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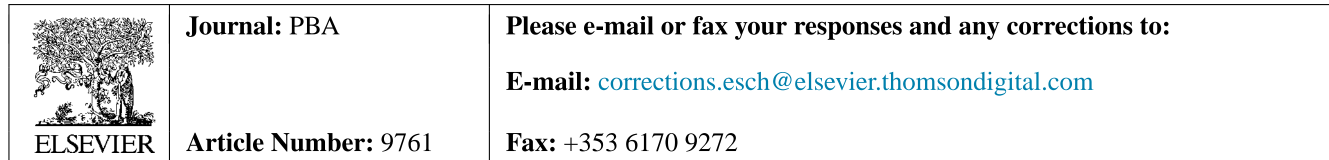

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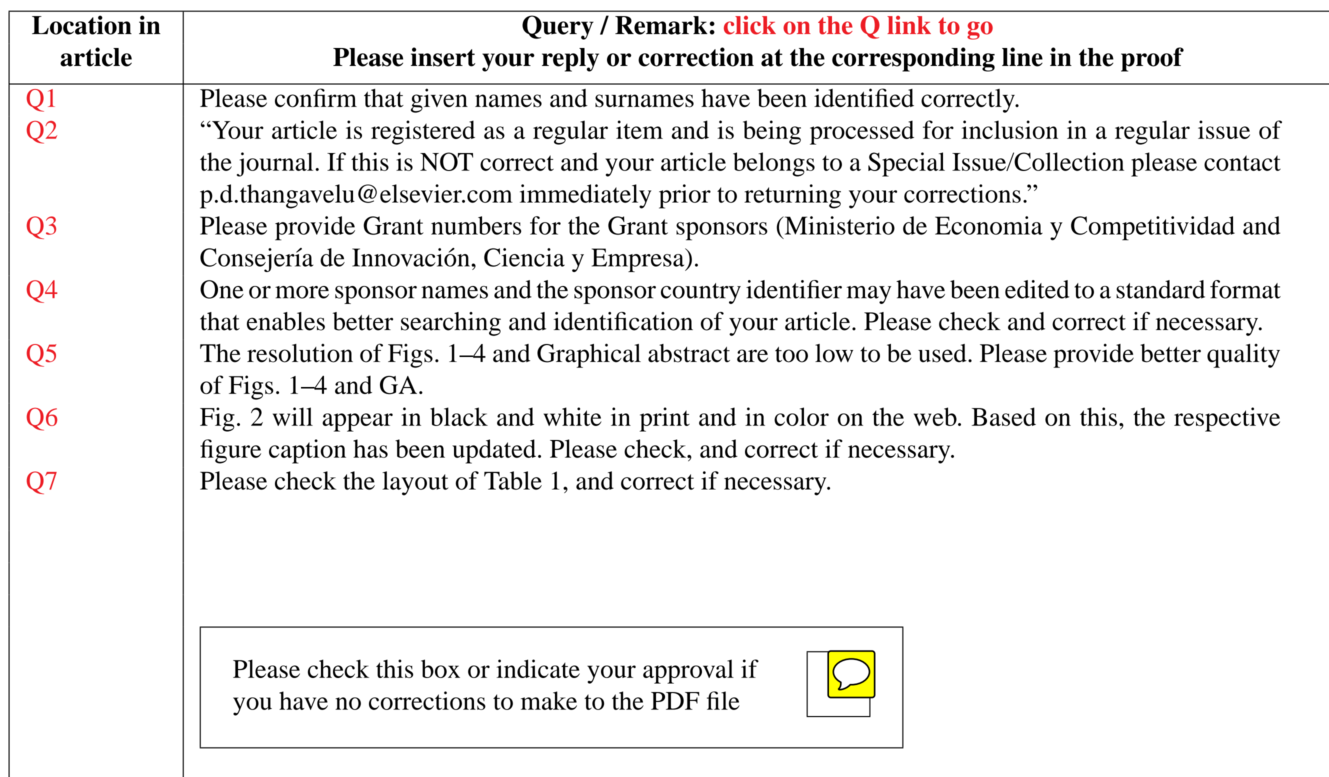

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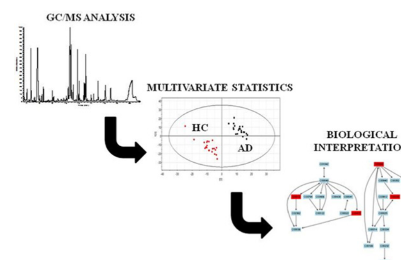
Metabolite profiling for the identification of altered metabolic pathways in Alzheimer's disease

Raúl González-Domínguez, Tamara García-Barrera**, José Luis Gómez-Ariza*



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Highlights

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Metabolite profiling for the identification of altered metabolic pathways in Alzheimer's disease

Raúl González-Domínguez, Tamara García-Barrera**, José Luis Gómez-Ariza*

- GC/MS profiling identified numerous altered metabolites in Alzheimer's disease.
- Pathway analysis was used to elucidate the underlying pathological mechanisms.
- AD could be associated with impaired metabolism of amino acids and hypometabolism.
- Novel potential markers were found in serum: pyroglutamate, adenosine, and others.

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Metabolite profiling for the identification of altered metabolic pathways in Alzheimer's disease

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ABSTRACT

Gas chromatography coupled to mass spectrometry is the most frequent tool for metabolomic profiling of low molecular weight metabolites. Its suitability in health survey is beyond doubt, given that primary metabolites involved in central pathways of metabolism are usually altered in diseases. The objective of this work is to investigate metabolic differences in serum between Alzheimer's disease patients and healthy controls in order to elucidate pathological mechanisms underlying to disease. Alterations in levels of 23 metabolites were detected, including increased lactic acid, α-ketoglutarate, isocitric acid, glucose, oleic acid, adenosine and cholesterol, as well as decreased urea, valine, aspartic acid, pyroglutamate, glutamine, phenylalanine, asparagine, ornithine, pipecolic acid, histidine, tyrosine, palmitic and uric acid, tryptophan, stearic acid and cystine. Metabolic pathway analysis revealed the involvement of multiple affected pathways, such as energy deficiencies, oxidative stress, hyperammonemia, and others. Moreover, it is noteworthy that some of these compounds have not been previously described in AD research, such as α-ketoglutarate, isocitrate pipecolic acid, pyroglutamate and adenosine, confirming the potential of this metabolomic approach in the search of novel potential markers for early detection of Alzheimer's disease.

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1. Introduction

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Metabolomics, based on the analysis of the entire set of metabolites in a tissue or biofluid, provides a comprehensive overview of the status of organisms, reflecting the interactions between genes, proteins and the environment. This integrative approach is a perfect tool to understand the biochemical and biological mechanisms occurring in complex systems, and for this reason, its application is rising in importance in health survey for the study of disease pathology, biomarkers discovery and drug development [1]. However, the comprehensive investigation of metabolome is very complicated due to its huge complexity and dynamics, since metabolome represents a vast number of components that belong to a wide variety of compound classes, very diverse in their physical and chemical properties, and present in a wide range of concentrations [2]. Moreover, metabolite distributions are subjected to high temporal and spatial variability [3]. Thus, there is no single way to perform a total metabolomic screening, but there are different complementary analytical platforms that can be applied, such as NMR [4], FT-IR [5] and mass spectrometry, this latter coupled to separation techniques or using direct infusion mass spectrometry (DIMS) [6]. Among them, gas chromatography coupled to mass spectrometry is one of the most frequently used tools for metabolomic profiling, especially in plant metabolomics [7], but widespread today to other fields as biomedical research [8]. Although NMR or DIMS may be preferred techniques for metabolic fingerprinting due to high throughput screening capability or minimal sample preparation requirements, GC–MS approaches present several analytical advantages making it an alternative to consider [9]. First of all, sensitivity is higher than in other conventional techniques employed in metabolomics, and above all, against techniques not

Abbreviations: AD, Alzheimer's disease; HC, healthy control; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; VIP, variable importance in the projection.

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based on mass spectrometry. In addition, chromatographic reproducibility and resolution exceed that provided by other hyphenated techniques such as LC–MS or CE–MS. Finally, it is possible a straightforward identification of peaks via libraries of mass spectra, due to the highly repeatable mass spectral fragmentation upon electron impact ionization [10]. On the other hand, the main downside of this technique is that analysis is restricted to volatile metabolites, usually after chemical derivatization. For all these reasons, GC–MS has been postulated as the technique of choice to get fingerprints of low molecular weight metabolites.

The ability of GC–MS to measure key primary metabolites such as amino acids, organic acids or saccharides, involved in essential pathways of organisms metabolism, makes it an interesting alternative in health survey, particularly in the discovery of disease biomarkers. This approach has been previously proposed for the study of different pathological situations, such as Parkinson's disease [11], cancer [12,13], diabetes [14], Huntington's disease [15] or Alzheimer's disease [16]. In this latter case, the discovery of biomarkers is of great importance because currently, diagnosis of Alzheimer's disease (AD) is based on the application of clinical criteria, since to date no biological markers have been established with the required reliability and specificity [17]. These criteria entail significant drawbacks, because when patients are diagnosed, dementia has already developed and the degree of brain damage is widespread. In this sense, the application of GC–MS metabolomics can be very useful, since AD is a multifactorial disorder in which many cellular processes are deregulated, causing alterations in several families of important low molecular mass metabolites such as amino acids [18], fatty acids [19] or neurotransmitters [20]. In this study, blood serum samples from AD patients and healthy controls were subjected to analysis by GC–MS, and metabolomic profiles were compared to evaluate the potential of technique for discrimination. For this purpose, after chemical derivatization and analysis, results were preprocessed for retention time alignment and intensity normalization, and finally multivariate analysis was carried out by partial least squares discriminant analysis (PLS-DA), in order to identify discriminant metabolites.

2. Materials and methods

2.1. Reagents and samples

Solvents employed were HPLC-grade. Methanol and pyridine were purchased from Aldrich (Steinheim, Germany), while ethanol was supplied by Merck (Darmstadt, Germany). Water was purified with a Milli-Q Gradient system (millipore, Watford, UK). Derivatizing agents, methoxylamine hydrochloride and N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), were obtained in Aldrich. A total of 44 blood serum samples were recruited by the Neurological Service of Hospital Juan Ramón Jiménez (Huelva, Spain), including healthy controls (HC) and Alzheimer's disease (AD) patients. Alzheimer's disease patients ($n = 23$, 8 male and 15 female, medium age 79.2 ± 5.9 y) were newly diagnosed of sporadic AD according to the criteria of the NINCDS-ADRDA [17], and only subjects that had not yet received any type of medication were included in the study. On the other hand, matched healthy controls in sex and age ($n = 21$, 9 male and 12 female, medium age 72.1 ± 5.4 y) were enrolled, after examination by neurologists to confirm the absence of neurological disorders, whom had not more than two reported cases of Alzheimer's disease in their families. Demographic characteristics of groups considered in the study can be found in supplementary material, including age, gender, co-morbidities, medication and family history of AD. Most subjects suffered other co-morbidities and were under different medical treatments, but there were not significant differences among AD and HC groups. 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 a refrigerator. The samples were immediately cooled and protected from light for 30 min to allow clot retraction to obtain serum after centrifugation (3500 rpm for 10 min). The serum was divided into aliquots and frozen at −80 °C until analysis. The study was performed in accordance with the principles contained in the Declaration of Helsinki. All persons gave informed consent for the extraction of peripheral venous blood and controls subjects were studied by neurologists to confirm the absence of neurological and cognitive disease.

2.2. Serum preparation

For the extraction of serum metabolites, proteins were precipitated following a modification of the procedure described elsewhere [21]: 100 μL of serum were mixed with 400 μL of 1:1 methanol/ethanol mixture in an Eppendorf tube and vortexed for 5 min at room temperature, followed by centrifugation at 4000 rpm for 10 min at 4 °C. The supernatant is transferred to another tube and dried under nitrogen stream. Then, derivatization was carried out according to the two step methodology proposed by Begley et al. [22]. For protection of carbonyl groups by methoxymation, dried extracts were redissolved in 50 μL of 20 mg mL^{-1} methoxyamine in pyridine, and after briefly vortexing were incubated at 80 °C for 15 min in a water bath. Subsequently silylation was performed by adding 50 μL of MSTFA and incubation at 80 °C for a further 15 min. Finally, extracts were centrifuged at 4000 rpm for 5 min and supernatant collected for analysis.

2.3. GC–MS analysis

Chromatographic analysis was performed in a Trace GC ULTRA gas chromatograph coupled to an ion trap mass spectrometer detector ITQ900 (Thermo Fisher Scientific), using a Factor Four capillary column VF-5MS 30 m × 0.25 mm ID, with 0.25 μm of film thickness (Varian). The GC column temperature was set to 100 °C for 0.5 min, and programmed to reach 320 °C at a rate of 15 °C per minute. Finally, this temperature is maintained for other 7 min, being the total time of analysis 22.17 min. The injector temperature was kept at 280 °C, and helium was used as carrier gas at a constant flow rate of 1 ml min^{-1}. For mass spectrometry detection, ionization was carried out by electronic impact (EI) with a voltage of 70 eV, by full scan mode in the m/z range 35–650, with an ion source temperature of 200 °C. For analysis, 1 μl of sample was injected in splitless mode.

2.4. Data processing

Raw data was processed following the pipeline described by Katajamaa and Oresic [23], which proceeds through multiple stages including feature detection, alignment of peaks and normalization. For this purpose, we employed the freely available software XCMS, included in the R platform (http://www.r-project.org). Files were converted into netCDF using the Thermo File Converter tool (Thermo Fisher Scientific) and subsequently, data were extracted using the matched filter method. This algorithm slices data into extracted ion chromatograms (XIC) on a fixed step size, and then each slice is filtered with matched filtration using a second-derivative Gaussian as the model peak shape [24]. The XCMS parameters were optimized according to the characteristics of data sets obtained in order to extract the maximum information as possible. Finally, the settings applied for were S/N threshold 2, full width at half-maximum (fwhm) 3, and width of the m/z range 0.1 (step parameter). After peak extraction, grouping and retention time correction of peaks (alignment) was accomplished in three

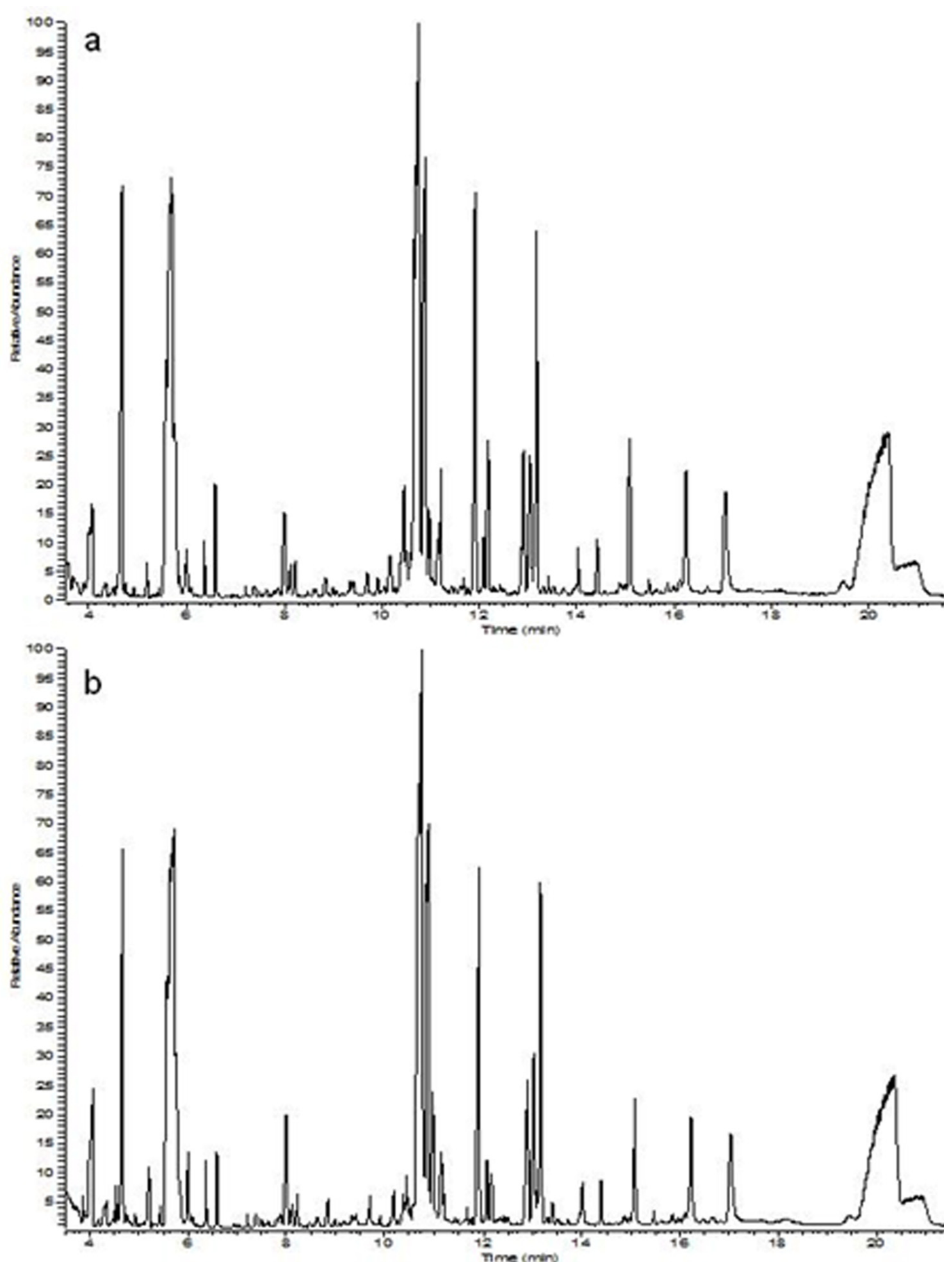

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Fig. 1. GC/MS chromatograms from (a) Alzheimer's disease and (b) healthy control samples.

176 iterative cycles with descending bandwidth (bw) from 5 to 1 s. Then, imputation of missing values was performed by returning to the raw spectral data and integrating the areas of the missing peaks which are below the applied signal-to-noise ratio threshold, using the fillPeaks algorithm. For data normalization, the locally weighted scatter plot smoothing (LOESS) normalization method was used, which adjusts the local median of log fold changes of peak intensities between samples in the data set to be approximately zero across the whole peak intensity range [25]. Finally, data were submitted to logarithmic transformation, in order to stabilize the variance of results. The preprocessed data were then exported as a .csv file for further data analysis by multivariate procedures. 187

188 *2.5. Data analysis*

189 Results were subjected to multivariate analysis by principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) in order to compare metabolomic profiles obtained, using the SIMCA-P™ software (version 11.5, published by UMetrics AB, Umeå, Sweden). Before performing statistical analysis, data was submitted to Pareto scaling, for reducing the relative importance of larger values, and logarithmic transformation, in order to approximate a normal distribution [26]. Performance of models was assessed by the R^2 and Q^2 values, provided by the software (indicative of class separation and predictive power of the model, respectively). Finally, metabolites responsible for discrimination were selected 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 1.5, indicative of significant differences among groups. These potential markers were identified using the NIST Mass Spectral Library (version 08), considering only those variables with a similarity index (SI) greater than 70%. 207

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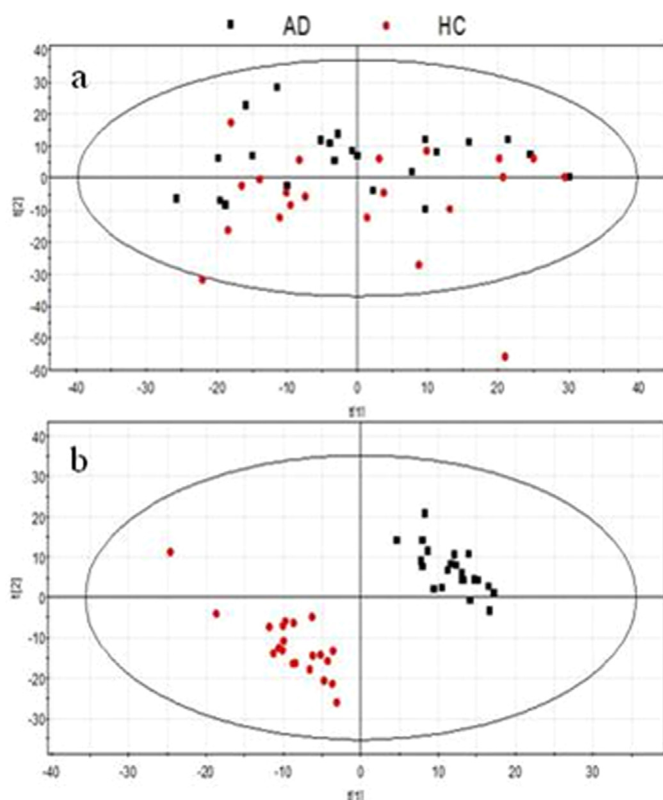

Q6 **Fig. 2.** Scores plots of PCA (a) and PLS-DA (b) models for GC–MS data. Black squares: AD patients; red circles: HC, healthy controls. (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

208 ### 2.6. Metabolic pathway analysis

209 Metabolic pathway analysis was subsequently performed to 210 identify and visualize the affected pathways on the basis of poten- 211 tial biomarkers detected. For this purpose, the MetPA web tool 212 was employed (http://metpa.metabolomics.ca), which conducts 213 pathway analysis through pathway enrichment analysis and path- 214 way topological analysis [27]. In this work, we select the *Homo* 215 *sapiens* library and use the default 'Hypergeometric Test' and 216 'Relative-Betweenness Centrality' algorithms for pathway enrich- 217 ment analysis and pathway topological analysis, respectively. In 218 order to identify the most relevant pathways, the impact-value 219 threshold calculated from pathway topology analysis was set to 220 0.1.

221 ## 3. Results

222 Serum metabolomic profiles obtained by GC–MS (Fig. 1) were 223 submitted to multivariate statistical analysis in order to evalu- 224 ate the potential of the methodology to discriminate between 225 AD patients and healthy control subjects. Thus, after raw data 226 pre-processing, Pareto scaling and logarithmic transformation, 227 principal component analysis (PCA) was applied to check trends, 228 outliers, and quality of the analysis. This unsupervised method was 229 not able to discriminate between groups (Fig. 2a), but showed a 230 clustering of samples in the scores plot without significant out- 231 liers according to the Hotelling T^2-range plot. Then, partial least 232 squares discriminant analysis (PLS-DA) was used in the same data 233 set to build a predictive model for classification of samples. PLS- 234 DA scores plots (Fig. 2b) displayed a clear separation between AD 235 patients and controls, being obtained good class separation value 236 and predictive power in a three components model (with $R^2 = 0.99$ 237 and $Q^2 = 0.74$). Metabolites responsible for discrimination were

Table 1
Potential biomarkers of AD identified by GC–MS metabolic profiling. Q7

RT (min)	Metabolite	Fold change
Decreased metabolites		
5.18	Valine	0.87
5.58	Urea	0.56
7.78	Aspartic acid	0.86
8.00	Pyroglutamic acid	0.85
8.63	Glutamine	0.82
8.87	Phenylalanine	0.72
9.08	Asparagine	0.85
10.20	Ornithine	0.76
10.97	Pipecolic acid	0.75
11.05	Histidine	0.83
11.13	Tyrosine	0.86
11.90	Palmitic acid	0.65
12.17	Uric acid	0.71
13.07	Tryptophan	0.73
13.17	Stearic acid	0.78
13.42	Cystine	0.88
Increased metabolites		
4.00	Lactic acid	1.51
8.30	α-Ketoglutarate	1.33
10.15	Isocitric acid	1.53
10.75	Glucose	1.92
13.02	Oleic acid	1.28
15.42	Adenosine	1.31
20.00	Cholesterol	1.90

then selected according to the variable importance in the projec- 238 tion (VIP > 1.5), indicative of significant differences among groups 239 (Table 1). Subsequently, altered biochemical pathways associated 240 with these abnormalities were identified by metabolic pathway 241 analysis. An overview of this analysis is shown in Fig. 3, where 242 each node represents a metabolic pathway and its size indicates the 243 impact of this pathway in response to Alzheimer's disease, reveal- 244 ing important impairments in energy metabolism and homeostasis 245 of amino acids. 246

4. Discussion 247

In a recent work, we demonstrated that serum metabolomics 248 using flow injection analysis with atmospheric pressure 249

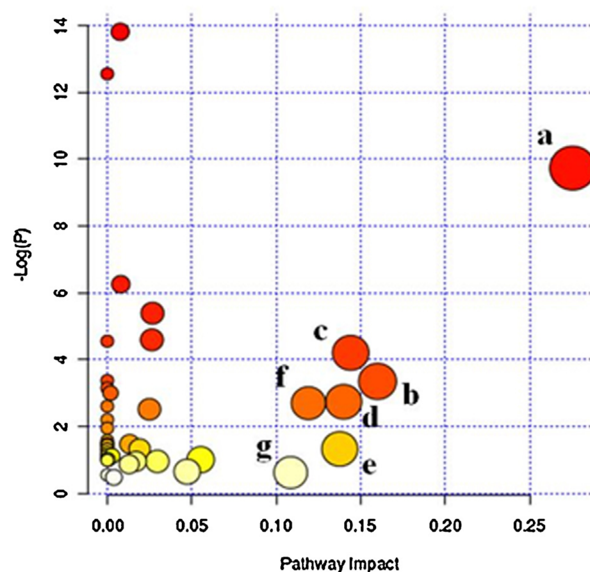

Fig. 3. Pathway analysis overview showing altered metabolic pathways in serum from AD patients. (a) Alanine, aspartate and glutamate metabolism; (b) arginine and proline metabolism; (c) TCA cycle; (d) histidine metabolism; (e) pyruvate metabolism; (f) phenylalanine metabolism; (g) tryptophan metabolism.

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photoionization mass spectrometry (FIA-APPI-MS) may be very useful for the study of pathological mechanisms associated with Alzheimer's disease, showing the involvement of numerous non-polar compounds (principally lipids) [28]. However, findings presented here highlight the potential of complementary GC–MS analysis for the comprehensive assessment of serum metabolome, demonstrating the implication of low molecular weight metabolites in the pathogenesis of this disorder. As shown in Table 1, the levels of 23 metabolites are significantly altered in AD patients compared with healthy controls: lactic acid, α-ketoglutarate, isocitric acid, glucose, oleic acid, adenosine and cholesterol increased in AD patients, while urea, valine, aspartic acid, pyroglutamate, glutamine, phenylalanine, asparagine, ornithine, pipecolic acid, histidine, tyrosine, palmitic and uric acid, tryptophan, stearic acid and cystine decreased in these samples. These metabolic differences in serum could be related to different perturbed cellular pathways (Fig. 3), and therefore it is possible to understand the biochemical processes underlying the pathology.

One of the most important changes observed in this study is the associated with an abnormal energy metabolism, corroborated by the increased levels of glucose, lactic acid, α-ketoglutarate and isocitrate in AD samples, and complementary decrease of aspartate and asparagine. Depletion in neuronal energy production by decreased rate of carbohydrate catabolism has been repeatedly documented in Alzheimer's disease [29], which is consistent with the increase found for serum levels of glucose (Table 1). In addition, this perturbed use of glucose by the neuron is also reflected in increased serum lactate, previously reported in CSF samples from AD patients in association with higher pyruvate levels [30]. On the other hand, this bioenergetic deficiency is also characterized by a dysfunction in mitochondrial activities [31]. In this sense, impairments in enzymes involved in tricarboxylic acid (TCA) cycle have been previously reported, with significant decreases in the activities of pyruvate dehydrogenase complex, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase complex, and increased activities of succinate dehydrogenase (complex II) and malate dehydrogenase in AD patients [32]. Furthermore, alterations in the levels of TCA intermediates or related compounds have also been described [33]. Thus, although to date it has not been previously reported an increase of α-ketoglutarate and isocitrate in AD serum samples, this situation is in complete agreement with the reduced activities of corresponding enzymes, α-ketoglutarate dehydrogenase and isocitrate dehydrogenase respectively, representing a possible new marker of aberrant energy metabolism in Alzheimer's disease. Finally, reduced serum levels were found for the amino acids aspartate, produced from oxalacetate, and its derivative asparagine. Nevertheless, contradictory results may be found with respect to this point in literature, considering plasma, cerebrospinal fluid and brain samples [34–36], so no clear conclusions can be drawn. Taking all these facts into account, we can conclude that mechanisms of energy production are completely perturbed in AD, affecting glycolysis, TCA cycle or synthesis of related amino acids, as schematized in Fig. 4.

Adenosine was other important metabolite contributing to the metabolomic differentiation of AD and HC samples. This nucleoside acts as neuromodulator and neuroprotector, controlling the release of glutamate [37]. It has been reported the up-regulation of adenosine receptors in the frontal cortex of AD patients [38], as well as the redistribution of these receptors, with a higher activity in neurons affected by β amyloid deposition or hyperphosphorilation of τ protein [39]. Thus, although high adenosine levels in brain have been related with normal ageing process [40], our experimental results indicate that an extra increase is produced in AD patients. Other discriminant metabolites found in GC–MS profiles related to the neurotransmitter system are the amino acids tryptophan, tyrosine, phenylalanine and glutamine, all of them decreased in AD

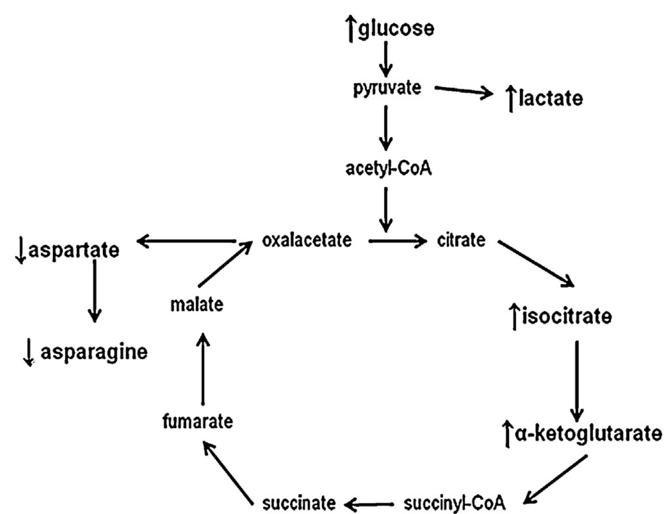

Fig. 4. Altered energy metabolism in AD. (↑) increased metabolites in AD samples; (↓) decreased metabolites in AD samples.

sera. Tryptophan is directly involved in two important pathways closely related to Alzheimer's disease, such as kynurenine pathway [41] and serotonergic system [42], and reduction of this amino acid in blood and cerebrospinal fluid of AD patients is well known [43,44]. On the other hand, phenylalanine and tyrosine participate in catecholamine metabolism for dopamine synthesis, and it is also well documented their decrease in serum, cerebrospinal fluid and brain from AD patients [18,34,35]. Finally, reduced glutamate neurotransmission is other characteristic hallmark of Alzheimer's disease [45], leading to low levels of corresponding neurotransmitters glutamate and glutamine (Table 1).

Several lipids also contributed for discriminating AD patients and healthy volunteers, including free fatty acids (increased oleic acid, and decreased palmitic and stearic acids in AD) or cholesterol. Fatty acids are bioactive molecules contributing to cell structure, energy storage and signal transduction, present at very high levels in neurons (50% of the neuronal membrane is composed by polyunsaturated fatty acids). An increasing number of studies have reported the potential relationship between fatty acids and Alzheimer's disease, and numerous works have demonstrated that long-chain n-3 fatty acids are inversely correlated with the development of Alzheimer's disease [46]. Moreover, has been shown that high levels of free fatty acids induce the amyloid deposition and tau hyperphosphorilation, contributing to the pathogenesis of AD [47]. However, previous works focused on analysis of fatty acids in brain, serum or plasma from Alzheimer's disease patients provided contradictory results [19,48,49]. Thus, the interpretation of altered levels of oleic, palmitic and stearic acids requires some caution, and probably further research is needed to establish accurate conclusions about the role of these compounds in AD development. On the other hand, the relationship between levels of cholesterol and AD progression is much more evident, since the increase of total cholesterol content in brain tissue has been correlated with the deposition of amyloid beta [50]. This situation of hyperlipidemia can affect to cerebrovascular system, leading decreased cerebral blood flow and finally, neuronal cell loss [51].

Ornithine and urea are two metabolites involved in urea cycle, responsible for nitrogen reutilization in conjunction with guanidine cycle [52], and controlling ammonia concentrations. Deficiencies in urea cycle may result in hyperammonemia, with deleterious effects on central nervous system [53]. In this sense, the role of this cycle in AD has been discussed, and was found that the expression of related genes is altered [54]. Therefore,

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the decrease in these two metabolites (Table 1) could be consistent with impaired nitrogen metabolism. Moreover, reduced ornithine levels could also be related with a perturbed status of polyamine system, given that overexpression of ornithine decarboxylase, responsible for the transformation of precursor ornithine in putrescine, is a common feature in AD [55].

It can also be noted that concentration of pyroglutamate decreases in AD serum, indicating the deregulation of the γ-glutamyl cycle (GGC). The main function of GGC is the transport of amino acids across the blood brain barrier by facilitative transport system L1, which may allow both desirable and undesirable amino acids to enter the brain by means of a carrier molecule of glutathione [56]. In this process a molecule of pyroglutamate is released, which is essential since stimulates sodium dependent carriers for the later removal of deleterious amino acids from brain. Thus, this cycle has a great importance in the regulation of cerebral glutamate, toxic to the brain at very low concentrations [57]. Changes in this cycle are known in AD brain, including decreases of the GSH/GSSG ratio, diminished γ-glutamylcysteine synthetase activity in de novo glutathione synthesis and increased activity of enzymes related to the GSH use such as glutathione peroxidase, γ-glutamyl transpeptidase, and glutathione S-transferase [58]. However, to our knowledge, is the first time that decreased concentration of pyroglutamate in serum is associated with AD.

The consequences of oxidative stress are especially important in neurodegenerative diseases, since brain is particularly susceptible to reactive oxygen species, which can attack neuronal lipids, proteins and nucleic acids, inevitably leading to neuronal dysfunction [59]. This pathological status is confirmed in metabolomic profiles by the reduction of three important compounds involved in the protection against oxidative stress: uric acid, cystine and histidine. Uric acid is one of the most common systemic plasma antioxidants, and its reduction in oxidative situations as AD has been previously reported [60]. On the other hand, cystine is the major form of cysteine at physiological conditions, being the limiting factor in the biosynthesis of glutathione. Reduced glutathione is the most important antioxidant for the defense of brain cells against oxidative stress and excitotoxicity of glutamate, and its deficit is associated to different disorders as AD [61]. Finally, histidine is an amino acid with antioxidant properties as other imidazole containing compounds such as carnosine or anserine, whose reduction has been previously related to AD [34].

Pipecolic acid is a degradation product of lysine in cerebral peroxisomes, which can act as neurotransmitter modulating GABAergic transmission, so that altered levels of this metabolite might be indicative of peroxisomal disease. The relation of this disorder with Alzheimer's disease has been previously described, since these organelles are responsible for the biosynthesis of docosahexaenic acid [62] and plasmalogens [63], both reduced in AD patients. Therefore, decrease of serum pipecolate levels can be considered as a new unequivocally marker of peroxisomal failure. Finally, valine was also reduced in serum samples from AD patients, in accordance with previous results in other neurodegenerative disorder as Huntington's disease [15]. Valine deficiency is related to neurological defects in rats [64], but its relation with AD is not clear.

5. Conclusions

The involvement of several low molecular weight metabolites in Alzheimer's disease has been found in the basis of the deregulation of numerous cellular processes, demonstrating the suitability of gas chromatography coupled to mass spectrometry for the elucidation of underlying pathological mechanisms. Selection of optimal experimental conditions for sample preparation, chromatographic analysis and data treatment allowed obtaining comprehensive metabolomic profiles of serum samples, useful for the discrimination of AD patients from healthy controls. Thus, up to 23 potential biomarkers were identified, which were related to different impairments associated with AD, such as hypometabolism, oxidative stress, changes in neurotransmission system, and others. Many of them have not been previously described (pipecolic acid, pyroglutamate, adenosine, α-ketoglutarate, isocitrate), so our findings provide a first step for the development of novel markers for early detection of Alzheimer's disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2014.10.010.

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