



Social and physical environments early in development predict DNA methylation of inflammatory genes in young adulthood

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Chronic inflammation contributes to a wide range of human diseases, and environments in infancy and childhood are important determinants of inflammatory phenotypes. The underlying biological mechanisms connecting early environments with the regulation of inflammation in adulthood are not known, but epigenetic processes are plausible candidates. We tested the hypothesis that patterns of DNA methylation (DNAm) in inflammatory genes in young adulthood would be predicted by early life nutritional, microbial, and psychosocial exposures previously associated with levels of inflammation. Data come from a population-based longitudinal birth cohort study in metropolitan Cebu, the Philippines, and DNAm was characterized in whole blood samples from 494 participants (age 20–22 y). Analyses focused on probes in 114 target genes involved in the regulation of inflammation, and we identified 10 sites across nine genes where the level of DNAm was significantly predicted by the following variables: household socioeconomic status in childhood, extended absence of a parent in childhood, exposure to animal feces in infancy, birth in the dry season, or duration of exclusive breastfeeding. To evaluate the biological significance of these sites, we tested for associations with a panel of inflammatory biomarkers measured in plasma obtained at the same age as DNAm assessment. Three sites predicted elevated inflammation, and one site predicted lower inflammation, consistent with the interpretation that levels of DNAm at these sites are functionally relevant. This pattern of results points toward DNAm as a potentially important biological mechanism through which developmental environments shape inflammatory phenotypes across the life course.

epigenetics | inflammation | ecological immunology | developmental origins of health | developmental origins of disease

Inflammation plays a central role in immune defenses against infectious disease (1), but dysregulated or chronic, low-grade inflammatory processes contribute to the pathophysiology of a wide range of diseases, including cardiovascular disease, type 2 diabetes, and autoimmune/atopic conditions (2, 3). Inflammatory activity is also central to normal pregnancy, but dysregulated inflammation can contribute to pregnancy complications and adverse birth outcomes (4, 5). The factors that influence the regulation of inflammation are therefore relevant to clinical practice, and they are of central interest to current research in genomics and immunology as well as epidemiology, demography, and psychobiology (6–11).

Recent empirical work has identified environments in infancy and early childhood as important determinants of inflammatory phenotypes, and concepts from life history theory and ecological immunology have provided a theoretical basis for anticipating these associations (12–14). Individuals born at lower birth weight, and infants who are breastfed for shorter durations, have

higher concentrations of C-reactive protein (CRP)—a key biomarker of inflammation—as adults (15–19). Higher levels of microbial exposure in infancy are associated with reduced levels of chronic inflammation in adulthood (18, 20), consistent with broader human and animal model literatures showing that microbial exposures during sensitive periods of immune development have lasting beneficial effects on the regulation of inflammation (21–23). In addition, an emerging body of research in developmental psychobiology reports that major psychosocial stressors (e.g., child neglect, extended parental absence) and socioeconomic adversity in childhood are associated with dysregulated and proinflammatory activity in adulthood (15, 24–26).

Although the underlying biological mechanisms that connect early nutritional, microbial, and psychosocial environments with adult inflammatory phenotypes are not known, epigenetic processes are likely candidates for preserving cellular memories of early experience in the immune system. Indeed, recent human studies suggest that socioeconomic and psychosocial environments in infancy and childhood leave a molecular imprint that has lasting effects on genomic regulation and the developing phenotype (27–30). Of the many marks contributing to the epigenome, DNA methylation (DNAm) has been the major focus of human research and involves the covalent linkage of methyl groups to cytosine residues primarily in the context of CpG dinucleotides. Methylation of sites in gene promoter regions

Significance

Environments in infancy and childhood influence levels of inflammation in adulthood—an important risk factor for multiple diseases of aging—but the underlying biological mechanisms remain uncertain. Using data from a unique cohort study in the Philippines with a lifetime of information on each participant, we provide evidence that nutritional, microbial, and psychosocial exposures in infancy and childhood predict adult levels of DNA methylation—biochemical marks on the genome that affect gene expression—in genes that regulate inflammation. We also show that DNA methylation in these genes relates to levels of inflammatory biomarkers implicated in cardiovascular and other diseases. These results suggest that epigenetic mechanisms may partially explain how early environments have enduring effects on inflammation and inflammation-related diseases.

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limits access of transcriptional machinery and is typically associated with reduced gene expression, whereas methylation within gene bodies often is associated with enhanced expression (31). The potential biological relevance of these processes is underscored by several studies documenting associations between DNAm and expression of genes involved in the regulation of inflammation as well as biomarkers of inflammation and risk for inflammation-related disorders (30, 32, 33).

Few studies possess the range and time depth of prospectively collected measures needed to test the hypothesis that nutritional, microbial, and psychosocial environments early in development predict patterns of DNAm in inflammatory genes in adulthood. In addition, current understandings of the regulation of inflammation are based almost exclusively on studies with animal models and with human participants residing in affluent, industrialized settings like the United States (34, 35). A broader perspective is important, as 81% of the world's population resides in low- and middle-income nations (36), where rates of inflammation-related diseases—including cardiovascular, metabolic, atopic, and autoimmune diseases—are rapidly rising (37). The limited variation in microbial, nutritional, and socioeconomic environments in samples drawn from affluent, industrialized settings may constrain investigations into important developmental determinants of inflammatory phenotypes (35).

We use data from a long-term birth cohort study in the Philippines to address both these limitations. Data collection began in 1983, and DNAm as well as concentrations of inflammatory biomarkers were assessed in blood samples collected in 2005, when participants were ~21 y old. In addition to the prospective design, an advantage of the study is the relatively wide range of ecological variation represented by the sample: Participants were born into areas that were classified as urban residential, congested urban poor, peri-urban, and remote rural (38). In 1983, approximately half the homes had electricity, more than three-quarters collected water from an open source, and less than half used a flush toilet (18). Furthermore, the study initially focused on detailed and frequent assessment of the contextual determinants of maternal and infant health. Beginning with the first interview of the mother during pregnancy and continuing through the offspring's infancy and childhood, careful attention was given to detailed measurement of proximate nutritional, microbial, psychosocial, and socioeconomic exposures of relevance to growth and development.

We implemented a hypothesis-driven investigation of the association between DNAm in young adulthood and early life environmental exposures. Analyses focused on methylation sites in 114 target genes shown previously to regulate inflammation, which were identified before statistical analyses. The rationale for our targeted analytic approach was twofold. First, limiting our analysis to genes involved in the regulation of inflammation takes advantage of existing knowledge and increases statistical power for detecting meaningful biological associations. Second, venous blood sampling provides access to the tissue of interest—immune cells that increase and decrease inflammation—where patterns of DNAm in regulatory regions are most likely to have functional relevance for inflammation due to the tissue-specific nature of epigenetic processes (39).

We hypothesized that measures of early life environmental exposures, identified in prior research as important to shaping inflammatory phenotypes, would be significant predictors of DNAm in young adulthood. To evaluate the functional relevance of associations between early environments and DNAm, we considered whether differentially methylated sites predicted concentrations of inflammatory biomarkers, measured concurrently in young adulthood. Our analyses identified 10 differentially methylated sites across nine genes, four of which were associated with a composite measure of inflammation. These results provide support for DNAm as a biological mechanism through which experiences early in life may have lasting effects on the regulation of inflammation.

Results

DNAm was characterized in 494 participants with the Illumina 450K array, using DNA extracted from cells in antecubital whole blood, collected by venipuncture when participants were 20.9 y of age (range, 20–22 y). Analyses focused on 222 variable probes in 114 genes (Tables S1 and S2) (40). Before statistical analysis, DNAm values were corrected for interindividual differences in blood cell type composition, bioinformatically derived from 450K DNAm profiles (41).

We hypothesized that measures of nutritional, microbial, psychosocial, and socioeconomic exposures early in life—selected before statistical analyses—would predict DNAm in inflammatory genes in adulthood (Table 1). For each probe, we fit linear regression models with parametric Bayes smoothing and included participant sex as a covariate. Multiple comparisons were accounted for by controlling false discovery rate (FDR) with the Benjamini and Hochberg step-up procedure (42), and probes with adjusted *P* values less than 0.15 are reported in Table 2. Overall, we identified 10 sites across nine genes where DNAm was associated with early life exposures (Fig. 1) (coefficients for all 222 probes are presented in Dataset S1).

Household socioeconomic status was indicated by the number of physical assets owned by the family in infancy and childhood, which provides a more stable measure of socioeconomic adversity than income reports in low-income settings (43). Having fewer household assets was associated with lower DNAm for a probe located in the first intron of *CIS* and higher DNAm for a probe in the promoter region of *GNG2*. Another measure of adversity in childhood—extended absence of the participant's mother or father (24)—was also associated with DNAm. Parental absence predicted higher DNAm in *EGR4*, in a site located in the 3' untranslated region in the second exon. Parental absence was not significantly associated with household assets (3.3 assets with parental absence vs. 3.0 without; $t = -1.47$, $P = 0.14$).

We considered the hypothesis that three measures of microbial exposures in infancy—frequency of infectious diarrhea, exposure to animal feces in the home, and season of birth—would predict DNAm. Previously, all three measures were shown to predict adult CRP in this population (18), and they are only moderately intercorrelated. The Philippines is a highly seasonal tropical environment, and the frequency of infectious diarrhea in infancy was positively correlated with birth in the dry season (Pearson $R = 0.14$, $P = 0.002$) and frequency of exposure to animal feces ($R = 0.11$, $P = 0.02$). Season of birth and exposure to animal feces were not correlated ($R = 0.01$, $P = 0.78$).

Exposure to animal feces significantly predicted DNAm for three sites across two genes: *CD8A* and *APBA2*. For each probe, increased frequency of fecal exposure was associated with less methylation. Both probes for *CD8A* are located in a CpG island in the promoter region, whereas the *APBA2* probe is located in the second intron, in a high-density CpG shore. Birth in the dry season predicted higher DNAm for a probe in the first exon, 5' UTR of *IL-1A*, and for another probe in the third intron of

Table 1. Distribution of independent variables for female and male participants

Variable	Female, <i>n</i> = 395	Male, <i>n</i> = 99	Total, <i>n</i> = 494
Birth weight, kg	3.003 (0.406)	3.075 (0.431)	3.017 (0.412)
Exclusively breastfed, d	62.1 (38.0)	61.9 (39.9)	62.0 (38.3)
Diarrhea, infancy, episodes	2.2 (1.6)	2.4 (1.8)	2.2 (1.7)
Fecal exposure, intervals	1.1 (1.2)	1.2 (1.2)	1.2 (1.2)
Born in dry season, %	20.8	22.2	21.1
Household assets, number	3.1 (1.5)	3.1 (1.8)	3.1 (1.6)
Parental absence, %	16.5	16.2	16.4

Mean (SD) values are presented for continuous variables. $n = 494$ for all variables except birth weight ($n = 487$) and gestational length ($n = 491$).

Table 2. Methylation sites significantly associated with exposures in infancy/childhood

Probe	Symbol	Gene region	Exposure	logFC	Adjusted P value
cg19434937	C1S	Body	Household assets (number of items)	0.0352	0.0093
cg08001559	GNG2	TSS200	Household assets (number of items)	-0.0303	0.0093
cg06804210	CD8A	TSS1500	Animal feces (number of intervals)	-0.0481	0.0196
cg12606911	CD8A	TSS1500	Animal feces (number of intervals)	-0.0835	0.0196
cg27083329	APBA2	Upstream	Animal feces (number of intervals)	-0.0630	0.0360
cg10014308	EGR4	3' UTR	Parental absence (1, 0)	0.1110	0.0391
cg02016764	TLR1	5' UTR	Breastfeeding duration (days)	0.0014	0.0702
cg27606396	IL-1A	5' UTR	Birth in dry season (1, 0)	0.0994	0.1020
cg26064794	PIK3C2B	Body	Birth in dry season (1, 0)	0.1026	0.1020
cg23163573	SULT1C2	5' UTR	Breastfeeding duration (days)	-0.0014	0.1162

PIK3C2B. Frequency of infectious diarrhea in infancy was not associated with DNAm in any of the target genes.

Mode of feeding is a defining component of the postnatal nutritional environment, and duration of exclusive breastfeeding predicted DNAm for two sites associated with *TLR1* and *SULT1C2*. Breastfeeding duration was positively associated with DNAm in the second intron 5' UTR of *TLR1* and negatively associated with DNAm in the first exon, 5' UTR of *SULT1C2*. There were no significant associations between participant birth weight (adjusted for gestational age) and DNAm in young adulthood at any of the sites considered.

To evaluate functional biological relevance, we tested for associations between DNAm at sites identified in Table 2 and inflammatory biomarkers measured in plasma collected during the same blood draw as the methylation analysis: CRP, IL-6, TNF α , IL-10, IFN γ , IL-1 β , and IL-8. Due to the level of intercorrelation across the markers (Table S3), we constructed a summary index averaging standardized values; this approach also reduced the probability of type 1 error. Higher DNAm at sites in *C1S* and *PIK3C2B* predicted significantly lower overall levels of inflammation, with a trend toward lower inflammation with higher DNAm in *EGR4* (Fig. 2). Inflammation was positively associated with DNAm in *TLR1*. Patterns of association between DNAm and each of the inflammatory markers in the index are presented as supplementary material (Table S4). The magnitude of many associations was substantial, with cytokine concentrations that differed by 8.3–75.0% for individuals with low versus high levels of DNAm at the identified sites (Fig. S1).

In these analyses, we inferred that differential methylation was contributing to differences in inflammatory biomarker production within our sample. However, the reverse process is

possible, in which increases in inflammation can affect DNAm in white blood cells (44). To evaluate this scenario, we reran our models with early life exposures and DNAm for the probes identified in Table 2, adding the inflammation index as a covariate. If inflammatory processes are more causally proximate to early life exposures, then associations between these exposures and DNAm should be attenuated in the presence of adjustment for inflammatory biomarkers. Coefficients were virtually identical, suggesting that inflammation does not account for the association between early exposures and DNAm (Table S5).

Individual genetic differences can contribute to patterns of DNAm and may confound associations with early environmental exposures (45). To determine whether this was a factor in our analysis, we reran our models including the top three principal components of genetic variation, as previously derived for this population (46). Adjusting for the principal components did not alter the magnitude or significance of associations reported in Table 2, indicating that our results are not likely to be confounded by genetic factors (Table S6).

Discussion

Developmental plasticity and ecological sensitivity are defining characteristics of the human immune system (12), but the mechanisms shaping the development and regulation of inflammation are poorly understood. Prior research, in a wide range of ecological settings, suggests that nutritional, microbial, and psychosocial exposures during sensitive periods of immune development have lasting effects on inflammation (10, 16, 18, 19). In this study, we use a prospective design to link these exposures with patterns of DNAm in inflammatory genes in young adulthood, providing evidence that DNAm may be an important

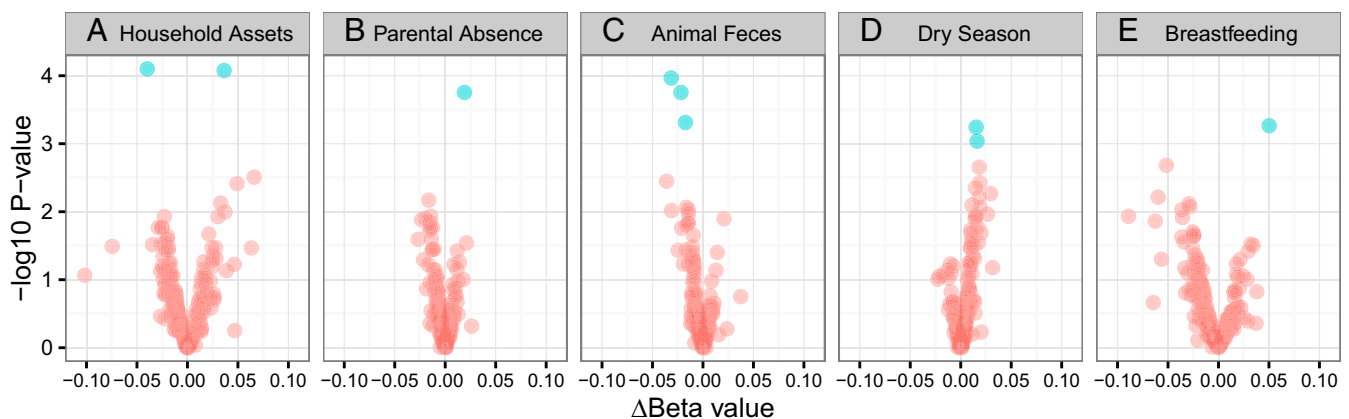


Fig. 1. Volcano plots of the associations between DNAm in young adulthood and variables measured early in development, including (A) household assets in childhood, (B) extended parental absence in childhood, (C) exposure to animal feces in infancy, (D) birth in the dry season, and (E) duration of exclusive breastfeeding. Each point represents a CpG site ($n = 222$), with the magnitude of association between each variable and DNAm on the x axis (delta beta) and $-\log_{10}$ of the uncorrected P value from the linear regression models on the y axis. Blue dots represent sites with adjusted P values < 0.15 .

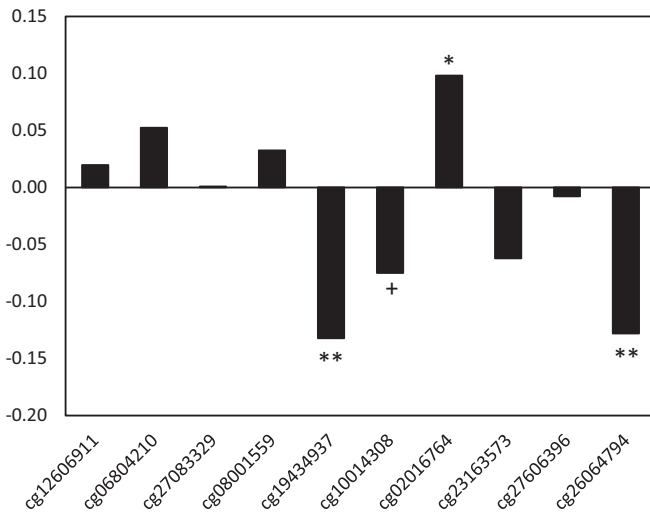


Fig. 2. Correlation between DNAm and inflammation index for probes identified in Table 2. Partial correlation coefficients are adjusted for participant sex and the presence of infectious symptoms at the time of blood collection. + $P = 0.1$; * $P < 0.05$; ** $P < 0.01$.

epigenetic mechanism through which early environments have enduring effects on inflammatory phenotypes. We also show that variations in DNAm relate to expression of inflammatory biomarkers implicated in a host of prevalent chronic diseases, underscoring the potential public health significance of these results.

Our analyses focused on target genes known to be involved in the regulation of inflammation, and we identified 10 CpG sites across nine genes that were predicted by developmental environments. The most statistically significant associations were for sites in the *C1S* and *GNG2* genes, which were predicted by household assets, a measure of socioeconomic adversity in infancy/childhood. *C1S* is a protein-coding gene that contributes to the production of C1, the first component of the classical pathway of the complement system, and *GNG2* produces proteins that are involved with transmembrane signaling mechanisms.

Similarly, we identified parental absence—a different type of childhood adversity—as a significant predictor of DNAm in the *EGR4* gene. *EGR4* is a protein coding gene and transcription factor that regulates cell proliferation and proinflammatory cytokine production. These results are consistent with several studies documenting associations between early life socioeconomic status and DNAm in adulthood, drawing on samples from the United States, United Kingdom, Canada, and Italy (30, 47–49), and with a recent study in the United States showing that higher levels of stress in infancy/early childhood are biologically embedded in the epigenome (27). Our study reports associations between early adversity and DNAm in adulthood in a non-Western, lower income setting. Furthermore, our findings are consistent with experimental animal models documenting durable effects of maternal separation on the regulation of inflammation (50–52).

A unique advantage of our study is the depth of prospectively collected data on more proximate environmental factors, beginning in infancy. As hypothesized, exposure to animal feces and season of birth—two proxy measures of the intensity and diversity of microbial encounters in infancy—predicted DNAm at five sites across four genes. Increased animal feces exposure was associated with reduced DNAm at two promoter sites in *CD8A* and one upstream site in a high-density CpG shore in *APBA2*. *APBA2* encodes a protein that interacts with amyloid precursor protein and is involved in signal transduction processes.

As part of the T-cell receptor complex, *CD8A* recognizes antigens and initiates phosphorylation cascades that lead to the activation of transcription factors such as NFAT, NF- κ B, and AP-1, all of which play important roles in the regulation of

inflammation. We document strong, negative associations between intensity of exposure to animal feces in infancy and DNAm at two sites in the *CD8A* promoter, ~300 bp apart, which are associated with a DNase hypersensitivity cluster. The potential significance of this finding is underscored by the consistent pattern of DNAm in two neighboring sites, the likely regulatory role of the gene region, and the established importance of the T-cell receptor complex to immune activity.

Birth in the dry season predicted higher DNAm in the first exon, 5' UTR region of *IL-1A* and the third intron of *PIK3C2B*. Both CpG sites are located in regions marked by increased chromatin accessibility, DNase hypersensitivity, and transcription factor binding sites. *IL-1A* produces a cytokine in the interleukin 1 family (IL-1 α) that is involved in several immune and inflammatory processes. *PIK3C2B* is a protein coding gene that plays roles in signal pathways related to cell proliferation, survival, and migration.

The pattern of associations between postnatal microbial exposures and methylation of inflammatory genes underscores the relevance of epigenetic processes to the “hygiene” or “old friends” hypotheses, which link the development of immunoregulatory pathways to microbial environments early in development (22, 53). More specifically, our findings are consistent with experimental animal models documenting negative associations between early exposure to microbes/microbial products and inflammatory processes later in life (54–56). Similarly, a relatively recent study in the Gambia has reported an association between season of birth and DNAm of metastable epialleles (57), while our analysis reports an association between season of birth and DNAm of genes involved in the regulation of inflammation.

Longer durations of breastfeeding in infancy have been associated with reduced inflammation in adulthood (19), and we identified sites in two genes where breastfeeding duration predicted levels of DNAm. Breastfeeding duration was negatively associated with DNAm in the 5' UTR region of *SULT1C2*, which encodes a protein in the SULT1 subfamily that facilitates sulfate conjugation of phenol-containing compounds. Breastfeeding duration predicted higher DNAm in the 5' UTR region of *TLR1*, which is a member of the Toll-like receptor (TLR) family that is centrally involved in pathogen recognition and the activation of innate immune processes, including inflammation. The TLR family is highly conserved and widely expressed on immune cells, and TLR1 forms a cluster with TLR2 and CD14 to recognize bacterial lipoproteins and transduce signals that lead to NF- κ B activation, cytokine production, and the inflammatory response (58). Prior research has documented associations between breastfeeding and DNAm in the *LEP* gene (59), while our study links breastfeeding with DNAm in immunoregulatory genes (19).

The range and time depth of measures in our study are major strengths, but the design does not include direct measures of gene expression, making it more difficult to infer the functional consequences of varying levels of DNAm at the identified sites. Similarly, the pattern of association with inflammatory biomarkers is not consistent for all sites, and we document associations between 4 of 10 DNAm sites and production of inflammatory biomarkers. Prior research has documented stronger associations between DNAm and inflammatory biomarkers in stimulated samples than in basal samples (47), suggesting that our tests of association between DNAm and inflammation in nonstimulated samples may represent a conservative estimate of the functional significance of the identified sites. Additional research is needed to determine the extent to which DNAm directly mediates transcriptional activity at these sites or, alternatively, whether it serves as a marker for other regulatory processes. The use of in vivo or ex vivo experimental protocols (e.g., vaccination, cell culture systems) to stimulate and measure the inflammatory response would provide better indicators of the inflammatory milieu, however the logistics of our field location precluded this approach. Despite these limitations, single measures of baseline concentrations of inflammatory biomarkers have been shown to predict increased risk for the onset of cardiovascular, metabolic,

and other diseases linked to inflammation (2, 3), attesting to their biological and clinical significance.

Another potential limitation of our study is the use of methylation data to estimate and adjust for blood cell composition in the absence of direct cell counts. Although this bioinformatic approach has been validated for use with whole blood samples (41, 60), the possibility of residual confounding by cell composition remains. In addition, we measured DNAm and inflammatory cytokines concurrently and conceptualized cytokine production as a downstream consequence of differential methylation in immunoregulatory genes. Because our study design precludes formal mediation analyses, we cannot rule out the possibility that increases in inflammation are causing changes in DNAm. Our tests of reverse causality suggest this is not the case, although they are limited by the cross-sectional measures and the mixed pattern of association between inflammatory biomarkers and DNAm in our sample. Lastly, we rely on proxy—rather than direct—measures of microbial exposure that are only moderately correlated. The distinct pattern of results for season of birth and exposure to animal feces may be attributable to this fact, and additional research is needed to determine the timing of microbial exposure and the types of exposures that impact DNAm in inflammatory genes.

Recent studies demonstrate that genetic polymorphisms can influence patterns of DNAm as well as moderate patterns of association between environmental exposures, DNAm, and phenotypic outcomes (45, 61). In our analyses, we determined that adjusting for principal components of genetic variation did not alter associations between early environmental exposures and DNAm, but this is a relatively limited test of genetic confounding that does not consider the potential role of specific genes in moderating patterns of association. Future research should consider the possibility of interactions between early environmental exposures and allelic variation in shaping DNAm and the regulation of inflammation.

Our results contribute to a rapidly growing body of literature investigating the importance of early life environments to the regulation of inflammation later in life (35). They point to DNAm as a potentially important biological mechanism through which nutritional, microbial, psychosocial, and socioeconomic exposures impact inflammatory phenotypes across the life course. These findings highlight specific genes that may be particularly promising foci for future research, and they contribute to the emerging emphasis on epigenetic processes in the developmental origins of health and disease (62–66).

Materials and Methods

Participants and Study Design. Analyses were implemented with a subset of participants in the Cebu Longitudinal Health and Nutrition Survey (CLHNS), an ongoing birth cohort study in the Philippines (see *SI Materials and Methods* for additional information on identification of study participants). The study began in 1983 with the recruitment of a community-based sample of 3,327 pregnant women, and home visits were made before birth, immediately following birth, and every 2 mo for 2 y (38). A follow-up survey in 2005 included the collection of venous blood, when the participants were 20–22 y of age. All data were collected and analyzed under conditions of informed consent with institutional review board approvals from the University of North Carolina at Chapel Hill, Northwestern University, and the University of British Columbia.

DNAm. Overnight fasting blood samples were collected into EDTA-coated vacutainer tubes and kept in coolers on ice packs for no more than 2 h during transport to a central facility, where samples were centrifuged to separate plasma and white blood cells before freezing at -70°C . Genomic DNA was treated with sodium bisulfite (Zymo Research), and converted DNA was applied to the Illumina HumanMethylation450 Bead Chip using the manufacturer's standard conditions (Illumina Inc.). Additional details on laboratory procedures, quality control analyses, and data processing are provided in *SI Materials and Methods*. Proportions of blood cell types were predicted using a previously established algorithm, and variance associated with cell composition was removed before statistical analyses (41, 60).

Data Analysis. The following independent variables were considered as measures of the early life nutritional, microbial, and psychosocial environment: birth weight (measured in the home immediately after delivery); duration of exclusive breastfeeding (based on maternal reports collected in the home during bimonthly interviews); frequency of infectious diarrhea, birth to 24 mo (maternal report during bimonthly interviews); exposure to animal feces, 6–12 mo (interviewer observation during bimonthly interviews); birth in the dry season (February through April; indicator of higher levels of microbial exposure in early infancy) (18); and household assets in infancy and childhood [sum of items, averaged across four surveys; extended absence of the participant's mother or father in childhood (up to ~ 11 y of age)] (additional details on variable construction and validation are provided in *SI Materials and Methods*).

Tests of association between early environments and DNAm were limited to highly variable methylation sites in target genes involved in the regulation of inflammation (Table S1). Target genes were identified using a web-based gene network and functional annotation tool (genemania.org) and review of prior human studies investigating the genetic and epigenetic control of inflammation (30, 48, 67–70) (additional details on target gene selection are provided in *SI Materials and Methods*). A total of 249 target genes were identified. Probes were matched to target genes based on proximity of the nearest transcription start site (71), resulting in 5,152 probes, which were then filtered to exclude probes for which variability in beta-values between the 10th and 90th percentiles was $<10\%$ (72). The final dataset included beta-values for 222 variable probes across 114 target genes (Table S2 and Dataset S2). Beta-values were converted to M-values before statistical analysis (73).

For hypothesis testing, probe-wise variance was determined by fitting linear regression models and applying a parametric empirical Bayes smoothing formula using the R bioconductor package *limma* (74). All models adjusted for participant sex. We accounted for multiple comparisons using the Benjamini and Hochberg step-up procedure for controlling FDR, with $q = 0.05$ (42). For exploratory purposes and to capture associations of potential biological relevance, we report all associations with adjusted P values < 0.15 . To evaluate the functional relevance of identified sites, we tested for associations between these sites and the following inflammatory biomarkers quantified in the same set of blood samples used to measure DNAm: CRP, IL-1 β , IL-6, IL-8, IL-10, IFN γ , and TNF α (see *SI Materials and Methods* for additional information on statistical analyses).

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