

Mitochondrial Dysfunction and Immune Activation are Detectable in Early Alzheimer's Disease Blood

Katie Lunnon^{a,b,1}, Zina Ibrahim^{a,b,1}, Petroula Proitsi^{a,b}, Anbarasu Lourdasamy^{a,b}, Stephen Newhouse^{a,b}, Martina Sattlecker^{a,b}, Simon Furney^{a,b,3}, Muzamil Saleem^{a,b}, Hilkkka Soininen^c, Iwona Kłoszewska^d, Patrizia Mecocci^e, Magda Tsolaki^f, Bruno Vellas^g, Giovanni Coppola^h, Daniel Geschwind^h, Andrew Simmons^{a,b}, Simon Lovestone^{a,b}, Richard Dobson^{a,b,2}, Angela Hodges^{a,b,2,*} and on Behalf of the AddNeuroMed Consortium

^a*Institute of Psychiatry, King's Health Partners Centre for Neurodegeneration Research, King's College London, London, UK*

^b*NIHR Biomedical Research Centre for Mental Health at South London and Maudsley NHS Foundation Trust and Institute of Psychiatry, King's College London, London, UK*

^c*Department of Neurology, University of Eastern Finland and Kuopio University Hospital, Kuopio, Finland*

^d*Medical University of Lodz, Lodz, Poland*

^e*Institute of Gerontology and Geriatrics, University of Perugia, Perugia, Italy*

^f*3rd Department of Neurology, "G. Papanicolaou" Hospital, Aristotle University of Thessaloniki, Thessaloniki, Greece*

^g*INSERM U 558, University of Toulouse, Toulouse, France*

^h*Department of Neurology, Programme in Neurogenetics, David Geffen School of Medicine, University of California at Los Angeles, CA, USA*

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Abstract. Alzheimer's disease (AD), like other dementias, is characterized by progressive neuronal loss and neuroinflammation in the brain. The peripheral leukocyte response occurring alongside these brain changes has not been extensively studied, but might inform therapeutic approaches and provide relevant disease biomarkers. Using microarrays, we assessed blood gene expression alterations occurring in people with AD and those with mild cognitive changes at increased risk of developing AD. Of the 2,908 differentially expressed probes identified between the three groups ($p < 0.01$), a quarter were altered in blood from mild cognitive impairment (MCI) and AD subjects, relative to controls, suggesting a peripheral response to pathology may occur very early. There was strong evidence for mitochondrial dysfunction with decreased expression of many of the respiratory complex I-V genes and subunits of the core mitochondrial ribosome complex. This mirrors changes previously observed in AD brain. A number of genes encoding cell adhesion molecules were increased, along with other immune-related genes. These changes are consistent with leukocyte activation and their increased the transition from circulation into the brain. In addition to expression

^{1,2}These authors contributed equally to the work.

³Present address: The Institute of Cancer Research, London, UK.

*Correspondence to: Angela Hodges, King's College London, Institute of Psychiatry, De Crespigny Park, London, UK. Tel.: +44 207 848 0772; Fax: +44 207 848 0632; E-mail: angela.k.hodges@kcl.ac.uk.

changes, we also found increased numbers of basophils in people with MCI and AD, and increased monocytes in people with an AD diagnosis. Taken together this study provides both an insight into the functional response of circulating leukocytes during neurodegeneration and also identifies potential targets such as the respiratory chain for designing and monitoring future therapeutic interventions using blood.

Keywords: Alzheimer's disease, blood, gene expression pattern analysis, inflammation, late onset, mild cognitive impairment, mitochondria

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INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia. It is characterized by slow progressive short term memory loss and the emergence of behavioral symptoms such as psychosis, agitation, apathy, and depression. Patients gradually lose their ability to maintain personal care and death usually occurs 10–15 years following diagnosis. A combination of an increasingly aging population and a current lack of disease-modifying treatments will lead to an overwhelmingly large number of people living with AD in the coming years with far reaching social and economic consequences [1]. Thus efforts to diagnose and treat disease early, before considerable damage has occurred, are a very high priority.

Probable AD is clinically diagnosed when robust symptoms emerge such as problems with memory, language, perceptual skills, attention, constructive abilities, orientation, problem solving, and functional abilities [2]. At present, diagnosis is only confirmed by postmortem histopathological assessment. Against this gold standard, clinical diagnostic accuracy can vary from fair to excellent (65–96%) [3–6], highlighting the reality of a disease which is clinically heterogeneous and similar to other disorders. Accurate in-life diagnosis requires a high threshold of disease. Subclinical disease has likely been present for decades before a formal AD diagnosis is made [7, 8]. The emergence of mild clinical symptoms such as memory complaints in individuals with intact cognition and normal daily living, which define Petersen's criteria of mild cognitive impairment (MCI) [9], may reflect the gradual and progressive appearance of plaques, neurofibrillary tangles, cellular dysfunction, and loss of specific neuronal cells which characterize AD. However, these symptoms can sometimes represent another neurological disease or be due to normal aging, as is the case for about 25% of people with MCI [10].

Plaques and neurofibrillary tangles are pathological hallmarks of AD and their generation is believed

to lead to the disruption of calcium homeostasis and neuronal synapses, loss of connectivity, increased reactive oxygen/nitrogen species, and altered plasticity in vulnerable brain regions, culminating in progressive neuronal cell death. Inappropriate neuroinflammation [11–13] and mitochondrial dysfunction [14–17] are consistent features of AD pathology in brain and are important mechanisms through which toxicity may be propagated.

Changes in AD are not just restricted to the brain. A number of genes have altered abundance in AD blood, some of which are sufficient to specifically and accurately classify disease [18–20]. Preliminary evidence suggests these changes may precede AD diagnosis [21] but this has yet to be extensively investigated in people with MCI. Mitochondria from peripheral blood cells such as platelets and lymphocytes are dysregulated [16, 22–24] and leukocytes from AD patients are far more responsive to activation signals [25]. Some AD blood cell vulnerabilities and disease-associated changes may be linked to changes in levels of amyloid- β (A β) peptides in plasma [26–29]. For example, when *ex vivo* blood cells from AD patients are treated with A β _{1–42}, there is a failure to upregulate MGAT3, a protein involved in A β clearance [30]. MGAT3 may be useful as a prognostic biomarker of disease deterioration [31]. Paralleling these alterations in blood cell activity, changes in circulating cytokines, chemokines, and other proteins, indicating activation of the peripheral innate immune system occur in people with MCI and AD [32–38].

A good strategy for further understanding disease and treatment development might be to identify important disease associated pathways in peripheral blood. Blood is an easily accessible tissue which can be used to assess changes occurring early in disease. Using microarrays and a unique collection of blood samples, with extensive demographic, clinical, and imaging data available, we have identified differentially expressed genes and altered biological processes in blood samples from people with MCI and AD. Changes in blood,

which we believe reflect the disease processes occurring in brain, could enable these cells to be used for future design and monitoring of treatments against disease-relevant biological processes.

METHODS AND MATERIALS

Subjects and samples

Blood samples for full blood cell count and mRNA analyses were taken from subjects participating in the AddNeuroMed study [39–41], involving six study sites across Europe (London, Kuopio, Lodz, Perugia, Thessaloniki, and Toulouse) or the London Dementia Case Register (London). All sample and data analyses were undertaken in London, with the exception of the microarrays, which were processed at the University of California in Los Angeles. Informed consent according to the Declaration of Helsinki (1991) and ethical approval were obtained at each study site, with all sites following an agreed standardized operating procedure for subject assessment and sample collection. Subjects were nominally assigned to an AD, MCI, or normal elderly control group as we have previously described [40, 42] and is summarized as follows. Cases with probable AD were identified from primary and secondary care services and diagnosed using the NINCDS-ADRDA criteria [2] and Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) [43]. MCI subjects were recruited from local memory clinics and as such the MCI cohort was expected to be composed largely of subjects with a likely AD-endpoint. All MCI subjects reported problems with memory, corroborated by an informant, but had normal activities of daily living as specified in the Petersen's criteria of amnesic MCI [44, 45] and scored 0.5 on the Total Clinical Dementia Rating Scale (CDR) or had a memory score of 0.5 or 1 [46]. Within 2 years of the baseline visit, some MCI subjects progressed to a clinical diagnosis of AD (MCI-AD), while others remained MCI (MCI-MCI). Normal elderly controls, defined as having no evidence of cognitive impairment, were recruited from primary care services and elsewhere.

Each subject underwent a semi-structured interview to collect demographic and medical information. They completed neuropsychological assessments including the Mini Mental State Examination [47], Global Deterioration Scale [48], and CDR [46]. Additionally, AD patients were assessed by the Alzheimer's Disease Assessment Scale-Cognitive subscale [49], the caregiver rated Neuropsychiatric Inventory scale [50], and

the caregiver rated Alzheimer's Disease Cooperative Study-Activities of Daily Living Inventory [51]. Control and MCI subjects were further assessed using the CERAD battery [52]. Subjects were excluded from the study if they were younger than 65 years, had significant neurological or psychiatric illness other than AD, significant systematic illness or organ failure, or a geriatric depression rating scale score $\geq 4/5$ [53]. Subject characteristics are summarized in Table 1.

Full blood cell count analysis

Approximately 3 ml of venous blood was collected in K₃EDTA vacutainer tubes (MidiMeds Limited). Full blood cell count analysis was performed at the Department of Haematological Medicine at King's College Hospital using an automated counter, according to standard clinical practice. Samples within the normal range $\pm 10\%$, were available from 125 normal elderly controls, 41 MCI subjects, and 73 AD subjects. Extreme values outside the normal range for any subject may represent an underlying episode of infection or peripheral inflammation and were therefore excluded from further analysis. Following log₁₀ transformation of the data, linear regression was carried out to assess the effect of disease status on individual leukocyte populations, adjusting for age, gender and other blood cell types. All statistical analyses were performed in SPSS or STATA.

Whole blood RNA extraction

Blood (~2.5 ml) was collected in PAXgene blood RNA vacutainer tubes (BD Diagnostics). The tube was inverted 8–10 times following blood collection, stored at -20°C for 24 h and then -80°C until RNA extraction. Where necessary, samples were shipped on dry ice to London prior to RNA extraction. RNA was extracted from all samples from their first visit and within two years of collection as follows. After thawing PAXgene tubes overnight at room temperature, total RNA was extracted using the PAXgene blood RNA kit (Qiagen), according to the manufacturer's protocol. The quality of the RNA was determined using the 2100 Bioanalyser (Agilent Technologies). The average RNA integrity number (RIN) across all samples was 8.8, standard deviation 0.7. Only samples with a RIN > 7.0 were used in the analysis.

Table 1
Subject characteristics of individuals used in Microarray and Blood cell count analyses

Microarray analysis	Control	MCI	MCI-AD	AD
Samples run on illumina BeadChips	116	83	44	113
Samples used in analysis ^a	105	78	42	104
Gender (M/F)	43/62	39/39	14/28	32/72
Age in years (\pm SD)	72.4 (6.4)	74.3 (5.6)	74.6 (6.8)	75.4 (6.7)
Disease duration in years (\pm SD)	n/a	n/a	n/a	3.6 (2.1)
MMSE (\pm SD)	29 (1.2)	27 (1.7)	26 (2.3)	21 (4.6)
ADAS-cog (\pm SD)	n/a	n/a	n/a	23 (10.0)
CDR sum of boxes (\pm SD)	0 (0.2)	1.3 (0.8)	2.1 (1.1)	6.5 (3.3)
Blood cell count analysis				
Samples with available blood cell count data	125	41	n/a	73
Gender (M/F)	45/80	24/17	n/a	32/41
Age in years (\pm SD)	77.7 (6.3)	79.0 (4.6)	n/a	80.3 (6.4)
Disease duration in years (\pm SD)	n/a	n/a	n/a	4.3 (3.8)
MMSE (\pm SD)	29.0 (1.2)	27.8 (1.5)	n/a	21.6 (5.4)
ADAS-Cog (\pm SD)	n/a	n/a	n/a	19.3 (9.4)
CDR sum of boxes (\pm SD)	0.2 (0.3)	1.3 (0.7)	n/a	6.3 (3.2)

^aMicroarray data from 1 AD and 1 Control were excluded from further analyses because of a discrepancy between XIST expression gender assignment and the original gender recorded for these participants; 20 samples were excluded due to an unacceptably low rate of detection on the BeadChip; 2 MCIs were removed from further analyses because one subsequently converted to a diagnosis of Frontotemporal Dementia, while the other case was subsequently diagnosed with a large meningioma. 1 AD was removed from further analyses due to a revoked diagnosis, and 1 AD was removed due to a low RNA Integrity Number (RIN). Control subjects who converted to an MCI diagnosis ($N=4$) and MCI subjects who converted to an AD diagnosis within 2 years post-sampling ($N=42$) were analysed as their status at sampling, unless indicated in the text; MMSE, Mini Mental State Examination; CDR, Clinical Dementia Rating scale; Alzheimer's Disease Assessment Scale-Cognitive subscale (ADAS-Cog).

Microarray expression analysis and pre-processing

A total of 356 (116 control, 127 MCI, and 113 AD) RNA samples (Table 1) were processed on Illumina Human HT-12 v3 Expression BeadChips (Illumina) according to the protocol supplied by the manufacturer. These chips contain 48,803 probes designed using data from RefSeq (Build 36.2, Rel 22) and the UniGene (Build 199) databases. cDNA was synthesized using 200 ng total RNA followed by amplification and biotinylation of cRNA and hybridization according to the protocol supplied with the Illumina Total-Prep RNA Amplification Kit (Ambion). Following hybridization, gene expression values were obtained using the Lumi package within Bioconductor which involves variance stabilization and quantile normalization [54]. Outlier BeadChips with very low detection rate ($<80\%$), discrepancy in *XIST* gene expression and recorded gender or serious co-morbid disease which came to light on follow-up visits, were removed from further analyses leaving 329 chips in the final analysis. Expression of *XIST* (ILMN_26235) is low or absent in males, since the *XIST* gene is expressed almost exclusively from the active X chromosome present only in females. Probes that did not vary across samples (largely representing non-expressed genes in blood) were removed from subsequent

analyses, leaving 19,161 probes. Gene expression data was adjusted for age, gender, RNA quality (RIN), and study site and the residuals used for subsequent analyses. Gene expression differences between diagnostic groups (control, MCI, and AD) were statistically evaluated using linear models for microarray analyses (Limma) [55]. *P*-values were adjusted for multiple testing according to the false discovery rate (FDR) procedure of Benjamini and Hochberg and differentially expressed genes were selected at $FDR < 0.01$ [56] (supplementary Table 1; available online: <http://www.j-alz.com/issues/30/vol30-3.html#supplementarydata04>). All statistical analyses were performed in the statistical environment R using Bioconductor packages.

Creation of weighted co-expression networks

We utilized the R package for weighted gene co-expression networking analysis (WGCNA) [57], to identify clusters (modules) of highly connected (correlated) genes. A full glossary of terms to describe the analysis is available in supplementary data and at the WGCNA web site (<http://www.genetics.ucla.edu/labs/horvath/CoexpressionNetwork/Rpackages/WGCNA/>). The underlying hypothesis is that such modules are likely to possess a common function [58]. We restricted the analysis to 9,970 of the 19,161 probes

after (i) removing genes that were not expressed in any sample, (ii) only using probes detected in at least 80% of samples in each diagnostic group, and (iii) limiting to those annotated with Entrez Ids.

We estimated the connectivity of probes using a pairwise Pearson correlation between all expressions profiles resulting in an adjacency matrix. We applied the topological overlap function to transform this matrix into a topological overlap matrix (TOM) adjacency (see glossary in supplementary data). This replaces the original adjacencies with an interconnected measure based on shared neighbors. To reduce noise, we further filtered the probes to only include those whose connectivity was above the median connectivity on the TOM, leaving 4,985 probes for subsequent analyses.

The modules were created dynamically by clustering the TOM using the Ward linkage clustering algorithm [59]. Each module is represented by an arbitrary color assigned by the analysis software according to size. We constructed signed networks, which preserve the sign of correlations (i.e., direction of expression change) among the expression profiles. The connection strength between two genes was regulated by a soft threshold $\beta = 15$, chosen by empirical assessment of the network topology [57]. Introducing a soft threshold allows generation of robust results by maintaining gene co-expression information. Modules were represented by a single expression profile, the module eigengene (ME), which allowed us to explore module behavior with respect to traits such as structural changes in the brain. MEs represent the first principal component for genes within each module. The MEs were used to estimate the module membership (MM) for each probe and thereby the extent to which a given probe conforms to the characteristics of the module. The correlation between individual gene expression values and ME is used to calculate MM [58].

Module-trait correlations

To understand the significance of the modules for disease, we correlated the MEs with discrete phenotypic traits including: MCI throughout study (MCI-MCI), MCI who were later diagnosed with AD within a two year follow-up (MCI-AD), AD at baseline (AD), and AD at baseline and/or follow-up (ALL AD) and Disease Severity, which discretises the severity of the disease using the following coding: control (0), MCI-MCI (1), MCI-AD (2), and AD (3). The MEs were also correlated with structural magnetic resonance imaging traits (sMRI) including brain

volume and cortical thickness, previously shown to be relevant in AD [42, 60–62] and available for the same individuals: right entorhinal volume, left entorhinal volume, total entorhinal volume, mean entorhinal thickness, ventricular volume, left hippocampal volume, right hippocampal volume, total hippocampal volume, and whole-brain volume. A full description of how the brain volume and cortical thickness measures were generated for these subjects has been detailed previously [61, 62]. The module-sMRI trait correlations were restricted to a subset of 239 subjects for which a full set of trait information was available. A correlation between each module and trait was considered significant at $p < 1 \times 10^{-3}$ (Fig. 3).

The gene significance (GS) of a gene describes the strength of the correlation between the probe and the trait in question while the MM quantifies the extent to which a gene conforms to the characteristics of a module (i.e., it correlates with the ME). The combination of MM and GS identifies genes which play important roles in a given network module and are furthermore significant for the clinical trait in question. The gene significance and module memberships for the genes with respect to the traits: MCI-MCI, MCI-AD, AD, ALL AD, and Disease Severity are listed in supplementary Tables 2–7. We plotted the MM against GS for traits that correlated with MEs and highlighted the genes that exhibit higher-than-median MM and GS with respect to the trait. The pink, brown, and turquoise modules were plotted against the MCI-MCI trait because their MEs show significant correlation with MCI-MCI in Fig. 3. For the same reason, the black, blue and red modules were plotted against the disease severity (DS) trait.

Gene enrichment analysis

We conducted enrichment analyses to describe the functional characteristics of probes assigned to each of the disease-associated modules. We tested for enrichment of Gene Ontology (GO) terms associated with biological processes, molecular functions, and cellular compartments. We also performed a gene set enrichment analysis against KEGG Pathways using probes with both higher-than-median MM and GS for each module, highlighted in green in Fig. 5. The test was performed using DAVID (<http://david.abcc.ncifcrf.gov/>), which calculates significance of probe over-representation in particular pathways [63]. We considered a pathway significant at $p < 5 \times 10^{-4}$. We also investigated whether groups of genes known to have direct disease-

association or association with increased disease risk, had higher-than-median MM and GS for any module. The candidate gene list is presented in supplementary Table 8 which includes the top 12 genes associated with increased AD risk collated from Gene-Wide Association Studies (GWAS) and available at <http://www.alzgene.org/> [64].

Module preservation between blood and brain

We first tested the blood modules for enrichment using a large collection of brain-related gene sets [65–86] where significance was computed using a hypergeometric test. To further investigate the extent by which disease associated changes present in peripheral blood reflect changes in AD brain we compared our blood modules with two human AD brain expression datasets in more detail [77, 87]. To assess module preservation, we used two statistical measures, the Z-statistic and the median rank. The value of the Z-statistic is directly proportional to how preserved the blood module is in the brain data set: the higher the value of a Z-statistic, the stronger the evidence that the module displays significantly higher preservation than expected by chance alone. Langfelder and colleagues suggests that a Z-statistic >10 indicates a highly-preserved module, $2 < \text{Z-statistic} < 10$ indicates a somewhat preserved module while a Z-statistic < 2 indicates very low confidence that the module is preserved [88]. Despite its usefulness, the Z-statistic tends to give a better rank to modules of larger sizes. We therefore also use the median rank, which is based on the observed preservation statistics (as opposed to the Z-statistics or p -value), and because it is less sensitive to the size of the module, enabled us to compare the relative preservation between modules. A module with lower median rank suggests greater preservation than a module with a higher median rank.

Preservation between blood and brain was explored for both statistics with respect to two network properties: network density and network connectivity. To ascertain preservation of network density, we examined the modules as a whole and determined whether densely-connected modules in blood remained densely connected in brain. To determine the preservation of network connectivity, we examined the patterns of connectivity at the probe level for each module in blood and provided a score which determined whether highly-connected probes central to the blood network (also termed hub genes) remained highly-connected in brain. Results are reported as Z-density, Z-connectivity and Z-summary (the average of Z-density and

Z-connectivity) and median rank density, median rank connectivity and median rank summary.

Blood cell types with over-represented changes in gene expression

To characterize whether particular blood cell populations were more affected in disease, probes with significantly altered expression in AD ($n = 2,270$ with $\text{FDR} < 0.01$) were mapped on to a list of probes previously reported to be enriched in particular blood cell types [89]. Over- or under-representation of significantly altered genes was tested using the Pearson's chi-squared test, or the Fisher's exact test if the number of probes was < 10 . To increase confidence in our results, we also tested whether more cell lineage probes attained a given p -value than would be expected by chance. This was achieved by randomly selecting 2,908 of the 19,161 probes used in the analysis and repeating the analysis for each cell-type specific list for 10,000 permutations. We further tested for just over-representation of significantly altered probes in particular blood cells in AD blood using a hypergeometric probability test.

RESULTS

Disease-associated gene expression differences

A large number of gene expression changes were observed in blood from MCI and AD subjects compared to normal elderly controls. Of the 2,908 differentially expressed probes identified between the three groups (FDR corrected $p < 0.01$), around a quarter were similarly altered in blood from MCI and AD subjects, relative to controls (Fig. 1 and supplementary Table 1). The probes which reached the greatest significance were predominantly down-regulated, although overall, there were roughly equal numbers of significantly up- and down-regulated probes below the nominal p -value threshold.

Across blood samples, expression patterns could be clustered together in to ten distinct modules, containing between 171 (magenta) to 1,179 (turquoise) probes (Fig. 2 and supplementary Tables 2–7). Modules are assigned an arbitrary color by the analysis software according to their size. Six of these modules had significant differences in mean ME expression between subject groups, with the red, black, pink, brown, blue, and turquoise modules achieving p -values 3.7×10^{-9} , 2.7×10^{-13} , 3.7×10^{-5} , 4.2×10^{-7} , 2.6×10^{-5} , and 8.6×10^{-9} , respectively,

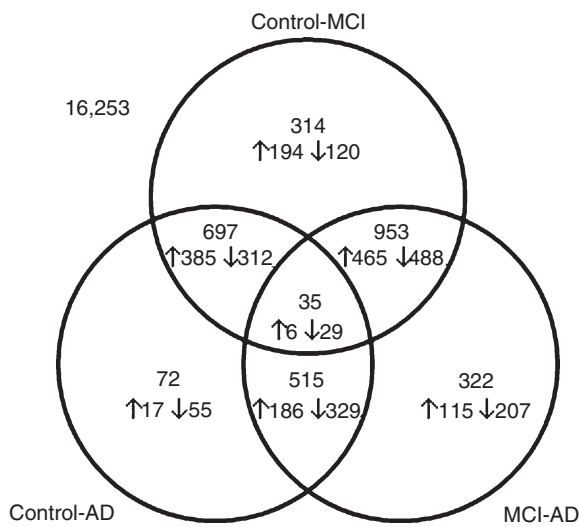


Fig. 1. Number of probes with differential expression between AD, MCI, and control blood samples, FDR corrected $p < 0.01$. Direction of change refers to an increase (↑) or decrease (↓) in expression of control relative to MCI blood (Control-MCI), control relative to AD (Control-AD), or MCI relative to control (MCI-AD), as indicated.

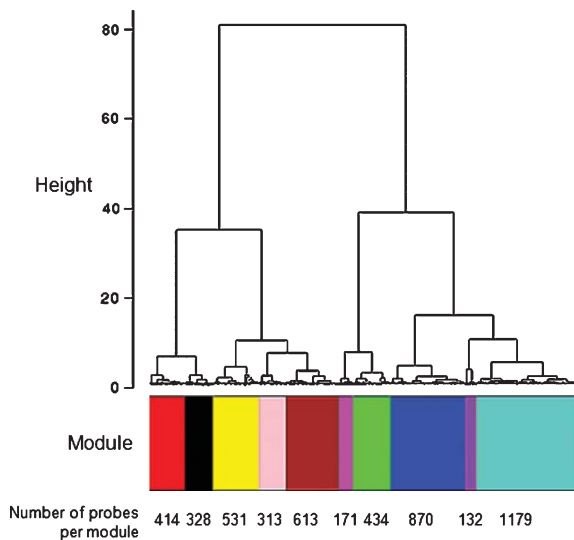


Fig. 2. Clustering dendrogram showing modules identified using blood expression data from control, MCI, and AD subjects. Modules are assigned an arbitrary color by the analysis software according to their size: Red, Black, Yellow, Pink, Brown, Magenta, Green, Blue, Purple, and Turquoise. The numbers of probes are indicated below each module.

in Kruskal Wallis tests. Of these, the red, black, pink, and brown modules were down-regulated in MCI-MCI, and/or MCI-AD, AD, or Disease Severity, relative to controls (Figs. 3 and 4). As expected, many of the structural MRI traits were positively

correlated with genes in these modules, whereby reductions in brain volume and cortical thickness correlated with reduced gene expression (Fig. 3). Where these modules differed from each other was in their relative levels of expression in AD subjects. Genes within the black and red modules continued to display reduced expression in blood from AD subjects, similar to that seen in MCI subjects (e.g., ALL AD, $r = -0.29$, correlation p -value $p = 8 \times 10^{-8}$, black module) (Figs. 3, 4a and b), while expression in the brown and pink modules abated in AD patients, achieving mean levels of expression more similar to control subjects (e.g., ALL AD, $r = -0.03$, correlation p -value $p = 0.6$, brown module) (Figs. 3, 4c and d).

The blue and turquoise modules displayed the opposite pattern. They were upregulated in MCI-MCI, MCI-AD, AD, and/or with Disease Severity relative to controls (Figs. 3 and 4). Many of the sMRI traits displayed negative correlation with these two modules (Fig. 3). The blue and turquoise modules differed in their relative levels of expression in AD subjects. Genes within the blue module continued to display upregulated expression in blood from AD subjects, similar to that seen in MCI subjects (ALL AD, $r = 0.21$, correlation p -value $p = 1 \times 10^{-4}$) (Figs. 3 and 4e), while expression in the turquoise module appeared to abate in AD patients, achieving mean levels of expression not significantly different from control subjects (All AD, $r = 0.056$, correlation p -value $p = 0.3$) (Figs. 3 and 4f).

The turquoise, brown, and pink modules displayed changes in MCI subjects which appeared to return toward normal levels in people clinically diagnosed with AD (Figs. 3 and 4). We therefore repeated the Kruskal Wallis tests after excluding the control group to test for MCI-AD differences. There were significant differences in the pink (p -value $= 8.7 \times 10^{-5}$), brown (p -value $= 7 \times 10^{-4}$), and turquoise (p -value $= 7 \times 10^{-4}$) modules between subjects with MCI and AD.

Functional identity of genes with altered expression

The GO enrichment test [90] for each of the disease-associated blood modules revealed important associations for the red, black, pink, brown, blue, and turquoise modules with particular GO terms, thus providing a functional characteristic to each module. For each module, GO terms with a p -value < 0.05 are summarized in Table 2. Overall, there were strong associations in the red module with lower

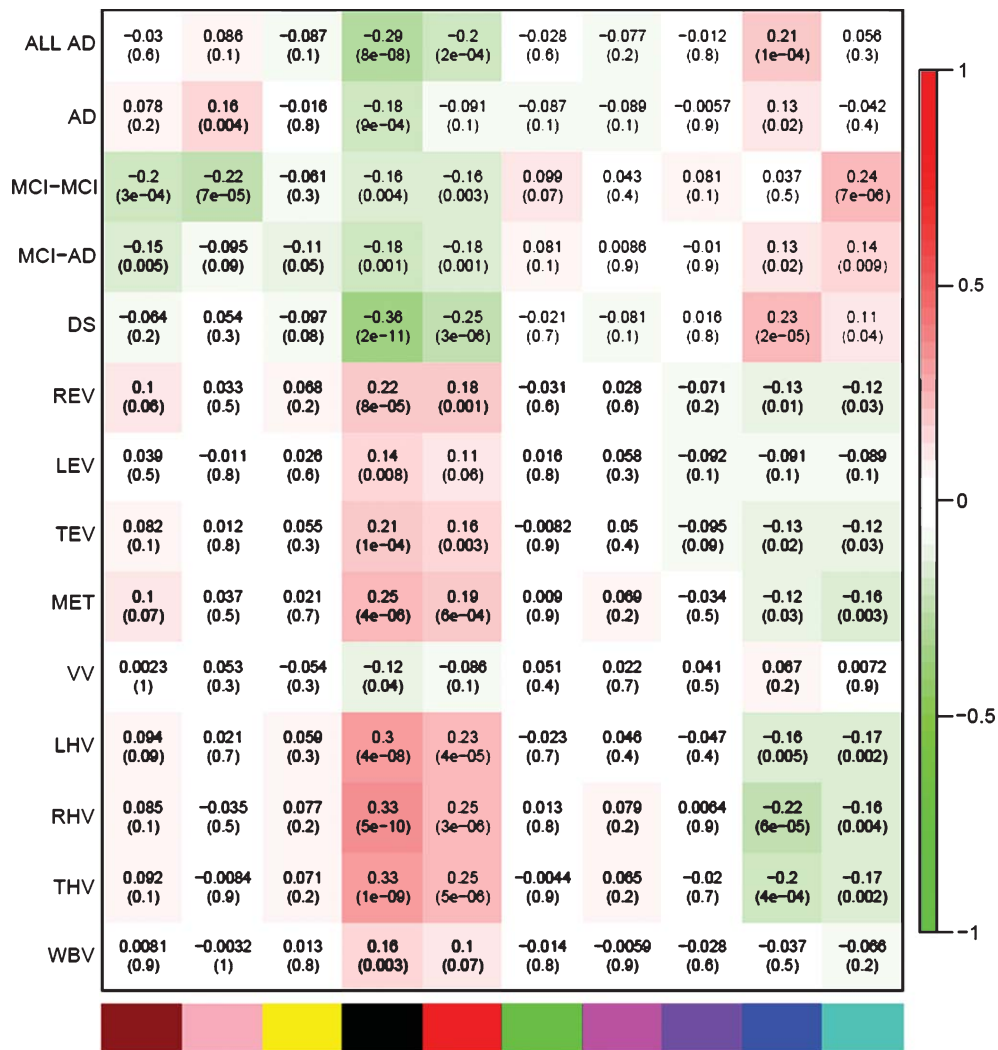


Fig. 3. Heatmap of module-trait correlations. The first four traits represent correlation of disease diagnosis (AD or MCI) relative to normal elderly controls, within each module. MCI was further divided into subjects who subsequently converted to an AD diagnosis within 2 years of sampling (MCI-AD) and those who did not (MCI-MCI). A further AD group (ALL AD) represents any subject who received a diagnosis of AD during the two years of the study. Disease Severity (DS) was also analyzed. The remaining traits represent structural imaging measures of regional brain volume or cortical thickness: right entorhinal volume (REV), left entorhinal volume (LEV), total entorhinal volume (TEV), mean entorhinal thickness (MET), ventricular volume (VV), left hippocampal volume (LHV), right hippocampal volume (RHV), total hippocampal volume (THV) and whole-brain volume (WBV). For each module-trait pair the top-most value is the Pearson correlation coefficient while the second (bracketed) value is the correlation p -value. The cell color represents the strength and direction of the correlation. Modules are assigned an arbitrary color by the analysis software according to their size.

ribosomal function and translation, the black module with decreased mitochondrial function and ribosomal function and translation, the pink module with lower secretory and endocytic function, the brown module with decreased metabolic processes, the blue module with altered immune function, and the turquoise module with activation of transcription, in disease.

To further explore the genes that were most representative of each module (module hubs) and were

also the most associated with disease-related traits, we correlated module membership (MM) and gene significance (GS), and selected probes which exhibited higher-than-median MM and GS and determined their enrichment in KEGG pathways (probes highlighted in green in Fig. 5). The scatter plots of GS and MM (Fig. 5) show concordance with the results presented in the heat map (Fig. 3): the more hub-like a probe is, the greater its correlation with the trait in

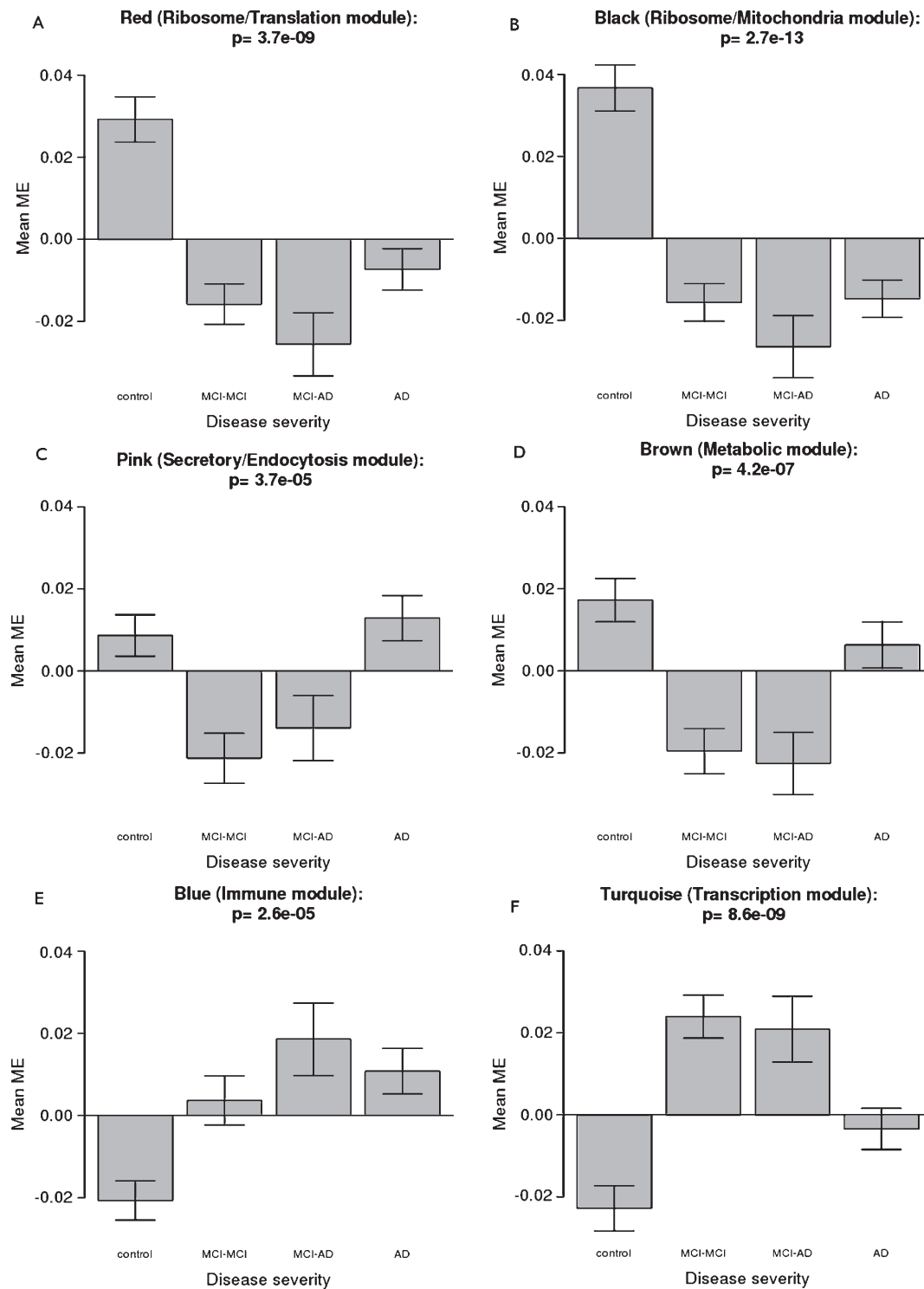


Fig. 4. Boxplots showing the mean module eigengene (ME) expression for the A) Ribosome/Translational, B) Ribosome/Mitochondrial, C) Secretory/Endocytosis, D) Metabolic, E) Immune, and F) Transcription-related modules. MEs represent the first principal component of the module gene members. Significance of differences in mean ME expression for each disease group determined by Kruskal Wallis tests. The mean MEs were compared across diagnostic category: normal elderly control, AD and MCI. MCI was further divided into subjects who subsequently converted to an AD diagnosis within 2 years of sampling (MCI-AD) and those who did not (MCI-MCI). The most significant modules ($p < 1 \times 10^{-4}$) are represented in A to F.

Table 2
GO enrichment test for each blood module revealing important associations between genes within each module

Module	GO term names
Red ^a	CC^b ribosome, ribonucleoprotein complex, cytosolic ribosome, mitochondrion, cytosolic small ribosomal subunit, cytosolic part, intracellular part, intracellular organelle, intracellular membrane-bounded organelle, small ribosomal subunit
<i>Ribosome/Translation</i>	BP^c cellular metabolic process, translation, metabolic process, cellular biosynthetic process, cellular macromolecule biosynthesis process, gene expression, macromolecule biosynthesis process, biosynthetic process, positive regulation of helicase activity, response to redox state MF^d structural constituent of ribosome, non-membrane spanning protein tyrosine phosphatase activity, oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor, NADPH: quinone reductase activity, structural molecule activity, serine-type peptidase activity, serine hydrolase activity, ATPase activity, peptidyl-prolyl cis-trans isomerase activity, transferase activity, transferring alkyl or aryl (other than methyl) groups
Black ^a	CC^b Ribosome, ribonucleoprotein complex, mitochondrion, mitochondrial part, mitochondrial inner membrane, organelle inner membrane, mitochondrial membrane, mitochondrial envelope, intracellular organelle, respiratory chain
<i>Ribosome/Mitochondria</i>	BP^c translation, oxidative phosphorylation, generation of precursor metabolites and energy, mitochondrial electron transport, NADH to ubiquinone, ATP synthesis coupled electron transport, mitochondrial ATP synthesis coupled electron transport, respiratory electron transport chain, cellular respiration, ribonucleoprotein complex biogenesis, electron transport chain MF^d structural constituent of ribosome, structural molecule activity, hydrogen ion transmembrane transporter activity, NADH dehydrogenase activity, NADH dehydrogenase (ubiquinone) activity, monovalent inorganic cation transmembrane transporter activity, oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor, oxidoreductase activity, acting on NADH or NADPH, RNA binding, oxidoreductase activity
Pink ^a	CC^b secretory granule membrane, endomembrane system, clathrin-coated vesicle Golgi apparatus, endosome membrane, synaptic vesicle
<i>Secretory/Endocytosis</i>	BP^c post-translational protein modification, protein amino acid phosphorylation, protein modification process, activation of innate immune response, regulation of synaptic plasticity, regulation of long-term neuronal synaptic plasticity, phosphate metabolic process, regulation of synaptic transmission, nuclear import, regulation of neuronal synaptic plasticity MF^d GDP binding, GTPase activity, cAMP binding, co-SMAD binding, catalytic activity ligase activity, purine nucleotide binding, phosphoprotein phosphatase activity, AMP binding, long-chain fatty acid-CoA ligase activity
Brown ^a	CC^b intracellular membrane-bounded organelle, membrane-bounded organelle, intracellular, intracellular part, Golgi apparatus, centriole, endosome membrane, organelle membrane, recycling endosome, intracellular organelle
<i>Metabolic</i>	BP^c amino sugar metabolic process, establishment of protein localization, steroid hormone receptor signaling pathway, protein transport, glucosamine metabolic process N-acetylglucosamine metabolic process, cellular aromatic compound metabolic process regulation of anti-apoptosis, intracellular receptor mediated signaling pathway MF^d protein-L-isoaspartate (D-aspartate) O-methyltransferase activity, solute:hydrogen symporter activity, nucleotide-sugar transmembrane transporter activity, O-methyltransferase activity, beta-N-acetylhexosaminidase activity, polynucleotide adenyltransferase activity, peptide hormone receptor binding, sugar:hydrogen symporter activity, 7 S RNA binding, hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides
Blue ^a	CC^b plasma membrane, membrane, plasma membrane part, intrinsic to membrane, integral to plasma membrane, intrinsic to plasma membrane, integral to membrane, membrane part, cell-substrate adherens junction
<i>Immune</i>	BP^c defense response, signal transduction, inflammatory response, response to wounding intracellular signaling pathway, response to stimulus, immune response, immune system process, response to stress, cell surface receptor linked signaling pathway MF^d signal transducer activity, receptor activity, transmembrane receptor activity, lipid binding, phospholipid binding, cytokine receptor activity, phosphotransferase activity, alcohol group as acceptor, enzyme activator activity, receptor signaling protein activity, cytokine binding
Turquoise ^a	CC^b nucleoplasm part, intracellular organelle part, nucleoplasm, nuclear lumen, nuclear part, protein complex, intracellular membrane-bounded organelle, membrane-bounded organelle, intracellular organelle, MLL1 complex
<i>Transcription</i>	BP^c none MF^d protein binding, transcription coactivator activity, transcription activator activity, transcription regulator activity, transcription cofactor activity, binding, transcription factor binding, molecular function, methyltransferase activity, transferase activity

^aTerms with a *p*-value <0.05 are summarized; ^bCC: Cellular Components; ^cBP: Biological Processes; ^dMF: Molecular Functions.

question. In the turquoise (transcription) and blue (immune) modules (Fig. 5E and F), probes highlighted in green are in the top-right because these modules showed a positive correlation with the trait, thus making high gene significance a positive value. For all other modules (Fig. 5A–D), high gene significance is negative as the modules showed negative correlation with the corresponding trait and therefore probes highlighted in green are in the bottom-right area instead.

The most significantly altered genes in MCI and AD blood were in the black (ribosome/mitochondria) module and compared to other modules, this module (probes filtered for high MM and GS) also had the most significant over-representation of genes enriched in KEGG pathways including the AD pathway (supplementary Tables 3 and 8 and supplementary Figure 1). A striking number of probes in this module coded for genes collectively associated with oxidative phosphorylation (OXPHOS), including subunits of the respiratory chain and ribosomal subunits required for translation of respiratory chain subunits (KEGG pathways Oxidative Phosphorylation, $p = 3.9 \times 10^{-5}$; Ribosome, $p = 6.6 \times 10^{-5}$; and Alzheimer's disease, $p = 1.8 \times 10^{-4}$, supplementary Figure 1 and supplementary Table 10). Genes attributable to the KEGG ribosome pathway were also enriched in the red (ribosome/translation) module ($p = 1.7 \times 10^{-7}$).

Following further analysis of the 77 genes assayed which form part of the mitochondrial respiratory chain (complex I to V) (OXPHOS), 32 were found to be significantly dysregulated in MCI and/or AD compared to control blood, with all but 3 genes showing decreased expression in disease (supplementary Table 8). Additionally, subunits of the mitochondrial ribosome expressed from the nuclear genome (MRP genes) and involved in translation of proteins for the respiratory chain complex from the mitochondrial genome, and/or ribosomal subunit genes involved in nuclear gene translation, were down-regulated in disease. Of the 78 MRP genes assayed, 28 had lower expression in MCI and/or AD compared to controls (supplementary Table 8 and supplementary Figure 1).

GO analysis also revealed a significant over-representation of immune-related genes in the blue (immune) module with the most significant trait and module-associated genes enriched in the Leukocyte transendothelial migration KEGG pathway, $p = 1.2 \times 10^{-4}$ (supplementary Tables 6 and 8 and supplementary Figure 1). Immune related genes were generally found in the blue (immune) and turquoise (transcription) modules, displaying modest upregulation of their expression in MCI and/or AD blood.

Most notable were genes which encode key adhesion molecules within the selectin (L-, P-, and E-selectin, P-selectin glycoprotein ligand 1), immunoglobulin superfamily (ICAMs, VCAM, and madCAM), integrin (CD11/CD18 and α -4) and chemokine gene families, which are crucial for leukocyte slowing, rolling and tethering in response to activated endothelial cell signals in blood microvessels (Fig. 5 and supplementary Tables 1–8). Both subunits of the LFA-1 (CD11a/CD18, *ITGAL/ITGB2*, α L β 2) and Mac-1 (CD11b/CD18, *ITGAM/ITGB2*, α M β 2) [91] complexes were upregulated. There were also changes in the genes encoding their downstream effectors such as Fyn and FGR. The cell-cell and extracellular matrix adhesion genes *ITGAX* and *ITGB7* were upregulated while *CLDN1*, a gene with adhesion properties important for maintaining tight junction integrity and regulating cell polarity was down-regulated. Members of the ADAM family of type I transmembrane glycoproteins, *ADAM8*, and *ADAM10*, were also upregulated. These are known to be involved in cell adhesion and processing of cytokines and adhesion molecules [92]. In particular, *ADAM8* has been shown to cleave L-selectin from the plasma membrane of activated neutrophils, and through this activity is predicted to facilitate transendothelial extravasation [93]. It is also possible that upregulation of ADAM genes may increase shedding of L-selectin to prevent rather than increase cell adhesion and resolve inflammation, as has been found for *ADAM17* [94].

Other noteworthy genes altered in the blood of MCI and/or AD people which might promote transendothelial extravasation of leukocytes include progranulin (*GRN*). The increase in expression of progranulin in blood from people with MCI is consistent with previous findings [95]. Progranulin can stimulate neutrophil and macrophage infiltration and neovascularization of wound tissue [96, 97] or alternatively display anti-inflammatory activity as an antagonist to TNF/TNFR signaling [98]. The expression of a number of pattern recognition receptors important for sensing a pathogenic or sterile stimulus [99], were either upregulated as in the case of three NLR family members (*NLRP1*, *NLRP12*, and *NLR5*) and C-type lectin domain receptors (*CLEC16A*) or down-regulated (*CLEC4D*, *CLEC4A*, and *CLEC7A* and *AIM2*), in MCI and/or AD subjects. Interestingly, *TLR2* and *TLR8*, which are part of the toll-like receptor family of pattern recognition receptors, were not altered in MCI subjects but were significantly upregulated in AD patients, along with upregulation of FGR which mediates TLR protein signals in addition to those of the LFA-1 and

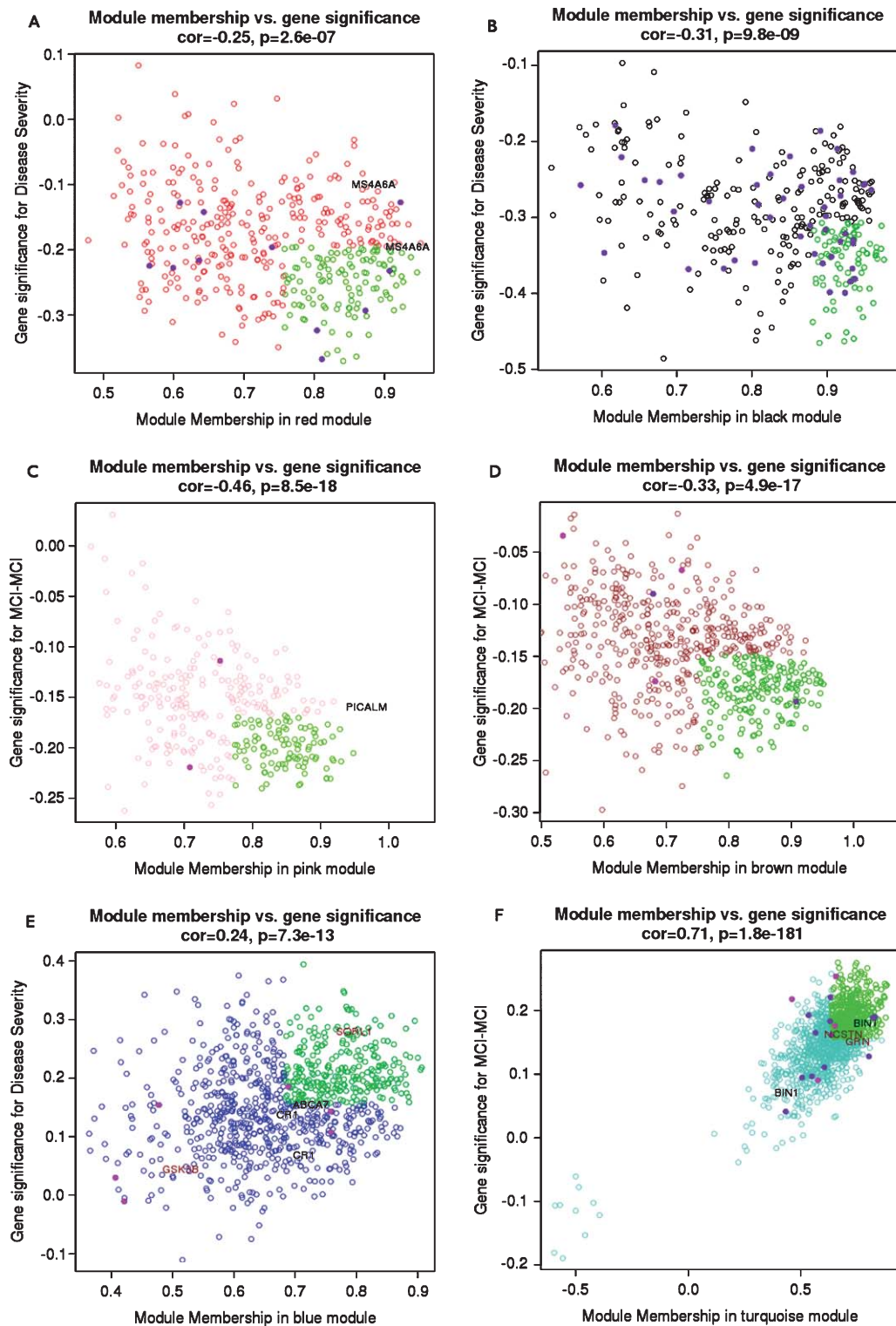


Fig. 5. (continued)

Mac-1 complexes. Some of these genes not only had a strong disease association but also had expression patterns characteristic of the modules in which they were allocated (Fig. 5).

Of the candidate disease genes analyzed, only *SORL1* (blue-immune module) and *BINI* (turquoise-transcription module) had higher-than-median module membership and trait significance, suggesting they may have significant influence over the behavior of other genes in their respective modules (Fig. 5). Other candidate genes including the disease associated genes *NCSTN* and *GRN* (turquoise-transcription module) and *GSK3B* (blue-immune module) and the AD susceptibility genes *CRI* (blue-immune module), *ABCA7* (blue-immune module), *PICALM* (pink-secretory/endocytosis module), and *MS4A6A* (red-ribosome/translation module) while assigned to modules either did not appear to be central to the characteristics of the module and/or did not display significant alteration in expression in disease.

Changes in blood cell number are unlikely to explain the disease associated gene expression differences observed in MCI and AD blood

Blood is a heterogeneous and dynamic tissue containing many different leukocyte populations. Leukocyte cell numbers can differ during disease and inflammation. In light of this, we compared the numbers of circulating leukocytes in blood from AD, MCI, and normal elderly control subjects to explore whether the gene expression changes we observe might reflect a shift in blood cell number or could potentially be explained by the activity of a particular cell type in blood. Of the three granulocyte populations investigated, only the number of basophils, a minor cell type in blood, was significantly different between control and disease blood samples. There were more basophils in blood from MCI and AD subjects compared to controls (Fig. 6A; Bonferonni-corrected $p < 0.017$). The numbers of neutrophils, eosinophils, and lymphocytes (Fig. 6B–D) were similar between diagnostic groups, while the numbers of monocytes, a particularly active

cell, were significantly lower in blood from MCI subjects compared to AD (Fig. 6E; Bonferonni-corrected $p < 0.017$). All subjects included in the analysis had blood cell numbers considered to be within the normal acceptable range for each cell type, indicating the changes we observed were small and not indicative of serious illness.

None of the leukocyte populations investigated had greater numbers of differentially regulated genes than any other cell type, given the total number of dysregulated between control, MCI and AD and the numbers of genes analyzed (supplementary Table 9). There were in fact fewer dysregulated genes representing activated granulocytes in MCI and B cells in AD than might be expected, suggesting activity in these cells was unlikely to explain a significant proportion of the gene expression differences we observed in disease (supplementary Table 9). Overall, our data suggests no one cell type is likely to be driving the gene expression changes we observe in disease and that the disease-related changes we report are not a simple reflection of a shift in cell numbers in disease.

Blood and brain module preservation

There was significant concordance between blood modules and brain-related modules generated from multiple published expression and protein datasets generated from brain tissue (supplementary Table 10). For example, the black (ribosome/mitochondria) module was significantly enriched with genes from brain modules, displaying a similar direction of change of expression in disease and preservation of module characteristics across datasets and species (supplementary Table 10). To explore this further, we investigated two well-powered studies of gene expression generated from hippocampus [77] and cortical regions [87] from control and AD patients. The black (ribosome/mitochondria) module showed high density preservation but was less preserved with respect to connectivity (Fig. 7), indicating a possible change in roles for the hub genes in the brain, although it should be noted that the two brain datasets alone did not

Fig. 5. The correlation between module membership (MM) and gene significance (GS) for each probe was computed. Scatterplots for the most significant traits and modules are presented, illustrating the relationship between MM and GS at the probe level and indicating how central a given probe is to each module and how associated it is with the traits we examined of disease severity (A, B, and E) or MCI relative to control (MCI-MCI) (C, D, and F). The following six modules were used for correlation: Red (ribosome/translation) (A), Black (ribosome/mitochondria) (B), Pink (secretory/endocytosis) (C), Brown (metabolic) (D), Blue (immune) (E), and Turquoise (transcription) (F). Probes highlighted in green exhibited higher-than-median module membership and trait significance, so are likely to be core module probes. Probes encoding mitochondrial protein subunits of complex I to V of the respiratory chain and protein subunits of the mitochondrial ribosome (listed in supplementary Table 8) are colored in purple, while probes encoding important immune-associated genes (listed in supplementary Table 8) are colored in dark pink. Candidate genes, which are listed in supplementary Table 8, are highlighted by gene name.

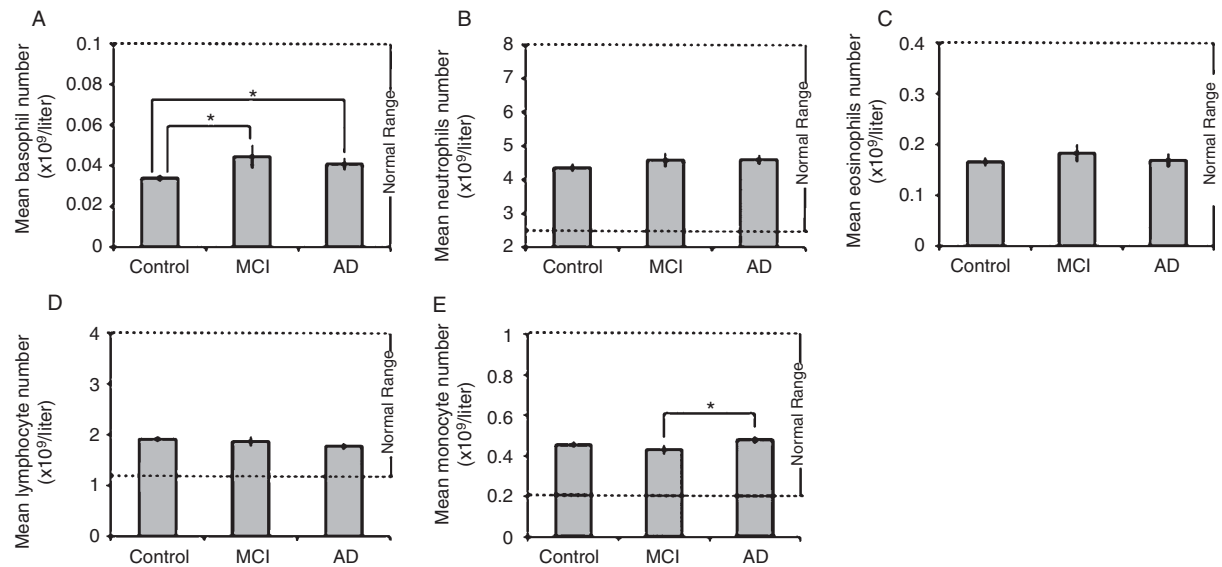


Fig. 6. A Full Blood Count analysis was undertaken to compare the numbers of various leukocyte populations in blood from AD, MCI, and normal elderly subjects. Three granulocyte populations were examined: basophils (A), neutrophils (B), and eosinophils (C) and two peripheral blood mononuclear populations, lymphocytes (D) and monocytes (E). Data within the normal range ($\pm 10\%$) was included in the analysis. Graphs represent mean leukocyte number ($\times 10^9/\text{liter}$) \pm SEM in normal elderly controls ($n = 125$), MCI ($n = 41$), and AD subjects ($n = 73$). Following \log_{10} transformation of the data, linear regression was carried out to assess the effect of disease status on individual leukocyte populations, adjusting for age, gender, and other blood cell types. *Significance, Bonferonni-corrected $p < 0.017$.

show particularly high connectivity concordance with each other (data not shown). In the hippocampus, the black (ribosome/mitochondria) module had Z-density and Z-summary >10 (threshold for high preservation) which was the highest among all the modules and had the highest median rank density statistics. In cortical tissue, the black (ribosome/mitochondria) module maintained Z-density >10 and ranked second highest with respect to median rank and median rank density. This pattern did not appear in the Z-connectivity and median rank connectivity statistics. Another module which showed some preservation across both data sets was the brown (metabolic) module. Again, the preservation patterns were stronger in the hippocampus than in cortical tissue. Connectivity of the brown (metabolic) module was more preserved than the black (ribosome/mitochondria) module. The red (ribosome/translation) module was only preserved in the hippocampal brain data, while the pink (secretory/endocytosis) module, which was associated with changes in MCI subjects only, showed only weak preservation in both brain datasets. Finally, the turquoise (transcription) and blue (immune) modules appeared to be unpreserved in the hippocampus, but at least for the turquoise (transcription) module,

more highly preserved in cortical tissue than in the hippocampus.

DISCUSSION

We have identified a large number of differentially expressed genes in blood samples from MCI and AD subjects compared to normal controls using microarray technology. These dynamic changes could be grouped into six disease-associated co-expression modules each containing genes with characteristic biological identities. There were strong associations in the red module with lower ribosomal function and translation, the black module with decreased mitochondrial function and translation, the pink module with lower secretory and endocytic function, the brown module with decreased metabolic processes, the blue module with altered immune function and the turquoise module with promotion of transcription in disease.

Disease was most strongly associated with lower expression of genes encoding respiratory chain subunits or ribosomal protein subunits which translate respiratory subunit genes from the mitochondrial genome. These genes were predominantly allocated to

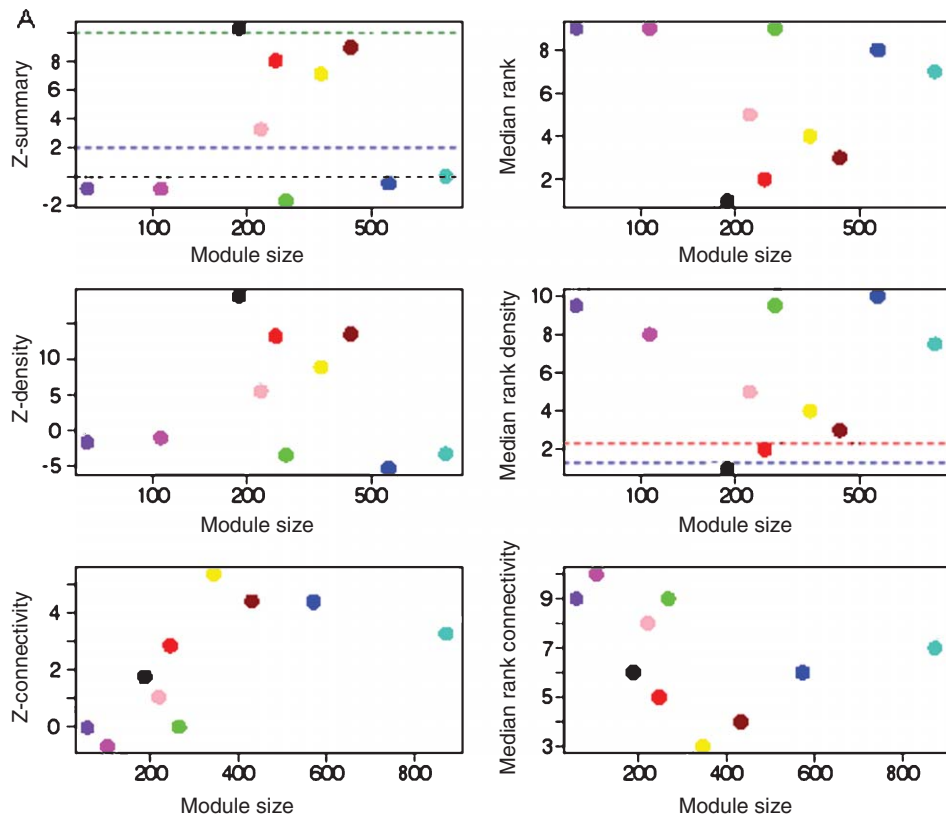


Fig. 7. The blood module preservation results across the two publicly available AD human brain gene expression data sets (Liang (A) and (B) Myers [77, 87]). The scatter plots describe the relation between the different modules and the six module-preservation statistics. For each plot, the x-axis corresponds to the module size while the y-axis is the value of the preservation statistics in question. Each plot contains the ten modules represented by their colors. The dotted lines represent the nominal strength of the relationship: the higher the value of a Z-statistic, the stronger the evidence that the module displays significantly higher preservation than expected by chance for Z-summary, Z-density, and median rank intensity.

the black (ribosome/mitochondria) module and were present in both AD patients and MCI subjects, including those known to have prodromal AD (MCI-AD). The production of ATP and respiratory chain activity therefore appears to be compromised early in disease.

Mitochondrial (respiratory chain) dysfunction is a consistent hallmark of AD

Platelets and lymphocytes have previously been found to have significantly reduced respiratory chain activity in MCI and AD subjects [16, 22–24]. In agreement with our findings, several studies have shown decreased cytochrome c activity (complex IV) in human platelets from AD patients [22, 100, 101] and lower abundance of complex IV proteins in blood from people with MCI [24]. Remarkably, the changes we observe in the respiratory chain in AD blood are

also well-preserved in brain in terms of density of the black module, particularly in the hippocampus [15, 77, 102], suggesting a shared disease response between these two tissues. Down-regulation of respiratory chain subunits and genes that regulate their transport in to the mitochondrion have been previously described in AD in a number of brain regions at both the RNA and protein levels [103]. Alterations to the OXPHOS/respiratory chain may in part provide an explanation for the significantly reduced glucose metabolism measured by FDG-PET which manifests very early in disease [104]. The overlap between blood and brain may represent a common response to the same disease-associated signal(s). A direct and indirect link between toxic A β species and respiratory chain activity has been well described in AD brain tissue [105], and it is plausible that the changes in circulating levels of A β in plasma [106] or the increase in A β

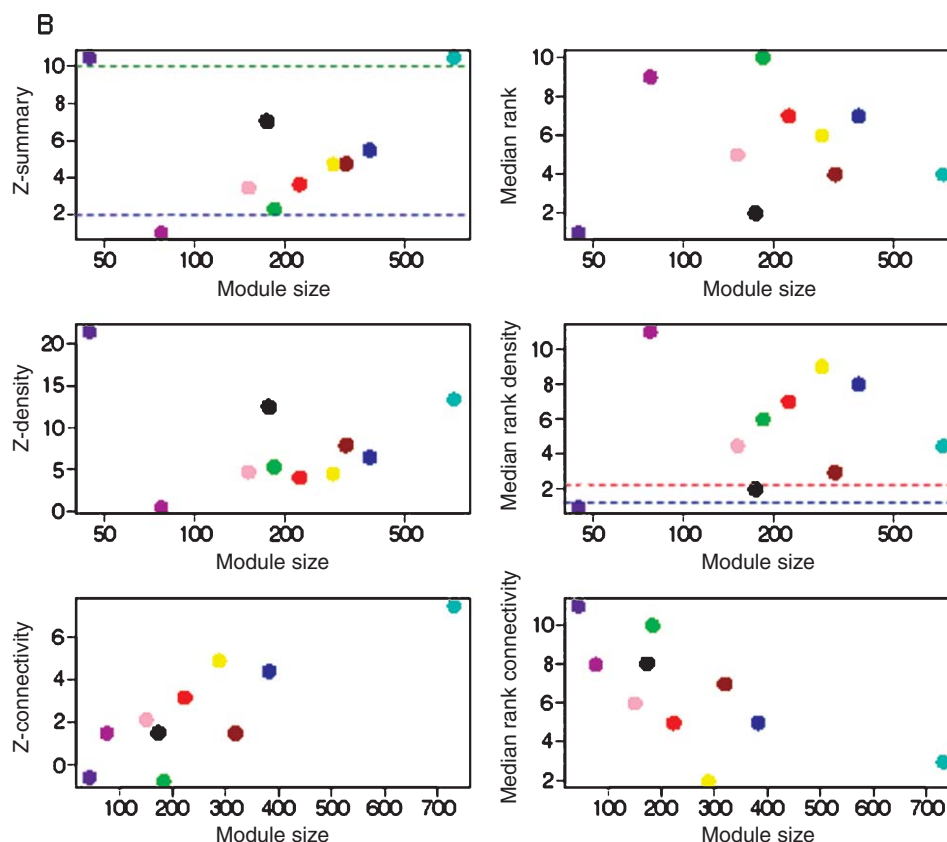


Fig. 7. (continued)

associated with blood vessels could provide the signal to blood cells in the periphery, resulting in the same genes being affected. Other more indirect signals may also be responsible.

Inherited mutations in respiratory chain subunits and assembly genes which we find down-regulated in the current study, including NDUSF4 [107], NSUSF3 [108], NDUFA1 [109, 110], UQCRB [111], UQCRCQ [112], and MRPS22 [113, 114], can cause loss of activity in complexes I, II, III, IV, and/or V and give rise to complex disease phenotypes which include Leigh syndrome, Leigh-like syndrome, fatal infantile lactic acidosis, neonatal cardiomyopathy with lactic acidosis, macrocephaly with progressive leukodystrophy, and unspecific encephalomyopathy [115]. Of particular relevance to the current study is the frequent occurrence of neurodegeneration in such diseases. The respiratory chain operates as a supercomplex involving complex interactions involving around 100 proteins derived from the nuclear and mitochondrial genomes, assembled in to five complexes. Each complex involves variable assemblies of components to enable precise

and dynamic control of thermodynamic and kinetic properties and thereby the modulation of functions including ATP generation [116]. It is difficult to predict with accuracy how a change in abundance of an individual protein subunit would affect the respiratory chain beyond the individual complex in which the affected subunit is located.

Nevertheless, compromised respiratory chain activity is expected to affect ATP production and increase cellular stress and generate excessive production of mitochondrial reactive oxygen species (mROS) which can damages cell components and lead to cell death. Mitochondria generate a significant amount of ROS when the respiratory chain, particularly complexes I and III, are compromised [117, 118]. mROS is believed to be one of the main events contributing to neuronal cell loss in AD and other related neurodegenerative disorders [119, 120]. Consistent with our findings, freshly isolated platelets [22] and lymphocytes [121, 122] from AD patients have decreased ATP levels, increased levels of ROS, and increased expression of genes associated with oxidative stress [18, 21], and

there is evidence of oxidative damage to plasma proteins, occurring even in mild disease [123]. Leukocytes are also more susceptible to respiratory chain inhibitors [121, 124–126] and have elevated DNA fragmentation and spontaneous apoptotic cell death [127–130]. Apoptosis of leukocytes in tissues is normally a critical mechanism for rapidly resolving inflammation and avoiding collateral inflammatory tissue damage by activated cells [131, 132]. We did not observe changes in genes encoding apoptotic markers in our samples, although activation of many of these genes is more likely to occur post-translationally and therefore would not be observed in our study. It will be important to establish if the changes we see lead to increased blood cell apoptosis in patients with MCI or AD and their increased clearance from circulation.

While excessive ROS can be detrimental, mROS is also an important intermediate signal for normal cell function. In the immune system, it can mediate signaling pathways including the RLR-dependent antiviral responses [133] and the TLR-dependent response [134], both of which signal through the NF- κ B, MAPK [135], ERR α –PGC1 β [136], and/or NLRP3 inflammasome [137] pathways to generate an immune response. mROS can enhance pro-inflammatory cytokine production and stimulate the oxidative burst in activated cells. The increase in activation markers we observe could therefore herald a healthy innate immune response characterized by increased leukocyte adhesion to the endothelium [138] and mobilization of these cells to the site of tissue injury. It will be important to establish whether the changes we find in mitochondrial genes are sufficient to increase mROS and support a healthy pro-inflammatory environment or alternatively, lead to excessive mROS production and a reduction in chemotactic behavior, oxidative burst, reduction in glycolysis, and cell clearance through apoptosis to prevent further immune activity [139, 140].

Immune activation is a prominent feature of AD

Our data support the presence of a chronic low grade innate immune response in people with MCI and AD. Important signaling, cell adhesion, and motility gene markers were upregulated, consistent with leukocytes having an activated phenotype, which is expected to increase endothelial cell association, leukocyte rolling, and transendothelial extravasation (diapedesis). These genes are predicted to be blood specific, so it is no surprise that the blue (immune) module which they mainly

belong to was not highly conserved in the brain. Activation of these genes is predicted to have arisen from a sterile immune response initiated in the brain [141] with the changes we observe likely to be part of a sustained and chronic peripheral response. Increased brain-associated inflammatory activity and systemic changes have been extensively described in AD. A β and/or dying neuronal cells are thought to trigger a local sterile immune response in AD brain [99, 142]. A β can bind to a multitude of receptors expressed on brain microglia, and can elicit the production of pro- and/or anti-inflammatory mediators such as cytokines and chemokines and ROS, which may exacerbate disease [143–147]. Alongside this cascade of events, alterations in the levels of acute phase proteins, in particular chemokines, pro-inflammatory cytokines [35, 148, 149], endothelial markers [150], and markers of complement activation [33, 151–153] have all consistently been found in blood early in AD and are thought to coincide with endothelial signals which in turn increase leukocyte migration into the brain, perhaps further propagating the inflammatory response [144, 154–157]. Polymorphisms in genes with inflammatory functions have emerged as important pathways which confer disease susceptibility in many GWAS studies [158–164]. Interestingly, variants of the sortilin-related receptor (*SORL1*) gene (a central hub in the blue-immune module), which was significantly upregulated in the blood of people with MCI, is a susceptibility gene for AD, albeit the increased risk is very small [165]. *SORL1* has been shown to functionally interact with A β PP, preventing homodimer formation which is believed in turn to affect secretase activity and A β PP processing, culminating in decreased production of amyloidogenic products [166]. A β peptides are abundant in human plasma and have been evaluated as possible biomarkers for AD. A net increase in *SORL1* expression, which we found in MCI blood, may in part be responsible for the decrease in A β peptides found in plasma from prodromal AD patients [167]. Furthermore, *SORL1* expression was lower in AD subjects compared to MCI blood, which coincides with the presence of higher A β peptides in people with AD [168]. As *SORL1* is a multifunctional protein thought to be important in the endocytic pathway, it may have additional roles in disease which could include controlling some of the immune-associated genes co-expressed in the blue (immune) module. It will be particularly interesting to test whether the AD-associated *SORL1* variants previously identified are able to exert a transacting influence over the expression of any other genes in the blue (immune) module.

Despite the presence of immune activation signals and markers of response, it is unclear whether the changes we see in disease represent diapedesis of leukocytes across the blood brain barrier. The degree of differential expression between control and disease blood was less significant for immune-related genes than for other differentially expressed genes. As the cells we sample in venous blood are still circulating they are not expected to have displayed sufficiently strong activation signals to enable them to have crossed the blood brain barrier (BBB). No study has yet reported an increase in the expression of the most important endothelial-specific adhesion molecules (ICAM-1, VCAM-1, and E selectin) at the BBB capillaries in AD, which would be necessary to bind the adhesion proteins we find increased in leukocytes at the RNA level [169]. There are, however, reports of clear disruption of the BBB and changes consistent with activation of endothelial cells and increased permeability, particularly in advanced disease [170–175], which is consistent with the profile we found. In some studies, significant numbers of monocytes [144, 154, 176–178] and T-lymphocytes [157, 179–182] are detectable in AD brain. However, as the mice used in these studies were grafted with GFP-expressing hematopoietic cells following irradiation, cell infiltration into the brain could be attributable to loss of BBB integrity as a consequence of irradiation [157, 183]. Other studies find very little evidence for significant leukocyte infiltration in the brain in the absence of disease [184]. It will be important to establish whether the activated phenotype we observed in blood cells very early in disease ultimately results in these cells being able to cross the BBB in patients, or merely reflects a priming of this process. Even if these cells do not actually cross the BBB, the markers of chronic activation we observed suggest these cells could be contributing to the pathology observed at the BBB in AD perhaps by producing damaging ROS.

Genes assigned to the pink, brown, and turquoise modules (associated with secretory/endocytic pathways, metabolism, and transcription, respectively) were altered in MCI blood, but generally returned to normal levels in AD patients. These changes may reflect protective or compensatory mechanisms activated when disease first emerges which abate when established clinical symptoms of disease develop. Interestingly, the two AD susceptibility genes *PICALM* and *BIN1* [158, 159, 162–164, 185], both believed to be important proteins involved in endocytic processing [186, 187], displayed this type of expression pattern. *BIN1* was central to the characteristics of the turquoise

(transcription) module displaying significantly greater expression in MCI subjects and lower expression in AD patients. *BIN1* may be an important regulator of expression of other transcription-associated genes in the turquoise (transcription) module, which would be consistent with a role previously described for this gene [188].

Changes are likely to be cell autonomous

None of the individual leukocyte cell populations analyzed had a higher proportion of cell marker genes altered in MCI or AD than any other cell type, despite MCI patients having significantly fewer monocytes than control and AD patients, and MCI and AD patients having higher numbers of basophils. This indicates no preferential activation of any one particular cell type in disease and suggests that our results do not merely reflect a change in the proportion of different cells in blood. Compared to other cells, B lymphocytes had relatively fewer gene expression changes in AD than might be expected by chance, suggesting that this branch of the adaptive immune system may not be activated in AD [99]. Similar changes in blood cell number have been reported previously [189]. Importantly, our findings suggest that the differentially expressed genes largely represent disease-associated cell autonomous changes and do not merely reflect changes in cell numbers, as there was no clear association between gene expression and individual blood cell populations.

The promise of blood for screening

The strong concordance between the respiratory changes in AD blood and brain suggests blood might be a good model for testing novel therapies, particularly those that target mitochondrial dysfunction. Neuroprotective strategies to neutralize oxygen radicals or alter respiratory chain activity to prevent oxidative damage in AD have been strongly pursued with many drugs such as Coenzyme Q derivatives and Dimebon now being tested in clinical trials. While clearly a relevant target in AD, results of clinical trials targeting mitochondrial dysfunction have thus far been very disappointing. A greater effort to screen promising candidates for disease efficacy in *ex vivo* blood cultures, or using the markers we have identified as outcome measures in *in vivo* screening studies, may perhaps improve the identification of promising candidates and provide data to help triage drugs to progress into clinical trials.

CONCLUSIONS

Overall, it is possible the weak inflammatory signal we find could be offset by the much stronger changes in respiratory chain activity which may be expected to lead to blood cell clearance from circulation to prevent collateral tissue damage. Our results are particularly exciting as they demonstrate mitochondrial and inflammatory changes in the periphery occur very early in AD, some of which are also known mechanisms occurring in AD brain. Fundamentally, these could be used as the basis for identifying people early in disease and for monitoring the efficacy of novel therapeutics for a range of disorders characterized by mitochondrial dysfunction in the CNS and blood. Resolving inflammation and preventing oxidative damage are very active areas being explored as possible targets for drug development in AD and our findings suggest blood could be a very good model in which to evaluate their efficacy.

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