

Manuscript Number: BIOCHI-D-14-00489R1

Title: DECIPHERING METABOLIC ABNORMALITIES ASSOCIATED WITH ALZHEIMER'S DISEASE IN SERUM FROM THE APP/PS1 MOUSE MODEL USING INTEGRATED METABOLOMIC APPROACHES

Article Type: Research Paper

Section/Category: Regular issue

Keywords: Metabolomics; APP/PS1 mice; Alzheimer's disease; serum

Corresponding Author: Prof. JOSE LUIS GOMEZ-ARIZA, Ph.D.

Corresponding Author's Institution: UNIVERSITY OF HUELVA

First Author: Raul Gonzalez-Dominguez, Ms

Order of Authors: Raul Gonzalez-Dominguez, Ms; Tamara Garcia-Barrera, Dr; Javier Vitorica, Dr; JOSE LUIS GOMEZ-ARIZA, Ph.D.

Manuscript Region of Origin: SPAIN

**Abstract:** The transgenic mouse APP/PS1 is widely employed by neuroscientists because reproduces well some of the neuropathological and cognitive deficits observed in human Alzheimer's disease. In this study, serum samples from APP/PS1 mice (n = 30) and wild-type controls (n = 30) were analyzed using a metabolomic multiplatform based on the combination of gas chromatography-mass spectrometry and ultra-high performance liquid chromatography-mass spectrometry, in order to obtain wide information about serum metabolome. Metabolic profiles showed significant differences between the groups of study, and numerous metabolites were identified as potential players in the development of Alzheimer-type disorders in this transgenic model. Pathway analysis revealed the involvement of multiple metabolic networks in the underlying pathology, such as deficiencies in energy metabolism, altered amino acid homeostasis, abnormal membrane lipid metabolism, and other impairments related to the integrity of the central nervous system. It is noteworthy that some of these metabolomic markers are in accordance with pathological alterations observed in human Alzheimer's disease, while others have not been previously described. Therefore, these results demonstrate the potential of metabolomics and the use of transgenic animal models to understand the pathogenesis of Alzheimer's disease.

Opposed Reviewers:

Dear Editor,

Please consider the manuscript entitled “DECIPHERING METABOLIC ABNORMALITIES ASSOCIATED WITH ALZHEIMER’S DISEASE IN THE APP/PS1 MOUSE MODEL USING INTEGRATED METABOLOMIC APPROACHES“ by R. Gonzalez-Dominguez, T. Garcia-Barrera, J. Vitorica and J.L.Gomez-Ariza for its publication in *Biochimie*.

This study considers the use of a metabolomic multiplatform based on the combination of gas chromatography-mass spectrometry and ultra-high performance liquid chromatography-mass spectrometry in order to obtain wide information about serum metabolome. The approach has been used in the transgenic mouse APP/PS1 widely employed by neuroscientists because reproduces some of the neuropathological and cognitive deficits observed in human Alzheimer’s disease. In this study, serum samples from APP/PS1 mice (n = 30) and wild-type controls (n = 30) were analyzed. Metabolic profiles showed significant differences between the groups of study, and numerous metabolites were identified as potential players in the development of Alzheimer-type disorders in this transgenic model. In this way, multiple pathways were involved in the underlying pathology, such as deficiencies in energy metabolism, altered amino acid homeostasis, abnormal membrane lipid metabolism, and other impairments related to the integrity of the central nervous system. It is noteworthy that some of these metabolomic markers are in accordance with pathological alterations observed in human Alzheimer’s disease, while others have not been previously described. Therefore, these results demonstrate the potential of this combined metabolomic approach and its possibilities to be used in the study of the pathogenesis of Alzheimer’s disease.

Yours sincerely,

J.L. Gómez-Ariza

## Reviewers' comments:

**Reviewer #1: Gonzalez-Dominguez and colleagues report on a shotgun metabolomic profiling of serum samples from APP/PS1 mouse model compared to WT animals. The metabolomic profiling was performed by both LC-MS and GC-MS. Reported results show an extended alteration of several pathways involved in aminoacid, lipid and sugar metabolism. The paper is clear and well written. The experimental outline is good, carefully described and well performed. Conclusions appear to be fully supported by the experimental evidences. The pathways analysis is clear.**

**I have just a few minor comments for the authors**

**1) The title should mention that you investigated the serum metabolome**

The title has been changed in order to mention the use of serum samples in this study.

**2) Page 3, Line 40. Please use Electon Ionization, not impact**

It was corrected.

**3) Page 4, Line 10. The description of the data thresholding is not clear at all. Could you be more clear here?**

This part was re-written.

**4) Page 4, Line 44. Is'nt 30ppm a little bit too high for metabolite identification? Standard is 5-10ppm. Did you recalibrate your spectra somehow? You surely got tens of putative IDs per each m/z, with this value. MS/MS spectra are available for a limited part of the metabolites. Can you comment or explain better this point?**

Although many metabolomic approaches based on ultra-high resolution systems employ mass accuracies below 10 ppm for database searching, it is not rare to find other metabolomic studies using higher values (*e.g.* Anal Chem 2014,86:9496; J Proteome Res 2010,9:4071; J Proteomics 2014,104:37). Anyway, this database search with a pre-established mass tolerance is only a preliminary filter to create a list of "candidate metabolites" (maybe tens, as you comment), but then confirmation is performed by means of the characterization of MS/MS spectra. When MS/MS spectra are not available in databases, which is very common for most lipid species, structural elucidation can be undertaken through the use of fragmentation patterns reported in literature, as described in section 2.7.

**5) Page 6-7. I think that a full metabolite list is too long. Can't you move it in the supplementary info section and report just a limited number of metabolites in the text?**

Table 1 has been replaced by another simpler one, and the original data were moved to the supplementary info section.

**6) I would emphasize Fig 2 a little bit more, and better explain its meaning. Maybe it should go along with Fig. 3**

Section 3.3 has been explained in more detail, and Fig. 2 and 3 have been come together in a single figure.

**7) I would shorten down the conclusion section a little bit, if posible.**

The Conclusion section has been shortened.

DECIPHERING METABOLIC ABNORMALITIES ASSOCIATED WITH  
ALZHEIMER'S DISEASE IN THE APP/PS1 MOUSE MODEL USING  
INTEGRATED METABOLOMIC APPROACHES

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

The authors declare no conflict of interest either financial, personal or other

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

DECIPHERING METABOLIC ABNORMALITIES ASSOCIATED WITH  
ALZHEIMER'S DISEASE IN THE APP/PS1 MOUSE MODEL USING  
INTEGRATED METABOLOMIC APPROACHES

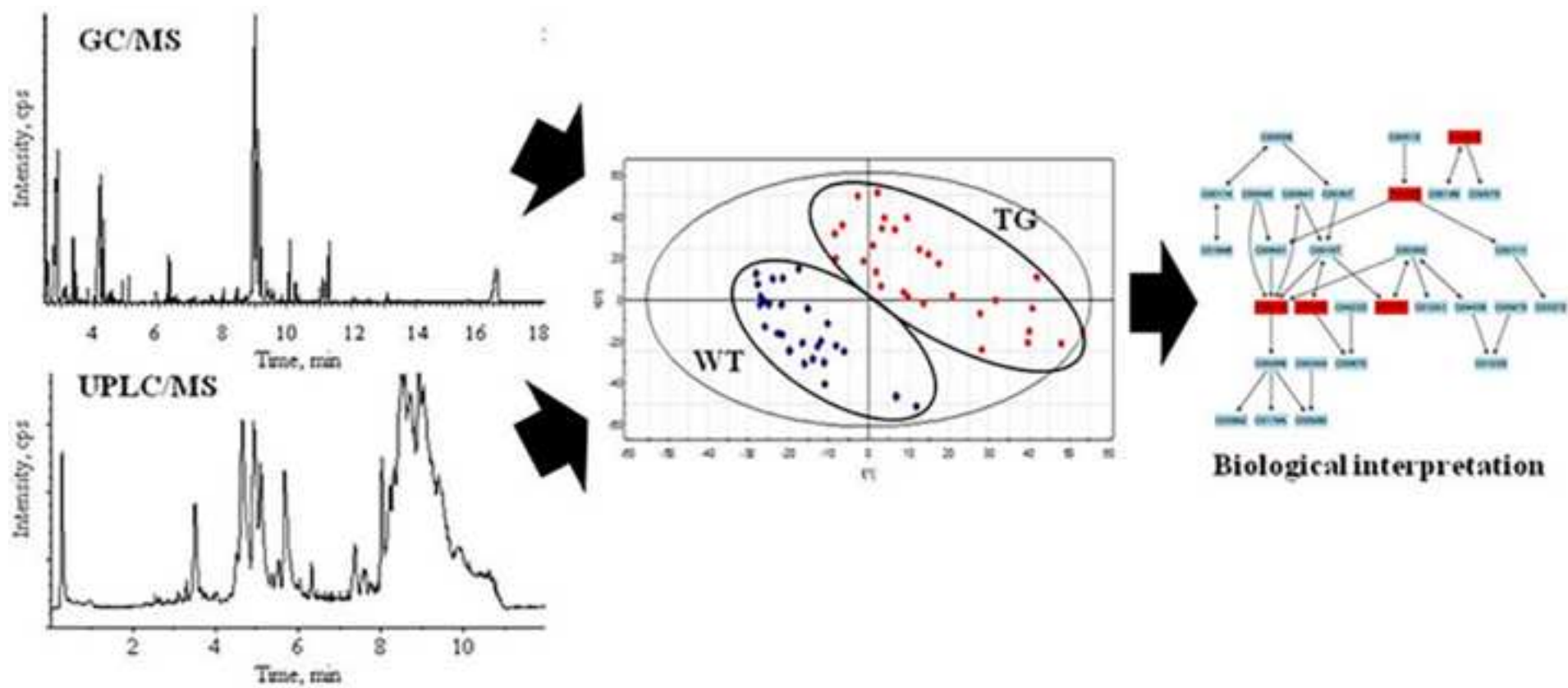
Raúl González-Domínguez, Tamara García-Barrera, Javier Vitorica, José Luis Gómez-Ariza

**ABSTRACT**

The transgenic mouse APP/PS1 is widely employed by neuroscientists because reproduces well some of the neuropathological and cognitive deficits observed in human Alzheimer's disease. In this study, serum samples from APP/PS1 mice (n = 30) and wild-type controls (n = 30) were analyzed using a metabolomic multiplatform based on the combination of gas chromatography-mass spectrometry and ultra-high performance liquid chromatography-mass spectrometry, in order to obtain wide information about serum metabolome. Metabolic profiles showed significant differences between the groups of study, and numerous metabolites were identified as potential players in the development of Alzheimer-type disorders in this transgenic model. Pathway analysis revealed the involvement of multiple metabolic networks in the underlying pathology, such as deficiencies in energy metabolism, altered amino acid homeostasis, abnormal membrane lipid metabolism, and other impairments related to the integrity of the central nervous system. It is noteworthy that some of these metabolomic markers are in accordance with pathological alterations observed in human Alzheimer's disease, while others have not been previously described. Therefore, these results demonstrate the potential of metabolomics and the use of transgenic animal models to understand the pathogenesis of Alzheimer's disease.

Highlights

- Combined GC/MS and UPLC/MS provides comprehensive metabolomic fingerprinting
- APP/PS1 mice showed characteristic metabolic signatures compared to wild type mice
- Metabolic pathway analysis allowed the elucidation of pathological mechanisms
- Impaired energy metabolism stands out as a key factor in pathogenesis of Alzheimer



# DECIPHERING METABOLIC ABNORMALITIES ASSOCIATED WITH ALZHEIMER'S DISEASE IN SERUM FROM THE APP/PS1 MOUSE MODEL USING INTEGRATED METABOLOMIC APPROACHES

Raúl González-Domínguez<sup>a,b,c</sup>, Tamara García-Barrera<sup>a,b,c</sup>, Javier Vitorica<sup>d,e,f</sup>, José Luis Gómez-Ariza<sup>a,b,c</sup>

<sup>a</sup>Department of Chemistry and CC.MM. Faculty of Experimental Sciences. University of Huelva. Campus de El Carmen. 21007 Huelva. Spain; <sup>b</sup>Campus of Excellence International ceiA3. University of Huelva. Spain; <sup>c</sup>Research Center of Health and Environment (CYSMA). University of Huelva. Campus de El Carmen. 21007 Huelva. Spain; <sup>d</sup>Department Bioquímica, Bromatología, Toxicología y Medicina Legal, Faculty of Pharmacy, University of Seville. 41012 Seville. Spain, <sup>e</sup>Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED). 41013 Seville. Spain, <sup>f</sup>Instituto de Biomedicina de Sevilla (IBiS)–Hospital Universitario Virgen del Rocío/CSIC/University of Seville. 41013 Seville. Spain

Corresponding authors:

Prof. J.L Gómez Ariza, Department of Chemistry and CC.MM. Faculty of Experimental Sciences. University of Huelva. Campus de El Carmen. 21007 Huelva. Spain. Tel.: +34 959 219968, fax: +34 959 219942, e-mail: [ariza@uhu.es](mailto:ariza@uhu.es)

Dr. T. Garcia-Barrera. Department of Chemistry and CC.MM. Faculty of Experimental Sciences. University of Huelva. Campus de El Carmen. 21007 Huelva. Spain. Tel.: +34 959 219962, fax: +34 959 219942, e-mail: [tamara@dqcm.uhu.es](mailto:tamara@dqcm.uhu.es)

## ABSTRACT

The transgenic mouse APP/PS1 is widely employed by neuroscientists because reproduces well some of the neuropathological and cognitive deficits observed in human Alzheimer's disease. In this study, serum samples from APP/PS1 mice (n = 30) and wild-type controls (n = 30) were analyzed using a metabolomic multiplatform based on the combination of gas chromatography-mass spectrometry and ultra-high performance liquid chromatography-mass spectrometry, in order to obtain wide information about serum metabolome. Metabolic profiles showed significant differences between the groups of study, and numerous metabolites were identified as potential players in the development of Alzheimer-type disorders in this transgenic model. Pathway analysis revealed the involvement of multiple metabolic networks in the underlying pathology, such as deficiencies in energy metabolism, altered amino acid homeostasis, abnormal membrane lipid metabolism, and other impairments related to the integrity of the central nervous system. It is noteworthy that some of these metabolomic markers are in accordance with pathological alterations observed in human Alzheimer's disease, while others have not been previously described. Therefore, these results demonstrate the potential of metabolomics and the use of transgenic animal models to understand the pathogenesis of Alzheimer's disease.

**KEYWORDS.** Metabolomics, APP/PS1 mice, Alzheimer's disease, serum

**ABBREVIATIONS.** AD: Alzheimer's disease; GC-MS: gas chromatography mass spectrometry; UHPLC-MS: ultra-high performance liquid chromatography mass spectrometry; TG: transgenic; WT: wild type; QC: quality control; PCA: principal component analysis; PLS-DA: partial least squares discriminant analysis; VIP: variable importance in the projection; HEPE: hydroxyl-eicosapentaenoic acid; LPC: lyso-phosphocholine; LPI: lyso-phosphoinositol; LPE: lyso-phosphoethanolamine; PI: phosphoinositol; PC: phosphocholine; PE: phosphoethanolamine; PPE: plasmenylethanolamine; PPC: plasmenylcholine; SM: sphingomyelin.

## 1. INTRODUCTION

Alzheimer's disease (AD), the most common neurodegenerative disorder worldwide, is characterized by an insidious onset and a progressive decline of cognitive functions. It is recognized that genetics plays an important role in AD, principally due to mutations in the genes of amyloid precursor protein (APP) and presenilin 1 and 2 (PS1 and PS2), which lead to the accumulation of A $\beta$  peptides [1]. However, the exact etiology and pathogenesis of this disorder is not clear, given that its study is hindered by the complex underlying biochemistry, the long presymptomatic period, the inability to study microscopic morphological changes in tissues until the final stage of the disease and the variability of clinical expression of symptoms. Thereby, nowadays AD can only be detected at advanced stages of disease by exclusion of other pathologies based on clinical criteria defined by the NINCDS-ADRDA, and a definitive diagnosis is only made when there is histopathological confirmation [2]. Therefore, early preclinical detection has become a primary focus of AD research. Numerous transgenic mouse models that mimic the main features of Alzheimer's disease have been developed to better understand the pathogenic mechanism of neurodegeneration and to test potential therapies [3]. In most of these models, the transgenic animals over-express mutated forms of human amyloid precursor protein, developing amyloid plaques with aging predominantly in the hippocampus and cortex. However, it has been demonstrated that co-expression of mutated human presenilin 1 or presenilin 2 in the model accelerates amyloid deposition. In this sense, the double mutant transgenic line APP/PS1 has been widely used in neuroscience studies given that reproduce some of the neuropathological and cognitive deficits observed in AD, with a phenotype characterized by early amyloid deposits and behavioral deficits [4].

Metabolomics presents a high potential in health survey and biomarkers discovery, because changes in specific groups of metabolites may be sensitive to pathogenically relevant factors. Thus, metabolomic profiling is emerging as a powerful tool for the characterization of complex phenotypes affected by both genetic and environmental factors [5]. Particularly, metabolomics plays a prominent role in biomarker identification of multifunctional disorders such as Alzheimer's disease, in which many heterogeneous cellular processes are involved. Numerous studies have been performed to assess the pathophysiological status of transgenic animals by postmortem analysis of brain tissue samples [6-9]. However, the use of peripheral biofluids has been only scarcely considered, although the discovery of a well-established peripheral biomarker easily accessible is of primary importance when considering the prevalence of this disease [10]. Jiang et al. found significant differences in endogenous metabolites from serum of the senescence-accelerated mouse, suggesting perturbed glucose and lipid metabolisms, and attenuated protective function of inosine [11]. In other studies, the metabolic profiles of both brain and plasma of transgenic mice were characterized and compared to those of wild-type mice [12-13]. Lower levels of metabolites were found in plasma samples, and they fluctuate more between the two groups than brain metabolites. However, the statistical models built using plasma metabolite profiles were more accurate than brain tissue despite the smaller number of factors. Finally, 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 [14-15].

In the study presented here, serum samples from APP/PS1 transgenic mice were analyzed by a metabolomic multiplatform combining gas chromatography-mass spectrometry (GC-MS) and ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS). No single analytical technique covers the entire spectrum of the metabolome, so the application of combined LC-MS and GC-MS technologies is becoming the most relevant tool for biomarkers discovery due to their complementarity. Gas chromatography-mass spectrometry has been traditionally employed for the profiling of low molecular weight metabolites with high sensitivity, peak resolution and reproducibility [16]. On the other hand, reversed-phase ultra-high performance liquid chromatography-mass spectrometry has become the main workhorse in metabolomics due to its high resolution and sensitivity, fast analysis and good potential for biomarker identification, which provides efficient retention and separation of relatively nonpolar metabolites across a large molecular weight range [17]. Thus, the application of this global metabolomic approach using two complementary platforms allowed a broad analytical coverage of serum metabolites, from small polar metabolites to lipids. Then, multivariate statistical analysis was used to differentiate serum metabolomic profiles of APP/PS1 mice from wild-type controls, and to identify perturbations in biochemical pathways related to pathological processes.

## 2. MATERIAL AND METHODS

### 2.1. ANIMAL HANDLING

1 Transgenic APP/PS1 mice (C57BL/6 background) were generated as previously described by Jankowsky  
2 et al., expressing the Swedish mutation of APP together with PS1 deleted in exon 9 [18]. On the other  
3 hand, age-matched wild-type mice of the same genetic background (C57BL/6) were purchased from  
4 Charles River Laboratory for their use as controls. In this study, male and female animals at 6 months of  
5 age were used for experiments (TG: N=30, male/female 13/17; WT: N=30, male/female 15/15). Animals  
6 were acclimated for 3 days after reception in rooms with a 12-h light/dark cycle at 20-25 °C, with water  
7 and food available *ad libitum*. Then, mice were anesthetized by isoflurane inhalation and blood was  
8 extracted by cardiac puncture. Blood samples were immediately cooled and protected from light for 30  
9 minutes to allow clot retraction, and then centrifuged at 3500 rpm for 10 minutes at 4°C. Serum was  
10 aliquoted in Eppendorf tubes and frozen at -80°C until analysis. Animals were handled according to the  
11 directive 2010/63/EU stipulated by the European Community, and the study was approved by the Ethical  
12 Committee of University of Huelva.

## 13 2.2. SERUM SAMPLES PREPARATION

14 For the extraction of metabolites, 100 µL of serum were mixed with 400 µL of methanol/ethanol (50%  
15 v/v) and vortexed for 5 min. Then, samples were centrifuged at 4000 rpm for 10 min at 4°C, and the  
16 supernatant was transferred to another tube to be dried under nitrogen stream. Finally, the resulting  
17 residue was reconstituted with methanol/water (80:20 v/v) containing 0.1% formic acid. An aliquot of this  
18 extract (50 µl) was split for derivatization before GC-MS fingerprinting, and the rest of the sample was  
19 transferred to the injection vial for LC-MS analysis. Derivatization was carried out according to the two  
20 step methodology proposed by Begley et al [19]. For this, 50µl of extracts were dried under nitrogen  
21 stream and redissolved in 50 µL of 20 mg mL<sup>-1</sup> methoxyamine in pyridine for protection of carbonyl  
22 groups by methoximation. After briefly vortexing, samples were incubated at 80°C for 15 min in a water  
23 bath. Then silylation was performed by adding 50 µL of MSTFA (N-methyl-N-trimethylsilyl  
24 trifluoroacetamide) and incubating at 80°C for a further 15 min. Finally, extracts were centrifuged at 4000  
25 rpm for one minute and supernatant was collected for analysis.

## 26 2.3. METABOLOMIC PROFILING BY GC-MS

27 Analyses were performed in a Trace GC ULTRA gas chromatograph coupled to an ion trap mass  
28 spectrometer detector ITQ 900 (Thermo Fisher Scientific), using a Factor Four capillary column VF-5MS  
29 30m×0.25mm ID, with 0.25 µm of film thickness (Varian). The GC column temperature was set to 100°C  
30 for 0.5 minutes, and programmed to reach 320°C at a rate of 15°C per minute. Finally, this temperature  
31 was maintained for other 2.8 minutes, being the total time of analysis 18 minutes. The injector  
32 temperature was kept at 280°C, and helium was used as carrier gas at a constant flow rate of 1 ml min<sup>-1</sup>.  
33 For mass spectrometry detection, ionization was carried out by electron ionization (EI) using a voltage of  
34 70 eV, and the ion source temperature was set at 200°C. Data were obtained acquiring full scan spectra in  
35 the m/z range 35-650. For analysis, 1 µl of sample was injected in splitless mode.

## 36 2.4. METABOLOMIC PROFILING BY UHPLC-MS

37 Serum was fingerprinted by ultra-high performance liquid chromatography (Accela LC system, Thermo  
38 Fisher Scientific) coupled to a quadrupole-time-of-flight mass spectrometry system equipped with  
39 electrospray source (QSTAR XL Hybrid system, Applied Biosystems). Chromatographic separations  
40 were performed in a reversed-phase column (Hypersil Gold C18, 2.1x50 mm, 1.9µm) thermostated at  
41 50°C, with an injection volume of 5µl. Solvents were delivered at a flow rate 0.5ml/min, using methanol  
42 (solvent A) and water (solvent B), both containing 10mM ammonium formate and 0.1% formic acid. The  
43 gradient elution program was: 0-1 min, 95% B; 2.5 min, 25% B; 8.5-10 min, 0% B; 10.1-12 min, 95% B.  
44 MS operated in positive and negative polarities, acquiring full scan spectra in the m/z range 50-1000 with  
45 1.005 seconds scan time. The ion spray voltage (IS) was set at 5000V and -2500V, and high-purity  
46 nitrogen was used as curtain, nebulizer and heater gas at flow rates about 1.48 L min<sup>-1</sup>, 1.56 L min<sup>-1</sup> and  
47 6.25 L min<sup>-1</sup>, respectively. The source temperature was fixed at 400°C, with a declustering potential (DP)  
48 of 100V/-120V, and a focusing potential (FP) of ±350V. To acquire MS/MS spectra, nitrogen was used as  
49 collision gas.

## 50 2.5. DATA PROCESSING

51 Raw data was processed following the pipeline described by Katajamaa et al., which proceeds through  
52 multiple stages including feature detection, alignment of peaks and normalization [20]. For this purpose,  
53 we employed the freely available software XCMS, included in the R platform (<http://www.r-project.org>).  
54 UHPLC-MS files were converted into mzXML format using the msConvert tool (ProteoWizard), while  
55 GC-MS files were converted into netCDF using the Thermo File Converter tool (Thermo Fisher  
56 Scientific). Subsequently, data were extracted using the matchedFilter method. This algorithm slices data  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 into extracted ion chromatograms (XIC) on a fixed step size (default 0.1 m/z), and then each slice is  
2 filtered with matched filtration using a second-derivative Gaussian as the model peak shape [21]. This  
3 filtration requires the optimization of two parameters in order to extract the maximum information as  
4 possible according to the characteristics of data sets obtained, named signal/noise threshold and full width  
5 at half-maximum (fwhm). The S/N threshold was set at 2 for both analytical approaches, while the  
6 optimal fwhm value was 10 for UHPLC-MS and 3 for GC-MS data, considering that lower peak widths  
7 are usually obtained in gas chromatography. After peak extraction, grouping and retention time correction  
8 of peaks (alignment) was accomplished in three iterative cycles with descending bandwidth (bw) from 10  
9 to 1 seconds in UHPLC-MS, and descending bw from 5 to 1 seconds for GC-MS. Then, imputation of  
10 missing values was performed by returning to the raw spectral data and integrating the areas of the  
11 missing peaks which are below the applied signal-to-noise ratio threshold, using the fillPeaks algorithm.  
12 For data normalization, the locally weighted scatter plot smoothing (LOESS) normalization method was  
13 used, which adjusts the local median of log fold changes of peak intensities between samples in the data  
14 set to be approximately zero across the whole peak intensity range [22]. Finally, data were submitted to  
15 logarithmic transformation, in order to stabilize the variance of results. The preprocessed data were then  
16 exported as a .csv file for further data analysis by multivariate procedures.

## 17 2.6. DATA ANALYSIS

18 Data were subjected to multivariate analysis by principal component analysis (PCA) and partial least  
19 squares discriminant analysis (PLS-DA) in order to compare metabolomic profiles obtained, using the  
20 SIMCA-PTM software (version 11.5, UMetrics AB, Umeå, Sweden). Before performing statistical  
21 analysis, data are usually scaled and transformed in order to minimize the technical variability between  
22 individual samples to extract the relevant biological information from these data sets [23]. For this, data  
23 was submitted to Pareto scaling, for reducing the relative importance of larger values, and logarithmic  
24 transformation, in order to approximate a normal distribution. Quality of the models was assessed by the  
25 R<sup>2</sup> and Q<sup>2</sup> values, supplied by the software, which provide information about the class separation and  
26 predictive power of the model, respectively. These parameters are ranged between 0 and 1, and they  
27 indicate the variance explained by the model for all the data analyzed (R<sup>2</sup>) and this variance in a test set  
28 by cross-validation (Q<sup>2</sup>). Finally, potential biomarkers were selected according to the Variable  
29 Importance in the Projection, or VIP (a weighted sum of squares of the PLS weight, which indicates the  
30 importance of the variable in the model), considering only variables with VIP values higher than 1.5,  
31 indicative of significant differences among groups. In addition, groups comparison was conducted by t-  
32 test using the STATISTICA 8.0 software (StatSoft, Tulsa, USA). Only p values below 0.05 were regarded  
33 as statistically significant.

## 34 2.7. IDENTIFICATION OF BIOMARKERS

35 Potential biomarkers detected by GC-MS were identified using the NIST Mass Spectral Library (version  
36 08). Alternatively, identification of metabolites from UHPLC-MS profiling was made matching the  
37 experimental accurate mass and tandem mass spectra (MS/MS) with those available in metabolomic  
38 databases (HMDB, METLIN and LIPIDMAPS), using a mass accuracy of 30 ppm. Furthermore, the  
39 identity of lipids was confirmed based on characteristic fragmentation patterns. Choline-containing  
40 phospholipids were detected as protonated and sodiated ions in positive ion mode, while in negative  
41 polarity these lipids formed demethylated ions or formate adducts. On the other hand, more abundant ions  
42 for ethanolamine species were [M+H]<sup>+</sup> and [M-H]<sup>-</sup>, in positive and negative modes respectively. Finally,  
43 phosphatidylinositols were detected in negative ion mode in form of deprotonated ions.  
44 Phosphatidylcholines (PCs) and lysophosphatidylcholines (LPCs) presented characteristic ions in positive  
45 ionization mode at m/z 184.07, 104.10 and 86.09, and two typical fragments due to the loss of  
46 trimethylamine (m/z 59) and phosphocholine (m/z 183 or 205, if the counterion is proton or sodium). In  
47 contrast, the product-ion spectra of ethanolamines were dominated by [M+H-141]<sup>+</sup> arising from  
48 elimination of the phosphoethanolamine moiety. Finally, in negative mode these distinctive signals were  
49 found at 168.04, 196.07 and 241.02, for choline, ethanolamine and inositol derived lipids, respectively.  
50 Furthermore, the fragmentation in the glycerol backbone and release of the fatty acyl substituents enabled  
51 the identification of individual species of phospholipids, as previously described [24]. For  
52 sphingomyelins, typical product ions appear at m/z 264 and 282 due to the fragmentation in the  
53 sphingosine moiety, and the cleavage of phosphocholine headgroup generates characteristic fragments at  
54 184.07 and 168.04 m/z, in positive and negative modes respectively [25]. Acylcarnitines were confirmed  
55 based on characteristic fragments of 60.08 and 85.03 m/z [26]. Finally, fatty acid amides [27],  
56 eicosanoids [28] and sphingoid bases [29] were also confirmed with characteristic fragments described in  
57 the literature.  
58  
59  
60  
61  
62  
63  
64  
65

## 2.8. METABOLIC PATHWAY ANALYSIS

Metabolic pathway analysis was subsequently performed to identify and visualize the affected metabolic pathways in APP/PS1 mice on the basis of potential biomarkers detected by GC-MS and UHPLC-MS profiling. For this purpose, the MetPA web tool was employed (<http://metpa.metabolomics.ca>), which conducts pathway analysis through pathway enrichment analysis and pathway topological analysis [30]. In this work, we select the *Mus musculus* library and use the default ‘Hypergeometric Test’ and ‘Relative-Betweenness Centrality’ algorithms for pathway enrichment analysis and pathway topological analysis, respectively. In order to identify the most relevant pathways, the impact-value threshold calculated from pathway topology analysis was set to 0.1.

## 3. RESULTS

### 3.1. MULTIVARIATE DATA ANALYSIS

The metabolomic approach based on the combination of GC-MS and UHPLC-MS profiling was applied to serum samples from APP/PS1 and wild-type mice in order to determine changes in metabolites associated with neurodegenerative processes. Due to the high complexity of metabolomic profiles obtained, multivariate data analysis based on projection methods was employed for the interpretation of results. Projection methods are based on the conversion of a multidimensional data table into a low-dimensional model through reduction of the high number of variables to obtain new variables or components (combinations of the originals) that are able to explain the variability of results [31]. These models make possible to extract and display the systematic variation in the data, and allow visualizing outliers, groupings and trends between different groups of samples, so they have become the main workhorse in chemometrics for metabolomics. In this way, after raw data processing, Pareto scaling and logarithmic transformation, data was submitted to multivariate statistics in order to build predictive models for classification of samples. As a first exploratory step, principal component analysis (PCA) was applied for a preliminary evaluation of the quality of the data before the application of any supervised statistical analysis. This unsupervised method was not able to discriminate between groups (Fig. 1A-C), as previously reported in other metabolomic studies with AD transgenic mice [11,14], but showed a clustering of samples in the scores plot without significant outliers (tested by inspection of Hotelling T<sup>2</sup>-range plots). Subsequently, partial least squares discriminant analysis (PLS-DA) was used in the same data set to sharpen the separation between groups. PLS-DA scores plots (Fig 1D-F) displayed a clear separation between transgenic (TG) and control (WT) mice. Furthermore, the statistics parameters confirmed the goodness of these models (GC-MS: R<sup>2</sup>=0.991 Q<sup>2</sup>=0.802; UHPLC-MS(+): R<sup>2</sup>=0.985 Q<sup>2</sup>=0.938; UHPLC-MS(-): R<sup>2</sup>=0.995 Q<sup>2</sup>=0.944).

### 3.2. SELECTION OF BIOMARKERS

After PLS-DA modeling, data was further analyzed to identify the significant metabolites that contributed to the separation of groups. For this purpose, statistically significant metabolites were selected by inspecting loadings plots from PLS-DA, considering only metabolites with VIP values higher than 1.5, as well as by t-test (p≤0.05). In Table 1 are listed the potential biomarkers identified by UHPLC-MS, their experimental mass and retention time, the ionization mode used for detection, the fold change (calculated by dividing the mean area for peaks in the APP/PS1 group by the mean area in the control group) and p value (data for individual lipids are presented in electronic Supplementary Material, Table S1). On the other hand, significant compounds identified by GC-MS are summarized in Table 2, including the retention time, the fold change and p value.

As can be observed, complementary results were obtained from both analytical techniques employed in this study. Metabolomic profiling by UHPLC-MS highlighted the importance of different classes of lipids in the development of features of Alzheimer’s disease displayed by the APP/PS1 mice, including phospholipids and lysophospholipids, eicosanoids, sphingomyelins and sphingoid bases, bile acids, and others. Alternatively, GC-MS showed that a number of low molecular weight metabolites were significantly perturbed in this mouse model, such as amino acids, hexoses or organic acids. Therefore, these results demonstrated that a broad number of metabolites can be detected by this metabolomic multiplatform, which provides a comprehensive overview of serum metabolome.

### 3.3. METABOLIC PATHWAY ANALYSIS

Pathway analysis was then performed to identify the altered biochemical networks associated with the metabolic perturbations presented in Tables 1-2, in order to elucidate the underlying pathological mechanisms occurring in the APP/PS1 mice. To this end, the MetPA web tool combines pathway enrichment analysis and pathway topology analysis to identify the most relevant metabolic pathways involved in the conditions under study, using pathways downloaded from the KEGG database. Thereby,

1 the pathway impact is calculated as the sum of the importance measures of the matched metabolites  
2 normalized by the sum of the importance measures of all metabolites in each pathway [30]. Finally,  
3 results from this pathway analysis can be visualized as a graphical output representing the p value for  
4 each metabolic pathway (log-transformed) vs. the calculated pathway impact, where each node represents  
5 a metabolic pathway and its size indicates the importance of this pathway in response to Alzheimer's  
6 disease (Fig. 2A). In this way, pathway analysis revealed that the most disturbed pathways were the  
7 metabolism of amino acids such as phenylalanine (a); glycine, serine and threonine (c); tryptophan (d);  
8 and tyrosine (h); metabolism of lipids as glycerophospholipids (b); sphingolipids (g); and ether lipids (l);  
9 perturbed inositol phosphate metabolism (k); and alterations related to energy metabolism, including  
10 glyoxylate and dicarboxylate metabolism (e); starch and sucrose metabolism (f); glycolysis (i); and TCA  
11 cycle (j).

#### 12 4. DISCUSSION

13 The APP/PS1 mouse model reproduces well some of the neuropathological and cognitive deficits  
14 observed in human Alzheimer, with a phenotype characterized by deposition of A $\beta$  plaques starting from  
15 the age of four months, glial activation, and deficits in cognitive functions at the age of six months [4].  
16 Furthermore, the study of the neurochemical profile and age-dependent metabolic changes exhibited by  
17 this transgenic line has demonstrated that these alterations precede cognitive dysfunctions [32], being  
18 very similar to those found in AD patients. Therefore, the use of this model may be of great help for the  
19 investigation of pathological mechanisms associated with Alzheimer's disease. Serum metabolomics  
20 based on mass spectrometry techniques, coupled to both gas chromatography and ultra-high performance  
21 liquid chromatography, revealed significant alterations in numerous metabolites. Furthermore, the  
22 application of metabolic pathway analysis allowed the identification of multiple abnormal processes  
23 underlying the pathology.

24  
25 It is noteworthy the presence of characteristic metabolomic signatures indicating a severe energy  
26 impairment, with altered serum levels of metabolites such as glucose, glucose-6-phosphate, glycerol-3-  
27 phosphate, 1,3-bisphosphoglycerate, inorganic phosphate, lactate,  $\beta$ -hydroxybutyrate, citrate, malate,  
28 succinate, oleyl-carnitine, stearic acid and creatinine. The reduction of the cerebral metabolic rate for  
29 glucose and oxygen is one of the main features of Alzheimer's disease, demonstrated by *in vivo* imaging  
30 using positron emission tomography [33]. This hypometabolic situation, occurring as a consequence of  
31 multiple metabolic defects, is finally reflected in perturbed levels of energy-related metabolites. Lower  
32 blood levels of glucose (Table 2) have been previously reported for different transgenic models of  
33 Alzheimer's disease [11-13], which suggests a change in carbohydrate and energy metabolism. In  
34 addition, the decrease observed in glycolytic intermediates such as glucose-6-phosphate and 1,3-  
35 bisphosphoglycerate, and the increase of glycerol-3-phosphate, precursor of glyceraldehyde-3-  
36 phosphate, also support an important dysfunction in glycolysis. In this sense, numerous alterations have  
37 been found in key glycolytic enzymes in AD, including increased glucose-6-phosphate dehydrogenase  
38 activity [34] and decreased glyceraldehyde-3-phosphate dehydrogenase activity [35], which is in  
39 agreement with our metabolomic findings. Serum concentration of inorganic phosphate is a determining  
40 factor in the regulation of energy metabolism and rate of oxygen consumption [36], so that reduced blood  
41 phosphate (Table 2) could be linked to a high demand by cells to stimulate metabolism of glucose.  
42 Mitochondrial dysfunction also plays a prominent role in energy deficiencies related to Alzheimer's  
43 disease. Significant impairments have been reported in multiple enzymes from the tricarboxylic acid  
44 cycle (TCA) [37], leading to abnormal levels of TCA intermediates and related compounds [38], in  
45 accordance with imbalances in serum concentrations of citrate, succinate and malate listed in Tables 1-2.  
46 In order to compensate these bioenergetic deficits caused by brain hypometabolism and impaired  
47 mitochondrial function, alternative pathways have been found significantly over-expressed in the  
48 APP/PS1 mice. The increase observed in serum lactate together with the reduction of glucose levels is  
49 consistent with a shift in energy metabolism toward anaerobic glycolysis. Under hypoxic conditions,  
50 neurons suffer adaptive processes to the reduced glucose consumption in order to maintain ATP levels,  
51 which are mediated by the hypoxia-inducible factor 1. This transcription factor induces the transcription  
52 of numerous genes related to hexoses metabolism including lactate dehydrogenase, but inhibits pyruvate  
53 dehydrogenase, thus promoting lactic fermentation instead of oxidative phosphorylation [39]. Thereby, a  
54 significant increase of lactate dehydrogenase activity has been previously found in AD brains, which  
55 produces an accumulation of lactate in brain [8,9,13] and blood [11,13]. Moreover, ketone body-driven  
56 energy production has been also proposed as a compensatory response when carbohydrate intake is low.  
57 Ketone bodies, principally acetoacetate and  $\beta$ -hydroxybutyrate, are produced from fatty acids in the liver  
58 and can be used to produce energy via conversion into acetyl-CoA and the TCA cycle. Yao et al.  
59 demonstrated that triple transgenic Alzheimer's mouse models use ketone bodies as alternative fuel to

1 sustain ATP generation, so that the decline in mitochondrial bioenergetics is accompanied by increased  
2 activity of 3-oxoacid-CoA transferase 1 (SCOT, required for ketone body utilization) [40]. This enhanced  
3 ketogenic pathway leads to decreased levels of ketone bodies such as  $\beta$ -hydroxybutyrate [13], in  
4 accordance with experimental data from GC-MS profiling in APP/PS1 mice (Table 2), and also supports  
5 the aforementioned increase in succinate levels, because it is a byproduct in the metabolism of ketone  
6 bodies by the action of SCOT. Another indicator of mitochondrial dysfunction observed in the APP/PS1  
7 mice is the decrease of serum oleyl-carnitine and complementary increase of stearic acid levels. Lower  
8 levels of L-carnitine have been reported in AD [41-42], suggesting a perturbed transport of fatty acids  
9 into the mitochondria. Furthermore, different enzymes related to the  $\beta$ -oxidation pathway have been  
10 found over-expressed in AD, such as hydroxyacyl-coenzyme A dehydrogenase [40] or short chain 3-  
11 hydroxyacyl-CoA dehydrogenase [43]. Therefore, this impaired metabolism of lipids could be involved in  
12 neurodegenerative energetic failures as a supplementary pathway to those previously described. Finally,  
13 the increase in creatinine levels (Table 2) implies an abnormal metabolism of creatine in the APP/PS1  
14 mice. Creatine plays a fundamental role in energy buffering and overall cellular bioenergetics by means  
15 of the creatine kinase/phosphocreatine system, being responsible for the transfer of energy from  
16 mitochondria to cytosol. Oxidative stress have been previously related to reduced creatine kinase activity  
17 in brain of AD patients, which finally results in reduced levels of creatine [41,42,44]. By contrast, levels  
18 of creatinine, the non-enzymatic degradation product of creatine and phosphocreatine, are usually higher  
19 in AD [9,42], which could indicate a dyshomeostasis of phosphocreatine system. Therefore, it could be  
20 concluded that deficits in bioenergetic metabolism is a key factor in pathogenesis of AD-type disorders  
21 found in the APP/PS1 mice, affecting multiple metabolic pathways such as glycolysis, TCA cycle,  $\beta$ -  
22 oxidation, ketogenic pathway and phosphocreatine system, as summarized in Figure 2B.

23 The decrease observed in adenosine monophosphate levels (Table 1) and the complementary increase of  
24 uric acid (Table 2) suggests an impaired purine metabolism. In this sense, Lin et al. found a perturbed  
25 catabolism of different purine derivatives toward the accumulation of uric acid in the cerebellum of  
26 transgenic CRND8 mice [45]. Moreover, the degradation of AMP may have important consequences in  
27 cellular energy homeostasis, since it plays a central role in glucose and lipid metabolism through the  
28 AMP-activated protein kinase, which is known to be decreased in AD brain [46]. Furthermore, the  
29 decrease of serum AMP levels could be related to an elevated adenosine monophosphate deaminase  
30 activity, which has been proposed as a potential source for ammonia production in Alzheimer's disease  
31 brain [47]. Hyperammonemia resulting from alterations in ammonia regulation, mainly due to urea cycle  
32 enzyme deficiencies, has deleterious effects on the central nervous system. In Alzheimer's disease, the  
33 alteration of urea cycle has been demonstrated on the basis of altered levels of expression in different  
34 enzymes and the corresponding genes [48]. Thus, abnormal content of urea and related amino acids has  
35 been previously reported in AD [44,49], in agreement with our metabolomic findings regarding reduced  
36 serum levels of urea and citrulline (Tables 1-2). Besides these metabolic alterations, other metabolites  
37 potentially involved in different pathways related to the integrity of the central nervous system were  
38 perturbed in serum from APP/PS1 mice, such as tyrosine, tryptophan, serotonin, fatty acid amides,  
39 monostearin and bile acids. Serotonin is a neurotransmitter derived from tryptophan involved in memory  
40 and learning, while tyrosine is the precursor of catecholamines such as dopamine or epinephrine.  
41 Thereby, reductions of serotonin, tryptophan and tyrosine levels denote a severe disturbance in  
42 monoaminergic neurotransmission systems, which confirm previous studies in AD subjects [41,44,50].  
43 Fatty acid amides are a family of lipid signaling molecules that can act as regulators of several cellular  
44 and physiological functions, demonstrating control over a variety of biological processes such as sleep  
45 regulation, modulation of monoaminergic systems, locomotion, inhibition of phospholipase A<sub>2</sub> and  
46 epoxide hydrolase, among other processes [51]. These lipids are metabolized by the fatty acid amide  
47 hydrolase (FAAH), the principal catabolic enzyme of endocannabinoid system. The over-expression of  
48 FAAH has been previously associated with AD [52], which might lead to decreased levels of palmitamide  
49 and stearamide observed in serum of APP/PS1 mice (Table S1). On the other hand, the reduction of  
50 circulating levels of monostearin could be considered as an indicator of altered expression of  
51 monoacylglycerol lipase, also involved in endocannabinoid system and over-expressed in AD brain [53].  
52 Therefore, reduced palmitamide, stearamide and monostearin could be considered as novel potential  
53 biomarkers of AD-type disorders in APP/PS1 mice indirectly related to endocannabinoid dysfunction,  
54 previously not described to our knowledge. Finally, decreased levels of deoxycholic, taurodeoxycholic  
55 and taurocholic acids were also observed in serum samples analyzed by UHPLC-MS (Table S1). In this  
56 context, ursodeoxycholic acid and tauroursodeoxycholic acid were demonstrated to be potent inhibitors of  
57 apoptosis [54], and they present neuroprotective action against amyloid deposition [55]. Therefore,  
58 although further studies have to be performed in order to confirm these results, these compounds  
59  
60  
61  
62  
63  
64  
65

1 highlight as interesting potential markers of AD, especially considering their role as important metabolic  
2 integrators and signaling factors [56].

3 Numerous lipids also contributed significantly to discrimination between APP/PS1 mice and WT-controls  
4 considering the high impact-value calculated from pathway topology analysis (Fig. 2A), including  
5 choline, ethanolamine and inositol derived phospholipids and lyso-phospholipids, plasmalogens,  
6 sphingomyelins and sphingoid bases, as well as cholesterol, which may indicate an abnormal metabolism  
7 of membrane lipids. Membrane breakdown in AD has been traditionally related to over-activation of  
8 phospholipases, principally phospholipase A<sub>2</sub>, leading to phospholipids degradation and resulting in the  
9 generation of second messengers involved in neurodegeneration [57]. However, this membrane  
10 destabilization process has been also associated with imbalance in the levels of saturated/unsaturated fatty  
11 acids contained in the structure of phospholipids [24,41]. In this study, decreased serum levels of different  
12 phospholipid species was observed, including cholines, ethanolamines, inositols and plasmalogens, most  
13 of them containing PUFAs in their structure, which is in accordance with previous studies in brain from  
14 transgenic mice of AD [6,7,58]. However, this is the first time that this finding is described in a peripheral  
15 sample as is serum. Furthermore, the release of fatty acids from the hydrolysis of these phospholipids by  
16 PLA<sub>2</sub> and subsequent oxidation supports the elevation of different eicosanoids in serum samples, such as  
17 prostaglandins (PG) and hydroxy-eicosapentaenoic acid (HEPE) (Table S1), which are important lipid  
18 mediators closely associated with neuronal pathways involved in AD neurobiology [59]. Moreover, lower  
19 concentrations of lyso-phospholipids were found in serum from APP/PS1 mice (Table S1), in agreement  
20 with previous studies in blood from human AD patients [24,29]. Therefore, results presented here  
21 demonstrate that phospholipids dyshomeostasis must be developed through similar pathways in both AD  
22 humans and transgenic mice. In addition, this altered metabolism of phospholipids in the APP/PS1 mice  
23 was also supported by other metabolomic changes in low molecular weight metabolites:  
24 phosphoethanolamine, myoinositol and myoinositol-1-phosphate. The decrease in phosphoethanolamine,  
25 the precursor of phosphatidylethanolamines, has been previously reported in postmortem AD brains [60],  
26 corroborating the evidence for a membrane defect in Alzheimer disease. On the other hand, perturbed  
27 levels of myoinositol and myoinositol-1-phosphate may be directly related to altered phosphatidylinositol  
28 metabolism and dysfunctions in the phosphoinositide signaling system. In this sense, previous reports  
29 have described increased levels of several isoenzymes of phosphoinositide-specific phospholipase C [61],  
30 as well as higher activity of myo-inositol monophosphatase in AD brains [62]. Therefore, an altered  
31 cellular homeostasis of myoinositol and their phosphorylated forms occurs in neurons, leading to the  
32 accumulation of free myoinositol, a conventional marker of astrogliosis and neuronal death, and decrease  
33 of myoinositol-1 phosphate in brains from both humans and animal models of AD [33,60].  
34

35 Sphingolipid metabolism also seems to play a major role in the dysfunctional management of cellular  
36 membranes in the APP/PS1 mice, as can be observed in Table S1 considering reduced levels of different  
37 species of sphingomyelins and sphingoid bases. In Alzheimer's disease, perturbed sphingomyelin  
38 metabolism has been proposed as a pivotal event in the dysfunction and degeneration of neurons, with  
39 increased SM levels in CSF [63] and decreased in blood [64], while results from post-mortem brain  
40 analyses are contradictory, showing increased [58] or decreased [65] total concentrations of  
41 sphingomyelins. On the other hand, lipidomic investigations in transgenic mouse brains showed  
42 consistent increases of SM levels [58,66], but this is the first time that altered circulating levels of  
43 sphingomyelins in serum samples is observed. Moreover, decreased levels of sphinganine,  
44 hexadecasphinganine and phytosphingosine also support a disturbed sphingolipid metabolism, as  
45 previously reported in human serum [29]. Cholesterol is also an essential constituent of lipid rafts, whose  
46 reduction in brain can produce serious alterations of the physicochemical structure of lipid raft  
47 microdomains [66]. However, elevated blood levels of cholesterol and other lipids have been proposed to  
48 increase the risk of developing AD [67], as observed in our study (Table 2). Hyperlipidemia is one of the  
49 most important vascular risk factors, which can affect to cerebrovascular system causing atrophy,  
50 structural changes in the blood-brain barrier and inflammation. In this way, it is recognized that subjects  
51 with vascular risk factors have an increased prevalence of both vascular dementia and Alzheimer's  
52 disease [68], caused by decreased cerebral blood flow that finally involves neuronal cell loss.  
53

54 Finally, decreased levels of amino acids glycine, threonine, proline and pyroglutamate were found by GC-  
55 MS profiling of serum samples (Table 2). By contrast, previous studies reported increased concentrations  
56 of several amino acids in brains from AD transgenic mice, including serine, threonine, valine, alanine,  
57 lysisne and leucine [7,8,9,12], which could indicate an important deregulation of the transport of amino  
58 acids across the blood brain barrier. Amino acids enter into the central nervous system by means of the  
59 sodium-independent system L1, which is regulated by the  $\gamma$ -glutamyl cycle [69]. In this process, amino  
60  
61  
62  
63  
64  
65

1 acids react with glutathione by the action of  $\gamma$ -glutamyl transpeptidase to form  $\gamma$ -glutamyl amino acids,  
2 which after enter cells are degraded to the corresponding amino acid, being liberated a molecule of  
3 pyroglutamate that is essential since stimulates sodium dependent carriers for the later removal of  
4 deleterious amino acids from brain. Changes in this cycle are known in AD brain, with a decrease of the  
5 GSH/GSSG ratio, diminished  $\gamma$ -glutamylcysteine synthetase activity and increased activity of enzymes  
6 related to the GSH use such as glutathione peroxidase,  $\gamma$ -glutamyl transpeptidase, and glutathione S-  
7 transferase [70]. In addition, reduced levels of pyroglutamate have been previously reported in AD brain  
8 [9], in accordance with our metabolomic results. Therefore, these data may suggest that disturbances in  
9 amino acids homeostasis play a critical role in the pathogenesis of AD in APP/PS1 mice.

## 10 5. CONCLUSIONS

11 In the present study, the APP/PS1 transgenic mice of Alzheimer's disease could be clearly differentiated  
12 from wild-type controls by metabolomic analysis of serum samples using combined GC-MS and  
13 UHPLC-MS. Subsequent application of metabolic pathway analysis allowed the elucidation of underlying  
14 pathological mechanisms occurring in the APP/PS1 mice, including failures in energy metabolism,  
15 perturbed homeostasis of membrane lipids and amino acids, as well as abnormal processes related to the  
16 integrity of the central nervous system, such as hyperammonemia, endocannabinoid dysfunction or  
17 monoaminergic alterations. Therefore, these findings stand out the potential of the APP/PS1 transgenic  
18 mice to study the pathogenesis and development of Alzheimer's disease, because many of these findings  
19 agree with pathological alterations observed in diseased humans.

20  
21 **Acknowledgements.** This work was supported by the projects CTM2012-38720-C03-01 from the  
22 Ministerio de Ciencia e Innovación and P008-FQM-3554 and P009-FQM-4659 from the Consejería de  
23 Innovación, Ciencia y Empresa (Junta de Andalucía). Raúl González Domínguez thanks the Ministerio de  
24 Educación for a predoctoral scholarship.

## 25 REFERENCES

- 26  
27 [1] Bettens K, Sleegers K, Van Broeckhoven C. Genetic insights in Alzheimer's disease. *Lancet Neurol*  
28 2013;2:92-104.
- 29 [2] McKahnn G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of  
30 Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of  
31 Health and Human Services Task Force on Alzheimer's disease. *Neurology* 1984;34:939-44.
- 32 [3] Hall AM, Roberson ED. Mouse models of Alzheimer's disease. *Brain Res Bull* 2012;88:3-12.
- 33 [4] Malm T, Koistinaho J, Kanninen K. Utilization of APPswe/PS1dE9 transgenic mice in research of  
34 Alzheimer's disease: Focus on gene therapy and cell-based therapy applications. *Int J Alzheimers Dis*  
35 2011;2011:517160.
- 36 [5] Lindon JC, Holmes E, Nicholson JK. Metabonomics and its role in drug development and disease  
37 diagnosis. *Expert Rev Mol Diagn* 2004;4:189-99.
- 38 [6] González-Domínguez R, García-Barrera T, Vitorica J, Gómez-Ariza JL. Region-specific metabolic  
39 alterations in brain of the APP/PS1 transgenic mice of Alzheimer's disease. *Biochim Biophys Acta Mol*  
40 *Basis Dis* 2014; 1842:2395-402.
- 41 [7] González-Domínguez R, García-Barrera T, Vitorica J, Gómez-Ariza JL. Metabolomic screening of  
42 regional brain alterations in the APP/PS1 transgenic model of Alzheimer's disease by direct infusion mass  
43 spectrometry. *J Pharm Biomed Anal* 2015;102:425-35.
- 44 [8] Salek RM, Xia J, Innes A, Sweatman BC, Adalbert R, Randle S, McGowan E, Emson PC, Griffin JL.  
45 A metabolomic study of the CRND8 transgenic mouse model of Alzheimer's disease. *Neurochem Int*  
46 2010;56:937-43.
- 47 [9] Trushina E, Nemutlu E, Zhang S, Christensen T, Camp J, Mesa J, Siddiqui A, Tamura Y, Sesaki H,  
48 Wengenack TM, Dzeja PP, Poduslo JF. Defects in mitochondrial dynamics and metabolomic signatures  
49 of evolving energetic stress in mouse models of familial Alzheimer's disease. *PLoS ONE* 2012;7:e32737.
- 50 [10] Patel S, Shah RJ, Coleman P, Sabbagh M. Potential peripheral biomarkers for the diagnosis of  
51 Alzheimer's disease. *Int. J. Alzheimers Dis* 2011;2011:572495.
- 52 [11] Jiang N, Yan X, Zhou W, Zhang Q, Chen H, Zhang Y, Zhang X. NMR-based metabolomic  
53 investigations into the metabolic profile of the senescence-accelerated mouse. *J Proteome Res*  
54 2008;7:3678-86.
- 55 [12] Hu ZP, Browne ER, Liu T, Angel TE, Ho PC, Chan ECY. Metabonomic profiling of TASTPM  
56 transgenic alzheimer's disease mouse model. *J Proteome Res* 2012;11:5903-13.
- 57 [13] Graham SF, Holscher C, McClean P, Elliott CT, Green BD. <sup>1</sup>H NMR metabolomics investigation of  
58 an Alzheimer's disease (AD) mouse model pinpoints important biochemical disturbances in brain and  
59 plasma. *Metabolomics* 2013;9:974-83.

- 1 [14] Fukuhara K, Ohno A, Ota Y, Senoo Y, Maekawa K, Okuda H, Kurihara M, Okuno A, Niida S, Saito  
2 Y, Takikawa O. NMR-based metabolomics of urine in a mouse model of Alzheimer's disease:  
3 identification of oxidative stress biomarkers. *J Clin Biochem Nutr* 2013;52:133-8.
- 4 [15] Gonzalez-Dominguez R, Castilla-Quintero R, Garcia-Barrera T, Gomez-Ariza JL. Development of a  
5 metabolomic approach based on urine samples and direct infusion mass spectrometry. *Anal Biochem*  
6 2014;465:20-7.
- 7 [16] Pasikanti KK, Ho PC, Chan ECY. Gas chromatography/mass spectrometry in metabolic profiling of  
8 biological fluids. *J Chromatogr B Analyt Technol Biomed Life Sci* 2008;871:202-11.
- 9 [17] Wilson ID, Nicholson JK, Castro-Perez J, Granger JH, Johnson KA, Smith BW, Plumb RS. High  
10 resolution "ultra performance" liquid chromatography coupled to oa-TOF mass spectrometry as a tool for  
11 differential metabolic pathway profiling in functional genomic studies. *J Proteome Res* 2005;4:591-8.
- 12 [18] Jankowsky JL, Fadale DJ, Anderson J, Xu GM, Gonzales V, Jenkins NA, Copeland NG, Lee MK,  
13 Younkin LH, Wagner SL, Younkin SG, Borchelt DR. Mutant presenilins specifically elevate the levels of  
14 the 42 residue beta-amyloid peptide in vivo: evidence for augmentation of a 42-specific  $\gamma$  secretase. *Hum*  
15 *Mol Genet* 2004;13:159-70.
- 16 [19] Begley P, Francis-McIntyre S, Dunn WB, Broadhurst DI, Halsall A, Tseng A, Knowles J,  
17 HUSERMET Consortium, Goodacre R, Kell DB. Development and performance of a gas  
18 chromatography-time-of-flight mass spectrometry analysis for large-scale nontargeted metabolomic  
19 studies of human serum. *Anal Chem* 2009;81:7038-46.
- 20 [20] Katajamaa M, Oresic M. Data processing for mass spectrometry-based metabolomics. *J Chromatogr*  
21 *A* 2007;1158:318-28.
- 22 [21] Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G. XCMS: Processing mass spectrometry  
23 data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Anal Chem*  
24 2006;78:779-87.
- 25 [22] Veselkov KA, Vingara LK, Masson P, Robinette SL, Want E, Li JV, Barton RH, Boursier-Neyret C,  
26 Walther B, Ebbels TM, Pelczar I, Holmes E, Lindon JC, Nicholson JK. Optimized preprocessing of ultra-  
27 performance liquid chromatography/mass spectrometry urinary metabolic profiles for improved  
28 information recovery. *Anal Chem* 2011;83:5864-72.
- 29 [23] van den Berg RA, Hoefsloot H CJ, Westerhuis JA, Smilde AK, van der Werf MJ. Centering, scaling,  
30 and transformations: improving the biological information content of metabolomics data. *BMC Genomics*  
31 2006;7:142.
- 32 [24] Gonzalez-Dominguez R, Garcia-Barrera T, Gomez-Ariza JL. Combination of metabolomic and  
33 phospholipid-profiling approaches for the study of Alzheimer's disease. *J Proteomics* 2014;104:37-47.
- 34 [25] Haynes CA, Allegood JC, Park H, Sullards MC. Sphingolipidomics: Methods for the comprehensive  
35 analysis of sphingolipids. *J Chromatogr B Analyt Technol Biomed Life Sci* 2009;877:2696-708.
- 36 [26] Vernez L, Hopfgartner G, Wenk M, Krahenbuhl S. Determination of carnitine and acylcarnitines in  
37 urine by high-performance liquid chromatography-electrospray ionization ion trap tandem mass  
38 spectrometry. *J Chromatogr A* 2003;984:203-13.
- 39 [27] Nichols KK, Ham BM, Nichols JJ, Ziegler C, Green-Church KB. Identification of fatty acids and  
40 fatty acid amides in human meibomian gland secretions. *Invest Ophthalmol Vis Sci* 2007;48:34-9.
- 41 [28] Murphy RC, Barkley RM, Berry KZ, Hankin J, Harrison K, Johnson C, Krank J, McAnoy A, Uhlson  
42 C, Zarini S. Electrospray ionization and tandem mass spectrometry of eicosanoids. *Anal Biochem*  
43 2005;346:1-42.
- 44 [29] Li N, Liu W, Li W, Li S, Chen X, Bi K, He P. Plasma metabolic profiling of Alzheimer's disease by  
45 liquid chromatography/mass spectrometry. *Clin Biochem* 2010;43:992-7.
- 46 [30] Xia J, Wishart DS. MetPA: a web-based metabolomics tool for pathway analysis and visualization.  
47 *Bioinformatics* 2010;26:2342-4.
- 48 [31] Trygg J, Holmes E, Lundstedt T. Chemometrics in metabonomics. *J Proteome Res* 2007;6:469-79.
- 49 [32] Chen SQ, Cai Q, Shen YY, Wang PJ, Teng GJ, Zhang W, Zang FC. Age-related changes in brain  
50 metabolites and cognitive function in APP/PS1 transgenic mice. *Behav Brain Res* 2012;235:1-6.
- 51 [33] Ferreira IL, Resende R, Ferreira E, Rego AC, Pereira CF. Multiple defects in energy metabolism in  
52 Alzheimer's disease. *Curr Drug Targets* 2010;11:1193-206.
- 53 [34] Russell RL, Siedlak SL, Raina AK, Bautista JM, Smith MA, Perry G. Increased neuronal glucose-6-  
54 phosphate dehydrogenase and sulfhydryl levels indicate reductive compensation to oxidative stress in  
55 Alzheimer disease. *Arch Biochem Biophys* 1999;370:236-9.
- 56 [35] Shalova IN, Cechalova K, Rehakova Z, Dimitrova P, Ognibene E, Caprioli A, Schmalhausen EV,  
57 Muronetz VI, Saso L. Decrease of dehydrogenase activity of cerebral glyceraldehyde-3-phosphate  
58 dehydrogenase in different animal models of Alzheimer's disease. *Biochim Biophys Acta*  
59 2007;1770:826-32.
- 60  
61  
62  
63  
64  
65

- 1 [36] Bose S, French S, Evans FJ, Balaban RS. Metabolic network control of oxidative phosphorylation:  
2 Multiple roles of inorganic phosphate. *J Biol Chem* 2003;278:39155-65.
- 3 [37] Bubber P, Haroutunian V, Fisch G, Blass JP, Gibson GE. Mitochondrial abnormalities in Alzheimer  
4 brain: mechanistic implications. *Ann Neurol* 2005;57:695-703.
- 5 [38] Redjems-Bennani N, Jeandel C, Lefebvre E, Blain H, Vidailhet M, Guéanta JL. Abnormal substrate  
6 levels that depend upon mitochondrial function in cerebrospinal fluid from Alzheimer patients.  
7 *Gerontology* 1998;44:300-4.
- 8 [39] Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC. HIF-1 mediates adaptation to hypoxia by  
9 actively down-regulating mitochondrial oxygen consumption. *Cell Metab* 2006;3:187-97.
- 10 [40] Yao J, Hamilton RT, Cadenas E, Brinton RD. Decline in mitochondrial bioenergetics and shift to  
11 ketogenic profile in brain during reproductive senescence. *Biochim Biophys Acta* 2010;1800:1121-6.
- 12 [41] González-Domínguez R, García-Barrera T, Gómez-Ariza JL. Using direct infusion mass  
13 spectrometry for serum metabolomics in Alzheimer's disease. *Anal Bioanal Chem* 2014;406:7137-48.
- 14 [42] González-Domínguez R, García A, García-Barrera T, Barbas C, Gómez-Ariza JL. Metabolomic  
15 profiling of serum in the progression of Alzheimer's disease by capillary electrophoresis-mass  
16 spectrometry. *Electrophoresis* 2014; 35:3321-30.
- 17 [43] Yang SY, He XY, Schulz H. 3-Hydroxyacyl-CoA dehydrogenase and short chain 3-hydroxyacyl-  
18 CoA dehydrogenase in human health and disease. *FEBS J* 2005;272:4874-83.
- 19 [44] González-Domínguez R, Garcia-Barrera T, Gomez-Ariza JL. Application of a novel metabolomic  
20 approach based on atmospheric pressure photoionization mass spectrometry using flow injection analysis  
21 for the study of Alzheimer's disease. *Talanta* 2015;131:480-9.
- 22 [45] Lin S, Kanawati B, Liu L, Witting M, Li M, Huang J, Schmitt-Kopplin P, Cai Z. Ultra high  
23 resolution mass spectrometry-based metabolic characterization reveals cerebellum as a disturbed region in  
24 two animal models. *Talanta* 2014;118:45-53.
- 25 [46] Cai Z, Yan LJ, Li K, Quazi SH, Zhao B. Roles of AMP-activated protein kinase in Alzheimer's  
26 disease. *Neuromol Med* 2012;14:1-14.
- 27 [47] Sims B, Powers RE, Sabina RL, Theibert AB. Elevated adenosine monophosphate deaminase  
28 activity in Alzheimer's disease brain. *Neurobiol Aging* 1998;19:385-91.
- 29 [48] Hansmann F, Sillaire A, Kamboh MI, Lendon C, Pasquier F, Hannequin D, Laumet G, Mounier A,  
30 Ayral AM, DeKosky ST, Hauw JJ, Berr C, Mann D, Amouyel P, Campion D, Lambert JC. Is the urea  
31 cycle involved in Alzheimer's disease? *J Alzheimers Dis* 2010;21:1013-21.
- 32 [49] González-Domínguez R, García-Barrera T, Gómez-Ariza JL. Metabolomic study of lipids in serum  
33 for biomarker discovery in Alzheimer's disease using direct infusion mass spectrometry. *J Pharm Biomed  
34 Anal* 2014;98:321-6.
- 35 [50] Storga D, Vrecko K, Birkmayer JGD, Reibnegger G. Monoaminergic neurotransmitters, their  
36 precursors and metabolites in brains of Alzheimer patients. *Neurosci Lett* 1996;203:29-32.
- 37 [51] Ezzili C, Otrubova K, Boger DL. Fatty acid amide signaling molecules. *Bioorg Med Chem Lett*  
38 2010;20:5959-68.
- 39 [52] Benito C, Nuñez E, Tolon RM, Carrier EJ, Rabano A, Hillard CJ, Romero J. Cannabinoid CB2  
40 receptors and fatty acid amide hydrolase are selectively overexpressed in neuritic plaque-associated glia  
41 in Alzheimer's disease brains. *J Neurosci* 2003;23:11136-41.
- 42 [53] Mulder J, Zilberter M, Pasquaré SJ, Alpár A, Schulte G, Ferreira SG, Köfalvi A, Martín-Moreno  
43 AM, Keimpema E, Tanila H, Watanabe M, Mackie K, Hortobágyi T, de Ceballos ML, Harkany T.  
44 Molecular reorganization of endocannabinoid signalling in Alzheimer's disease. *Brain* 2011;134:1041-60.
- 45 [54] Ramalho RM, Viana RJS, Low WC, Steer CJ, Rodrigues CMP. Bile acids and apoptosis modulation:  
46 an emerging role in experimental Alzheimer's disease. *Trends Mol Med* 2008;14:54-62.
- 47 [55] Lo AC, Callaerts-Vegh Z, Nunes AF, Rodrigues CMP, D'Hooge R. Tauroursodeoxycholic acid  
48 (TUDCA) supplementation prevents cognitive impairment and amyloid deposition in APP/PS1 mice.  
49 *Neurobiol Dis* 2013;50:21-9.
- 50 [56] Thomas C, Pellicciari R, Pruzanski M, Auwerx J, Schoonjans K. Targeting bile-acid signalling for  
51 metabolic diseases. *Nat Rev Drug Discov* 2008;7:678-93.
- 52 [57] Farooqui AA, Ong WY, Horrocks LA. Biochemical aspects of neurodegeneration in human brain:  
53 involvement of neural membrane phospholipids and phospholipases A<sub>2</sub>. *Neurochem Res* 2004;29:1961-  
54 77.
- 55 [58] Chan RB, Oliveira TG, Cortes EP, Honig LS, Duff KE, Small SA, Wenk MR, Shui G, Di Paolo G.  
56 Comparative lipidomic analysis of mouse and human brain with Alzheimer disease. *J Biol Chem*  
57 2012;287:2678-88.
- 58 [59] Frisardi V, Panza F, Seripa D, Farooqui T, Farooqui AA. Glycerophospholipids and  
59 glycerophospholipid-derived lipid mediators: A complex meshwork in Alzheimer's disease pathology.  
60 *Prog Lipid Res* 2011;50:313-30.

- [60] Klunk WE, Panchalingam K, McClure RJ, Stanley JA, Pettegrew JW. Quantitative <sup>1</sup>H and <sup>31</sup>P MRS of PCA extracts of postmortem Alzheimer's disease brain. *Neurobiol Aging* 1996;17:349-57.
- [61] Shimohama S, Sasaki Y, Fujimoto S, Kamiya S, Taniguchi T, Takenawa T, Kimura J. Phospholipase C isoenzymes in the human brain and their changes in Alzheimer's disease. *Neuroscience* 1998;82:999-1007.
- [62] Shimohama S, Tanino H, Sumida Y, Tsuda J, Fujimoto S. Alteration of myo-inositol monophosphatase in Alzheimer's disease brains. *Neurosci Lett* 1998;245:159-62.
- [63] Kosicek M, Zetterberg H, Andreasen N, Peter-Katalinic J, Hecimovic S. Elevated cerebrospinal fluid sphingomyelin levels in prodromal Alzheimer's disease. *Neurosci Lett* 2012;516:302-305.
- [64] Han X, Rozen S, Boyle SH, Hellegers C, Cheng H, Burke JR, Welsh-Bohmer KA, Doraiswamy PM, Kaddurah-Daouk R. Metabolomics in early Alzheimer's disease: identification of altered plasma sphingolipidome using shotgun lipidomics. *PLoS ONE* 2011;6:e21643.
- [65] Cutler RG, Kelly J, Storie K, Pedersen WA, Tammara A, Hatanpaa K, Troncoso JC, Mattson MP. Involvement of oxidative stress-induced abnormalities in ceramide and cholesterol metabolism in brain aging and Alzheimer's disease. *Proc Natl Acad Sci* 2004;101:2070-5.
- [66] Fabelo N, Martín V, Marín R, Santpere G, Aso E, Ferrer I, Díaz M. Evidence for premature lipid raft aging in APP/PS1 double-transgenic mice, a model of familial Alzheimer disease. *J Neuropathol Exp Neurol* 2012;71:868-81.
- [67] Pappolla MA, Bryant-Thomas TK, Herbert D, Pacheco J, Fabra Garcia M, Manjon M, Girones X, Henry TL, Matsubara E, Zambon D, Wolozin B, Sano M, Cruz-Sanchez FF, Thal LJ, Petanceska SS, Refolo LM. Mild hypercholesterolemia is an early risk factor for the development of Alzheimer amyloid pathology. *Neurology* 2003;61:199-205.
- [68] Breteler MMB. Vascular risk factors for Alzheimer's disease: An epidemiologic perspective. *Neurobiol Aging* 2000;21:153-60.
- [69] Lee WJ, Hawkins RA, Peterson DR, Viña JR. Role of oxoprolin in the regulation of neutral amino acid transport across the blood-brain barrier. *J Biol Chem* 1996;271:19129-33.
- [70] Zhu Y, Carvey PM, Ling Z. Age-related changes in glutathione and glutathione-related enzymes in rat brain. *Brain Res* 2006;1090:35-44.

## FIGURE LEGENDS

**Fig 1.** Scores plots of PCA (A-C) and PLS-DA (D-F) models for GC-MS, UHPLC-ESI(+)/MS and UHPLC-ESI(-)/MS data

**Fig 2.** (A) Pathway analysis overview showing altered metabolic pathways in serum from APP/PS1 mice. (a) phenylalanine metabolism; (b) glycerophospholipid metabolism; (c) glycine, serine and threonine metabolism; (d) tryptophan metabolism; (e) glyoxylate and dicarboxylate metabolism; (f) starch and sucrose metabolism; (g) sphingolipid metabolism; (h) tyrosine metabolism; (i) glycolysis; (j) TCA cycle; (k) inositol phosphate metabolism; (l) ether lipids metabolism. (B) Overview of serum metabolomic changes in APP/PS1 mice related to bioenergetic metabolism; (↑) increased; (↓) decreased. Abbreviations: Glc, glucose; Glc-6-P, glucose-6-phosphate; G-3-P, glycerol-3-phosphate; 1,3-BPG, 1,3 bisphosphoglycerate; Pi, inorganic phosphate; βHB, β-hydroxybutyrate.

**Table 1.** Potential biomarkers identified by UHPLC-MS profiling of serum samples

metabolite	mass (Da)	RT (min)	ion mode	fold change	p value
Phosphoethanolamine	141.022	0.30	P	0.83	0.0196
Adenosine monophosphate	347.056	0.30	P	0.61	0.0016
Citrulline	175.101	0.30	N	0.80	0.0021
Citric acid	192.031	0.35	N	0.66	0.0044
C18:1-carnitine	425.341	4.77	P	0.94	0.0439
Monostearin	358.291	5.67	P	0.70	0.0475
Bile acids	Table S1			↓	-
Eicosanoids	Table S1			↑	-
Fatty acid amides	Table S1			↓	-
Sphingoid bases	Table S1			↓	-
Lyso-phospholipids	Table S1			↓	-
Phospholipids	Table S1			↓	-
Sphingomyelins	Table S1			↓	-

Abbreviations: P: positive mode, N: negative mode.

**Table 2.** Potential biomarkers identified by GC-MS profiling of serum samples

<b>metabolite</b>	<b>RT (min)</b>	<b>fold change</b>	<b>p value</b>
Lactic acid	2.75	1.69	0.0249
$\beta$ -hydroxybutyric acid	3.42	0.52	0.0427
Urea	4.13	0.50	0.0001
Phosphoric acid	4.25	0.89	0.0350
Glycine	4.47	0.41	0.0003
Succinic acid	4.53	3.35	0.0069
Threonine	5.05	0.65	0.0134
Malic acid	5.92	0.83	0.0481
Pyroglutamic acid	6.23	0.58	0.0357
Creatinine	6.55	1.87	0.0475
Proline	6.68	0.58	0.0436
Glycerol-3-phosphate	8.02	1.12	0.0464
Citric acid	8.45	0.57	0.0284
Glucose	8.87	0.51	0.0002
Tyrosine	9.35	0.73	0.0410
Myoinositol	10.25	1.14	0.0453
Uric acid	10.37	1.55	0.0448
Glucose-6-phosphate	11.02	0.91	0.0483
Tryptophan	11.15	0.62	0.0238
Stearic acid	11.28	1.11	0.0462
Myoinositol-1-phosphate	12.13	0.73	0.0372
Serotonin	12.52	0.43	0.0147
1,3 Bisphosphoglycerate	15.55	0.67	0.0475
Cholesterol	16.45	1.19	0.0043

Fig 1

[Click here to download high resolution image](#)

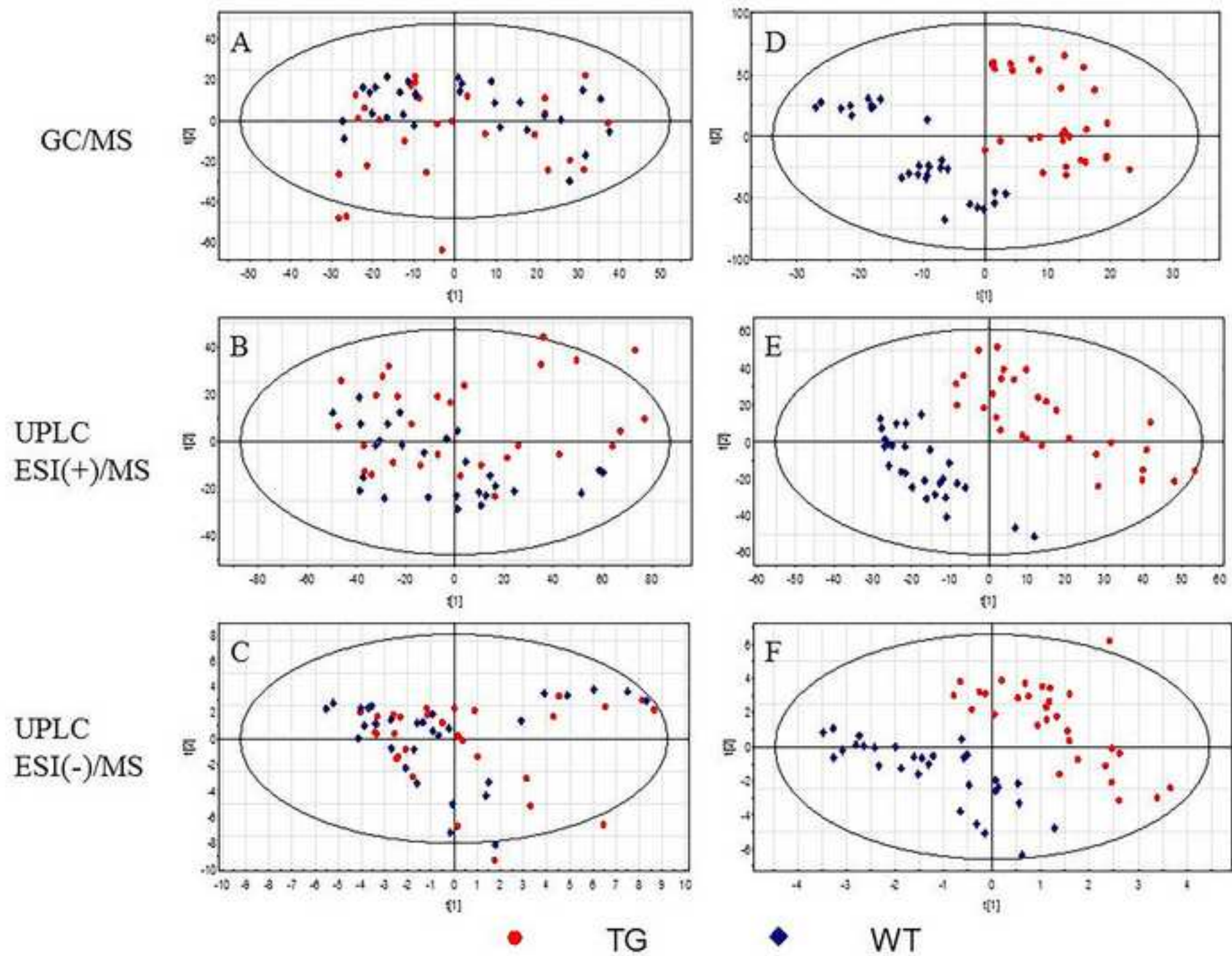
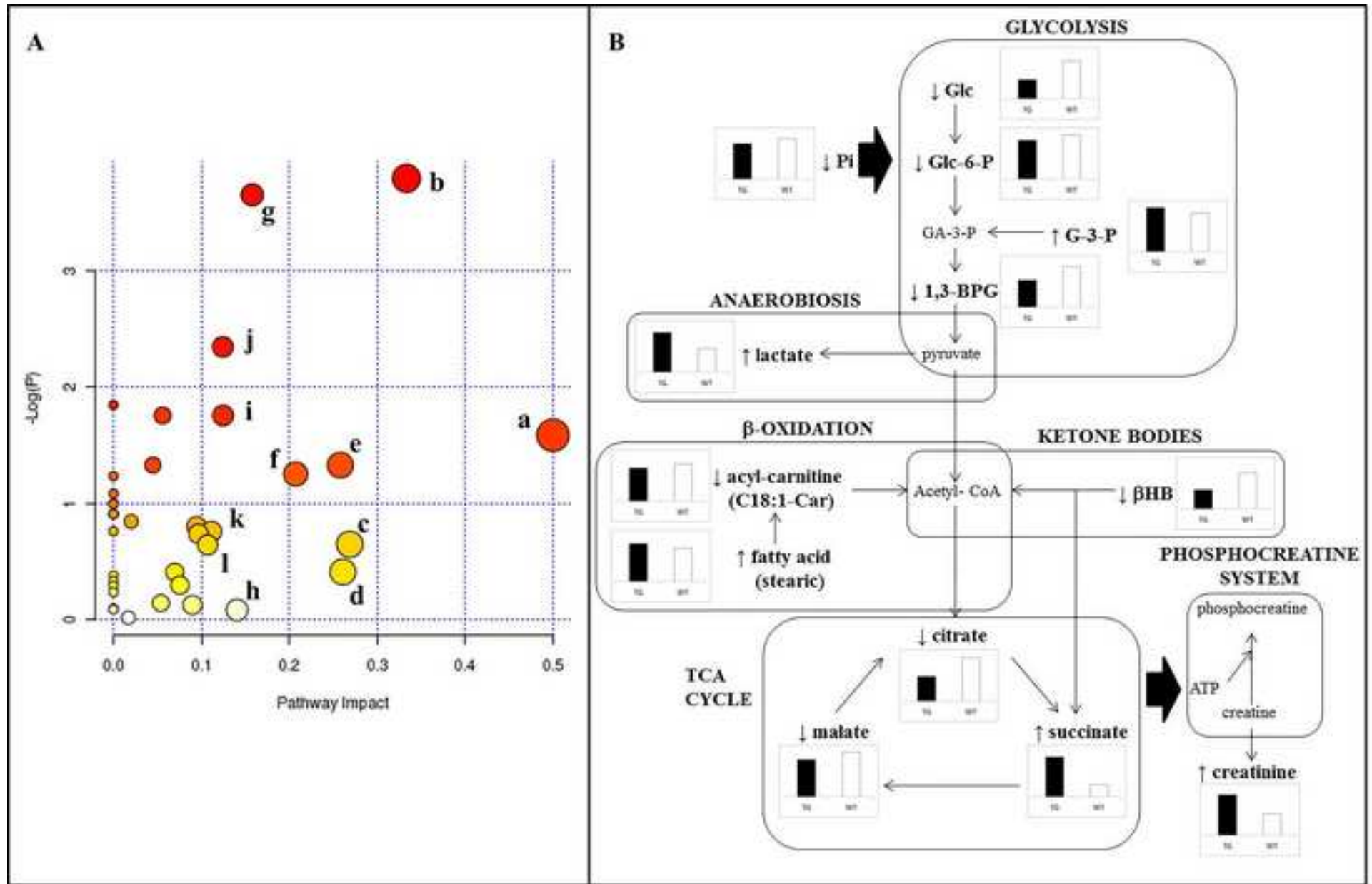


Fig 2

[Click here to download high resolution image](#)



**Supplementary Material**

[Click here to download Supplementary Material: Supplementary material.docx](#)