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Immune gene regulation is associated with age and environmental adversity in a nonhuman primate

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Abstract

Phenotypic aging is ubiquitous across mammalian species, suggesting shared underlying mechanisms of aging. Aging is linked to molecular changes to DNA methylation and gene expression, and environmental factors, such as severe external challenges or adversities, can moderate these age-related changes. Yet, it remains unclear whether environmental adversities affect gene regulation via the same molecular pathways as chronological, or 'primary', aging. Investigating molecular aging in naturalistic animal populations can fill this gap by providing insight into shared molecular mechanisms of aging and the effects of a greater diversity of environmental adversities – particularly those that can be challenging to study in humans or laboratory organisms. Here, we characterised molecular aging – specifically, CpG methylation – in a sample of free-ranging rhesus macaques living off the coast of Puerto Rico (*n* samples = 571, *n* individuals = 499), which endured a major hurricane during our study. Age was associated with methylation at 78,661 sites (31% of all sites tested). Age-associated hypermethylation occurred more frequently in areas of active gene regulation, while hypomethylation was enriched in regions that show less activity in immune cells, suggesting these regions may become de-repressed in older individuals. Age-associated hypomethylation also co-occurred with increased chromatin accessibility while hypermethylation showed the opposite trend, hinting at a coordinated, multi-level loss of epigenetic stability during aging. We detected 32,048 CpG sites significantly associated with exposure to a hurricane, and these sites overlapped age-associated sites, most strongly in regulatory regions and most weakly in quiescent regions. Together, our results suggest that environmental adversity may contribute to aging-related

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molecular phenotypes in regions of active gene transcription, but that primary aging has specific signatures in non-regulatory regions.

KEYWORDS
aging, DNA methylation, environmental adversity, gene expression, gene regulation

1 | INTRODUCTION

Aging is ubiquitous across mammals and is accompanied by gradual declines in physiological systems across the body: from reproduction to sensory systems to immunity (Nussey et al., 2013). This suggests that there are shared underlying mechanisms at the molecular or cellular level that might be associated with, or even drive, the aging process. Indeed, molecular mechanisms, such as epigenetic alterations and genomic instability, have been identified almost exclusively based on human or laboratory animal (e.g. rodents) studies, and little research has focused on naturalistic mammalian populations (Jones et al., 2015; López-Otín et al., 2013, 2023) (but see Pinho et al., 2022; Wilkinson et al., 2021). Doing so is important for understanding the generalisability and evolution of the aging process across mammals (Bronikowski et al., 2011; Laine et al., 2023; Nussey et al., 2013). Further, the aging process can be modified by external insults and adversities, and naturalistic populations offer unique opportunities to investigate the effects of diverse environmental perturbations that are challenging to study in or do not affect humans and laboratory animals (Snyder-Mackler et al., 2020).

One unified and well-studied mechanism of aging is changes to the epigenome – including DNA methylation, which is a gene regulatory mechanism that canonically suppresses gene expression (Lea et al., 2018; Moore et al., 2013). Due to its gene regulatory role, changes in DNA methylation with aging can both generate damage, contributing to the aging process, and be part of the damage-repair response (Seale et al., 2022; Sriraman et al., 2020). For example, the progressive loss of cell- and tissue-specific DNA methylation is expected to drive age-related loss of tissue and cellular specificity and function in humans and mice (Dmitrijeva et al., 2018; Hernando-Herraez et al., 2019; Thompson et al., 2010). Yet, DNA methylation also suppresses deleterious genomic elements such as transposable elements (TEs) that contribute to molecular damage, inflammation and immune dysregulation (Gorbunova et al., 2021; Pal & Tyler, 2016).

These molecular changes may mechanistically link environmental adversities to variation in the timing of onset and trajectories of various age-related phenotypes, such as the relationship between social adversity and increased morbidity and mortality in humans and other primates (Anderson et al., 2021; Cunliffe, 2016; Holt-Lunstad et al., 2015; Poganik et al., 2023; Snyder-Mackler et al., 2019; Tung et al., 2016). At the molecular level, age-associated damage that is exacerbated by environmental insults is recognised as 'secondary aging'. This stands in contrast to 'primary aging', which is the inevitable accumulated molecular damage over chronological time that results from diminished repair capacity (Holloszy, 2000; Seale et al., 2022). Yet, how these two types of aging influence gene regulation and the extent to which environmental adversity mechanistically contributes to secondary aging remains unclear (Seale et al., 2022).

DNA methylation is expected to be a key link between sources of secondary aging such as environmental adversities and downstream phenotypes, as it is environmentally responsive and stable across long periods of time (Ciccarone et al., 2018; Tobi et al., 2014; Unternaehrer et al., 2012; Zannas et al., 2015). However, most studies to date that have investigated the molecular consequences of environmental adversities using DNA methylation have focused on single epigenetic biomarkers, such as composite epigenetic clocks, or DNA methylation near candidate genes (i.e. glucocorticoid/stress response pathway) (Seale et al., 2022; Unternaehrer et al., 2012; Zannas et al., 2015). This means that we know little about how aging and environmental adversity affect DNA methylation at individual CpG sites throughout the genome, and consequently have limited ability to differentiate the pathways linked to primary versus secondary aging (Seale et al., 2022). For example, it remains unclear the extent to which secondary aging caused by environmental perturbations recapitulates primary aging effects on the methylome, including the specificity of their genomic targets (i.e. location) and their potential to generate deleterious consequences (Jones et al., 2015).

Here, we tested the extent to which primary and secondary aging affect DNA methylation via distinct molecular pathways and processes by probing the similarities between age-related and environmentally-induced differences in methylation. We studied the long-term, free-ranging rhesus macaque population of Cayo Santiago, in which individuals are tracked longitudinally and experience variation in naturally occurring environmental adversity during their lifetime. Specifically, we investigated primary and secondary aging on DNA methylation by assessing the impact of an adversity known to have biological and social impacts on these macaques – exposure to Hurricane Maria (Gonzalez et al., 2023; Newman et al., 2023; Testard et al., 2021, 2024; Watowich et al., 2022). Hurricane Maria struck Puerto Rico, and the nearby island of Cayo Santiago, as a category 4 storm in September 2017 and caused substantial damage to both islands. Most of the research infrastructure on Cayo Santiago was destroyed, along with over 60% of the vegetation, which remains far below pre-storm levels even 5 years after the storm (Testard et al., 2021, 2024; Watowich et al., 2022). Macaques adjusted their social behaviour following the hurricane, becoming more socially tolerant

on average which allowed greater access to scarce shade that is important for thermoregulation. This social tolerance predicted survival after, but not before, the storm (Testard et al., 2024). At the genomic level, we previously found that macaques sampled after the storm had transcriptomic ages an average of 2 years older than their chronological age, while there was no difference in pre-hurricane samples, and that effects of hurricane-exposure broadly recapitulated those of primary aging on the peripheral blood transcriptome (Watowich et al., 2022). Together, this suggests that the storm and its aftermath may accelerate facets of biological aging.

To investigate how primary aging and secondary aging via the environmental adversity of a major hurricane affect DNA methylation, we combined detailed demographic data with 571 peripheral blood samples collected from 499 animals to address four goals. First, we described how DNA methylation levels vary across the lifespan and following a major natural disaster to characterise the effects of primary aging and exposure to natural disaster on the methylome. Second, we quantified the extent to which signatures associated with natural disaster were distinct from or similar to (i.e. secondary aging) signatures of primary aging. Third, we used transcriptomic data to probe the relationship between primary and secondary age effects on DNA methylation and gene expression. Finally, while our sampling design consisted primarily of cross-sectional samples, we had repeated samples from 67 individuals, allowing us to compare the effects of age and hurricane exposure cross-sectionally to longitudinal intra-individual changes in methylation and gene expression.

2 | METHODS

2.1 | Study subjects and experimental design

Whole blood and behavioural data were collected from rhesus macaques (*Macaca mulatta*) living on the island of Cayo Santiago, a long-running field site established in 1938. Macaques are monitored daily by staff scientists from the Caribbean Primate Research Center who record births and other major life history events, providing highly detailed and accurate record of animals' birth dates. Whole blood samples and information about the animal's body condition and weight are collected during the annual trap and release period on Cayo Santiago, which occurs approximately 1–3 months before the breeding season (2010–2012: January–March, 2012–2013: November–February and 2015–2018: October–December). Age and sex-matched individuals were identified for biological sampling before the trapping period each year. Our study drew on samples collected from 2010 to 2018, although animals were not sampled in 2017, as Hurricane Maria devastated the island's infrastructure weeks before the sampling period was scheduled. When conditions allowed, we resumed biological sampling in 2018, with the aim of collecting samples from animals matched for age and sex to pre-hurricane samples. More details on sample collection is provided in Watowich et al. (2022).

2.2 | Biological sampling

Samples included in this study were collected during the annual trap and release period from animals living on Cayo Santiago. Veterinary staff drew whole blood from sedated animals into one 3 mL BD Vacutainer® EDTA tube, which was later used for DNA methylation data generation (Chiou et al., 2020; Goldman et al., 2022). Blood in the EDTA tube was immediately refrigerated for ~1–3 h before transport to the Puerto Rican mainland and long-term storage in −80°C. The DNA methylation dataset is comprised of 470 blood samples from 421 animals (median age of 9.09 years; *n* females = 260, *n* males = 210) from 2010 to 2016, and from 101 animals (median age = 7.17 years; *n* females = 56, *n* males = 45) in 2018, after Hurricane Maria. Detailed metadata for each sample in the DNA dataset can be found in Table S1. The RNA-seq dataset was generated from whole blood collected in 2.5 mL PAXgene Blood RNA Tubes (PreAnalytiX GmbH). The RNAseq dataset is comprised of 435 blood samples from 357 animals (median age of 6.94 years; *n* females = 174, *n* males = 261) sampled from 2013 to 2016 and 108 animals sampled in 2018 (median age = 6.05 years; *n* females = 57, *n* males = 51). For a detailed description of the RNA dataset blood sampling and handling and metadata for the RNAseq dataset, see (Watowich et al., 2022). As part of ongoing population management, a subset of animals in 2016 were transported from Cayo Santiago to the Sabana Seca Field Station where the standard blood collection procedures were performed (2016: DNA dataset: *n* = 89, RNA dataset: *n* = 95). Due to the limited infrastructure on Cayo Santiago following Hurricane Maria and as part of the same population management, animals sampled in 2018 were also trapped on Cayo Santiago and sampled at Sabana Seca (2018: DNA dataset: *n* = 101, RNA dataset: *n* = 108).

2.3 | DNA/RNA extraction and sequencing and data preprocessing

Detailed methods of sample extraction, sequencing, and initial data quality control are provided in the Supplemental Methods. Briefly, we generated the DNA methylation dataset by extracting DNA from EDTA-anticoagulated whole blood and measuring CpG methylation using reduced representation bisulphite sequencing (RRBS) (Meissner et al., 2005). RNA was extracted from RNA-stabilised whole blood stored in PAXgene tubes and sequenced using a 3′ RNA sequencing approach, TM3′Seq (Pallares et al., 2020). RRBS and RNAseq reads were mapped to the rhesus macaque reference genome (Mmul_10). We quantified the number of methylated and unmethylated CpGs in each sample using Bismark (Krueger & Andrews, 2011). CpG sites with data in greater than 100 individuals and variably methylated sites (>10%, <90% methylated) were retained for further analysis (*n* = 253,076 CpGs). For the RNAseq data, we excluded lowly expressed genes and seven haemoglobin and ribosomal RNA subunit genes from the analysis, resulting in 7009 genes we carried through our analysis.

2.4 | Modelling the effects of age and adversity on DNA methylation

CpG methylation was modelled using a binomial mixed modelling framework, to model methylated and total counts as a function of age, Hurricane Maria exposure (coded as binary: 0/1), sex, and controlling for relatedness (generated from the Cayo Santiago population pedigree) and technical effects of DNA extraction batch. Modelling was performed using MACAU implemented in the PQLseq package in R (Lea et al., 2015; Sun et al., 2018; Sun et al., 2019, p. 20). For each main variable modelled (i.e. age, sex and Hurricane Maria exposure), per cent variance explained was calculated using the following equation (Figure S3):

$$\text{beta}(x)^2 * \text{var}(x) / \left(\left((\text{beta}(y)^2 * \text{var}(y)) + (\text{beta}(n..)^2 * \text{var}(n..)) \right) + \text{sigma}^2 \right)$$

We also investigated the relationship between DNA methylation and gene expression from a dataset that was previously generated and partially overlapping our DNA methylation dataset (Figure 1a) (Watowich et al., 2022). We quantified the relationship between DNA methylation and gene expression in several ways which are detailed in the Appendix S1 (section *Correlation between DNA methylation and gene expression*).

2.5 | Defining differentially methylated regions

Regions of multiple CpGs that vary across aging in a coordinated manner – differentially methylated regions (DMRs) – are expected to be more functionally relevant and impactful on gene regulation than single CpG sites (Lea et al., 2016). We defined differentially methylated regions as regions with higher than expected density of significantly age-associated (or, separately, hurricane-associated) sites and coordinated methylation patterns. To identify DMRs, we focused on 'focal' CpG sites (FDR < 5%) for our variable of interest (i.e. age) and scanned for (i) strictly significant (FDR < 5%), (ii) loosely significant (FDR < 10%) and (iii) all other CpGs within ±2000 base pairs of the focal site. We then performed 10 permutations for each variable of interest to determine the median number of loosely significant CpGs significantly within DMRs by random chance (Lea et al., 2016). We retained DMRs that had more than the median number of CpGs associated with the variable of interest at the relaxed threshold significance threshold determined by the permutations. Further, putative DMRs were removed if fewer than 75% of the effect sizes for all CpGs in the DMR or CpGs that were significant at the relaxed threshold were in the same direction (https://github.com/mwatowich/Immune-gene-regulation-is-associated-with-age-and-environmental-adversity-in-a-nonhuman-primate). DMRs were merged if they overlapped but we removed three DMRs that were longer than 10,000 base pairs, as these were 13× less CpG-dense than DMRs shorter than 10,000 base pairs. The effect size attributed to the DMR was the median effect size of all CpG sites within the DMR. We annotated whether DMRs intersected with different chromatin states and regulatory regions in the same way as individual CpG sites.

2.6 | Enrichment analyses overview

We performed enrichment analyses to examine putative biological function of CpG sites and DMRs. We annotated CpGs in several ways which are described briefly here but detailed in the Appendix S1. First, we annotated CpGs by the genomic regions they fell in, such as promoters, gene bodies, CpG shores, islands and enhancers or other chromatin states annotated from the Roadmap Epigenomics Project ChromHMM models of human primary mononuclear cells and used the web browser-based liftOver program to lift coordinates to the macaque mmul_10 genome (Ernst & Kellis, 2012; Kent et al., 2002; Quinlan & Hall, 2010). Second, we annotated genomic regions by chromatin accessibility, both from ATAC-seq data generated in rhesus macaque PBMCs (Snyder-Mackler et al., 2019) and characterised in human PBMCs (Márquez et al., 2020). Third, we annotated CpGs within TEs using the RepeatMasker annotation track from UCSC's Genome Browser database for the mmul_10 genome (Karolchik et al., 2004; Kent et al., 2002). Fourth, we performed several gene set enrichment analyses which are detailed in the Appendix S1. Next, we focused our analysis on the DMR-level rather than the level of single CpG sites and asked whether age-associated DMRs were more likely to be (i) in regions known to be differentially methylated with aging from human epigenome-wide association studies (Li et al., 2019; Xiong et al., 2022) or (ii) enriched for transcription factor binding motifs (Castro-Mondragon et al., 2022).

2.7 | Correlation between cross-sectional analysis and repeated samples

While the majority of our DNA methylation dataset consisted of cross-sectional samples – and we thus analysed the dataset as cross-sectional, controlling for repeated samples using random effects models – we had 67 animals that were sampled at least two time points during our data collection. This allowed us to perform several analyses to determine whether samples obtained from the same individual over time recapitulated trends found from our cross-sectional analyses. To do so, we determined the frequency that repeated samples were in the same direction as expected from the cross-sectional study results (repeated samples in DNA dataset = 67; RNA = 89). We determined the proportion of methylation for repeated samples for each age-associated DMR and subtracted the proportion methylated at the oldest time points from that of the youngest. We performed this analysis at the DMR-level as we expected that DMR methylation would be more stable than single CpG sites. For each DMR, we calculated the per cent of individuals with a positive difference in proportion of methylation between oldest and youngest time points. Pearson's correlation

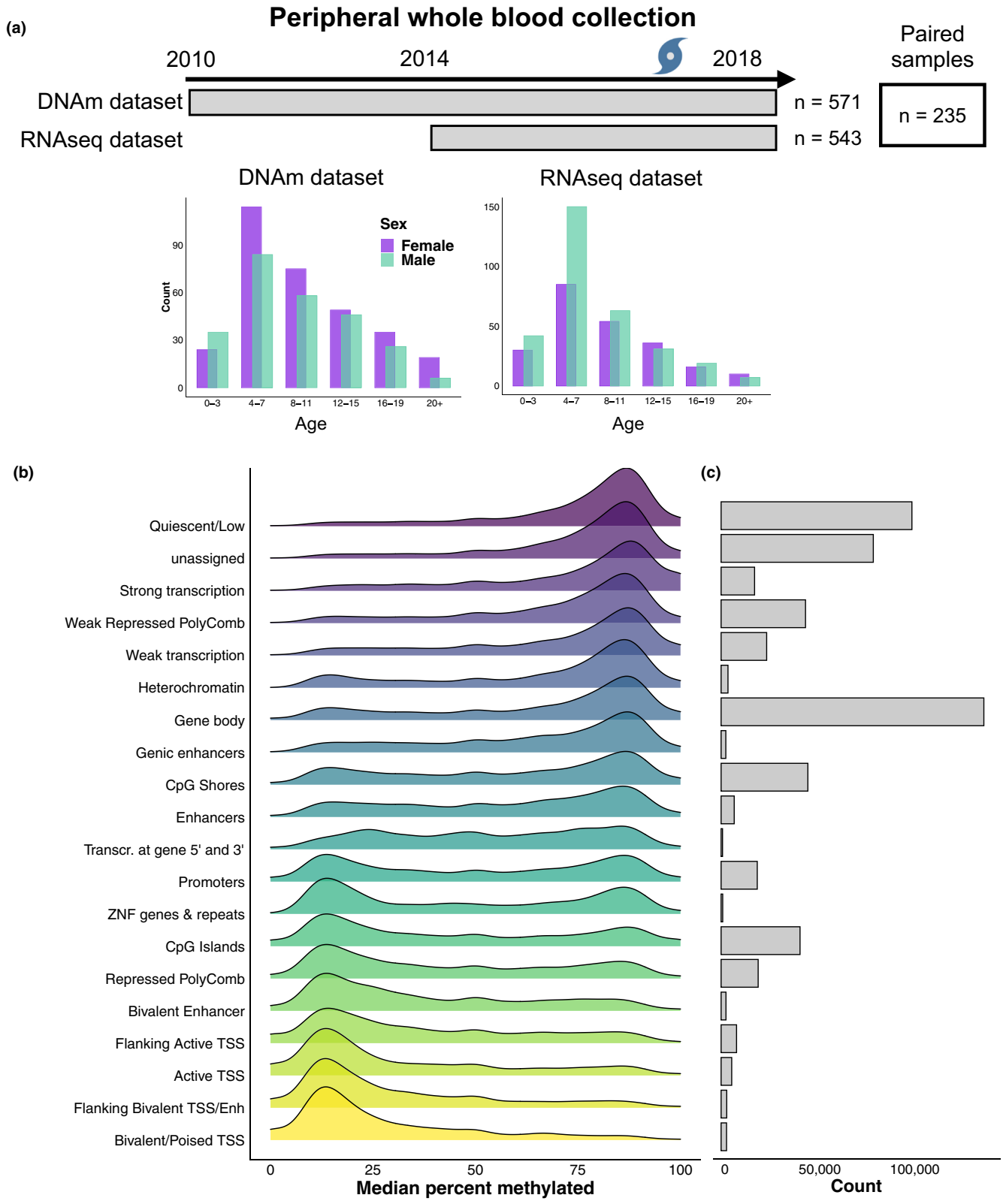

FIGURE 1 Sampling design and RRBS coverage. (a) Schematic summary of the sampling design. Peripheral whole blood was collected from rhesus macaques on Cayo Santiago from 2010 to 2018 and stabilised RNA was collected from 2014 to 2018. Sampling did not occur in 2017 due to Hurricane Maria. Both datasets include animals from infancy to old age, with an approximately even split of females and males. Paired DNA methylation and RNA sequencing data was available for 235 samples. (b) The median per cent methylation of sites and the (c) number of sites within each genomic region in the filtered RRBS dataset.

tests were used to test the association between the per cent of individuals with a positive change (more methylation when older) and the standardised betas for a given variable from the models of cross-sectional samples. We performed this analysis for age-associated DMRs and Hurricane Maria-associated DMRs. We also performed the same analysis for our gene expression dataset, subtracting gene expression levels in older repeated samples from those in the younger sample for the same individual. For the gene expression dataset, we used the same modelling design as DNA methylation – a model of gene expression as a function of age, sex, Hurricane Maria exposure, technical effects and kinship using our entire gene expression dataset ($n = 543$). We compared standardised effect sizes of age to the per cent of genes in repeated samples that changed in the expected direction.

3 | RESULTS

3.1 | Age and hurricane exposure are associated with global immune cell hypomethylation

First, we identified differential DNA methylation associated with age and exposure to a hurricane at 253,076 CpG sites across the genome in a sample of animals spanning their natural lifespan, using a cross-sectional modelling approach ($n = 573$; 0.1–29 years; information about retained sites is in Figure 1a–c). As expected, chronological age was associated with many differences in DNA methylation levels across the genome – 31% of tested CpG sites were significantly associated with age ($n = 78,661$; FDR < 5%; Table S2). Consistent with prior studies in humans and other mammals, we identified ~4× as many sites that were less methylated (i.e. 'hypomethylated') in older animals than sites that were more methylated with age (i.e. 'hypermethylated'; n sites hypomethylated = 63,931, n hypermethylated = 14,730; Figure 2a) (Jones et al., 2015). Exposure to Hurricane Maria was significantly associated with differential methylation of 32,048 CpG sites (Table S2). Similar to age, the vast majority of these sites (~90%, $n = 29,234$) were less methylated in animals sampled after the storm, while 2814 were hypermethylated in the post-storm sample in comparison to the age and sex-matched samples collected prior to the storm (Figure 2b).

As CpGs are often clustered together and because aggregate methylation of proximate sites is expected to exert stronger effects than single CpGs, we grouped proximate age-associated CpG sites into 'DMRs' (see Section 2.5). We identified 5006 DMRs associated with age (hereafter 'age DMRs'), and these DMRs showed a similar distribution of hypo- to hypermethylation as single sites (n hypermethylated age DMRs = 3659, n hypermethylated = 1347). Age DMRs contained a median of 6 CpG sites, a median of 5 CpGs associated with age at a relaxed threshold (FDR < 10%) and a median of 4 CpGs associated with age at a stringent threshold (FDR < 5%). We also quantified 2349 DMRs associated with hurricane exposure. Hurricane DMRs also followed the distribution of effect sizes

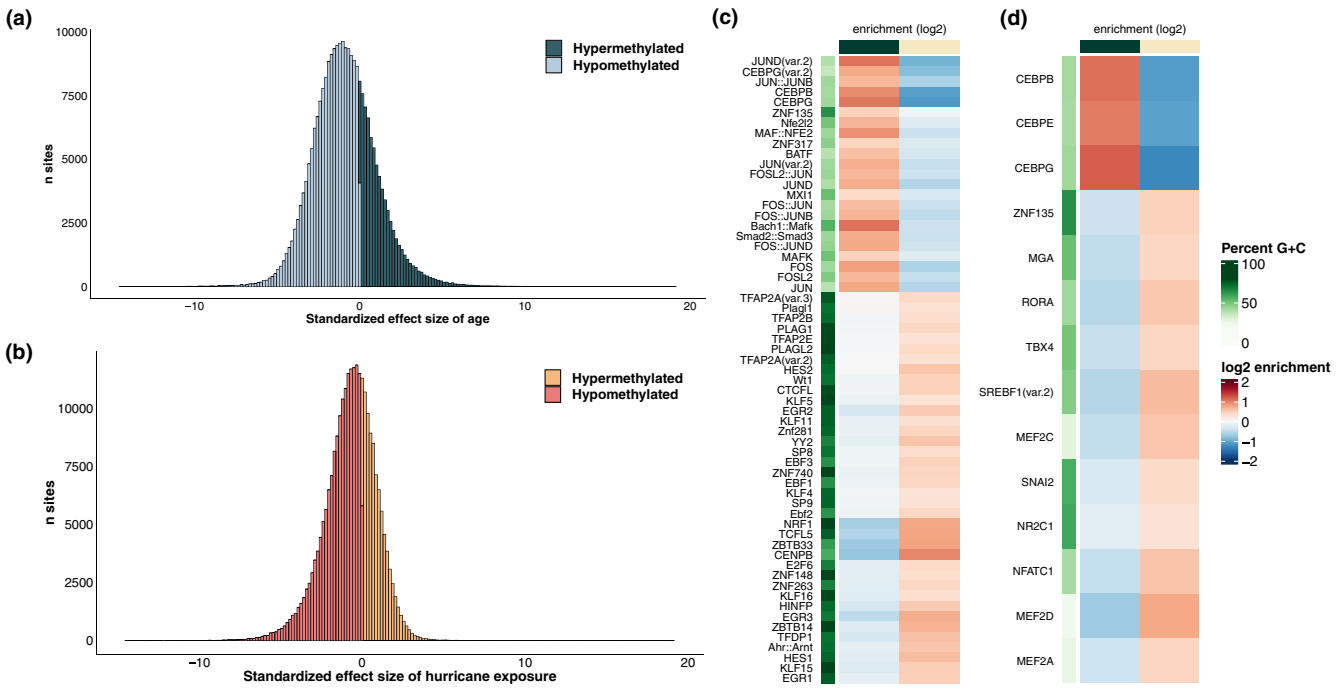

FIGURE 2 Age and hurricane exposure are associated with global hypomethylation. The distribution of standardised effect size of (a) age and (b) hurricane exposure. (c) Age and (d) hurricane-associated DMR transcription factor-binding site enrichment. The green bar on the x-axis shows enrichment statistics for hypomethylated DMRs while the white bar represents hypermethylated DMRs.

we observed at the single-site level, with 2327 being hypomethylated in hurricane-exposed animals and 22 being hypermethylated. Hurricane-associated DMRs contained a median of 7 CpG sites, a median of 5 at a relaxed FDR threshold of 10% and a median of 4 that passed a strict FDR threshold of 5%.

To confirm that the age-associated effects we observed showed effects consistent with those reported by previous studies, we tested whether our age-associated DMRs were more likely to overlap loci found to be significantly associated with age in human studies and indeed observed significant enrichment of age DMRs and age-related sites on both the 450K and EPIC arrays (450K: OR = 2.59, $p = 3.78 \times 10^{-27}$, OR = 3.29, $p = 5.86 \times 10^{-74}$) (Li et al., 2019; Xiong et al., 2022).

Genes in and near hypomethylated age DMRs – in which less methylation would canonically predict upregulation of gene expression – trended towards being enriched for inflammatory pathways, including the interferon alpha and gamma responses, IL-6/JAK/STAT3 signalling, inflammatory response and TNFa signalling via NFkB (Table S3), though no gene sets passed a false discovery rate threshold of 5%. To understand whether certain regulatory networks were potentially affected by aging, we next investigated whether age-associated methylation near DMRs were enriched for specific regulatory factors [i.e. transcription factor-binding site (TFBS) motifs]. Strikingly, hypomethylated DMRs were enriched for motifs of TFs associated with inflammation and the oxidative stress response (Figure 2c). For example, hypomethylated DMRs were enriched for Bach1, a major regulator of the oxidative stress response, NFE2L2, which partially regulates the NLRP3 inflammasome in conjunction with c-Jun, and five members of the AP-1 complex (JUND, JUN, MAFK, FOS and FOSL2), which is increasingly recognised to contribute to age-related inflammation (Figure 2c) (Garces de los Fayos Alonso et al., 2018).

Conversely, genes in and near hypermethylated age DMRs (i.e. canonically downregulated) trended to be enriched for genes in the peroxisome pathway, DNA repair, MYC targets V1, PI3K/Akt/mTOR and the G2/M DNA damage checkpoint pathways, which are pathways often associated with repair processes and age-related immune dysregulation, although no processes passed a false discovery rate threshold of 5% (Table S3). Hypermethylated age DMRs were enriched for several TFs in the ZBTB family, which is implicated in development and lineage commitment, and NRF1, which activates expression of metabolic genes and cell growth, as well as several zinc finger proteins (Figure 2c) (Cassandri et al., 2017; Cheng et al., 2021). Interestingly, ZBTB33 was also enriched among hypermethylated age DMRs. *ZBTB33* encodes the transcriptional regulator KAISO, which can promote histone deacetylation and heterochromatin formation, mechanisms proposed to suppress gene expression of inflammation, cell proliferation and apoptosis (Chen et al., 2015).

Hypomethylated hurricane DMRs trended to be enriched for hallmarks associated with inflammation (i.e. interferon responses and IL-6 signalling) (Table S4). Hypomethylated hurricane-associated DMRs were enriched for three transcription factors in the CEBP family, which play an important role in the inflammatory response and myeloid differentiation, regulate acute-phase cytokine genes, and can have an antiproliferative effect on T cells (Figure 2d). As there were few hypermethylated hurricane DMRs, we compared hypomethylated DMRs to hypermethylated DMRs and DMRs not significantly associated with the hurricane. The transcription factors enriched for hypermethylated and background hurricane DMRs appeared to have less specificity in their function, but included three members of the MEF2 family, which is involved in cell and tissue differentiation and proliferation (Figure 2d) (Pon & Marra, 2015).

3.1.1 | Age and hurricane exposure are associated with similar suites of CpG sites

Next, we quantified the extent to which the effects of age and hurricane exposure affected similar CpG sites in three ways. First, we asked whether age and hurricane-associated CpG sites were more likely to overlap than expected by chance and found that 37% of hurricane-associated CpG sites were also significantly associated with age, a significant enrichment between site-level effects (OR = 1.35, $p = 1.75 \times 10^{-124}$). Second, we also observed that age and hurricane effects were almost 2× more likely to overlap than expected by chance at the DMR-level (OR = 3.56, $p = 4.65 \times 10^{-168}$), with 51% of hurricane DMRs also being characterised as age DMRs ($n = 1194$). Third, we further probed the overlap of age and adversity effects by asking whether age-associated DMRs were more likely to overlap sites associated with childhood adversity from human studies, and, conversely, if hurricane-associated DMRs were enriched for sites found to be associated with aging from human studies (see Appendix S1 section *EWAS enrichment* for details). Indeed, we found that age DMRs were more likely to overlap sites associated with childhood adversity (OR = 1.4, $p = .01$), and hurricane DMRs were more likely than expected to overlap sites associated with age from human studies, both for the 450 K and EPIC arrays (450 K OR = 1.76, $p = 1.37 \times 10^{-8}$; EPIC OR = 1.69, $p = 1.23 \times 10^{-11}$) (Li et al., 2019; Xiong et al., 2022). Additionally, the results of age and hurricane gene set enrichment were moderately correlated ($r = .38$, $p = .01$) and more hallmarks than expected were affected in the same direction by age and hurricane exposure (OR = 4.61, $p = .03$; Figure S1), suggesting that age and hurricane exposure may broadly affect similar biological pathways.

3.1.2 | DNA methylation predicts gene expression

DNA methylation regulates gene expression, with higher methylation levels canonically repressing expression, and we investigated the extent to which this relationship was observed at multiple levels of the methylome for the effects of age and hurricane exposure. Age-associated hypomethylated sites were less likely to fall in or within 50 kb of blood-expressed genes (OR = 0.82, $p = 1.51 \times 10^{-96}$), while hypermethylated age-associated sites were more

likely (OR = 1.3, $p = 2 \times 10^{-55}$). As canonically expected, the effects of age at the single CpG site level were negatively correlated with effects of age on gene expression. This negative relationship was observed among (i) all gene-CpG pairs (i.e. CpG sites within 50 kb of a gene, $r = -.02$, $p = 9.26 \times 10^{-27}$) and more strongly among (ii) CpG site-gene pairs that were both significantly associated with age ($r = -.068$, $p = 1.064 \times 10^{-11}$). When we specifically investigated this relationship in promoters, effects of age at CpG sites in promoters were more negatively correlated with gene expression of the nearby gene than among all sites ($r = -.1$, $p = 4.4 \times 10^{-13}$) and this effect was even stronger among promoter CpG site-genes pairs where the site and gene were both significantly associated with age ($r = -.34$, $p = 2.87 \times 10^{-7}$). Finally, the association between age effects of promoter CpG sites and downstream genes was strongest for sites closest to transcription start sites (TSS) and weaker for more distal promoter CpG sites (Figure 3). For example, CpGs significantly associated with age and within 500 base pairs of age-associated gene TSS were more strongly correlated ($r = -.65$, $p = 3.11 \times 10^{-7}$) than CpGs between 0 and 1000 bp from TSS ($r = -.43$, $p = .02$).

Hurricane-associated CpGs were more likely to fall in and near (±50 kb) genes expressed in whole blood (OR = 1.5, $p = 1.59 \times 10^{-242}$), specifically near genes significantly associated with hurricane exposure (OR = 1.11, $p = 1.89 \times 10^{-3}$; hurricane-associated genes determined from mixed-effects linear models detailed in Watowich et al., 2022 and passing a FDR < 10%). Hurricane-associated sites also showed the expected negative relationship with gene expression when we tested all site-gene pairs ($r = -.0048$, $p = .04$) and pairs for which the CpG site and gene were significantly associated with hurricane exposure ($r = -.08$, $p = 8.30 \times 10^{-3}$). We did not observe a statistically significant relationship for hurricane-associated sites within promoter regions and the expression of downstream genes.

3.2 | Effects of primary aging and environmental adversity co-occur but have divergent signatures dependent on genomic region

3.2.1 | Older age is associated with hypermethylation of active regulatory states and less methylation in quiescent regions

We next tested whether sites associated with age and hurricane exposure were more likely to occur in particular regions of the genome, based on the direction of effect. Here, we tested genomic regions including gene bodies, promoters, CpG islands, CpG shores and unassigned regions (i.e. not otherwise categorised), as well as chromatin states based on ChromHMM states from human peripheral blood mononuclear cells. Consistent with previous observations in humans, hypermethylated age-associated CpG sites were more likely to fall in regions of active transcription, including CpG islands, promoters, gene bodies, enhancers, and regions annotated as active TSS according to their histone profiles, trends that were recapitulated at the DMR level (Figure 4a; CpG islands: OR = 7.44, $p < 10^{-25}$, promoters: OR = 1.97, $p = 5.95 \times 10^{-134}$, gene bodies: OR = 1.44, $p = 2.14 \times 10^{-97}$, enhancers: OR = 1.15, $p = 4.11 \times 10^{-3}$, active TSS: OR = 1.83, $p = 2.86 \times 10^{-35}$; Table S5; *DMR results* Table S6).

Hypomethylated age-associated sites were more likely to fall in putatively non-regulatory or weakly regulatory regions, such as regions annotated as weak repressed polycomb, weak transcription, genic enhancers, unassigned regions and – overwhelmingly – quiescent regions (weak repressed polycomb: OR = 1.05, $p = 3.20^{-5}$, weak transcription: OR = 1.06, $p = 1.11 \times 10^{-4}$, genic enhancers: OR = 1.11, $p = 2.75 \times 10^{-2}$, unassigned: OR = 1.67, $p < 10^{-25}$, quiescent regions: OR = 1.83, $p < 10^{-25}$; Figure 4a; Table S5). Strikingly, over 40% of age-associated sites were located in quiescent regions ($n = 34{,}551$) and 94% of these were hypomethylated. Quiescent

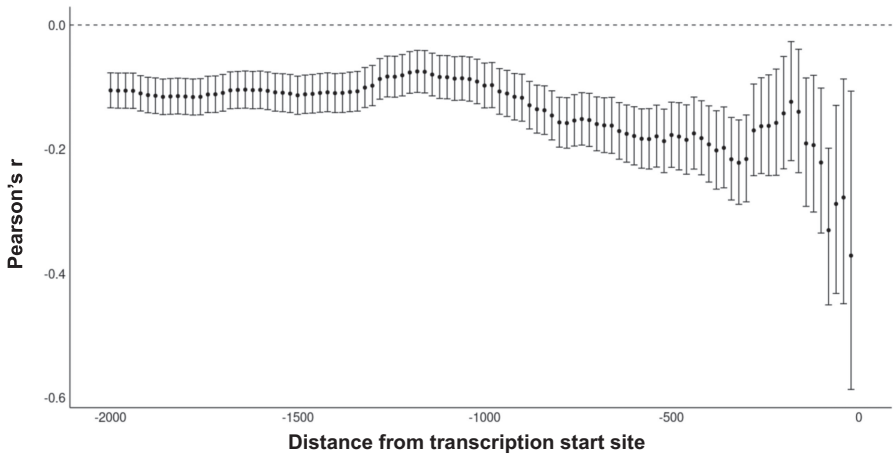

FIGURE 3 Correlation of the effects of age between CpG sites in promoter regions and downstream genes. Points show the median Pearson's *r* value (among all CpGs) for the correlation between the effects of age on CpG methylation and gene expression, with 95% confidence intervals shown. Bins represent 20 base pair units.

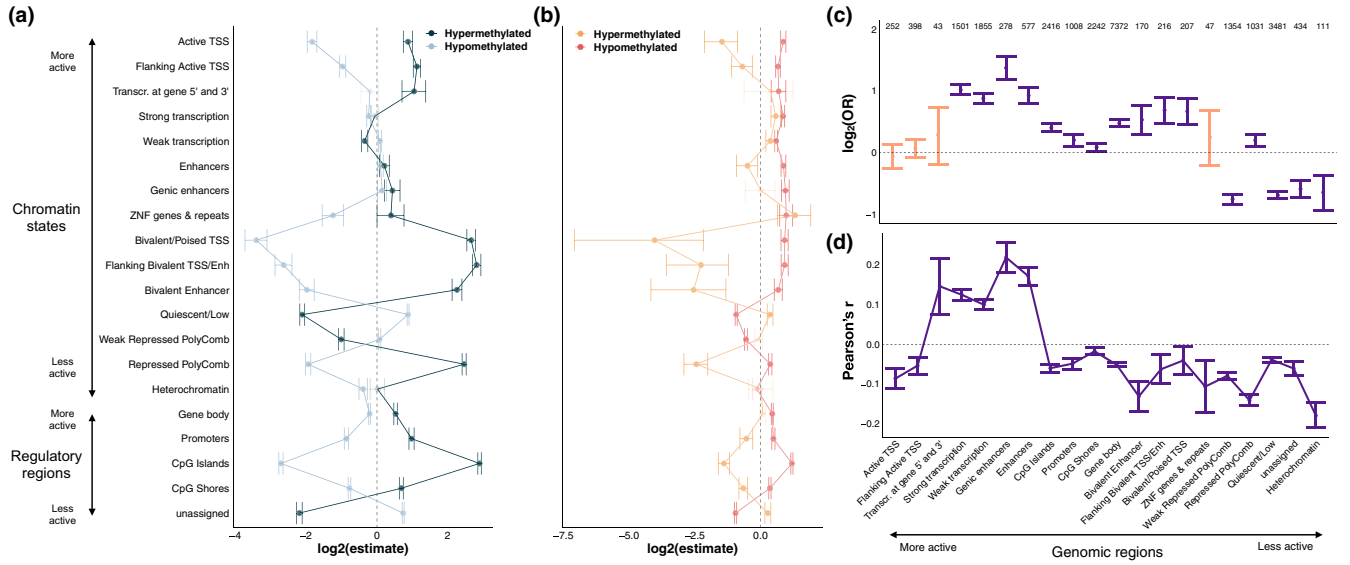

FIGURE 4 Primary and secondary age effects are enriched and more correlated in areas of active gene regulation. Enrichment of hyper- and hypomethylated (a) age-associated and (b) hurricane-associated sites in different genomic regions and chromatin states. (c) Enrichment of overlapping significant effects of age and hurricane exposure. The number of CpG sites in each region is noted at the top of the plot. (d) Correlation between the effects of age and hurricane exposure is shown for all CpG sites within each genomic region. On all plots, confidence intervals crossing the dotted line at zero are not significantly enriched or correlated and are denoted with transparency or a lighter colour.

regions are putatively non-regulatory regions that are devoid of histone modifications and constitutively highly methylated (Hoffman et al., 2013). The loss of methylation in constitutively highly methylated regions is hypothesised to enable increased activity of deleterious elements and contribute to immune dysregulation with aging (Jones et al., 2015; Seale et al., 2022). We predicted that age-associated hypomethylated sites in quiescent regions may be in or near genes implicated in immune dysregulation and found that genes hypomethylated in quiescent regions were enriched for positive regulation of apoptotic processes ($p = 1.8 \times 10^{-3}$), interferon-gamma production ($p = 8.77 \times 10^{-3}$), negative regulation of cell migration ($p = 3.72 \times 10^{-3}$), negative regulation of cytokine production ($p = 8.19 \times 10^{-3}$) and inflammatory processes ($p = 4.46 \times 10^{-03}$; Table S7). We note that many other processes were significantly enriched among hypomethylated genes, including several processes related to transcriptional regulation, positive regulation of apoptotic processes ($p = 1.80 \times 10^{-3}$) and regulation of cellular response to stress ($p = 1.80 \times 10^{-3}$; Table S7).

3.2.2 | Hurricane exposure is associated with hypomethylation in active regulatory states and hypermethylation in quiescent regions

While hurricane-associated CpG sites were globally hypomethylated – as were age effects – the genomic regions in which differential methylation occurred were strikingly different. Sites hypomethylated in post-hurricane samples were enriched in nearly all areas of the genome associated with active transcription and gene regulation, such as promoters, enhancers, gene bodies and areas near TSS, and were only depleted in quiescent regions, weak repressed polycomb and regions not otherwise annotated (Figure 4b; Table S8), and this was recapitulated at the DMR-level (Table S9). Approximately half of hypermethylated CpG sites in the post-hurricane samples were located in quiescent regions ($n = 1288$, OR = 1.28, $p = 2.66 \times 10^{-10}$). Hypermethylated hurricane-associated sites were also enriched in genomic regions associated with strong and weak transcription, and regions not otherwise annotated (strong transcription: OR = 1.49, $p = 5.11 \times 10^{-9}$, weak transcription: OR = 1.29, $p = 3.87 \times 10^{-5}$, unassigned: OR = 1.21, $p = 3.15 \times 10^{-6}$; Figure 4b; Table S8).

3.2.3 | Age and hurricane exposure-associated effects overlap in active regulatory regions

We next tested where age and hurricane effects were most likely to overlap and found substantial enrichment in areas associated with active gene regulation. Specifically, sites significantly associated with age and hurricane exposure were more likely to overlap in enhancers, gene bodies, promoters, CpG shores and islands, as well as chromatin states associated with strong and weak transcription and near bivalent/poised TSS (Figure 4c; Table S10). Age and hurricane effects were less likely to overlap in quiescent and heterochromatin regions, as well as weak repressed polycomb complexes (Figure 4c; Table S10).

Following our observation that primary age effects and the effects of hurricane exposure co-occurred in particular genomic

regions with greater frequency than expected, we next asked whether these effects were differently correlated among regions. Specifically, we quantified the correlation among effects of environmental adversity and chronological age. Among all sites, the effects of hurricane exposure were slightly negatively correlated with those of age ($r = -.07$, $p = 7.48 \times 10^{-275}$). Within specific chromatin or regulatory regions, the correlation between the effects of hurricane exposure and age was generally modest. Interestingly, the association was strongest and generally positive in enhancers and chromatin regions associated with transcription, and negative among less active regulatory regions. Together, this may suggest that the effects of secondary aging may be more similar to primary aging within genomic contexts associated with active gene regulation (Figure 4d; Table S11).

3.2.4 | Age and hurricane exposure have distinct signatures in repetitive elements

One mechanism that has been suggested to partially explain immunosenescence and dysregulation with aging is increased activity of deleterious elements such as TEs. In particular, it is expected that in genomic regions that are constitutively highly methylated and become less methylated with age, the epigenetic suppression of deleterious elements is reduced (López-Otín et al., 2023; Pal & Tyler, 2016). Thus, we investigated whether age-associated hypomethylated sites were enriched in or near (±50 kb) TEs, which could potentially indicate a pathway for greater TE activation with increasing age. We observed that 84,282 CpG sites (33.3%) were in TEs, including 28,684 age-associated sites and 7936 hurricane-associated sites. Of the TE types we observed, CpGs in our dataset primarily overlapped long tandem repeats ($n = 13{,}729$) and short and long interspersed retrotransposable elements ($n = 53{,}073$ and $n = 9773$ respectively). TEs were enriched in putatively non-regulatory regions, including quiescent regions, heterochromatin and chromatin states associated with weak transcription (Table S12). We found that sites in TEs were more likely to be significantly associated with aging than expected (OR = 1.24, $p = 1.64 \times 10^{-110}$). Consistent with prior studies, age-associated CpG sites in TEs were more likely to be hypomethylated than expected (OR = 1.69, $p < 10^{-25}$); this effect was even stronger when we limited our test to only significantly age-associated sites (OR = 5.41, $p < 10^{-25}$; Figure 5a).

Hurricane-associated CpG sites were less likely to overlap TEs than expected by chance (OR = 0.63, $p = 1.21 \times 10^{-275}$). Hurricane-associated sites in TEs were less likely to be hypomethylated after the hurricane (OR = 0.56, $p < 10^{-25}$) and, in fact, were more likely to be hypermethylated (OR = 1.51, $p = 7.39 \times 10^{-26}$; Figure 5a). Notably, sites associated with both age and hurricane exposure were less likely to overlap TEs than expected by chance (OR = 0.81, $p = 1.80 \times 10^{-25}$), suggesting that differences in TE methylation may be signatures of primary, but not secondary, aging.

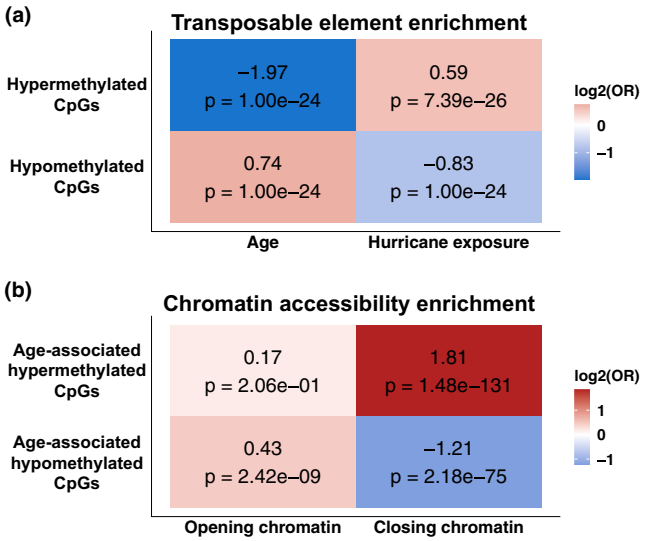

FIGURE 5 Enrichment of CpG sites in transposable elements and variably accessible chromatin regions. (a) CpGs that are differentially methylated with aging (left) or hurricane-exposure (right) overlap TEs more or less than expected by chance depending on the direction of methylation (e.g. hyper- or hypomethylation with age/hurricane-exposure). For example, in the top-left quadrant of (a), CpGs significantly hypermethylated with age are less likely to be located within TEs. (b) The extent to which age-associated hypo- and hypermethylated CpG sites are enriched/depleted for overlap with chromatin regions found to open or close with age in human PBMCs (chromatin accessibility characterised by Márquez et al., 2020).

3.2.5 | Age-associated methylation is coordinated with other epigenetic modifications

DNA methylation is one of several epigenetic modifications involved in gene regulation. To better understand how changes in DNA methylation coordinate with other epigenomic modifications, we used data generated from independent datasets. First, we asked whether age and hurricane-associated sites were enriched within or outside of regions of accessible chromatin, generated from rhesus macaque PBMCs (Snyder-Mackler et al., 2019). Hypermethylated age-associated sites were more likely to be located in areas of accessible chromatin (OR = 1.62, $p = 6.47 \times 10^{-65}$), while hypomethylated sites were depleted in accessible chromatin regions (OR = 0.57, $p = 2.51 \times 10^{-192}$). Fitting with our chromatin state enrichment analysis, hypomethylated hurricane-associated sites were more likely to overlap regions of open chromatin (OR = 1.74, $p = 1.58 \times 10^{-156}$), while hypermethylated sites were not statistically enriched.

Like other epigenetic modifications, chromatin conformation can change with age (Márquez et al., 2020) and can alter the structure and relative accessibility of the genome to molecules such as transcription factors. However, it remains unresolved the extent to which changes in chromatin accessibility and DNA methylation act independently or overlap – either to compound or negate one another – to alter genome accessibility during aging. We therefore

investigated the extent to which differential methylation overlapped age-dependent changes in chromatin accessibility identified in humans (Márquez et al., 2020). Hypermethylated age-associated CpGs were nearly two-times more likely to be in regions of chromatin that closed with increasing age (OR = 3.52, $p = 3.71 \times 10^{-132}$; Figure 5b), suggesting that previously accessible chromatin becomes less active with age due to twin processes of methylation and heterochromatin formation. Interestingly, in no genomic regions that we tested was there a depletion of hypermethylation and closing chromatin, demonstrating that these processes co-occur consistently in all areas of the genome we tested. Conversely, sites significantly hypomethylated with increasing age were more likely to be in chromatin regions that became more accessible with age (OR = 1.35, $p = 1.82 \times 10^{-9}$; Figure 5b). Together, our results suggest that age-associated changes in these two epigenetic modifications are highly correlated across the genome and, given their extensive coordination, these effects may compound and exacerbate dysregulation accrued during aging.

3.3 | Within-individual gene regulatory changes reflect cross-sectional estimates

With the repeated samples in our DNA methylation and gene expression datasets, we asked whether the results of our cross-sectional analyses recapitulated intra-individual DNA methylation changes across aging and hurricane exposure. We performed these analyses at the DMR level as we expected that the coordinated methylation changes in DMRs is likely more stable at two time points than that of single sites. Age effects from the cross-sectional analysis (i.e. standardised beta of age) were correlated ($r = .32$, $p = 5.38 \times 10^{-117}$; Figure 6a) with the proportion of repeated samples that showed increased methylation in animals sampled when they were younger compared to when they were older (n individuals = 67; years between sampling: median = 3.78, range = 0.78–7.86). Effects of exposure to Hurricane Maria from the cross-sectional analysis was also correlated with intra-individual methylation levels in animals sampled before versus after the storm (n individuals = 23, $r = .21$, $p = 1.19 \times 10^{-24}$; Figure 6b). We extended this analysis to our gene expression dataset which had 172 repeated samples from 81 individuals, with a median of 1.03 years between repeated samples and a range of 0.79–4.84 years. We found that, similarly to DNA methylation, the standardised effect size of age from the cross-sectional analysis was correlated with intra-individual gene expression changes ($r = .28$, $p = 1.82 \times 10^{-127}$; Figure 6c). This association became stronger when we tested only genes significantly associated with age (FDR < 10%) from the cross-sectional models ($r = .48$, $p = 7.63 \times 10^{-69}$). Notably, the effects of Hurricane Maria exposure and change in gene expression between pre- and post-hurricane Maria samples were highly correlated (n individuals with pre- and post-hurricane expression = 21, $r = .38$, $p = 2.68 \times 10^{-238}$), which increased among the 260 genes significantly associated with Hurricane Maria exposure (FDR < 10%) in the cross-sectional model ($r = .77$, $p = 5.26 \times 10^{-52}$; Figure 6d). Observing correlations between longitudinal methylation or gene expression changes and the results of cross-sectional models allows us to infer that our cross-sectional analyses are indeed capturing true variation in how aging and natural disaster induce immune cell genomic alterations.

4 | DISCUSSION

By integrating DNA methylation, gene expression, and demographic data from a naturalistic population of rhesus macaques studied at a long-term field site, we were able to characterise primary and secondary age effects. Specifically, we investigated the extent to which secondary aging via environmental adversity recapitulated the effects of primary aging at the epigenomic level in a population exposed to natural environmental variation. We found that, at the level of the methylome, primary aging effects were largely concentrated in quiescent and heterochromatin regions, and exhibited extensive hypomethylation in older individuals, consistent with prior studies in humans (Jones et al., 2015; Seale et al., 2022). We also found that DNA methylation may be responsive to extreme natural disasters and their aftermath. CpG sites associated with environmental adversity from a major hurricane were extensively hypomethylated and were primarily concentrated in areas associated with active gene regulation. Broadly, we observed that the effects of environmental adversity largely overlapped and were positively correlated with age-associated effects in areas of active gene regulation, such as enhancers, promoters, and gene bodies, but diverged in less active gene-regulatory regions, such as quiescent and heterochromatin regions.

Interestingly, we observed two potential pathways by which primary aging in rhesus macaques may generate immune dysregulation or genomic damage. First, we found that quiescent chromatin states were overwhelmingly more likely to be hypomethylated with age and that these sites were in and nearby genes involved in inflammatory pathways, suggesting potential de-repression of these genes with age. Further, we observed coordinated loss of methylation in regions that also became more accessible with age, and thus joint activation of two epigenetic modifications and potentially greater expression of deleterious elements or genes. Second, we found that TEs were far more likely to be hypomethylated in older individuals, suggesting that the epigenetic landscape becomes more permissible for TE mobilisation (Andrenacci et al., 2020; Gorbunova et al., 2021). The mobilisation of TEs can cause double-stranded DNA breaks, promoting genomic instability and triggering the inflammatory response, which may compound the effects of primary aging (Gorbunova et al., 2021). Interestingly, we observed broad hypermethylation of TEs in animals exposed to Hurricane Maria. This is surprising given that adverse conditions are generally expected to reduce epigenetic silencing capacity (Pappalardo et al., 2021). However, epigenetic-level changes in TE methylation following stressful events is not well-understood and appears to be highly dependent on the adversity and the TE type (Horváth et al., 2017; Pappalardo et al., 2021). For example, human

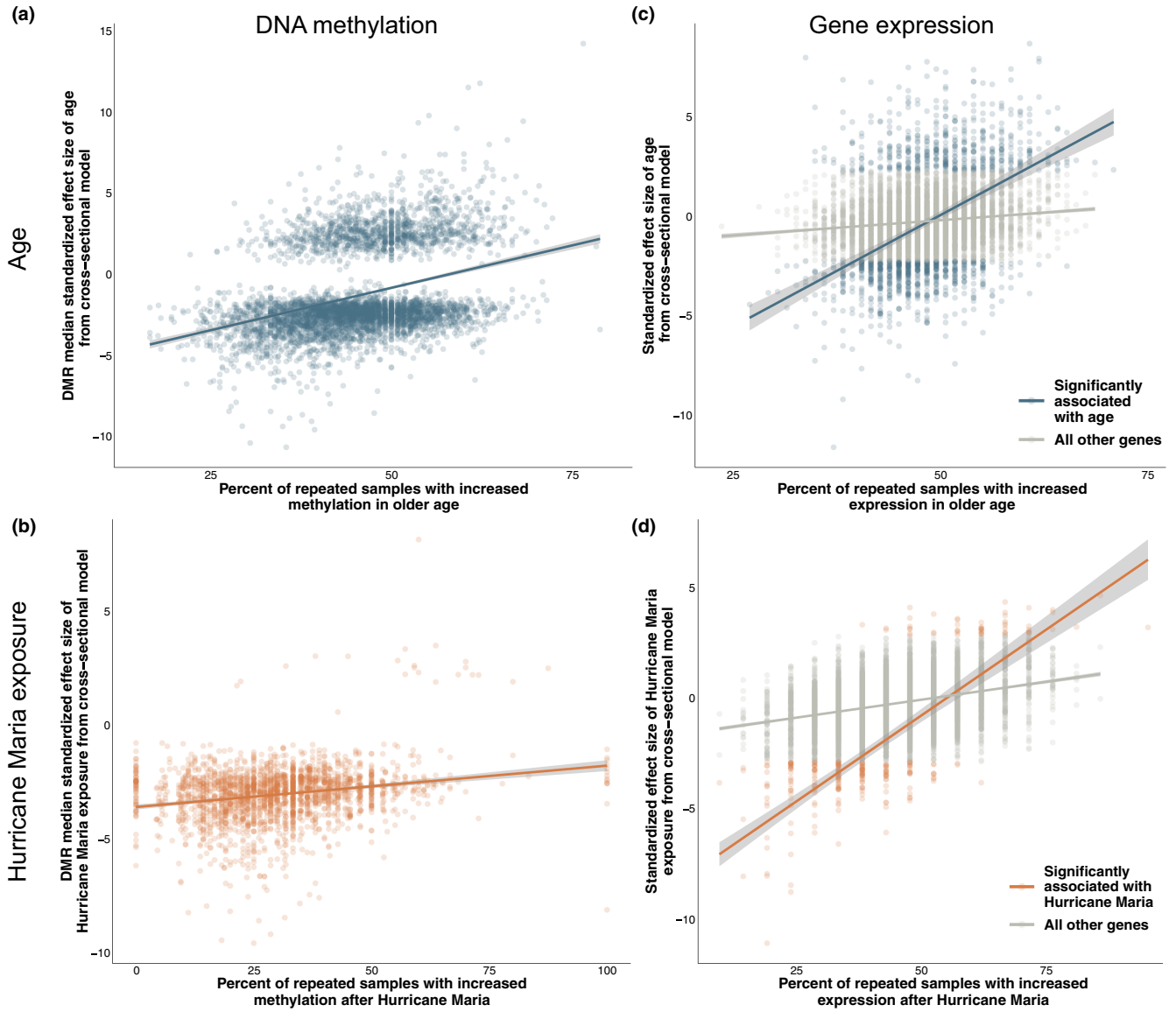

FIGURE 6 Cross-sectional results are correlated with intra-individual changes in DNA methylation and gene expression across aging and exposure to extreme natural disaster. (a) The per cent of repeated samples (i.e. same individual) that had greater proportion methylation at older age, compared to the standardised effect sizes of age for CpG sites. (b) The per cent of repeated samples with more methylation in post-hurricane samples compared to samples taken pre-hurricane, versus the median standardised effect size of Hurricane Maria exposure. The per cent of repeated samples with higher gene expression at older age (i.e. post-hurricane), versus the standardised effect size of age (c) and hurricane exposure (d).

neuroblastoma cells exhibited hypermethylation of LINE-1 elements when treated with morphine – which can induce oxidative stress – for short periods of time, but hypomethylation of LINE-1 elements when exposed for longer periods of time (Trivedi et al., 2014). This highlights the contextual and temporal dependence of epigenomic changes in response to stressors, which is currently not well-understood. It is possible that the global hypermethylation of TEs we observe is a time-dependent response that functions as an adaptive repair mechanism and future studies should seek to measure differential methylation at multiple time points following environmental perturbations.

Our findings also support gene regulatory effects of promoter methylation on downstream gene expression, particularly for the effects of primary aging. CpG sites in promoter regions associated with age were much more strongly correlated with gene expression than non-age-associated CpG sites or sites outside of promoter regions. CpG sites closer to TSSs tended to be more strongly associated with aging and also more highly correlated with downstream gene expression, suggesting that the sites close to the TSSs exert a greater influence on gene expression. We observed the expected canonically negative relationship between methylation and gene expression for hurricane-associated CpG sites near genes. However, we did not observe a significant relationship between hurricane-associated sites within promoter regions and the expression of downstream genes. This possibly suggests that the effects of

secondary aging are more stochastic in relation to gene regulation than those of primary aging, or could indicate a lack of power in our study to detect a relationship between the methylation of this subset of promoters and the expression of downstream genes.

We note that our study had some limitations. Methylation patterns vary between cell types and will likely be affected by changes in cell composition. In this study, we were unable to fully disentangle cell-intrinsic methylation changes from changes in cell composition, yet we found that cell composition changed as expected with age in this population (detailed in the Appendix S1 and results *Cell type heterogeneity* sections, Figure S2). Previously, we have found that immune cell-specific marker genes in animals sampled after Hurricane Maria showed generally similar patterns to those of age, suggesting that the hurricane and age had similar effects on cell composition (Watowich et al., 2022). Yet, future studies should measure cell-specific methylation levels to disentangle cell-specific and compositional effects on methylation. We also note that there may be non-linear age-related epigenomic changes that are not captured by our modelling strategy and future studies should explore the extent to which DNA methylation exhibits non-linear trajectories across aging and the overlap of these effects with secondary aging patterns.

While our study was largely cross-sectional, approximately one quarter of our samples were from animals repeatedly sampled at least 9 months apart. Among both the DNA methylation and gene expression datasets, we found moderate correlation of mean differences in methylation from the cross-sectional models and intra-individual changes in methylation and gene expression levels. Longitudinal sampling designs remain the gold-standard for detecting age-related changes and due to the opportunistic nature of our study, it is possible that animals sampled after the hurricane differed in ways that affected our results. However, the correlation we observed between the results from our cross-sectional study to those from the subset of longitudinal samples, highlights that cross-sectional designs should not be discounted and can, as we have done here, detect some variance that can likely be attributed to aging or environmental exposure. Indeed, that we observed correlation between intra-individual changes in methylation and gene expression across aging and hurricane exposure allows us to infer that our cross-sectional results are capturing true age-related (and hurricane-related) variation. Further, including both repeated and cross-sectional samples allows us to disentangle the effects of the hurricane from those of aging, which would be confounded in a purely longitudinal study.

In conclusion, we find that a strong environmental perturbation – surviving a major hurricane – was associated with DNA methylation patterns broadly similar to those of primary aging in genomic regions associated with active gene regulation, but diverged in putatively non-regulatory regions, suggesting damage-generating pathways specific to primary aging. While environmental insults leading to secondary aging can be detrimental and advance disease-progression, our findings suggest that environmental adversity contributes to specific aspects of secondary aging and partially, but not fully, overlaps with primary aging. Therefore, it may be possible to both disentangle how secondary aging advances immune dysregulation to inform strategies to mitigate negative consequences for survivors of extreme environmental hardships and to simultaneously reduce deleterious effects of primary and secondary aging by identifying moderators of aging that affect areas in which primary and secondary aging overlap.

AUTHOR CONTRIBUTIONS

M.M.W., M.J.M., C.B.R.U., J.E.H., M.I.M., J.P.H., L.J.N.B., M.L.P., A.J.L. and N.S.-M. designed research; M.M.W., C.E.C., K.L.C., E.A.G., M.J.M., R.M.P., S.P. and J.E.H. performed research; M.M.W., A.J.L. and N.S.-M. analysed data; and M.M.W., A.J.L. and N.S.-M. wrote the paper with input from all authors.

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DATA AVAILABILITY STATEMENT

RNA sequencing reads are available in the National Center for Biotechnology Information Short Read Archive (SRA project number 715739) (ID 715739—BioProject—NCBI, n.d.). DNA reads will be uploaded to SRA and made available upon publication under BioProject ID PRJNA610241(ID 610241—BioProject—NCBI, n.d.). Macaque chromatin accessibility data are available in the NCBI Sequence Read Archive, https://www.ncbi.nlm.nih.gov/bioproject (BioProject ID PRJNA476378) (ID 476378—BioProject—NCBI, n.d.; Snyder-Mackler et al., 2019).

BENEFIT-SHARING STATEMENT

Benefits Generated: This research demonstrates the utility of open-source genomic materials. Further benefits from this research include the sharing of our results on public databases as described above.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

ETHICS STATEMENT

All work with animals adheres to the joint Principles for the Ethical Treatment of Nonhuman Primates as set forth by the American Society of Primatologists and International Primatological Society. This research was reviewed and approved by the Institutional Animal Care and Use Committees of the University of Puerto Rico, Medical Sciences Campus (assurance number A400117).

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