



Altered metabolic pathways in a transgenic mouse model suggest mechanistic role of amyloid precursor protein overexpression in Alzheimer's disease

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Abstract

Introduction The mechanistic role of amyloid precursor protein (APP) in Alzheimer's disease (AD) remains unclear.

Objectives Here, we aimed to identify alterations in cerebral metabolites and metabolic pathways in cortex, hippocampus and serum samples from Tg2576 mice, a widely used mouse model of AD.

Methods Metabolomic profilings using liquid chromatography-mass spectrometry were performed and analysed with MetaAnalyst and weighted correlation network analysis (WGCNA).

Results Expressions of 11 metabolites in cortex, including hydroxyphenyllactate—linked to oxidative stress—and phosphatidylserine—lipid metabolism—were significantly different between Tg2576 and WT mice (false discovery rate < 0.05). Four metabolic pathways from cortex, including glycerophospholipid metabolism and pyrimidine metabolism, and one pathway (sulphur metabolism) from hippocampus, were significantly enriched in Tg2576 mice. Network analysis identified five pathways, including alanine, aspartate and glutamate metabolism, and mitochondria electron transport chain, that were significantly correlated with AD genotype.

Conclusions Changes in metabolite concentrations and metabolic pathways are present in the early stage of APP pathology, and may be important for AD development and progression.

Keywords Network analysis · Dementia · Amyloid-beta · Oxidative stress · Lipids metabolism · Glutamate

1 Introduction

At least 50 million people globally have dementia, and Alzheimer's disease (AD) is the most prevalent type of dementia (World Alzheimer Report 2018, 2018). Dementia is also the fifth leading cause of death worldwide (Masters et al., 2015). The current lack of disease modifying treatments may be because AD patients are diagnosed well into disease development, and pathology is already advanced, and this cannot

be reversed (Mehta et al., 2017; Petersen, 2009). Therefore, the ability to identify and treat AD patients in the preclinical stage is critical to successfully interfere with AD development and progression. By investigating the mechanisms associated with the presymptomatic stage of AD, potential therapeutic targets and/or biomarkers may emerge.

Recently, metabolomic analysis has become a prominent tool for hypothesis generation and the study of mechanisms for a number of diseases and disorders (Dettmer et al., 2007; Nagana et al., 2013). This is mainly due to its unique ability to profile all the identifiable metabolites within the samples of interest and compare it to the reference samples (Birkemeyer et al., 2005). Liquid chromatography-mass spectrometry (LC-MS) instrumentation in particular is rapidly improving and in the past decade, it has been shown that the data generated from LC-MS has higher quality, consistency and reproducibility than it has ever been (Aebbersold & Mann, 2003; Kuhn et al., 2012). Metabolomic analysis of blood samples may provide insights on the changes in metabolic processes that

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are associated with AD. However, whole blood contains cellular metabolites which may overshadow changes in the non-cellular metabolite levels and therefore it is common for metabolomic study to investigate serum or plasma metabolite levels instead of whole blood (Chaleckis et al., 2016). Additionally, recent technological advances combined with novel methodologies in metabolomics have managed to overcome biological problems associated with blood-based samples such as high variability in the metabolite concentration, thus making LC–MS a technique that provides both high specificity and sensitivity for metabolites (Anderson et al., 2004; Gonzalez-Dominguez et al., 2017; Pernemalm & Lehtio, 2014).

Access to tissue samples at specific timepoints during AD development is also an important aspect for the investigation of metabolomic pathways that are associated with the pathogenesis of AD. Currently, it is not possible to obtain samples from human patients because presymptomatic AD cannot yet be diagnosed in humans (Weller & Budson, 2018). Metabolomic profiling of transgenic mouse models of AD can provide insights into changes in the metabolic pathways that are associated with specific AD pathology, such as APP overexpression (Wilkins & Trushina, 2017). Therefore, in this metabolomic analysis study, the Tg2576 mouse model of AD was utilised. Tg2576 is one of the most characterised mouse models of AD that display APP pathology (Hsiao et al., 1996) by overexpressing human APP with the Swedish mutation (K760N/M671L). In this model, amyloid- β (A β) plaques are observed from approximately 9 months of age, while cognitive deficits appear from approximately 10 months old (Hall & Roberson, 2012). Aged Tg2576 displays an array of symptoms that are relevant to clinical profile of human AD patients (Van Dam & De Deyn, 2011). Here we chose to use 6-month-old Tg2576 mice, which are at a presymptomatic stage, to investigate metabolomic changes that are associated with early stages of AD development. Cortex, hippocampus and serum samples were of particular interest. The cortex is one of the first brain regions to be affected by A β pathology (Calderon-Garciduenas & Duyckaerts, 2017; Thal et al., 2002), and so changes in the metabolomic profile that are associated with early stage of AD pathology should be observable in this region. Additionally, since hippocampus is highly involved with cognitive functions and memory (Bird & Burgess, 2008)—classic symptoms of dementia—it would be interesting to study how human mutant APP overexpression pathology can affect the metabolomic profile in this area of the brain. We also chose to study serum in an attempt to identify potential metabolomic biomarkers associated with early stages of AD.

In addition to metabolite analysis, we also explored the utilisation of Weighted Gene Coexpression Network Analysis (WGCNA) in the context of metabolomic data. WGCNA is a powerful method that has been extensively used for

transcriptomic analysis, however it can also be implemented for metabolomic characterisation (DiLeo et al., 2011; Fukushima et al., 2011; Novais et al., 2019). This open source pipeline performs coexpression analysis using the tools and concepts of graph theory. Under the premise that strongly correlated metabolites are likely to be functionally associated, WGCNA integrates the concentration variation of metabolites across samples into a network, which is then used to elucidate relationships between metabolite groups and sample traits. These metabolomic and network analyses provide a global profiling of all identifiable metabolites in samples without observational biases that are typically associated with focused studies of metabolism.

2 Methods

2.1 Animals

Six-month-old mixed-sex Tg2576 mice and c57 \times SJL (WT) littermates ($n=7$ /group) were obtained from our colonies at the Department of Medicine (Royal Melbourne Hospital), University of Melbourne, Parkville, Australia. All experiments were conducted following approval from the Florey Neuroscience Institute Animal Ethics Committee (#15-003).

2.2 Tissue collection

Mice were euthanised via lethal injection of pentobarbitone (80 mg/kg) (Provet, VIC, Australia) and cardiac puncture was performed to collect blood from each animal. The blood was allowed to clot in an anticoagulant-free tube for 15 min at room temperature and then centrifuged at 1500 $\times g$ in 20 °C for 10 min. The supernatant was then collected as serum and frozen with liquid nitrogen before being stored at – 80 °C until processing. The brains were rapidly dissected into cortex and hippocampus and the samples were snap-frozen in liquid nitrogen before being stored at – 80 °C until processing.

2.3 Materials

LC–MS-grade acetonitrile was from Burdick and Jackson (Muskegon, MI, USA). Reverse osmosis purified Milli-Q water used in LC–MS analysis was from Millipore water purification system (Merck, Darmstadt, Germany). Ammonium carbonate and internal standards consisting of CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate); CAPS (3-(cyclohexylamino)-1-propanesulfonic acid); PIPES (1,4-piperazinediethanesulfonic acid) and TRIS (2-amino-2-(hydroxymethyl)-1,3-propanediol) were purchased from Sigma Aldrich.

2.4 Sample preparation

Frozen brain samples were crushed into powder using a steel multisample biopulveriser (Daintree Scientific, Australia) and quickly weighed, while not allowing the samples to thaw. Then 20 μl of cold extraction solvent was added per 1 mg of powdered tissue. The extraction solvent consisted of 80% methanol and internal standards at 1 μM concentration. The samples were then sonicated for 30 min in an ice bath, followed by vortexing at 4 $^{\circ}\text{C}$ for 15 min. The samples were then centrifuged at 14800 $\times g$ in 4 $^{\circ}\text{C}$ for 10 min. Finally, 100 μl of the supernatant was transferred to LC–MS vials and stored at – 80 $^{\circ}\text{C}$ until further processing.

For serum samples, 25 μl was extracted with 100 μl of cold methanol containing 1 μM of internal standards. The samples were vortexed at 4 $^{\circ}\text{C}$ for 30 min, then centrifuged at 14800 $\times g$ speed at 4 $^{\circ}\text{C}$ for 10 min. 100 μl of supernatant was transferred to LC–MS vials and the samples were stored at – 80 $^{\circ}\text{C}$ until the analysis. Blank samples were prepared so that background noise could be analysed. Pooled quality control sample was prepared by combining 5–10 μl of each sample for tissue and serum analysis separately.

2.5 LC–MS metabolomic analysis

Details of the LC–MS metabolomic analysis and data processing have been described previously (Creek et al., 2016). Briefly, LC–MS data was acquired on Q-Exactive Orbitrap mass spectrometer (Thermo Scientific, Waltham, Massachusetts, United States) coupled with high-performance liquid chromatography (HPLC) system Dionex Ultimate® 3000 RS (Thermo Scientific). Chromatographic separation was performed on a ZIC-pHILIC column (5 μm , polymeric, 150 \times 4.6 mm, SeQuant®, Merck). The mobile phase (A) was 20 mM ammonium carbonate and (B) acetonitrile.

2.6 Data processing

The acquired LC–MS data was processed in an untargeted fashion using open source software IDEOM (Creek et al., 2012), which initially used *ProteoWizard* to convert raw LC–MS files to *mzXML* format and *XCMS* to pick peaks to convert to *peakML* files. *Mzmatch.R* was subsequently used for the alignment of samples and the filtering (Scheltema et al., 2011). IDEOM interface in MS Excel (Creek et al., 2012) was, again, utilised for feature identification, organisation and data quality evaluation.

2.7 Raw data analysis and metabolomic profiling

Univariate and multivariate analyses were performed using *Metaboanalyst 4.0* (Chong et al., 2018). The datasets were quantile normalised, log transformed (Durbin et al., 2002) and auto-scaled (van den Berg et al., 2006). These parameters ensured the best normal distribution for the data. For the univariate analysis, unpaired *t*-test was performed and fold-changes were calculated in order to identify the differences in abundance of each metabolite between Tg2576 and WT. The *p*-values acquired from the *t*-test were corrected for multiple comparisons with the Benjamini–Hochberg method (Benjamini & Hochberg, 1995), which yielded false discovery rate (FDR). The significance threshold between groups was set to FDR < 0.05.

For the multivariate analysis, principle component analysis (PCA) was performed to detect outliers and partial least-square discriminant analysis (PLS-DA) was performed to identify metabolites that were driving the separation between the groups. A metabolite with variable importance in projection (VIP) scores of > 1 was considered to be an important feature. Leave-one-out cross validation (LOOCV) was performed to examine the fit quality of the model (Westervhuis et al., 2008). In LOOCV, the difference between R^2 and Q^2 values should be approximately less than 0.3 (Eriksson et al., 2013). A metabolite was deemed as significantly affected by genotype when its FDR < 0.05 and its variable importance in projection (VIP) score > 1.

2.8 Pathway enrichment analysis

Pathway enrichment analysis was performed separately from the raw data analysis using the *Metaboanalyst 4.0* online tool (Chong et al., 2018) with *Mus musculus* species (KEGG database) as reference (Kanehisa & Goto, 2000). The normalised peak intensity data from the Tg2576 samples were compared to WT littermates. To be qualified as an important pathway, the metabolic pathway must have FDR < 0.05 and the pathway impact score > 0.2.

2.9 Network analysis (WGCNA)

Weighted Correlation Network Analysis (WGCNA) is a systems biology methodology which can be used with metabolomics data to investigate the correlation between metabolites based on their abundance across the samples. This approach will identify previously unknown groups of metabolites that act in concert in the context of AD pathology. For detailed description of the WGCNA package please refer to (Langfelder & Horvath, 2008). Using the general framework of WGCNA, correlation-based networks characterising the metabolome of the Tg2576 and WT littermates were constructed. Briefly, the raw LC–MS peak intensity

values were quantile normalised and log transformed, followed by auto scaling, and the zero values were omitted by replacing them with one-half of the detection threshold value. A dendrogram was created via hierarchical clustering of the samples in order to identify and remove outliers. A series of soft thresholding values were used to determine the optimal power at which the normalised data fit the scale-free topology model. Using a soft thresholding power of 7, an adjacency matrix was constructed reflecting the pair-wise correlation coefficients between all the detected metabolites. The correlation network was then built based on the adjacency matrix, where each node corresponds to a single metabolite, and the edges between the nodes represent the correlation between the peak intensity values of the given metabolite across all samples. The metabolite modules were then identified through clustering analysis and pathway enrichment analysis was performed. We also examined the sample trait correlation (Tg2576 vs WT) to the modules identified in the network.

3 Results

3.1 Raw data analysis

The normalised metabolomic data acquired from the cortex, hippocampus and the serum can be seen in Supplementary Figs. 1, 2 and 3. Multivariate analysis, in the form of unsupervised principle component analysis (PCA) was performed on the normalised peak intensity data from each sample set. This allowed the visualisation and comparison of the individual Tg2576 and WT data from cortex, hippocampus and serum in order to identify outliers. Score plots from PCA identified one outlier from the cortex samples as can be seen in Fig. 1a—this sample was removed from subsequent analyses. Data from the hippocampus (Fig. 1b) and serum (Fig. 1c) showed appropriate data grouping, which indicated consistency in the metabolomic profiles of the samples within each group.

Supervised partial least-square discriminant analysis (PLS-DA) was performed to identify metabolites that are significantly different between groups. PLS-DA showed no overlap between Tg2567 and WT groups in all three sample types (Fig. 2). The results from PLS-DA were validated using leave-one-out cross validation (LOOCV) (Supplementary Fig. 4). The LOOCV successfully validated the PLS-DA results from the cortex samples (Supplementary Fig. 4A and Supplementary Table 1). LOOCV indicated that the multivariate analysis results from hippocampus and serum samples had high risks of being overfitted (Supplementary Fig. 4B and C) and therefore these two sets of data could not be visualised via PLS-DA.

3.2 Metabolomic profiling

In the cortex samples, PLS-DA identified 11 metabolites with VIP scores > 1 and FDR-adjusted p -value < 0.05 (Table 1). Average fold-changes were also calculated for each metabolite. Average fold-change > 1 indicates an increase in the level of the metabolite, while fold-change < 1 indicates decrease. The most significantly altered metabolite was the 3–4-Hydroxyphenyllactate (FDR = 0.045 & VIP scores = 1.97), which was found to be decreased (average fold-change = 0.60) compared to the WT control. Levels of metabolites that are linked to the glycerophospholipids pathway such as Phosphatidylethanolamine (44:3) (FDR = 0.045, VIP scores = 1.92 & average fold-change = 1.56) and Lysophosphatidylethanolamines (18:0) (FDR = 0.045 & VIP scores = 1.92, average fold-change = 1.30) were also significantly increased compared to the controls.

3.3 Pathway enrichment analysis

Four significantly enriched metabolic pathways, including Glycerophospholipid metabolism (FDR = 0.0271 & Pathway impact = 0.560) and Glycerolipid metabolism (FDR = 0.0271 & Pathway impact = 0.374), were identified in the cortical samples of Tg2576 mice (Table 1 and Supplementary Fig. 6). In addition, one significantly enriched pathway from the hippocampal samples was also identified, the sulphur metabolism pathway (FDR = 0.0237 & Pathway impact = 0.213). There were no significantly enriched pathways detected in the serum samples.

3.4 Network analysis

In order to investigate the metabolite-group correlations, WGCNA was conducted on the metabolite peak intensity data from each of the three tissues types. By hierarchically clustering all samples from each tissue type into a sample tree, we identified and eliminated one WT sample from cortical dataset (Fig. 3a), two samples (TG and WT) from hippocampal dataset (Fig. 3b) and one WT sample from serum dataset (Fig. 3c) as outliers (Supplementary Fig. 7). The coexpression networks were then constructed and 12 modules were identified in the cortical dataset, 10 modules in hippocampal dataset and eight modules in serum dataset. In the cortical dataset, three modules were positively correlated with TG samples ($p < 0.05$), and one module was negatively correlated with TG samples (Fig. 4a). In the hippocampal dataset, one module was significantly positively correlated with the TG samples (Fig. 4b). Lastly, analysis of serum dataset revealed one module that was significantly negatively correlated with TG genotype (Fig. 4c). The modules were then functionally annotated by performing pathway enrichment analysis of the member metabolites for each identified

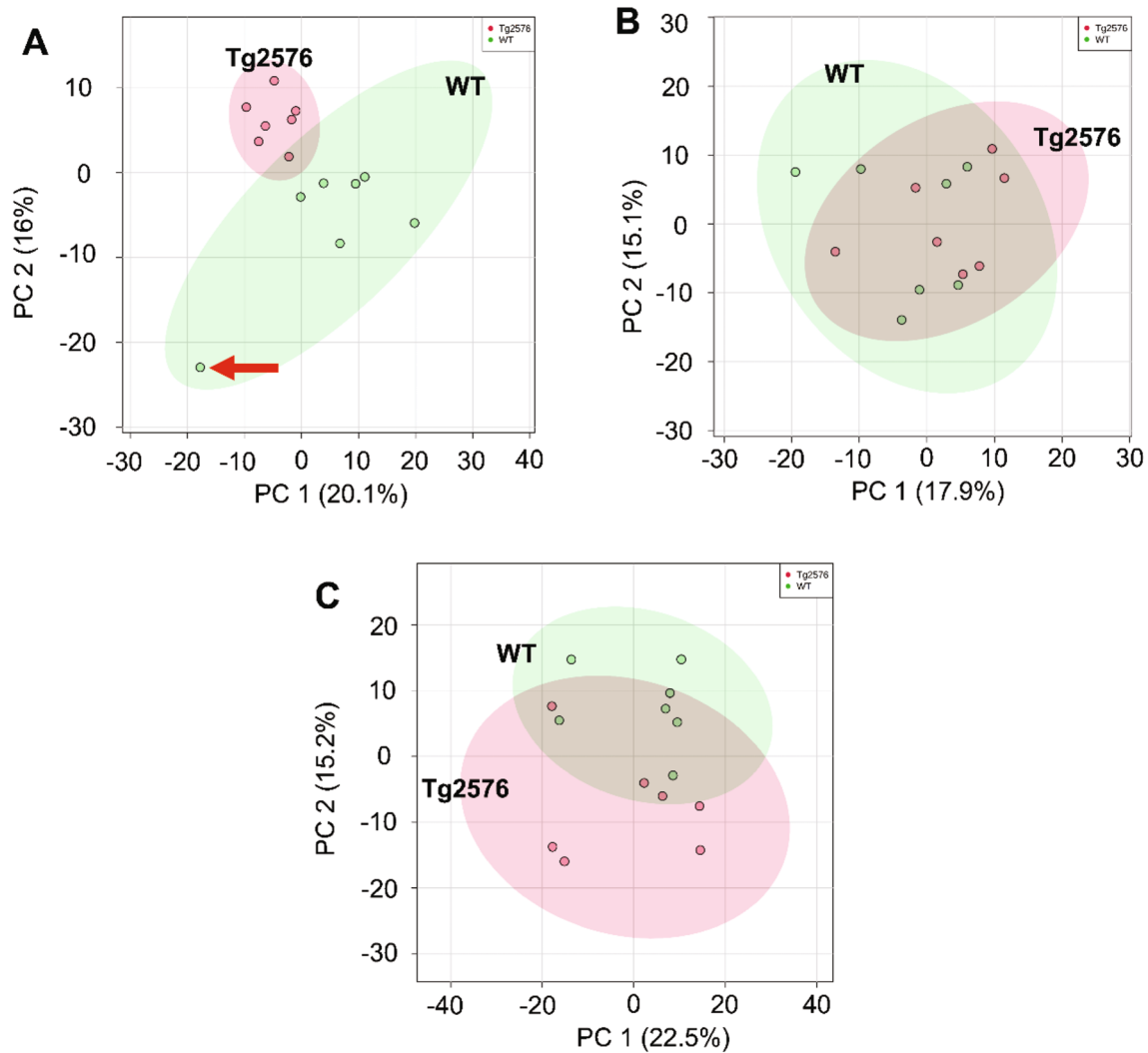


Fig. 1 Identification of the outlier using the principle component analysis (PCA) on the metabolomic data acquired from the 6-month-old Tg2576 and WT littermates. Principle component (PC) 1 and 2 were plotted against each other as these two components represent the

module. The functional enrichment analysis revealed significant enrichment in three pathways from the cortical dataset (Table 2), two pathways from the hippocampal dataset (Table 2) and one pathway “aminoacyl t-RNA biosynthesis” (FDR = 0.0449) in the serum dataset.

4 Discussion

Currently, AD can only be diagnosed with certainty post-mortem by staining the brain for A β plaques and neurofibrillary tangles (McKhann et al., 1984). Because of this, identifying early mechanisms and metabolic pathways that are associated with the pathogenesis of AD might enable patients to be diagnosed before substantial brain pathology

majority of the variances a PCA identified one WT sample to be an outlier from the cortex samples (red arrow). The metabolomic data collected from this mouse was removed from subsequent analysis. There were no outliers in the b hippocampus and the c serum samples

emerges, potentially facilitating therapeutic intervention. Here, 6-month-old Tg2576 mice were used, as this age represents the presymptomatic stage of the A β pathology in AD (Hsiao et al., 1996). Metabolomic analysis was the chosen method for this investigation due to its ability to provide global profiling of the metabolites, pathway enrichment and network correlation between disease and control samples (Fiehn, 2002).

We identified metabolites and metabolic pathways that were altered in Tg2576 mice compared with WT mice, each of which may be related to mechanisms associated with the APP pathology in AD. Firstly, we observed that metabolites which are linked to oxidative stress were significantly altered by mutant APP overexpression phenotype. The metabolite level with the most pronounced

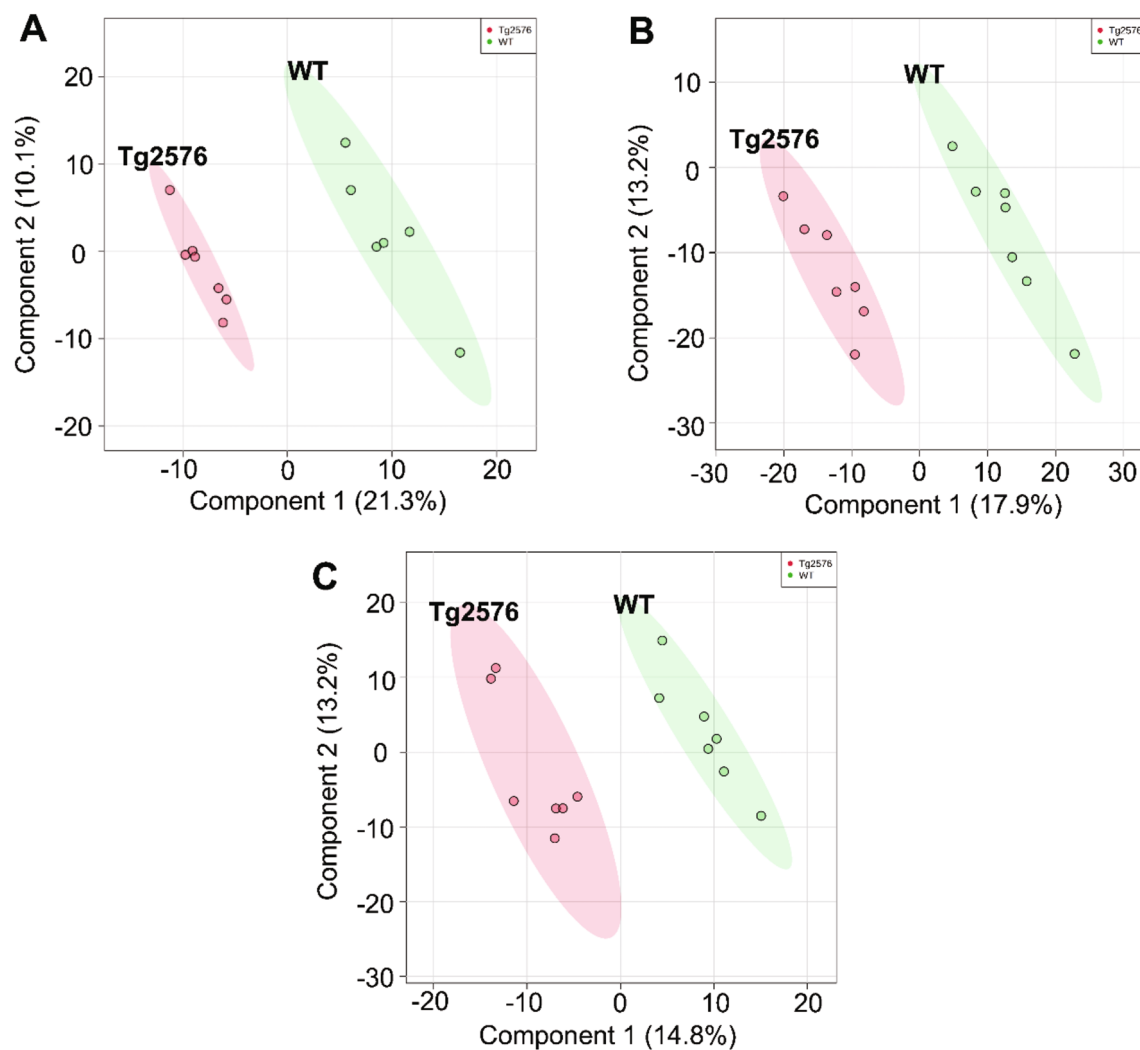


Fig. 2 Multivariate analysis using the partial least square-discriminant analysis (PLS-DA) on the peak intensity data. PLS-DA is a classification-based statistical model, which sharpens the separation between groups of observations. The PLS-DA scores plots show no

overlap between the data collected from **a** cortex; **b** hippocampus and **c** serum samples of Tg2576 and WT groups, suggesting that the metabolomic profiles of the samples from Tg2576 are distinguishable from WT and therefore further analysis may be performed

difference was the hydroxyphenyllactate (KEGG ID: C03672). Hydroxyphenyllactate is classified as a phenolic compound and has been shown to exhibit anti-oxidant effects by reducing production of mitochondrial reactive oxygen species (Beloborodova et al., 2012; RiceEvans et al., 1997). We found a significant decrease in the level of hydroxyphenyllactate in Tg2576 mice. Npi-methyl-L-histidine (KEGG ID: C01152), a derivative of L-histidine, was also decreased in the cortex of the Tg2576. This metabolite is directly linked to the catabolism of carnosine (Kanehisa & Goto, 2000), and carnosine has been reported to exhibit antioxidant effects (Kohen et al., 1988). This could indicate a decrease in the synthesis of carnosine from L-histidine due to an early metabolic dysfunction in AD (Cararo et al., 2015; Fonteh et al., 2007), which may lead

to an increase in oxidative stress. Finally, deoxyguanosine monophosphate (dGMP) (KEGG ID: C00362) was identified by the metabolomic analysis. Similar to hydroxyphenyllactate and npi-methyl-L-histidine, dGMP also has a role in oxidative stress (Henle et al., 1996). Furthermore, dGMP is also involved in purine metabolism and purine metabolism disorder has been reported in AD (Ansoleaga et al., 2015). It was found that the level of dGMP decreases as the AD stage progresses (Ansoleaga et al., 2015). Since Hydroxyphenyllactate, npi-methyl-L-histidine and dGMP are all associated with oxidative stress, this may indicate that the increase in oxidative stress in the brain is one of the earliest events that occurs in the presymptomatic stage of AD. The role of oxidative stress in AD is supported by the current literature (Lin & Beal, 2006; Nunomura et al.,

Table 1 The list of differentially expressed metabolites and the significantly enriched pathways between Tg2576 and WT in cortex samples

(A) Metabolite name	Average fold change	p value	FDR	VIP score	Associated pathway
3–4-Hydroxyphenyllactate	0.60	0.00025	0.045	1.97	Tyrosine metabolism
Phosphatidylethanolamine (44:3)	1.56	0.00048	0.045	1.92	Glycerophospholipids
Lysophosphatidylethanolamines (18:0)	1.30	0.00052	0.045	1.92	Glycerophospholipids
Phosphatidylserine (36:2)	1.25	0.00059	0.045	1.90	Glycerophospholipids
<i>O</i> -Propanoylcarnitine	0.71	0.00061	0.045	1.90	Oxidation of branched fatty acids
Npi-methyl-L-histidine	0.60	0.00066	0.045	1.90	Histidine metabolism
Phosphatidylserine (40:7)	1.28	0.00070	0.045	1.89	Glycerophospholipids
<i>O</i> -Butanoylcarnitine	0.65	0.00090	0.045	1.87	Fatty acyl carnitines
2'-Deoxyguanosine 5'-monophosphate	0.53	0.00097	0.045	1.87	Purine metabolism
Diglyceride (P-32:1)	1.91	0.0010	0.045	1.86	Diradylglycerols
Phosphatidylserine (44:12)	1.44	0.0011	0.045	1.86	Glycerophospholipids
(B) Metabolic pathway	p value		FDR		Pathway impact score
Glycerophospholipid metabolism	0.000757		0.0271		0.560
Glycerolipid metabolism	0.00125		0.0271		0.374
Glycine, serine & threonine metabolism	0.00233		0.0379		0.366
Pyrimidine metabolism	0.00431		0.0467		0.464

(A) A metabolite was identified as significantly different when its FDR-adjusted p -value < 0.05 and variable importance in projection (VIP) score > 1 . Average fold change was calculated to identify both the amplitude and the direction of the change in the metabolite levels in the Tg2576 group compared to the WT group. The metabolic pathway that is associated with each metabolite is also listed. (B) Metabolic pathways with an FDR-adjusted p -value < 0.05 and pathway impact score > 0.2 were identified as significantly enriched. This analysis identified four significantly enriched metabolic pathways from the data acquired from the cortex samples

2001) and therefore increase in oxidative stress in the brain can be one of the earliest indicators of AD.

In addition to oxidative stress, six significantly altered metabolites that are related to lipid metabolism were also identified: three from the phosphatidylserine (PS) (KEGG ID: C02737) group; two from the phosphatidylethanolamine (PE) (KEGG ID: C00350) group; and one from the diglyceride (KEGG ID: C00165) group. PS and PE metabolites are classified as phospholipids and are involved in many important metabolic pathways such as glycine, serine, threonine and glycerophospholipid metabolism (Kanehisa & Goto, 2000). In late-onset AD, changes in the level of PS metabolites have been reported (Sarrafpour et al., 2019; Tokuoka et al., 2019; Wells et al., 1995), however these changes have not been previously observed in the presymptomatic stage of AD. In addition to their roles in metabolic pathways, PS also play important roles in neural transduction, being involved in signalling protein interactions, membrane-bound receptor modulation and regulation of exocytosis (Kim et al., 2014). PS can also increase the affinity of AMPA receptor in rat brain membranes (Baudry et al., 1991) and therefore the significant increase in the levels of PS detected here might indicate an increase in the membrane hyperexcitability in the presymptomatic stage of AD.

Lastly, metabolites that are associated with energy production (Schooneman et al., 2013) including *o*-butanoylcarnitine (KEGG ID: C02862) and *o*-propanoylcarnitine (KEGG ID: C03017) were also identified here. These two metabolites are classified as acylcarnitine and changes in the level of acylcarnitines can be an indicator of metabolic disorders (Koeberl et al., 2003; Millington & Stevens, 2011). It has been documented that metabolic dysfunction is a prominent feature in the AD brain (Blonz, 2017) and disorders such as diabetes can be associated with AD (Craft, 2009).

Pathway enrichment analysis identified five pathways that were significantly enriched in AD compared to controls. The glycerolipid metabolism pathway and the glycerophospholipid metabolism pathways were both enriched in the cortex of Tg2576 mice. This may be relevant to the glycerolipid & glycerophospholipid metabolic disorder which has been observed in AD patients (Wood, 2012). The enrichment in glycerolipid and glycerophospholipid metabolism pathways might occur because these two classes of lipids are the major constituent of neuronal and mitochondria membranes and in AD, both neurodegeneration (Masters et al., 2015) and mitochondrial dysfunction are present (Lin & Beal, 2006). This finding complements well with the results from individual metabolites, as we identified six metabolites that are associated with lipid metabolism.

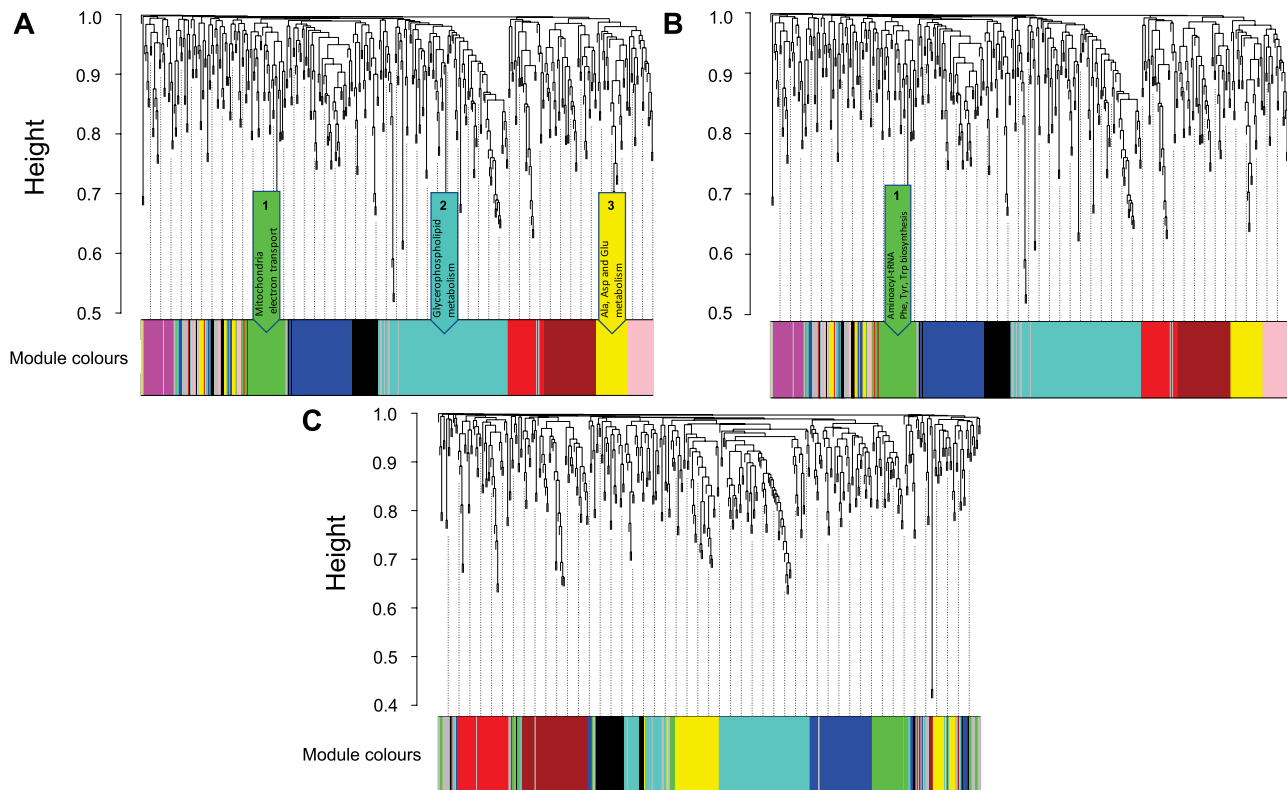


Fig. 3 Cluster dendrogram of all annotated metabolites identified in **a** cortex, **b** hippocampus and **c** serum of Tg2576. The metabolites were clustered into modules (colour coded) based on their abundance correlation across all samples in the dataset. The parameters of the

adjacency matrix and the module detection were set as follows: soft thresholding power=7; minimum module size=10; and type of network = signed

Another pathway that was highlighted by our analysis was the glycine, serine and threonine metabolism pathway. This pathway is involved in the energy production process in the brain as it can produce metabolic intermediates that can be directly utilised by glycolysis and the Krebs cycle (Kanehisa & Goto, 2000). AD has been linked to energy production deficiency (Blonz, 2017) and the results from our metabolomics analysis support the role of energy production deficits in the presymptomatic stage of AD. Furthermore, pyrimidine metabolism pathway was also identified as significantly enriched and recently, the literature has associated pyrimidine metabolism with AD (Muguruma et al., 2020). It has been demonstrated that AD pathology could alter pyrimidine metabolism (Pesini et al., 2019) and indeed, pyrimidine metabolism plays a big role in mitochondria (Wang, 2016). Therefore, the impairment in pyrimidine metabolism might indicate mitochondrial dysfunction in AD (Murphy & Hartley, 2018).

In the hippocampus samples, sulphur metabolism pathway was identified as significantly enriched. This discovery is supported by the current literature, which demonstrated that the disruption in sulphur metabolism is linked to AD (Griffin & Bradshaw, 2017; Townsend et al., 2004). It has

been reported that the levels of sulphur-containing amino acids such as cysteine were increased in the hippocampal region of AD patients (Xu et al., 2016). Additionally, the increase in plasma homocysteine have been associated with AD (Townsend et al., 2004) and the rise in plasma homocysteine level may precede AD symptoms (Seshadri, 2006). Here it can be seen that the results from the cortex and hippocampus are different. This is possibly because at 6-month-old, the cortex of Tg2576 is more affected by APP overexpression than the hippocampus.

In agreement with the results from metabolite analysis and pathway enrichment analysis, WGCNA identified mitochondrial dysfunction and glycerophospholipid metabolism to be positively correlated with AD genotype (cortex, green and turquoise modules, Table 2). Mitochondrial electron transport chain is important for the production of adenosine triphosphate (ATP) and reactive oxygen species (ROS) (Zhao et al., 2019) and therefore, dysfunction in this pathway could indicate energy deficit and overproduction of ROS. Moreover, early APP pathology is associated with altered lipid metabolism as evidenced in this study. Metabolites associated with glycerophospholipid metabolism pathway were significantly different in Tg2576 mice compared

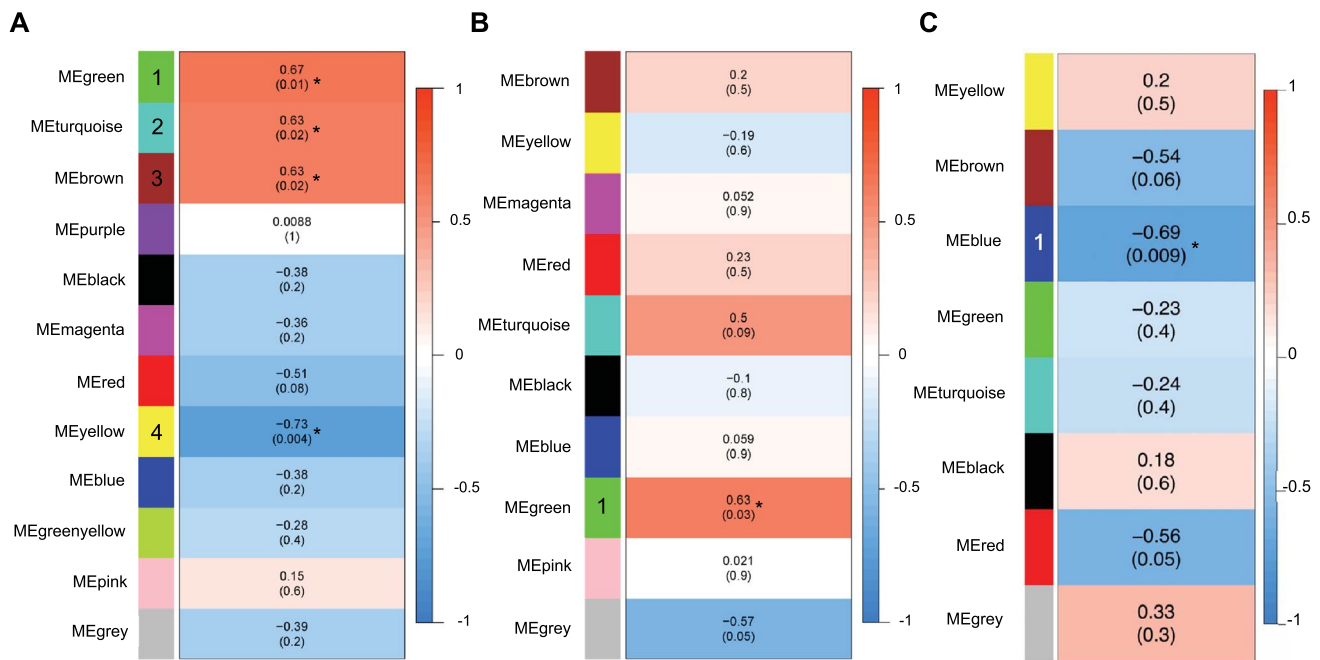


Fig. 4 The relationship between sample traits and modules in **a** cortex, **b** hippocampus and **c** serum datasets. The number in each module represent the positive or negative Pearson correlation, with the associated *p*-value in brackets, between the module eigengene (ME)

and Tg2576 samples. The green, turquoise, brown and yellow modules in the cortex, the green module in hippocampus and the blue module in the serum were significantly correlated with the AD genotype

Table 2 The metabolic pathways in the (A) cortex and the (B) hippocampus that are significantly correlated to the AD genotype as calculated by WGCNA

Module colour	Metabolic pathway	p value	FDR
(A)			
Green	Mitochondria electron transport chain	0.00042	0.041
Turquoise	Glycerophospholipid metabolism	0.00044	0.037
Yellow	Alanine, aspartate and glutamate metabolism	0.000063	0.0053
(B)			
Green	Aminoacyl-tRNA biosynthesis	0.00022	0.019
Green	Phenylalanine, tyrosine and tryptophan biosynthesis	0.00089	0.037

The significance threshold was set to FDR < 0.05

to WT. Additionally, at the metabolic pathway level, glycerophospholipid and glycerolipid pathways were identified as significantly enriched.

Alanine, Aspartate and Glutamate metabolism (cortex, yellow module, Table 2) has also been identified to be negatively correlated with AD genotype. While alanine and aspartate are very important in the CNS, there is no clear evidence of their association with the pathogenesis of AD (Ellison et al., 1986; Kori et al., 2016). On the other hand, changes in glutamate levels are known to be highly associated with AD, motivating the use of memantine as a therapeutic option, which targets the glutamatergic pathway (McShane, et al., 2019). This finding is very important because it implies that the change in the level of glutamate in AD may happen during the presymptomatic stage of AD.

Therefore, pathways that regulate glutamate homeostasis in the brain, such as the glutamate-glutamine cycle (Danbolt, 2001), may have the potential to be an early pharmacological target for the treatment of AD. The current literature would suggest that astrocytic glutamate transporters such as glutamate transporter 1 might be such candidate for drug target (Fontana, 2015).

Finally, aminoacyl-transfer RNA biosynthesis and phenylalanine, tyrosine and tryptophan biosynthesis were identified as significantly positively correlated with AD samples (hippocampus, green module, Table 2). Changes in the level of aminoacyl-transfer RNA has been associated with AD and mild cognitive impairment (MCI) in the literature (Ding et al., 2005; Xu et al., 2019). Similarly, changes in the levels of phenylalanine, tyrosine

and tryptophan biosynthesis have also been reported in AD (Griffin and Bradshaw, 2017). These results suggest that pathways involving amino acid metabolism could be affected early in the APP pathology. The functional annotation of the modules identified by WGCNA as well as pathway enrichment analysis was limited by the small size of metabolite databases and ambiguities in identifiers/aliases for particular isomers of a given metabolite as well as the small sample size of this study.

5 Conclusion

We have identified several metabolites that are linked to lipid metabolism and oxidative stress which are affected early in disease development in the Tg2576 mouse model of AD which exhibits mutant human APP overexpression pathology. Additionally, we report here that pathways such as Alanine, Aspartate and Glutamate metabolism are associated with AD and these findings can be the future basis of research in this area. However, we found no significant change in the metabolomic profile of the serum samples. This might mean that any metabolite change in the serum may not be detectable in the early stage of APP pathology. The results from this investigation have identified potential targets for future research focusing on metabolic disturbances. This might lead to new approaches to diagnose and treat patients in the presymptomatic stage of AD. It also allowed for new hypotheses on the mechanisms and pathways involved in the pathogenesis of AD to be generated and provided more insights into the current hypotheses.

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Authors' contributions HD conceptualized and designed the study, prepared the material and collected the data. Analysis were performed by HD and AH. The first draft of the manuscript was written by HD and all authors commented on previous versions of the manuscript. PK and NJ provided the resources, supervision and funding acquisition. All authors read and approved the final manuscript.

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Availability of data and materials The metabolomics and metadata reported in this paper are available via Metabolights (www.ebi.ac.uk/metabolights/MTBL.S2280) study identifier MTBL.S2280 (Haug et al., 2020).

Declarations

Conflict of interest The authors have no conflict of interest—either financial or otherwise—associated with this research.

Research involving human and/or animal participants This article does not contain any studies involving human participants performed by any of the authors. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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