International Journal of Biochemistry and Cell Biology 93 (2017) 148–155

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Metabolomic analysis identifies altered metabolic pathways in Multiple Sclerosis

Simone Poddighe[a,b,*], Federica Murgia[a], Lorena Lorefice[c], Sonia Liggi[a,1], Eleonora Cocco[c], Maria Giovanna Marrosu[c], Luigi Atzori[a,*]

[a] Department of Biomedical Sciences, University of Cagliari, Italy
[b] Unité de Chimie Environnementale et Interactions sur le Vivant, Université du Littoral Côte d'Opale, France
[c] Department Medical Sciences and Public Health, University of Cagliari, Italy

ARTICLE INFO

Keywords:
Multiple sclerosis
Metabolomics
Biomarkers
Biomarker discovery
Metabolite profiling
Gas cromatography–mass spectrometry

ABSTRACT

Multiple sclerosis (MS) is a chronic, demyelinating disease that affects the central nervous system and is characterized by a complex pathogenesis and difficult management. The identification of new biomarkers would be clinically useful for more accurate diagnoses and disease monitoring. Metabolomics, the identification of small endogenous molecules, offers an instantaneous molecular snapshot of the MS phenotype. Here the metabolomic profiles (utilizing plasma from patients with MS) were characterized with a Gas cromatography-mass spectrometry-based platform followed by a multivariate statistical analysis and comparison with a healthy control (HC) population. The obtained partial least square discriminant analysis (PLS-DA) model identified and validated significant metabolic differences between individuals with MS and HC ($R2X = 0.223$, $R2Y = 0.82$, $Q2 = 0.562$; $p < 0.001$). Among discriminant metabolites phosphate, fructose, *myo*-inositol, pyroglutamate, threonate, L-leucine, L-asparagine, L-ornithine, L-glutamine, and L-glutamate were correctly identified, and some resulted as unknown. A receiver operating characteristic (ROC) curve with AUC 0.84 ($p = 0.01$; CI: 0.75–1) generated with the concentrations of the discriminant metabolites, supported the strength of the model. Pathway analysis indicated asparagine and citrulline biosynthesis as the main canonical pathways involved in MS. Changes in the citrulline biosynthesis pathway suggests the involvement of oxidative stress during neuronal damage. The results confirmed metabolomics as a useful approach to better understand the pathogenesis of MS and to provide new biomarkers for the disease to be used together with clinical data.

1. Introduction

Multiple sclerosis (MS) is a chronic, inflammatory disease of the central nervous system (CNS) characterized by demyelination and simultaneous axonal and neuronal degeneration that occurs from the earliest clinical stages of the disease (Lassmann et al., 2007). MS pathology and immunopathogenesis are extremely complex and are widely believed to be driven by heterogeneous and multifaceted mechanisms involving both adaptive and innate immune systems (Lassmann et al., 2007), oxidative damage (Fischer et al., 2013; Haider et al., 2011), and mitochondrial injury (Campbell et al., 2011; Mahad et al., 2008). The relevance of each factor varies in relation to the type and location of the lesions (Haider et al., 2016), disease course, and disease evolution (Mahad et al., 2015). The complexity of MS is also related to different clinical manifestations, radiologic features and drug responses, resulting in complex management of the disease (Bermel et al., 2013; Bielekova et al., 2005; Lublin et al., 2014).

The identification of biomarkers can be clinically useful for a more accurate diagnosis, prognosis, treatment choice and disease monitoring (Villoslada and Baranzini, 2012). In recent years, there have been advances in molecular biology, cellular immunology, and the new "omics" (genomics, transcriptomics, proteomics, and metabolomics), which focus on exploring the processes underlying disease pathogenesis to provide a list of possible MS biomarkers (Tumani et al., 2008; Villoslada, 2010).

In the plethora of omics, metabolomics concerns the identification and quantification of small endogenous molecules; i.e., the metabolites, in a biological system (Hollywood et al., 2006; Psychogios et al., 2011; Wishart et al., 2008). Because the metabolites represent the final product of the physiological processes in a living organism (Mangalam and Poisson, 2013; Nicholson and Lindon, 2008; Zhang et al., 2013), the profiling of the metabolome in tissues and biofluids offers an

* Corresponding authors at: Department of Biomedical Sciences, University of Cagliari, Italy.
E-mail addresses: s.poddighe@unica.it (S. Poddighe), latzori@unica.it (L. Atzori).
[1] Present address: Department of Biochemistry, University of Cambridge, UK.

http://dx.doi.org/10.1016/j.biocel.2017.07.004
Received 10 November 2016; Received in revised form 17 May 2017; Accepted 12 July 2017
Available online 16 July 2017

instantaneous molecular image of the phenotype (Cocco et al., 2016; Reinke et al., 2014a). Among the several analytical techniques available, nuclear magnetic resonance (NMR) and gas chromatography coupled with mass spectrometry are the most commonly used methods in the metabolomics field. In particular, these techniques have been used to investigate a number of pathologies and neurological disorders, including MS (Hatano et al., 2016; Kork et al., 2012; Mehrpour et al., 2013; Regenold et al., 2008; Reinke et al., 2014a; Sato et al., 2012). However, the metabolomic studies on MS published to date are more focused on cerebrospinal fluid (CSF) rather than blood serum/plasma. To cover this knowledge gap, we (Cocco et al., 2016) recently conducted a study using ^{1}H NMR-based metabolomics with blood plasma samples from patients with MS.

Aim of the study was to integrate previous results with NMR by using Gas chromatography coupled with mass spectrometry to better define the metabolomic profile and discriminate a group of MS patients from healthy controls (HC).

2. Materials and methods

2.1. Composition of the cohort and sample collection

The cohort consisted of 65 participants (n = 32 MS patients; n = 33 HC) from the original pool recruited by Cocco et al. (2016). The patients had a definite MS diagnosis according to the revised McDonald criteria (McDonald et al., 2001; Polman et al., 2011, 2005); were free from other significant comorbidities; and had been therapy-free for at least 90 days (i.e., disease modifying drugs and steroids) (Cocco et al., 2016). The HC matched the MS patients demographically and ethnically and were volunteers recruited from healthcare staff and relatives and friends of the patients. Fasting blood samples (10 mL) were withdrawn from each subject, and plasma was immediately separated via centrifugation (10,000 rpm). Samples were immediately stored at −80 °C until the analysis.

2.2. Standard protocol approvals, registrations, and patient consents

The study was approved by our institutional Ethics Committee, and written informed consent was obtained from each participant. Experimentation and methods were performed according to the approved guidelines. The study was conducted in accordance with the principles of Good Clinical Practice.

2.3. Sample preparation and metabolite extraction

Plasma samples were thawed and then centrifuged (4 °C, 10 min, 4500 rpm). Metabolites were extracted by adding 2400 μL of working solution (methanol/chloroform 1:1 plus 350 μL of H_2O) to 800 μL of each sample. After vortexing, samples were then centrifuged at room temperature (30 min, 4000 rpm) to obtain separation of the hydrophilic and hydrophobic phases. An aliquot of 700 μL of water-phase supernatant per sample was transferred in a clean Eppendorf tube and then concentrated overnight to dryness in a speed-vacuum. Blank extractions were also made to avoid chemical noise due to laboratory equipment and the chemicals used for derivatization. Blanks were processed following the same procedures used for the samples.

2.4. Derivatization of samples

Dried samples were derivatized in a two-step process of methoximation followed by silylation, according to a previous described protocol (Caboni et al., 2016) with small variations. First, a 100 μL of methoxyamine hydrochloride in pyridine solution (10 mg/mL) was added to the extract (17 h). Next, 100 μL of N-trimethylsilyltrifluoroacetamide (MSTFA) was added and vortexed (R.T., 1 h). The samples were then diluted in 600 μL hexane plus undecane (25 ppm), which was used as retention standard (R.S.) to evaluate retention time reproducibility. Diluted samples were then filtered (PTFE 0.45 μmø) and transferred to an auto-sampler glass vial.

2.5. Untargeted Gas cromatography–mass spectrometry analysis and acquisition of spectra

The derivatized extracts were placed into the autosampler of an Agilent 7890 A gas chromatograph coupled with an Agilent 5975C mass spectrometer in a random sequence in order to minimize experimental bias due to class membership. Blank samples were distributed at the beginning, end and during acquisition. The 1 μL aliquots of the samples were injected splitless by an autosampler onto a HP-5MS capillary column (5%-phenyl-methylpolysiloxane; 30 m, 0.25 mm i.d., 0.25 μm film thickness). The initial oven temperature was set at 50 °C (held 3 min) and increased at 10 °C/min to 250 °C for a total run of 35 min. Acquisitions were performed in electron impact mode and full scan monitoring mode (m/z 50–800). The injector and ion source temperatures were set at 200 °C and 250 °C, respectively. Helium was used as carrier gas in constant pressure mode (7.6522 psi).

2.6. Data processing

Obtained chromatograms of blanks and samples were exported in the netCDF format using MSD Chemstation E.02.02.1431 and processed using R version 3.2.1 (R Developement Core Team, 2015). In particular, the library XCMS (Smith et al., 2006) version 1.44.0 was used for peak extraction, grouping and retention time correction. The CentWave algorithm (Tautenhahn et al., 2008) was used to perform peak density and wavelet based feature detection with a chromatographic peak width between 5 s and 10 s, a maximal tolerated m/z deviation in consecutive scans of 0.1 in ppm, a signal-to-noise cutoff of 1, and a minimum difference in m/z for peaks with overlapping retention times of 0.01. Moreover, mass traces were retained if they contained at least 5 peaks with intensities higher than 100. The retention time correction across samples was performed with a centre-star strategy using the Obiwarp method (Prince and Marcotte, 2006) with a profstep (step size to be used for the generation of profiles from the raw data) of 0.1 ppm. Peaks belonging to different samples were grouped together according to overlapping m/z bins and similar retention times using the density method (Smith et al., 2006). The grouping step was performed using a width of overlapping m/z bins of 0.25 and a bandwidth of the Gaussian smoothing kernel of 3. Only peaks belonging to at least 20% of the samples in either the pathologic or control groups were retained for further analyses. This grouping passage will always yield some groups that do not contain peaks from all samples. Hence, the method “chrom” of the function fillpeaks was used to integrate row data into the groups for the missing samples. Successively, the library CAMERA (Kuhl et al., 2012) version 1.24.1 was utilized to annotate isotopes and cluster peaks into pseudospectra (i.e., metabolites) according to the extracted ion chromatogram (EIC) correlation between peaks inside a sample with a threshold of 0.75. The matrix containing all samples, the features found across samples and their grouping into pseudospectra were processed with in-house python scripts to: 1) eliminate signals present in the blanks representing the R.S.as well as contamination and 2) normalise the data using total area normalisation in order to determine the relative concentration for each peak in a sample based on the total area of the peaks found in the sample. These relative concentrations, expressed as a ratio of the peak area to the total sample area (peak area/total sample area), were used for all the subsequent analyses. Trimethylsilylated metabolite identities were checked using the NIST library, and the obtained spectra were matched with those available on the Human Database Metabolome (HMDB) or via injection of derivatized pure standards using the same acquisition parameters as described above. Details of the identification of discriminant metabolites are reported in Table S1.