DNA Methylation and Protein Markers of Chronic Inflammation and Their Associations With Brain and Cognitive Aging

This is an open access article distributed under the terms of the Creative Commons Attribution License 4.0 (CC BY), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Neurology® Published Ahead of Print articles have been peer reviewed and accepted for publication. This manuscript will be published in its final form after copyediting, page composition, and review of proofs. Errors that could affect the content may be corrected during these processes.
Author(s):
Eleanor L.S. Conole, BSc, MRes\textsuperscript{1,2,3}; Anna J. Stevenson, BSc, MSc, PhD\textsuperscript{2,4}; Susana Muñoz Maniega, BSc, PhD\textsuperscript{1,3}; Sarah E. Harris, BSc, PhD\textsuperscript{1}; Claire Green, BSc, MSc\textsuperscript{5}; Maria del C. Valdés Hernández, BSc, PhD\textsuperscript{1,3}; Mathew A. Harris, BSc, PhD\textsuperscript{1,5}; Mark E. Bastin, BSc, PhD\textsuperscript{1}; Joanna M. Wardlaw, CBE, MB ChB(Hons), MD, FRCR, FRCP, FMedSci, FRSE\textsuperscript{1,3}; Ian J. Deary, BSc, PhD, MBChB, FMedSci, FRCPsych, FRCPEd, FBA, FRSE, OBE\textsuperscript{1}; Veronique E. Miron, BSc, PhD\textsuperscript{4,6}; Heather C. Whalley, BSc, PhD\textsuperscript{1,5}; Riccardo E. Marioni, BSc, MSc, PhD\textsuperscript{1,2}; Simon R. Cox, BSc, MSc, PhD\textsuperscript{1}

Corresponding Author:
Eleanor L.S. Conole
eleanor.conole@ed.ac.uk

Affiliation Information for All Authors: 1. Lothian Birth Cohorts group, Department of Psychology, University of Edinburgh, Edinburgh EH8 9JZ, UK; 2. Centre for Genomic and Experimental Medicine, Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh EH4 2XU, UK; 3. Centre for Clinical Brain Sciences, University of Edinburgh, Edinburgh EH16 4SB, UK; 4. UK Dementia Research Institute, Edinburgh Medical School, University of Edinburgh, Edinburgh, UK; 5. Division of Psychiatry, Royal Edinburgh Hospital, University of Edinburgh, Edinburgh, EH10 5HF, UK; 6. The Queen's Medical Research Institute, Edinburgh BioQuarter, University of Edinburgh, Edinburgh, EH16 4TJ;

Contributions:
Eleanor L.S. Conole: Drafting/revision of the manuscript for content, including medical writing for content; Study concept or design; Analysis or interpretation of data
Anna J. Stevenson: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data
Susana Muñoz Maniega: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data
Sarah E. Harris: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data
Claire Green: Drafting/revision of the manuscript for content, including medical writing for content
Maria del C. Valdés Hernández: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data
Mathew A. Harris: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data
Mark E. Bastin: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data
Joanna M. Wardlaw: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data
Ian J. Deary: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data
Veronique E. Miron: Drafting/revision of the manuscript for content, including medical writing for content; Analysis or interpretation of data
Heather C. Whalley: Drafting/revision of the manuscript for content, including medical writing for content; Analysis or interpretation of data
Riccardo E. Marioni: Drafting/revision of the manuscript for content, including medical writing for content; Study concept or design; Analysis or interpretation of data
Simon R. Cox: Drafting/revision of the manuscript for content, including medical writing for content; Study concept or design; Analysis or interpretation of data

Copyright © 2021 The Author(s). Published by Wolters Kluwer Health, Inc. on behalf of the American Academy of Neurology.
Conole 3

Publication History: This manuscript was pre-published in MedRxiv doi: https://doi.org/10.1101/2020.10.08.20205245

Number of characters in title: 114
Abstract Word count: 303
Word count of main text: 4457
References: 50
Figures: 6
Tables: 1

Supplemental: STROBE checklist e-methods eTable 1 eTable 2 eTable 3 eTable 4 eTable 5 eTable 6 eTable 7 eTable 8 eTable 9 eTable 10 eTable 11 eTable 12 eTable 13 eFigure 1 eFigure 2 eFigure 3 eReferences

Statistical Analysis performed by: Eleanor L.S. Conole, BSc, MRes, Riccardo E. Marioni, BSc, MSci, PhD Simon R. Cox, BSc, MSci, PhD

Search Terms: Cognitive aging, Cohort studies, Public health, Volumetric MRI, Class II

Acknowledgements: We thank the Lothian Birth Cohort 1936 members who took part in this study, and Lothian Birth Cohort 1936 research team members who collected, entered and checked data used in this manuscript. The Lothian Birth Cohort 1936 study acknowledges the financial support of NHS Research Scotland (NRS), through Edinburgh Clinical Research Facility.

Study Funding: This research was funded in whole, or in part, by the Wellcome Trust [Grant number:108890/Z/15/Z]. For the purpose of open access, the author has applied a CC BY public copyright licence to any Author Accepted Manuscript version arising from this submission. The LBC1936 and this research are supported by Age UK (Disconnected Mind project) and by the UK Medical Research Council [MRC; G0701120, G1001245, MR/M013111/1, MR/K026992/1]. Methylation typing was supported by Centre for Cognitive Ageing and Cognitive Epidemiology (Pilot Fund award), Age UK, The Wellcome Trust Institutional Strategic Support Fund, The University of Edinburgh, and The University of Queensland. The Olink Proteomics assays were supported by a National Institutes of Health (NIH) research grant R01AG054628. This work was in part conducted in the Centre for Cognitive Ageing and Cognitive Epidemiology, which is supported by the Medical Research Council and Biotechnology and Biological Sciences Research Council (MR/K026992/1) and which supports IJD. E.L.S.C. and A.J.S. are supported by funding from the Wellcome Trust 4-year PhD in Translational Neuroscience [108890/Z/15/Z to E.L.S.C.; 203771/Z/16/Z to A.J.S]. M.E.B., I.J.D. and S.R.C. are also supported by a National Institutes of Health (NIH) research grant R01AG054628. S.R.C is supported by a Sir Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society (Grant Number 221890/Z/20/Z).

Disclosures: Disclosures: E. L. S. Conole reports no disclosures relevant to the manuscript, A. J. Stevenson reports no disclosures relevant to the manuscript, S. E. Harris reports no disclosures relevant to the manuscript, SM Maniega reports no disclosures relevant to the manuscript, C. Green reports no disclosures relevant to the manuscript, M. Valdés-Hernández reports no disclosures relevant to the manuscript, M. A. Harris reports no disclosures relevant to the manuscript, M. E. Bastin reports no disclosures relevant to the manuscript, J. M. Wardlaw reports no disclosures relevant to the manuscript, I. J. Deary reports no disclosures relevant to the manuscript, V. E. Miron reports no disclosures relevant to the manuscript, H. C. Whalley reports no disclosures relevant to the manuscript, R. E. Marioni reports no disclosures relevant to the manuscript, S. R. Cox reports no disclosures relevant to the manuscript.
Abstract

Objective: To investigate chronic inflammation in relation cognitive ageing by comparison of an epigenetic and serum biomarker of C-Reactive Protein and their associations with neuroimaging and cognitive outcomes.

Methods: At baseline, participants (N = 521) were cognitively normal, around 73 years of age (M = 72.4, SD = 0.716), and had inflammation, vascular risk (cardiovascular disease history, hypertension, diabetes, smoking, alcohol consumption and BMI) and neuroimaging (structural and diffusion MRI) data available. Baseline inflammatory status was quantified by a traditional measure of peripheral inflammation – serum C-Reactive Protein (serum CRP) – and an epigenetic measure (DNA methylation signature of CRP; DNAm CRP). Linear models were used to examine the inflammation-brain health associations; mediation analyses were performed to interrogate the relationship between chronic inflammation, brain structure and cognitive functioning.

Results: We demonstrate that DNAm CRP shows significantly (on average 6.4-fold) stronger associations with brain health outcomes than serum CRP. DNAm CRP is associated with total brain volume ($\beta = -0.197$, 95% CI [-0.28, -0.12], $p_{FDR} = 8.42 \times 10^{-6}$), grey matter volume ($\beta = -0.200$, 95% CI [-0.28, -0.12], $p_{FDR} = 1.66 \times 10^{-5}$) and white matter volume ($\beta = -0.150$, 95% CI [-0.23, -0.07], $p_{FDR} = 0.001$) and regional brain atrophy. We additionally find that DNAm CRP has an inverse association with global and domain-specific (speed, visuospatial and memory) cognitive functioning, and that brain structure partially mediates this CRP-cognitive association (up to 29.7%), dependent on lifestyle and health factors.

Conclusions: These results support the hypothesis that chronic inflammation may contribute to neurodegenerative brain changes which underlie differences in cognitive ability in later life and highlight the potential of DNA methylation proxies for indexing chronic inflammatory status.
Classification of Evidence: This study provides Class II evidence that a DNA methylation signature of CRP levels is more strongly associated with brain health outcomes than serum CRP levels.

Introduction

Low-level, systemic chronic inflammation has emerged as a hallmark and potential driver for individual differences in brain ageing\(^1^\text{-}^5\). Yet while chronic inflammation has been consistently linked to dementia\(^6^\text{-}^8\), studies investigating peripheral inflammatory markers in non-clinical groups show disparity with respect to cognitive outcomes\(^9^\text{-}^{14}\), and have not yet clarified the magnitude and regional extent of brain structural associations\(^13^\text{-}^{18}\).

One reason for this inconsistency is that there are no standard biomarkers for chronic inflammation, and to date many studies have relied upon blood biomarkers of acute inflammation such as C-Reactive Protein (CRP). A significant caveat of this approach is assuming baseline inflammation status from highly phasic protein levels, which are subject to swift and rapid concentration changes in blood plasma\(^19\). This introduces significant noise at the epidemiological level\(^20,21\) (see Figure 1D) and few studies take repeat measures of serum CRP\(^12\) or attempt to correct for within-person fluctuations. A more accurate reflection may come from an epigenetic approach: DNA methylation (DNAm) profiles have been identified in inflammatory diseases\(^22,23\) and inflammation-related disease outcomes\(^24,25\) and are theorised to provide more stable reflections of inflammatory exposure\(^24,26\text{-}^{28}\). In the same cohort as in the present study, a DNAm proxy of CRP exhibited greater longitudinal stability and stronger associations with cognitive functioning than serum CRP levels\(^11,28\).

Here, we predict that a DNAm signature of CRP will show significantly stronger associations with brain health outcomes than its serological counterpart. Our objective is to examine the
relationship between chronic inflammation, brain structure and cognitive ageing in a large community-dwelling sample of older age adults.

**Materials and Methods**

**Participants**

The Lothian Birth Cohort 1936 (LBC1936) comprises of individuals who were surviving members of the Scottish Mental Survey 1947 (SMS1947), born in 1936, and who were living in Edinburgh and the surrounding area (the Lothians) when the study began in 2004. Full details of the recruitment procedures and protocols have been previously published. Participants took part in four waves of testing in later life (at mean ages 70, 73, 76 and 79 years) as part of an investigation into the determinants of cognitive ageing. At each wave, subjects were interviewed and tested individually by a trained psychologist and a research nurse during a visit to the Wellcome Trust Clinical Research Facility (http://www.wtcrf.ed.ac.uk), Western General Hospital, Edinburgh. This visit included cognitive and other psychological assessments, physical examinations, extensive history taking and blood analyses. From Wave 2 onwards, neuroimaging data is also available.

The current study on chronic inflammation is cross-sectional (all variables described here were collected in 2007, at Wave 2 of the LBC1936 study) and addresses the following primary research questions:

1. Does an epigenetic inflammation (DNAm CRP) measure show stronger associations with brain structure and function than serum C-Reactive Protein levels? [Class II evidence]
2. Does an epigenetic inflammation measure (DNAm CRP) show stronger associations with white matter microstructure than serum C-Reactive Protein levels? [Class II evidence]

3. To what extent can alterations in brain structure explain the association between inflammation and cognitive ability? [Class II evidence]

Participants were free of neurodegenerative diagnoses at baseline and were excluded if they had a self-reported history of stroke, Parkinson’s, Alzheimer’s disease or had an MMSE < 24, indicating mild cognitive impairment. We additionally excluded participants with serum CRP level >10mg/L, suggestive of acute infection or illness at the time of blood draw. After exclusions, a total of 521 participants had complete inflammation, cognitive, neuroimaging and relevant health data. For further details on data availability and attrition, see Figure 2 and eTable 1 of the supplementary document.

**Brain imaging data**

Structural and diffusion tensor (DTI) MRI acquisition and processing in LBC1936 were performed according to an open-access protocol\(^\text{29}\). A 1.5T GE Signa HDx clinical scanner (General Electric, Milwaukee, WI, USA) was used to collect structural T1-(voxel size = 1 × 1 × 1.3mm), T2-(voxel size = 1 × 1 × 2 mm), T2*- (voxel size = 1 × 1 × 2mm), and FLAIR-weighted images (voxel size = 1 × 1 × 4 mm); for full details on MRI sequence parameters, refer to table 1. in the open access protocol article\(^\text{29}\). Local processing and QC of cortical reconstruction and segmentation was performed using FreeSurfer v5.1 on T1-weighted volumes. Full information on brain imaging acquisition, QC and variables used in analyses is detailed in supplementary eMethods.

**C-Reactive Protein data**

Serum CRP was measured from whole-blood samples using a high sensitivity assay (enzyme-linked immunosorbent assay; R&D Systems, Oxford, UK).
**DNA methylation preparation and DNAm CRP score**

Genome-wide DNA methylation was measured in blood samples using the Illumina Human MethylationEPIC BeadChip at the Edinburgh Clinical Research Facility Genetics Core; the epigenetic measure of chronic inflammation was calculated for each participant as described previously. Briefly, a DNAm CRP score was assembled for each participant in Wave 2 of LBC1936; this was created by means of a weighted composite score, based on a discovery meta-analysis (9 cohorts, n = 8,863) and a replication meta-analysis (4 cohorts, n = 4,111) of CRP-EWAS studies. Methylation beta values were derived for the 7 CpG sites shown to have the strongest association with serum CRP levels, then multiplied by their standardised regression weights and added together. Given that all regression weights from the EWAS were negative, a higher DNAm CRP score (i.e. closer to 0) corresponds to a higher inflammatory profile. Relative weights for the 7 CpGs are included in the supplementary document (eTable 2).

**Cognitive ability data**

All participants in the LBC1936 underwent a detailed battery of standardised cognitive tests. From these, participant scores for three distinct cognitive domains (visuospatial ability, processing speed and verbal memory) alongside a general fluid-type cognitive ability score \((g_f)\) were created based upon well-fitting, hierarchical structural equation models tested in our previously published work\(^{30}\); relevant cognitive tests and individual weightings can be found in the supplementary document (eTable 3).

**Lifestyle variables**

Building on from previous work that looked at the impact of vascular risk factors on cognitive-ageing\(^{31}\), we selected the most pertinent variables available to us in the LBC1936 cohort that may influence or confound the relationship between inflammation, brain health and cognitive ageing. Lifestyle variables included body mass index (BMI; kg/m\(^2\)), calculated...
from height and weight at the time of interview (see eMethods for details), alongside variables relating to self-reported health and disease history: cardiovascular disease history (CVD); hypertension; diabetes; smoking status (coded as current smoker [1] versus ex/non-smoker [0]) and alcohol use (coded as drinker [1] versus non-drinker [0]). Regular anti-inflammatory drug use was also collected at baseline and coded as: on medication [1]; not on medication [0].

Statistical Analyses
Statistical analyses were performed in R version 3.6.1 (https://www.rproject.org). Alpha was 0.05 for all analyses and results were corrected for multiple comparisons using the false discovery rate (FDR)\textsuperscript{32}. Standardized coefficients are reported throughout to facilitate comparison of associations. Serum measures of CRP were log-transformed to correct a positively skewed distribution. WMH volume was log transformed, after which it showed an approximately normal distribution. All global MRI volumetric measures (total brain; TB, grey matter; GM, normal appearing white matter; NAWM, white matter hyperintensity; WMH) were corrected for intracranial volume (ICV) and expressed as a ratio of ICV. For volumetric brain associations, differences between association magnitudes (serum CRP vs DNAm CRP associations) were assessed using the Williams’ test\textsuperscript{33} for dependent groups with overlapping correlations (cocor.indep.groups.overlaps) as implemented in the ‘cocor’ R package (https://cran.r-project.org/web/packages/cocor/cocor.pdf). We ensured that models showed acceptably low multicollinearity (variance inflation was ascertained using ‘vif’ in the ‘car’ package in R; https://cran.r-project.org/web/packages/car/car.pdf). Pairwise bivariate associations were assessed between markers of inflammation, neuroimaging and lifestyle covariates using Pearson correlation. All models were adjusted for age and sex. Details of individual analyses are as follows.
Volumetric brain associations with inflammation

Linear regression models were used to identify the proportion of phenotypic variance explained by DNAm CRP and to determine whether this was independent of the serum CRP signal for each brain health phenotype. Logistic regressions were conducted for self-reported disease history variables with binary outcomes (disease/no disease).

Regional brain analyses

Localized associations between DNAm CRP score and vertex-wise cortical volume, area and thickness were performed using linear regression, controlling for age, sex, and ICV. We used the SurfStat MATLAB toolbox (http://www.math.mcgill.ca/keith/surfstat) for Matrix Laboratory R2012a (The MathWorks, Inc., Natick, MA, USA). The resulting statistical maps (t-maps) were corrected for multiple comparisons using FDR with a q-value of 0.05 across all 327,684 vertices on the cortical surface.

Sensitivity analyses

Ancillary mixed-effects models including interaction terms (e.g., inflammation * age, inflammation * sex, inflammation * anti-inflammatory drug use) investigated whether the association of chronic inflammation with brain health outcomes was modified or confounded by age, sex, or the use of anti-inflammatory medication (eTable 4). Similarly, we included lifestyle and health covariates in a fully-adjusted model (alongside age and sex) to determine whether individual aspects of health and lifestyle had an impact on the association of inflammation with brain-health phenotypes (eTable 5).

Mediation analyses

We ran mediation analyses in a structural equation modelling (SEM) framework using the R ‘lavaan’ package (https://cran.r-project.org/web/packages/lavaan/lavaan.pdf). This simultaneously characterised associations among CRP, brain and cognitive metrics, and also specifically tested the hypothesis that brain structure would partly and significantly mediate
associations between measures of CRP and cognitive ability. Both single and multiple mediator models were specified (see Figure 5A-C as example). Single mediator models provided information on the proportion of CRP-cognitive associations attributable to individual neuroimaging metrics. By contrast, in Multiple mediator models, brain structural variables were entered simultaneously as covarying mediators (see path diagram, Figure 5C). This allowed us to quantify the proportion of variance in CRP-cognitive associations uniquely explained by each facet of brain structure (GM, NAWM, WMH, gFA, gMD). The primary estimates of interest in this study are the degree of change (mediation) in the direct path (c to c’) between inflammation measures (DNAm CRP or serum CRP) and cognitive ability (gf or processing speed or visuospatial ability or verbal memory) when the indirect path from inflammation to cognitive ability via brain structure (a x b) is included. A significant mediation of the c path (to c’) is denoted by the statistical significance of this indirect effect. Bootstrapping was used calculate the standard errors. Multiple comparisons were corrected for by FDR correction. These mediations were re-run when accounting for self-reported health variables as covariates: in Model 1 age and sex were covariates; in Model 2, they were age, sex, BMI, hypertension, diabetes, smoking status and alcohol use. To account for missing data bias, we took account of all available data, using full information maximum likelihood estimation. Model fit was evaluated based on root mean squared error approximation (RMSEA), the comparative fit index (CFI), the standardized root mean square residual (SRMR) and the Tucker–Lewis index (TLI). We considered a model an acceptable fit when it respected the following thresholds: RMSEA ≤ 0.05; SRMR ≤ 0.06; CFI ≥ 0.97; TLI ≥ 0.95 as recommended by.

Data Availability Statement

The data analysed in this study is not publicly available as it contains data that could compromise participant consent and confidentiality, but can be requested via a data access request to the Lothian Birth Cohorts research group.
Standard Protocol Approvals, Registrations and Patient Consents

Ethical permission for the LBC1936 was obtained from the Multi-Centre Research Ethics Committee for Scotland (MREC/01/0/56) and the Lothian Research Ethics Committee (LREC/2003/2/29). Written informed consent was obtained from all participants. All necessary patient/participant consent has been obtained and the appropriate institutional forms have been archived.
Results

**DNAm CRP is associated with global and regional brain volume**

We studied 521 eligible older adults (aged ~ 73 years; refer to Figure 2 and eTable 1) and looked at epigenetic vs serum inflammation associations across a range of neuroimaging and cognitive measures (Table 1). To index chronic inflammation, an epigenetic measure of CRP (DNAm CRP) was assembled for each participant (see Methods). The correlation between the DNAm CRP score and serum log(CRP) was moderate ($r = 0.29$, 95% CI [0.28, 0.4]), and the DNAm CRP score showed a stronger correlation with serum CRP than any one of its composite CpGs$^{11}$.

We found that higher inflammatory burden, indexed by DNAm CRP scores, associated with poor cognitive and neuroimaging brain health outcomes (Table 1). DNAm CRP exhibited significantly larger (6.4-fold, on average) associations with brain structural MRI metrics (including global grey and white matter atrophy, poorer white matter microstructure and increased white matter hyperintensity burden) than serum CRP. These DNAm CRP-associated brain structural changes were independent of anti-inflammatory drug-use, age, or sex (eTable 4). Participants with a higher inflammatory burden on average had greater overall brain atrophy, with higher DNAm CRP associating with lower total brain volume ($\beta = -0.197$, 95% CI [-0.28, -0.12], $p_{FDR} = 8.42 \times 10^{-6}$), grey matter volume ($\beta = -0.200$, 95% CI [-0.28, -0.12], $p_{FDR} = 1.66 \times 10^{-5}$) and white matter volume ($\beta = -0.150$, 95% CI [-0.23, -0.07], $p_{FDR} = 0.001$). Models which included additional health and lifestyle covariates (BMI, smoking, alcohol consumption, hypertension, diabetes and cardiovascular disease history) attenuated the relationship between DNAm CRP and brain health outcomes by up to 40% (eTable 5). Of these, the associations between DNAm CRP with white matter measures (WMH, NAWM) were the most attenuated (34-40%). Out of the lifestyle and health factors
accounted for, smoking appeared to have the greatest influence on the attenuation (as illustrated in supplementary eFigure 2).

After examining global brain structural alterations, we looked at specific regional cortical brain associations with higher inflammation levels. We found regional heterogeneity in the patterning of associations between CRP measures and cortical metrics: atrophy in frontal, anterior lateral and medial temporal lobes were associated with higher DNAm CRP (Figure 3B); inflammation associations with brain cortical thickness are presented in the supplementary document (eFigure 1). Overall, these results emphasise that the DNAm-CRP score associates with lower cortical volume of specific brain regions (lateral and medial temporal regions of the brain), which show overlap with those of serum CRP and unique variance (Figure 3F), with DNAm CRP reflecting atrophy above and beyond the serum CRP score.

**DNAm CRP is associated with white matter microstructure in specific white matter tracts**

Next, we investigated whether higher DNAm CRP was related to lower white matter microstructure based on global and regional diffusion MRI (dMRI) measures by looking at inflammation associations with white matter tract fractional anisotropy and mean diffusivity. While serum CRP-dMRI associations were null in all cases (all pFDR > 0.089) (eTables 6-7), higher DNAm CRP scores predicted overall lower general fractional anisotropy (gFA) ($\beta = -0.162$, $p_{FDR} = 6.94 \times 10^{-4}$) and higher general mean diffusivity (gMD) ($\beta = 0.124$, $p_{FDR} = 0.010$). For specific white matter tracts, the strongest associations were seen for the arcuate fasciculus and uncinate fasciculus, with lower FA and higher MD with higher DNAm CRP (see Figure 4; eTables 6-7). For global measures of WM tract integrity (gFA, gMD), accounting for health and lifestyle covariates did not substantially alter the magnitude
or significance of these associations (eTable 5); however, at the level of individual WM tracts, the relationship between DNAm CRP and FA and MD was attenuated when lifestyle factors were included in the models (eTables 8-9); this is illustrated in supplementary eFigure 3.

**Brain structure partly mediates the association of DNAm CRP with cognitive ability**

As higher DNAm CRP levels were associated with lower cognitive performance both here (Table 1) and previously\(^11\), we quantified the degree to which brain structural differences contribute to the inflammation-cognition association, and which facets show the strongest unique contributions to this relationship using an SEM framework. Bivariate associations between all variables (inflammation, brain structure, cognitive ability, and lifestyle measures) are provided in eTable 10. While TB volume, GM volume, NAWM volume and WMH volume all emerged as significant mediators in single SEM models (percentage attenuation 14-21%; eTable 11), multiple mediator models were used to test the degree to which each global MRI metric contributed uniquely to mediation of the same association (Figure 5D; eTable 12). Here, the sum total of MRI measures significantly mediated the association between DNAm CRP and general cognitive ability ($\beta = -0.047 [-0.076,-0.018]$, pFDR = 0.002; percentage attenuation 29.7%). The unique contributions to this variance were largest for NAWM volume ($\beta = -0.03 [-0.053, -0.023]$, pFDR = 0.012) indicating that the loss of white matter may contribute to inflammation-associated differences in cognitive functioning in older age. Out of the individual cognitive domains, processing speed was the most significantly mediated by the sum total of MRI metrics ($\beta = -0.058 [-0.090,-0.027]$, pFDR = 0.001; percentage attenuation 41%). Again, NAWM emerged as the largest unique contribution to this variance ($\beta = -0.037 [-0.053, -0.007]$, pFDR = 0.006); eTable 13. Similarly, visuospatial ability was significantly mediated by the sum total of MRI metrics ($\beta = -0.036 [-0.063,-0.010]$, pFDR = 0.013; percentage attenuation 37%), with NAWM
accounting for the largest unique contribution to this effect ($\beta = -0.030 [-0.063, -0.010]$, pFDR = 0.012). While verbal memory was significantly mediated by the sum total of MRI metrics ($\beta = -0.031 [-0.057, -0.007]$, pFDR = 0.026; percentage attenuation 33%), there were no significant contributions from individual MRI metrics (eTables 12-13).

Finally, with the addition of lifestyle and health covariates to our models, no aspect of brain structure remained a significant mediator of the associations between DNAm CRP and general cognitive ability $\beta_{\text{mediation}} = -0.023 [-0.049, 0.003]$, pFDR = 0.167 (see Figure 5, eTables 11-12) or any of the individual cognitive domains (eTable 12).
Discussion

Only recently has there been a push for integrated multi-omics approaches to better characterise chronic inflammation\(^3,26\). DNA methylation profiles may act as promising peripheral biomarkers for cognitive-ageing differences at the population level, given their relative stability in the short-term, and their joint modulation by both genetic and lifestyle traits. Elsewhere, DNAm markers of inflammation have proved informative in predicting a range of age-related health outcomes, from cardiovascular disease to depression\(^23,24,36\), but few studies have applied this same approach to cognitive ageing differences in healthy cohorts. As chronic inflammation is considered to be an insidious, cumulative, and often undetected contributor to cognitive ageing\(^1,3–5,14\), the importance of such epigenetic markers may be their utility to index inflammatory load with greater reliability than phasic protein measures. In this study, DNAm CRP was more robustly associated with a range of cognitive and neuroimaging metrics than serum CRP, supporting our original hypothesis. We discovered that DNAm CRP shows consistently stronger associations with brain structure than serum CRP (on average, 6.4 fold greater), that these associations are not regionally homogeneous across the brain’s cortex, and that specific aspects of brain structure partly mediate (up to 29.7%) associations between an epigenetic signature of CRP and cognitive functioning. Our results highlight the potential of epigenetic approaches to indexing inflammation in population cohorts and suggest that chronic inflammation may contribute to both focal and global brain structural changes which underlie differences in cognitive ageing.

We found regional heterogeneity in the patterning of associations between CRP measures and cortical metrics, indicating differential regional vulnerability to chronic inflammation. Reductions in brain cortical volume and thickness in frontal, anterior lateral and medial temporal lobes were associated with increased DNAm CRP. Consistently, previous studies...
report structural changes associated with inflammatory markers in the temporal and frontal cortices\textsuperscript{17,18}. Atrophy in these regions is implicated in cognitive decline\textsuperscript{37}, and differential patterns of pro-inflammatory receptor distribution may underlie why some brain regions are more vulnerable to inflammation than others. For example, in Alzheimer’s disease (AD) patients, pro-inflammatory cytokine receptor density and expression are increased in regions of neurodegeneration, including the medial frontal and temporal cortices\textsuperscript{38}. Higher inflammation levels have also been related to progression of atherosclerosis, with evidence for differential effects of CRP in different beds of the arterial brain supply\textsuperscript{39,40}. These findings suggest that raised levels of inflammatory mediators may contribute to localised brain atrophy via their differential expression in brain tissue and cerebrovasculature.

Overall, the results of our mediation analyses indicate that chronic inflammation’s detrimental impact on white matter above and beyond other brain structural features may underlie the inflammation-associated differences in cognitive functioning in older age. While numerous studies have found associations between reduced white matter volume and raised inflammatory markers both in healthy cohorts\textsuperscript{14,17,41}, and those with chronic inflammatory conditions\textsuperscript{42}, few have looked at inflammation, brain structure and cognitive function concurrently\textsuperscript{13}. Fewer still have attempted to more robustly characterise chronic inflammation beyond assessing serum inflammatory protein-profiles – although a notable exception comes from recent work, where the same DNAm CRP signature was found to be significantly associated with widespread reductions in white matter integrity beyond that of serum CRP\textsuperscript{36}. In agreement with this study, but in an older-age cohort, we found that higher DNAm CRP related to increased WMH burden, reduced NAWM volume and ostensibly poorer white matter microstructure (lower FA and higher MD). In particular, the white matter tracts of arcuate fasciculus and uncinate fasciculus showed the most consistent significant
relationships with DNA methylation (DNAm) CRP levels (across both FA and MD), alongside significantly lower FA in the anterior thalamic radiation, which are consistent with studies assessing the effects of vascular risk on microstructure with advanced age\textsuperscript{31}.

In agreement with longstanding findings from neurocognitive studies – where, consistently, inflammation is more strongly associated with declines in processing speed than other cognitive domains\textsuperscript{9,10} – our results indicate that some cognitive domains (processing speed) may be more mediated by the brain structural consequences of chronic inflammation than others (verbal memory, visuospatial ability). Processing speed has been strongly linked to white matter integrity at both global and regional levels\textsuperscript{43} and many of the downstream impacts of neuroinflammatory processes directly affect white matter integrity (to include demyelination, de-afferentation and gliosis; see Figure 6 and eFigure 5)\textsuperscript{14}. As such, chronic inflammation’s contribution to diffuse and global WM loss may disproportionately impact cognitive functions which require the coordination of brain regions (e.g., processing speed), compared to more functionally localised ones (e.g., verbal memory). However, we caveat that we have not formally compared the magnitude of these attenuations, and judge that this greater degree of attenuation is likely to be a general shared process\textsuperscript{30} plus some degree of noise, given that variance across cognitive domains is shared at the general level\textsuperscript{30,44}.

The attenuation seen in inflammation-brain health associations when lifestyle factors were accounted for is to be expected given what is known about inflammation and vascular risk factors on brain-health outcomes\textsuperscript{3,31}. Vascular inflammation is considered to be a shared mechanism linking cardiometabolic factors (to include hypertension and smoking) with poor cognitive outcomes\textsuperscript{31,40,45}. Figure 6 models this relationship and illustrates how increased inflammation in the periphery can result in neurodegenerative processes via blood brain...
Conole 20

barrier (BBB) dependent and independent pathways. The regional areas of brain loss that were particularly associated with DNA methylation (DNAm) CRP are also areas where others have shown increased BBB leakage in persons at risk of AD. Peripheral markers of inflammation such as CRP have been consistently linked to the risk of cerebrovascular and cardiovascular events, and there is evidence that atherosclerotic and thrombotic presentations signify a chronic inflammatory process. The methylation CRP score was more strongly associated with modelled VRFs (hypertension, CVD history, diabetes, alcohol consumption and smoking) than serum CRP. Given that the 7 CpGs which make up the DNA methylation score reside in inflammation and vascular-related genes, these DNAm CRP-brain MRI associations may be capturing the impact of upstream inflammatory activity above and beyond that of serum CRP levels, which may explain why some VRFs show greater association with the DNA methylation score. Upstream inflammatory cytokines such as interleukin-6 (IL6) and tumour necrosis factor alpha (TNF-α) have been shown to be associated with increased risk of dementia where serum CRP levels showed no association, such as in the Rotterdam Study, Whitehall II longitudinal cohort study and The Framingham Study. Variation and measurement error in serum CRP levels may be confounding the relationship between chronic inflammation and related health and lifestyle triggers and exacerbators. Our results add to the evidence base that DNAm-based predictors of inflammation may act as a quantifiable archive of the longitudinal effects of these exposures, and other unaccounted for health and genetic profiles, that serum CRP levels fail to capture.

Strengths of this study include the large sample size, array of multi-modal data, and that inflammation, lifestyle, cognitive and structural brain variables were measured in the same individuals at about the same time. Compared to recent EWAS-neuroimaging research, this
study is exceptionally well-powered with 521 participants after exclusions. We believe that, alongside as the narrow age range, the homogeneity of LBC1936 cohort (e.g., all participants are of Scottish ancestry) may have minimized any potentially strong confounding effects that factors such as mixed ethnicity and geography might have had in a more heterogenous sample. This cross-sectional nature however means we are unlikely to capture the effect of more age-related changes in inflammatory profile and cognitive decline. Although we endeavoured to remove participants with cognition-related pathology, these were screened via self-reported diagnoses, and we may be missing undiagnosed or subclinical incident neurodegenerative pathology. Similarly, while we have identified a range of health and lifestyle variables which we could influence inflammatory load (BMI, diabetes, CVD history, smoking, alcohol consumption, hypertension), there are many non-modelled variables that could contribute to this effect, as discussed in depth elsewhere.

Finally, a clear limitation of the study is that our epigenetic surrogate of inflammation was measured in blood rather than brain tissue. While brain-based biomarkers are the optimal choice for investigating cognitive outcomes, it is impractical to profile such methylomes in brain tissue in living human subjects. Furthermore, the use of post-mortem brain tissue samples has its own problems (in particular, the stability of global DNAm following death) and cannot reliably reflect the plastic state of methylomes in vivo. Future studies should consider examining a wider range of DNAm inflammatory markers (DNAm levels of interleukins, prostaglandins, neurotrophins); looking at DNAm inflammatory markers in younger participants (where there is likely greater variation in baseline inflammation levels); looking at DNAm inflammatory markers in specific brain pathology cases (e.g. multiple sclerosis patients); as well as examining how peripheral inflammatory and
neuroinflammatory DNAm patterns equate, and how each relates to cellular differences within the brain to give rise to the structural alterations we observe here\textsuperscript{50}.

Our findings do not establish causality but support the hypothesis that chronic systemic inflammation may contribute to neurodegenerative brain changes which underlie differences in cognitive ability in later life. Previous studies exploring this relationship may underestimate the brain and cognitive sequelae of chronic inflammation by relying on single measurements of phasic serum proteins. By utilising an epigenetic inflammation measure, which integrates information from multiple immune-related CpG sites, we may provide a more reliable measure of chronic inflammation and thus a more comprehensive overview of the consequences of chronic inflammation on brain structure and function. Reliable monitoring of inflammatory exposure could enable clinicians to review the efficacy of drug and lifestyle interventions to attenuate inflammation levels with a view to improving cognitive outcomes.

\textbf{Main text word count: 4457 words}
### Tables:

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Serum CRP (model 1)</th>
<th>DNAm CRP (model 2)</th>
<th>( \Delta ) association magnitudes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \beta )</td>
<td>SE</td>
<td>( p )</td>
</tr>
<tr>
<td>TB</td>
<td>-0.033</td>
<td>0.040</td>
<td>0.579</td>
</tr>
<tr>
<td>GM</td>
<td>-0.026</td>
<td>0.042</td>
<td>0.670</td>
</tr>
<tr>
<td>NAWM</td>
<td>-0.027</td>
<td>0.042</td>
<td>0.657</td>
</tr>
<tr>
<td>WMH</td>
<td>0.018</td>
<td>0.042</td>
<td>0.740</td>
</tr>
<tr>
<td>gFA</td>
<td>-0.055</td>
<td>0.045</td>
<td>0.347</td>
</tr>
<tr>
<td>gMD</td>
<td>0.025</td>
<td>0.045</td>
<td>0.677</td>
</tr>
<tr>
<td>visuospatial ability</td>
<td>-0.082</td>
<td>0.038</td>
<td>0.069</td>
</tr>
<tr>
<td>processing speed</td>
<td>-0.088</td>
<td>0.038</td>
<td>0.054</td>
</tr>
<tr>
<td>verbal memory</td>
<td>-0.046</td>
<td>0.038</td>
<td>0.347</td>
</tr>
<tr>
<td>gf</td>
<td>-0.098</td>
<td>0.038</td>
<td>0.027</td>
</tr>
</tbody>
</table>

Table 1. Cross-sectional associations between serum CRP and DNAm CRP with neuroimaging and cognitive outcomes.
References:


Table and Figure Legends:

Table 1. Cross-sectional associations between serum CRP and DNAm CRP with neuroimaging and cognitive outcomes

Cross-sectional associations of inflammation measures and neuroimaging and cognitive phenotypes. Bold typeface denotes p < 0.05. Asterisks indicate significant differences between serum and DNAm CRP regressions. TB: total brain, GM: grey matter, NAWM: normal-appearing white matter, WMH: white matter hyperintensity, gf: general cognitive ability; gFA: general fractional anisotropy, gMD: general mean diffusivity. All p values reported are FDR corrected.

Fig. 1. Chronic inflammation increases with age and may contribute to variance in cognitive ability

(A) Schematic demonstrating life-span curves for chronic inflammation. Inflammatory load tends to increase with age; lifestyle, genetics and health conditions can all influence susceptibility to chronic inflammation and account for variance in inflammation levels between individuals.1,3 (B) Chronic inflammation can be measured by inflammatory proteins taken from a blood sample, such as serum levels and DNA methylation (DNAm) proxies of C-Reactive Protein (CRP). (C) Life-span curves for cognitive ability outlining how there is considerable inter-individual heterogeneity in rate and timing of cognitive decline, with some people on more accelerated cognitive ageing trajectories than others.4 (D) Trajectories of LBC1936 participants respective inflammation scores over age, as outlined in11, illustrating comparative stability of DNAm inflammation marker compared to serum CRP. ©Eleanor Conole.
Fig. 2. Flowchart depicting the step-by-step selection process of the Lothian Birth Cohort 1936 (LBC1936) subjects included in the final sample for data analyses
Fig. 3. DNAm CRP shows stronger and more widespread associations with global and regional brain structure than serum CRP

(A) Regional cortical volume regressed against serum CRP (A,D) and DNAm CRP (B,E) n = 521. Colours denote the magnitude (T-maps; top) and significance (Q values; bottom) of the negative associations between inflammation and brain cortical volume. Panel (C) shows the percentage attenuation for the significant associations between DNAm-CRP and cortical volume when also controlling for serum CRP. Conjunction plot (E) shows the spatial extent of independent contributions and overlap (red) in cortical loci that exhibit FDR-corrected unique associations with simultaneously-modelled serum (pink) and epigenetic (blue) inflammation measures; results are corrected for sex, age and ICV. TB: total brain, GM: grey matter, NAWM: normal-appearing white matter, WMH: white matter hyperintensity, gf, general cognitive ability; gFA: general fractional anisotropy, gMD: general mean diffusivity.
Fig. 4. DNAm CRP is associated with white matter microstructure in specific white matter tracts

(A) Standardised regression coefficients for associations between white matter tract-averaged fractional anisotropy (FA; left), and mean diffusivity (MD; right). Bars show standardized coefficients and standard errors. Asterisks indicate where associations are significantly larger for DNAm than for serum (*P<0.05, **P<0.01) (B) Illustration of the respective white matter tracts measured using probabilistic neighbourhood tractography in one LBC1936 study participant.
Fig.5. Brain structure partly mediates the association of DNAm CRP with cognitive ability

Top panel (A,B) displays single mediator models, bottom panel (C,D) displays multiple mediator models (A) Model 1 structural equation model path diagrams showing that in model 1, the association between DNAm CRP and general cognitive ability (path c) was significantly and partially mediated by normal appearing white matter volume (path ab = -0.033, p = 0.001), attenuating the c path by 21% (path c'), and (C) 29.7% by multiple MRI variables (ab = -0.047, p = 0.002) (B) Single mediator models indirect effect size and standard error bars. (D) Multiple mediator models indirect effect size and standard error bars. Light bars show model 1 (includes covariates age and sex), dark bars show model 2 which contains additional health covariates (age + sex + BMI + hypertension + smoking status + alcohol use + CVD history + diabetes), *p <0.05; ** p < 0.01.
Fig. 6. Mechanisms of neurodegeneration via increased systemic chronic inflammation.

(A) Chronic inflammation is pertinent to brain ageing in that inflammatory mediators in the periphery can damage the blood brain barrier (BBB), permitting entry into the brain where they go on to disrupt neurons and glia and perpetuate a chronic inflammatory state. This directly contributes to various neurodegenerative pathways (illustrated) which lead to brain cell death. (B) Suggested mechanisms by which the causes of inflammaging (immunosenescence, lifestyle, clinical health) and related consequences may drive brain health (structural and cognitive) outcomes (C) Study model: chronic inflammation is a key driver of cognitive decline through its effects on brain structure. Left shows generic directed acyclic graph for mediation analysis [left panel] and for the study example [right panel]. C, confounder; A, exposure; M, mediator; Y, outcome. BBB: Blood-brain barrier; CRP: C-reactive protein; IL6: interleukin-6; IL1β: interleukin-1β; TNF-α: tumour necrosis factor-α; PGE2: prostaglandin E2; BDNF: Brain derived neurotrophic factor; IGF-1: insulin-like growth factor 1; SASP: senescence associated secretory phenotype; ROS: reactive oxygen species. ©Eleanor Conole, created with BioRender.com
DNA Methylation and Protein Markers of Chronic Inflammation and Their Associations With Brain and Cognitive Aging

Eleanor L.S. Conole, Anna J. Stevenson, Susana Muñoz Maniega, et al.

*Neurology* published online November 17, 2021
DOI 10.1212/WNL.0000000000012997

This information is current as of November 17, 2021

Updated Information & Services
including high resolution figures, can be found at:
http://n.neurology.org/content/early/2021/11/17/WNL.0000000000012997.full

Subspecialty Collections
This article, along with others on similar topics, appears in the following collection(s):
Class II
http://n.neurology.org/cgi/collection/class_ii
Cognitive aging
http://n.neurology.org/cgi/collection/cognitive_aging
Cohort studies
http://n.neurology.org/cgi/collection/cohort_studies
Public health
http://n.neurology.org/cgi/collection/public_health
Volumetric MRI
http://n.neurology.org/cgi/collection/volumetric_mri

Permissions & Licensing
Information about reproducing this article in parts (figures, tables) or in its entirety can be found online at:
http://www.neurology.org/about/about_the_journal#permissions

Reprints
Information about ordering reprints can be found online:
http://n.neurology.org/subscribers/advertise