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Serum analysis by ^{1}H Nuclear Magnetic Resonance spectroscopy: a new tool for distinguishing neuromyelitis optica from multiple sclerosis

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Abstract

Background: Neuromyelitis optica (NMO) and multiple sclerosis (MS), two inflammatory demyelinating diseases, are characterized by different therapeutic strategies. Currently, the only biological diagnostic tool available to distinguish NMO from MS is the specific serum autoantibody that targets aquaporin 4, but its sensitivity is low.

Objective: To assess the diagnostic accuracy of metabolomic biomarker profiles in these two neurological conditions, compared to control patients.

Methods: We acquired serum spectra (47 MS, 44 NMO and 42 controls) using proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. We used multivariate pattern recognition analysis to identify disease-specific metabolic profiles.

Results: The ^{1}H-NMR spectroscopic analysis evidenced two metabolites, originating probably from astrocytes, scyllo-inositol and acetate, as promising serum biomarkers of MS and NMO, respectively. In 87.8% of MS patients, scyllo-inositol increased 0.15 to 3-fold, compared to controls and in 74.3% of NMO patients, acetate increased 0.4 to 7-fold, compared to controls. Using these two metabolites simultaneously, we can discriminate MS versus NMO patients (sensitivity, 94.3%; specificity, 90.2%).

Conclusion: This study demonstrates the potential of ^{1}H-NMR spectroscopy of serum as a novel, promising analytical tool to discriminate populations of patients affected by NMO or MS.

Keywords

Acetate, biomarkers, metabolomics, multiple sclerosis, neurodegeneration, neuromyelitis optica, nuclear magnetic resonance, scyllo-inositol

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Introduction

Neuromyelitis optica (NMO) is an inflammatory demyelinating disease of the central nervous system (CNS), characterized by severe attacks of optic neuritis and myelitis. Whether NMO is a variant of multiple sclerosis (MS) or a separate disease has long been debated.[1,2] Today, NMO is recognized as a distinct clinical entity, distinguished from classical MS by clinical, neuroimaging and serological criteria.[3] The presence of a highly specific serum immunoglobulin G autoantibody marker, NMO-IgG, differentiates NMO from MS and has helped define a NMO spectrum.[4] NMO-IgG targets aquaporin-4 (AQP4), the main channel that regulates water homoeostasis in the CNS[5]; however, the sensitivity of this antibody is relatively low,[6] between 50–70%, so there is definitely a need to find a better marker of this disease.[7,8] As treatments usually used in NMO and MS differ substantially, the differential diagnosis between these two diseases is of uttermost importance.[9]

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To date, very few studies have been conducted by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy on serum[10,11] and cerebrospinal fluid (CSF) samples[10,12–14] of MS patients, and no study reports on NMO patients' serum samples using this technique.

In this manuscript, we present the ^{1}H-NMR metabolomic approach to look for metabolic markers capable of differentiating NMO from MS and healthy controls.[7]

Materials and methods

Patient population

A cohort consisting of 47 MS and 44 NMO patients, all with a relapsing–remitting form, was included in this study. We clinically assessed these patients in the Department of Neurology of the Strasbourg University Hospitals in France, between 2005 and 2011. The NMO patients fulfilled the revised criteria of Wingerchuk[3] and the MS patients satisfied the criteria of McDonald, revised in 2005.[8] The control group consisted of 42 healthy subjects, matched for age and gender.

The MS patients were either not treated or treated by natalizumab, and the NMO patients were not treated or treated by various immunosuppressants, including: mycophenolate mofetil, azathioprine, cyclophosphamide or rituximab.

Serum samples

We collected blood samples in 5-mL dried tubes and transported them on ice, before being promptly centrifuged at 2000 rpm for 10 min and aliquoted. All samples were stored at −80°C and analyzed after a maximum of two freeze-thaw cycles. 18 µL of each patient's serum (previously thawed at room temperature) were added into a 30-µL disposable KelF insert, and then 8 µL of deuterium oxide were added to provide a lock frequency.

Metabolomic analysis

Data acquisition

We recorded proton High Resolution Magic Angle Spinning Nuclear Magnetic Resonance (^{1}H HRMAS NMR) spectra[15] at 277K and at a speed of 3502 Hz, on a Bruker Avance III 500 MHz system. A one-dimensional (1D) NMR spectrum, using a Carr-Purcell-Meiboom-Gill pulse sequence[16] with 1024 transients was acquired for each serum sample. Further experimental details can be found in our previous work.[17,18]

Multivariate statistical analysis

The spectral region between 4.7 and 0.5 ppm of each 1D NMR spectrum was automatically binned into 421 regions of 0.01-ppm each, using the AMIX 3.8 software (Bruker GmbH, Germany). This process generated an X data matrix, containing as columns the chemical shift values and as rows the patient's identity.

Data sets were then imported into the SIMCA P 11.0 software (Umetrics AB, Umeå, Sweden) and preprocessed, using unit variance scaling of the X columns.[19] The X matrix was then analyzed using principal component analysis (PCA). This procedure quickly evaluates the quality of the data and identifies possible outliers. After the PCA analysis, we conducted partial least-square discriminant analysis (PLS-DA), in order to build a model that optimized the separation between different classes of patients. PLS-DA[19] is a technique used to find the fundamental relationship between two matrices, X and Y (Y represents only the patient's class). A PLS-DA model tries to find the multidimensional direction in the X space that explains the maximum multidimensional variance direction in the Y space. The axis represented on a PLS-DA model corresponds to a linear combination of the variables (the buckets of the NMR spectrum or the metabolite concentrations, in our case). The first component of the PLS-DA model (horizontal axis) is the most important to explain this separation. The exact number of components of a PLS-DA model is determined by cross-validation.[20]

Metabolite quantification

We quantified the two most discriminant metabolites, using an external reference standard of lactate and the Pulcon principle.[21] Spectra were first automatically realigned and locally baseline-corrected for these two spectral regions of interest, by an in-house program[18] using MATLAB 7.0 (Mathworks, Natick, MA, USA). We then normalized the spectra, according to sample weight and peaks of interest that were automatically defined. Results were calculated in $nmol.mg^{-1}$ and then transformed to be expressed in $\mu mol.L^{-1}$ of serum. For each of the two metabolites, the regions of interest in ppm were defined with the following chemical shifts: scyllo-inositol (ScI; (3.346; 3.362), CH) and acetate (Ace; (1.915; 1.925), CH_3).

Univariate statistical analyses

We performed receiver operating characteristic (ROC) curve analysis to test for the sensitivity/specificity of scyllo-inositol and acetate. We calculated the areas under the ROC curves, their statistical comparison (P value) cut-off values, sensitivity and specificity with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). We also used a non-parametric Mann-Whitney U test to search for a correlation between the concentrations of the two relevant metabolites and patients' characteristics, i.e. positivity or negativity of anti-AQP4 antibody (Ab) status, disease duration and presence of a treatment. The level of significance was set $p = 0.05$.

Table 1. Clinical characteristics of the patients.

	Controls	NMO patients	MS patients
Number of patients	42	44	47
Female to male (*n*)	25 : 17	32 : 12	33 : 14
Average age at recruitment (years)	39.35 ± 13.47	43.89 ± 12.28	38.47 ± 9.84
Disease duration (years)	NA	8.27 ± 9.16	8.35 ± 5.24
Anti-AQP4 Ab status negative/positive	NA	22 : 22	NA
Number of patients following each treatment	NA	no treatment (*n* = 11)	no treatment (*n* = 5)
Treatment type (*n*)		mycophenolate mofetil (*n* = 14)	natalizumab (*n* = 39)
		azathioprine (*n* = 9)	interferon beta (*n* = 3)
		cyclophosphamide (*n* = 6)	
		mitoxantrone (n = 2)	
		rituximab (*n* = 1)	
		methotrexate (*n* = 1)	
		corticotherapy (*n* = 1)	

AQP4: aquaporin-4, MS: multiple sclerosis; NA: not applicable; NMO: neuromyelitis optica.

Results

Table 1 summarizes the characteristics of the MS patients, the NMO patients and the control group.

Preliminary multivariate statistical analyses

PCA analysis of the NMR data applied to the 4.7–0.5 ppm chemical shift range and using the entire patient population was not able to discriminate either of the two pathologies from healthy serum samples in a statistically-significant manner. We identified 16 outliers, characterized by either EDTA pollution or large lipoprotein signals, by this analysis. The PLS-DA analysis of the NMR data was performed on 117 serum samples (from 39 NMO, 41 MS and 37 controls). This procedure allowed us to identify the main metabolites at the origin of the discrimination, for the three different hypotheses tested: NMO versus MS, NMO versus control and MS versus control.

PLS-DA model for discriminating NMO patients from MS patients

Serum samples were first assigned to a Y column matrix as being either NMO (value of 0) or MS serum samples (value of 1). The analysis generated a 2-component PLS-DA model, characterized by a faithful representation[19] of the Y data ($R^2Y = 0.85$) and by a very good cumulative confidence criterion of prediction ($Q^2 = 0.80$). The score plot of the PLS-DA model (Figure 1(a)) showed a very clear separation of the two sets of serum samples. The MS group was characterized by a higher concentration of scyllo-inositol and glutamine; while the NMO group contained more acetate, glutamate, lactate and lysine.

PLS-DA model for discriminating MS patients from control subjects

The analysis generated a 2-component PLS-DA model, characterized by the following parameters: $R^2Y = 0.84$ and $Q^2 = 0.68$. The score plot of the PLS-DA model (Figure 1(b)) showed a clear separation of the two sets of serum samples. The MS group was characterized by a higher concentration of scyllo-inositol and lysine, while the control group contained more glutamine and valine.

PLS-DA model for discriminating NMO patients from control subjects

The analysis generated a 2-component PLS-DA model characterized by the following parameters: $R^2Y = 0.77$ and $Q^2 = 0.72$. The score plot of the PLS-DA model (Figure 1(c)) showed a clear separation of the two sets of serum samples. The NMO group contained a higher concentration of acetate, glutamate and lactate; while the control group contained more glutamine, lysine and valine.

The most discriminant metabolites, scyllo-inositol (MS group) and acetate (NMO group), were selected as candidate biomarkers for a distinction between MS and NMO. These two metabolites were then subsequently quantified in all 117 serum samples.

Serum metabolic profile of MS patients: Scyllo-inositol

Figure 2(a) reports the concentrations of circulating scyllo-inositol in MS patients, NMO patients and control subjects. We showed that scyllo-inositol in MS patients (average concentration 254.1 ± 8.7 µmol/L) had a substantial 2.4-fold increase, in comparison with the concentrations measured in the serum of NMO patients (average

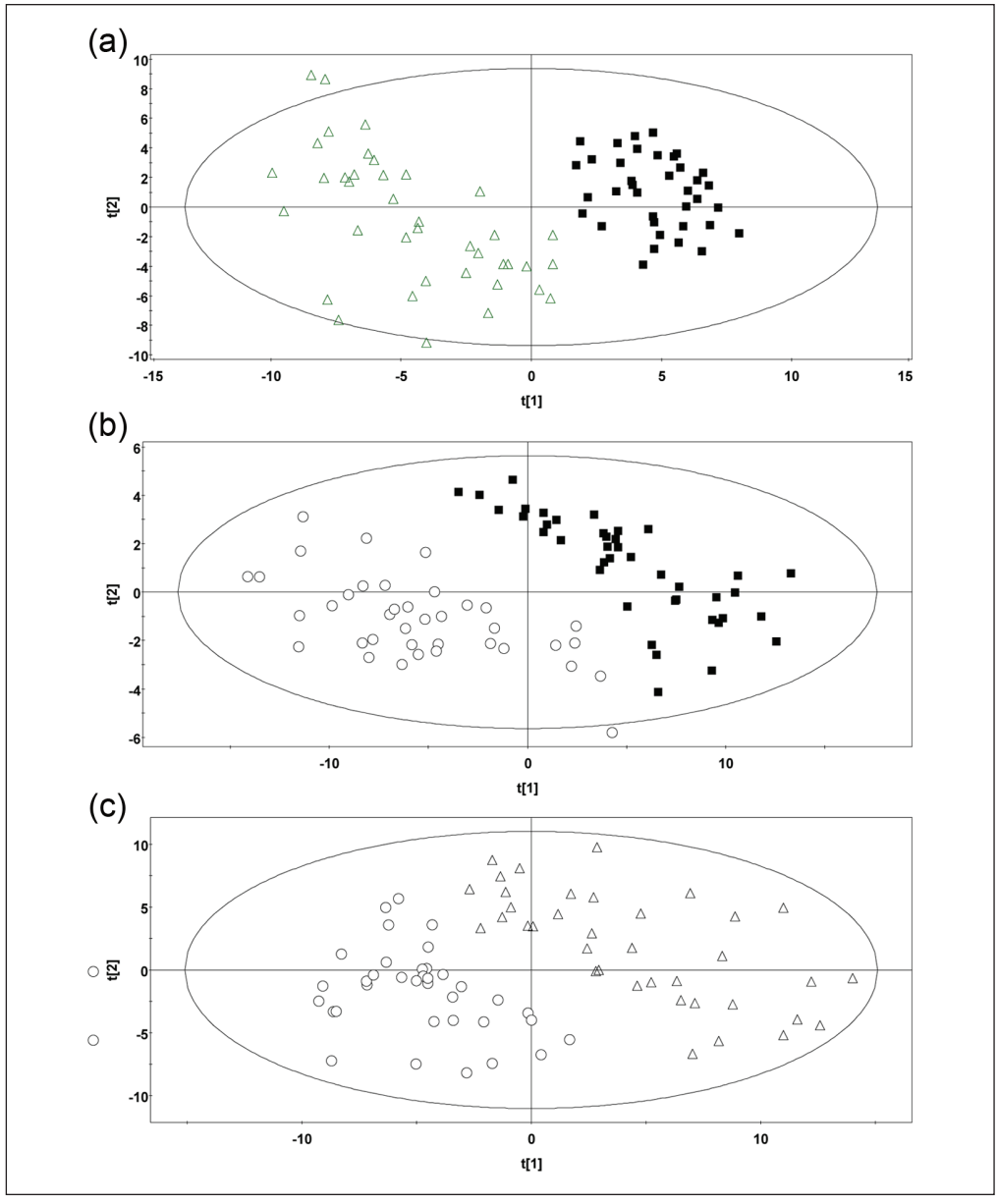

Figure 1. Multivariate modeling of NMO patients, MS patients and controls.

PLS-DA score plots obtained when classifying serum samples from: 1(a) NMO patients (empty triangles) and MS patients (black boxes) ($R^2Y = 0.85$; $Q^2 = 0.80$); 1(b) control subjects (empty circles) and MS patients ($R^2Y = 0.84$; $Q^2 = 0.68$); 1(c) control subjects and NMO patients ($R^2Y = 0.77$; $Q^2 = 0.72$), using, for each model, the most discriminant metabolites.

For each model presented, there was very good clustering of each of the two classes. In the model presented in 1(a), the most discriminant metabolites were acetate, glutamate, lactate and lysine for the NMO group; and scyllo-inositol and glutamine for the MS group, respectively. In the model presented in 1(b), the most discriminant metabolites were glutamine and valine for the control group, and scyllo-inositol and glutamine for the MS group. In the model presented in 1(c), the most discriminant metabolites were glutamine and lysine for the control group; and acetate, glutamate and lactate for the NMO group.

Axes represent the two first principal components responsible for the maximum of variance among the considered data. The *X* axis represents the first principal component and *Y* axis, the second one.

MS: multiple sclerosis; NMO: neuromyelitis optica; PLS-DA: partial least square discriminant analysis.

concentration 105.7 ± 6.6 µmol/L) and a significant 2.6-fold increase in comparison with the serum concentrations measured in control patients (average concentration 95.3 ± 4.1 µmol/L).

Scyllo-inositol was not specifically correlated with the AQP4 status among the NMO population. The ROC analysis performed on the scyllo-inositol concentration, for discriminating NMO from MS patients, allowed us to determine a cut-off value of 190.3 µmol/L for this metabolite (AUROC, 0.982; Se, 87.8%; Sp, 95%). Among the 39 NMO patients and the 41 MS patients, 37 and 36 subjects were correctly classified, respectively.

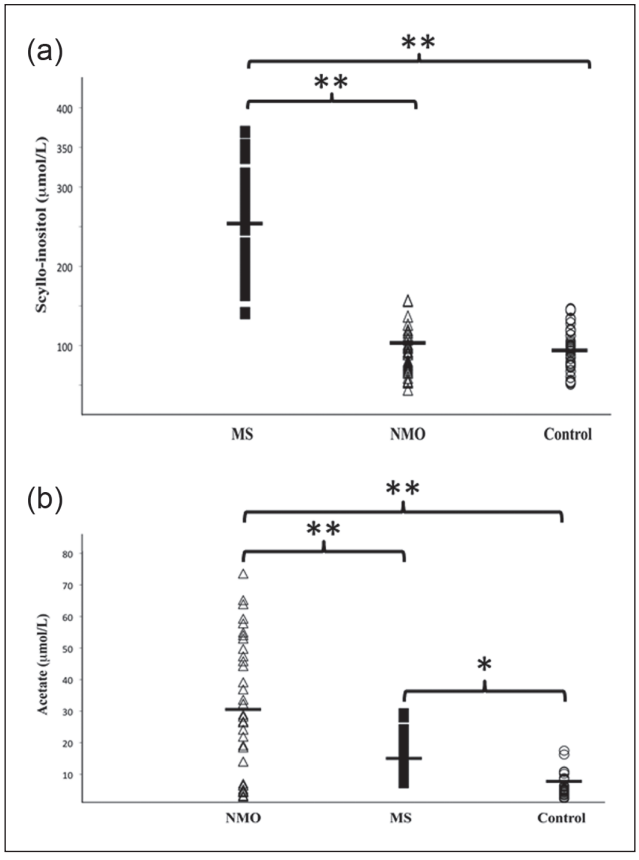

Figure 2. Scatter plot showing the concentrations of scyllo-inositol (2(a)) and acetate (2(b)) recorded in the serum of 37 healthy controls (empty circles), 41 MS patients (black boxes) and 39 NMO patients (empty triangles). Horizontal bars indicate the mean values calculated in the three different groups, two asterisks indicate a p-value < 10^{-4} and one asterisk indicates a p-value < 10^{-2} (Mann-Whitney U-test). Note in 2(a), the significant elevation of scyllo-inositol in MS patients compared to the two remaining groups, and in 2(b) the significant elevation of acetate in the NMO group, as indicated by the different asterisks. In 87.8% of the MS patients, scyllo-inositol increased between 0.15 and 3-fold, compared to controls; and in 74.3% of the NMO patients, acetate increased between 0.4 and 7-fold, compared to the controls.
MS: multiple sclerosis; NMO: neuromyelitis optica.

The ROC analysis performed for discriminating MS patients from healthy subjects allowed us to determine a cut-off value of 145.2 µmol/L (AUROC, 0.99; Se, 97.6%; Sp 97.3%) for this metabolite.

We found no specific clinical or biological pattern among the patients whom were wrongly classified.

Serum metabolic profile of NMO patients: Acetate

Figure 2(b) reports the concentrations of circulating acetate in NMO patients, MS patients and control subjects. Acetate in NMO patients (average concentration, 31.1 ± 3.6 µmol/L of serum) showed a substantial 1.8-fold increase, in comparison with the concentrations measured in serum of MS patients (average concentration, 17.3 ± 1.1 µmol/L of serum) and a significant 3.4-fold increase in comparison with the concentrations measured in control sera (average concentration, 9.1 ± 1.0 µmol/L of serum). The concentrations were not correlated with the disease duration nor the type of treatment followed by the MS or NMO patients. They were not correlated either with the anti-AQP4 antibody status among the NMO population.

The ROC analysis performed for discriminating NMO from control patients allowed us to determine a cut-off value of 10.9 µmol/L for this metabolite (AUROC, 0.829; Se, 86.5%; Sp, 72.5%). Among the 39 NMO and 37 healthy subjects, 29 and 33 subjects were correctly classified, respectively.

The ROC analysis performed for discriminating NMO from MS patients allowed us to determine a cut-off value of 18.5 µmol/L (AUROC, 0.67; Se, 75.6%; Sp, 67.5%) for this metabolite.

We found no specific clinical nor biological pattern among the patients whom were wrongly classified. These two metabolites are highlighted on representative ^{1}H NMR spectra of each of the three different groups considered (Figure 3).

Additional value of considering both scyllo-inositol and acetate concentrations as putative biomarkers for distinguishing NMO from MS patients

A multivariate PLS-DA model based solely on the serum concentrations of scyllo-inositol and acetate in NMO and MS patients was developed. The analysis generated a 2-component PLS-DA ($R^2Y = 0.74$; $Q^2 = 0.73$). The score plot of the PLS-DA model (Figure 4) showed a very clear separation between the two sets of serum samples. An extensive cross-validation embedded in a Monte-Carlo resampling approach was used during the construction of the model, in order to build a confusion matrix that allowed a direct visualization of the performances of the model.[22,23] The confusion matrix resulting from this analysis revealed a sensitivity of 94.3% and a specificity of 90.2% for NMO patients. Among the 39 NMO and the 41 MS patients, 37 and 37 subjects were correctly classified, respectively.

This multivariate approach allows taking into account the two biomarkers simultaneously, which is not obvious by using a ROC curve.

Discussion

The previous results suggested that the serum concentrations of two small metabolites, scyllo-inositol and acetate, have a high potential for distinguishing NMO from MS. The metabolomic changes occurring in the serum of these two patient types has never been explored previously, even

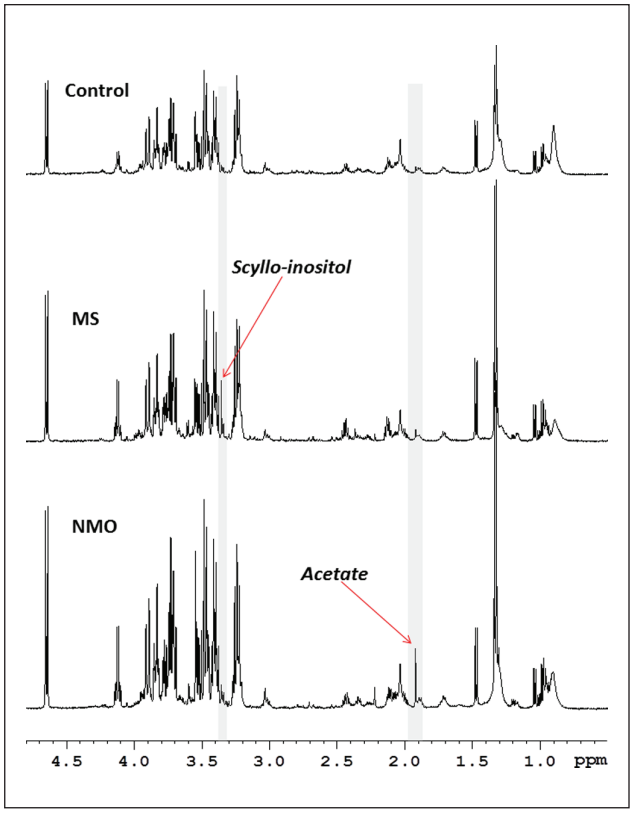

Figure 3. Representative ^{1}H NMR spectra of control subjects, NMO patients and MS patients with a particular focus on scyllo-inositol, represented by a singlet resonating at 3.36 ppm; and acetate, represented by a singlet resonating at 1.92 ppm. Note the elevated acetate that distinguishes the NMO patient from the MS and control ones and the elevated scyllo-inositol in the MS patient.
^{1}H NMR: proton nuclear magnetic resonance. MS: multiple sclerosis; NMO: neuromyelitis optica.

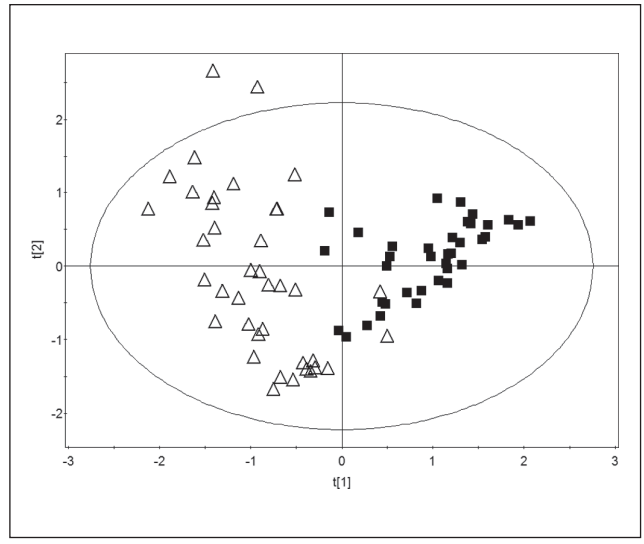

Figure 4. PLS-DA score plot obtained when classifying serum samples from NMO patients (empty triangles) and MS patients (black boxes), using solely their respective circulating concentrations of acetate and scyllo-inositol ($R^2Y = 0.74$; $Q^2 = 0.73$). Note the satisfying discrimination between the two classes, considered when taking account solely the respective concentrations of these two metabolites.
MS: multiple sclerosis; NMO: neuromyelitis optica; PLS-DA: partial least-square discriminant analysis.

though such diagnostic markers could be easily assessed at a low cost. The two metabolites found by our untargeted analysis are particularly relevant, because of their link with the alterations in brain metabolism.

Scyllo-inositol and its stereoisomer, myo-inositol, are the two most ubiquitous forms of inositol, present in the brain, liver and kidney. Scyllo-inositol is the second largest isomer in abundance after myo-inositol, which contributes over 90% to the total.[24] The two isoforms were found in the same tissues and a positive correlation between their concentrations was reported, suggesting a metabolic relationship between the two metabolites. It is not clear whether brain scyllo-inositol and myo-inositol are synthesized in situ, or whether they are produced elsewhere and transported into the brain. Scyllo-inositol was recently tested as a therapeutic agent to prevent the formation of amyloid deposits in Alzheimer's disease.[25] The results of brain in vivo magnetic resonance spectroscopy studies suggest an increase of scyllo-inositol in patients with mitochondrial enzyme deficiency, infection by human immunodeficiency virus (HIV), certain brain tumors and a decrease in hepatic encephalopathy.[24] In inflammatory diseases of the CNS, spectroscopy studies found that MS was associated with an increase of myo-inositol concentrations in the brain,[26] while NMO patients presented normal myo-inositol concentrations in normal-appearing white matter in the brain[27] and decreased myo-inositol concentrations within inflammatory lesions of the spinal cord.[28] No change in scyllo-inositol concentrations in the brain was reported, but it is not clear whether scyllo-inositol was correctly evaluated. The increase in myo-inositol has been suggested as a marker of the diffuse glial proliferation in MS brains, which takes place after the demyelination and neuronal damages. This glial proliferation is not observed in NMO, in which the immune attack targets the astrocytes, causing astrocyte necrosis and cavitation rather than astrogliosis.[29] It is not clear why scyllo-inositol, but not myo-inositol concentrations increased in the serum of MS patients. A specific increase in synthesis of scyllo-inositol by MS patients or an increased release/decreased uptake by the brain may explain this difference.

The involvement of the second metabolite, acetate, in brain metabolism and neurotransmitter synthesis (especially in the transformation of glutamate into glutamine) is already known. Acetate is preferentially metabolized by astrocytes, due to specific transport into these cells and so its interest as a marker of astrocyte metabolism has already been suggested.[30] Brain spectroscopy studies report an increase of acetate concentrations in amyotrophic lateral sclerosis,[31] but not in NMO nor MS patients.[26,27] Some

studies also report an increase of acetate concentrations in the CSF of MS patients, especially during their relapses.[10,12] A previous study measured acetate in serum, but found no difference between MS and control patients.[10] Glial abnormalities are found in both MS and NMO, but the astrocytes are particularly damaged in NMO. The latter disease is even described as an astrocytopathy, because of the extensive astrocyte loss probably related with anti-AQP4 antibodies.[29] Accordingly, we found that MS and NMO patients presented respectively mild and marked changes in acetate concentration, in serum. These changes may be related to a respectively mild or marked impairment of acetate uptake by astrocytes.

In clinical practice, scyllo-inositol could be very interesting for discriminating NMO from MS, with a better sensitivity (95%) than that of anti-AQP4 antibodies (50–70%). The specificity (87.8%) was lower than that of anti-AQP4 (> 90%), but remains high. Scyllo-inositol would be also useful to discriminate MS from control subjects. Acetate could be used to discriminate NMO from control subjects, but the large variation of its concentration resulted in a lower sensitivity (72.5%); however, it remained higher than that of anti-AQP4 antibodies. The specificity was relatively good (89.2%).

Other biomarkers, such as the analysis of magnetic resonance imaging (MRI) abnormalities patterns, have been proposed for discriminating NMO from MS. In a recent article, Matthews et al.[32] reported that MRI criteria (at least 1 lesion adjacent to the body of the lateral ventricle and in the inferior temporal lobe; or the presence of a subcortical U-fiber lesion; or a Dawson's finger-type lesion) allow the discrimination of MS from NMO with a sensitivity of 92% and a specificity of 96%.[32] A study using an ultrahigh field (7 T) MRI[33] found that 91% of lesions of NMO patients are not traversed by a venule, but this work included no MS nor healthy subject groups and gave no estimation of sensitivity nor specificity. One of the major advantages of scyllo-inositol and acetate is that they can be easily and quickly assessed in serum, after a simple blood draw. Their measurement could be also less expensive than MRI or anti-AQP4 antibody analysis. Concerning valine, our results are in accordance with the results of Monaco et al.[34] and Mehrpour et al.[11] They also find a decrease in its concentration in the MS group, compared to the control group.[11,34]

These results were obtained in a small group of patients, because of the relative rarity of NMO; however, the patients and controls were matched for age and gender, and the patients were under various therapies in each group, to prevent the impact of a particular condition. We checked the absence of correlation between the metabolites' concentrations and the duration of the disease or the presence of treatment: especially, the increase in acetate was not related to therapy by glatiramer acetate. Neither a particular diet nor a metabolic abnormality was reported in any of our three groups.

Conclusion

This study demonstrated for the first time the potential of ^{1}H NMR spectroscopy of serum as a novel, promising analytical tool to discriminate two populations of patients, affected by NMO or MS. More specifically, two metabolites were found to be particularly important for a clear discrimination between these two pathologies and a control group: scyllo-inositol, which discriminates MS patients from other subjects (NMO patients and control subjects) and acetate, which discriminates NMO patients from other subjects (MS patients and control subjects).

The next step will be a simultaneous validation of the PLS-DA models and of the two biomarkers (scyllo-inositol and acetate) on a larger cohort of patients. A determination of a cut-off value for the respective serum concentrations of these two metabolites, which could help clinicians choose the most appropriate therapeutic protocol to treat their NMO or MS patients, will also have to be validated.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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