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Multifaceted Analysis of Cerebrospinal Fluid and Serum from Progressive Multiple Sclerosis Patients: Potential Role of Vitamin C and Metal Ion Imbalance in the Divergence of Primary Progressive Multiple Sclerosis and Secondary Progressive Multiple Sclerosis

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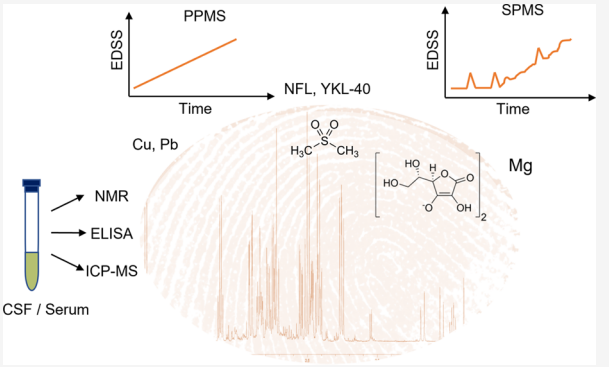
ABSTRACT: The progressive forms of multiple sclerosis (MS) primary progressive MS (PPMS) and secondary progressive MS (SPMS) are clinically distinguished by the rate at which symptoms worsen. Little is however known about the pathological mechanisms underlying the differential rate of accumulation of pathological changes. In this study, ^{1}H NMR spectroscopy was used to measure low-molecular-weight metabolites in paired cerebrospinal fluid (CSF) and serum of PPMS, SPMS, and control patients, as well as to determine lipoproteins and glycoproteins in serum samples. Additionally, neurodegenerative and inflammatory markers, neurofilament light (NFL) and chitinase-3-like protein 1 (CHI3L1), and the concentration of seven metal elements, Mg, Mn, Cu, Fe, Pb, Zn, and Ca, were also determined in both CSF and serum. The results indicate that the pathological changes associated with progressive MS are mainly localized in the central nervous system (CNS). More so, PPMS and SPMS patients with comparable disability status are pathologically similar in relation to neurodegeneration, neuroinflammation, and some metabolites that distinguish them from controls. However, the rapid progression of PPMS from the onset may be driven by a combination of neurotoxicity induced by heavy metals coupled with diminished CNS antioxidative capacity associated with differential intrathecal ascorbate retention and imbalance of Mg and Cu.



KEYWORDS: *multiple sclerosis, NMR; metabolomics; metallomics, oxidative stress; antioxidant, vitamin C, copper, magnesium, lead*

INTRODUCTION

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS), which predominantly affects young adults, with gender distribution skewed toward females. Despite the elusiveness of the etiology of MS, it is regarded as a confluence of genetics and environmental factors. The clinical presentations of MS vary significantly between and within patients. Hence, its generalized clinical classifications are based on the regularity and the rate of deterioration of symptoms. While some MS patients exhibit an accelerated worsening of symptoms from the onset of the disease, referred to as primary progressive MS (PPMS), others experience alternating bouts of symptoms and remissions known as relapsing−remitting MS (RRMS). The majority of the latter group eventually reach a point of continuous worsening of symptoms and are classified as secondary progressive MS (SPMS).[1,2] MS should therefore be regarded as a progressive disease regardless of the initial presentations.

The progressive forms of MS (PPMS and SPMS) have been considered as varied phenotypic presentations of the same disease. Some studies have shown that from the onset of progression, not only are disability milestones among PPMS and SPMS patients reached at approximately the same time but also disease activity outcome measures are similar within specified timeframes.[3]

The pathophysiology of MS has generally been attributed to autoimmune and inflammatory reactions against the CNS facilitated by the infiltration of the CNS by autoreactive

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lymphocytes. Hence, neuroinflammation is regarded as an integral pathological feature of MS. Some studies have found plausible etiopathological associations between MS and nonspecific inflammatory biomarkers such as chitinase-3-like protein 1 (CHI3L1), also known as YKL-40.[4] Others have found a correlation between cerebrospinal fluid (CSF) and serum concentrations of YKL-40 and the extent of disability among MS patients.[5,6] While systemic inflammation, which is evident in the serum concentrations of inflammatory markers, may be necessary for the initiation of the pathological cascade that leads to MS, it is not clear whether peripheral inflammation is necessary for the evolution of pathological changes in progressive MS.

Quite recently, the glycosylation products of serum proteins by *N*-acetylglucosamine/*N*-acetylgalactosamine or *N*-acetylneuraminic acid, known as GlycA and GlycB, and a function that defines the area between GlycA and GlycB, consisting of acetyl groups of unbound *N*-acetylneuraminic acid, *N*-acetylgalactosamine, and *N*-acetylglucosamine, referred to as GlycF, have emerged as nonspecific nuclear magnetic resonance (NMR)-based markers of systemic inflammation.[7,8] The longer half-life of GlycA makes it a better marker of chronic systemic inflammation compared to C-reactive protein (CRP) and other serum cytokines. GlycA and GlycB have been investigated in diseases that consist of chronic inflammation[9−12] and neurological conditions such as cognitive impairment[13] or Parkinson's disease.[14] However, to the best of our knowledge, research is yet to explore the role of GlycA, GlycB, and GlycF in the pathology of MS. Determining the variations in serum concentrations of these glycoproteins and their derived parameters could clarify the role of systemic inflammation in MS.

Metal ions have also been implicated in the pathophysiology of various neurological diseases. Their modus operandi has been attributed to neurotoxicity, oxidative stress, and unavailability to serve as cofactors in enzymatic reactions. However, the association between the metals and disease is not straightforward since the distribution of metal elements in the brain exhibits regional, hemispheric, and age-dependent differences.[15] Hence, while CSF concentrations of metals could serve as surrogates for CNS concentrations, they may not reflect regional and hemispheric differences. This is particularly important in establishing relationships between the functional systems of the extended disability status scale (EDSS) and the regional distribution of metals in the CNS of MS patients. While some metal elements such as cerebral iron and vascular calcification could be noninvasively measured with magnetic resonance imaging and computed tomography, other metals lack equivalent in vivo measurements. They could, however, be indirectly measured from CSF with analytical techniques, such as inductively coupled plasma mass spectrometry (ICP-MS). A prior study of trace elements in the blood of MS patients revealed reduced concentrations of Be, Cu, Cr, Co, Ni, Mg, and Fe, while Pb was increased in MS patients relative to controls.[16] However, in this study, the subtype of MS studied was not indicated. Therefore, to the best of our knowledge, studies comparing the levels of certain metals in the two progressive forms of MS have not been reported to date.

Despite all these research efforts, a gap remains in the complete understanding of the pathophysiological mechanisms underlying progressive MS. More so, since different studies have addressed different aspects of the disease pathology, there is no unifying theory to explain the pathophysiology of progressive MS. While some studies focused on metabolic differences, others have been based on either inflammatory or neurodegenerative biomarkers. In some instances, the different categories of MS have been combined, resulting in inconsistent results. Considering the heterogeneity of MS, we have applied a multifaceted approach to investigate the similarities and differences in the pathological contributors to the progressive forms of MS.

MATERIALS AND METHODS

Study Subjects, Sample Collection, and Storage

CSF and serum samples were collected from 22 SPMS, 18 PPMS, at the time of disease progression confirmation, and 13 controls at the Neurology Unit of the "Virgen de la Macarena" University Hospital in Seville, using standardized protocols. All samples were collected, coded, and stored in the hospital biobank, from where they were sent, anonymized, to the NMR Unit for analysis. All study subjects signed the informed consent form for research on biobanked samples, which was approved by the Ethics Committee of the Hospital, prior to participation in the study. MS patients were classified according to the 2017 revised McDonald criteria.[2] Control subjects were patients whose diagnosis required the collection of CSF samples but whose conditions were not considered to be neurodegenerative in nature. The absence of unrecognized neurodegeneration or neuroinflammation among controls was subsequently confirmed with low concentrations of neurofilament light (NFL) and YKL-40 in their CSF. All samples were stored at −80 °C until analysis.

None of the PPMS patients were under any treatment at the time of sample collection, and only four of them were taking a combination of B vitamin supplementations (B12, B6, and B1). As for the SPMS patients, only 4 of the 22 patients were under treatment, 1 with interferon beta-1b and 3 with natalizumab. Furthermore, one of the patients under natalizumab was also supplemented with B vitamins, and another patient was only supplemented with the same combination of B vitamins. The rest of the SPMS patients had been without any type of treatment for at least a year.

NMR Sample Preparation and Spectral Acquisition

Prior to NMR analysis, samples were gradually thawed on ice. 450 μL of CSF and 300 μL of serum were, respectively, mixed with 150 and 300 μL of their corresponding buffers containing 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid acid as the chemical shift reference. Spectra were acquired on a Bruker Avance III 600 MHz spectrometer, using a three-channel Z-gradient reverse detection probe (Prodigy TCI cryoprobe). The acquisition sequences included 1D Nuclear Overhauser Effect SpectroscopY (NOESY), for both serum and CSF, 1D Carr−Purcell−Meiboom−Gill (CPMG), which provides T_2 edited spectra for the suppression of signals from macromolecules in serum, 2D *J*-resolved (JRES) to aid in the identification of metabolites, and 1D diffusion edited spectra (Diff), which filter out the small molecular weight metabolites to enable the quantification of macromolecules such as glycoproteins and lipoproteins. All spectra were acquired with pre-saturation of the water signal. The acquisition was fully automated under standard operating procedures, including temperature adjustment and stabilization, ^{1}H channel tuning, shimming, and radiofrequency pulse calibration.

The NMR buffered samples were again stored at −80 °C for ELISA and ICP-MS analysis.

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ELISA

Quantification of CSF NFL was performed with the Uman Diagnostics NF-Light ELISA kit (Umea, Sweden) according to the manufacturer's protocol using a final dilution factor of 1:2. CSF and serum YKL-40 levels were also quantified according to the manufacturer's protocol for YKL-40 (Invitrogen, Thermo Fisher Scientific) with dilution factors of 1:200 and 1:150 for CSF and serum, respectively. All samples were subjected to the same freeze−thaw cycle to ensure comparability of the results. In addition, samples were randomized to nullify potential differences due to a batch effect in all analyses and an edge effect during ELISA.

ICP-MS

Trace metal elements were also measured in both CSF and serum with ICP-MS based on a modified protocol from ref 17. 200 μL of NMR diluted samples was mixed with 200 μL of HNO_3 and 100 μL of H_2O_2. The mixture was briefly vortexed and digested for 90 min at 65 °C. The final volume was raised to 10 mL with deionized water and vortexed, which was ready for ICP-MS analysis.

Data Analysis

Spectral Processing and Quantification of Metabolites, Lipoproteins, and Glycoproteins. Both untargeted metabolic fingerprinting and profiling were performed on the NMR spectra. NOESY and CPMG spectra were pre-processed using *TopSpin 3.5.pl7* (Bruker BioSpin, Ettlingen, Germany). The pre-processing included Fourier transformation after 0.3 Hz exponential filtering, baseline correction, and the addition, as the concentration reference, of the Electronic REference To access In vivo Concentrations (ERETIC), implemented as ERETIC 2 in *TopSpin 3.5.pl7*. The spectra were then converted to text files after deleting unwanted spectral regions, such as water signals in both CSF and serum and the imidazole pH indicator in the CSF samples, using MestReNova (v12.0.4, Mestrelab Research S.L.). The spectral matrices were then processed using scripts written in R v. 3.6.1, including peak picking using the R library Speaq 2.6.[18] Statistically significant peaks based on the Kruskal−Wallis test were assigned by first identifying other signals that belong to the same metabolite using an in-house implemented concatenation of statistical methods, such as ratio analysis NMR spectroscopy[19] and statistical total correlation spectroscopy.[20] Peaks that showed statistically significant differences in crowded spectral regions but did not have related peaks that were clearly isolated or those with intensities very close to noise level, thus hindering precise identification, were excluded. Peak assignments to specific metabolites were confirmed using the human metabolome database and Chenomx (v8.4, Chenomx Inc., Edmonton, Canada). The assigned metabolites were quantified with Chenomx using ERETIC as the concentration reference. The accurate quantification of metabolites requires, in addition to a reliable concentration reference, avoiding T_1 and T_2 relaxation effects. Thus, fairly long recycle times (~7 s) were used in all cases to minimize T_1 effects. As for T_2 effects, they were avoided in CSF samples by using a pulse-acquisition-type sequence (1D NOESY). However, in the case of serum samples, a CPMG sequence was used to reduce the contribution of macromolecules, which implies that certain signal loss and, therefore, a slight underestimation of metabolite concentrations occur due to T_2 relaxation effects. Yet, this underestimation of metabolite concentrations is the same across samples, and therefore, comparisons between different groups or subjects remain valid. Glycoproteins and lipoproteins were quantified from diffusion-edited spectra. Briefly, lipoprotein analysis was performed by using the Liposcale test (IVD-CE, Biosfer Teslab, Reus), a previously reported 2D ^{1}H NMR test based on an approach for lipoprotein profile characterization including lipid content, size, and particle number of the lipoprotein classes, very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL), and the main lipoprotein subclasses.[21] The methyl signal was deconvoluted by using nine Lorentzian functions to determine the lipid concentration of the large, medium, and small subclasses of the main lipoprotein classes (VLDL, LDL, and HDL) and their size-associated diffusion coefficients. Complementarily, we analyzed the region of the ^{1}H NMR spectrum where the glycoproteins resonate (2.15−1.90 ppm) using several analytical functions according to a previously published procedure.[22] For each function, the total area (proportional to the concentration), height, position, and bandwidth were determined. The area of GlycA provided the concentration of acetyl groups of protein-bound *N*-acetylglucosamine and *N*-acetylgalactosamine, and the area of GlycB, those of *N*-acetylneuraminic acid. The GlycF area arises from the concentration of the acetyl groups of *N*-acetylglucosamine, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid unbound to proteins (free fraction). Height-to-weight ratios of GlycA and GlycB, a parameter describing the aggregation state of the sugar−protein bonds, were also reported to be higher for inflammation-associated processes.

Statistical Analysis

Statistical analysis and visualization of metabolite concentrations, glycoproteins, lipoproteins, NFL, YKL-40, metal elements, and demographic data were performed using R, SPSS v.20, and Metaboanalyst v.5.0. The gender distribution was analyzed with Pearson's Chi-Square, while the age at inclusion was analyzed with one-way analysis of variance. EDSS was analyzed with an independent sample *t*-test with the assumption of equal variance based on Levene's test for equality of variance, while equal variance was not assumed for the disease duration for the same test. All concentrations were Pareto scaled prior to multivariate analysis. Due to the relatively high number of variables relative to the number of observations, sparse partial least squares discriminant analysis (sPLSDA) with leave-one-out cross-validation was used to identify the metabolites with the greatest contribution to group separation. Since some of the concentration data were not normally distributed based on Kolmogorov−Smirnov and Shapiro−Wilk tests, nonparametric tests, such as the Kruskal−Wallis test for multiple comparisons and the Mann−Whitney *U* test for pairwise comparisons, were performed. Descriptive statistics for the biomarkers are reported as the median and interquartile range (IQR), while those for the demographic data are reported as the mean and standard deviation (SD). Due to multiple comparisons and unless otherwise stated, *p*-values lower than 0.01 were considered to be statistically significant, while *p*-values higher than 0.01 but less than 0.05 were considered as trends. Spearman's correlation coefficients were also calculated for relevant metabolites. Receiver operator characteristic (ROC) curves were also constructed for significant metabolites using SPSS v.20. The multidimensional scaling method ALSCAL was used to project the significant features in two dimensions with the dissimilarity matrix based on the Euclidean distance.

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RESULTS

Demographic Data

Information about the demographic distribution of samples in this study is provided in Table 1. No significant association was observed between the gender distribution and disease categories ($X^2 = 1.239$, $p = 0.538$). Also, there was no significant difference in the age at inclusion among the controls and the MS patients. However, at an alpha level of 0.05, the disease duration and EDSS scores were significantly higher in SPMS compared to PPMS ($p < 0.001$ and $p = 0.027$, respectively).

Table 1. Demographic Data

	CTR	PP	SP	*p*-value
gender (F:M)	10:3	11:7	13:9	0.538
%(F)	76.9%	61.1%	59.1%	
age at inclusion, mean (SD)	33.38 (10.3)	49.33 (9.6)	46.14(9.66)	0.303
disease duration, mean (SD)	NA	5.89(6.22)	18.18(9.76)	<0.001
EDSS, mean (SD)	NA	4.61(1.29)	5.7(1.64)	0.027

High-Resolution ^{1}H NMR Spectra

The 1D ^{1}H NMR spectra acquired with a liquid nitrogen-cooled cryoprobe showed excellent reproducibility and high sensitivity, allowing an accurate quantification of metabolites even in the low micromolar range (Figures 1 and 2).

Multivariate Analysis

The score plot of the first two components of sPLSDA (Figure 3A) revealed a large overlap between PPMS and SPMS, while the controls were clearly separated. However, only 26.5% of the variability within the data was explained by the first two components. The biomarkers that contributed the most to the group separation in the first component are shown in Figure 3B.

Pairwise Analysis

Pairwise comparison of CSF metabolites in progressive forms of MS and controls at the alpha level of 0.01 in Table 2 shows that citrate, dimethylamine, formate, and methylmalonate were significantly increased in the CSF of the two progressive forms of MS relative to controls. However, the concentrations of these metabolites did not differ significantly between PPMS and SPMS. While lactate was significantly higher in PPMS compared to controls, there was a trend toward higher concentrations in SPMS compared to controls. Additionally, alanine, dimethyl sulfone, and guanidinoacetate were significantly higher in SPMS compared to controls, but no differences were observed for these metabolites in PPMS compared to controls. In conclusion, all these metabolites were selectively and significantly increased in one or both progressive forms of MS but showed no differences between both MS groups. On the other hand, ascorbate and glycolate showed statistically significant differences between SPMS and PPMS. Interestingly, the CSF concentration of ascorbate was significantly lower in PPMS compared to SPMS and controls, while no difference was found between controls and SPMS. Conversely, the concentration of glycolate was significantly lower in SPMS compared to PPMS and controls but showed no difference between PPMS and controls. Besides the metabolic changes, the NFL polypeptide and the YKL-40 glycoprotein also showed increased concentrations in both MS forms compared to controls, although there were no differences between PPMS and SPMS. Intriguingly, the increased levels of

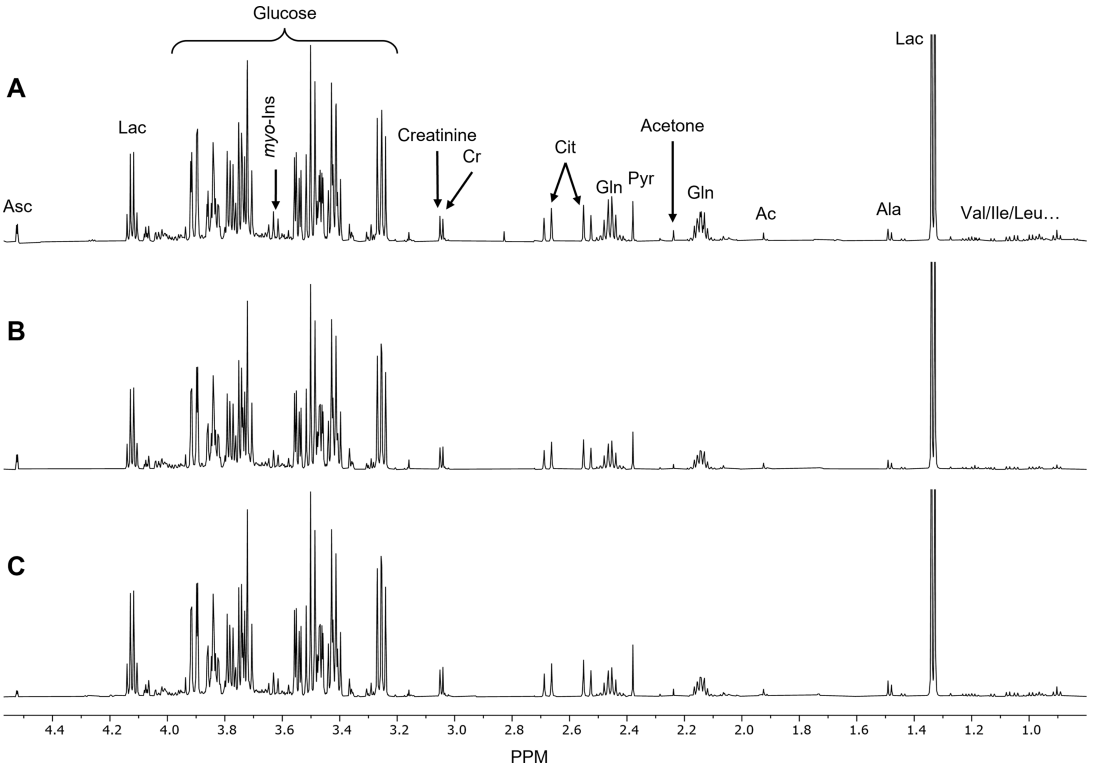

Figure 1. Representative ^{1}H NMR spectra from the CSF of (A) control, (B) SPMS, and (C) PPMS patients. Most abundant metabolites are labeled in the upper spectra. Asc: ascorbate; Lac: lactate; myo-Ins: myo-inositol; Cr: creatine; Cit: citrate; Gln: glutamine; Pyr: pyruvate; Ac: acetate; Ala: alanine; Val: valine; Ile: isoleucine; Leu: leucine.

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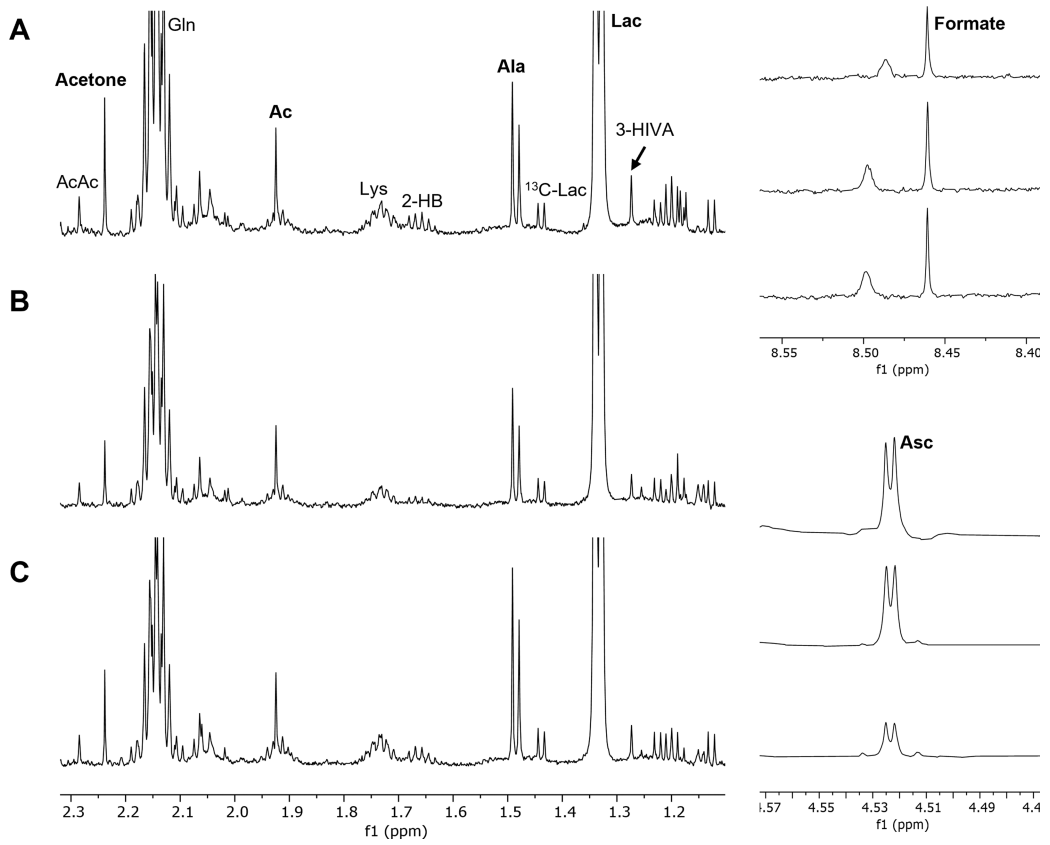

Figure 2. Zoomed spectral regions from the spectra in Figure 1 showing some relevant metabolites (bold). (A) Control, (B) SPMS, and (C) PPMS. AcAc: acetoacetate; Gln: glutamine; Ac: acetate; Lys: lysine; 2-HB: 2-hydroxybutyrate; Ala: alanine; D-Lac: D-lactate; Lac: L-lactate; 3-HIVA: 3-hydroxyisovalerate; Asc: ascorbate. Concentration of the displayed metabolites in the control sample: AcAc = 9.2 μM; acetone = 12.3 μM; Gln = 0.486 mM; Lys = 33.6 μM; Ala = 41.61 μM; Lac = 1.485 mM; 3-HIVA = 4.9 μM; formate = 45.9 μM; Asc = 0.225 mM.

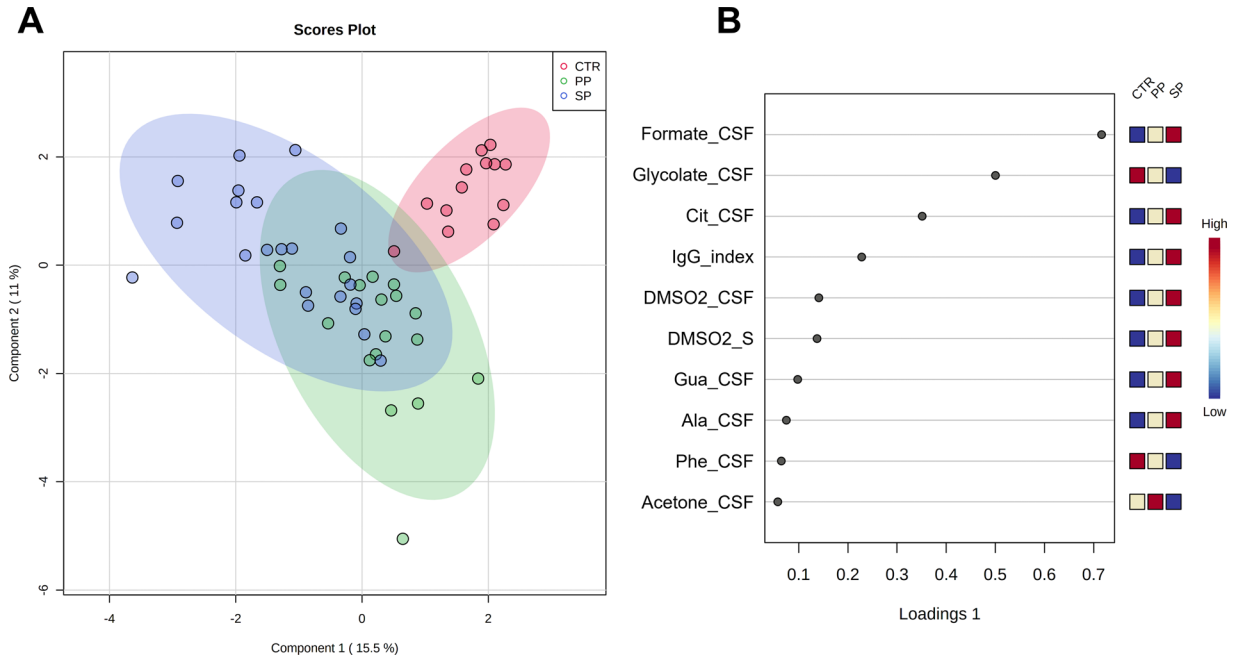

Figure 3. Multivariate analysis by sPLSDA. (A) Score plot and (B) loading plot. Cit: citrate; DMSO2: dimethyl sulfone; Gua: guanidinoacetate; Ala: alanine; Phe: phenylalanine.

YKL-40 in the CSF of the MS groups were not concomitant with the increased levels in the serum of the same patients.

Regarding the concentrations of serum metabolites (Table 3), only dimethyl sulfone was significantly higher in SPMS compared to controls, while phenylalanine was significantly

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Table 2. Descriptive Statistics and Pairwise Comparison of CSF Biomarkers[a]

biomarker	CTR Median (IQR)	PPMS Median (IQR)	SPMS Median (IQR)	CTR-PPMS	Δ	CTR-SPMS	Δ	PPMS-SPMS	Δ
Acetate (mM)	**0.0179** (0.0115)	**0.0264** (0.0432)	**0.0801** (0.0875)			0.017(tr)	↑		
Acetone (mM)	**0.0116** (0.0075)	**0.0111** (0.0057)	**0.0083** (0.0036)			0.044(tr)	↓	0.01	↓
Alanine (mM)	**0.0276** (0.0138)	**0.0361** (0.0176)	**0.0398** (0.0109)			**0.006**	↑	0.014(tr)	↑
Ascobate (mM)	**0.1533** (0.0303)	**0.0807** (0.0871)	**0.1459** (0.0784)	**0.006**	↓			**0.004**	↑
Citrate (mM)	**0.2046** (0.0369)	**0.2758** (0.0858)	**0.292** (0.1187)	**<0.001**	↑	**<0.001**	↑		
Dimethyl sulfone (mM)	**0.007** (0.0048)	**0.01** (0.0058)	**0.0111** (0.0043)			**0.007**	↑		
Dimethylamine (mM)	**0.0016** (0.0005)	**0.0023** (0.0011)	**0.0022** (0.0009)	**0.009**	↑	**0.015**	↑		
Formate (mM)	**0.0396** (0.0095)	**0.0586** (0.0204)	**0.108** (0.0746)	**<0.001**	↑	**<0.001**	↑	0.047(tr)	↑
Glycolate (mM)	**0.0192** (0.0056)	**0.0165** (0.0059)	**0.0127** (0.0053)			**0.001**	↓	**0.001**	↓
Guanidinoacetate (mM)	**0.0274** (0.01)	**0.031** (0.0108)	**0.0356** (0.0126)			**0.005**	↑		
Lactate (mM)	**1.3085** (0.2473)	**1.5014** (0.3294)	**1.4686** (0.2427)	**0.008**	↑	0.018(tr)	↑		
Methylmalonate(mM)	**0.0071** (0.0016)	**0.0085** (0.0023)	**0.0085** (0.0018)	**0.008**	↑	**0.006**	↑		
NFL (pg/mL)	**307.35** (83.21)	**755.73** (1109.49)	**633.51** (627.12)	**0.001**	↑	**0.005**	↑		
YKL-40 (ng/mL)	**50.20** (37.48)	**120.39** (104.02)	**82.37** (129.67)	**<0.001**	↑	**0.003**	↑		
Mg (μg/L)	**27524.98** (2507.07)	**26003.80**(1009.44)	**26932.00**(2540.77)	**<0.001**	↓			0.036(tr)	↑
Cu (μg/L)	**25.46** (10.86)	**64.13**(54.51)	**30.61**(43.31)	**<0.001**	↑			0.030(tr)	↓
Pb (μg/L)	**20.70**(3.18)	**26.73**(16.37)	**22.02**(8.21)	**0.002**	↑			0.034(tr)	↓
IgG-index	**0.49**(0.06)	**0.67**(0.32)	**0.81**(0.99)	**<0.001**	↑	**<0.001**	↑		
IgM-index	**0.05**(0.05)	**0.12**(0.10)	**0.07**(0.08)	0.021(tr)	↑				

[a]CTR—control, PPMS—primary progressive multiple sclerosis, SPMS—secondary progressive multiple sclerosis, IQR—interquartile range, Mg—magnesium, Cu—copper, Pb—lead, Δ—change in the concentration, tr—trend, green arrows—decreased concentration, red arrows, increased concentration, black arrows—trend; downward and upward arrows indicate decreased and increased concentrations, respectively, hyphen—no significant change.

Table 3. Descriptive Statistics and Pairwise Comparison of Serum Biomarkers[a]

biomarker	CTR median (IQR)	PPMS median (IQR)	SPMS median (IQR)	CTR-PPMS	Δ	CTR-SPMS	Δ	PPMS-SPMS	Δ
citrate (mM)	**0.0706** (0.04)	**0.0838** (0.0363)	**0.0823** (0.0349)	0.014(tr)	↑				
dimethyl sulfone (mM)	**0.0071** (0.004)	**0.0085** (0.0061)	**0.0102** (0.003)			**0.004**	↑		
phenylalanine (mM)	**0.0522** (0.01)	**0.0450** (0.0177)	**0.048650** (0.01)	0.028(tr)		**0.006**	↓		
Glyc-F (μmol/L)	**245.85** (84.77)	**243.76** (57.89)	**279.30** (44.37)					0.014	↑
VLDL-TG mg/dL	**44.44** (40.37)	**67.18** (58.2)	**74.86** (59.16)			0.024 ns	↑		

[a]CTR—control, PPMS—primary progressive multiple sclerosis, SPMS—secondary progressive multiple sclerosis, IQR—interquartile range, VLDL-TG—very-low-density lipoprotein−triglycerides, Δ—change in the concentration, tr—trend, green arrows—decreased concentration, red arrows, increased concentration, black arrows—trend; downward and upward arrows indicate decreased and increased concentrations, respectively, hyphen—no significant change.

lower in SPMS compared to controls and showed a trend in PPMS compared to controls. None of the lipoprotein or glycoprotein parameters measured from the diffusion-edited ^{1}H NMR spectra differed significantly in any of the comparisons. However, very-low-density lipoprotein−triglycerides (VLDL-TG) showed an upward trend in SPMS compared to controls. In addition, GlycF showed a higher trend in SPMS compared to PPMS.

Finally, out of the seven metal elements measured in CSF, three of them, Mg, Cu, and Pb, showed significant differences. The most significant differences were found between PPMS and controls, with Mg being lower in PPMS ($p < 0.001$), while Cu and Pb were significantly higher in this group ($p < 0.001$ and $p = 0.002$, respectively). The average concentrations of these metals in the CSF of SPMS lie between those of controls and PPMS. Hence, the comparison between the PPMS and SPMS showed a trend similar to the differences observed between the controls and PPMS groups for Mg, Cu, and Pb ($p = 0.036$, 0.03, and 0.34, respectively).

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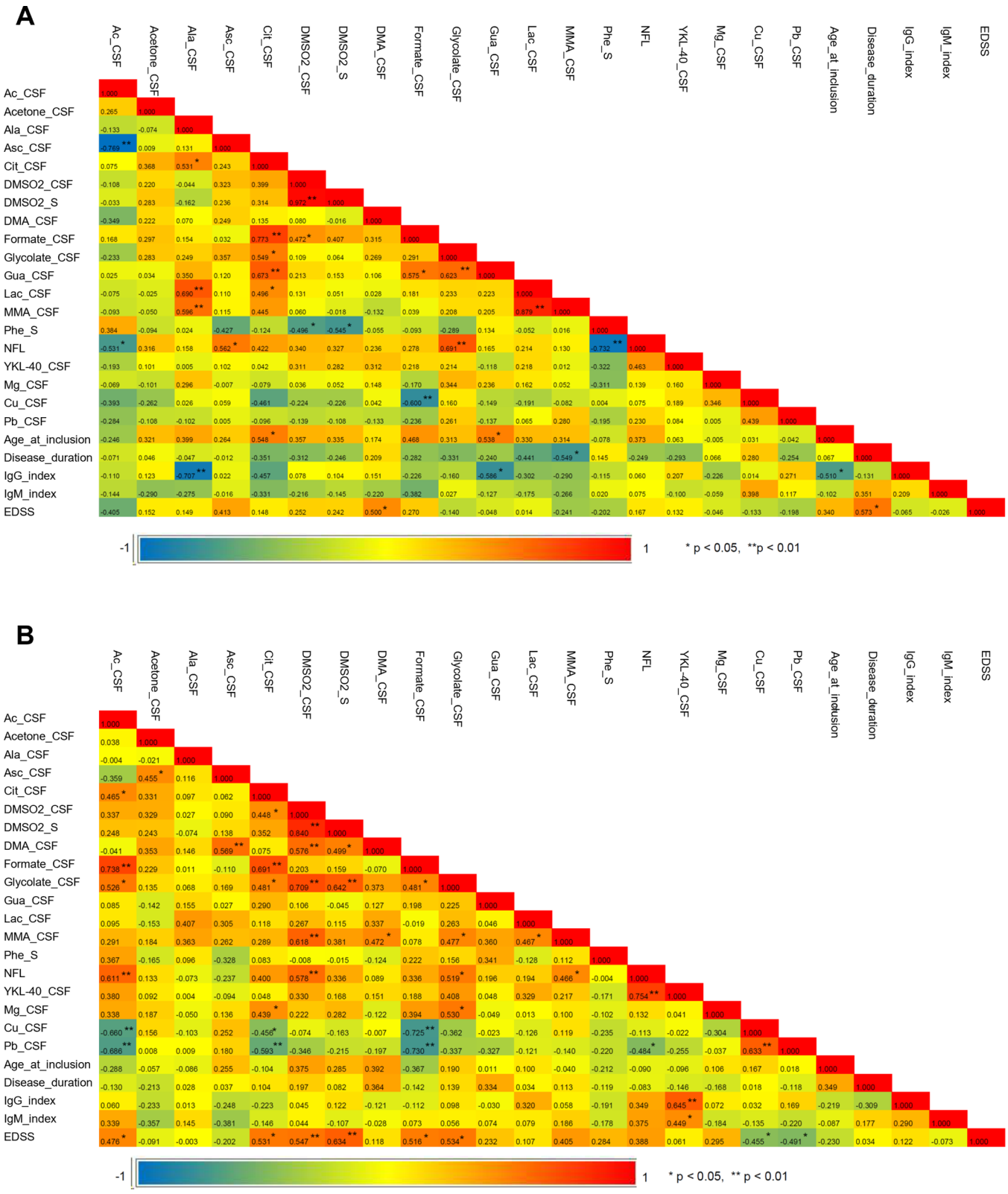

Figure 4. Correlation matrices for (A) PPMS and (B) SPMS. Ac: acetate; Ala: alanine; Asc: ascorbate; Cit: citrate; DMSO2: dimethyl sulfone; Gua: guanidinoacetate; DMA: dimethylamine; Gua: guanidinoacetate; Lac: lactate; MMA: methylmalonate; Phe: phenylalanine; NFL: neurofilament light; YKL-40: chitinase-3-like protein 1; EDSS: extended disability status scale.

Correlation Analysis

Metabolites with significant differences between controls and MS categories exhibited some correlation with each other, as shown in the correlation matrices in Figure 4. However, since a correlation does not necessarily mean causation, only correlations that are statistically significant at the alpha level of 0.01 are discussed.

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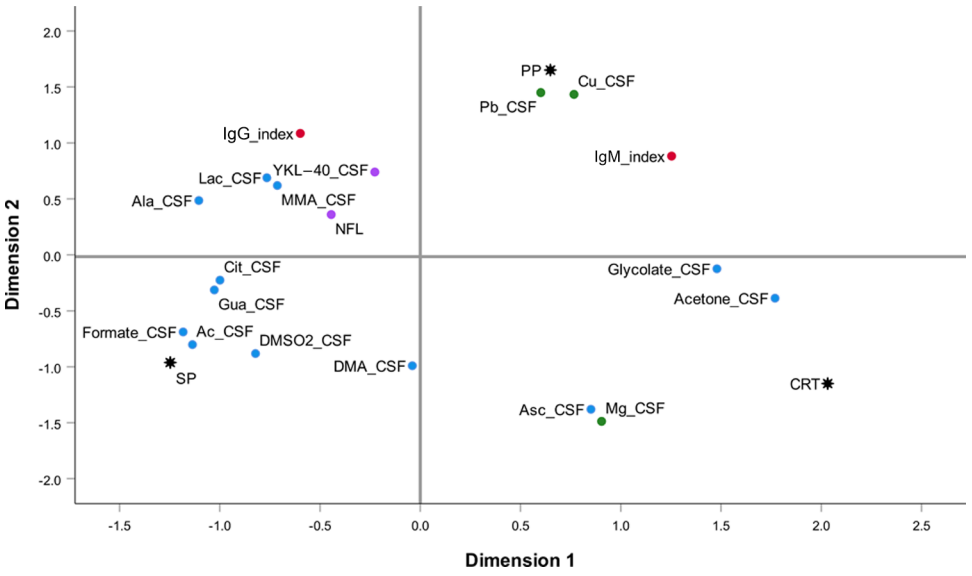

Figure 5. ALSCAL analysis showing biomarkers related to the various categories compared. PP—primary progressive MS, SP—secondary progressive MS, CTR—controls, Lac—lactate, MMA—methylmalonate, Ala—alanine, NFL—neurofilament light, Cit—citrate, Gua—guanidinoacetate, Ac—acetate, DMSO2—dimethyl sulfone, DMA—dimethylamine, Asc—ascorbate, Pb—lead, Cu—copper.

In general, a relatively higher number of significant correlations were observed between biomarkers in SPMS than in PPMS. Notable among SPMS correlations were strong, positive correlations between EDSS and DMSO2_CSF (r = 0.55, p = 0.008) and EDSS and DMSO2_S (r = 0.63, p = 0.002). EDSS also showed strong positive correlations with Cit_CSF (r = 0.53, p = 0.011), formate_CSF (r = 0.52, p = 0.014), and glycolate_CSF (r = 0.53, p = 0.011), respectively. Moreover, CSF and serum DMSO2 also exhibited a strong positive correlation with each other (r = 0.84, p < 0.001) and with glycolate_CSF (r = 0.71, p < 0.001 and r = 0.64, p = 0.001, respectively). In addition, DMSO_CSF showed a strong positive correlation with DMA_CSF (r = 0.58, p = 0.005), MMA_CSF (r = 0.62, p = 0.002), and NFL (r = 0.58, p = 0.005). Strong positive correlations also occurred between NFL and YKL-40_CSF (r = 0.75, p < 0.001). YKL-40_CSF also showed a strong positive correlation with IgG_index (r = 0.65, p = 0.001). There was an interesting positive correlation between Cu_CSF and Pb_CSF (r = 0.63, p = 0.002). This resulted in corresponding similar correlation patterns between these metals and other biomarkers. Thus, there were strong negative correlations between Cu_CSF, on the one hand, and Ac_CSF (r = −0.66, p = 0.001), and formate_CSF (r = −0.73, p < 0.001), on the other hand. Similarly, there was a strong negative correlation between Pb_CSF, on the one hand, and Ac_CSF (r = −0.69, p < 0.001), formate_CSF (r = −0.73, p < 0.001), and Cit_CSF (r = −0.59, p = 0.004), on the other hand.

In contrast to the number of significant positive correlation patterns observed between EDSS and other biomarkers in SPMS, only two significant strong positive correlations were observed for EDSS in PPMS. These correlations occurred between EDSS and DMA_CSF (r = 0.50, p = 0.034) and disease duration (r = 0.57, p = 0.013). Similar to the CSF observation, there was a strong positive correlation between CSF and serum concentrations of DMSO2 (r = 0.97, p < 0.001). Strong positive correlations also occurred between Cit_CSF and formate_CSF (r = 0.77, p < 0.001), Gua_CSF (r = 0.67, p = 0.002), glycolate_CSF (r = 0.55, p = 0.018), and Ala_CSF (r = 0.53, p = 0.023), respectively. Other relevant strong positive correlations were observed between NFL and glycolate_CSF (r = 0.69, p = 0.002) and Asc_CSF (r = 0.56, p = 0.015), respectively. Some strong negative correlations were observed between Ac_CSF and Asc_CSF (r = −0.77, p < 0.001) and NFL (r = −0.53, p = 0.023). There was also a strong negative correlation between NFL and Phe_S (r = −0.73, p = 0.001). Phe_S also showed strong negative correlations with DMSO2 in CSF and serum, respectively. Strong negative correlations were also observed between IgG_index and Ala_CSF (r = −0.71, p = 0.001), Gua_CSF (r = −0.59, p = 0.011), and age_at_inclusion (r = −0.51, p = 0.030).

ALSCAL

ALSCAL analysis showed the summary of the associations that exist between the significant biomarkers and the categories compared, revealing both specific and nonspecific biomarkers for PPMS and SPMS (Figure 5). While PPMS is closely associated with IgM_index, Pb, and Cu, SPMS is closely associated with CSF concentrations of formate, acetate, DMSO2, dimethylamine, guanidinoacetate, and citrate. However, the nonspecific biomarkers for progressive MS include IgG_index and CSF concentrations of NFL, YKL-40, lactate, alanine, and methylmalonate. Among the biomarkers that shared the same quadrant with controls, ascorbate and Mg were closer to the SPMS quadrant, while glycolate and acetone were in close proximity to the PPMS quadrant.

ROC Analysis

ROC analysis, shown in Figure 6 and Table 4, revealed that CSF concentrations of IgG, formate, citrate, and NFL were better at separating PPMS and SPMS from controls than the rest of the biomarkers in CSF. However, only CSF ascorbate, acetone, and glycolate marginally separated PPMS and SPMS. None of the serum metabolites performed better in terms of their discriminatory ability than the CSF metabolites, except serum dimethyl sulfone, which performed better than the corresponding CSF concentrations.

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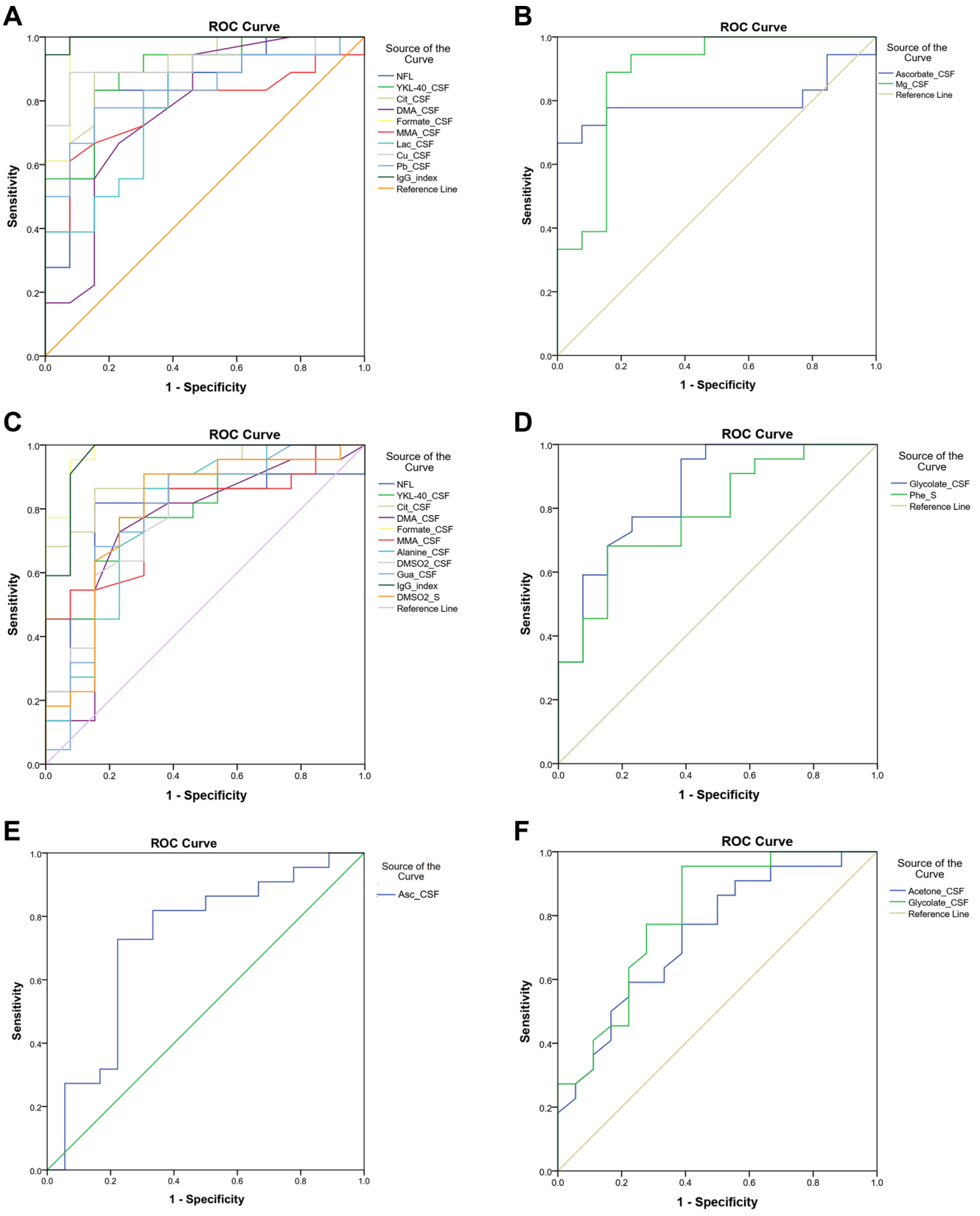

Figure 6. ROC analysis of metabolites that significantly differed between controls, PPMS, and SPMS. (A) Controls vs PPMS, increased biomarkers; (B) controls vs PPMS, decreased biomarkers; (C) controls vs SPMS, increased biomarkers; (D) controls vs SPMS, decreased biomarkers; (E) PPMS vs SPMS, increased metabolites; and (F) PPMS vs SPMS, decreased metabolites.

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Table 4. ROC Analysis of Significantly Different Biomarkers in CSF and Serum[a]

	controls vs PPMS				controls vs SPMS				PPMS vs SPMS			
biomarker	cutoff	AUC	sensitivity	specificity	cutoff	AUC	sensitivity	specificity	cutoff	AUC	sensitivity	specificity
CSF												
NFL	352.36	84.2	83.3	84.6	364.15	78.7	81.8	84.6				
YKL-40	66.85	89.3	83.3	84.6	56.67	80.1	77.3	76.9				
citrate	0.2233	91.2	88.9	84.6	0.2232	90.6	86.4	84.6				
dimethylamine	0.0019	77.8	72.2	69.2	0.0019	74.7	77.3	69.2				
formate	0.0454	97.0	100	92.3	0.0465	97.9	95.5	92.3				
methylmalonate	0.0077	78.2	77.8	69.2	0.0078	78.3	81.1	69.2				
lactate	1.4043	78.2	77.8	69.2								
ascorbate	0.1294	79.5	77.8	84.6					0.1285	72.7	72.7	77.8
acetone									0.0097	73.9	63.6	66.7
glycolate					0.0171	85.5	95.5	61.5	0.0150	80.2	77.3	72.2
alanine					0.0327	78.3	86.4	69.2				
dimethyl sulfone					0.0076	77.6	90.9	61.5				
guanidinoacetate					0.0277	78.5	90.9	61.5				
Mg	26619.9	88.0	88.9	84.6								
Cu	35.88	91.5	88.9	93.0								
Pb	21.42	83.8	77.8	76.9								
IgG_index	0.605	99.6	94.4	100	0.505	96.5	100	84.6				
Serum												
dimethyl sulfone					0.0078	79.5	90.9	69.2				
phenylalanine					0.5175	78.3	77.3	61.5				

[a]AUC—Area under the curve

DISCUSSION

Although MS is a complex disease with heterogeneous clinical presentations, researchers have been separately addressing different aspects of the disease spanning inflammation, energy metabolism, oxidative stress, general metabolism, and neurodegeneration. In this study, we have taken a multifaceted approach to decipher the similarities and differences between progressive MS and controls. From the demographic data, it is evident that the observations in this study can neither be attributed to differences in the gender distribution nor the age at inclusion since there were no significant differences in these variables between the MS patients and controls. However, gender differences could exist within each category. The majority of the changes in biomarkers were observed in CSF but not in serum, indicating the localization of the pathophysiological changes in the CNS. While the average concentration of the majority of metabolites is higher in SPMS compared to PPMS and controls, the average concentrations of the CSF biomarkers for neuroinflammation (YKL-40), neurodegeneration (NFL), and metal elements (Pb and Cu) in SPMS tend to lie between the controls and PPMS. These observations suggest that while SPMS is a metabolically active disease that progresses gradually over a longer period of time, the pathological insults induced in PPMS are rather more intense at the beginning, causing severe disability within a relatively short period. Hence, PPMS might be considered a severe form of SPMS initiated by the convergence of aggressive exogenous pathological triggers and genetic predisposition, which rapidly overwhelms endogenous defense mechanisms, such as response to oxidative stress. The longer duration of SPSS due to the transition from RRMS is responsible for observing a higher average disability, EDSS, among SPMS patients compared to controls and PPMS. Moreover, a significant number of the main biomarkers exhibit strong correlations with disease severity or EDSS in SPMS compared to PPMS. Such relationships between disease severity and biomarkers probably evolved from the gradual evolution of the SPMS from RRMS. On the contrary, the aggressive nature of PPMS is evidenced by the absence of strong correlations between major discriminatory biomarkers and disease severity or EDSS. However, this aggressive initiation of PPMS lays the foundation for a pathophysiologic cascade that results in increasing disability within a short period of time. The evidence for this allusion is based on the fact that the disease duration is one of the two variables that showed a significant positive correlation with EDSS in PPMS.

Pathological Triggers for Progressive MS

Different potential pathological triggers have been proposed for MS, ranging from genetic predisposition or vitamin D deficiency to viral infections. Evidence from our research suggests that in addition to these plausible pathological triggers, the toxic effects of heavy metals and the changes in the concentration of essential metal elements in the CNS could alter the clinical course of patients. We have observed a higher concentration of Pb and a reduction in the concentration of Mg and Cu in the CSF of PPMS compared to SPMS and controls.

Pb is a heavy metal with no known biological role but a reputable neurotoxic effect even at very low doses.[23] The accumulation of Pb after exposure has been reported to be several orders of magnitude higher in the brain than in the blood and could have long-lasting neurological consequences.[24] It is therefore not surprising that while the concentration of Pb in serum in this study was below the detection limit in most of the samples in all categories, its concentration in CSF was high enough to exhibit differences, especially in PPMS compared to SPMS and controls. On the other hand, the neurotoxic effect of Pb depends either on variations in the regional distribution of Pb[25] and/or variations in regional vulnerability to the toxic effects of Pb,[26] which means that different individuals may respond differently to similar concentrations of Pb. Therefore, although the average concentration of Pb in the CSF of SPMS

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was only marginally higher than that in controls, susceptibility variations to the neurotoxic effects of Pb among individuals imply that Pb could contribute to the pathophysiologic changes in SPMS as well. Moreover, the accumulation of Pb in the body has been found to be dependent on both environmental exposure and polymorphism of the vitamin D receptor (VDR),[27] while allelic differences in VDR genes have been associated with the progressive forms of MS.[28] Since there is no evidence of occupational exposure in the study subjects, the increased concentration of Pb in the CSF of PPMS compared to the SPMS and controls could have been due to genetic polymorphism of VDRs, which probably aided the gradual accumulation of Pb in the CNS of PPMS patients. Further studies are needed to confirm this hypothesis. A possible mechanism through which Pb could contribute to the pathophysiology of progressive MS is via the alteration of cytokine production. This proceeds by dysregulating the balance between type 1 and type 2 T helper cells (Th1 and Th2), hence inducing an autoimmune response.[29] In addition, Pb-induced oxidative stress has been reported to overwhelm both non-enzymatic antioxidant defense activation and protection against lipid peroxidation regardless of whether the exposure has been chronic or sub-chronic.[30] Therefore, the lack of protection against lipid peroxidation might have been heightened in the initial stages of PPMS, resulting in the accelerated deterioration of symptoms. Besides the direct effects of Pb neurotoxicity, some genetic variations of VDRs aid the uptake of Pb, which has been implicated in the pathophysiology of MS.[31] It has also been established that Pb also replaces calcium in the mitochondrion,[32] triggering mitochondrial destruction and eventual cell death. Different combinations of these factors may unilaterally trigger or, in combination with other factors, promote the pathological cascade at various rates in progressive MS.

Interestingly, our results show significant positive correlations between Pb and Cu in SPMS. At the same time, both metals exhibit significant negative correlations with acetate, citrate, and formate (a biomarker for oxidative stress). Cu has been demonstrated to exhibit both protective and neurotoxic effects in other diseases.[33] Its protective role occurs via the protection against oxidative stress, which seems to be the case in SPMS. An increase in Cu in progressive MS, in particular SPMS, is most likely a consequence of MS or a protective mechanism rather than a cause. An increase in Pb induces oxidative stress, which is counteracted by increased antioxidative mechanisms, which probably led to an increase in the concentration of Cu since Cu is a component of cytochrome oxidase. Hence, cell death from mitochondrial destruction and breakdown of cytochrome oxidase could lead to an elevated CSF concentration of Cu. This could be a result of the disruption of the electron transport chain.[34] Restoring Cu homeostasis in the CNS could serve therapeutic purposes in progressive MS, especially SPMS.

Unlike Pb, Mg is a metal element with important biological functions, including being an integral component of some enzymes. Mg, for instance, serves as a principal cofactor in a wide array of enzymatic reactions, and it is particularly essential for reactions associated with energy metabolism. Mg is also involved in maintaining stable cell membranes and moderates oxidative stress. Mg deficiency, therefore, leads to the loss of its antioxidant properties, thereby promoting oxidative stress.[35] Studies have shown that peripheral Mg does not influence CNS concentrations in healthy individuals with an intact blood−brain barrier (BBB)[36,37] but is marginally increased in individuals with alterations in the BBB.[38] Hence, the observation of differences only in the CSF but not in the serum indicates partial independence of the intrathecal Mg concentration from systemic Mg obtained by ingestion.

In conclusion, while the rapid onset of PPMS may be due to an aggressive pathological insult to the CNS, the lag in the progression of SPMS from RRMS might be due to the gradual accumulation of pathological changes, which are initially counterbalanced by compensatory defense mechanisms but eventually become overwhelmed.

Pathophysiological Similarities between SPMS and PPMS

Regardless of the longer disease duration in SPMS compared to PPMS, the average EDSS in SPMS was only approximately 1 point higher than that in PPMS, indicating a comparable level of disability. Corresponding similarities in the level of neuroinflammation and neurodegeneration were also observed for both PPMS and SPMS, as indicated by the comparatively higher concentrations of CSF YKL-40 and NFL in both groups compared to controls. Elevated intrathecal YKL-40 is known to be associated with chronic active lesions, especially in SPMS.[39] However, the absence of concomitant differences in serum concentrations of YKL-40 highlights the independence of CNS inflammation from peripheral inflammation. This assertion has been corroborated by the absence of significant differences in serum concentrations of emerging NMR-based glycoprotein biomarkers of chronic inflammation, such as GlycA and GlycB, as well as HDL, LDL, VLDL, and triglycerides. These observations are in tune with previous studies, which have also reported that CNS YKL-40 does not influence the serum or plasma concentration of YKL-40 references.[37,38] A possible converse influence of peripheral YKL-40 on CNS YKL-40 has not yet been reported. Nonetheless, the variation pattern exhibited by NFL is similar to what has been observed for YKL-40.

Although there were no significant differences between SPMS and PPMS in relation to the concentrations of both NFL and YKL-40, the average concentrations of both biomarkers were marginally higher in PPMS compared to SPMS. On the contrary, the metabolites that were significantly higher in progressive MS than in controls but were not significantly different between PPMS and SPMS were on average marginally higher in SPMS than in PPMS. This could be an indication that while neuronal damage in PPMS is severe compared to SPMS and results from aggressive pathological triggers, the pathological changes in SPMS tend to be gradual changes that accumulate over longer periods of relapses and active metabolic changes.

Similar Metabolic Changes Are Associated with the Pathophysiology of Progressive MS

While several metabolites significantly changed between the CSF of the progressive MS groups compared to the control group, only a few differed between PPMS and SPMS. The higher concentrations of citrate, dimethyl sulfone, formate, and methylmalonate observed in both PPMS and SPMS render them general biomarkers for MS, in particular the progressive forms. Although alanine, dimethyl sulfone, and guanidinoacetate were only significantly different between SPMS and controls, the concentrations of these metabolites in PPMS were marginally distributed between the controls and SPMS such that they neither exhibited differences between PPMS and controls nor PPMS and SPMS. The marginal distribution of the concentrations of these metabolites renders them accessory biomarkers for SPMS but not PPMS. These observations suggest that

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similar metabolic pathways are dysregulated in the progressive forms of MS but at different rates.

Besides altered metabolic pathways, the accumulation of metabolic waste products is also a consequence of the MS pathology. Dimethyl sulfone, which was significantly higher in the CSF of the progressive MS groups compared to controls and in the serum of SPMS compared to controls, is a waste product of metabolic changes in the brain.[40] Its concentration is associated with the rate of metabolic activities in the brain. The observation of strong positive correlations between the CSF and serum concentrations of dimethyl sulfone in all cases suggests that these metabolic waste products could have been transported from the CSF into the peripheral circulation. Hence, the observed increase in the concentration could be an indicator of increased cerebral metabolic activity associated with MS, especially SPMS. The severity of disability as measured by EDSS mirrors an increase in the CSF and serum concentrations of dimethyl sulfone only among SPMS patients, alluding to the presence of the active chronic CNS inflammation previously mentioned. This observation suggests that while PPMS is induced and probably maintained by a stronger initial pathological trigger, the pathological cascade in SPMS relies on the continuous accumulation of pathological insults during the relapse that preceded the progression. The accumulation of dimethyl sulfone in serum could serve as a surrogate biomarker for both active inflammation and disability among SPMS patients.

Metabolic Differences between SPMS and PPMS

While the pathological cascade of progressive MS might have been triggered by the neurotoxic effect of heavy metals like Pb and fueled by Mg deficiency and the breakdown of the electron transport system, the reduction in the ability to respond to oxidative stress could further deepen the divergence in the clinical course of progressive MS. One of the major responders to oxidative stress in the body is ascorbate. Ascorbate or vitamin C is a water-soluble vitamin that humans do not endogenously synthesize; hence, it is obtained from exogenous sources by ingestion. Since a significantly higher ascorbate concentration was observed only in the CSF of controls and SPMS but not in their matched serum samples, this could indicate that the differences were due to differences in the retention of ascorbate in the CNS but not due to differences in ingestion. There is experimental evidence that vitamin C retention in the brain exhibits regional variations, which in turn influence the response to oxidative stress.[41] The fact that the concentration of ascorbate was higher in the CSF of SPMS patients compared to PPMS patients could evidence that the ability to retain ascorbate in the brain might play a protective role, delaying the worsening of symptoms among SPMS compared to PPMS.

On the contrary, glycolate has an opposite trend compared to ascorbate, being significantly lower in SPMS compared to controls and PPMS. Being a xenobiotic, the possible role of glycolate in the mechanism of MS is unclear. However, it is regarded as a principal precursor of oxalate in hepatocytes, and its reduced concentration has been observed in the plasma of RRMS animal models.[42] Taken together, the CSF concentrations of ascorbate and glycolate could serve as biomarkers to differentiate between SPMS and PPMS. Nevertheless, glycolate may be more reliable for diagnostic purposes since ascorbate is unstable in vitro.

The differential intrathecal retention of ascorbate might be the key to unlocking the rate at which patients with progressive forms of MS diverge to their different disease courses. An increase in the rate of flow of CSF results in a decreased concentration of ascorbate in serum.[43] In contrast to the study by Reiber et al., in which there were no observable differences in the ascorbate concentration in CSF between the controls and the MS patients, our study clearly separated the MS patients into PPMS and SPMS. SPMS and controls showed similar CSF ascorbate concentrations in opposition to the lower concentrations observed in PPMS. Since there was no significant concomitant difference in the concentration of ascorbate in serum, the lower concentrations observed in the CSF of PPMS compared to the controls and SPMS could be attributed to differentially expressed or defective sodium-dependent vitamin C transporters among PPMS patients. This observation may also be useful in the design of treatments for the progressive forms of MS.

Association between Ascorbate Antioxidation in SPMS and PPMS

Interestingly, although there were no significant associations between ascorbate, NFL, and the major metabolites implicated in MS among the controls, there was a moderate negative association between ascorbate, formate, and NFL among SPMS patients. This is probably due to the antioxidative propensity of ascorbate, protecting against oxidative stress and slowing down eventual neurodegeneration. This might have contributed to the delayed worsening of symptoms among SPMS patients as compared to the rapid deterioration observed among PPMS patients. Eventually, the antioxidative protective resistance provided by ascorbate would be progressively overwhelmed by the accumulative effect of neurodegeneration resulting from other pathophysiological mechanisms, resulting in the switch from relapsing−remitting to secondary progression.

The result from this study accentuates the fact that PPMS and SPMS may be metabolically related and also suggests that differences in CNS antioxidation may be a major determinant of the clinical fate of patients with progressive MS.

Importantly, the control group in this study is very homogeneous, as shown on the sPLDA score plot, with very low levels of neurodegeneration and neuroinflammation, as indicated by the low concentrations of NFL and YKL-40. Some observations contrast those in previous studies, which is a reflection of the differences in the composition of the control groups used in other studies. Also, our MS groups are clearly defined and separated into PPMS and SPMS, which has promoted the revelation of previously unobserved differences.

Potential Therapeutic Implications

The observations from this study have presented the opportunity for evaluating the therapeutic implications of biomarkers that significantly differed between progressive MS and controls. Considering the hypothesis that the pathological triggers of PPMS are probably aggressive environmental factors such as Pb acting on genetically vulnerable individuals and the overwhelming effects of Pb toxicity on defense mechanisms, preventive strategies could be of utmost importance. The preventive role of vitamin D, for instance, has been demonstrated in previous studies through the observation of lowered risk of developing MS in adulthood based on perinatal vitamin D intake[44] and childhood to early adolescence exposure to sunlight.[45] When individuals who are genetically susceptible to VDR-dependent Pb accumulation are identified early enough, preventive measures could be put in place to reduce exposure. More so, by taking advantage of the competition between

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vitamin D and Pb for VDRs, genetically vulnerable individuals could be placed on vitamin D supplements to lower the accumulation of Pb in the CNS, hence preventing its toxic effect. However, clinical trials are required to determine the appropriate dose of vitamin D since the prior study has also revealed that higher doses of the biochemically active form of vitamin D, calcitriol, could potentially cause hypercalcemia.[46] Also, the development of safe methods to remove Pb from the CNS before it causes irreversible damage could be useful as well.

The antioxidant properties of vitamin C and Mg could be leveraged in the clinical management of MS. In addition to its antioxidant properties, ascorbate also has the ability to promote myelin formation,[47] which could be an adjuvant in stem cell therapies. The independence of intrathecal ascorbate and Mg from peripheral concentrations implies that BBB permeability in MS patients may be required to benefit from the intravenous application of ascorbate and Mg. In the same light, ascorbate and Mg ingestion might not play a preventive role against MS unless their transport into the CNS is augmented or they are intrathecally applied. Investigating the factors that promote the intrathecal retention of ascorbate and Mg could have therapeutic or preventive potential against MS.

Therapeutic strategies aimed at arresting mitochondrial damage might halt the neuronal damage in MS.

Due to the existence of competition between vitamin D and Pb, the efficacy of vitamin D supplementation for the prevention or treatment of MS will depend on the timing of its application. The best option would be to avoid the accumulation of Pb by avoiding vitamin D deficiency since Pb is usually stored in bones and takes longer to be cleared.

CONCLUSIONS

Taking it all together, the evidence so far buttresses the idea that both PPMS and SPMS are different presentations of the same disease with similar metabolic alterations. Nonetheless, whether a patient rapidly progresses to PPMS or delays rapid deterioration for a longer period in the case of SPMS may result from the combination of the magnitude of the initial pathological triggers, such as oxidative stress induced by a possible VDR-dependent accumulation of Pb in the CNS, the differential response to oxidative stress, and the reduced antioxidative capacity of the CNS mediated by the concentrations of ascorbate and Mg in the CNS. Differences in the magnitude of Pb exposure coupled with the predisposition to Pb accumulation may be responsible for the initial divergence in the clinical course of PPMS and SPMS. While SPMS remains a metabolically active disease, the deleterious initial triggers of PPMS set off a dominos pathological cascade responsible for rapid deterioration within a short period of time. The utility of ascorbate as a diagnostic biomarker might be limited by its absolute exogenous dietary source in addition to its instability in vitro. Nonetheless, it could serve as a potential therapeutic agent. The antioxidant properties of ascorbate may be leveraged as an auxiliary therapeutic agent for SPMS. The efficiency and efficacy of intrathecal delivery and oral vitamin C supplementation on MS may need to be investigated. Further studies are required to determine whether the reduced intrathecal ascorbate retention capacity of individuals with PPMS is a cause or consequence and to elucidate the mechanism by which ascorbate retention influences the rate of progression of progressive MS symptoms.

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Author Contributions

P.K.P., M.I.G.S., and M.L.G.M. were responsible for the conceptualization and design of the study. M.I.G.S. and M.L.G.M. raised funding sources. P.K.P. carried out the experiments, data collection, most of the data analysis, and wrote the manuscript. S.E. was in charge of patient enrollment and clinical data collection. M.G.V. and I.H. carried out sample collection, processing, and preservation. F.M. performed the immunological data analysis. N.A. carried out the characterization of glycoproteins and lipoproteins. L.B. assisted and supervised the statistical analysis. M.L.G.M. reviewed and edited the article to its final form. All authors have given their approval to the final version of the manuscript.

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Notes

The authors declare the following competing financial interest(s): N.A. is stock owner of Biosfer Teslab and has a patent of the lipoprotein profiling described in the present manuscript. The rest of the authors declare no conflict of interest.

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The datasets generated and analyzed during the current study are not publicly available because other papers that rely on these datasets are currently being prepared. However, the datasets will be available from the corresponding author on reasonable request once these papers have been published.

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the "Hospital Virgen de la Macarena" (Date: November 29, 2017/No 43170030).

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

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