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RESEARCH PAPER

Using direct infusion mass spectrometry for serum metabolomics in Alzheimer's disease

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Abstract Currently, there is no cure for Alzheimer's disease and early diagnosis is very difficult, since no biomarkers have been established with the necessary reliability and specificity. For the discovery of new biomarkers, the application of omics is emerging, especially metabolomics based on the use of mass spectrometry. In this work, an analytical approach based on direct infusion electrospray mass spectrometry was applied for the first time to blood serum samples in order to elucidate discriminant metabolites. Complementary methodologies of extraction and mass spectrometry analysis were employed for comprehensive metabolic fingerprinting. Finally, the application of multivariate statistical tools allowed us to discriminate Alzheimer patients and healthy controls, and identify some compounds as potential markers of disease. This approach provided a global vision of disease, given that some important metabolic pathways could be studied, such as membrane destabilization processes, oxidative stress, hypometabolism, or neurotransmission alterations. Most remarkable results are the high levels of phospholipids containing saturated fatty acids, respectively, polyunsaturated ones and the high concentration of whole free fatty acids in Alzheimer's serum samples. Thus, these results represent an interesting approximation to understand the pathogenesis of disease and the identification of potential biomarkers.

Keywords Alzheimer's disease · Direct infusion mass spectrometry · Metabolomics · Biomarkers

Abbreviations

AD	Alzheimer's disease
AUC	Area under the curve
Ch	Choline
CV	Coefficient of variation
DHA	Docosahexaenoic acid
DIMS	Direct infusion mass spectrometry
ESI	Electrospray ionization
FC	Fold change
FFA	Free fatty acid
GPCh	Glycerophosphocholine
HC	Healthy control
LPC	Lyso-phosphatidylcholine
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
PC	Phosphatidylcholine
PLS-DA	Partial least squares discriminant analysis
PPC	Choline plasmalogen
PPE	Ethanolamine plasmalogen
PUFA	Polyunsaturated fatty acid
ROC	Receiving operating characteristic
SFA	Saturated fatty acid
TG	Triglyceride
VIP	Variable importance in the projection

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Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder, characterized by an insidious onset and progressive decline of cognitive functions. Many heterogeneous cellular processes have been associated with pathological alterations in the brain of patients with Alzheimer's disease, such as β-amyloid deposition and hyperphosphorylation of τ protein [1], oxidative stress [2], mitochondrial dysfunction [3], metal dyshomeostasis [4], lipid dysregulation [5], and others. For this reason, metabolomic approaches, which provide the simultaneous measure of many untargeted metabolites, may have a high potential in the study of this multifunctional disease. The comprehensive analysis of organisms' response to a perturbation in the metabolome is a good tool to understand biological mechanisms occurring in complex systems, surpassing classical biochemical approaches focused on single analytes and other omics such as genomics and proteomics. Thereby metabolomics, defined as the study of the concentrations and fluxes of metabolites and their identification, is emerging as a promising technique in health survey, study of disease pathology, discovery of biomarkers, and drug development [6]. This experimental approximation to AD is extremely important, since nowadays diagnosis can only be performed by exclusion of other pathologies based on clinical criteria [7]. Furthermore, Alzheimer's disease is only definitively diagnosed after postmortem histopathological verification, which confirms 10 to 15 % of misleading diagnoses, and with low specificity against other dementia [8]. Therefore, the discovery of sensitive and specific biomarkers is crucial in order to detect key features of the disease. However, the identification of reliable biomarkers is hindered by the fact that patient classification relies on clinical diagnosis which is not always accurate, especially at early stages of the disease, and control groups are likely to contain individuals with preclinical AD [9]. Furthermore, given the multifactorial nature of the disease, it is unlikely that a single biomarker will meet the needs for clinical diagnosis, while a panel of biomarkers may offer the appropriate sensitivity, specificity, and positive and negative predictive values.

Mass spectrometry has become the most important analytical platform for metabolomics in recent years, offering high sensitivity and selectivity, and the potential to identify and quantify compounds. Metabolomic analyses are normally performed by coupling high-resolution separation techniques to the MS detector, which provides a great analytical performance when studying complex samples. The main advantages of hyphenated approaches are the ability to resolve isobaric compounds, reliable quantification due to reduced matrix effects, as well as improved identification of metabolites through MS/MS experiments and prediction of retention/migration times [10]. Nevertheless, a major drawback of these conventional separation methods is the low sample throughput, so other complementary approaches are emerging in order to reduce the time of analysis. Kuehnbaum et al. [11] described a multiplexed separation platform for plasma metabolomics based on multisegment injection-capillary electrophoresis-mass spectrometry, which increases sample throughput up to one order of magnitude while improving overall data fidelity. Alternatively, the use of ambient ionization techniques has extended the utility of mass spectrometry to direct analysis of complex solid materials or liquid aerosols without the need for sample pretreatment or extraction [12]. However, the most common fingerprinting tool in metabolomics is direct infusion mass spectrometry (DIMS), based on the direct introduction of sample extracts containing whole metabolites into the mass spectrometer, which avoids the conventional time-resolved introduction of metabolites into the MS after chromatographic separation, improving analysis rapidity and reproducibility, nontargeted metabolite coverage, and, consequently, high-throughput screening capability [13], although this approach has the disadvantage of metabolites interferences, especially in complex matrices as serum. However, this screening method may provide broad information about the biological system under study, very valuable for disease diagnosis and the study of pathogenesis. In Alzheimer's research, different platforms have been proposed for metabolomic profiling of biological samples to map potential biomarkers, such as LC-MS [14–17], GC-MS [14, 15], or CE-MS [18]. In these studies, cerebrospinal fluid (CSF) is usually the biofluid of choice as its composition is directly related to metabolite production in the brain, but the use of noninvasive samples such as blood serum or plasma is gaining importance in order to get easier and cheaper methods for diagnosis. However, the use of shotgun metabolomics has not been yet reported for AD diagnosis, except in recent studies with brain of transgenic mice [19, 20], which could contribute to deepen into our understanding of the pathological mechanisms occurring in this disorder.

The aim of this study is to use DIMS in order to characterize metabolic abnormalities occurring in Alzheimer's disease. For this purpose, blood serum was the selected sample, since it is easy to obtain and very suitable in clinical applications, instead of other matrices such as brain biopsy or CSF. Serum samples of AD patients and healthy controls (HC) were analyzed by DIMS, and data obtained was subsequently treated by multivariate statistical tools for discrimination of groups. Altered metabolites identified could be associated with biochemical pathways involved in the development of Alzheimer's disease.

Methods

Serum samples

Blood samples from AD patients (N=22, age 78.5±5.0, male/female 10/12) and healthy controls (N=18, age 70.7±4.1, male/female 7/11) were obtained by venipuncture of the

antecubital region after 8 h of fasting. Patients were newly diagnosed of sporadic Alzheimer's disease by the Neurologic Service of Hospital Juan Ramón Jiménez (Huelva, Spain), according to the criteria of the NINCDS-ADRDA [7], and only subjects that had not yet received any type of medication were included in the study. 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 (3,500 rpm for 10 min). The serum was divided into aliquots in Eppendorf tubes and frozen at −80 °C until analysis. Demographic characteristics of subjects enrolled in the study are listed in the Electronic Supplementary Material Table S1), including age, gender, comorbidities, and medication. It is noteworthy that, given the advanced age, most subjects suffered other comorbidities and were under different medical treatments, but there were no significant differences among the three groups considered. The study was performed in accordance with the principles contained in the Declaration of Helsinki and approved by the ethical committee of the University of Huelva. 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.

Sample treatment and analysis

Metabolomic analysis of serum was performed by extracting samples in a two-stage sequential procedure, followed by analysis with high-resolution tandem mass spectrometry, using electrospray (ESI) source in both positive and negative ionization modes. For the extraction of metabolites, 100 μL of serum were mixed with 400 μL of methanol/ethanol (50 %) and stirred for 5 min, followed by centrifugation at 4,000 rpm for 10 min at 4 °C. The supernatant was transferred to another tube, and the precipitate was kept for further treatment. Then, supernatant was dried under nitrogen stream and the resulting residue reconstituted with 80 μL of methanol and 20 μL of water (polar extract). On the other hand, the precipitate isolated in the first step was extracted with 400 μL of chloroform/methanol (50 %) by stirring during 5 min, followed by centrifugation at 10,000 rpm for 10 min at 4 °C. Finally, the resulting supernatant was taken to dryness under nitrogen stream and reconstituted with 100 μL of 60:40 dichloromethane/methanol (lipophilic extract). For analysis by mass spectrometry, 0.1 % formic acid was added to the extracts if positive ionization mode is used. In the case of negative ionization, the addition of any reagent is not necessary. The mass spectrometry experiments were performed in a QSTAR XL Hybrid system (Applied Biosystems, Foster City, CA, USA) using the electrospray (ESI) source. The samples were introduced into the mass spectrometer at 5 μL min^{-1} flow rate using an integrated apparatus pump and a 1,000 μL volume Hamilton syringe. For accurate mass measurement, the TOF analyzer is calibrated before analyzing each batch of samples using renin and taurocholic acid as standards (in positive and negative modes, respectively). Data were obtained in both positive and negative ionization modes, acquiring full scan spectra for 0.2 min in the m/z range 50–1,100 with 1.005-s scan time. In positive mode, the ion spray voltage (IS) was set at 3,300 V, and high-purity nitrogen was used as curtain and nebulizer gas at flow rates about 1.13 and 1.56 L min^{-1}, respectively. The source temperature was fixed at 60 °C, with a declustering potential (DP) of 60 V and a focusing potential (FP) of 250 V. For ESI(−), only few parameters were modified, respectively, ESI(+) method, with an ion spray voltage at −4,000 V, a declustering potential (DP) of −100 V, and a focusing potential (FP) of −250 V. To acquire the MS/MS spectra, nitrogen was used as collision gas. Furthermore, quality control (QC) samples were prepared by pooling equal volumes of each sample and analyzed throughout the sequence run, which allows monitoring instrumental drifts along the analysis period [21].

Data analysis

Metabolomic data were submitted to peak detection by Markerview™ software (Applied Biosystems) in order to filter the mass spectrometry results, and to carry out the reduction into a two-dimensional data matrix of spectral peaks and their intensities. For this, the peak search was done with a mass tolerance of 0.1 Da, and a minimum response of ten counts was considered for filtering. Then, data was processed by partial least squares discriminant analysis (PLS-DA) in the SIMCA-P™ software (version 11.5, published by UMetrics AB, Umeå, Sweden) in order to find differences between the groups of study (AD patients and healthy controls). Before performing statistical analysis, data are usually scaled and transformed in order to minimize the technical variability between individual samples to extract the relevant biological information from these data sets [22]. For this, data were submitted to Pareto scaling, for reducing the relative importance of larger values, and logarithmic transformation, in order to approximate a normal distribution. PLS-DA models were cross-validated in seven rounds by the leave-one-out method, and the statistical performance of these models was assessed by the R^2 and Q^2 values, 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, discriminant compounds 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 2.0, indicative of significant differences among groups. These metabolites were validated by *t* test with Bonferroni correction for multiple testing (*p* values below 0.05), using the STATISTICA 8.0 software (StatSoft, Tulsa, USA). Furthermore, the potential biomarkers were subjected to receiver operating characteristic (ROC) analysis to assess their diagnostic ability. The ROC curve analysis was performed using the GraphPad Prism software (version 6.04, Intuitive Software for Science, San Diego, CA), and the area under the curve (AUC) was used as a metric of sensitivity and specificity of these biomarkers. Thereby, a marker is excellent when AUC ranges from 0.9 to 1, good if AUC is 0.8 to 0.9, moderate if AUC is 0.8 to 0.7, and poor if below 0.7 [23].

Identification of metabolites

Potential markers were identified matching the experimental accurate mass and tandem mass spectra (MS/MS) with those available in metabolomic databases (HMDB, METLIN, and LIPIDMAPS), using a mass accuracy of 50 ppm. Furthermore, individual lipids were confirmed based on characteristic fragmentation patterns previously described. Choline-containing phospholipids were detected as protonated ($[M+H]^+$) and sodiated ($[M+Na]^+$) ions in positive ion mode, while in negative polarity, these lipids formed adducts with chloride ($[M+Cl]^-$) or demethylated ions ($[M-CH_3]^-$). On the other hand, more abundant ions for ethanolamine species were $[M+H]^+$ and $[M-H]^-$, in positive and negative modes, respectively. Phosphatidylcholines presented characteristic ions in positive ionization mode at *m/z* 184.07, 104.10, and 86.09, and two typical fragments due to the loss of trimethylamine (*m/z* 59) and phosphocholine (*m/z* 183 or 205, if the counterion is proton or sodium). Conversely, the product-ion spectra of ethanolamines were dominated by $[M+H-141]^+$, arising from the elimination of the phosphoethanolamine moiety. Finally, in negative mode, these distinctive signals were found at 168.04 and 196.07, for choline- and ethanolamine-derived lipids, respectively [24]. Furthermore, the fragmentation in the glycerol backbone and release of the fatty acyl substituents enabled the identification of individual species of phospholipids, as previously described [25].

Results

Metabolomic profiles

The high-throughput metabolomic approach used in this work, based on the combination of a two-step extraction procedure with complementary analysis by ESI(+)/ESI(−) modes, provided a comprehensive snapshot of serum metabolites. Protein precipitation with organic solvents (methanol/ethanol) allows the extraction of hydrophilic metabolites, but fails to extract the lipophilic components, which may remain adsorbed to protein precipitate. For this reason, a second extraction step was applied by using a chloroform/methanol mixture in order to recover lipid compounds. Thereby, four complementary metabolomic profiles were acquired for each blood serum sample, for polar and lipophilic extracts in both positive and negative modes of analysis (see Electronic Supplementary Material Fig. S1). Therefore, a large number of metabolites can be studied combining the different spectral profiles, which provide a very characteristic metabolic fingerprinting of serum samples. Furthermore, matrix-based ion suppression effects were evaluated by analyzing several samples at different dilution factors, and by comparing the resulting TIC of infusion profiles and the number of peaks [26]. The occurrence of matrix effects is an important limitation in MS-based metabolomics that can be attenuated by optimizing the dilution factor because the relationship between signal intensity and metabolite concentration is generally linear at higher sample dilutions. In this study, the maximum sensitivity was achieved when samples are reconstituted in 100 μL (as described in "Sample treatment and analysis" section), because in higher concentrated samples, the total number of ions is increased causing ion suppression, while at higher dilutions the beneficial effect of diminished ion suppression is already reduced. Anyway, the large amount of different compounds in serum helps to regulate these matrix effects, so it can be assumed that ion suppression would affect all samples equally, making any fingerprint comparison meaningful [26].

Comparison of metabolomic profiles

In order to differentiate between patients and healthy controls, a partial least squares discriminant analysis (PLS-DA) was performed employing the intensities of signals. Models built with both ESI(+) and ESI(−) data, analyzing polar and lipophilic extracts, provided a good classification of samples in two groups, as shown the scores plots (Fig. 1). Furthermore, these models, as well as the model created with all the data compiled together, yielded satisfactory values for the quality parameters R^2 and Q^2, with a variance explained around 99 % and variance predicted above 50 % (see Electronic Supplementary Material Table S2). A large number of signals could be used for class discrimination, according to the variable importance in the projection (VIP>2). The most striking results can be associated with altered levels of different phospholipids, including phosphatidylcholines and lyso-phosphatidylcholines, as well as choline and ethanolamine plasmalogens (Table 1). Other compounds perturbed were

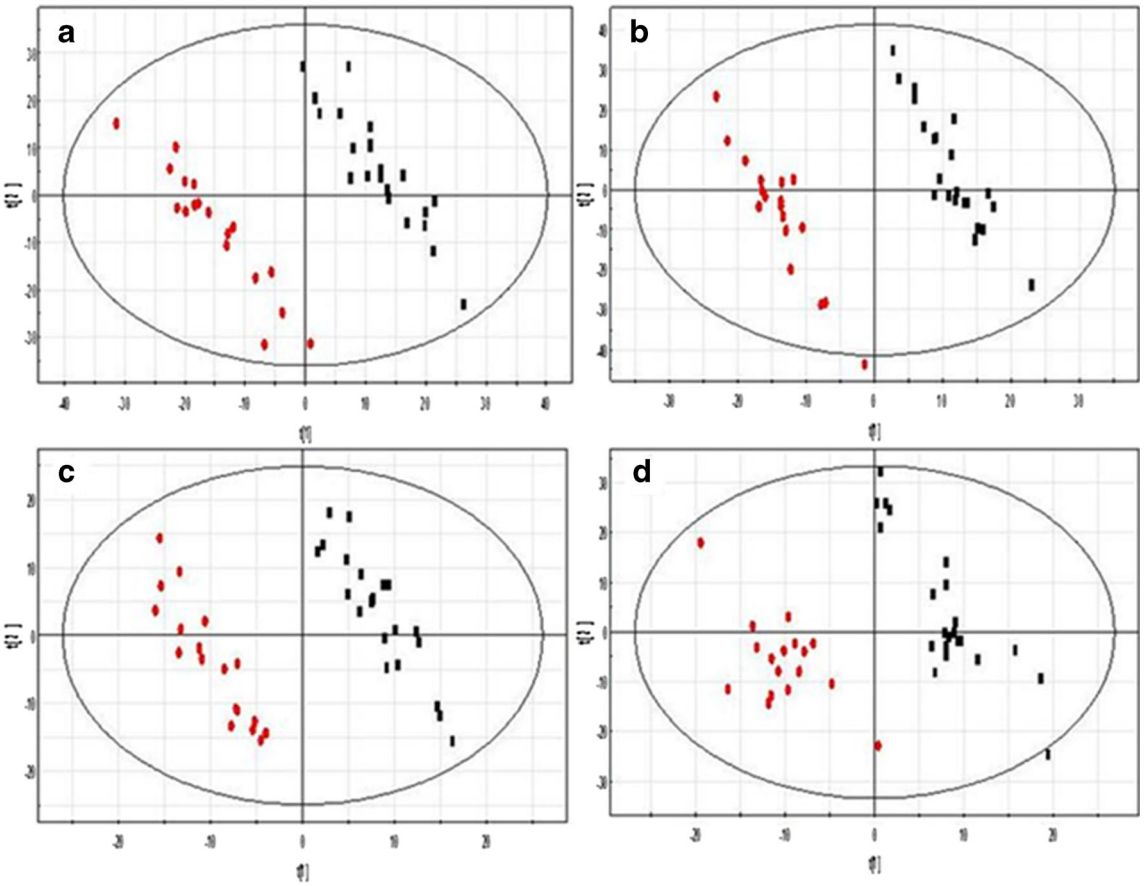

Fig. 1 Scores plots of the PLS-DA for ESI^+ (**a**, **b**) and ESI^- data (**c**, **d**), with polar (**a**, **c**) and lipophilic (**b**, **d**) extracts. *Black squares* AD, *red circles* HC

free fatty acids, eicosanoids, and different low molecular weight metabolites (Table 2), as well as a cluster of signals from ESI+spectra of lipophilic extracts (*m*/*z* 850–900) assigned to triglycerides (TG), which were slightly increased in AD (with VIP values ranged between 1.9 and 2.1). However, individual TG species were not identified, since overexpression was found in the whole of these lipids (Fig. 2). The reproducibility of the method was good, with coefficients of variation measured in QC samples below 20 % for most the metabolites identified. Furthermore, it should be noted that all these compounds presented AUC values higher (or close) than 0.7, indicative of good diagnostic power.

Discussion

Potential of direct infusion mass spectrometry for serum metabolomics

Despite the considerable number of previous metabolomic studies focused on the investigation of Alzheimer's disease, there is still a lack of understanding about pathological processes underlying to this neurodegenerative disorder. In order to deal with the high complexity of the metabolome, different complementary platforms have been applied in this field. NMR-based metabolomics has been traditionally employed due to its potential for high-throughput fingerprinting and minimal requirements for sample preparation [27, 28]. However, only medium to high abundance metabolites can be detected with this approach and the identification of individual metabolites is challenging in complex mixtures, so very limited metabolic information can be obtained [29]. Most authors used hyphenated approaches based on mass spectrometry for the elucidation of metabolic signatures associated with AD, including LC/MS, GC/MS, and CE/MS [16–18, 30, 31]. Furthermore, a few works also considered the combination of complementary platforms to extend the range of analyzable metabolites [14, 15, 32]. Nevertheless, the potential of serum metabolomics by direct infusion mass spectrometry is still unexplored in AD research.

Therefore, the aim of this work was the evaluation of a high-throughput DIMS-based methodology in terms of metabolite coverage, rapidity, and instrumental simplicity for the screening of serum metabolic abnormalities in AD. This fingerprinting tool enabled the analysis of multiple metabolites in a single run, including low molecular weight metabolites as well as both polar and neutral lipids,

Table 1 Discriminant phospholipids in serum from AD patients

Compound	Formula	MS fragments	Δm (ppm) P/N	Extract	FC	CV (%)	VIP	*P* value	AUC
Lyso-phospholipids									
LPC(16:0)	$C_{24}H_{50}NO_7P$	P: 480.318 ($-CH_3$), 255.23, 168.04	18.5	POL	1.25	7.3	2.14	$2.4 \cdot 10^{-2}$	0.70
LPC(18:1)	$C_{26}H_{52}NO_7P$	P: 522.352 ($+H^+$), 339.29, 184.07, 104.10, 86.09 N: 556.323 ($+Cl^-$), 281.25, 168.04	−6.5/9.9	POL	0.65	7.9	2.26	$2.6 \cdot 10^{-4}$	0.81
LPC(18:0)	$C_{26}H_{54}NO_7P$	P: 524.359 ($+H^+$), 341.31, 184.07, 104.10, 86.09 N: 558.337 ($+Cl^-$), 283.26, 168.04	−23.1/6.8	POL	0.75	14.6	2.15	$5.9 \cdot 10^{-4}$	0.79
LPC(20:5)	$C_{28}H_{48}NO_7P$	P: 564.311 ($+Na^+$), 359.26, 184.07, 104.10, 86.09	8.7	LIP	0.82	10.9	2.05	$3.1 \cdot 10^{-2}$	0.65
Phospholipids									
PPE(16:0/22:6)	$C_{43}H_{74}NO_7P$	N: 746.511 ($-H^+$), 327.23, 196.07	−2.7	LIP	0.85	19.7	2.09	$3.5 \cdot 10^{-2}$	0.68
PPE(18:1/20:4)	$C_{43}H_{76}NO_7P$	N: 748.521 ($-H^+$), 303.23, 196.07	−10.3	LIP	0.75	11.5	2.59	$1.5 \cdot 10^{-2}$	0.74
PPE(18:0/20:4)	$C_{43}H_{78}NO_7P$	N: 750.544 ($-H^+$), 303.23, 196.07	−0.4	LIP	0.84	9.7	2.10	$2.9 \cdot 10^{-2}$	0.68
PPE(18:1/22:6)	$C_{45}H_{76}NO_7P$	N: 772.523 ($-H^+$), 327.23, 196.07	−7.4	LIP	0.85	23.2	2.23	$1.3 \cdot 10^{-2}$	0.67
PPE(18:0/22:6)	$C_{45}H_{78}NO_7P$	N: 774.528 ($-H^+$), 327.23, 196.07	−21.0	LIP	0.82	13.5	3.34	$3.0 \cdot 10^{-2}$	0.69
PC(16:0/18:3)	$C_{42}H_{78}NO_8P$	P: 778.551 ($+Na^+$), 313.27, 335.26, 184.07, 104.10, 86.09	6.8	POL	1.53	6.2	2.21	$4.7 \cdot 10^{-4}$	0.83
PC(16:0/18:2)	$C_{42}H_{80}NO_8P$	P: 780.544 ($+Na^+$), 313.27, 337.27, 184.07, 104.10, 86.09 N: 792.549 ($+Cl^-$), 255.23, 279.23, 168.04	−9.5/21.9	POL	1.70	12.3	2.68	$8.0 \cdot 10^{-6}$	0.89
PC(16:0/18:1)	$C_{42}H_{82}NO_8P$	P: 782.563 ($+Na^+$), 313.27, 339.29, 184.07, 104.10, 86.09 N: 794.556 ($+Cl^-$), 255.23, 281.25, 168.04	−5.1/11.1	POL	1.47	13.6	2.24	$9.7 \cdot 10^{-4}$	0.80
PC(16:0/18:0)	$C_{42}H_{84}NO_8P$	P: 784.573 ($+Na^+$), 313.27, 341.31, 184.07, 104.10, 86.09	−12.4	POL	1.27	4.4	2.21	$2.6 \cdot 10^{-2}$	0.70
PC(16:0/20:5)	$C_{44}H_{78}NO_8P$	P: 802.540 ($+Na^+$), 313.27, 359.26, 184.07, 104.10, 86.09 N: 814.541 ($+Cl^-$), 255.23, 301.22, 168.04	5.4/30.8	LIP	0.53	23.8	2.45	$1.9 \cdot 10^{-3}$	0.82
PC(18:2/18:2)	$C_{44}H_{80}NO_8P$	P: 804.549 ($+Na^+$), 337.27, 184.07, 104.10, 86.09	−3.0	POL	1.77	10.1	2.36	$2.2 \cdot 10^{-4}$	0.84
PC(18:2/18:1)	$C_{44}H_{82}NO_8P$	P: 806.565 ($+Na^+$), 337.27, 339.29, 184.07, 104.10, 86.09 N: 818.559 ($+Cl^-$), 279.23, 281.25, 168.04	−2.5/14.4	POL	1.70	11.8	2.41	$2.2 \cdot 10^{-4}$	0.84
PC(18:2/18:0)	$C_{44}H_{84}NO_8P$	P: 808.581 ($+Na^+$), 337.27, 341.31, 184.07, 104.10, 86.09 N: 820.575 ($+Cl^-$), 279.23, 283.26, 168.04	−2.1/14.7	POL	1.44	2.4	2.07	$4.0 \cdot 10^{-3}$	0.77
PPC(16:0/22:6)	$C_{46}H_{80}NO_7P$	P: 812.562 ($+Na^+$), 385.27, 184.07, 104.10, 86.09	6.8	LIP	0.81	6.2	2.63	$1.1 \cdot 10^{-3}$	0.78
PPC(16:0/22:5)	$C_{46}H_{82}NO_7P$	P: 814.563 ($+Na^+$), 387.29, 184.07, 104.10, 86.09	−11.2	LIP	0.85	6.6	2.02	$2.7 \cdot 10^{-2}$	0.75
PPC(18:1/22:6)	$C_{48}H_{82}NO_7P$	P: 816.584 ($+H^+$), 385.27, 184.07, 104.10, 86.09	−7.6	LIP	0.83	12.3	2.54	$1.7 \cdot 10^{-3}$	0.83
PC(16:0/22:6)	$C_{46}H_{80}NO_8P$	P: 828.552 ($+Na^+$), 313.27, 385.27, 184.07, 104.10, 86.09 N: 840.553 ($+Cl^-$), 255.23, 327.23, 168.04	0.7/25.5	LIP	0.68	11.5	2.31	$1.7 \cdot 10^{-2}$	0.74
PC(18:1/20:4)	$C_{46}H_{82}NO_8P$	P: 830.568 ($+Na^+$), 339.29, 361.27, 184.07, 104.10, 86.09 N: 842.561 ($+Cl^-$), 281.25, 303.23, 168.04	1.2/16.4	LIP	0.73	13.8	2.48	$1.6 \cdot 10^{-3}$	0.80
PC(18:0/22:6)	$C_{48}H_{84}NO_8P$	P: 856.581 ($+Na^+$), 341.31, 385.27, 184.07, 104.10, 86.09	−2.0	LIP	0.78	21.2	2.19	$3.8 \cdot 10^{-2}$	0.71

Δm mass error, *FC* fold change, *CV* coefficient of variation in QCs, *VIP* variable importance in the projection, *AUC* area under the curve, *P* positive mode, *N* negative mode, *POL* polar extract, *LIP* lipophilic extract, *LPC* lyso-phosphocholine, *PC* phosphocholine, *PPC* choline-plasmalogen, *PPE* ethanolamine-plasmalogen

Table 2 Discriminant low molecular weight metabolites in serum from AD patients

Compound	Formula	MS fragments	Δm (ppm) P/N	Extract	FC	CV (%)	VIP	*P* value	AUC
Caprylic acid	$C_8H_{16}O_2$	N: 143.110 ($-H^+$)	15.4	LIP	1.27	12.1	2.05	$3.8{\cdot}10^{-2}$	0.71
Capric acid	$C_{10}H_{20}O_2$	N: 171.138 ($-H^+$)	−6.4	LIP	1.29	11.1	2.03	$2.8{\cdot}10^{-2}$	0.69
Lauric acid	$C_{12}H_{24}O_2$	N: 199.174 ($-H^+$)	18.1	LIP	1.45	17.6	2.11	$2.2{\cdot}10^{-2}$	0.75
Myristic acid	$C_{14}H_{28}O_2$	N: 227.201 ($-H^+$)	−3.1	LIP	1.47	15.2	2.25	$6.2{\cdot}10^{-3}$	0.74
Palmitoleic acid	$C_{16}H_{30}O_2$	N: 253.222 ($-H^+$)	18.6	LIP	1.68	6.7	2.13	$2.9{\cdot}10^{-2}$	0.70
Palmitic acid	$C_{16}H_{32}O_2$	N: 255.236 ($-H^+$)	11.8	LIP	1.22	10.1	2.04	$2.8{\cdot}10^{-2}$	0.68
Linoleic acid	$C_{18}H_{32}O_2$	N: 279.238 ($-H^+$)	17.9	LIP	1.52	12.8	2.03	$1.9{\cdot}10^{-2}$	0.69
Docosahexaenoic acid	$C_{22}H_{32}O_2$	N: 327.237 ($-H^+$)	12.2	LIP	0.78	13.2	2.12	$1.1{\cdot}10^{-2}$	0.72
Leukotriene B4	$C_{20}H_{32}O_4$	P: 301.209 ($M+H^+-2H_2O$), 283.22, 189.16, 119.10, 105.08	−27.6	LIP	1.16	23.1	2.07	$3.4{\cdot}10^{-2}$	0.69
Prostaglandin	$C_{20}H_{30}O_4$	P: 317.199 ($M+H^+-H_2O$), 299.21, 185.08	−40.0	LIP	1.19	18.1	2.02	$2.0{\cdot}10^{-2}$	0.71
Choline	$C_5H_{13}NO$	P: 104.105 ($+H^+$), 60.08	−19.2	POL	1.26	8.5	2.30	$2.8{\cdot}10^{-2}$	0.72
Valine	$C_5H_{11}NO_2$	P: 118.084 ($+H^+$), 72.08, 55.05	−19.4	POL	0.63	4.8	2.17	$2.8{\cdot}10^{-3}$	0.76
Creatine	$C_4H_9N_3O_2$	P: 132.073 ($+H^+$), 90.05	−28.8	POL	0.66	8.8	2.07	$2.3{\cdot}10^{-3}$	0.78
Glutamine	$C_5H_{10}N_2O_3$	P: 147.080 ($+H^+$), 130.05, 84.05	−2.7	POL	0.88	7.8	2.11	$2.8{\cdot}10^{-3}$	0.72
Glutamate	$C_5H_9NO_4$	P: 148.056 ($+H^+$), 130.05, 102.05, 84.05	−29.7	POL	0.84	6.2	2.35	$1.0{\cdot}10^{-2}$	0.68
Dopamine	$C_8H_{11}NO_2$	P: 154.082 ($+H^+$), 137.06, 91.05	−27.9	POL	0.89	9.7	2.03	$3.6{\cdot}10^{-2}$	0.67
Histidine	$C_6H_9N_3O_2$	N: 154.061 ($-H^+$), 137.04, 93.04	−7.8	POL	0.81	10.1	2.99	$4.9{\cdot}10^{-3}$	0.72
Carnitine	$C_7H_{15}NO_3$	P: 162.116 ($+H^+$), 103.04, 85.02, 60.08	21.6	POL	0.76	5.2	2.26	$8.7{\cdot}10^{-3}$	0.76
Arginine	$C_6H_{14}N_4O_2$	P: 175.115 ($+H^+$), 158.09, 116.07, 70.06, 60.05 N: 173.108 ($-H^+$), 131.09	−22.8/20.8	POL	0.84	8.7	2.98	$5.5{\cdot}10^{-3}$	0.74
N-acetyl glutamine	$C_7H_{12}N_2O_4$	P: 189.082 ($+H^+$), 172.06, 130.05, 84.05	−26.4	POL	0.89	9.8	2.11	$3.1{\cdot}10^{-2}$	0.72
Glucose	$C_6H_{12}O_6$	P: 203.049 ($+Na^+$), 85.02, 61.02 N: 215.035 ($+Cl^-$), 71.01, 59.01	−17.7/10.2	POL LIP	1.24	10.4	2.47	$1.8{\cdot}10^{-4}$	0.86
GPCh	$C_8H_{20}NO_6P$	P: 280.091 ($+Na^+$), 104.02, 86.08	−3.6	POL	1.46	5.3	2.02	$1.0{\cdot}10^{-2}$	0.73

Δm mass error, *FC* fold change, *CV* coefficient of variation in QCs, *VIP* variable importance in the projection, *AUC* area under the curve, *P* positive mode, *N* negative mode, *POL* polar extract, *LIP* lipophilic extract, *GPCh* glycerophosphocholine

as summarized in Tables 1 and 2. Furthermore, the lack of a time-consuming separation step before MS detection allows faster analysis of samples (less than 30 s for DIMS against several minutes for other methods published elsewhere), which reduces instrumental drift along batch analysis and consequently increases intersample reproducibility, improving the accuracy of subsequent data analysis [29]. In this sense, quality of statistical models built in this study was comparable or higher to those previously described in other works, with a variance explained (R^2) around 99 % and variance predicted (Q^2) above 50 %. Finally, it is also noteworthy that direct infusion of samples facilitates the experimental design of metabolomic studies, in terms of greater instrumental simplicity and easier data processing. Problems associated with gradual deterioration or clogging of chromatographic columns or electrophoretic capillaries are avoided, which increases robustness of the analytical procedure and reduces costs. In addition, significant effort and expertise is required for preprocessing of data from conventional profiling methods, which proceeds through multiple stages including feature detection, alignment of peaks, and normalization [33], while preprocessing of DIMS data is limited to peak filtration. However, it should be noted that DIMS also presents important drawbacks associated with the lack of

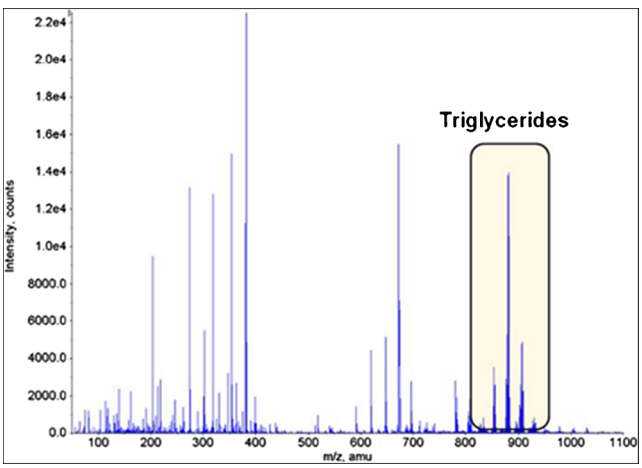

Fig. 2 Triglycerides from lipophilic extracts, increased in AD

resolution for the differentiation of isobars and difficulty of quantification due to ion suppression. In order to overcome problems associated with isobaric interferences, the use of high-resolution systems has become the main workhorse for accurate MS-fingerprinting, including time-of-flight (TOF), Fourier transform ion cyclotron resonance (FTICR), and especially the hybrid system Q-TOF, which allows more accurate mass measurement than single TOF instruments and structural elucidation by MS/MS experiments [34]. On the other hand, although ion suppression is a potential problem in any MS-based metabolomic platform, there is no evidence that it presents a more detrimental effect on flow infusion fingerprinting than in hyphenated approaches [13]. Anyway, the large amount of different compounds in biological samples helps to regulate matrix effects, so that ion suppression becomes a constant factor imposed uniformly in all samples, as previously described in the "Results" section.

In summary, direct infusion mass spectrometry stands out as a suitable tool for fast and comprehensive "first pass" screening of metabolic abnormalities. This fingerprinting methodology was successfully applied to a cohort study in serum samples from Alzheimer's disease patients and healthy controls, demonstrating the involvement of numerous discriminant metabolites as discussed in the next section.

Metabolic abnormalities associated with Alzheimer's disease

The high-throughput metabolomic approach based on direct infusion mass spectrometry allowed the elucidation of numerous metabolic abnormalities, which may help to understand the biochemical processes and pathology associated with AD. Therefore, metabolites listed in Tables 1 and 2 can be classified into different groups, based on the biochemical pathway dysregulated in AD, which are discussed as follows.

Membrane destabilization

Abnormalities in lipids from membrane are well-known processes previously described in brains from AD and other neurodegenerative diseases [35, 36]. These disorders are related to anomalous metabolism of phospholipids originated by a complex sequence of cellular events in hypoxic neurons, which involve release of glutamate and influx of large amounts of calcium into neurons. As a consequence, pathological overactivation of catabolic enzymes is induced, principally phospholipase A_2 [37]. This enzymatic stimulation causes phosphatidylcholine (PC) degeneration, yielding lyso-phosphatidylcholines (LPC), which rapidly hydrolyze to glycerophosphocholine (GPCh), phosphocholine (PCh), and, finally, choline (Ch), with the release of free fatty acids (FFAs) [38].

Thereby, it has been reported that membrane breakdown in AD leads to decreased total levels of phosphocholines and the accumulation of their degradation products in brain and CSF samples [39, 40]. However, although our experimental data showed increased levels of GPCh and Ch in AD (Table 2), alterations in LPC and PC are not consistent with this rationale, since the behavior of these compounds is not similar for all of them, and depends on the type o fatty acid linked to the molecular moiety, decreasing polyunsaturated fatty acid (PUFA) phosphatidylcholines and increasing saturated fatty acid (SFA) containing ones (Table 1). This fact could be related to Conquer et al.'s findings [41] who reported low levels of PUFA-containing phospholipids in plasma of AD patients, causing membrane destabilization and contributing to AD pathogenesis. More recently, Mapstone et al. [42] found a similar decrease of plasma phospholipids with a high unsaturation degree, corroborating this hypothesis. In addition, this downregulation of unsaturated phospholipids is consistent with the reduction of free PUFAs associated with this disorder [43], which can contribute to membrane damage given that neuronal phosphocholines are rich in PUFAs, principally docosahexaenoic (DHA) and araquidonic acid (AA). However, the increase of saturated fatty acid phosphocholines in serum from AD patients has not been previously reported to our knowledge, which could be a complementary process consistent with destabilization of neuronal membranes.

A similar behavior can be observed with lyso-phosphatidylcholines, which show increased levels of saturated respect unsaturated fatty acid compounds (Table 1). Only a few reports can be found about these lipids in AD, in which lower total concentrations were found [44], attributed to overactivation of lyso-phospholipid acyltransferase, which recycles lyso-phospholipids produced in membrane breakdown into phospholipids [45]. However, any of these works have established distinctions between the nature of bound fatty acids (saturated or unsaturated) in relation to neuronal membrane stability, as found in our analyses.

Additional data that confirms this hypothesis is provided by the altered levels of FFAs found in the ESI(−) analysis. Numerous studies demonstrated that long-chain n-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are inversely correlated with the development of Alzheimer's disease [46], which agrees with the lower levels of DHA in AD shown in Table 2. On the other hand, it has long been known that total free fatty acid levels increase during hypoxia [47]. Moreover, more recently, it has been shown that high levels of free fatty acids induce amyloid deposition and tau hyperphosphorylation, contributing to pathogenesis of AD [48]. This fact, associated with the mentioned process of membrane destabilization releasing fatty acids, could explain that whole saturated FFAs are increased in AD. However, previous studies in brain, serum, or plasma from Alzheimer's disease patients provided

contradictory results about levels of fatty acids [32, 49–51], so the interpretation of these changes requires some caution.

Finally, another family of compounds related with the membrane breakdown process are plasmalogens, which are a major phospholipid constituent of human neural membranes and serve as reservoir of prostaglandin and thromboxane precursors, and in addition, are precursors of an analog of platelet-activating factor, and may act as antioxidants. Previously, low levels of ethanolamine plasmalogens have been reported in brain and serum from Alzheimer's disease patients [52, 53], as well as decreased choline plasmalogens in brain [54]. Similarly, a decrease in plasmalogens is observed in our results, both in ethanolamine and choline plasmalogens (Table 1).

In this way, we can conclude that not only overactivation of phospholipases causes membrane destabilization processes, well known from long ago, but also imbalance in the levels of saturated/unsaturated fatty acids contained in the structure of phospholipids are involved in this abnormal situation, leading to cell apoptosis. Possibly, the decrease of PUFA-containing phosphocholines suggests the implication of oxidative stress in the progressive degradation of brain phospholipids in AD, since they are rich in readily oxidizable arachidonic and docosahexaenoic acids. On the other hand, their replacement by saturated ones may be consistent with destabilization of neuronal membranes, since PUFAs play important roles in the membrane structure and permeability [55]. These facts agree with our experimental findings that show low levels of PUFAs-containing phospholipids and plasmalogens in AD; and high concentrations of degradation products as GPCh, Ch, and FFA, as well as SFA-containing phospholipids. Therefore, the potential of blood serum samples have been demonstrated to investigate these cerebral biochemical changes since, to date, there are only a few studies of these metabolites in this matrix [30, 56, 57].

Oxidative stress

Because of its high-metabolic rate, the brain is particularly susceptible to reactive oxygen species (ROS) [2]. The free radical species can attack neuronal lipids, proteins, and nucleic acids, which inevitably leads to neuronal dysfunction. Thus, oxidative stress is another important process involved in AD, which explains decreased levels of antioxidant compounds in patients with the disease, such as vitamins and carotenoids [58], and others [59]; in addition, increased markers of protein, lipid, and nucleic acid oxidation have been also found [60–63]. The results in Table 2 show decreased levels of several antioxidant compounds in AD, as the amino acid histidine [64] and the quaternary ammonium compound carnitine, this later with neuroprotective effects [65]. Furthermore, the occurrence of high lipid oxidation can be supported by the increase in eicosanoids levels (prostaglandins and leukotrienes), produced by oxidation of araquidonic acid, traditional markers of oxidative stress [66].

Hypometabolism

The energy requirements of brain tissue are very high in order to maintain ion gradients across the plasma membrane, being the oxidative phosphorylation the main energy source, due to the limited glycolytic capacity of neurons. However, the metabolism of energy production is depleted in AD, provoking reductions in the cerebral metabolic rate of glucose [67] and mitochondrial dysfunction [3]. The low glucose consumption produces high levels of this compound in AD, as have been found in both ESI^+ and ESI^- analysis, using polar and lipophilic extracts. On the other hand, decreases in creatine and carnitine were also found in relation to this situation of abnormal energetic metabolism. Creatine plays a fundamental role in energy buffering and overall cellular bioenergetics through the creatine kinase/phosphocreatine system, being responsible for the transfer of energy from mitochondria to cytosol [68]. In the case of carnitine, several physiological functions can be highlighted, such as transport of fatty acids into mitochondria for β-oxidation, oxidation of pyruvate, and others [69]. Thus, low levels of these compounds could be considered as indicative of mitochondrial impairment.

Neurotransmitters alterations

Previous studies indicated the involvement of neurotransmitters in pathology of AD, since their synthesis pathways are altered, causing synaptic failure that leads to neurodegeneration. Several systems are dysregulated, such as cholinergic [70], glutamatergic [71], GABAergic [72], serotonergic [73], or dopaminergic [74] systems, with a low occurrence of corresponding neurotransmitters. These results are supported by results from Table 2 that show decreased levels of certain neurotransmitters in AD. Thus, we can observe decreased levels of glutamate and related metabolites such as glutamine (formed in a process responsible for detoxifying ammonia from brain) or *N*-acetyl glutamine (acetylated analogue of glutamine), which are in accordance to previous results obtained in transgenic mice brains [75]. In addition, other neurotransmitter reduced in AD was dopamine, well known to be related to Parkinson's disease, but also disturbed in AD samples, indicating changes in the catecholamine metabolism [64].

Vascular risk

In the present study, we find slightly increased levels of triglycerides in AD (Fig. 2) that, together with the large amount of saturated free fatty acid previously commented, suggests a situation of hyperlipidemia, one of the most

important vascular risk factors. Vascular disorders can affect the cerebrovascular system, causing atrophy, structural changes in the blood–brain barrier, inflammation, etc. These changes lead to decreased cerebral blood flow, which finally involves neuronal cell loss. Thus, AD and vascular diseases are closely related [76]. In this sense, several studies have reported the relationships between Alzheimer's disease and high levels of lipids, principally triglycerides and cholesterol [77].

Other biomarkers

Other potential biomarkers detected were amino acids valine and arginine, both decreased in AD samples (Table 2). While arginine is involved in processes of detoxification of nitrogen, valine is a branched-chain amino acid that supplies energy to the muscles during intense physical activity. Only a few works study the levels of these metabolites in AD, but no clear conclusion can be drawn [64, 78, 79], so further studies have to be performed in order to confirm these results.

Conclusions

The use of a metabolomic approach based on direct infusion mass spectrometry gave fast and simple fingerprinting of blood serum samples, suitable for the study of Alzheimer's disease. Application of statistical analysis (PLS-DA) allowed a clear separation between samples from AD patients and healthy controls, and in addition some altered compounds could be identified. These compounds were found to participate in several cellular processes involved in AD. The most important findings were observed in relation to membrane breakdown processes, in which both phospholipids (phosphocholines and plasmalogens) as well as their degradation products (lyso-phosphocholines and free choline) or free fatty acids are involved. Other metabolites indicated alterations in energy metabolism (e.g., glucose and creatine) or neurotransmission (e.g., glutamate and dopamine), or could be related to pathological situations such as oxidative stress (e.g., histidine and prostaglandins) and vascular risk (triglycerides). Thus, this study supposes a holistic approximation to Alzheimer's disease, suitable for understanding the biochemical pathology underlying this disorder. Moreover, it should be noted that some of these discriminant metabolites have not been previously described in AD to our knowledge, such as high serum levels of phospholipids containing saturated fatty acids (PC and LPC), respectively, polyunsaturated ones, and the high concentration of whole free fatty acids. Finally, the investigation in serum samples instead of other matrix more studied as brain or CSF (more representative of the neurodegenerative status) is of great interest, enabling future clinical application. The main strength of this study is the careful selection of participants in order to match experimental groups on demographic factors (age, sex, and 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. However, the small cohort size employed limits the potential of this pilot study, so 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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