

Manuscript Number: JPBA-D-14-01586R1

Title: APPLICATION OF METABOLOMICS BASED ON DIRECT MASS SPECTROMETRY ANALYSIS FOR THE ELUCIDATION OF ALTERED METABOLIC PATHWAYS IN SERUM FROM THE APP/PS1 TRANSGENIC MODEL OF ALZHEIMER'S DISEASE

Article Type: Full Length Article

Keywords: Metabolomics; APP/PS1 mice; DI-ESI-MS; FI-APPI-MS; Alzheimer's disease

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Abstract: Metabolomic analysis of brain tissue from transgenic mouse models of Alzheimer's disease has demonstrated a great potential for the study of pathological mechanisms and the development of new therapies and biomarkers for diagnosis. However, in order to translate these investigations to the clinical practice it is necessary to corroborate these findings in peripheral samples. To this end, this work considers the application of a novel metabolomic platform based on the combination of a two-steps extraction procedure with complementary analysis by direct infusion electrospray mass spectrometry and flow infusion atmospheric pressure photoionization mass spectrometry for a holistic investigation of metabolic abnormalities in serum samples from APP/PS1 mice. A number of metabolites were found to be perturbed in this mouse model, including increased levels of di- and tri-acylglycerols, eicosanoids, inosine, choline and glycerophosphoethanolamine; reduced content of cholesteryl esters, free fatty acids, lysophosphocholines, amino acids, energy-related metabolites, phosphoethanolamine and urea, as well as abnormal distribution of phosphocholines depending on the fatty acid linked to the molecular moiety. This allowed the elucidation of possible pathways disturbed underlying to disease (abnormal homeostasis of phospholipids leading to membrane breakdown, energy-related failures, hyperammonemia and hyperlipidemia, among others), thus demonstrating the utility of peripheral samples to investigate pathology in the APP/PS1 model.

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Dear Editor,

Please find enclosed the revised version of the manuscript entitled:
APPLICATION OF METABOLOMICS BASED ON DIRECT MASS
SPECTROMETRY ANALYSIS FOR THE ELUCIDATION OF ALTERED
METABOLIC PATHWAYS IN SERUM FROM THE APP/PS1 TRANSGENIC
MODEL OF ALZHEIMER'S DISEASE, by Raúl González-Domínguez, Tamara
García-Barrera, Javier Vitorica and José Luis Gómez-Ariza

The different comments and suggestions from the referees have been considered and
included in the new version.

Yours sincerely,

Prof. José Luis Gómez Ariza

Reviewer #1:

The Title - APPLICATION OF METABOLOMICS BASED ON DIRECT MASS SPECTROMETRY ANALYSIS FOR THE ELUCIDATION OF ALTERED METABOLIC PATHWAYS IN SERUM FROM THE APP/PS1 TRANSGENIC MODEL OF ALZHEIMER'S DISEASE - seems accurate and appropriate.

Abstract- Acceptable, including graphic

Highlights - Overall not acceptable.

Ist statement (Combined DI-ESI-MS and FI-APPI-MS provides comprehensive metabolic fingerprinting) cannot be proved, as the size and composition of the mouse serum metabolome is not known how can the authors make the hubristic statement that they are providing comprehensive metabolic fingerprinting?

The second statement is incomplete and requires the addition of disease to the Alzheimer at the end (Samples from APP/PS1 mice allows investigating pathological mechanisms of Alzheimer....) Also, as the disease in mouse is NOT the same as the human disease, but a MODEL of it, they cannot say emphatically that this model allows pathological mechanisms of Alzheimers disease to be investigated without a LOT more proof that it accurately conforms to the human condition.

The 3rd statement (We detected alterations in energy metabolites, amino acids and numerous lipids) is probably correctly based on the data produced by the study.

The 4th statement (APP/PS1 mice stand out as a suitable model for studying Alzheimer's disease) is not fully supported by the translational data required showing the equivalence of these mice with human disease.

Highlights were rewritten.

Keywords - acceptable

Abbreviations -acceptable

Introduction - reasonably comprehensive, and whilst I do not necessarily agree with all of the statements made I am aware that this represents differences of opinion, and not necessarily errors on the part of the authors! Overall acceptable.

Experimental. How was the serum obtained (i.e were blood samples left to clot at room temperature or in the fridge, for how long, how were the formed components of the blood removed in terms of centrifugation. Were samples stored frozen until prepared for analysis and if so at what temperature?

The procedure for obtaining serum samples is described in section 2.1 (Animal Handling).

What was the ratio of ethanol/methanol used for precipitation.

The ratio of the mixture methanol/ethanol was 1:1 (v/v).

Centrifugations conditions are given in rpm, this is not acceptable either, g or RCF must be provided (or the centrifuge rotor size).

Centrifugation conditions have been changed ($\times g$).

It would be good to have a statement indicating that the samples were randomized before analysis, and also to indicate at what spacing the QC samples were during the run (every 10 samples?).

This statement has been introduced at the end of section 2.2.

The methods used for the identification of the metabolites are borderline in terms of the claims made being based on database searching (which is not a means of identification in metabolomics)

and mass spectrometric arguments (which are better) but there is no comparison with authentic standards spiked into representative samples. A good number of the compounds provisionally identified are in fact commercially available and good practice would have been to get them and perform a more convincing confirmatory analysis.

Low molecular weight metabolites were confirmed with commercial standards when available. However the identification of individual lipids only could be performed by means of MS/MS experiments, given that they are not available. This has been clarified in the revised manuscript.

Multivariate statistical data analysis seems acceptable.

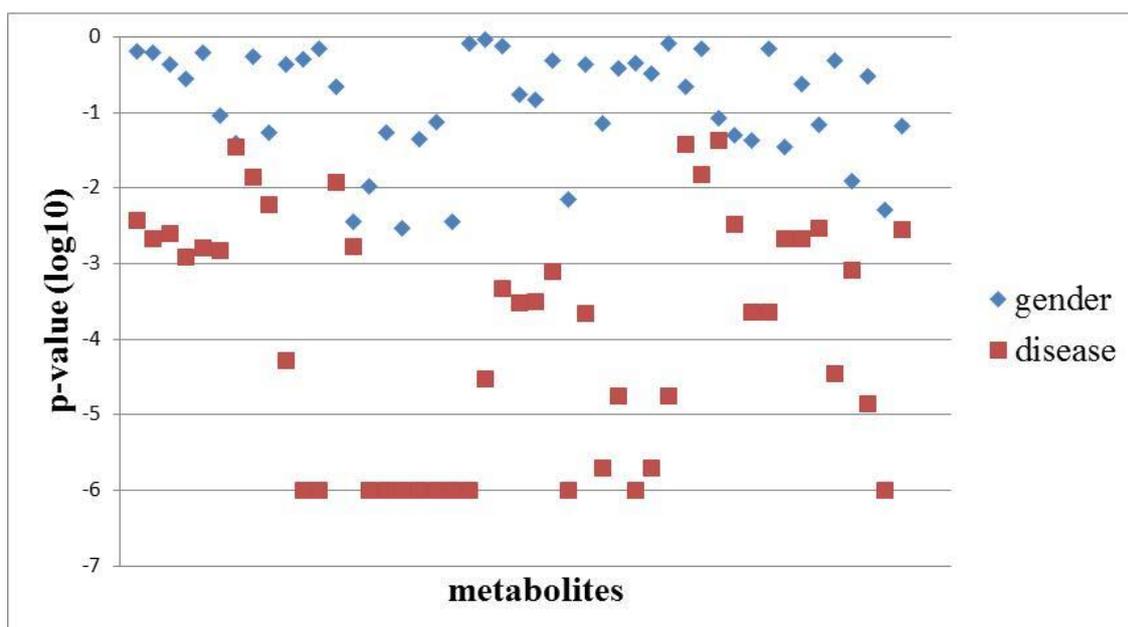
Results & Discussion - Given the lack of a complete metabolite list for serum, and the several orders of magnitude over which metabolites are known to be present I would dispute the authors claims of comprehensiveness made in the first paragraph of the R&D section as being unsupported. The best that they can claim is that they have taken pains to ensure that it is a comprehensive as they can achieve, but that is not the same thing. However, it is clear that the analytical methodology is working well, and the authors use of QCs in this context is to be commended, and good enough so as not to need excessive claims to be made.

We claimed that the methodology used in this work provides comprehensive metabolic profiles because we could detect alterations in multiple metabolites with very diverse physico-chemical properties (triglycerides, phospholipids, low molecular weight compounds) by using complementary ionization sources (ESI, APPI). Anyway, the first sentence of the R&D section has been changed in line with your comments:

A high-throughput metabolomic approach based on the combination of a two-steps extraction procedure with complementary analysis by DI-ESI-MS and FI-APPI-MS was used in this work to perform a rapid and comprehensive screening of serum metabolites in the APP/PS1 transgenic model.

We any gender-based differences noted (or indeed looked for?). The presence or absence of gender-related effects is worth a comment.

The evaluation of gender-specific metabolic changes in the APP/PS1 model was out of the scope of this manuscript. Anyway, we have performed t tests according to gender for discriminant metabolites presented in Table 1, and p-values obtained were much lower to those listed in the table for the comparison APP/PS1 vs. WT, evidencing that the effect of gender on metabolic profiles is much less important than the disease state (see attached figure).



It would be useful to have the %CV in the QC samples of the important metabolites identified in this work provided somewhere. The evidence is that the QCs are showing good concordance through the analysis, but this is a representation of the global metabolite profile, and does not mean that all the metabolites are behaving with the same degree of repeatability.

Values of CV (%) in QCs for discriminant metabolites have been computed and added to Table 1.

The discussion seems to me to be generally based on the findings, and whilst I may have different opinions on the significance of some of them I do think the authors are entitled to propose their interpretation of the results.

Conclusions. I do think that in the last sentence the authors claim too much for the model.

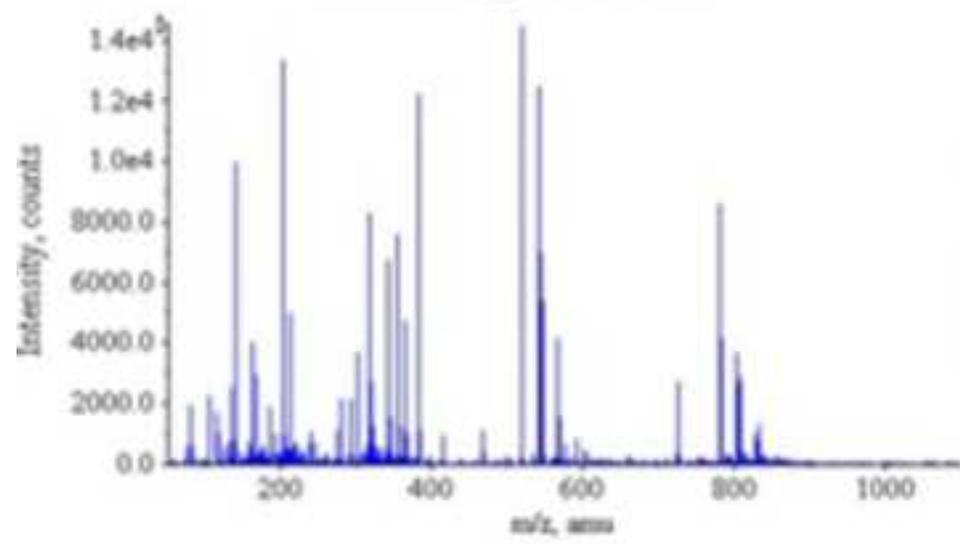
As mentioned along the entire Discussion section, we found numerous similarities between metabolic alterations detected in serum from APP/PS1 mice and previous metabolomic works with AD patients, evidencing that this transgenic mouse could be considered as a suitable model for studying AD pathology. The last sentence of the Conclusions section was rewritten in these terms.

References - comprehensive

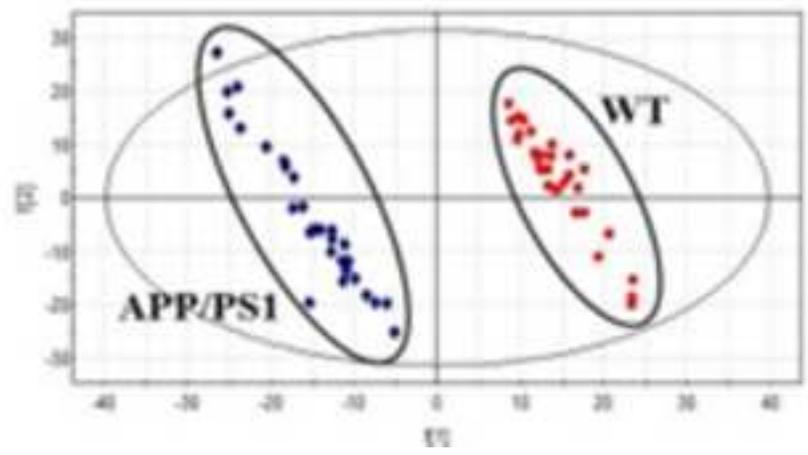
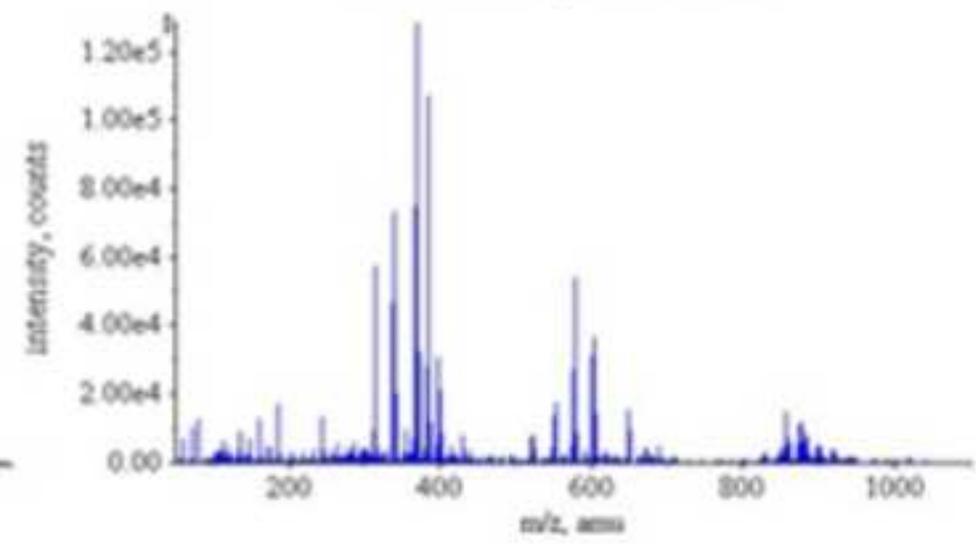
Table -acceptable

Figures - acceptable

DI-ESI-QTOF-MS



FI-APPI-QTOF-MS



APPLICATION OF METABOLOMICS BASED ON DIRECT MASS SPECTROMETRY ANALYSIS FOR THE ELUCIDATION OF ALTERED METABOLIC PATHWAYS IN SERUM FROM THE APP/PS1 TRANSGENIC MODEL OF ALZHEIMER'S DISEASE

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

- Serum metabolic profiles of APP/PS1 mice were characterized by combined ESI/APPI-MS
- Alterations in energy metabolites, amino acids and lipids were detected
- APP/PS1 mice allow studying pathological mechanisms as model of Alzheimer disease

1 **APPLICATION OF METABOLOMICS BASED ON DIRECT MASS SPECTROMETRY**
2 **ANALYSIS FOR THE ELUCIDATION OF ALTERED METABOLIC PATHWAYS IN SERUM**
3 **FROM THE APP/PS1 TRANSGENIC MODEL OF ALZHEIMER'S DISEASE**

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21
22 **ABSTRACT**

23 Metabolomic analysis of brain tissue from transgenic mouse models of Alzheimer's disease has
24 demonstrated a great potential for the study of pathological mechanisms and the development of new
25 therapies and biomarkers for diagnosis. However, in order to translate these investigations to the clinical
26 practice it is necessary to corroborate these findings in peripheral samples. To this end, this work
27 considers the application of a novel metabolomic platform based on the combination of a two-steps
28 extraction procedure with complementary analysis by direct infusion electrospray mass spectrometry and
29 flow infusion atmospheric pressure photoionization mass spectrometry for a holistic investigation of
30 metabolic abnormalities in serum samples from APP/PS1 mice. A number of metabolites were found to
31 be perturbed in this mouse model, including increased levels of di- and tri-acylglycerols, eicosanoids,
32 inosine, choline and glycerophosphoethanolamine; reduced content of cholesteryl esters, free fatty acids,
33 lysophosphocholines, amino acids, energy-related metabolites, phosphoethanolamine and urea, as well as
34 abnormal distribution of phosphocholines depending on the fatty acid linked to the molecular moiety.
35 This allowed the elucidation of possible pathways disturbed underlying to disease (abnormal homeostasis
36 of phospholipids leading to membrane breakdown, energy-related failures, hyperammonemia and
37 hyperlipidemia, among others), thus demonstrating the utility of peripheral samples to investigate
38 pathology in the APP/PS1 model.

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40 **KEYWORDS.** Metabolomics, APP/PS1 mice, DI-ESI-MS, FI-APPI-MS, Alzheimer's disease

41
42 **ABBREVIATIONS.** AD: Alzheimer's disease; TG: transgenic; WT: wild type; QC: quality control; DI-
43 ESI-MS: direct infusion electrospray mass spectrometry; FI-APPI-MS: flow infusion atmospheric
44 pressure photoionization mass spectrometry; PCA: principal component analysis; PLS-DA: partial least
45 squares discriminant analysis; VIP: variable importance in the projection; ROC: receiver operating
46 characteristic analysis; AUC: area under the curve; CV: coefficient of variation; PE:
47 phosphoethanolamine; GPE: glycerophosphoethanolamine; HEPE: hydroxy-eicosapentaenoic acid; PG:
48 prostaglandin; LTB4: leukotriene B4; LPC: lyso-phosphocholine; PC: phosphocholine; DAG:
49 diacylglycerol; CE: cholesteryl ester; TAG: triacylglycerol.

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1. INTRODUCTION

Animal models are nowadays one of the most important tools in biomedical research for the investigation of disease pathophysiology, the discovery of potential biomarkers and the *in vivo* evaluation of novel therapeutic treatments [1]. The ability to create transgenic models has had a profound impact on areas such as Alzheimer's disease (AD) research, where genetics plays a prominent role. Thereby, the discovery of genes associated with pathology of this disorder, such as amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2) genes, has allowed the development of numerous mouse models for studying AD [2]. These models offer an outstanding opportunity to investigate the early pathological disease mechanisms, for which the application of metabolomic techniques presents a great potential. Metabolomics, defined as the comprehensive measurement of metabolites from a biological system in a high-throughput manner, allows monitoring disease perturbations because alterations in specific groups of metabolites may be sensitive to pathogenically relevant factors, reflecting changes downstream of genomic, transcriptomic and proteomic fluctuations. Furthermore, since metabolic pathways are conserved through evolution, and are essentially similar in rodents and humans, the metabolic signatures identified in AD mouse models could be directly translated into human studies [3]. Metabolomic analysis can be performed in a variety of easily accessible biofluids such as cerebrospinal fluid, blood or urine, thus highlighting the clinical utility of this approach. However, although numerous studies have been performed to assess the pathophysiological status of transgenic animals by postmortem analysis of brain tissue samples [4-9], the use of peripheral samples has been only scarcely considered. In this sense, Graham et al. analyzed comparatively brain and plasma samples from APP/PS1 and wild type mice using ¹H-NMR with the aim of discovering new AD biomarkers [10]. The statistical models built using plasma profiles were more accurate than those obtained with brain tissue, despite the smaller number of metabolites detected, demonstrating the potential of blood metabolomics for non-invasive investigation of metabolic abnormalities underlying to AD. Similarly, metabolomic profiling of brain and plasma samples from the TASTPM mice also revealed a number of metabolites perturbed in both matrices, which could be related to altered metabolic pathways previously associated with human AD [11]. Jiang et al. found significant differences in endogenous metabolites from serum of the senescence-accelerated mouse, suggesting perturbed glucose and lipid metabolism, and attenuated protective function of inosine [12]. Moreover, metabolomics has been also applied to examine changes in urinary metabolites of transgenic AD mice, demonstrating the utility of a very simple and easily available sample in clinical laboratory as is urine for the search of potential biomarkers [13-15].

In this work we applied a novel metabolomic multi-platform based on direct mass spectrometry analysis for the characterization of metabolic abnormalities in serum from the APP/PS1 transgenic mice. These fingerprinting tools, based on direct introduction of sample extracts containing whole metabolites into the mass spectrometer, avoid the conventional time-resolved detection of metabolites after chromatographic or electrophoretic separation, improving analysis rapidity and reproducibility, non-targeted metabolite coverage and consequently, high-throughput screening capability [16]. Furthermore, the availability of complementary atmospheric pressure ionization sources may help to delve into the high physico-chemical complexity of the metabolome. Thus, the metabolomic approach used here comprises a multistage extraction procedure for releasing a broad range of metabolites from serum samples, combined with MS analysis in positive and negative ionization modes and the complementary use of electrospray (ESI) and atmospheric pressure photoionization (APPI). Direct infusion electrospray mass spectrometry (DI-ESI-MS) has proved to be a suitable tool for fast and comprehensive "first pass" screening of metabolic abnormalities, which has been successfully applied to AD research in previous investigations using human serum [17-19] and brain tissue from transgenic mice [5,6,9]. On the other hand, González-Domínguez et al. described in a recent work that flow injection atmospheric pressure photoionization mass spectrometry (FI-APPI-MS) may complement ESI-MS for the analysis of little polar or non-polar metabolites, and it is able to differentiate serum samples from AD patients and healthy control subjects [20]. Therefore, we hypothesized that the application of this high-throughput approach combining complementary ionization techniques to the APP/PS1 mice could provide a profound understanding about metabolic alterations detectable in a peripheral sample as is blood serum, which is of great interest to allow the study of disease pathology in a non-invasive manner and for the discovery of reliable biomarkers.

2. MATERIALS AND METHODS

2.1. ANIMAL HANDLING

121 Transgenic APP/PS1 mice (C57BL/6 background) were generated as previously described by Jankowsky
122 et al., expressing the Swedish mutation of APP together with PS1 deleted in exon 9 [21]. On the other
123 hand, age-matched wild-type mice of the same genetic background (C57BL/6) were purchased from
124 Charles River Laboratory for their use as controls. In this study, male and female animals at 6 months of
125 age were used for experiments (TG: N=30, male/female 13/17; WT: N=30, male/female 15/15). Animals
126 were acclimated for 3 days after reception in rooms with a 12-h light/dark cycle at 20-25 °C, with water
127 and food available *ad libitum*. Then, mice were anesthetized by isoflurane inhalation and blood was
128 extracted by cardiac puncture. Blood samples were immediately cooled in fridge and protected from light
129 for 30 minutes to allow clot retraction, and then centrifuged at 1500×g for 10 minutes at 4°C. Serum was
130 aliquoted in Eppendorf tubes and frozen at -80°C until analysis. Animals were handled according to the
131 directive 2010/63/EU stipulated by the European Community, and the study was approved by the Ethical
132 Committee of University of Huelva.

133 134 **2.2. SERUM PREPARATION**

135 Serum samples were extracted following a two-stage sequential procedure described elsewhere [18]. For
136 this, 100 µL of serum were mixed with 400 µL of methanol/ethanol (1:1, v/v) and stirred for 5 min,
137 followed by centrifugation at 2000×g for 10 min at 4°C. The supernatant was transferred to another tube,
138 and the precipitate was kept for further treatment. Then, supernatant was dried under nitrogen stream and
139 the resulting residue reconstituted with methanol/water (80:20, v/v) containing 0.1% formic acid (aqueous
140 extract). On the other hand, the precipitate isolated in the first step was extracted with 400 µL of
141 chloroform:methanol (1:1, v/v) by stirring during 5 min, followed by centrifugation at 13000×g for 10
142 minutes at 4 °C. Finally, the resulting supernatant was taken to dryness under nitrogen stream and
143 reconstituted with 100 microliters of dichloromethane:methanol (60:40, v/v) containing 10 mM
144 ammonium formate (organic extract). Furthermore, quality control (QC) samples were prepared by
145 pooling equal volumes of each sample and analyzed throughout the sequence run, which allows
146 monitoring instrumental drifts along the analysis period [22]. Samples were randomized before analysis,
147 and QC samples were injected every 10 individual serum samples.

148 149 **2.3. METABOLOMIC ANALYSIS**

150 Mass spectrometry experiments were performed in a quadrupole-time-of-flight mass spectrometer, model
151 QSTAR XL Hybrid system (Applied Biosystems, Foster City, CA, USA), using the electrospray (ESI)
152 and atmospheric pressure photoionization (APPI) sources according to the methods previously optimized
153 by González-Domínguez et al. [18,20]. For accurate mass measurement, the TOF analyzer was calibrated
154 before analyzing each batch of samples using renin and taurocholic acid as standards (in positive and
155 negative modes, respectively).

156 157 **2.3.1. METABOLOMIC ANALYSIS BY DI-ESI-MS**

158 Samples were introduced into the mass spectrometer at 5 µL min⁻¹ flow rate using an integrated apparatus
159 pump and a 1000 µL volume Hamilton syringe. Data were obtained in both positive and negative
160 ionization modes, acquiring full scan spectra for 0.2 minutes in the m/z range 50-1100 with 1.005 seconds
161 scan time. In positive mode, the ion spray voltage (IS) was set at 3300 V, and high-purity nitrogen was
162 used as curtain and nebulizer gas at flow rates about 1.13 L min⁻¹ and 1.56 L min⁻¹, respectively. The
163 source temperature was fixed at 60°C, with a declustering potential (DP) of 60 V and a focusing potential
164 (FP) of 250 V. For ESI(-) only few parameters were modified respect ESI(+) method, with an ion spray
165 voltage at -4000 V, a declustering potential (DP) of -100 V and a focusing potential (FP) of -250 V. To
166 acquire MS/MS spectra, nitrogen was used as collision gas.

167 168 **2.3.2. METABOLOMIC ANALYSIS BY FI-APPI-MS**

169 For FI-APPI analysis, serum samples were introduced by flow injection using an Accela LC system
170 (Thermo Fisher Scientific) equipped with autosampler and quaternary pump. In addition, a model KDS
171 100 syringe pump from KD scientific (New Hope, PA, USA) was employed to deliver the dopant for
172 photospray ionization. Methanol was used as flow injection solvent at 50/100 µl min⁻¹, in positive and
173 negative ion mode respectively, while toluene (dopant) was delivered at 20/40 µl min⁻¹, in both ionization
174 modes. Data were obtained both in positive and negative ion modes, injecting 10 µl of sample, and
175 acquiring full scan spectra in the m/z range 50-1100 with 1.005 seconds of scan time. The ion spray
176 voltage (IS) was set at 1500 V and -2300 V in positive and negative modes respectively, with declustering
177 potential (DP) of ±50 V and focusing potential (FP) of ±250 V. The source temperature was maintained at
178 400°C, and the gas flows (high-purity nitrogen) were fixed at 1.13 L min⁻¹ for curtain gas, 1.50 L min⁻¹
179 for nebulizer gas, 3.0 L min⁻¹ for heater gas, and 1 L min⁻¹ for lamp gas. To acquire MS/MS spectra,
180 nitrogen was used as collision gas.

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2.4. 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, all peaks above the noise level (10 counts, determined empirically from experimental spectra) were selected and binned in intervals of 0.1Da. Finally, data were normalized according to the total area sum. Then, data were subjected to multivariate analysis by partial least squares discriminant analysis (PLS-DA) in order to compare metabolomic profiles obtained, using the SIMCA-P™ software (version 11.5, UMetrics AB, Umeå, Sweden). 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 [23]. For this, data was submitted to Pareto scaling, for reducing the relative importance of larger values, and logarithmic transformation, in order to approximate a normal distribution. Quality of the models was assessed by the R^2 and Q^2 values, supplied by the software, which provide information about the class separation and predictive power of the model, respectively. These parameters are ranged between 0 and 1, and they indicate the variance explained by the model for all the data analyzed (R^2) and this variance in a test set by cross-validation (Q^2). Finally, potential biomarkers were selected according to the Variable Importance in the Projection (VIP: a weighted sum of squares of the PLS weight, which indicates the importance of the variable in the model) with confidence intervals derived from jack-knifing. Only variables with VIP values higher than 1.5 were considered, 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 below 0.7 [24].

2.5. METABOLITES IDENTIFICATION

Potential biomarkers were identified matching the experimental accurate mass and tandem mass spectra (MS/MS) with those shown in metabolomic databases (HMDB, METLIN and LIPIDMAPS), and then confirmed with commercial standards when available. On the other hand, the identity of individual lipids was confirmed based on characteristic fragmentation patterns previously described. Phosphatidylcholines presented characteristic ions in positive ionization mode at m/z 184.073, 104.107 and 86.096, and two typical fragments due to the loss of trimethylamine (m/z 59) and phosphocholine (m/z 183, 205 or 221, if the counterion is proton, sodium or potassium). In contrast, a distinctive signal was found at 168.041 in negative mode [25]. 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 [26]. Fragmentation of glycerolipids (di- and tri-acylglycerols) occurs through the release of fatty acids generating different types of ions (named A, B, C and D), which show characteristic m/z values according to the fatty acid attached to the glycerol backbone [27]. On the other hand, cholesteryl esters can be easily identified by means of an abundant fragment ion at m/z 369.351 produced upon collision induced dissociation [28]. Finally, acylcarnitines [29] and eicosanoids [30] were also confirmed with characteristic fragments described in the literature

3. RESULTS AND DISCUSSION

3.1. COMPARISON OF METABOLOMIC FINGERPRINTS

A high-throughput metabolomic approach based on the combination of a two-steps extraction procedure with complementary analysis by DI-ESI-MS and FI-APPI-MS was used in this work to perform a rapid screening of serum metabolites in the APP/PS1 transgenic model. In a previous study we reported that protein precipitation with organic solvents allows the extraction of hydrophilic metabolites from serum samples, but fails to extract the lipophilic components that may remain adsorbed to protein precipitate, so that a second extraction step is necessary to recover lipid compounds [18]. Furthermore, it was demonstrated that direct analysis of these extracts by ESI/APPI-MS provides a comprehensive snapshot of serum metabolites with high sensitivity and reproducibility, and without significant matrix-based ion suppression effects [18,20]. In the present study, this procedure was applied to serum samples from transgenic APP/PS1 and wild-type mice, and metabolomic fingerprints obtained were subsequently subjected to multivariate statistics in order to evaluate the classification power of the approach. As a first exploratory step, principal component analysis (PCA) was applied for a preliminary evaluation of data quality. A good clustering of quality control samples was observed in the scores plot (Fig 1 a-b, for ESI

241 and APPI data, respectively), indicative of stability during the analyses, without significant outliers
242 according to the Hotelling T^2 -range plot (not shown). Then, partial least squares discriminant analysis
243 (PLS-DA) demonstrated a clear classification of APP/PS1 and control mice along the first latent variable
244 (Fig 1c-d, for ESI and APPI data, respectively), which yielded satisfactory values for the quality
245 parameters R^2 and Q^2 , with regards to both DI-ESI-MS ($R^2 = 0.991$, $Q^2 = 0.761$) and FI-APPI-MS data
246 ($R^2 = 0.997$, $Q^2 = 0.834$). In addition, QC samples were correctly predicted in the center of the scores
247 plots for PLS-DA models, demonstrating the robustness of the analytical procedure [22].
248

249 3.2. IDENTIFICATION OF POTENTIAL BIOMARKERS

250 Metabolites responsible for discrimination between APP/PS1 and control animals in PLS-DA models
251 were identified by inspecting the corresponding loadings plots, and then were validated by t-test (p value
252 below 0.05). These potential biomarkers are listed in Table 1 along with the experimental mass, the
253 ionization mode used for detection (ESI or APPI, in positive or negative modes), the extract where they
254 were found (aqueous or organic extract), the fold change (FC, calculated by dividing the mean area for
255 peaks in the APP/PS1 group by the mean area in the control group), p-value, the area under the curve
256 (AUC) determined by ROC analysis, and the coefficient of signal variation (CV) observed in quality
257 control samples. From these findings it is noteworthy the utility of using complementary ionization
258 techniques to obtain a more comprehensive characterization of serum metabolome, because while ESI-
259 MS showed the implication of numerous polar metabolites in pathology of the APP/PS1 mice, APPI-MS
260 mainly focuses in less polar compounds. The most notable perturbation was the increase of different
261 eicosanoids in serum from the APP/PS1 mice, including leukotriene B4 (LTB4), hydroxy-
262 eicosapentaenoic acid (HEPE) and prostaglandins (PG), with fold changes in the range 1.6-3.8. Other
263 lipid compounds significantly altered were increased diacylglycerols (FC \approx 1.4) and triacylglycerols (FC
264 = 1.3-1.6), decreased levels of free fatty acids (FC = 0.6-0.7), lysophosphocholines (FC = 0.5-0.7) and
265 cholesteryl esters (FC \approx 0.8), and abnormal distribution of phosphocholines depending on the fatty acid
266 linked to the molecular moiety. Furthermore, numerous low molecular weight metabolites were also
267 altered in serum samples from the APP/PS1 mice, such as amino acids, catabolites of phospholipids or
268 energy-related metabolites, among others. Then, these metabolites were subjected to receiver operating
269 characteristic analysis in order to evaluate their potential as biomarkers for diagnosis. Most of lipids
270 identified showed only a modest diagnostic power, with AUC values in the range 0.7-0.8, including fatty
271 acids, di- and tri-acylglycerols, phosphocholines and cholesteryl esters. However, other metabolites stand
272 out as strong biomarkers for AD-type disorders in the APP/PS1 model, such as lysophosphocholines,
273 eicosanoids, urea, serine, phosphoethanolamine or tyrosine, with AUC > 0.9. Finally, it is also remarkable
274 the excellent analytical reproducibility measured in QC samples, with coefficients of variation below 10%
275 for all metabolites identified. Therefore, it could be concluded that the metabolomic platform presented in
276 this study shows a high potential for the comprehensive assessment of metabolic alterations in serum
277 samples from APP/PS1 mice, which is of great utility for the search of new biomarkers and the study of
278 pathological mechanisms underlying to these metabolic disorders, as described in the next section.
279

280 3.3. BIOLOGICAL HYPOTHESIS

281 Metabolic alterations detected in this study (Table 1) offer the opportunity to elucidate possible
282 biochemical pathways affected in the APP/PS1 mice, thus allowing a better understanding about disease
283 pathology. Furthermore, these findings demonstrate the potential of using peripheral samples such as
284 blood serum in order to characterize AD-mechanisms, providing complementary results to those observed
285 in brain tissue, the matrix commonly employed in previous metabolomics investigations with AD models
286 [4-9]. It is noteworthy that serum from APP/PS1 mice showed reduced levels of different amino acids
287 (serine, valine, threonine, pyroglutamate, histidine, tyrosine, tryptophan), which may suggest that
288 perturbations of amino acid metabolism play a critical role in the pathogenesis of AD in this mouse
289 model. Metabolisms of serine, valine and threonine are interrelated through the amino acid threonine, an
290 indirect precursor of valine that connects the glycine, serine and threonine metabolism pathway to
291 biosynthesis of branched-chain amino acids. Interestingly, Hu et al. found that levels of these three amino
292 acids are increased in the brain of TASTPM mice [11], in agreement with other metabolomic studies
293 performed in AD brains [4,7,9], so the reduction observed in serum samples (Table 1) could indicate an
294 important deregulation of their transport across the blood brain barrier. In this sense we also observed a
295 significant decrease of pyroglutamic acid levels, an essential compound for the regulation of the entry of
296 amino acids into the brain via the γ -glutamyl cycle, whose dyshomeostasis is indicative of aberrant
297 functioning of this metabolic process. Thereby, previous studies already reported lower levels of
298 pyroglutamate in brain [7], CSF [31] and blood [32,33] from AD patients, corroborating results presented
299 here. On the other hand, reduced serum histidine could point to impaired synthesis of carnosine and/or
300 histamine, important biomolecules associated with oxidative stress [34] and failures in neurotransmission

301 [35] respectively, whose reduction has been previously related to AD pathogenesis [18,19,33,36].
302 Moreover the reduction of tryptophan and tyrosine levels, precursors of serotonin and catecholamines
303 respectively, might denote a severe disturbance in monoaminergic neurotransmission systems, which
304 confirm previous studies in AD subjects [37-38]. Decreased urea content (Table 1) supports a disturbed
305 homeostasis of ammonia via the urea cycle, which may elicit deleterious effects on the central nervous
306 system and has been closely related to AD pathology [39]. Thereby, an abnormal content of urea and
307 related amino acids has been previously found in serum [19-20,33] and brain [8-9] of AD, accompanied
308 by altered levels of expression in different enzymes and the corresponding genes [40]. Other studies
309 reported an increased concentration of inosine in response to an accelerated degradation of nucleotides in
310 brain from human patients [41] and APP/PS1 mice [9], which is finally reflected in peripheral blood as
311 revealed our metabolomic fingerprinting platform. However, Jiang et al. surprisingly described the lack of
312 inosine in serum from the SAMP8 mice [12], demonstrating the disparity between different existing
313 models of AD and the need to select the correct one to mimic metabolic features detected in human AD.
314 Finally, we also observed a significant decrease of three energy-related metabolites in serum from the
315 APP/PS1 mice: glucose, creatine and carnitine. Lower blood levels of glucose have been reported in
316 different transgenic models of Alzheimer's disease [10-12], denoting a profound disturbance in
317 carbohydrate and energy metabolism. On the other hand, reduced levels of creatine could be behind
318 impaired phosphocreatine system due to mitochondrial dysfunction, as González-Domínguez et al.
319 described in serum from human AD patients [18,20,36]. Furthermore, the decrease of free carnitine could
320 have important consequences in the transport of fatty acids into mitochondria for β -oxidation, which is in
321 accordance with previous studies in brain [42], CSF [43] and serum [18,36] from AD patients.

322
323 Numerous lipid compounds were also significantly perturbed in serum from APP/PS1 mice, as can be
324 observed in Table 1. The most notable finding was an abnormal metabolism of phospholipids, regarding
325 altered levels of phosphocholines and lysophosphocholines, as well as other related compounds such as
326 eicosanoids, diacylglycerols and other catabolites. Enhanced degradation of phospholipids from neural
327 membranes has been described as a key pathological hallmark in Alzheimer's disease, whose origin
328 seems to take place in the over-activation of phospholipases, principally phospholipase A₂ [44]. However,
329 recent studies performed in serum samples from human AD patients also point to the implication of an
330 altered fatty acid composition of phospholipids in this membrane destabilization process, decreasing
331 phospholipids containing polyunsaturated fatty acids (PUFA) and increasing saturated species [18,45]. In
332 this sense we detected a substantial decrease of different phosphocholines (PC) in serum of the APP/PS1
333 model, in accordance with previous studies in brain from transgenic mice of AD [46-48], demonstrating
334 the potential of serum to reflect metabolic changes occurring in brain. Nevertheless, some individual
335 phosphocholine-species containing stearic acid (PC-18:0/18:2 and PC-18:0/22:6) were surprisingly over-
336 expressed in these serum samples, which has been previously described only once in brain tissue from
337 this APP/PS1 mouse model and might suggest a profound membrane remodeling [9]. Furthermore, this
338 abnormal serum composition of phosphocholines was accompanied by an overall decrease of
339 lysophosphocholine (LPC) levels, which could be attributed to anabolic stimulation of lysophospholipids
340 metabolism, in agreement with previous studies in human AD [45,49]. The production of
341 lysophospholipids by the action of phospholipase A₂ over membrane phospholipids also leads to the
342 liberation of free fatty acids, principally arachidonic acid, which may be easily oxidized producing
343 eicosanoids. Thereby, the accumulation of these lipid oxidation compounds (Table 1) can be considered
344 as a suitable marker of oxidative stress and membrane breakdown. Alternatively, there is also evidence
345 for a role of phospholipases C and D in processes associated with degradation of phospholipids in AD,
346 although they have been much less studied [50-51]. These enzymes hydrolyze phosphodiester bonds in
347 the hydrophilic group of phospholipids, thus releasing diacylglycerols as final products. In this context,
348 González-Domínguez et al. recently described the elevation of total levels of diacylglycerols in serum
349 from AD patients as potential marker of over-activated phospholipase C/D [19-20], but this is the first
350 time that is observed in serum from transgenic mice. To conclude, this altered metabolism of
351 phospholipids in the APP/PS1 mice was also supported by other metabolomic changes in several low
352 molecular weight metabolites. Choline and glycerophosphoethanolamine, catabolites resulting from
353 degradation of phospholipids, were elevated in serum samples in accordance with numerous previous
354 studies [18,52,53]. By contrast, phosphoethanolamine levels were decreased, as previously observed in
355 postmortem AD brains [54], corroborating the evidence for a membrane defect in Alzheimer disease.

356
357 Besides the abovementioned changes, abnormalities in lipid metabolism were also reflected in other
358 compounds not directly related to membrane destabilization processes, including free fatty acids,
359 cholesteryl esters and triacylglycerols. Lower levels of myristic and palmitoleic acid were found in serum
360 from the APP/PS1 mice, in agreement with previous investigations in blood from AD patients [32,55].

361 However, although it is well known that polyunsaturated fatty acids are closely involved in pathogenesis
362 of AD since they are highly concentrated in neural membranes, the role of fatty acids detected in the
363 present work has not been elucidated. On the other hand, decreased serum content of cholesteryl esters
364 could denote impaired cholesterol metabolism, corroborating findings described by Fabelo et al. in cortex
365 from APP/PS1 mice [56]. Finally, the accumulation of triacylglycerols suggests a severe hyperlipidemia,
366 one of the most important vascular risk factors that have been associated with the development of AD.
367 Thus, several studies have reported relationships between Alzheimer's disease and high levels of lipids,
368 principally triglycerides, in both human [18] and mice blood [57].
369

370 4. CONCLUSIONS

371 This work describes for the first time the application of a metabolomic multi-platform based on direct
372 mass spectrometry analysis with the aim to investigate metabolic alterations in serum from the APP/PS1
373 transgenic mice of Alzheimer's disease. The combination of DI-ESI-MS and FI-APPI-MS provided a
374 comprehensive characterization of serum metabolome, thus allowing a better understanding about AD-
375 related mechanisms in this transgenic model. Multivariate statistics revealed disturbances in numerous
376 metabolites, which could be associated with perturbed amino acids metabolism, energy-related failures,
377 abnormal homeostasis of membrane phospholipids leading to membrane breakdown, hyperammonemia
378 and hyperlipidemia, or abnormal metabolism of cholesterol, among others. The most notable finding is
379 that many of these alterations have been previously described only in brain tissue, thus demonstrating the
380 potential of peripheral blood serum for the investigation of disease pathology in a non-invasive manner
381 and, subsequently, the discovery of reliable biomarkers. Finally, it is also noteworthy that perturbations
382 detected in this work showed close similarities with previous metabolomic studies in AD patients, as
383 described in the Discussion section, reinforcing the role of APP/PS1 mice in AD research.
384

385 **Acknowledgements.** This work was supported by the projects CTM2012-38720-C03-01 from the
386 Ministerio de Ciencia e Innovación and P008-FQM-3554 and P009-FQM-4659 from the Consejería de
387 Innovación, Ciencia y Empresa (Junta de Andalucía). Raúl González Domínguez thanks the Ministerio de
388 Educación for a predoctoral scholarship (AP2010-4278).
389

390 REFERENCES

- 391 [1] P. McGonigle, B. Ruggeri. Animal models of human disease: Challenges in enabling translation.
392 *Biochem. Pharmacol.* 2014, 87, 162-171.
- 393 [2] A.M. Hall, E.D. Roberson. Mouse models of Alzheimer's disease. *Brain Res. Bull.* 2012, 88, 3-12.
- 394 [3] E. Trushina, M.M. Mielke. Recent advances in the application of metabolomics to Alzheimer's
395 disease. *Biochim. Biophys. Acta* 2014, 1842, 1232-1239.
- 396 [4] R.M. Salek, J. Xia, A. Innes, B.C. Sweatman, R. Adalbert, S. Randle, E. McGowan, P.C. Emson, J.L.
397 Griffin. A metabolomic study of the CRND8 transgenic mouse model of Alzheimer's disease.
398 *Neurochem. Int.* 2010, 56, 937-943.
- 399 [5] S. Lin, H. Liu, B. Kanawati, L. Liu, J. Dong, M. Li, J. Huang, P. Schmitt-Kopplin, Z. Cai.
400 Hippocampal metabolomics using ultrahigh-resolution mass spectrometry reveals neuroinflammation
401 from Alzheimer's disease in CRND8 mice. *Anal. Bioanal. Chem.* 2013, 405, 5105-5117.
- 402 [6] S. Lin, B. Kanawati, L. Liu, M. Witting, M. Li, J. Huang, P. Schmitt-Kopplin, Z. Cai. Ultra high
403 resolution mass spectrometry-based metabolic characterization reveals cerebellum as a disturbed region in
404 two animal models. *Talanta* 2014, 118, 45-53.
- 405 [7] E. Trushina, E. Nemutlu, S. Zhang, T. Christensen, J. Camp, J. Mesa, A. Siddiqui, Y. Tamura, H.
406 Sesaki, T.M. Wengenack, P.P. Dzeja, J.F. Poduslo. Defects in mitochondrial dynamics and metabolomic
407 signatures of evolving energetic stress in mouse models of familial Alzheimer's disease. *PLoS ONE*
408 2012, 7, e32737.
- 409 [8] R. González-Domínguez, T. García-Barrera, J. Vitorica, J.L. Gómez-Ariza. Region-specific metabolic
410 alterations in the brain of the APP/PS1 transgenic mice of Alzheimer's disease. *Biochim. Biophys. Acta*
411 2014, 1842, 2395-2402.
- 412 [9] R. González-Domínguez, T. García-Barrera, J. Vitorica, J.L. Gómez-Ariza. Metabolomic screening of
413 regional brain alterations in the APP/PS1 transgenic mouse model of Alzheimer's disease by direct
414 infusion mass spectrometry. *J. Pharm. Biomed. Anal.* 2015, 102, 425-435.
- 415 [10] S.F. Graham, C. Holscher, P. McClean, C.T. Elliott, B.D. Green. ¹H NMR metabolomics
416 investigation of an Alzheimer's disease (AD) mouse model pinpoints important biochemical disturbances
417 in brain and plasma. *Metabolomics* 2013, 9, 974-983.
- 418 [11] Z.P. Hu, E.R. Browne, T. Liu, T.E. Angel, P.C. Ho, E.C.Y. Chan. Metabonomic profiling of
419 TASTPM transgenic alzheimer's disease mouse model. *J. Proteome Res.* 2012, 11, 5903-5913.

420 [12] N. Jiang, X. Yan, W. Zhou, Q. Zhang, H. Chen, Y. Zhang, X. Zhang. NMR-based metabonomic
421 investigations into the metabolic profile of the senescence-accelerated mouse. *J. Proteome Res.* 2008, 7,
422 3678-3686.

423 [13] K. Fukuhara, A. Ohno, Y. Ota, Y. Senoo, K. Maekawa, H. Okuda, M. Kurihara, A. Okuno, S. Niida,
424 Y. Saito, O. Takikawa. NMR-based metabolomics of urine in a mouse model of Alzheimer's disease:
425 identification of oxidative stress biomarkers. *J. Clin. Biochem. Nutr.* 2013, 52, 133-138.

426 [14] R. Gonzalez-Dominguez, R. Castilla-Quintero, T. Garcia-Barrera, J.L. Gomez-Ariza. Development
427 of a metabolomic approach based on urine samples and direct infusion mass spectrometry. *Anal.*
428 *Biochem.* 2014, 465, 20-27.

429 [15] J. Peng, K. Guo, J. Xia, J. Zhou, J. Yang, D. Westaway, D.S. Wishart, L. Li. Development of isotope
430 labeling liquid chromatography mass spectrometry for mouse urine metabolomics: quantitative
431 metabolomic study of transgenic mice related to Alzheimer's disease. *J. Proteome Res.* 2014, 13, 4457-
432 4469.

433 [16] J. Draper, A.J. Lloyd, R. Goodacre, M. Beckmann. Flow infusion electrospray ionisation mass
434 spectrometry for high throughput, non-targeted metabolite fingerprinting: a review. *Metabolomics* 2013,
435 9, S4-S29.

436 [17] R. Gonzalez-Dominguez, T. Garcia-Barrera, Gomez-Ariza JL. Metabolomic approach to
437 Alzheimer's disease diagnosis based on mass spectrometry. *Chem. Papers* 2012, 66, 829-835.

438 [18] R. González-Domínguez, T. García-Barrera, J.L. Gómez-Ariza. Using direct infusión mass
439 spectrometry for serum metabolomics in Alzheimer's disease. *Anal. Bioanal. Chem.* 2014, 406, 7137-
440 7148.

441 [19] R. González-Domínguez, T. García-Barrera, J.L. Gómez-Ariza. Metabolomic study of lipids in
442 serum for biomarker discovery in Alzheimer's disease using direct infusion mass spectrometry. *J. Pharm.*
443 *Biomed. Anal.* 2014, 98, 321-326.

444 [20] R. González-Domínguez, T. García-Barrera, J.L. Gomez-Ariza. Application of a novel metabolomic
445 approach based on atmospheric pressure photoionization mass spectrometry using flow injection analysis
446 for the study of Alzheimer's disease. *Talanta* 2015, 131, 480-489.

447 [21] J.L. Jankowsky, D.J. Fadale, J. Anderson, G.M. Xu, V. Gonzales, N.A. Jenkins, N.G. Copeland,
448 M.K. Lee, L.H. Younkin, S.L. Wagner, S.G. Younkin, D.R. Borchelt. Mutant presenilins specifically
449 elevate the levels of the 42 residue beta-amyloid peptide in vivo: evidence for augmentation of a 42-
450 specific γ secretase. *Hum. Mol. Genet.* 2004, 13, 159-170.

451 [22] T. Sangster, H. Major, R. Plumb, A.J. Wilson, I.D. Wilson. A pragmatic and readily implemented
452 quality control strategy for HPLC-MS and GC-MS-based metabonomic analysis. *Analyst* 2006, 131,
453 1075-1078.

454 [23] R.A. van den Berg, H.C.J. Hoefsloot, J.A. Westerhuis, A.K. Smilde, M.J. van der Werf. Centering,
455 scaling, and transformations: improving the biological information content of metabolomics data. *BMC*
456 *Genomics* 2006, 7, 142.

457 [24] J. Xia, D.I. Broadhurst, M. Wilson, D.S. Wishart. Translational biomarker discovery in clinical
458 metabolomics: an introductory tutorial. *Metabolomics* 2013, 9, 280-299.

459 [25] M. Pulfer, R.C. Murphy. Electrospray mass spectrometry of phospholipids. *Mass Spectrom. Rev.*
460 2003, 22, 332-364.

461 [26] C. Wang, S. Xie, J. Yang, Q. Yang, G. Xu. Structural identification of human blood phospholipids
462 using liquid chromatography/quadrupole-linear ion trap mass spectrometry. *Anal. Chim. Acta* 2004, 525,
463 1-10.

464 [27] R. González-Dominguez, T. García-Barrera, J.L. Gómez-Ariza. Iberian ham typification by direct
465 infusion electrospray and photospray ionization mass spectrometry fingerprinting. *Rapid Commun. Mass*
466 *Spectrom.* 2012, 26, 835-844.

467 [28] G. Liebisch, M. Binder, R. Schifferer, T. Langmann, B. Schulz, G. Schmitz. High throughput
468 quantification of cholesterol and cholesteryl ester by electrospray ionization tandem mass spectrometry
469 (ESI-MS/MS). *Biochim. Biophys. Acta* 2006, 1761, 121-128.

470 [29] L. Vernez, G. Hopfgartner, M. Wenk, S. Krahenbuhl. Determination of carnitine and acylcarnitines
471 in urine by high-performance liquid chromatography–electrospray ionization ion trap tandem mass
472 spectrometry. *J. Chromatogr. A* 2003, 984, 203-213.

473 [30] R.C. Murphy, R.M. Barkley, K.Z. Berry, J. Hankin, K. Harrison, C. Johnson, J. Krank, A. McAnoy,
474 C. Uhlson, S. Zarini. Electrospray ionization and tandem mass spectrometry of eicosanoids. *Anal.*
475 *Biochem.* 2005, 346, 1-42.

476 [31] E. Trushina, T. Dutta, X.M.T. Persson, M.M. Mielke, R.C. Petersen. Identification of altered
477 metabolic pathways in plasma and CSF in mild cognitive impairment and Alzheimer's disease using
478 metabolomics. *PLoS ONE* 2013, 8, e63644.

479 [32] G. Wang, Y. Zhou, F.J. Huang, H.D. Tang, X.H. Xu, J.J. Liu, Y. Wang, Y.L. Deng, R.J. Ren, W.
480 Xu, J.F. Ma, Y.N. Zhang, A.H. Zhao, S.D. Chen, W. Jia. Plasma metabolite profiles of Alzheimer's
481 disease and mild cognitive impairment. *J. Proteome Res.* 2014, 13, 2649-2658.

482 [33] R. González-Domínguez, T. García-Barrera, J.L. Gómez-Ariza. Metabolite profiling for the
483 identification of altered metabolic pathways in Alzheimer's disease. *J. Pharm. Biomed. Anal.* 2015, 107,
484 75-81.

485 [34] A.R. Hipkiss. Could carnosine or related structures suppress Alzheimer's disease? *J. Alzheimers Dis.*
486 2007, 11, 229-240.

487 [35] S. Nuutinen, P. Panula. Histamine in neurotransmission and brain diseases. *Adv. Exp. Med. Biol.*
488 2010, 709, 95-107.

489 [36] R. González-Domínguez, A. García, T. García-Barrera, C. Barbas, J.L. Gómez-Ariza. Metabolomic
490 profiling of serum in the progression of Alzheimer's disease by capillary electrophoresis-mass
491 spectrometry. *Electrophoresis* 2014, 35, 3321-3330.

492 [37] H. Tohgi, T. Abe, S. Takahashi, M. Kimura, J. Takahashi, T. Kikuchi. Concentrations of serotonin
493 and its related substances in the cerebrospinal fluid in patients with Alzheimer type dementia. *Neurosci.*
494 *Lett.* 1992, 141, 9-12.

495 [38] D. Storga, K. Vrecko, J.G.D. Birkmayer, G. Reibnegger. Monoaminergic neurotransmitters, their
496 precursors and metabolites in brains of Alzheimer patients. *Neurosci. Lett.* 1996, 203, 29-32.

497 [39] V. Felipo, R.F. Butterworth. Neurobiology of ammonia. *Prog. Neurobiol.* 2002, 67, 259-279.

498 [40] F. Hansmannel, A. Sillaire, M.I. Kamboh, C. Lendon, F. Pasquier, D. Hannequin, G. Laumet, A.
499 Mounier, A.M. Ayrat, S.T. DeKosky, J.J. Hauw, C. Berr, D. Mann, P. Amouyel, D. Campion, J.C.
500 Lambert. Is the urea cycle involved in Alzheimer's disease? *J. Alzheimers Dis.* 2010, 21, 1013-1021.

501 [41] N.R. McFarland, T. Burdett, C.A. Desjardins, M.P. Frosch, M.A. Schwarzschild. Postmortem brain
502 levels of urate and precursors in Parkinson's disease and related disorders. *Neurodegener. Dis.* 2013, 12,
503 189-198.

504 [42] S.F. Graham, C. Holscher, B.D. Green. Metabolic signatures of human Alzheimer's disease (AD):
505 ¹H NMR analysis of the polar metabolome of post-mortem brain tissue. *Metabolomics* 2014, 10, 744-753.

506 [43] C. Ibáñez, C. Simó, P.J. Martín-Álvarez, M. Kivipelto, B. Winblad, A. Cedazo-Mínguez, A.
507 Cifuentes. Toward a predictive model of Alzheimer's disease progression using capillary
508 electrophoresis-mass spectrometry metabolomics. *Anal. Chem.* 2012, 84, 8532-8540.

509 [44] A.A. Farooqui, W.Y. Ong, L.A. Horrocks. Biochemical aspects of neurodegeneration in human
510 brain: involvement of neural membrane phospholipids and phospholipases A₂. *Neurochem. Res.* 2004, 29,
511 1961-1977.

512 [45] R. Gonzalez-Dominguez, T. Garcia-Barrera, J.L. Gomez-Ariza. Combination of metabolomic and
513 phospholipid-profiling approaches for the study of Alzheimer's disease. *J. Proteomics* 2014, 104, 37-47.

514 [46] J.K. Yao, T.M. Wengenack, G.L. Curran, J.F. Poduslo. Reduced membrane lipids in the cortex of
515 Alzheimer's disease transgenic mice. *Neurochem. Res.* 2009, 34, 102-108.

516 [47] X. Han, D.M. Holtzman, D.W. McKeel Jr. Plasmalogen deficiency in early Alzheimer's disease
517 subjects and in animal models: molecular characterization using electrospray ionization mass
518 spectrometry. *J. Neurochem.* 2001, 77, 1168-1180.

519 [48] R.B. Chan, T.G. Oliveira, E.P. Cortes, L.S. Honig, K.E. Duff, S.A. Small, M.R. Wenk, G. Shui, G.
520 Di Paolo. Comparative lipidomic analysis of mouse and human brain with Alzheimer disease. *J. Biol.*
521 *Chem.* 2012, 287, 2678-2688.

522 [49] N. Li, W. Liu, W. Li, S. Li, X. Chen, K. Bi, P. He. Plasma metabolic profiling of Alzheimer's
523 disease by liquid chromatography/mass spectrometry. *Clin. Biochem.* 2010, 43, 992-997.

524 [50] S. Shimohama, Y. Sasaki, S. Fujimoto, S. Kamiya, T. Taniguchi, T. Takenawa, J. Kimura.
525 Phospholipase C isoenzymes in the human brain and their changes in Alzheimer's disease. *Neuroscience*
526 1998, 82, 999-1007.

527 [51] J.K. Jin, N.H. Kim, Y.J. Lee, Y.S. Kim, E.K. Choi, P.B. Kozlowski, M.H. Park, H.S. Kim, D.S. Min.
528 Phospholipase D1 is up-regulated in the mitochondrial fraction from the brains of Alzheimer's disease
529 patients. *Neurosci. Lett.* 2006, 407, 263-267.

530 [52] W.E. Klunk, K. Panchalingam, R.J. McClure, J.A. Stanley, J.W. Pettegrew. Quantitative ¹H and ³¹P
531 MRS of PCA extracts of postmortem Alzheimer's disease brain. *Neurobiol. Aging* 1996, 17, 349-357.

532 [53] A. Walter, U. Korth, M. Hilgert, J. Hartmann, O. Weichel, M. Hilgert, K. Fassbender, A. Schmitt, J.
533 Klein. Glycerophosphocholine is elevated in cerebrospinal fluid of Alzheimer patients. *Neurobiol. Aging*
534 2004, 25, 1299-1303.

535 [54] D.W. Ellison, M.F. Beal, J.B. Martin. Phosphoethanolamine and ethanolamine are decreased in
536 Alzheimer's disease and Huntington's disease. *Brain Res.* 1987, 417, 389-392.

537 [55] D.C. Wang, C.H. Sun, L.Y. Liu, X.H. Sun, X.W. Jin, W.L. Song, X.Q. Liu, X.L. Wan. Serum fatty
538 acid profiles using GC-MS and multivariate statistical analysis: potential biomarkers of Alzheimer's
539 disease. *Neurobiol. Aging* 2012, 33, 1057-1066.
540 [56] N. Fabelo, V. Martín, R. Marín, G. Santpere, E. Aso, I. Ferrer, M. Díaz. Evidence for premature lipid
541 raft aging in APP/PS1 double-transgenic mice, a model of familial Alzheimer disease. *J. Neuropathol.*
542 *Exp. Neurol.* 2012, 71, 868-881.
543 [57] Y. Tajima, M. Ishikawa, K. Maekawa, M. Murayama, Y. Senoo, T. Nishimaki-Mogami, H.
544 Nakanishi, K. Ikeda, M. Arita, R. Taguchi, A. Okuno, R. Mikawa, S. Niida, O. Takikawa, Y. Saito.
545 Lipidomic analysis of brain tissues and plasma in a mouse model expressing mutated human amyloid
546 precursor protein/tau for Alzheimer's disease. *Lipids Health Dis.* 2013, 12, 68.

547
548 **Figure captions**

549 **Fig 1** Scores plots of statistical models for metabolomic data from serum samples; a: PCA for DI-ESI-MS
550 data, b: PCA for FI-APPI-MS data, c: PLS-DA for DI-ESI-MS data, d: PLS-DA for FI-APPI-MS data.
551 APP/PS1 mice: red dots, wild-type mice: blue diamonds, quality control samples: black squares
552

553 **Table 1.** Discriminant metabolites identified in serum from APP/PS1 mice

compound	mass	ionization mode	extract	fold change	p value	AUC	CV %
Low molecular weight metabolites							
urea	60.033	ESI+	A	0.34	$1.0 \cdot 10^{-6}$	0.96	7.9
choline	103.097	ESI+	A/O	1.34	$1.5 \cdot 10^{-3}$	0.74	5.9
serine	105.046	APPI+	A	0.43	$1.8 \cdot 10^{-5}$	0.92	7.5
valine	117.081	APPI-	A	0.81	$2.8 \cdot 10^{-3}$	0.72	7.8
threonine	119.062	ESI+/ESI- /APPI-	A	0.66	$3.5 \cdot 10^{-5}$	0.86	8.0
pyroglutamate	129.045	APPI+	O	0.74	$3.0 \cdot 10^{-5}$	0.82	7.9
creatine	131.071	APPI+	O	0.60	$3.5 \cdot 10^{-2}$	0.67	9.2
PE	141.022	ESI+	A/O	0.58	$1.8 \cdot 10^{-5}$	0.93	8.2
histidine	155.073	APPI+	O	0.69	$1.2 \cdot 10^{-2}$	0.76	7.0
carnitine	161.105	ESI+	O	0.77	$2.5 \cdot 10^{-3}$	0.72	5.2
glucose	180.067	ESI-/APPI+	A/O	0.64	$5.3 \cdot 10^{-5}$	0.83	5.7
tyrosine	181.076	ESI+/ESI-	A	0.56	$1.4 \cdot 10^{-5}$	0.90	6.8
tryptophan	204.087	ESI+	O	0.80	$8.3 \cdot 10^{-4}$	0.75	5.1
GPE	215.059	ESI+	A	1.79	$1.0 \cdot 10^{-6}$	0.85	6.7
inosine	268.085	ESI-	A/O	1.91	$1.7 \cdot 10^{-3}$	0.71	6.2
Free fatty acids							
myristic acid	228.214	APPI-	O	0.73	$3.7 \cdot 10^{-3}$	0.72	7.9
palmitoleic acid	254.230	ESI-/APPI-	O	0.61	$2.1 \cdot 10^{-3}$	0.74	8.4
Eicosanoids							
HEPE	318.222	ESI-	A/O	1.64	$1.0 \cdot 10^{-6}$	0.99	8.1
PG (series 2)	334.210	ESI-	A/O	2.54	$1.0 \cdot 10^{-6}$	0.95	9.5
LTB4	336.235	ESI-	A/O	3.44	$1.0 \cdot 10^{-6}$	0.99	7.9
PG (series 3)	350.211	ESI-	A/O	3.79	$2.0 \cdot 10^{-5}$	0.92	8.9
Lysophosphocholines							
LPC(16:1)	493.319	ESI+	A/O	0.73	$1.0 \cdot 10^{-6}$	0.85	12.1
LPC(16:0)	495.343	ESI+	A/O	0.55	$1.0 \cdot 10^{-6}$	0.91	5.1
LPC(18:2)	519.330	ESI+	A	0.73	$1.0 \cdot 10^{-6}$	0.95	5.0
LPC(18:1)	521.358	ESI+	A	0.56	$1.0 \cdot 10^{-6}$	0.90	9.3
LPC(20:4)	543.345	ESI+	A	0.61	$1.0 \cdot 10^{-6}$	0.92	4.9
LPC(22:6)	567.341	ESI+	A	0.59	$1.0 \cdot 10^{-6}$	0.91	5.4
Phosphocholines							
PC(16:0/16:0)	733.571	ESI+	O	0.76	$4.7 \cdot 10^{-4}$	0.79	3.6
PC(16:0/18:1)	759.585	ESI+/ESI-	A/O	0.71	$3.2 \cdot 10^{-4}$	0.81	3.7
PC(16:0/18:0)	761.598	ESI+/ESI-	A/O	0.75	$3.0 \cdot 10^{-4}$	0.78	8.4
PC(16:0/20:4)	781.562	ESI+/ESI-	A/O	0.76	$7.9 \cdot 10^{-4}$	0.74	6.7
PC(18:2/18:0)	785.599	ESI+	A	1.59	$2.0 \cdot 10^{-6}$	0.85	5.4
PC(18:1/20:4)	807.579	ESI+/ESI-	A/O	0.75	$2.2 \cdot 10^{-4}$	0.77	6.5
PC(18:0/22:6)	833.606	ESI+	A	1.78	$1.0 \cdot 10^{-6}$	0.85	7.4

Diacylglycerols							
DAG(18:3/18:2)	614.481	APPI+	O	1.41	$1.4 \cdot 10^{-2}$	0.68	8.1
DAG(18:3/18:1)	616.516	APPI+	O	1.44	$6.0 \cdot 10^{-3}$	0.71	5.6
Cholesteryl esters							
CE(16:1)	622.581	ESI+	O	0.77	$1.2 \cdot 10^{-3}$	0.76	4.7
CE(16:0)	624.595	ESI+	O	0.85	$1.6 \cdot 10^{-3}$	0.75	4.4
Triacylglycerols							
TAG(16:1/16:0/20:4)	852.742	ESI+/APPI+	O	1.29	$3.8 \cdot 10^{-2}$	0.68	5.5
TAG(16:0/16:0/20:4)	854.747	ESI+/APPI+	O	1.36	$1.5 \cdot 10^{-2}$	0.69	6.1
TAG(16:1/16:1/22:6)	874.712	APPI+	O	1.27	$4.2 \cdot 10^{-2}$	0.67	3.3
TAG(16:1/16:0/22:6)	876.739	ESI+/APPI+	O	1.44	$3.3 \cdot 10^{-3}$	0.70	3.8
TAG(16:0/16:0/22:6)	878.754	ESI+	O	1.64	$2.3 \cdot 10^{-4}$	0.77	4.3
TAG(16:0/16:0/22:5)	880.768	ESI+	O	1.40	$2.3 \cdot 10^{-4}$	0.78	5.4
TAG(16:1/18:2/22:6)	900.709	APPI+	O	1.58	$2.1 \cdot 10^{-3}$	0.71	3.8
TAG(16:0/18:2/22:6)	902.720	APPI+	O	1.43	$2.1 \cdot 10^{-3}$	0.72	5.7
TAG(18:1/22:6/22:6)	976.773	APPI+	O	1.39	$2.9 \cdot 10^{-3}$	0.71	4.2

554 Abbreviations: PE, phosphoethanolamine; GPE, glycerophosphoethanolamine; HEPE, hydroxy-
555 eicosapentaenoic acid; PG, prostaglandin; LTB₄, leukotriene B₄; LPC, lyso-phosphocholine; PC,
556 phosphocholine; DAG, diacylglycerol; CE, cholesteryl ester; TAG, triacylglycerol; A, aqueous extract;
557 O, organic extract.

Figure 1
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