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TITLE

Prenatal exposures and DNA methylation in newborns: A Pilot Study in Durban, South Africa

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SHORT RUNNING TITLE

Neonatal DNA Methylation in South Africa

KEYWORDS

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ABSTRACT

The *in utero* environment has the potential to influence epigenetic programming and subsequently the health of offspring. Even though pregnant women living in urban Africa are exposed to multiple chemicals and infectious agents that may impact their developing children, the neonatal epigenome has not been studied in these regions. We assessed whether prenatal exposures to air pollution and maternal human immunodeficiency virus (HIV) are associated with changes to DNA methylation throughout the epigenome using a pilot sample from the Maternal and Child Environmental (MACE) birth cohort, of which 36% of the mothers are HIV positive. Families living in a high air pollution region (south Durban, n=11) and a low air pollution region (north Durban, n=11) with comparable socioeconomic characteristics were selected for analysis. DNA methylation was quantified in cord blood plasma DNA at >430,000 CpG sites using the Infinium HumanMethylation450 BeadChip. Sites associated with living in south Durban or maternal HIV infection ($p < 0.001$) were more likely to be hypomethylated and located in CpG islands. Top differentially methylated sites by region of Durban were enriched in pathways related to xenobiotic metabolism, oxygen and gas transport, and sensory perception of chemical stimuli when performing gene set enrichment testing with LRpath. Differentially methylated sites by maternal HIV status were enriched in cytochrome P450s, pathways involved in detection of chemical stimuli, metabolic processes, and viral regulation and processing. Given the small sample size of the study, future work examining the impact of prenatal exposures to air pollution, maternal infection, and antiviral treatment on the epigenome and downstream health implications is merited in Sub-Saharan African populations.

ENVIRONMENTAL IMPACT STATEMENT:

South Durban is a heavily industrialized area in South Africa that is known to have high ambient air pollution and greater prevalence of respiratory complications (e.g., childhood asthma) among residents compared with the non-industrial north Durban. Prenatal air pollution exposure has the potential to impact epigenetic programming and influence disease risk in the offspring throughout the lifespan. Thus, we compared DNA methylation at >430,000 sites across the epigenome in newborns from south Durban with those from north Durban. In addition to air pollution exposure, we also examined associations of DNA methylation with maternal HIV infection, as co-exposures to both the virus and antiviral treatment are relevant health concerns for neonates from many African cities including Durban.

INTRODUCTION

In utero exposures lead to epigenetic perturbations that influence early development and in some cases persist throughout the life course, contributing to complex diseases. For example, prenatal and early postnatal exposures to airborne pollutants, cigarette smoke, and allergens are associated with changes to the offspring's epigenome and subsequent risk for childhood asthma (see reviews (1,2)). In a Belgian birth cohort study, global hypomethylation of placenta DNA and altered mitochondrial DNA methylation were associated with maternal particulate matter (PM_{2.5}) exposure, and the latter mediated the relationship between PM_{2.5} and decreased total mitochondrial DNA (3,4). In an American mother-child cohort, prenatal polycyclic aromatic hydrocarbon (PAH) exposure was associated with hypermethylation of acyl-CoA synthetase long-chain family member 3 (*ACSL3*), and this hypermethylation was associated with parental report of asthma by the time children were five years of age (5). As the epigenome is most vulnerable to external exposures during early development, fetuses are vulnerable to epigenetic changes which may influence later-in-life adverse health outcomes (6).

Even though Sub-Saharan African families in urban areas often face multiple stressors (e.g., violence, poverty), infectious agents (e.g., HIV), chemical exposures (e.g. air pollution, e-waste) and malnutrition, there are few epigenetic studies focused on these populations (7). To date, epigenetic epidemiological studies of mothers and children in Sub-Saharan Africa have identified differentially methylated genes in rural Gambian babies and children associated with periconceptual nutrition status (8-11) or maternal aflatoxin exposure during pregnancy (12). In South African, children with fetal alcohol syndrome had altered methylation at imprinted genes compared with healthy children (13). In the Democratic Republic of Congo, maternal stress from ongoing war was associated with differential methylation – including at key genes involved in

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3 stress response – among newborns and their mothers (14-16). While these studies are providing
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5 needed insight into epigenetically labile regions of the genome and key environmental factors in
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7 African populations, they only stem from three major study populations in rural Gambia, South
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9 Africa, and the eastern Democratic Republic of Congo. To our knowledge no studies have
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11 assessed the impact of *in utero* air pollution exposure or maternal HIV infection on newborn or
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13 children's epigenomes in Sub-Saharan Africa.
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17 In the present pilot study, the recruitment area includes south Durban in KwaZulu-Natal,
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19 South Africa, which has been regarded as one of the most industrialized and polluted regions in
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21 Southern Africa (17). A previous study documented a higher prevalence of respiratory outcomes
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23 such as asthma and airway hyper-responsiveness among children in this area compared to north
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25 Durban, a less industrialized region (18). South Durban is characterized by higher oxides of
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27 nitrogen (NO_x), sulfur dioxide, and volatile organic compounds among other pollutants
28
29 compared to north Durban, though both regions have similar ambient PM₁₀ and PM_{2.5} which
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31 hover near WHO guidelines for maximum annual exposure (17). In addition, KwaZulu-Natal has
32
33 the highest HIV prevalence in South Africa (16.9% in 2012) with the highest risk of exposure
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35 among black African females aged between 20 and 34 years (19). All HIV positive pregnant
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37 women receiving antenatal care, regardless of their CD4 count, are given a fixed dose
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39 combination of antiretroviral therapy from 14 weeks of pregnancy that continues throughout the
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41 breastfeeding period (20). Pregnant women in this area thus encounter multiple environmental
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43 insults that may impact the growing fetus (e.g., air pollution, maternal HIV infection, and
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45 antiviral medication), yet the impact on the epigenome remains to be characterized.
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53 The aim of this pilot study is to assess changes to the neonatal DNA methylome
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55 following *in utero* exposures to airborne pollutants and maternal HIV (infection and treatment).
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3 We hypothesize that both factors will alter DNA methylation levels throughout the epigenome
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5 quantified with the Infinium HumanMethylation450 BeadChip. We address this hypothesis in a
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7 pilot sample from the Maternal and Child Environmental (MACE) birth cohort, comparing 11
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9 newborns from the high ambient pollution south Durban to 11 newborns from the lower
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11 pollution north Durban region.
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MATERIALS AND METHODS

MACE Cohort

For the pilot stage of the MACE birth cohort study, 100 women were recruited during the third trimester of pregnancy from four public antenatal clinics in close proximity to Air Quality Monitoring Stations in eThekweni Municipality, which is located in KwaZulu-Natal province of South Africa. Half of the participants resided in a heavily industrialized and polluted region (south Durban) and half resided in a lesser polluted area (north Durban) with similar socioeconomic characteristics (21). Women with pregnancy complications (e.g., hypertension, diabetes), multiples, or late first antenatal visits (>34 weeks) were excluded. Further information on recruitment was previously detailed (21,22). Mothers provided informed consent before participating including for genetic analyses, and ethical approval was obtained from the Biomedical and Research Ethics Committee of the University of KwaZulu-Natal.

A subset of mother-child pairs was selected for epigenome-wide analysis (12 each from north and south Durban with the best DNA quality) from among 30 providing cord blood samples. Cord blood samples were limited among the 100 participants due to logistical and compliance limitations at the hospitals where the mothers gave birth.

Demographic and Exposure Data

Demographic (e.g., maternal education, race, age, marital status) and exposure information (e.g., smoking history, exposure to environmental tobacco smoke, occupational chemical exposures) was collected via questionnaire. HIV status of the mothers was assessed at the antenatal clinics. Gestational age, birth weight, and birth outcomes were obtained from patient records. Hospital staff estimated gestational age based on first day of the last menstrual

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3 period, and birth weight was measured in the delivery room by trained nurses. Mothers did not
4
5 receive folate supplementation during pregnancy except for one taking prenatal vitamins. Air
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7 pollution exposure is approximated by both region of residency and NO_x concentrations.
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9 Individual exposures to NO_x were estimated based on land use regression modeling (23,24) for
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11 all subjects except one (missing necessary data).
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17 *DNA Isolation and Treatment*

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20 DNA was isolated from umbilical cord blood plasma, as whole blood from the
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22 participants was not archived, using the QIAamp DNA Kit (Qiagen), stored at -80°C at the
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24 University of KwaZulu-Natal, and shipped overnight on dry ice to the U.S. Double-stranded
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26 DNA concentration was determined via the Qubit fluorimetric system. The EZ DNA
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28 Methylation kit (Zymo Research Corporation, Irvine, CA) was used for bisulfite conversion of
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30 500 ng of genomic DNA which converts unmethylated cytosines and leaves methylated
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32 cytosines unchanged (25). The protocol recommended for downstream Illumina Infinium
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34 analysis was followed.
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41 *Epigenome-Wide DNA Methylation Analysis*

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44 DNA methylation at >485,000 CpG sites throughout the genome was interrogated with
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46 the Illumina Infinium HumanMethylation450 BeadChip ('450K') (26). Amplification and
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48 hybridization of bisulfite converted DNA samples to the beadchips were performed according to
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50 manufacturer's protocol. Twenty-four samples were run across three batches (beadchips). Image
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52 data was scanned and recorded via the Illumina iScan with the Methylation NXT scan setting.
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55 Raw data from image files was read into R version 3.1 using both ChAMP and methylumi
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3 packages (27,28). Raw data was then processed to remove samples and probes with poor quality
4 control, and to adjust for batch effects and biases inherent in the 450K platform. Probes with low
5 detection ($p > 0.01$ or bead count < 3 in at least 2 samples) were removed. One subject from the
6 north Durban group that failed to pass quality controls was also excluded. Additional probes
7 were excluded if they: 1) hybridized to the X and Y chromosomes, 2) were known to be cross-
8 reactive (29), or 3) had a SNP within the query site or single base extension position with a
9 minor allele frequency $> 4\%$ in Africans (or worldwide if African data was not available) leaving
10 a final dataset of 433,782 CpG sites. Dye bias adjustment and background correction were
11 performed using the methylumi package (27). In the ChAMP package (30), beta-mixture quantile
12 normalization was performed to correct for probe type differences (31). The ComBat function
13 was used to correct for batch effects by beadchip (32). Probe locations were annotated with the
14 IlluminaHumanMethylation450kanno.ilmn12.h19 package (33).

34 *Statistical Analysis*

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36 All statistics were performed with the R Project for Statistical Computing version 3.1.
37 Univariate statistics for demographic and exposure variables were computed for all subjects
38 (excluding a racial outlier, Supplemental Figure S1) with high quality 450K data ($n=22$) and
39 stratified by air pollution or maternal HIV exposure group. Fisher exact tests were used to
40 compare demographic and exposure characteristics between groups (north vs. south Durban and
41 maternal HIV positive vs. negative status). All statistical analyses of methylation data utilized
42 'M-Values' which are logit transformed versions of the pre-processed and adjusted beta values
43 from 433,782 CpG sites. Unsupervised clustering by all sites and by the 1000 most variable sites
44 via a multi-dimensional scaling (MDS) algorithm were performed and examined by key
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3 categorical variables (e.g., sex, race, region, maternal HIV status, parental smoking). One outlier
4 subject was identified that differed from the rest (the only white subject, Supplemental Figure
5 S1), and all downstream analyses were run without this subject (n=22 in reported analyses).
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10 Multivariable analysis was performed to find differentially methylated sites (DMS) by air
11 pollution exposure or maternal HIV status. The limma package was used to compare methylation
12 at all sites by air pollution exposure group (north Durban, n=11 vs. south Durban, n=11) or
13 maternal HIV status (14 negative vs. 8 positive) while adjusting for gestational age and sex (34).
14
15 The following covariates were not considered because they were too unbalanced for reliable
16 statistical modeling (e.g., one family in a category): maternal vitamin or supplement use, marital
17 status, mother currently smoking, or mother exposed to smoke at work. The following covariates
18 were tested but not included in the final model as they were associated with methylation at <5
19 CpG sites at an FDR of 10%: maternal age, previous pregnancies, pre-pregnancy weight, smoke
20 exposure at home, paternal smoking status, maternal smoking in the past, apgar score, delivery
21 type, and use of labor medication(s). Adjusted models with air pollution exposure and HIV status
22 modeled separately or simultaneously were used to identify DMS. Estimates of NO_x were also
23 modeled as continuous in subjects with the data (n=21).
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41 Along with identifying DMS by air pollution exposure or maternal HIV status, we
42 examined trends in the data (e.g., hypo- versus hyper-methylation, enrichment in regulatory
43 regions or CpG islands) for CpG sites with p<0.001 or log-fold change>1 or <-1 in exposure and
44 HIV models. Chi-square tests with p-values estimated from 2000 Monte-Carlo simulations were
45 performed to assess enrichment/depletion in annotated regions compared to expected proportion
46 based on all probes.
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3 We performed gene set enrichment testing using the publically-available LRpath program
4 (35). This program uses logistic regression and pre-defined sets of genes (“concepts”) that are
5 related by molecular or biological function or pathway to test the hypothesis that certain concepts
6 have higher significance values (based on raw p-values) for differential methylation compared
7 with other concepts. We utilized log-fold change and p-value data from HIV or air pollution
8 exposure models for 328,040 probes that mapped to known genes (35). Enriched concepts with
9 10-1000 genes per concept were screened from the databases of KEGG, GO Biological Process,
10 and GO Molecular Function concepts. Overall, this logistic-regression based approach relates the
11 odds of belonging to a given concept with the raw p-value for differential methylation while
12 taking into account the size (e.g., number of genes) of each concept.
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RESULTS

Study Population Characteristics

Twenty-two mother-child pairs, 11 from north Durban and 11 from south Durban, were included in the epigenome-wide 450K analysis. Of these, 68% of the newborns were female, 21 families identified as African and 1 as Coloured. 95% of mothers were unmarried, and 86% had never smoked. Table I summarizes these and other characteristics of the study subjects and stratifies results by residency region or maternal HIV status. Demographic variables did not vary significantly by air pollution or maternal HIV exposure groups. NO_x levels were higher in south Durban compared with the north (36.1±6.0 vs. 17.8±5.4 ppb, p<0.001 for T test).

HumanMethylation450 BeadChip (450K)

Models adjusting for gestational age and gender were run to search for DMS among 433,782 CpG sites by 1) air pollution exposure group, 2) NO_x levels (n=21 in these models), or 3) maternal HIV status. Results (top DMS) were compared in models assessing any exposure alone or models with HIV and air pollution exposure together. No DMS were identified that were significant at an FDR level of 10%. Given the small sample size, nineteen top DMS by air pollution exposure group and/or NO_x levels selected by raw p-value are detailed in Supplemental Table I, including sites in intergenic regions and sites in known genes (e.g., *NOVA2*, *SELK*, *CNTD2*). Twelve of the top DMS by maternal HIV status (p<0.0001 in multiple models) are displayed in Supplemental Table II, including intergenic CpG sites and sites in genes (e.g., *POLDIP3*, *RHOBTB3*). No DMS by HIV status remained significant at a FDR of 10%.

No sites were significant following FDR correction. We examined trends in methylation across all sites or groups of sites by air pollution exposure or maternal HIV status (Figures 1 and

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3 2). When comparing methylation of samples from south and north Durban, CpG sites were more
4 likely to be hypomethylated (62.0% of all sites, 77.1% of sites with nominal $p < 0.001$, and 57.4%
5 of sites with log-fold change greater than the absolute value of one [herein referred to as effect
6 size > 1]). Likewise, children with HIV positive mothers had more hypomethylated sites (69.2%
7 of all sites, 86.4% of CpG sites with $p < 0.001$, and 65.8% of sites with effect size > 1 ; see Figure
8 1). Enrichment of sites with $p < 0.001$ or effect size > 1 in specific chromosomes, annotation
9 relative to CpG islands, in DNase I hypersensitive sites, in enhancer regions, in well-
10 characterized differentially methylated regions (DMRs; such as tissue-specific or imprinted
11 gene-specific DMRs), or in specific regulatory features (e.g., promoter or gene associated) was
12 also assessed (data not shown). Among sites with $p < 0.001$ for air pollution exposure group, there
13 was a significant enrichment of sites in CpG islands (46.2% compared to 31.7% of total probes;
14 Figure 2), in DNase I hypersensitive sites (16.4 vs. 12.6% of all probes), and in DMRs (11.4%
15 vs. 8% of total probes). When looking at top sites by effect sizes, only the significant DMR
16 enrichment remained. Similar analyses by maternal HIV status revealed enrichment of sites with
17 $p < 0.001$ in CpG islands (52.0% vs. 31.7% expected; Figure 2), DNase I hypersensitive sites
18 (19.0% vs. 12.6% expected), DMRs (16.3% vs. 8% expected), and a paucity of probes in
19 enhancer regions (14.8% vs. 22.4% expected). When comparing probes with effect sizes > 1 , only
20 the enhancer depletion remained significant (χ^2 test, $p = 0.02$).
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48 *Pathway Analysis*

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50 The program LRpath was used to search databases of gene function and gene pathways
51 (KEGG and GO) for concepts enriched amongst the most DMS (35). Thirty-four concepts or
52 pathways were enriched by air pollution exposure group with (FDR < 0.1 see Supplemental Table
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3 III for full details; 22 pathways had FDR <0.05, Table II). Enriched functions and pathways
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5 include those related to xenobiotic metabolism (e.g., cytochrome P450), oxygen and gas
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7 transport, and olfactory and sensory perception of chemical stimuli. Pathways enriched amongst
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9 DMS by maternal HIV status include 67 with FDR <10% (Supplemental Table IV) and 26 <5%
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11 (Table III). Differential methylation in pathways involved in sensory perception and detection of
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13 chemical stimuli, cytochrome P450, and various metabolic processes was enriched. Eleven gene
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15 groups related to viral regulation and processing and the innate immune response to viral
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17 infections were enriched for higher methylation among individuals whose mothers had HIV with
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19 FDR 10%.
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DISCUSSION

In a pilot study using the South African birth cohort, MACE, we observed DNA methylation changes associated with prenatal exposures to airborne pollutants and maternal HIV infection using an epigenome-wide platform, the Infinium HumanMethylation450 BeadChip. To our knowledge, this is one of the first studies in an urban Sub-Saharan African cohort to assess the neonatal epigenome, a much needed area of research given the range of environmental factors encountered by African populations including infectious diseases, chemical exposures, and malnutrition. The increasing prevalence of non-communicable diseases in African populations, which may partially be explained by environmentally-induced epigenetic perturbations early in life as posited by the Developmental Origins of Health and Disease (DOHaD) hypothesis, further necessitates research in this area (7,36).

No significant DMS were observed with an FDR level of 10%. As this was a pilot study with a small sample size, it is possible but not guaranteed that increasing the sample size would reveal significant DMS at a 10% FDR level. Even so within this pilot dataset, intriguing trends in the data by raw p-value, direction and annotation relative to CpG islands among top DMS, and enriched pathways among the sites using LRpath were observed for both group comparisons (air pollution and maternal HIV status). When comparing direction of methylation difference between the high air pollution exposure group or the maternal HIV positive group with their respective reference groups, hypomethylation was more commonly observed (Figure 1). Hypomethylation is typically linked to increased gene expression, especially within CpG islands and promoter regions (37). Interestingly, the top DMS by air pollution or maternal HIV exposure group were more likely to be found within CpG islands and other regulatory regions (DNase I hypersensitive sites (38) and well-characterized DMRs). Hypomethylation is also problematic in

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3 intergenic regions and leads to genomic instability (39-41). Air pollution and antiviral treatments
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5 may modify the DNA methylome via oxidative stress which reduces the efficiency of the one-
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7 carbon metabolism pathway, thereby reducing the amount of available methyl donors necessary
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9 to establish and maintain DNA methylation (42,43). Given that wide-spread modification of the
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11 neonatal DNA methylome in regulatory regions could impact gene expression throughout
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13 development and potentially beyond and genome-wide hypomethylation can lead to genomic
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15 instability, the combined impact of maternal air pollution and HIV exposures on the epigenome
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17 and downstream health effects merits further study in a larger cohort.

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22 In terms of specific CpG sites associated with air pollution exposure, none survived
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24 correction for multiple testing. Top DMS by raw p-value (Supplemental Table I) were found in
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26 genes with potential relevance to early development and response to exposure including the
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28 antioxidant selenoprotein *SELK*, *GGT5* which is involved in leukotriene synthesis, and the
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30 osteogenesis-regulator *PBX1*. Pathway analysis via LRpath revealed significantly enriched gene
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32 concept groups after correction for multiple hypothesis testing that may confer continued
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34 protection against environmental agents (e.g., air pollution) encountered *in utero*. For example,
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36 pathways involved in xenobiotic metabolism and detection of chemical stimuli were enriched
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38 amongst samples from the high air pollution region. Pregnant women and fetuses have altered
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40 biotransformation capabilities, which are an increased risk for the developing fetus in terms of
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42 epigenetic reprogramming in response to environmental insults (6,44). Our study adds to the
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44 growing body of literature that suggests transplacental exposure to components of ambient
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46 pollution modifies the epigenome and may contribute to risk for adverse outcomes in childhood
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48 such as asthma (3-5,45). Previous studies have provided evidence for the impact of ambient
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50 pollution components (e.g., PAHs, PM_{2.5}, PM₁₀) on DNA methylation at global markers
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3 (repetitive elements) in adult blood leukocytes (46,47) and at the *FOXP3* gene in children's
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5 regulatory T cells, which was related to immunity and atopic status in the same children (48).
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8 Similar to trends observed in the present study, primarily hypomethylated DMS were discovered
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10 among both healthy and asthmatic children in a heavily polluted area of the Czech Republic
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12 compared with a less polluted region using an epigenome-wide platform examining >27,000
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14 CpG sites (49).

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17 In terms of specific sites associated with maternal HIV status, the top represented genes
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19 by nominal p-value included transcription and translation regulators (*TULP2*, *ZNF398*,
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21 *POLDIP3*) and a modifier of mitochondrial DNA topology (*TOP1MT*), though none were
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23 significant with a FDR of 10%. Twenty-seven pathways were enriched according to LRpath
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25 analysis, specifically for hypomethylation with a FDR level of 5% (Table III), and enriched
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27 concepts primarily involved detection of chemical stimuli, olfactory signaling, xenobiotic
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29 metabolism, and other metabolic functions. Increased expression in these pathways may enable
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31 offspring to cope with exposures to maternal antiviral therapies. Additional concepts enriched
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33 with an FDR of 10% include processes involved in viral processing and regulation, and this may
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35 be a result of treatment exposure or a response to viral exposure. The few previous studies
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37 examining the impact of HIV and maternal antiviral therapies on the neonatal epigenome suggest
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39 that antiviral treatment and not infection itself is the driver of change (50,51). Heterochromatin
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41 differences were observed in lymphocytes from children with prenatal nucleoside reverse
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43 transcriptase inhibitor (NRTI) exposure compared with children without NRTI exposure
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45 regardless of maternal HIV status (51). Likewise, maternal anti-viral therapy was associated with
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47 DNA hypomethylation at repetitive elements, a global marker, in newborn peripheral blood
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49 mononuclear cells (PBMCs) compared with newborns from untreated HIV-positive mothers
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3 (50). Of the eight HIV positive mothers in the MACE cohort, at the time of birth seven took
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5 Nevaripine (a non-NRTI), one additionally took azidothymidine (a NRTI), and one was
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7 untreated. The impact of common antiviral medication used by African populations with high
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9 HIV prevalence on children's health through perinatal epigenetic perturbation needs further
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11 exploration.
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15 Evidence for the functional and phenotypic impact of exposure-induced epigenetic
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17 changes is growing. In a longitudinal study, an association between prenatal PAH exposure and
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19 *ACSL3* hypermethylation in umbilical cord blood leukocytes was linked to asthma development
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21 in children by five years of age (5). In a cross-sectional study, blood leukocyte DNA methylation
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23 at *TNF* mediated the association between phthalate metabolite levels and asthma in children (52).
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25 Thus, the need to better understand the impact of multiple co-exposures and stressors (e.g., to
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27 nutritional deficiencies, maternal antiviral therapies, air pollution, additional environmental
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29 chemicals, socioeconomic status-related stress) on the neonatal epigenome and the downstream
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31 implications for development of disease later in life (i.e., DOHaD) is imperative. Furthermore,
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33 development of epigenetic biomarkers by establishing DMS reflective of certain exposures or
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35 outcomes would be useful for research, diagnostic, and potentially therapeutic purposes in
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37 regions with limited resources for full-scale epigenome analyses.
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44 While we are unique in assessing epigenetic changes by both prenatal air pollution and
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46 maternal HIV exposure in a South African pilot cohort using an epigenome-wide platform, this
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48 study had several limitations. Along with small sample size, due to archived resources available
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50 for the study, DNA was isolated from cord blood plasma. Thus, our ability to compare findings
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52 to studies using blood leukocytes or to extrapolate findings to other tissues of the body is
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54 extremely limited. Top DMS by nominal p-value could not be validated for functional relevance
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3 (e.g., gene expression analysis) as the samples were not preserved and stored for RNA isolation.
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6 Our estimation of air pollution exposure (by region or by NO_x levels) did not include specific
7
8 levels of certain air pollutants that have been shown to impact the epigenome (e.g., PAHs,
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10 PM_{2.5}). Additionally, we were not able to tease apart the epigenetic differences due to maternal
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12 HIV infection versus antiviral therapy.
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17 CONCLUSION

19
20 In conclusion, epigenetic modification by prenatal exposures to common stressors in
21
22 urban African populations such as air pollution and maternal HIV (infection or treatment) may
23
24 lead to epigenetic modifications in neonates. Due to limitations of this study, we recommend
25
26 further examination of these key *in utero* exposures, their impact on the epigenome, and the
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28 continued impact on downstream growth and disease development in future Sub-Saharan African
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30 cohorts.
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FIGURE LEGENDS

Figure 1. Enrichment of Hypomethylated Sites among High Air Pollution and Maternal

HIV Exposure Groups. A statistically significant enrichment of hypomethylated sites was observed when comparing cord blood plasma DNA methylation from individuals A) residing in a high air pollution region (south Durban) compared with the low pollution north Durban and B) with maternal HIV exposure compared with uninfected mothers. Relationships were assessed in models adjusting for gestational age, child's sex, air pollution group, and maternal HIV status (n=22). Percentage of hypomethylated (direction=negative) and hypermethylated (positive) sites are displayed from the following groups of CpG sites: 1) all sites (n=433,782), 2) $p < 0.001$ (n=420 for air pollution, 689 for HIV), or effect size > 1 (log fold-change < -1 or > 1 ; n=781 for air pollution analysis, 514 for HIV). A reference line is drawn at 50%.

Figure 2. Enrichment of Differentially Methylated Sites (DMS) in CpG Islands among High

Air Pollution and Maternal HIV Exposure Groups. Among the top DMS ($p < 0.001$) by air pollution exposure region (420 CpG sites) or maternal HIV status (689 sites) in models adjusting for both exposures, gestational age, and sex, there was a statistically significant shift in annotation related to CpG islands compared to the expected proportions based on all probes ('All CpG Sites'). Specifically, DMS were over-represented in CpG islands and depleted in the 'open sea' for both *in utero* exposures.

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TABLES

Table I. Study Population Characteristics

		Subjects with 450K Data			Stratified by Air Pollution Exposure Region						Stratified by Maternal HIV Status						
		N	%	Mean (SD)	North (Low)			South (High)			Negative			Positive			
		N	%	Mean (SD)	N	%	Mean (SD)	N	%	Mean (SD)	N	%	Mean (SD)	N	%	Mean (SD)	
Child	Males	7	32		2	18		5	45		5	36		2	25		
	Females	15	68		9	82		6	55		9	64		6	75		
	<i>Race</i>																
	African	21	95		11	100		10	91		13	93		8	100		
	Coloured	1	5		0	0		1	9		1	7		0	0		
	Gestational Age (weeks)				38.8 (1.4)			39.2 (1.7)			38.5 (0.9)			38.8 (1.5)			38.9 (1.0)
	Birth Weight (g)				3174 (430)			3140 (410)			3207 (467)			3257 (501)			3058 (193)
Maternal	Age (years)				26.1 (6.6)			27.0 (6.6)			25.3 (6.9)			24.3 (5.6)			28.6 (7.9)
	<i>Marital Status</i>																
	Married	1	5		0	0		1	9		1	7		0	0		
	Unmarried	21	95		11	100		10	91		13	93		8	100		
	<i>Previous Pregnancy</i>																
	Yes	10	45		4	36		6	55		6	43		4	50		
	No	12	55		7	64		5	45		8	57		4	50		
	<i>Ever Smoke</i>																
	Yes	3	14		2	18		1	9		3	21		0	0		
	No	19	86		9	82		10	91		11	79		8	100		
	<i>Smoking in the Home</i>																
	Yes	6	27		3	27		3	27		4	29		2	25		
	No	16	73		8	73		8	73		10	71		6	75		
<i>HIV Status</i>																	
Negative	14	64		6	55		8	73		14	100		0	0			
Positive	8	36		5	45		3	27		0	0		8	100			
Air Pollution Exposure	<i>Region</i>																
	North Durban (low)	11	50		11	100		0	0		6	43		5	63		
	South Durban (high)	11	50		0	0		11	100		8	57		3	38		
NOx (ppb)				26.5 (10.9)			17.8 (5.4)			36.1 (6.0)			26.5 (11.4)			27.4 (9.7)	

Table II. Top Gene Ontology or Function Pathways Enriched in Air Pollution Exposure Analysis. Concepts significant at a 5% FDR level using LRpath gene set enrichment testing are displayed.

Concept Name	Concept Database	# Genes in Concept	FDR q-value	Direction [†]
Ascorbate and aldarate metabolism	KEGG	19	0.003	↑
oxygen transport	GOBP	11	0.005	↓
Pentose and glucuronate interconversions	KEGG	24	0.007	↓
Drug metabolism - cytochrome P450	KEGG	62	0.013	↓
Drug metabolism - other enzymes	KEGG	39	0.017	↓
oxygen binding	GOMF	32	0.019	↓
Retinol metabolism	KEGG	54	0.024	↓
Other types of O-glycan biosynthesis	KEGG	37	0.024	↓
gas transport	GOBP	15	0.025	↓
detection of chemical stimulus involved in sensory perception	GOBP	357	0.025	↑
RNA splicing, via endonucleolytic cleavage and ligation	GOBP	10	0.029	↓
detection of chemical stimulus	GOBP	388	0.029	↑
cellular glucuronidation	GOBP	13	0.029	↓
uronic acid metabolic process	GOBP	14	0.029	↓
glucuronate metabolic process	GOBP	14	0.029	↓
Metabolism of xenobiotics by cytochrome P450	KEGG	63	0.030	↓
sensory perception of chemical stimulus	GOBP	403	0.031	↑
glucuronosyltransferase activity	GOMF	22	0.031	↓
olfactory receptor activity	GOMF	320	0.031	↑
detection of stimulus involved in sensory perception	GOBP	396	0.032	↑
negative regulation of intrinsic apoptotic signaling pathway in response to DNA damage	GOBP	23	0.033	↑

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detection of chemical stimulus involved in sensory perception of smell	GOBP	320	0.047	↑
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† Up direction signifies that most genes in the concept were hypermethylated in the south Durban group compared with the north Durban group.
Vice versa for the down direction.

Table III. Top Gene Ontology or Function Pathways Enriched in Maternal HIV Status Analysis. Concepts significant at a 5% FDR level using LRpath gene set enrichment testing are displayed.

Concept Name	Concept Database	# Genes in Concept	FDR q-value	Direction [†]
olfactory receptor activity	GOMF	320	3.83E-36	↓
detection of chemical stimulus involved in sensory perception	GOBP	357	7.59E-36	↓
detection of chemical stimulus involved in sensory perception of smell	GOBP	320	1.91E-35	↓
sensory perception of chemical stimulus	GOBP	403	1.91E-35	↓
detection of stimulus involved in sensory perception	GOBP	396	3.19E-35	↓
sensory perception of smell	GOBP	344	8.15E-35	↓
detection of chemical stimulus	GOBP	388	1.62E-34	↓
Olfactory transduction	KEGG	337	1.33E-30	↓
detection of stimulus	GOBP	553	1.06E-29	↓
sensory perception	GOBP	758	5.31E-24	↓
G-protein coupled receptor activity	GOMF	705	9.80E-22	↓
Ascorbate and aldarate metabolism	KEGG	19	3.32E-06	↓
Retinol metabolism	KEGG	54	7.46E-06	↓
Pentose and glucuronate interconversions	KEGG	24	7.46E-06	↓
Metabolism of xenobiotics by cytochrome P450	KEGG	63	1.81E-05	↓
glucuronosyltransferase activity	GOMF	22	1.83E-05	↓
Starch and sucrose metabolism	KEGG	38	3.49E-05	↓
Drug metabolism - cytochrome P450	KEGG	62	3.49E-05	↓
Steroid hormone biosynthesis	KEGG	47	3.49E-05	↓
Porphyrin and chlorophyll metabolism	KEGG	33	7.42E-05	↓
Other types of O-glycan biosynthesis	KEGG	37	1.86E-04	↓
Drug metabolism - other enzymes	KEGG	39	0.001	↓
cellular glucuronidation	GOBP	13	0.002	↓
uronic acid metabolic process	GOBP	14	0.002	↓

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glucuronate metabolic process	GOBP	14	0.002	↓
Taste transduction	KEGG	48	0.045	↓

† Up direction signifies that most genes in the concept were hypermethylated in the maternal HIV positive group compared with the negative group. Vice versa for the down direction.

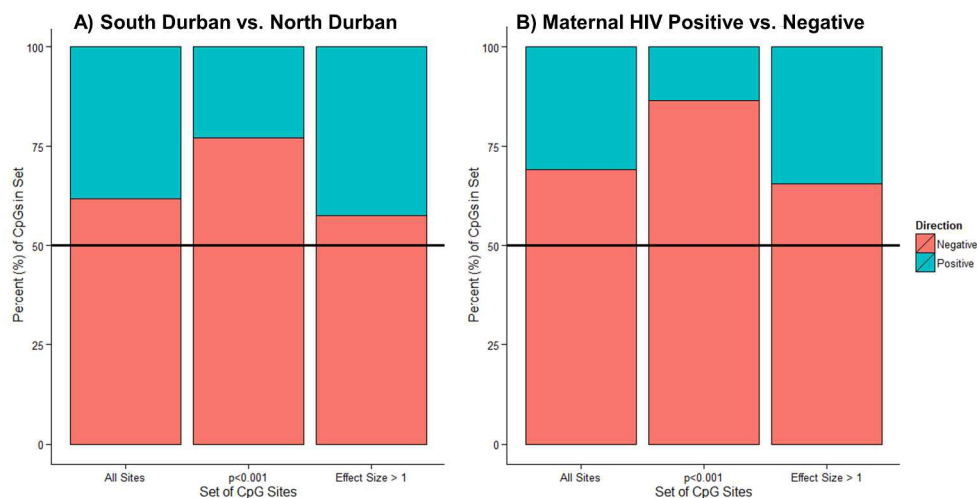


Figure 1. Enrichment of Hypomethylated Sites among High Air Pollution and Maternal HIV Exposure Groups. A statistically significant enrichment of hypomethylated sites was observed when comparing cord blood plasma DNA methylation from individuals A) residing in a high air pollution region (south Durban) compared with the low pollution north Durban and B) with maternal HIV exposure compared with uninfected mothers. Relationships were assessed in models adjusting for gestational age, child's sex, air pollution group, and maternal HIV status (n=22). Percentage of hypomethylated (direction=negative) and hypermethylated (positive) sites are displayed from the following groups of CpG sites: 1) all sites (n=433,782), 2) p<0.001 (n=420 for air pollution, 689 for HIV), or effect size >1 (log fold-change <-1 or >1; n=781 for air pollution analysis, 514 for HIV). A reference line is drawn at 50%.
321x164mm (243 x 243 DPI)

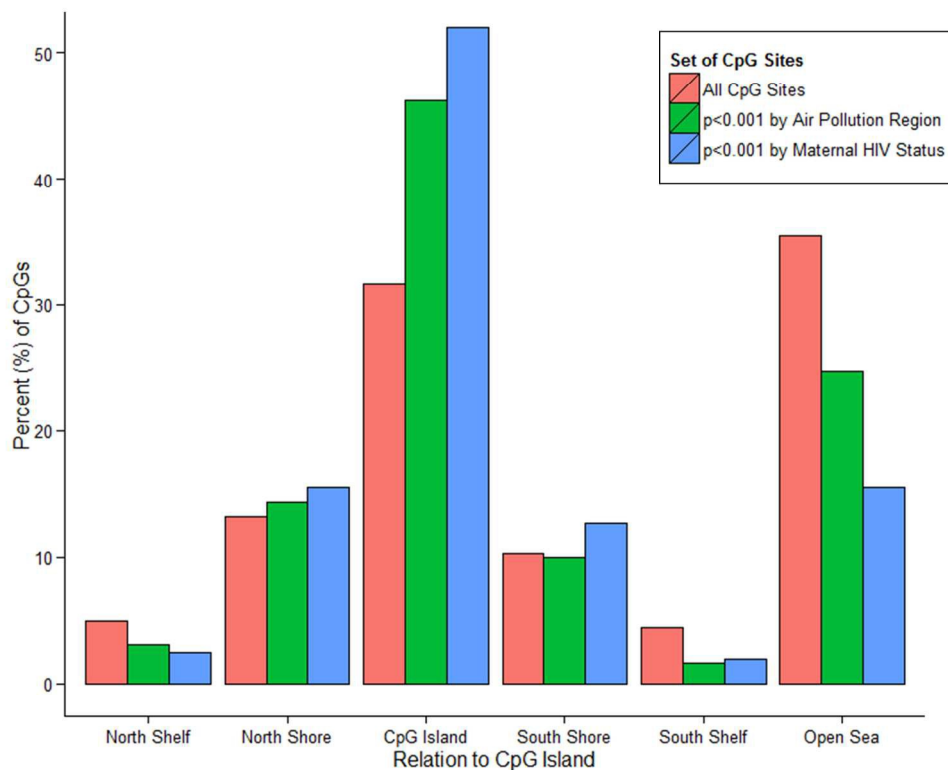


Figure 2. Enrichment of Differentially Methylated Sites (DMS) in CpG Islands among High Air Pollution and Maternal HIV Exposure Groups. Among the top DMS ($p < 0.001$) by air pollution exposure region (420 CpG sites) or maternal HIV status (689 sites) in models adjusting for both exposures, gestational age, and sex, there was a statistically significant shift in annotation related to CpG islands compared to the expected proportions based on all probes ('All CpG Sites'). Specifically, DMS were over-represented in CpG islands and depleted in the 'open sea' for both *in utero* exposures.

203x165mm (300 x 300 DPI)