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Research Article

Metabolomic profiling of serum in the progression of Alzheimer’s disease by capillary electrophoresis–mass spectrometry

There is high interest in the discovery of early diagnostic biomarkers of Alzheimer’s disease, for which metabolomics exhibits a great potential. In this work, a metabolomic approach based on ultrafiltration and analysis by CE-MS has been used to obtain representative fingerprints of polar metabolites from serum samples in order to distinguish between patients with Alzheimer’s disease, mild cognitive impairment, and healthy controls. By the use of partial least squares discriminant analysis it was possible to classify patients according to the disease stage and then identify potential markers. Significant increase was observed with progression of disease in levels of choline, creatinine, asymmetric dimethyl-arginine, homocysteine-cysteine disulfide, phenylalanyl-phenylalanine, and different medium chain acylcarnitines. On the other hand, asparagine, methionine, histidine, carnitine, acetyl-spermidine, and C5-carnitine were reduced in these serum samples. In this way, multiple essential pathways were found implicated in the underlying pathology, such as oxidative stress or defects in energy metabolism. However, the most interesting results are related to the association of several vascular risk factors with Alzheimer’s disease.

Keywords: Alzheimer’s disease / CE-MS / Metabolomics / Mild cognitive impairment

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1 Introduction

The increasing incidence of Alzheimer’s disease (AD), the most common neurodegenerative disorder worldwide characterized by a progressive decline of cognitive functions (or dementia), makes the search of new biomarkers imperative. Nowadays, diagnosis of AD relies on a combination of neuropsychological tests and the exclusion of other neurological, psychiatric, or systemic diseases by means of physical, neurological, and laboratory examinations, usually according to the clinical criteria of the National Institute of Neurological and Communicative Disorders and the Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) [1]. However, this clinical diagnosis only detects AD at advanced stages of disease, too late to carry out pharmacological interventions. Furthermore, AD can only be definitively diagnosed after postmortem histopathological verification, which confirms 10–15% of misleading diagnoses, and with low specificity against other dementias [2]. For this reason, discovery of biomarkers is needed for early and accurate diagnosis as well as for helping in new drug development [3]. In addition, they should be useful for monitoring disease progression from mild cognitive impairment (MCI), a syndrome characterized by memory impairments that precedes the clinical definition of dementia in severity and shares many features with early AD [4]. Thereby, current data suggest that conversion from MCI to AD occurs at a rate of 10–15% per year, with ~80% conversion by the sixth year of follow-up [5]. Moreover, AD like any other chronic condition may have other

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medical and psychiatric comorbidities that need to be known and addressed [6]. Therefore, a successful investigation of AD pathogenesis requires a careful selection of patient's cohort in order to homogenize dispersing factors such as comorbidities or medication. Current strategies for the search of biomarkers in AD include neuroimaging techniques, such as structural magnetic resonance imaging for measurement of brain atrophy, magnetic resonance spectroscopy to quantify metabolic abnormalities in brain, fluorodeoxyglucose positron emission tomography measurements of decline in cerebral metabolic rate for glucose, and PET measurements of amyloid-β burden [7]. However, the cost and time associated with imaging approaches make clinical biomarkers in biological fluids an attractive alternative. Thus, several classic markers can be related to AD, such as cerebrospinal fluid levels of Aβ peptides and tau protein [8], increased production of F2-isoprostanes due to oxidative stress [9] or inflammatory markers [10], among others. Nevertheless, to date, none of these biomarkers have been established with the necessary reliability and specificity. Discovery of reliable biomarkers for early diagnosis requires sophisticated analytical approaches in order to delve into the high complexity of AD. In this sense, metabolomics has particular relevance for this purpose because metabolites directly reflect interactions between genes, proteins, and the environment, so they are the biological level most closely associated with phenotypic changes [11]. A great challenge in metabolomics is the need of multiple complementary analytical platforms to get global metabolome coverage due to its physicochemical complexity, heterogeneity, and dynamic. Thus, numerous metabolomic studies have been performed in the last years for the investigation of AD [12], mainly dominated by nuclear magnetic resonance and MS, the last one normally coupled to gas or LC [13]. In AD research, cerebrospinal fluid (CSF) is usually the biofluid of main interest due to its physicochemical complexity, heterogeneity, and dynamic. However, the use of non-invasive samples such as blood serum or plasma has a great interest in order to get easier and cheaper methods for diagnosis, without any risk for the patient. Thereby, UHPLC/MS has been employed for metabolic profiling of plasma samples to map potential biomarkers of AD and monitoring its progression [23–25]. In another study, GC-MS and LC-MS were used for the profiling of small polar metabolites and lipids, respectively, to assess AD progression in a prospective survey involving three diagnostic groups, healthy controls, MCI, and AD [26]. Tukiainen et al. studied serum samples from MCI patients by a three-molecular-window approach for 1H NMR spectroscopy, demonstrating the coexistence of inflammation and metabolic syndrome with this disorder [27]. Moreover, lipidomics is also emerging in AD research, given the great importance of lipids in pathogenesis of AD [28, 29]. Finally, it is also noteworthy the great number of targeted approaches focused on the study of different classes of metabolites such as amino acids [30], phospholipids [31, 32] or steroids [33]. However, besides the common analytical platforms mentioned above, CE-MS can provide orthogonal and complementary metabolomic information because it is suitable for separation of highly polar and ionic compounds, usually not resolved by GC or LC [34, 35]. In addition, some attractive features of CE are its small sample requirement with minimum treatment, and high resolution obtained. However, its lower robustness compared with chromatographic methods has traditionally relegated to the background the use of CE-MS in metabolomics, despite its considerable potential for a complementary characterization of metabolite profiles. In this way, to our knowledge, this approach has only been considered only once in the study of AD progression, for metabolomic profiling of CSF samples to examine metabolic differences among subjects with different cognitive status [36]. Alternatively, Tsuruoka et al. conducted a metabolomic analysis of serum and saliva from different neurodegenerative dementias, including AD, but the study suffered from several limitations to be addressed such as low number of samples (n = 10, with only three cases of AD), which were uneven in age and gender distribution [37].

The present study shows the application of a metabolomic approach based on CE-ESI-TOF-MS for the search of potential biomarkers of AD progression in serum. Multivariate statistics demonstrated the validity of serum fingerprints obtained for the discrimination between healthy controls, mild cognitive impairment, and Alzheimer patients. Finally, several discriminant compounds were identified by performing two-class comparisons, which could be related to early or advanced neurodegenerative processes (MCI versus HC and AD versus HC, respectively) and progression of the disease (AD versus MCI). These potential biomarkers could help to understand the biochemical processes underlying pathology associated with AD.

2 Materials and methods

2.1 Chemicals

Methanol, acetonitrile (HPLC-grade), and formic acid (MS-grade) were purchased from Sigma-Aldrich (Steinheim, Germany), and water was purified with a Milli-Q Plus 185 system (Millipore, Bedford, USA). Standards of metabolites for identification of potential biomarkers were obtained from Sigma (choline chloride, creatinine, creatine, carnitine chloride, octanoyl-carnitine chloride, decanoyl-carnitine chloride, asparagine, methionine, histidine, cysteine, homocysteine, N-methyl-lysine, N-acetyl-ornithine, N\textsuperscript{O},N\textsuperscript{N}'-dimethylarginine, cystathionine, carnosine, phenylalanine-phenylalanine, glycidylglutamate). Methionine sulphone (internal standard, IS) was
from Sigma, while purine and HP921 (standard reference solutions) were obtained from Agilent Technologies (Palo Alto, CA, USA). Finally, homocysteine-cysteine disulfide (Hcy-Cys) was prepared in-house by incubating equimolar solutions of reduced homocysteine and cysteine at room temperature over 24 h without further purification, as described elsewhere [38].

2.2 Blood serum samples

In the present study, a total number of 93 subjects were recruited by the Neurological Service of Hospital Juan Ramón Jiménez (Huelva, Spain), including healthy controls (HC), MCI, and AD patients. Blood samples were obtained by venipuncture of the antecubital region after 8 h of fasting and collected in BD Vacutainer SST II tubes with gel separator and advance vacuum system, previously cooled in refrigerator. Samples were immediately cooled and protected from light for 30 min to allow clot retraction, and then centrifuged at 3500 rpm for 10 min. Serum was aliquoted and frozen at −80°C until analysis. Alzheimer’s disease patients (n = 42, 18 male and 24 female, medium age 79.9 ± 5.7 years) were newly diagnosed of sporadic AD according to the criteria of the NINCDS-ADRDA [1], and only subjects that had not yet received any type of medication were included in the study. Mild cognitive impairment patients (n = 14, 7 male and 7 female, medium age 77.1 ± 5.4 years) reported cognitive decline and impairment on objective cognitive tasks, but they were not demented and did not meet the NINCDS-ADRDA requirements for a possible or probable diagnosis of Alzheimer [39]. Finally, matched healthy controls in sex and age (n = 37, 14 male and 23 female, medium age 72.2 ± 5.3 years) were enrolled, after examination by neurologists to confirm the absence of neurological disorders, whom had not more than two reported cases of AD in their families. Demographic characteristics of groups considered in the study are listed in Supporting Information, including age, gender, comorbidities, medication, and family history of AD. It is noteworthy that most subjects suffered other comorbidities and were under different medical treatments, but there were not significant differences among the three groups considered (i.e. AD, MCI, and HC). In addition, although individual values are not recorded, there were no significant differences in common biochemical parameters among these groups. The study was performed in accordance with the principles contained in the Declaration of Helsinki and approved by the Ethical Committee of University of Huelva.

2.3 Sample preparation and analysis

Metabolomic profiling of serum samples was performed following a modification of procedure described by Naz et al., based on deproteinization of serum by ultrafiltration (30 kDa cutoff) and analysis by CE-TOF-MS [40]. For extraction of metabolites, 100 L of serum were mixed with 100 µL of extractant, containing 0.4 ppm methionine sulfone (IS), 0.2 M formic acid and 5% acetonitrile. After brief stirring, sample was transferred to Centrifree® ultrafiltration device (Merck Millipore, Ireland) and centrifugated at 2000 rpm for 70 min at 4°C. Finally, filtrate was collected for metabolomic analysis. Furthermore, quality control (QC) samples were prepared by pooling equal volumes of each sample, which allows monitoring instrumental drifts along the analysis period [41]. Serum profiling was carried out by CE (Agilent 7100) coupled to a TOF-MS system equipped with electrospray source (Agilent 6224). Coupling was performed via sheath–liquid interface with a CE-ESI sprayer (Agilent G1607) and a HPLC pump (Agilent 1200) to deliver sheath liquid. Separations occur in a 100 cm long fused-silica capillary with 50 µm of internal diameter (Agilent Technologies), using a solution of 0.8 M formic acid in 10% methanol as BGE. Before the first use, new capillaries were conditioned with 1 M sodium hydroxide, 2 M ammonium hydroxide, and BGE. For analysis, capillary is precleaned by flushing BGE for 5 min and then, samples are hydrodynamically injected at 50 mbar for 50 s, followed by injection of BGE for 20 s at 100 mbar. Finally, separation is performed at 25 mbar of pressure with a voltage of 30 kV during 35 min. MS operated in positive polarity acquiring full-scan spectra in the m/z range 85–1000. The capillary voltage was set to 3500 V, with 100 V of fragmentor voltage and 65 V of skimmer voltage. Nitrogen was used as drying and nebulizer gas, whose temperature was fixed at 200°C. Drying gas was supplied at 10 L/min, while nebulizer gas pressure was programmed to prevent drops in current [42]. Thus, nebulizer gas was switch off during the first minute after injection and then, a pressure of 10 psi was applied. The sheath liquid was methanol/water (1:1, v/v) containing 1 mM formic acid and reference standards for mass accuracy monitoring (0.5 nM purine and 0.75 nM HP921), delivered at a flow rate of 0.6 mL/min with a 1:100 split.

2.4 Data processing

The raw data were preprocessed by the molecular feature extraction tool in MassHunter Qualitative Analysis Software (Agilent Technologies) for cleaning background noise and unrelated ions. The molecular feature extraction algorithm uses the accuracy of the measurements for grouping-related ions by charge state envelope, isotopic distribution, and/or the presence of adducts and dimmers, and then creates a list of all possible components (or features) described by mass, retention time and abundance [43]. Thus, processing was performed by applying an abundance cutoff of 200 counts and enabling the search of different ion species (M+H+, M+Na+, M+NH4+, M-H2O). In addition, for isotope grouping, the peak spacing tolerance was set to 0.0025 m/z, and the charge states were limited to 2. Then, alignment, normalization, and filtering were conducted using the Mass Profiler Professional software (Agilent Technologies). Firstly, data is filtered by selecting features into the range 1.0–25.0 m/z, and then, peaks
are aligned applying a retention time window of 2 min and a mass window of 20 ppm. Finally, data are normalized according to the intensity of IS.

2.5 Data analysis

First of all, data were filtered in Mass Profiler Professional to remove nonreproducible signals before to perform statistical analysis. Thus, features were filtered by choosing masses present in at least 75% of samples in one of the compared groups, and then, features were again filtered on sample variability, selecting only variables with a coefficient of variation less than 50% within each group. Then, the data matrix containing these filtered features was processed by partial least squares discriminant analysis (PLS-DA) in SIMCA-P\textsuperscript{TM} software (version 11.5, Umetrics, Umeå, Sweden), in order to find differences between the groups of study. For this, data was submitted to Pareto scaling, for reducing the relative importance of larger values, and logarithmic transformation, in order to approximate a normal distribution [44]. In addition, quality of the model was assessed by the $R^2$ and $Q^2$ values, supplied by the software, which provide information about the class separation and predictive power of the model, respectively. These parameters are ranged between 0 and 1, and they indicate the variance explained by the model for all the data analyzed ($R^2$) and this variance in a test set by cross-validation ($Q^2$). Finally, potential biomarkers of disease and its progression were found by two-class comparisons, AD versus HC, MCI versus HC (markers of advanced and early disease, respectively), and AD versus MCI (markers of disease progression). For this purpose, univariate statistical analyses (t-test, $p \leq 0.05$) were performed with Bonferroni correction for multiple testing, and loadings plots from PLS-DA were inspected to select altered metabolites according to the Variable Importance in the Projection, or VIP (a weighted sum of squares of the PLS weight, which indicates the importance of the variable in the model), considering only variables with VIP values higher than 2, indicative of significant differences among groups.

2.6 Metabolites identification

Potential biomarkers were putatively identified by matching the experimental accurate mass with those available in metabolomic databases (HMDB, METLIN, KEGG, LIPID, MAPS), using a mass accuracy of 20 ppm. Then, when available, standards were injected to confirm the identification according to experimental migration time.

3 Results and discussion

3.1 Serum metabolomic profiling by CE-MS

A metabolomic approach based on ultrafiltration and analysis by CE-ESI-TOF-MS with sheath-liquid interface has been used to obtain representative fingerprints of polar metabolites from serum samples. In a previous study, it was demonstrated the suitability of ultrafiltration for serum treatment before CE-MS analysis, comparing with other methods such as precipitation of proteins with organic solvents or SPE [40]. The extractant solution was optimized to recover as many metabolites as possible from serum samples. The best sensitivity was obtained by adding 5% acetonitrile, which allowed the liberation of metabolites absorbed in proteins, and 0.2 M formic acid, which facilitated the separation of metabolites in normal mode CE and the detection by ESI-MS in positive mode. Furthermore, methionine sulfone was used as IS to correct instrumental drift in sensitivity along the sequence analysis by data normalization and to evaluate the variability in migration times (less than 2 min in this study, corrected by data alignment). Thereby, further analysis by a rapid and simple method based on CE-MS provided good sensitivity and reproducibility for global serum metabolic profiling. However, the use of programmed nebulizing gas pressure in this work also allowed increasing separation efficiency of this methodology. The programmed nebulizing gas pressure strategy is based on switching off the nebulizer gas during the injection period, in order to avoid the entrance of air bubbles due to the suction afforded by the nebulizing pressure when the tip of CE capillary is being exchanged between sample and BGE vials [42]. This finally results in increased current stability, which provides higher reproducibility as well as resolution, given that drops in current generate a loss of separation efficiency due to the induction of a parabolic profile. Furthermore, this gain in resolution allowed introducing larger volumes of sample respect to the method proposed by Naz et al. (from 35 s of hydrodynamical injection to 50 s), maintaining a similar separation between peaks but increasing sensitivity. A typical serum metabolomic profile obtained with this approach is shown in Fig. 1. Although a seemingly simple electropherogram is obtained with a low number of peaks, the deconvolution and subsequent data processing showed the presence of 535 different metabolic features, demonstrating the potential of CE-MS for high-throughput
metabolomic analysis of polar metabolites from serum samples.

3.2 Classification models

The metabolomic approach previously described was applied to serum samples from AD and mild cognitive impairment patients, as well as healthy controls, to evaluate the capability of CE-MS for discriminating between these groups. For this purpose, after data preprocessing (deconvolution, alignment, normalization and filtering), the resulting data matrix containing 535 time-aligned metabolic features was subjected to PLS-DA in order to perform samples classification. Furthermore, logarithmic transformation and Pareto scaling were used to extract relevant biological information from these large data sets, reducing the technical variability between individual samples [44]. First, a PLS-DA model was obtained comparing the three study groups (AD, MCI, HC) in order to visualize groupings and trends. As can be observed in Fig. 2, samples were clearly clustered according diagnosis. Furthermore, in order to assess the reliability of the methodology in terms of stability and repeatability, quality control samples analyzed throughout the sequence run were predicted in the model. Thereby, quality controls were closely clustered in the center of the plot (as expected, since QCs are prepared by pooling equal volumes of all samples), showing the low analytical variability between different runs (Fig. 2). Finally, two-class comparisons were performed to simplify the search of potential markers according the stage of disease (AD versus HC, MCI versus HC, AD versus MCI), as previously reported in other metabolomic investigations considering AD and MCI [18, 25, 45]. Groups could be totally discriminated, as inferred from scores plots and $R^2$ values, close to 1 in the three models (Fig. 3). Furthermore, model validation yielded acceptable $Q^2$ measures around 0.3 using an iterative n-fold strategy.

3.3 Selection of biomarkers

Statistically significant metabolites were selected by inspecting loadings plots from PLS-DA models (two-class comparisons) as well as by univariate statistics ($t$-test, $p \leq 0.05$). Only those variables that showed a RSD in QC samples lower than 25% were considered as significant, in order to remove metabolic features with a high analytical variability that might mask the biological variability. Then, standards were used to confirm the identity of some of these classifiers, while other compounds could be only tentatively allocated due to the unavailability of commercial standards. For this purpose, migration times of the discriminant metabolites in serum samples, standards, and standard spiked serum samples were compared under identical instrumental conditions for a final confirmation. In addition, this procedure allowed to discard some metabolites wrongly assigned based on their molecular mass, such as N-methyl-lysine ($M = 160.1204$), N-acetyl-ornithine ($M = 174.1007$), glycyl-glutamate ($M = 204.0755$), and cystathionine ($M = 222.0589$), whose retention times in samples did not match with that of standards. In Table 1 are listed the potential biomarkers identified, their migration times (MT) with the corresponding RSD before alignment, the experimental accurate masses, the mass error, the percentage of change for each comparison, the coefficient of signal variation observed in QC samples, and $p$ value. Furthermore, bar plots and the 95% confidence interval for these putative biomarkers are presented in Fig. 4. As can be observed, major differences were found between AD patients and healthy controls, showing the potential of this metabolomic approach to map potential markers of AD. Alternatively, the comparison MCI versus HC allowed selecting those classifiers that suffer changes in the onset of disease, so they could be considered
Table 1. Biomarkers found by CE-MS analysis

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mass (Da)</th>
<th>∆m (ppm)</th>
<th>MT (min)</th>
<th>RSD in MT</th>
<th>Change (%)</th>
<th>RSD in QC</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AD versus HC</td>
<td>MCI versus HC</td>
<td>AD versus MCI</td>
</tr>
<tr>
<td>Choline</td>
<td>103.0999</td>
<td>1.92</td>
<td>12.61</td>
<td>2.3%</td>
<td>+19.5</td>
<td>+51.9</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine</td>
<td>113.0591</td>
<td>1.77</td>
<td>13.34</td>
<td>2.6%</td>
<td>+15.0</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Creatine</td>
<td>131.0695</td>
<td>0</td>
<td>16.18</td>
<td>3.0%</td>
<td>NS</td>
<td>NS</td>
<td>−40.4</td>
</tr>
<tr>
<td>Asparagine</td>
<td>132.0529</td>
<td>−4.54</td>
<td>19.11</td>
<td>3.6%</td>
<td>−10.0</td>
<td>−13.1</td>
<td>NS</td>
</tr>
<tr>
<td>Proline betaine</td>
<td>143.0845</td>
<td>−0.70</td>
<td>22.60</td>
<td>4.1%</td>
<td>+23.3</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Methionine</td>
<td>149.0510</td>
<td>0</td>
<td>19.52</td>
<td>3.6%</td>
<td>−14.7</td>
<td>−16.2</td>
<td>NS</td>
</tr>
<tr>
<td>Histidine</td>
<td>155.0586</td>
<td>0.64</td>
<td>13.33</td>
<td>2.8%</td>
<td>−12.1</td>
<td>−17.8</td>
<td>NS</td>
</tr>
<tr>
<td>Carnitine</td>
<td>161.1555</td>
<td>1.86</td>
<td>15.65</td>
<td>2.7%</td>
<td>−39.4</td>
<td>−50.5</td>
<td>NS</td>
</tr>
<tr>
<td>Gly Val</td>
<td>174.1007</td>
<td>1.72</td>
<td>21.81</td>
<td>5.2%</td>
<td>−42.0</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>N-acetyl-spermidine</td>
<td>187.1678</td>
<td>−3.80</td>
<td>11.73</td>
<td>2.2%</td>
<td>−13.3</td>
<td>−21.2</td>
<td>NS</td>
</tr>
<tr>
<td>Asymmetric dimethyl-Arginine</td>
<td>202.1419</td>
<td>−5.44</td>
<td>14.47</td>
<td>2.9%</td>
<td>+16.4</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Leu Pro</td>
<td>228.1471</td>
<td>−1.31</td>
<td>17.71</td>
<td>2.1%</td>
<td>+22.0</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>C5-Carnitine</td>
<td>245.1628</td>
<td>0.41</td>
<td>17.62</td>
<td>2.8%</td>
<td>−24.9</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Hcy-Cys</td>
<td>254.0392</td>
<td>−1.18</td>
<td>19.52</td>
<td>3.6%</td>
<td>+45.8</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Glu Gin</td>
<td>275.1112</td>
<td>−1.82</td>
<td>23.40</td>
<td>4.2%</td>
<td>+27.2</td>
<td>+37.6</td>
<td>NS</td>
</tr>
<tr>
<td>C8-carnitine</td>
<td>287.2087</td>
<td>−3.48</td>
<td>18.61</td>
<td>3.2%</td>
<td>+38.9</td>
<td>+40.2</td>
<td>NS</td>
</tr>
<tr>
<td>Phe Phe</td>
<td>312.1428</td>
<td>2.56</td>
<td>20.37</td>
<td>3.7%</td>
<td>+26.5</td>
<td>+56.5</td>
<td>NS</td>
</tr>
<tr>
<td>C10:1-carnitine</td>
<td>313.2233</td>
<td>−6.39</td>
<td>19.03</td>
<td>1.9%</td>
<td>+34.4</td>
<td>NS</td>
<td>+34.6</td>
</tr>
<tr>
<td>C10:0-carnitine</td>
<td>315.2399</td>
<td>−3.17</td>
<td>19.19</td>
<td>3.3%</td>
<td>+18.5</td>
<td>+15.3</td>
<td>NS</td>
</tr>
<tr>
<td>Tripeptide</td>
<td>361.1581</td>
<td>−4.71</td>
<td>13.34</td>
<td>3.6%</td>
<td>+15.1</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Methionine Sulfone (IS)</td>
<td>181.0412</td>
<td>3.31</td>
<td>23.16</td>
<td>4.0%</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

a) Biomarker putatively identified, nonconfirmed with standard.
NS, nonsignificant change.

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3.4 Biological meaning

Neurodegenerative failures associated with AD progression are related to multiple cellular abnormalities, as revealed metabolomic profiling of serum samples by CE-MS. These metabolic changes could be proposed as potential biomarkers for diagnosis of AD, but also allow establishing the perturbed as precursors in the early development of neurodegenerative failures. Finally, profiles from AD and MCI patients were very similar, and only several metabolites were significantly altered between these two stages of disease. However, these metabolites have a great relevance for the study of disease progression, since these metabolic changes could be related to the decline of cognitive functions.
biological processes underlying the pathology. Thereby, pathway analysis of metabolites shown in Table 1 demonstrates the multifunctional character of this disorder, as described below. Oxidative stress is considered a primary factor in the pathogenesis of AD, which initiates in MCI brain and contributes significantly to the progression of AD [46]. Thus, endogenous antioxidant levels have been described as important markers of AD [47]. In our study, this pathological status was confirmed by the decrease of histidine levels in AD patients (Table 1), an amino acid with antioxidant properties as other imidazole containing compounds such as carnosine or anserine, whose reduction has been previously related to AD [48]. On the other hand, changes observed in different metabolites such as creatine, creatinine, asparagine, carnitine, and acyl-carnitines point to impaired mitochondrial function [49]. Creatine plays a fundamental role in energy buffering and overall cellular bioenergetics by means of the creatine kinase/phosphocreatine system, being responsible for the transfer of energy from mitochondria to cytosol [50]. As can be seen in Table 1, creatine was lower in serum from AD patients respect to MCI ones, in agreement with previous findings in CSF [36] and hippocampus [51]. In addition, increased serum levels of creatinine (Table 1), the nonenzymatic degradation product of creatine and phosphocreatine, also support the hypothesis of abnormal metabolism of creatine in the development of AD. Carnitine is involved in several physiological functions such as transport of fatty acids into mitochondria for β oxidation, oxidation of pyruvate, and other processes, so that a change in endogenous levels may have important consequences in energy metabolism. In this sense, free carnitine was lower in serum from AD and MCI subjects (Table 1), as previously reported in CSF [36] and plasma samples [52], but in addition, important changes were also observed in some short and medium chain acyl-carnitines, whose association with AD has not been described to date. On the one hand, elevated octanoyl-, decenoyl-, and decanoyl-carnitines are characteristic of medium chain acyl-CoA dehydrogenase deficiency, an inherited disorder of fatty acid oxidation that impairs the breakdown of medium-chain fatty acids into acetyl-CoA [53]. Therefore, reduced metabolism of medium chain fatty acids could be involved in neurodegenerative hypometabolism as a supplementary pathway to those already described of decreased rate of carbohydrate catabolism or impaired tricarboxylic acid cycle [54]. On the other hand, the decrease observed in CS-carnitine (Table 1) could be related to increased activity of short chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD), which is involved in AD pathogenesis through modulation of intraneuronal steroid pools in brain, which causes a local estrogen deficiency [55]. Finally, reduced serum asparagine in both AD and MCI patients might indicate an altered biosynthesis from oxaloacetate in the early development of disease, probably due to impaired tricarboxylic acid cycle [56]. Choline was significantly increased in AD and MCI patients respect to healthy controls, in agreement with previous results in brain [57] and CSF [36]. The increase in free choline and related compounds such as phosphocholine and glycerophosphocholine in AD has been traditionally related to breakdown of phosphatidylcholines during neurodegeneration, due to abnormal metabolism of membrane phospholipids [31, 58]. However, choline is also involved in metabolism of betaine and methionine acting as methyl donor, so its dyshomeostasis may be also related to impaired transmethylation pathway, as reflects the altered levels of methionine (Table 1). This pathway is required for the production of S-adenosylmethionine (SAM), the major DNA methylating agent, using methionine and folate as precursors. In AD, abnormal transmethylation mechanisms have been associated with polymorphisms of genes participating in the cycle and reduced levels of folate [59], SAM [60,61] and methionine [62–64], in agreement with our findings. Other three potential markers found in the present study could be related to vascular disorders: homocysteine-cysteine disulfide (Hcy-Cys), asymmetric dimethyl-arginine (ADMA) and phenylalanine-phenylalanine (Phe-Phe). It is recognized that subjects with vascular risk factors have an increased prevalence of AD [65], so the measurement of these risk indicators can provide a new insight into the development of cognitive impairment. Homocysteine-cysteine disulfide is one of the most common low molecular weight oxidized compounds of homocysteine, representing the major circulating form of this amino acid at physiological conditions [66], so its increase in serum reflects a situation of hyperhomocysteinemia. Thus, although homocysteine plays a central role in methionine metabolism and synthesis of cysteine, its overexpression has emerged as a major vascular risk factor closely related to pathogenesis of AD [67, 68]. On the other hand, asymmetric dimethyl-arginine is an important endogenous inhibitor of nitric oxide synthase, produced by arginine methylation of proteins and further liberation during protein degradation in the course of physiological protein turnover [69]. Nitric oxide (NO) is a key regulator in the cardiovascular, immune and neuronal systems, and its depletion has been associated with Alzheimer’s disease, so that plasma levels of ADMA have been shown to be elevated in AD patients [70]. Furthermore, there is evidence that increased plasma concentrations of homocysteine impair endothelial function by increasing the plasma concentration of ADMA [71]. Thus,
homocysteine appears to participate in the biosynthesis of ADMA through the implication of SAM as methyl donor to arginine and due to its inhibiting role over the activity of endothelial dimethylaminohydrolase, which hydrolyses ADMA. In relation with dimethyl-arginine and its precursor arginine, N-acetyl spermidine was found decreased in serum from AD and MCI patients (Table 1). Acetyl spermidine is an intermediate involved in the synthesis of polyamines, the second major pathway in which arginine takes part [72]. In AD, amyloid beta deposition is known to upregulate polyamine metabolism by increasing ornithine decarboxylase activity and polyamine uptake by initiating free radical damage [73], leading to altered levels of polyamines in body fluids and brain [74–76]. Therefore, together to the increased levels of ADMA, dyshomeostasis of polyamines system highlights the great importance of arginine metabolism in pathogenesis of disease. Finally, the dipeptide phenylalanyl-phenylalanine was upregulated in both mild and severe disease (Table 1), which has been also previously associated with different types of cancer [77, 78], but whose biochemical role has not been described. However, Krumsie et al. demonstrated in a recent study that Phe-Phe is a peptide derived from a vasoactive oligopeptide cleaved by the angiotensin converting enzyme [79]. This enzyme plays a central role in the renin-angiotensin system, cleaving angiotensin precursors and other functional oligopeptides into active peptides that causes vasoconstriction and increases blood pressure. Alterations in blood pressure due to dysregulation of renin-angiotensin system components lead to hypertension, which contributes to the development and progression of AD [80]. Thus, elevation of serum phenylalanyl-phenylalanine could be considered as a novel marker of vascular risk in AD. Taking all this into account, vascular risk factors appear to be closely related to AD pathogenesis through the involvement of essential metabolic pathways such as trasmethylation, the arginine-NO cycle and the angiotensin system, as schematized in Fig. 5. To conclude, numerous peptides were identified as possible markers of AD, which could correspond to abnormal protein turnover, including both protein synthesis and protein degradation, in relation to the numerous proteolytic processes associated with AD biochemistry [81]. However, these compounds could only be identified tentatively due to the absence of commercial standards, so further studies have to be performed in order to confirm these results.

4 Concluding remarks

A metabolomic approach based on CE-MS has been employed for the study of AD pathogenesis and its progression from mild cognitive impairment. The methodology provided several potential biomarkers for diagnosis, some of which have not been previously described, such as the dipeptide phenylalanyl-phenylalanine or different acylcarnitines. Thus, pathobiochemistry of this neurodegenerative disorder could be associated with multiple factors, as oxidative stress, vascular risk factors, or hypometabolism, among others. The main strength of this study was a careful selection of participants in order to match experimental groups on demographic factors (age, sex, comorbidities) and the inclusion of subjects that had not yet received any type of medication, in order to study early markers of disease and avoid confusing metabolic alterations derived from the treatment. Furthermore, a significantly large population (n = 93) was enrolled in this study, comparable or even higher than that used in previous works focused on AD progression from MCI [18, 20, 24, 25, 45]. Nevertheless, a second validation phase should be performed on a larger number of samples in order to confirm our findings and demonstrate the potential of these discriminant metabolites as potential biomarkers for diagnosis.

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5 References