Integrated methylome and phenome study of the circulating proteome reveals markers pertinent to brain health

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Characterising associations between the methylome, proteome and phenome may provide insight into biological pathways governing brain health. Here, we report an integrated DNA methylation and phenotypic study of the circulating proteome in relation to brain health. Methylome-wide association studies of 4058 plasma proteins are performed (N = 774), identifying 2928 CpG-protein associations after adjustment for multiple testing. These are independent of known genetic protein quantitative trait loci (pQTLs) and common lifestyle effects. Phenome-wide association studies of each protein are then performed in relation to 15 neurological traits (N = 1,065), identifying 405 associations between the levels of 191 proteins and cognitive scores, brain imaging measures or APOE e4 status. We uncover 35 previously unreported DNA methylation signatures for 17 protein markers of brain health. The epigenetic and proteomic markers we identify are pertinent to understanding and stratifying brain health.
The health of the ageing brain is associated with risk of neurodegenerative disease\(^1,^2\). Relative brain age—a measure of brain health calculated using multiple volumetric brain imaging measures—has recently been shown to predict the development of dementia\(^3\). Structural brain imaging and performance in cognitive tests are well-characterised markers of brain health\(^4\), which clearly associate with potentially modifiable traits such as body mass index (BMI), smoking and diabetes\(^5\). Understanding the interplay between environment, biology and brain health may therefore inform preventative strategies.

Multiple layers of omics data indicate the biological pathways that underlie phenotypes. Proteomic blood sampling can track peripheral pathways that may impact brain health, or record proteins secreted from the brain into the circulatory system. Although proteome-wide characterisation of cognitive decline and dementia risk\(^6,^10\) have been facilitated at large scale by SOMAscan\(^\text{®}\) protein measurements, there is a need to further integrate omics to characterise brain health phenotypes. Epigenetic modifications to the genome record an individual’s response to environmental exposures, stochastic biological effects, and genetic influences. Epigenetic changes include histone modifications, non-coding RNA, chromatin remodelling, and DNA methylation (DNAm) at cytosine bases, such as 5-hydroxymethylcytosine. These are implicated in changes to chromatin structure and the regulation of gene expression at large scale. Epigenetic modifications to the genome record an individual’s response to environmental exposures, stochastic biological effects, and genetic influences. Epigenetic changes include histone modifications, non-coding RNA, chromatin remodelling, and DNA methylation (DNAm) at cytosine bases, such as 5-hydroxymethylcytosine. These are implicated in changes to chromatin structure and the regulation of gene expression at large scale.

Modifications to DNAm at CpG sites play differential roles in influencing gene expression at the transcriptional level\(^1\). Additionally, DNAm accounts for inter-individual variability in circulating protein levels\(^9,^16\). Recently, through integration of DNAm and protein data, we have shown that epigenetic scores for plasma protein levels—known as EpiScores—associate with brain morphology and cognitive ageing markers\(^8\) and predict the onset of neurological diseases\(^9\). These studies highlight that while datasets that allow for integration of proteomic, epigenetic and phenotypic information are rarely-available, they hold potential to advance risk stratification. Integration may also uncover candidate biological pathways that may underlie brain health.

Associations between protein levels and DNA methylation at CpG sites are known as protein quantitative trait methylation loci (pQTLs) and can be quantified by methylome-wide association studies (MWAS) of protein levels. The largest MWAS of protein levels to date assessed 1,123 SOMAmers protein measurements in the German KORA cohort (\(n = 944\))\(^12\). In that study, Zaghloul et al. reported 98 pQTLs that replicated in the QM Biolab cohort (\(n = 344\)), with significant associations between DNA methylation and immune-associated locus NLRC5 and seven immune-related proteins (\(P < 2.5 \times 10^{-7}\)). This suggested that DNA methylation not only reflects variability in the proteome but is closely related to chronic systemic inflammation. Hillary et al. have also assessed epigenetic signatures for 281 SOMAmers protein measurements that were previously associated with Alzheimer’s disease, in the Generation Scotland cohort that we utilised in this study\(^13\). However, proteome-wide assessment of pQTLs has not been tested against a comprehensive spectrum of brain health traits.

Here, we conduct an integrated methylome- and phenome-wide assessment of the circulating proteome in relation to brain health (Fig. 1), using 4058 protein level measurements (Annotation information provided in Supplementary Data 1). We characterise CpG–protein associations (pQTLs) for these proteins in 774 individuals from the Generation Scotland cohort using EPIC array DNAm at 772,619 CpG sites. We then identify which of the 4058 protein levels associate with one or more of 15 neurological traits (seven structural brain imaging measures, seven cognitive scores and APOE e4 status) in 1065 individuals from the same cohort where the pQTM data are a nested subset. By overlapping these datasets, we probe the epigenetic signatures of proteins that are related to brain health. For these signatures, we map potential underlying genetic components and chromatin interactions that may play a role in protein level regulation.

### Results

**Methylome-wide studies of 4058 plasma proteins**

We conducted MWAS to test for pQTM associations between 772,619 CpG sites and 4058 circulating protein levels (corresponding to 4235 SOMAmers; Supplementary Data 1). The MWAS population included 774 individuals from Generation Scotland (mean age 60 years [SD 8.8], 56% Female; Supplementary Data 2), 143 principal components explained 80% of the cumulative variance in the 4235 measurements (Supplementary Fig. 1 and Supplementary Data 3). A threshold for multiple testing based on these components was applied across all MWAS (\(P < 0.05/(143 \times 772,619) = 4.5 \times 10^{-10}\)).

![Fig. 1 | Methylome and phenome study of the plasma proteome in relation to brain health study design.](https://example.com/fig1.png)

In our basic model adjusting for age, sex and available genetic PTL effects from Sun et al.\(^19\) 238,245 pQTLs (2107 cis and 236,138 trans, representing 0.005% of tested associations) had \(P < 4.5 \times 10^{-10}\) (Supplementary Data 4). In our second model that further adjusted for Houseman-estimated white blood cell proportions\(^6\), there were 3,213 associations (453 cis and 2,760 trans) that had \(P < 4.5 \times 10^{-10}\) (Supplementary Data 5). Smoking status and BMI are known to have well-characterised DNA methylation signatures\(^22,^23\); fully-adjusted models were

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therefore further adjusted for these factors. There were 2,928 associations (451 cis and 2477 trans) in the fully-adjusted models (Supplementary Data 6). 2847 pQTM associations were significant in all models. There were 191 unique proteins with associations in the fully-adjusted models, corresponding to 195 SOMAmer measurements (two SOMAmers were present for CLEC11A, GOLM1, ICAM5 and LRP11).

Figure 2 summarises these findings. Genomic in-flation statistics for these 195 SOMAmer measurements (fully-adjusted MWAS) are presented in Supplementary Data 7. In a sensitivity analysis, restriction of the threshold for cis pQTMs from 10 Mb to 1 Mb from the transcription start site of the gene encoding the protein yielded 409 cis pQTMs (a reduction of 42 pQTMs) in the fully-adjusted MWAS. A summary of known pQTLs20 and a record of whether these were available for adjustment is provided in Supplementary Data 8. Characterising the genomic location of the findings, 46% of cis and 29% of trans pQTMs in the fully-adjusted MWAS involved CpGs positioned in either a CpG Island, Shore or Shelf (Supplementary Data 6).

Pleiotropic pQTM associations in the fully-adjusted MWAS

Pleiotropy was observed for both CpG sites and protein levels (Fig. 3). Nineteen proteins had 10 or more pQTMs in the fully-adjusted MWAS (Supplementary Data 9). Of the 2928 pQTMs in the fully-adjusted MWAS, 987 involved Pappalysin-1 (PAPPA) and there were a further 1116 pQTMs that involved the Proteoglycan 3 Precursor protein. The remaining 825 pQTMs involved 189 unique protein levels, with 434 cis and 391 trans associations (Fig. 2). Principal components analyses indicated high correlations between CpGs associated with the pleiotropic proteins PAPPA and PRG3, whereas the CpGs involved in the remaining 825 pQTMs were largely uncorrelated (Supplementary Fig. 2). pQTM frequencies for the 1837 unique CpGs selected in the fully-adjusted models, with their respective genes and EWAS catalog24 lookup of epigenome-wide significant (\(P < 3.6 \times 10^{-8}\)) phenotypic associations is presented in Supplementary Data 10. Of these CpGs, sites within the NLRC5, SLC7A11 and PARP9 gene regions exhibited the highest levels of pleiotropy (Fig. 3). The pleiotropic findings for PAPPA and cg07839457 (NLRC5 gene) replicated previous MWAS results from Zaghlool et al.14 (944 individuals, with 1123 protein SOMAmers). Of the 98 pQTMs identified by Zaghlool et al., 81 were comparable (both the protein and CpG sites from the 98 pQTMs were available across both MWAS). Of these 81 pQTMs, 26 replicated at our significance threshold (\(P < 4.5 \times 10^{-10}\)) with the same direction of effect, a further 16 replicated at the epigenome-
wide significance threshold \((P < 3.6 \times 10^{-6})\)\(^{25}\) and a further 39 replicated at nominal \(P < 0.05\) (Supplementary Data 11 and Supplementary Fig. 3). When accounting for 26 pQTM s that were previously reported by Zaghloul et al. and 10 pQTM s that were previously reported by Hillary et al.\(^{14,19}\), 2892 of the 2928 fully-adjusted pQTM s were previously unreported. Of these 2892 pQTM s, 1109 involved the levels of 41 proteins that were measured by Zaghloul et al. (973 pQTM s for PAPPA and 136 additional pQTM s for the levels of 40 proteins), whereas 1783 pQTM s involved the levels of proteins that were previously unmeasured (1116 pQTM s for PRG3 and 667 further pQTM s for 148 proteins).

### Proteome associations with brain health phenotypes

We next conducted a proteome-wide association study of brain health characteristics (protein PheWAS of brain imaging, cognitive scoring and \(APOE\) e4 status, alongside age and sex; Fig. 4). Distribution plots for the seven cognitive scores and seven brain imaging phenotypes are presented in Supplementary Figs. 4, 5. A maximum sample of 1065 individuals was available (mean age 59.9 years [SD 9.6], 59% Female; Supplementary Data 2); all 774 individuals from the pQTM study were included in these analyses. A threshold for multiple testing adjustment was calculated based on 143 independent components that explained >80% of the 4235 SOMAmer levels (Supplementary Data 3 and Supplementary Fig. 1). This equated to \(P < 0.05/143 = 3.5 \times 10^{-4}\). The levels of 587 plasma proteins were associated with age and 545 were associated with sex, with 222 proteins common to both phenotypes (Supplementary Data 12). When comparable associations from three studies (with \(N > 1000\)) were tested\(^{20,26,27}\), 97% of age and 98% of sex associations replicated in one or more of studies (Supplementary Data 12).

There were 191 unique protein markers that had a total of 405 associations with brain health characteristics (Supplementary Fig. 6 and Fig. 4a). These consisted of 95 brain imaging (Supplementary...
Fig. 4 | Phenome-wide associations studies (PheWAS) of 4058 plasma proteins and brain health. a Number of protein marker associations with \( P < 3.5 \times 10^{-4} \) for each of the 15 traits related to brain health in the phenome-wide protein association studies (protein PheWAS). These studies included a maximum sample of 1065 individuals with protein measurements from Generation Scotland and tested for associations between 15 phenotypes and the levels of 4058 plasma proteins via linear mixed effects regression. Cognitive score (green), brain imaging (light blue) and APOE e4 status (dark blue) associations are summarised. Full summary statistics for the 405 associations with \( P \) values are presented in Supplementary Data 17. All associations were generated through linear regression and were adjusted for multiple testing correction. b Heatmap of standardised beta coefficients for 77 of the 405 protein PheWAS associations (\( P < 3.5 \times 10^{-4} \) indicated by an asterisk). These include three proteins that had associations with both APOE e4 status and one or more cognitive scores, in addition to 22 proteins that had associations with both a brain imaging measure and a cognitive score. Negative and positive beta coefficients are shown in blue and red, respectively. A heatmap describing the full 405 associations for APOE e4 status, cognitive scores and brain imaging measures is available in Supplementary Fig. 6. All associations were generated through linear regression and were adjusted for multiple testing correction.
such as ASB9 and NCAN were found to be consistently identified across multiple brain imaging traits as markers of poorer and better brain health, respectively (Supplementary Data 16). While many of the associations for brain imaging measures identified proteins that were distinct from those found for cognitive scores and APOE e4 status, 22 protein markers were associated with both a cognitive score and a brain imaging trait (Fig. 4b and Supplementary Data 18). A principal components analysis of the 22 protein levels was conducted. The first five components had an eigenvalue >1 and a cumulative variance of >80% was explained by the first 10 components. These are both commonly-used thresholds for deciding how many principal components to retain (Supplementary Fig. 11). Three APOE e4 status markers (ING4, APOB and CRP) were also associated with cognitive scores (Fig. 4b).

Replication of protein PheWAS associations
Six of the 14 APOE e4 status associations replicated previous SOMAmer protein findings (N SOMAmer = 4785 and N participants = 227) 10, and eight previously unreported relationships involved NEFL, ING4, PAF, MENT, TMCC3, CRP, FAM20A and PEF1. Several of the markers for eight previously unreported relationships involved NEFL, ING4, PAF, SOMAmer-based, whole proteome PheWAS studies of the brain imaging and cognitive score traits we have profiled in a healthy ageing population that were not enriched for neurodegenerative diseases. However, replication of associations from several studies 8,30,31 was found for a small subset of associations (Supplementary Data 19).

Integration of the brain health proteome with our pQTM dataset
Differential DNA signatures were explored for the 191 protein markers that had P < 3.5 × 10−4 in associations with either cognitive scores, brain imaging measures or APOE e4 status in the protein PheWAS. Of the 191 proteins, 17 had pQTMs in the fully-adjusted MWAS. Higher levels of 15 of these proteins were associated with poorer brain health, while AMY2A and CST5 were associated with better brain health via multiple pQTMs. There were a total of 35 pQTMs involving 31 unique CpGs that were located within 20 distinct genes (Supplementary Data 20), with 15 trans (Fig. 5) and 20 cis associations. All pQTMs were previously unreported. The 20 cis pQTMs involved the levels of CHI3L1, I188R1, SIGLEC5, OLFM2, UCHD1, CRHBP, AMY2A and CFHR1 proteins. The trans pQTMs involved the levels of SCUBE1, RBL2, TNFRSF1B, CST5, HEXB, ACY1, CTRAM, SMPD1 and RB5 protein.

Of the 20 cis pQTMs, 11 involved CpGs in different genes to the protein-coding gene on the same chromosome, whereas the remaining 9 pQTMs involved CpGs located within the protein-coding gene. Several CpG sites were associated with multiple protein levels in the trans pQTMs (Fig. 5). DNA methylation at site cg06690548 in the SLCA11 gene was associated with RBPS, ACY1 and SCUBE1 levels. The cg11294350 site in the HPT1 gene was associated with HEXB and SMPD1 levels. The cg07839457 site in the NLRC5 gene was associated with the levels of CTRAM and TNFRSF1B. There was also a protein that had several trans associations with multiple CpG sites; pQTMs were identified between circulating RBL2 levels and cg01132052, cg0539861, cg18457916, cg27294008 and cg18404041, within the NEK4/ITIH3/ITIH gene region of chromosome 3.

Functional mapping of neurological pQTMs
A lookup that integrated information from the GoDMC and eQTLGen databases assessed whether pQTMs were partially driven by an underlying genetic component. This identified methylation quantitative trait loci (eQTLs) for CpGs that were associated with CHI3L1, I188R1 and SIGLEC5 levels and were also expression quantitative trait loci (eQTLs) for the respective proteins (Supplementary Data 20). Further visual inspection of the distributions for the 35 pQTMs indicated that trimodal distributions—suggestive of unaccounted SNP effects—were present for CpGs involved in seven of the pQTMs (Supplementary Fig. 12).

Tissue expression profiles for the 33 genes that were linked to either CpGs or proteins in the 35 neurological pQTMs are summarised in Supplementary Fig. 13. Gene set enrichment for these 33 genes identified enrichment for immune effector pathways in a subset of 11 genes, whereas a cluster of four genes (SMPD1, HEXB, AMY2A and AMY2B) were enriched for amylase and hydrolase activity (Supplementary Fig. 14).

Of the 35 pQTMs, seven had CpGs that were located in either a CpG Shore or Shelf position and there were 13 that were located either 1500 bp or 200 bp from the TSS of the protein-coding gene (Supplementary Data 20). Fifteen pQTMs involved CpGs that were located in the gene body and 7 were located in either the first exon or UTR regions (Supplementary Data 20).

Promoter-capture Hi-C and ChIP-sequencing integration were used to assess the interactions and chromatin states of our pQTMs and associated CpG loci. This analysis focused on 11 of the 20 cis pQTMs that involved CpGs on the same chromosome as the protein-coding gene, but was located in a different gene. Mapping information is presented for the seven proteins involved in these pQTMs in Supplementary Figs. 15–21. In all instances, we found evidence of spatial colocalisation of these genes using promoter-capture Hi-C data from brain hippocampal tissue. We attempted to contextualise these sites further with ChIP-seq (ENCODE project) analyses of active chromatin marks H3K27ac and H3K4me1 and repressive chromatin H3K4me3 and H3K27me3 in both peripheral blood mononuclear cells (PBMCs) and brain hippocampus. ChIP-seq data suggested that in many instances there were shared regulatory regions that existed across both blood and hippocampal samples that were hubs for local promoter interactions. For example, promoter loops were found linking the STO2Z and CRHBP genes, with a signature of activating (H3K4me1 and H3K27ac) and silencing (H3K27me1 and H3K4me3) marks (normally considered bivalent chromatin) that may form the basis for shared regulation of this gene locus.

Discussion
We have conducted a large-scale integration of the circulating proteome with indicators of brain health and blood-based DNA methylation. We characterised 191 protein markers that were associated with either brain imaging measures, cognitive scores or APOE e4 status in an ageing population. We also report methylome-wide characterisations for the SOMAscan panel V.4 (4038 protein measurements) in a nested subset of this population. By overlapping these datasets, we uncovered 35 methylation signatures for 17 protein markers of brain health. We delineated pQTM CpGs that had evidence of underlying genetic influence and characterised the potential for chromatin interactions for genes involved in cis pQTMs. As this population consists of older individuals that were not enriched for neurodegenerative diseases, the markers we identify are likely indicators of healthy brain ageing.

Many of the 191 proteins identified in the protein PheWAS were part of inflammatory clusters with shared functions in acute phase response, complement cascade activity, innate immune activity and cytokine pathways. Tissue expression analyses suggested that a large proportion of the 191 protein markers were not expressed in the brain; this supports work suggesting that sustained peripheral inflammation influences general brain health 11,12 and accelerates cognitive decline.14 We also found evidence of expression quantitative trait loci that are linked to brain-derived proteins that may enter the bloodstream as biomarkers. SLIT and NTRK like Family Member 1 (SLITRK1),
Neurocan (NCAN) and IgLON family member 5 (IGLON5) were examples of proteins expressed in brain for which higher levels associated with either larger grey matter volume, larger whole brain volume, or higher general fractional anisotropy. SLITRK1 localises at excitatory synapses and regulates synapse formation in hippocampal neurons\(^3\). NCAN is a component of neuronal extracellular matrix and is linked to neurite growth\(^3\). IGLON5 has been implicated in maintenance of blood–brain barrier integrity and an anti-IGLON5 antibody disease involves the deterioration of cognitive health\(^4\). Taken together, the protein markers identified in the PheWAS may, therefore, reflect pathways that could be targeted to improve brain health.

Integration of our fully-adjusted protein MWAS dataset revealed 35 associations between DNAm and 17 protein markers of brain health (Fig. 6; Supplementary Data 20). All 35 associations were previously unreported. While this study is focused on blood DNAm—limiting generalisation to brain DNAm—many of the 35 pQTM involved CpGs and proteins that have been previously implicated in neurological processes. DNAm at site cg06690548 (located in the SLC7A11 gene) was of particular interest; differential DNAm at this CpG in blood has been identified as a causal candidate for Parkinson’s disease (\(N>900\) cases and \(N>900\) controls)\(^5\). Xc- is the cystine-glutamate antiporter encoded by SLC7A11, which facilitates glutamatergic transmission, oxidative stress defence and microglial response in the brain\(^6\) and is a target for the neurodegeneration-associated environmental neurotoxin β-methylamino-L-alanine\(^7\). Analyses in the wider Generation Scotland cohort suggests that cg06690548 is a site associated with alcohol consumption\(^8\). The proteins associated with cg06690548 in the subset of this cohort that we assessed (ACY1, SCUBE1 and RBP5)

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**Fig. 5 | Trans pQTM associations involving protein markers of brain health.** Circular plot showing 15 trans pQTM associations between DNAm at 11 CpG sites and the levels of nine protein markers of brain health that had \(P<4.5\times10^{-10}\) (Bonferroni threshold for multiple testing adjustment) in the fully-adjusted MWAS. Chromosomal positions are given on the outermost circle. Details of the full set of 35 pQTM for protein markers of brain health are provided in Supplementary Data 20 with \(P\) values. Results were generated through linear regression models.
have known links to liver function. DNA at cg06690548 in blood has also been recently implicated in the largest MWAS of amyotrophic lateral sclerosis (ALS) to date (6763 cases, 2943 controls). Given that ACY1, SCUBE1 and RBP5 were markers for lower processing speed and higher relative brain age, the CpG sites we identify in this study—such as cg06690548—may be important plasma markers for mediation of environmental risk on brain health that merit further exploration. cg06690548 lies within the first intron of SLC7A11, indicating that this site is of potential functional significance.

The presence of NLRC5-associated CpGs and various other inflammatory proteins in our neurological protein pQTMs suggests that the methylome may capture an inflammatory component of brain health. Many of the genes corresponding to CpGs and proteins involved in the 35 pQTMs were enriched for immune effector processes and were not expressed in brain. However, some genes did show evidence for brain-specific expression, such as acid sphingomyelinase (SMGD) and Hexosaminidase Subunit Beta (HEXB). The HEXB and SMGD proteins associated with DNA at cg11294350 in the CHPT1 gene, are involved in neuronal lipid degradation in the brain and have been associated with the onset of a range of neurodegenerative conditions. RBL2 is another protein that had partial expression signals across brain regions; the NEK4/ITIH3/ITIH1 region was the location for five CpGs with differential DNA levels. This region is implicated in schizophrenia and bipolar disorder by several large-scale, genome-wide association studies (GWAS).

Similarly, the RBL2 locus has been associated with intelligence,
cognitive function and educational attainment in GWAS (n > 260,000 individuals)\textsuperscript{4,5,7,8}.

Given that this study utilised CpGs from the Illumina EPIC array, 15 of the 31 unique CpGs did not have mQTL characterisations in public databases, which primarily comprise results from the earlier 450K array. However, our plots showing pQTM associations suggested that for several CpGs (such as cg11294350 that associated with SMPD1 and HEXB), there may be a partial genetic component influencing DNAm. As mQTLs tend to explain 15–17% of the additive genetic variance of DNAm\textsuperscript{9}, it is possible that the signals we isolate in these instances are partially driven by genetic loci, but are also likely driven by unmeasured environmental and biological influences. In the case of SIGLEC5, IL1SR1 and CH3L, mQTLs were identified that were also eQTLs, providing evidence that mQTLs for these CpG sites were possible regulators of protein expression.

Integration of promoter-capture Hi-C chromatin interaction and ChIP-seq databases\textsuperscript{10} provided evidence for long-range interaction relationships for cis pQTM with CpGs in different gene regions that are proximal to the protein-coding gene of interest. This suggests that in such instances, the pQTM may reflect regulatory relationships in the 3-dimensional genomic neighbourhood. The pQTM therefore direct us towards pathways that can be tested in experimental contexts. Positional information suggested that many of the implicated CpGs involved in neurological pQTM lay within 1500 bp of the TSS of the respective protein-coding gene. While positional information of CpGs is thought to infer whether DNAm is likely to play a role in the expression regulation of nearby genes, this is still somewhat disputed. Some studies suggest that transcription factors regulate DNAm\textsuperscript{11} and differential methylation at gene body locations predicts dosage of functional genes\textsuperscript{12}. Additionally, the DNAm signatures of proteins we quantify represent widespread differences across blood cells that are related to circulating protein levels and are therefore not derived from the same cell-types as proteins. Despite this limitation, previous work supports DNAm scores for proteins as useful markers of brain health, suggesting there is merit in integrating DNAm signatures of protein levels in disease stratification\textsuperscript{13}.

Our study has several limitations. First, though full replication of our results was not possible, our replication of pQTMd identified by Zaghloul et al.\textsuperscript{14} reinforces inflammation signalling as intrinsic to the methylome signature of blood proteins. This also suggests that pQTM may be common across ancestries. Second, we observed a substantial inflation for PAPP5 and PRG3 proteins. While comprehensive adjustment for estimated immune cells was performed and the remainder of CpGs involved in pQTM did not show high correlations (Supplementary Fig. 2), concurrently measured blood components such as haemoglobin, red blood cells and platelets were not available. Future studies should seek to resolve signals with more detailed blood-cell phenotyping and immune cell estimates\textsuperscript{15}. Third, 89% of the proteins identified in our protein PhEWS did not have epigenetic pQTM; this may be due to 1) the presence of pathways relating to neurological disease that are not reflected by blood immune cell DNAm, 2) underpowered analyses, or 3) the presence of indirect associations that are not captured by our MWAS approach. Fourth, the extent of non-specific and cross-aptamer binding with SOMAmeter technology has not been fully resolved\textsuperscript{16}. Fifth, there are likely unknown genetic influences on pQTM. Further characterisation of pQTLs and advances in multicom modelling techniques\textsuperscript{17} will aid in the separation of genetic and environmental influences on epigenetic signatures. Sixth, differences in blood and brain DNAm and pQTLs are emerging; these indicate that blood-based markers may not fully align to biology of brain degeneration\textsuperscript{18,19}. However, our ChIP-seq analysis of chromatin regulation suggested that some regulatory states may persist between blood and brain. Seventh, profiling DNAm signatures alone cannot capture the full role of the epigenome in brain health. Integration of more diverse epigenetic markers will be critical to further resolve these relationships. Finally, though we have incorporated a wide portfolio of brain health measures, we recognise that these are not extensive. Increasing triangulation across modalities, as we have shown here, will be useful in identifying candidate markers.

In conclusion, by integrating epigenetic and proteomic data with cognitive scoring, brain morphological and APOE e4 status, we identify 191 protein markers of brain health. We characterise DNAm signature for all 4058 proteins included in the study, uncovering 35 associations between differential DNAm and the levels of 17 of the protein markers of brain health. These data identify candidate targets for the preservation of brain health and may inform risk stratification approaches.

### Methods

#### The Generation Scotland sample population

A YouTube video providing an overview of this study and detailing how summary statistics can be accessed is available at: https://www.youtube.com/channel/UCexQFfTIItF25yJ7hXuumQ. The Stratifying Resilience and Depression Longitudinally (STRADL) cohort used in this study is a subset of N = 1185 individuals from Generation Scotland: The Scottish Family Health Study (GS). Generation Scotland constitutes a large, family-structured, population-based cohort of >24,000 individuals from Scotland\textsuperscript{20}. Individuals were recruited to GS between 2006 and 2011. During a clinical visit detailed health, cognitive, and lifestyle information was collected in addition to biological samples. Of the 21,525 individuals contacted for participation, N = 1185 completed additional health assessments and biological sampling 5 years after GS baseline\textsuperscript{21}. Of these, N = 1,065 individuals had proteomic data available and N = 778 of these had DNAm data available. Four individuals from this subset were excluded from the DNAm sample due to having incomplete depression status information, leaving 774 individuals available for analyses. Supplementary Data 2 summarises the demographic characteristics across the two groups, with descriptive statistics for phenotypes.

#### Proteomic measurement

SOMAscan\textsuperscript{®} V.4 technology was used to quantify plasma protein levels. This aptamer-based assay facilitates the simultaneous measurements of multiple Slow Off-rate Modified Aptamers (SOMAmers)\textsuperscript{22}. SOMAmers were processed for 1065 individuals from the STRADL subset of Generation Scotland. Briefly, binding between plasma samples and target SOMAmers was achieved during incubation and quantification was recorded using a fluorescent signal on microarrays. Quality control steps included hybridisation normalisation, signal calibration and median signal normalisation to control for inter-plate variation. Full details of quality control stages are provided in Supplementary Information. In the final dataset, 4235 SOMAmeter epitope measures were available in 1065 individuals and these corresponded to 4058 unique proteins (classified by common Entrez gene names). Supplementary Data 1 provides annotation information for the 4235 SOMAmeter measurements that were available.

#### DNAm measurement

Measurements of blood DNAm in the STRADL subset of GS subset were processed in two sets on the Illumina EPIC array using the same methodology as those collected in the wider Generation Scotland cohort\textsuperscript{23–25}. Quality control details are provided in Supplementary Information. Briefly, samples were removed if there was a mismatch between DNAm-predicted and genotype-based sex and all non-specific CpG and SNP probes (with allele frequency >5%) were removed from the methylation file. Probes which had a beadcount of less than 3 in more than 5% of samples and/or probes in which >1% of samples had a detection P > 0.01 were excluded. After quality control, 793,706 and 773,860 CpG were available in sets 1 and 2, respectively. These sets were truncated to include a total of 772,619
common probes and were joined together for use in the MWAS, with 476 individuals included in set 1 and 298 individuals in set 2. DNAm-specific technical variables (measurement batch and set) were adjusted in all MWAS models.

Phenotypes in Generation Scotland
All phenotypes in Generation Scotland MWAS and PheWAS samples are summarised in Supplementary Data 2. An epigenetic score for smoking exposure, EpiSmokEr⁷⁹ was calculated for all individuals with DNAm. The meffil⁴⁴ implementation of the Houseman method was used to calculate estimated white blood cell proportions for Sets 1 and 2. Blood reference panels were sourced from Reinius et al.⁹ with accession GSE35069. The blood gse35069 complete panel was used to imputed measures for Monocytes, Natural killer cells, B cells, Granulocytes, CD4 T cells and CD8 T cells. Eosinophil and Neutrophil estimates were also sourced through the blood gse35069 panel. Body mass index (body weight in kilograms, divided by squared height in metres) was available for all individuals, alongside depression status (defined as a research version of the Structured Clinical Interview for DSM disorders (SCID) assessment), which was coded as a binary variable of no history of depression (0) or lifetime episode of depression (1). Five individuals did not have depression status information and were excluded from MWAS and PheWAS analyses, where appropriate. APOE e4 status was available for 1050 individuals. APOE e4 status was coded as a numeric variable (e2e2 = 0, e2e3 = 0, e3e3 = 1, e3e4 = 2, e4e4 = 2). Fifteen e2e4 individuals were excluded due to small sample size.

Scores from five cognitive tests (Supplementary Fig. 4; Supplementary Data 2) measured at the clinic visit for the STRADL subset of GS were considered. Cognitive scores were measured at the baseline clinic visit⁶⁸ and full details are provided in Supplementary Information. Briefly, these included the Wechsler Logical Memory Test (maximum possible score of 50), the Wechsler Digit Symbol Substitution Test (maximum possible score of 133), the verbal fluency test (based on the Controlled Oral Word Association task), the Mill Hill Vocabulary test (maximum possible score of 44) and the Matrix Reasoning test (maximum possible score of 15). Outliers were defined as scores >3.5 standard deviations above or below the mean and were removed prior to analysis. The first unrotated principal component combining logical memory, verbal fluency, vocabulary and digit symbol tests was calculated as a measure of general cognitive ability (g). General fluid cognitive ability (gf) was extracted using the same approach, but with the vocabulary test (a crystallised measure of intelligence) excluded from the model. While highly similar to g, the gf score is exclusive to measures such as memory and processing capability that are considered fluid. gf may therefore be of greater relevance for assessing cognitive decline in ageing individuals.

The derived brain volume measures (Supplementary Fig. 5; Supplementary Data 2) were recorded at two sites (Aberdeen and Edinburgh)⁶⁷. Data processing used the resources provided by the Edinburgh Compute and Data Facility (http://www.ecdf.ed.ac.uk/). Brain volume data included total brain volume (ventricle volumes excluded), global grey matter volume, white matter hyperintensity volume and total intracranial volume. Intracranial volume was treated as a covariate to adjust for head size in all tests including brain volume associations. The derived global white matter integrity measures included gFA and global mean diffusivity. The protocols applied to derive the brain volume measures from T1-weighted scans, and white matter integrity measures from diffusion tensor imaging scans were measured at baseline⁴⁵,⁴⁶ and full details are provided in Supplementary Information. Brain Age was estimated using the software package brainageR (Version 2.1; https://doi.org/10.5281/zenodo.3476365, available at https://github.com/james-cole/brainageR), which uses machine learning and a large training set to predict age from whole-brain voxel-wise volumetric data derived from structural T1 images³. This estimate was regressed on chronological age to produce a measure of Relative Brain Age (residuals from the linear model). Outliers for all imaging variables were defined as measurements >3.5 standard deviations above or below the mean and were removed prior to analyses.

Phenome-wide association analyses
Prior to running protein PheWAS analyses, protein levels were transformed by rank-based inverse normalisation and scaled to have a mean of zero and standard deviation of 1. Models were run using the lmekin function in the coxme R package (Version 2.2-16)⁷⁷. This modelling strategy allows for mixed-effects linear model structure with adjustment for relatedness between individuals. Models were run in the maximum sample of 1065 individuals, with the 4235 protein levels as dependent variables and phenotypes as independent variables. Continuous variables were scaled to mean of zero and variance one and missing data were excluded from lmekin models. Each model adjusted for age and sex (male = 1, reference female = 0). A random intercept was fitted for each individual and a kinship matrix was included as a random effect to adjust for relatedness. Diagnosis of depression (case = 1, reference control = 0) at the STRADL clinic visit was included as a covariate in all models, due to known selection bias for depression phenotypes in STRADL⁶⁸. Clinic study site and protein lag group (storage time before proteomic sequencing) were included as covariates in all models. For the analyses with age and sex as the predictors of interest, two beta coefficients for age and sex were extracted from the same model structure. In the remaining PheWAS models, either numerical APOE e4 status variable (e2 = 0, e3 = 1, e4 = 2), cognitive test scores or brain imaging phenotypes were included in addition to the described covariates as scaled predictors. The beta coefficients were extracted for the phenotype in each protein-phenotype association. All analyses of brain volume measures included further adjustment for intracranial volume (ICV) and study site as main effects, in addition to the interaction between these variables. ICV was used to account for head size. Processing batch, and presence or absence of manual intervention during quality control were also included as covariates for volumetric brain imaging associations. The Pcomp function in the stats R package (Version 3.6.2)⁸⁰ was used to generate principal components for the 4,235 SOMAmers measurements (N = 1065). 143 components explained >80% of the cumulative variance in protein levels (a commonly-used threshold for the retention of principal components); Supplementary Fig. 1 and Supplementary Data 3). These 143 components were used to derive the PheWAS multiple testing adjustment threshold of P < 0.05 / 143 = 3.5 × 10⁻⁴. This method was chosen due to the presence of high intercorrelations within the protein data.

Epigenome-wide association study of protein levels
Prior to running the MWAS, protein levels for 774 individuals with complete phenotypic information were log transformed and regressed on age, sex, study site, lag group, 20 genetic principal components (generated from multidimensional scaling of genotype data from the Illumina 610-QuadV1 array) and known pQTL effects (from a previous genome-wide association study of 4034 SOMAmers targeting 3622 proteins from Sun et al.)⁷⁻. Residuals from these models were then rank-based inverse normalised and taken forward as protein level data. Methylation data were in M-value format and were pre-adjusted for age, sex, processing batch, methylation set and depression status⁷⁻. A second model further adjusted for estimated white blood cell proportions (Monocytes, CD4 T cells, CD8 T cells, B cells, Natural Killer cells, Granulocytes and Eosinophils). While Neutrophil estimates were available, they were excluded due to high correlation (r > 0.95) with Granulocyte proportions (Supplementary Fig. 22). Finally, the fully-
adjusted model further regressed DNAm onto an epigenetic score for smoking, EpiSmokEr and BMI.

Omics-data-based complex trait analysis (OSCA) Version 0.41 was used to run EWAS analyses. Within OSCA, a genetic relationship matrix (GRM) was constructed for the STRADL population. A threshold of 0.05 was used to identify 120 individuals likely to be related based on their genetic similarity. For this reason, the MA method was used to calculate associations between individual CpG sites and protein levels, with the addition of the GRM as a random effect to adjust for relatedness between individuals. CpG sites were the dependent variables and the 4235 proteins were the independent variables.

For fully-adjusted models did not converge (NAGLU, CFHR2, MST1, PILRA) and were excluded. A threshold for multiple testing correction ($P < 4.5 \times 10^{-10}$) was based on 143 independent protein components with cumulative variance >80% (Supplementary Table 1 and Supplementary Data 3). ($P < 0.05/(143 \times 772,619)$ CpG sites). A more conservative threshold based on total number of SOMAmers was also considered ($P < 0.05/(4235 \times 772,619) = 1.5 \times 10^{-16}$) and is detailed in Supplementary Data 4-6. pQTM sites were classified as cis if the CpG was on the same chromosome as the protein-coding gene and fell within 10 Mb of the transcriptional start site (TSS) of the protein gene. pQTM involving a CpG located on a different chromosome to the protein-coding gene, or >10 Mb from the TSS of the protein gene were classed as trans.

Chips plots were created with the circilize package (Version 0.4.12). BioRender.com was used to create Figs. 1, 2, 3 and 6. All analyses were performed in R (Version 4.0.3).

Functional mapping and tissue expression analyses

Functional mapping and annotation (2014) gene set enrichment and tissue expression analyses were conducted for genes corresponding to protein markers that were identified through the PheWAS study, in addition to genes linked to either CpGs or proteins in the neurological pQTM subset. Protein-coding genes were selected as the background set and ensemble v92 was used with a false discovery rate adjusted $P < 0.05$ threshold for gene set testing. For the genes corresponding to protein markers in the PheWAS a minimum overlapping number of genes was set to 3. The STRING database was queried to build a protein interaction network based on all proteins that had associations in the PheWAS. mQTL and eQTL lookups were performed using the GoDMC and eQTLGen databases, respectively. UCSC database searches were used to profile the positional information relating to CpGs in the pQTMs.

Although inter-chromosomal chromatin interactions are unlikely to be stable and persistent, seven proteins with cis pQTM involving CpGs located intra-chromosomally to the proximal protein-coding gene were considered for ChIP-seq and promoter-capture Hi-C mapping to interrogate local chromatin interactions and states that might form the basis for co-regulation of these loci. ChIP-seq data from PBMCs and brain hippocampus were selected from the ENCODE project, with accession identifiers available in Supplementary Data 21. Processed promoter-capture Hi-C data for brain hippocampal tissue was selected from Jung et al., and are available at NCBI Geo with accession GSE86189. Data concerning both promoter-promoter interactions and promoter-other interactions were concatenated and all regions subsequently visualised on the WashU epigenome browser.

Ethics declarations

All components of GS received ethical approval from the NHS Tayside Committee on Medical Research Ethics (REC Reference Number: 05/S1401/89). GS has also been granted Research Tissue Bank status by the East of Scotland Research Ethics Service (REC Reference Number: 20/ES/0021), providing generic ethical approval for a wide range of uses within medical research. All participants included in the current study provided informed consent for the use of their data for biomedical research.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The fully-adjusted MWAS summary statistics for 4231 protein levels generated in this study have been deposited in the MRC-IEU EWAS catalogue. These files are also available through a Zenodo repository at https://doi.org/10.5281/zenodo.6801458. Datasets generated in this study are made available in Supplementary Data files 1–21. The raw data from Generation Scotland are not available due to them containing information that could compromise participant consent and confidentiality. Generation Scotland is run as a Resource for the research community. Requests to use the Resource are made from: Academic collaborators: employees who are party to the Generation Scotland Collaboration Agreement, or researchers or employees of an academic institution or the NHS. Commercial organisations: specific arrangements have been defined to allow commercial organisations to access Generation Scotland resources. Data can be obtained from the data owners. Instructions for accessing Generation Scotland data can be found here: https://www.ed.ac.uk/generation-scotland/for-researchers/access; the GS Access Request Form can be downloaded from this site. Completed request forms must be sent to access@generationscotland.org to be approved by the Generation Scotland Access Committee.

For any further correspondence and material requests please contact Dr Riccardo Marioni at riccardo.marioni@ed.ac.uk. Source data are provided with this paper.

Code availability

All R code used in this study is available with open access at the following Github repository: https://github.com/DannGaddi/Epigenome-and-phenome-wide-study-of-brain-health-outcomes.

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Author contributions
D.A.G., and R.E.M., were responsible for the conception and design of the study. D.A.G. carried out the data analyses. D.A.G., and R.E.M., drafted the article. S.R.C., and H.W., advised on methodology. R.F.H., and D.L.M., contributed to methodology and data analyses. R.I.McG., S.M., R.M.W., L.S., D.L.M., R.M.W., A.C., A.H., C.H., K.E., D.J.P., H.W., A.M.M., and S.R.C., contributed to data methylation and proteomic data collection and preparation. A.S., M.C.B., M.A.H., E.V.B., J.D.S., S.X., C.G., and J.M.W processed the brain imaging data. D.A.O., G.M.T., and C.R., provided scientific counsel. T.C., and N.R., consulted on chromatin analyses. R.E.M., supervised the project. All authors read and approved the final manuscript.

Competing interests
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Additional information
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