference in homocysteine levels between SPMS or PPMS patients and controls [51].

In line with previous researches, we found that elevated homocysteine levels were positively associated with MS, implying that hyperhomocysteinemia may be a risk factor for this neuroinflammatory illness [7, 10]. Homocysteine has been found to be toxic to brain cells and to cause neuronal damage via a variety of mechanisms. First, homocysteine may predispose neurons to oxidative stress via sulfhydryl group oxidation, resulting in the production of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide [11, 60]. Second, through stimulating N-methyl-D-aspartate receptors (NMDA), excitotoxicity is promoted, resulting in DNA damage in neurons, inducing apoptosis and cell death [12, 61, 62]. Third, high homocysteine levels cause inflammation in the CNS [63], impair T and B lymphocyte responses [64], and decrease S-adenosyl methionine (SAM) levels, which are required for myelin basic protein (MBP) methylation [65]. Methylation-related modifications may disrupt myelin structures by causing hypomethylation of MBP, a critical component of myelin in the CNS [66].

Additionally, this study found that adiponectin, age, sex, BMI, the *MTHFR* 677C>T variant, homocysteine, vitamin D3, folate, and CRP explain 54.4% of the variance in MS and its severity. The latter index combines the MS diagnosis, EDSS, and MSSS scores to create a composite score that represents the severity of MS, including disability and disability progression.

Previous studies reported contradictory findings regarding folate levels in MS. Some studies found that MS patients had lower folate levels than controls [29, 30], whereas others found no difference between patients with MS and controls [32, 67, 68]. No significant difference in folate levels was found between MS patients and controls in meta-analyses [7, 51, 59]. However, two of these meta-analyses [7, 59] omitted crucial variables such as sex, age, disease phase and/or severity, and/or ethnic origin of study populations. A case-control study and meta-analysis revealed no significant difference in folate levels or the frequency of folate deficiency between MS patients and controls [9].

The functioning folate-vitamin B12 methylation pathway is required for the continual repair of myelin [69]. Folate and vitamin B12 are required for the methionine-synthase enzyme to perform its function of converting homocysteine to methionine. Both 5-methyltetrahydrofolate and methyl-vitamin B12 are required for homocysteine methionine synthesis [70]. It is widely recognized that vitamin B12 and folate deficiency can result in elevated homocysteine levels. However, previous studies included in a meta-analysis [51] demonstrated a high degree of heterogeneity, which may contribute to the apparent discrepancy in data relating folate and MS. Additionally, some studies specifically excluded participants who had not taken folic acid supplementation, while others did not account for confounding variables, such as food and medication use, which may interfere with the association between folate and MS.

In contrast to some previous studies [29, 30], we detected significantly higher folate levels in MS patients than in controls. Three possible explanations exist. First, MS patients may have decreased folate receptor (FR)- β expression than controls. Healthy cells obtain folate (or folic acid as a supplement) by the use of reduced folate carriers and/or the proton-coupled folate transporter, both of which are required for normal cell survival and proliferation. However, during inflammation, activated macrophages ingest folate predominantly via the beta isoform of FR (FR- β), which has roughly 1000 times the affinity for folate as the reduced folate carrier [71, 72]. According to animal tests and tissue autopsy from MS patients vs. controls, a recent study [73] demonstrated that macrophages express FR- β during the active phase of MS. Because all of our MS patients were in clinical remission of the illness, there would be less FR- β expression in the cells and thus more folate available in the circulation than in controls. Additionally, macrophages expressing functional FR- β are abundant in both CNS and peripheral inflammatory sites [74].

The second hypothesis is supported by the observation that plasma homocysteine is negatively correlated with the expression of FR [75]. Additionally, previous study showed that low levels of homocysteine enhance FR- β expression, but excessive high concentrations have the opposite effect [76]. Because our MS patients had elevated homocysteine levels, we can presume that they have lower FR- β expression, resulting in decreased folate internalization into cells and increased folate levels in the blood. Thirdly, the *MTHFR* 677C>T variant may be associated with the development of autoantibodies against FR- β [77]. These authors discovered that plasma levels of FR- β autoantibodies were considerably higher in women with the TT genotype of the *MTHFR* 677C>T than in women with the CC genotype [78, 79]. By extrapolation, because the TT genotype was more prevalent in MS patients than in controls, folate transport may be impaired. While all three of the aforementioned hypotheses are plausible, the current study did not allow us to understand the precise mechanism by which higher folate levels were detected in our cohort of patients with MS compared to controls.

Inflammation, homocysteine, MS disability and disability progression

Regarding the combination of a panel of biomarkers associated with MS, our findings indicated that adiponectin levels and male sex were negatively associated with MS, whereas folate and homocysteine, IAI, and age were positively associated with MS. Studies have established a strong association between elevated homocysteine levels and inflammation in both human and animal models [80, 81]. Homocysteine elevations promote inflammatory responses in the mouse brain by activation of microglia and increased production of pro-inflammatory cytokines such as IL-1 β and TNF- α [82]. Homocysteine has been linked to inflammation via a variety of mechanisms, including the expression of adhesion molecules, leukocyte adhesion, endothelial dysfunction, oxidative stress, and decreased nitric oxide bioavailability [83].

MS is not driven by a single cytokine, but rather by a complex interplay of pro- and anti-inflammatory cytokines, as demonstrated in human and animal researches [84]. Taking this into account, we analyzed a wide inflammatory and anti-inflammatory cytokine profile, expressed as the IAI, a score computed as the first principal component of the major cytokines produced by M1, Th1, Th17, Th2, and Treg cells, as well as TNF- α + sTNFR1 + sTNFR2 values. We found that MS is associated with elevated IAI levels, consistent with previous studies, highlighting the critical significance of an imbalance between inflammatory and anti-inflammatory responses as a fundamental element in the pathophysiology of MS [85–87]. Additionally, we demonstrated higher TNF- α , IL-17, and IFN- γ levels in MS patients compared to controls in previous research [88]. Another study shown that changes in the EDSS over a 16-month period were related with changes in IL-17 (positively) and IL-4 (negatively), regardless of the clinical forms of MS, treatment modality, smoking status, age, or SAH. Additionally, this investigation found that, in addition to homocysteine, IL-6 and IL-4 levels were positively associated with progressive forms of MS vs. RRMS, whereas 25(OH)D was negatively associated with RRMS [89].

Adiponectin is the most abundant anti-inflammatory adipokine in plasma and regulates the pro-inflammatory nuclear factor kappa B (NF- κ B) signaling pathway [90], decreases the expression of pro-inflammatory cytokines TNF- α , IL-6, and IFN- γ , and increases the expression of anti-inflammatory molecules such as IL-10 and IL-1 receptor antagonist (IL-1Ra) [91], highlighting its role in MS-related inflammation modulation [92].

We also found that age, IAI, and CRP levels were associated with moderate/high disability in MS (EDSS ≥ 3). Additionally, IAI and CRP were positively associated with disability progression (MSSS), whereas vitamin D3 was negatively associated. These findings corroborate previous studies [89, 93, 94] indicating that vitamin D3 deficiency is associated with disability progression in MS patients. Vitamin D3 has significant immunomodulatory effects and has been associated to the regulation of the inflammatory response [93, 95], including inhibition of the NF- κ B pathway [96], downregulation of pro-inflammatory cytokines such as TNF- α , IL-6, IL-12, and IFN- γ , and upregulation of anti-inflammatory Treg and Th2 cells and their cytokines [97]. These findings demonstrated that a variety of pathways contribute to disease progression regardless of relapses.

Although all of the MS patients were clinically in remission, we found disease progression. Although at least 12 drugs have been approved as disease-modifying treatments for MS, the major challenge for clinicians is identifying the subjects most likely to develop an aggressive, rapidly progressing form of the disease at the onset of the disease in order to initiate high-impact treatments before severe disability develops. Simultaneously, patients with mild forms should avoid overtreatment, which has significant benefits for safety, quality of life, and total resource allocation [98, 99].

At least four limitations apply to the findings in this paper. First, the case-control design does not allow inferences on causal relationship between the variables evaluated. Second, key critical lifestyle variables, such as dietary intake and vitamin B12 levels, were not controlled. Third, the study of a single specific genetic variant (*MTHFR* 677C>T) excludes an assessment of the complicated link between the multifaceted etiology of MS and other genetic variables. Fourth, the study included individuals with a variety of clinical forms of MS and were treated with a variety of MS medications; nevertheless, all patients were in the disease's remission clinical phase, and the results were adjusted for clinical forms and MS therapy. Despite these limitations, some strengths should be highlighted, including the integration of MS patient data with new composite measures of established biomarkers, such as IAI, TNF- α + its receptors, and MS-EDSS-MSSS, the latter of which may more accurately reflect the MS clinical course. Additionally, the study used robust methods for genotyping *MTHFR* 677C>T, measuring laboratory biomarkers, and developing in-depth statistical models.

Conclusion

Taken together, these findings provide important insights into the role of the *MTHFR* 677C>T variant, homocysteine, folate, and the inflammatory response in the underlying pathophysiological mechanisms of MS. While the *MTHFR* 677C>T variant was not shown to be related with MS, disability, or disability progression, the TT genotype has an indirect effect on MS susceptibility and disability via homocysteine but not folate. These findings emphasize the intricate interaction of genetic factors with inflammatory and anti-inflammatory mechanisms in the susceptibility, disability, and disability progression of MS, and suggest additional potential new targets that may help define the concept of precision medicine in MS management and patient care.

Declarations

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Conflict of interest

There is no conflict of interest to declare. None of the authors are involved in the publication process or have a financial or other beneficial interest in the products or concepts mentioned in the submitted manuscript.

Availability of data and material

The data and materials are available.

Authors' contributions

1) conception and design of the study: ANCS, EMVR, MM; 2) acquisition of data: CMR, SRO, TF, DFA; 3) laboratory analysis: CMR, TF, DFA, MABL; 4) statistical analysis: MM; 5) analysis and interpretation of data: CMR, SRO, ANCS, MM, EMVR; 5) drafting of the manuscript, tables and figures: CMR, SRO, MM, EMVR; 6) manuscript review: CMR, SRO, MM, EMVR. All authors have read and approved the final manuscript.

Compliance with ethical standards

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.

Human rights

All procedures performed in this study involving human participants were in accordance with the ethical standards of the Institution and/or National Research Committee and with the World Medical Association 1964 Helsinki Declaration.

Standards for reporting

The manuscript was prepared taken into account the recommendations of the guidelines hosted by the Strengthening the Reporting of Observational studies in Epidemiology (STROBE). STROBE is used for observational studies (cohort, case-control, or cross-sectional designs) according to the STROBE statement (www.strobe-statement.org)

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent for publication

The studied participants were informed about the present research, and a written consent form was taken from all of them before their enrollment. Moreover, all the authors and co-authors participated and contributed sufficiently in the research, and all of them concur with the submission. The manuscript has been approved by the responsible authorities where the work was carried out. The authors also concur that, if accepted, the manuscript shall not be published elsewhere in the same form in either the same or any other language, without the consent of the Editor-in Chief of **Molecular Neurobiology**.

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Figures

Figure 1

Results of Partial Least Squares (PLS) path analysis performed in healthy controls. This PLS analysis shows the causal relationships between the MTHFR C677T variant (entered as recessive and additive models), age and sex (entered as independent variables) and homocysteine (Hcy) (entered as final dependent variable). Folate was entered as a partial mediator allowing for mediated effects of the genotypes, age and sex on Hcy levels. MTHFRco: additive model (CC=-1; CT=0; TT=+1); MTHFRrec: recessive model (CC + CT vs. TT genotypes); Associations are defined as pathways coefficients with p-values. The white figures in the circles indicate the explained variance.

Figure 2

Results of Partial Least Squares (PLS) path analysis performed in all patients with multiple sclerosis (MS) and healthy controls combined. This PLS analysis shows the relationship between MTHFR 677C>T variant, demographic and metabolic data, immune activation index (IAI), homocysteine, folate plasma levels, and MS-EDSS-MSSS [an index of MS and its severity, entered as a latent vector extracted from MS diagnosis, disability (EDSS) and disability progression (MSSS)]. IAI was entered as a latent vector extracted from macrophage M1, T helper (Th)1, Th17, and Th2+T regulatory (Treg) cytokine levels. The other variables were entered as single indicators. Associations are defined as pathways coefficients with p-values. The figures in the circles indicate the explained variance. MTHFRco: additive model of the MTHFR 677C>T variant (CC=-1; CT=0; TT=1); MTHFRrec: recessive model of the MTHFR 677C>T variant (CC + CT vs. TT genotypes); BMI: body mass index; Hcy: homocysteine levels; CRP: C-reactive protein; MS: multiple sclerosis; EDSS: Expanded Disability Status Score; MSSS: Multiple Sclerosis Severity Score; Adipo: adiponectin; M1: macrophage M1 cytokines as interleukin (IL)-6 and tumor necrosis factor (TNF)- α ; Th1: T helper 1 cytokines as IL-2 and interferon (IFN)- γ ; Th17: T helper 17 cytokines as IL-17 and IL-6; Th2reg: T helper 2 and T regulatory cytokines as IL-4 and IL-10.