# Alzheimer's disease (AD)-like pathology has discontinuous effects on the brain and blood metabolome.

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## Abstract

The pathogenesis of Alzheimer's disease (AD) is complex involving multiple contributing factors, including amyloid β (Aβ) peptide accumulation, inflammation and oxidative stress. The extent to which AD pathology impacts upon the metabolome is not understood, nor is it known how disturbances may change over the disease process. Here both the brain and plasma metabolome of APP/PS1 double transgenic mice and wild type (WT) controls were profiled longitudinally (6, 8, 10, 12 and 18 months) for the first time. A total of 187 metabolites including amino acids, biogenic amines, phospholipids and acylcarnitines were quantified using a targeted metabolomic methodology. Multivariate statistical analysis (orthogonal projection to latent structures-discriminant analysis) produced metabolomic models that easily distinguishing APP/PS1 from WT ($R2 \leq 0.994$; $Q2 \leq 0.890$). Metabolic pathway analysis found perturbed polyamine metabolism in both brain and blood. In particular, putrescine, spermidine and spermine were significantly increased in APP/PS1 mouse brain (6-8 months) and blood plasma (10-12 months). There were disturbances in essential amino acids, branched chain amino acids and also in the neurotransmitter serotonin. Severe imbalances in phospholipid homeostasis were evident at two specific time points. In APP/PS1 brain at 8 months 79% phosphotidylcholines (PCs) species were significantly increased. Contrastingly, in APP/PS1 plasma at 12 months 88% PCs were significantly decreased. A number of LysoPCs, sphingolipids and acylcarnitines were similiarly disturbed. In summary, AD-like pathology impacts greatly on both the brain and blood metabolome but metabolites appear to be affected in a discontinuous manner and with brain disturbances ~~precede~~ preceding those in blood.

## Introduction

Dementia mainly affects older people, with prevalence roughly doubling every five years over the age of 65. Alzheimer's disease (AD) is a progressive and fatal neurodegenerative disorder and the most common form of dementia, accounting for 60-80 percent of all dementia cases. AD is clinically characterised by progressive memory loss, mood changes, and problems with communication and reasoning [1]. The familial form of AD (FAD), which is early-onset (<65 years of age), is caused by mutations in genes encoding amyloid precursor protein (APP) and presenilins 1 and 2 (PS1 and PS2) which lead to the accumulation of β-amyloid (Aβ) [2-5]. AD is pathologically characterised by the accumulation of extracellular Aβ and the occurrence of abnormal tau filaments in neurons which leads to senile plaques and neurofibrillary tangles (NFTs), respectively [6-8]. Transgenic mouse models with gene mutations for APP and/or PS1 are widely used in experimental studies to investigate the pathophysiological role of Aβ in early-onset AD patients. The APPswe/PS1deltaE9 strain is has been extensively characterised and utilised these mice develop Aβ plaques at around 5-6 months although production of Aβ has been shown to occur as early as 3 months in the form of both Aβ(1-40) and (1-42) [9]. These mice display progressive age-related impairments in memory which can be seen as early as 7 months of age [9, 10]. In behavioural tests the mice show deficits in measuring spatial navigation and reference learning [10]. Although APP/PS1 mice do not mimic all facets of human AD, they do enable longitudinal investigations not normally possible a clinical situation.

Metabolomics is the scientific investigation of chemical processes involving metabolites. Metabolomic techniques can monitor the disturbances in metabolic pathways that reflect changes downstream from genomic, transcriptomic and proteomic systems comprehensively and simultaneously in a high-throughput manner [11, 12]. There is considerable potential as a discovery platform for identifying novel diagnostic biomarkers for AD. Metabolomic studies have been undertaken in APP/PS1 mice [13-19], however, the majority of these studies (including our own [16]) suffer from flaws commonly befalling many metabolomics

investigations. The current study re-evaluated the earlier approaches to undertake a more robust metabolomics evaluation of this important model of AD. It is evident that many studies do not give adequate consideration to the optimal experimental design. Many studies have apparently arbitrary sample sizes with no calculation of optimal statistical power. Also a large number of studies examine a single time point - providing only the narrowest of windows from which to view and obtain reliable biological information. Furthermore, the majority of studies examine one sample type in isolation and do not examine if biochemical alternations are more widespread. Finally, all potential sources of biological variation are not always minimised in the experimental design. For example, even the gender of animals and subject has a strong influence on the metabolome [20, 21]. This study undertook a targeted and quantitative methodology with optimal sample size pre-calculated to achieve 100% statistical power. A total of 187 metabolites were measured in both brain and blood samples from female animals and this included amino acids, biogenic amines, phospholipids and acylcarnitines.

Previous metabolomic studies have revealed a number of biochemical disturbances in APP/PS1 mice. Previous studies using *in vivo* proton magnetic resonance spectroscopy ($^{1}$H MRS) found decreases in N-acetylasparatate (NAA) and glutamate, and an increase in myo-inositol concentrations in APP/PS1 mice [13, 14]. Glycolytic pathways involving the Kreb's cycle, and neurotransmitter and amino acid metabolism, were found to be significantly affected in APP/PS1 mouse brain [15]. Furthermore, $^{1}$H NMR metabolomic studies found altered ascorbate, creatine, γ-aminobutyric acid and NAA in APP/PS1 mouse brain, and altered acetate, citrate, glutamine and methionine in blood plasma [16]. A recent study applying GC-MS and UPLC-MS investigated the metabolic perturbations in five brain regions of APP/PS1 mice at 6 months of age [17]. Region-specific alterations were observed for some metabolites associated with abnormal fatty acid composition of phospholipids and sphingomyelins, or differential regulation of neurotransmitter amino acids (e.g. glutamate,

glycine, serine, N-acetyl-aspartate). Disturbances in phospholipids, energy deficiencies, altered homeostasis of amino acid and oxidative stress in APP/PS1 mouse spleen and thymus were also observed [18]. One study employing HPLC-ELSD compared the cortical levels of cholesterol and phospholipid (PL) subclasses at ages 4 and 9 months [19], and found that membrane lipids of APP/PS1 mice including cholesterol and PL were significantly decreased at 9 months [19]. Among PL subclasses, phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylcholine (PC) were selectively reduced [19]. Despite the fact that metabolomics studies have pinpointed some metabolites affected by the development of AD-like pathology the findings are often conflicting, fragmented and incongruent. The aim of this study was to longitudinally study an important and widely used transgenic AD model over much of its lifespan, and to monitor disturbances close to the initial pathological insult as well as those which arise within the blood circulation.

## Methods

### Brain tissue and plasma from APPswe/PS1deltaE9 mouse

APPswe/PS1DE9 (APP/PS1) mice used in this study were obtained from the Jackson lab (USA). Heterozygous males were bred with wild-type (WT) C57/Bl6 females bought locally (Harlan, UK). APP/PS1and WT mice were housed under identical conditions and fed the same rodent maintenance diet (____, ___). APP/PS1 mice are a transgenic C57BL/6J mouse model co-expressing the Swedish mutation (K595N/M596L) and the deltaE9 PS-1 exon deletion (mutated human presenilin-1) [22]. Offspring were tail snipped and genotyped using PCR. PCR used primers specific for the APP sequence (Forward "GAATTCCGACATGACTCAGG", Reverse: "GTTCTGCTGCATCTTGGACA"). Mice not expressing the transgene were used as wild-type controls. For this study, female APP/PS1dE9 mice, aged 6, 8, 10, 12 and 18 months and, age matched WT female C57BL/6littermate controls (n = 8-9) were used. Mice were fasted for 16 h, deeply

**Comment [BDG1]:** Details of the diet used. Christian/Paula

anaesthetised with pentobarbitol, and blood samples were collected into heparinised tubes, centrifuged for 30 seconds at 13,000 x g (IEC Micromax RF) and the resulting plasma were stored at -80˚C prior to metabolomic investigations. Whole mouse brain was also collected and snap-frozen in liquid nitrogen and stored at -80˚C until further use.

**Brain tissue extraction**

Mouse brain samples were collected in individual tubes to avoid cross-contamination, then lyophilized and milled to a fine powder, and 25 mg (±0.5 mg) added to 300 µL of solvent (85% ethanol and 15% PBS buffer) in a 2 mL sterile Eppendorf tube. The samples were sonicated (5 min), vortexed (30 sec), centrifuged at (10,000g; 4°C; 5 min) and the supernatant retained for analysis.

**Targeted metabolomics**

Quantitative mass spectrometry-based metabolomic profiling was performed using the Biocrates AbsoluteIDQ p180 (BIOCRATES, Life Science AG, Innsbruck, Austria), as previously described [23, 24]. The AbsoluteIDQ p180 kit provides simultaneous quantification of amino acids, acylcarnitines, sphingomyelins, phosphatidylcholines, hexose (glucose), and biogenic amines in many biological samples. The samples were processed according to the manufacturer's instructions and analysed on a triple-quadrupole mass spectrometer (Xevo TQ-MS, Waters Corporation, USA). The measurements were made in a 96-well format, and seven calibration standards were integrated in the kit. Human EDTA plasma samples spiked with standard metabolites were used as quality control samples to assess reproducibility of the assay. Briefly, 10 µL of mouse plasma samples and 10 µL of supernatant of brain extractions (prepared as described above) were used for targeted metabolomics analysis. The amino acids and biogenic amines were derivatised using phenylisothiocyanate (PITC) in the presence of isotopically labelled internal standards,

followed by separation UPLC and quantification on a triple-quadrupole mass spectrometer (Xevo TQ-MS, Waters Corporation, USA) operating in the multiple reaction monitoring (MRM) mode. All the other metabolites were quantified on the same mass spectrometer without column separation by the flow injection analysis (FIA) operating in MRM mode. Metabolite concentrations were calculated and expressed as µmol/L.

### Statistical analysis

The concentrations of 187 metabolites were exported to Simca 13 (Umetrics, Umea, Sweden) for multivariate analysis. Data were log transformed, pareto-scaled and grouped into APP/PS1 and WT prior to analysis using orthogonal projection to latent structures-discriminant analysis (OPLS-DA). Statistical differences in metabolite concentrations were analysed by Mann-Whitney U-test. Blood and brain metabolite changes were cross-referenced to identify changes which were common to both sample types. Metabolic pathway analysis was performed using online metabolomics tools (Metaboanalyst [25] and Vanted (version 2.0.1) [26]). Heat map visualisations of data were created using PermutMatrix version 1.9.3.0 [27].

## Results

### Multivariate analysis of mouse brain and plasma sample at different age

The 187 metabolites concentrations quantified by mass spectrometry in both mouse brain and plasma samples underwent both univariate and multivariate analysis. Univariate analysis (Mann-Whitney U-test) provided an overview of how metabolites in each metabolite class were affected at each age and in each sample type, which is detailed in Table 1. Multivariate analysis (OPLS-DA) was used to build a model differentiating WT (green) and APP/PS1 (blue) samples at each ages (Figure 1). The scores plot (Figure 1) displays the

results of model building. R2 (cumulative) and Q2 (cumulative) were used to determine the validity of models. R2 indicates the variation described by all components in the model and Q2 is a measure of how accurately the model can predict class membership. It does this by leaving out 1/7th of the data from the model and then predicting their class membership. The explained variance (R2) in all models was very satisfactory with values greater than 0.9 in all age groups. However, predictive ability (Q2) varied substantially with age group in both brain and plasma. Models with the greatest predictive ability were at 8 months (Q2 = 0.836) and 12 months (Q2 = 0.890), for brain and plasma respectively.

**Disturbances in metabolic pathways.**

Cross-referencing of blood and brain metabolite changes identified some amino acids and polyamines altered in both sample types. Metabolic pathway analysis revealed the biochemical relationships of these metabolites and the ages at which they were affected (Figure 2 and 3). Significant increases in the polyamines putrescine, spermidine and spermine ($p<0.05$) in APP/PS1 mice were detected in both brain and plasma. Disturbances of these metabolites occurred earlier (6-8 months) in brain than in plasma (10-12 months). Brain citrulline was lower ($p<0.05$) in APP/PS1 mice at 6 and 10 months, and this metabolite was also lower in plasma but at 12 months ($p<0.01$) and 18 months ($p<0.05$). Threonine levels were lower in APP/PS1 brain tissue (10 months; $p<0.05$) and in plasma (12 months; $p<0.01$). Serine and glutamine were also significantly altered ($p<0.05$) in both brain and plasma of APP/PS1 mice. Contrastingly, arginine was only significantly altered ($p<0.05$) in brain tissue, whilst glutamine and proline were only changed ($p<0.05$) in plasma.

**Disturbances in amino acids**

The concentrations of a number of amino acids were significantly different in APP/PS1 mice compared with WT. Cross-referencing of brain and blood changes found five consistent

amino acids alterations: histidine, leucine, phenylalanine, valine and lysine (Figure 4). The direction of change was the same in both sample types and for histidine, leucine and phenylalanine the disturbances were always occurred earlier in brain than plasma. Some changes were quite profound for example leucine was 51.6% higher ($p<0.01$) in APP/PS1 brain at 10 months. Interestingly all of the five amino acids are classed as essential and also leucine and valine make up two of the three branched-chain amino acids (BCAAs), therefore we assessed the overall changes in BCAAs and the ratio of essential amino acids to non-essential amino acids (EAA:NEAA; Supplementary Table 1). For APP/PS1 mice the EAA:NEAA ratio was significantly higher in both brain (18 months) and plasma (12 & 18 months), and total BCAAs was higher in the brain (10 months) and plasma (12 months).

A host of other changes in amino acid and biogenic amines were also identified (Supplementary Table 2). There were many more disturbances were detected in plasma than in brain tissue. Plasma levels of asparagine, isoleucine (a BCAA), tyrosine, serotonin and taurine were significantly higher in APP/PS1 mice. Of these serotonin was profoundly increased 72.7% (8 months) and 52.5% (10 months). Plasma levels of asymmetric dimethylarginine (ADMA) and α-aminoadipic acid were significantly lower in APP/PS1 mouse plasma. At 12 months α-aminoadipic acid was more than 60% ($p<0.01$) lower in APP/PS1 mice. Disturbances in both methionine and methionine sulfoxide correlated well, both of which were higher at 10 months ($p<0.05$) and lower at 12 months ($p<0.01$) in APP/PS1 mice.

**Phospholipid alterations**

The alteration of 101 membrane lipids metabolites including 14 lysophosphatidylcholines (LysoPCs), 73 phosphatidylcholines (PCs) and 14 sphingomyelins (SPHs) were assessed for both plasma and brain tissue samples. Changes in plasma and brain were visualised

using heat maps (Figure 5). Metabolites in red are higher in APP/PS1 and those in green are lower. 1 PC (PC aa C36:0) was not measureable in brain and 5 SPHs (SM (OH) C22:1, SM (OH) C24:1, SM C20:2, SM C26:0 and SM C26:1) were not measurable either in brain tissue or in plasma. The heat map revealed very considerable and widespread increases in PC levels in APP/PS1 brain tissue at 8 months of age. Also very evident was the widespread decrease in phospholipid levels in APP/PS1 plasma at 12 months. From 72 PCs measured in brain 58 were significantly higher ($p<0.05$) in APP/PS1 mice at 8 months. From 73 PCs measured in plasma 64 were significantly lower ($p<0.05$) in APP/PS1 mice at 12 months. Of the 14 LysoPCs, 3 LysoPCs (LysoPC a C16:1, LysoPC a C17:0 and LysoPC a C28:0) were significantly higher in APP/PS1 brain at 8 months. A total of 9 LysoPCs (LysoPC a C14:0, LysoPC a C16:0, LysoPC a C16:1, LysoPC a C17:0, LysoPC a C18:1, LysoPC a C18:2, LysoPC a C20:3, LysoPC a C20:4 and LysoPC a C24:0) were significantly lower in APP/PS1 plasma ($p<0.05$) at 12 months. Furthermore, of the 9 measured SPHs, 5 SPHs (SM (OH) C16:1, SM C16:0, SM C18:0, SM C24:0 and SM C24:1) significantly higher in APP/PS1 brain at 8 months and 3 SPHs (SM (OH) C14:1, SM C16:1 and SM C24:0) were significantly lower in APP/PS1 plasma ($p<0.05$) at 12 months.

**Acylcarnitine alterations**

Of the 40 acylcarnitines quantified 18 were significantly different in brain tissue and 18 were significantly different in plasma (Figure 6). Only 9 acylcarnitines were significantly altered in both brain and plasma (C2, C4:1, C6:1, C7-DC, C10:1, C10:2, C18:1, C18:1-OH and C18:2) and only one of these (C10:2) exhibited the same direction of change in both sample types. Of all the acylcarnitines, C4:1 and C6:1, were most different being more than 60% lower ($p<0.01$) in APP/PS1 plasma at 8 months.

## Discussion

This is the first high-throughput targeted metabolomic analysis to longitudinally investigate the metabolic disturbances of APPswe/PS1deltaE9 (APP/PS1) mice. The aim was to identify the specific metabolite changes consequential to the AD-like pathology, and how these changes are affected over the disease course. The study also assessed which neurometabolite changes are mirrored within the blood circulation. A total of 187 metabolites were quantified including amino acids, biogenic amines, phospholipids and acylcarnitines, and one of the key findings is that the majority of metabolite perturbations in the APP/PS1 model are discontinuous. The majority of perturbations do not persist, as might have been expected in chronic and progressive disease pathology. Our findings suggest that there are key periods when wide-ranging metabolic disturbances occur, and the data contained herein could be highly informative, both for future experimentation with this animal model, but also in terms of interpreting metabolomics findings from human volunteers.

The targeted metabolomics approach undertaken correctly classified APP/PS1 mice from WT with high specificity and sensitivity. Some multivariate statistical models were particularly impressive, for example, plasma metabolites at 12 months predicted APP/PS1 mice with 89% accuracy, and brain metabolites at 8 months predicted with 84% accuracy. This is a significant improvement on the predictive abilities of our earlier NMR-based profiling approaches - which predicted APP/PS1 mice (12 months) with 62% and 74% for brain and plasma samples, respectively [16]. It was evident that predictive scores were poorer at 6 months when Aβ plaque formation is in the early stages and memory impairment is minimal [9, 28]. Significant alterations in amino acid and phospholipid concentrations were key factors distinguishing between APP/PS1 and WT. Many of these significantly altered at 6-8 months in brain and 10-12 months in plasma. Within the amino acids we found that the arginine/polyamine pathway was discontinuously disturbed in both brain and plasma. Increased levels of three polyamines, putrescine, spermidine and spermine, have once been reported in brain tissue from AD patients [29, 30]. The underlying explanation for this is that

Aβ causes up-regulation of polyamine uptake and increased ornithine decarboxylase activity, which leads to increased polyamine synthesis [31, 32]. Polyamines, like spermidine and spermine are positive modulators of the N-Methyl-D-Aspartate (NMDA) receptor. Thus, increased polyamine synthesis causes dysfunction of the NMDA receptor leading to the neuronal excitotoxicity which occurs in AD [33]. Here we found evidence that brain polyamine elevations occur early in the pathology. Putrescine which precedes both spermidine and spermine in the biochemical pathway is the first to be elevated (6 months) in APP/PS1 mice. This elevation of putrescine could stem from the observed rise in its precursor metabolite, arginine. This is then followed by an increase in both brain spermidine and spermine at 8 months. These observations were mirrored in blood plasma where we found a very similar trend, albeit much later. Putrescine was elevated at 10 months and spermidine and spermine at both 10 and 12 months. It remains to be proven whether the polyamine disturbances detected in the circulation are the corollary of the observed cerebral disturbances. It is entirely possible that these arise peripherally or systemically in the APP/PS1 mouse. This must still be determined, however, putrescine is deceased in the thymus of APP/PS1 mice and is entirely absent from the spleen, which at least rules out these organs as sources for elevated blood polyamine levels [18]. Despite this, there are clear comparisons which can be drawn with human subjects. Our recent study which undertook untargeted metabolomics analysis in human plasma found that both stable MCI subjects and MCI subjects who converted to AD there was a significant increase in arginine compared with cognitively normal age-matched controls [34]. Furthermore, the fate of this arginine differed substantially between groups. In subjects who converted to AD putrescine was channelled towards the production of spermidine and spermine, whereas in stable MCI subjects putrescine was diverted towards the production of N-acetylputrescine or 4-aminobutanal (not measured in the present study). The findings here therefore strengthen the validity of this particular mouse model because the elevated putrescine, spermidine and spermine observed here translate nicely to findings in human AD patients who also have elevated circulating levels of these 3 polyamines. Certainly it does appear that there is some

selectivity towards the polyamine pathway in AD since other metabolites downstream of arginine (e.g. creatinine, sarcosine, ornithine, proline or hydroxyproline) are either not affected or are very inconsistently affected. Further investigation of polyamine metabolism is a worthwhile line of inquiry in AD research. Particularly promising is the finding that the chemical inhibition of the polyamine system counteracts beta-amyloid induced memory impairments, apparently by modulating extrasynaptic NMDA receptor signalling [35].

Beyond these biochemical pathways, a host of other metabolite disturbances were evident in the APP/PS1 mouse. First, we observed a number changes in the levels of amino acids. Five amino acid concentration changes (histidine, leucine, lysine, phenylalanine and valine) were consistent in both brain and blood. Here we also noted an approximately 2-4 month time-lag between disturbances in the brain and disturbances in the plasma. Significantly, all five of these are essential amino acids and they included two of the three branched-chain amino acids (BCAAs). The third BCAA isoleucine was elevated in plasma. The extent of these amino acid disturbances was sufficiently large enough to impact on both the overall ratio of essential amino acids to non-essential amino acids (EAA:NEAA), and the overall levels of BCAAs. It is known that at least one third of the amino groups of brain glutamate are derived from the BCAAs [36]. Leucine alone was elevated by more than 50% in APP/PS1 brain and ~~contrubutes~~ contributes 25% of gluamate's amino groups [36]. However, we did not find consistent evidence that glutamate was affected here. It could be suggested EEA and BCAA changes are a consequence of the neuronal destruction or degenerative brain pathology, which occurs in this model [28]. We also found a number of other important metabolites which were altered in APP/PS1 plasma including the neurotransmitter serotonin which was increased by >70% at 8 months and >50% at 10 months. It is not entirely clear why there were significant decreases in plasma asymmetric dimethylarginine (ADMA) and α-aminoadipic acid in APP/PS1 mice, but since both of these are intermediates in amino acid metabolism they could be a consequence of the wider amino acid disturbances. Previous studies found ADMA to be higher in plasma from AD patients [37, 38] whilst others found

decreased ADMA in CSF from AD patients [38, 39]. As far as we are aware, changes in α-aminoadipic acid have not been reported before but the potential relevance that it is a NMDA receptor ligand. In fact the L-enantiomeric form exerts an agonist activity, while the D-enantiomeric form acts as an antagonist [40, 41]. Since NMDA has been implicated in both cell death and neuronal excitotoxicity in AD [42], it may be worth examining how the individual D-and L-enantiomers of α-aminoadipic acid are affected by AD pathology. Methionine and methionine sulfoxide correlated closely, both of which were elevated at 10 months ($p<0.05$) and reduced at 12 months ($p<0.01$) in APP/PS1 mice. This is potentially supported by a study which found modest non-significant increases in methionine in serum from MCIs subjects but significantly reduced levels in AD patients [43]. Also noteworthy is that the levels of methionine sulfoxide reductase (Msr) enzymes are elevated by 40% in APP/PS1 mice at 9 months of age [44] which may suggest that upregulation of Msr in the early stages of AD may be an attempt to mitigate oxidative stress, ultimately failing in the later stages of the disease.

Detailed glycerophospholipid and sphingolipid lipid profiling of the APP/PS1 mouse was also undertaken in this study. Glycerophospholipids (PCs and LysoPCs) are the major class of complex lipids playing essential roles in neural membrane formation and intraneuronal signal transduction [45, 46]. PCs are the most abundant glycerophospholipids which have a choline polar headgroup attached to the phosphate group. The LysoPCs result from partial hydrolysis of phosphatidylcholines, which removes one of the fatty acid groups. This hydrolysis is generally thought to result of the enzymatic action of phospholipase A2 (PLA2) [46]. Our results demonstrate that there are 2 particular time periods when PCs undergo considerable and wide-spread flux. Firstly, in the APP/PS1 brain at 8 months PCs increase across the board (58 from 72 significantly increased). Secondly and contrastingly, in APP/PS1 plasma, PCs decrease across the board at 12 months (64 from 73 are significantly decreased). Whilst such detailed profiling has not been undertaken is this model before the

aggregate levels of PCs in APP/PS1 mouse brain have been reported to be lower at 9 months of age [19]. It has also been noted that total phosphatidylcholine diacyl (PCaa) levels tend to be lower in APPswe brain [47]. However our findings are in broad agreement with a previous study which demonstrated the APP/PS1 mouse brain exhibits increased sphingomyelins (SPHs) and PCs at 9 months [48]. The potential relevance of such a finding is that neural membrane PCs are hydrolysed preferentially by the enzyme $PLA_2$ to generate LysoPCs and free fatty acids [49-51], and the importance of $PLA_2$ in AD pathogenesis is a hotly debated topic [52-54]. Taken together, the data from these studies suggests that $PLA_2$ activity (particularly $cPLA_2$ and $iPLA_2$) is reduced, resulting in reduced phospholipid turnover in early AD pathogenesis. This could contribute to cognitive dysfunction and neuropathology in the early stages of the disease. As the disease worsens, $cPLA_2$ and $sPLA_2$ levels (and accordingly the metabolism of phospholipids) become elevated in AD brains [55, 56]. This may explain why we observed a general increase in PCs in the brain at 8 months and why this increase is later eliminated.

One third of all the sphingomyelin (SPH) species measured in APP/PS1 mouse brain were significantly elevated at 8 months of age. SPHs are precursors for ceramide production. Their accumulation induces apoptosis and seems to worsen neurodegeneration by increasing amyloid beta biosynthesis and promoting gamma-secretase processing of amyloid precursor protein [57-59]. Our findings closely resemble other work showing that global brain levels of ceremides, sphingolipids and related molecules are increased in the cerebral cortex of $APP^{SL}$/PS1Ki and APP/PS1 mice especially after the age of 6 months [60]. Interestingly, the concentrations of sphingomyelin were nearly constant between 3 & 6 months old $APP^{SL}$/PS1Ki mice [60]. We also found substantive changes in acylcarnitine species. From the 40 acylcarnitines analysed, significant differences were observed for 18 acylcarnitines in both brain and plasma from APP/PS1 mice. However, it must be stated that we could not detect any particular overall pattern in the acylcarnitine alterations.

In conclusion, this study has delineated some of the key metabolomic impairments occurring longitudinally over the lifespan of the APP/PS1 mouse and this could provide an insight into the different phases of metabolic disturbances which may occur in human AD. There appear to be key periods in which the model undergoes widespread metabolic flux, initially occurring in the brain followed 2 to 4 months later with changes in circulating metabolites. Disturbances are frequently discontinuous in nature, neither being progressively nor persistently affected (as might previously have been surmised from previous studies examining one or two time points). The depiction of how polyamine metabolism is disturbed potentially has future therapeutic relevance for the development of inhibitors of the polyamine system. Furthermore, the observed fluxes particularly in phosphotidylcholine species both in the CNS and circulation provides new opportunities for studying and potentially tracking the pathophysiology of AD.

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**Table 1 – Overview of Brain and Blood metabolites significantly altered in APP/PS1 mice**

| Age (months) | 6 | | 8 | | 10 | | 12 | | 18 | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Metabolites** | **Brain** | **Plasma** | **Brain** | **Plasma** | **Brain** | **Plasma** | **Brain** | **Plasma** | **Brain** | **Plasma** |
| **Amino acids and biogenic amines (42)** | 6 [14%] | 2 [5%] | 4 [10%] | 4 [10%] | 4 [10%] | 17 [40%] | 2 [5%] | 11 [26%] | 4 [10%] | 2 [5%] |
| **Acylcarnitines (40)** | 1 [3%] | 5 [13%] | 6 [15%] | 7 [18%] | 8 [20%] | 6 [15%] | 4 [10%] | 5 [13%] | 9 [23%] | 8 [20%] |
| **Phosphotidylcholines (PCs) (73)** | 0 [0%] | 1 [<1%] | 58 [79%] | 2 [3%] | 0 [0%] | 1 [<1%] | 5 [7%] | 64 [88%] | 1 [<1%] | 1 [<1%] |
| **Lyso-phosphotidylcholines (LysoPCs) (17)** | 2 [12%] | 1 [6%] | 3 [18%] | 1 [6%] | 1 [6%] | 1 [6%] | 2 [12%] | 9 [53%] | 3 [18%] | 1 [6%] |
| **Sphingolipids (SPHs) (15)** | 1 [7%] | 0 [0%] | 5 [33%] | 0 [0%] | 2 [13%] | 0 [0%] | 5 [33%] | 3 [20%] | 2 [13%] | 0 [0%] |
| **Total (187)** | 10 [5%] | 9 [5%] | 76 [41%] | 14 [7%] | 15 [8%] | 25 [13%] | 18 [10%] | 92 [49%] | 19 [10%] | 12 [6%] |

Numbers in parenthesis indicate the total number of metabolites measured in each metabolite class. Shown in the Table are the number of metabolites significantly different in APP/PS1 mice vrs. WT (Mann-Whitney U test). Values in square brackets are the percentage of measured metabolites affected.

**Figure Legends**

**Figure 1. Multivariate statistical models arising from targeted metabolomics data**. Left: OPLS-DA scores plot classifying the brain samples from WT (green) and APP/PS1 (blue) mice aged (A) 6 months, (B) 8 months, (C) 10 months, (D) 12 months and (E) 18 months. Right: OPLS-DA scores plot classifying the plasma samples from WT (green) and APP/PS1 (blue) mice aged (F) 6 months, (G) 8 months, (H) 10 months, (I) 12 months and (J) 18 months.

**Figure 2. Age-dependent changes in metabolic pathways in brain samples.** Data are presented as mean ± SEM (n=8). Mann-Whitney U-tests showed significant differences between WT (black) and APP/PS1 (red) mice. *p<0.05, **p<0.01 and ***p<0.001.

**Figure 3. Age-dependent changes in metabolic pathways in blood plasma samples.** Data are presented as mean ± SEM (n=8). Mann-Whitney U-tests showed significant differences between WT (black) and APP/PS1 (red) mice. *p<0.05 and **p<0.01.

**Figure 4. Significant changes in amino acid concentrations.** Graphs show changes of individual amino acids in (A) brain and (B) plasma of APP/PS1 mice relative to WT mice (6, 8, 10, 12 and 18 months). Statistical differences were determined using the Mann-Whitney U-test (*p<0.05, **p<0.01 and ***p<0.001).

**Figure 5. Phosophlipid disturbances in the APP/PS1 mouse.** Heat maps show the changing phospholipid profiles (14 lysophosphatidylcholine (LysoPCs), 76 Phosphatidylcholine (PCs) and 15 sphingomyelin (SPHs)) of WT and APP/PS1 mice. Left: brain. Right: plasma. Each individual row represents a sample, and each individual column represents a phospholipid. Red pixels indicate increasing concentrations and green pixels decreasing concentrations (see colour scale above the heat map). Yellow boxes indicate the divergent changes detected in brain and plasma at 8 and 12 months, respectively. Heat map visualisations were produced with PermutMatrix graphical interface after Z-score normalization and Pearson's dissimilarity was used as distance measure.

**Figure 6. Significant changes in acylcarnitine concentrations.** Graphs show changes in individual acylcarnitines in brain and plasma of the APP/PS1 mice relative to WT mice (6, 8, 10, 12 and 18 months). Statistical significances were determined using the Mann-Whitney U-test (*p<0.05, **p <0.01 and ***p<0.001).

**Figure 1**

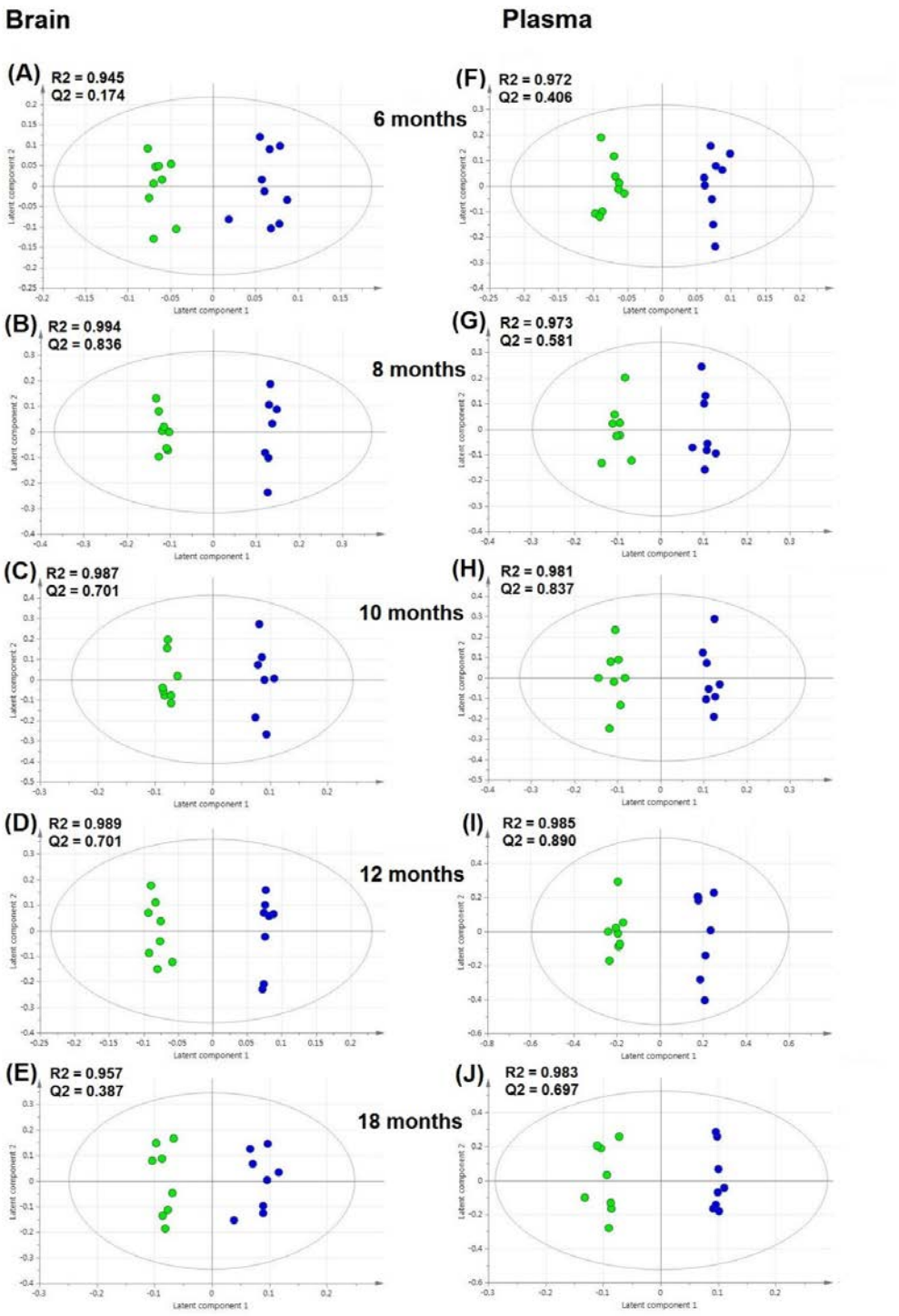

Brain
Plasma
(A) R2 = 0.945 Q2 = 0.174
(F) R2 = 0.972 Q2 = 0.406
6 months
(B) R2 = 0.994 Q2 = 0.836
(G) R2 = 0.973 Q2 = 0.581
8 months
(C) R2 = 0.987 Q2 = 0.701
(H) R2 = 0.981 Q2 = 0.837
10 months
(D) R2 = 0.989 Q2 = 0.701
(I) R2 = 0.985 Q2 = 0.890
12 months
(E) R2 = 0.957 Q2 = 0.387
(J) R2 = 0.983 Q2 = 0.697
18 months
Latent component 1
Latent component 2

**Figure 2**

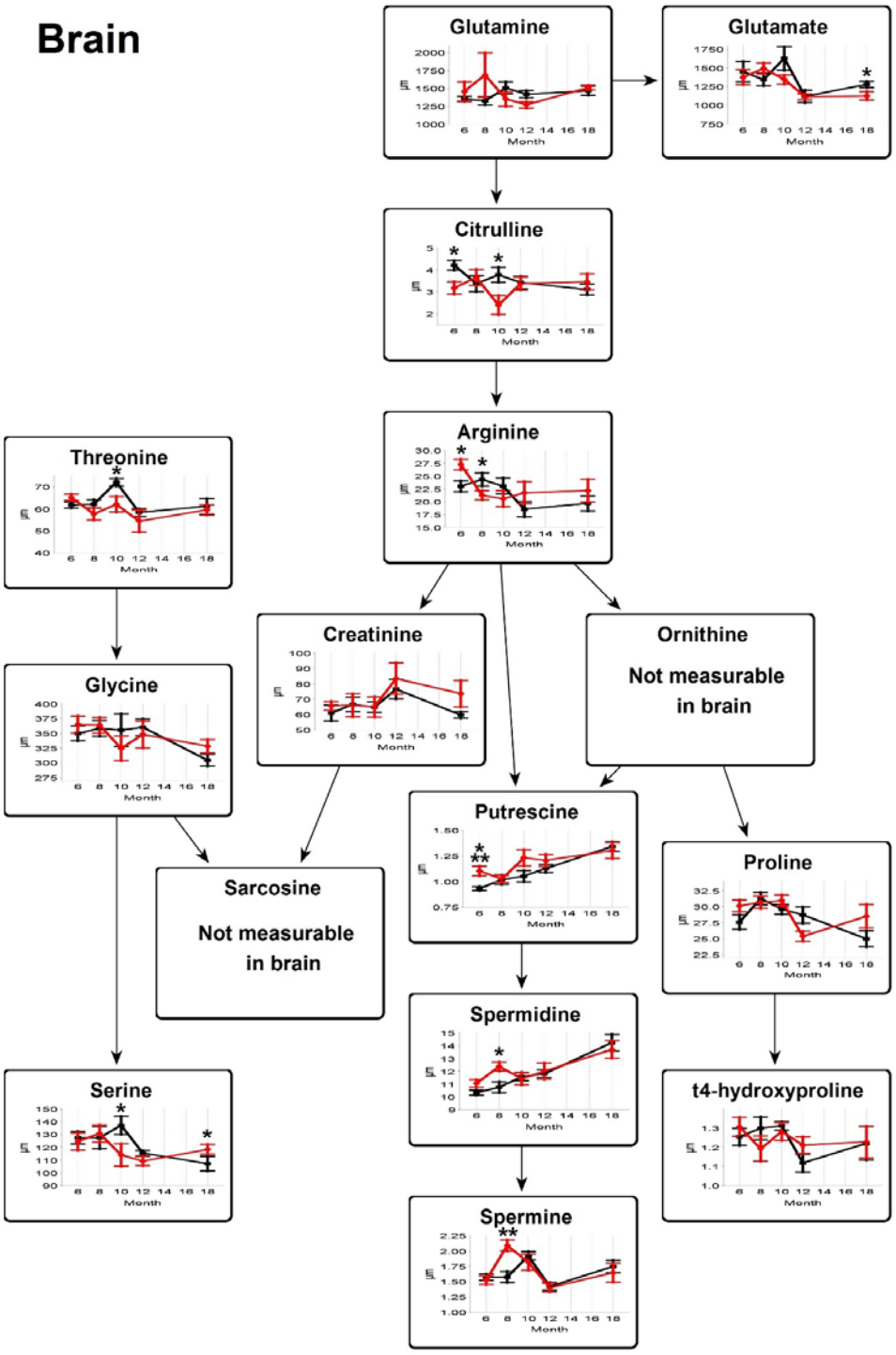

Brain
Glutamine
Glutamate
Citrulline
Arginine
Threonine
Creatinine
Ornithine
Not measurable
in brain
Glycine
Sarcosine
Not measurable
in brain
Putrescine
Proline
Spermidine
Serine
t4-hydroxyproline
Spermine
Month

**Figure 3**

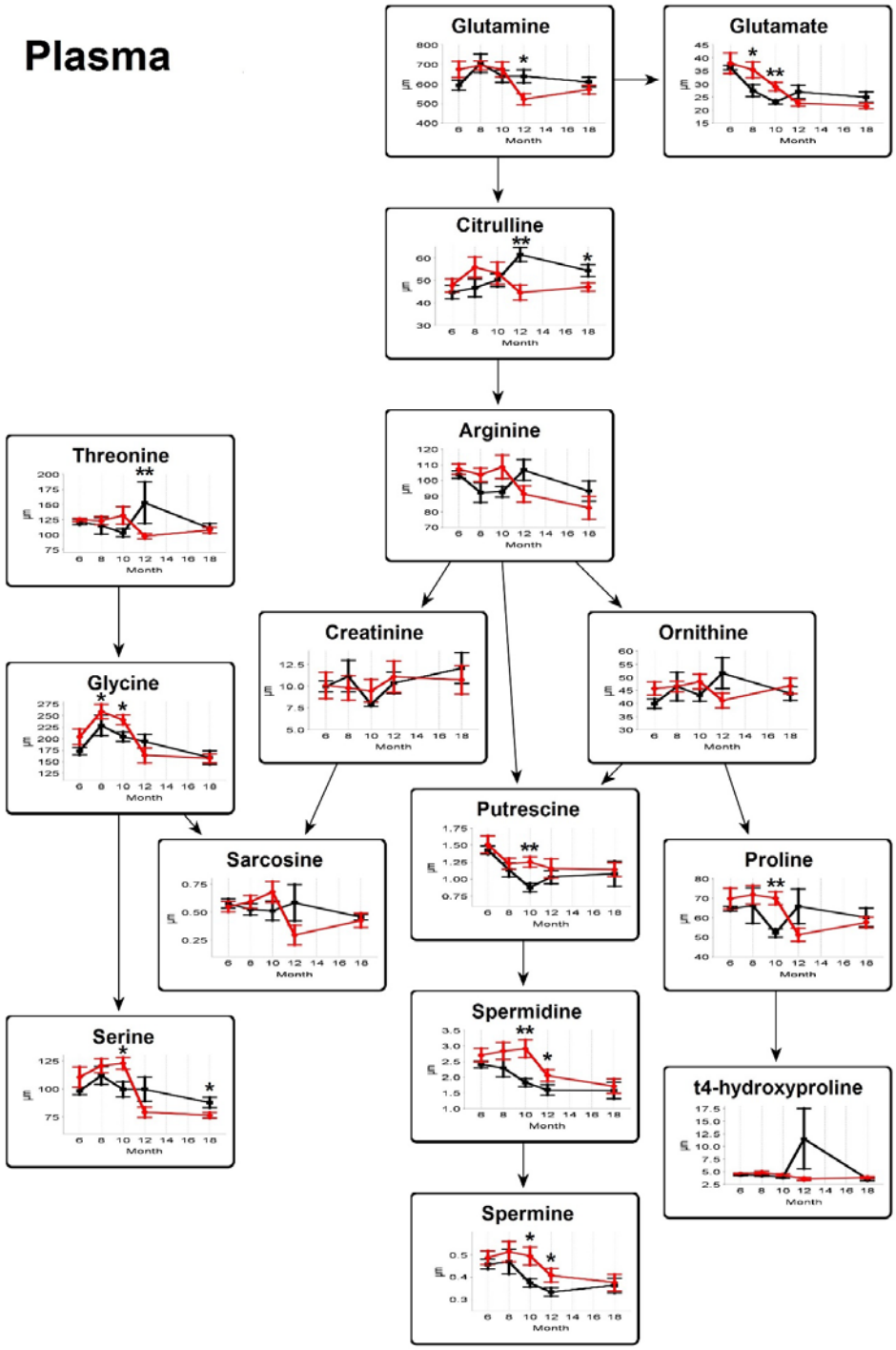
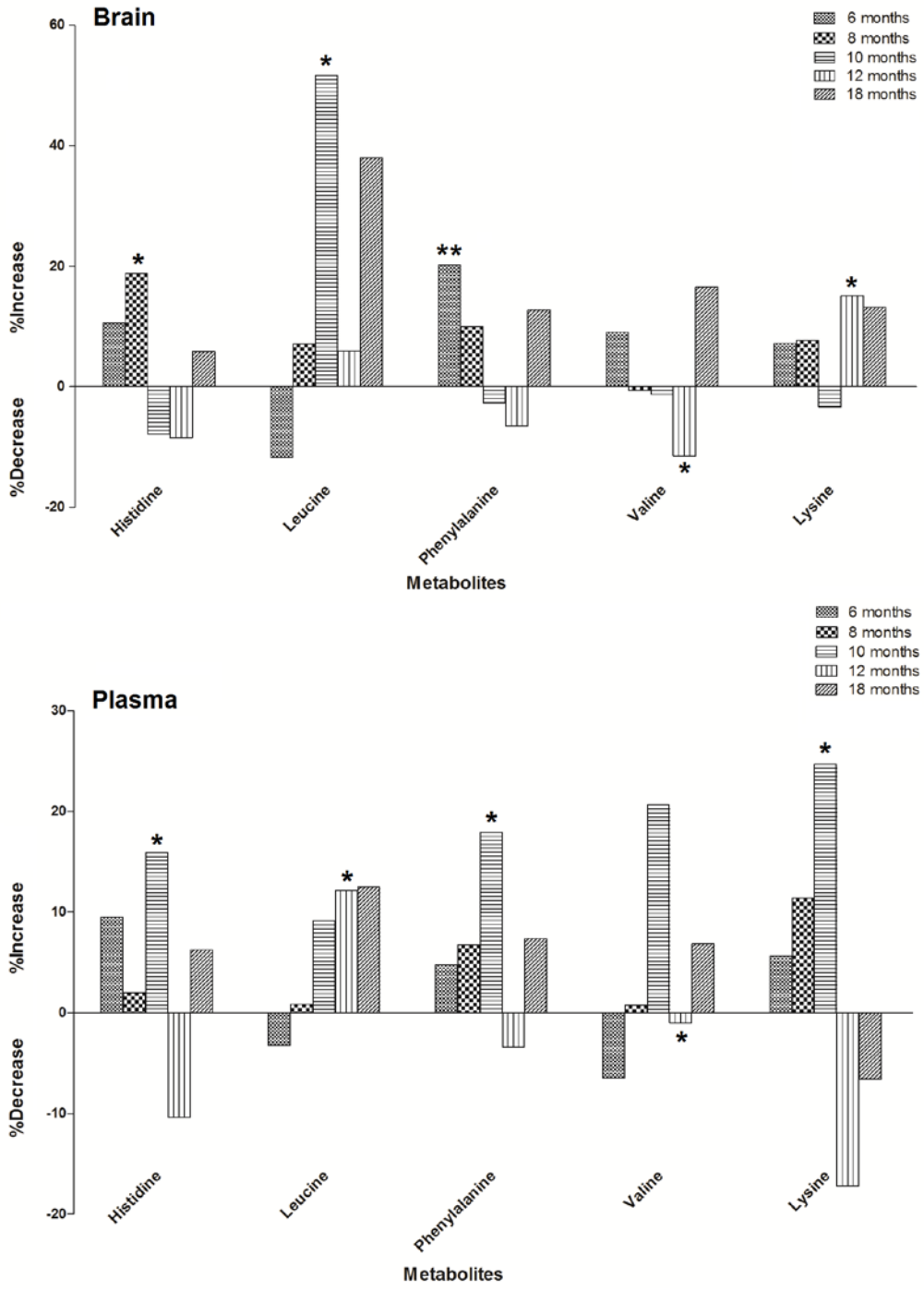

Plasma
Glutamine
Glutamate
Citrulline
Arginine
Threonine
Creatinine
Ornithine
Glycine
Sarcosine
Putrescine
Proline
Serine
Spermidine
t4-hydroxyproline
Spermine
Month

**Figure 4**




**Comment [BDG2]:** -Change Brian to Brain
-Changes the stars to asterisks
-Can we move Lysine to last in the order of both.
- Some small ticks/dividers between the amino acids would help. Or maybe just a wider gap.

**Figure 5**

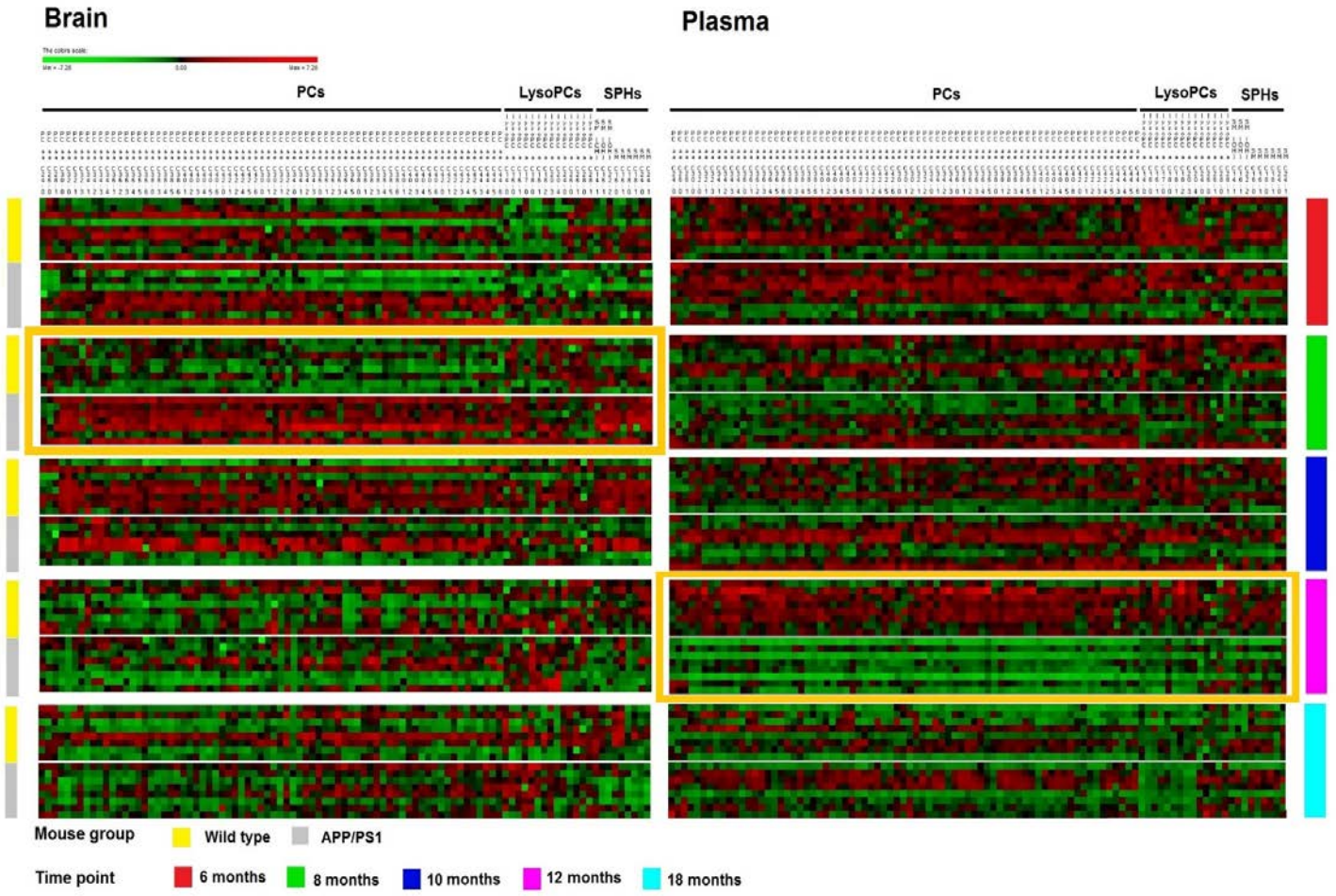
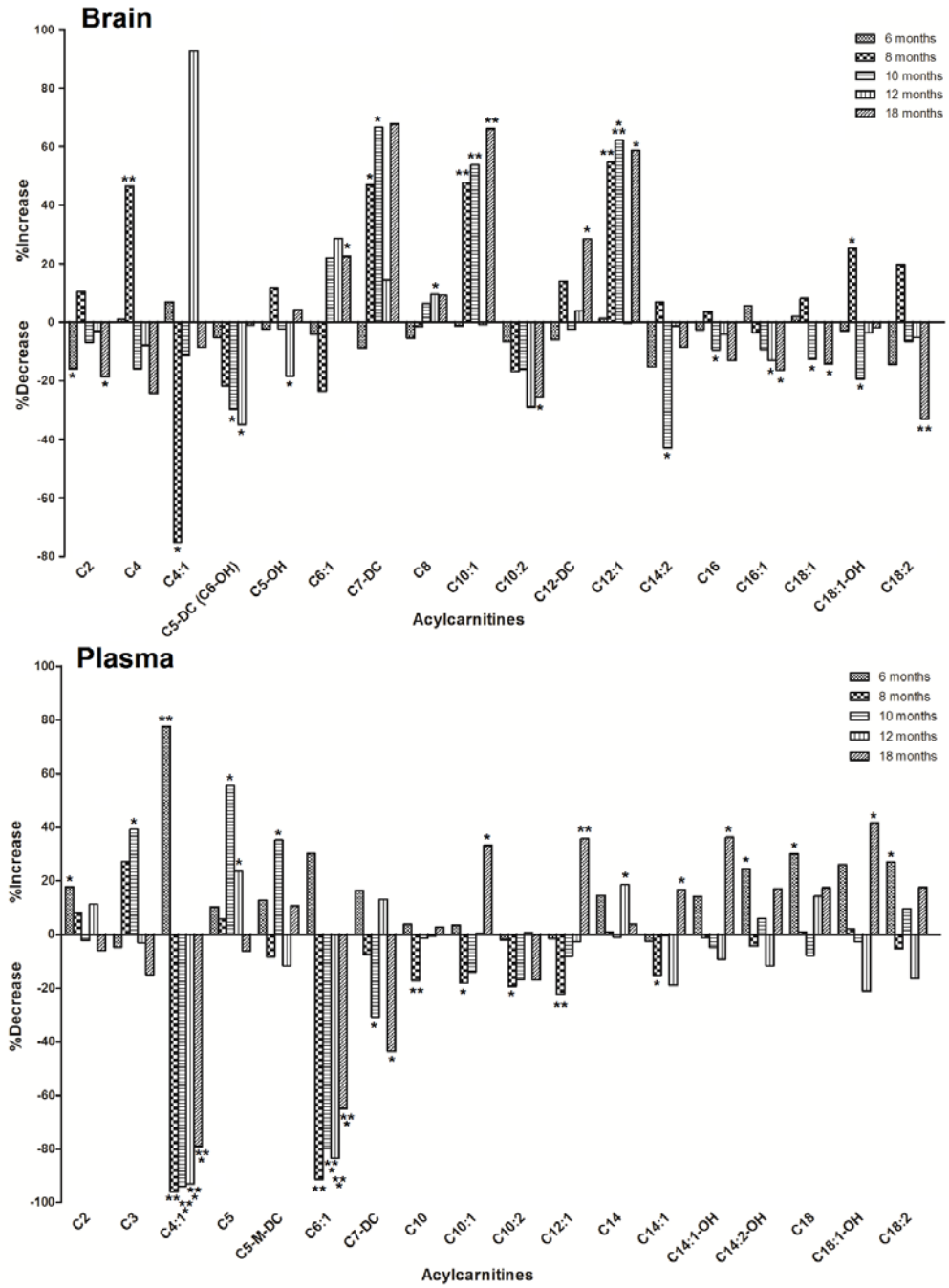

Brain
Plasma
PCs
LysoPCs
SPHs
PCs
LysoPCs
SPHs
Mouse group
Wild type
APP/PS1
Time point
6 months
8 months
10 months
12 months
18 months

**Figure 6**

**Comment [BDG3]:** Should have the Figures labelled for brain and plasma. Apologies if you did this already in another file and I have missed it.



**Supplementary Table 1 – Alterations in essential and branched chain amino acids in APP/PS1 mice.**

| | | EAA:NEAAs | | BCAAs | |
|---|---|---|---|---|---|
| | | **WT** | **APP/PS1** | **WT** | **APP/PS1** |
| **Brain** | **6 months** | 0.049±0.003 | 0.050±0.002 | 74.67±6.63 | 72.67±8.54 |
| | **8 months** | 0.051±0.002 | 0.048±0.002 | 78.07±3.60 | 78.47±7.27 |
| | **10 months** | 0.046±0.002 | 0.051±0.002 | 65.74±1.77 | **77.66±5.73*** |
| | **12 months** | 0.048±0.003 | 0.049±0.001 | 64.53±9.05 | 61.95±4.40 |
| | **18 months** | 0.046±0.002 | **0.051±0.002*** | 57.95±7.17 | 73.52±3.32 |
| **Plasma** | **6 months** | 0.53±0.01 | 0.47±0.02 | 395.52±7.60 | 374.27±14.54 |
| | **8 months** | 0.46±0.01 | 0.45±0.01 | 377.89±13.23 | 381.54±17.87 |
| | **10 months** | 0.46±0.02 | 0.47±0.03 | 343.59±14.41 | 394.31±30.46 |
| | **12 months** | 0.48±0.04 | **0.52±0.01*** | 328.01±49.11 | **331.90±10.42*** |
| | **18 months** | 0.46±0.01 | **0.52±0.02**** | 307.55±15.86 | 338.32±20.41 |

Values are presented as mean ± SEM. EAA:NEAAs – ratio of essential amino acids to non-essential amino acids; BCAAs – Branched chain amino acids (total concentration in μM); Statistical differences were determined using the Mann-Whitney U-test (*p<0.05, **p<0.01).

## Supplementary Table 2 – Other significant alternations in amino acid and biogenic amine concentrations.

| | Age (months) | % change in APP/PS1 mice | p-value |
|---|---|---|---|
| **Brain** | | | |
| **Alanine** | 6 | + 13.08 | 0.019 |
| **Trypotophan** | 6 | + 30.53 | 0.019 |
| | 18 | + 33.06 | 0.046 |
| **Histamine** | 18 | – 5.04 | 0.046 |
| **Plasma** | | | |
| **α-Aminoadipic acid** | 12 | -68.17 | 0.001 |
| **Asparagine** | 10 | + 37.96 | 0.016 |
| **Isoleucine** | 12 | + 14.98 | 0.012 |
| **Methionine** | 10 | + 35.91 | 0.021 |
| | 12 | – 33.67 | 0.003 |
| **Tyrosine** | 8 | + 31.73 | 0.009 |
| | 10 | + 30.02 | 0.016 |
| **ADMA** | 10 | – 23.40 | 0.021 |
| **Methionine sulfoxide** | 10 | + 73.13 | 0.016 |
| | 12 | – 33.44 | 0.009 |
| **Serotonin** | 8 | + 72.72 | 0.005 |
| | 10 | + 52.52 | 0.027 |
| **Taurine** | 6 | + 17.99 | 0.024 |
| | 10 | + 27.85 | 0.009 |

Changes in APP/PS1 mice are expressed as percentages compared with WT. '+' indicates an increase and '–' a decrease. P-values were calculated using the Mann-Whitney U-test.