

¹H-NMR analysis provides a metabolomic profile of patients with multiple sclerosis

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ABSTRACT

Objective: To investigate the metabolomic profiles of patients with multiple sclerosis (MS) and to define the metabolic pathways potentially related to MS pathogenesis.

Methods: Plasma samples from 73 patients with MS (therapy-free for at least 90 days) and 88 healthy controls (HC) were analyzed by ¹H-NMR spectroscopy. Data analysis was conducted with principal components analysis followed by a supervised analysis (orthogonal partial least squares discriminant analysis [OPLS-DA]). The metabolites were identified and quantified using Chenomx software, and the receiver operating characteristic (ROC) curves were calculated.

Results: The model obtained with the OPLS-DA identified predictive metabolic differences between the patients with MS and HC ($R^2X = 0.615$, $R^2Y = 0.619$, $Q^2 = 0.476$; $p < 0.001$). The differential metabolites included glucose, 5-OH-tryptophan, and tryptophan, which were lower in the MS group, and 3-OH-butyrate, acetoacetate, acetone, alanine, and choline, which were higher in the MS group. The suitability of the model was evaluated using an external set of samples. The values returned by the model were used to build the corresponding ROC curve (area under the curve of 0.98).

Conclusion: NMR metabolomic analysis was able to discriminate different metabolic profiles in patients with MS compared with HC. With the exception of choline, the main metabolic changes could be connected to 2 different metabolic pathways: tryptophan metabolism and energy metabolism. Metabolomics appears to represent a promising noninvasive approach for the study of MS.

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GLOSSARY

ANOVA = analysis of variance; **AUC** = area under the curve; **EAE** = experimental autoimmune encephalomyelitis; **HC** = healthy controls; **IPA** = Ingenuity Pathway Analysis; **KP** = kynurenine pathway; **LSD** = least significant difference; **MS** = multiple sclerosis; **NMO** = neuromyelitis optica; **OPLS-DA** = orthogonal partial least squares discriminant analysis; **PCA** = principal components analysis; **PLS-DA** = partial least squares discriminant analysis; **ROC** = receiver operating characteristic; **TRP** = tryptophan; **TSP** = trimethylsilyl propanoic acid.

Multiple sclerosis (MS) is a chronic disease of the CNS characterized by high levels of heterogeneity in pathologic, clinical, and radiologic features, as well as drug responses.^{1–4} Despite substantial advances, the pathogenesis of the disease remains elusive, and no definitive therapy exists. In addition, the effectiveness of the therapeutic response varies between patients and cannot be predicted in advance.

MS diagnoses are based on the evolving McDonald criteria,^{5–7} which are centered on a combination of clinical, MRI, neurophysiologic, and CSF parameters; in some cases, the diagnosis is made because no better explanation exists. Moreover, some patients remain in the “limbo” of radiologically or clinically isolated syndromes² for a prolonged period of time. Because early treatment is widely recognized as the best option to prevent long-term disability, a correct and early diagnosis is extremely important.⁸

Supplemental data
at Neurology.org/nn

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From this perspective, the lack of a single predictive or diagnostic test remains a great obstacle in the management of MS at most stages. Consequently, the availability of biomarkers that reliably capture the different aspects of the disease could be extremely useful.⁹

Metabolomics is a “hypothesis-generating” method with the potential to identify new biomarkers and to capture an instant functional picture of an organism. Metabolomics consists of the comprehensive study of the “metabome” in a biological system via the detailed analysis of metabolites in tissues and biofluids without a priori selection of a specific pathway.^{10,11}

¹H-NMR spectroscopy is one of the analytical tools used in metabolomics; it offers the opportunity to detect and quantify a variety of metabolites within a sample. Furthermore, it is highly reproducible.¹²

The metabolomics approach has been applied in various neurologic diseases with interesting results.¹³ To date, very few studies have been conducted with ¹H-NMR spectroscopy using blood^{14–17} or CSF samples^{15,18–21} from patients with MS, and the results are controversial.

We analyzed the blood samples from a large group of patients with MS and healthy controls (HC) using ¹H-NMR and pattern recognition analysis to define a discriminatory metabolomic profile able to distinguish patients with MS from HC.

METHODS Population and sample collection. We included patients affected with definite MS according to the McDonald criteria^{5–7} who had been therapy-free for at least 90 days (i.e., disease-modifying drugs and steroids) and who were free from other significant medical conditions. The HC were demographically and ethnically matched and were recruited from volunteers, health personnel, and spouses and friends of the patients.

Standard protocol approvals, registrations, and patient consents. The study was conducted in accordance with the principles of good clinical practice. The institutional ethics committee approved the study, and written informed consent was obtained from each participant.

Sample preparation and acquisition. Ten mL of blood was collected from each participant, and the plasma samples were stored at -80°C until analysis.

The plasma samples were thawed and centrifuged at 2,500g for 10 minutes at 4°C. An 800- μL aliquot was added to 2,400 μL of a chloroform/methanol 1:1 solution plus 350 μL of distilled water (D2O) solution. The samples were vortexed (1 minute) and

centrifuged (30 minutes at 1,700g) at room temperature. The hydrophilic and hydrophobic phases were obtained. The water phase was concentrated overnight using a speed vacuum instrument. The water phase was resuspended in D2O and trimethylsilyl propanoic acid (TSP) 5.07 mM. TSP was added to provide an internal reference for the chemical shifts (0 ppm), and 650 μL of the solution was transferred to a 5-mm NMR tube.

The samples were analyzed with a Varian UNITY INOVA 500 spectrometer (Agilent Technologies, Inc., Santa Clara, CA), which was operated at 499 MHz equipped with a 5-mm triple resonance probe with z-axis-pulsed field gradients and an autosampler with 50 locations. One-dimensional ¹H-NMR spectra were collected at 300 K with a presat pulse sequence to suppress the residual water’s signal. The spectra were recorded with a spectral width of 6,000, a frequency of 2 Hz, an acquisition time of 1.5 seconds, a relaxation delay of 2 milliseconds, and a 90° pulse of 9.2 microseconds. The number of scans was 256. Each free induction decay was zero-filled to 64,000 points and multiplied by a 0.5-Hz exponential line-broadening function. The spectra were manually phased and baseline-corrected. The chemical shifts were referred to the internal standard, TSP (at $\delta = 0.0$ ppm), using MestReNova software (version 8.1; Mestrelab Research S.L., A Coruña, Spain).

Data processing and multivariate analysis. Data processing and multivariate analysis was mainly performed as previously described.²² Each spectrum was divided into consecutive “bins” 0.04 ppm wide. The spectral area under investigation was the region between 0.6 and 8.6 ppm. In order to remove variations in the presaturation of the residual water resonance and spectral regions of noise, the regions between 4.64 and 5.2 ppm and between 5.28 and 6.6 ppm were excluded. The integrated area within each bin was normalized to percent values to minimize the effects of the different concentrations of plasma samples. This generated a final dataset consisting of a 154×161 matrix. The values in the columns represent the normalized area of each bin (variables), and the values in the rows represent the samples (participants). A multivariate statistical analysis was performed on the matrix generated using SIMCA-P software (v13.0; Umetrics, Malmö, Sweden). In order to emphasize all metabolite signals and reduce the spectral noise, the variables were Pareto-scaled.

Initially, data analyses were conducted using principal components analysis (PCA), which is important in order to explore the sample distributions without classification. To identify potential outliers, the DmodX and Hotelling T² tests were applied.

Partial least squares (PLS-DA) and orthogonal partial least squares discriminant analyses (OPLS-DA) were subsequently applied. PLS-DA and OPLS-DA maximize the discrimination between samples assigned to different classes. The variance and the predictive ability (R2X, R2Y, Q2) were established. In addition, a permutation test ($n = 200$) was performed to validate the models.

The scores from each OPLS-DA model were subjected to a cross-validated analysis of variance (ANOVA) to test for significance ($p < 0.05$).

The most significant variables were extracted by the loading plot from each model and quantified using the Chenomx NMR Suite 7.1 (Chenomx Inc., Edmonton, Alberta, Canada).²³ Custom library entries were created for unidentified resonances to carry them through the analysis for relative concentration comparisons. These concentrations were used for a 1-way multivariate ANOVA before conducting a series of follow-up ANOVAs with SPSS 22 software (SPSS, Armonk, NY). In particular, Wilks λ test was used to test whether there were differences between the means of the identified subject groups in a combination of

Table 1 Demographic and clinical features of patients with multiple sclerosis and healthy controls

		MS (n = 73)		HC (n = 88)		p Value
Female/male		46/27		61/27		NS
Age, y, mean \pm SD (range)		39.6 \pm 10.1 (20-61)		44.3 \pm 14.1 (20-77)		NS
MS phenotype	Total, N	Clinical activity, N	MRI activity, N	Age at onset, y, mean \pm SD	Disease duration, y, mean \pm SD	EDSS score, mean \pm SD
Relapsing-remitting	61	28	18	32.6 \pm 5.3	5.2 \pm 4.1	2.3 \pm 1.2
Progressive	12	2	0	33.4 \pm 4.1	11.6 \pm 7.4	5.4 \pm 1.2

Abbreviations: EDSS = Expanded Disability Status Scale; HC = healthy controls; MS = multiple sclerosis; NS = nonsignificant.

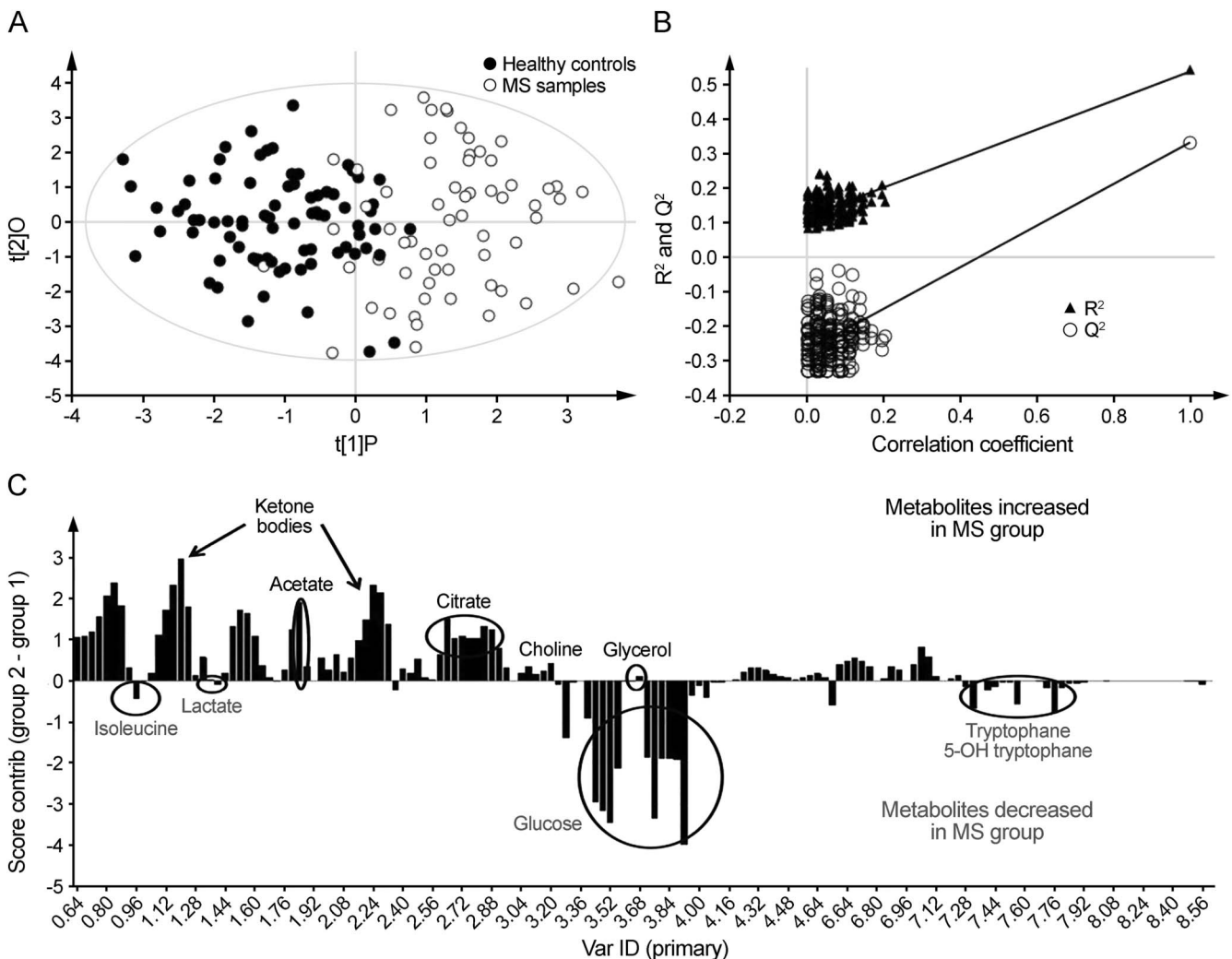
Clinical activity was defined by the presence of relapses and brain MRI activity by the presence of gadolinium-enhancing lesions and/or new or unequivocally enlarging T2 lesions in the last 6 months.²

dependent variables. A Fisher least significant difference (LSD) test was performed to explore and compare the concentration means of each metabolite in the 2 groups.

An analysis using receiver operating characteristic (ROC) curves was generated with MATLAB software (R2012b;

MathWorks, Natick, MA) to test the specificity and sensitivity of the method. The Mahalanobis distance scale was used to plot the ROC curves.

Metabolic pathways were built using the Ingenuity Pathway Analysis software (IPA; QIAGEN, Redwood City, CA); only

Figure 1 OPLS-DA model healthy controls vs patients with MS built using bins as variables

(A) Distribution of healthy controls (black circles) and multiple sclerosis (MS) samples (white circles) with orthogonal partial least squares discriminant analysis (OPLS-DA) ($R^2X = 0.615$, $R^2Y = 0.619$, $Q^2 = 0.476$; $p < 0.001$). (B) Validation of the corresponding partial least squares discriminant analysis model via a permutation test. (C) The contribution plot generated by the spectral differences of the 2 groups identified the metabolic changes.

Table 2 Metabolites that discriminate the HC and MS classes

Metabolite	HC	MS	Fold change	p Value
5-Hydroxytryptophan	+	-	-1.77	<0.001
Citrate	+	-	-1.05	NS
Glucose	+	-	-1.15	<0.05
Glycerol	+	-	-1.21	0.05
Isoleucine	+	-	-1	NS
Lactate	+	-	-1.03	NS
Tryptophan	+	-	-2.40	<0.001
3-Hydroxybutyrate	-	+	1.45	<0.01
Acetate	-	+	1.11	NS
Acetoacetate	-	+	3.57	<0.001
Acetone	-	+	9.34	<0.001
Alanine	-	+	1.5	0.01
Choline	-	+	1.27	0.01
Glutamate	-	+	1.13	NS
Malonate	-	+	1.19	NS
Methylmalonate	-	+	1.05	NS
Scyllo-inositol	-	+	1.05	NS

Abbreviations: HC = healthy controls; MS = multiple sclerosis; NS = nonsignificant.

Fold change was calculated as ratio between MS and HC concentrations. Calculation of the *p* value refers to univariate analysis of the concentrations.

metabolites with significantly different ($p < 0.05$) concentrations between HC and patients with MS were used.

RESULTS Seventy-three patients with MS and 88 HC were included. Clinical and demographic features of the participants are summarized in table 1. The age and sex distributions were similar between the 2 groups.

PCA was performed (data not shown). The DmodX test identified the presence of moderate outliers that may not affect the model. The Hotelling T² test did not identify any strong outliers; therefore, all samples were used for the subsequent analyses.

The OPLS-DA (figure 1A) identified a differential distribution of the HC and MS samples (R2X = 0.615, R2Y = 0.619, Q2 = 0.476; $p < 0.001$). Validation of the corresponding PLS-DA model was performed with a permutation test (figure 1B).

An additional validation test was performed via the blind introduction of an external test set of 20 samples (10 from each class). Class membership depends on matching the value of the unknown observation, and a value close to one class indicates membership in the class. Two MS samples were not correctly classified. The values returned by the model as yPREDps were used to build the corresponding

ROC curve (area under the curve [AUC] of 0.93). The contribution plot generated by the spectral differences of the 2 groups identified the metabolic changes (figure 1C).

From the NMR profile, it was possible to identify 41 molecules belonging to different classes, such as sugars, amino acids, etc. Seventeen metabolites discriminating between patients with MS and HC were identified and quantified using Chemomx software (listed in table 2).

After removal of the outlier samples, OPLS-DA was performed on a training set model, generated by randomly excluding 10 HC and 10 patients with MS from each class and using the matrix of the 17 metabolites identified and quantified (R2X = 0.427, R2Y = 0.615, Q2 = 0.583; $p < 0.001$) (figure 2A). Validation of the corresponding PLS-DA model was performed with a permutation test (figure 2B). The contribution plot generated by the quantified metabolites indicated the differences in patients with MS and HC (figure 2C). A validation test was performed by using the external set of 10 samples from each class, and the values returned by the model as yPREDps were used to build the corresponding ROC curve (AUC of 0.98).

The matrix that contained the concentrations of the discriminant metabolites for each patient was analyzed with SPSS using an ANOVA with multivariate and univariate tests. Wilks λ indicated a difference in the metabolite concentrations between the 2 groups: $F_{17,143} = 11.39$, $p < 0.0005$. The individual *p* values of the discriminant metabolites are shown in table 2.

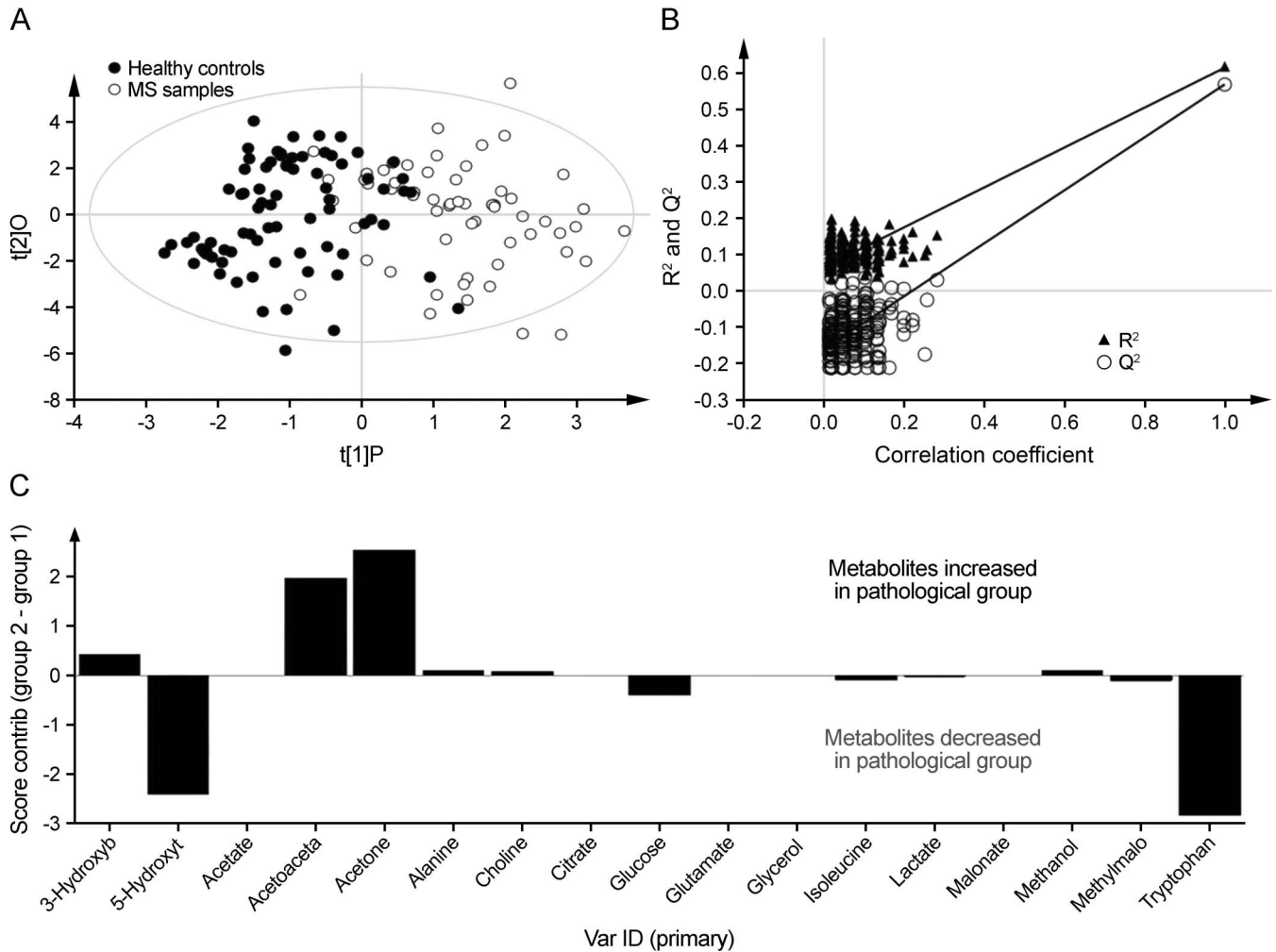
Fisher LSD test indicated that glucose, 5-OH-tryptophan, and tryptophan (TRP) were lower in the patients with MS; 3-OH-butyrate, acetoacetate, acetone, alanine, and choline were higher in the same clinical group. The box plots of the individual metabolites are provided (figure e-1 at Neurology.org/nn). Repeating the analysis excluding progressive patients did not show any difference (figure e-2).

The ROC curves (figure 3) were subsequently built considering all 17 metabolites (AUC 0.78). To define the minimal number of metabolites needed to discriminate the 2 groups, only the metabolites with a $p < 0.05$ (5-OH-TRP, acetoacetate, acetone, glucose, TRP, 3-OH-butyrate, choline, and alanine) and then with a $p < 0.01$ (5-OH-TRP, acetoacetate, acetone, TRP, and 3-OH-butyrate) were used (figure 3).

The significant metabolites allowed identification of the main canonical pathways, the biofunctions, and diseases by IPA (figure 4).

DISCUSSION We performed a metabolomics study with pattern recognition methods on blood in a large cohort of patients with MS and HC using ¹H-NMR.

Figure 2 OPLS-DA model healthy controls (black circles) vs patients with MS (white circles) built using the concentrations of metabolites



(A) The orthogonal partial least squares discriminant analysis (OPLS-DA) of the training set model after the removal of the outliers ($R^2X = 0.427$, $R^2Y = 0.615$, $Q^2 = 0.583$; $p < 0.001$). (B) Validation of the partial least squares discriminant analysis model via a permutation test. (C) The contribution plot generated by the quantified metabolites with the indication of their different ratios in patients with multiple sclerosis (MS) and healthy controls.

This approach enables the simultaneous identification and quantification of a wide range of endogenous and exogenous metabolites in tissues or biofluid samples, which results in a holistic view of the functional state of the organism.¹² In addition, the pattern recognition approach categorizes the samples on the basis of the metabolic differences and similarities and recognizes the metabolic fingerprint of a disease or a peculiar condition. Therefore, it is also possible to detect the metabolites responsible for the differences and to identify novel pathways involved in a disease.¹²

One concern with metabolomics could arise from the high sensitivity of the approach (i.e., lifestyle may modify findings), which could limit its use in prevalence studies. Recent data obtained from the EPIC-Potsdam study,²⁴ which analyzed the intra- and interperson metabolic variations in 100 healthy individuals over a 4-month period, demonstrated high reliability for most

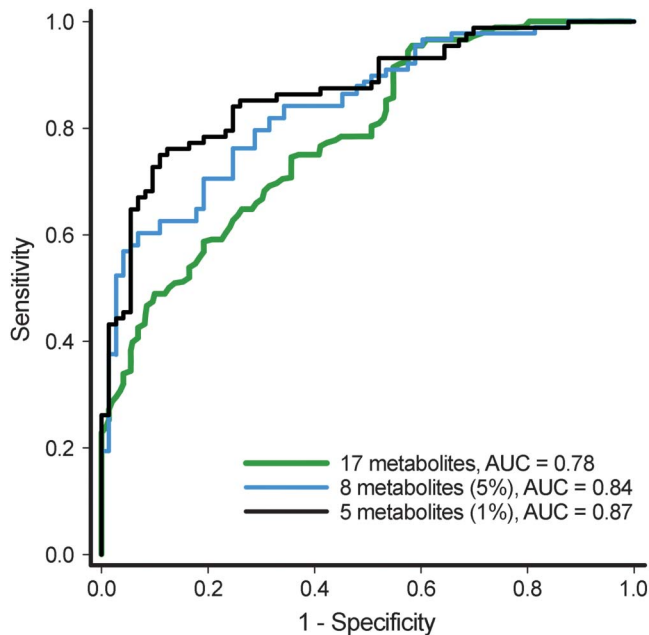
metabolites tested. Thus, a single analysis can potentially be used in risk assessments.²⁴

In this respect, we built a model able to discriminate patients with MS from HC on the basis of the degree of metabolic similarities and differences between the participants who belonged to the same or different categories, respectively. The model appears to be robust, because when an external set of samples was blindly introduced, it was capable of correctly recognizing all but 2 samples.

Moreover, considering the well-known sex bias in MS occurrence,²⁵ the analysis was repeated separately for each sex. Apart from the increased lactate concentration in men (both those with MS and HC), the 2 models were similar to the overall model, which suggests that the metabolic differences identified were associated with the disease (data not shown).

A possible limit of our approach is due to the intrinsic characteristics of NMR-based technique.

Figure 3 ROC curve to test the performance of the selected metabolites



Receiver operator characteristic (ROC) curves with the 17, 8, and 5 identified metabolites to define the minimal number of variables needed to discriminate patients with multiple sclerosis and healthy controls. AUC = area under the curve.

This technique is excellent for quantification and reproducibility of the data, but there is a lack of sensitivity. In order to be detected by NMR, concentrations of metabolites need to be high (as opposed to mass spectrometry). This could explain the relatively limited number of metabolites found to be discriminants.

The chemometric analysis indicated that a combination of 8 metabolites primarily drives the separation of the patients with MS and HC in our model.

In particular, glucose, 5-OH-TRP, and TRP were lower in the patients with MS, whereas 3-OH-butyrate, acetoacetate, acetone, alanine, and choline had higher concentrations in the patients with MS. In agreement with our results, choline was increased in a metabolomics study of CSF obtained from patients with MS.²¹ It is also one of the main metabolites increased according to brain MRI spectroscopy performed in patients with MS.²⁶ Choline has also been associated with the demyelinating process in the CNS.

The remaining metabolites could be assigned to 2 different metabolic pathways: TRP metabolism (5-OH-TRP and TRP) and energy metabolism (glucose, alanine, OH-butyrate, acetoacetate, and acetone).

5-OH-TRP and TRP were 1.7- and 2.4-fold lower in the patients with MS vs the HC. Consistent with our observation, decreased TRP concentrations in plasma and CSF obtained from patients with MS have been reported,^{27–29} which suggests a potential involvement of TRP in MS.

TRP is an essential amino acid, and it is the key metabolite of 2 pathways, including the synthesis of the neurotransmitter 5-hydroxytryptamine (serotonin) and the kynurenine pathway (KP), which ultimately lead to the production of nicotinamide adenine dinucleotide. In particular, the KP performs regulatory mechanisms of the immune response, and its metabolites can exhibit both neuroprotective and neurotoxic properties.^{30,31} Thus, this pathway is exceptionally interesting in an inflammatory disease of the CNS. The influence of the KP in MS has recently been demonstrated in a metabolomics study of blood samples from the experimental autoimmune encephalomyelitis (EAE) model.³² In this case, no direct changes in TRP levels were identified. However, the plasma levels of kynurenine acid were reduced, and the indolepropionate and indoleacrylate concentrations were increased, which suggests an alteration in TRP metabolism.³²

The indolamine 2,3-dioxygenase enzyme, which metabolizes TRP in the first step of the KP, drives immune dysfunction by suppressing T cell proliferation and altering the Th17/Treg balance.³⁰ Thus, we can hypothesize a role of TRP in MS pathogenesis through the KP.³⁰

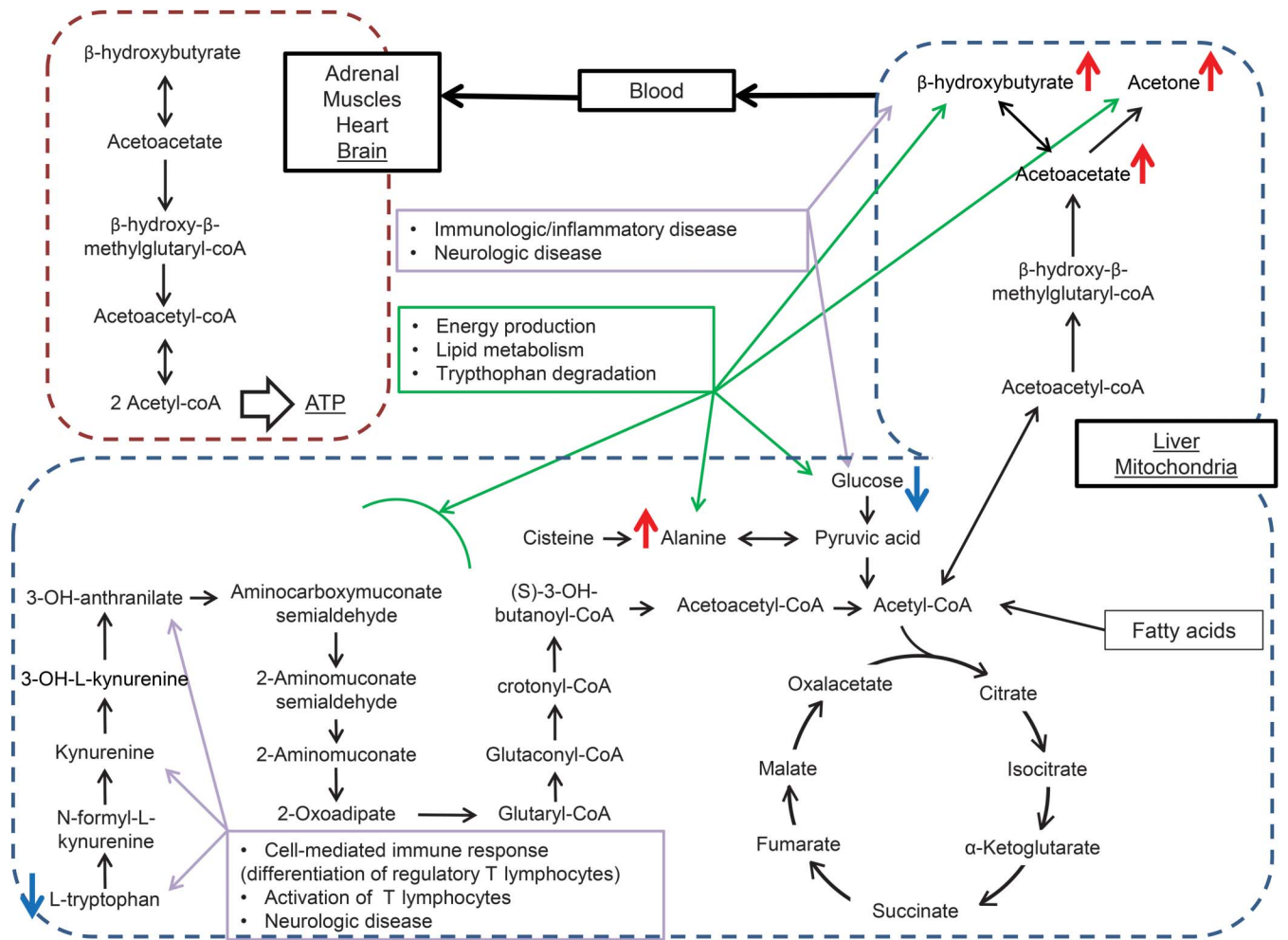
The metabolites that enter the energy pathways belong to 2 different arms: the pyruvate production pathway (alanine) and the “energy shift” pathway (glucose, acetone, acetoacetic acid, and β -hydroxybutyric acid).

We identified an increased alanine concentration in the patients with MS. Considering this amino acid is used as a source for pyruvate production and energy metabolism or for the de novo synthesis of macromolecules within neuronal and immune cells,³³ it could be involved in MS pathogenesis.

In addition, glucose reductions and increases in 3-OH-butyrate, acetoacetate, and acetone in the blood of patients with MS were identified, which suggests a contribution of the “energy shift” in MS. In conditions of low glucose or carbohydrate concentrations, ketone bodies (acetone, acetoacetic acid, and β -hydroxybutyric acid) are produced in mitochondria through fatty acid catabolism. Acetoacetic acid and β -hydroxybutyric acid cross the blood-brain barrier and can be used by cells as an energy source (i.e., converted to acetyl coenzyme A to participate in the citric acid cycle in mitochondria), and acetone is eliminated by excretion. Both β -hydroxybutyric acid and acetoacetate levels were increased in the blood of the patients with MS; it is noteworthy that a ketogenic diet has been proposed in many neurodegenerative diseases.³⁴ It was also tested in an EAE model with positive results.³⁵

We observed a nonsignificant increase in acetate in the patients with MS. Other reports did not identify

Figure 4 Relevant pathways involved in MS



Ingenuity Pathway Analysis showed the main canonical pathways, biofunctions, and diseases identified with significant metabolites. Metabolites playing a key role are associated to arrows indicating the relative change in patients with multiple sclerosis (MS) compared with healthy controls (blue arrow: decrease; red arrow: increase). Among the canonical pathways and cellular functions, the tryptophan degradation and the energy production were the most relevant (box and lines in green). Decrease of glucose in patients with MS leads to a decrease in the production of acetyl coenzyme A (acetyl-CoA), a fundamental precursor of the Krebs cycle and the most important pathway in energy production. To overcome the lack of glucose, the β -oxidation of fatty acids in mitochondria is induced. This leads to an increased production of ketone bodies (3-OH-butyrate, acetoacetate, acetone) in patients with MS. Ketone bodies can migrate into the bloodstream for use in peripheral tissues where they are metabolized for the production of ATP, especially 3-OH-butyrate. In particular, tryptophan and some of the intermediaries of this pathway (kynurenine, 3-OH-anthranilate) are implicated in the cell-mediated immune response (differentiation of regulatory T lymphocytes, activation of T lymphocytes). Moreover, the pathways analysis identifies correlation between tryptophan, glucose, and 3-OH-butyrate with immunologic/inflammatory disease and neurologic disease (box and lines in violet).

metabolite differences in the blood¹⁵ or a reduction in the CSF.^{19,36} Nevertheless, other studies performed with blood or CSF samples have demonstrated increased acetate.^{14,18,37} A very recent study¹⁴ analyzed the blood of 47 patients with MS, 44 patients with neuromyelitis optica (NMO), and 42 HC using NMR. The authors identified a 1.9-fold increase in acetate in the patients with MS vs the HC, but it was mainly increased in patients with NMO (1.8-fold and 3.4-fold higher than patients with MS and HC). Acetate is preferentially metabolized in astrocytes, and it is involved in neurotransmitter synthesis and brain metabolism. Thus, the changes observed could be due to different degrees of acetate uptake impairment by astrocytes.

It is noteworthy that instead of the great variability of the results obtained in previous studies, which have largely been attributed to the heterogeneity of the disease, the metabolic changes in energy metabolism pathways are consistent in the majority of studies in both human and animal models, supporting their roles in MS pathogenesis.

As previously discussed, some authors¹⁴ identified higher blood levels of acetate in both patients with MS and patients with NMO compared with HC; however, the primary metabolite able to discriminate patients with MS vs both patients with NMO and HC was a marker of glial proliferation, *scyllo*-inositol (increased 2.4- and 2.6-fold compared with patients

with NMO and HC, respectively). In our samples, *scyllo*-inositol was only slightly increased in patients with MS compared with the HC. It is possible that the different results were due to differences in the MS populations studied. In particular, the MS population studied recently¹⁴ was largely (42/47) treated (39 with natalizumab and 3 with interferon β). In contrast, we considered only therapy-free patients. Because metabolic changes can be induced by exposure to exogenous factors such as drugs and metabolomics can also be used to follow metabolic changes induced by specific treatments, this could have led to the different results.

As demonstrated by our results, metabolomics has the advantage of enabling the study of several metabolites simultaneously, which thereby allows the evaluation of multiple biomarkers in a single experiment and the creation of a metabolic model able to discriminate patients vs HC. Moreover, it is noteworthy that Dickens et al.¹⁷ determined that serum metabolite profiles were able to distinguish patients with MS with different subtypes and stages, which supports the utility of the metabolomics approach for the identification of biomarkers in MS.

In addition, the differential metabolites might facilitate the understanding of the pathophysiology of MS. More specifically, a definite ¹H-NMR metabolic profile of patients with MS was described, and 2 metabolic pathways were identified. This study requires replication in other populations, the results should be confirmed and completed by mass spectrometry, and correlations between blood and CSF metabolic profiles must be examined.³⁸

The study highlights the potential of ¹H-NMR examination of blood as a novel analytical tool to discriminate patients with MS and HC. Therefore, metabolomics appears to be a promising noninvasive approach to identify new biomarkers and has the potential to be integrated with other tools for the diagnosis of MS and to provide a better understanding of the disease pathogenesis.

AUTHOR CONTRIBUTIONS

Dr. Cocco conceptualized the study, gave an interpretation of the data, and drafted the manuscript. Dr. Murgia ran the NMR experiments, analyzed the data, and contributed to writing and revising the manuscript. Dr. Loreface obtained the samples, designed the study, analyzed the data, and revised the manuscript. Dr. Barberini conceptualized the study, analyzed the data, gave an interpretation of the data, and revised the manuscript. Dr. Poddighe assisted with running the NMR experiments, gave an interpretation of the data, and revised the manuscript. Dr. Frau gave an interpretation of the data and revised the manuscript. Dr. Fenu gave an interpretation of the data and revised the manuscript. Dr. Coghe gave an interpretation of the data and revised the manuscript. Dr. M.R. Murru gave an interpretation of the data and revised the manuscript. Dr. R. Murru gave an interpretation of the data and revised the manuscript. Dr. Del Carratore analyzed the data, gave an interpretation of the data, and revised the manuscript. Prof. Atzori designed the study, analyzed the data, and revised the manuscript. Prof. Marrosu conceptualized

the study, gave an interpretation of the data, and drafted the manuscript.

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DISCLOSURE

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