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## Distinctive RNA Expression Profiles in Blood Associated with Alzheimer's Disease after Accounting for White Matter Hyperintensities

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### Abstract

**Background**—Defining the RNA transcriptome in Alzheimer's Disease (AD) will help understand disease mechanisms and provide biomarkers. Though the AD blood transcriptome has been studied, effects of white matter hyperintensities (WMH) were not considered. This study investigated the AD blood transcriptome and accounted for WMH.

**Methods**—RNA from whole blood was processed on whole-genome microarrays.

**Results**—293 probe sets were differentially expressed in AD versus controls, 5 of which were significant for WMH status. The 288 AD-specific probe-sets classified subjects with 87.5% sensitivity and 90.5% specificity. They represented 188 genes of which 29 have been reported in prior AD blood and 89 in AD brain studies. Regulated blood genes included MMP9, MME (Nepriylisin), TGF $\beta$ 1, CA4, OCLN, ATM, TGM3, IGFR2, NOV, RNF213, BMX, LRRN1, CAMK2G, INSR, CTSD, SORCS1, SORL1 and TANC2.

**Conclusions**—RNA expression is altered in AD blood irrespective of WMH status. Some genes are shared with AD brain.

### Keywords

Alzheimer's disease; RNA; white matter hyperintensities; blood; brain

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#These authors contributed equally to this study.

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## 1. Introduction

The diagnosis of Alzheimer's disease (AD) is based mainly on clinical criteria. Though research guidelines recommend confirmation using PET imaging or CSF biomarkers, high cost and low patient compliance make introducing these in the typical clinical setting challenging. Peripheral blood is accessible, and provides information on systemic factors involved in disease. It has been suggested that blood-based biomarkers could be useful for evaluating diagnosis, pathogenesis and progression of AD<sup>1-5</sup>.

Clinical dementia phenotypes, however, are complex syndromes manifested by various diseases potentially leading to both false positive and negative associations. For example, cerebrovascular disease (CVD) and white matter hyperintensities (WMH) commonly co-occur with AD, and contribute to the dementia syndrome. In fact, mixed AD and CVD pathologies may be the most common presentation of the AD phenotype in community populations. Further understanding of the genetic underpinning of AD, therefore, should account for CVD and WMH. This approach has been used to understand the effects of SORL1 on dementia risk<sup>6</sup>.

White matter hyperintensities (WMH) are commonly found on brain MRI T2-weighted and fluid-attenuated inversion recovery images and are associated with advancing age, vascular risk factors, stroke risk, incident cognitive impairment and incident dementia<sup>7</sup>.

Neuropathological and amyloid PET imaging studies show that WMH are not correlated with AD pathology, but associated with cerebral arteriosclerosis, supporting the relationship between WMH and CVD<sup>7</sup>. Our previous study suggested that WMH found in normal aging and in AD patients are associated with distinctive RNA expression profiles in peripheral blood that are not secondary to AD and may be related in part to oxidative stress<sup>8</sup>.

RNA expression in blood of patients with AD has been assessed in several recent independent studies<sup>1-4, 9, 10</sup>. However, none has considered the possible effects of WMH on RNA expression in blood as a confounder for AD gene profiles<sup>8</sup>. The aim of this study was to examine RNA expression in blood of AD patients with and without WMH as compared to controls. We determined that 188 AD genes were not affected by WMH, and of these 29 had been previously reported in AD blood and 89 had previously been reported in AD brain.

## 2. Methods

Subjects (n=17 AD (9 WMH<sup>+</sup>), and n=21 non-AD controls (11 WMH<sup>+</sup>)) were recruited from the Alzheimer's disease (AD) Center at University of California at Davis (UCD). The UCD institutional review board approved this study.

AD diagnosis was made according to the NINCDS and Communication Disorders and Stroke/AD and Related Disorders Association (NINDS-ADRDA) criteria. AD with and without WMH, as well as controls (cognitively normal subjects with and without WMH) were included in the present study<sup>8</sup>.

Whole blood was collected from each subject and mRNA hybridized on Affymetrix Human U133 Plus 2.0 Arrays. A multivariate ANCOVA was performed on AD status with

adjustments for potential confounders, including batch, sex, age, heart disease, hyperlipidemia, education and presence of WMH. Differentially expressed genes (DEGs) were identified based on significance of  $p < 0.005$  and absolute fold-change ( $|FC|$ ) expression  $> 1.2$ . The results of the gene sets enrichment analysis (GSEA) and functional enrichment analysis were filtered for FDR  $p$ -value  $< 0.05$ . Prediction analysis was performed as described <sup>8</sup>.

Microarray datasets for AD blood transcriptome studies were downloaded from Gene Expression Omnibus (GEO). The major datasets re-analyzed in this study included GSE6613 <sup>10</sup> and GSE4229 <sup>4</sup> from GEO. In addition, DEG lists were retrieved directly from published papers <sup>1-3</sup>. We also conducted a re-analysis of our independent blood microarray study in a Chinese cohort <sup>5</sup>. For the re-analysis, DEGs were identified using the method of RankProd ( $|FC| > 1.5$ ,  $FDR < 0.05$ ). Detailed methods are presented in Supplementary Material 1.

### 3. Results

#### 3.1 Demographics

Demographic information for AD and control subjects with and without WMH is provided in Table 1 and Supplementary Table 1A. A closely matched proportion of the subjects in the AD and non-AD (control) groups had coexisting WMH. To evaluate the influence of each possible confounder on the RNA expression profile, we conducted a one-way ANOVA on the demographic information (Table 1). There were no significant differences in age, gender, race, years of education, history of hyperlipidemia, or heart disease between those with and without AD. There was a significant difference in history of diabetes (Table 1).

#### 3.2 Distinctive RNA expression profile in the blood for AD

ANCOVA was performed for AD versus non-AD to control for expression changes caused by potential confounders, including batch, gender, age, heart disease, hyperlipidemia, diabetes and WMH, since they are known risk factors for developing AD/WMH, were different between the groups and/or because could affect gene expression. The ANCOVA showed 293 differentially expressed probe sets between the AD and non-AD subjects. Among them, 5 genes were also regulated by WMH <sup>8</sup>. To avoid confounding effects of WMH, these 5 genes were removed from the gene list, which resulted in a DEG list of 288 probe sets (188 annotated genes) exclusively regulated by AD (Supplementary Table 1B). These 288 probe sets were used for AD classification analyses described below.

#### 3.3 Discrimination of AD vs controls regardless of the WMH status

Three different approaches were used to determine how well the 288 probe sets could differentiate AD vs non-AD subjects. We conducted a Principal Components Analysis (PCA) on all the subjects regardless of the coexisting WMH using the 288 probe sets. AD subjects were separated from non-AD subjects, the AD with and without WMH forming a separate centroid from non-AD with and without WMH (Figure 1). The three principal components accounted for 60% of the total variance. To further evaluate the 288 probe set classifier, a non-supervised cluster analysis was performed (Figure 2). The AD and non-AD

subjects generally cluster separately, and do not cluster based upon WMH status (Figure 2). Prediction Analysis of Microarray (PAM) was also performed using 10-fold leave-one-out cross-validation in the derivation cohort, and showed sensitivity for AD diagnosis of 87.5% and specificity of 90.5% (Supplementary Figure 1).

### 3.4 Genes with most significantly altered gene expression levels

Among the 288 DEGs between AD and controls, the largest FCs were for TGM3 (transglutaminase 3, FC=2.18), NOV (nephroblastoma overexpressed, FC=1.96), BMX (BMX non-receptor tyrosine kinase, FC=1.93), CA4 (carbonic anhydrase IV, FC=1.88), MMP9 (matrix metalloproteinase 9, FC=1.82), LRRN1 (leucine rich repeat neuronal 1, FC=1.77), INSC (inscrutable homolog, FC=1.74), and OCLN (occludin, FC=-1.74).

### 3.5 Gene functions/ pathways

Functional enrichment analysis of the 188 genes showed up-regulation of membrane-bound vesicle pathways, endocytosis, cytoskeleton, actomyosin, apoptosis, and protein kinase cascades; and down-regulation of transcription (Supplementary Table 1C). Canonical pathway analysis using the Ingenuity database showed cell cycle, nervous system signaling, hypoxia - angiogenesis, inflammatory signaling and cancer signaling were over represented (Supplementary Table 1D).

### 3.6 Comparison with blood and brain transcriptome from other AD studies

The comparative analysis of AD transcriptome was based on datasets from five published studies and our own independent microarray experiment on a Chinese cohort<sup>5</sup>. We also conducted another analysis of the data from this study using the method of rank product and a stringent threshold of  $|FC| > 1.5$  (FDR < 0.05). In total, we found 44 genes regulated in AD blood in this study that had been either reported in previous studies of AD blood, or were confirmed using our separate analytical method (Supplementary Table 1E). Of these 44 genes, 29 have been reported to be regulated in blood of AD patients in previous studies (Supplementary Table 1E)<sup>1-5, 9, 10</sup>. In addition, of these 29 genes regulated in blood in this and previous studies, 15 have been reported to be regulated in AD brain (Table 2).

Among the 15 genes, most were regulated similarly in blood and brain (e.g. TGFB1, NDE1 and HIST1H4C), while others showed discordant regulation in blood and brain (e.g. ATP6V0D10) - that is being up regulated in one tissue and down regulated in the other. In addition, of the 15 genes regulated in AD blood and brain, MME, MMP9, CAMK2G and TGFB1 were identified as AlzGene (reported in previous genetic studies).

### 3.7 Cellular Functions of the 44 overlapping genes

In order to summarize the 44 genes that have been replicated from previous AD studies and/or in from both our analytical approaches, we manually constructed a pathway map based on information from Entrez Gene (Figure 3). Genes involved in survival/death signaling were mainly up-regulated. ZFP106 and NOV participate in insulin signaling, MAP4K5 in insulin and TNF receptor signaling, and TGFB1 is upstream of the TGFB signaling pathway. TBKBP1 participates in TNF/NF $\kappa$ b signaling, ATM responds to DNA damage, LRRN1 is involved in cell proliferation, and INSC is involved in cell division and

nervous system development. HIVEP3 is involved in apoptosis, PDCL3 modulates caspase activation, and RAF1 is a MAP3K kinase involved in apoptosis, and regulation of actin cytoskeleton. PDLIM7, FGD3 and NDE1 regulate the cytoskeleton (Figure 3).

Several genes related to immune response and catabolism were up-regulated. MX2 is up-regulated by interferon-alpha, SFTPD is involved in phagosome function, ATG16L2 participates in autophagy, ATP6V0D1 is a lysosomal proton transporter, KIF13A is involved in endosome to lysosome transport and IGF2R is involved insulin response and in trafficking of lysosomal enzymes. MME interacts with and degrades amyloid beta, MMP9 degrades extracellular matrix at the blood brain barrier and is also involved in A $\beta$  degradation. TGM3 has cross-linking activity which may be involved in the aggregation of A $\beta$  and tau (Figure 3).

Many of the down-regulated genes were linked to transcriptional regulation. SMARCA2 is involved in transcriptional activation, CNOT1 is involved in RNA degradation, DENND4A regulates the transcription factor c-myc, UBN1 is involved in transcriptional repression, and RBM34 is a RNA binding protein. Sec62 is involved in post-translational protein translocation and ABCB10 is a mitochondrion-associated membrane transporter. OCLN is part of vascular endothelial cell tight junctions (Figure 3).

### 3.8 Genes with altered expression in AD blood and in AD brain studies

In our previous studies we conducted comprehensive analyses of the AD brain transcriptome<sup>11, 12</sup>. For the present analyses we included all of the published studies on AD brain, as well as genes recorded in the AlzGene database for GWAS findings in AD. When comparing our DEG list of 288 probe sets (188 genes) with these datasets, we found that 89 genes regulated in blood in this study were altered in brain transcriptome and/or identified as AlzGene (Supplementary Table 1F). This is a high percentage considering that the 288 genes/probes represented only 188 unique genes with clear annotation. Among the 89, some genes of interest were IL6R, IL17R, ANKRD13D, SORCS1, PFDN2, PREP, HES1, CRISPLD2, MYH11, SYMPK, and several genes were identified as AlzGenes, including: SORCS1, SORL1, INSR, CTSD and TANC2

## 4. Discussion

This is the first study to identify differences of RNA expression in blood of Alzheimer's disease (AD) patients compared to controls that considers the possible confounding effects of WMH. Though there are only a few overlapping genes between the two entities, it is important to consider co-morbid conditions when searching for AD biomarkers. There were several remarkable findings. First, of the 188 genes regulated in AD blood in this study, 89 have been reported to be altered RNA expression in AD brain or have been confirmed to have increased risk for clinical AD based on genetic studies. Second, of the 188 genes regulated in AD blood in this study 44 genes were reported in previous AD blood studies or were replicated using an independent analytical method; and, 15 of these were previously reported to have altered expression in AD brain. Of the genes regulated in AD blood a number have been implicated in AD pathogenesis including MMP9, MME (Nepriylsin) and Transforming growth factor 1, and several have been reported in previous genetic

association studies including: MMP9, MME, TGF $\beta$ 1, CAMK2G, SORCS1, SORL1, INSR, CTSD and TANC2.

### Significance of regulated RNAs in AD blood

The majority of AD biomarker studies have examined proteins from CSF as a direct expression of brain pathology. Studies of peripheral blood have been more difficult to replicate. In this study RNA from whole blood was examined, an important difference from most previous studies. The RNA is almost all intracellular, and thus represents expression of RNA in blood leukocytes and immature platelets and red blood cells. Thus, the data reported represents changes mostly in immune cells in AD patients, and supports previous reports of changes in the immune system in AD. The following discussion will focus mainly on a few molecules that have been reported in previous AD studies.

**MMP9 (Metalloproteinase 9)** participates in NGF-induced  $\alpha$ -secretase cleavage of A $\beta$ PP. Thus, it contributes to the shift of A $\beta$ PP processing towards the non-amyloidogenic pathway precluding the formation of neurotoxic A $\beta$  peptides. The apolipoprotein E4 fragment affects matrix metalloproteinase 9 in brain cell lines and estrogen activates MMP9 to increase beta amyloid degradation. MMP9 not only degrades A $\beta$  but also affects cytoskeleton organization, and also plays a key role in opening the blood brain barrier following cerebral ischemia and other acute injuries to brain, and MMP9 RNA expression increases in blood leukocytes following ischemic stroke in humans. MMP9 levels are reported to correlate with brain amyloid burden, though no differences in activity in blood were reported in AD<sup>13</sup>. MMP9 levels are elevated in CSF of AD patients and help differentiate AD from other diseases<sup>14</sup> and MMP9 protein and enzymatic activity are increased in AD brain. Variable blood findings in the literature may relate in part to whether levels of pre-mRNA, mRNA, pre-protein, active protein or MMP9 enzymatic activity were measured.

**MME, membrane metallo-endopeptidase (Nepilysin, NEP)**, is a zinc metallopeptidase which is a major A $\beta$ -degrading peptide. Genetic variations in the gene have been associated with increased AD risk in some but not all studies. Though systemic neprilysin can decrease peripheral beta amyloid levels, this has been reported to decrease brain A $\beta$  in some studies<sup>15</sup> and not in others<sup>16</sup>. The levels of the A $\beta$ -degrading enzyme neprilysin are reduced in human dementia pugilistica cases. Though MME/neprilysin is high in blood in this and other studies, levels of NEP and NEP2 have been reported to be lower, higher or unchanged in AD brain<sup>17</sup>. Notably, Neprilysin protects against cerebral amyloid angiopathy and A $\beta$ -induced degeneration of cerebrovascular smooth muscle cells<sup>18</sup>. In addition, peripheral delivery of a CNS targeted neprilysin reduces A $\beta$  toxicity in one mouse model of Alzheimer's disease<sup>19</sup> and decreases plaque formation but not memory deficits in another mouse model<sup>20</sup>. Another key role of NEP in amyloid processing is emphasized by the fact that the intracellular domain of the amyloid precursor protein binds to the NEP promoter and induces its expression and NEP degrades both beta amyloid 42 and 40<sup>21</sup>. NEP is expressed in amyloid plaques, dystrophic neurites and astrocytes but not microglia of sporadic AD but not familial AD cases<sup>22</sup>.



**TGF**—Transforming growth factor signaling has been implicated in AD. TGF stimulates A $\beta$  uptake into microglia via SMAD 3 and decreases amyloid plaque burden in AD mouse models<sup>23</sup>. The CC genotype of TGFB1 may increase the risk of late-onset AD<sup>24</sup>. TGFB1 levels are altered in human AD brain<sup>25</sup> and are increased in brain of transgenic AD mouse models<sup>26</sup>. Levels of TGFB1 have been reported to be altered in AD blood but may relate to stage of the disease<sup>27</sup>. Elevated CSF levels of TGFB1 have also been reported in AD<sup>28</sup>. Amyloid-beta peptide suppresses TGFB1-induced MMP2 production via Smad 7, and TGFB1 potentiates amyloid-beta generation in astrocytes and transgenic mice. Moreover, TGFB1 over expressing mice develop an amyloid angiopathy similar to that seen in AD<sup>29</sup>.

**RNF213** has E3 ligase and AAA+ATPase activities and genetic variants (SNPs) in this gene have a strong association with Moya Moya disease. This is of interest since vascular abnormalities can precede development of amyloid plaques and behavioral deficits in AD patients and AD animal models<sup>30</sup>. Knockdown of RNF213 in zebrafish causes irregular wall formation in large arteries and abnormal sprouting of vessels.

**Occludin (OCLN)** is highly down regulated in blood cells in this study. It is a key protein that forms part of the tight junctions between brain endothelial cells that control blood-brain barrier (BBB) function. Aging and high cholesterol decrease BBB function and OCLN levels in normal mouse brain. A $\beta$ <sub>1-42</sub> decreases OCLN expression in brain endothelial cells<sup>31</sup>, though there is no direct evidence for changes of tight junction proteins in AD brain<sup>32</sup>.

The **Ataxia Telangiectasia mutated** gene (**ATM**) is involved in controlling the cell cycle, and aberrant entry of neurons into the cell cycle has been one mechanism proposed for neuronal cell death in AD brain<sup>33</sup>. A $\beta$ <sub>1-42</sub> and DNA-damaging drugs increase ATBF1 levels in cultured neurons which activates ATM signaling responsible for neuronal death through the binding of ATBF1 to phosphorylated ATM<sup>33</sup>.

**Transglutaminase 3 (TGM3)** was up regulated over two fold in AD blood in this study. Transglutaminases (TGs) are Ca<sup>2+</sup>-dependent enzymes that catalyze modifications of glutaminy (Q) residues including a number of amine-bearing compounds, including lysyl (K) residues and polyamines in brain. TGMs have been implicated in the pathogenesis of AD, Huntington's and other neurodegenerative diseases. Both A $\beta$  and tau are substrates of TGM cross-linking activity which modulates the protein aggregation process in AD brains<sup>34</sup>.

**IGFR2 and BMX**—Insulin growth factor receptor 2 is altered in AD brain<sup>35</sup> and is significantly up regulated in blood in this study. Insulin signaling is reported to be altered in AD brain in general, and global decreases of brain blood flow and glucose metabolism precede symptoms in AD<sup>36</sup>. There is significant evidence for aberrant IGFR1 signaling in AD brain<sup>37</sup>. IGFR2 levels are specifically up regulated in neurons most vulnerable to forming amyloid plaques in mouse AD models<sup>38</sup>. BMX is also markedly increased in AD blood in this study (1.9 fold), and it interacts with IGFR pathways and is elevated following brain trauma. NOV and NAP4K5 are both regulated in AD blood in this study and they are also involved in insulin signaling (Figure 3).



Of the canonical pathways significantly regulated in AD blood, it was notable that decreased regulation of transcription was among them. This would appear to be parsimonious with the decreases of blood flow and glucose metabolism that occur in AD brain.

**CA4**—Carbonic anhydrase 4 plays a critical role in pH regulation and long-term synaptic transformation, and is associated with mental retardation, AD and Down syndrome. CA4 is highly associated with brain blood vessels and not other vascular beds and in brain is the major astrocyte carbonic anhydrase. Carbonic anhydrase 4 is also one of the major RNAs that change expression in blood following ischemic stroke. Carbonic anhydrase II (CA2) is decreased in brain of a mouse AD model, is increased in human AD brain<sup>39</sup>, and carbonic anhydrase II is elevated in plasma of AD patients, particularly in males<sup>40</sup>. Any relationship between CA2 and CA4 is unknown.

**NOV/CCN3 (Nephroblastoma over expressed gene)**—Matricellular proteins of the CCN family (CYR61/CTGF/NOV) regulate and are regulated by cytokines including TGF (see above), which is also regulated in AD blood in this study. NOV also regulates extracellular matrix enzymes via integrins and proteoglycans, and can up regulate MMP9 – which also increases in AD blood in this study.

**ICAM-4** (intracellular adhesion molecule-4, Landsteiner-Wiener blood group) and **NSMAF** (Neutral sphingomyelinase (N-SMase) activation associated factor). ICAM-1 and sphingolipids have been proposed as blood-based biological marker candidates of microvascular pathology in AD, where plasma concentrations of ICAM-1 are elevated in AD and sphingolipids are significantly altered in mild AD or during the prodementia stage of mild cognitive impairment<sup>41</sup>. ICAM-4's function is not well understood. It has structural relatedness to ICAM-1, -2 and -R, and in our data is down-regulated, contrary to the ICAMs associated with AD. The neutral sphingomyelinase (N-SMase) activation associated factor (NSMAF) is up-regulated in our data. Sphingolipid metabolism has been associated with AD neuropathology, contributing to amyloid-beta production in AD<sup>42</sup>.

**ITGB3** (integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)). This gene showed up-regulation in AD in this study. It is a baseline platelet activation biomarker, where activated glycoprotein IIb-IIIa was shown to be significantly higher in patients with AD with fast vs slow cognitive decline during 1-year follow up<sup>43</sup>.

## 5. Conclusions

This is the first study to consider possible confounding effects of WMH on the AD blood transcriptome. A list of 288 probe sets representing 188 genes expressed in whole blood differentiates AD from controls using several analytical methods irrespective of WMH status. A number of the genes regulated in this study have been reported to be regulated in other studies of both AD blood and AD brain, and strongly support a systemic effect of AD that includes altered RNA expression of peripheral immune cells. Further validation in an independent cohort to specifically test the sensitivity and specificity of the identified genes to predict AD is needed.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

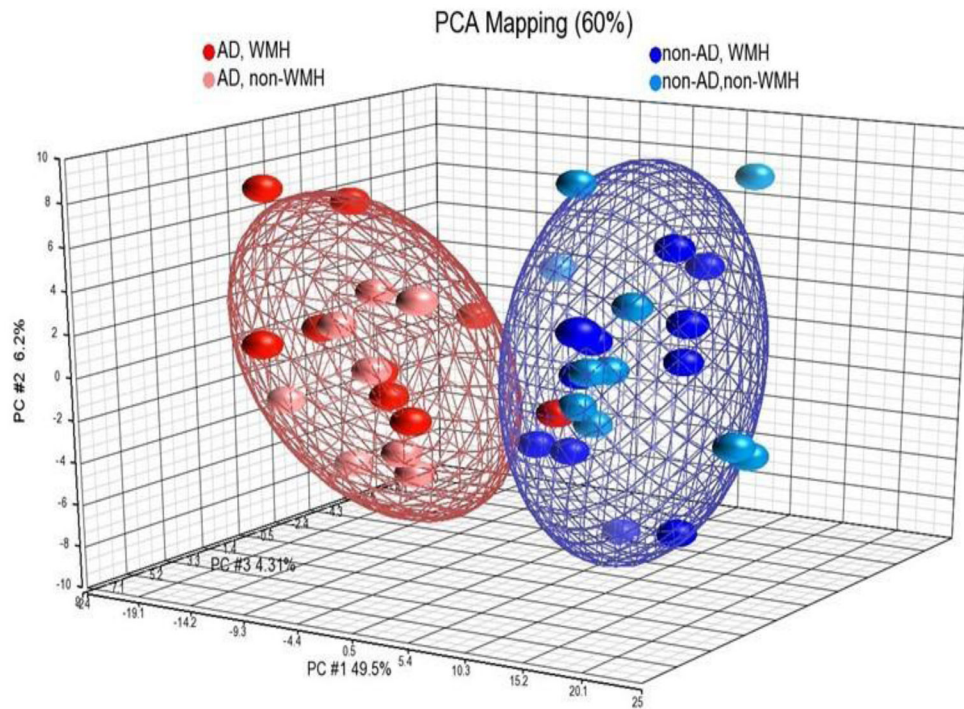
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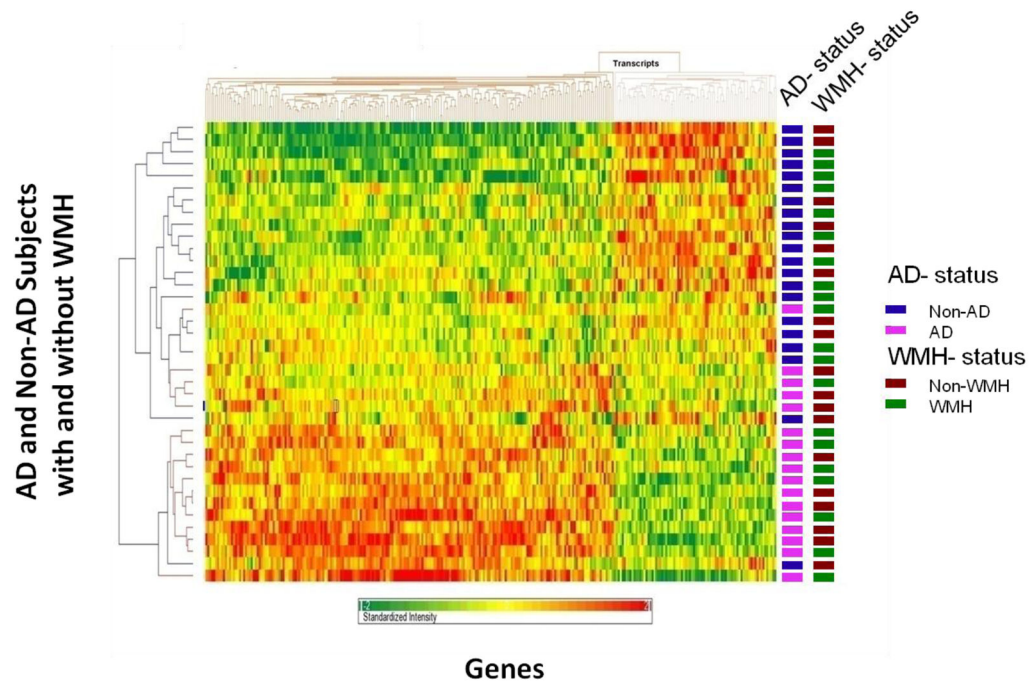
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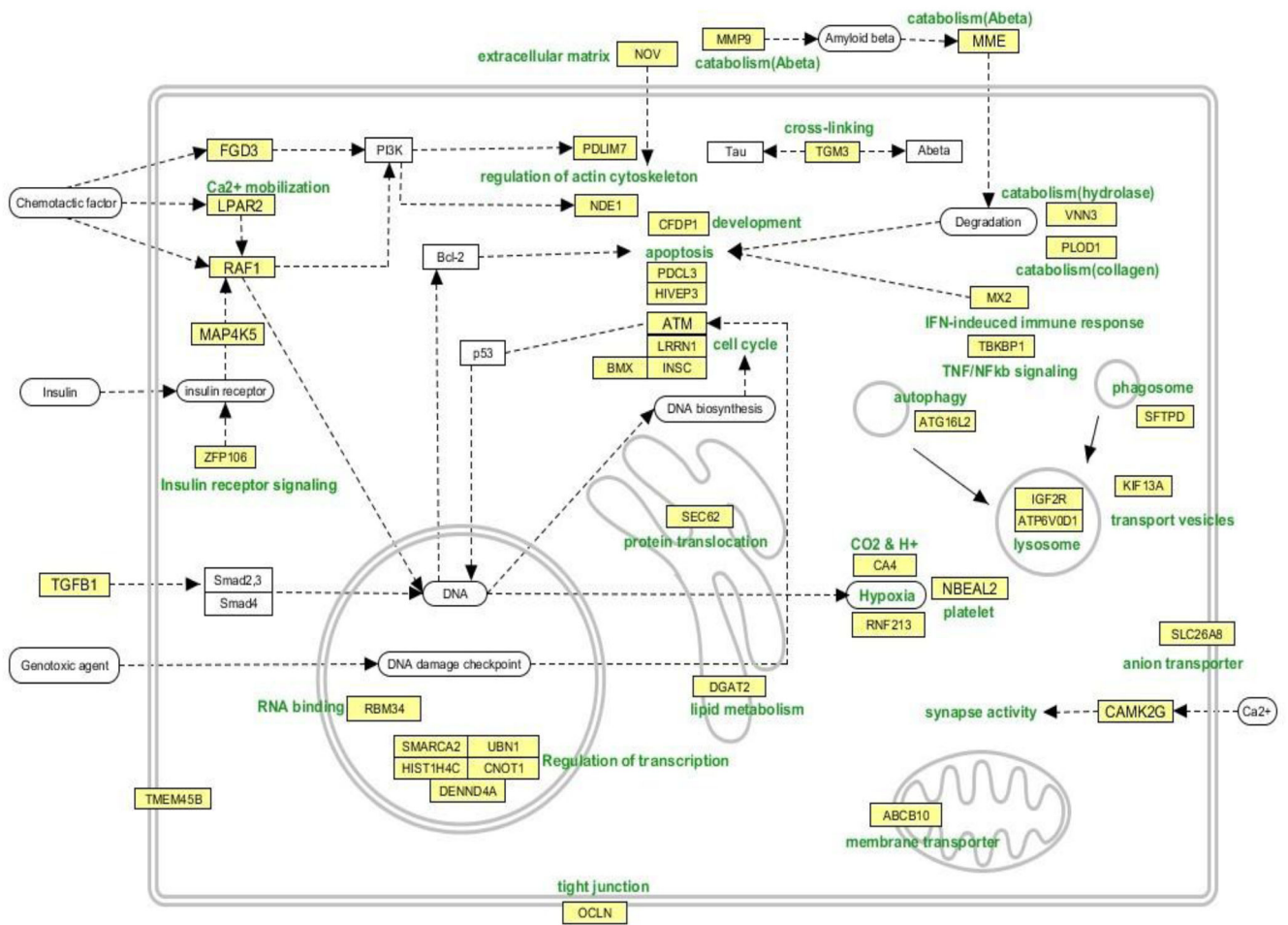
**Figure 1.** Principal Components Analysis (PCA) on the 288 probe sets that were differentially expressed between AD and control subjects (non-AD). AD subjects with WMH (AD, WMH – red dots) and AD without WMH (AD, non-WMH, pink dots) are included within the same centroids, and are separated from the centroids for control non-AD with WMH (non-AD, WMH – blue dots) and control non-AD without WMH (non-AD, non-WMH, light blue dots). The PCA accounts for 60% of the variance.





**Figure 2.**

Cluster analysis for the 288 probe sets that separated AD from control (Non-AD). Note that subjects with WMH (WMH) and without WMH (Non-WMH) do not cluster separately. Subjects are on the Y axis, and genes/probe sets are on the X axis. Dark red represents a 2 fold increase in expression and dark green a 2 fold decrease in expression compared to controls.



**Figure 3.** Cellular functions for the 44 genes listed in Supplementary Table 1D. These genes represent those that were regulated in AD blood in this study (ANCOVA,  $p < 0.005$ ,  $|FC| > 1.2$ ) and which were reported to be regulated in other AD blood studies, or were shown to be regulated in this study also using the rank product method and a  $|FC|$  threshold of  $> 1.5$ .