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Alpha-2 macroglobulin in Alzheimer's disease: a marker of neuronal injury through the RCAN1 pathway

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Abstract

Preclinical changes that precede the onset of symptoms and eventual diagnosis of Alzheimer's disease (AD) are a target for potential preventive interventions. A large body of evidence suggests that inflammation is closely associated with AD pathogenesis and may be a promising target pathway for such interventions. However, little is known about the association between systemic inflammation and preclinical AD pathophysiology. We first examined whether the acute-phase protein, alpha-2 macroglobulin (A2M), a major component of the innate immune system, was

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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associated with cerebrospinal fluid (CSF) markers of neuronal injury in preclinical AD and risk of incident AD in the predictors of cognitive decline among normal individuals (BIOCARD) cohort. We find that A2M concentration in blood is significantly associated with CSF concentrations of the neuronal injury markers, tau and phosphorylated tau, and that higher baseline serum A2M concentration is associated with an almost threefold greater risk of progression to clinical symptoms of AD in men. These findings were replicated in the Alzheimer's Disease Neuroimaging (ADNI) study. Then, utilizing a systems level approach combining large multi-tissue gene expression datasets with mass spectrometry-based proteomic analyses of brain tissue, we identified an A2M gene network that includes regulator of calcineurin (RCAN1), an inhibitor of calcineurin, a well-characterized tau phosphatase. A2M gene and protein expression in the brain were significantly associated with gene and protein expression levels of calcineurin. Collectively these novel findings suggest that A2M is associated with preclinical AD, reflects early neuronal injury in the disease course and may be responsive to tau phosphorylation in the brain through the RCAN1-calcineurin pathway.

INTRODUCTION

The detailed characterization of the preclinical phase of Alzheimer's disease (AD) represents a major advance that is allowing researchers to test potential preventive treatments to modify the trajectory of AD during presymptomatic stages of the disease.[1,2] The preclinical stages of AD are characterized by a temporal ordering of cerebrospinal fluid (CSF) and neuroimaging biomarkers that are thought to reflect the early accumulation of brain amyloid, emergence of synaptic dysfunction and appearance of markers of neuronal injury.[3] These preclinical changes preceding the onset of clinical symptoms and eventual diagnosis of AD have together been described as the 'AD pathophysiological signature'.[4]

A large body of evidence suggests that inflammatory and immune-regulatory processes are intrinsic to AD pathogenesis and are important both in the appearance of core pathological features of AD as well as in triggering the onset of clinical symptoms.[5,6] The close association between inflammation and AD pathogenesis has led to several efforts targeting inflammation as a potential strategy for disease modification in AD.[7] Although previous clinical trials of anti-inflammatory agents in patients with established AD have failed,[8–10] there is currently renewed interest in strategies that target inflammation during the early, preclinical stages before the onset of clinical symptoms of mild cognitive impairment (MCI) and AD.[11] However, despite evidence implicating inflammation in AD pathogenesis, little is known about the association between systemic inflammation and the 'preclinical AD pathophysiological signature.' A comprehensive understanding of how specific inflammatory pathways are associated with AD pathogenesis, including the molecular mechanisms that may drive such associations, is critical to targeting these pathways for the prevention of AD.

The acute-phase protein alpha-2 macroglobulin (A2M) is a major component of the innate immune system, functions as a pan-protease inhibitor and is a chaperone protein. Prior evidence indicates that A2M is capable of binding to misfolded, aggregation-prone client proteins and may have an important role in AD pathogenesis.[12–14] Two principal lines of

evidence from human studies suggest that A2M may have a key role in the neuroinflammatory response to AD pathogenesis. These include its co-localization with amyloid plaques in AD,[15–17] and proteomic studies showing an increase in plasma A2M concentration in AD patients relative to controls.[18–21] Moreover, early candidate gene analyses suggested that polymorphic variation in the A2M gene are associated with increased risk of AD, although these were not replicated in larger studies.[22,23]

In the current study, we first examined associations between blood A2M and CSF measures of preclinical AD pathophysiology in a well-characterized longitudinal cohort—predictors of cognitive decline among normal individuals (that is, the BIOCARD study). Then, based on these findings, we explored whether blood concentrations of A2M predicted risk of progression from normal cognition to the onset of clinical symptoms of MCI. Next, we used data from the Alzheimer's Disease Neuroimaging Initiative (ADNI) to replicate our index findings in an independent sample. Finally, using a systems level approach combining multi-tissue gene expression and mass spectrometry-based proteomic analyses of brain tissue, we identified plausible molecular mechanisms that may underlie associations between A2M and CSF markers of preclinical AD as well as disease progression.

Figure 1 provides a summary of the study aims and design.

MATERIALS AND METHODS

BIOCARD study (primary analyses)

The BIOCARD study was used to examine associations between A2M and CSF measures of preclinical AD pathophysiology as well as risk of MCI/AD (Figure 1a). The BIOCARD study was initiated by the National Institutes of Health (NIH) in 1995 and continued by Johns Hopkins University in 2009. The study was designed to follow participants who were cognitively normal at baseline but at high risk of AD, to better understand the preclinical stage of AD[3] that typically precedes the onset of MCI/AD symptoms by several years. Annual clinical and cognitive evaluations of the BIOCARD participants included a comprehensive neuropsychological battery, family history of dementia, history of symptom onset and a clinical dementia rating. Consensus diagnosis criteria and protocol have been described previously,[24] and followed the NINCDS/ADRDA criteria for AD dementia[25,26] and the Petersen criteria for MCI.[27] For participants with MCI or AD, age of onset of clinical symptoms was estimated primarily based on the report of the subject and collateral obtained during the clinical dementia rating interview.

CSF was collected in participants approximately every 2 years. CSF data collection protocol in the BIOCARD study has been described in detail previously,[28] and followed protocols similar to the ADNI. Blood draws for serum A2M assays began in 2001. Details on assay protocol are included in Supplementary Table 1. Additional BIOCARD study design and participant recruitment details are described elsewhere.[24,29] All study participants provided written informed consent, and the Johns Hopkins School of Medicine Institutional Review Board approved all study protocols.

Of the 303 BIOCARD participants with a baseline A2M blood draw, CSF was collected concurrently (defined as within 180 days of baseline A2M blood draw) in 274 participants. See Supplementary Figure 1 for a schematic summary of inclusion/exclusion criteria and sample size. Demographic characteristics of the BIOCARD sample are included in Supplementary Table 2.

ADNI study (replication analyses)

The ADNI study (adni.loni.usc.edu) was used as an independent sample to replicate findings from BIOCARD (Figure 1a). Data used in the preparation of this article were obtained from the ADNI database (adni.loni.usc.edu). The ADNI was launched in 2003 as a public–private partnership, led by Principal Investigator Michael W. Weiner, MD. The primary goal of ADNI has been to test whether serial magnetic resonance imaging, positron emission tomography, other biological markers, and clinical and neuropsychological assessment can be combined to measure the progression of MCI and early AD.

Details on study design and participant recruitment, sample collection of plasma A2M and CSF, and consensus diagnosis criteria are included in Supplementary Table 3. The ADNI study included baseline plasma A2M samples on 566 participants, and concurrent CSF data on 353 of those participants. Demographic characteristics of the ADNI sample are included in Supplementary Table 2.

Gene expression data: Genotype-Tissue Expression Project

Publicly available gene expression data acquired from the genotype-tissue expression (GTEx) project[30] was used to explore the association between blood and brain A2M gene expression (Figure 1b). The GTEx project is a public repository that enables researchers to explore correlations in gene expression across multiple healthy tissues including blood and brain. Details have been described previously.[31] All donors provided written informed consent and study protocols were approved by the NIH National Human Genome Research institute. We assembled a dataset of RNA sequence (RNASeq) expression data to explore matched blood and brain gene expression levels from 921 samples across 13 brain regions. A list of brain regions and associated participant sample sizes are included in Supplementary Table 2. See Supplementary Figure 2 for a schematic summary of inclusion/exclusion criteria and sample size.

Gene expression data: Gene Expression Omnibus

Publicly available gene expression data acquired from the National Center for Biotechnology Information (NCBI) (GEO) (http://www.ncbi.nlm.nih.gov/geo/)[32] was used to identify global gene networks driving A2M gene expression (Figure 1c.I) and brain-specific gene expression correlations (Figure 1c.II) based on *a priori* specified gene(s) identified from the global gene expression analysis. GEO datasets were restricted to healthy and normal samples (identified through keyword search and manually confirmed). All GEO data is required to conform to NIH Genomic Data Sharing policy that provides protections for publicly available data on human subjects. The final dataset for network analysis included 4755 samples from 70 different tissues. The final dataset for brain-specific analyses included 281 samples selected from 7 brain regions. A list of sample sizes by organ/brain

region and specific tissue sampling locations are included in Supplementary Table 4. GEO sample and series accession numbers are available by request. See Supplementary Figure 2 for a schematic summary of inclusion/exclusion criteria and sample size.

Protein expression data: Baltimore Longitudinal Study of Aging Proteomic data from brain tissue acquired from autopsy samples in a subset of participants from the Baltimore Longitudinal Study on Aging (BLSA) was used to test correlations in protein levels between A2M and *a priori* specified protein(s) emerging from the brain gene expression analysis. BLSA is a prospective cohort study of community dwelling participants that began in 1958.[33,34] Written informed consent was obtained at each visit, and the study was approved by the local Institutional Review Board and the National Institute on Aging.

We examined the proteome of the middle frontal gyrus of 47 deceased BLSA participants with a range of AD pathology including those with clinical and postmortem pathological diagnosis of AD, individuals who were cognitively normal during life but with significant AD pathology confirmed via postmortem histopathology (that is, 'asymptomatic AD' described in detail previously[35]) and normal controls free of symptoms and AD pathology. Demographic characteristics of the sample are included in Supplementary Table 2. Label-free quantification of the brain proteome by LC–MS/MS was performed using a ThermoFisher Scientific (San Jose, CA, USA) Q-Exactive Plus mass spectrometer). Label-free quantitation analysis of raw data was performed using MaxQuant 1.5.3.28 software (Max Planck Institute of Biochemistry, Martinsried, Germany). Additional details on collection of proteomic data, raw data availability, and analysis parameters are provided through the Synapse AMP-AD portal (https://www.synapse.org/#!Synapse:syn3606086).

Statistical analysis

A2M and AD: associations with preclinical pathophysiology (CSF markers of AD)—Linear mixed effects regression models were used to explore associations between blood A2M concentration and CSF measures, including Aβ1-42, t-tau and p-tau, in the BIOCARD study sample. We replicated these analyses using the ADNI study. All CSF measures in BIOCARD and ADNI were log-transformed to meet normality assumptions. Considering epidemiological and biological evidence indicating that sex differences may have an important role in the pathology and progression of AD[36,37] as well as in the modulation of immune and neuroinflammatory responses,[38,39] all analyses were run separately for the total, male and female samples.

Model 1 explored associations between baseline A2M concentration and baseline and longitudinal change in CSF measures, including Aβ1-42, t-tau and p-tau. Estimated effect sizes for A2M in BIOCARD were expressed in units of 10 mg dl^{-1}. As described in Supplementary Table 3, the ADNI Biomarkers Consortium performed a number of quality control steps, including box-cox transformations and imputation for missing data and samples below assay sensitivity, to generate cleaned data; we further multiplied measures by 10 to allow for easier comparison to BIOCARD results. Additional model details including model covariates and interpretation of the main coefficients of interest are included in Supplementary Table 5. All models included covariates age, race and sex (excluded in sex-specific models). Considering prior evidence suggesting that A2M levels may be associated

with infections,[13] we included total white blood cell count measured at the time of the A2M assays, as an additional covariate in our primary analyses in the BIOCARD study.

Model 2 explored associations between change in A2M concentration and longitudinal change in CSF measures. This model was identical to model 1, other than the replacement of the main predictor (baseline A2M) with a binary predictor variable indicating whether A2M concentration was maintained/increased or decreased over time. Additional model details are included in Supplementary Table 5.

To further explore the potential of blood A2M concentration to identify individuals with abnormal levels of CSF pathology, we examined A2M concentration in relation to hypothetical preclinical AD groups defined by severity of abnormality in CSF AD biomarker concentrations in the BIOCARD study sample. These preclinical groups have been described in detail previously.[3,40,41] Specific CSF biomarker cutoffs were based on methodology used previously.[42] We tested for differences in serum A2M concentration by sex and conversion status using non-parametric (spearman rank correlation) tests. We additionally explored differences in baseline blood A2M concentration by diagnosis (normal, MCI and AD) in the ADNI study. We tested for differences between groups using spearman rank correlation tests.

In addition to CSF Aβ1-42, t-tau and p-tau, we also explored associations between A2M and neurofilament light (NFL) and neurogranin (Ng), two novel CSF markers of neuronal injury in AD[43,44] assayed in the ADNI study. We used Pearson correlations to test these associations.

A2M and AD: associations with risk of MCI/AD—Cox regression models (model 3) were used to explore whether baseline blood A2M concentration predicted the risk of developing clinical symptoms of MCI, before the diagnosis of MCI or AD in BIOCARD and conversion to MCI or AD in ADNI. Owing to the sample size constraints, in BIOCARD, a binary variable of 'high' or 'low' baseline serum A2M concentration (median split) was used as the main predictor variable, and age at onset of clinical symptoms was used as the outcome variable for participants who later developed MCI; for participants who remained cognitively normal, age censored at the last evaluation was used as the outcome variable.[28] Similar to prior studies,[28] participants diagnosed as 'impaired non-MCI' ($n = 42$) at their final visit were included with subjects diagnosed as normal ($n = 177$). Sensitivity analyses were also performed excluding individuals diagnosed as 'impaired non-MCI.' Because of the larger sex-specific sample size, in ADNI we were able to use a continuous variable for A2M as the main predictor variable; date of diagnosis was used as the outcome variable. Included participants were either normal or MCI at baseline ($n = 454$); participants with dementia at baseline were excluded ($n = 112$). Additional model details including model covariates and interpretation of the main coefficients of interest are included in Supplementary Table 5.

We additionally explored associations between baseline A2M concentration and baseline and longitudinal change in cognitive performance, including measures of executive function, attention and psychomotor speed, language, memory and learning, in the BIOCARD study.

This model (model 4) was identical to model 1, other than the outcomes. Model details are included in Supplementary Table 5.

A2M and AD: molecular mechanisms—Pearson correlations were used in the GTEx gene expression dataset to test whether peripheral (blood) A2M gene expression reflected A2M gene expression in the hippocampus and other brain regions. A2M gene co-expression analyses in the GEO dataset were performed using the ExplainBio web tool (http://www.explainbio.com; developed by SV).[45] The tool uses a recursive algorithm to build a network where the expression of the target gene (A2M) is modeled as a linear combination of other genes (source genes). Supplementary Table 6 provides a step-by-step summary of the algorithm used by the ExplainBio web tool.

On the basis of *a priori* specified genes identified in the above gene expression network analysis, we used Pearson correlations to explore log gene co-expression associations in the brain-specific GEO dataset, both across the whole brain as well as within the entorhinal cortex and hippocampus, regions especially vulnerable to tau pathology and neurodegeneration.[46–48]

To validate correlations found in the brain-specific gene co-expression analyses described above, we examined protein-level associations between A2M and *a priori* specified protein(s) of interest in brain tissue derived from the autopsy sample of the BLSA. Log_2 of label-free quantitation normalized protein intensities reported by MaxQuant for the participant sample was bootstrap regressed against age and postmortem interval of each individual; Pearson correlations were used to explore correlations between proteins of interest. Protein-level analyses were run separately for male and female samples.

All associations and group differences were assessed using two-tailed *t*-tests with a significance level of 0.05. All software code used to generate results is available by request.

RESULTS

Demographic characteristics of study samples are presented in Supplementary Table 2. BIOCARD participants were all normal at baseline. Males had significantly higher education and significantly lower baseline serum A2M concentration compared with females. Most ADNI participants were MCI at baseline, had lower education and were older than BIOCARD participants. Males in ADNI were significantly older, had significantly higher education and were more likely to be MCI at baseline compared with females. Average total white blood cell count in the BIOCARD study did not vary by sex, and only 4 participants (out of 303) had an elevated total white blood cell count (defined as > 10 K μl^{-1}).

As observed in our primary analyses, male participants in ADNI also had lower plasma A2M concentration compared with females. Figure 2e shows results exploring differences in A2M among normal, MCI and AD individuals in ADNI. We found significantly higher plasma A2M concentration in MCI compared with normal, and AD compared with normal individuals. A2M concentration by diagnosis and *P*-values are included in Supplementary Table 9.

A2M and AD: associations with preclinical pathophysiology (CSF markers of AD)

Results for the linear mixed effects regression model 1 testing cross sectional associations between blood A2M concentration and CSF biomarkers are shown in Figures 2a and b for BIOCARD and Figures 2c and d for ADNI. In both the BIOCARD and ADNI samples, we found that higher baseline A2M was associated with higher baseline CSF t-tau in the total sample. In the ADNI sample, we additionally found that higher baseline A2M was also associated with higher baseline p-tau in the total sample and higher baseline t-tau in females only. We found no significant associations between baseline A2M concentration and longitudinal changes in CSF measures in either sample. Full model results for the total, male, and female samples in BIOCARD and ADNI are included in Supplementary Table 7.

For model 2, in the total and male samples in BIOCARD, we found that independent of the level of baseline A2M concentration, participants who maintained or increased A2M levels over time showed a decreased annual rate of change in CSF p-tau compared with participants whose A2M levels decreased over time. In the ADNI sample, we found a similar marginally significant association between longitudinal changes in A2M concentration, and rate of change in CSF t-tau in the total and female samples. Full model results for the total, male and female samples in BIOCARD and ADNI are included in Supplementary Table 8.

Figure 2f shows results exploring differences in A2M among hypothetical preclinical AD groups in BIOCARD. We found that among individuals who later converted to MCI/AD, serum A2M concentration increased with severity of the concurrent CSF AD biomarker profile. The SNAP category, which is defined only by abnormal neurodegeneration biomarkers (that is, abnormal t-tau or p-tau[41]), showed the highest serum A2M concentration. Participants in the SNAP category had significantly higher A2M concentration than those in stage 0 and stage 1; participants in the stage 2 category had significantly higher A2M concentration than those in stage 0. A2M concentration by stage and *P*-values are included in Supplementary Table 9.

Supplementary Figure 3 shows associations in the ADNI sample between A2M and NFL and Ng, two novel CSF markers of neurodegeneration. We found significant correlations (Ng: $r = 0.12$; $P = 0.032$; NFL: $r = 0.12$; $P = 0.120$), although weaker than correlations with CSF t-tau concentration.

A2M and AD: associations with risk of MCI/AD

Figures 3a and b show model 3 (Cox regression model) results and Kaplan–Meier survival curves in BIOCARD. The mean time from baseline A2M assessment to the date of final visit or onset of clinical symptoms was 8.59 years (s.d. = 3.87 years). Higher serum concentration of A2M at baseline was significantly associated with a threefold greater risk of clinical symptoms of MCI in males only. Results from Cox regression models in sensitivity analyses were comparable when participants diagnosed as 'impaired non-MCI' were excluded (results not shown). Figures 3c and d show parallel results in ADNI. The mean time from baseline A2M assessment to the date of final visit or conversion to MCI/AD was 3.39 years (s.d. = 2.69). Higher plasma A2M concentration at baseline was significantly associated with

risk of conversion to MCI/AD in males only. Full model results for the total, male and female samples in BIOCARD and ADNI are included in Supplementary Table 10.

Results for model 4 exploring associations between baseline A2M concentration and cognitive performance in BIOCARD are included in Supplementary Table 11. We found in the total and male samples that greater baseline A2M concentration was associated with accelerated worsening in executive function (Trails B) over time.

A2M and AD: molecular mechanisms

Results of correlations between matched blood and brain samples from the GTEx dataset are shown in Figure 4a. We found that peripheral A2M gene expression was significantly correlated with brain A2M gene expression in several regions including the hippocampus (Figure 4b) (P-values ranging from < 0.001 to 0.02).

Results for the GEO multi-tissue gene network analysis are shown in Figure 5a. The final global gene co-expression model selected nine genes as having the highest direct contributions to predicting A2M gene expression. Given our findings on the associations between serum A2M concentration and CSF tau/p-tau, we were particularly interested in exploring genes known to modulate tau levels and/or tau phosphorylation. Of the nine genes comprising the A2M gene network, the regulator of calcineurin (RCAN1) gene is known to inhibit calcineurin, a well-characterized calcium and calmodulin-dependent serine/threonine tau phosphatase in the brain,[49–51] and was thus of most interest as a probable candidate gene to explain these associations. Molecular functions of the other genes in the A2M gene network previously shown to be associated with AD, including the slit guidance ligand 2 (SLIT2; associated with brain–blood barrier dysfunction),[52,53] are included in Supplementary Table 12. We have additionally included global brain log gene expression correlations between A2M and SLIT2 in Supplementary Figure 4.

On the basis of our findings from the gene network analysis, we then examined both global and regional brain gene expression correlations between A2M, RCAN1 and calcineurin, including the three calcineurin catalytic subunits, each encoded by a separate gene (PPP3CA, PPP3CB and PPP3CC). We found that global brain gene expression of A2M was significantly correlated with global brain gene expression of RCAN1 ($r = 0.206$; $P < 0.001$) and global brain gene expression of PPP3CA ($r = -0.453$; $P < 0.001$), PPP3CB ($r = -0.358$; $P < 0.001$) and PPP3CC ($r = -0.357$; $P < 0.001$). We additionally found significant region-specific correlations between hippocampal gene expression of A2M and PPP3CA ($r = -0.613$; $P < 0.001$) and PPP3CB ($r = -0.577$; $P < 0.001$), as well as entorhinal cortical gene expression of A2M and PPP3CA ($r = -0.418$; $P = 0.002$) and PPP3CC ($r = -0.607$; $P < 0.001$). Scatterplots showing these results are included in Figure 5b and correlations and P-values are included in Supplementary Table 13.

On the basis of our findings from global and regional brain gene expression analyses, we examined associations at the protein level between A2M and components of the RCAN1-calcineurin pathway that were detectable by our label-free proteomic analyses of brain tissue. In brain tissue samples obtained from the middle frontal gyrus in individuals representing a range of AD pathology, we found a significant negative correlation between

A2M and PPP3CB protein levels in the total ($r = -0.30$; $P = 0.04$) and male samples ($r = -0.49$; $P = 0.006$) (Figure 5c).

DISCUSSION

We have demonstrated that serum concentration of the acute-phase protein, A2M, is associated with CSF markers of neuronal injury in preclinical AD and predicts clinical symptoms of MCI in males. We replicated these results in an independent sample showing that plasma A2M concentration is associated with CSF tau and predicts conversion to MCI and AD in males. To the best of our knowledge, these results represent the first report of an association between a systemic marker of inflammation and the preclinical AD pathophysiological signature. Our findings have several translational implications and open up new areas of investigation into the role of the inflammatory response during early stages of AD pathogenesis.

The finding that blood A2M concentrations reflect the severity of neuronal injury, assessed by CSF levels of tau and p-tau, raises the possibility that blood A2M concentration may be of utility in the initial non-invasive screening or stratification of participants in clinical trials targeting neuronal injury in AD/MCI. Our findings that blood A2M concentration reflects the severity of preclinical disease stage and is associated with increasing cognitive impairment (normal < MCI < AD) further emphasize this potential. The observation that the A2M response in preclinical AD is distinct in males versus females may be critical in understanding sex differences in AD risk and incidence[36,54] as well as in the rational design of clinical trials targeting inflammation in AD (for example, ref. 55). If as suggested by our results, the primary acute-phase response to neuroinflammation in AD is sex-specific, it would be especially important to test the utility of sex-specific dosing regimens in such clinical trials.

It is important to also note that modulating the protein chaperone activity of A2M has been proposed as a plausible therapeutic approach in diseases characterized by accumulation of misfolded proteins such as AD.[56] In addition, epidemiologic and clinical trial evidence indicates that suppressing inflammation may prevent the accumulation of AD neuropathology.[57,58] Our findings showing a mechanistic link between A2M and tau phosphorylation provide additional evidence for exploring such experimental and therapeutic approaches in preclinical models of AD.

To identify the mechanistic basis for our findings implicating peripheral A2M levels in the pathophysiology of preclinical AD, we applied a systems level strategy combining large multi-tissue gene expression datasets as well as mass spectrometry-based proteomic analyses of brain tissue. Our gene expression analyses first tested whether peripheral A2M gene expression was significantly associated with A2M gene expression in the brain. A significant challenge in interpreting studies describing associations between systemic/peripheral immune or inflammatory processes and AD is whether such changes in blood reflect parallel changes in the brain.[59–62] Our findings that peripheral A2M gene expression is highly correlated with A2M gene expression in brain regions vulnerable to tau-mediated

neurodegeneration, is critical to our interpretation of associations between serum A2M and preclinical AD pathogenesis.

We then showed that within the brain, the A2M gene is part of a co-expression network that includes calcineurin, a well-established tau phosphatase enzyme, as well as its regulator gene, RCAN1. The observation that A2M gene expression may be influenced by both calcineurin and RCAN1 is significant considering previous studies implicating calcineurin and RCAN1 in AD pathogenesis. These include the finding that a single nucleotide polymorphism in the regulatory subunit of calcineurin is associated with elevated CSF p-tau and faster disease progression in AD.[49,63,64] Moreover, protein levels of calcineurin are lower in AD brains and are inversely correlated to both clinical and pathological measures in AD.[49] Similarly, dysregulation of RCAN1 has been implicated in AD pathogenesis and aberrant RCAN1 expression has been shown to promote tau phosphorylation and neuronal apoptosis leading to neuronal loss and formation of neurofibrillary tangles.[48] Together, these results uncover a brain network of tau-phosphorylation sensitive genes (that is, RCAN1, and the calcineurin subunits-PPP3CA, PPP3CB, PPP3CC) associated with A2M. We propose that A2M may be upregulated in the preclinical AD brain and blood in response to tau phosphorylation, a key trigger of neurodegeneration within the hippocampus and entorhinal cortex, and a crucial molecular event thought to mediate the onset of cognitive impairment in AD.[65]

To extend and validate our gene expression results, we next explored associations between A2M and components of the RCAN1/calcineurin network at the protein level in the brain. Using unbiased label-free quantification by mass spectrometry, we were able to detect A2M and PPP3CB, a catalytic subunit of calcineurin, at the protein level in the middle frontal gyrus in AD and control samples from the BLSA. Our findings confirmed our gene expression results and showed that brain protein levels of A2M are also negatively correlated with those of calcineurin. Although the primary goal in our gene expression and proteomic analyses was to elucidate plausible mechanisms underlying the associations between A2M and tau/p-tau, there are likely to be other biological pathways intrinsic to AD pathogenesis that may also be associated with A2M. It is relevant to note here that one of the novel A2M network genes discovered in our primary analyses is SLIT2 (Figure 5a; Supplementary Figure 4), which has recently been implicated in the regulation of blood brain barrier permeability and associated with AD-like abnormalities in a transgenic mouse model.[52,53] Abnormalities in brain–blood barrier integrity are in turn, closely linked to vascular dysfunction in AD.[66–68] Our findings thus provide a rationale for further mechanistic studies linking peripheral inflammatory responses with brain–blood barrier abnormalities and vascular dysfunction in AD. Another promising area of further investigation would be to study associations between A2M and emerging novel CSF markers of neurodegeneration such as NFL and Ng.[43,69] Exploratory analyses included in Supplementary Figure 3 provide additional evidence implicating A2M in the inflammatory response to neuronal injury.

Our study raises several questions that merit further investigation. Although A2M is a well-established extracellular chaperone capable of clearing misfolded, aggregated proteins including Aβ, recent evidence suggests that it may also function as an intracellular chaperone.[70] This finding, in combination with our results suggesting that A2M may

respond to tau phsphorylation states, opens up a novel line of investigation to further study A2M-tau interactions *in vivo*. Our results additionally suggest the importance of better understanding mechanisms underlying the sex-specific A2M response in preclinical AD. Although these studies are beyond the scope of our current analyses, it is striking that consistent with our CSF results, the protein-level associations between A2M and calcineurin in the brain appear to be driven primarily by males. Moreover, these results are consistent with previous animal studies that show a sex-specific A2M response to a systemic inflammatory challenge, wherein the acute-phase response is delayed or blunted in female rats compared with males.[71–73] Together with previous animal and human studies that indicate a greater vulnerability in females to neurofibrillary degeneration,[74–76] our current results further underscore the importance of studying mechanisms related to sex-specific inflammatory responses in AD pathogenesis.

The strengths of our study include the use of the well-characterized BIOCARD cohort; a study sample with longitudinal clinical and cognitive assessments that was cognitively normal at baseline and enriched for a family history of dementia. To confirm that our observations in the BIOCARD study were not specific to this highly selected sample, we performed an independent replication study in a larger sample consisting of older individuals, that is, the ADNI study. Such independent replication analyses are especially important in the context of blood biomarker studies in AD where several promising results in small studies are not replicated in subsequent analyses.[77,78] A second strength of our study is the use of a systems levels approach combining multi-tissue gene expression data with mass spectrometry-based proteomic analyses to propose a mechanistic explanation of our findings relating A2M in the pathogenesis of AD.

Our study has limitations. Both the BIOCARD and ADNI cohorts consist primarily of Caucasian participants and therefore the generalizability of our results in more diverse samples merits further consideration.

In conclusion, we have demonstrated that serum A2M concentration is associated with preclinical AD, reflects early neuronal injury in the disease course and may be responsive to tau phosphorylation in the brain through the RCAN1-calcineurin pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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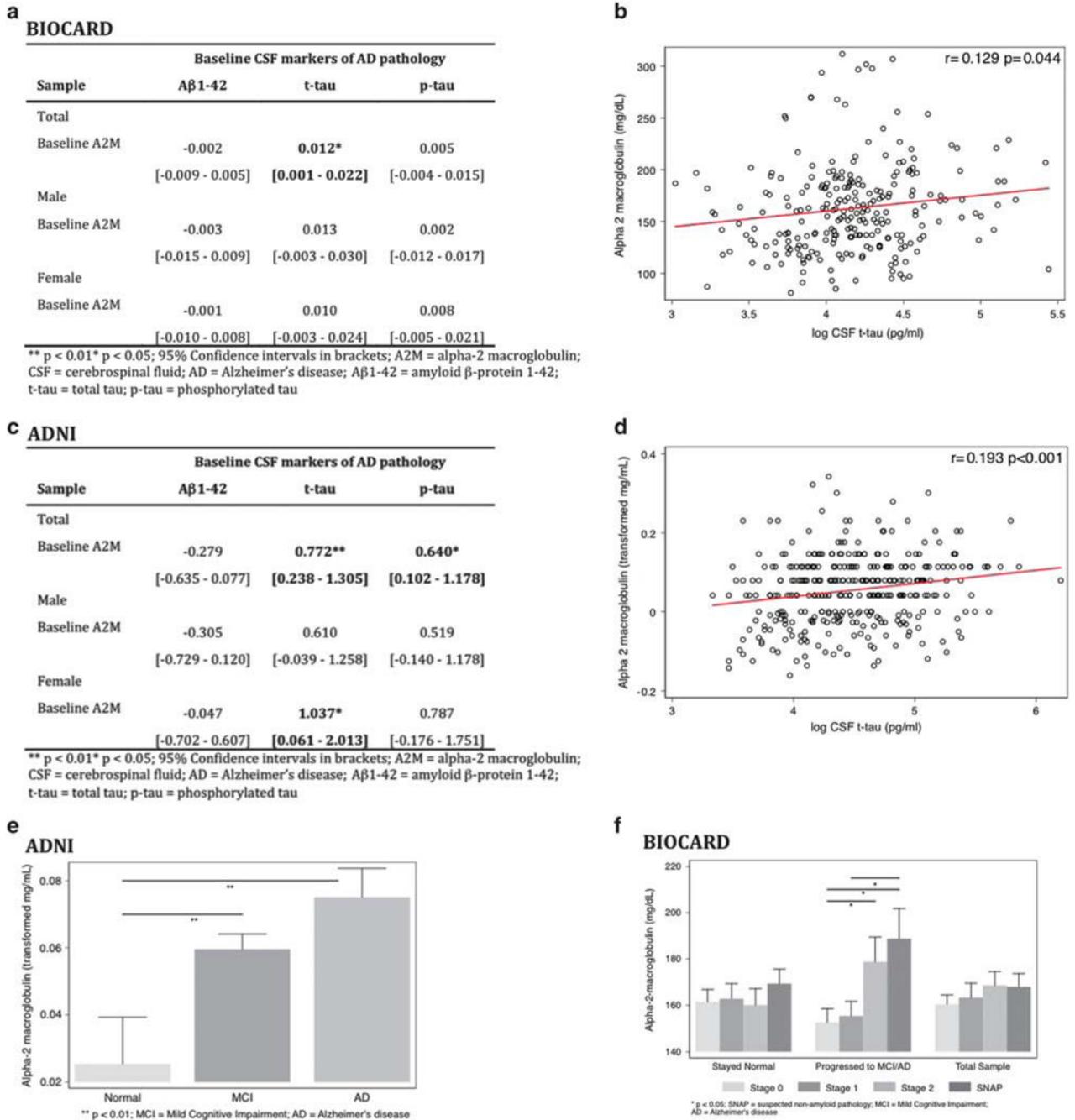
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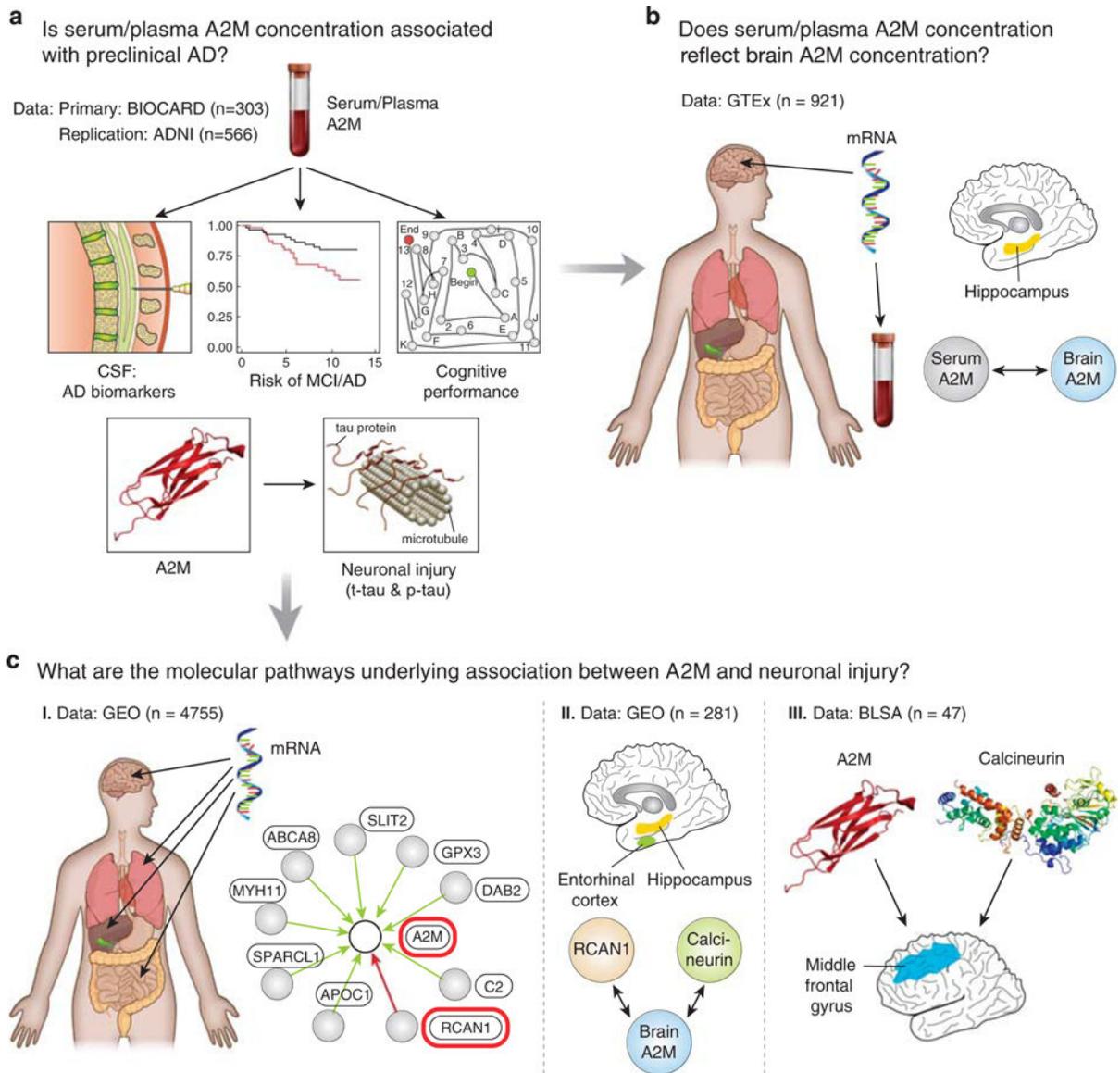
Figure 1.
Schematic representation of the study design. (**a**) Using the BIOCARD and Alzheimer's Disease Neuroimaging (ADNI) studies we explored associations between alpha-2 macroglobulin (A2M) and cerebrospinal fluid measures of preclinical Alzheimer's disease (AD) pathophysiology and risk of mild cognitive impairment (MCI)/AD. (**b**) Using publicly available gene expression data acquired from the GTEx project, we explored the association between blood and brain A2M gene expression. (**c**) Using publicly available gene expression data acquired from GEO, we explored global gene networks driving A2M gene expression (I) and brain-specific gene expression correlations (II). Protein expression data from autopsy samples in the Baltimore Longitudinal Study of Aging (BLSA) was used to validate gene expression findings (III). CSF, cerebrospinal fluid.

a BIOCARD

Sample	Baseline CSF markers of AD pathology		
	Aβ1-42	t-tau	p-tau
Total			
Baseline A2M	-0.002	0.012*	0.005
	[-0.009 - 0.005]	[0.001 - 0.022]	[-0.004 - 0.015]
Male			
Baseline A2M	-0.003	0.013	0.002
	[-0.015 - 0.009]	[-0.003 - 0.030]	[-0.012 - 0.017]
Female			
Baseline A2M	-0.001	0.010	0.008
	[-0.010 - 0.008]	[-0.003 - 0.024]	[-0.005 - 0.021]

** $p < 0.01$* $p < 0.05$; 95% Confidence intervals in brackets; A2M = alpha-2 macroglobulin; CSF = cerebrospinal fluid; AD = Alzheimer's disease; Aβ1-42 = amyloid β-protein 1-42; t-tau = total tau; p-tau = phosphorylated tau

c ADNI

Sample	Baseline CSF markers of AD pathology		
	Aβ1-42	t-tau	p-tau
Total			
Baseline A2M	-0.279	0.772**	0.640*
	[-0.635 - 0.077]	[0.238 - 1.305]	[0.102 - 1.178]
Male			
Baseline A2M	-0.305	0.610	0.519
	[-0.729 - 0.120]	[-0.039 - 1.258]	[-0.140 - 1.178]
Female			
Baseline A2M	-0.047	1.037*	0.787
	[-0.702 - 0.607]	[0.061 - 2.013]	[-0.176 - 1.751]

** $p < 0.01$* $p < 0.05$; 95% Confidence intervals in brackets; A2M = alpha-2 macroglobulin; CSF = cerebrospinal fluid; AD = Alzheimer's disease; Aβ1-42 = amyloid β-protein 1-42; t-tau = total tau; p-tau = phosphorylated tau

b r= 0.129 p=0.044 (Alpha 2 macroglobulin (mg/dL) vs log CSF t-tau (pg/ml))

d r= 0.193 p<0.001 (Alpha 2 macroglobulin (transformed mg/mL) vs log CSF t-tau (pg/ml))

e ADNI — Alpha-2 macroglobulin (transformed mg/mL) by Normal, MCI, AD
** $p < 0.01$; MCI = Mild Cognitive Impairment; AD = Alzheimer's disease

f BIOCARD — Alpha-2-macroglobulin (mg/dL) by Stayed Normal, Progressed to MCI/AD, Total Sample; Stage 0, Stage 1, Stage 2, SNAP
* $p < 0.05$; SNAP = suspected non-amyloid pathology; MCI = Mild Cognitive Impairment; AD = Alzheimer's disease

Figure 2.
Associations between alpha-2 macroglobulin (A2M) and cerebrospinal fluid (CSF) measures of preclinical Alzheimer's disease (AD) pathophysiology. (**a**) Coefficient estimates from model 1 (linear mixed effects models) in BIOCARD; baseline A2M was significantly associated with concurrently measured t-tau in the total sample. (**b**) Scatter plot and linear fit line (red) of baseline CSF t-tau and baseline serum A2M concentrations in BIOCARD. (**c, d**) Replication results from model 1 using plasma A2M from the Alzheimer's Disease Neuroimaging Initiative (ADNI) study. (**e**) Differences in plasma A2M by diagnosis in

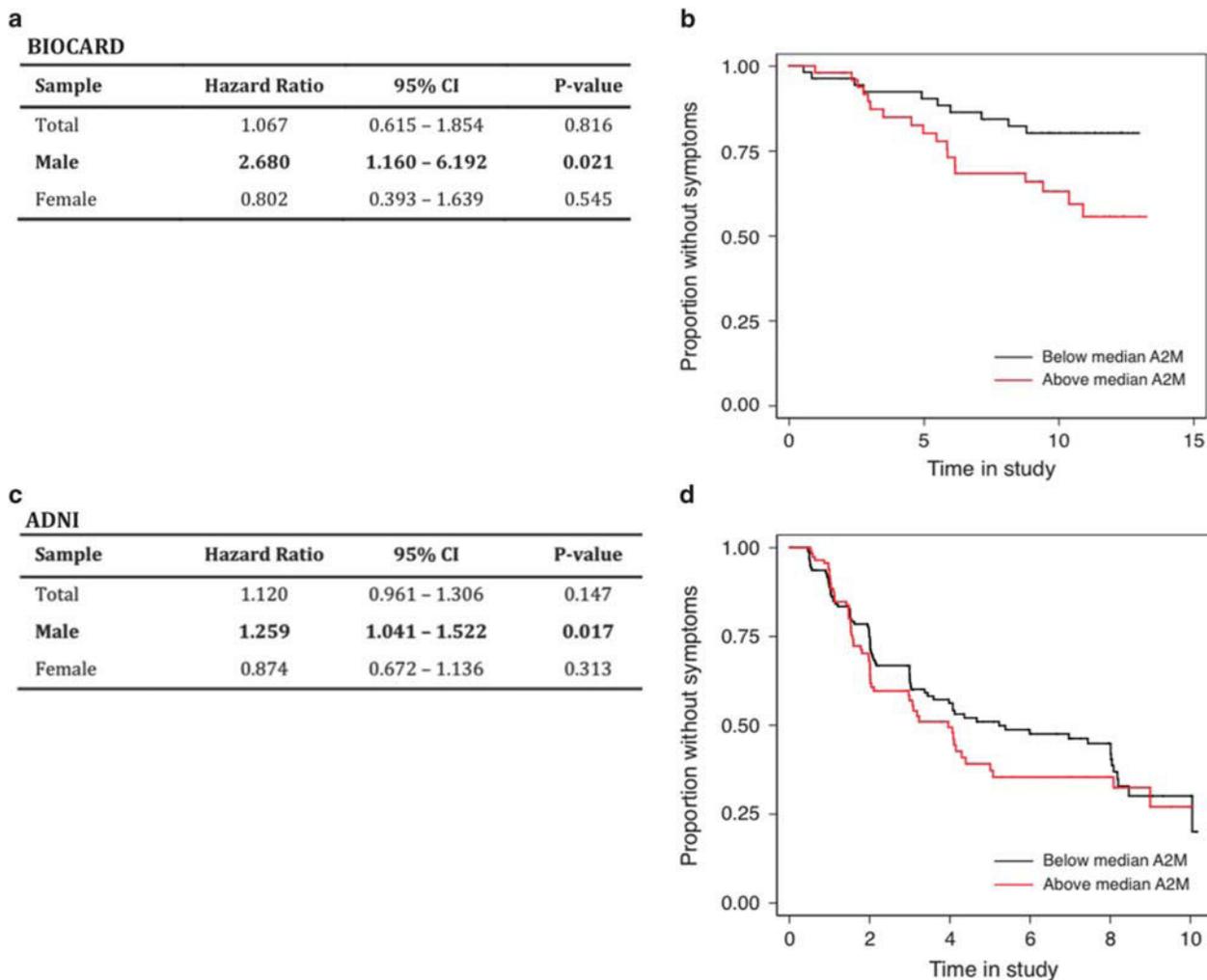ADNI; participants with mild cognitive impairment and AD had significantly higher A2M than normal individuals. (**f**) Differences in serum A2M among hypothetical preclinical AD groups; participants in the SNAP category that is, only (abnormal t-tau and p-tau) had significantly higher A2M than those in stage 0 and stage 1; participants in the stage 2 category had significantly higher A2M concentration that those in stage 0.

a

BIOCARD
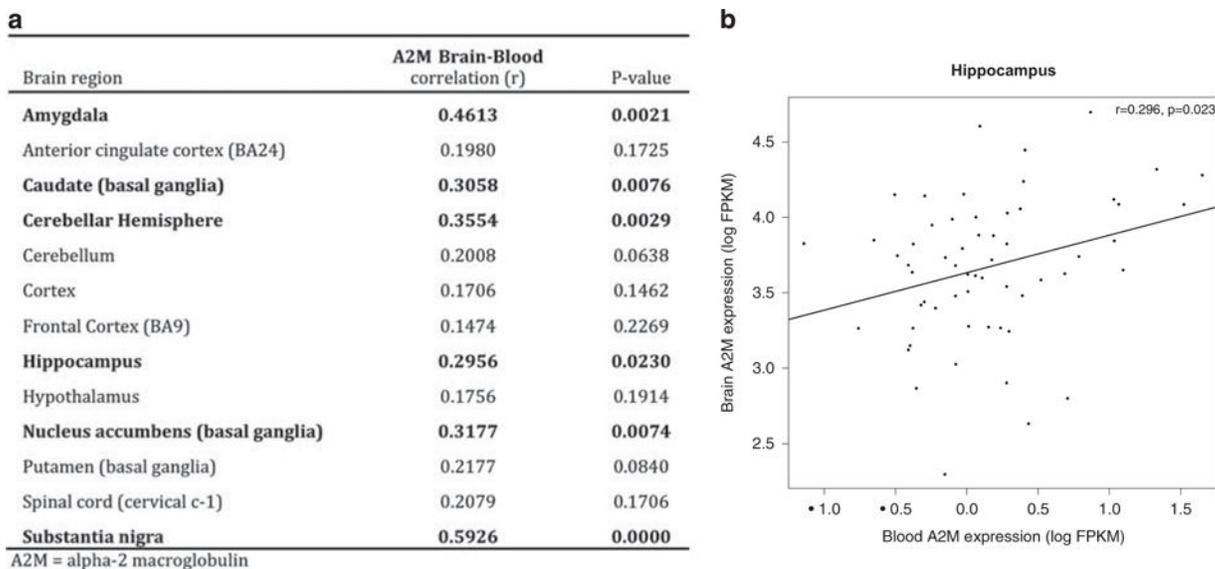
Sample	Hazard Ratio	95% CI	P-value
Total	1.067	0.615 – 1.854	0.816
Male	**2.680**	**1.160 – 6.192**	**0.021**
Female	0.802	0.393 – 1.639	0.545

b

c

ADNI

| Sample	Hazard Ratio	95% CI	P-value
Total	1.120	0.961 – 1.306	0.147
Male	**1.259**	**1.041 – 1.522**	**0.017**
Female	0.874	0.672 – 1.136	0.313

d

Figure 3.
Associations between alpha-2 macroglobulin (A2M) and risk of mild cognitive impairment (MCI)/Alzheimer's disease (AD). (**a**) Coefficient estimates from model 2 (Cox regression models); serum concentration of A2M at baseline was significantly associated with risk of clinical symptoms of MCI in males. (**b**) Kaplan–Meier survival curves indicating the probability of onset of clinical symptoms for participants with higher versus lower baseline A2M concentration. (**c, d**) Replication results from model 2 using plasma A2M concentrations in the Alzheimer's Disease Neuroimaging (ADNI) study.

a

Brain region	A2M Brain-Blood correlation (r)	P-value
Amygdala	**0.4613**	**0.0021**
Anterior cingulate cortex (BA24)	0.1980	0.1725
Caudate (basal ganglia)	**0.3058**	**0.0076**
Cerebellar Hemisphere	**0.3554**	**0.0029**
Cerebellum	0.2008	0.0638
Cortex	0.1706	0.1462
Frontal Cortex (BA9)	0.1474	0.2269
Hippocampus	**0.2956**	**0.0230**
Hypothalamus	0.1756	0.1914
Nucleus accumbens (basal ganglia)	**0.3177**	**0.0074**
Putamen (basal ganglia)	0.2177	0.0840
Spinal cord (cervical c-1)	0.2079	0.1706
Substantia nigra	**0.5926**	**0.0000**

A2M = alpha-2 macroglobulin

b

Figure 4.

Associations between brain and blood alpha-2 macroglobulin (A2M) gene expression. (**a**) Results of Pearson's correlations between brain and blood gene expression; expression was significantly positively correlated in six regions. (**b**) Scatter plot and linear fit of blood and brain A2M gene expression in the hippocampus.
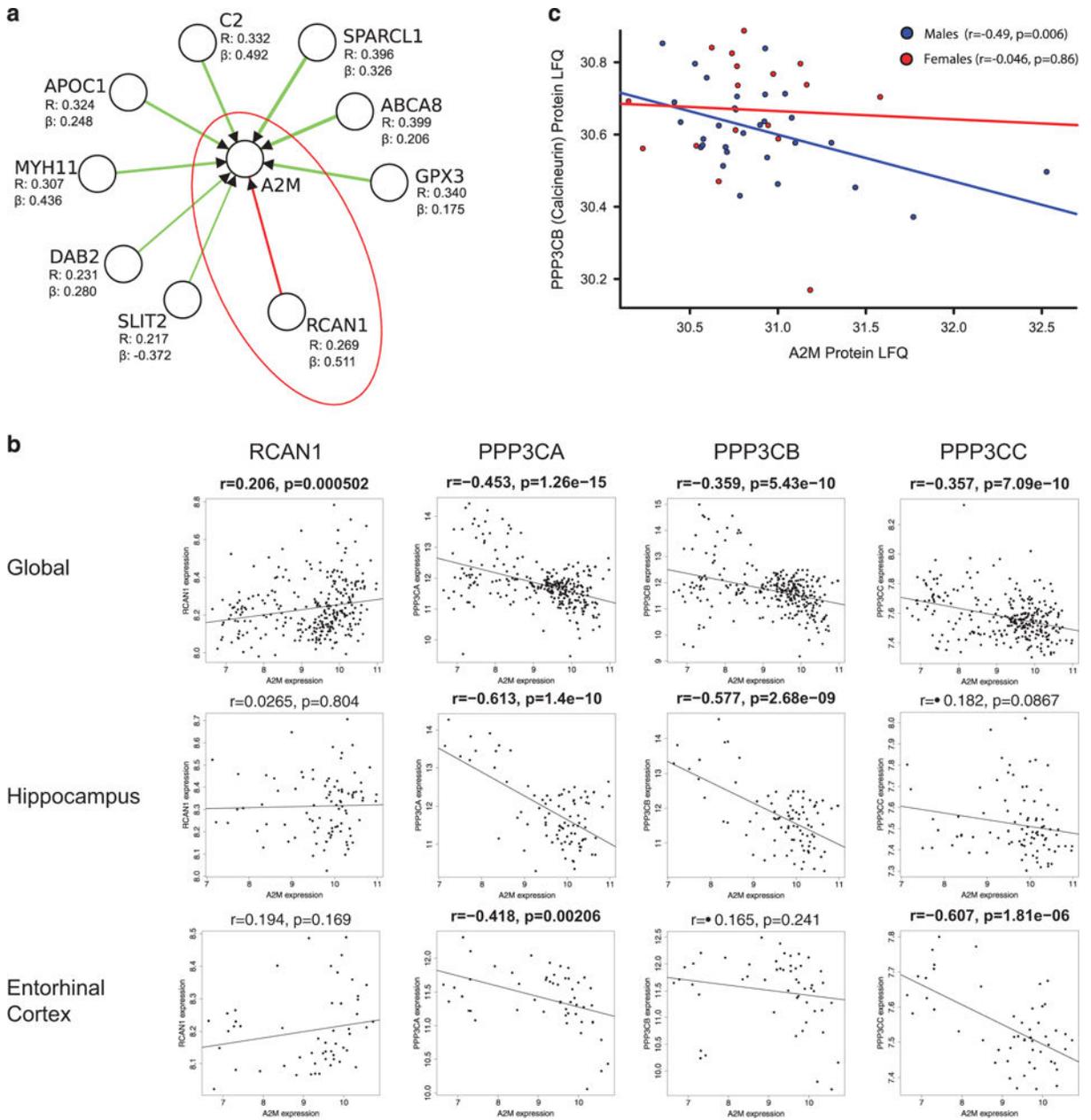
Figure 5.

Molecular mechanisms explaining associations between alpha-2 macroglobulin (A2M) and Alzheimer's disease (AD). (**a**) Global gene networks driving A2M gene expression; of the nine genes comprising the A2M gene network, the regulator of calcineurin gene (RCAN1) was most likely to explain associations between A2M and t-tau and p-tau. (**b**) Results (significant results in bold) of global and regional brain log gene expression correlations between A2M and RCAN1 and A2M and three catalytic subunits of calcineurin (PPP3CA, PPP3CB and PPP3CC); gene expression of A2M was significantly associated with RCAN1 and calcineurin. (**c**) Results of brain tissue protein-level correlations between A2M and

calcineurin (PPP3CB); A2M was significantly correlated with PPP3CB in the male sample (blue) but not the female sample (red).