Supplemental Material

Associations between Maternal Tobacco Smoke Exposure and the Cord Blood CD4+ DNA Methylome

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**Figure S5.** Dot and boxplots for the 33 CpGs that were identified as differentially methylated in MACHS (A), which replicated (%methylation difference in the same direction and raw p-value<0.05) in the WakeMed SMKE EPIC array study of cord blood CD4$^+$ cells (B). The name of the EPIC array CpG is shown on the x-axis, and the %methylation level is shown on the y-axis. Horizontal lines within each boxplot indicate the median %methylation value for each CpG. The interquartile range is represented by the upper and lower boundaries of the boxplot. The vertical lines (“whiskers”) at the top and bottom of each boxplot indicate the boundaries of 1.5 times the interquartile range. Points beyond these whiskers are outliers. Newborns who were exposed to maternal tobacco smoke in utero are indicated in blue, while unexposed newborns are indicated in pink.

**References**

**Additional File**- Excel Document
Replication analyses were conducted using data from two different studies of tobacco smoke exposure, which also profiled DNA methylation patterns in CD4+ cells.

The first study, SMKE, profiled DNA methylation using Illumina’s Infinium MethylationEPIC array in cord blood CD4+ cells isolated from a subset of 30 newborns exposed (n=14) versus unexposed (n=16) to maternal tobacco smoke, who were recruited from the WakeMed hospital in Raleigh, North Carolina. Non-smoking mothers from this study were lifetime non-smokers. Cord blood mononuclear cells were isolated using Ficoll-Paque PLUS (Sigma-Aldrich), and CD4+ antibody-coated magnetic beads (Invitrogen Dynabeads) were used to isolate CD4+ T cells. DNA/RNA was extracted using the Qiagen All Prep DNA/RNA/miRNA kit (Qiagen, 80224), according to the manufacturer’s instructions. Participant characteristics are shown in Table S1. DNA methylation was analyzed on Illumina’s Infinium MethylationEPIC array as per manufacturer’s instructions. Raw methylation image files were processed using the minfi package in R (Aryee et al. 2014). Background correction and dye-bias equalization was performed via the normal-exponential out-of-band (noob) correction method. The methylation level at each CpG was reported as the beta-value \([\beta = \text{intensity of the methylated allele (M)} / (\text{intensity of the unmethylated allele (U)} + \text{intensity of the methylated allele (M)} + 100)]\). Beta-values were then transformed to obtain the log ratio, defined as \(\log[\beta/(1 – \beta)]\), or M. Robust linear regression was used to evaluate the association between DNA methylation (M) at each CpG and smoking status while adjusting for potential confounders, including gestational age, infant sex, mother’s ethnicity (non-Hispanic black, non-Hispanic white, and Hispanic other), and sample batch. A total of 485 CpGs that were identified as differentially methylated (false discovery rate-adjusted \(p<0.05\)) in the Maternal and Child Health Study (MACHS) are represented on the EPIC array and could therefore be queried in the SMKE study.

The second set of replication analyses were conducted using results from a study of adult smokers (n=59) and lifetime non-smokers (n=72) who were recruited at the National Institute of Environmental Health Sciences Clinical Research Unit (NIEHS CRU), which has been described previously (Wan et al. 2018). DNA methylation was measured in CD4+ cells for all participants using Illumina’s Infinium HumanMethylation450 array, and was also measured in CD4+ cells from a subset of participants using Illumina’s EPIC array (n=9 smokers, n=11 non-smokers), and also in CD4+ cells from a subset of female participants, using reduced representation bisulfite sequencing (RRBS) (n=9 smokers, n=10 non-smokers). The 450k and EPIC array data were processed and analyzed using the same methods as those described above for the SMKE study, and statistical models were similarly adjusted for age, sex, and ethnicity. RRBS was carried out as reported previously in Wan et al. (Wan et al. 2018). Briefly, DNA from CD4+ cells was digested with MspI, bisulfite converted, made into RRBS libraries, and sequenced on the Illumina NextSeq platform. Bismark version 0.14.3 was used to align the reads to the hg19 assembly and methylation percentages were derived for each CpG, excluding any sites that had fewer than 10 reads or occurred at a single nucleotide polymorphism as reported previously (Su et al. 2016). RRBS DMRs were determined using the method of Wan et al. (Wan et al. 2018). Demographic characteristics for each set of NIEHS CRU participants are shown in Tables S2-S4.

CpG sites that were found to be differentially methylated (\(p_{\text{FDR}}<0.05\)) by maternal tobacco smoke exposure status in MACHS, which are represented on the 450K (n=399) and EPIC (n=485) arrays, were queried in the SMKE and NIEHS CRU EPIC array results using Practical Extraction and Reporting Language (PERL) script to match chromosomal positions. Raw \(p\)-values<0.05 were considered statistically significant for the replication study. Directions of
association were also compared. Similarly, DMRs that were represented in the NIEHS CRU RRBS results ($n=9$) were identified using chromosome position. Raw $p$-values $<0.05$ were considered statistically significant. Directions of effect were also compared.
**Table S1. Demographic Characteristics of the WakeMed SMKE EPIC Study Participants**

|                                | Unexposed (n=16) | Smoke Exposed (n=14) |
|--------------------------------|------------------|----------------------|
|                                | Mean ± SD or n (%) | Mean ± SD or n (%)   |
| Maternal Age, years            | 27.3 (5.2)       | 28.5 (4.8)           |
| Gestational Age, days          | 274.6 (6.2)      | 274.6 (6.2)          |
| Birth Weight, grams            | 3375.9 (281.8)   | 3113.9 (379.7)       |
| Baby's Sex                     |                  |                      |
| Female                         | 8 (50.0)         | 5 (35.7)             |
| Male                           | 8 (50.0)         | 9 (64.3)             |
| Ethnicity                      |                  |                      |
| Non-Hispanic Black             | 8 (50.0)         | 5 (35.7)             |
| Non-Hispanic White             | 8 (50.0)         | 8 (57.1)             |
| Hispanic Other                 | 0 (0.0)          | 1 (7.1)              |
| Cord Blood Cotinine, ng/mL     | 2 (0.0)          | 104.3 (103.5)        |
| Cigarettes Per Day             | 0 (0.0)          | 9.9 (5.6)            |
| Years Smoked                   | 0 (0.0)          | 12.3 (7.5)           |
| Caesarean Section              | 8 (50.0)         | 10 (71.4)            |
| Progesterone Use During Pregnancy| 1 (6.3)          | 0 (0.0)              |
| Any ETS Exposure*              | 2 (12.5)         | 12 (85.7)            |

*Environmental tobacco smoke (ETS) exposure is defined as any tobacco smoke exposure inside or outside the home.*
|                          | Non-Smokers (n=11) | Smokers (n=9) |
|--------------------------|--------------------|--------------|
|                          | Mean ± SD or n (%) | Mean ± SD or n (%) |
| Age, years               | 42.1 (11.6)        | 40.7 (9.8)   |
| Gender                   |                    |              |
| Female                   | 6 (54.5)           | 5 (55.6)     |
| Male                     | 5 (45.5)           | 4 (44.4)     |
| Ethnicity                |                    |              |
| Non-Hispanic Black       | 6 (54.5)           | 5 (55.6)     |
| Non-Hispanic White       | 5 (45.5)           | 4 (44.4)     |
| Cotinine, ng/mL          | 2.0 (0.0)          | 178.5 (58.8) |
| Cigarettes Per Day       | 0 (0.0)            | 15.8 (10.0)  |
| Pack-Years               | 0 (0.0)            | 20.2 (22.6)  |
| Any ETS Exposure<sup>a</sup> | 2 (18.2)          | 8 (88.9)    |

<sup>a</sup>Environmental tobacco smoke (ETS) exposure is defined as any tobacco smoke exposure inside or outside the home.

Abbreviations Used: NIEHS CRU, National Institute of Environmental Health Sciences Clinical Research Unit
|                          | Non-smokers (n=72) | Smokers (n=59) |
|--------------------------|--------------------|---------------|
| **Age, years**           | 37.6 (9.9)         | 42.0 ± 9.4    |
| **Gender**               |                    |               |
| Female                   | 27 (45.8)          | 32 (54.2)     |
| Male                     | 45 (54.2)          | 27 (45.8)     |
| **Ethnicity**            |                    |               |
| Hispanic Black           | 2 (2.8)            | 0 (0.0)       |
| Non-Hispanic Black       | 30 (41.7)          | 31 (52.5)     |
| Hispanic White           | 0 (0.0)            | 1 (1.7)       |
| Non-Hispanic White       | 34 (47.2)          | 23 (39.0)     |
| Hispanic Other           | 0 (0.0)            | 1 (1.7)       |
| Non-Hispanic Other       | 6 (8.3)            | 3 (5.1)       |
| **Serum Cotinine, ng/mL**| 2.3 (1.4)          | 231.2 (165.4) |
| **Cigarettes per day**   | 0 (0.0)            | 14.1 (7.7)    |
| **Pack-Years**           | 0 (0.0)            | 17.3 (14.8)   |
| **Any ETS exposure**     | 3 (5.1)            | 42.0 (71.2)   |

*Environmental tobacco smoke (ETS) exposure is defined as any tobacco smoke exposure inside or outside the home.

**Abbreviations Used:** NIEHS CRU, National Institute of Environmental Health Sciences Clinical Research Unit
**Table S4.** Demographic Characteristics of the NIEHS CRU RRBS Study Participants

|                      | Non-smokers (n=10) | Smokers (n=9) |
|----------------------|--------------------|---------------|
| Mean ± SD or n (%)   | Mean ± SD or n (%) |               |
| Age, years           | 46 ± 6             | 46 ± 8        |
| Gender               | Female 10 (100)    | 9 (100.0)     |
|                      | Male 0 (0.0)       | 0 (0.0)       |
| Ethnicity            | Black 5 (50.0)     | 5 (55.6)      |
|                      | White 5 (50.0)     | 4 (44.4)      |
| Serum Cotinine, ng/mL| 2 ± 0              | 217 ± 102     |
| Cigarettes Per Day   | 0 (0.0)            | 22 ± 11       |
| Pack-Years           | 0 (0.0)            | 34 ± 24       |

Abbreviations Used: NIEHS CRU, National Institute of Environmental Health Sciences Clinical Research Unit; RRBS, reduced representation bisulfite sequencing
Table S5. Enrichment of Hypomethylated and Hypermethylated DMRs Identified in MACHS in Regulatory Regions of Interest\textsuperscript{a}

|                      | Observed | Expected | \( p\)-value\textsuperscript{b} |
|----------------------|----------|----------|-------------------------------|
| HYPERMETHYLATED      |          |          |                               |
| All Enhancers        | 5        | 2        | \(8.3 \times 10^{-4}\)        |
| T Cell Enhancers     | 0        | 0        | \(>0.99\)                      |
| CD4\textsuperscript{*} DNase Sensitive Regions (ENCOD... | 17       | 3        | 0.01                          |
| CD4\textsuperscript{*} Transcription Factor Binding S... | 3        | 13       | 0.02                          |
| CD4\textsuperscript{*} CTCF Binding Sites            | 4        | 1        | 0.05                          |
| HYPO...               |          |          |                               |
| All Enhancers        | 6        | 2        | 0.05                          |
| T Cell Enhancers     | 2        | 0        | \(9.4 \times 10^{-3}\)        |
| CD4\textsuperscript{*} DNase Sensitive Regions (ENCOD... | 18       | 3        | \(4.8 \times 10^{-9}\)        |
| CD4\textsuperscript{*} Transcription Factor Binding S... | 3        | 21       | \(7.4 \times 10^{-11}\)       |
| CD4\textsuperscript{*} CTCF Binding Sites            | 7        | 1        | \(1.7 \times 10^{-4}\)        |

\(\textsuperscript{a}\)Comparing maternal tobacco smoke-exposed to unexposed newborns

\(\textsuperscript{b}\)\( p\)-value is from Fisher’s Exact Test

Abbreviations Used: DMR, differentially methylated region; ENCODE, Encyclopedia of DNA Elements; Maternal and Child Health Study
**Table S6. FANTOM5 Enhancers Overlapping DMRs Identified in MACHS\(^a\) and Their Predicted Targets**

| Enhancer | %Methylation Difference | Tissues/Cells with Highest, Significant Overrepresentation of Enhancer | Genes          | Distance | Correlation between Promoter and Enhancer Activity\(^b\) |
|----------|-------------------------|---------------------------------------------------------------------|----------------|----------|------------------------------------------------------|
| Chr1:173379915-173380349 | -17.2 | Natural Killer Cell, Lymphocyte from B Cell Lineage, Monocyte | PRDX6, KLHL20 | 65728/64, 304035 | 0.86/0.85, 0.79 |
| Chr8:141108929-141109987* | -11.7 | Brain, Olfactory Region, T Cell | KCNK9 | 394087 | 0.73 |
| Chr9:36154326-36154976 | -29.6 | Basophil, Mast Cell, Monocytes | GLIPR2, RNF38 | 17650, 246318/454 | 0.64, 0.60/0.58 |
| Chr10:134549634-134549842 | -11.3 | Spinal Cord, Brain | KNDC1 | 423706 | 0.77 |
| Chr12:77810111-77811133 | 17.1 | Testis | CLEC4A | 495124/41 | 0.75/0.58 |
| Chr16:1519780-1519909 | 10.5 | Monocyte, Dendritic Cell | BAIAP3, MAPK8IP3 | 136156, 236394 | 0.58, 0.76 |
| Chr16:85368480-85368758 | 8.7 | Mast Cell | GSE1 | 278222/319419 | 0.71, 0.74 |
| Chr17:154480-154575 | 24.1 | Brain, Kidney | -- | NA | NA |
| Chr17:14641682-14642082 | 8.1 | Monocyte, Basophil, Amniotic Epithelial Cell | HS3ST3B1 | 437473 | 0.72 |
| Chr17:76233617-76233864 | -17.4 | Spinal Cord, Brain | TMEM235, CYTH1 | 6029, 485879/907 | 0.83, 0.68/0.68 |
| Chr21:44104688-44105340* | -15.0 | Natural Killer Cell, T Cell | ABCG1, NDUFV3 | 465920, 194796 | 0.66, 0.92 |

\(^a\)DMRs were identified by merging neighboring CpGs with a false discovery rate-adjusted \(p<0.05\), identified by beta-binomial regression models, adjusted for maternal working status and infant sex, using the RADMeth program.

\(^b\)The FANTOM5 consortium predicts targets of enhancers by examining correlations between enhancers and all robust FANTOM5 promoter pairs within 500 kb and then filters these pairs for correlations with \(p\)-values<1.0x10\(^{-5}\) after adjusting for the false discovery rate.

*Active in T cells

Abbreviations Used: DMR, differentially methylated region; FANTOM5, functional annotation of the mammalian genome; MACHS, Maternal and Child Health Study.
Table S7. Replication Results for Six CpGs Contained within One of the 20 DMRs with the Largest %Methylation Differences that Could be Queried in the WakeMed SMKE Study

| Position           | EPIC CpG     | MACHS %Methylation Difference | Average Coverage | SMKE %Methylation Difference | SMKE P-Value |
|--------------------|--------------|-------------------------------|------------------|-------------------------------|--------------|
| chr4:1607110      | cg08089543   | 21.0                          | 6.5              | -0.3                          | 0.76         |
| chr4:1607290      | cg27207756   | 32.9                          | 2.4              | 2.2                           | 0.55         |
| chr5:177209284    | cg26673648   | -47.0                         | 2.0              | -0.6                          | 0.82         |
| chr9:36154749     | cg19097407   | -34.7                         | 7.6              | 8.7                           | 0.44         |
| chr10:123100180   | cg07044115   | 42.4                          | 7.5              | -1.9                          | 0.52         |
| chr17:27359874    | cg03597174   | 18.3                          | 5.1              | -3.1                          | 0.03         |

Abbreviations Used: DMR, differentially methylated region; MACHS, Maternal and Child Health Study
**Table S8.** DMRs identified in MACHS\(^a\) that overlap DMRs identified by Bauer et al. 2016

| Bauer DMR                     | %Methylation Difference | #CpGs | MACHS DMR             | %Methylation Difference | #CpGs\(^b\) | Genomic Region | Nearest Gene | Distance |
|-------------------------------|-------------------------|-------|-----------------------|-------------------------|-------------|----------------|--------------|----------|
| chr1:149293953-149294204      | 11.5                    | 11    | chr1:149293953-149293970 | 12.2                    | 3           | 3' End         | TRNA_Val    | 0        |
| chr14:93153235-93153358       | -32.9                   | 9     | chr14:93153343-93153502 | 14.2                    | 5           | Intron         | RIN3        | 0        |
| chr20:58713541-58714104       | 12.6                    | 28    | chr20:58713718-58713922 | -15.0                   | 8           | Promoter       | MIR646HG    | 0        |
| chr7:5183707-5184308          | 13.7                    | 27    | chr7:5183954-5184155   | -15.9                   | 7           | Promoter       | ZNF890P     | 0        |
| chr8:41593896-41594261        | 13.7                    | 16    | chr8:41593896-41594262 | -15.1                   | 8           | Intron         | ANK1, NKKX6-3 | 0    |
| chr9:72026853-72027406        | 10.8                    | 29    | chr9:72027018-72027281  | 17.8                    | 8           | Intergenic     | APBA1       | 15,167   |

\(^a\)DMRs were identified by merging neighboring CpGs with a false discovery rate-adjusted \(p<0.05\), identified by beta-binomial regression models, adjusted for maternal working status and infant sex, using the MethPipe software.

\(^b\)Number of CpGs within the DMR that had both a raw and false discovery rate-adjusted \(p<0.05\).

Abbreviations Used: DM, differentially methylated; DMR, differentially methylated region; MACHS, Maternal and Child Health Study.
Figure S1. Dot and boxplots for the 10 CpGs with the smallest p-values and absolute %methylation differences between 10-11%. The CpG position is shown on the x-axis and the %methylation level is shown on the y-axis. Horizontal lines within each boxplot indicate the median %methylation level for each CpG. Interquartile ranges are represented by the upper and lower boundaries of the boxplots. The vertical lines ("whiskers") at the top and bottom of the boxplots indicate the boundaries of 1.5 times the interquartile range. Points beyond these whiskers are outliers. Newborns who were exposed to maternal tobacco smoke in utero are indicated in blue, while unexposed newborns are indicated in pink.
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blue and pink, respectively. The height of each bar indicates the %methylation difference between groups.
Figure S3. Histograms showing distributions for the A) %methylation differences within differentially methylated regions (DMRs), B) base pair lengths of DMRs, and C) number of differentially methylated CpG sites (raw and false discovery rate-adjusted $p<0.05$) within DMRs.
A

![Bar chart showing fraction of Obs, Exp, and p-value for hypermethylated and hypomethylated DMRs in promoter, exon, intron, 3' end, and intergenic regions.]

B

| Region   | Obs | Exp | p-value |
|----------|-----|-----|---------|
| Promoter | 13  | 5   | 1.6 x 10^-7 |
| Exon     | 8   | 5   | 0.15    |
| Intron   | 74  | 77  | 0.65    |
| 3' End   | 8   | 5   | 0.15    |
| Intergenic | 82  | 93  | 0.10    |

C

| Region   | HYPERMETHYLATED  | HYPOMETHYLATED  |
|----------|-------------------|------------------|
| Promoter | 18.1 (10.6, 31.3) | -14.4 (-29.4, -10.5) |
| Exon     | 16.3 (12.6, 21.1) | -12.7 (-26.0, -10.1) |
| Intron   | 14.3 (10.0, 39.2) | -15.1 (-30.5, -10.2) |
| 3' End   | 7.3 (12.2, 35.3)  | -14.4 (-26.4, -10.1) |
| Intergenic | 15.5 (10.0, 39.7) | -15.4 (-39.8, -10.2) |

D

| Region   | HYPERMETHYLATED  | HYPOMETHYLATED  |
|----------|-------------------|------------------|
| Promoter | 13  | 5   | 2.6 x 10^-4 |
| Exon     | 12  | 4   | 7.0 x 10^-4 |
| Intron   | 85  | 63  | 0.81    |
| 3' End   | 9   | 4   | 0.02    |
| Intergenic | 56  | 80  | 1.4 x 10^-4 |
Figure S4. (A) Proportion of differentially methylated regions (DMRs) that were hypermethylated (black) and hypomethylated (gray) in the maternal tobacco smoke exposed, compared with unexposed, group by genomic region, (B) corresponding enrichment tests (Fisher’s exact test), comparing the number of DMRs overlapping each genomic region with a set of similar-sized regions randomly selected from the genome, and (C) median (range) %methylation differences by genomic region.
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and bottom of each boxplot indicate the boundaries of 1.5 times the interquartile range. Points beyond these whiskers are outliers. Newborns who were exposed to maternal tobacco smoke *in utero* are indicated in blue, while unexposed newborns are indicated in pink.
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