Title:

Genetic risk in endolysosomal network genes correlates with endolysosomal dysfunction across neural cell types in Alzheimer's disease

Mamde S*[1], Rose SE*[2], Prater KE*[3], Cochoit A[3], Lin YF[4], Smith I[3], Johnson CS[3], Reid AN[3], Qiu W[4], Strohbehn S[5], Keene CD[2], Rolf BA[5], Lin KZ[6], Lee SI[4], Garden GA[7], Blue EE[5], Young JE[#2,8], Jayadev S[#3,5,8]

1. Department of Cellular and Molecular Medicine, University of California San Diego, San Diego, CA
2. Department of Laboratory Medicine and Pathology, University of Washington, Seattle WA
3. Department of Neurology, University of Washington, Seattle WA
4. Paul G Allen School of Computer Science and Engineering, University of Washington, Seattle WA
5. Department of Medicine, Division Medical Genetics, University of Washington, Seattle WA
6. Department of Biostatistics, School of Public Health, University of Washington, Seattle WA
7. Department of Neurology, University of North Carolina, Chapell Hill, NC
8. Institute of Stem Cell and Regenerative Medicine, University of Washington, Seattle WA

***** authors contributed equally to this work
# co-corresponding authors

Correspondence:
Jessica Young PhD: jeyoung@uw.edu
Suman Jayadev MD: sumie@uw.edu

Running Title: Cell-type specific molecular and functional effects of AD risk variants

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ABSTRACT

Late-onset Alzheimer's disease (LOAD) has a complex genomic architecture with risk variants in multiple pathways, including the endolysosomal network (ELN). Whether genetic risk in specific pathways correlates with corresponding biological dysfunction remains largely unknown. We developed an endolysosomal pathway-specific polygenic risk score (ePRS) using 13 established AD GWAS loci containing ELN genes. We investigated the association between ePRS and AD neuropathology, then examined cell-specific endolysosomal morphology and transcriptomic profiles in post-mortem dorsolateral prefrontal cortex samples from donors stratified by ePRS burden. We found that the ePRS was significantly associated with AD diagnosis and neuropathological measures, comparable to a pathway-agnostic PRS despite representing far fewer loci. High ePRS correlated with increased neuronal endosome volume, number and perinuclear aggregation, as well as enlarged microglial lysosomes, independent of AD pathology. Single-nucleus RNA sequencing revealed cell-type transcriptomic changes associated with ePRS status, including glutamatergic signaling, protein homeostasis, responses to DNA damage and immune function. Neurons, astrocytes, oligodendrocytes, and microglia showed varied gene expression patterns associated with ePRS burden.

Conclusions: This study provides evidence that AD genetic risk variants harboring ELN genes correlate with endolysosomal dysfunction in human brain tissue. These findings suggest that pathway-specific genetic risk contributes to corresponding cellular pathology in AD and nominates candidate mechanisms by which ELN AD variants contribute to pathogenesis.

INTRODUCTION

Recent clinical trials targeting amyloid beta removal in late-onset Alzheimer's disease (LOAD) have shown modest potential benefits, and there is room for more effective interventions. Drug development in LOAD has been hindered by an incomplete understanding of its mechanisms, largely due to the disease's genetic, pathologic and mechanistic heterogeneity. While amyloid beta is present in AD brains and thought to be necessary for the development of the AD phenotype, the progressive degeneration leading to cognitive decline results from multiple cellular responses across several disease phases[3]. Elucidating these aspects of disease progression may reveal additional intervention targets.

Genome-Wide Association Studies (GWAS) and candidate gene approaches have revealed the complex genomic architecture underlying LOAD[3-8] by identifying single nucleotide polymorphisms (SNPs) which tag discrete regions of chromosomes or loci, associated with AD. Genes within these risk loci give pathogenic clues to etiology; genomic regions correlated to AD may harbor genes relevant to AD pathophysiology. Notably, GWAS of LOAD involve hundreds of thousands of individuals but detailed molecular studies of human brain tissue are not currently feasible at that scale due to limitations in tissue availability and resources. Therefore, human genetic variation presents a significant challenge to investigating LOAD pathogenesis when attempting to derive mechanistic insights from patient samples in a reasonably sized cohort. In this study, we developed approaches to this challenge that would leverage, rather than be limited by human variation.

The aggregate burden of common genetic risk identified in GWAS can be estimated using polygenic risk scores (PRS) which calculate a cumulative load of AD genetic SNPs weighted by factors including odds ratio, that predict an association of variant burden with risk for AD incidence [9-11], as well as various AD biomarkers, neuropathology and endophenotypes[12-19]. GWAS have identified variant haplotypes with genes in immune, synaptic function, lipid metabolism, endolysosomal pathways among others[20] nominating those pathways as candidate AD drivers. Whether variant burden in a specific pathway actually correlates to observable pathway changes is largely not known and remains a significant gap in the goal to map the mechanisms linking variant to disease pathogenesis. The endolysosomal network (ELN) plays important cell-type specific roles in brain homeostasis and is already strongly linked to AD pathogenesis[21-23]. However, it is not known if endolysosomal network risk loci contribute to observed ELN pathology

in AD, and if they confer additional gene regulatory or functional changes relevant to AD pathology. Further, the cell type specific consequences of AD variants are not well established yet clearly important to fully understanding pathophysiology. Determining whether or not having a higher burden of ELN risk portends a more likely role for ELN dysfunction could have important implications for future development of targeted therapies.

We hypothesized that a higher burden of ELN risk loci drives endolysosomal pathology in AD and this could be assessed when comparing to AD without high ELN genetic risk burden. In the context of AD pathology we predicted gene expression profiles would be distinct between high and low ELN burden in donors with neuropathologically confirmed AD. To test our hypotheses, we investigated the correlation of an endolysosomal polygenic risk score (ePRS), representing 13 established AD GWAS loci which contain genes of the endolysosomal network, with cell-type specific cellular and molecular phenotypes in neuropathologically confirmed AD and control brain tissue.

Gene	rsID	β	*p*-value
BIN1	rs6733839	0.0650	1.01E-37
BIN1	rs744373	0.0476	1.68E-19
CD2AP	rs10948363	0.0311	3.11E-08
CD33	rs3865444	0.0164	4.49E-03
CLU	rs9331896	0.0245	1.74E-06
EPHA1	rs11771145	-0.0188	2.28E-04
HLA-DRB5	rs9271192	-0.0257	5.31E-06
MEF2C	rs190982	0.0113	3.94E-02
MS4A6A	rs983392	-0.0326	2.37E-09
MS4A4E	rs670139	0.0243	1.28E-06
PICALM	rs10792832	0.0344	1.48E-10
PTK2B	rs28834970	0.0244	3.89E-06
SORL1	rs11218343	-0.0468	8.14E-05

Table 1:

Thirteen variants identified through AD GWAS used to create the ePRS score.

RESULTS

Pathway Specific PRS predicts AD status and AD neuropathologic measures

PRS combine the effects of many SNPs into a single measure of accumulated risk, and their association with AD risk varies by which variants are included and how their signals are

combined [25]. PRS limited to AD GWAS hits implicating genes within specific pathways can also provide useful insight. For example, endocytosis-focused PRS are associated with risk of AD and mild cognitive impairment [19], AD biomarkers [12, 15, 16], and multiple cognitive measures[12].
We hypothesized that the cumulative burden of ELN-affiliated risk alleles impacts cell type-specific ELN function, thus contributing to LOAD pathogenesis. To test this hypothesis, we first developed an ELN pathway-specific polygenic risk score (ePRS) combining information from 13 AD GWAS SNPs (Table 1) and contrasted it with a pathway-agnostic PRS focused on common AD GWAS hits with relatively strong effect sizes (Table 1 + rs7274581, rs10838725, rs6656401, rs8093731, rs17125944, rs35349669, rs2718058, rs10498633, rs1476679)[4, 26, 27]. We calculated both PRS in a local sample of clinically and neuropathologically confirmed AD cases and controls and the AD Sequencing Project data (ADSP; n = 10967 cases, 16432 controls). We tested their association with AD neuropathology after adjusting for sex and *APOE* ε4 carrier status in the UW sample and sex, ε2, ε4, population structure, and relatedness in the ADSP. We found that the significant association between ePRS and multiple AD neuropathology measures in the UW and ADSP samples was comparable to the pathway-agnostic PRS despite representing ~50% of the AD GWAS loci (p-value < 0.05; Figure 1).

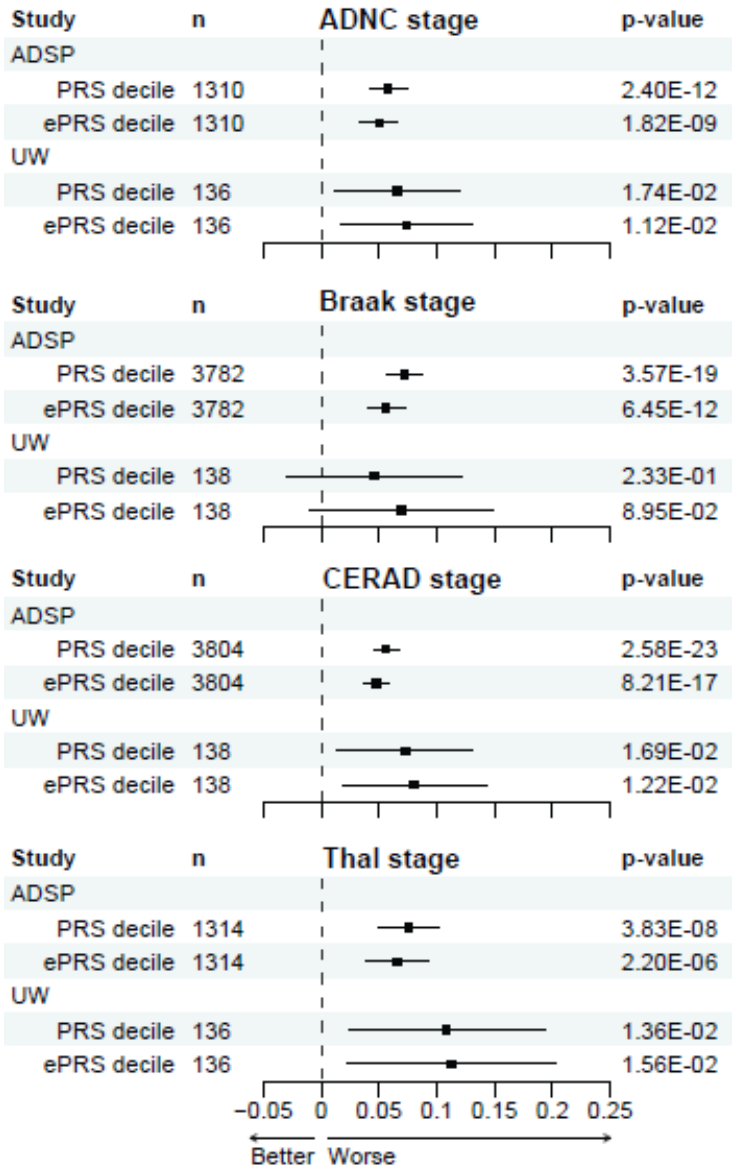

Figure 1

Association between ePRS, PRS, and AD neuropathology. X-axis: effect size (point) and 95% confidence interval (line) for association with ADNC [1], Braak [2], CERAD [2], and Thal [24] scores. Covariates include sex + ε4 carrier status (UW) or sex, ε2 and ε4 dose, population structure, and relatedness (ADSP).

ePRS is associated with cell type specific endolysosomal cytopathology

The relationship between abnormal and enlarged early endosome pathology and AD is well established in the literature, both in post-mortem brain and model systems[28-31]. We analyzed

early endosomes in neurons from high and low ePRS cases from our tissue cohort (Table 2) using quantitative immunofluorescence and confocal microscopy. Due to the abundance of these organelles in neurons, we analyzed early endosome changes in the somatodendritic and perinuclear neuronal regions separately. Figure 2 A-D shows representative neurons at different ADNC stages (0-3). We observed a higher number of endosomes in neurons with high ePRS (Fig 2E,F), a finding seen in other neurodegenerative disease studies[32], and suggestive of altered function. Increased perinuclear early endosome aggregation with high ePRS was observed across ADNC levels; for example, the representative AD case with low ePRS in Figure 2C has smaller and fewer endosomes in the perinuclear region than the high ePRS individual who also has AD pathology (Figure 2D). This relationship between increasing ePRS and somatodendritic EE density remained significant even when intermediate-high AD cases only were included in the model ("ADNC 2/3 cases", red). Interestingly, the high ePRS genetic background was also significantly correlated with enlarged endosome size in the perinuclear region in cases with intermediate to high AD pathology although not in the somatodendritic region (Fig 2E-F, red bars). Perinuclear aggregation of endolysosomes has been observed in LOAD hippocampal neurons[33] and we also noted striking aggregation of early endosomes in the perinuclear region of high ePRS cases with AD pathology (Fig 2G).

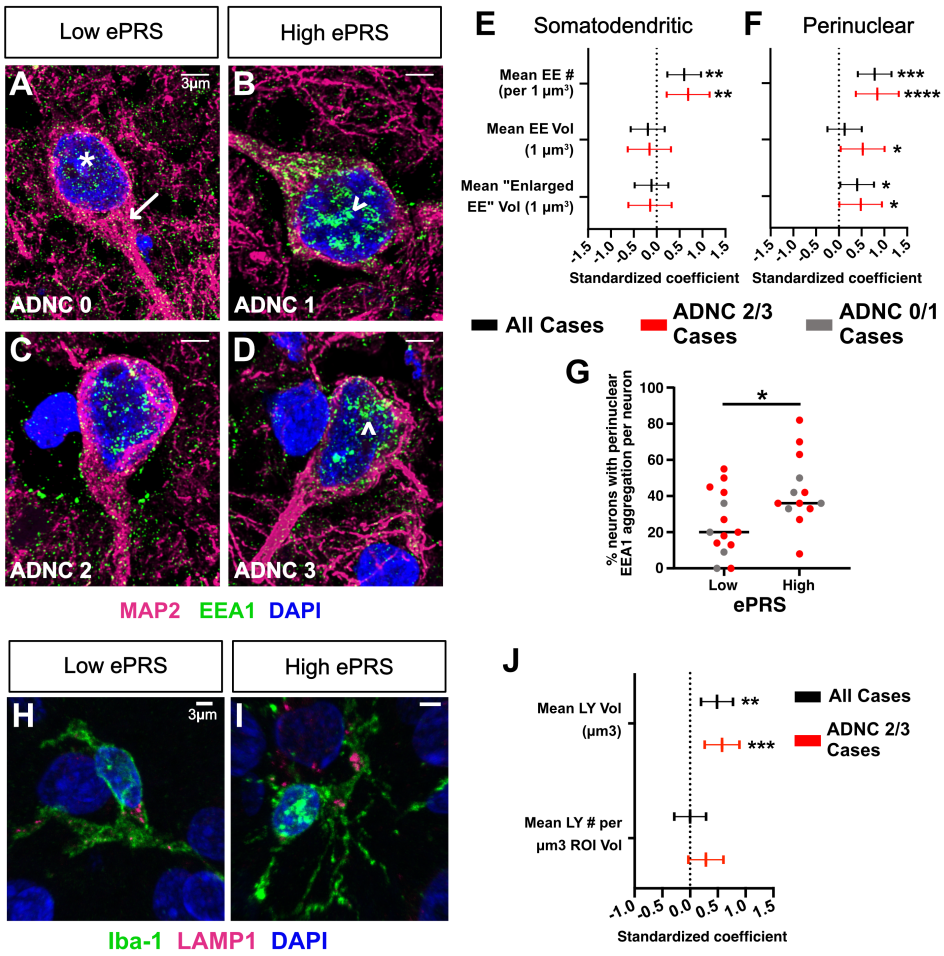

Figure 2:

ePRS shifts the size and number of early endosomes in the perinuclear compartment in post-mortem cortical neurons and the size of lysosomes in post-mortem cortical microglia. A-D) Representative pyramidal neurons from postmortem parietal cortex. MAP2 (magenta) labels somatodendritic region of the neuron compartment, EEA1 (green) labels early endosomes (EEs), and DAPI (blue) labels nuclei. Perinuclear region of interest (ROI) noted with asterisk; somatodendritic ROI noted with arrow. A, C) Neurons from low ePRS cases have minimal EE aggregation in the perinuclear region, shown in both A) no-low AD (ADNC 0/1) and C) AD (ADNC 2/3) cases. B, D) Neurons in high ePRS cases have distinct increase in perinuclear EE aggregation (white arrowheads), shown in both B) no-low AD (ADNC 0/1) and D) AD (ADNC 2/3) cases. E-F) Correlation between ePRS and EE morphology metrics using a linear regression model. E) High ePRS correlates with a significant increase in somatodendritic EE number in the full cohort ("All cases", black). F) High ePRS correlates with a significant increase in perinuclear EE number and size in the full cohort ("All cases", black). G) The percentage of neurons imaged per case with clear perinuclear EE aggregation (neurons assigned yes/no for presence of aggregation) was significantly increased in the high ePRS cases compared to low ePRS. ADNC 2/3 cases in red to show that AD cases are represented in both low and high ePRS groups. H) Representative image of a microglia in an individual with low ePRS and AD pathology. I) Representative image of a microglia with high ePRS and AD pathology. J) High ePRS is correlated with increased mean lysosomal volume in both all cases and individuals with AD pathology. Scale bars are 3 µm in all images. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Microglial lysosomes are larger in high ePRS individuals.

Quantitative analysis of lysosome volume and number per microglia was performed on LAMP1 immunostaining of Iba-1 positive cells in post-mortem human DLPFC tissues (Fig 2H, I) (Table 3). We found that similar to endosome morphology in neurons, microglial lysosome correlated to ePRS. LAMP1 immunoreactivity was used to assess lysosomal content. We analyzed mean lysosomal volume, and the number of lysosomes present per micron of microglial cell while adjusting for age, sex, post-mortem interval and *APOE* genotype. When comparing high versus low ePRS cases regardless of AD pathology, or in just cases with high AD pathology high ePRS was significantly associated with larger lysosomal mean volume but not the number of lysosomes (Fig 2J).

ELN genes in ePRS variant loci are expressed across brain cell types in AD DLPFC tissue.

Single-cell/nucleus omics studies of affected AD brain tissue have dramatically improved our understanding of cellular interactions and responses to pathology[34-37]. They serve as platforms for hypothesis generation, enabling exploration of cell-type specific pathways and gene networks that may be obscured in bulk tissue RNA sequencing. We performed single nucleus sequencing (snRNAseq) from 11 low ePRS, high ADNC and 12 high ePRS, high ADNC DLPFC donors (Table 4). The output of the snRNAseq analysis from the 23 donors demonstrated good representation of all the standard brain cell types (Fig 3A; Emani et al., 2024). We found roughly similar composition of cell types between high and low ePRS donors, though with a statistically significantly smaller cluster of microglia in high ePRS (Fig 3B). We first established the expression profile of the endolysosomal genes associated with the ePRS variants. As expected, there was variable expression across cell types (Fig 3C). *PICALM* and *MEF2C* were expressed in many cell types, most highly in microglia, while other genes were more selectively expressed such as *CD2AP* or *CLU*. The differential pattern of expression underscores that there may be cell-type biased effects of ePRS AD variant burden.

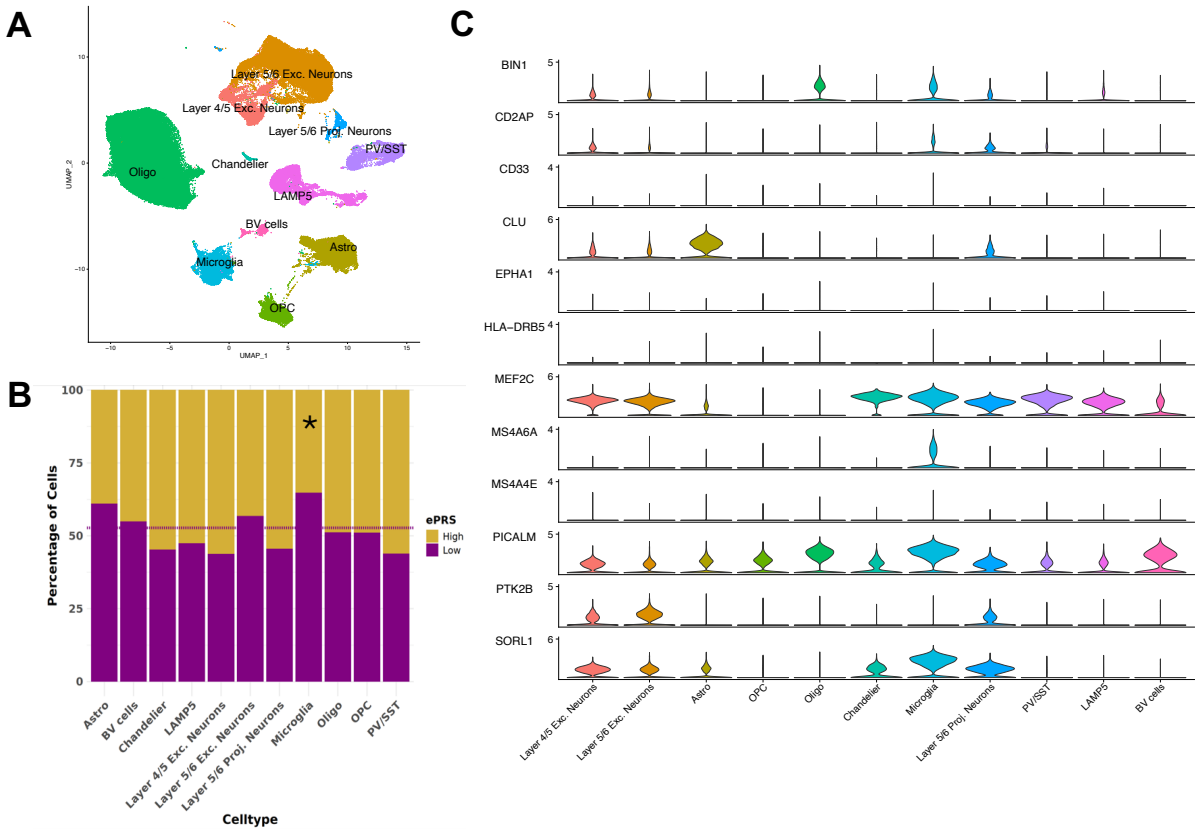

Figure 3.

Cell-type composition and endolysosomal gene expression patterns across brain cell populations. A) UMAP of the 149,533 nuclei from 23 high ADNC donors (11 low ePRS and 12 high ePRS). B) Cell composition comparison between high (gold) and low (purple) ePRS donors (Parvalbumin/SST neurons “PV/SST”; blood vessel cells “BV”; Excitatory Neurons “Exc Neuron”; Lamp5 inhibitory neuron “Lamp5”; Chandelier inhibitory neuron “Chandelier”). Microglia proportion is significantly decreased in high ePRS ($p<0.05$). C) Expression patterns of endolysosomal genes within ePRS loci across cell types. *MEF2C* and *PICALM* are broadly expressed across multiple cell types, while *CLU* shows highest expression in astrocytes, and *SORL1* is predominantly expressed in neurons and oligodendrocytes. The width of each violin plot indicates relative frequency of expression levels, with color denoting cell type as shown in panel A.

DLPFC brain nuclei demonstrate shared and distinct transcriptomic changes associated with ePRS status

We performed pseudobulk analyses within each cluster (cell-type) to generate DEGs and GSEA and begin to characterize the overall biological pathway differences associated with ePRS score

despite the cohort all having similar high AD pathology score. Across all cell types we observed that the high ePRS cohort showed decreased enrichment of Gene Ontology Biological Processes (GOBP) related to “macroautophagy” and “synaptic vesicle lumen acidification”, suggesting that increased ELN variant burden may impact those endolysosomal functions broadly (Fig 4A). Pathways related to protein homeostasis such as "protein refolding" were similarly depressed in high ePRS donors. Evidence of metabolic changes correlating to ePRS was suggested by negative enrichment of electron transport chain pathways and enrichment in glycolysis pathways in select cell types (Fig 4A). These findings identify common alterations in certain endolysosomal, metabolic processes and protein processing pathways across neuron and glial cell types associated with higher ePRS risk allele burden. These changes are likely occurring beyond those that develop as a result of AD pathology as all individuals in this dataset have AD pathology.

Neurons: Several excitatory and inhibitory neuronal subtypes across all layers of cortex were identified. The majority of excitatory neurons (clusters 1,6,8,11) expressed genes associated with layers 4-6 of the cortex[35]. Using pseudobulk analyses we identified genes significantly enriched in high ePRS donors in cluster 1 excitatory neurons including transcription factors or DNA-binding proteins involved in cell differentiation and neuronal fate such as kelch like family member 4 (*KLHL4*), and AT-rich interaction domain family 3B (*ARID3B*)[38, 39]. Genes that regulate neuronal excitability and AMPA receptor trafficking to and from the membrane were altered in cluster 1, including ORAI Calcium Release-Activated Calcium Modulator 2 (*ORAI2*), a component of an inward-rectifying calcium channel[40, 41]. Consistent with these observations, excitatory neurons in cluster 1 and cluster 6 demonstrated enrichment of calcium ion GOBP gene sets in high ePRS individuals (Fig 4A). Additionally, high ePRS excitatory neurons upregulated a distinct set of glutamatergic signaling genes related to synaptic organization such as *SHANK2 and SHANK3*[42, 43] and presynaptic neurotransmitter release, *UNC13A*[44]. Inhibitory neurons were classified as LAMP5 expressing (cluster 3), parvalbumin/somatostatin expressing (cluster 7) or chandelier neurons (cluster 10)[35]. LAMP5 expressing inhibitory neurons showed significant enrichment of the “positive regulation of synaptic transmission glutamatergic” pathway in high ePRS individuals. Genes driving this enrichment included those encoding an NMDA receptor subunit, *GRIN2* and AMPA receptor regulating subunit *CACNG2*[45, 46]. Thus excitatory and inhibitory neuron cell types both appear to respond to high ePRS by modulating glutamatergic signaling gene expression but in a cell-type specific profile. This suggested that LAMP5 Inhibitory neurons may be increasing gene expression to receive glutamatergic input,

while excitatory neurons may be modifying both input and output signaling components. OPCs similarly showed enrichment of the same glutamatergic signaling pathway in high ePRS.

To better understand the distribution of key pathway components across cell populations, we assessed the relative expression of gene modules using the Seurat module score approach and both GOBP and Reactome databases (Fig 4B). The '"Positive Regulation of Synaptic Transmission Glutamatergic" module revealed heterogeneous expression across neural populations, with both neuronal subtypes showing expression as expected, but interestingly,

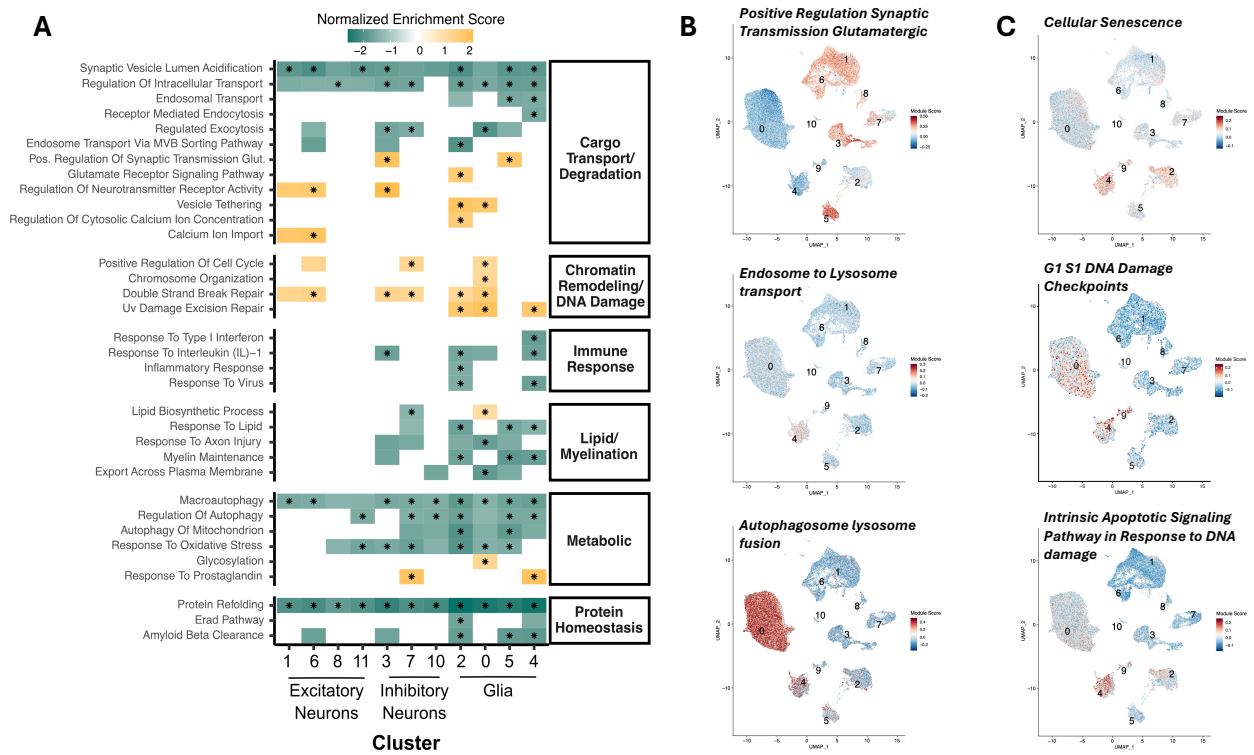

Figure 4:

High ADNC cases demonstrate shifts in gene expression associated with ePRS risk burden. A) Pathway analysis of genes shifting expression together in high ePRS individuals versus low ePRS individuals across the different cell types (columns) reveals categories of gene set enrichment sets that were significantly different between high and low ePRS. Positive enrichment in high ePRS is indicated in yellow, negative enrichment in green. Some pathways, such as protein refolding and macroautophagy, are differentially depressed across cell types in high ePRS while others such as vesicle, lipid, and DNA-related pathways are significantly enriched in high ePRS in glial populations. Pathways demarcated with a colored square are trending at the $q<0.1$ level while a * indicates that a pathway was significant at $q<0.05$. (OPC oligodendrocyte progenitor cell; Exc. excitatory; Inh. inhibitory). Cluster 2: astrocytes, Cluster 0: oligodendrocytes, Cluster 5: OPC, Cluster 4: microglia. Module score visualizations showing expression distribution of B) ELN gene sets and DNA damage, senescence, apoptotic pathways across cell populations in different cell types. Module scores are represented as a gradient from blue (low expression) to red (high expression).

also robust expression in OPCs (cluster 5). It is known that OPCs may synapse with glutamatergic synapses[47], and these data suggest perhaps more so in the context of high ePRS. Further analysis of endolysosomal transport modules revealed cell type-specific expression patterns for GOBP terms, "Endosome to Lysosome Transport" and "Autophagosome-Lysosome Fusion" modules (Fig 4B) were generally lower in neuronal populations compared to glia.

In addition to synaptic and endolysosomal pathways, we found that excitatory and inhibitory neurons in high ePRS individuals showed significant upregulation of DNA damage repair and chromatin remodeling pathways (Fig 4A). Interestingly, visualizing the modules associated with DNA damage, and downstream consequences of DNA damage such as senescence, apoptosis and cell cycle arrest; (GOBP "Intrinsic apoptotic signaling pathway by p53", Reactome, "Cellular Senescence" and " G1 S DNA Damage Checkpoint"), we found higher module scores in glia compared to neuronal populations. Thus while high ePRS showed preferential enrichment of DNA damage pathways, glial cells appear to maintain a baseline higher expression of these modules.

<u>Astrocytes:</u> Astrocytes (cluster 2) GSEA analysis demonstrated that many of the representative pathways in each biological category were significantly altered in astrocytes by ePRS variant burden (Fig 4B). These pathways include "vesicle tethering" and "glutamate receptor signaling", as well as "transport via multivesicular body (MVB) sorting" pathways in the cargo and vesicle trafficking category. Similar to other cell types, pathway analysis also identified pathways involved in DNA repair such as "double strand break repair" and "UV damage excision repair" that are up in high ePRS individuals compared to low ePRS individuals in astrocytes. Multiple immune-related pathways were altered in astrocytes including "response to virus" that was only shared with microglia. Immune pathways are lower in high ePRS individuals than low ePRS individuals. These findings suggest that immune response, metabolism, cargo transport/degradation, protein homeostasis, and DNA repair processes are all altered in astrocytes by the increased burden of ePRS risk alleles.

<u>Oligodendrocytes and OPCs:</u> Oligodendrocytes (cluster 0) made up the largest population in our dataset. Gene expression in oligodendrocytes was significantly altered by ePRS. Genes more highly expressed in low ePRS included RAS oncogene family members *RAB39A* and *RAB3A* which are both related to vesicle formation and trafficking[48, 49]; *RAB3A* has been implicated in oligodendrocyte maturation and may participate in myelination programs[49]. Like astrocytes,

gene sets associated with metabolism differentially enriched in low ePRS included “response to oxidative stress” and “macroautophagy”. Oligodendrocytes demonstrated increased enrichment of all representative DNA repair and chromatin organization pathways in high ePRS individuals. “Vesicle tethering” and “lipid response” pathways were enriched in high ePRS while other myelin-related functions including “response to axon injury” and “export across plasma membrane” were lower in high ePRS individuals. Regulation of exocytosis was most strongly enriched in high ePRS oligodendrocytes compared to low ePRS. This is notable as exocytosis is key to myelin formation and expansion[50] and requires regulated transport and release of cargo including proteolipid protein to myelin sheaths[51, 52]. Module scores analysis showed relatively much lower enrichment for this gene set in oligodendrocytes compared to other cell types, supporting the premise that lower baseline expression of a pathway in a particular cell type does not necessarily indicate reduced biological importance or vulnerability to perturbation. Oligodendrocyte progenitor cells (OPCs; cluster 5) shared many pathway signatures with oligodendrocytes, but with some additional shifts associated with ePRS. Notably, OPCs showed significant enrichment for synaptic glutamatergic gene sets similar to inhibitory neurons, suggesting a potential effect of ePRS burden on OPCs responding to neuronal activity. “Amyloid beta clearance” and “endosomal transport” pathways were significantly lower in high ePRS individuals in OPCs. Similar to inhibitory neurons, as discussed above, OPCs showed significant enrichment for synaptic glutamatergic gene sets.

Microglia: Composition analyses had suggested possibly a modest decrease in microglial (cluster 4) proportions in high ePRS. Microglial demonstrated a less marked shift in individual genes as assessed by pseudobulk measures, based on ePRS. Pathways significantly altered demonstrate a profile similar to astrocytes, with increased “UV damage excision repair” and decreased “response to virus” and “amyloid beta clearance” in high ePRS individuals (Fig 4A). High ePRS microglia showed relative decrease of enrichment for “receptor mediated endocytosis”, suggesting that higher ELN burden may alter the regulation of internalization in the context of AD pathology. Microglia also demonstrated a unique signature of “response to prostaglandin” pathway more enriched in high ePRS individuals.

Together, these findings suggest that ePRS burden affects ELN pathways as well as a broader panel of functional pathways regulating neuronal excitability and cellular metabolism across cell types. Module score analyses reveal that even in cell types with lower baseline expression of

specific pathways, genetic risk factors can drive significant changes, highlighting the cell type-specific consequences of endolysosomal network genetic burden.

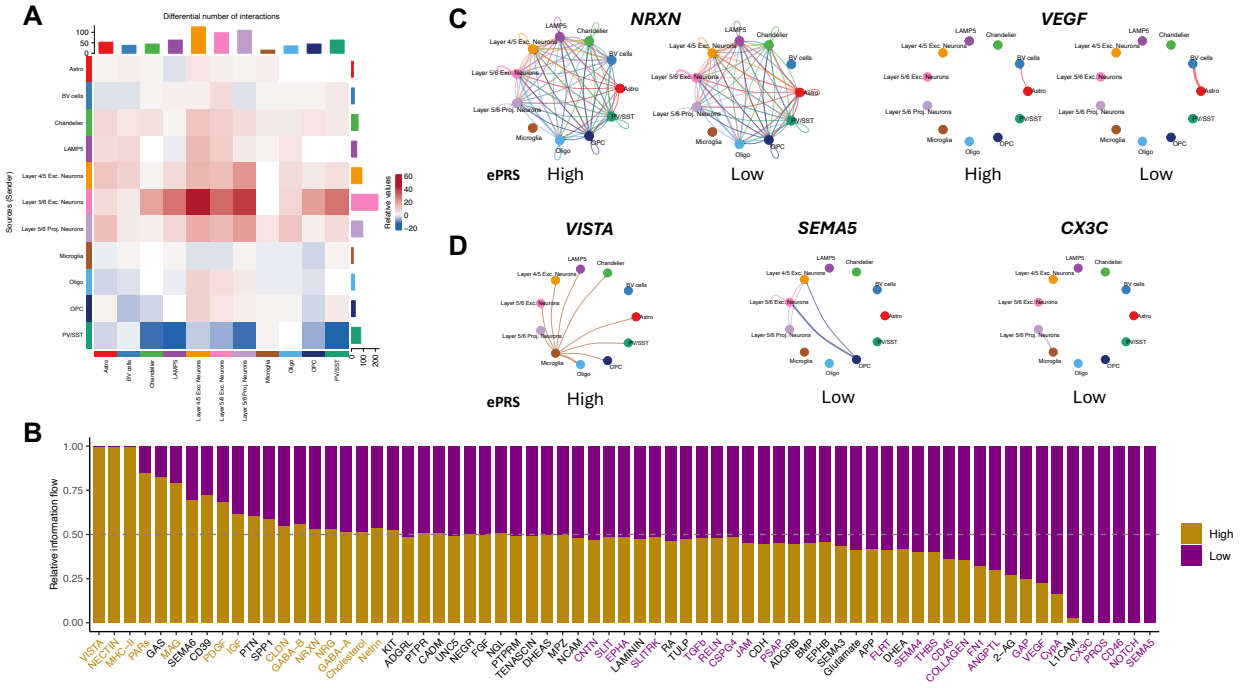

Figure 5:

ELN variant burden influences interactions between brain cell types in high ADNC donors. A) Heatmap of interactons between cell types. Rows indicate “senders” of signal, columns are “receivers”. Red indicates increased interaction number in high ePRS while blue indicates decreased interaction number. Color intensity represents the magnitude of difference. Excitatory neuron populations show increased self and cross-population communication in high ePRS (red), while PV/SST inhibitory neurons exhibit reduced interaction diversity (blue). Both OPCs and oligodendrocytes maintain communicaton with excitatory neurons though interact less with each other in high ePRS. B) Relative contribution of major signaling pathways in high versus low ePRS conditions. Pathways are ordered by the degree of difference between conditions, with gold representing high ePRS and purple representing low ePRS. C-D) Network visualization of representative pathways in low versus high ePRS conditions. Edge thickness and color intensity represent interaction strength between cell types. C) NRXN (left) shows enhanced connectivity among excitatory neurons in high ePRS, while VEGF (right) shows enhanced connectivity between astrocytes and blood vessel cells (BV). Some cell-cell communication networks were unique to or significantly biased towards high or low ePRS. D) VISTA signaling is detected only in high ePRS (left). In contrast, SEMA5 (Semaphorin) and Fractalkine signaling networks are primarily active in low ePRS, with SEMA5 showing strong OPC-neuron interactions and Fractalkine microglia-neuron communication patterns that absent in high ePRS.

Cell-Cell Communication is distinct between high and low ePRS donors

To begin to understand the intercellular signaling and communication patterns that may be altered by ePRS in AD donors, we applied the CellChat (V2) computational tool to infer cell-cell communication networks[53]. The CellChat V2 version includes existing databases such as CellPhoneDB and NeuronChatDB[54]. The methods identify the “senders” and “receivers” of signaling and incorporate other data of known mediators of that signaling network. We compared signaling patterns between high and low ePRS individuals, all of whom had high AD pathology, again to enable identification of differences more likely to be related to ELN variant burden.

High ePRS was associated with differentially higher number of interactions primarily from Layer 5/6 and Layer 5/6 Near Projection neurons both in communicating to other excitatory neurons as well as inhibitory neuronal populations (Fig 5A). In contrast, high ePRS oligodendrocytes demonstrated decreased interactions with other glial cells, particularly OPCs. We examined specific pathways and found variable effects of ePRS on cell-cell communication. For example, NRXN signaling, an important component of synaptic signaling was enriched in high ePRS, consistent with pseudobulk and correlation gene expression analyses (Fig 4). In contrast, VEGF signaling between astrocytes and the cluster identified as blood vessel cells, was more prominent in low ePRS individuals (Fig 5C).

ePRS correlates to changes in gene expression changes in ADNC donors across cell types

Pseudobulk DEG analysis, a relatively conservative method that corrects for Type 1 error, demonstrated significant differences in degrees of gene and pathway expression across cell types based on ePRS status. While our initial analyses used a binary approach comparing high versus low ePRS quartiles, we also investigated how the continuous ePRS measure correlates with gene expression. We calculated Pearson's correlation coefficient between donor ePRS and gene expression CPM (counts per million). Despite the discontinuous ePRS distribution we observed robust transcriptomic associations with ePRS score in multiple cell types.

Figure 6A shows gene expression heat maps from four different cell types. Donors (columns in heatmap) are arranged by increasing ePRS score left to right (Fig 6A). Patterns of coordinated gene expression appear to correlate with ePRS burden in all cell types (shown is oligodendrocyte, astrocyte and neuronal clusters, microglia and others are in Supplementary). Statistical analysis identified significant correlations ($r > 0.6$, $p < 0.01$) between ePRS and expression of key genes in multiple functional categories (Fig 6B). These included endolysosomal genes such as *RAB7A* and *VPS35* which are positively correlated to ePRS and both critical regulators of endosomal trafficking. In contrast, we found negative correlation of expression of *COPA,* a gene involved in retrograde vesicular trafficking[55] (Fig 6B). Genes involved in protein homeostasis demonstrated correlation to ePRS in multiple cell types such as co-chaperone *DNAJC12*[56], proteosome component *FXBO7*[57] and *ITMB2B*, a gene whose product interacts with APP and is involved in mitochondrial homeostasis[58]. Chromatin remodeling and cell cycle genes such as transcription factor *CTCF* in LAMP5 neurons and *CCND1* in OPCs also showed positive correlation with ePRS consistent with our GSEA findings of altered DNA damage pathways (Fig 6B). In addition we found positive correlation of genes involved in glutamatergic signaling such as *KCNB1* (r: 0.71, p value 0.00013) and *SHANK2* (r: 0.67, p value 0.00054) in Layer 5/6 (Supplemental data). Expression patterns and direction of association were similar to the biological pathways we found in the GSEA from binary comparison further supporting the notion that ePRS may interact with responses to DNA damage, chromatin remodeling and immune pathways.

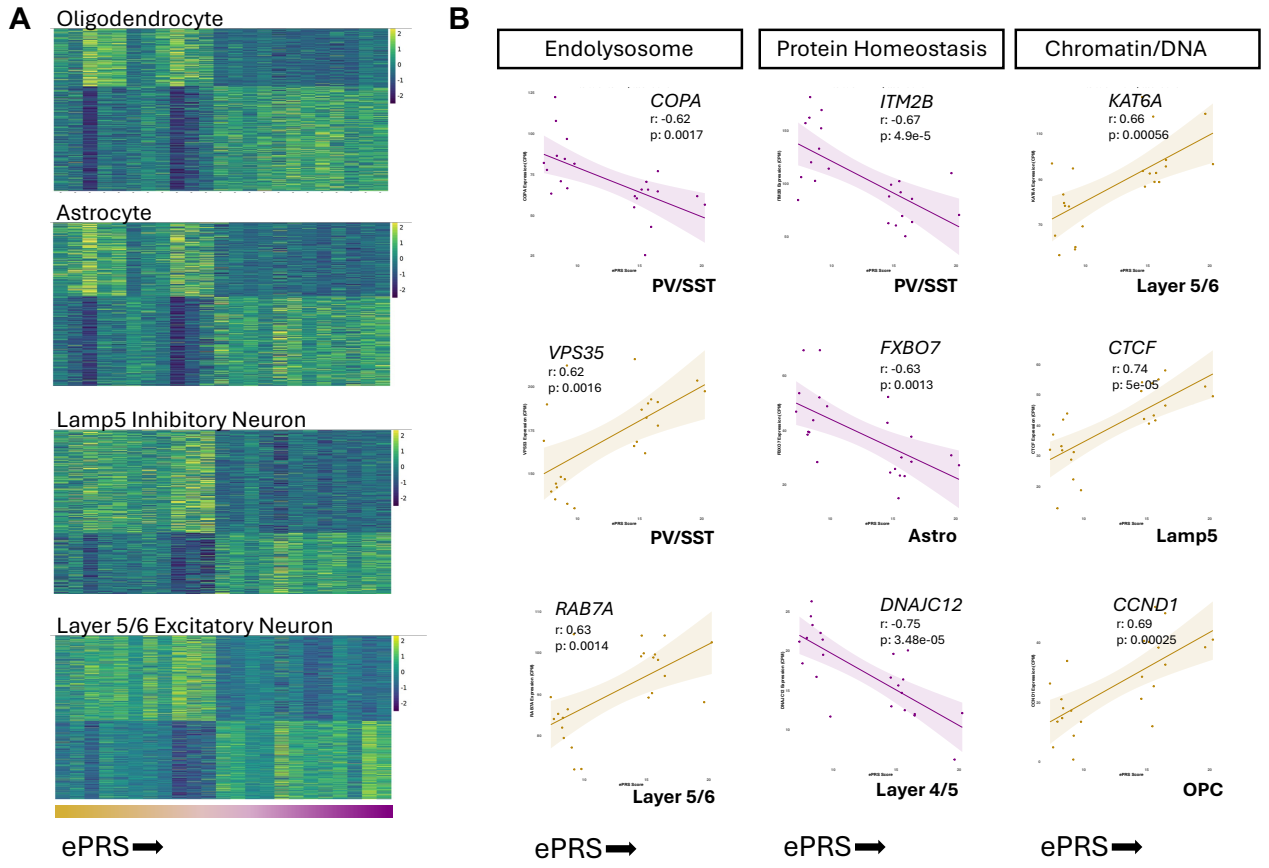

Figure 6:

ePRS correlates to gene expression in neural cell types. A) Heat map displaying gene expression (rows) for each individual (columns) ordered by ePRS demonstrating progressive transcriptomic change with increasing ePRS. Four cell types are shown as examples. B) Correlation plots showing significant relationships between ePRS scores (x-axis) and gene expression (y-axis, counts per million) for representative genes in three biological pathways: endolysosomal function, protein homeostasis, and chromatin/DNA regulation. Each point represents an individual donor, with regression lines and 95% confidence intervals shown. Pearson correlation coefficients (r) and p-values are indicated for each relationship.

DISCUSSION

Here we report the first study in human donor brain tissue, to our knowledge, that assesses the cell type specific molecular and cytopathological features associated with a pathway specific AD polygenic risk score that is also sensitive to predicting AD diagnosis and pathology. We show that a PRS representing endolysosomal pathway AD risk variants is associated with endolysosomal changes across cell types in pathologically confirmed AD. A strength of our approach is the application of complementary analyses with different statistical limitations. We

employed pseudobulk for differential expression, a comparatively more conservative method that minimizes false positives as well as single-cell methods such as gene to ePRS correlation and cell-cell communication inference. These analyses revealed similar themes and associations giving confidence to our findings despite the limitations to smaller sample number and the inherent heterogeneity of human brain samples.

The ELN is an established candidate mechanistic driver of AD and other neurodegenerative diseases[22]. The discovery of AD risk variants harboring ELN genes gave human genetics support to the premise as well as potential leads to the molecular pathways linking genetic risk to neural cell dysfunction. The ELN is a fundamental component of cellular homeostasis and some cell types have developed specialized functions dependent on the ELN such as synaptic receptor recycling, exocytosis, lysosomal degradation in phagocytes[22, 23, 59-62]. Therefore the first step in leveraging genomic variation to reveal AD mechanisms is to better understand the cell-type specific changes in ELN related genes and pathways with cell-type specific resolution. Establishing these associations is critical to mapping how the molecular effects of AD variants contribute to AD pathogenesis.

ELN cytopathology as an early feature of AD has been well established for years[28]. However, to what degree abnormal endosome and lysosome morphology is simply a secondary consequence of proteopathy progression rather than contributor to the disease process is not fully understood. Despite this, studies in tissue show endosomal pathology in MCI and Down's syndrome subjects prior to the development of amyloid plaques and hiPSC models of AD-related endosome dysfunction similarly show enlarged endosomes in cells that do not yet have much amyloid pathology[28, 31, 63] The discovery of common AD risk alleles harboring ELN genes and AD rare variants in ELN genes (*SORL1, ABCA7*) provides strong human genetic evidence supporting a pathogenic role for intrinsic changes to ELN function. Here we show that common genetic risk in ELN loci have significant correlations with endolysosomal morphology and gene expression in pathways dependent on ELN function, such as autophagy, synaptic function and immune response. Importantly, these changes vary within the diverse cell types of the central nervous system, highlighting both the importance of this pathway for brain homeostasis and the complexity of developing it as a therapeutic target. Our findings of increased numbers, aggregation and enlargement of early endosomes in neurons suggests deficiencies in function of these important organelles. Indeed, other work by our group and others show altered neuronal endosomal function in models lacking key endosomal genes[64, 65].

Importantly, some of the endosomal pathologies we observe are present in low ADNC subjects, further suggesting that they are driven by genetics rather than amyloid pathology. Interestingly, we find strong transcriptomic signatures showing altered glutamatergic signaling in high vs. low ePRS neuronal populations. This corroborates work in model systems showing that depletion of endosomal trafficking complexes, such as retromer, or endosomal genes, such as *SORL1,* leads to altered localization of AMPARs and deficits in long-term potentiation, the cellular basis of memory[65-67]. Additionally upregulation of glutamatergic signaling in excitatory and inhibitory neurons could suggest that altered endolysosomal function may lead to compensatory changes in synaptic transmission machinery.

The enlarged endosomes in neurons and lysosomes in microglia align with expression changes in trafficking genes like *VPS35* and *RAB7A,* suggesting that genetic risk directly impacts both the physical organization and molecular machinery of the endolysosomal system of multiple cell types. While microglia demonstrated enlarged lysosomes in high ePRS individuals though showed fewer differential genes between high and low ePRS. It is possible that microglia are particularly susceptible to modest changes caused by ePRS loci. Enlarged lysosomes may reflect impaired acidification, degradation[68], pathways differentially impacted by ePRS as measured by gene expression. There was a modest reduction in microglia numbers in the high ePRS cohort, which if not driven by technical issues, could suggest changes in microglial survival, proliferation or recruitment. Both astrocytes and microglia showed enrichment in apoptotic pathways which could explain cell number differences though this will need to be validated though intact tissue studies such as immunohistochemistry. The enrichment of apoptotic and senescence pathways in conjunction with DNA damage responses suggests that ePRS may influence responses to accumulating DNA damage. Whether this would also be the case in normal aging is an important question and relates to earlier work suggesting endolysosomal and immune risk variant burden is associated with cognitive health in aging in a unique population of centenarians[13]. Notably, our method did not enrich for microglia and therefore the numbers of microglia per person averaged ~350 nuclei, typical for nuclei approaches that do not enrich for or against cell types, which limits resolution of microglial states. Thus, the “microglia” cluster itself is likely heterogeneous and within it different microglial states which may reduce sensitivity to ePRS driven changes. It is possible that ePRS may impact specific microglial states or proportion of states which may be revealed with enrichment or spatial techniques[69].

Module score analysis enabled better understanding of GSEA and gene expression patterns. We could appreciate that some pathways may be expressed across multiple cell types though differentially impacted by ePRS status in a cell-type specific manner. Though many pathways are expressed across multiple cell types, the effect of ePRS was more prominent in some cell types more than others, or conversely that ePRS was associated with broad effects on a pathway that had variable enrichment across cell types. For instance the glutamatergic signaling module was active in excitatory and inhibitory neurons and OPCs, though GSEA showed significant enrichment of this pathway in inhibitory neurons suggesting differential vulnerability to ELN pathway changes. In contrast, macroautophagy showed consistent negative enrichment in high ePRS cell types suggesting a possible fundamental consequence of ELN dysfunction. Module score analysis meanwhile revealed higher baseline expression of the macroautophagy gene set in microglia (Supplemental Data) which may reflect their specialized roles in clearance.

CellChat analyses highlighted differences in strength and number of connections between neural cells. While both CellChat and GSEA are based solely on gene expression, CellChat also takes into account downstream targets of the signaling pathway and renders further hypotheses generating analyses[53]. PV/SST neurons showed increased strength of signaling though fewer number of interactions in high ePRS (Fig 5). We had noted in the pseudobulk analysis that genes driving the enrichment of glutamatergic signaling in these inhibitory neurons included postsynaptic elements, *GRIN2A/B*, which could again suggest compensatory increases in receiving glutamatergic signaling as a compensatory mechanism. We observed increased neuronal-oligodendrocyte and OPC communication. This cross talk between neurons and oligodendrocyte/OPC could reflect compensatory attempts at remyelination, modulation of neuronal activity or other interactions which contribute to vulnerability or resilience. For instance, neuronal signaling can induce exocytosis of myelin membrane[52] from oligodendrocytes while OPCs can induce exocytosis of neuronal lysosomes and related metabolism[70].

Endolysosomal dysfunction has been associated with various forms of programmed cell death[71]. The co-occurrence of altered DNA damage and chromatin remodeling pathways with endolysosomal dysfunction across multiple cell types in high ePRS individuals suggests a potential connection between AD ELN variants and cellular stress responses that warrants further investigation. Notably protein homeostasis pathways such as protein folding, chaperone

mediated processes were uniformly depressed in the high ePRS (or enriched in the low ePRS) which could indicate that impaired ELN function and related disruption of protein handling could also contribute to cell stress.

There are limitations to this study. New loci have emerged in recent AD GWAS, while fine-mapping and colocalization experiments have implicated additional genes at established loci[72]. Genes influence the ELN (ex., *TREM2*[73]) and should be considered as additions to our ePRS and will be in future iterations of PRS. Several loci have multiple independent signals that may not be adequately captured by the SNPs in our ePRS. For example, while *CLU* rs9331896 and *PTK2B* rs28834970 are often considered the same locus, the two SNPs are not in LD in 1000 Genomes European samples[74, 75]. Confident linking of ELN genes to the tagging SNPs used in GWAS will require additional epigenetic analyses with emerging single cell chromatin accessibility, methylation and 3D chromatin conformation methods.
This study is focused on donors with only high AD pathology and therefore reflects end-stage disease. Ideally, a larger cohort across the spectrum of pathology can provide even greater insight into the earlier and later changes that correlate to ELN burden. We attempted to control for secondary effects of AD neurodegenerative processes on the ELN in our assessment of ePRS by conducting high ADNC only comparisons across ePRS. Whether by neuropathology or gene expression, we find that despite the same neuropathological burden and diagnosis, higher ePRS appears to drive endolysosomal changes in neurons and microglia.

Conclusions

Our work demonstrates that an AD endolysosomal variant pathway-specific genetic risk score does correlate to cytopathologic and molecular changes that are associated with endolysosomal function in those with higher ELN variant risk (high ePRS). While genes in the ELN that are within AD risk loci have supported the ELN role in AD pathogenesis (in addition to findings in vitro and in animal studies), direct assaying of ELN function proxies such as morphology and cell gene expression in relation to ELN variant risk had not yet been established. This information is valuable to dissecting the molecular consequences of these non-coding variants and the mechanisms by which they contribute to AD, including how they may lead to local or distal gene regulation, either directly or indirectly. The effects of high ePRS resulted in shared and distinct molecular patterns in different cell types. It will be essential to leverage human neural cell models and determine whether this relationship is observed in neurons, glia and

other cell types carrying varying burdens of ELN variants, where external factors such as the lived experience are removed. Once a biomarker confirmed diagnosis is made, the ePRS could be used to stratify clinical trials participants into endolysosome targeting therapies. It is likely that various ELN components will have differential import to different cell types and identifying which are most targetable, occur earliest, and most applicable across patients will be similarly important to translating the rapidly gathering evidence implicating the ELN in AD into clinically actionable findings.

METHODS

*Tissues***.** Brain tissue was collected from human donors post-mortem after appropriate consent was obtained during life. All tissues were obtained for processing from the Precision Neuropathology Core at the University of Washington. Cases with diagnoses of non-AD tauopathies, FTLD and ALS were excluded, as well as cases with known genetic chromosomal abnormalities. Additionally, comorbid pathology cortical stroke, history of malignancy, cerebral infection, were exclusionary (Demographic data for each analysis is provided in Tables 2-4 and de-identified individual level data in Supplemental data). All cases had cognitive data available, as well as rigorous neuropathology work-ups in accordance with consensus guidelines including AD Neuropathologic Change (ADNC) scoring. ADNC reflects severity of hallmark AD amyloid-beta plaques and phosphor-tau neurofibrillary tangles, assessed histologically. An ADNC Not ("0") score indicates no evidence of AD pathology. At the ADNC Low ("1") stage AD pathology is present but not sufficient for AD diagnosis. ADNC 2 and 3 scores are more tightly correlated with dementia and clinical presentation of AD in clinico-pathologic correlation studies. In accordance with common practice in LOAD neuropathologic studies we designate ADNC 2-3 cases " AD" group ADNC 0-1 cases as "controls"[1, 76-78].

<u>Genotyping</u>

Four mm punches from fresh frozen cerebellum or dorsolateral prefrontal cortex (DLPFC) were lysed and genomic DNA (gDNA) extracted using the GeneJET gDNA Purification Kit (Thermo Scientific #K0722). One microgram of gDNA was sequenced using the Infinium Global Diversity Array 8 v1.0 to detect common single-nucleotide polymorphisms (SNP) and sequenced at the Northwest Genomics Center, Seattle, WA.

<u>Development and calculation of the ePRS.</u>

Endolysosomal pathway-specific polygenic risk score (ePRS[79]) was developed combining information from 13 AD GWAS SNPs correlated to ELN genes (**Table 1**) using the explained-variance approach[4, 10].

Immunohistochemistry

Immunostaining and IMARIS analysis of endosome and lysosomes. Immunohistochemistry was performed for endosome and lysosome morphology in fresh frozen DLPFC (neuronal endosomes) or paraffin embedded DLPFC (microglia lysosomes) based upon the optimal tissue type for the endolysosomal antibody.

1. *Neuronal endosome morphology analysis from postmortem brain tissue:*

Postmortem brain tissue from 14 low ePRS and 13 high ePRS donors with PMI <8 hours and available fresh frozen inferior parietal lobule tissue were immunolabeled for endosome morphology (Table 2). The immunohistochemistry, imaging and image analysis methods used for quantifying early endosome morphology in postmortem brain tissue are described in detail in previously published work[80]. Major steps are described in brief here.

Fresh frozen inferior parietal lobule samples were cut at 5 um thickness and fixed with 4% PFA. Tissue was blocked in 5% normal goat serum (NGS, Jackson ImmunoResearch Laboratories # 005-000-121) + 2.5% bovine serum albumin (BSA, Sigma-Aldrich # A3294) + 0.1% Triton X-100 (Fisher Bioreagents # BP151-100). Primary antibodies used were mouse anti-EEA1(BD Biosciences # 610456) and chicken anti-MAP2 (Abcam # ab92434) diluted 1:500. Secondary antibodies used were goat anti-Chicken IgY (H+L) secondary antibody Alexa Fluor™ 594 (Invitrogen # A11042) and goat anti-Mouse IgG (H+L) Secondary Antibody Alexa Fluor™ 488 (Invitrogen # A11029) diluted 1:1000. Autofluorescence was quenched with TrueBlack (Biotium # 23007) 1:20 according to manufacturer guidelines.

Neuron IHC cohort		Low ePRS (n = 14)	High ePRS (n = 13)
ePRS	Mean +/- SD	8.0 +/- 1.5	15.7 +/- 1.6
Age at death	Mean +/- SD (yrs.)	90.0 +/- 8.1	83.2 +/- 12.4
PMI	Mean +/- SD (hrs.)	4.4 +/- 1.2	6.0 +/- 1.0
Sex	Female % (#)	71% (10)	62% (8)
	Male % (#)	29% (4)	38% (5)
Cognitive status	Normal cognition % (#)	50% (7)	62% (8)
	Dementia % (#)	50% (7)	38% (5)
APOE	E2/E3 % (#)	7% (1)	0% (0)
	E3/E3 % (#)	71% (10)	77% (10)
	E3/E4 % (#)	21% (3)	23% (3)
	E4/E4 % (#)	0% (0)	0% (0)
ADNC	0 % (#)	21% (3)	8% (1)
	1% (#)	17% (1)	23% (3)
	2 % (#)	36% (5)	38% (5)
	3 % (#)	36% (5)	31% (4)

Table 2

Demographics of cohort immunolabeled for neuronal endosomes.

Confocal imaging - Neuron

MAP2-positive pyramidal neurons within the cortical gray matter were imaged on the Leica TCS SP8 confocal microscope with a 63x/1.40 oil lens. Z-stack images were captured in the Leica Application Suite X (LAS X 3.5.5.19976) at 5x zoom with LIGHTNING adaptive deconvolution post-processing, using image acquisition settings as described in previous publication[80]. Total z-size was 1.68 µm with 14 steps and a z-step size of 0.13 µm. An average of ten neurons were imaged per case (range 8-13, due to variability in the size of cortex sampled and proportion of gray matter present).

Image analysis and endosome quantification – Neuron

Endosomal morphology was analyzed from confocal Z-stacks using Bitplane Imaris software (Oxford Instruments). EEA1-positive endosome puncta were identified and segmented in Imaris to quantify the total puncta counts and individual puncta volumes. This quantification was performed within two neuronal regions of interest (ROIs): the MAP2-positive somatodendritic

ROI and the perinuclear ROI. The perinuclear ROI boundaries were achieved by expanding a surface from the DAPI-positive nucleus to capture the subpopulation of early endosomes localized most closely with the nucleus.

In order to investigate changes in the early endosome size specifically within the largest subset of early endosomes, we defined an “Enlarged early endosome” subpopulation using the traditional definition of a statistical outlier: early endosomes were considered “Enlarged EEs” if their volume was above 2 standard deviations from the mean volume calculated using ADNC 0-1/low ePRS cases (cases most likely to reflect an early endosome size distribution found in healthy aged controls).

A univariate linear regression model was used to assess relationship between ePRS and early endosome morphology output variables (Mean EE volume, Mean EE # per unit ROI volume, Mean “Enlarged EE” volume, and proportion of “Enlarged EEs” compared to total EE #), and adjusted for confounding factors age, sex, PMI, and *APOE* genotype. Statistical analyses were performed on the full cohort (n = 27; ADNC low-high cases) as well as an “AD-only” subcohort (n = 19, ADNC intermediate-high cases). The Mann-Whitney test was used for comparison of early endosome morphologies in low versus high ePRS cases and in non-AD versus AD cases.

2. *Microglial lysosome morphology analysis from post-mortem human brain tissue:* Immunohistochemistry of microglia and lysosomal markers was performed on formalin-fixed paraffin embedded tissue from the dorsolateral prefrontal cortex of 13 low ePRS and 13 high ePRS donors (Table 3) following previously published protocols[69] with the addition of the LAMP1 antibody to Iba-1 and DAPI (Lamp-1: Thermo 14-1079-80, 1:250). An Alexa-fluor Donkey anti-Mouse 555 antibody was used for the LAMP1 (ThermoFisher A31570, 1:500).

Microglia IHC cohort		Low ePRS (n = 13)	High ePRS (n = 13)
ePRS	Mean +/- SD	8.2 +/- 1.6	15.8 +/- 1.6
Age at death	Mean +/- SD (yrs.)	92.5 +/- 5.8	81.5 +/- 11.2
PMI	Mean +/- SD (hrs.)	4.4 +/- 1.3	5.9 +/- 1.6
Sex	Female % (#)	62% (8)	54% (7)
	Male % (#)	38% (5)	46% (6)
Cognitive status	Normal cognition % (#)	62% (8)	54% (7)
	Dementia % (#)	38% (5)	46% (6)
APOE	E2/E3 % (#)	8% (1)	8% (1)
	E3/E3 % (#)	69% (9)	62% (8)
	E3/E4 % (#)	23% (3)	31% (4)
	E4/E4 % (#)	0% (0)	0% (0)
ADNC	0 % (#)	15% (2)	23% (3)
	1 % (#)	8% (1)	8% (1)
	2% (#)	31% (4)	15% (2)
	3 % (#)	38% (5)	38% (5)

Table 3

Demographics of cohort immunolabeled for microglial lysosomes.

Confocal Imaging - Microglia

Slides were imaged using a 40x oil lens on the Leica SP8 laser confocal microscope to create Z-stacks with a step size of 0.47µm, 2.2x zoom, 600 Hz speed, and 1024x1024 format.

Image analysis and lysosome quantification - Microglia

The IMARIS software version 10.1 was used to generate surfaces for Iba-1 using a standardized machine-learning segmentation parameter. The machine-learning parameter was created by selecting the background and foreground across z-stacks. Areas without Iba-1 signal or with noise were selected as background, while areas with Iba-1 signal were selected as foreground. Using the "train and predict" feature, we generated a 3D surface of the Iba-1 signal. Microglia were analyzed if DAPI was fully enclosed in the Iba-1 surface and the whole microglia was present inside the image (no processes cut off by the image edge). When the Iba-1 surface was identified to be less than 7 microns distant from a process, the two surfaces were united.

Other background Iba-1 signals were eliminated. The LAMP1 signal contained within the microglial surfaces was identified and surfaces were created for the LAMP1 signal using a similar machine learning approach. For LAMP1, surfaces under 1μm^3 in volume were confirmed to be largely single fluorescent puncta, whereas surfaces larger than 1μm^3 in volume were assessed to be largely dual or clusters of fluorescent puncta. Lysosomes were therefore analyzed in two groups: volumes under 1μm^3, and clusters with volumes between 1 and 15μm^3.

A univariate linear regression model was used to assess relationship between ePRS and lysosome morphology output variables (Mean lysosome volume, Mean lysosome # per unit ROI volume), and adjusted for confounding factors age, sex, PMI, and *APOE* genotype.

Single-nucleus transcriptomic studies

Nuclei isolation and enrichment

For single-nucleus RNA-seq (snRNA-seq), methods largely followed those of Prater, Green et al. (2023). Fresh frozen human DLPFC samples from 11 low ePRS and 12 high ePRS (Table 2) were isolated as previously described[69]. Briefly, four 2-mm punches of DLPFC gray matter were collected using a biopsy punch (Thermo Fisher Scientific) into a 1.5-ml microcentrifuge tube on dry ice. The nuclei in nuclei suspension solution was layered onto 900 μl of percoll/myelin gradient buffer[81]. The gradient was centrifuged at 950*g* for 20 min at 4 °C without break and with slow acceleration. The supernatant with myelin was aspirated, and the nuclei pellet was resuspended in resuspension buffer at a concentration of 1,000 nuclei per microliter and proceeded immediately to snRNA-seq. All samples were processed using the 10X Genomics Chromium Next GEM Single Cell 3' Kit v3.1 (#1000268) with a target capture of 10,000 nuclei. Samples were sequenced using either the Illumina NovaSeq 6000 or NovaSeq X platform through the Northwest Genomics Center.

snRNAseq cohort		Low ePRS (n = 11)	High ePRS (n = 12)
ePRS	Mean +/- SD	8.5 +/- 0.7	16.2 +/- 1.8
Age at death	Mean +/- SD (yrs.)	89.5 +/- 8.0	84.8 +/- 12.5
PMI	Mean +/- SD (hrs.)	4.7 +/- 1.4	6.1 +/- 1.7
Sex	Female % (#)	64% (7)	58% (7)
	Male % (#)	36% (4)	42% (5)
Cognitive status	Normal cognition % (#)	45% (5)	42% (5)
	Dementia % (#)	55% (6)	58% (7)
APOE	E3/E3 % (#)	64% (7)	58% (7)
	E3/E4 % (#)	36% (4)	33% (4)
	E4/E4 % (#)	0% (0)	8% (1)
ADNC	2 % (#)	36% (4)	42% (5)
	3 % (#)	64% (7)	58% (7)

Table 4:

Demographics of snRNAseq cohort.

Alignment, quality control. Methods largely followed those previously published[69]. CellRanger 7.0.2 was used to align the samples using the 2020-A genome provided by 10X Genomics. Analysis was performed in R (R Development Core Team, 2010). Droplets from the samples were combined using Seurat version 4.0.0. The dataset was thresholded to remove droplets containing fewer than 350 genes and droplets with more than 1% mitochondrial reads. Ambient RNA and doublets were detected as previously described[69]. After QC, the total number of nuclei was 149,533 with 2579.71 average genes/nucleus. A clustering resolution of 0.1 was used after reducing the dimensions of the dataset to 34 principal components.

Normalization, Clustering, and Differential Gene Expression testing with GSEA. Analysis proceeded as previously described with noted exceptions[69]: Seurat v4.0.0 was used for all analyses. Harmony was used for batch correction in these datasets. For cell type identification in unsorted datasets and prior to subsetting microglia from other cell types after enrichment, marker gene expression from BICCN were used to detect cell types in clusters[35]. All datasets

were clustered using the Louvain algorithm with multilevel refinement. GSEA using ClusterProfiler used the 2024 versions of the pathway datasets for GO-BP, Reactome, and Wikipathways. To assess differences in cell-type composition between ePRS groups, we calculated the percentage of cells from each condition (high vs. low ePRS) within each annotated cell type. Cell-type proportions were computed and results were visualized using stacked bar plots. Statistical differences in cellular composition were assessed using a chi-square test.

Single cell analyses:

Violin plots: (Fig 3A) Gene expression distributions were visualized using violin plots generated with the VlnPlot_scCustom() function from the scCustomize package in R.

Cell-Cell communication Inference

Cell–cell communication analysis was conducted using CellChat V2 to compare signaling interactions between high ePRS and low ePRS donors across all cell types[53]. CellChat V2 was run separately on each donor group to infer ligand–receptor interactions and compute communication probability scores. The results from both groups were then integrated for comparative analysis. The analysis followed the approach outlined in the CellChat tutorial using all the default parameters.

To identify differentially active signaling pathways between High ePRS and Low ePRS donors, we used the rankNet function in CellChat with default parameters. A Wilcoxon rank-sum test was performed to assess statistical significance, and pathways with an adjusted p-value < 0.05 were considered differentially enriched. For network visualization, we applied a communication probability threshold of 0.05 to focus on high-confidence interactions.This analysis followed the approach outlined in the CellChat comparison tutorial.

Module Scoring To assess pathway activity, we computed module scores using the AddModuleScore function from Seurat (v5.0.2)[82] with default parameters (nbin=25, ctrl=100). Gene sets were obtained from the Molecular Signatures Database (MSigDB v7.4), focusing on the GO Biological process, Reactome collections.

Correlation of ePRS to cell-type specific gene expression

To identify genes with expression levels correlated with ePRS in each cell type, we calculated Pearson correlation coefficients between pseudo-bulk counts per million (CPM) and donor

ePRS. Due to variability in cell numbers captured per donor, we applied filtering criteria to donors and genes before correlation analysis. Only genes that were expressed with at least one count in at least one donor were included. Pearson correlation coefficients were computed between gene expression levels and donor ePRS for each cell type. Genes with a correlation coefficient greater than 0.6 and an unadjusted p-value < 0.01 were considered significant.

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