

**METABOLOMIC RESEARCH ON THE ROLE OF INTERLEUKIN-4 IN ALZHEIMER'S
DISEASE**

^{a,b,c}Raúl González-Domínguez, ^{a,b,c}Tamara García-Barrera*, ^{d,e,f}Javier Vitorica, ^{a,b,c}José Luis Gómez-Ariza*

^aDepartment of Chemistry and CC.MM. Faculty of Experimental Sciences. University of Huelva. Campus de El Carmen. 21007 Huelva. SPAIN; ^bCampus of Excellence International ceiA3. University of Huelva. SPAIN; ^cResearch Center of Health and Environment (CYSMA). University of Huelva. Campus de El Carmen. 21007 Huelva. SPAIN; ^dDepartment Bioquímica, Bromatología, Toxicología y Medicina Legal, Faculty of Pharmacy, University of Seville. 41012 Seville. SPAIN, ^eCentro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED). 41013 Seville. SPAIN, ^fInstituto de Biomedicina de Sevilla (IBiS)–Hospital Universitario Virgen del Rocío/CSIC/University of Seville. 41013 Seville. SPAIN

Corresponding authors:

Prof. J.L Gómez Ariza, Tel.: +34 959 219968, fax: +34 959 219942, e-mail: ariza@uhu.es

Dr. T. Garcia-Barrera, Tel.: +34 959 219962, fax: +34 959 219942, e-mail: tamara@dqcm.uhu.es

Abbreviated title: Metabolomics in the APP/PS1/IL4-KO mice

Acknowledgements: This work was supported by the projects CTM2012-38720-C03-01 from the Ministerio de Ciencia e Innovación, and projects P012-FQM-0442 and P009-FQM-4659 from the Consejería de Innovación, Ciencia y Empresa (Junta de Andalucía). Raúl González Domínguez thanks the Ministerio de Educación for a predoctoral scholarship.

1
2
3
4 **ABSTRACT**
5
6

7 **Inflammation plays a prominent role in the pathogenesis of Alzheimer’s disease,**
8 **affecting both brain and the peripheral system. Thus, modulation of inflammation**
9 **in animal models of this neurodegenerative disorder may be of great interest to**
10 **elucidate the pathological mechanisms underlying this inflammatory component.**
11 **To this end, a metabolomic investigation on a triple transgenic mouse model**
12 **obtained by crossing the APP/PS1 mice with interleukin-4 knockout mice (a model**
13 **of impaired immune function) was performed for the first time. Serum samples**
14 **from transgenic mice and wild type animals were analyzed by direct infusion mass**
15 **spectrometry followed by multivariate statistics in order to identify altered**
16 **metabolites. Subsequently, metabolic pathway analysis allowed the elucidation of**
17 **potential pathological mechanisms associated with the development of Alzheimer-**
18 **type disorders in response to interleukin-4 deficiency, such as impaired**
19 **homeostasis of histamine, altered metabolism of amino acids (threonine, aspartate**
20 **and tyrosine), deregulated urea cycle and increased production of eicosanoids.**
21 **Therefore, this work demonstrates the potential of this triple transgenic model**
22 **with modulated immunity for the study of pathological mechanisms associated**
23 **with inflammation in Alzheimer’s disease.**
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 **KEYWORDS.** Metabolomics, APP/PS1 mice, interleukin-4, direct infusion mass spectrometry,
54 Alzheimer’s disease
55
56
57
58
59
60
61
62
63
64
65

INTRODUCTION

Inflammation is a primary pathological hallmark of Alzheimer's disease (AD), generated in response to the deposition of senile plaques containing β -amyloid peptides, formation of neurofibrillary tangles and neurodegeneration (Akiyama et al., 2000). The major players involved in these inflammatory processes are thought to be the activation of microglia and astrocytes, and possibly to a lesser extent the neurons, leading to the production of many inflammatory mediators in the brain such as cytokines, growth factors, prostaglandins, leukotrienes, thromboxanes, complement factors or cell adhesion molecules, among others (Tuppo and Arias, 2005). However, considerable evidence supports that inflammation in AD is not only localized in the central nervous system, but there are also significant disturbances in the periphery (Butchart and Holmes, 2012). Thus, the immune system and inflammatory processes have become a primary topic in AD research in the last years for the discovery of potential markers of diagnosis, the elucidation of pathological mechanisms underlying to disease and the development of novel therapeutic approaches.

Numerous transgenic mouse models that mimic the main features of Alzheimer's disease have been developed to better understand the pathogenic mechanisms of neurodegeneration and to test potential therapies (Hall and Roberson, 2012). Nevertheless, these mice usually do not present significant neuronal loss, so it has been theorized that the addition of the inflammatory component of AD could generate best models to investigate the disease (Birch et al., 2014). Glial modulation in these mouse models of AD via stimulation of a variety of receptors has been considered for the elucidation of the role of microglia activation in the development of disease, including genetic manipulation of scavenger receptors (Frenkelet al., 2013), Toll-like receptors (Jin et al., 2008) or chemokine receptors (Naert and Rivest, 2011). Moreover, different studies have demonstrated that over-expression of pro-inflammatory mediators in transgenic mice of AD may potentiate pathology by increasing A β generation, aggregation and by affecting its clearance, in the same way that their deletion causes a significant improvement. Thereby, common inflammatory targets investigated in these transgenic models are interleukins (Vom Berg et al., 2012), tumor necrosis factor (He et al., 2007) or nitric oxide synthase (Colton et al., 2006). Among these mediators, there is growing evidence that interleukin-4 (IL-4) plays a prominent role in pathology of Alzheimer's disease. This interleukin is an anti-inflammatory cytokine produced by mature

1 T type 2 helper cells (Th2), mast cells or basophils, involved in T-cell proliferation and in higher
2 functions of the normal brain, such as memory and learning (Gadani et al., 2012). Previous studies
3 demonstrated an association between single nucleotide polymorphisms of the IL-4 gene and risk for AD
4 development (Ribizzi et al., 2010), as well as reduced IL-4 production in peripheral blood mononuclear
5 cells from AD patients (Reale et al., 2008). Furthermore, it is proved that induction of IL-4 production
6 reduces A β deposition and attenuates AD pathogenesis in different transgenic mice (Kijota et al., 2010;
7 Kawahara et al., 2012). In fact, several studies proposed that amelioration of AD pathology in patients
8 treated with acetylcholinesterase inhibitors is due to increased IL-4 expression (Reale et al., 2004; Gambi
9 et al., 2004), supporting a pivotal immunomodulatory effect of IL-4 in AD.

10 Considering this apparent close relation between interleukin-4 and Alzheimer's disease, the objective of
11 this study was the elucidation of pathological mechanisms underlying to AD in response to IL-4
12 depletion. For this purpose, we performed a serum metabolomic investigation into a transgenic line
13 obtained by crossing the APP/PS1 mice with IL-4 knockout mice. The IL-4 deficient mouse is a standard
14 model of impaired immune function, which presents a disturbed production of Th2 cytokines and
15 immunoglobulins E and G1 (Kühn et al., 1991; Kopf et al., 1993), so that we hypothesized that the triple
16 transgenic mice APP/PS1/IL4-KO must develop enhanced AD pathology. Serum samples from wild type
17 animals and transgenic models (APP/PS1/IL4 and APP/PS1) were subjected to metabolomic analysis by
18 direct infusion mass spectrometry (DIMS), and then multivariate statistics was employed to find
19 metabolites responsible for discrimination. Furthermore, pathway analysis allowed to identify metabolic
20 perturbations associated with these pathological processes.

21 **MATERIALS AND METHODS**

22 **ANIMAL HANDLING**

23 Two transgenic mouse lines were compared in this study (C57BL/6 background). The double transgenic
24 mice APP/PS1 were generated as previously described by Jankowsky et al., overexpressing the Swedish
25 mutation of APP, together with PS1 deleted in exon 9 (Jankowsky et al., 2004). Furthermore, these
26 APP/PS1 mice were crossed with IL-4 knockout mice (Kühn et al., 1991) to create homozygotic
27 APP/PS1/IL4-KO mice. On the other hand, age-matched wild-type mice of the same genetic background
28 (C57BL/6) were used as controls. In this study, male and female animals at 6 months of age were used for
29 experiments (N=7, male/female 5/2, for each group). Animals were acclimated for 3 days after reception
30 in rooms with a 12-h light/dark cycle at 20-25 °C, with water and food available *ad libitum*. Then, mice

1 were anesthetized by isoflurane inhalation and blood was extracted by cardiac puncture. Blood samples
2 were immediately cooled and protected from light for 30 minutes to allow clot retraction, and then
3 centrifuged at 3500 rpm for 10 minutes at 4°C. Serum was aliquoted in Eppendorf tubes and frozen at -
4 80°C until analysis. Animals were handled according to the directive 2010/63/EU stipulated by the
5 European Community, and the study was approved by the Ethical Committee of University of Huelva.
6
7

8 **METABOLOMIC ANALYSIS**

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

Metabolomic analysis was performed by extracting serum samples in a two-stage sequential procedure, followed by direct infusion analysis with high resolution tandem mass spectrometry, using the electrospray (ESI) source in both positive and negative ionization modes, as described elsewhere (González-Domínguez et al., 2014a). For protein precipitation and the extraction of polar metabolites, 100 µL of serum were mixed with 400 µL of methanol/ethanol (1:1 v/v), vortexed for 5 min and centrifuged at 4000 rpm for 10 min at 4°C. The supernatant was transferred to another tube, and the precipitate was kept for further treatment. Then, supernatant was dried under nitrogen stream and the resulting residue was reconstituted with 100 µL of methanol/water (80:20 v/v) containing 0.1% formic acid (aqueous extract). On the other hand, the precipitate isolated in the first step was again extracted in order to recover lipophilic compounds adsorbed into the protein pellet. For this purpose, the precipitate was mixed with 400 µL of chloroform:methanol (1:1 v/v) and then stirred during 5 min, followed by centrifugation at 10000 rpm for 10 minutes at 4°C. Finally, the resulting supernatant was taken to dryness under nitrogen stream and reconstituted with 100 µL of 60:40 dichloromethane:methanol containing 10mM ammonium formate and 0.1% formic acid (organic extract). Furthermore, quality control (QC) samples were prepared by pooling equal volumes of each sample, which allows monitoring the stability and performance of the system along the analysis period (Sangster et al., 2006).

Mass spectrometry experiments were performed in a quadrupole-time-of-flight mass spectrometry system (QTOF-MS), model QSTAR XL Hybrid system (Applied Biosystems, Foster City, CA, USA), using the electrospray (ESI) source. Samples were directly introduced into the mass spectrometer at 5 µL min⁻¹ flow rate using an integrated apparatus pump and a 1000µL volume Hamilton syringe. Data were obtained in both positive and negative ionization modes, acquiring full scan spectra during 0.2 minutes in the m/z range 50-1100, with 1.005 seconds of scan time. In positive mode, the ion spray voltage (IS) was set at 3300V, and high-purity nitrogen was used as curtain and nebulizer gas at flow rates about 1.13 L min⁻¹ and 1.56 L min⁻¹, respectively. The source temperature was fixed at 60°C, with a declustering

1 potential (DP) of 60V and a focusing potential (FP) of 250V. For ESI(-)/MS only a few parameters were
2 modified respect to the ESI(+)/MS method, with an ion spray voltage at -4000V, a declustering potential
3 (DP) of -100V and a focusing potential (FP) of -250V. To acquire MS/MS spectra, nitrogen was used as
4 collision gas.
5
6

7 **DATA ANALYSIS**

8
9
10 Metabolomic fingerprints were submitted to peak detection using the Markerview™ software (Applied
11 Biosystems) in order to filter the mass spectrometry results, and to carry out the reduction of raw data into
12 a two-dimensional data matrix containing spectral peaks and their intensities. For this, all peaks above the
13 noise level (10 counts, determined empirically from experimental spectra) were selected and binned in
14 intervals of 0.1Da, and then data were normalized according to the total area sum in order to correct inter-
15 sample variability due to instrumental drifts. Subsequently, data were subjected to multivariate analysis
16 by principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) in order
17 to compare metabolomic profiles obtained, using the SIMCA-PT™ software (version 11.5, UMetrics AB,
18 Umeå, Sweden). Before performing statistical analysis, data are usually scaled and transformed in order
19 to minimize the technical variability between individual samples to extract the relevant biological
20 information from these data sets (van den Berg et al., 2006). For this purpose, we performed Pareto
21 scaling for reducing the relative importance of larger values, and logarithmic transformation, in order to
22 approximate a normal distribution. Quality of the statistical models was assessed by the R^2 and Q^2 values,
23 which provide information about the class separation and predictive power of the model, respectively.
24 These parameters are ranged between 0 and 1, and they indicate the variance explained by the model for
25 all the data analyzed (R^2) and this variance in a test set by cross-validation (Q^2). Potential biomarkers
26 were selected by two-class comparisons: APP/PS1/IL4 vs. WT, APP/PS1 vs. WT, APP/PS1/IL4 vs.
27 APP/PS1. To this end, loadings plots from PLS-DA models were inspected to select altered metabolites
28 according to the Variable Importance in the Projection, or VIP (a weighted sum of squares of the PLS
29 weight, which indicates the importance of the variable in the model), considering only variables with VIP
30 values higher than 1.5, indicative of significant differences among groups. In addition, groups comparison
31 was also conducted by one-way analysis of variance with Bonferroni correction for multiple testing, using
32 the STATISTICA 8.0 software (StatSoft, Tulsa, USA). Only p values below 0.05 were regarded as
33 statistically significant.
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 **IDENTIFICATION OF DISCRIMINANT METABOLITES**

1 Discriminant metabolites were first putatively identified by matching the experimental accurate mass and
2 tandem mass spectra (MS/MS) with those shown in metabolomic databases (HMDB and METLIN).
3
4 Then, this identification was confirmed with commercial standards when available, according to the
5
6 guidelines from the Metabolomics Standards Initiative (MSI). For this, standards were analyzed using
7
8 identical experimental conditions to those described for the primary metabolomic analysis, and then
9
10 fragmentation patterns and accurate masses obtained were compared to those observed in real serum
11
12 samples. Standards of metabolites for this identification of potential biomarkers were obtained from
13
14 Sigma (urea, histamine, threonine, aspartic acid, urocanic acid, dopamine, citrulline, tyrosine).
15

16 **METABOLIC PATHWAY ANALYSIS**

17
18 Metabolic pathway analysis was performed to identify and visualize the affected pathways in transgenic
19
20 mice on the basis of potential biomarkers detected. For this purpose, the MetPA web tool was employed
21
22 (<http://metpa.metabolomics.ca>), which conducts pathway analysis through pathway enrichment analysis
23
24 and pathway topological analysis (Xia and Wishart, 2010). In this work, we select the *Mus musculus*
25
26 library and use the default 'Hypergeometric Test' and 'Relative-Betweenness Centrality' algorithms for
27
28 pathway enrichment analysis and pathway topological analysis, respectively.
29
30

31 **RESULTS**

32
33
34 Metabolic fingerprints obtained by direct infusion mass spectrometry analysis of serum samples from
35
36 APP/PS1/IL4, APP/PS1 and wild type mice were submitted to multivariate statistics in order to evaluate
37
38 the potential of this high-throughput metabolomic approach for the investigation of mechanisms
39
40 associated with the development of AD-type disorders in response to deficient interleukin-4. After peak
41
42 detection, c.a. 5000 molecular features were observed in DIMS profiles for each ionization mode, and all
43
44 of them were used for statistical modelling. An initial principal components analysis (PCA) was applied
45
46 to check trends and outliers (Fig. 1A), and then partial least squares discriminant analysis (PLS-DA) was
47
48 performed to separate the study groups (Fig. 1B). Transgenic mice (APP/PS1/IL4 and APP/PS1) were
49
50 clearly separated from control animals in the first latent variable, while a distinct classification of
51
52 transgenic mice with/without IL-4 depletion was observed along the second latent variable (Fig. 1B).
53
54 Statistical parameters also confirmed the goodness of this model for sample classification, with a variance
55
56 explained (R^2) of 99.8% and variance predicted (Q^2) of 72.5%. Furthermore, the robustness of the
57
58 methodology was assessed in terms of the clustering of quality control samples in the scores plots. For
59
60
61
62
63
64
65

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

this purpose, quality control samples were prepared by pooling equal volumes from each sample and analyzed throughout the sequence run. As can be observed, QCs were tightly grouped in the PCA plot (Fig. 1A), and they were correctly predicted in the center of the scores plots for PLS-DA models (Fig. 1B), indicative of stability during the analyses (Sangster et al., 2006). Then, three additional PLS-DA models were generated according two-groups comparisons in order to facilitate the interpretation of results: APP/PS1/IL4 vs. WT (Fig. 1C, $R^2=0.998$, $Q^2=0.794$), APP/PS1 vs. WT (Fig. 1D, $R^2=1$, $Q^2=0.729$), APP/PS1/IL4 vs. APP/PS1 (Fig. 1E, $R^2=0.998$, $Q^2=0.517$). Discriminant metabolites influencing the differentiation between the three study groups were identified by inspecting loadings plots from these PLS-DA models (two-class comparisons) as well as by ANOVA. These classifiers are listed in Table 1, together with their ID numbers in HMDB database, the experimental mass, the ionization mode used for detection, (P: positive mode; N: negative mode), the extract where they were found (A: aqueous extract; O: organic extract), and the fold change and p-value for each comparison. These findings showed that a number of metabolites are significantly perturbed in both APP/PS1/IL4 and APP/PS1 mice, including different amino acids (threonine, aspartic acid, tyrosine), eicosanoids (HEPE, prostaglandins, leukotriene B4) and other compounds (urea, citrulline, histamine, 1-methylhistamine, urocanic acid, dopamine), while only dopamine allowed to differentiate between the two transgenic lines. However, it is noteworthy that some of these metabolites suffered a gradual change between the three groups considered in this study (from WT to APP/PS1, and finally APP/PS1/IL4), as represented in the bar plots of Fig. 2. Therefore, it can be concluded that metabolic alterations detected in this work could be behind pathological mechanisms associated with the appearance of AD disorders in the APP/PS1 mice and their aggravation in response to IL-4 depletion. Finally, these altered metabolites were subjected to metabolic pathway analysis in order to elucidate the biochemical networks potentially impaired in these transgenic mice. An overview of this analysis is shown in Fig. 3, where each node represents a metabolic pathway and its size indicates the impact of this pathway in response to disease. Thus, the most important disturbances could be related to dyshomeostasis of different amino acids, such as altered biosynthesis of phenylalanine, tyrosine and tryptophan (a), and abnormal metabolism of histidine (b), tyrosine (c), alanine, aspartate and glutamate (d), arginine and proline (f), as well as perturbed arachidonic acid metabolism (e).

DISCUSSION

1 Numerous efforts have been made in the last years to identify metabolic failures associated with
2 pathological mechanisms underlying to Alzheimer's disease. To this end, different authors have
3 previously addressed the metabolomic investigation of several mouse models of AD using both brain
4 tissue and blood samples, such as theG APP/PS1_{ΔE9} (González-Domínguez et al. 2014b; 2015a),
5 TASTPM (Hu et al., 2012; Forster et al., 2012), CRND8 (Salek et al., 2010; Lin et al., 2013; Lin et al.,
6 2014), APP/PS1_{M146L} (Woo et al., 2010; Trushina et al., 2012), APP_{Tg2576} (Dedeoglu et al., 2004; Lalande
7 et al., 2014) or SAMP8 (Jiang et al., 2008; Wang et al., 2014), among others. Thereby, multiple
8 associations have been described between Alzheimer's disease and metabolic perturbations such as
9 oxidative stress, mitochondrial dysfunction, abnormal lipid metabolism or inflammatory processes. The
10 main objective of this work was to investigate a new triple transgenic line of AD characterized by
11 deficient production of the anti-inflammatory interleukin 4 in order to determine the specific contribution
12 of inflammation to AD development, and to elucidate the metabolic mechanisms underlying these
13 pathological processes. For this purpose, we employed a screening tool previously optimized based on
14 direct infusion mass spectrometry, whose potential has been demonstrated in terms of wide metabolome
15 coverage and fast analysis (González-Domínguez et al., 2014a). The application of this metabolomic
16 approach revealed significant alterations in levels of 13 metabolites: 1-methylhistamine, prostaglandins
17 (series 2 and 3), HEPE, and leukotriene B4 were increased in transgenic mice, while urea, histamine,
18 threonine, aspartate, urocanic acid, dopamine, citrulline and tyrosine were decreased in these samples.
19 Furthermore, these perturbations could be linked to different metabolic pathways (Fig. 3), allowing the
20 elucidation of biochemical processes underlying the pathology.

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

Reduced levels of histamine and urocanic acid, as well as increased 1-methylhistamine (Table 1) suggest a perturbed metabolism of histidine in these transgenic mice, as revealed metabolic pathway analysis. In this sense, previous works reported decreased content of this amino acid in brain (Mazurkiewicz-Kwilecki and Nsonwah, 1989), cerebrospinal fluid (Ibañez et al., 2012) and serum (González-Domínguez et al., 2014c; 2014d; 2015b) from AD patients, probably due to impaired biosynthesis of histamine. This compound is involved in the inflammatory response against pathogens by increasing the production of pro-inflammatory cytokines, but in addition may play an important role in neurotransmission failures in Alzheimer's disease (Naddafi and Mirshafiey, 2013). Thereby, AD has been previously associated with a loss of histaminergic neurons in the tuberomamillary nucleus (Nakamura et al., 1993), leading to a decline of histamine levels in different brain areas (Mazurkiewicz-Kwilecki and Nsonwah, 1989; Panula

1 et al., 1998), in agreement with our findings in serum samples. However, this work describes for the first
2 time that reduced histamine is accompanied by altered serum levels of related metabolites (i.e. 1-
3 methylhistamine and urocanic acid), supporting profound failures in the histaminergic system. Moreover,
4 these changes were slightly accentuated in the IL-4 knockout mice compared with the APP/PS1 model
5 (Fig. 2), indicating an aggravation of pathology. Metabolism of interleukin 4 and histamine are
6 interconnected through immunoglobulin E, given that IL-4 induces the production of IgE by B-cells,
7 which in addition is involved in the release of histamine during the immunologic response against
8 allergens (Gould et al., 2003). Therefore, the down-regulation of histamine synthesis in the APP/PS1/IL4
9 mice could be due to reduced liberation of IgE in response to IL-4 depletion (Kühn et al., 1991).

10 Metabolomic fingerprints also showed decreased levels of different amino acids and related compounds
11 in transgenic mice (threonine, aspartate, tyrosine, dopamine), highlighting the importance of a proper
12 metabolism of amino acids for the immune system. Thereby, it is recognized that threonine and aspartate
13 are important immunostimulants that promote growth of thymus and contribute to the modulation of
14 immune function by controlling proliferation and activation of lymphocytes (Li et al., 2007). Moreover,
15 dopamine and other tyrosine-derived compounds also may influence the immune system, by reducing the
16 synthesis of proinflammatory cytokines and inducing the production of anti-inflammatory mediators, as
17 well as by regulation of lymphocyte proliferation, platelet aggregation and the phagocytic activity of
18 neutrophils (Basu and Dasgupta, 2000).

19 Abnormal content of metabolites involved in the urea cycle has been previously related to impaired
20 ammonia detoxification in AD (González-Domínguez, 2014c; 2015c), in accordance with reduced levels
21 observed in this study for urea and citrulline in serum from APP/PS1 and APP/PS1/IL4 mice. The
22 accumulation of ammonia in Alzheimer's disease (hyperammonemia) and consequent dyshomeostasis of
23 urea-related metabolites has been associated with reduced glutamine synthetase activity (Seiler, 2002) and
24 enzymatic deficiencies in the urea cycle (Hansmann et al., 2010). However, the extra decrease found in
25 the IL-4 knockout mice compared with the APP/PS1 mice (although not statistically significant, Fig. 2)
26 suggest an implication of interleukin 4 in this process. In this context, it is noteworthy that IL-4 promotes
27 alternative activation of macrophages into M2 cells, leading to a differential utilization of arginine due to
28 the release of proline and arginase, the enzyme that catalyzes the conversion of arginine into ornithine and
29 urea (Gordon, 2003). Thus, we hypothesized that IL-4 depletion in the APP/PS1 model may aggravate the
30 already perturbed urea cycle.
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

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

Finally, a considerable accumulation of several eicosanoids was also found in serum from these transgenic models, including prostaglandins (PG), hydroxy-eicosapentaenoic acid (HEPE) and leukotriene B4 (LTB4), with the highest fold changes with regard to wild type controls (Table 1). The increase of these pro-inflammatory mediators in brain and biofluids has been repeatedly described in both human AD (Bazan et al., 2002; Trushina et al., 2013) and transgenic models (Piro et al., 2012; Lin et al., 2013; Lin et al., 2014). This inflammatory process has been traditionally associated with the over-activation of phospholipases, principally PLA₂, leading to the release of polyunsaturated fatty acids from the hydrolysis of phospholipids (Farooqui et al., 2004), and subsequent oxidation by cyclooxygenases and 5-lipoxygenase (Manev et al., 2011), generating a complex meshwork of lipid mediators closely associated with neuronal pathways involved in AD neurobiology (Frisardi et al., 2011). However no substantial differences were observed between the APP/PS1 and APP/PS1/IL4 mice (Fig. 2), despite the demonstrated anti-inflammatory effects of interleukin 4 (Hart et al., 1989), suggesting that IL-4 does not play a relevant role in the production of eicosanoids in pathogenesis of Alzheimer's disease.

CONCLUDING REMARKS

In this study, we demonstrated that depletion of interleukin 4 may potentiate the pathology in the APP/PS1 mouse model of Alzheimer's disease. Metabolomic fingerprinting of serum samples from transgenic mice (APP/PS1 and APP/PS1/IL4-KO) and wild type controls using direct infusion mass spectrometry revealed significant alterations in levels of 13 metabolites (urea, histamine, threonine, 1-methylhistamine, aspartic acid, urocanic acid, dopamine, citrulline, tyrosine, HEPE, prostaglandins and leukotriene B4), which could be related to abnormalities in different metabolic pathways. Thus, the most important failures might be associated with impaired biosynthesis of histamine, altered metabolism of amino acids, deregulated urea cycle and increased production of pro-inflammatory eicosanoids. Therefore, these findings highlight the potential of this triple transgenic model (APP/PS1/IL4) for the study of pathological mechanisms associated with inflammation in Alzheimer's disease.

Acknowledgements. This work was supported by the projects CTM2012-38720-C03-01 from the Ministerio de Ciencia e Innovación, and projects P012-FQM-0442 and P009-FQM-4659 from the Consejería de Innovación, Ciencia y Empresa (Junta de Andalucía). Raúl González Domínguez thanks the Ministerio de Educación for a predoctoral scholarship.

Conflict of interest. The authors declare no conflict of interest.

1
2 **Compliance with ethical requirements.** Animals were handled according to the directive 2010/63/EU
3 stipulated by the European Community, and the study was approved by the Ethical Committee of
4 University of Huelva.
5
6

7 8 **REFERENCES** 9

- 10 Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G. M., et al. (2000). Inflammation and
11 Alzheimer's disease. *Neurobiol Aging*, 21, 383-421.
12
13 Basu, S. & Dasgupta, P. S. (2000). Dopamine, a neurotransmitter, influences the immune system. *J*
14 *Neuroimmunol*, 102, 113-124.
15
16 Bazan, N. G., Colangelo, V., & Lukiw, W. J. (2002). Prostaglandins and other lipid mediators in
17 Alzheimer's disease. *Prostaglandins Other Lipid Mediat*, 68-69, 197-210.
18
19 Birch, A. M., Katsouri, L., & Sastre, M. (2014). Modulation of inflammation in transgenic models of
20 Alzheimer's disease. *J Neuroinflammation*, 11, 25.
21
22 Butchart, J., & Holmes, C. (2012). Systemic and central immunity in Alzheimer's disease: therapeutic
23 implications. *CNS Neurosci Ther*, 18, 64-76.
24
25 Colton, C. A., Vitek, M. P., Wink, D. A., Xu, Q., Cantillana, V., Previti, M. L., et al. (2006). NO synthase
26 2 (NOS2) deletion promotes multiple pathologies in a mouse model of Alzheimer's disease. *Proc Natl*
27 *Acad Sci USA*, 103, 12867-12872.
28
29 Dedeoglu, A., Choi, J. K., Cormier, K., Kowall, N. W., & Jenkins, B. G. (2004). Magnetic resonance
30 spectroscopic analysis of Alzheimer's disease mouse brain that express mutant human APP shows
31 altered neurochemical profile. *Brain Res*, 1012, 60-65.
32
33 Farooqui A. A., Ong W. Y., & Horrocks L. A. (2004). Biochemical aspects of neurodegeneration in
34 human brain: involvement of neural membrane phospholipids and phospholipases A2. *Neurochem Res*,
35 29, 1961-1977.
36
37 Forster, D. M., James, M. F., & Williams, S. R. (2012). Effects of Alzheimer's disease transgenes on
38 neurochemical expression in the mouse brain determined by ¹H MRS *in vitro*. *NMR Biomed*, 25, 52-58.
39
40 Frenkel, D., Wilkinson, K., Zhao, L., Hickman, S. E., Means, T. K., Puckett, L. et al. (2013). Scar1
41 deficiency impairs clearance of soluble amyloid-beta by mononuclear phagocytes and accelerates
42 Alzheimer's-like disease progression. *Nat Comm*, 4, 2030.
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 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
- Frisardi, V., Panza, F., Seripa, D., Farooqui, T., & Farooqui, A. A. (2011). Glycerophospholipids and glycerophospholipid-derived lipid mediators: A complex meshwork in Alzheimer's disease pathology. *Prog Lipid Res*, 50, 313-330.
- Gadani, S. P., Cronk, J. C., Norris, G. T., & Kipnis, J. (2012). Interleukin-4: A cytokine to remember. *Immunol*, 189, 4213-4219.
- Gambi, F., Reale, M., Iarlori, C., Salone, A., Toma, L., Paladini, C., et al. (2004). Alzheimer patients treated with an AchE inhibitor show higher IL-4 and lower IL-1 beta levels and expression in peripheral blood mononuclear cells. *J Clin Psychopharm* 24, 314-321.
- González-Domínguez, R., García-Barrera, T., & Gomez-Ariza, J. L. (2014a). Using direct infusion mass spectrometry for serum metabolomics in Alzheimer's disease. *Anal Bioanal Chem*, 406, 7137-7148.
- González-Domínguez, R., García-Barrera, T., Vitorica, J., & Gómez-Ariza, J. L. (2014b). Region-specific metabolic alterations in the brain of the APP/PS1 transgenic mice of Alzheimer's disease. *Biochim Biophys Acta*, 1842, 2395-2402.
- González-Domínguez, R., García-Barrera, T., & Gómez-Ariza, J. L. (2014c). Metabolomic study of lipids in serum for biomarker discovery in Alzheimer's disease using direct infusion mass spectrometry. *J Pharm Biomed Anal*, 98, 321-326.
- González-Domínguez, R., García, A., García-Barrera, T., Barbas, C., & Gómez-Ariza, J. L. (2014d). Metabolomic profiling of serum in the progression of Alzheimer's disease by capillary electrophoresis - mass spectrometry. *Electrophoresis*, 35, 3321-3330.
- González-Domínguez, R., García-Barrera, T., Vitorica, J., & Gómez-Ariza, J. L. (2015a). Metabolomic screening of regional brain alterations in the APP/PS1 transgenic model of Alzheimer's disease by direct infusion mass spectrometry. *J Pharm Biomed Anal*, 102, 425-435.
- González-Domínguez, R., García-Barrera, T., & Gomez-Ariza, J. L. (2015b). Metabolite profiling for the identification of altered metabolic pathways in Alzheimer's disease. *J Pharm Biomed Anal*, 107, 75-81.
- González-Domínguez, R., García-Barrera, T., & Gomez-Ariza, J. L. (2015c). Application of a novel metabolomic approach based on atmospheric pressure photoionization mass spectrometry using flow injection analysis for the study of Alzheimer's disease. *Talanta*, 131, 480-489.
- Gordon, S. (2003). Alternative activation of macrophages. *Nat Rev Immunol*, 3, 23-35.
- Gould, H. J., Sutton, B. J., Bevil, A. J., Bevil, R. L., McCloskey, N., Coker, H. A., et al. (2003). The biology of IgE and the basis of allergic disease. *Annu Rev Immunol*, 21, 579-628.

- 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
- Hall, A. M., & Roberson, E. D. (2012). Mouse models of Alzheimer's disease. *Brain Res Bull* 88, 3-12.
- Hansmannel, F., Sillaire, A., Kamboh, M. I., Lendon, C., Pasquier, F., Hannequin, D., et al. (2010). Is the urea cycle involved in Alzheimer's disease? *J Alzheimers Dis*, 21, 1013-1021.
- Hart, P. H., Vitti, G. F., Burgess, D. R., Whitty, G. A., Piccoli, D. S. & Hamilton, J. A. (1989). Potential antiinflammatory effects of interleukin 4: suppression of human monocyte tumor necrosis factor alpha, interleukin 1, and prostaglandin E2. *Proc Natl Acad Sci U S A*, 86, 380-33807.
- He, P., Zhong, Z., Lindholm, K., Berning, L., Lee, W., Lemere, C., et al. (2007). Deletion of tumor necrosis factor death receptor inhibits amyloid beta generation and prevents learning and memory deficits in Alzheimer's mice. *J Cell Biol*, 178, 829-841.
- Hu, Z. P., Browne, E. R., Liu, T., Angel, T. E., Ho, P. C., & Chan, E. C. Y. (2012). Metabonomic profiling of TASTPM transgenic alzheimer's disease mouse model. *J Proteome Res*, 11, 5903-5913.
- Ibáñez, C., Simó, C., Martín-Álvarez, P. J., Kivipelto, M., Winblad, B., Cedazo-Mínguez, A., et al. (2012). Toward a predictive model of Alzheimer's disease progression using capillary electrophoresis–mass spectrometry metabolomics. *Anal Chem*, 84, 8532-8540.
- Jankowsky, J. L., Fadale, D. J., Anderson, J., Xu, G. M., Gonzales, V., Jenkins, N. A., et al. (2004). Mutant presenilins specifically elevate the levels of the 42 residue beta-amyloid peptide in vivo: evidence for augmentation of a 42-specific g secretase. *Hum Mol Genet*, 13, 159-170.
- Jiang, N., Yan, X., Zhou, W., Zhang, Q., Chen, H., Zhang, Y., et al. (2008). NMR-based metabonomic investigations into the metabolic profile of the senescence-accelerated mouse. *J Proteome Res*, 7, 3678-3686.
- Jin, J. J., Kim, H. D., Maxwell, J. A., Li, L., & Fukuchi, K. (2008). Toll-like receptor 4-dependent upregulation of cytokines in a transgenic mouse model of Alzheimer's disease. *J Neuroinflammation*, 5, 23.
- Kawahara, K., Suenobu, M., Yoshida, A., Koga, K., Hyodo, A., Ohtsuka, H., et al. (2012). Intracerebral microinjection of interleukin-4/interleukin-13 reduces beta-amyloid accumulation in the ipsilateral side and improves cognitive deficits in young amyloid precursor protein 23 mice. *Neuroscience*, 207, 243-260.
- Kiyota, T., Okuyama, S., Swan, R. J., Jacobsen, M. T., Gendelman, H. E., & Ikezu, T. (2010). CNS expression of anti-inflammatory cytokine interleukin-4 attenuates Alzheimer's disease-like pathogenesis in APP + PS1 bigenic mice. *FASEB J*, 24, 3093-3102.

- 1
2 Kopf, M., Le Gros, G., Bachmann, M., Lamers, M. C., Bluethmann, H., & Köhler, G. (1993). Disruption
3 of the murine IL-4 gene blocks Th2 cytokine responses. *Nature*, 362, 245-248.
- 4 Kühn, R., Rajewsky, K., & Müller, W. (1991). Generation and analysis of interleukin-4 deficient mice.
5 *Science*, 254, 707-710.
- 6
7 Lalande, J., Halley, H., Balayssac, S., Gilard, V., Déjean, S., Martino, R., et al. (2014). ¹H NMR
8 metabolomic signatures in five brain regions of the A β PPswe Tg2576 mouse model of Alzheimer's
9 disease at four ages. *J Alzheimers Dis*, 39, 121-143.
- 10
11 Li, P., Yin, Y. L., Li, D., Kim, S. W., & Wu, G. (2007). Amino acids and immune function. *Br J Nutr*, 98,
12 237-252.
- 13
14 Lin, S., Liu, H., Kanawati, B., Liu, L., Dong, J., Li, M. et al. (2013). Hippocampal metabolomics using
15 ultrahigh-resolution mass spectrometry reveals neuroinflammation from Alzheimer's disease in CRND8
16 mice. *Anal Bioanal Chem*, 405, 5105-5117.
- 17
18 Lin, S., Kanawati, B., Liu, L., Witting, M., Li, M., Huang, J. et al. (2014). Ultra high resolution mass
19 spectrometry-based metabolic characterization reveals cerebellum as a disturbed region in two animal
20 models. *Talanta*, 118, 45-53.
- 21
22 Manev, H., Chen, H., Dzitoyeva, S., & Manev, R. (2011). Cyclooxygenases and 5-lipoxygenase in
23 Alzheimer's disease. *Prog Neuropsychopharmacol Biol Psychiatry*, 35, 315-319.
- 24
25 Mazurkiewicz-Kwilecki, I. M., & Nsonwah, S. (1989). Changes in the regional brain histamine and
26 histidine levels in postmortem brains of Alzheimer patients. *Can J Physiol Pharmacol*, 67, 75-78.
- 27
28 Naddafi, F., & Mirshafiey, A. (2013). The neglected role of histamine in Alzheimer's disease. *Am J*
29 *Alzheimers Dis Other Demen*, 28, 327-336.
- 30
31 Naert, G., & Rivest, S. (2011). CC chemokine receptor 2 deficiency aggravates cognitive impairments
32 and amyloid pathology in a transgenic mouse model of Alzheimer's disease. *J Neurosci*, 31, 6208-6220.
- 33
34 Nakamura, S., Takemura, M., Ohnishi, K., Suenaga, T., Nishimura, M., Akiguchi, I., et al. (1993). Loss
35 of large neurons and occurrence of neurofibrillary tangles in the tuberomammillary nucleus of patients
36 with Alzheimer's disease. *Neurosci. Lett*, 151, 196-199.
- 37
38 Panula, P., Rinne, J., Kuokkanen, K., Eriksson, K.S., Sallmen, T., Kalimo, et al. (1998). Neuronal
39 histamine deficit in Alzheimer's disease. *Neuroscience*, 82, 993-997.
- 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

- 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
- Piro, J. R., Benjamin, D. I., Duerr, J. M., Pi, Y. Q., Gonzales, C., Wood, K. M., et al. (2012). A dysregulated endocannabinoid-eicosanoid network supports pathogenesis in a mouse model of Alzheimer's disease. *Cell Rep* 1, 617-623.
- Reale, M., Iarlori, C., Feliciani, C., & Gambi, D. (2008). Peripheral chemokine receptors, their ligands, cytokines and Alzheimer's disease. *J Alzheimers Dis*, 14, 147-159.
- Reale, M., Iarlori, C., Gambi, F., Feliciani, C., Salone, A., Toma, L., et al. (2004). Treatment with an acetylcholinesteraseinhibitor in Alzheimer patients modulates the expression and production of the pro-inflammatory and anti-inflammatory cytokines. *J Neuroimmunol*, 148, 162-171.
- Ribizzi, G., Fiordoro, S., Barocci, S., Ferrari, E., & Megna, M. (2010). Cytokine polymorphisms and Alzheimer disease: possible associations. *Neurol Sci*, 31, 321-325.
- Salek, R. M., Xia, J., Innes, A., Sweatman, B. C., Adalbert, R., Randle, S., et al. (2010). A metabolomic study of the CRND8 transgenic mouse model of Alzheimer's disease. *Neurochem Int*, 56, 937-943.
- Sangster, T., Major, H., Plumb, R., Wilson, A. J., & Wilson, I. D. (2006). A pragmatic and readily implemented quality control strategy for HPLC-MS and GC-MS-based metabonomic analysis. *Analyst*, 131, 1075-1078.
- Seiler N. (2002). Ammonia and Alzheimer's disease. *Neurochem Int*, 41, 189-207.
- Trushina, E., Nemutlu, E., Zhang, S., Christensen, T., Camp, J., Mesa, J., et al. (2012). Defects in mitochondrial dynamics and metabolomic signatures of evolving energetic stress in mouse models of familial Alzheimer's disease. *PLoS ONE*, 7, e32737.
- Trushina, E., Dutta, T., Persson, X. M. T., Mielke, M. M., & Petersen, R. C. (2013). Identification of altered metabolic pathways in plasma and CSF in mild cognitive impairment and Alzheimer's disease using metabolomics. *Plos ONE*, 8, e63644.
- Tuppo, E. E., & Arias, H. R. (2005). The role of inflammation in Alzheimer's disease. *Int J Biochem Cell Biol*, 37, 289-305.
- van den Berg, R. A., Hoefsloot, H. C. J., Westerhuis, J. A., Smilde, A. K., & van der Werf, M. J. (2006). Centering, scaling, and transformations: improving the biological information content of metabolomics data. *BMC Genomics*, 7, 142.

1 Vom Berg, J., Prokop, S., Miller, K. R., Obst, J., Kalin, R. E., Lopategui-Cabezas, I., et al. (2012).
 2 Inhibition of IL-12/IL-23 signaling reduces Alzheimer's disease-like pathology and cognitive decline.
 3 Nat Med, 18, 1812-1819.
 4
 5 Wang, H., Lian, K., Han, B., Wang, Y., Kuo, S. H., Geng, Y., et al. (2014). Age-related alterations in the
 6 metabolic profile in the hippocampus of the senescence-accelerated mouse prone 8: a spontaneous
 7 Alzheimer's disease mouse model. J Alzheimers Dis, 39, 841-848.
 8
 9 Woo, D. C., Lee, S. H., Lee, D. W., Kim, S. Y., Kim, G. Y., Rhim, H. S., et al. (2010). Regional
 10 metabolic alteration of Alzheimer's disease in mouse brain expressing mutant human APP-PS1 by 1H
 11 HR-MAS. Behav Brain Res, 211, 125-131.
 12
 13 Xia, J., & Wishart, D. S. (2010). MetPA: a web-based metabolomics tool for pathway analysis and
 14 visualization. Bioinformatics, 26, 2342-2344.
 15
 16
 17
 18
 19
 20
 21
 22
 23
 24
 25
 26
 27
 28
 29

Table 1 Discriminant metabolites identified by DIMS in two class comparisons: APP/PS1/IL4 vs. WT (IL/WT), APP/PS1 vs. WT (TG/WT), APP/PS1/IL4 vs. APP/PS1 (IL/TG)

metabolite	ID (HMDB)	mass (Da)	ion mode	extract	fold change (p-value)		
					IL/WT	TG/WT	IL/TG
urea [#]	HMDB00294	60.033	P	A	0.32 (2.4·10 ⁻⁴)	0.37 (5.4·10 ⁻⁴)	NS
histamine [#]	HMDB00870	111.072	P	A	0.56 (6.7·10 ⁻⁴)	0.77 (4.8·10 ⁻²)	NS
threonine [#]	HMDB00167	119.055	P/N	A/O	0.62 (1.1·10 ⁻⁴)	0.59 (4.6·10 ⁻⁵)	NS
1-methylhistamine	HMDB00898	125.099	P	A	2.07 (7.5·10 ⁻³)	2.12 (5.3·10 ⁻³)	NS
aspartic acid [#]	HMDB00191	133.034	P	A	0.54 (1.9·10 ⁻⁴)	0.66 (2.8·10 ⁻³)	NS
urocanic acid [#]	HMDB00301	138.043	P	O	0.57 (1.8·10 ⁻³)	NS	NS
dopamine [#]	HMDB00073	153.075	P	A/O	0.59	0.77	0.77

					(2.0·10 ⁻⁶)	(1.1·10 ⁻³)	(7.7·10 ⁻³)
citrulline [#]	HMDB00904	175.099	P	A	0.56 (1.0·10 ⁻⁶)	0.64 (9.0·10 ⁻⁶)	NS
tyrosine [#]	HMDB00158	181.069	P/N	A	0.52 (5.0·10 ⁻⁵)	0.52 (4.6·10 ⁻⁵)	NS
HEPE*	-	318.222	N	A/O	4.38 (1.6·10 ⁻³)	4.57 (1.0·10 ⁻³)	NS
PG (series 2)*	-	334.210	N	A/O	3.24 (6.0·10 ⁻⁴)	3.02 (1.5·10 ⁻³)	NS
LTB4	HMDB01085	336.235	N	A/O	2.89 (1.9·10 ⁻³)	3.13 (6.7·10 ⁻⁴)	NS
PG (series 3)*	-	350.211	N	A/O	1.96 (8.8·10 ⁻⁴)	2.25 (6.5·10 ⁻⁵)	NS

**isomers of eicosanoids were undistinguishable by MSMS experiments. Metabolites marked with [#] were confirmed with commercial standards. Abbreviations: HEPE, hydroxyeicosapentaenoic acid; PG, prostaglandin; LTB4, leukotriene B4; P, positive mode; N, negative mode; A, aqueous extract; O, organic extract; NS, non significant.*

FIGURE CAPTIONS

Fig. 1 Scores plots of multivariate statistical models for DIMS data. PCA (A) and PLS-DA (B) models discriminating wild type controls (WT), APP/PS1 and APP/PS1/IL4 mice, with tight clustering of quality control samples (QC). Two-class comparisons: APP/PS1/IL4 vs. WT (C), APP/PS1 vs. WT (D) and APP/PS1/IL4 vs. APP/PS1 (E). QC: green stars (circled); WT: black squares; APP/PS1: red dots; APP/PS1/IL4: blue diamonds

Fig. 2 Bar plots and 95% confidence interval for the discriminant metabolites identified by DIMS. WT: wild type; TG: APP/PS1; IL: APP/PS1/IL4

Fig. 3 Pathway analysis overview showing altered metabolic pathways in serum from transgenic mice APP/PS1/IL4 and APP/PS1. (a) phenylalanine, tyrosine and tryptophan biosynthesis; (b) histidine metabolism; (c) tyrosine metabolism; (d) alanine, aspartate and glutamate metabolism; (e) arachidonic acid metabolism; (f) arginine and proline metabolism

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

Figure 1
[Click here to download high resolution image](#)

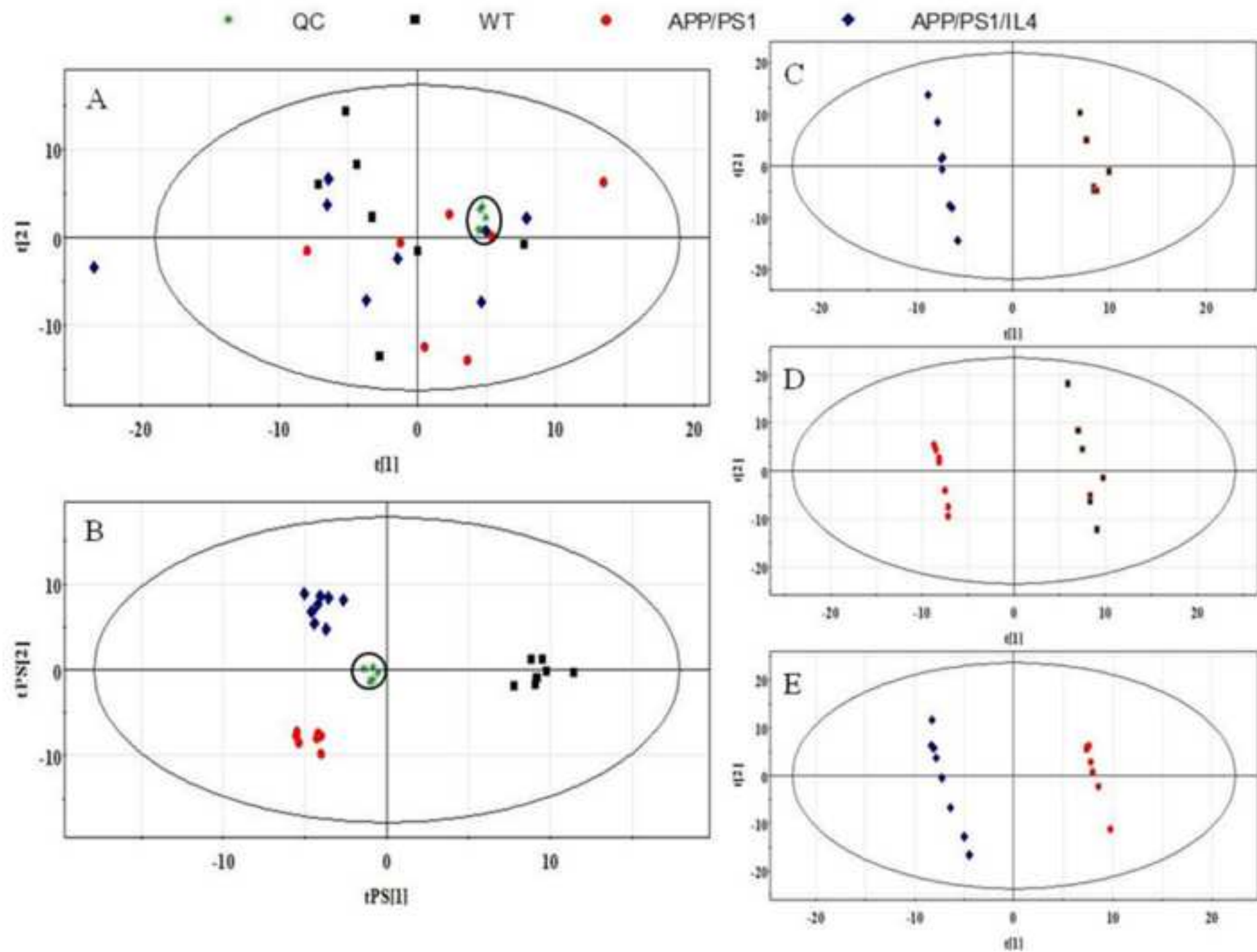


Figure 2
[Click here to download high resolution image](#)

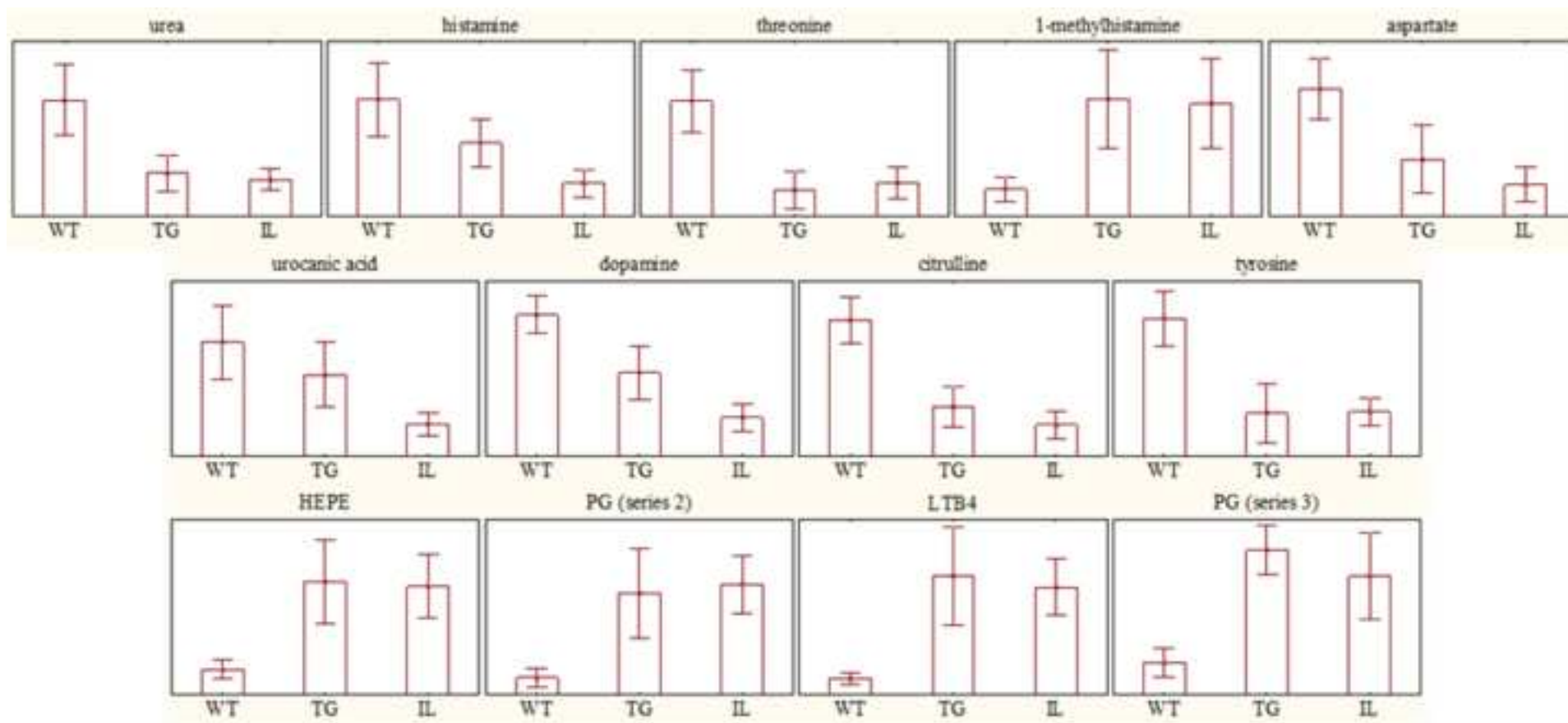


Figure 3

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

