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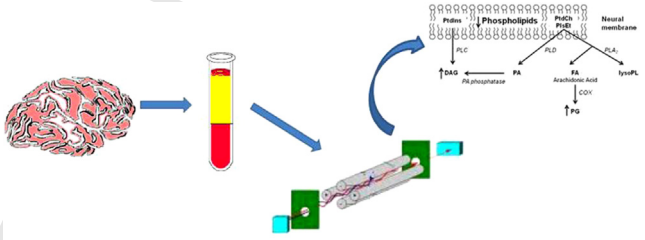
## Graphical Abstract

*Journal of Pharmaceutical and Biomedical Analysis xxx (2014) xxx–xxx*

**Metabolomic study of lipids in serum for biomarker discovery in Alzheimer's disease using direct infusion mass spectrometry**

R. González-Domínguez, T. García-Barrera**, J.L. Gómez-Ariza*



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## Highlights

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R. González-Domínguez, T. García-Barrera**, J.L. Gómez-Ariza*

- Direct infusion mass spectrometry allows comprehensive lipidomic fingerprinting.
- Numerous lipids and metabolites are altered in serum of Alzheimer's disease.
- Potential biomarkers can be associated with important hallmarks of Alzheimer's disease.
- Membrane breakdown highlights as a key factor in development of Alzheimer's disease.
- Several novel biomarkers were found: diacylglycerols, oleamide and other metabolites.

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# Metabolomic study of lipids in serum for biomarker discovery in Alzheimer's disease using direct infusion mass spectrometry

Q1 R. González-Domínguez[a,b,c], T. García-Barrera[a,b,c,**], J.L. Gómez-Ariza[a,b,c,*]

[a] Department of Chemistry and CC.MM., Faculty of Experimental Science, University of Huelva, Campus de El Carmen, 21007 Huelva, Spain
[b] Campus of Excellence International ceiA3, University of Huelva, Spain
[c] Research Center of Health and Environment (CYSMA), University of Huelva, Campus de El Carmen, 21007 Huelva, Spain

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ABSTRACT

In this study, we demonstrated the potential of direct infusion mass spectrometry for the lipidomic characterization of Alzheimer's disease. Serum samples were extracted for lipids recovery, and directly analyzed using an electrospray source. Metabolomic fingerprints were subjected to multivariate analysis in order to discriminate between groups of patients and healthy controls, and then some key-compounds were identified as possible markers of Alzheimer's disease. Major differences were found in lipids, although some low molecular weight metabolites also showed significant changes. Thus, important metabolic pathways involved in neurodegeneration could be studied on the basis of these perturbations, such as membrane breakdown (phospholipids and diacylglycerols), oxidative stress (prostaglandins, imidazole and histidine), alterations in neurotransmission systems (oleamide and putrescine) and hyperammonaemia (guanidine and arginine). Moreover, it is noteworthy that some of these potential biomarkers have not been previously described for Alzheimer's disease.



## 1. Introduction

Multiple pathological disorders have been associated with Alzheimer's disease (AD), involving abnormal protein aggregation in brain (amyloid β plaques and tangles of hyperphosphorylated τ protein) [1] and other processes such as oxidative stress [2], mitochondrial dysfunction [3], neurotransmission changes [4], and others. In this context, the importance of metabolites for studying the pathogenesis of diseases has been demonstrated, since the metabolome is the biological level closer to phenotype [5]. Particularly, lipids are very useful targets since play important roles in biological systems, so the global characterization of these compounds in a large-scale, or lipidomics, has a high potential in health survey [6]. In AD, lipids can be linked to several hallmarks of disease [7], principally dysregulation of membrane lipids, oxidative stress and vascular changes. Breakdown of cellular membranes is one of the most characteristic features of neurodegeneration, associated with abnormal metabolism of membrane lipids [8]. In this sense, alterations in two families of compounds have been described; (i) phospholipids, such as phosphocholines, phosphoethanolamines and plasmalogens [9], and (ii) sphingolipids and related compounds, such as sphingomyelins, ceramides or sulfatides [10]. On the other hand, brain is particularly susceptible to oxidative damage because of the high concentration of polyunsaturated fatty acids (PUFAs) and high oxygen consumption rates. Thus, the contribution of oxidative stress to AD also has consequences on the lipidomic profile, leading to the accumulation of typical markers of lipid oxidation. An important group are the eicosanoids, oxidation products of araquidonic acid through different enzymatic pathways [11]. Furthermore, the attack of reactive oxygen species (ROS) causes lipid peroxidation, generating isoprostanes (free radical peroxidation of araquidonic acid) [12], neuroprostanes (from docosahexaenoic acid) or aldehydes such as 4-hydroxynonenal and malondialdehyde [13]. Finally, AD has been also associated with several vascular risk factors, such as the epsilon 4 allele of the apolipoprotein E (ApoE), elevated homocysteine levels, hyperlipidemia, obesity or diabetes. These vascular defects could cause abnormalities in the vascular system, specifically in cerebrovascular system (atrophy, structural changes in the blood–brain barrier and inflammation), which result in decreased cerebral blood flow that finally involves neuronal loss [14]. In this sense, the contribution of high

* Corresponding author at: Department of Chemistry and CC.MM., Faculty of Experimental Sciences, University of Huelva, Campus de El Carmen, 21007 Huelva, Spain. Tel.: +34 959 219968; fax: +34 959 219942.
** Corresponding author at: Department of Chemistry and CC.MM., Faculty of Experimental Sciences, University of Huelva, Campus de El Carmen, 21007 Huelva, Spain. Tel.: +34 959 219962; fax: +34 959 219942.
*E-mail addresses:* raul.gonzalez@dqcm.uhu.es (R. González-Domínguez), tamara@dqcm.uhu.es (T. García-Barrera), ariza@uhu.es (J.L. Gómez-Ariza).

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levels of triglycerides, cholesterol, lipoproteins or fatty acids has been previously reported as one of the most important vascular factors in AD. For all these reasons, the characterization of global changes in lipids and their metabolites can be interesting in order to understand the role of these compounds in physiopathology of AD.

The study and identification of lipidomic biomarkers requires analytical techniques with high sensitivity and selectivity, and wide range of applicability to analyze the large number of molecules existing, with very different structures and functions. In this sense, mass spectrometry with soft ionization technologies as electrospray (ESI) or atmospheric pressure chemical ionization (APCI) is emerging in this field [15]. This platform offers capability for both quantitative and qualitative analyses and it may be coupled to separation techniques, principally chromatography and capillary electrophoresis. On the other hand, direct infusion of samples into the spectrometer is also possible, providing faster analysis and higher reproducibility, but it presents the disadvantage of isobaric interferences. For this, the analysis of complex samples requires the use of high resolution and accuracy instruments such as time of flight (TOF-MS), Fourier transform ion cyclotron resonance (FTICR-MS) or Orbitrap [16]. Moreover, the hybrid system Q-TOF-MS, which allows more accurate mass measurement than single TOF instrument and structural elucidation by MS/MS experiments [17], is gaining great importance in recent years in metabolomics [18,19], and particularly in lipidomics on the basis of multi-dimensional mass spectrometry-based shotgun lipidomics, or MDMS-SL [20–22].

The present work represents a lipidomic approximation to Alzheimer's disease based on direct infusion mass spectrometry analysis. Metabolic changes in blood serum samples of AD patients respect to healthy controls were evaluated by ESI-Q-TOFMS fingerprinting, demonstrating the involvement of different classes of lipids and individual molecular species of these compounds, as well as low molecular mass metabolites.

## 2. Material and methods

### 2.1. Reagents and samples

Methanol and chloroform (HPLC-grade) were purchased from Aldrich (Steinheim, Germany), and ammonium acetate was supplied by Merck (Darmstadt, Germany). Blood samples were obtained by venipuncture of the antecubital region after 8 h of fasting, from 22 patients (10 male and 12 female, medium age 78.5 ± 5 y) newly diagnosed of sporadic Alzheimer's disease (AD), according to the criteria of NINCDS-ADRDA [23], and 18 matched healthy controls, HC (7 male and 11 female, medium age 70.7 ± 4.1 y). All samples were 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 in Eppendorf tubes 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. Sample treatment

Extraction of serum samples was performed following a procedure derived from the method proposed by Bligh and Dyer [24], employing a mixture of chloroform and methanol. In addition, since neutral lipids are not readily ionized by ESI, addition of ammonium ions was selected for analysis in positive ion mode. In the case of negative ionization, any additive was employed. For extraction, 50 μL of serum are mixed with 150 μL of methanol, containing 30 mM ammonium acetate for ESI(+) and pure methanol for ESI(−) analysis. After stirring during 1 min in vortex, which causes the precipitation of proteins, the extract is combined with 200 μL of chloroform and again stirred for another minute. Finally, sample is centrifuged at 10,000 rpm and 4 °C during 10 min, and organic phase is taken for analysis.

### 2.3. Instrumentation

The experiments were performed in a QSTAR XL Hybrid system (Applied Biosystems, Foster City, CA, USA) using an electrospray (ESI) source. The samples were introduced into the mass spectrometer using an integrated apparatus pump and a 1000 μL volume Hamilton syringe at flow rate 5 μL min$^{-1}$. Data were obtained both in positive and negative ion mode, acquiring full scan spectra for 0.2 min in the $m/z$ range 50–1100 with 1.005 s scan time. In positive mode, the ion spray voltage (IS) was set at 3300 V, the curtain gas flow at 1.13 L min$^{-1}$ and the nebulizer gas flow at 1.56 L min$^{-1}$. The source temperature was fixed at 60 °C, with a declustering potential (DP) of 60 V and a focusing potential (FP) of 250 V. In ESI(−), only few parameters were modified respect ESI(+) method, with an ion spray voltage at −4000 V, a declustering potential (DP) of −100 V and a focusing potential (FP) of −250 V. To acquire MS/MS spectra, nitrogen was used as collision gas.

### 2.4. Data analysis

To carry out statistical analysis, spectra were submitted to peak picking and matching of peaks across samples in order to reduce the results into a two-dimensional data matrix of spectral peaks and peak intensities, by using Markerview™ software (Applied Biosystems). Then, SIMCA-P™ software (version 11.5, published by UMetrics AB, Umeå, Sweden) was employed for statistical processing. Partial least squares discriminant analysis (PLS-DA) was performed to build predictive models in order to find differences between the groups of study (AD patients and healthy controls) and further study of potential biomarkers. Quality of the model 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).

### 2.5. Compounds identification

Identification of significant compounds was made matching the experimental accurate mass and tandem mass spectra with those available in metabolomic databases (HMDB, METLIN, KEGG and LIPIDMAPS), using a mass accuracy of 50 ppm. Moreover, different classes of lipids were confirmed based on characteristic fragmentation patterns reported in literature. Phosphatidylcholines and plasmenylethanolamines presented characteristic ions in negative ionization mode at $m/z$ 168.04 and 196.07, respectively [25]. In addition, the fragmentation in the glycerol backbone and release of the fatty acyl substituents enabled the identification of individual species of phospholipids [26]. Finally, diacylglycerols [27], fatty acid amides [28] and eicosanoids [29] were also confirmed with characteristic fragments described in the literature.

## 3. Results

### 3.1. Metabolomic profiles

Mass spectra of serum extracts provided abundant biochemical information, considering the high number of signals that



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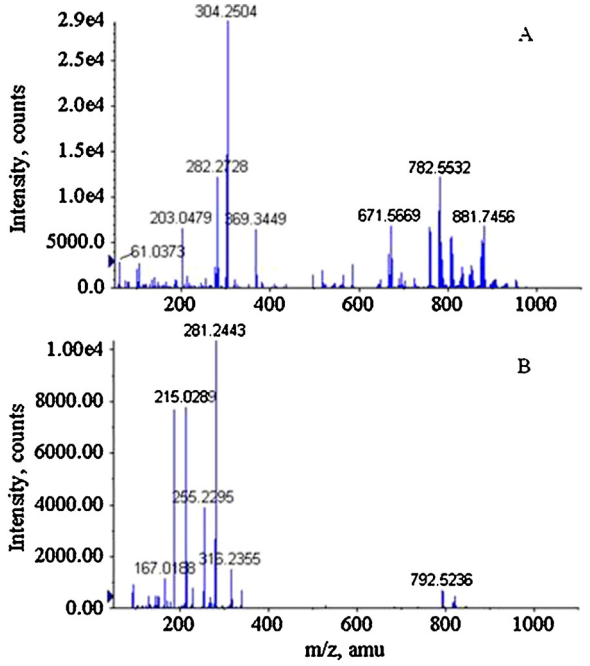


**Fig. 1.** ESI spectra of serum extracts in positive (A) and negative (B) modes.

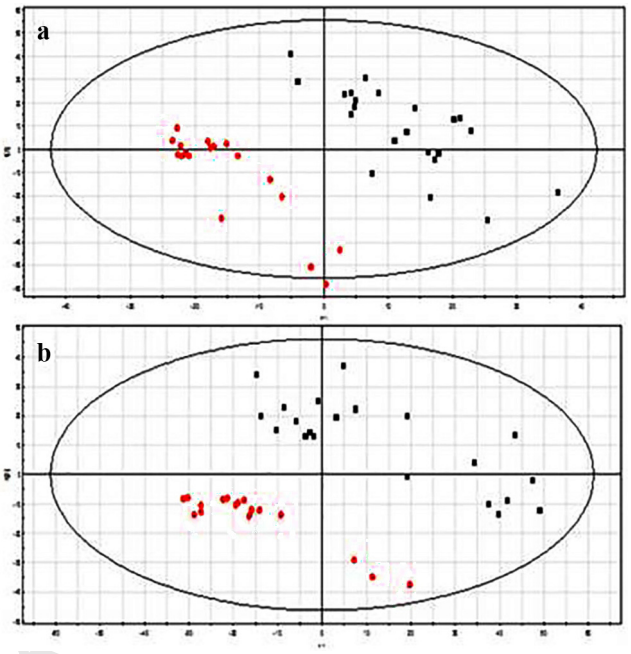


**Fig. 2.** Scores plots of the PLS-DA for ESI+ (a) and ESI− data (b). Black squares: AD patients; red circles: HC, healthy controls. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

could be detected. Furthermore, application of both positive and negative ionization modes allowed analyzing different subsets of metabolome according to the chemical nature of compounds, as reflected in the complementary profiles obtained (Fig. 1A and B). Therefore, a large number of metabolites can be studied combining the different spectral profiles, which provides a very characteristic metabolic fingerprinting of serum samples.

### 3.2. Multivariate analysis

In order to discriminate between samples from Alzheimer's disease (AD) and healthy controls (HC), multivariate data analysis was employed. Partial least squares discriminant analysis (PLS-DA) was performed to carry out the classification of the samples, building models that provide a clear separation between groups, visible in the scores plots both for positive and negative ionization modes (Fig. 2). Four components models clearly differentiate AD from HC samples, with high class separation predictive values ($R^2$ = 0.999 in both models). Moreover, cross-validation of these models was also successful, with $Q^2$ values around 0.9.

### 3.3. Selection of potential biomarkers

The most discriminant signals were selected according to the Variable Importance in the Projection (VIP, predictive parameter provided by the software that indicates the importance of the variable in the model), for later study by MS/MS and identification with metabolomic databases. Selecting only variables with VIP values greater than 2.0, numerous signals assigned to lipid compounds were identified as potential biomarkers in positive and negative ionization analyses (Tables 1 and 2). Furthermore, several low molecular weight metabolites also presented alterations in AD samples respect to healthy controls, both in positive and negative modes, listed in Table 3.

## 4. Discussion

Biomarkers identified could be associated with different pathologies of AD, as shown in this section. Thus, interpretation

**Table 1**
Lipid biomarkers of AD in ESI+ analysis; (↑) increased in AD, (↓) decreased in AD.

| *m/z* | Compound | Change |
|---|---|---|
| *Diacylglycerols (DAGs)* | | |
| 591.49 | C16:0/C18:3-DG (+H) | |
| 614.55 | C16:0/C18:0-DG (+$NH_4$) | |
| 619.53 | C18:3/C18:0-DG (+H) | |
| 634.52 | C16:0/C20:4-DG (+$NH_4$) | ↑ |
| | C18:2/C18:2-DG (+$NH_4$) | |
| | C18:1/C18:0-DG (+$NH_4$) | |
| *Eicosanoids* | | |
| 317.24 | Prostaglandins, PGs (M+$H^+$-$H_2O$) | ↑ |
| *Fatty acid amides (FAAs)* | | |
| 299.31 | Oleamide (+$NH_4^+$) | ↓ |

**Table 2**
Phospholipids as biomarkers of AD in ESI− analysis (decreased in AD).

| *m/z* | Compound |
|---|---|
| *Phosphocholines (PtdCh)* | |
| 802.55 | C18:3/C20:4-PC (−$H^+$) |
| 806.55 | C18:2/C20:3-PC (−$H^+$) |
| | C18:1/C20:4-PC (−$H^+$) |
| 814.51 | C16:0/C20:5-PC (+$Cl^-$) |
| 840.52 | C16:0/C22:6-PC (+$Cl^-$) |
| | C18:2/C20:4-PC (+$Cl^-$) |
| | C18:1/C20:5-PC (+$Cl^-$) |
| 842.53 | C16:0/C22:5-PC (+$Cl^-$) |
| | C18:1/C20:4-PC (+$Cl^-$) |
| | C18:0/C20:5-PC (+$Cl^-$) |
| 868.55 | C18:0/C22:6-PC (+$Cl^-$) |
| 870.55 | C18:0/C22:5-PC (+$Cl^-$) |
| *Ethanolamine plasmalogens (PlsEt)* | |
| 748.53 | P18:1/C20:4-PlsEt (−$H^+$) |
| 774.55 | P18:0/C22:6-PlsEt (−$H^+$) |



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**Table 3**
Other biomarkers of AD in ESI+ analysis; (↑) increased in AD, (↓) decreased in AD.

| | m/z | Compound | Change |
|---|---|---|---|
| ESI+ | 60.04 | Guanidine (+H) | ↓ |
| | 89.06 | Putrescine (+H) | ↓ |
| | 69.05 | Imidazole (+H) | ↓ |
| | 209.11 | Kynurenine (+H) | ↑ |
| | 126.02 | Taurine (+H) | ↓ |
| ESI− | 154.06 | Histidine (−H) | ↓ |
| | 173.10 | Arginine (−H) | ↓ |

of alterations can provide very valuable biochemical information about the disease.

### 4.1. Membrane breakdown

Metabolism of membrane phospholipids is disturbed in neurodegenerative disorders, producing changes in membrane properties such as permeability, fluidity and alterations in ion homeostasis. This process is caused by overactivation of phospholipases, leading to phospholipid degradation and resulting in the generation of second messengers, which are associated with neurodegeneration [30]. In Alzheimer's disease, abnormalities in membrane phospholipids are principally related to overactivation of phospholipase $A_2$, or $PLA_2$ [31], which catalyzes the hydrolysis of the ester bonds liberating fatty acids (FA) and lyso-phospholipids. This enzymatic stimulation leads to decreased total levels of phospholipids and accumulation of their degradation products [9,32]. Moreover, the release and oxidation of arachidonic acid, one of the most abundant fatty acids contained in neural phospholipids, produces several lipid mediators closely associated with neuronal pathways involved in AD, such as eicosanoids or peroxidation products as 4-hydroxy-2-nonenal [33]. On the other hand, there are also evidences for a role of phospholipases C and D in AD, although they have been much less studied. PLC hydrolyzes the phosphodiester bond at the sn-3 position forming 1,2-diacylglycerol (DAG) and a free base, while PLD cleaves phospholipids into phosphatidic acid (PA) that can be latter converted to diacylglycerol by PA phosphatases. Therefore, both enzymatic reactions yield DAGs as final product, which have unique functions as a basic component of membranes, intermediates in lipid metabolism and key element in lipid-mediated signaling [34]. In this context, previous reports have described increased levels of several isoenzymes of phosphoinositide-specific PLC [35,36] as well as PLD [37] in AD brains, and it has been demonstrated the involvement of PLD in APP trafficking and Aβ generation [38]. As a consequence, this abnormal metabolism results in important biochemical changes in brain, which is reflected in peripheral blood serum, as shown in Tables 1 and 2. A considerably decrease in diverse phosphocholine and ethanolamine-plasmalogen species was observed (Table 2), but only in PUFA-containing phospholipids, indicating that membrane destabilization processes could be also related to imbalance in the levels of saturated/unsaturated fatty acids contained in the structure of phospholipids, as recently reported [39]. In addition, the release of arachidonic acid from the hydrolysis of these phospholipids by $PLA_2$ and subsequent action of cyclooxygenases (COXs) supports the elevation of serum prostaglandin levels (Table 1), which are important markers of oxidative stress. Finally, the possible overactivation of phospholipases C and D in AD was also demonstrated, since it was found an increase in diacylglycerols (Table 1), not described to date in serum from AD patients. Thus, membrane breakdown highlights as a key factor in pathogenesis of AD, affecting numerous metabolites as summarized in Fig. 3.

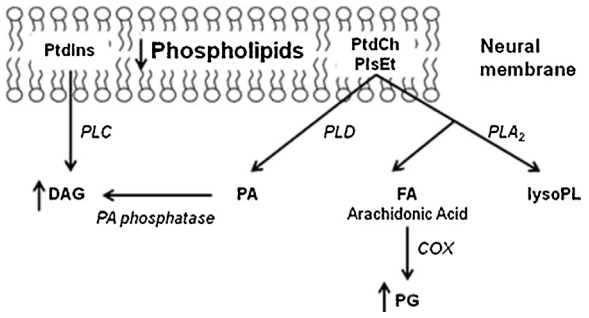


**Fig. 3.** Membrane phospholipids degradation by phospholipases, showing compounds up- (↑) and down-regulated (↓).

### 4.2. Oxidative stress

Oxidative stress is other important process implicated in neurodegenerative diseases because the high-metabolic rate of brain [2], resulting in decreased levels of antioxidant compounds and increased markers of protein, lipid, and nucleic acid oxidation. In this sense, alterations observed in imidazole and histidine (decreased, Table 3) and prostaglandins levels (increased, Table 1) could be related with this oxidative damage. It has been previously reported a decrease in imidazole containing aminoacids in plasma, urine and cerebrospinal fluid (CSF) of AD patients [40]. Histidine, carnosine and anserine are antioxidant compounds involved in the protection against oxidative damage due to the presence of the imidazole ring in their structures, which can provide chelating properties for divalent ions, prevention of lipid peroxidation and acting as quencher of 4-hydroxy-2-nonenal and malonaldehyde. However, no data about decreased levels of free imidazole in AD (Table 3) has been previously reported. On the other hand, the appearance of several markers of oxidative stress in AD samples, such as prostaglandins (Table 1), is a typical finding in neurodegenerative disease research [11], as it has been described in the previous section.

### 4.3. Hyperammonaemia

Significant low levels of guanidine and arginine were observed in AD (Table 3), not described previously to our knowledge, suggesting an alteration in the guanidine cycle. This cycle, in conjunction with urea cycle, is the responsible for nitrogen reutilization [41], and control of the ammonia concentrations in the organism. In brain, ammonia concentrations are maintained at low values in healthy persons due to the action of a series of enzymes, principally glutamine synthetase. However, disorders in urea-guanidine cycle may cause high amounts of ammonia, or hyperammonaemia, which have deleterious effects on the central nervous system [42]. In Alzheimer's disease the alteration of urea cycle has been recently discussed, and it has been found that enzymes of this cycle and the corresponding genes have altered levels of expression [43]. Thus, the involvement of hyperammonaemia in AD appears clear, and alterations in this regulatory cycle were demonstrated considering the low levels of guanidine and arginine shown in Table 3.

### 4.4. Other alterations in central nervous system

Other metabolites involved in different processes related to the integrity of the central nervous system (CNS) were altered in AD samples, such as putrescine, taurine and oleamide (decreased), and kynurenine (increased). Putrescine is a polyamine with several functions within the CNS, including nerve growth and regeneration, modulation of N-methyl-D-aspartate (NMDA) receptor or protection over stress. Amyloid beta deposition is responsible for the

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up-regulation of polyamine metabolism, with increased polyamine uptake and elevated ornithine decarboxylase (ODC) activity [44]. Thus, it was found that changes in polyamine system causes a decrease in putrescine levels in brain of AD patients [45], which is in agreement with results in blood serum shown in Table 3. Taurine is an amino acid present at high concentrations in the mammalian brain with several roles in neurotransmission, neuromodulation, osmoregulation, control of calcium influx, and cell excitability. It has been demonstrated its potential role in preventing the neurotoxicity of beta amyloid and glutamate receptor agonists [46], and in this sense, it have already been reported lower levels in CSF of AD patients [47]. On the other hand, oleamide is an amidated lipid normally found in the brain and blood of mammals, including humans [48], where interacts with several neurotransmission systems, enhancing the action of serotonin and GABA receptors [49]. Modulation of memory by this lipid has been reported in rats [50], and it has been demonstrated its improving effects over choline acetyltransferase *in vitro*, whose reduced activity is closely related to AD [51]. However, there are not studies about physiological levels of oleamide in AD, but the decrease observed in Table 1 for this compound could be related to analogous decrease in neurotransmitters serotonin and GABA, previously found by other authors [52,53]. Finally, it is known the implication of up-regulated kynurenine pathway (KP) in Alzheimer's disease, by overexpression of indole 2,4 dioxygenase [54]. In this way, high conversion of tryptophan (TRP) into kynurenine (KYN) is found, which finally leads to altered synthesis of related neuroactive compounds as kynurenic or quinolinic acids. Thus, experimental increase observed for kynurenine in AD (Table 3) may be related to other previous results, such as increased KYN/TRP quotient and quinolinic acid levels, accompanied by a decrease of kynurenic acid levels [55].

## 5. Conclusions

The important role of lipids in Alzheimer's disease pathogenesis has been previously reported, and was confirmed by results obtained in the present study. It has been demonstrated that comprehensive metabolic fingerprints can be obtained by direct infusion mass spectrometry of serum samples extracted with a mixture of chloroform and methanol. This methodology allows simple, fast and reliable comprehensive metabolomic fingerprinting of serum, a sample of high clinical value. In addition the application of this method to samples from AD patients and healthy controls allowed their discrimination. We observed alterations in the levels of diacylglycerols, prostaglandins and phospholipids that can be related to metabolic disorders associated to AD, such as oxidative stress and membrane breakdown. It was also observed a decrease of oleamide, involved in the suitable operation of central nervous system. Moreover, other metabolites of lower molecular weight involved in hyperarmmonaemia (guanidine, arginine), oxidative stress (histidine, imidazole) or polyamine system (putrescine) also exhibited changes of expression in AD, and may be candidates to be used as biomarkers of the disease.

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