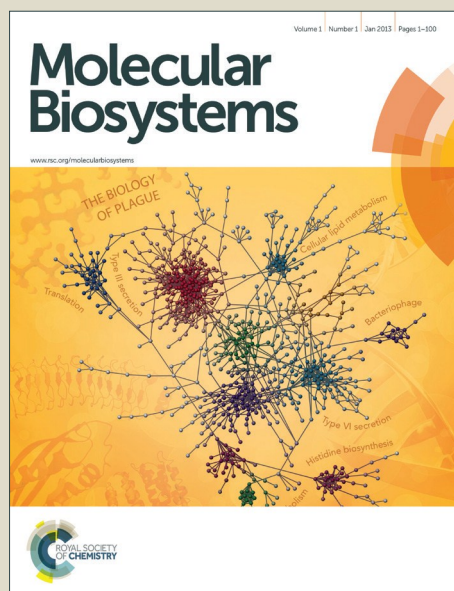


Molecular BioSystems

Accepted Manuscript



This article can be cited before page numbers have been issued, to do this please use: D. Pieragostino, M. D'Alessandro, M. Di Iorio, C. Rossi, M. Zucchelli, A. Urbani, C. di Iorio, A. Lugaresi, P. Sacchetta and P. Del

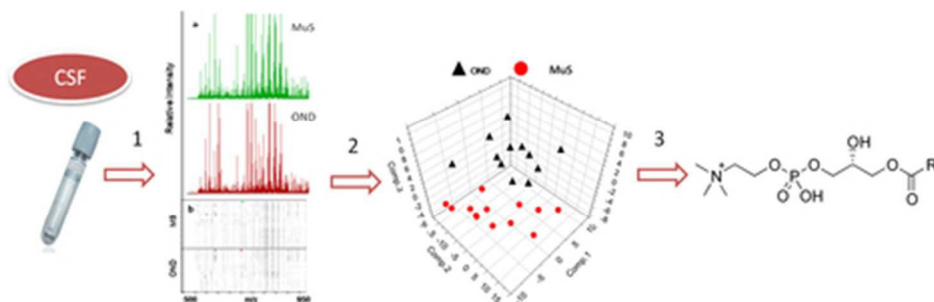


This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the [Information for Authors](#).

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard [Terms & Conditions](#) and the [Ethical guidelines](#) still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.



Graphical Abstract: 1) MALDI-TOF analysis of lipid species in Multiple Sclerosis (MuS) and OND patients. 2) Distinctive biochemical alteration associated with MuS. 3) Characterization and validation of differences.
39x19mm (300 x 300 DPI)

An integrated metabolomics approach for the research of new cerebrospinal fluid biomarkers of multiple sclerosis

Damiana Pieragostino^{1,2}, Michele D'Alessandro^{1,2}, Maria di Ioia^{2,3}, Claudia Rossi^{1,2}, Mirco Zucchelli^{2,4}, Andrea Urbani⁵, Carmine Di Ilio^{1,2}, Alessandra Lugaresi^{2,3}, Paolo Sacchetta^{1,2} and Piero Del Boccio^{1,2}

¹ Department of Medical, Oral and Biotechnological Sciences, University "G. d'Annunzio" of Chieti- Pescara, Chieti, Italy.

² Analytical Biochemistry and Proteomics Unit, Research Centre on Aging (Ce.S.I), University "G. d'Annunzio" of Chieti-Pescara, Chieti, Italy.

³ Department of Neurosciences and Imaging, University "G. d'Annunzio" of Chieti-Pescara, Chieti, Italy.

⁴ School of Medicine and Health Sciences, University "G. d'Annunzio" of Chieti-Pescara, Chieti, Italy.

⁵ Department of Experimental Medicine and Surgery, University of Tor Vergata, Rome, Italy.

Keywords: Mass Spectrometry; Lipidomics; Metabolomics; CSF; Multiple Sclerosis; Biomarkers.

*Corresponding Author:

Dr. Piero Del Boccio, PhD
Department of Medical, Oral and Biotechnological Sciences, University "G. d'Annunzio" of Chieti-
Pescara, Chieti, Italy.

e-mail: p.delboccio@unich.it

Phone: +39 0871 541593

Fax: +39 0871 541598

Abstract

Multiple Sclerosis (MuS) is a disease due to an autoimmune attack against myelin components in which non proteic mediators may play a role. Recent research in metabolomics and lipidomics has been driven by rapid advances in technologies such as mass spectrometry and computational methods. They can be used to study multifactorial disorders like MuS, highlighting the effects of disease on metabolic profiling, regardless of the multiple trigger factors.

We coupled MALDI-TOF-MS untargeted lipidomics and targeted LC-MS/MS analysis of acylcarnitines and aminoacids to compare cerebrospinal fluid metabolites in 13 MuS subjects and in 12 patients with Other Neurological Diseases (OND). After data processing and statistical evaluation, we found 10 metabolites that significantly ($p < 0.05$) segregate the two clinical groups. The most relevant result was the alteration of phospholipids levels in MuS and the correlation between some of them with clinical data. In particular lysophosphatidylcholines ($m/z=522.3$ Da, 524.3 Da) and an unidentified peak at $m/z=523.0$ Da correlated to the Link index, lysophosphatidylinositol ($m/z=573.3$ Da) correlated to EDSS and phosphatidylinositol ($m/z=969.6$ Da) correlated to disease duration. We also found high levels of glutamate in MuS. In conclusion, our integrated mass spectrometry approach showed high potentiality to find metabolic alteration in cerebrospinal fluid. These data, if confirmed in an wider clinical study, could open the door for the discovery of novel candidate biomarkers of MuS.

Introduction

Multiple Sclerosis (MuS) is a chronic demyelinating disease of the Central Nervous System (CNS) characterized by inflammation and axonal degeneration. It is the most common cause of neurologic disability in young adults in the Western world affecting mostly people between 20 and 40 years of age, with a female-male ratio which has increased, in the last decades from 2:1 to 3:1¹. In most cases MuS has a relapsing-remitting (RR) course².

MuS is commonly considered an acquired autoimmune pathology in genetically susceptible individuals when environmental risk factors, such as viral infections, vitamin D deficiency and ultraviolet radiation, occurred³. While the etiology of MuS is still unclear, a favored hypothesis suggests that one factor contributing to the development of autoreactive T-cells in MuS is a cross-reactive immune response between viral components and CNS antigens (“antigenic mimicry”) ^{4, 5}. Among possible mediators are proteins, metabolites and lipids thought to be involved in disease mechanisms. Lipids, in particular, represent important targets since they are implicated in many signalling routes and antigen reactions. Recent studies suggest a role for lipids in the autoimmune process and recent evidence have shown lipid metabolism alterations in the CNS of MuS subjects^{6,7}. Metabolomic and lipidomic approaches revealed useful in identifying metabolic alterations in Cerebrospinal Fluid (CSF) in animal model of MuS and in neurodegenerative diseases in general. In a previous work of Gonzalo et al, through untargeted approach of metabolomics and lipidomics, several discriminant metabolites were found between MuS and non-MuS patients. Among these differential molecules, the lipid oxidation marker 8-iso-prostaglandine F2 α was found increased in MuS patients. Also autoantibodies against lipoxidized proteins were increased in MuS, suggesting an enhanced autoimmune response underlying the progression of the disease⁸. Moreover, when the limits of metabolomics will be exceeded, it will be possible the comprehension of pathogenetic pathways facilitating the development of specific disease treatments. Sphingosine is the molecular backbone of sphingolipids which are the most abundant lipids in the myelin sheath and Fingolimod, for example, is a sphingosine 1-phosphate receptor (S1PR) modulator, today used as a new oral

drug for MuS⁹. Furthermore, metabolic homeostasis is deeply modified during pregnancy, which is a typical remission period in MuS course^{10, 11}. Therefore, determining the major epitopes of the different encephalitogenic myelin and neuronal factors implicated in MuS is of major significance not only for devising immuno-specific therapeutic approaches to MuS, but also for understanding the pathophysiology and etiology of the disease. Moreover it's demonstrated that carnitine, involved in fatty acids metabolism, and its derivatives are present at different concentrations in various body fluids and tissues and that they can be implicated in various diseases characterized by upregulated or impaired immune responses^{12, 13}. The therapeutic rationale in MuS derives from the demonstration of a reduction of nitroxidative stress in CSF in active MuS patients treated with acetylcarnitine¹⁴. However a current Cochrane review on the efficacy of carnitine in MuS-related fatigue conclude for an insufficient evidence of a therapeutic advantage of carnitine over placebo or active comparators¹⁵. Recently, a metabolomics study by Noga MJ et al. demonstrated a significant change in amino acids metabolism in CSF during Experimental Allergic Encephalomyelitis (EAE). They found altered levels of metabolites related to pathways including nitric oxide synthesis, altered energy metabolism, polyamine synthesis and levels of endogenous antioxidants¹⁶.

On the spur of these recent findings, here we report a metabolomics investigation of CSF including carnitines, amino acids and lipid profiles in MuS subjects and patients with OND by combining untargeted and targeted metabolomics strategies. Aim of our study was to identify new candidate biomarkers for MuS diagnosis, in order to better understand the possible link between metabolic alterations and clinical features of the disease.

MATERIALS AND METHODS

Ethics Statement

The study design was made following the guidelines for Good Clinical practice (GCP) of the local Ethics Committee that approved the study (Ethic committee of “G. d’Annunzio” University and ASL N.2 Lanciano-Vasto-Chieti, Italy), and following the ethical standards in conformity with

those laid down in the Declaration of Helsinki (World Medical Association, 1997). All patients were informed about the procedures and provided written informed consent to participate in the study. In order to protect human subject identity a number code was employed for specimen identification.

Patients

13 patients with Relapsing Remitting MuS, in accordance with the 2010 Polman's criteria¹⁷, were included in this study. Clinical diagnosis was confirmed by cerebral and spinal cord magnetic resonance imaging (MRI) studies and by the presence of oligoclonal bands in CSF. The Expanded Disability Status Scale (EDSS) score was obtained at the time of lumbar puncture. To be enrolled in the study patients should have not been treated with steroids in the month before study entry and should have been never treated with immunomodulatory or immunosuppressive drugs both for MuS and for other diseases. 12 CSF samples of patients with other neurological diseases (OND) were used as control group. The diagnosis in each of these patients was defined according to individual disease diagnostic criteria. **Table S1** in supplementary materials summarizes clinical and demographic features of the enrolled patients. The disease duration is defined as the time elapsed (expressed in days) between the first symptom to the day of CSF collection. All patients were selected in order to obtain age, sex and ethnicity (white people) matched cohorts of subjects. Patients with other disease course and comorbidities were excluded. CSF samples were collected at the MuS Center of Chieti (Italy).

Sample Collection

CSF samples, taken by a routine lumbar puncture at L3/L4 or L4/L5 interspace, were always collected in the morning on the first day of patient observation. Each sample (around 3 mL) was centrifuged at 10000g at 4°C, for 10 minutes. The supernatant was divided into aliquots and stored at -80°C. Around 2 ml of CSF from each subject was used for diagnosis. Only 300 microliters of

CSF per patient were employed in this study (200 μL for untargeted lipidomics and 10 μL for targeted amino acids and carnitines determination).

Lipid extraction procedure and MALDI-TOF-MS analysis

200 μL of CSF per patient were used for lipid extraction. Total lipids were extracted using a modified “Bligh and Dyer” method¹⁸. Briefly, 100 μL of a saturated solution of $(\text{NH}_4)\text{SO}_4$ (Sigma Aldrich) for protein precipitation were added to 2 different aliquots of 100 μL of CSF. After vortexing and centrifugation (15 min at 10000 g) 500 μL of MeOH, 250 μL of CH_3Cl and 250 μL of H_2O were added to supernatant (180 μL) and vortexed (all solvent purchased by Sigma Aldrich). After centrifugation for 15 min at 10000 g the CH_3Cl phase of two aliquots of the same sample were bring together, dried and sealed to be stored at $-80\text{ }^\circ\text{C}$. Total lipids extract were re-suspended in 50 μL of MeOH, vortexed, centrifuged for 15 min at 10000 g and used for MALDI-TOF-MS analysis. Each CSF sample was fortified with 10 μL of Dimyristoylphosphatidylcholine (DMPC) (Avanti Polar Lipids, inc USA) at 2.5 $\mu\text{g}/\text{mL}$, used as internal standard for mass accuracy verification. After spectra acquisition the signals of the DMPC and the signal of an endogenous well characterized signal were used to verify the instrumental mass accuracy (see **Figure S1A** for details). In order to compare groups, the variability of quantification and the extraction recovery were assessed by using quality controls of CSF fortified with standard DMPC at different concentration levels (2.5, 5, 10 and 25 $\mu\text{g}/\text{mL}$). As reported in Figure S1B supplementary materials, the method shown good linearity response with an RSD% below 20% for each concentration level. The matrix solution was prepared by using a saturated solution of 2,5-dihydroxybenzoic acid (DHB) in Acetone/Chloroform (9:1) according with Fujiwaki et al.¹⁹ Extracted lipids from CSF were mixed 1:1 with the matrix solution and 0.5 μL of the resulting solution were spotted on MTP Ground steel 384 (Bruker Daltonics). All analyses were performed with a Autoflex Speed MALDI-TOF-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in the mass range 300-3000 Da. Instrument parameters were tuned in order to obtain the highest resolution and sensitivity in the

mass range used. All mass spectra were acquired in positive Reflectron mode at a voltage of 19; 16.72 and 8.54 kV for the first and second ion extraction stages and lens, respectively. Every single acquisition run was composed by 500 laser pulses at 1000 Hz. The most abundant lipid signals were characterized by fragmentation experiments by LIFT tandem mass spectrometry analysis. Analyses were performed using the following acquisition settings: ion source 1: 6.0 kV; ion source 2: 5.3 kV; lens: 3.0 kV; reflector 1: 27.0 kV; reflector 2, 11.70 kV; lift 1: 19.0 kV; lift 2: 4.25 kV; pulsed ion extraction 120 ns.

Lipid identification was carried out by database search (mainly by: “Lipid Maps”) using their accurate mass measured and by the mass of characteristic fragments obtained in LIFT experiments.

Figure S2, S3 and S4 in Supplementary material show the fragmentation spectrum of the signals at $m/z=522$ Da, 524 Da and 734 Da reported in **Table 2** as differential metabolites.

LC-MS/MS Analysis of amino acids, free carnitine and acylcarnitines

A targeted metabolic fingerprint strategy by Liquid Chromatography-tandem Mass Spectrometry (LC/MS/MS) in multiple reaction monitoring (MRM) mode was performed for the determination of amino acids, free carnitine and acylcarnitines levels in CSF samples. CSF (9.6 μ L) was transferred into 1.5 mL tubes (Eppendorf, Hamburg, Germany) and then extracted with a solution containing the stable isotope labeled internal standards for each analyte of interest, according to the principle of isotope dilution internal standardization. The stable isotope labeled internal standards, as well as the extraction solution, were obtained from the NeoBase Non-derivatized MSMS Kit (Perkin Elmer Life and Analytical Sciences, Turku, Finland). The tubes were then capped and vortex mixed. The samples were centrifuged (22582 g at 4 °C for 15 minutes), and the supernatant was analyzed by direct infusion mass spectrometry (DIMS) as already reported²⁰.

The NeoBase non derivatized MSMS Kit is validated for blood spots for the determination of absolute concentrations of amino acids and acylcarnitines. As done in other studies^{21, 22} working with different biological matrixes such as plasma and serum, the use of the kit was intended to

reveal relevant alterations in amino acids and acylcarnitines profiles in CSF patients samples. Anyway, once accepted the use of the kit for different biological matrixes other than for blood spot to determine alterations in metabolites profile, we decided to extract 9.6 μL of CSF for each sample, after testing the reproducibility at three different volume of sample. A quality control (QC) CSF pool was prepared from the CSF patients samples, then extracted and analyzed as described for the CSF samples in study. A total of 10 QC CSF pool were analyzed during the run. Method accuracy was accessed for each analyte, precision being evaluated as repeatability in terms of coefficient of variation (CV) for the QC samples. The calculated mean CV for the amino acids and acylcarnitines were between 3.9-13% and 6.1-11.5%, respectively.

The DIMS analysis for the evaluation of metabolic profile in CSF samples was performed using a LC-MS/MS system consisting of an Alliance HT 2795 HPLC Separation Module coupled to a Quattro Ultima Pt ESI tandem quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA). The instrument operated in positive electrospray ionization mode using MassLynx V4.0 Software (Waters) with auto data processing by NeoLynx (Waters Corporation, Milford, MA, USA). Autosampler injections of 30 μL were made into the ion source directly by a narrow peek tube, and the mobile phase was methanol/water 75:25 (v/v) plus 0.01% oxalic acid (Perkin Elmer). The total run time was 1.8 min, injection-to-injection. The mass spectrometer ionization source settings were optimized for maximum ion yields for each analyte. Capillary voltage was 3.25 kV, source temperature was 120 $^{\circ}\text{C}$, desolvation temperature was 350 $^{\circ}\text{C}$, and the collision cell gas pressure was 3-3.50 e-3 mbar Argon. In Supplementary material **Table S2-S4** are reported the detailed list of analytes.

Data Processing and Statistical Methods

All CSF raw MALDI mass spectra acquired were processed using SpecAlign free software, version 2.4.1, developed by Dr Jason Wong at the University of Oxford (<http://powcs.med.unsw.edu.au/research/adult-cancer-program/services-resources/specalign>). This

procedure allowed peak deconvolution and alignment, denoise and Total Ion Count (TIC) normalization to give a table of mass pairs with associated relative intensities for all the detected MALDI peaks for each CSF sample analyzed. The relative intensities of lipid signals obtained by untargeted MALDI-TOF-MS analysis were underwent to multivariate processing. This data matrix was exported, UV-scaled, mean centred and used for partial least squares discriminant analysis (PLS-DA) using SIMCA-P + 11.0 (Umetrics AB, Umeå, Sweden). The most influential variables responsible for the separation between classes are the variables having the greatest influence in PLS-DA. A parameter named VIP (Variable Importance in the Projection), was employed to reflect the variables importance in the discriminant analysis. Results of the PLS-DA analysis was a list of all variables (lipids) sorted by their discriminatory power (VIP). We used this results as explorative and select the major discriminant lipid signals (VIP >2), obtained by PLS-DA elaboration, for a statistical re-evaluation applying the Student's t-test when normality was accepted, otherwise the Mann Whitney U-test was applied. From this processing of untargeted data we obtained a list of significant variables listed ad potential biomarkers.

For targeted analysis, all LC-MS/MS data were underwent to D'Agostino and Pearson omnibus normality test in order to determine the normality of each variable measured in each group. When normality was accepted the Student's t-test was employed, otherwise the Mann Whitney U-test was used for comparing the groups. All statistical elaborations were performed by using GraphPad Prism (GraphPad Software, Inc. USA). Correlation analysis with clinical parameters to lipids, carnitines and amino acids levels were performed by Statistica 7.0 (StatSoft DemoVersion). GraphPad Prism was employed for ROC curve analysis.

RESULTS

Metabolic profiling in CSF by targeted LC-MS/MS analysis

We investigated amino acids, free carnitine and acylcarnitines levels in CSF samples by LC-MS/MS in a targeted metabolomic strategy. Samples were randomized and analyzed by DIMS as

described in the methods section. **Table 1** shows CSF relative abundance of short and medium acylcarnitines and amino acids obtained by DIMS analysis in OND and MuS subjects. Long-chain acylcarnitines were not detectable in CSF. As showed in **Table 1** only glutamate resulted differential between the two clinical groups investigated ($p < 0.05$, t-test).

CSF lipidomics by MALDI-TOF-MS

Lipid pool extracted from CSF of each patient was randomized and subjected to MALDI-TOF-MS analysis. **Figure 1 Panel a** shows the average MALDI-TOF mass spectra obtained by the two clinical groups investigated. **In panel b** was reported the virtual gel showing the lipid profiling of each CSF analyzed. The more populated range of lipid signals in the spectrum was between $m/z=500$ Da and 1000 Da. Fragmentation analysis of the main peaks (data not shown) indicated that the most abundant ions contain phosphocoline as head group at $m/z=184$ Da, mainly sphingomyelins (SM) and phosphatidylcholines (PC)²³. Once analyzed all CSF samples, an automatic elaboration of all spectra acquired was performed. After denoising and signals alignment, an average data matrix was obtained containing 642 m/z signals ascribable to lipid molecular species, with an intensity normalized vs the Total Ion Count (TIC). Data were displayed in a final reference matrix where all molecules studied were reported as variables (642) linked to each observation (patient) in a compatible format for the multivariate analysis with Simca-P software. To explain the maximum separation between defined class samples in the data set, the 642 variables were processed with Partial Least Squares Discriminant Analysis (PLS-DA). In **Figure 2** was reported the PLS-DA score plot of the first three components where the two group of patients were visualized and separated, based on their lipidomics pattern. However, the model with greater predictive power was constituted by nine components describing a 78% of variation in X ($R^2X=78.6\%$) and 99% of the variation in the response Y (class) ($R^2Y = 98.9\%$) with a $Q^2Y=0.68$. In order to avoid over-parametrization due to the fact that the model use to many variables in respect to the analyzed cases, we validated this exploratory results by univariate analysis as

described below. Simca-P software generates a list of all the variables analysed sorted by a score number named VIP. The VIP score reflects the variable's contribution on classification, and can be used to discover the most relevant differential variables responsible for group separation. We used the results of multivariate analysis as explorative and selected the major discriminant lipid signals for a statistical re-evaluation. The software indicates $VIP > 1$ significant for discrimination. In this study the differential lipids with a VIP value > 2 (two fold the nominal cut off) were taken in consideration for an independent statistical re-evaluation (Mann–Whitney U test/t-test). Variables with a $p < 0.05$ obtained from hypothesis testing were taken into consideration as putative biomarkers.

Potential biomarkers identified

In **Table 2** were reported all differential ($p < 0.05$) metabolites highlighted in this study and in particular nine lipid species and glutamate. Among all the differential lipid signals some of them were tentatively identified through database search of the accurate mass measured and, when possible, by fragmentation analysis (for mass tolerance in the database search it was chosen 15 ppm which is about 2 times higher than the mass accuracy measured for DMPC standard, considering the large range in ion intensity, during the fast acquisition of the TOF). **Figure 3**, shows the distribution of each discriminant metabolite in two groups of patients studied. Three lyso phospholipids at $m/z = 522.3$ Da (a), 524.3 Da (b) and 573.3 Da (c) identified as two lysophosphatidylcholine (LPC) and lysophosphatidylinositol (LPI), respectively and the signals at 734.5 Da (d) and 969.6 Da (e) identified as phosphatidylcholine (PC) and phosphatidylinositol (PI) respectively, as well as two unidentified species at $m/z = 523.0$ Da (f) and 523.9 Da (g), are significantly increased in MuS people. Otherwise the signals at 673.4 Da (h) identified as phosphatidic acid (PA) and 727.0 Da (i) (unidentified signal) resulted decreased in MuS patients vs OND subjects. Considering all amino acids and carnitine species analysed, the glutamate (l) in CSF of MuS patients resulted significantly elevated vs OND patients. In **Table 3** was summarized the

discriminatory power of each potential biomarker highlighted, showing a receiver operating characteristic curve (ROC) data based on the relative intensity of each biomarker analyzed. The accuracy of each biomarker in classifying the two groups is outlined by the sensitivity and the specificity in the reclassification of MuS patients. Eight compounds, taken singularly, show significant reclassification and, most of them, showed high specificity in classifying MuS group.

Correlation with clinical parameters

To study the potential of the detected metabolites to identify ongoing disease activity we evaluated the relation of the signal intensity values of the discriminatory compounds with clinical parameters shown in **Table S1** of Supplementary material. The intensity of two LPC species at $m/z=522.3$ Da, 524.3 Da and an unidentified signal at $m/z=523.0$ Da (elevated in MuS patients) correlated positively with the Link index as shown in **Figure 4** (a-c). The intensity of the signal at $m/z = 969.6$ Da (elevated in MuS) identified as PI, correlated positively with disease duration (**Figure 4**, d), while the lyso isoform (LPI) at $m/z=573.3$ Da (elevated in MuS) showed a negative correlation with the EDSS (**Figure 4**, e). Other variables studied did not show significant correlations with the clinical parameters considered.

DISCUSSION

In this study we reported a targeted LC-MS/MS of carnitines and amino acids and MALDI-TOF-MS untargeted lipidomics profiling in order to improve knowledge of metabolic pathways modulation in the CSF of MuS patients. Therefore we looked at a possible correlation of the metabolomics data with clinical parameters to study their relationship to disease pathophysiology. By targeted analysis we studied 36 acyl carnitines and 19 amino acids species, while by untargeted analysis we obtained hundreds of peaks associated to lipid species. The choice of high throughput screening by MALDI-TOF-MS technology was driven to obtain high sensitivity to analyze CSF lipids and to be able to carry out identification of unknown compounds, as well as to apply a robust

methodology easy to transfer into clinical practice. Our main objective is to identify new candidate biomarkers for MuS diagnosis, in order to better understand the possible link between metabolic alterations and clinical features of the disease. The potential biomarkers, in the lipid screening, were tentatively identified by accurate mass and, when possible, by fragmentation experiments. Lipids containing phosphocholines were assigned with a better fashion due to their major susceptibility to ionization of the quaternary nitrogen in the head group, giving a typical m/z at the fragment ($m/z=184$ Da). Ten metabolites that significantly ($p<0.05$) segregate the two clinical groups analyzed were obtained. The most relevant result of this approach was the detection of an altered level of specific phospholipids in MuS when compared to OND subjects. In particular we found a significantly increased level of LPC (18:1/0:0), LPC (18:0/0:0) and LPI (16:0/0:0) in MuS people and other m/z signals (523.9 Da and 523.04 Da) unidentified but probably associated to similar compounds in CSF. Moreover we identified the signal at $m/z=734.5$ Da as a PC specie that resulted higher in MuS than OND people. LPC (18:1/0:0), LPC (18:0/0:0) and the unidentified lipid at $m/z=523.04$ Da are well correlated ($p<0.05$) to the Link Index (also known as “IgG Index”) a parameter that indicates high levels of intrathecal IgG synthesis. The intrathecal IgG synthesis is a common event in MuS. Interestingly, our previously published data demonstrated a significant decrease of this specific LPC species (LPC-18:1/0:0 and LPC-18:0/0:0) in serum of MuS patients in respect healthy control subjects²⁴, while similar levels were found between MuS and OND. Here we obtained overexpression of these metabolites in CSF of MuS, a cerebral compartment physiologically independent from the serum, suggestive for a possible function of these lipids as candidate biomarkers, reflecting intrathecal synthesis and CNS inflammation²⁵.

We considered a single OND control group even if disease parameters are quite variables in the considered clinical cohort. However, aware of the limitation of such choice, we think our finding are strengthened from the heterogeneity of OND group highlighting a specific trend in MuS. Moreover, we investigated the lipid composition between gender in the MuS and OND groups, showing not significant differences between males and females, demonstrating that there is not

gender influence on the lipid profile in CSF (**Figure S5**). Unfortunately, we don't have information about the body mass index of patients and this can be considered a biasing factor, even if the CSF should not be strongly influenced by this index like the serum. However, we attempted to insert a homogeneous group of patients.

PC is the major phospholipid specie of eukaryotic membranes and removal of one of the fatty acids results in LPC usually through the enzymatic action of a phospholipase A2 (PLA2). Several studies suggested altered levels of PCs in neurodegenerative diseases concluding that secretory PLA2 activity in the CSF might serve as a valuable biomarker of neuroinflammation as demonstrated in Alzheimer disease^{26, 27}. In EAE, the blockade of cytosolic PLA(2) α was highly efficacious in ameliorating disease course probably reducing T cell proliferation, proinflammatory cytokine production, preventing activation of CNS-resident microglia and increasing oligodendrocyte survival. The authors, administrating a cPLA(2) α inhibitor in a relapsing-remitting model of EAE, completely protected mice from subsequent relapses²⁸. The therapeutic effect of Fingolimod is probably also due to inhibition of cPLA(2) α activity, as previously demonstrated, directly in CNS²⁹. Here we can speculate that the pathological overstimulation of PLA2 determines cutting of PC from the membranes, resulting in accumulation of LPC species into the damaged tissue, confirmed by high levels of circulating LPCs in CSF of MuS patients.

Moreover LPC is recognized as an important factor underlying signal transduction and plays a functional role in various diseases by LPC specific G-protein-coupled receptors³⁰. LPC are released into the brain in pathological conditions linked to high levels of pro-inflammatory cytokines Interleukin 1b (IL-1b)³¹. LysoPC released from apoptotic cells could also act as chemotactic factor for monocytic cells and primary macrophages³². It was demonstrated both in vivo and in vitro that LPC induces deramification of murine microglia. In particular LPC (16:0) and (18:0) are able to induce IL-1b release, an important pro-inflammatory cytokine, from microglial cells through activation of P2X7 receptor³³. This is consistent with the timing of CSF withdrawal in MuS patients during a relapse. Another interesting results of our study was the identification of LPI (16:0/0:0)

species and high molecular weight PI that are elevated in MuS subjects vs OND patients, even if in this case the identification was obtained without fragmentation data due to low intensity of these signals in the spectra. The levels of LPI in MuS negatively correlated to EDSS score indicating that an increased level of this metabolite in CSF may be associated to a protective role against disease's progression and severity of symptoms. On the other hand we found a positive correlation of PI levels in CSF with disease's duration; this result may also reflect a role of the metabolite in a broadening of neurodegeneration due to inflammation³⁴. These data may seem contradictory, since EDSS value, usually, is proportional to disease's duration, but they might reflect different roles of these metabolites. However, it is arduous to discuss a possible involvement of LPI and its high expression in the CSF of MuS patients considering that our preliminary evidence pertains to a limited group of patients. However we may speculate that the role of such metabolite may be compared with other LPIs for biological activities. Consistently with this observation there is fascinating new evidence indicating the involvement of LPIs in neuroprotection, mediating modulation of microglia function through G protein-coupled receptor 55 (GPR55)³⁵. Actually, the physiological roles of GPR55 and its possible involvement in the pathophysiology are emerging. Recent studies established LPI as activator of GPR55 and its implication in pain transmission, where cannabinoids are potent inhibitors of such machine³⁶. In summary, LPI is rising as a key modulator of cell proliferation, migration, and function, and holds important pathophysiological implications due to its high levels in diseased tissues³⁷.

Regarding all aminoacids and acyl-carnitines quantified in CSF, glutamate levels seem to be slightly increased in MuS during the acute phase of inflammation, although it is known that glutamate is increased also in other neurological diseases, thus it cannot be considered a specific marker of MuS. However, this result is in agreement to recent findings that demonstrated the role of glutamate, the first excitatory neurotransmitter of CNS, in MuS and EAE^{38, 39}. It was already reported that glutamate levels were elevated in CSF of MuS patients and these levels correlated to disease's severity⁴⁰. Interestingly, this result was in agreement to high levels of LPC in CSF

considering that glutamate release, calcium influx, and activation of cellular PLA2 were reported as important steps initiating membrane breakdown⁴¹. These considerations could indicate a potential use of glutamate as biomarker of MuS severity even in patients without lesions in NAWM. Moreover in according with Tejani et al carnitine levels do not seem to be influenced by the disease¹⁵. In conclusion, even though OND patients show metabolic patterns similar to MuS subjects, some of these features are distinctive and can be considered specific for MuS. In **Table 3** the discriminatory power of each potential biomarker was summarized, Eight compounds, taken singularly, show significant reclassification and, most of them, showed high specificity in classifying MuS group. Even if the discriminatory power of each compound is not excellent, taken together, the pattern, can represent a specific cerebral metabolic alteration in MuS disease. The limit of this study is the lack of external validation of the results, consisting in a computing prediction for an independent set of test observations. However, a confirmation of these preliminary results in a more wider clinical study could lead to a better understanding of the metabolic (dys)homeostasis in the pathogenesis of MuS, giving the opportunity of new functional biomarkers and new promising targets for therapeutic interventions.

Acknowledgement

This study was supported by the Italian Ministry of Health, Grant number : GR-2010-2307655.

Maria di Ioia is supported by a FISM- Fondazione Italiana Sclerosi Multipla- training fellowship

Cod. 2012/B/2

Tables

Table 1: CSF of short, medium, and long-chain acylcarnitines and amino acids in OND and MuS subjects expressed as relative abundance \pm SEM. The compounds not listed below were not detectable in CSF.

Compound (abbreviation name)	MuS	OND	p-value
C0	4.75(\pm 1.00)	4.64(\pm 1.71)	0.85
C2	1.50(\pm 0.39)	1.52(\pm 0.78)	0.93
C3	0.08(\pm 0.02)	0.10(\pm 0.07)	0.26
C4	0.07(\pm 0.01)	0.08(\pm 0.03)	0.77
C5OH/C4DC [§]	0.08(\pm 0.01)	0.09(\pm 0.03)	0.27
C5DC/C6OH [§]	0.17(\pm 0.05)	0.17(\pm 0.06)	0.97
C6DC	0.09(\pm 0.03)	0.08(\pm 0.03)	0.55
PRO	61.33(\pm 13.90)	55.07(\pm 13.51)	0.27
VAL	52.06(\pm 6.61)	50.17(\pm 10.52)	0.59
LEU/ILE/PRO-OH [§]	42.87(\pm 8.24)	40.41(\pm 12.75)	0.57
ORN	25.07(\pm 6.33)	23.91(\pm 6.92)	0.67
MET	10.16(\pm 2.06)	11.28(\pm 3.97)	0.38
PHE	18.08(\pm 4.03)	16.97(\pm 4.35)	0.51
ARG	57.04(\pm 10.65)	54.27(\pm 12.36)	0.55
CIT	24.03(\pm 6.84)	24.25(\pm 6.69)	0.94
TYR	16.86(\pm 4.95)	17.61(\pm 5.33)	0.72
GLY	757.74(\pm 259.78)	687.75(\pm 243.43)	0.49
ALA bis	248.04(\pm 36.44)	259.50(\pm 64.61)	0.59
SER	8.29(\pm 1.94)	8.47(\pm 2.56)	0.84
THR	51.50(\pm 5.90)	47.97(\pm 6.16)	0.16
ASN	3.42(\pm 0.76)	3.13(\pm 0.89)	0.38
ASP	6.50(\pm 1.18)	5.82(\pm 1.09)	0.15
LYS/GLN [§]	5985.08(\pm 846.54)	5714.12(\pm 1296.35)	0.54
GLU	26.66(\pm 2.37)	23.58(\pm 4.49)	0.04*
HIS	57.18(\pm 9.77)	55.84(\pm 14.48)	0.79

* indicates statistical test with a p-value $<$ 0.05.

[§] isomeric compounds having the same m/z and measured as a single sum value.

Table 2: differential metabolites resulted from metabolomics analysis. Ten metabolites with a $p < 0.05$ of the independent statistical test in the discrimination of the two clinical groups analyzed.

Abbreviation	observed	calc m/z	molecular formula	Identification tools	p-value	Trend in MuS
^δ LPC(18:1(9Z)/0:0)	522.361	522.3554	C26H53NO7P	Lipid Maps; MS/MS	0.015	up
^δ unidentified	523.040	nd	nd		0.004	up
^δ unidentified	523.993	nd	nd		0.017	up
^δ LPC(18:0/0:0))	524.382	524.3711	C26H54NO7P	Lipid Maps; MS/MS	0.03	up
^δ LPI(16:0/0:0)	573.311	573.3035	C25H50O12P	Lipid Maps	0.009	up
^δ PA ^β	673.475	673.4803	C37H70O8P	Lipid Maps	0.034	down
^δ unidentified	727.021	nd	nd		0.022	down
^δ PC ^β	734.559	734.5695	C40H81NO8P	Lipid Maps; MS/MS	0.037	up
^δ PI ^β	969.651	969.6427	C53H94O13P	Lipid Maps	0.039	up
^α Glutamate	-	-	-	-	0.04	up

^δ Variables derived from lipidomics analysis, six of them were tentatively identified by database search and fragmentation analysis.

^αGlutamate was analyzed by LC-MS/MS.

^β Our data do not provide information about backbone structure of lipids, thus the ID is not univocal when the compound can have more combinations of instauration's positions and fatty acid substitutions. In these cases the ID returned from database was selected from a list of probable species with identical MW.

Lipid Maps: on-line database resource at <http://www.lipidmaps.org/tools> for lipid identification.

MS/MS: fragmentation analysis by tandem MS. Spectra were reported in Supporting Information **Figure S2, S3 and S4.**

Trend in MuS: Abundance of each analyte in MuS CSF compared to the abundance in OND CSF.

nd : not determined

Table 3: Diagnostic power of candidate biomarkers.

Biomarker	Cutoff (Relative Intensity)	*Specificity (%)	*Sensitivity (%)	AUC	p-value
LPC(18:1(9Z)/0:0)	> 2391	83,33	69,23	0,80	0,010
unidentified (m/z=523.040)	> 1007	83,33	76,92	0,87	0,001
unidentified (m/z=523.993)	> 404.8	91,67	53,85	0,79	0,012
LPC(18:0/0:0))	> 208.4	83,33	69,23	0,76	0,029
LPI(16:0/0:0)	> 199.7	83,33	61,54	0,81	0,007
PA	< 256.2	66,67	61,54	0,72	0,064
unidentified (m/z=727.021)	< 242.7	58,33	92,31	0,72	0,057
PC	> 1441	83,33	61,54	0,74	0,044
PI	> 135.4	75,00	69,23	0,73	0,050
Glutamate	> 25.72	75,00	53,85	0,71	0,081

* Sensitivity and specificity values were chosen (along with their 95% confidence interval) by selecting a possible cutoff between MuS and non-MuS. The cutoff were selected considering the better compromise of Sensitivity and Specificity with a major likelihood ratio returned by the software (Graphpad Prism). The likelihood ratio equals sensitivity/(1.0-specificity).

Figure captions

Figure 1: lipidomic results in CSF by MALDI-TOF-MS. The lipid species show m/z signals between 500 and 1000 m/z . a) Average MALDI-TOF mass spectrum of CSF obtained by 13 MuS patients (upper spectrum) and by 12 OND patients (bottom spectrum). b) Virtual gel showing the cases vs the m/z signals of each CSF sample analyzed. The horizontal line distinguishes the two clinical groups. This image was made by CinProTools software (Bruker Daltonics, Germany).

Figure 2: multivariate analysis of metabolomics data. Partial least squares discriminant analysis (PLS-DA) score plot ($t[1]/[t2] / [t3]$) of the first three components, from metabolomics data derived from OND subjects (black triangles) and MuS patients (red circles). The most significant variables that drive the separation between the two clinical groups are reported in Table 2.

Figure 3: distribution of each discriminant metabolite in two clinical groups studied. The compounds reported in panels a-g are significantly increased in MuS people. The signals at $m/z=673.4$ (h) and $m/z=727.0$ (i) resulted decreased in MuS patients vs OND subjects. The level of glutamate (l) in CSF of MuS patients resulted significantly elevated vs OND patients.

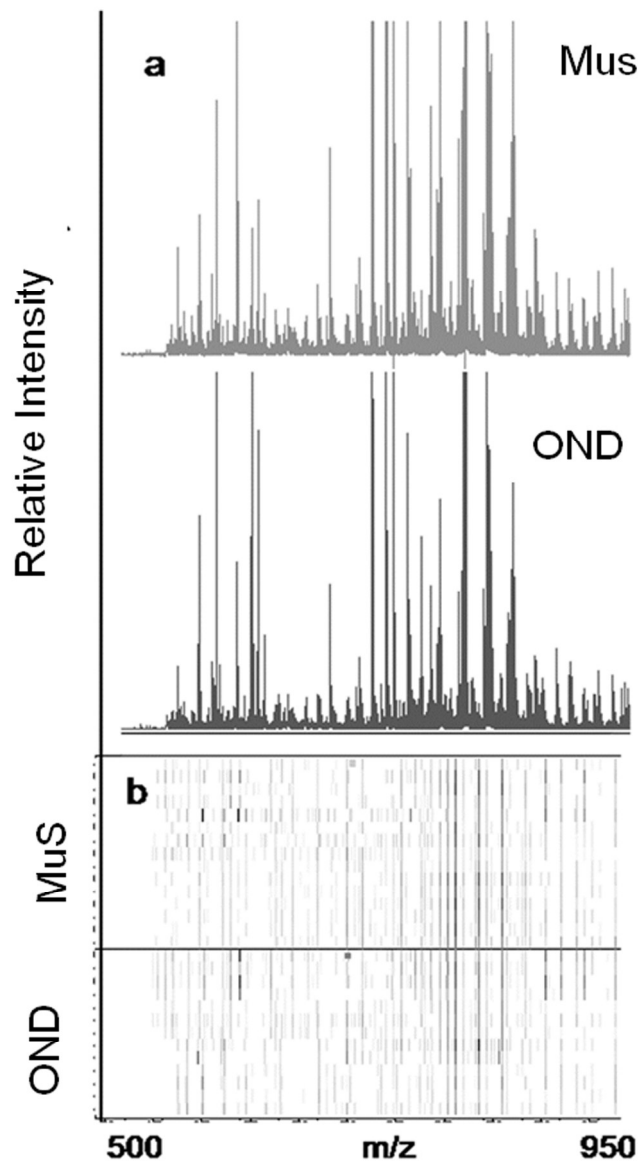
* indicates univariate test with a p -value < 0.05 .

Figure 4: correlation of Link Index, disease duration and EDSS with specific peak intensities in CSF. (a-c) Correlation of m/z at 522.3, 524.3 and 523.0 with Link Index. (d) Correlation of the signal at $m/z = 969.6$ with Disease Duration. (e) Correlation of the signal at $m/z = 573.3$ with EDSS values. Spearman correlation coefficients (r) are indicated in the figure.

References

1. H. Tremlett, Y. Zhao, P. Rieckmann and M. Hutchinson, *Neurology*, 2010, **74**, 2004-2015.
2. F. D. Lublin and S. C. Reingold, *Neurology*, 1996, **46**, 907-911.
3. R. Milo and E. Kahana, *Autoimmunity reviews*, 2010, **9**, A387-394.
4. M. Sospedra and R. Martin, *Annual review of immunology*, 2005, **23**, 683-747.
5. R. Hohlfeld and H. Wekerle, *Proceedings of the National Academy of Sciences of the United States of America*, 2004, **101 Suppl 2**, 14599-14606.
6. D. Wheeler, V. V. Bandaru, P. A. Calabresi, A. Nath and N. J. Haughey, *Brain : a journal of neurology*, 2008, **131**, 3092-3102.
7. F. J. Quintana, M. F. Farez, V. Viglietta, A. H. Iglesias, Y. Merbl, G. Izquierdo, M. Lucas, A. S. Basso, S. J. Khoury, C. F. Lucchinetti, I. R. Cohen and H. L. Weiner, *Proceedings of the National Academy of Sciences of the United States of America*, 2008, **105**, 18889-18894.
8. H. Gonzalo, L. Brieva, F. Tatzber, M. Jove, D. Cacabelos, A. Cassanye, L. Lanau-Angulo, J. Boada, J. C. Serrano, C. Gonzalez, L. Hernandez, S. Peralta, R. Pamplona and M. Portero-Otin, *Journal of neurochemistry*, 2012, **123**, 622-634.
9. J. A. Cohen and J. Chun, *Annals of neurology*, 2011, **69**, 759-777.
10. C. L. Jensen, *The American journal of clinical nutrition*, 2006, **83**, 1452S-1457S.
11. A. Tsui and M. A. Lee, *Current opinion in obstetrics & gynecology*, 2011, **23**, 435-439.
12. G. Famularo, C. De Simone, V. Trinchieri and L. Mosca, *Annals of the New York Academy of Sciences*, 2004, **1033**, 132-138.
13. C. Lebrun, H. Alchaar, M. Candito, V. Bourg and M. Chatel, *Multiple sclerosis*, 2006, **12**, 321-324.
14. V. Calabrese, G. Scapagnini, A. Ravagna, R. Bella, D. A. Butterfield, M. Calvani, G. Pennisi and A. M. Giuffrida Stella, *Neurochemical research*, 2003, **28**, 1321-1328.
15. A. M. Tejani, M. Wasdell, R. Spiwak, G. Rowell and S. Nathwani, *The Cochrane database of systematic reviews*, 2012, **5**, CD007280.
16. M. J. Noga, A. Dane, S. Shi, A. Attali, H. van Aken, E. Suidgeest, T. Tuinstra, B. Muilwijk, L. Coulier, T. Luider, T. H. Reijmers, R. J. Vreeken and T. Hankemeier, *Metabolomics : Official journal of the Metabolomic Society*, 2012, **8**, 253-263.
17. C. H. Polman, S. C. Reingold, B. Banwell, M. Clanet, J. A. Cohen, M. Filippi, K. Fujihara, E. Havrdova, M. Hutchinson, L. Kappos, F. D. Lublin, X. Montalban, P. O'Connor, M. Sandberg-Wollheim, A. J. Thompson, E. Waubant, B. Weinshenker and J. S. Wolinsky, *Annals of neurology*, 2011, **69**, 292-302.
18. E. G. Bligh and W. J. Dyer, *Canadian journal of biochemistry and physiology*, 1959, **37**, 911-917.
19. T. Fujiwaki, M. Tasaka, N. Takahashi, H. Kobayashi, Y. Murakami, T. Shimada and S. Yamaguchi, *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, 2006, **832**, 97-102.
20. V. Sirolli, C. Rossi, A. Di Castelnuovo, P. Felaco, L. Amoroso, M. Zucchelli, D. Ciavardelli, C. Di Ilio, P. Sacchetta, S. Bernardini, A. Arduini, M. Bonomini and A. Urbani, *Blood transfusion = Trasfusione del sangue*, 2012, **10 Suppl 2**, s78-88.
21. L. Di Liberato, A. Arduini, C. Rossi, A. Di Castelnuovo, C. Posari, P. Sacchetta, A. Urbani and M. Bonomini, *Journal of nephrology*, 2014.
22. S. Rizza, M. Copetti, C. Rossi, M. A. Cianfarani, M. Zucchelli, A. Luzi, C. Pecchioli, O. Porzio, G. Di Cola, A. Urbani, F. Pellegrini and M. Federici, *Atherosclerosis*, 2014, **232**, 260-264.
23. C. Lohmann, E. Schachmann, T. Dandekar, C. Villmann and C. M. Becker, *Journal of neurochemistry*, 2010, **114**, 1119-1134.
24. P. Del Boccio, D. Pieragostino, M. Di Ioia, F. Petrucci, A. Lugaesi, G. De Luca, D. Gambi, M. Onofri, C. Di Ilio, P. Sacchetta and A. Urbani, *Journal of proteomics*, 2011, **74**, 2826-2836.

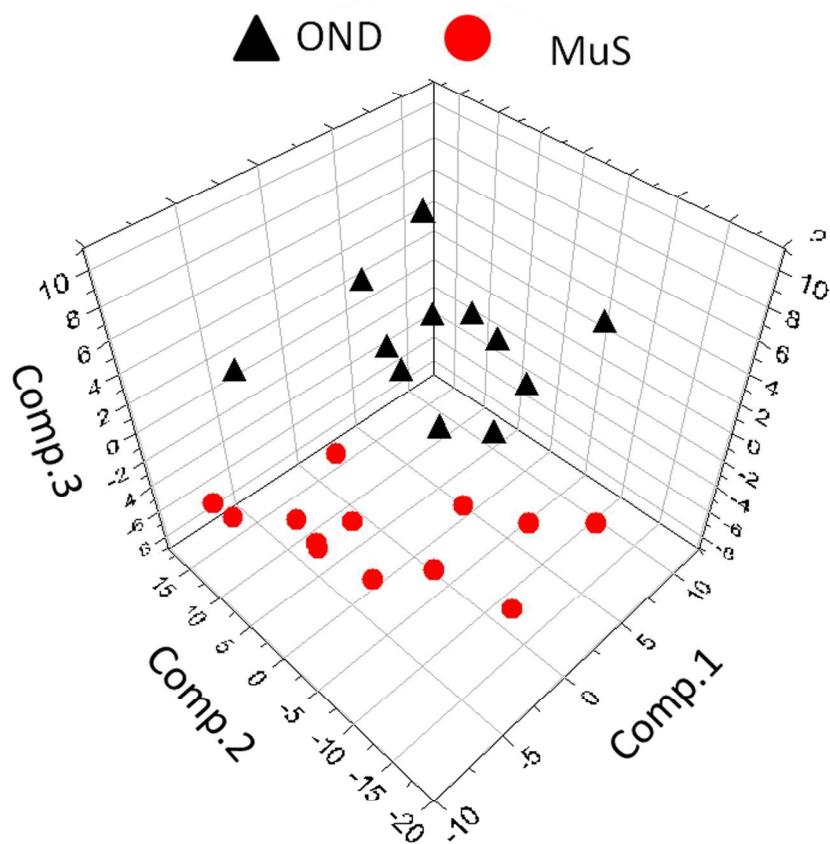
25. K. M. Brennan, F. Galban-Horcajo, S. Rinaldi, C. P. O'Leary, C. S. Goodyear, G. Kalna, A. Arthur, C. Elliot, S. Barnett, C. Linington, J. L. Bennett, G. P. Owens and H. J. Willison, *Journal of neuroimmunology*, 2011, **238**, 87-95.
26. S. Chalbot, H. Zetterberg, K. Blennow, T. Fladby, I. Grundke-Iqbal and K. Iqbal, *Clinical chemistry*, 2009, **55**, 2171-2179.
27. A. Walter, U. Korth, M. Hilgert, J. Hartmann, O. Weichel, M. Hilgert, K. Fassbender, A. Schmitt and J. Klein, *Neurobiology of aging*, 2004, **25**, 1299-1303.
28. P. Thakker, S. Marusic, N. L. Stedman, K. L. Lee, J. C. McKew, A. Wood, S. J. Goldman, M. W. Leach, M. Collins, V. K. Kuchroo, S. F. Wolf, J. D. Clark and M. Hassan-Zahraee, *Journal of immunology*, 2011, **187**, 1986-1997.
29. S. G. Payne, C. A. Oskeritzian, R. Griffiths, P. Subramanian, S. E. Barbour, C. E. Chalfant, S. Milstien and S. Spiegel, *Blood*, 2007, **109**, 1077-1085.
30. Y. Asaoka, M. Oka, K. Yoshida, Y. Sasaki and Y. Nishizuka, *Proceedings of the National Academy of Sciences of the United States of America*, 1992, **89**, 6447-6451.
31. J. H. Kabarowski, Y. Xu and O. N. Witte, *Biochemical pharmacology*, 2002, **64**, 161-167.
32. K. Lauber, E. Bohn, S. M. Krober, Y. J. Xiao, S. G. Blumenthal, R. K. Lindemann, P. Marini, C. Wiedig, A. Zobywalski, S. Baksh, Y. Xu, I. B. Autenrieth, K. Schulze-Osthoff, C. Belka, G. Stuhler and S. Wesselborg, *Cell*, 2003, **113**, 717-730.
33. T. Schilling, F. Lehmann, B. Ruckert and C. Eder, *The Journal of physiology*, 2004, **557**, 105-120.
34. J. M. Frischer, S. Bramow, A. Dal-Bianco, C. F. Lucchinetti, H. Rauschka, M. Schmidbauer, H. Laursen, P. S. Sorensen and H. Lassmann, *Brain : a journal of neurology*, 2009, **132**, 1175-1189.
35. S. Kallendrusch, S. Kremzow, M. Nowicki, U. Grabiec, R. Winkelmann, A. Benz, R. Kraft, I. Bechmann, F. Dehghani and M. Koch, *Glia*, 2013, **61**, 1822-1831.
36. S. Anavi-Goffer, G. Baillie, A. J. Irving, J. Gertsch, I. R. Greig, R. G. Pertwee and R. A. Ross, *The Journal of biological chemistry*, 2012, **287**, 91-104.
37. V. Gangadharan, D. Selvaraj, M. Kurejova, C. Njoo, S. Gritsch, D. Skoricova, H. Horstmann, S. Offermanns, A. J. Brown, T. Kuner, A. Tappe-Theodor and R. Kuner, *Pain*, 2013, **154**, 2801-2812.
38. C. Bolton and C. Paul, *Mediators of inflammation*, 2006, **2006**, 93684.
39. G. Mandolesi, A. Musella, A. Gentile, G. Grasselli, N. Haji, H. Sepman, D. Fresegna, S. Bullitta, F. De Vito, G. Musumeci, C. Di Sanza, P. Strata and D. Centonze, *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 2013, **33**, 12105-12121.
40. A. Tisell, O. D. Leinhard, J. B. Warntjes, A. Aalto, O. Smedby, A. M. Landtblom and P. Lundberg, *PloS one*, 2013, **8**, e61817.
41. J. Klein, *Journal of neural transmission*, 2000, **107**, 1027-1063.



lipidomic results in CSF by MALDI-TOF-MS. The lipid species show m/z signals between 500 and 1000 m/z .

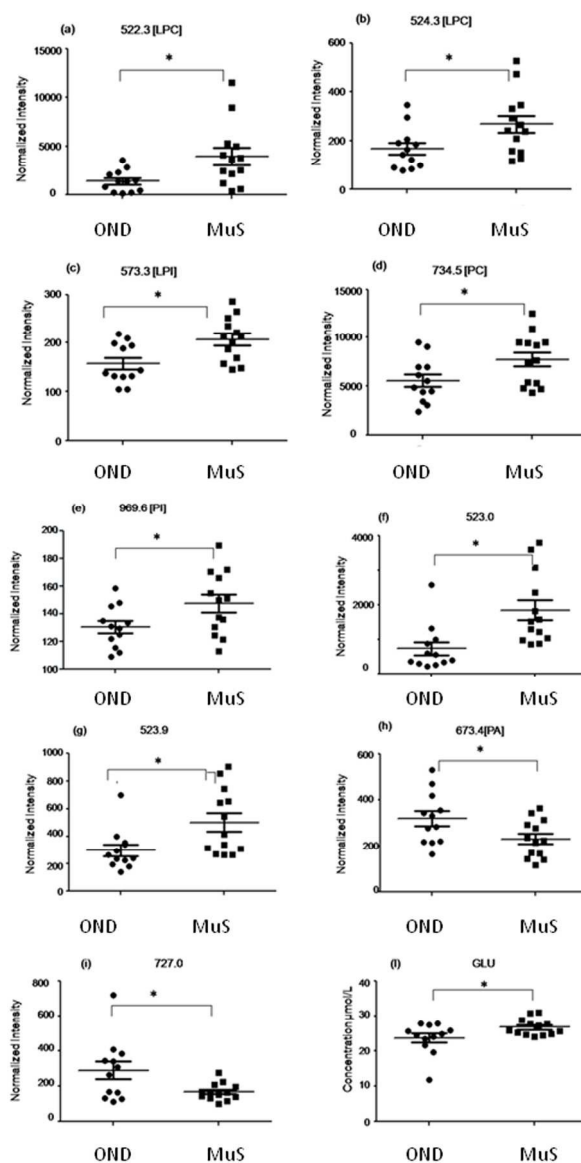
a) Average MALDI-TOF mass spectrum of CSF obtained by 13 MuS patients (upper spectrum) and by 12 OND patients (bottom spectrum). b) Virtual gel showing the cases vs the m/z signals of each CSF sample analyzed. The horizontal line distinguishes the two clinical groups. This image was made by CinProTools software (Bruker Daltonics, Germany).

139x234mm (600 x 600 DPI)



Partial least squares discriminant analysis (PLS-DA) score plot ($t[1]/[t2] / [t3]$) of the first three components, from metabolomics data derived from OND subjects (black triangles) and MuS patients (red circles). The most significant variables that drive the separation between the two clinical groups are reported in Table 2.

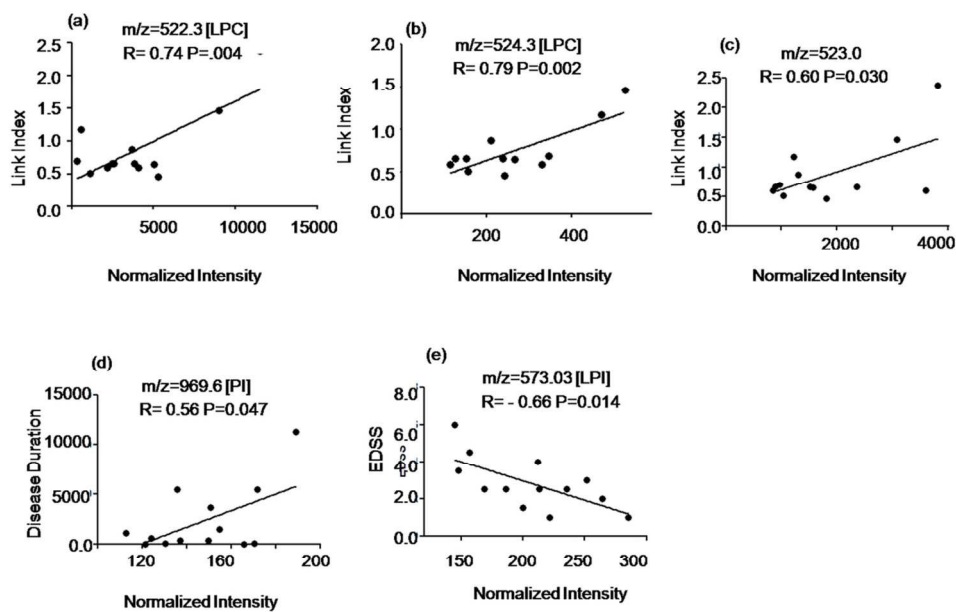
75x68mm (600 x 600 DPI)



distribution of each discriminant metabolite in two clinical groups studied. The compounds reported in panels a-g are significantly increased in MuS people. The signals at $m/z = 673.4$ (h) and $m/z = 727.0$ (i) resulted decreased in MuS patients vs OND subjects. The level of glutamate (j) in CSF of MuS patients resulted significantly elevated vs OND patients.

* indicates univariate test with a p-value < 0.05.

166x332mm (600 x 600 DPI)



correlation of Link Index, disease duration and EDSS with specific peak intensities in CSF. (a-c) Correlation of m/z at 522.3, 524.3 and 523.0 with Link Index. (d) Correlation of the signal at $m/z = 969.6$ with Disease Duration. (e) Correlation of the signal at $m/z = 573.3$ with EDSS values. Spearman correlation coefficients (r) are indicated in the figure.
54x35mm (600 x 600 DPI)