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Brain, Behavior, and Immunity 110 (2023) 322–338

Contents lists available at ScienceDirect

Brain Behavior and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Full-length Article

Immuno-epigenetic signature derived in saliva associates with the encephalopathy of prematurity and perinatal inflammatory disorders

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ARTICLE INFO

Keywords:
Perinatal
Inflammation
DNA methylation
Epigenetics
Encephalopathy of Prematurity
White Matter
Preterm Birth
Necrotizing Enterocolitis
Neonatal Sepsis
Diffusion Tensor Imaging
Neurodevelopment
Multiomics

ABSTRACT

Background: Preterm birth is closely associated with a phenotype that includes brain dysmaturation and neurocognitive impairment, commonly termed Encephalopathy of Prematurity (EoP), of which systemic inflammation is considered a key driver. DNA methylation (DNAm) signatures of inflammation from peripheral blood associate with poor brain imaging outcomes in adult cohorts. However, the robustness of DNAm inflammatory scores in infancy, their relation to comorbidities of preterm birth characterised by inflammation, neonatal neuroimaging metrics of EoP, and saliva cross-tissue applicability are unknown.

Methods: Using salivary DNAm from 258 neonates (n = 155 preterm, gestational age at birth 23.28 – 34.84 weeks, n = 103 term, gestational age at birth 37.00 – 42.14 weeks), we investigated the impact of a DNAm surrogate for C-reactive protein (DNAm CRP) on brain structure and other clinically defined inflammatory exposures. We assessed i) if DNAm CRP estimates varied between preterm infants at term equivalent age and term infants, ii) how DNAm CRP related to different types of inflammatory exposure (maternal, fetal and postnatal) and iii) whether elevated DNAm CRP associated with poorer measures of neonatal brain volume and white matter connectivity.

Results: Higher DNAm CRP was linked to preterm status (-0.0107 ± 0.0008, compared with −0.0118 ± 0.0006 among term infants; $p < 0.001$), as well as perinatal inflammatory diseases, including histologic chorioamnionitis, sepsis, bronchopulmonary dysplasia, and necrotising enterocolitis (OR range |2.00 | to |4.71|, $p < 0.01$). Preterm infants with higher DNAm CRP scores had lower brain volume in deep grey matter, white matter, and hippocampi and amygdalae (β range |0.185| to |0.218|). No such associations were observed for term infants. Association magnitudes were largest for measures of white matter microstructure among preterms, where elevated epigenetic inflammation associated with poorer global measures of white matter integrity (β range | 0.206| to |0.371|), independent of other confounding exposures.

Conclusions: Inflammatory-related DNAm captures the allostatic load of inflammatory burden in preterm infants. Such DNAm measures complement biological and clinical metrics when investigating the determinants of neurodevelopmental differences.

1. Introduction

Preterm infants are at an increased risk of elevated inflammation, related health complications, and adverse neurodevelopment compared to infants born at term (Back, 2015; Bennet et al., 2018; Hagberg et al., 2015; Inomata et al., 2014; Shah et al., 2008; Stoll et al., 2004; Humberg et al., 2020). While the aetiology of these outcomes is multifactorial, inflammation is considered to be a key component linking preterm birth

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https://doi.org/10.1016/j.bbi.2023.03.011
Received 19 October 2022; Received in revised form 12 February 2023; Accepted 16 March 2023
Available online 21 March 2023

and poor neurodevelopmental and mental health outcomes via its effects on cerebral maturational processes (Malaeb and Dammann, 2009; Reiss et al., 2022; Kelly et al., 2016; Favrais et al., 2011). Neonatal neuroimaging has identified neurostructural hallmarks of preterm birth commonly referred to as Encephalopathy of Prematurity (EoP), including dysmaturation of cortical and deep grey matter, atypical white matter development and disrupted connectivity (Boardman and Counsell, 2020). Recent advances in epigenetics may permit new ways to characterise sustained inflammation and reveal new insights into the relationship between inflammatory exposures, inflammation and neonatal brain and health outcomes.

Preterm infants are more susceptible to sustained inflammation than term infants and can be subject to multiple inflammatory stimuli during the perinatal period (Leviton et al., 2012; Dammann and Leviton, 2014). Alongside maternal lifestyle-related exposures (Chahal et al., 2017), various complications during pregnancy such as preeclampsia and histologic chorioamnionitis (Sullivan et al., 2021; Dammann et al., 2016) can induce both maternal and fetal inflammatory responses and increase the risk of a sustained pro-inflammatory state postnatally (Sullivan et al., 2021; Dammann et al., 2016; Yoon et al., 1997; Anblagan et al., 2016; Han et al., 2019). Preterm infants are additionally at higher risk for developing severe inflammatory conditions in the first few weeks of life, which may in turn perpetuate inflammation (Humberg et al., 2020) – including bronchopulmonary dysplasia (BPD), necrotizing enterocolitis (NEC), severe retinopathy of prematurity (ROP) and episodes of sepsis (Bassler et al., 2009). Preterm infants often present with multiple chronic conditions at once (Singh et al., 2019), putting them at higher risk of a greater allostatic load of inflammation (Leviton et al., 2012; Singh et al., 2019; Korzeniewski et al., 2014; Barnett et al., 2018).

Though numerous studies report associations between inflammation and cognitive outcomes in preterm populations (Humberg et al., 2020; Suleri et al., 2022; Kuban et al., 2017; O'Shea et al., 2013), research linking inflammatory biomarkers with neurostructural measures yield inconsistent findings (Inomata et al., 2014; Shah et al., 2008; Kelly et al., 2016; Yoon et al., 1997; Anblagan et al., 2016; Travis et al., 2015; Lee et al., 2021; Sullivan et al., 2020; Wu et al., 2021). Gaining a clearer understanding of the pathways via which sustained inflammation in very early life may precipitate well characterised cognitive and neurostructural outcomes requires novel approaches. The relative inconsistency of work to date is likely due to heterogeneity in study design: there is substantial variation in the demographic characteristics of study samples; the degree to which residual confounding factors are controlled; and the presence or absence of term control groups. Moreover, the relative nascency of the neonatal neuroimaging field (Korom et al., 2022) contributes to substantial variation in the acquisition and selection of brain outcome measures, and there is marked anatomic variation in early life, which can confound investigation of structure–function relationships (Dimitrova et al., 2020).

Above these factors, we argue that the measures used to characterise inflammation in the first place may account for the greatest source of ambiguity in the inflammation-brain structure literature. In both clinical and research settings, there is a historical reliance on sampling phasic inflammation-related protein measures from blood to signpost inflammation. Of these, C-Reactive Protein (CRP) is the most widely adopted (Brown et al., 2019;), although there are criticisms of this approach (Bower et al., 2012; DeGoma et al., 2012). Accurate characterisation of sustained (and not transient or acute) inflammation arguably requires repeated sampling or follow up examinations, which few studies endeavour to profile (Macallister et al., 2019). Single recordings of CRP levels, such as those typically obtained for clinical purposes, may lead to misclassifications of baseline inflammation levels when used in population research contexts due to the high variation in baseline CRP within individuals over time (Bower et al., 2012; DeGoma et al., 2012; Bogaty et al., 2013; Nash et al., 2013). In the case of PTB, CRP levels are considered a less than ideal capture of inflammatory processes because the hepatic enzymatic machinery that generates CRP is maturation-dependent over the 2nd and 3rd trimesters of pregnancy, with studies suggesting that reference levels of CRP indicative of elevated inflammation should be dependent on gestational age (Macallister et al., 2019; Borowski et al., 2022; Chiesa et al., 2011; Chiesa et al., 2001; Hofer et al., 2012; Hofer and Resch, 2011; Matoba et al., 2009). Drawing blood from preterm infants (for research purposes) also has ethical implications, as this is an intrusive procedure which would require repeated cannulations to achieve a baseline reading. Because of these drawbacks, there is a precedent to find alternative ways to capture inflammatory burden to fully characterise its impact.

Our previous work demonstrated that DNA methylation (DNAm) markers of inflammation may provide more stable readouts of cumulative inflammatory exposure (Stevenson et al., 2018; Stevenson et al., 2020) and shed greater insight into the consequences of inflammation on brain structure (Conole et al., 2021; Green et al., 2021). DNAm is an epigenetic mechanism that can act as an interface by which environmental exposures influence gene function. DNAm is dynamic during fetal development, both in terms of the developing immune system (Martino et al., 2011) and brain (Spiers et al., 2015) and may mediate the impact of maternal, fetal and postnatal exposures on brain development (Ozanne and Constância, 2007). In the context of preterm birth, only a limited number of studies have investigated DNAm changes (Konwar et al., 2018; Liu et al., 2013; Merid et al., 2020; Sparrow et al., 2016; Winchester et al., 2022; Fumagalli et al., 2018; Chen et al., 2015; Wheater et al., 2022; Camerota et al., 2021; Everson et al., 2020) – of these, few examine DNAm in relation to neonatal neuroimaging metrics (Sparrow et al., 2016; Chen et al., 2015; Wheater et al., 2022). Additionally, though some of these studies have examined DNAm in relation to postnatal health outcomes (Everson et al., 2020; Massaro et al., 2021), no study to date has examined inflammation, DNAm, and neuroimaging concurrently in the neonatal period.

Here, using a cohort of 258 infants (103 term, 155 preterm), we examine (Back, 2015) how a salivary DNAm signature of the inflammatory marker C-reactive protein (DNAm CRP) relates to preterm birth (Bennet et al., 2018) how this signature associates with maternal, fetal and postnatal inflammatory exposures both individually and in aggregate, and (Hagberg et al., 2015) how variance in this measure relates to global measures of MRI brain volume, diffusion MRI (dMRI) correlates of connectivity, and regional variation in individual white matter tracts.

2. Methods

2.1. Study population

Preterm (gestational age at birth < 37 weeks) and term born infants delivered at the Royal Infirmary of Edinburgh, UK were recruited to the Theirworld Edinburgh Birth Cohort, a longitudinal study designed to investigate the effect of preterm birth on brain development (Boardman et al., 2020). Cohort exclusion criteria were major congenital malformations, chromosomal abnormalities, congenital infection, overt parenchymal lesions (cystic periventricular leukomalacia, hemorrhagic parenchymal infarction) or post-hemorrhagic ventricular dilatation. Ethical approval has been obtained from the National Research Ethics Service, South East Scotland Research Ethics Committee (11/55/0061, 13/SS/0143 and 16/SS/0154). Informed consent was obtained from a person with parental responsibility for each participant. DNAm data were available from 258 neonates, 214 of whom also had successful structural and diffusion MRI acquisition.

2.2. Study variables

Inflammatory exposures were coded as binary variables (1 = present, 0 = absent) and were grouped as follows: maternal (pertaining to mother / maternal exposure), fetal (affecting placenta or fetus) or neonatal (affecting infant after birth). Table 1 presents participant characteristics of these categories. Histologic chorioamnionitis (HCA)

Table 1
Demographic and clinical features of study sample (n = 258). P values denote significant difference between term and preterm groups.

Characteristics & clinical features	Term infants (n = 103)	Preterm infants (n = 155)	P value
Sex: Female (%)	44 (43)	75 (48)	0.2166
Gestational age at birth/weeks (range)	39.7 (37.00 – 42.14)	28.84 (23.28 – 34.84)	<0.001
Gestational age at scan/weeks (range)	42.27 (39.84 – 47.14)	40.56 (37.70–45.14)	<0.001
Birth weight/g (range)	3482 (2346 – 4670)	1177 (500 – 2100)	<0.001
Birth weight z-score (range)	0.43 (-2.30 – 2.96)	−0.19 (-3.13 – 1.58)	<0.001
DNAm CRP (mean, SD)	−0.012 (0.001)	−0.011 (0.001)	<0.001
Maternal / fetal			
Maternal age (years)	33.7 (19–48)	31.1 (17–44)	<0.001
Antenatal corticosteroid administration in pregnancy, n (%)	2 (1.9)	148 (95.5)	<0.001
MgSO4 administration in pregnancy, n (%)	0 (0)	112 (72.3)	<0.001
Smoked during pregnancy (%)	2 (1.9)	29 (19.0)	<0.001
Histologic chorioamnionitis, n (%)	6 (5.8)	49 (31.6)	<0.001
Preeclampsia, n (%)	7 (6.8)	22 (14.2)	<0.001
Neonatal			
Necrotizing enterocolitis, n (%)	0 (0)	10 (6)	N/A
Bronchopulmonary dysplasia, n (%)	0 (0)	48 (31)	N/A
Retinopathy of prematurity, n (%)	0 (0)	8 (5)	N/A
Sepsis, n (%)	0 (0)	36 (23)	N/A

was defined via placental histopathology, as reported previously (Sullivan et al., 2021; Anblagan et al., 2016). Incidence of any neonatal sepsis (either late onset or early onset sepsis) was defined as detection of bacterial pathogen from blood culture, or physician decision to treat for $\geq$ 5 days in the context of growth of coagulase negative staphylococcus from blood or a negative culture. Necrotising enterocolitis (NEC) was defined as stages two or three according to the modified Bell's staging for NEC. Bronchopulmonary dysplasia (BPD) was defined by the requirement for supplemental oxygen at 36 weeks gestational age. Birthweight z-scores were calculated according to International Fetal and Newborn Growth Consortium for the 21st Century (INTERGROWTH-21st) standards. Further details on variable selection and classification are provided in supplementary methods.

2.3. DNA extraction and methylation measurement and pre-processing

Saliva was obtained on the same day of MRI acquisition which was at term equivalent age (TEA) for preterm infants and shortly after birth for term infants (Table 1). Saliva was collected in Oragene OG-575 Assisted Collection kits, by DNA Genotek, and DNA extracted using prepIT.L2P reagent (DNA Genotek, ON, Canada). DNA was bisulfite converted and methylation levels were measured using Illumina HumanMethylationEPIC BeadChip (Illumina, San Diego, CA, USA) at the Edinburgh Clinical Research Facility (Edinburgh, UK). The arrays were imaged on the Illumina iScan or HiScan platform and genotypes were called automatically using GenomeStudio Analysis software version 2011.1 (Illumina). Details of DNAm pre-processing have been outlined previously (Wheater et al., 2022); for full details, refer to **supplementary methods**.

2.4. Inflammatory-related methylation signature

For each individual (n = 258), a weighted linear signature (DNAm CRP) was obtained by multiplying the methylation proportion at a given CpG by the effect size from a previous epigenome wide association study (EWAS) of CRP (Ligthart et al., 2016) (**supplementary** Table 1), and then summing these values (see equation (1) below).

$$b_1 cpg_1 + b_2 cpg_2 + \ldots + b_7 cpg_7$$

where "cpg" is the normalised methylation value for the LBC1936 participant at a given site and "b" is the effect size from Ligthart et al., (2016). The original CRP EWAS examined peripheral blood DNAm profiles in relation to circulating CRP levels across multiple adult cohort study groups (see Fig. 1). This method to generate an inflammatory-related DNAm score has been used previously in various population cohorts to index cumulative inflammation (Stevenson et al., 2020; Conole et al., 2021; Green et al., 2021; Barker et al., 2018).

2.5. MRI acquisition

This study incorporates data from two phases of MRI acquisition which is reflected in the flowchart of the study sample (**supplementary** Figure S3**)**. The data acquisition of this study has been reported previously (Wheater et al., 2022).

In the first phase (n = 93), structural and dMRI were performed in neonates using a MAGNETOM Verio 3 T clinical MRI scanner (Siemens Healthcare GmbH, Erlangen, Germany) and 12-channel phased-array head coil. For dMRI, A protocol consisting of 11 baseline volumes (b = 0 s/mm^2 [b0]) and 64 diffusion-weighted (b = 750 s/mm^2) single-shot spin-echo echo planar imaging (*EPI*) volumes acquired with 2 mm isotropic voxels (TR/TE 7300/106 ms) was used; 3D T1-weighted (T1w) MPRAGE (TR/TE 1650/2.43 ms) with 1 mm isotropic voxels was acquired.

For the second phase (n = 121), structural and dMRI were performed neonates using a MAGNETOM Prisma 3 T clinical MRI scanner (Siemens Healthcare GmbH, Erlangen, Germany) and 16-channel phased-array pediatric head and neck coil. This was used to acquire dMRI in two separate acquisitions: the first consisted of 8 b0 and 64 volumes with b = 750 s/mm^2; the second consisted of 8 b0, 3 volumes with b = 200 s/mm^2, 6 volumes with b = 500 s/mm^2 and 64 volumes with b = 2500 s/mm^2. An optimal angular coverage for the sampling scheme was applied (Caruyer et al., 2013). In addition, an acquisition of 3 b0 volumes with an inverse phase encoding direction was performed. All dMRI volumes were acquired using single-shot spin-echo planar imaging (*EPI*) with 2-fold simultaneous multi-slice and 2-fold in-plane parallel imaging acceleration and 2 mm isotropic voxels; all three diffusion acquisitions had the same parameters (TR/TE 3500/78.0 ms). Images affected by motion artifact were re-acquired multiple times as required; dMRI acquisitions were repeated if signal loss was seen in 3 or more volumes. 3D T2-weighted SPACE images (T2w) (TR/TE 3200/409 ms) with 1 mm isotropic voxels and 3D T1w MPRAGE (TR/TE 1970/4.69 ms) with 1 mm isotropic voxels were also acquired.

Infants were fed and wrapped and allowed to sleep naturally in the scanner without sedation. Pulse oximetry, electrocardiography and temperature were monitored. Flexible earplugs and neonatal earmuffs (MiniMuffs, Natus) were used for acoustic protection. All scans were supervised by a doctor or nurse trained in neonatal resuscitation. Structural images were reported by an experienced pediatric radiologist (A.J.Q), and each acquisition was inspected contemporaneously for motion artefact and repeated if there had been movement while the baby was still sleeping; dMRI acquisitions were repeated if signal loss was seen in 3 or more volumes.

As details on dMRI pre-processing have been previously outlined (Blesa et al., 2021) please refer to **supplementary methods** for specifics**.** T2w images from phase 2 were processed using the dHCP pipeline

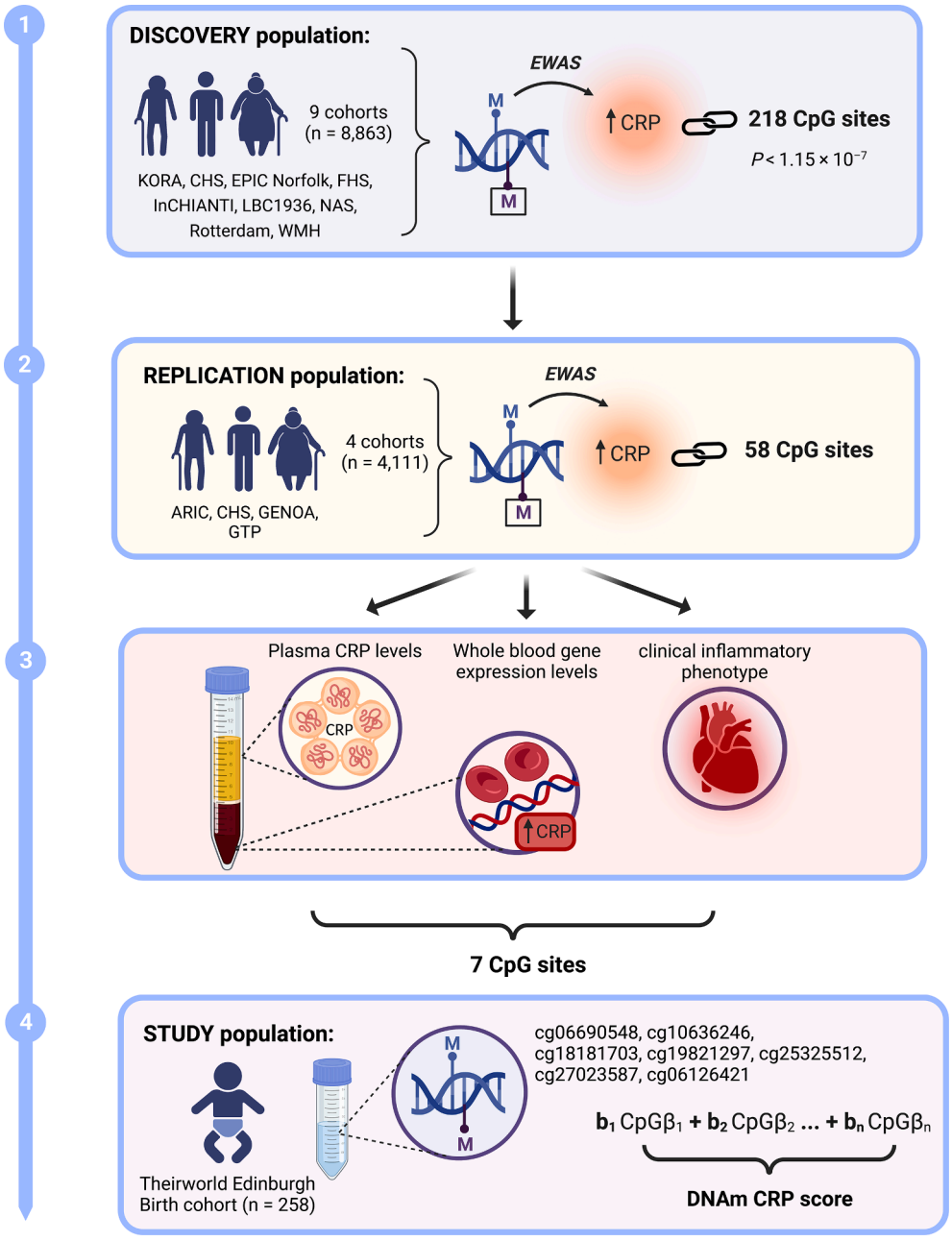

Fig. 1. DNAm CRP signature pipeline. The inflammatory-related DNAm signature used in this study is comprised of immune-related CpG sites identified from a multi-cohort EWAS of CRP (Ligthart et al., 2016) which examined DNAm in relation to circulating CRP levels in peripheral blood. Relative weights for the CpG sites of interest are reported in supplementary Figure 1.

(Makropoulos et al., 2018). The T1w images from phase 1 were processed using specific software for brain skull-stripping and tissue segmentation (Doshi et al., 2013). The phase 1 pipeline relies on some atlases, for these purposes, 10 subjects from the phase 2 that have both T1w and T2w were selected. The volumes extracted include cortical grey matter, deep grey matter, white matter, hippocampi and amygdalae, cerebellum, brainstem, cerebrospinal fluid (CSF) and ventricles.

From the diffusion images we calculated the tensor – fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD), and radial diffusivity (RD) – and the NODDI (intracellular volume fraction [NDI] maps) (Zhang et al., 2012). All the subjects were registered to the Edinburgh Neonatal Atlas (ENA50) using DTI-TK (Zhang et al., 2012; Blesa et al., 2020). The diffusion tensor derived maps of each subject (FA and MD) were calculated after registration; NDI was then propagated to the template space using the previously calculated transformations. The data was skeletonized using the ENA50 skeleton and then multiplied by a custom mask. Finally, the peak width of the histogram of values computed within the skeletonized maps was calculated as the difference between the 95th and 5th percentiles. Global values of white matter microstructure reported in this study are the peak width of skeletonised metrics (PSFA, PSMD, PSRD, PSAD, PSNDI), which have been derived from the same pipeline previously used to characterise brain structural

differences between preterm and term infants (Blesa et al., 2020).

2.6. Tract segmentation and extraction of tract-averaged dMRI metrics

As above, details of individual white matter tract segmentation and subsequent extract of tract-averaged dMRI metrics have been outlined previously from infants from this study sample (Vaher et al., 2022). Briefly, FA and MD were derived for the left and right hemispheric tracts of the arcuate fasciculus (AF), anterior thalamic radiation (ATR), cingulum cingulate gyrus (CCG), corticospinal tracts (CST), inferior fronto-occipital fasciculus (IFOF), inferior longitudinal fasciculus (ILF), uncinate fasciculus (UNC) and genu and splenium of the corpus callosum (CC).

2.7. Statistical analysis

All statistical analyses were performed in R (version 4.0.5) (R Core Team, 2020).

2.7.1. Selection of covariates and confounding variables

In comparing participant demographics between term infants (n = 103) and preterm infants (n = 155), p-values derived from the *t*-test (for continuous variables) and Chi-square test or Fischer's Exact test if the count was below 5 (categorical variables) (Table 1) (Kim, 2017). In all models examining the association between DNAm CRP with health outcomes and brain MRI metrics, we adjusted for infant sex, gestational age at birth, gestational age at scan/ DNAm sampling, and birthweight Z score. Scanner variable was included when data included two phases of MRI acquisition. We also tested for an interaction between gestational age × DNAm CRP and infant sex × DNAm CRP in sensitivity analyses, where a significant interaction would indicate differences in association magnitudes at different gestational ages / between males and females. To quantify the amount of variance in each brain imaging biomarker accounted for by DNAm CRP, for all neuroimaging associations we report both the adjusted R^2 and the incremental R^2 (Tzoulaki et al., 2009), the latter of which was calculated by comparing the R^2 of each model with that from a baseline model (reported as **Model H_0**) R^2 in which the MRI measure was modelled with covariates only (e.g., *Model H_0 = MRI metric ~ sex + birthweight + gestational age age + gestational age at scan + scanner*).

After examining global associations, we wanted to control for variables that could either cause the raised DNAm CRP (the exposure), variance in MRI metrics (the outcome), or both. We ran bivariate correlations and from these we included all common correlates of the exposure and the outcome in our second regression model (Model H_2) to eliminate alternative explanations of the outcome due to confounding (**supplementary** Figure 2). In these models, neither antenatal treatment of corticosteroids and MgSO4 for anticipated preterm birth were included to circumvent issues of multicollinearity, since they were given to the majority of mothers (96 % and 72 % respectively) in the preterm group and were highly correlated with preterm status ($r = 0.71 – 0.93$, $p < 0.001$). The magnitude of effects are classified as small, medium, or large when the standardized coefficients are 0.1, 0.3, or 0.5, respectively as classified by Cohen (Cohen, 1992).

Finally, accounting for the fact that inflammatory risk factors are positively correlated, we included all inflammatory risk factors in one multiple linear regression alongside DNAm CRP signature for each MRI variable of interest. This allowed us to account for unique contribution of DNAm CRP in the context of inflammatory-related exposures to variance in brain structural outcomes. For further details on the selection of covariates in this study, see supplementary methods.

2.7.2. Multiple inflammatory hits and DNAm CRP

We next performed investigations to assess whether DNAm was related to number of inflammatory episodes experienced. Due to small numbers of individual inflammatory risk factors and the frequent overlap of episodes experienced in the preterm group, we created binary outcome measures based on combinations of inflammatory risk factors or conditions experienced, combining infants that experienced three or more morbidities into a single group, resulting in four possible levels for the risk score of 0, 1, 2, or 3 + alongside a term control reference (0 inflammatory episodes). Results are presented firstly unadjusted (model H_1), then adjusted for gestational age at birth, infant sex and birthweight z-score (model H_2), and then adjusted for gestational age at birth, infant sex and birthweight z-score as well as administration of MgSO4 and corticosteroids in pregnancy (model H_3), given these have been identified as potential confounders of the relationship between inflammation and health outcomes in previous studies (Lingam and Robertson, 2018; Odufalu et al., 2022). Other potential covariates, such as gestational diabetes and maternal age, were not found to significantly associate with inflammation (supplementary Figure 1), so were not controlled for in these models. Results are presented as odds ratios (OR) and 95 % confidence intervals (CI) for categorical outcome measures.

2.7.3. Dnam CRP and global brain structure associations

To determine the effect of inflammation (DNAm CRP) on neuroimaging outcomes, data were analysed using regression models, controlling for factors considered relevant to inflammation and EoP outcomes (see supplementary methods for details on selection procedure of covariates). For baseline models, these controlled factors were: gestational age at birth, gestational age at scan, infant sex, MRI scanner, and birthweight Z score; visual inspection of diagnostic plots suggested no regression assumptions were violated (an example is provided in **supplementary** figure 4). We aimed to contextualise these associations with clinical health data. Inflammatory risk factors were added simultaneously as covariates into a second model (models H_2 and H_4) in addition to the standard covariates of gestational age at birth, gestational age at scan, birthweight Z score and sex (baseline models, H_1 and H_3). As no term infants had postnatal inflammatory episodes (sepsis, NEC, ROP, BPD), analyses were stratified according to term or preterm status; an overview of these models is provided in **supplementary** figure 5. Brain volume metrics were corrected for ICV. In models testing global brain structural metrics such as brain volumes and PSMD and PSFA, MRI scanner was included as a binary covariate as MRI data from both phases of data collection were included (refer to **supplementary** figure 3, study sample flowchart). All continuous variables were standardised using z-score scaling to obtain standardised effect sizes (β). P-values were corrected for multiple testing using the false discovery rate (FDR) method and significance was deemed FDR corrected p-value (pFDR) < 0.05. 95 % CIs are reported throughout.

2.7.4. Dnam CRP and dMRI white matter tract associations

dMRI measures of white matter appear to be highly correlated (e.g. high FA in an individual tract such as the arcuate fasciculus is often accompanied by high FA across all other white matter tracts in that individual), a property that persists from early infancy through to older age (Vaher et al., 2022). As a result of this, it is common to derive general factors (g-factors) of white matter microstructure to characterise global white matter microstructure. One PCA was conducted for FA and MD parameters across the 16 tracts to quantify the proportion of shared variance between them; in each analysis, each subject was described by 16 features, computed as the tract-averaged values of FA or MD across each tract (**supplementary Figure S6**). The first unrotated principal component (PC) scores were extracted as the single-metric g-factors, gFA and gMD (scree plot and PCA variable contributions illustrated in **supplementary Figure S7**).

As with global brain structural metrics, two models were used:

$$\text{Model } H_1\text{: dMRI metric} \sim \text{DNAm CRP} + \text{gestational age at scan} + \text{gestational age at birth} + \text{infant sex} + \text{birthweight} \quad (1)$$

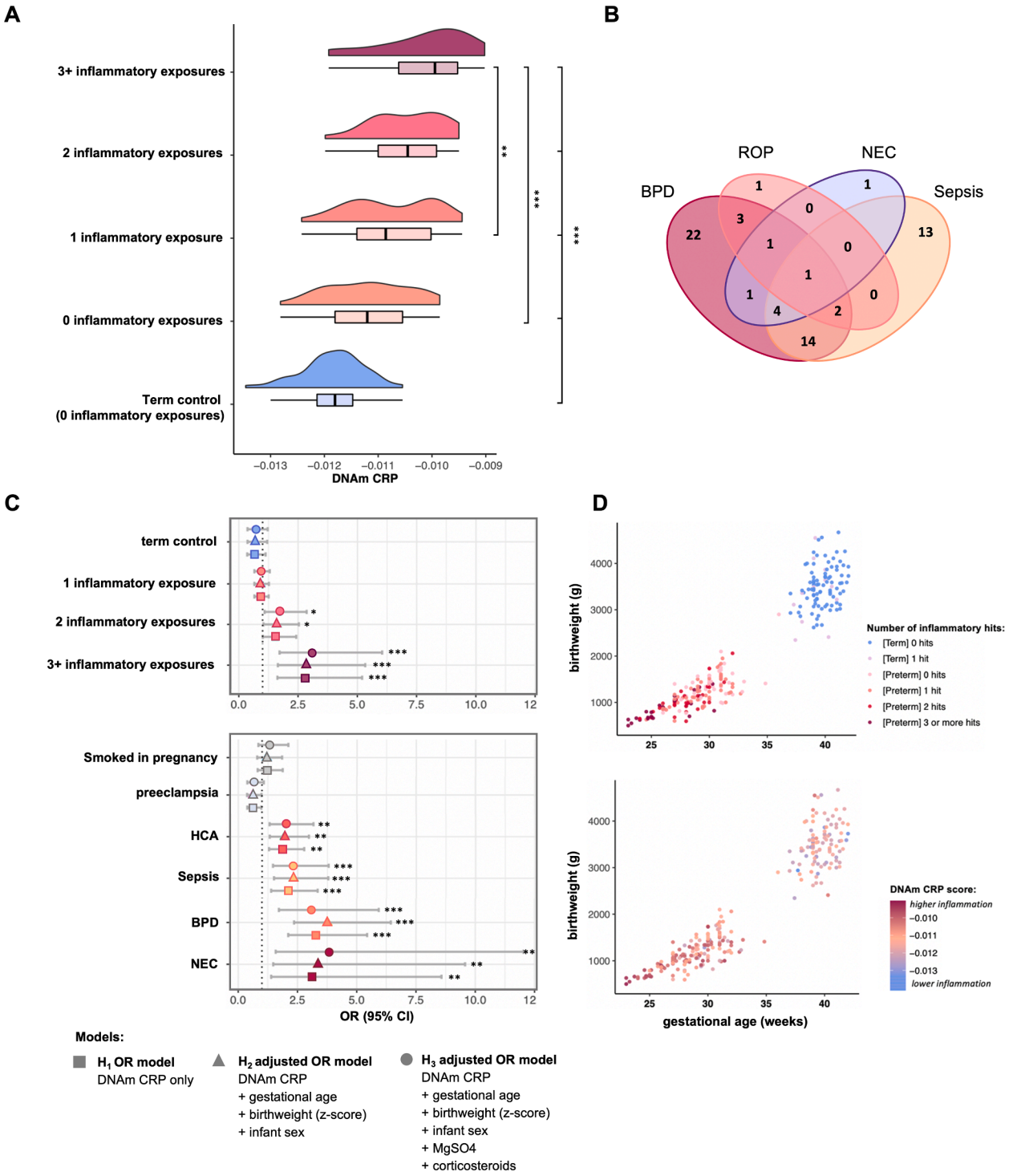

Fig. 2. Multiple inflammatory hits associate with raised DNAm CRP (A) distributions of DNAm CRP according to number of inflammatory episodes experienced by infant (B) Venn diagram showing the overlap postnatal inflammatory morbidities in study sample (C) Odds ratios and 95 % confidence intervals for contribution of DNAm CRP to inflammatory exposures, asterisks (*) indicate statistically significant (FDR-corrected $p < 0.05$) (D) Scatter plots of the relationships between gestational age and birthweight, coloured according to number of inflammatory episodes/exposures (top panel) and DNAm CRP (bottom panel). Models are based on full sample (n = 258); for further details see supplementary Figure 8.

$$\text{Model } H_2: \text{dMRI metric} \sim \text{DNAm CRP} + \text{gestational age at scan} + \text{gestational age at birth} + \text{infant sex} + \text{birthweight} + \text{all inflammatory risk factors} \quad (2)$$

In comparison to global MRI volumetric metrics and PSFA, PSMD, PSAD and PSRD, all individual tract associations, gFA, gMD and PSNDI were limited to neuroimaging data from phase 2 of the study, hence no scanner variable was included in these analyses.

2.8. Data and code availability

Requests for original image and anonymised data will be considered through the BRAINS governance process (https://www.brainsimagebank.ac.uk). Raw DNAm data are available upon request from Theirworld Edinburgh Birth Cohort, University of Edinburgh (https://www.tebc.ed.ac.uk/2019/12/data-access-and-collaboration), while DNAm and metadata are not publicly available, generated DNAm CRP signatures are included alongside scripts for data analysis. All brain volumetric metrics were obtained using the scripts provided in https://github.com/amakropoulos/structural-pipeline-measures. The segmented tracts in the ENA50 template space are available online: https://git.ecdf.ed.ac.uk/jbrl/ena. Code for primary data analysis and figures are available at https://github.com/EleanorSC/TEBC_DNAmCRP and code for tract propagation and average calculation are available at https://git.ecdf.ed.ac.uk/jbrl/neonatal-gfactors.

3. Results

3.1. Participant characteristics

The study group consisted of 258 neonates: 155 participants were preterm and 103 were controls born at full term, see Table 1 for participant characteristics and **supplementary** figure 3 for a flowchart of data acquisition. Among the preterm infants, 48 (31 %) had bronchopulmonary dysplasia, 10 (6 %) developed necrotising enterocolitis, 8 (5 %) developed ROP, 49 had HCA (32 %), 22 (15 %) were born to women whose pregnancy was complicated by preeclampsia, and 36 (23 %) had an episode of postnatal sepsis. Of the 258 participants with DNAm data, 214 also had MRI data. Correlations between all variables are provided in **supplementary** figure 1.

3.2. Multiple inflammatory hits increase risk of elevated inflammation

Preterm Infants in the sample for whom DNAm data and composite neonatal inflammatory risk scores were available (n = 155), had high prevalence (n = 112, 72 %) of experiencing at least one of the documented inflammatory exposures (Fig. 2B), which included incidence of smoking during pregnancy, preeclampsia, HCA, sepsis, BPD, NEC or ROP. A small subset of these infants experienced three or more of these exposures (n = 24, 15 %).

There was an association between number of inflammatory episodes and the inflammatory-related DNA methylation signature, with higher DNAm CRP in infants who had experienced greater exposure to inflammation. DNAm CRP was associated with higher odds of several perinatal morbidities including HCA, sepsis, BPD, and NEC. These relationships remained significant following adjustment for gestational age at birth, birthweight, and infant sex as well as perinatal variables of administration of corticosteroids and MgSO4 in pregnancy (Fig. 2C, **supplementary** Table S3). The association of DNAm CRP with ROP was no longer significant after controlling for MgSO4 and corticosteroid administration (model H_3). DNAm CRP was also associated with three or more inflammatory episodes. There was no significant association of DNAm CRP with maternal smoking in pregnancy or preeclampsia. Infants with increasing numbers of complications were more likely to be gestationally younger at birth and have lower birthweights (Fig. 2**D**). Furthermore, preterm infants had significantly higher DNAm CRP (Table 1, $p < 0.001$). When examining DNAm CRP alongside clinical inflammatory exposures (**supplementary** Table S4), there was no significant difference between term infants with no inflammatory episodes vs those with one (a breakdown of the 14 infants who had an inflammatory exposure recorded is provided in **supplementary** Figure S5; of note, term infants only had antenatal inflammatory exposures, as no term infants went on to develop a postnatal inflammatory condition, or experience neonatal sepsis). The largest difference was found between term infants with no inflammatory episodes and preterm infants with 3 or more inflammatory risk-factors ($p < 0.001$).

3.3. Dnam CRP and brain volumes

Overall, magnitudes of associations between DNAm CRP and global MRI brain volumes were modest, explaining a small amount of additional variance beyond covariates (of infant sex, gestational age at birth, birthweight z-score, gestational age at scan, scanner variable). After examining inflammation-brain structure associations across all infants (n = 214; **supplementary** Table S5), we stratified analyses into term (n = 87) and preterm (n = 127) subgroups to examine group differences (Fig. 3B). Term infants displayed no significant brain structural associations with DNAm CRP, whereas preterm infants with higher DNAm CRP displayed brain volume reductions in deep grey matter, white matter, and hippocampi and amygdalae (Fig. 3B).

Analyses were repeated to include interactions between DNAm CRP and both sex and gestational age (**supplementary Figure S9**). While null findings were observed with the former ($p > 0.05$; **supplementary** Table S7), within the preterm cohort there was evidence for interactions with gestational age at birth (**supplementary** Table S8). Higher DNAm CRP was consistently associated with lower brain volume in infants of lower gestational ages (i.e. extremely preterm infants tended to have higher DNAm CRP and correspondingly smaller global brain volume measures). In contrast, there was no significant interaction between gestational age and DNAm CRP within the term sub-group ($p > 0.05$; **supplementary** Table S9).

In fully adjusted models (Fig. 4, **supplementary** Table S11), where analyses were conducted separately for a term control model (n = 87) and preterm subgroup (n = 127) where aggregate inflammatory risk factors were examined separately (models H_3-H_{12}) there remained a significant association of DNAm CRP with deep grey matter volume (β = -0.206, p = 0.008), white matter volume (β = -0.346, p = 0.0006), and cerebellum volume (β = -0.201, p = 0.013). For most brain metrics, the strength of the association between DNAm CRP and MRI metric was increased when additional inflammatory covariates were included in the model (percentage increase for deep grey matter volume = 6 %, white matter volume = 39 %, and cerebellum volume = 27 %). Individually modelling risk factors revealed that this increase was mostly driven by controlling for incidence of sepsis, whereas brain structural associations were most attenuated by controlling for incidence of BPD (Fig. 4).

3.4. Global white matter microstructure associations with DNAm CRP

Preterm infants with higher DNAm CRP had poorer measures of white matter tract integrity. This was seen at the global level for all peak width of skeletonised white matter microstructure metrics (Table 2); PSFA, PSMD, PSRD, PSAD; β range $|0.186|$ to $|0.341|$, incremental R^2 2.7 – 9 %). In term infants, there were no significant associations (**supplementary** Table S10).

As with global brain volumetric measures, there were significant interactions between gestational age and DNAm CRP across measures of white matter integrity excepting PSRD: PSFA (interaction β = 0.225; main effect β = -0.212), PSMD (interaction β = -0.257; main effect β = 0.371) and PSAD (interaction β = -0.271; main effect β = 0.232), indicating infants at younger gestational ages were more likely to have poor white matter integrity with high DNAm CRP. When controlling for inflammatory risk factors, DNAm CRP associations between PSRD and

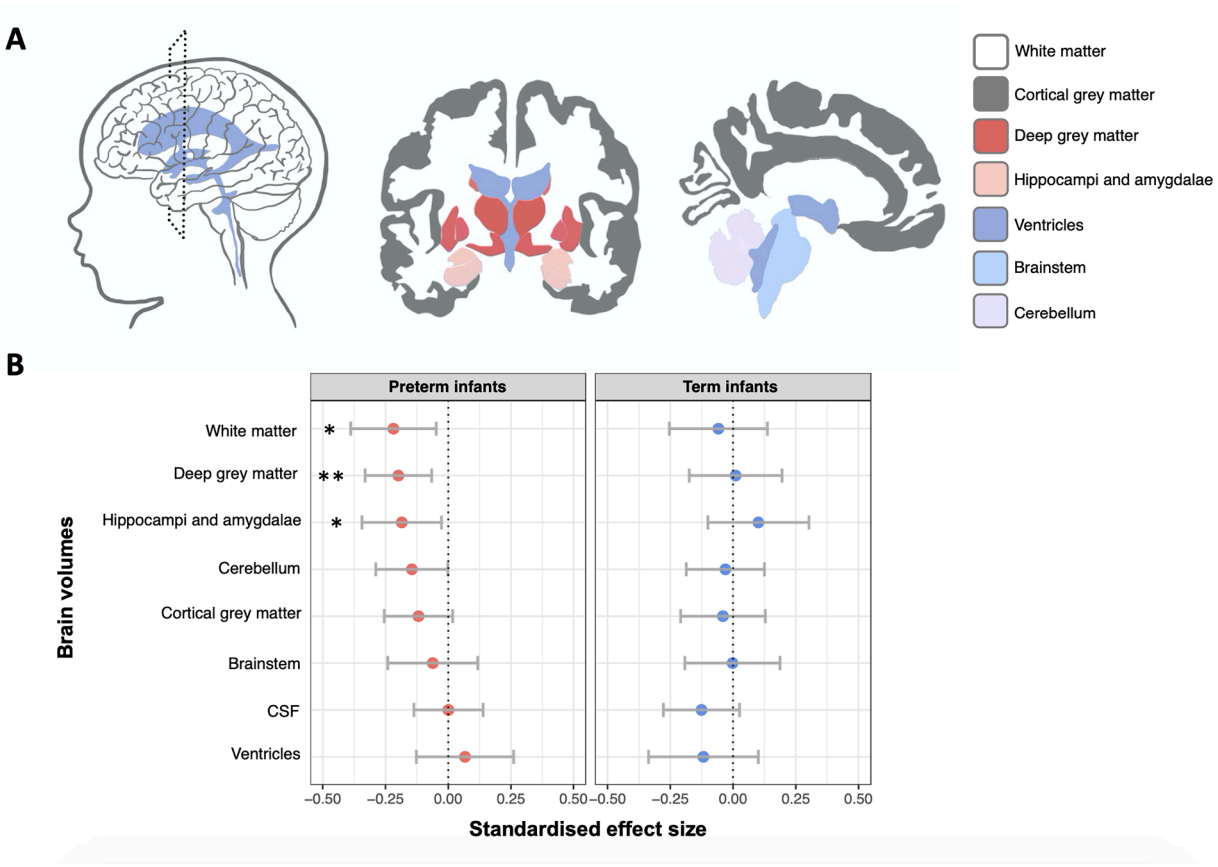

Fig. 3. Association of DNAm CRP with brain volumes (A) schematic drawn by ggseg package (Mowinckel & Vidal-Piñeiro, 2020) of reconstructed MRI brain volumetric measures (B) Standardized regression coefficients for DNAm CRP associations between brain volumetric measures for preterm infants (red circles) and preterm infants (blue circles). Points show standardized coefficients and 95 % confidence intervals. Asterisks (*) indicate statistically significant (FDR-corrected $p < 0.05$), (**) indicates $p_{FDR} < 0.01$. All models are controlled for infant sex, gestational age at birth, gestational age at scan and birthweight Z score and scanner variable.

PSAD were no longer significant.

Within a smaller subgroup of this sample, individual tract FA and MD as well as neurite density index data was available (Phase 2, refer to **supplementary** figure 3 for study flow diagram). PCA-derived single-metric g-factors, gFA and gMD (scree plot and PCA variable contributions illustrated in **supplementary Figure S7**) were almost exactly correlated with those previously reported in a larger sample of Theirworld Edinburgh Birth cohort infants (Vaher et al., 2022). When examining the association between DNAm CRP and global white matter measures in this subsample, the most striking association was seen with differences in a general factor of fractional anisotropy, gFA (β = -0.52 [95 % CI −0.304, −0.736], $p = 1.48 \times 10^{-5}$, incremental $R^2 = 22$ %) and mean diffusivity, gMD ($\beta = 0.423$ [95 % CI 0.661, 0.191], $p = 7.79 \times 10^{-4}$, incremental $R^2 = 14$ %). These effect sizes were attenuated by controlling for additional inflammatory risk factors (model H_2) but remained significant (β range $|0.35|$ to $|0.37|$, $p < 0.05$). No significant associations were found between DNAm CRP with PSNDI.

3.5. Individual white matter tract associations with DNAm CRP

We next examined associations between DNAm CRP and individual tract-averaged FA and MD. In all models, term infants displayed no significant tract associations with DNAm CRP (**supplementary Figure S10**). In preterm infants, altered FA was present in both hemispheric tracts of the AF, CST, IFOF, ILF, UNC and CCG (Fig. 5 shows tract-averaged fractional anisotropy for each of the 16 tracts for the term and preterm neonates). Some tract associations were specific to hemisphere such as decreased FA in the left (but not right) ATR. Equally, altered FA and MD was present in only the genu (and not splenium) of the corpus callosum. A breakdown of all dMRI results is reported in **supplementary Table S12.** However, after adjusting for additional inflammatory risk factors, (in order of effect size) only FA in the right corticospinal tract (15.4 %), right AF (10 %), and right CCG (8.7 %) remained significant. For tract MD, bilateral increases in MD were observed in the AF, CST, IFOF, ATR and CCG. Hemispheric specific associations were found for the left ILF, left ATR and genu of the corpus callosum. Of these associations, AF and ILF were no longer significant when accounting for additional inflammatory exposures.

4. Discussion

In this study, we integrated data from placenta, saliva and brain MRI in a large cohort of 258 infants to characterise the association of inflammation with brain structure. Previous research has typically relied on C-Reactive Protein (CRP) to measure inflammation, yet CRP's fluctuating nature, variability within short timeframes (which can lead to individuals displaying transiently elevated or lowered CRP levels in population studies), and the developmental-dependant physiology of hepatocytes, renders it an inadequate tool for profiling inflammation in preterm populations. We demonstrate that a composite buccal-cell DNA

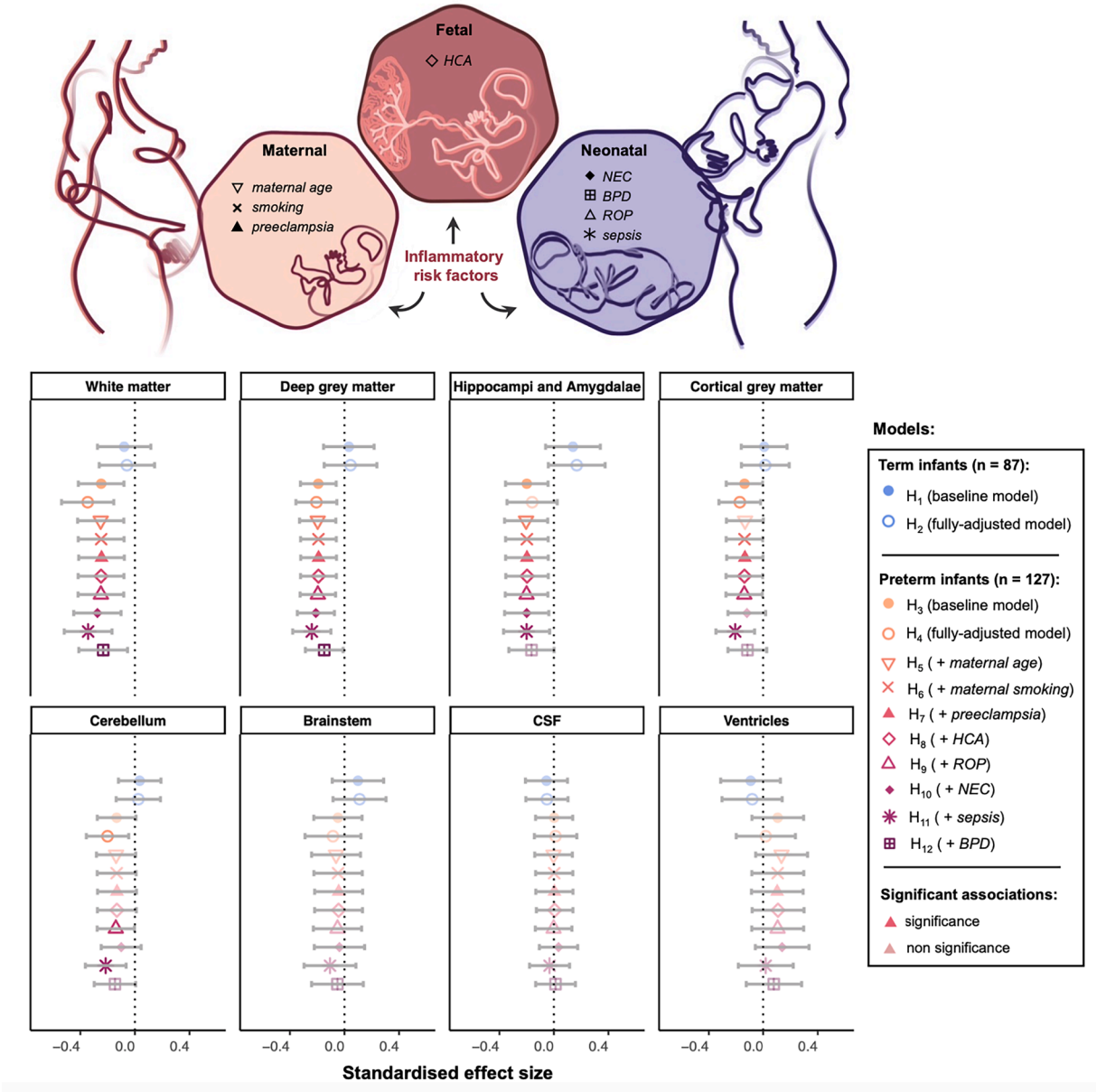

Fig. 4. Associations between DNAm CRP and brain volumes and the impact of inflammatory risk factors on associations. Top panel displays schematic of different perinatal inflammatory exposures controlled for, including smoking in pregnancy, preeclampsia, histologic chorioamnionitis (HCA), bronchopulmonary dysplasia (BPD), necrotizing enterocolitis (NEC), severe retinopathy of prematurity (ROP) and neonatal sepsis. Bottom panel displays standardized regression coefficients for DNAm CRP associations between brain volumetric measures, where points show standardized coefficients and 95 % confidence intervals. The alpha of points denotes whether associations were significant, with pFDR < 0.05 associates outlined by dark points and non-significant associations faded points. The two main models, H_1 and H_3, represent baseline models run on term ($n = 87$) and preterm ($n = 127$) subgroups respectively; these, and all subsequent models, controlled for infant sex, gestational age at birth, gestational age at scan and birthweight Z score and scanner variable. Models H_2 and H_4 control for all inflammatory risk factors in aggregate in term and preterm infants respectively. The additional shapes show the degree to which these associations, in preterms, were influenced by accounting for allied inflammatory exposures individually (H_3-H_{12}, corresponding with supplementary Table S11).

methylation measure of inflammation trained in adult peripheral blood samples associates with comorbidities of preterm birth that are characterised by a pro-inflammatory state and widespread differences in brain structure among preterm infants. The inflammatory-related DNAm signature was particularly associated with white matter dysmaturation at term-equivalent age both globally and at the level of individual white matter tract microstructure, associations that largely remained significant when accounting for inflammatory exposures. These data motivate further research into the potential of immune-DNAm markers for translational medicine in the neonatal period as diagnostic tools for identifying those at risk for inflammatory-related morbidities and neurodevelopmental impairment.

4.1. *Inflammatory-related DNAm biomarkers*

There has been increasing interest in using methylation data to

Table 2
Associations between DNAm CRP and global white matter microstructure and the impact of inflammatory risk factors on associations in preterm infants; standardized regression coefficients for DNAm CRP associations between global white matter microstructure metrics for preterm infants. Betas (standardized coefficients) and 95 % confidence intervals are reported. Bold text indicate statistically significant association (FDR-corrected $p < 0.05$). Model H_1 controls for infant sex, gestational age at birth, gestational age at scan birthweight Z score and scanner variable; model H_2 additionally controls for inflammatory risk factors and associated morbidities (maternal smoking in pregnancy, preeclampsia, HCA, sepsis, BPD, NEC and ROP).

		beta	lower CI	upper CI	p	r^2	additional r^2	n
Model H_1	PSFA	**−0.186**	−0.324	−0.048	**0.009**	0.540	0.027	127
	PSMD	**0.341**	0.166	0.517	**2.17E-04**	0.256	0.090	127
	PSRD	**0.312**	0.122	0.501	**0.002**	0.130	0.075	127
	PSAD	**0.201**	0.030	0.372	**0.023**	0.294	0.031	127
	gFA[a]	**−0.520**	−0.736	−0.305	**1.48E-05**	0.441	0.216	64
	gMD[a]	**0.426**	0.191	0.661	**0.001**	0.333	0.145	64
	PSNDI[a]	−0.089	−0.321	0.142	0.452	0.354	0.006	64
Model H_2	PSFA	**−0.215**	−0.375	−0.055	**0.009**	0.577	0.026	127
	PSMD	**0.206**	0.009	0.403	**0.042**	0.357	0.024	127
	PSRD	0.175	−0.041	0.392	0.115	0.222	0.017	127
	PSAD	0.093	−0.095	0.280	0.336	0.414	0.005	127
	gFA[a]	**−0.371**	−0.617	−0.124	**0.005**	0.573	0.073	64
	gMD[a]	**0.355**	0.082	0.627	**0.014**	0.477	0.067	64
	PSNDI[a]	−0.123	−0.419	0.173	0.420	0.385	0.008	64

[a] scanner variable is controlled for when examining PS metrics but not gFA, gMD and PSNDI (single-scanner sample).

advance our understanding of the causes and consequences of preterm birth as outlined in several reviews (9,79,80). Only recently has attention turned to exposures in the perinatal period, the role of epigenetics *in utero* for neurodevelopment, and the potential of peripherally sampled DNAm to capture the impact of environmental exposure in relation to brain and cognitive outcomes (Barker et al., 2018). Among these developments are the use of methylation risk scores of exposure, which integrate information from multiple CpG sites to provide a record of exposure or to capture a complex trait (Bakulski and Fallin, 2014; Yousefi et al., 2022). This approach has been used to examine maternal smoking (Odintsova et al., 2021; Reese et al., 2017; Richmond et al., 2015; Richmond et al., 2018) glucocorticoid, and prenatal folate exposure during pregnancy (Bakulski et al., 2021; Suarez et al., 2020), alongside environmental exposures such as pollution (Suarez et al., 2020). Examining DNAm proxies of inflammation is uncommon, but given the health associations with inflammatory-related DNAm in adult cohorts (Conole et al., 2021; Green et al., 2021; Somineni et al., 2019), and the shared nature of epigenetic changes between mothers and infants (Camerota et al., 2021; Sasaki et al., 2022), we hypothesised that variance in the neonatal methylome could reflect a convergence of amassed inflammatory burden from different perinatal origins.

4.2. Dnam CRP associates with gestational age and multiple inflammatory exposures

Our findings suggest that epigenetics offers a solution to the traditional limitations of assessing inflammatory burden in infancy (which include the phasic nature of CRP responses, particularly in preterm infants where CRP may be an inappropriate marker owing to the development-related physiology of the liver, and unethical serial sampling in vulnerable neonates).

Preterm infants displayed higher DNAm CRP than term infants, and associations between DNAm CRP and postnatal health and brain outcomes were restricted to the preterm infants. This novel finding that gestational age at birth correlates strongly with inflammatory-related DNAm ($r = -0.62$, $p < 0.001$) aligns with previous observations of elevated inflammatory protein concentrations with lower gestational ages and prematurity (Humberg et al., 2020; Dammann and Leviton, 2014), lending further weight to the validity of this measure for carrying clinical significance. Equally, finding that inflammation-related alterations in brain structure were more pronounced in preterms who were gestationally younger (supplementary Figure S9) highlights the dose-dependent effect on brain structure, whereby increasing prematurity (lower gestational age) is associated with higher inflammatory burden and related structural consequences. These findings are in line with previous studies which demonstrate that extremely preterm infants are at enhanced risk of neurodevelopmental impairment, with sustained postnatal inflammation significantly increasing this risk (Leviton et al., 2012; Korzeniewski et al., 2014; Barnett et al., 2018; Glass et al., 2018). We speculate that the enhanced vulnerability of extremely preterm infants is due to rapid developmental changes during the second and third trimester of pregnancy for both the developing immune system and brain – in particular, the disruptive impact of inflammation on neurogenesis, neuronal migration, synaptogenesis and myelination (Hagberg et al., 2015). As these processes are highly dynamic during these periods and early postnatal life, preterm infants are both more susceptible to sustained inflammation and neurodevelopmental disruption. Relative differences in immune system maturity and neurodevelopmental processes between extremely preterm infants, preterm and term infants likely explain these findings.

Our finding that multiple inflammatory hits contributed to higher DNAm CRP strengthens the hypothesis that DNAm may index the allostatic load of inflammation during neonatal intensive care. In both preclinical studies and cohort groups, preterm infants with multiple inflammatory episodes or morbidities display an increased risk for brain structural abnormalities compared to infants who had only one inflammatory episode or condition recorded (Glass et al., 2018; Yanni et al., 2017; Fleiss et al., 2015). We also find stronger relationships between DNAm CRP and postnatal inflammatory factors (NEC, BPD, sepsis) than antenatal factors (preeclampsia, maternal smoking), indicating early-life exposures contribute to greater variance in DNAm than maternal inflammation (as depicted in Fig. 2C). The multi-hit hypothesis of sustained inflammation (Leviton et al., 2012; Barnett et al., 2018; Yanni et al., 2017) suggests that postnatal health complications related to preterm birth can perpetuate a chronic inflammatory state, with timing of insults a key factor for why preterm infants are more susceptible than term infants to sustained inflammation (Ophelders et al., 2020).

Previous work has shown that that a paediatric buccal-cell derived epigenetic age acceleration measure (PedBE) is associated with adverse

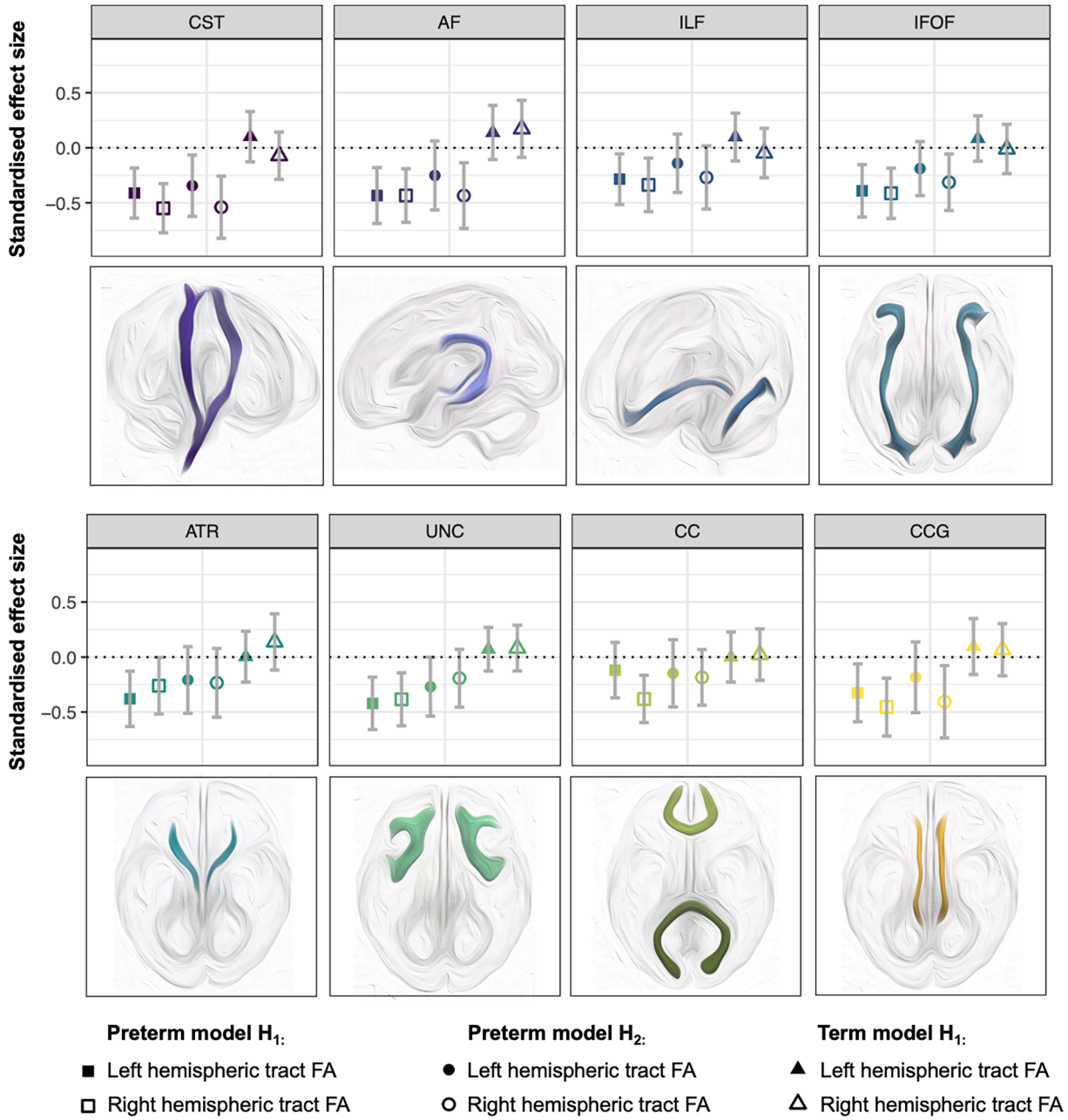

Fig. 5. DTI-tract associations with DNAm CRP. Standardized regression coefficients for DNAm CRP associations between tract fractional anisotropy (FA) for preterm infants (squares), preterm infants in models controlling for additionally inflammatory risk factors (circles) and term infants (triangles). Filled shapes are left tracts and open shapes are right hemispheric tracts, except in the case of the CC where filled shapes are the splenium and open shapes are the genu of the corpus callosum. Points show standardized coefficients and 95% confidence intervals. All models are controlled for sex, gestational age at birth, gestational age at scan and birthweight Z score. Model H_2 (circles) additionally controls for inflammatory risk factors (maternal smoking during pregnancy, preeclampsia, HCA, neonatal sepsis, BPD, NEC, ROP). For MD associations see supplementary Figure S10.

neonatal brain growth and neurodevelopmental outcomes among children born very preterm with a neonatal infection (Gomaa et al., 2022). Of interest here is that none of the CpG sites which constitute the DNAm CRP used in this study were identified as top hits within the previous EWAS in this cohort examining methylation associated with gestational birth (Wheater et al., 2022) or the PedBE (Gomaa et al., 2022) gestational clock (built from 94 CpG sites), indicating that the DNAm CRP score is capturing something unique over residual gestational age-related differences, and further affirming our hypothesis that sustained inflammation may be driving differences in brain dysmaturation above and beyond preterm status itself.

The CpG sites which constitute the score are involved in vascular and immune function (**supplementary Table S1**). In the original EWAS by Ligthart et al (2016), the top CpG hits relating to circulating CRP levels mapped to the genes AIM2 and SOCS3; the former of which is an inflammasome receptor, upstream of CRP, involved in the processing of interleukins responsible for the induction of CRP from hepatocytes. The importance of this locus in inflammation has been reinforced by

candidate gene studies and further EWAS work (Miller et al., 2018; Myte et al., 2019), including the latest work on 22,000 participants (Wielscher et al., 2022). The second of these top hits, SOCS3 (cg18181703) has been significantly associated with a general factor of cognitive ability (Stevenson et al., 2020), as well as processing speed (Marioni et al., 2018) and brain structural characteristics such as WMH burden and global grey and white matter atrophy (Conole et al., 2021), suggesting the importance of this locus in driving cognitive changes. How this maps to neurodevelopment and cognitive changes earlier in life is however unclear, and scope to study this association may be challenging owing to the variance in neurodevelopmental testing scores in preterm infants in early infancy (van Beek et al., 2022), with some evidence for standard neurodevelopmental testing paradigms underestimating impairment and developmental delay following preterm birth (Anderson et al., 2010; Vohr et al., 2012). SOCS3 is involved in pro-inflammatory cytokine pathways (Rottenberg and Carow, 2014), is implicated in demyelination via oligodendrocyte disruption (Emery et al., 2006), has been previously implicated in a murine-model of preterm-related WM injury (Boccazzi et al., 2021) and has been found to be upregulated in the microglia of AD patients (Walker et al., 2015). This evidence suggests that aberrant methylation of cg18181703 may play an important intermediary role linking peripheral inflammatory processes with white matter maturation in the preterm neonate. This locus may also be particularly susceptible and responsive to differences in health and lifestyle as this site has also been linked to metabolic differences across the lifecourse such as BMI (Ali et al., 2016) and incident type 2 diabetes (Chambers et al., 2015), conditions themselves which have been linked to elevated CRP levels. More studies that conduct EWAS of inflammation in neonatal cohorts is needed, as the largest multi-cohort EWASs to date have been conducted in adult populations (Ligthart et al., 2016; Wielscher et al., 2022). Overall, our findings indicate that epigenetic modifications are an essential mechanism by which inflammatory risk factors could lead to long-term disruptions in both immune and brain development (Ozanne and Constância, 2007; Fleiss and Gressens, 2012), and this work highlights the utility of profiling such changes alongside other clinical and biological data.

4.3. White and deep grey matter dysmaturation in preterms with elevated inflammation

The most striking finding from this study is the association of DNAm CRP with widespread variances in brain structure in preterm but not term infants (Figs. 3-5). We observe larger effect sizes for associations of DNAm CRP with global white matter microstructure (gFA and gMD) than white matter volume in a sub-population of these infants. This may be because diffuse white matter injury antedates overall reductions in white matter volume, with DNAm CRP-DTI associations capturing a more subtle dysmaturation of programmed development (Skiöld et al., 2010). Both global white matter volume, microstructure and regional white matter integrity were lower in preterms with elevated DNAm CRP, with infants at younger gestational ages more prone to elevated inflammation and related poor white matter integrity. These findings echo the results of prior studies (Korzeniewski et al., 2014; Volpe, 2019; Favrais et al., 2011; Dubner et al., 2019), and are overall consistent with the theory that alterations in white matter microstructure are largely a consequence of dysregulation of white matter development driven by inflammation (Dubner et al., 2019).

In addition to white matter, volume reductions were observed in the hippocampi and amygdale and deep grey matter with increased DNAm CRP, though the former did not remain significant after accounting for additional inflammatory risk factors (**supplementary Table S11**). The association of elevated DNAm CRP with lower deep grey matter volume is consistent with previous research that finds that preterms infants exhibit deep grey matter loss relative to term infants (Padilla et al., 2015; Inder et al., 2005; Boardman et al., 2006). Given the relationship we outline here between preterm birth and inflammatory load, inflammation may be a key driver of these differences, both via its direct effects on brain structure and its contribution to related damage such as sensitisation to hypoxia ischaemia, excitotoxic insults and other early-life stressors (Bennet et al., 2018; Lammertink et al., 2022). These widespread alterations in brain structure are particularly interesting given the evidence base for inflammation relating to cognitive impairment, as studies have shown that both hippocampal volume and thalamic volume loss accompanying white matter microstructural alterations are linked to neurodevelopmental outcomes in early childhood (Boardman et al., 2010; Beauchamp et al., 2008; Ball et al., 2013). Inflammation-related grey matter loss is considered a consequence of dysregulated neuronal development, with inflammatory mediators disrupting processes such as dendritic arborization and cortico-thalamic connectivity (Favrais et al., 2011). As consolidation of thalamocortical connections happens in the third trimester of pregnancy, deep grey matter structures may be vulnerable to inflammatory stimuli (Volpe et al., 2011).

We also observed regional variance in how DNAm CRP associates with white matter tracts, a finding consistent with previous studies that indicate that certain white matter tracts are more vulnerable to inflammatory-adjacent events such as hypoxia ischemia (Kostović et al., 2014) traumatic brain injury (Malaeb and Dammann, 2009), intraventricular haemorrhage (Leviton et al., 2013) and cerebral palsy (Lin et al., 2010). Different white matter tracts develop at different rates *in utero* and display distinct transient growth periods of increased axonal development. These windows of plasticity have been outlined as particularly vulnerable to perturbation (Leviton and Gressens, 2007; Ment et al., 2009), with inflammation disrupting the developmental lineage of oligodendrocytes, resulting in hypomyelinated axons (Back, 2015; Favrais et al., 2011; Back et al., 2001; Majnemer et al., 2000). Developmental growth periods of certain white matter tracts may therefore underscore regional vulnerability to elevated inflammation, with younger tracts likely to have higher proportions of pre-myelinating oligodendrocytes vulnerable to inflammatory mediators. However, we caution that we lack the statistical power to reliably detect differences between the magnitude of associations in regional white matter structure, and instead interpret these findings as evidence of the pervasive and widespread impact of inflammation on the development of white matter. Correspondingly, though there were differences between the association significance for the left and right hemispheres for several of the delineated tracts, these unilateral findings are in keeping with previous studies of similar sample size (Inomata et al., 2014; Alexandrou et al., 2014); as the magnitudes were similar (with overlapping confidence intervals), this did not indicate a strong basis for laterality of effects.

4.4. Strengths and limitations

To our knowledge, this is the first time an epigenetic measure of inflammation has been examined in a preterm cohort in relation to brain health outcomes. The effect sizes reported in this study are consistent with that of previous epidemiologic studies of DNAm and early life outcomes (Breton et al., 2017). The sample size (n = 258 for inflammatory exposure, and n = 121–214 for neuroimaging associations), is akin to that of previous work examining inflammation and brain structure in preterm infant populations (Shah et al., 2008; Kelly et al., 2016; Sullivan et al., 2020; Glass et al., 2008), and in many cases more substantial, with the vast majority of prior work conducted in sample sizes of<100 infants (Inomata et al., 2014; Yoon et al., 1997; Anblagan et al., 2016; Travis et al., 2015; Lee et al., 2021; Volpe, 2019; Alexandrou et al., 2014; Basu et al., 2015). There is a distinct scarcity of detailed methylation alongside multi-modal neuroimaging data (Wheater et al., 2020; Lancaster et al., 2018), particularly in neonatal cohorts (Fumagalli et al., 2018; Chen et al., 2015; Sparrow et al., 2016), making this a valuable contribution to the DNAm-neuroimaging field.

Although the weights for the predictor were trained in adult blood

samples, we observed similar associations between DNAm CRP and brain structural outcomes to those in previous studies of adults (Conole et al., 2021; Green et al., 2021). Given we have now applied this method to buccal-cell DNAm, it is encouraging to see similar associations between DNAm CRP and brain structural outcomes, especially given that DNAm is highly tissue specific (Davies et al., 2012), and previous research has reported on cross-tissue differences in magnitude and direction of effects for other traits (Walton et al., 2016). This cross-tissue approach (where weights were originally created from blood-based DNAm, and later applied to saliva-based composite signatures) has also been adopted in other studies (Suarez et al., 2020; Blostein et al., 2022). While future studies would ideally measure CRP directly from serum or blood spots in infants to enable direct cross-tissue comparisons, saliva has the advantage of being one of the most accessible tissue samples for infant populations, and may be more suitable than other peripheral samples (such as blood) when examining brain and cognitive outcomes owing to the brain and buccal cell shared ectodermal origins (Lowe et al., 2013; Braun et al., 2019). While we suggest that future studies consider a larger range of DNAm signatures (based off other inflammatory mediators implicated in PTB, such as IL6), we do not consider the lack of direct comparison between DNAm CRP and serum CRP a serious limitation of this study. We have previously shown that DNAm CRP outperforms serum CRP in anticipating brain structural and cognitive outcomes when derived from peripheral blood samples in adult population samples (Stevenson et al., 2020; Conole et al., 2021; Green et al., 2021). Additionally, within preterm populations, the use of CRP as a reliable measure of inflammation, or predictor of infection, is debated. A significant advantage of DNAm is that it may provide a historical archive of exposure that can be leveraged in instances where other clinical or biological data was not originally collected. To obtain useful baseline inflammation levels, serial sampling of CRP is encouraged; however, this is both impractical and ethically disputed in preterm neonates (given the number of needling episodes and volume of blood required to obtain average readings). There is evidence that preterm infants display dysregulated serum CRP responses compared to term infants, with studies cautioning its reliability as a biomarker of inflammation in this population group (Macallister et al., 2019; Borowski et al., 2022). The threshold values for raised CRP levels are dependent on gestational age at birth, with infants born too early displaying more phasic responses in serum CRP profiles which are less reflective of severity of infection. A recent systematic review (20 studies; n = 1,615 infants) concluded that CRP in neonates was an unreliable measure of inflammatory responses and should be avoided with regards to profiling sustained inflammation, or for subsequent guiding treatment (Brown et al., 2019;). Furthermore, irrespective of preterm status, the use of sampling CRP postnatally is complicated by the non-specific rise in CRP levels related to the stress of delivery itself (Chiesa et al., 2001; Hofer et al., 2012). In the absence of direct comparison with inflammatory mediators from blood samples, the strong correlates with clinical inflammatory conditions (both fetal and postnatal) is affirming, and we have taken steps to account for possible sources of confounding (**supplementary** Fig. 2).

Neuroimaging studies are notoriously heterogenous in their design given the array of different MRI acquisition techniques, processing pipelines and chosen outcome measures. The choice of neuroimaging features is even more relevant in the context of preterm birth to adequately address the motivating research questions (Korom et al., 2022). Here, our choice of neuroimaging features was guided by established characterizations of EoP in preterm infants, namely water content and dendritic/axonal complexity and dysmaturation within the white matter, and grey matter volume (Blesa et al., 2020). While we consider this comprehensive characterisation of brain structure from NODDI and dMRI data a significant strength of this study, we acknowledge that microstructure measures such as FA and MD in older cohorts are commonly considered surrogates of white matter integrity or myelination, the white-matter pathways in this study are still developing at the time of gestational age at scan (range 37.70–45.14 weeks), and as such may not reflect permanent differences. Longitudinal follow up is therefore encouraged for future studies designed to examine the implications of sustained inflammation in preterms for neurodevelopmental outcomes and lifecourse brain health.

We do not attempt to discuss the causality of the relationship between DNAm CRP with brain structure, though the causality of such associations is a persistent topic of debate in epigenetic epidemiological research and has been discussed in depth in reviews (Yousefi et al., 2022; Cecil et al., 2022; Birney et al., 2016). Future work examining transcriptomic changes on the same peripheral samples from which DNAm data is collected, as well as statistical approaches like two-step mendelian randomization, are important developments to unpick causality of these relationships. Studies investigating whether these differences in DNAm remain, amplify, or attenuate with age are advised, as well as how sensitive these signatures are in the context of intervention (anti-inflammatory medications and treatments, as well as lifestyle interventions such as the cessation of smoking in pregnancy). As infants born before 37 weeks gestation display reduced protection against immune-mediated disturbances (e.g., absent placental trophic and hormonal factors, immature responses to free radicals), examining these analytes in relation to DNAm signatures could demarcate specific pathways that confer added vulnerability for preterm brain dysmaturation. There is also precedent to examine whether composite methylation proxies of inflammation differ across psychopathology or specific neurological cases such as Cerebral Palsy or neurodevelopmental disorders such as Fragile X syndrome, autism and ADHD, given examples of other poly-epigenetic signatures of psychiatric disorders (Cecil et al., 2022). Future work that focuses on such DNAm dynamics in relation to these outcomes in ongoing longitudinal studies of infants born preterm is therefore of interest, as well as replication in different population samples.

Finally, DNAm was sampled in neonates postnatally. While this is rational when examining variances in DNAm and brain structural differences in infants, future studies that examine both maternal and infant DNAm could examine the degree to which exposures are shared or specific to parent and offspring. Equally, multiple DNAm sampling during pregnancy could elucidate key critical periods of susceptibility to inflammation by parsing out exposures specific to trimester or months of pregnancy, affording new insights into the spatiotemporal patterning of brain development in relation to dynamic immune changes in the perinatal period.

4.5 Future directions.

The use of DNAm biomarkers in neonatology has the potential to provide a more comprehensive picture of the health risks associated with preterm birth and can potentially be used to inform the clinical management of at-risk pregnancies, preterm infants and later-life health outcomes. The increasing evidence for the association between DNA methylation and clinical features of patients, such as the ability to stratify patients on the basis of disease subtypes (Somineni et al., 2019; Aref-Eshghi et al., 2020; Good et al., 2021; He et al., 2020), reflect dose-dependent exposure to certain substances such as smoking and alcohol (Langdon et al., 2021; Colicino et al., 2021; Yousefi et al., 2019) demonstrates the general utility of DNA methylation signatures as biomarkers of various disease-endpoints, diagnoses and exposures. The clinical potential of DNAm is further supported by the stability and robustness of DNA methylation and the convenience of DNAm sampling. Extending this paradigm to investigate how DNAm can be leveraged to investigate interactions at the maternal-fetal interface, exposures in the perinatal period, and early-life outcomes has lagged behind this progress (Cecil et al., 2022), though there are recent studies that demonstrate the utility of DNAm in indexing prenatal exposures and early-life outcomes (Camerota et al., 2021; Everson et al., 2020; Bakulski et al., 2021; Abrishamcar et al., 2022).

In the context of neonatology, DNA methylation-based biomarkers may offer an opportunity to develop predictive and prognostic tools to

optimise neonatal care. Inflammation-associated DNA methylation signatures such as those explored in this study could provide insight into the relative risk of adverse outcomes and act as a means to monitor how intervention, treatment and lifestyle effectively ameliorate the risk of these. The potential of profiling DNAm to track health trajectories in pregnancy and anticipate PTB is of particular translational value for identifying the relatively 'silent' and sudden onset triggers for preterm labour, such as preeclampsia, HCA, premature rupture of the membranes (PRROM) or a signpost risk of pregnancy-related disease (such as hyperemesis gravidarum, acute fatty liver of pregnancy, cholestasis) that can cause complications to both mother and infant. Overall, robust indexes inflammatory burden would enable better obstetric management to anticipate preterm birth, mitigate morbidity risk (through timely administration of prenatal steroids, magnesium sulphate, tocolytics and optimal delivery procedures), and thus improve the health and long-term outcomes for many children. Furthermore, the identification and validation of further inflammation-associated methylation markers, particularly those connected to specific pregnancy complications and exposures and postnatal complications, may provide insights into the developmental pathways that affect risk of sustained inflammation.

5. Conclusion

Inflammatory-related DNAm is associated with risk of postnatal health outcomes and brain dysmaturation. Our results indicate that multiple inflammatory-related hits from different origins (pertaining to maternal, fetal, and postnatal exposures) may be captured by changes to the DNA methylation profiles of infants and may help to explain variances in brain structure in preterm populations, circumventing limitations of traditional measures of inflammation. As early birth is associated with sudden change in immune-related risks, which coincide with the developing immune system and windows of neurodevelopmental plasticity, it is theorised that preterm infants are at greater risk of inflammation-related disruption of white matter. Our work here provides new layers to this theory, with epigenetic markers of inflammation associating with diffuse and global brain – and particularly white matter – alterations in preterm but not term infants, indicating that sustained inflammation may be a key driver of neurodevelopmental disruption. In summary, the association of an DNA methylation signature with inflammatory outcomes, and inflammation-relevant neural phenotypes, supports the use of methylation data in integrative, multimodal approaches toward disease stratification in the perinatal period.

Funding

This research was funded in whole, or in part, by the Wellcome Trust (Grant No 108890/Z/15/Z and 221890/Z/20/Z). For the purpose of open access, the authors have applied a CC BY public copyright licence to any Author Accepted Manuscript version arising from this submission.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: R. E.M has received a speaker fee from Illumina and is an advisor to the Epigenetic Clock Development Foundation and Optima Partners.

Data availability

Data will be made available on request.

Acknowledgements

Some of the participants were scanned in the University of Edinburgh Imaging Research MRI Facility at the Royal Infirmary of Edinburgh which was established with funding from The Wellcome Trust, Dunhill Medical Trust, Edinburgh and Lothians Research Foundation, Theirworld, The Muir Maxwell Trust and other sources. We thank Thorsten Feiweier at Siemens Healthcare for collaborating with dMRI acquisitions (Works-in-Progress Package for Advanced EPI Diffusion Imaging). The authors are grateful to the families who consented to take part in the study and to all the University's imaging research staff for providing the infant scanning.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2023.03.011.

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