Metabolic Brain Disease (2019) 34:1401–1413
https://doi.org/10.1007/s11011-019-00456-7

ORIGINAL ARTICLE

Disability in multiple sclerosis is associated with age and inflammatory, metabolic and oxidative/nitrosative stress biomarkers: results of multivariate and machine learning procedures

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Received: 11 October 2018 / Accepted: 3 July 2019 / Published online: 13 July 2019

Abstract

The aim of this study was to evaluate the immune-inflammatory, metabolic, and nitro-oxidative stress (IM&NO) biomarkers as predictors of disability in multiple sclerosis (MS) patients. A total of 122 patients with MS were included; their disability was evaluated using the Expanded Disability Status Scale (EDSS) and IM&NO biomarkers were evaluated in peripheral blood samples. Patients with EDSS ≥3 were older and showed higher homocysteine, uric acid, advanced oxidized protein products (AOPP) and low-density lipoprotein (LDL)-cholesterol and higher rate of metabolic syndrome (MetS), while high-density lipoprotein (HDL)-cholesterol was lower than in patients with EDSS <3; 84.6% of all patients were correctly classified in these EDSS subgroups. We found that 36.3% of the variance in EDSS score was explained by age, Th17/T regulatory (Treg) and LDL/HDL ratios and homocysteine (all positively related) and body mass index (BMI) (inversely related). After adjusting for MS treatment modalities, the effects of the LDL/HDL and zTh17/Treg ratios, homocysteine and age on disability remained, whilst BMI was no longer significant. Moreover, carbonyl proteins were associated with increased disability. In conclusion, the results showed that an inflammatory Th17 profile coupled with age and increased carbonyl proteins were the most important variables associated with high disability followed at a distance by homocysteine, MetS and LDL/HDL ratio. These data underscore that IM&NO pathways play a key role in increased disability in MS patient and may be possible new targets for the treatment of these patients. Moreover, a panel of these laboratory biomarkers may be used to predict the disability in MS.

Keywords Multiple sclerosis · Disability · Inflammation · Oxidative stress · Homocysteine · Biomarkers

Introduction

Multiple sclerosis (MS) is a chronic inflammatory immune-mediated disease that affects the central nervous system (CNS) and is the major cause of disability in adults. Many different pathological mechanisms are involved in MS, including autoimmune inflammation, demyelination, neurodegeneration with axonal and death and gliosis in the brain and spinal cord. Genetic, immunological, hormonal, environmental, and epigenetic factors are most likely responsible for the heterogeneity of neurological symptoms disseminated in time and space, as well as disease progression (Versini et al. 2014). The autoimmune inflammation in early MS is primarily mediated by adaptive immune responses and involves autoreactive T cells, B cells, and antibodies, while the later,

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chronic stages of MS are characterized by a compartmentalized immune response in the CNS with activated microglia and macrophages (Sospedra and Martin 2016). The adaptive immune response in MS is characterized by the imbalance in the expression of T helper (Th)1, Th2, Th17 and T regulatory (Treg) cytokines, as well as chemokines and their receptors (Mikulkova et al. 2011; Sospedra and Martin 2005, 2016).

Mounting evidence suggests that autoreactive Th1 and Th17 cells generated in the periphery have the ability to enter to the CNS by crossing the brain blood barrier (BBB). There, they persist and generate an inflammatory cascade and increase reactive oxygen species (ROS) and reactive nitrogen species (RNS) leading to formation of lesions and neurologic deficits (Das Sarma et al. 2009; Murphy et al. 2010). Pro-inflammatory and anti-inflammatory cytokine levels are associated with changes in MS disease activity (Graber et al. 2007). Interleukin (IL)-17A produced by a number of different cell types plays a critical role in the pathogenesis of MS by affecting the BBB breakdown, inducing the infiltration of immune cells into the CNS compartment, amplifying inflammatory responses and by the generation of cytotoxic mediators that result in demyelination and neuronal damage (Kolbinger et al. 2016). Moreover, there is ample evidence that Treg cells, which are characterized by secretion of IL-10 and transforming growth factor (TGF)-β, are important for controlling inflammatory mechanisms in animal models of experimental autoimmune encephalomyelitis (EAE). In MS, a reduced number and activity of Treg were described (Haas et al. 2005).

Moreover, oxidative and nitrosative stress play a major role in the development and progression of chronic and autoimmune diseases, including MS (Dichi et al. 2014). The increased production of ROS and RNS leads to high production of oxidized substrates, such as lipids, proteins and nucleic acid, that contribute to immune dysregulation and aggravate the clinical course of the MS (Sayre et al. 2008). Excessive nitro-oxidative stress and decreased anti-oxidant levels are found in MS patients and are associated with disability progression (Oliveira et al. 2012).

Changes in metabolic pathways and metabolic comorbidities are involved in the disease course of MS and disability (Marrie et al. 2010; Weinstock-Guttman et al. 2011; Oliveira et al. 2014). There is strong evidence that homocysteine, an inflammatory marker of risk for cardiovascular disease (Faeh et al. 2006) is able to induce BBB disruption (Kamath et al. 2006). Studies in animal models demonstrated that homocysteine is neurotoxic and increased risk of neurodegenerative diseases (Bonetti et al. 2016; Longoni et al. 2018). Increased levels of homocysteine were associated with cognitive impairment in neurodegenerative disorders, such as Alzheimer's disease and MS (Seshadri 2006; Russo et al. 2008), and were associated with MS clinical progression (Teunissen et al. 2008; Oliveira et al. 2018).

Disability accumulation over time in MS patients is slow but persistent, often leading to a decreased mobility and physical activity, resulting in more weakness, fatigue and increased risk of metabolic syndrome (MetS). On the other hand, MetS may trigger MS in susceptible individuals and is considered one of the risk factors (Reale and Sanchez-Ramon 2017). Insulin resistance, hypertension, hypercholesterolemia, and peripheral vascular disease were independently associated with increased risk of disability (Marrie et al. 2010; Oliveira et al. 2014). Worsening disability was associated with high total cholesterol, low-density lipoprotein (LDL)-cholesterol, and triglycerides in patients with MS, whilst high-density lipoprotein (HDL)-cholesterol was associated with low levels of acute inflammatory activity (Weinstock-Guttman et al. 2011).

MS is a heterogeneous disease and the search for more adequate biomarkers predicting disease course is currently an active area of research. The concomitant evaluation of immune-inflammatory (Th1, Th17 and Treg response), metabolic pathways (MetS, lipid metabolism, homocysteine) and oxidative stress biomarkers may provide more accurate predictive models for the evaluation of disease disability in MS patients. Hence, the aim of the present study was to evaluate immune-inflammatory, metabolic and nitro-oxidative stress (IM&NO) biomarkers as predictors of disability in MS patients.

Materials and methods

Subject data

The study included 122 MS patients consecutively recruited from the Demyelinating Diseases Outpatient of the State University of Londrina, Londrina, Paraná, South of Brazil. The MS diagnosis was established according to the McDonald criteria (Polman et al. 2011), and the patients were classified with relapsing-remitting MS (RRMS) ($n = 103$) or progressive MS ($n = 19$), including primary progressive MS (PPMS, $n = 3$) and secondary progressive MS (SPMS, $n = 16$). The majority of the patients were treated with interferon (IFN)-β ($n = 76$), and others with glatiramer acetate ($n = 30$), natalizumab (n = 7), and fingolimod ($n = 2$). Patients without treatment (n = 7) were those who abandoned therapy or were drug naïve at the time of blood collection. Disability was evaluated using the EDSS score (Kurtzke 1983) and the patients were categorized with mild (EDSS <3) and moderate/severe (EDSS ≥3) disability (Kallaur et al. 2017). 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. The patients were not on a special diet or on the use of antioxidants.

Baseline characteristics and the MS therapy used before the enrollment of MS patients were obtained using a standard questionnaire on study admission. The ethnicity was self-reported as Caucasians and non-Caucasians (Black, Afro-Brazilian, and Asiatic) (IBGE: Brazilian Institute of Geography and Statistics 2011; Suarez-Kurtz et al. 2012). Body mass index (BMI), waist circumference, systolic blood pressure (SBP) and diastolic blood pressure (DBP) values were measured as previously described (Kallaur et al. 2017; Oliveira et al. 2017). Type 2 diabetes mellitus (T2DM) was defined as a fasting serum glucose ≥126 mg/dL, a non-fasting serum glucose ≥200 mg/dL and/or use of hypoglycemic medication (American Diabetes Association 2014). MetS was defined as previously reported (Grundy et al. 2005).

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, biochemical and immunological biomarkers

Venous blood samples were obtained after fasting for 12 h with ethylenediaminetetraacetic acid (EDTA) as anticoagulant and without anticoagulant. The samples were consecutively and anonymously coded and centrifuged at 2,500 rpm for 15 min. Further, plasma and serum aliquots were stored at -80^0 C until use. Uric acid, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, and glucose were evaluated using enzymatic colorimetric assay with a biochemical autoanalyzer (Dimension® EXL200, Siemens Healthcare Diagnostics Ltd., Newark, DE, USA). Homocysteine levels were determined by chemiluminescent microparticle immunoassay (CMIA, Architect, Abbott Laboratory, Abbott Park, IL, USA). IL-6, IL-17 and IL-10 levels were determined using microspheres multiplex immunoassay (Novex Life Technologies, Frederick, MD, USA) for Luminex platform (MAGPIX®, Luminex Corp., Austin, TX, USA). All laboratory analysis were performed according to the manufacturer's instructions and their reference values.

Oxidative and nitrosative stress biomarkers

The oxidative and nitrosative stress biomarkers were determined as reported elsewhere (Oliveira et al. 2012; Oliveira et al. 2016). Briefly, lipid hydroperoxides were evaluated by tert-butyl hydroperoxide-initiated chemiluminescence (CL-LOOH), as described (Gonzalez Flecha et al. 1991), using the GloMax® 20/20 Luminometer (Promega Corporation, Madison, WI, USA). Advanced oxidation protein products (AOPP) and protein carbonyl levels were determined as described previously (Witko-Sarsat et al. 1998; Reznick and Packer 1994, respectively) using colorimetric assay (Microplate reader LMR-96, Loccus, Cotia, São Paulo, Brazil). Nitric oxide (NO) was estimated by measuring NO metabolites (NOx) nitrites (NO_2^-) and nitrates (NO_3^-) using colorimetric assay, according to Navarro-Gonzálvez et al. (1998) and the results were obtained by microplate reader (LMR-96, Loccus, Cotia, São Paulo, Brazil).

Statistical analysis

Continuous variables were expressed as mean and ± standard deviation (± SD) and categorical variables were expressed as absolute number (n) and percentage (%). Analysis of variance (ANOVAs) was employed to check differences in continuous variables among EDSS groups dichotomized using a cut off value ≥3. Analysis of contingence tables (x^2 tests) was used to assess the associations between these EDSS groups and other nominal variables. Correlations between scale variables were computed with Pearson's product moment correlation coefficients. Computed z unit-weighted composite scores were used to assess indices of atherogenicity based on HDL-cholesterol and LDL-cholesterol levels, and indices of Th17 profile using the cytokine levels, as reported previously (Maes and Carvalho 2018).

Univariate general linear model (GLM) analysis was employed to assess the effects of independent variables (including biomarkers, MetS, drug state) on the dependent variables (the EDSS score), while adjusting for age, BMI and sex. Hierarchical binary logistic regression analysis was used to delineate the most significant explanatory variables predicting the group with EDSS scores ≥3 (with EDSS <3 as reference group). We used Nagelkerke values as estimates of effect size and computed Odds ratio (OR) and 95% confidence intervals (95% CI). A multilayer perceptron (MLP) neural network (NN) analyses was used to separate patients with EDSS scores ≥3 from those with scores <3 and to assess the complex non-linear relationships between EDSS scores and biomarkers and other variables, such as MetS and age. We used automated feedforward architecture models with biomarkers MetS, age, sex, duration of illness as input variables, while the output layer contains the EDSS subgroups (dichotomized) or EDSS test scores (scale variables) as output variables. Two hidden layers were considered with a variable number of nodes. As a stopping rule, we used one consecutive step with no further decrease in the error term. The relative number of cases assigned to the training (to estimate the network parameters), testing (to prevent overtraining) and holdout (to evaluate the final network) sets were 7, 3 and 3, respectively. We computed error terms for the training and testing samples as well as relative errors in the three samples. The rate of incorrect predictions and the partitioned confusion matrices for categorical output variables (EDSS groups) in the three samples and the area under the receiving operating curves (AUC ROC) or the

correlation between the model-predicted EDSS score and the actual EDSS score were computed. Finally, we assessed the (relative) importance of the input variables in sensitivity analyses. The EDSS score was used both as a continuous and also as a binary-split dichotomized dependent variable in regression and NN analyses. The former method because the use of continuous dependent variables is statistically more adequate than using its dichotomized values (Altman and Royston 2006; Naggara et al. 2011). The second method was employed because an EDSS score ≥ 3 is a recognized cut-off point denoting increased disability and, thus, is important in the clinical practice. All data were analyzed using IBM SPSS windows version 25 and Statistica 12.

Results

Subjects characteristics

Table 1 shows the demographic, clinical as well as the biomarker characteristics of subjects according to the EDSS score ≥ 3 versus those with EDSS score < 3. We did not use p-corrections for false discovery rate because the results of Table 1 and the correlation matrices between the variables shown in Table 1 were used to delineate the explanatory variables that were consequently used in the multivariate statistical analyses presented in Tables 3 and 4. Three patients did not have data on EDSS score. Patients with higher EDSS score were significantly older than those with lower EDSS score. There were no significant differences in sex ratio (female/male), ethnicity, smoking and alcohol use, T2DM frequency, BMI, waist circumference, cytokines, NOx, CL-LOOH, and carbonyl proteins between both groups. Nevertheless, subjects with EDSS ≥3 showed higher homocysteine, uric acid, AOPP and LDL-cholesterol levels and higher rate of MetS, while HDL-cholesterol levels were significantly lower than those with EDSS <3.

There were significant positive correlations between age and AOPP (r = 0.234, $p = 0.010$), NOx (r = 0.234, p = 0.010), carbonyl proteins (r = 0.188, $p = 0.039$), homocysteine (r = 0.224, $p = 0.015$ and LDL-cholesterol (r = 0.333, $p < 0.001$), but not with the other biomarkers. There were significant sex-related differences in uric acid and homocysteine, but not with the other biomarkers. Uric acid (mean ± SD = 4.7 ± 1.1 versus 3.8 ± 1.2 mg/dL; F = 14.91, df = 1/120, p < 0.001) and homocysteine (13.9 ± 4.6 versus 11.7 ± 3.7 μmol/L; F = 8.26, df = 1/117, $p = 0.005$) were significantly higher in males than females. HDL-cholesterol was significantly lower in men than women (45.3 ± 13.2 versus 57.6 ± 18.7 mg/dL; F = 13.61, df = 1/116, p < 0.001). Duration of illness was significantly associated with EDSS score (r = 0.368, p < 0.001, $n = 119$) and NOx (r = 0.317, p < 0.001, $n = 121$), but not with the other biomarkers.

Use of z unit-weighted composite scores

Consequently, we have computed z unit-weighted composite scores to assess indices of atherogenicity based on HDL-cholesterol and LDL-cholesterol levels, reflecting Castelli index risk factor 1 as z value of total cholesterol (z cholesterol) - zHDL-cholesterol (zcholesterol/HDL). We also computed zLDL-zHDL as an atherogenic marker reflecting Castelli risk index 2. Table 2 shows that both Castelli risk indices were strongly correlated with the new z unit-weighted composite scores. Likewise we computed z unit-weighted composite scores reflecting Th17 profile activity as z natural logarithmic of IL-6 (zLnIL-6) + zLnIL-17 (zTh17). Table 2 shows that this z composite score is similar to the first principal component (PC) extracted from both LnIL-6 and LnIL-17 values. Finally, we have also computed an index reflecting Th17/Treg ratio as zLnIL6 + zLnIL17 − zlnIL-10 (zTh17/Treg). The latter ratio was strongly correlated with the first PC extracted from LnIL-6 and LnIL-17/ LnIL10 (see Table 2).

Age was significantly associated with zcholesterol/HDL (r = 0.235, $p = 0.10$, $n = 118$) and zLDL/HDL (r = 0.239, $p = 0.009$, n = 118), but not with the other z-unit weighted composite scores. The zLDL/HDL ratio was significantly higher in men than women (+0.49 ± 1.35 versus − 0.24 ± 1.29; F = 7.83, df = 1/116, $p = 0.006$), but there were no significant sex differences in the other z unit-weighted composite scores. Duration of illness was significantly and positively correlated with zcholesterol/HDL (r = 0.266, $p = 0.004$, $p = 118$) and zLDL/HDL (r = 0.282, $p = 0.002$, n = 118).

Prediction of the EDSS score

Correlation analyses showed significant relationships between the EDSS score and age (r = 0.377, $p < 0.001$), AOPP (r = 0.190, $p = 0.039$), homocysteine (r = 0.332, p < 0.001) and LDL-cholesterol (r = 0.246, $p = 0.008$) but not with the other biomarkers. There were significant correlations between the EDSS score and zcholesterol/HDL (r = 0.291, p = 0.002), zLDL/HDL (r = 0.331, p < 0.001), zTh17 (r = 0.202, $p = 0.037$) and zTh17/Treg (r = 0.267, p = 0.006). Consequently, we have used zTh17, zTH17/Treg, zcholesterol/HDL, zLDL/HDL, MetS and the significant biomarkers (see Table 1) together with age, sex and BMI as explanatory variables in GLM and binary logistic regression analyses. After defining the best model, we also examined the possible impact of the other biomarkers as well as demographic data (including ethnicity, diabetes mellitus, smoking and alcohol use) and the drug state of the MS patients.

Table 3 regression #1 shows the outcome of univariate GLM analysis with EDSS score as dependent variable and zTh17, zTh17/Treg, zcholesterol/HDL, zLDL/HDL, homocysteine, uric acid, AOPP, MetS together with age, sex and BMI as explanatory variables. The EDSS score was best

Table 1 Clinical, demographic and biomarker characteristics of patients with multiple sclerosis categorized according to their disability evaluated using Expanded Disability Scale Score (EDSS)

Variable	EDSS <3 ($n = 49$)	EDSS ≥3 ($n = 70$)	F/X^2	df	p value
EDSS	0.99 (0.85)	4.79 (1.56)	239.69	1/117	<0.001
Age (years)	37.3 (11.3)	46.8 (12.3)	18.51	1/117	<0.001
Sex (F/M)	35 (71.4)/14 (28.6)	46 (65.7)/24 (34.3)	0.43	1	0.511
Caucasian/Non-Caucasian	41 (83.7)/8 (16.3)	55 (78.6)/15 (21.4)	0.48	1	0.488
BMI (kg/m^2)	26.0 (5.3)	25.7 (4.9)	0.14	1/117	0.707
Waist circumference (cm)	88.4 (13.4)	92.5 (13.1)	2.77	1/112	0.099
Smoking (No/Yes)	45 (91.8)/4 (8.2)	59 (84.3)/11 (15.7)	1.49	1	0.222
Alcohol use (No/Yes)	42 (85.7)/7 (14.3)	63 (90.0)/7 (10.0)	0.51	1	0.475
T2DM (No/Yes)	46 (93.9)/3 (6.1)	63 (90.0)/7 (10.0)	0.56	1	0.453
MetS (No/Yes)	43 (87.8)/6 (12.2)	42 (61.8)/26 (38.2)	9.68	1	0.002
Glucose mg/dL	89.6 (2.8)	90.3 (2.3)	0.03	1/117	0.855
Total cholesterol mg/dL	182.9 (6.0)	195.0 (5.1)	2.37	1/114	0.127
Triglycerides (mg/dL)	108.6 (8.7)	13.4 (7.6)	1.62	1/114	0.205
HDL-cholesterol (mg/dL)	58.2 (18.5)	50.9 (17.1)	4.45	1/113	0.037
LDL-cholesterol (mg/dL)	102.9 (33.4)	119.2 (33.1)	6.78	1/113	0.010
Uric acid (mg/dL)	3.82 (1.06)	4.31 (1.35)	4.53	1/113	0.035
Homocysteine (μmol/L)	11.4 (2.9)	13.3 (4.7)	5.82	1/114	0.017
Hydroperoxide (cpm)	174 (91)	176 (92)	0.05	1/116	0.829
Carbonyl Protein (nmol mL^{-1} mg^{-1} total protein)	3.07 (1.34)	3.29 (1.20)	0.84	1/116	0.362
AOPP (μmol/L of chloramine-T equivalents)	118.7 (41.2)	136.7 (48.6)	5.13	1/116	0.025
NOx (μM)	11.7 (3.7)	13.2 (3.7)	0.39	1/116	0.533
IL-6 (pg/mL)	22.2 (79.7)	19.7 (93.6)	1.14	1/105	0.289
IL-17 (pg/mL)	11.2 (19.9)	27.0 (122.3)	0.82	1/105	0.367
IL-10 (pg/mL)	22.3 (62.2)	26.8 (117.2)	0.05	1/105	0.819
Clinical form					
RRMS	48 (97.9)	52 (74.3)	12.040	1	0.001
ProgMS (PPMS + SPMS)	1 (2.1)	18 (25.7)			

EDSS Expanded Disability Status Scale, *EDSS <3* mild disability, *EDSS ≥3.0* moderate/high disability, *F/X²* the results of analyses of variance. *χ2* results of analyses of contingency tables. Continuous variables were expressed as mean and ± standard deviation (SD) and categorical variables were expressed as absolute number (n) and percentage (%). *df* degree of freedom, *BMI* body mass index, *T2DM* type 2 diabetes mellitus, *MetS* metabolic syndrome, *AOPP* advanced oxidation protein product, *NOx* nitric oxide metabolites, *IL* interleukin, *HDL* high-density lipoprotein cholesterol, *LDL* low-density lipoprotein cholesterol, *RRMS* relapsing-remitting multiple sclerosis, *ProgMS* progressive clinical forms of multiple sclerosis, *PPMS* primary progressive multiple sclerosis, *SPMS* secondary progressive multiple sclerosis

predicted by age, zLDL/HDL, zTh17/Treg and homocysteine (all positively related) and BMI (inversely correlated). These variables independently predicted 36.3% of the variance in the EDSS scores. None of the other biomarkers yielded a significant effect, while sex ($F = 1.16$, $df = 1/95$, $p = 0.285$), ethnicity ($F = 2.11$, $df = 1/95$, $p = 0.150$), smoking ($F = 0.00$, $df = 1/95$, $p = 0.964$), alcohol use ($F = 1.48$, $df = 1/95$, $p = 0.227$), MetS ($F = 1.27$, $df = 1/94$, $p = 0.263$) and T2DM ($F = 0.03$, $df = 1/95$, $p = 0.860$) did not result in significant effects after considering the five variables entered in model regression #1. In order to examine whether the effects of these five significant predictors would change after controlling for the drug state of the patients we have entered the use of drugs as additional predictor variables, namely no drugs ($n = 7$), IFN-β ($n = 76$), glatiramer ($n = 30$) and natalizumab ($n = 7$). There were only two patients taking fingolimod and therefore the latter was excluded from the analysis. Table 3 regression #2 shows that controlling for the drug state did not change the effects of zLDL/HDL, zTh17/Treg, homocysteine and age, although BMI was no longer significant.

Table 4 regression # 1 shows the outcome of binary logistic regression analysis with the group with EDSS ≥3 (versus those with EDSS <3) as dependent variables and MetS, age, sex and BMI as explanatory variables. We found that age (positively), MetS (positively) and BMI (inversely) significantly predicted high EDSS score with a Nagelkerke value of 0.294. After introducing zTh17, zTh17/Treg, zcholesterol/HDL, zLDL/HDL, homocysteine, uric acid and AOPP in the same analysis (regression #2), we found that age, zLDL/HDL and zTh17/Treg better predicted the EDSS score with a

Table 2 Description of the z-unit weighted composite scores of inflammatory and metabolic biomarkers used in the present study

z-unit score	Index	Correlation
zcholesterol/HDL	Castelli risk index 1	r = 0.955, p < 0.001, *n* = 118
zLDL/HDL	Castelli risk index 2	r = 0.944, p < 0.001, *n* = 118
zTh17 profile	PC IL-6 and IL-17 (Th17 profile)	r = 1.000, p < 0.001, *n* = 110
zTh17/Treg	PC IL-6 and IL-17/IL-10 (Treg profile)	r = 0.701, p < 0.001, *n* = 110

zcholesterol/HDL z value of total cholesterol - high-density lipoprotein cholesterol, *zLDL/HDL* z value of low-density lipoprotein cholesterol - high-density lipoprotein cholesterol, *zTh17profile* z natural logarithmic (Ln) of interleukin (IL)-6 (zLnIL-6) + zLnIL-17, *zTh17/Treg* zLnIL6 + zLnIL17 − zLnIL-10, *PC* principal component, *Treg* T regulatory cells

Nagelkerke value of 0.330. MetS (Wald = 1.04, df = 1, p = 0.307) and BMI (Wald = 2.31, df = 1, p = 0.128) were no longer significant in this regression analysis. Entry of the other variables in regression #2 (namely sex, ethnicity, smoking and alcohol use, T2DM, or the biomarkers CL-LOOH, uric acid, NOx, AOPP and carbonyl proteins) did not change the significance level of these three predictors and showed that none of these variables had a significant effect. Entering the drug state of the patients (four groups) as a categorical variable showed that the significances of zTh17/Treg, zLDL/HDL and age were unaffected, while the drug state had no significant effect.

Results of neural network procedures

Table 5 shows the results of two different MLP/NN models predicting EDSS scores (output variables) using the biomarkers and demographic or clinical data as input variables. In MLP/NN model #1 we used the EDSS score as a scale variable (continuous variable) and biomarkers together with age, sex, BMI, MetS and duration of illness as input variables. Automatic architecture training of the MLP/NN delineated two hidden layers with three units in layer one (with hyperbolic tangent as activation function) and two units in layer two (with identity as activation factor for the output layer). The differences in sum of square error between the training and testing samples show that the MLP/NN model has learnt to generalize from the trend. The relative error was relatively stable across the training, testing and holdout samples. The predicted EDSS score was significantly and positively correlated with the actual EDSS score (r = 0.616, p < 0.001). Figure 1a shows the relative and normalized importance of the input variables predicting the EDSS score. Homocysteine, zLDL/HDL and zTh17/Treg were the most important determinants of the predictive power of MLP/NN model #1, followed at a distance by duration of illness.

In MLP/NN model #2, Table 5, we trained the MLP/NN using the dichotomized EDSS score as output variable and the same input variables as in MLP/ NN model #1. Automatic architecture training model delineated two hidden layers with two units in layer one (activation function: hyperbolic tangent) and two units in layer two (activation factor in the output layer: softmax). The differences in cross-entropy error between the training and testing samples show that the model learnt to generalize from the trend. The rate of incorrect predictions was 21.2% in the training sample, 21.7% in the testing sample and 15.4% in the holdout sample. Table 5 shows also the partitioned confusion matrices. In the training sample, the sensitivity of the model was 92.0% and specificity 66.7%, while in the testing sample it was 93.3% and 50.0%, respectively. The accuracy of the holdout sample was somewhat better with a sensitivity of 94.4% and a specificity of 62.5%, whilst the AUC ROC curve was 0.842. Figure 1b shows the relative and normalized importance of the input variables predicting the dichotomized EDSS score. As showed, zTh17/Treg, carbonyl proteins and age were the most important determinants of the predictive power of high EDSS followed at a distance by homocysteine, MetS and LDL/HDL ratio.

Discussion

The major finding of this study is that disability in MS patients is strongly associated with age, an inflammatory Th17 immune response profile, carbonyl proteins, as well as MetS, homocysteine and LDL/HDL ratio. This study established a model predicting increased risk towards high EDSS indicating that age, the LDL/HDL ratio (Castelli index 2) and a Th17/Treg profile are the most important features of disability independently of other confounding factors. Moreover, we used MLP/NN models to predict the EDSS score, both as a scale and a dichotomized variable, in MS patients and detected that of the top-6 features in each model 4 variables corresponded among both models, namely Th17/Treg, zLDL/HDL, homocysteine and age. Nevertheless, when we used the dichotomized EDSS score, also carbonyl protein was among the top features. Phrased differently, the cumulative effects of Th17 activation, a pro-atherogenic index, homocysteine, age and protein oxidation are important predictors.

Our data showing that patients with EDSS ≥3 were significantly older than those with EDSS <3 are in agreement with previous studies, which demonstrated an association between

Table 3 Results of univariate general linear model (GLM) analysis with Expanded Disability Scale Score as dependent variable and immune-inflammatory, metabolic, and oxidative stress biomarkers as explanatory variables

Regression	Explanatory variables	*F*	df	*p* value	R^2	*F*	df	*p* value	R^2
#1	Age (+)	12.25	1/96	0.001	0.113	10.92	5/96	<0.001	0.363
	BMI (−)	5.28	1/96	0.024	0.052				
	zLDL/HDL (+)	5.31	1/96	0.023	0.052				
	zTh17/Treg (+)	11.08	1/96	0.01	0.103				
	Homocysteine (+)	7.30	1/96	0.008	0.071				
#2	Age (+)	10.30	1/91	0.002	0.102	9.50	8/91	<0.001	0.455
	BMI	1.30	1/91	0.257	0.014				
	zLDL/HDL (+)	9.81	1/91	0.002	0.097				
	zTh17/Treg (+)	5.11	1/91	0.026	0.053				
	Homocysteine (+)	7.42	1/91	0.008	0.075				
	Drug state	5.37	1/91	0.002	0.150				

All the results of analyses of variance (*F* values); *df* degree of freedom, *BMI* body mass index, *Treg* T regulatory cells, *zLDL/HDL* z value of low-density lipoprotein cholesterol - high-density lipoprotein cholesterol, *zTh17/Treg* z natural logarithmic value of interleukin (IL) 6 + z natural logarithmic value of IL17 – z natural logarithmic value of IL-10, (−): negatively associated; (+): positively associated

increased age and disability progression (Trojano et al. 2002; Scalfari et al. 2011). Previous studies show that many aspects of the clinical course of MS, including clinical symptoms (Tremlett et al. 2006), occurrence of relapses (Tremlett et al. 2008) and disability progression, are associated with age (Trojano et al. 2002; Confavreux and Vukusic 2006; Tremlett et al. 2006; Scalfari et al. 2011). Furthermore, a five-year follow-up progression in the EDSS demonstrated that increased age was a strong predictor of the EDSS and pyramidal dysfunction scores but was not significantly associated with the actual changes in the EDSS, pyramidal, sensitive, or cerebellar symptoms (Kallaur et al. 2016).

Initially, it was postulated that an imbalance between Th1 cytokines, such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α and IL-2 and Th2 cytokines, such as IL-4 and IL-10 was the major mediator of inflammation in the CNS in patients with MS (Sredni-Kenigsbuch 2002; Imitola et al. 2005; Langrish et al. 2005), which damages oligodendrocytes, myelin sheaths and neurons/axons, resulting in demyelination and axonal loss (Imitola et al. 2005). Further, mounting evidence showed the role of other $CD4^+$ T cell lineages in MS inflammatory mechanisms, such as Th17 and Treg cells through the production of IL-17, IL-21, IL-22, and granulocyte-macrophage colony-stimulating factor (GM-CSF) by Th17 cells, and IL-10 and TGF- β by Treg cells (Rostami and Ciric 2013). Reduced number and activity of Treg cells have been described in MS, creating an imbalance between the Th17/Treg cells and their cytokines (Hemmer

Table 4 Result of binary logistic regression analysis with an increased EDSS score (≥3) as dependent variable and those with lower EDSS score (<3) as reference group

Regression	Explanatory Variables	Wald	df	*p* value	OR	CI 95%	X^2	df	*p* value	Nagel
≠1	Age (+)	11.23	1	0.001	1.07	1.03–1. 11	28.16	3	<0.001	0.294
	MetS (+)	6.84	1	0.009	5.42	1.53–19.20				
	BMI (−)	4.20	1	0.040	0.90	0.82–0.99				
≠2	Age (+)	7.68	1	0.006	1.07	1.03–1.11	29.23	3	<0.001	0.330
	zLDL/HDL (+)	5.08	1	0.024	1.69	1.17–2.45				
	zTh17/Treg (+)	7.18	1	0.007	1.78	1.08–2.93				
≠3	Age (+)	8.71	1	0.003	1.07	1.02–1.11	36.77	6	<0.001	0.407
	zLDL/HDL (+)	9.99	1	0.002	1.99	1.30–3.04				
	zTh17/Treg (+)	3.89	1	0.049	1.69	1.00–2.85				
	Drug state	1.18	3	0.759	–	–				

df degree of freedom, *OR* odds ratio, *CI* confidence interval, *MetS* metabolic syndrome, *BMI* body mass index, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *Treg* T regulatory cells

zLDL/HDL z value of low-density lipoprotein cholesterol – z value of high-density lipoprotein cholesterol, *zTh17/Treg* z natural logarithmic (Ln) value of interleukin (IL) 6 + z Ln value of IL17 – z Ln value of IL-10

Table 5 Multilayer Perceptron (MLP) Neural Network (NN) models with the EDSS score (continuous or dichotomized variable) as output variables and age, sex, duration of illness, metabolic syndrome and IM&NO biomarkers as input variables

NN models	Model Output Input	NN ≠ 1 Scale EDSS All (n = 14 variables)	NN ≠ 2 EDSS ≥3 All (n = 14 variables)
	SS/CE error	1.418	27.356
Training	Relative error or % incorrect	0.644	21.2%
	prediction (sensitivity-specificity)	–	(92.0% – 66.7%)
	SS/CE error	0.602	10.337
Testing	Relative error or % incorrect	0.669	21.7%
	prediction (sensitivity-specificity)	–	(93.3% – 50.0%)
Holdout	Relative error or % incorrect prediction (sensitivity-specificity)	0.653	15.4%
		–	(94.4% – 62.5%)
	AUC ROC	–	0.842

EDSS Expanded Disability Status Scale, *IM&NO* immune-inflammatory, metabolic, and oxidative stress biomarkers, *AUC ROC* area under the receiving operating curves, *SS/CE* sum of squares or cross-entropy error

et al. 2015; Sospedra and Martin 2016). Therefore, the demyelination and neurodegeneration that occur in the MS are driven by Th1/Th2 and Th17/Treg imbalance, which Th1 and Th17 cytokine profiles mediate the inflammatory damage mechanisms whereas Th2 and Treg cytokine profiles mediate a compensatory anti-inflammatory and protective mechanisms in MS (Hemmer et al. 2015; Sospedra and Martin 2016).

The role of the Treg response through IL-10 and TGF-β cytokines is to dampen an overzealous inflammatory response caused by Th1 and Th17 immune responses. In the present study, the positive association between the z-unit weighted composite score zTh17/Treg with moderate/severe disability in MS patients obtained in different statistical models (Table 4) underscores the important role of T17 cells in the physiopathology of MS. Moreover, this result showed that the Treg-mediated anti-inflammatory response, by itself, was unable to restore the immune homeostasis and that this failure in the compensatory immune-regulatory reflex system (CIRS) as recently reviewed (Maes and Carvalho 2018) and, consequently, may result in a uncontrolled inflammation that contributed to the disability and disease progression in this cohort

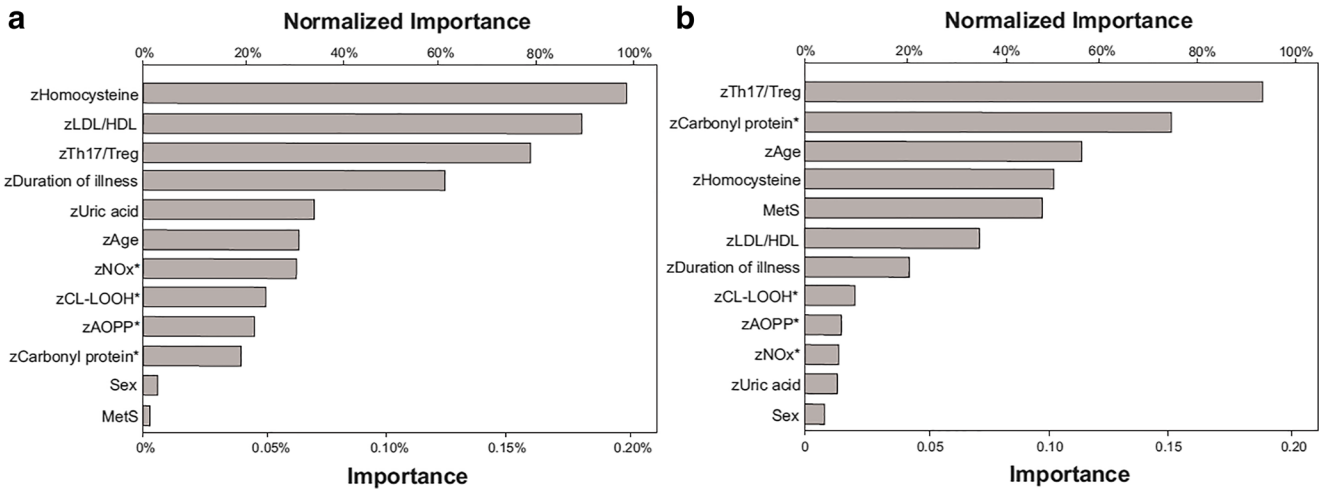

Fig. 1 Results of two different multilayer perceptron (MLP) neural network (NN) models of analysis showing the relative and normalized importance of the input variables predicting the disability using Expanded Disability Scale Score (EDSS). **a**: Model #1 used EDSS as continuous variable and immune inflammatory, metabolic and oxidative stress biomarkers, together with age, sex, body mass index, metabolic syndrome (MetS), and duration of illness as input variables. As showed, the homocysteine, zLDL/HDL and zTh17/Treg were the most important determinants of the predictive power of MLP/NN model #1, followed at a distance by duration of illness. **b:** Model #2 used dichotomized EDSS (EDSS <3 and EDSS ≥3) as output variable and the same input variables as in MLP/ NN model #1. As showed, zTh17/Treg, carbonyl proteins and age were the most important determinants of the predictive power of higher EDSS followed at a distance by homocysteine, MetS and LDL/HDL ratio. *z-scores obtained on Ln transformations. AOPP: advanced oxidation protein product; NOx: nitric oxide metabolites; CL-LOOH: hydroperoxide; Treg; T regulatory; HDL: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol

of MS patients. Altogether, this result suggests that Th17/Treg score may be considered an important biomarker to predict the disability progression in MS patients.

Possibly, in different clinical forms of MS, or in different clinical phases, either Th1 or Th17 cells are the main drivers of pathological processes. This might also be the case in the evolution of individual CNS lesions, with one Th1 lineage initiates the pathologic mechanisms and Th17 lineage perpetuates them. Therefore, the Th17 cells will continue to be of intense interest for therapeutic targeting in MS and new approaches could be developed, hopefully with great benefit to MS patients (Rostami and Ciric 2013).

Moreover, the present study showed that higher levels of homocysteine were obtained in patients with moderate/severe disability than those with wild disability, underscoring the association between hyperhomocysteinemia and disease progression, independently of confounding factors, when MS patients were evaluated using the Multiple Sclerosis Severity Score (MSSS) (Oliveira et al. 2018). Hyperhomocysteinemia was also associated with the presence of MS (Ramsaransing et al. 2006; Moghaddasi et al. 2013), as well as with high disability (Guzel et al. 2016). Homocysteine is known to be toxic to the CNS and hyperhomocysteinemia may be a risk factor for neurodegenerative conditions (Seshadri 2006; Zhu et al. 2011). The CNS may be particularly sensitive to extracellular homocysteine, as it promotes excitotoxicity via stimulation of N-methyl-D-aspartate receptors (NMDA), and damages neuronal DNA, thereby triggering apoptosis (Ho et al. 2002; Škovierová et al. 2015). Different mechanisms are involved in the neurotoxic effect of homocysteine on neuronal and glial cells (Škovierová et al. 2015, 2016; Longoni et al. 2018). On neuronal cells, homocysteine acts as an agonist for both groups of glutamate receptors, NMDA and non-NMDA, and the overstimulation of these receptors results in increased level of cytoplasmic calcium, higher production of free radicals and activation of caspases leading to apoptosis (Škovierová et al. 2016). Moreover, homocysteine promotes oxidative stress. An in vitro effect of homocysteine in rat hippocampus showed that it significantly increases lipid peroxidation parameter and decreases total antioxidant plasma capacity, both in a dose-dependent manner, but did not change antioxidant enzymes (Streck et al. 2003). On glial cells, hyperhomocysteinemia in vitro showed to be a potent gliotoxic agent capable of inducing the death of human glial cells already at concentrations reached in brain during hyperhomocysteinemia (Škovierová et al. 2015). Moreover, homocysteine induced in adult astrocytes changes in cellular morphology/cytoskeleton proteins, pro-inflammatory TNF-α, IL-1β, and IL-6 cytokines release, cellular antioxidant defenses, nuclear factor κ B (NF-κB) transcriptional activity, and heme oxygenase 1 (HO-1) expression levels (Longoni et al. 2018). Therefore, it is conceivable to suggest that association between hyperhomocysteinemia and increased disability and progression of the disease may be explained by direct neurotoxic effects of homocysteine via excitotoxic mechanism thereby contributing to neurodegeneration.

The results of the current study also showed that the presence of MetS and increased levels of LDL-cholesterol were significantly associated with moderate/severe disability. MetS is a global public health challenge and a complex condition represented by a combination of risk factors, such as central obesity, dyslipidemia, hypertension, and disturbed glucose metabolism, a cluster of interconnected factors which lead to an increased risk of cardiovascular diseases and T2DM (Reaven 1997; Kassi et al. 2011). Previous research has shown that the prevalence of MetS is higher than national averages for individuals with autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus (Versini et al. 2014). However, conflictin results are also reported, such as that the rate of 30% of MetS obtained in a sample of 130 MS patients with moderate/severe disability (EDSS score ≥ 3), with no gender difference, did not differ from the general population (Pinhas-Hamiel et al. 2015).

A prospective cohort design in patients with MS found that high levels of total cholesterol were associated with increased disability, and increased total cholesterol/HDL ratio was associated with annual accumulating disability (Tettey et al. 2014). A placebo-controlled trial carried out with MS patients showed that those using lipid-lowering statins had a lower change in EDSS after two years than the placebo group (Chataway et al. 2013). Further, these authors showed that, compared with placebo, simvastatin 80 mg per day reduced the annualized rate of whole-brain atrophy (the primary outcome) by 43.0%, considered a positive effect given that longitudinal studies have shown a relation between atrophy progression and disability. These authors also noted a small, but significant, effect in two the secondary clinical outcomes, such as the mean score at 24 months of EDSS evaluated by a physio and multiple sclerosis impact scale 29 (MSIS-29) by a patient reported viewpoint, supporting a true effect of dyslipidemia on disease progression (Chataway et al. 2014).

Regarding the lipid profile of the present cohort of MS patients, our results are in agreement with the findings that patients with acute transverse myelitis (ATM) and patients with MS showed higher serum triglycerides, total cholesterol, and LDL-cholesterol levels than healthy controls. Moreover, longer disease duration was associated with high triglycerides while high HDL-cholesterol was associated with a trend toward low disability scores (Liu et al. 2016).

Mounting evidence indicates that serum cholesterol and other risk factors for cardiovascular diseases intensify normative trajectories of age-related cognitive decline and may have impact on brain networks (Spielberg et al. 2017). These authors demonstrated a deleterious effect of cholesterol on brain health and suggested that cholesterol accelerates the impact of

age on neural trajectories by disrupting connectivity in circuits implicated in integrative processes and behavioral control (Spielberg et al. 2017).

Some lines of evidence also indicates that nitro-oxidative stress plays a major role in the pathogenesis of MS by contributing to myelin and oligodendroglia degeneration (Ferretti et al. 2005). We previously demonstrated that individuals with MS have persistent oxidative stress, even when the disease remained in clinical remission, and that oxidative stress may play an important role in the physiopathology of disability progression (Oliveira et al. 2012). Increased AOPP and progression of MS were significantly and positively associated with increase of ferritin; furthermore, the combination of serum ferritin levels and oxidative stress markers were responsible for 13.9% of the MS diagnosis and the results suggested that ferritin could aggravate oxidative stress in patients with MS and contribute to progression of disease (Ferreira et al. 2017).

Our results showed that AOPP was higher in MS with moderate/severe disability (EDSS ≥3) than those with mild disability (EDSS <3), while carbonyl proteins was associated with the dichotomized EDSS score. Differences between both indices of protein oxidation may be explained by differences in formation. AOPP, a group of oxidatively modified proteins that contain pentosidine, dityrosine and carbonyls, is formed as a consequence of hypochlorous acid stress which may proceed via elevated activity of myeoloperoxidase coupled with increased peroxide and peroxynitrite production (Huang et al. 2013; Gryszczyńska et al. 2017). The formation of protein carbonyl is earlier than the AOPP and is the consequence of many more oxidative processes and therefore reflects overall oxidation of proteins (Weber et al. 2015; Gryszczyńska et al. 2017). Carbonylation refers to the non-enzymatic addition of aldehydes or ketones to specific amino acid residues and constitutes the most common oxidative modification of proteins affecting the function and/or metabolic stability of the modified proteins (Levine 2002). While AOPP is an irreversible process, carbonylation is a reversible one. The formation of protein carbonyls seems to be a common phenomenon during oxidation, and their quantification can be used to measure the extent of oxidative modification (Dalle-Donne et al. 2003).

Our previous study showed that RRMS patients exhibited higher plasma levels of lipid peroxidation (CL-LOOH) and carbonyl proteins, while lower plasma levels of NOx, TRAP, and sulfhydryl (SH) groups than controls. Moreover, patients with moderate/severe disability showed higher CL-LOOH than controls and a positive correlation was observed between CL-LOOH and EDSS, as well as between carbonyl proteins and EDSS (Oliveira et al. 2012). Previous findings also showed that plasma and cerebrospinal fluid AOPP levels were higher and SH group levels were lower in patients with clinically isolated syndrome (CIS) and with RRMS than those obtained in controls. Furthermore, regarding to disability of patients with CIS and RRMS, plasma and cerebrospinal fluid AOPP levels were positively correlated with EDSS, while SH group levels was inversely correlated with EDSS (Ljubisavljevic et al. 2013). Taken together, these results suggest that oxidative and nitrosative stress pathways could be possible new targets of integrative therapies for patients with MS.

Carbonylation of brain proteins has been implicated in the etiology and/or progression of several neurodegenerative disorders including Alzheimer's disease (Aksenov et al. 2001), Parkinson's disease (Floor and Wetzel 2002), amyotrophic lateral sclerosis (Ferrante et al. 1997), as well as MS (Bizzozero et al. 2005; Zheng and Bizzozero 2010). Elevated levels of carbonyl proteins are generally a sign not only of oxidative stress but also of disease-derived protein dysfunction and the assay of carbonyl protein has a major advantage over lipid peroxidation products as markers of oxidative stress because oxidized proteins are generally more stable (Floor and Wetzel 2002). Moreover, a positive correlation was observed between carbonyl group level and disability in SPMS patients (Morel et al. 2017).

We have shown previously that the immune-inflammatory and oxidative stress mechanisms, as well as the *TNFβ* + 252 G/A (rs909253) genetic variant play a role in the pathophysiology of MS and disease progression and that biomarkers of these pathways are predictors of high disability and are associated with different aspects of disease progression (Kallaur et al. 2017). We demonstrated that IL-10, TNF-α, IFN-γ, AOPP, and NOx levels were higher while IL-4 was lower in MS patients with moderate/severe disability compared with those with mild disability. The actual accumulating disability observed during five-year follow-up were positively associated with TNF-α and IFN-γ. In that study, increased IFN-γ values were associated with high pyramidal symptoms, increased IL-6 with sensitive symptoms while increased carbonyl protein and IL-10 but lowered albumin levels predicted cerebellar symptoms. Moreover, the TNFB1/B2 genotype of *TNFβ* + 2552 G/A (rs909253) variant decreased the risk towards progression of pyramidal symptoms (Kallaur et al. 2017).

Conclusions

To our knowledge, no studies have evaluated the association between Castelli indices 1 and 2 surrogates, such as total cholesterol/HDL-cholesterol ratio and LDL/HDL ratio, respectively, as well as the association between IM&NO biomarkers and MS disability. The results demonstrated here underscore that IM&NO pathways play a key role in increased disability in MS patient and may be possible new targets for the treatment of them. Moreover, the results contribute to the

identification of a laboratory biomarker panel that may be used in predicting the disability in MS patients.

Author's contributions Conception and research design: Edna Maria Vissoci Reiche and Andrea Name Colado Simão; Manuscript writing and discussion of results: Edna Maria Vissoci Reiche, Andrea Name Colado Simão; Tamires Flauzino; Data collection: Damacio Ramón Kaimen-Maciel, Wildea Lice de Carvalho Jennings Pereira, Tamires Flauzino, Daniela Frizon Alfieri, Ana Paula Kallaur, and Sayonara Rangel Oliveira, which contributed equally; Laboratory analysis: Tamires Flauzino, Daniela Frizon Alfieri, Marcell Alysson Batisti Lozovoy; Statistical analysis: Andrea Name Colado Simão, Michael Maes. All authors have read and approved the final manuscript.

Financial support This study was partially and financially supported by Novartis Biosciences S.A for the development of the research according to the Researcher's Initiative Study CFTY720DBR07T. The authors do not receive any reimbursement or financial benefits and declare that they have no competing interests. Novartis Biosciences S.A. played no role in the design, methods, data management or analysis or in the decision to publish. The study was also supported by grants from Coordination for the Improvement of Higher Level of Education Personnel (CAPES) of Brazilian Ministry of Education; Institutional Program for Scientific Initiation Scholarship (PIBIC) of the National Council for Scientific and Technological Development (CNPq).

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 of the individuals invited were informed in detail about the research and gave written Informed Consent.

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