Identification of plasma proteins relating to brain neurodegeneration and vascular pathology in cognitively normal individuals

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Abstract
Introduction: This study aims to first discover plasma proteomic biomarkers relating to neurodegeneration (N) and vascular (V) damage in cognitively normal individuals and second to discover proteins mediating sex-related difference in N and V pathology.

Methods: Five thousand and thirty-two plasma proteins were measured in 1061 cognitively normal individuals (628 females and 433 males), nearly 90% of whom had magnetic resonance imaging measures of hippocampal volume (as N) and white matter hyperintensities (as V).
1 | INTRODUCTION

The National Institute on Aging and Alzheimer’s Association (NIA-AA) have proposed classifying Alzheimer’s disease (AD) based on biomarkers of amyloid pathology (A), tau pathology (T), and neurodegeneration (N).1 The flexibility of the AT(N) system could be expanded to incorporate new biomarkers that track brain vascular (V) damage, leading to ATV(N).1 Both neurodegeneration and vascular pathology can be measured by several different magnetic resonance imaging (MRI) measures. For example, neurodegeneration can be measured by brain atrophy. Multiple lines of evidence showed that hippocampal atrophy is closely associated with AD.2–4 Vascular pathology can be measured by white matter hyperintensities (WMHs), which have been associated with an increased risk for developing AD and dementia.5–7

There is increasing evidence to suggest an influence of biological sex on neuroimaging biomarkers in AD pathogenesis. For example, brain atrophy rates for those with mild cognitive impairment (MCI) and AD dementia were faster in females compared to males.8–11 In contrast, increased WMHs led to faster progression to MCI or AD only among males.12,13 Although the differences are likely due to sex hormones,14 the exact mechanisms that underlie these sex-related differences are still unclear. Gaining a more detailed picture of the causes of sex-related differences could yield important clues about the pathophysiology of AD and eventually lead to sex-specific preventative or therapeutic strategies.

Compared to MRI measures, blood-based biomarkers show promise as a simple and potentially cost-effective option for the early detection, classification, and monitoring of AD pathology. With this in mind, the present study had two main objectives: first, to identify plasma biomarkers related to neurodegeneration and vascular pathology in cognitively normal individuals; second, to identify proteins mediating sex-related differences in neurodegeneration and vascular damage. To do this, we used SomaLogic’s Somascan assay to measure 5032 proteins in plasma from 1061 cognitively healthy individuals. Using a range of statistical approaches, we identified different proteins associated with markers of neurodegeneration and vascular damage. Furthermore, using causal mediation analysis, we found evidence for several proteins that may mediate sex-related differences in neurodegeneration and vascular pathology (Figure 1).

2 | METHODS

2.1 | Participants

The participants in this study were recruited as part of the Stratifying Resilience and Depression Longitudinally (STRADL) study, which re-contacted participants from the Generation Scotland: Scottish Family Health Study (GS). The GS study is a large, family-structured, population-based cohort study of more than 24,000 individuals from across Scotland. Recruitment took place between 2006 and 2011 with a clinical visit during which detailed health and cognitive test data were collected along with biological samples (blood, urine, saliva). Full details of the STRADL cohort and GS protocol are published elsewhere.15–17 Briefly, blood was collected by venepuncture into standard polypropylene ethylenediaminetetraacetic acid (EDTA) test tubes followed by centrifugation; the obtained plasma was aliquoted in 500 μL aliquots, and stored at −20 °C for future analyses.17 Ethical approval for STRADL was formally obtained from the National Health Service Tayside committee and all participants provided their written informed consent.

We selected a total of 1061 cognitively normal individuals including 628 females and 433 males from STRADL study. Plasma and general demographic information were available for all subjects (including family history of AD and apolipoprotein E [APOE] ε4 genotype data). Four cognitive tests covering multiple cognitive domains were also assessed for each subject. These tests included digit symbol, verbal fluency, Mill Hill vocabulary scale, and Wechsler memory test.15 A general cognitive ability score was generated via extracting the first unrotated component from a principal component analysis of the four tests. Brain structural MRI scans were available for 927 participants to obtain hippocampal volume. Visually inspecting and rating fluid-attenuated inversion recovery (FLAIR) scans were available for 940 subjects to obtain Fazekas score, an index of WMHs.
1061 cognitively normal individuals with brain MRI measurement | Plasma | SOMA scan assay (5032 proteins)

n=628
n=433

Differential protein expression
Proteins associated with MRI

Protein co-expression network
Protein modules associated with MRI

Causal mediation analysis
Identifying proteins mediating sex-related differences in neurodegeneration and vascular pathology

FIGURE 1 Overview of the study design: 5032 proteins were measured in 1061 cognitively normal individuals who had magnetic resonance imaging (MRI) measurement. Proteome-wide association study of MRI and protein co-expression network analysis revealed potential proteins for causal mediation analysis, leading to the finding of proteins mediating sex-related differences in brain neurodegeneration and vascular damage.

2.2 Plasma analyses

Plasma proteins were measured using the SOMA scan assay platform (SomaLogic Inc.). SOMA scan is an aptamer-based assay allowing for the simultaneous measurement and quantification of, in the version used here, 5032 proteins. The assay uses chemically modified nucleotides to transform a protein signal into a nucleotide signal that can be quantified using relative fluorescence on microarrays. Raw data processing and initial quality control (QC) led to 4235 proteins for final analysis. The abundance of each protein was log-transformed, then the effects of sample collection site and plasma storage time on proteins were removed by linear regression and the residuals were used for all subsequent analyses.

2.3 MRI acquisition and analyses

Participants were scanned at two centers: the Ninewells Hospital in Dundee and at the Aberdeen Royal Infirmary in Aberdeen. Participants in Dundee were scanned using a Siemens 3T Prisma-FIT (Siemens Healthineers) with a 20-channel head and neck coil and a back-facing mirror (software version VE11, gradient with max amplitude 80 mT/m and maximum slew rate 200 T/m/s). In Aberdeen, participants were imaged on a 3 T Philips Achieva TX series MRI system (Philips Healthcare) with a 32-channel phased-array head coil with a back-facing mirror (software version 5.1.7; gradients with maximum amplitude 80 mT/m and maximum slew rate 100). Both study centers followed the same protocol including structural sequences. 3T MRI scans were anonymized at the time of acquisition and scanning site was included as a covariate in statistical analyses. Full details of the imaging sequences and parameters are published elsewhere.

2.3.1 Hippocampal volume

T1 structural measures were processed using FreeSurfer version 5.3 to quantify the volumes of 14 subcortical structures as well as the volumes, surface area, and thickness of 34 cortical regions per hemisphere.
RESEARCH IN CONTEXT

1. Systematic review: Plasma proteins are studied as candidate biomarkers to predict brain neurodegeneration and vascular pathology in Alzheimer’s disease (AD), while few studies focus on prodromal stage of AD. Furthermore, there is increasing evidence to suggest brain sex differences in AD pathogenesis, while the exact mechanisms that underlie these sex-related differences are still unclear.

2. Interpretation: Our findings offer new insights into changes in individual proteins and protein networks linked to neurodegeneration and vascular pathology in the prodromal stage of AD. Furthermore, we identified several plasma proteins mediated sex-related differences in brain neurodegeneration and vascular damage. Our study is the largest plasma proteomic study in prodromal AD in terms of the number of proteins assayed and sample size, to our knowledge.

3. Future directions: It suggests that blood proteins can predict brain neurodegeneration and vascular pathology in the prodromal stage of AD. Furthermore, the nominated proteins are tractable targets for further mechanistic studies on brain sex differences, with potential to lead to more effective sex-specific preventative or therapeutic strategies.

Statistical analyses were completed using R (version 3.3.2). To compare baseline cohort characteristics between males and females, we used Mann-Whitney U tests and Chi-square tests to compare continuous and binary variables, respectively.

2.4 I Statistical analysis

Statistical analyses were completed using R (version 3.3.2). To compare baseline cohort characteristics between males and females, we used Mann-Whitney U tests and Chi-square tests to compare continuous and binary variables, respectively.

2.4.1 I Protein differential expression analysis (DEA)

We used partial Spearman correlation to test the association of proteins with hippocampal volume and WMHs (Fazekas scores), adjusting for age, sex, and APOE ε4 genotype. Additionally, imaging batch, number of image edits per individual, assessment center, and standardized intracranial volume (ICV) were set as covariates for hippocampal volume analyses. We performed such analyses in all individuals as well as in subgroups stratified by sex. P values were corrected using a false discovery rate (FDR) for multiple testing. Proteins that were differentially expressed at a nominal significance level of $P < .05$ were included in pathway analysis using WebGestalt software (http://www.webgestalt.org/). Briefly, differentially expressed proteins were included as the "protein list" and all proteins measured by SOMAscan assay were used as "background." This enrichment analysis was performed on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

2.4.2 I Weighted gene correlation network analysis (WGCNA)

We first used the R package WGCNA to construct a co-expression network from the proteins. The effects of age and sex on proteins were adjusted for by linear regression and the resulting residuals were used for analysis. WGCNA clustering is based on calculating correlations between paired variables, soft-threshold transforming them with a power function ($|cor|^\beta$), and using the result as adjacency matrix between variables. The final step applies hierarchical clustering to this adjacency matrix. We applied this algorithm with default parameters, except for the following settings: soft threshold power beta $\beta = 4$, minimum module size $= 10$ proteins, merge cut height $= 0.2$. The resulting modules or groups of co-expressed proteins were used to calculate module eigenproteins. The eigenprotein-based connectivity ($kME$) was used to represent the strength of a protein’s correlation with other protein module members. Proteins with high intramodular $kME$ in the
## Causal mediation analysis

We used R package regmedint\(^{24}\) to investigate how the relationship between an exposure variable and an outcome variable relate to a third intermediate variable, namely the mediator. Here, we wanted to test if proteins (as mediator, \(M\)) mediate the relationship between sex (as exposure, \(X\)) and MRI measurement (as outcome, \(Y\)). A mediator needs to meet the following three criteria\(^25\) (Figure S2A in supporting information): (1) A change in levels of the exposure variable significantly affects the changes in the outcome (i.e., total effect of \(X\) on \(Y\) is significant). (2) There is a significant relationship between the mediator and the outcome (i.e., Path from \(M\) to \(Y\)). (3) A change in levels of the exposure variable significantly affects the changes in the mediator (i.e., Path from \(X\) to \(M\)).

There was a significant difference between females and males in terms of hippocampal volume and WMHs after adjusting for covariates (Figure S2B and C; criteria 1). For mediators, we selected proteins significantly associated with hippocampal volume or WMHs (criteria 2). We also checked that these proteins are significantly expressed between females and males (criteria 3). We reported those proteins whose natural indirect effect is significant and the direction is consistent with natural direct effect.

## RESULTS

### 3.1 Subject demographics

Demographic information of subjects is shown in Table 1. The male group was slightly older than the female group. No significant difference was observed in the distribution of APOE \(\varepsilon 4\) carriers, education, and self-reported family history (mother or father reported as having AD). In terms of MRI measures, females had a smaller hippocampal volume than males on average, while no difference was observed in WMHs.

### 3.2 Proteins significantly associated with hippocampal volume and WMHs

Using partial Spearman correlation, we found nominally significant associations (\(P < .05\)) between 377 proteins and hippocampal volume in all individuals. Seventeen of these were significant after FDR correction for multiple testing (FDR \(P < .05\); Figure 2A, Table S1 in supporting information). KEGG pathway analysis of the 228 proteins with significantly negative associations (\(P < .05\)) revealed three pathways that were cell adhesion molecules (CAMs) and complement and coagulation cascades (Figure 2B). Conversely, the 149 proteins with significantly positive associations (\(P < .05\)) indicated two pathways that included cytokine–cytokine receptor interaction, axon guidance, and metabolic pathways (Figure 2B).

Stratifying by sex, we found 328 proteins showed nominally significance in females and 2 of them were significant after FDR correction. In contrast, 172 proteins showed nominally significance in males while no proteins were significant after FDR correction.

For WMHs, 178 proteins showed nominal significance and 2 of them remained significant after FDR correction in all individuals.

### Table 1

Demographics of participants included in the analysis by sex

| Characteristics                  | Female (n = 628) | Male (n = 433) | \(P\) value |
|----------------------------------|-----------------|---------------|------------|
| Age mean (SD), y                 | 59.3 ± 9.7      | 60.7 ± 9.3    | 0.02       |
| APOE \(\varepsilon 4\) N (%)     | 170 (27%)       | 118 (27%)     | 0.99       |
| Education mean (SD), y           | 4.5 (1.5)       | 4.5 (1.6)     | 0.83       |
| Mother with AD                   | 46 (7.3%)       | 27 (6.2%)     | 0.57       |
| Father with AD                   | 24 (3.8%)       | 15 (3.5%)     | 0.89       |
| Hippocampal volume (SD) in mm\(^3\) | 8155 (744)     | 8659 (936)    | <0.001     |
| WMHs (SD)                        | 2.27 (0.92)     | 2.21 (0.88)   | 0.35       |

Abbreviations: AD, Alzheimer’s disease; APOE, apolipoprotein E; SD, standard deviation; WMHs, white matter hyperintensities.

Note: Percentage of cases is shown in parentheses for APOE \(\varepsilon 4\) carriers and mother or father with AD.
FIGURE 2 Volcano plot of proteins associated with (A) hippocampal volume and (C) white matter hyperintensities (WMHs). Those proteins with significantly negative correlations (\(P < .05\)) are shown in blue, while the proteins with significantly positive correlations (\(P < .05\)) are noted in red. Enriched Kyoto Encyclopedia of Genes and Genomes pathways of proteins with significantly negative (blue) and positive (red) associations with (B) hippocampal volume and (D) WMHs. DE, differential expression; FDR, false discovery rate.

(FIGURE 2C, Table S1). KEGG pathway analysis of the proteins with significantly negative and positive associations (\(P < .05\)) revealed one and five enriched pathways, respectively (FIGURE 2D). None of the proteins were significant after FDR correction in either females or males. Of the 17 hippocampal volume-related and 2 WMH-related proteins (FDR \(P < .05\)), 8 of them were significantly associated with general cognitive score (Figure S3 in supporting information).

3.3 Plasma protein co-expression network analysis reveals modules linked to hippocampal volume and WMHs

We first performed a network-based analysis of the plasma proteome using WGCNA. We found eight modules (M) of co-expressed proteins and ranked them based on size from largest (M1 turquoise module; \(n = 2694\) proteins) to smallest (M8 pink module; \(n = 13\) proteins; Table S2 in supporting information). Figure 3A shows the clustering of these modules’ concordance according to similarities in expression patterns. We further investigated the biological significance of proteins in each module and found that the modules were enriched with various pathways after FDR correction (Table S3 in supporting information), such as renal cell carcinoma (M1 turquoise module), cytokine–cytokine receptor interaction (M2 blue module), metabolism of xenobiotics by cytochrome P450 (M4 yellow module), complement and coagulation cascades (M5 green module), cholesterol metabolism (M6 red module), and pancreatic secretion (M8 pink module).

We then assessed the module correlations to hippocampal volume and WMHs in all individuals as well as only in females and males. As shown in Figure 3B, across all individuals and after FDR correction, the M4 yellow and M6 red modules had a negative and positive correlation with hippocampal volume, respectively. Furthermore, such correlations remained significant in males and females and their direction of association were consistent. In addition, the M5 green module was positively correlated with hippocampal volume in males.

For WMHs, none of the modules passed FDR in all individuals. However, in females, the M1 turquoise and M6 red modules had negative correlations with WMHs. In males, the M1 turquoise and M3 brown modules were positively associated with WMHs while the M5 green module was negatively associated with WMHs (Figure 3B). Furthermore, modules that were positively correlated with hippocampal volume were negatively associated with WMHs such as the M5 green and M6 red modules, indicating the consistency between such associations because hippocampal atrophy and larger WMHs are associated with increased AD risk. Figures 3C and D showed the hub proteins within the M5 green and M6 red modules, respectively.
FIGURE 3 | Protein modules correlating to hippocampal volume and white matter hyperintensities (WMHs). A, Weighted gene correlation network analysis (WGCNA) of the plasma proteome. This algorithm generated eight modules (M) of co-expressed proteins. Modules are clustered in the network dendrogram based on their relatedness. B, Analysis of the association of modules with hippocampal volume and WMHs. * and ** denote significant correlations $P < 0.05$ and $P < 0.001$ after false discovery rate (FDR) correction, respectively. (C) and (D) Hub proteins noted within the M5 green and M6 red modules, respectively. F, female; M, male; N, neurodegeneration; V, vascular damage.

3.4 | Correlation of protein networks with cognitive test scores and AD risk

We further investigated the module correlations to cognitive test measures and AD risk. We found that the M4 yellow and M8 pink modules had negative correlations and M5 green module had a positive correlation with general cognitive score after FDR correction (Figure 4A-C). These results are in concordance with MRI correlations as the M4 yellow and M5 green modules had negative and positive correlations with hippocampal volume, respectively. The AD risk was decided by both family history and APOE ε4 genotype as described previously, leading to 662 low risk, 315 moderate risk, and 32 high risk individuals. We found that the M2 blue and M6 red modules showed significant increase and decrease in higher risk compared to low-risk individuals, respectively (Figure 4D and E). Of these, the M6 red module is in concordance with MRI correlations as it had positive and negative correlations with hippocampal volume and WMHs, respectively.

3.5 | Causal mediation analysis reveals proteins mediating brain sex differences

We selected proteins obtained from DEA for further causal mediation analysis. Overall, we considered 17 hippocampal volume-related and two WMH-related proteins (FDR $P < .05$). Of the 17 proteins, 4 of them showed significant natural indirect effect with hippocampal volume (Table 2), indicating they mediated sex-related differences in brain neurodegeneration. Furthermore, two of them were in the M5 green module, which had positive correlations with hippocampal volume only in males (Figure 3B) and NCAM-1 was the hub protein in the M5 green module (Figure 3C), further indicating the consistency
across DEA and co-expression network analysis. For WMHs, one protein (Apo B) showed a significant natural indirect effect (Table 2), meaning it mediated sex-related difference in vascular damage. This protein is in the M6 red module, which had a negative correlation with WMHs only in females (Figure 3B).

### 4 | DISCUSSION

In this study, we used SOMAscan to measure 5032 plasma proteins from 1061 cognitively normal individuals (female n = 628 and male n = 433). We performed both proteome-wide DEA and protein co-expression network analysis to gain insights into changes in individual proteins as well as networks of proteins relating to neurodegeneration and vascular markers in cognitively healthy individuals. We identified different proteins and modules associated with markers of neurodegeneration and vascular pathology, respectively. Using causal mediation analysis, we further confirmed that four proteins mediated sex-related difference in brain neurodegeneration and one protein mediated such difference in vascular damage. Of the five proteins, three of them were in the modules that were associated with neurodegeneration or vascular damage only in male or female and one protein (NCAM-1) was the hub protein in the module, suggesting that our findings are robust across multiple computational analyses. In addition, most neurodegeneration- and vascular pathology-related proteins and modules were also associated with cognitive score and their direction of change was in concordance with MRI correlations.
It is important to identify blood-based biomarkers relating to neurodegeneration, particularly in the preclinical stage of AD. Most clinical trials for AD have been unsuccessful to date. The failure of such trials was partially caused by the fact that participants enrolled in such trials were relatively late in the disease process. Targeting treatment to earlier pre-symptomatic or prodromal stages of the disease might have more success. With this in mind we sought to identify blood biomarkers relating to neurodegeneration in cognitively normal individuals collected from a population-based cohort that resembles the real-world situation of participants’ recruitment in clinical trials. We identified 17 proteins that were significant after FDR correction for multiple testing in the full cohort; two proteins were FDR significant in females compared to no proteins in males. This might be because brain atrophy is small in cognitively normal individuals and that the subgroup analysis is too small to detect such differences. Nevertheless, because these proteins were associated with neurodegeneration in the preclinical stage, they could potentially help when recruiting cognitively normal individuals with neurodegeneration for clinical trials with further validation.

The importance of WMHs in AD is increasingly recognized as they are related to risk for developing AD. Some studies showed that there is an association between WMHs and AD pathological hallmarks such as amyloid beta (Aβ) plaques, tau, and neurodegeneration. Here, we only found two proteins that were associated with WMHs after FDR correction for multiple testing. This might be because the participants recruited in our study were relatively young whereas WMHs tend to be observed with aging. Current treatment of WMHs includes pharmacological approaches (i.e., blood pressure medications and statins) as well as nonpharmacological approaches such as lifestyle modifications and risk factor management. Given the associations between WMHs and vascular risk factors, it is imperative to target vascular health throughout the life course as a prevention strategy. The proteins identified here could potentially help to select cognitively normal individuals with vascular pathology at a very early stage as well as to monitor the intervention outcomes throughout the life course.

Current findings on the associations of AD hallmarks in plasma with neurodegeneration and vascular pathology have generated new enthusiasm. For example, various studies found that neurofilament light chain (NFL) was closely associated with neurodegeneration. Furthermore, NFL was also associated with vascular pathology in an age-dependent manner. Another marker, phosphorylated tau (p-tau)181 was found to be a good marker for predicting and monitoring neurodegeneration. These two biomarkers are well recognized as they have been validated in multiple cohorts across different laboratories. In comparison, the identified biomarkers from our study need further validation in independent longitudinal cohorts. Furthermore, it is important to conduct head-to-head comparison studies in the future not only to compare the performance of our identified biomarkers to both NFL and p-tau181 but also to check whether they could add extra value on top of these two well-recognized biomarkers.

The increasing recognition of sex differences in the brain and AD risk has highlighted the urgent need for biomarkers that more comprehensively reflect the complex mechanisms underlying these differences. Examination of these differences may shed light on the pathophysiology of AD that differs between the sexes and ultimately lead to more effective interventions and precision medicine.

Of the five proteins, neural cell adhesion molecule (NCAM) is a part of a family of cell-surface glycoproteins that play key roles in normal brain development, including axonal/dendritic growth and branching, and synaptic plasticity. The levels of NCAM-1 have been shown to alter in AD patients’ blood, cerebrospinal fluid (CSF), and brain tissue. Furthermore, it interacted with amyloid precursor protein (APP) and promoted neurite outgrowth, indicating that it could be a potential therapeutic target for AD treatment. Apolipoprotein B (Apo B) forms the primary protein component of atherogenic lipoprotein particles. Lower levels of this protein was associated with better maintenance of cognitive abilities. Furthermore, it was highly associated with Aβ and tau pathology in subjective cognitive decline individuals, indicating that Apo B may be a potential biomarker for the preclinical stage of AD. This protein has been shown to increase in CSF of presymptomatic and affected persons carrying familial AD mutations as well as to reduce Aβ and reverse cognitive impairment in AD mouse model. The other two proteins were EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1), a member of the fibulin family that mediates cell–cell and cell–matrix interactions; follistatin-related protein 3 (FSTL3), an important physiological regulator of activin; and other TGFβ superfamily members. These findings not only confirm the role of previously reported proteins in AD, but also identify new biomarkers relating to early neurodegeneration pathology such as EFEMP1 and FSTL3. Furthermore, these findings provide tractable targets for further mechanistic studies of sex-related neurodegeneration and vascular pathology in the preclinical stage of AD, eventually leading to sex-specific preventative or therapeutic strategies.

Pathway analysis of hippocampal volume-related and WMH-related proteins revealed five and six significantly enriched pathways, respectively. Some of them have been reported being associated with AD such as axon guidance, MAPK signaling pathway, and cell adhesion molecules (CAMs), further demonstrating the relatedness of these proteins with AD. Furthermore, most neurodegeneration and vascular damage-related proteins and protein modules were associated with cognitive score and their direction of association were in concordance with brain MRI correlations. Various studies have reported that brain atrophy and increased WMHs lead to cognitive decline. Our findings further demonstrated that proteins relating to brain pathology were also associated with lower cognitive test scores.

There are three limitations for our study. First, the population in this study is of European ancestry, predominantly of Scottish ancestry, so validation in independent cohorts and particularly in other ethnic
groups is needed to see if the results are generalizable. Second, our study is cross-sectional and longitudinal studies are required to determine the role of nominated proteins in sex differences in brain pathology and risk of AD. Third, although the individuals in this study are well characterized on cognition, APOE ε4 genotype, and family history, there is a lack of Aβ plaques and tau tangles to confirm the stage of AD. Therefore, the terminology of preclinical AD needs to be interpreted with caution. Further studies on individuals defined by both neuropathology and clinical symptoms are needed to confirm the results.

Despite this, our study is the largest we are aware of to report plasma biomarkers indicative of both neurodegeneration and vascular damage in cognitively normal individuals in terms of the number of proteins assayed as well as sample size. By applying different statistical approaches, we identified individual proteins and protein networks linked to N and V in the poorly understood preclinical stage of AD. Furthermore, we demonstrated that four proteins mediated sex-related differences in brain neurodegeneration and one protein in vascular damage. These nominated proteins can not only serve as biomarkers to help recruit cognitively normal individuals with neurodegeneration and vascular damage for clinical trials but also as predictive biomarkers to monitor possible intervention outcomes. Furthermore, these proteins provide tractable targets for further mechanistic studies of sex differences in brain pathology and AD risk, with potential to lead to more effective sex-specific preventative or therapeutic strategies.

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CONFLICTS OF INTEREST
S.R.C received payment from the Society of Biological Psychiatry (plenary at SOBP2021). R.E.M. is an advisor to the Epigenetic Clock Development Foundation and has received a speaker fee from Illumina. A.C. is member of Edinburgh MVM Research Ethics Committee. J.M.W is involved in European Stroke Organisation Guideline on Covert Small Vessel Disease 2021 and European Stroke Organisation Chair of Conference Planning Group 2021 and 2022. D.S. helped to set up CAPE study. A.M. received speaker fees from Janssen and Illumina. S.L. is an employee of Janssen Medical UK and Co-founder of Akrivia Health Ltd. He is also named as an inventor on biomarker intellectual property protected by Proteome Sciences and Kings College London unrelated to the current study and within the past 5 years has advised for Optum labs, Merck, SomaLogic, and been the recipient of funding from AstraZeneca and other companies via the IMI funding scheme. N.B is a member of Mehta Family Centre for Engineering in Medicine, Kanpur, India as well as the editorial board of Stem Cells. A.N.H. is the main PI of a project funded by J&J, and another projects funded by GSK, all unrelated to this study. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ETHICS STATEMENT
Ethical approval for the GS:SFHS study was obtained from the Tayside Committee on Medical Research Ethics (on behalf of the National Health Service).
DATA AVAILABILITY STATEMENT
Access to and use of GS and STRADL data must be approved by the GS
Access Committee under the terms of consent. Full details of the applica-
tion process can be found at www.generationscotland.org.

REFERENCES
1. Jack CR Jr, Bennett DA, Blennow K, Carrillo MC, Framework NIA-
AAResearch. Toward a biological definition of Alzheimer’s disease. Alzheimers Dement. 2018;14:535-562.
2. Apostolova LG, Green AE, Babakchanian S, et al. Hippocampal atro-
phy and ventricular enlargement in normal aging, mild cognitive
impairment (MCI), and Alzheimer disease. Alzheimer Dis Assoc Dis-
order. 2012;26(1):17-27.
3. Schröder J, Pantel J. Neuroimaging of hippocampal atrophy in early
recognition of Alzheimer’s disease—a critical appraisal after two
two decades of research. Psychiatry Res Neuroimaging. 2016;247:71-78.
4. Seab JP, Jagust WJ, Wong ST, Roos MS, Reed BR, Budinger TF. Quantita-
tive NMR measurements of hippocampal atrophy in Alzheimer’s dis-
ease. Magn Reson Med. 1988;8:200-208.
5. Debette S, Schilling S, Duperron MG, Larsson SC. Markus HS. Clin-
ical significance of magnetic resonance imaging markers of vascu-
lar brain injury: a systematic review and meta-analysis. JAMA Neurol.
2019;76:81-94.
6. Prins ND, Scheltens P. White matter hyperintensities, cognitive
impairment and dementia: an update. Nat Rev Neurol. 2015;11:157-
165.
7. Wardlaw JM, Valdés Hernández MC, Muñoz-Maniega S. What are
white matter hyperintensities made of? Relevance to vascular cogni-
tive impairment. J Am Hear Assoc. 2015;4:1140.
8. Sundermann EE, Biegön A, Rubín LH, Lipton RB, Landau S, Maki PM.
Does the female advantage in verbal memory contribute to under-
estimating Alzheimer’s disease pathology in women versus men?. J
Alzheimers Dis. 2017;56:947-957.
9. Sundermann EE, Biegön A, Rubín LH, et al. Better verbal memory in
women than men in MCI despite similar levels of hippocampal atrophy.
Neurology. 2016;86:1368-1376.
10. Hua X, Hilbar DP, Lee S, et al. Sex and age differences in atrophic
rates: an ADNI study with n = 1368 MRI scans. Neurobiol Aging.
2010;31:1463-1480.
11. Aredekan BA, Convit A, Bachman AH. Analysis of the MIRIAD
data shows sex differences in hippocampal atrophy progression. J
Alzheimers Dis. 2016;50:847-857.
12. Burke SL, Hu T, Fava NM. Sex differences in the development of mild
cognitive impairment and probable Alzheimer’s disease as predicted
by hippocampal volume or white matter hyperintensities. J Women
Aging. 2019;31:140-164.
13. Kim S, Kim MJ, Kim S, et al. Gender differences in risk factors for transition
from mild cognitive impairment to Alzheimer’s disease: a CREDOS
study. Compr Psychiatry. 2015;62:114-122.
14. Mielke MM, Vemuri P, Rocca WA. Clinical epidemiology of Alzheimer’s
disease: assessing sex and gender differences. Clin Epidemiol.
2014;6:37-48.
15. Smith BH, Campbell A, Linksted P, et al. Cohort Profile: generation
Scotland: Scottish Family Health Study (GS:SFHS). The study, its par-
ticipants and their potential for genetic research on health and illness.
Int J Epidemiol. 2013;42:689-700.
16. Navrady LB, Wolters MK, Machtyne RJ, et al. Cohort profile: stratifi-
cying resilience and depression longitudinally (STRADL): a question-
aire follow-up of Generation Scotland: Scottish family health study
(GS:SFHS). Int J Epidemiol. 2018;47:13-14g.
17. Habota T, Sandu A-L, Walter GD, et al. Cohort profile for the Strati-
Fyng resilience and depression longitudinally (STRADL) study:
a depression-focused investigation of Generation Scotland, using
detailed clinical, cognitive, and neuroimaging assessments. Welcome
Open Res. 2019;4:185.
18. Gold L, Ayers D, Bertino J, et al. Aptamer-based multiplexed proteome

technology for biomarker discovery. PLoS One. 2010;5:e15004.
19. Green C, Shen X, Stevenson AJ, et al. Structural brain correlates of
serum and epigenetic markers of inflammation in major depressive dis-
order. Brain Behav Immun. 2021;92:39-48.
20. Fischl B, et al. FreeSurfer. Neuroimage. 2012;62 (2): 774-781.
21. Desikan RS, Ségonne F, Fischl B, et al. An automated labeling system
for subdividing the human cerebral cortex on MRI scans into gyral
based regions of interest. Neuroimage. 2006;31:968-980.
22. Fazekas F, Chawluk JB, Alavi A, Hurtig HI, Zimmerman RA. MR signal
anomalities at 1.5 T in Alzheimer’s dementia and normal aging. AJR Am
J Roentgenol. 1987;149:351-356.
23. Langfelder P, Horvath S. WGCNA: an R package for weighted correla-
tion network analysis. BMC Bioinformatics. 2008;9:559.
24. Yoshida K, Mathur M, Glynn RJ, Conducting Regression-based Causal
Mediation Analysis Using the R Package“ regmedint” 2020.
25. Valeri L, VanderWeele TJ. Mediation analysis allowing for exposure–
mediator interactions and causal interpretation: theoretical assump-
tions and implementation with SAS and SPSS macros. Psychol Methods.
2013;18:137.
26. Cummings J, Lee G, Ritter A, Zhong K. Alzheimer’s disease drug
development pipeline: 2018. Alzheimer’s Dement (New York, N Y).
2018;4:195-214.
27. Golde TE, DeKosky ST, Galasko D. Alzheimer’s disease: the right drug,
the right time. Science (80-.). 2018;362:1250-1251.
28. Marnane M, O’O Al-Jawadi, Mortazavi S, et al. Periventricular white
intensities are associated with elevated cerebral amyloid. Neurology.
2016;86:535-543.
29. Lee S, Viquer F, Zimmerman ME, et al. White matter hyperintensities are
a core feature of Alzheimer’s disease: evidence from the dominantly
inherited Alzheimer’s network. Ann Neurol. 2016;79:929-939.
30. Gaubert M, Lange C, Garnier-Crussard A, et al. Topographic pat-
terns of white matter hyperintensities are associated with multimodal
neuroimaging biomarkers of Alzheimer’s disease. Alzheimers Res Ther.
2011;13:29.
31. Garnier-Crussard A, Bougacha S, Wirth M, et al. White matter hyper-
intensities across the adult lifespan: relation to age, Aβ load, and cog-
nition. Alzheimers Res Ther. 2020;12:127.
32. Habes M, Ers G, Toledo JB, et al. White matter hyperintensities and
imaging patterns of brain aging in the general population. Brain.
2016;139:1164-1179.
33. Edwards JD, Ramirez J, Callahan BL, et al. Antihypertensive treatment
is associated with MRI-derived markers of neurodegeneration and
impaired cognition: a propensity-weighted cohort study. J Alzheimers
Dis. 2017;59:1113-1122.
34. Moll van Charante EP, Richard E, Eurelings LS, et al. Effectiveness
of a 6-year multidomain vascular care intervention to prevent
dementia (preDIVA): a cluster-randomised controlled trial. Lancet.
2016;388:797-805.
35. ten Dam VH, van den Heuvel DM, van Buchem MA, et al. Effect of
pravastatin on cerebral infarcts and white matter lesions. Neurology.
2005;64:1807-1809.
36. Sexton CE, Betts JF, Demnitz N, Dawes H, Ebmeier KP, Johansen-
Berg H. A systematic review of MRI studies examining the relationship
between physical fitness and activity and the white matter of the age-
ning brain. Br J Sports Med. 2005;39:417-422.
37. Gardener H, Sarmesou N, Gu Y, et al. Mediterranean diet and white
matter hyperintensity volume in the Northern Manhattan Study. Arch
Neurol. 2012;69:251-256.
38. Espeland MA, Erickson K, Neilberg RH, et al. Brain and white
matter hyperintensity volumes after 10 years of random assign-
m ent to lifestyle intervention. Diabetes Care. 2016;39:764-
771.

SHI ET AL.
39. Mielle MM, Syrijanen JA, Blennow K, et al. Plasma and CSF neurofilament light: relation to longitudinal neuroimaging and cognitive measures. Neurology. 2019;93:e252-60.
40. Mattsson N, Cullen NC, Andreasson U, Zetterberg H, Blennow K. Association between longitudinal plasma neurofilament light and neurodegeneration in patients with Alzheimer’s disease. JAMA Neurol. 2019;76:791-799.
41. Lewczuk P, Ermann N, Andreasson U, et al. Plasma neurofilament light as a potential biomarker of neurodegeneration in Alzheimer’s disease. Alzheimer’s Res Ther. 2018;10:71.
42. Walsh P, Sudre CH, Fiford CM, et al. The age-dependent associations of white matter hyperintensities and neurofilament light in early- and late-stage Alzheimer’s disease. Neurobiol Aging. 2021;97:10-17.
43. Moscoco A, Grothe MJ, Ashton NJ, et al. Longitudinal associations of blood phosphorylated Tau181 and neurofilament light chain with neurodegeneration in Alzheimer’s disease. JAMA Neurol. 2021;78:396-406.
44. Hansson O, Cullen N, Zetterberg H, Blennow K, Mattsson-Carlsgen N. Plasma phosphorylated tau181 and neurodegeneration in Alzheimer’s disease. Ann Clin Transl Neurol. 2021;8:259-265.
45. Ferretti MT, Iulita MF, Cavedo E, et al. Sex differences in Alzheimer’s disease—the gateway to precision medicine. Nat Rev Neurol. 2018;14:457-469.
46. Nebel RA, Aggarwal NT, Barnes LL, et al. Understanding the impact of sex and gender in Alzheimer’s disease: a call to action. Alzheimers Dement. 2018;14:1171-1183.
47. Kleene R, Schachner M. Glycans and neural cell interactions. Nat Rev Neurosci. 2004;5:204-208.
48. Gnanapavan S, Grant D, Illes-Toth E, Lakdawala N, Keir G, Giovannoni G. Neural cell adhesion molecule–description of a CSF ELISA method and evidence of reduced levels in selected neurological disorders. J Neuroimmunol. 2010;225:118-122.
49. Todaro L, Puricelli L, Gioseffi H, et al. Neural cell adhesion molecule in human serum. Increased levels in dementia of the Alzheimer’s type. Neurobiol Dis. 2004;15:387-393.
50. Aisa B, Gil-Bea FJ, Solas M, et al. Altered NCAM expression associated with the cholinergic system in Alzheimer’s disease. J Alzheimers Dis. 2010;20:659-668.
51. Chen K, Lu H, Gao T, Xue X, Wang C, Miao F. Synergic interaction between amyloid precursor protein and neural cell adhesion molecule promotes neurite outgrowth. Oncotarget. 2016;7:14199-14206.
52. Chen KP, Dou F. Selective interaction of amyloid precursor protein with different isoforms of neural cell adhesion molecule. J Mol Neurosci. 2012;46:203-209.
53. Reynolds CA, Gatz M, Prince JA, Berg S, Pedersen NL. Serum lipid levels and cognitive change in late life. J Am Geriatr Soc. 2010;58:501-509.
54. Hu H, Tan L, Bi YL, et al. Association of serum Apolipoprotein B with cerebrospinal fluid biomarkers of Alzheimer’s pathology. Ann Clin Transl Neurol. 2020;7:1766-1778.
55. Kamata T, Katsube K, Michikawa M, Yamada M, Takada S, Mizusawa H. R-spondin, a novel gene with thrombospondin type 1 domain, was expressed in the dorsal neural tube and affected in Wnts mutants. Biochim Biophys Acta. 2004;1676:51-62.
56. Ringman JM, Schulman H, Becker C, et al. Proteomic changes in cerebrospinal fluid of presymptomatic and affected persons carrying familial Alzheimer’s disease mutations. Arch Neurol. 2012;69:96-104.
57. Park SY, Kang JY, Lee T, Nam D, Jeon CJ, Kim JB. SPON1 can reduce amyloid beta and reverse cognitive impairment and memory dysfunction in Alzheimer’s disease mouse model. Cells. 2020;9(5):1275.
58. Zhang Y, Marmorstein LY. Focus on molecules: fibulin-3 (EFEMP1). Exp Eye Res. 2010;90:374-375.
59. Schneyer A, Sidis Y, Xia Y, et al. Differential actions of follistatin and follistatin-like 3. Mol Cell Endocrinol. 2004;225:25-28.
60. Zhang L, Qi Z, Li J, et al. Roles and mechanisms of axon-guidance molecules in Alzheimer’s disease. Mol Neurobiol. 2021;1-18.
61. Ogishima S, Mizuno S, Kikuchi M, et al. A map of Alzheimer’s disease-signaling pathways: a hope for drug target discovery. Clin Pharmacol Ther. 2013;93:399-401.
62. Nagappan-Chettiar S, Johnson-Venkatesh EM, Umehori H. Activity-dependent proteolytic cleavage of cell adhesion molecules regulates excitatory synaptic development and function. Neurosci Res. 2017;116:60-69.
63. Wirth M, Madison CM, Rabinovici GD, Oh H, Landau SM, Jagust WJ. Alzheimer’s disease neurodegenerative biomarkers are associated with decreased cognitive function but not β-amyloid in cognitively normal older individuals. J Neurosci. 2013;33:5553-5563.
64. Van Den Berg E, Geerlings MI, Biessels GJ, Nederkoorn PJ, Klokpenborg RP. White matter hyperintensities and cognition in mild cognitive impairment and Alzheimer’s disease: a domain-specific meta-analysis. J Alzheimer’s Dis. 2018;63:515-527.
65. Alber J, Alladi S, Bae HJ, et al. White matter hyperintensities in vascular contributions to cognitive impairment and dementia (VCID): knowledge gaps and opportunities. Alzheimers Dement (N Y). 2019;5:107-117.
66. Benson G, Hildebrandt A, Lange C, et al. Functional connectivity in cognitive control networks mitigates the impact of white matter lesions in the elderly. Alzheimers Res Ther. 2018;10:109.
67. Kloppenborg RP, Nederkoorn PJ, Geerlings MI, van den Berg E. Presence and progression of white matter hyperintensities and cognition: a meta-analysis. Neurology. 2014;82:2127-2138.

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