

1 A comparison of blood and brain- 2 derived ageing and inflammation- 3 related DNA methylation signatures 4 and their association with microglial 5 burdens 6

7 Anna J. Stevenson^{1,2}, Daniel L. McCartney¹, Gemma L. Shireby³, Robert F. Hillary¹, Declan King^{2,4},
8 Makis Tzioras^{2,4}, Nicola Wrobel⁵, Sarah McCafferty⁵, Lee Murphy⁵, Barry W. McColl^{2,4}, Paul
9 Redmond⁶, Adele M. Taylor⁶, Sarah E. Harris^{6,7}, Tom C. Russ^{6,8,9}, Eilis J Hannon³, Andrew M.
10 McIntosh⁹, Jonathan Mill³, Colin Smith¹⁰, Ian J. Deary^{6,7}, Simon R. Cox^{6,7}, Riccardo E. Marioni^{1,6*}, Tara
11 L. Spires-Jones^{2,4*}

12 ¹ Centre for Genomic and Experimental Medicine, Institute of Genetics and Molecular Medicine,
13 University of Edinburgh, Edinburgh, EH4 2XU, UK

14 ² Centre for Discovery Brain Sciences, University of Edinburgh, Edinburgh, EH8 9JZ, UK

15 ³ University of Exeter Medical School, University of Exeter, Exeter, EX2 4TE, UK

16 ⁴ UK Dementia Research Institute, University of Edinburgh, Edinburgh, EH16 4SB, UK

17 ⁵ Edinburgh Clinical Research Facility, Western General Hospital, Edinburgh, EH4 2XU, UK

18 ⁶ Lothian Birth Cohorts, University of Edinburgh, Edinburgh, EH8 9JZ, UK

19 ⁷ Department of Psychology, University of Edinburgh, Edinburgh, EH8 9JZ, UK

20 ⁸ Alzheimer Scotland Dementia Research Centre, 7 George Square, University of Edinburgh,
21 Edinburgh, EH8 9JZ, UK

22 ⁹ Division of Psychiatry, University of Edinburgh, Royal Edinburgh Hospital, Edinburgh, EH10 5HF, UK

23 ¹⁰ Centre for Clinical Brain Sciences, University of Edinburgh, Chancellor's Building, 49 Little France
24 Crescent, Edinburgh EH16 4SB, UK

25

26 *Corresponding author

27

28

29

30

31

32

33

34

35 Abstract

36

37 Inflammation and ageing-related DNA methylation patterns in the blood have been linked to a
38 variety of morbidities, including cognitive decline and neurodegenerative disease. However, it is
39 unclear how these blood-based patterns relate to patterns within the brain, and how each
40 associates with central cellular profiles. In this study, we profiled DNA methylation in both the blood
41 and in five *post-mortem* brain regions (BA17, BA20/21, BA24, BA46 and hippocampus) in 14
42 individuals from the Lothian Birth Cohort 1936. Microglial burdens were additionally quantified in
43 the same brain regions. DNA methylation signatures of five epigenetic ageing biomarkers
44 ('epigenetic clocks'), and two inflammatory biomarkers (DNA methylation proxies for C-reactive
45 protein and interleukin-6) were compared across tissues and regions. Divergent correlations
46 between the inflammation and ageing signatures in the blood and brain were identified, depending
47 on region assessed. Four out of the five assessed epigenetic age acceleration measures were found
48 to be highest in the hippocampus (β range=0.83-1.14, $p \leq 0.02$). The inflammation-related DNA
49 methylation signatures showed no clear variation across brain regions. Reactive microglial burdens
50 were found to be highest in the hippocampus ($\beta=1.32$, $p=5 \times 10^{-4}$); however, the only association
51 identified between the blood- and brain-based methylation signatures and microglia was a
52 significant positive association with acceleration of one epigenetic clock (termed DNAm PhenoAge)
53 averaged over all five brain regions ($\beta=0.40$, $p=0.002$). This work highlights a potential vulnerability
54 of the hippocampus to epigenetic ageing and provides preliminary evidence of a relationship
55 between DNA methylation signatures in the brain and differences in microglial burdens.

56

57

58

59

60

61

62

63

64

65

66 1. Introduction

67

68 Ageing is characterised by a progressive deterioration of physiological integrity and is a key risk
69 factor for a multitude of diseases. A pervasive feature of ageing is a persistent, or chronic, systemic
70 inflammation (1). This process is characterised by a subtle elevation of inflammatory mediators in
71 the periphery, in the absence of evident precipitants or disease states. Chronic inflammation has
72 been identified as a common feature in the preponderance of neurodegenerative diseases and is
73 increasingly recognised as a potential mediator of cognitive impairment in older age (2). There is,
74 however, still a paucity of understanding of the biological mechanisms involved in chronic
75 inflammation and how peripheral and central inflammatory mechanisms relate.

76 Recently, the link between inflammation and the epigenetic mechanism of DNA methylation (DNAm)
77 has begun to be addressed (3, 4). DNAm is typically characterised by the addition of a methyl group
78 to a cytosine, in the context of a cytosine-guanine (CpG) dinucleotide. It has been implicated in the
79 regulation of gene expression and can itself be influenced by both genetic and environmental factors
80 (5, 6). Genome-wide DNAm patterns in the blood have been leveraged to index lifestyle traits, such
81 as smoking (7, 8), and have been used to investigate diverse physical and mental health-related
82 phenotypes, including cognitive functioning (9). In addition to this, by exploiting the manifest
83 alterations in DNAm patterns with ageing, several DNAm-based markers of age have been
84 developed, which attempt to provide surrogate measures of biological ageing (10-13). These
85 ‘epigenetic clocks’ have been used to provide a measure of biological age acceleration, or
86 deceleration, by establishing the difference between an individual’s chronological and epigenetic
87 age. Positive age acceleration quantified in the blood has been associated with an increased risk of
88 mortality and a variety of age-related morbidities, including with a lower cognitive ability (14-16). In
89 addition to this, recently, we found that blood-based DNAm proxies for two inflammatory mediators
90 – C-reactive protein (CRP) and interleukin-6 (IL-6) – were inversely associated with cognitive ability in
91 older adults with larger effect sizes compared to the biomarkers themselves (17, 18).

92 While these findings suggest that an accelerated biological age, and raised DNAm inflammation
93 patterns associate with poorer cognitive functioning, it is important to note that these studies
94 analysed blood tissue. While the blood represents a practical, accessible source by which to
95 investigate such outcomes, DNAm is known to confer both cell-type and tissue-specific patterns (19).
96 For analyses of brain-based traits such as cognitive ability, brain samples offer the optimal disease-
97 relevant tissue; however, given the obvious limitations of access to such tissue, much of the research
98 assessing the association between differential DNAm and disorders of the central nervous system
99 has been conducted in peripheral whole blood (20, 21). While this approach can provide informative

100 peripheral markers of central aberration or disease, it is important to investigate the relevant target
101 tissue to characterise both how peripheral and central patterns equate, and how each relates to
102 cellular differences within the brain. Microglia are the primary tissue-resident immune cells of the
103 central nervous system and have critical roles in homeostasis and neuroinflammation. Aged
104 microglia have been shown to be more responsive to pro-inflammatory stimuli compared to naïve
105 microglia, and evidence suggests the cells are particularly sensitive to both acute and chronic
106 systemic inflammation detected via peripheral-central signalling pathways (22, 23). Microglia have
107 additionally been implicated in age-related neurological dysfunction; however, as yet, it is unclear
108 how inflammation and age-related DNAm patterns in both the periphery and the brain itself relate
109 to microglial burdens.

110 In this study, we utilise data from 14 participants of the Lothian Birth Cohort 1936. These individuals
111 have blood-based DNAm data available at up to 4 time-points between the ages of 70-79 years and
112 additionally donated *post-mortem* brain tissue to the study. In the brain, we profiled DNAm and
113 quantified microglial burdens in five regions (primary visual cortex [BA17], inferior temporal gyrus
114 [BA20/21], anterior cingulate cortex [BA24], dorsolateral prefrontal cortex [BA46], and
115 hippocampus). DNAm CRP and IL-6 profiles, along with five different DNAm age acceleration
116 measures, were characterised in the blood and in each brain region to investigate the relationship
117 between peripheral and central age- and inflammation-related methylation patterns and how these
118 relate to inflammatory processes in the brain. Given the small sample size of this study, the results
119 presented here represent preliminary patterns; however, this data, and the methodology employed,
120 provides a framework upon which future, larger scale, work can be based.

121

122 2. Methods

123

124 2.1 The Lothian Birth Cohort 1936

125

126 The Lothian Birth Cohort 1936 (LBC1936) is a longitudinal study of ageing. Full details on the study
127 protocol and data collection have been described previously (24, 25). Briefly, the cohort comprises
128 1,091 individuals born in 1936 most of whom completed a study of general intelligence – the
129 Scottish Mental Survey – in 1947 when they were aged around 11 years. Participants who were
130 living in Edinburgh and the surrounding area were re-contacted around 60 years later with 1,091
131 individuals consenting to join the LBC1936 study. At Wave 1 of the study participants were around
132 70 years old (mean age: 69.6 ± 0.8 years) and they have since completed up to four additional

133 assessments, triennially. At each assessment, participants have been widely phenotyped with
134 detailed physical, cognitive, epigenetic, health and lifestyle data collected. A tissue bank for *post-*
135 *mortem* brain tissue donation was established at Wave 3 of LBC1936 in collaboration with the
136 Medical Research Council-funded University of Edinburgh Brain Banks. To date, ~15% of the original
137 LBC1936 sample have given consent for *post-mortem* tissue collection. At the time of this study,
138 samples from 14 individuals were available.

139 2.2 Ethics

140

141 Ethical permission for LBC1936 was obtained from the Multi-Centre Research Ethics Committee for
142 Scotland (MREC/01/0/56), the Lothian Research Ethics Committee (Wave 1: LREC/2003/2/29) and
143 the Scotland A Research Ethics Committee (Waves 2, 3 and 4: 07/MRE00/58).

144 Use of human tissue for *post-mortem* studies was reviewed and approved by the Edinburgh Brain
145 Bank ethics committee and the medical research ethics committee (the Academic and Clinical
146 Central Office for Research and Development, a joint office of the University of Edinburgh and NHS
147 Lothian, approval number 15-HV-016). The Edinburgh Brain Bank is a Medical Research Council
148 funded facility with research ethics committee (REC) approval (16/ES/0084).

149

150 2.3 DNA methylation preparation

151

152 2.3.1 Blood

153

154 DNAm from whole blood was quantified at 485,512 CpG sites using the Illumina Human Methylation
155 450k BeadChips at the Edinburgh Clinical Research Facility. Full details of the quality control steps
156 have been described previously (26, 27). Briefly, raw intensity data were background-corrected and
157 normalised using internal controls. Samples with inadequate bisulphite conversion, hybridisation,
158 staining signal or nucleotide extension were removed upon manual inspection. Further, probes with
159 a low detection rate ($p > 0.01$ in $> 5\%$ of samples), samples with a low call rate ($< 450,000$ probes
160 detected at $p < 0.01$), samples exhibiting a poor match between genotype and SNP control probes,
161 and samples with a mismatch between methylation-predicted, and recorded, sex were additionally
162 excluded. This left a total of 450,276 autosomal probes. In analyses comparing blood and brain
163 DNAm signatures, the last blood measurement before death was used and models were adjusted for
164 the interval between the blood draw and death (see **Supplementary Table 1**; mean interval: 2.5
165 years, SD: 1.5).

166 2.3.2 Brain

167

168 Brains were removed at *post-mortem* and cut into coronal slices. Regions of interest dissected, as
169 detailed previously (28). Tissue samples from cortical regions BA17, BA20-21, BA24, BA46 and
170 hippocampus, were collected and snap frozen. From these sections ~25mg of tissue was processed
171 for DNA extraction. DNA extraction was performed using a DNeasy kit (Qiagen) and DNAm was
172 profiled using Illumina MethylationEPIC BeadChips at the Edinburgh Clinical Research Facility.
173 Samples were processed randomly. Quality control steps were performed as follows: the
174 *wateRmelon* pfilter() function (29) was used to remove samples in which >1% of probes had a
175 detection p-value of >0.05, probes with a beadcount of <3 in >5% of samples, and probes in which
176 >1% of samples had a detection p-value of >0.05. Probes mapping to polymorphic targets, cross-
177 hybridising probes and probes on the X and Y chromosomes were additionally removed. The
178 performance of 15 normalisation functions was assessed, following the protocol described by Pidsley
179 *et al.* (29). The top-ranking method was *danet* which equalises background from type 1 and type 2
180 probes, performs quantile normalisation of methylated and un-methylated intensities
181 simultaneously, and then calculates normalised methylation β -values. The normalised dataset
182 comprised 69 samples (14 individuals, 5 regions, 1 missing hippocampal sample) and 807,163
183 probes.

184 2.4 Derivation of DNA methylation signatures

185

186 2.4.1 Epigenetic age acceleration

187

188 Methylation-based epigenetic age acceleration estimates were obtained from the online Horvath
189 DNAm age calculator (<https://dnamage.genetics.ucla.edu/>)(11). Normalised DNAm data was
190 uploaded to the calculator using the 'Advanced Analysis' option. This output provides four different
191 age acceleration measures: intrinsic epigenetic age acceleration (IEAA) (11); extrinsic epigenetic age
192 acceleration (EEAA) (12); DNAm PhenoAge acceleration ($\text{AgeAccel}_{\text{Pheno}}$)(10); and DNAm GrimAge
193 acceleration ($\text{AgeAccel}_{\text{Grim}}$)(13). IEAA is defined as the residuals resulting from the regression of
194 estimated epigenetic age based on the Horvath epigenetic clock on chronological age, fitting
195 estimated proportions of immune cells. IEAA is designed to capture cell-intrinsic epigenetic ageing,
196 independent of age-related changes in blood cellular composition. EEAA is estimated firstly by
197 calculating a weighted average of Hannum's methylation age with three cell types — naive cytotoxic
198 T cells, exhausted cytotoxic T cells and plasmablasts. EEAA is defined as the residuals resulting from
199 the univariate regression of this weighted estimate on chronological age and correlates with age-

200 related changes in the blood cellular composition. Though these measures are most appropriate for
201 use in the blood as they account for blood cell proportions, the correlation between these and the
202 unadjusted measures are both >0.97 , suggesting they are very similar. Rather than aiming to predict
203 chronological age, DNAm PhenoAge was designed to capture an individual's 'phenotypic age' – a
204 composite set of clinical measures associated with mortality. Regressing DNAm PhenoAge onto
205 chronological age provides the acceleration measure: $\text{AgeAccel}_{\text{Pheno}}$. Similarly, DNAm GrimAge was
206 designed to predict mortality based on a linear combination of age, sex, and DNAm-based surrogates
207 for smoking and seven plasma proteins. $\text{AgeAccel}_{\text{Grim}}$ provides the measure of epigenetic age
208 acceleration from this clock. In addition to the epigenetic age acceleration measures, the online
209 calculator provides an estimate of the proportion of neurons in each sample, derived using the cell
210 epigenotype specific (CETS) algorithm (30).

211 Recently, a novel epigenetic clock ($\text{DNAmClock}_{\text{Cortical}}$) was developed to optimally capture brain-
212 specific epigenetic ageing (31). This clock was trained on 9 human cortex methylation datasets of
213 tissue from individuals unaffected by Alzheimer's disease (total $n=1,397$, age range=1-104 years).
214 The model selected 347 DNAm sites and the clock was then tested in an external cohort,
215 outperforming other epigenetic clocks for age prediction within the brain. The sum of DNAm levels
216 at these sites weighted by their regression coefficients provided the cortical $\text{DNAmClock}_{\text{Cortical}}$ age
217 estimate. The residuals resulting from regressing $\text{DNAmClock}_{\text{Cortical}}$ age on chronological age provided
218 the age acceleration measure for this epigenetic clock ($\text{AgeAccel}_{\text{Cortical}}$).

219 *2.4.2 Inflammation signatures*

220

221 DNAm scores for the acute-phase inflammatory mediator C-reactive protein (CRP) and the pro-
222 inflammatory cytokine interleukin-6 (IL-6) were derived as described previously (17, 18, 32). The
223 DNAm CRP score was obtained using data from a large epigenome-wide association study (EWAS) of
224 CRP (3). This EWAS identified 7 CpG sites with strong evidence of a functional association with
225 circulating CRP. One of these CpGs (cg06126421) was not available on the EPIC array, therefore the
226 sum of DNAm levels at the remaining 6 CpG sites weighted by their regression coefficients from the
227 EWAS provided the DNAm CRP score (32) (**Supplementary Table 2**). The IL-6 score was derived from
228 an elastic net penalised regression model using the Wave 1 LBC1936 blood methylation and Olink®
229 IL-6 data (Olink® inflammation panel, Olink® Bioscience, Uppsala, Sweden) (17). This approach
230 identified 12 CpG sites that optimally predicted circulating IL-6. In the current study, the elastic net
231 regression was re-run omitting individuals providing *post-mortem* brain samples ($n=863$). This model
232 returned a set of 13 CpG sites (inclusive of the 12 CpGs from the original model). The DNAm IL-6

233 score in both blood and brain were thus derived from the sum of DNAm levels at these 13 CpG sites
234 weighted by their regression coefficients (**Supplementary Table 3**).

235

236 2.5 Immunohistochemistry, thresholding and burden quantification

237

238 Fixed tissue sections (4 μ m) from cortical regions BA17, BA20-21, BA24, BA46 and hippocampus were
239 processed for immunohistochemistry. Staining was carried out as described previously (33). Briefly,
240 microglial lysosomes were stained using CD68 (mouse anti-human monoclonal primary antibody,
241 Dako M0876, 1:100, citric acid in pressure cooker pre-treatment). Immunohistochemistry was
242 performed using standard protocols, enhanced with the Novolink Polymer detection system and
243 visualised using 3,3'-diaminobenzidine (DAB) with 0.05% hydrogen peroxide as chromogen. Tissue
244 was counterstained with haematoxylin for 30 seconds to visualise cell nuclei.

245 Stains were visualised using a ZEISS Imager.Z2 stereology microscope using MBF Biosciences Stereo
246 Investigator software. All 6 layers of cortical grey matter were included in analysis. Cortical grey
247 matter was outlined at 1.5X objective magnification and tile scans were acquired at 5X for
248 quantification. Glia were quantified using in-built software that captures immuno-positive objects
249 using an automated thresholding algorithm based on colour and size. Objects smaller than 10 μ m²
250 were not considered true staining and were thus excluded in the burden analysis. The threshold and
251 exposure remained consistent throughout all analysis. Neurolucida Explorer was used to quantify
252 the total area of the region of interest and that of the outlined objects. A percentage burden was
253 then calculated by dividing the stained area by the total tissue area.

254 2.6 Statistical analyses

255

256 Spearman correlations were calculated between the inflammation, and epigenetic age acceleration,
257 measures in the blood and each brain region using the last available blood-based measure prior to
258 death. Linear mixed effects models were used to investigate the regional heterogeneity in the
259 epigenetic age acceleration variables and the DNAm inflammation scores in the brain. BA17 was set
260 as the reference as this region is typically not affected until the latter stages of neurodegenerative
261 diseases that impact cognitive functioning, such as Alzheimer's disease. Models were adjusted for
262 age at death, *post-mortem* interval, sex, and proportion of neurons, with participant ID fitted as a
263 random effect on the intercept. Linear mixed effects models were additionally used to assess the
264 association between the DNAm signatures in both the blood and the brain and CD68⁺ microglial
265 burdens. Here, an interaction term between the brain region and DNAm score was included to test if

266 any effects were region dependent. The same covariates and random effect as above were included.
267 Models assessing blood-based signatures were additionally adjusted for the interval between their
268 measurement and death. In each regression analysis, continuous variables were scaled to have a
269 mean of zero and unit variance. We considered a statistical significance threshold of $p < 0.05$. We
270 additionally discuss how results change at a more conservative Bonferroni-corrected level of
271 significance ($p < 0.05/41 = 0.001$).

272 3. Results

273

274 3.1 Cohort demographics

275

276 *Post-mortem* details for each individual included in the study are presented in **Supplementary Table**
277 **1**. Summary statistics for each of the variables included in analyses is presented in **Table 1**. Age at
278 death ranged from 77.6 to 82.9 years (mean=80.3, SD=1.56). Five of the 14 (36%) individuals were
279 female.

280 3.2 DNAm inflammation signatures

281

282 The Spearman correlation between the last blood DNAm CRP score and the mean brain DNAm CRP
283 score was 0.06. This blood-brain correlation varied by region, ranging from -0.52 in BA17 to 0.46 in
284 BA46 (**Supplementary Figure 1**).

285 A boxplot of the DNAm CRP score in the five brain regions is presented in **Figure 1**. No significant
286 differences were identified in the analysis by region (**Supplementary Table 4**), indicating none of the
287 assessed regions had a significantly different DNAm CRP score compared to BA17.

288 The correlation between the last blood DNAm IL-6 score and the mean brain DNAm IL-6 score was
289 0.04, ranging from -0.12 in the hippocampus to 0.27 in BA46 (**Supplementary Figure 2**).

290 A boxplot of the DNAm IL-6 score in the five brain regions is presented in **Figure 1**. In the analysis by
291 region, the DNAm IL-6 score was found to be significantly lower in BA24 ($\beta = -0.86$, $SE = 0.35$, $p = 0.017$),
292 BA46 ($\beta = -0.82$, $SE = 0.30$, $p = 0.009$) and the hippocampus ($\beta = -1.002$, $SE = 0.32$, $p = 0.003$) compared to
293 BA17 (**Supplementary Table 4**).

294 3.3 DNAm age acceleration

295

296 The correlations between the last blood DNAm age acceleration and the mean age acceleration in
297 the brain were -0.04 for IEAA, 0.48 for EEAA, 0.39 for AgeAccel_{Grim}, and 0.30 AgeAccel_{Pheno}.

298 Correlation plots between the last blood DNAm age acceleration measure and the DNAm age
299 acceleration in the brain split by region are presented in **Supplementary Figures 3-6**. The coefficients
300 for AgeAccel_{Grim}, AgeAccel_{Pheno} and EEAA were all positive, ranging from 0.09 between AgeAccel_{Pheno}
301 in the blood and in BA46, to 0.78 between the last blood EEAA and EEAA in BA17. IEAA showed a
302 negative correlation between the last blood measurement and the measure in BA20/21 ($r=-0.27$),
303 BA24 ($r=-0.14$) and BA46 ($r=-0.25$) but a positive correlation in the hippocampus ($r=0.30$) and BA17
304 ($r=0.49$). For EEAA, some of the positive correlations appear largely driven by an individual with a
305 high last blood measure (38.2) which corresponded with high measures in each of the brain regions
306 (**Supplementary Figure 4**). This individual additionally had consistently high last blood measures in
307 each of the other epigenetic age acceleration measures assessed (range: 6.6-25.4).

308 Boxplots of the five different epigenetic age acceleration measures in each of the five brain regions
309 are presented in **Figure 2**. The hippocampus displayed the highest DNAm age acceleration compared
310 to BA17 for each of the assessed measures except for AgeAccel_{Grim} which was highest in BA24
311 (**Supplementary Table 5**; AgeAccel_{Cortical}: $\beta=0.901$, $SE=0.19$, $p=2.6 \times 10^{-5}$; AgeAccel_{Pheno}: $\beta=1.14$,
312 $SE=0.27$, $p=1 \times 10^{-4}$; IEAA: $\beta=0.83$, $SE=0.34$, $p=0.02$; EEAA: $\beta=0.99$, $SE=0.24$, $p=1 \times 10^{-4}$). The result for
313 EEAA remained similar when the individual with consistently high measures across all regions was
314 removed ($\beta=1.22$, $SE=0.30$, $p=1.4 \times 10^{-4}$).

315 3.4 Microglial burdens

316

317 A boxplot of the CD68⁺ microglial burdens in each of the five brain regions and a representative
318 imaging of the staining is presented in **Figure 3**. The microglial burden was found to be significantly
319 higher in the hippocampus compared to BA17 ($\beta=1.32$, $SE=0.4$, $p=5 \times 10^{-4}$), with the plot suggesting
320 large variance in this region compared to the others.

321 The associations between both the DNAm age acceleration variables and the DNAm inflammation
322 signatures with microglial burdens are presented in **Supplementary Table 6**. Here, a higher mean
323 AgeAccel_{Pheno} in the brain associated with an increased microglial burden ($\beta=0.40$, $SE=0.14$, $p=0.002$).
324 No other significant associations were identified (all $p \geq 0.1$) and there were no significant
325 interactions found between any of the methylation scores and brain region.

326 4. Discussion

327

328 In this study, we took advantage of blood and *post-mortem* brain tissue available in 14 individuals in
329 LBC1936 to investigate the relationship between peripheral and central inflammation- and age-
330 related DNAm signatures and how they relate to neuroinflammatory processes. Due to the small

331 sample size the results of this work are preliminary; however some potentially interesting patterns
332 were identified. We found heterogeneous correlations between both the age acceleration, and
333 inflammation-related, methylation signatures in the blood and the brain depending on the region
334 assessed. Of the inflammatory signatures, the DNAm CRP score did not show significant variation
335 across the brain regions, while the DNAm IL-6 score was found to be slightly lower in BA24, BA46
336 and hippocampus than in BA17. Other than for AgeAccel_{Grim}, epigenetic age acceleration was found
337 to be significantly higher in the hippocampus than in BA17. Reactive microglial burdens, identified
338 through CD68 immunostaining, were additionally found to be higher in the hippocampus, consistent
339 with previous findings in a smaller sample of the LBC1936 cohort (33). However, the only association
340 identified between the DNAm signatures (age acceleration or inflammation proxies) and microglial
341 load was a positive association with the mean brain-based DNAm AgeAccel_{Pheno}.

342 It is recognised that DNAm patterns at individual CpG sites in the blood and the brain are often
343 disparate (34). We found that DNAm scores for CRP and IL-6 comprising multiple CpG sites displayed
344 heterogeneous, region-specific correlations when comparing the blood- and brain-derived
345 signatures. This suggests that blood DNAm patterns may proxy methylation in some areas of the
346 brain better than others. Additionally, it cautions against the use of a single sample of post-mortem
347 brain tissue as representative of the brain in aggregate, as it appears there is additional
348 heterogeneity in methylation patterns even within the same tissue source. The DNAm age
349 acceleration measures additionally displayed discrepant blood-brain correlations dependant on
350 region. However, all the assessed measures showed positive blood-brain correlations in each region,
351 to a greater or lesser degree, excepting IEAA. IEAA is based on the Horvath clock which is regarded
352 as a pan-tissue model (11), whereas the other three peripheral measures were derived solely on
353 blood DNAm data. Estimates from the Horvath clock have previously been found to be consistent
354 across tissue types, making it surprising that IEAA showed the most inconsistent blood-brain
355 correlation. A recent study has, however, suggested that the age prediction ability of the Horvath
356 clock begins to deteriorate in older age (>60 years), possibly due to saturation of methylation levels
357 at some loci (35). This may have impacted our results given both blood and brain tissue were
358 gathered from 70 years onwards. The blood-brain correlations identified here suggest significant
359 heterogeneity between the tissues, contingent on region; however, it should be noted that the
360 mean interval between methylation assessed in the blood and in the brain was 2.5 years which
361 reflects a period where methylation alterations are possible (36).

362 In the regional analyses of DNAm signatures in the brain, no real differences emerged in the
363 assessment of the DNAm CRP score. On the other hand the DNAm IL-6 score seemed to be lower in
364 BA24, BA46 and the hippocampus compared to BA17, possibly suggesting a disparity in the DNAm

365 inflammation signatures across the brain. CRP itself does not typically cross the blood-brain barrier
366 (BBB) although its pro-inflammatory effects may lead to an increased paracellular permeability of
367 the BBB (37). Additionally, when using post-mortem blood tissue there is a possibility of blood
368 contamination due to the lack of perfusion at *post-mortem*. Conversely, IL-6 can cross the BBB
369 through the brain's circumventricular organs and is additionally expressed in the brain itself.
370 However, the DNAm signatures of CRP and IL-6 were both created in blood and have not yet been
371 validated in brain tissue. Work to assess other blood-calibrated predictors within in brain tissue is
372 currently ongoing. It seems likely that brain tissue may exhibit different alterations in methylation in
373 response to inflammation that were not captured by the two DNAm inflammatory marker proxies
374 utilised here. In contrast to the inflammatory results, a higher DNAm age acceleration in the
375 hippocampus was found for each of the assessed measures apart from AgeAccel_{Grim}. This was true
376 both for the cortex-specific clock as well as for the measures developed in the blood (AgeAccel_{Pheno}
377 and EEAA) or in multiple tissues (IEAA). This consistency implies that the hippocampus may
378 represent a region more susceptible to biological ageing than other areas of the neocortex. Age-
379 related decline in hippocampal volume is well established (38) and it is one of the earliest, and most
380 profoundly, affected regions in Alzheimer's disease, suffering insidious synapse loss and neuronal
381 cell death culminating in a substantial atrophy as the disease progresses (39). While none of the
382 individuals included in this study had a diagnosis of Alzheimer's disease prior to their death, the
383 hippocampus can suffer substantial deterioration before clinical dementia becomes evident. The
384 accelerated epigenetic ageing noted here is perhaps capturing the vulnerability of this region.

385 Equivalent to this finding, we identified a higher percentage burden of CD68⁺ microglia in the
386 hippocampus compared to BA17. CD68 is a marker of phagocytic activity and is typically used to
387 classify reactive microglia. Microglia are important in the maintenance of integrity and function
388 within the central nervous system; however, aged microglia have been shown to be more responsive
389 to pro-inflammatory stimuli compared to the naïve cell-type. This altered phenotype can lead to
390 exaggerated neuro-inflammation in response to peripheral or central immune challenges which can
391 precipitate neuro-toxicity, and thus, degeneration (40, 41). The only association identified between
392 the DNAm signatures and microglial load was a positive association with the mean brain
393 AgeAccel_{Pheno}; however we did not find any significant interaction between the DNAm signatures and
394 region. The DNAm PhenoAge clock was trained on a set of nine haematological and biochemical
395 measures that were found to optimally predict an individual's 'phenotypic age' including four
396 immune cell profiles (lymphocyte percent, mean cell volume, red cell distribution width and white
397 blood cell count) alongside CRP and albumin (10). Despite being developed on blood DNAm data, the
398 predominantly inflammatory and immune composition of this clock may mean that AgeAccel_{Pheno} is

399 better able to capture process associated with inflammation even outwith the blood. In this regard,
400 it may have outperformed the DNAm CRP and IL-6 score due to the inclusion of a composite set of
401 phenotypes, which may more accurately index systemic inflammation compared to a single
402 inflammatory surrogate.

403 This study provides a rarely-available assessment of data from blood, alongside *post-mortem* brain
404 tissue methylation profiles and histology from the same individuals. Alongside this, profiling DNAm
405 in multiple regions of the brain allowed us to investigate the heterogeneity of methylation patterns
406 within the same tissue type. This study is limited by the small number of individuals for which data
407 was available, leading to a lack of statistical power and the potential for both type 1 and type 2
408 errors. We considered $p < 0.05$ as the threshold for statistical significance in the analyses. However,
409 the following associations fail to pass a strict Bonferroni-corrected threshold ($p \leq 0.001$): the
410 differences of the DNAm IL-6 score across the brain regions, the IEAA measure being highest in the
411 hippocampus compared to BA17, and the association of DNAm AgeAccel_{Pheno} with the CD68+
412 microglia burden. This, again, highlights that the results presented here should be taken as
413 preliminary patterns until analyses can be repeated in larger sample sizes. In regards to the
414 microglial burdening, we used only one antibody (CD68) which limited definitive identification of
415 labelled cells as parenchymal microglia. CD68 stains the lysosomes of ostensibly reactive microglia;
416 however, the antibody can additionally stain infiltrating macrophages. Capturing both the microglia
417 and macrophage burden still provides a relevant read-out of the cellular inflammatory status;
418 however, further characterisation of the microglial phenotype, including generating a reactive:total
419 ratio would be desirable to glean a better understanding of their specific relationship to DNAm
420 signatures. Further to this, the burden metric used to quantify microglia could reflect differences in
421 sizes of the cells as well as in total numbers. An additional aspect to bear in mind when utilising *post-*
422 *mortem* tissue in methylation studies is the stability of global DNAm following death and the
423 biological implications of this (42, 43). We attempted to account for the potential impact of this by
424 adjusting analyses for *post-mortem* intervals; however as *post-mortem* changes in DNAm are not yet
425 well characterised it cannot be ruled out that this confounded results. Finally, *post-mortem* studies
426 will always be retrospective in nature, rendering it impossible to discern causal or consequential
427 events.

428 In summary, using a well-characterised cohort of 14 individuals, we identified divergent correlations
429 between the blood and brain in DNAm inflammation-related and age acceleration measures
430 depending on region assessed. The hippocampus was found to display the highest DNAm age
431 acceleration in four out of five assessed measures, potentially reflecting its inherent susceptibility to
432 biological ageing and pathological processes compared to other cortical regions. The hippocampus

433 additionally showed the highest burden of reactive microglia. Whilst an accelerated DNAm
434 PhenoAge associated with an elevated microglial load across the brain, no region-specific
435 associations were identified. Our results provide some initial indications of the blood-brain
436 relationships in DNAm patterns and how these relate to central processes; however further work is
437 needed to verify these results in larger sample sizes and to investigate how these patterns associate
438 with cognitive function and neurodegenerative disease.

439

440 5. Acknowledgements

441

442 The authors thank all LBC1936 study participants and research team members who have
443 contributed, and continue to contribute, to ongoing studies. LBC1936 is supported by Age UK
444 (Disconnected Mind program) and the Medical Research Council (MR/M01311/1). Blood
445 methylation typing was supported by Centre for Cognitive Ageing and Cognitive Epidemiology (Pilot
446 Fund award), Age UK, The Wellcome Trust Institutional Strategic Support Fund, The University of
447 Edinburgh, and The University of Queensland. This work was in part conducted in the Centre for
448 Cognitive Ageing and Cognitive Epidemiology, which is supported by the Medical Research Council
449 and Biotechnology and Biological Sciences Research Council (MR/K026992/1) and which supports
450 IJD.

451 AJS and RFH are Translational Neuroscience PhD students funded by Wellcome (203771/Z/16/Z to
452 AJS; 108890/Z/15/Z to RFH). REM and DLM_cC are supported by an Alzheimer's Research UK major
453 project grant (ARUK-PG2017B-10). TSJ is supported by the European Research Council (ERC) under
454 the European Union's Horizon 2020 research and innovation programme (Grant agreement No.
455 681181) and the UK Dementia Research Institute which receives its funding from DRI Ltd, funded by
456 the UK Medical Research Council, Alzheimer's Society, and Alzheimer's Research UK. PMV is
457 supported by the Australian National Health and Medical Research Council (1113400) and the
458 Australian Research Council (FL180100072). SRC is supported by the Medical Research Council
459 (MR/R024065/1) and the US National Institutes of Health (R01AG054628).

460

461

462

463

464

465 References

- 466 1. Franceschi C, Bonafè M, Valensin S, Olivieri F, De Luca M, Ottaviani E, et al. Inflamm-aging.
467 An evolutionary perspective on immunosenescence. *Annals of the New York Academy of Sciences*.
468 2000;908:244-54.
- 469 2. Amor S, Puentes F, Baker D, van der Valk P. Inflammation in neurodegenerative diseases.
470 *Immunology*. 2010;129(2):154-69.
- 471 3. Ligthart S, Marzi C, Aslibekyan S, Mendelson MM, Conneely KN, Tanaka T, et al. DNA
472 methylation signatures of chronic low-grade inflammation are associated with complex diseases.
473 *Genome biology*. 2016;17(1):255.
- 474 4. Gonzalez-Jaramillo V, Portilla-Fernandez E, Glisic M, Voortman T, Ghanbari M, Bramer W, et
475 al. Epigenetics and Inflammatory Markers: A Systematic Review of the Current Evidence.
476 *International Journal of Inflammation*. 2019;2019:6273680.
- 477 5. Beck S, Rakyan VK. The methylome: approaches for global DNA methylation profiling. *Trends*
478 *in Genetics*. 2008;24(5):231-7.
- 479 6. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates
480 intrinsic and environmental signals. *Nature genetics*. 2003;33 Suppl:245-54.
- 481 7. McCartney DL, Stevenson AJ, Hillary RF, Walker RM, Bermingham ML, Morris SW, et al.
482 Epigenetic signatures of starting and stopping smoking. *EBioMedicine*. 2018;37:214-20.
- 483 8. Liu C, Marioni RE, Hedman ÅK, Pfeiffer L, Tsai PC, Reynolds LM, et al. A DNA methylation
484 biomarker of alcohol consumption. *Molecular Psychiatry*. 2018;23(2):422-33.
- 485 9. Marioni RE, McRae AF, Bressler J, Colicino E, Hannon E, Li S, et al. Meta-analysis of
486 epigenome-wide association studies of cognitive abilities. *Molecular Psychiatry*. 2018;23(11):2133-
487 44.
- 488 10. Levine ME, Lu AT, Quach A, Chen BH, Assimes TL, Bandinelli S, et al. An epigenetic biomarker
489 of aging for lifespan and healthspan. *Aging*. 2018;10(4):573-91.
- 490 11. Horvath S. DNA methylation age of human tissues and cell types. *Genome biology*.
491 2013;14(10):R115.
- 492 12. Hannum G, Guinney J, Zhao L, Zhang L, Hughes G, Sada S, et al. Genome-wide methylation
493 profiles reveal quantitative views of human aging rates. *Molecular cell*. 2013;49(2):359-67.
- 494 13. Lu AT, Quach A, Wilson JG, Reiner AP, Aviv A, Raj K, et al. DNA methylation GrimAge strongly
495 predicts lifespan and healthspan. *Aging*. 2019;11(2):303-27.
- 496 14. Marioni RE, Shah S, McRae AF, Ritchie SJ, Muniz-Terrera G, Harris SE, et al. The epigenetic
497 clock is correlated with physical and cognitive fitness in the Lothian Birth Cohort 1936. *International*
498 *journal of epidemiology*. 2015;44(4):1388-96.
- 499 15. Beydoun MA, Shaked D, Tajuddin SM, Weiss J, Evans MK, Zonderman AB. Accelerated
500 epigenetic age and cognitive decline among urban-dwelling adults. *Neurology*. 2020;94(6):e613.
- 501 16. Hillary RF, Stevenson AJ, Cox SR, McCartney DL, Harris SE, Seeboth A, et al. An epigenetic
502 predictor of death captures multi-modal measures of brain health. *Molecular Psychiatry*. 2019.
- 503 17. Stevenson AJ, Gadd DA, Hillary RF, McCartney DL, Campbell A, Walker RM, et al. Creating
504 and validating a DNA methylation-based proxy for Interleukin-6. *medRxiv*.
505 2020:2020.07.20.20156935.
- 506 18. Stevenson AJ, McCartney DL, Hillary RF, Campbell A, Morris SW, Bermingham ML, et al.
507 Characterisation of an inflammation-related epigenetic score and its association with cognitive
508 ability. *Clinical Epigenetics*. 2020;12(1):113.
- 509 19. Mendizabal I, Yi SV. Whole-genome bisulfite sequencing maps from multiple human tissues
510 reveal novel CpG islands associated with tissue-specific regulation. *Hum Mol Genet*. 2016;25(1):69-
511 82.
- 512 20. Chuang Y-H, Paul KC, Bronstein JM, Bordelon Y, Horvath S, Ritz B. Parkinson's disease is
513 associated with DNA methylation levels in human blood and saliva. *Genome medicine*. 2017;9(1):76-
514 .

- 515 21. Di Francesco A, Arosio B, Falconi A, Micioni Di Bonaventura MV, Karimi M, Mari D, et al.
516 Global changes in DNA methylation in Alzheimer's disease peripheral blood mononuclear cells.
517 *Brain, Behavior, and Immunity*. 2015;45:139-44.
- 518 22. Cunningham C, Wilcockson DC, Campion S, Lunnon K, Perry VH. Central and Systemic
519 Endotoxin Challenges Exacerbate the Local Inflammatory Response and Increase Neuronal Death
520 during Chronic Neurodegeneration. *The Journal of Neuroscience*. 2005;25(40):9275.
- 521 23. Norden DM, Godbout JP. Review: microglia of the aged brain: primed to be activated and
522 resistant to regulation. *Neuropathol Appl Neurobiol*. 2013;39(1):19-34.
- 523 24. Deary IJ, Gow AJ, Taylor MD, Corley J, Brett C, Wilson V, et al. The Lothian Birth Cohort 1936:
524 a study to examine influences on cognitive ageing from age 11 to age 70 and beyond. *BMC geriatrics*.
525 2007;7:28.
- 526 25. Taylor AM, Pattie A, Deary IJ. Cohort Profile Update: The Lothian Birth Cohorts of 1921 and
527 1936. *International journal of epidemiology*. 2018;47(4):1042-r.
- 528 26. Shah S, McRae AF, Marioni RE, Harris SE, Gibson J, Henders AK, et al. Genetic and
529 environmental exposures constrain epigenetic drift over the human life course. *Genome research*.
530 2014;24(11):1725-33.
- 531 27. Zhang Q, Marioni RE, Robinson MR, Higham J, Sproul D, Wray NR, et al. Genotype effects
532 contribute to variation in longitudinal methylome patterns in older people. *Genome medicine*.
533 2018;10(1):75.
- 534 28. Samarasekera N, Al-Shahi Salman R, Huitinga I, Klioueva N, McLean CA, Kretzschmar H, et al.
535 Brain banking for neurological disorders. *Lancet Neurol*. 2013;12(11):1096-105.
- 536 29. Pidsley R, Y Wong CC, Volta M, Lunnon K, Mill J, Schalkwyk LC. A data-driven approach to
537 preprocessing Illumina 450K methylation array data. *BMC Genomics*. 2013;14(1):293.
- 538 30. Guintivano J, Aryee MJ, Kaminsky ZA. A cell epigenotype specific model for the correction of
539 brain cellular heterogeneity bias and its application to age, brain region and major depression.
540 *Epigenetics*. 2013;8(3):290-302.
- 541 31. Shireby GL, Davies JP, Francis PT, Burrage J, Walker EM, Neilson GWA, et al. Recalibrating the
542 epigenetic clock: implications for assessing biological age in the human cortex. *Brain*. 2020.
- 543 32. Barker ED, Cecil CAM, Walton E, Houtepen LC, O'Connor TG, Danese A, et al. Inflammation-
544 related epigenetic risk and child and adolescent mental health: A prospective study from pregnancy
545 to middle adolescence. *Development and psychopathology*. 2018;30(3):1145-56.
- 546 33. Tzioras M, Easter J, Harris S, McKenzie C-A, Smith C, Deary I, et al. Assessing amyloid- β , tau,
547 and glial features in Lothian Birth Cohort 1936 participants post-mortem. *Matters*.
548 2017;3(10):e201708000003.
- 549 34. Hannon E, Lunnon K, Schalkwyk L, Mill J. Interindividual methylomic variation across blood,
550 cortex, and cerebellum: implications for epigenetic studies of neurological and neuropsychiatric
551 phenotypes. *Epigenetics*. 2015;10(11):1024-32.
- 552 35. El Khoury LY, Gorrie-Stone T, Smart M, Hughes A, Bao Y, Andrayas A, et al. Systematic
553 underestimation of the epigenetic clock and age acceleration in older subjects. *Genome biology*.
554 2019;20(1):283.
- 555 36. Zaimi I, Pei D, Koestler DC, Marsit CJ, De Vivo I, Tworoger SS, et al. Variation in DNA
556 methylation of human blood over a 1-year period using the Illumina MethylationEPIC array.
557 *Epigenetics*. 2018;13(10-11):1056-71.
- 558 37. Elwood E, Lim Z, Naveed H, Galea I. The effect of systemic inflammation on human brain
559 barrier function. *Brain Behav Immun*. 2017;62:35-40.
- 560 38. Raz N, Lindenberger U, Rodrigue KM, Kennedy KM, Head D, Williamson A, et al. Regional
561 brain changes in aging healthy adults: general trends, individual differences and modifiers. *Cerebral*
562 *cortex (New York, NY : 1991)*. 2005;15(11):1676-89.
- 563 39. Braak H, Braak E, Bohl J. Staging of Alzheimer-related cortical destruction. *European*
564 *neurology*. 1993;33(6):403-8.

- 565 40. Perry VH, Holmes C. Microglial priming in neurodegenerative disease. *Nature reviews*
566 *Neurology*. 2014;10(4):217-24.
- 567 41. Luo X-G, Ding J-Q, Chen S-D. Microglia in the aging brain: relevance to neurodegeneration.
568 *Molecular Neurodegeneration*. 2010;5(1):12.
- 569 42. Pidsley R, Mill J. Epigenetic studies of psychosis: current findings, methodological
570 approaches, and implications for postmortem research. *Biological psychiatry*. 2011;69(2):146-56.
- 571 43. Sjöholm LK, Ransome Y, Ekström TJ, Karlsson O. Evaluation of Post-Mortem Effects on Global
572 Brain DNA Methylation and Hydroxymethylation. *Basic & clinical pharmacology & toxicology*.
573 2018;122(2):208-13.
- 574

575 **Table 1.** Summary of the variables assessed in the 14 Lothian Birth Cohort 1936 participants.

576 The brain variables refer to the mean across all five regions.

577 DNAm=DNA methylation; CRP= C-reactive protein; IL-6= interleukin-6; IEAA=intrinsic epigenetic age

578 acceleration; EEAA= extrinsic epigenetic age acceleration; CD68=Cluster of Differentiation 68.

579

| Variable | Mean | SD |
|------------------------------|-------------|----------------------|
| Sex (% female) | 35.71 | - |
| Age at death (years) | 80.33 | 1.56 |
| Age at last blood draw | 77.88 | 1.67 |
| <i>Brain</i> | | |
| DNAm CRP score | -0.014 | 6.1×10^{-4} |
| DNAm IL-6 score | -0.66 | 0.11 |
| AgeAccel _{Cortical} | -0.52 | 6.12 |
| AgeAccel _{Grim} | -0.31 | 2.32 |
| AgeAccel _{Pheno} | 0.053 | 5.71 |
| IEAA | -0.049 | 3.97 |
| EEAA | -0.55 | 3.38 |
| CD68 burden (%) | 0.34 | 0.38 |
| <i>Blood</i> | | |
| DNAm CRP score | -0.014 | 1.2×10^{-3} |
| DNAm IL-6 score | -0.75 | 0.18 |
| AgeAccel _{Grim} | 6.68 | 6.53 |
| AgeAccel _{Pheno} | 3.23 | 8.48 |
| IEAA | 1.23 | 5.62 |
| EEAA | 2.99 | 11.20 |

580

581

582

583

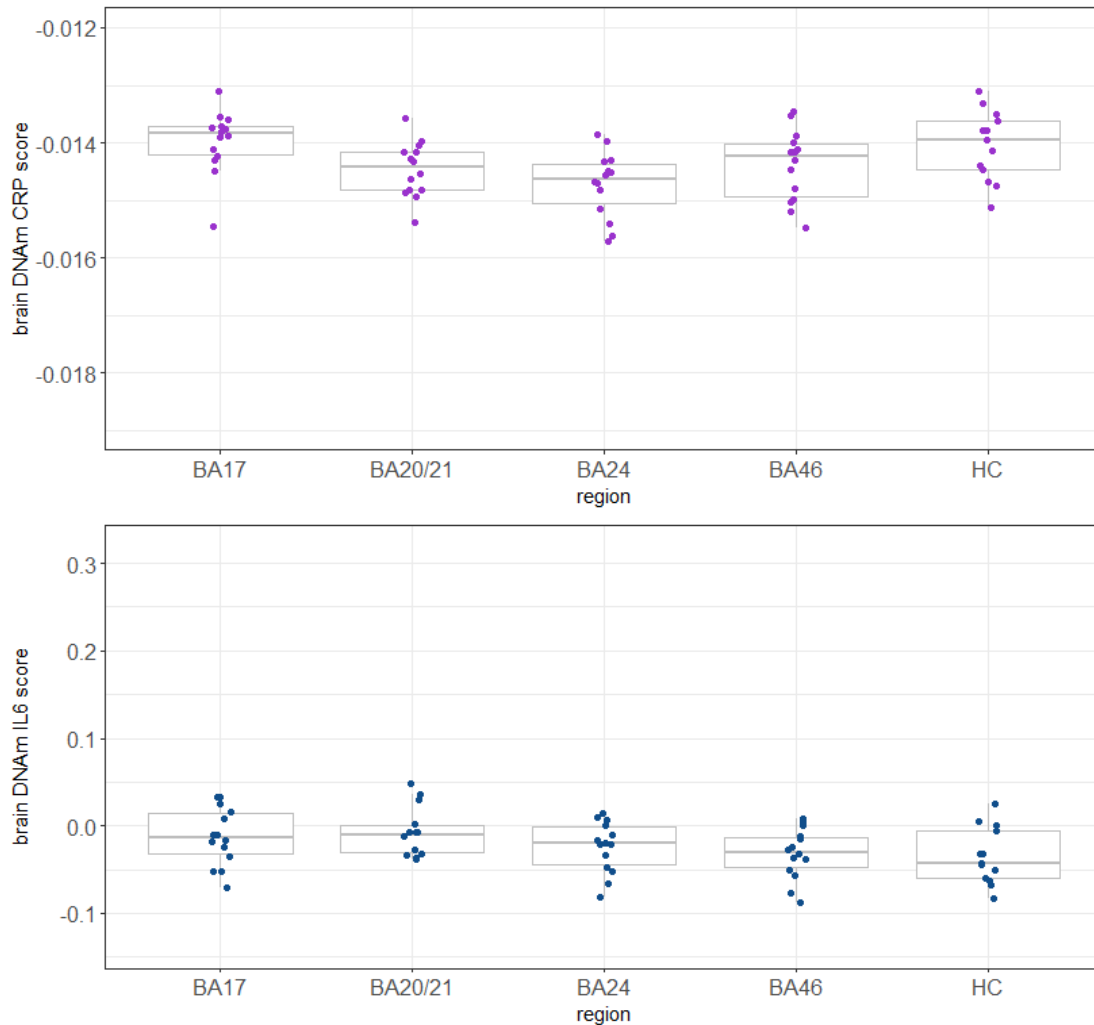
584

585

586

587 **Figure 1.** The DNAm CRP and IL-6 score in each of the five regions of the brain.

588 BA=Brodmann area; HC=hippocampus; DNAm=DNA methylation; CRP=C-reactive protein; IL-
589 6=interleukin-6.



590

591

592

593

594

595

596

597

598

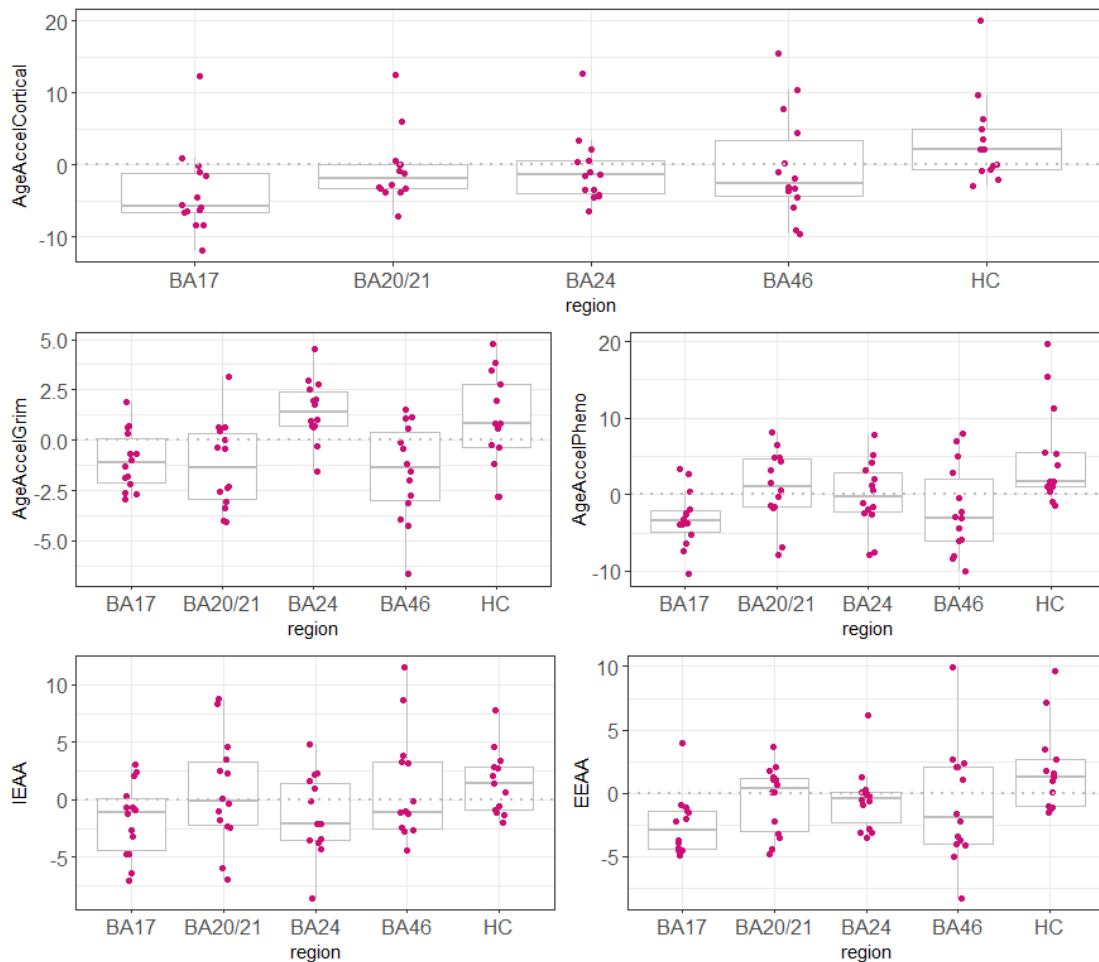
599

600 **Figure 2.** DNAm age acceleration measures across the five brain regions. The dashed grey lines
601 represent where the mean difference is zero.

602 IEAA=intrinsic epigenetic age acceleration; EEAA=extrinsic epigenetic age acceleration;
603 BA=Brodmann area; HC=hippocampus.

604

605

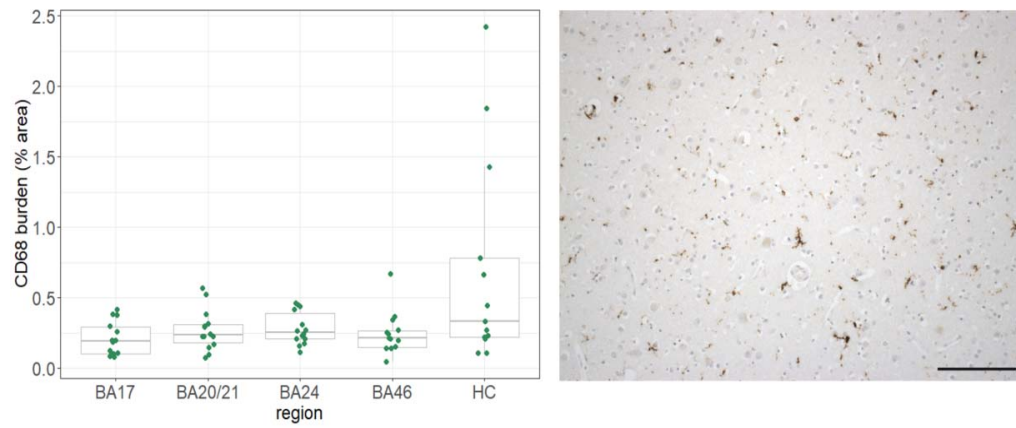


606
607

608 **Figure 3.** CD68⁺ microglial burdens over the five brain regions and representative staining.

609 BA=Brodmann area; HC=hippocampus. Scale bar=150 μ m.

610



611

612