Single nuclear transcriptional signatures of dysfunctional brain vascular homeostasis in Alzheimer's disease

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Abstract

Brain perfusion and normal blood brain barrier integrity are reduced early in Alzheimer's disease (AD). We performed single nucleus RNA sequencing of vascular cells isolated from AD and control brains to characterise pathological transcriptional signatures. We found that endothelial cells (EC) are enriched for expression of genes associated with susceptibility to AD. EC transcriptional signatures identified mechanisms for impaired β-amyloid clearance. Evidence for immune activation was found with upregulation of interferon signalling genes in EC and in pericytes (PC). Transcriptional signatures suggested dysregulation of vascular homeostasis and angiogenesis with upregulation of pro-angiogenic signals (*HIF1A*) and metabolism in EC, but downregulation of homeostatic growth factor pathways (VEGF, EGF, insulin) in EC and PC and of extracellular matrix genes in fibroblasts (FB). Our genomic dissection of vascular cell risk gene enrichment suggests a potentially causal role for EC and defines transcriptional signatures associated with microvascular dysfunction in AD.

Introduction

Alzheimer's disease (AD) is the most common form of dementia[1], characterized by extracellular deposits of toxic forms of β-amyloid (Aβ) protein, intracellular neurofibrillary tangles (NFTs) and neurodegeneration. Large-scale genomic association studies have suggested specific molecular processes responsible for susceptibility to disease[2-4]. The non-neuronal cells in which these genes are predominantly expressed are candidates for early "causal" roles in the initiation of the pathological cascades of AD[5].

Brain microvasculature appears to play a major role in AD pathophysiology[6-8]. Cells constituting the blood brain barrier (BBB) contribute to the clearance of Aβ and other toxic species from the central nervous system (CNS) and allow the selective exclusion of potentially inflammatory or toxic blood proteins from the brain and control of immune cell trafficking[9]. Vascular pericytes are responsible for regulating brain perfusion and contribute to the regulation of endothelial permeability and immune activation[7,10]. Multiple *in vivo* imaging and *post mortem* neuropathological studies, as well as studies of preclinical models, provide evidence for impaired regulation of cerebral blood flow and maintenance of the integrity of the blood brain barrier (BBB) in early AD[11-14]. Recent work has begun to elucidate the transcriptional mechanisms[15-17].

We have performed an integrated analysis of our own single-nuclei RNA sequencing (snRNAseq) data with that from a previously published dataset[18] to quantitatively define the enrichment of brain microvascular cells for the expression of AD risk genes as a test of their potential causal contributions to disease genesis[5]. We then explored the functional roles of AD risk genes by assessing functional enrichment of genes co-expressed with them in vascular cells. Differential expression and gene co-expression analyses allowed characterisation of specific genes and pathways altered in AD. A cell-cell communication analysis further defined signalling mechanisms supporting vascular homeostasis and angiogenesis that are impaired with AD. Together, our results provide a transcriptomic

mechanistic description for major features of the vascular pathophysiology observed *in vivo* with AD.

Results

Endothelial cells are enriched in genes associated with genetic risk for AD

Our analyses were based on data from 57 different brain samples from donors with AD (n=31) or non-diseased controls (NDC, n=26). Fluorescence-activated sorting (FACS) of nuclei isolated before snRNAseq removed neuronal and oligodendrocyte nuclei to achieve a better representation of the less abundant brain cell types of interest. Data was integrated using LIGER[19] and clustered with UMAP[20] (Figure 1A). AD and NDC donor nuclei and nuclei from different datasets, brain regions and sexes were well-mixed after integration (Figure S1). Nuclei numbers did not differ significantly between AD and the NDC samples. Feature plots of canonical cell markers identified major brain cell types in the integrated dataset (Figure S2). Endothelial cells (EC) expressed marker genes *FLT1, VWF, NOSTRIN (*Figure S3A*), CLDN5* and *IFI27*[17,21] (Figure 1C). Specific expression of *COL1A1, COL12A1, COL6A1* (Figure S3B) and *COL5A1* was used to identify fibroblasts (FB) (Figure 1C). A separate, heterogeneous cluster of vascular mural cell nuclei expressed *PDGFRB, RGS5 and GRM8 (*characteristic of PC[17]) (Figure S3C) and *ACTA2* and *MYH11* (highly expressed in smooth muscle cells (SMC)[17]) (Figure 1C). To distinguish PC from SMC nuclei, we re-clustered the EC, FB and vascular mural cell (PC and SMC) nuclei from the total dataset (Figure 1B) to separate those expressing high levels of *ACTA2* and *MYH11* with very low levels of *RGS5* and *GRM8* (corresponding to SMC) from those expressing high levels of *RGS5* and *GRM8* with very low levels of *ACTA2* and *MYH11* (corresponding to PC) (Figure 1C and S4). We confirmed our cluster annotations by demonstrating significant mutual overrepresentations of our cluster markers and those reported previously in human[17] (Figure S5A) and mouse[17,21] (Figure S5B) single nuclei or single cell RNA sequencing studies. To further characterize the identity of the FB population, we tested the overrepresentation of previously described meningeal and

perivascular FB markers[15] and found that our FB markers were more significantly enriched in perivascular fibroblast markers (Fisher's exact test (FET) for overrepresentation: perivascular FB markers, $p = 2.82x10^{-77}$; meningeal FB markers, $p = 6.36x10^{-19}$).

Well-annotated genes associated with genetic risk of AD[2-4] were expressed in nuclei from all four vascular cell types (Figure 1F): 52/61 AD risk genes tested were found in at least one of the vascular cells studied, although less than half of these genes were expressed in 5% or more of nuclei (EC, 21/61; FB, 21/61; SMC, 17/61; PC, 19/61). 14/61 of these genes were expressed in at least 5% of the nuclei across all *four* cell types (*ADAM10, APOE, CD2AP, CELF1, CLU, CNTNAP2, FERMT2, IQCK, MEF2C, PICALM, SORL1, SPPL2A, USP6NL, WWOX*).

We employed MAGMA.Celltyping to test for the significance of the enrichment of vascular nuclei across the larger set of genomic loci associated with AD[5]. First, we analysed a dataset that included all the canonical cell types of the brain (Figure S6-S8). This showed that the AD risk gene expression enrichment is greatest in microglia, as has been reported previously[5] (Figure 1D). Vascular cells also were relatively enriched, albeit less than microglia. To partition enrichment amongst the individual vascular cell types, the analysis was repeated with vascular cell data alone. Only EC were significantly enriched for expression of AD risk genes (Figure 1E). Brain small vessel disease and MRI brain white matter hyperintensities (WMH) are associated with risk of AD[22,23]. To test whether risks for small vessel disease were responsible for the EC enrichment for AD genetic risk, we re-assessed enrichment for the latter after statistically controlling for WMH risk gene expression[24]. The results remained virtually unchanged (Figure 1E): the genetic risk for AD associated to the EC transcriptome thus appears to be independent of that for WMH. However, when the analysis was repeated after controlling for the microglial enrichment, the vascular enrichment largely disappeared, suggesting that similar AD risk gene sets are enriched in microglia and vascular (Figure 1D).

Differential gene expression (DGE) identifies transcriptional signatures of dysfunctional angiogenesis in AD

We employed a mixed-effects model in MAST[25] to discover genes differentially expressed in AD relative to NDC for each of the cell types. Greater numbers of genes were downregulated (90 genes, EC; 47 genes, FB; 47 genes, PC; FDR 0.1), than upregulated (73 genes, EC; 25 genes, FB; 25 genes, PC) in EC, FB and PC (Figure 2A-C). We did not find significant differentially expressed genes (DEG) in the SMC.

Pathological angiogenic transcriptional signatures in AD

Proangiogenic *HIF1A* was overexpressed in EC in AD (Figure 2A). However, the expression of multiple functionally related genes (e.g., *SPRED2, SHC2, KSR1, RASGRF2, DAB2IP, RASAL2, DUSP16, VCL* and *EGFR)* involved in VEGFR2, EGFR and insulin receptor-mediated pathways were downregulated. Pathological angiogenic gene expression signatures also were found in FB with downregulation of the expression of VEGF, FGF, EGF and IGF pathway genes (including *SPRED2*, *DAB2IP* and *SPTBN1*), *DTX2*, a regulator of Notch signalling[26] and the sialyltransferase gene, *ST3GAL6* (Figure 2B, E). Gene expression in PC highlighted a strikingly mixed angiogenic signature with upregulation of the angiogenic Wnt/β-catenin signalling pathway and downregulation of EGF/EGFR signalling pathway genes including *RPS6KA2, ASAP1, MEF2D* and *EGFR* (Figure 2C, F).

Clues to additional mechanisms responsible for loss of BBB integrity in AD were found with the differentially expressed gene signatures. For example, genes contributing to adherens junction assembly (*VCL, TBCD* and *PIP5K1C*) were variably differentially expressed in EC (*VCL* and *TBCD* were downregulated, whereas *PIP5K1C* was upregulated). As noted above, Wnt/β-catenin pathway genes, including *TCF4* and *APC,* expression of which also support blood brain barrier integrity[27], are significantly upregulated with AD in PC. Finally, *LAMC1,* encoding for the gamma laminin subunit, was significantly downregulated in FB.

Differential expression of risk genes with functional roles in amyloid processing and immune response in AD

Risk genes with functional roles related to amyloid processing were differentially expressed in AD. *ADAM10*, which encodes the constitutive α-secretase that governs non-amyloidogenic pathway β-amyloid precursor protein processing, was significantly upregulated in PC. *PICALM*, encoding a clathrin assembly protein modulating clearance of Aβ, was downregulated in EC. Risk genes related to immune responses also were differentially expressed with AD. Increased expression of the inhibitory complement receptor *CD46* gene and decreased expression of *IRAK3,* which encodes an IL-1 receptor associated kinase were found in PC. In EC, we also found increased expression of *IFITM3,* which regulates interferon pathway inflammatory responses[28] and can potentiate gamma secretase activity[29].

Co-expression modules for angiogenesis, lysosomal processing and interferon activation are differentially regulated with AD

To identify gene co-expression modules differentially expressed with AD, we first performed gene co-expression network analyses separately for EC, FB and PC pooled across AD and NDC (MEGENA[30]). We then determined which gene co-expression modules were differentially associated with AD (limma[31]). SMC were not included in this analysis because of the relatively low number of nuclei available for analysis and consequent sparse co-expression representation. Our results defined cell-specific gene regulation pathways associated with AD.

Reduced expression of co-expression modules enriched for angiogenesis and vascular homeostasis

Although fold-changes varied, expression of modules enriched for multiple angiogenic or vascular homeostasis pathways were generally decreased with AD, most prominently for EC (Figure 3), e.g., Module 119, which is enriched in the EGF/EGFR signalling pathway (e.g., including *EGFR*, *IQSEC1*, *INPP5D*, *NEDD4* and *IQGAP1*) and several hub genes (e.g., the G-protein activator and adhesion G protein coupled receptor genes, *DOCK9* and *ADGRL4,* respectively, and the hypoxia inducible transcription factor, *EPAS1)* with roles in angiogenesis[32,33]. Expression also was reduced for module 47, which is enriched in insulin-

like growth factor 1 receptor (IGF1R) (*IGF1R*, *PSMD5*, *PSMD1*, *TSC1*, *RASAL2*) and Ras signalling cascade (*PSMD5*, *PSMD1*, *RASAL2*) genes. Expression of several genes in module 47 with recognised functional roles in angiogenesis (e.g., *RASAL2* and *PALD1*[34]) were significantly independently downregulated in AD.

While the fold-changes were relatively low, FB modules functionally related to vascular homeostasis also were significantly downregulated, e.g., Module 2, which was significantly enriched in NOTCH signalling genes (Figure 3E), including *NOTCH1* and *NOTCH2*, as well as the regulators of NOTCH expression[26], *ARRB1* and *DTX2,* both of which also were independently significantly differentially reduced in expression with AD. Module 2 also is significantly enriched in genes individually downregulated in AD (FET adjusted $p=1.28x10^{-8}$). Some of them (*SPRED2*, *DAB2IP*, *ARRB1*, *SPTBN1)* encode for proteins that are downstream components of several growth factor signalling pathways (e.g., FGFR1-4, VEGFR2, EGFR) or genes for components (*COL5A1* and *COL1A2*) of the extracellular matrix (ECM)[35]. Angiogenesis pathway enriched modules were downregulated in PC, as well, e.g., module 19 (Figure 3F), in which several genes showing individually significantly reduced expression in AD (*EGFR*, *ZBTB16*, *IRAK3*, *TMTC1*, *MAOA*) and genes involved in EGFR signalling (*EGFR, MAPK1, FOXO3*) are found or module 15, which is enriched in PI3K-Akt signalling pathway genes (*ANGPT2, COL6A2*, *DDIT4*, *COL6A1*, *BCL2*, *PDGFA*, *PPP2R3B*, PPP2R3A) genes[36]. A recent report has shown the *Angpt2* knock out potentiates BBB leakage in a preclinical Aβ mouse model[37].

However, despite the decreased expression of modules enriched for many angiogenic pathways central to angiogenesis with AD, we also found modules enriched in metabolic pathways supporting angiogenesis in EC and PC and extracellular matrix genes in FB, the expression of which was *increased* in AD. Module 661, the top upregulated module in AD, includes *TPI1* that encodes for the triosephosphate isomerase, an enzyme implicated in glycolysis and gluconeogenesis[38]. Module 41, the expression of which is increased with AD, includes genes encoding for the acyl-CoA dehydrogenase that executes the first step of the β-oxidation of fatty acids (*ACAD8, ACADS)* and genes of the butanoate metabolism pathway

(*BDH2, ACSM3, and ACADS*)[39], both of which are highly upregulated in AD. Module 12, also increased in expression with AD, is amongst several in EC that were enriched for pathways coding for proteins of the mitochondrial respiratory chain complexes, including *ATP5PF*, *ATP5PD, ATP6 NDUFB10* and *NDUFS3*. The most highly upregulated module in PC, module 512, was enriched in oxidative phosphorylation genes (*NDUFA4L2, ATP5MC2, ATP5F1D*) and for lipid metabolism pathway genes (*PDHA1*, *PRKAB1*)[40,41]. Fibroblasts play a central role in the development of the basement membrane. Extracellular matrix collagen genes (*COL3A1, COL5A2, COL5A3, COL11A1, COL21A1*), the fibronectin 1 gene (*FN1)*, proteoglycan and glycosaminoglycan metabolism-related genes (*CHSY3, GXYLT2, GPC6, CHST15, TIAM1, KDR, PLCE1, COL21A1, FLNB, ANK3, TP53*) were enriched in modules upregulated with AD in FB, e.g., in branches of modules 128, 135, 164, 408, 488 and 775. Modules 408 and 391, also upregulated with AD in FB, were enriched in solute carrier genes involved in nutrient and metabolite transfer across the blood brain barrier[42].

Increased expression of APOE and lysosomal pathway enriched modules in FB and PC with AD

Module 396, the most highly upregulated module in FB with AD, was enriched in the AD risk gene *APOE* and other cholesterol metabolism-related genes (*e.g., AGT),* as well as genes with individually increased expression in AD that are related to pathways for lytic vacuole functions (including *CACNG7*, *VPS28*, *CTSO, RRAGC, NPC2, GAA, HPS4, CTNS, RAB9A, VAMP4, VPS16* and *RAB7A*). Expression of module 512, which is enriched for lipid metabolism (with *PDHA1* and *PRKAB1*) and lysosome (with *PSAP, CLTB* and *CTSF*) pathways, increased in PC, whereas other lipid processing and metabolic gene pathways were downregulated with AD, notably in module 500 (enriched for the sphingolipid signalling pathway genes *AKT3, MAPK14, PLCB1* and *NSMAF*). FB modules 2 (enriched for “regulation of lipid metabolism by PPAR-α” and “metabolism of lipids and lipoproteins” pathways) and modules 468 (enriched for “response to lipid”) and 284 (enriched for the “ABC transporters in lipid homeostasis” pathway) also were downregulated with AD.

Module enrichments suggest pathological amyloid processing and immune responses in AD

There was evidence for upregulation of Aβ production and immune responses with AD. Module 41, which was highly upregulated in EC, contains *PSEN2* and *APH1A*, which encode for components of the gamma secretase[43]. We also found increased expression of interferon-related co-expression modules in EC (module 661, the most highly upregulated modules includes *C2* and *TRIM5* which is a interferon type I-stimulated gene[44]) and in PC (module 161 includes *RNASEL, EIF4A3, AAAS* and *IFIT1*) with AD. The most highly upregulated module in PC (Module 512) is enriched for pathways for ferroptosis (*GPX4, FTH1)*, which promotes release of oxidised lipid species that generate pro-inflammatory damage-associated molecular patterns (DAMPs) able to trigger the innate immune system[45]. However, modules enriched for other immune response pathways were *downregulated* with AD, e.g., EC module 455, which is enriched for IL-6 signalling (*NLK, IL6ST, JAK1*), and PC module 19, enriched for cytokine signalling, and modules 32 and 500, which are enriched for Toll-like response genes. This was most striking for FB, for which the largest number of immune response modules differentially expressed was identified (modules 468 and 154 enriched for interferon-alpha responses, module 2 enriched for the Toll-like receptor 9 (TLR9) cascade, module 169 enriched for cytokine signalling (*IL15, KIT, RAF1)* and module 16 enriched for JAK-STAT signalling), all of which were downregulated with AD.

Two-layer neighbourhood analysis of risk genes suggests cell-specific mechanisms of AD susceptibility

Cell-specific enrichments for risk genes expression provide insights into the genesis AD[5]. Cell specific co-expression signatures can suggest specific functional roles for the risk genes in susceptibility. To explore those relevant to the cerebral microvasculature, we identified genes having the most direct expression correlations (those within a two-layer neighbourhood, i.e., any gene that is either directly connected to an AD risk gene or through at most one other gene) with AD risk genes in the vascular cell-specific co-expression networks generated from

both AD and NDC[30,46]. To discover relationships specifically relevant to disease genesis, we determined the overrepresentation of genes differentially expressed with AD in the neighbourhoods of each GWAS gene in the cell-specific co-expression networks (Figure 4A).

AD risk genes *PICALM, SORL1* and *INPP5D* had the largest neighbourhoods (230, 167 and 121 differentially expressed genes, respectively) in the EC co-expression network. The neighbourhood of *PICALM* was enriched in IL-6 signalling genes (*IL6ST, STAT3, JAK1*), as well as semaphorin (*SEMA5A*, *ARHGEF11*, *SEMA6D*, *ITGA1*, *MYH11*, *PLXNC1*) and NOTCH signalling-related genes (*TNRC6C, B4GALT1, TFDP2, POFUT1, MAMLD1, TNRC6A*). The *SORL1* neighbourhood also was enriched in genes for pathways involved in immune response (e.g., T-cell activation, TYROBP causal network and cytokine response-related pathways) and proteoglycan metabolism (chondroitin sulfate biosynthesis and proteoglycan metabolism) pathway genes encoding proteins essential for vascular extracellular matrix formation. The functional enrichment of the *INPP5D* neighbourhood showed an overrepresentation of individual differentially expressed genes that were downregulated in insulin- and EGF/EGFR-signalling pathways (Figure 4B).

While the co-expression neighbourhoods of AD risk genes *WWOX*, *CLU and CCN2* expressed in FB (e.g., including individually differentially regulated genes *ITPR2*, *ROBO1*, *LHFPL6* and *SLC38A1 [WWOX]*; *PALD1, ZBTB46, PDZD2, SIL1* [CLU]; *SPTBN1*, *ZMIZ1*, *KLF7*, *CACNA2D3 [CCN2]*) and PC were enriched for genes involved in a range of functions, the neighbourhood of *WWOX* also was enriched in Notch signalling pathway genes and both this neighbourhood and that of *IQCK* were enriched in ECM-related pathways (Figure 4C). In PC, the neighbourhood of *MEF2C* transcription factor (108 genes) (one of the largest) included genes involved in the EGF/EGFR signalling pathway (*EPS8*, *MEF2A*, *MEF2C*, *PLCE1*, *RAF1*) and Toll-like receptor pathways (e.g. *MEF2A*, *PPP2CB*, *MEF2C* and *RIPK2*) (Figure 4D).

Pathological growth factor and ECM signalling between vascular cells with AD

We applied CellChat to identify differentially expressed receptor-ligand pairs responsible for cell-cell signalling (both autocrine and paracrine) in AD[47]. 759 potential ligand-receptor

pairs implicating 48 distinct signalling pathways were identified amongst genes expressed EC, PC and FB (Figure 5). We first focused on assessment of interaction pairs that involved DEG and pathways enriched in co-expression modules specifically with AD. The analysis provided insight into mechanisms contributing to dysregulated angiogenesis with AD. Several VEGF-related signalling pairs were detected only in NDC. Signalling via INHBA (FB/PC) – ACVR1B and ACVR1A (EC) also was found only in NDC. By contrast, evidence for TGFB3 (EC) – TGFBR1_R2 (FB) and the TGFB3 (EC) – ACVR21B_TGFBR2 (FB) signalling was found only in AD samples.

Growth factor signalling important for angiogenesis and vascular homeostasis also was generally decreased with AD. Receptor expression for the EGF(EC/PC)-EGFR(EC/PC), EGF(EC/PC) - EGFR_ERBB2(PC), NTF3(FB/PC) - NTRK3(FB) communication pairs was reduced. Decreased expression of FB *LAMC1* should reduce autocrine and EC/PC CD44 signalling. Increased expression of *CD46* in PC with AD may modulate NOTCH signalling in an autocrine manner (CD46 (PC)- JAG1(PC))[48]. Autocrine NEGR1(FB)- NEGR1(FB) signalling also increases in AD. Vascular cell interactions with the ECM were reduced with decreased integrin expression on EC affecting multiple ligand-receptor communication pairs: COL1A2 (EC/FB) - ITGB1 (EC), COL1A2 (FB) -ITGA2 (EC), COL4A1 (FB/PC) - ITGA2 (EC) or COL4A2 (EC/FB) - ITGB1 (EC), COL4A4 (PC) - ITGA2_ITGB1(PC) and COL6A3 (FB) - ITGA2_ITGB1 (EC). Both lower EC and PC integrin and FB laminin expression in AD also should reduce the many laminin-ECM interactions identified (LAMC1 (FB) - ITGA1_ITGB1 (EC/PC) - ITGA2_ITGB1 (EC), LAMA3 (EC) - ITGA2_ITGB1(EC), LAMA4 (FB/PC) - ITGA2_ITGB1(EC), LAMB1 (FB) - ITGA2_ITGB1(EC), LAMC1 (FB) - ITGA2_ITGB1 (EC/PC), LAMC3 (EB/PC) - ITGA2_ITGB1 (EC), LAMC3 (FB/PC) - ITGA2_ITGB1 (EC/PC), LAMC1 (FB) - ITGA6_ITGB1(EC/PC), LAMC1 (FC) - ITGA7_ITGB1 (EC/PC)).

Expression of vascular endothelial angiogenic pathway genes is related to Aβ plaque and phosphorylated Tau (pTau) load

To explore how vascular transcriptomic pathology may evolve over the course of AD, we performed an exploratory analysis of gene expression as a regression of the individual brain regional Aβ and pTau densities in the 24/57 brain samples used in the analyses above for which quantitative IHC was available. We limited this regression analysis to the EC, the most abundant of the cell populations, to minimise Type I errors. We found 28 genes were significantly (adjusted $p<0.1$) differentially expressed with greater brain regional Aβ plaque density and 75 genes differentially expressed with greater pTau density. Genes showing significant regression with Aβ density in EC were enriched in VEGF signalling pathway components (*CYFIP1*, *PXN*, *CTNNA1*, *CALM1*). By contrast, genes that showed significant regression against pTau were enriched in EGFR, IGF1R and insulin receptor signalling pathways (*PPP2R5E*, *NF1*, *CALM1*, *DUSP16*, *AP2M1*, *RBX1*).

Discussion

Brain vascular structural pathology and physiological dysfunction is characteristic of both preclinical models expressing brain Aβ and of AD[13,49]. We found that, although EC, SMC and PC all express AD risk genes, EC appear to be uniquely *significantly* enriched amongst vascular cells, suggesting a quantitatively more important role in "causally" mediating AD susceptibility. Our results provided evidence that pathological regulation of angiogenic gene expression contributes to the early vascular impairments in AD: we found upregulation of the pro-angiogenic *HIF1A* and increased expression of mitochondrial oxidative and fatty acid oxidation genes, metabolic drivers of angiogenesis[50], in conjunction with *downregulation* of vascular developmental and homeostatic pathways involving EGF/EGFR, VEGF/VEGFR and insulin and IGF signalling in EC and PC and reduced expression of laminin and collagen IV genes in FB. The correlation of these expression differences in angiogenic growth factor pathways in EC with both the Aβ plaque and pTau burden suggests that impairments of vascular growth factor pathway signalling worsen progressively through the course of the disease. Analyses of cell-cell communication provided novel evidence for decreased signalling

though VEGFR2 and components of the ECM to EC, decreased expression of the LDLR pathway for clearance of Aβ from the CNS by EC, interferon pathway activation and increased inflammatory responses in EC and PC. Together, these processes would be expected to reduce clearance of Aβ while also, via inflammatory activation, increasing pathological Aβ production and processing[29]. Specific evidence for this was found with upregulation in EC of *PSEN2* and *APH1A*, which encode for components of the gamma secretase.

A recent preprint also reported that GWAS risk genes were enriched in EC and vascular mural cells and suggested an "evolutionary shift" of AD risk gene expression from a singular predominance in microglia in the mouse to microglial and vascular cells in humans[15]. Our analysis extends this by showing that, among vascular cells, risk genes associated with AD are enriched significantly in EC, suggesting an involvement of EC in the early genesis of AD[5]. Moreover, we showed that the risk genes enriched for expression in EC overlap substantially with those in microglia (Figure 1D).

Co-expression and two-layer neighbourhood analyses provided insights into some possible functional roles for proteins encoded by AD risk genes expressed in the vascular cells. For example, our results showed lower expression of *PICALM* in EC with AD, suggesting a mechanism by which vascular clearance of Aβ is reduced in AD[27,51]. Functionally less well characterised AD GWAS genes, *WWOX* and *IQCK,* have large neighbourhoods in the FB and PC co-expression networks associated with enrichment for pathways supporting maintenance of BBB integrity[9]. Finally, *INPP5D* had consistently one of the largest two-layer neighbourhoods across the vascular cell types, whereas the enriched pathways associated with them were cell type specific (e.g., EGF/EGFR in EC and ECM components in FB).

Innate immune responses are central to AD pathogenesis and progression but have not been well defined in the microvasculature to date[29,52]. We found evidence for cell-specific differences in vascular inflammatory responses to AD in EC with upregulation of interferon signalling genes in EC. PC also appear to play a role in vascular inflammatory mediation of early AD. Recently identified risk genes *CD46,* encoding a serine protease which mediates

inactivation of complement proteins, and *IRAK3,* encoding a homeostatic mediator of innate immune responses[53] were upregulated and downregulated, respectively, in PC.

However, the most strikingly differentially expressed gene sets in AD are involved in angiogenesis and vascular homeostasis. VEGF/VEGFR and insulin signalling pathways[54] in EC and EGF/EGFR signalling in EC and PC were downregulated with AD[55] and negatively correlated with Aβ pathology, despite upregulation of other genes (e.g. *HIF1A)* associated with pro-angiogenic regulation[28]. These results add to prior evidence of *dysfunctional angiogenesis* in AD[16,43,49]. We have extended descriptions by showing that, despite angiogenic signals (e.g., upregulation of *HIF1A*) and metabolic adaptations, major downstream effector pathways fail to respond at the transcriptional level.

Previous reports also have implicated dysfunction of both EC and PC in AD[56]. Our observations emphasise the extent to which pathology of *FB* also contribute to vascular abnormalities in AD, with NOTCH signalling as a candidate mechanism central for this. Multiple genes encoding for ECM components were downregulated in FB, which should contribute to BBB dysfunction[57,58], impair angiogenesis[35] and lead to altered expression of tight junction proteins in EC[58] via reduced signalling between ECM proteins in FB and integrins in EC[59].

Although we have made a number of novel observations, our analyses had limitations which need to be addressed in future work. Our data was generated from nuclei from multiple brain regions and thus could address robustly only those transcriptomic differences that were common to all of these regions in AD, even given that we took the confound of brain region into account as a fixed effect in the statistical models. A second limitation was that we assessed the total extracted populations of nuclei without seeking to identify and separately study cells expressed from specific vascular anatomic zones[15,17]. Nevertheless, the high overlap of our cellular markers and the cellular markers from human and mouse brain vessel-associated cells in previous reports provides some confidence that all major cell types were represented. Third, like other recent studies, our conclusions are based on relatively sparse (10X Genomics Single Cell 3' Gene Expression assay) sequencing of the nuclear transcriptome,

which may be biased relative to those from the whole cell, potentially reducing the power to detect transcripts from some genes[60]. Use of larger numbers of nuclear and co-expression-based analyses, which rely less on detection of absolute expression levels than do single gene differential expression analyses, may have reduced the impact of this although the impact of this limitation, but this is difficult to assess without future, more comprehensive transcriptional analyses of the whole cells.

Impairment of angiogenesis and vascular homeostasis, reduced endothelial Aβ clearance with reduced expression of *PICALM* and increased production of Aβ by EC with upregulation of interferon (*IFITM3*) and γ-secretase component genes all will act to increase toxic Aβ concentrations in the brain[29]. The extraordinary length of the brain capillary network (~650 km) and its large surface area (~120 cm^2/g) suggest that even small relative effects could contribute substantially to increasing the overall Aβ burden in the CNS[61]. The identification of significant EC enrichment in AD risk genes also suggests that their specific contribution to inflammatory activation and reduced Aβ clearance are early, potentially "causal" factors in the onset of sporadic, late onset AD. Our work suggests specific mechanisms by which small vessel disease from many causes (e.g., metabolic disease, hypertension, smoking) could potentiate early AD and add to the rationale for AD prevention through interventions for control of modifiable cardiometabolic risk factors[62]. More generally, our results suggest that EC therapeutic targets related to angiogenic, inflammatory and Aβ clearance pathways deserve prioritisation in the search for treatments able to slow or prevent the onset of AD.