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**Research Article**

# Duration of exposure to epidural anesthesia at delivery, DNA methylation in umbilical cord blood and their association with offspring asthma in Non-Hispanic Black women

**Yaxu Wang[1], Jung-Ying Tzeng[1,2,3], Yueyang Huang[1], Rachel Maguire[2,4], Cathrine Hoyo[4] and Terrence K. Allen[5,*]**

[1]Bioinformatics Research Center, North Carolina State University, Raleigh, NC 27607, USA, [2]Center for Human Health and the Environment, North Carolina State University, Raleigh, NC 27695-7633, USA, [3]Department of Statistics, North Carolina State University, Raleigh, NC 27607, USA, [4]Department of Biological Sciences, North Carolina State University, Raleigh, NC 27695, USA, [5]Department of Anesthesiology, Duke University School of Medicine, Durham, NC 27710, USA
*Correspondence address. Department of Anesthesiology, Duke University School of Medicine, Durham, NC 27710, USA. Tel: +919-668-2024; Fax: +919-681-4698; E-mail: terrence.allen@duke.edu

## Abstract

Epidural anesthesia is an effective pain relief modality, widely used for labor analgesia. Childhood asthma is one of the commonest chronic medical illnesses in the USA which places a significant burden on the health-care system. We recently demonstrated a negative association between the duration of epidural anesthesia and the development of childhood asthma; however, the underlying molecular mechanisms still remain unclear. In this study of 127 mother–child pairs comprised of 75 Non-Hispanic Black (NHB) and 52 Non-Hispanic White (NHW) from the Newborn Epigenetic Study, we tested the hypothesis that umbilical cord blood DNA methylation mediates the association between the duration of exposure to epidural anesthesia at delivery and the development of childhood asthma and whether this differed by race/ethnicity. In the mother–child pairs of NHB ancestry, the duration of exposure to epidural anesthesia was associated with a marginally lower risk of asthma (odds ratio = 0.88, 95% confidence interval = 0.76–1.01) for each 1-h increase in exposure to epidural anesthesia. Of the 20 CpGs in the NHB population showing the strongest mediation effect, 50% demonstrated an average mediation proportion of 52%, with directional consistency of direct and indirect effects. These top 20 CpGs mapped to 21 genes enriched for pathways engaged in antigen processing, antigen presentation, protein ubiquitination and regulatory networks related to the Major Histocompatibility Complex (MHC) class I complex and Nuclear Factor Kappa-B (*NFkB*) complex. Our findings suggest that DNA methylation in immune-related pathways contributes to the effects of the duration of exposure to epidural anesthesia on childhood asthma risk in NHB offspring.

**Key words:** epidural anesthesia; labor analgesia; asthma; health disparities; DNA methylation; mediation analysis

## Introduction

Neuraxial anesthesia, which includes epidural anesthesia, is an effective technique for intrapartum pain relief for laboring women. Neuraxial anesthesia is used by ∼70% of women in the USA for labor analgesia potentially exposing millions of newborns to its effects [1, 2]. Administration of neuraxial anesthesia also varies substantially by ethnicity, with Non-Hispanic Black (NHB) women less likely to receive this analgesic when compared with Non-Hispanic White (NHW) women as a labor pain management modality [3, 4]. Despite its widespread use, ethnic disparities in exposure and safety profile, studies investigating the long-term effects of neuraxial anesthesia on childhood outcomes are lacking but are desperately needed. In fact, recent epidemiological studies with sample sizes ranging from 123 175 to 479 178 participants investigating an association between epidural anesthesia for labor analgesia and neurocognitive outcomes, specifically autism spectrum disorders, have produced mixed results while adjusting for maternal sociodemographic, prepregnancy, pregnancy and perinatal covariates, raising the possibility that epidural anesthesia may have effects far in excess of the short-term outcomes that have previously been investigated [1, 5–7]. Indeed, increasing the duration of exposure to epidural anesthesia has been associated with a lower risk of asthma in children at 5 years of age, presumably because it attenuates the maternal physiological stress response to labor [8]. However, mechanistic insights linking epidural anesthesia exposure—drugs administered at the period of extreme maternal stress at and around the time of delivery—and the reduced odds of developing childhood asthma remain unknown [8].

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**Table 1:** Characteristics of study population and univariate analysis

| | | NHB ($N = 75$, 59.1%) | | | NHW ($N = 52$, 40.9%) | | |
|---|---|---|---|---|---|---|---|
| | | Asthma ($N = 20$) | Normal ($N = 55$) | $P$-value[a] | Asthma ($N = 7$) | Normal ($N = 45$) | $P$-value[a] |
| Child's gender (%) | Female | 8 (40.0) | 30 (54.5) | | 1 (14.3) | 19 (42.2) | |
| | Male | 12 (60.0) | 25 (45.5) | 0.268 | 6 (85.7) | 26 (57.8) | 0.187 |
| Gestational weight gain[b] (%) | <Adequate | 3 (15.0) | 7 (12.7) | | 0 | 7 (15.6) | |
| | Adequate | 3 (15.0) | 16 (29.1) | 0.376 | 0 | 11 (24.4) | 1.000 |
| | >Adequate | 14 (70.0) | 32 (58.2) | 0.978 | 7 (100.0) | 27 (60.0) | 0.996 |
| Maternal age at delivery (years) (SD) | | 25.25 (6.52) | 27.51 (5.96) | 0.163 | 27.71 (4.61) | 31.38 (5.99) | 0.134 |
| Gestational age in weeks (SD) | | 38.25 (1.75) | 38.32 (2.17) | 0.889 | 38.71 (1.57) | 39.31 (1.36) | 0.292 |
| Parity (%) | 0 | 5 (25.0) | 7 (12.7) | | 2 (28.6) | 19 (42.2) | |
| | 1 | 5 (25.0) | 18 (32.7) | 0.222 | 2 (28.6) | 15 (33.3) | 0.823 |
| | 2 | 7 (35.0) | 11 (20.0) | 0.879 | 3 (42.9) | 9 (20.0) | 0.248 |
| | >2 | 3 (15.0) | 19 (34.5) | 0.077 | 0 | 2 | 0.996 |
| Maternal education (%) | <High school | 4 (20.0) | 9 (16.4) | 0.713 | 1 (14.3) | 1 (2.2) | |
| | High schoolor more | 16 (80.0) | 46 (83.6) | | 6 (85.7) | 44 (97.8) | 0.178 |
| Maternal smoking (%) | No | 10 (50.0) | 31 (56.4) | | 1 (14.3) | 35 (77.8) | |
| | Yes | 10 (50.0) | 24 (43.6) | 0.625 | 6 (85.7) | 10 (22.2) | **0.007** |
| Maternal asthma (%) | No | 15 (75.0) | 46 (83.6) | | 6 (85.7) | 40 (88.9) | |
| | Yes | 5 (25.0) | 9 (16.4) | 0.399 | 1 (14.3) | 5 (11.1) | 0.807 |
| Maternal obesity[c] (%) | No | 12 (60.0) | 35 (63.6) | | 6 (85.7) | 34 (75.6) | |
| | Yes | 8 (40.0) | 20 (36.4) | 0.774 | 1 (14.3) | 11 (24.4) | 0.559 |
| Breastfeeding (%) | No | 11 (55.0) | 31 (56.4) | | 3 (42.9) | 12 (26.7) | |
| | Yes | 9 (45.0) | 24 (43.6) | 0.916 | 4 (57.1) | 33 (73.3) | 0.386 |
| Delivery route (%) | Vaginal | 14 (70.0) | 36 (65.5) | | 3 (42.9) | 33 (73.3) | |
| | Cesarean | 6 (30.0) | 19 (34.5) | 0.712 | 4 (57.1) | 12 (26.7) | 0.120 |
| Birth weight (%) | <4 kg | 19 (95.0) | 50 (90.9) | | 6 (85.7) | 41 (91.1) | |
| | ≥4 kg | 1 (5.0) | 5 (9.1) | 0.569 | 1 (14.3) | 4 (8.9) | 0.656 |

All categorical variables were summarized by mean (SD), and continuous variables were summarized by sample size (%).
[a]$P$-values correspond to univariate logistic regression. Analysis was conducted in the NHB and NHW groups separately. For categorical variables, the first level was used as a reference.
[b]Gestational weight gain is defined by the categories of the Institute of Medicine.
[c]Maternal obesity is defined by maternal BMI > 30 kg/m$^2$.

Childhood asthma remains one of the most common chronic medical illnesses in the USA and it places a significant burden on the health-care system with annual direct medical costs estimated to be in excess of $6 billion dollars annually [9]. The sex distribution of childhood asthma is complex and variable, but up to 5 years of age, the incidence of asthmatic wheezing is nearly two-fold higher in boys than in girls [10]. Moreover, asthma is nearly twice as prevalent in NHB children than in NHW children [11]. Additionally, NHB children experience higher rates of asthma-related emergency room visits, hospitalizations and deaths when compared with NHW children [11]. Despite the significant burden asthma places on the health-care system, the established risk factors fail to fully explain the prevalence of asthma, and underlying mechanisms leading to its development are poorly understood, particularly in ethnic minorities.

Although multiple genetic variants have been identified from genome-wide association studies, these loci only explain a modest proportion of the asthma risk [12]. Emerging research now suggests that epigenetic mechanisms may play a role in the pathogenesis, acknowledging that early life exposures may contribute significantly to the development of asthma [13]. A recent epigenome-wide association study has identified nine CpG sites that were differentially methylated in newborn cord blood samples and were associated with childhood asthma [13]. However, of the eight newborn cohorts contributing to this analysis, only two were from non-European ancestry limiting the relevance of these findings in ethnic minority populations in whom the prevalence of asthma is higher. Furthermore, the included cohorts did not address the role of exposure to epidural anesthesia at delivery, which, although widely used, is less commonly administered to NHB women when compared with NHW women as a labor pain management modality [3, 4].

Given the ethnic disparities in asthma prevalence and epidural anesthesia utilization together with epidural anesthesia having been shown to partly attenuate the maternal physiological stress response to labor, we hypothesized that the duration of exposure to epidural anesthesia would reduce the risk of asthma development in NHB children via epigenetic mechanisms [14–21]. To test this hypothesis, we used existing maternal, pediatric, anesthetic and umbilical cord blood epigenetic marker data from the Newborn Epigenetics STudy (NEST) to examine the association between the duration of exposure to epidural anesthesia and childhood asthma development by race/ethnicity strata and identify potentially any novel newborn umbilical cord blood epigenetic markers that might mediate these effects, particularly in ethnic minorities.

## Results

### Characteristics of Study Population and Univariate Analysis

The characteristics of our study population are represented in Table 1. Of the 127 mother–child pairs, 75 (59.1%) were NHB, and 52 (40.9%) were NHW. About 15.7% of children born to NHB women were diagnosed with asthma, whereas only ∼5.5% of children born to NHW were diagnosed with asthma. The mean (SD) age of asthma onset in the offspring was 6.8 (1.3) years. Our preliminary analyses revealed an association between maternal

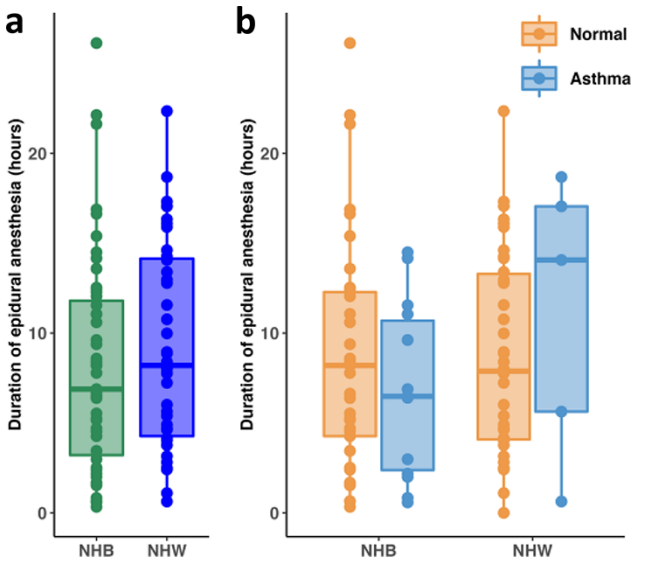


**Figure 1:** Boxplot of the duration of epidural anesthesia during delivery in the NHB and NHW groups. The points indicate the duration of maternal exposure to epidural anesthesia (in hours) during delivery. (a) Overall distribution of the duration of epidural anesthesia in NHB and NHW. (b) Grouped by the childhood asthma.

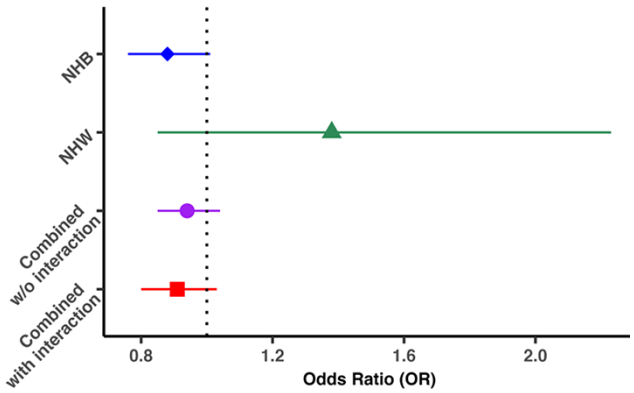


**Figure 2:** Association between the duration of exposure to epidural anesthesia and childhood asthma in different analysis scenarios. Multiple logistic regression was used to evaluate the association, adjusting for spinal administration, gender, maternal asthma, maternal smoking, maternal obesity, maternal age at delivery, maternal gestational weight gain, maternal education, birth weight, breastfeeding status, delivery route and gestational age. In the combined analysis, we also adjusted for maternal ethnicity. In the combined analysis with interactions, we further included an interaction term between maternal ethnicity and epidural duration. The points indicate the OR of developing asthma per 1-h increase of the duration of epidural anesthesia under given analysis scenarios. Horizontal lines are 95% CIs. Vertical dotted line indicates the position with OR equal to 1

smoking and childhood asthma in the NHW population ($P < 0.007$) as previously reported [22, 23]. While we did not observe significant associations between childhood asthma and sex, maternal asthma, maternal obesity, maternal gestational weight gain, maternal education, birth weight, breastfeeding status, delivery route, or gestational age (Table 1), these factors were nonetheless adjusted for in subsequent analysis considering previously reported associations [24–28]. Supplementary Table S1 shows the difference of the characteristics between NHB and NHW women.

## Distribution of the Duration of Exposure to Epidural Anesthesia at Delivery

Figure 1 shows the distribution of the duration of exposure to epidural anesthesia at delivery in NHB and NHW women. Overall, the duration of maternal exposure to epidural anesthesia was longer for NHW [median = 8.20 h, interquartile range (IQR) = 4.27–14.14 h] compared to NHB (median = 6.88 h, IQR = 3.22–11.80 h, Wilcoxon rank sum test $P$-value = 0.14, Fig. 1a). Surprisingly, children without asthma that were born to NHW women had a shorter median duration of exposure to epidural anesthesia (median = 8.00 h, IQR = 4.15–13.40 h) than those diagnosed with asthma (median = 14.10 h, IQR = 5.63–17.00 h), whereas children without asthma that were born to NHB women had a longer median duration of exposure to epidural anesthesia (median = 8.20 h, IQR = 4.27–12.30 h) than those diagnosed with asthma (median = 6.48 h, IQR = 2.38–10.70 h, Fig. 1b). The dose of drugs administered and the duration of epidural and spinal anesthetics are summarized in Supplementary Table S2.

## Association between the Duration of Exposure to Epidural Anesthesia and Childhood Asthma

These differences in the distribution of the duration of exposure to epidural anesthesia and childhood asthma between NHB and NHW persisted after adjusting for potential confounders—maternal asthma, maternal smoking, maternal obesity, maternal age at delivery, maternal gestational weight gain, maternal education status, birth weight, gender of the offspring, breastfeeding status, delivery route, spinal anesthesia administration and gestational age at delivery (Fig. 2). The duration of exposure to epidural anesthesia was not associated with asthma risk among participants when the NHB and NHW population were combined. However, the duration of exposure to epidural anesthesia was associated with a marginally lower risk of asthma for children in NHB [odds ratio (OR) = 0.88, 95% confidence interval (CI) = 0.76–1.01], for each 1-h increase in the exposure to epidural anesthesia (Fig. 2). Since parity may shorten the length of the second stage of labor, and the rates of multiparity were also different between the two groups, we performed a sensitivity analysis adjusting for parity [29]. Similar results were observed for the association of the duration of exposure to epidural anesthesia with the risk of asthma in the NHB group (OR = 0.85, 95% CI = 0.73–1.00). This association was not apparent in NHW, with OR (95% CI) of 1.38 (0.85–2.23) suggesting a lack of a risk-reducing effect and intriguingly possibly an opposite effect of the duration of exposure to epidural anesthesia on the development of childhood asthma in the NHW population.

## Mediation Analysis of Umbilical Cord Blood DNA Methylation

Next, we identified cytosine methylation markers (measured in umbilical cord blood–derived DNA) associated with the duration of exposure to epidural anesthesia and determined if these methylation markers mediated the association between the duration of exposure to epidural anesthesia and childhood asthma in NHB, with the joint significant method that was used to obtain mediation $P$-values [30]. As expected with our limited sample size, none of the CpG sites survived the Bonferroni correction for multiple comparisons at the 0.05 level of significance. Thus, we focused on the 20 most significant CpGs (see Supplementary Table S3a),

**Table 2:** Causal mediation analysis of top 20 CpGs with the smallest mediation *P*-values in NHB

| CpG | Chr:Position | Nearest Gene | Δ($\beta$-values)[a] | Total Effect (*P*-value) | Indirect Effect (*P*-value) | Direct Effect (*P*-value) | Ratio of Indirect Effect to Total Effect |
|---|---|---|---|---|---|---|---|
| cg17437770 | chr10:43250800 | *RASGEF1A*[b] | −0.0536 | −0.0225 (0.0472) | 0.0120 (0.0052) | −0.0345 (0.0076) | −0.4962 |
| cg14921485 | chr6:158650606 | *SYTL3*[b] | −0.0283 | −0.0233 (0.0584) | −0.0131 (0.0064) | −0.0102 (0.3624) | 0.5490 |
| cg10383829 | chr6:28864419 | *RPL13*[b] | 0.0071 | −0.0243 (0.0548) | 0.0118 (0.0064) | −0.0361 (0.0080) | −0.4453 |
| cg02068388 | chr5:107979173 | *FER*[b] | 0.0089 | −0.0270 (0.0208) | −0.0124 (0.0092) | −0.0146 (0.1456) | 0.4576 |
| cg20864636 | chr9:130025713 | *GARNL3* | −0.0050 | −0.0188 (0.0828) | −0.0129 (0.0092) | −0.0059 (0.5940) | 0.6431 |
| cg07857243 | chr16:77822441 | *VAT1L* | 0.0099 | −0.0206 (0.0772) | −0.0107 (0.0096) | −0.0099 (0.3712) | 0.4902 |
| cg12792775 | chr14:26076574 | *LINC02306*[b] | 0.0177 | −0.0227 (0.0548) | 0.0103 (0.0100) | −0.0330 (0.0136) | −0.4201 |
| cg00300216 | chr2:6992700 | *CMPK2* | 0.0145 | −0.0224 (0.0560) | 0.0104 (0.0104) | −0.0328 (0.0072) | −0.4228 |
| cg04930571 | chr7:1279790 | *UNCX*[b] | 0.0072 | −0.0256 (0.0296) | 0.0110 (0.0108) | −0.0366 (0.0080) | −0.4041 |
| cg12732864 | chr6:31598424 | *PRRC2A* | −0.0122 | −0.0197 (0.0816) | −0.0097 (0.0112) | −0.0100 (0.3612) | 0.4659 |
| cg02690968 | chr11:35683923 | *TRIM44* | 0.0149 | −0.0227 (0.0468) | 0.0105 (0.0116) | −0.0332 (0.0088) | −0.4329 |
| cg20944143 | chr1:202972455 | *CYB5R1*[b] | −0.0310 | −0.0236 (0.0332) | −0.0094 (0.0116) | −0.0143 (0.1788) | 0.3831 |
| cg13036546 | chr6:30460576 | *HLA-E* | 0.0218 | −0.0250 (0.0484) | 0.0108 (0.0116) | −0.0358 (0.0084) | −0.3963 |
| cg14381948 | chr7:154684893 | *DPP6* | −0.0396 | −0.0197 (0.0764) | −0.0117 (0.0120) | −0.0080 (0.4768) | 0.5630 |
| cg12594615 | chr2:101643137 | *TBC1D8* | 0.0194 | −0.0200 (0.0572) | 0.0122 (0.0124) | −0.0322 (0.0068) | −0.5617 |
| cg20025238 | chr7:128829789 | *SMO* | 0.0099 | −0.0196 (0.0588) | 0.0105 (0.0124) | −0.0301 (0.0052) | −0.4852 |
| cg15929078 | chr6:33267996 | *TAPBPRGL2* | 0.0271 | −0.0219 (0.0656) | −0.0103 (0.0132) | −0.0116 (0.3208) | 0.4519 |
| cg08354908 | chr6:32803009 | *TAP2* | −0.0043 | −0.0237 (0.0380) | 0.0096 (0.0136) | −0.0332 (0.0096) | −0.3762 |
| cg22424615 | chr12:53404170 | *EIF4B* | 0.0116 | −0.0201 (0.0576) | −0.0145 (0.0136) | −0.0057 (0.5216) | 0.7089 |
| cg07018435 | chr1:246860007 | *SCCPDH*[b] | −0.0727 | −0.0203 (0.0820) | −0.0102 (0.0140) | −0.0101 (0.3572) | 0.4742 |

[a]Δ($\beta$-values) were the difference between the mean residualized cord blood DNA methylation $\beta$-values of children diagnosed with asthma and children not diagnosed with asthma.
[b]Genes were located nearby the corresponding CpGs within 50 kb.

after which there was an inflection point of the ordered mediation *P*-values obtained using the joint significant method (Supplementary Fig. S1). For these top 20 CpG sites, we performed a more comprehensive mediation analysis, and the effect sizes were summarized in Table 2.

Table 2 shows that all of the 20 top CpGs had mediation effects with *P*-value ≤0.014. For example, cg17437770, mapping nearby *RasGEF Domain Family Member 1A*(*RASGEF1A*), significantly mediated the association between the duration of exposure to epidural anesthesia and asthma risk. The total effect [log(OR) = −0.0225 (*P*-value 0.0472)] indicates that the duration of exposure to epidural anesthesia is associated with a decreased risk of childhood asthma when not accounting for the methylation status at cg17437770. The indirect effect of the duration of exposure to epidural anesthesia via cg17437770 on asthma, i.e. log(OR) = 0.0120 (*P*-value 0.0052), suggests that hypomethylation of the cg17437770 mediates these associations. The ratio of the indirect effect to the total effect, also known as the mediation ratio, is −0.4962, where the absolute value indicates that the relative magnitude of indirect effect via cg17437770 is ∼50% of the total effect. Similarly, the indirect effect of the duration of exposure to epidural anesthesia contributed by cg14921485 mapping near *Synaptotagmin Like 3*(*SYTL3*) on asthma risk is log(OR) = −0.0131 (*P*-value 0.0064), suggesting the same direction as the total effect. The mediation ratio (0.5490) also suggested that ∼55% of the total effect is mediated via cg14921485.

As seen in Table 2, of the 20 CpGs investigated, 10 CpGs indicated consistent mediation, i.e. the same direction of indirect and direct effects; the 10 CpGs with consistent mediation also showed the same direction of indirect and total effects, with an average mediation ratio equal to 0.519. The 10 CpGs with inconsistent mediation effects had an average mediation ratio of −0.444. The 20 CpGs were scattered in different genomic positions with no sign of high correlation [median $(r^2) = 0.016$, where $r$ is the pairwise Pearson correlation coefficient; see Fig. S2].

Since inconsistent mediation may result in a nonsignificant total effect because the direct and indirect effects cancel each other out [31], we replicated the mediation analysis in the NHW group despite not observing any significant association between the duration of exposure to epidural anesthesia and childhood asthma in this group. However, none of the CpGs tested showed any significant mediation effect at a nominal significance level of 0.1 (Supplementary Table S3b).

## Association between Umbilical Cord Blood DNA Methylation and the Duration of Exposure to Epidural Anesthesia in NHB Group

To explore how the duration of exposure to epidural anesthesia may affect umbilical cord blood DNA methylation, we revisited the epigenome-wide association results of Model (2). In total, 2066 CpGs were associated with the duration of exposure to epidural anesthesia with nominal *P*-values <0.01 (see Supplementary Table S4), but again, none survived the Bonferroni multiple test correction level of 0.05, leading us to again focus on the 20 CpGs with the strongest associations.

## Functional Analysis

For the 21 genes mapped by the top 20 CpGs showing the most significant mediation effects between the duration of exposure to epidural anesthesia and childhood asthma in NHB, we identified canonical pathways, Gene Ontology (GO) terms and diseases significantly enriched by these genes [false discovery rate (FDR) < 0.05] using Ingenuity Pathway Analysis (IPA) and Enrichr. From the GO library, we identified enriched molecular function terms, including MHC class I protein binding and transporter 1, ATP binding cassette subfamily B member binding, and cellular component terms involving the MHC protein complex (Fig. 3a). Combining GO, IPA and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, we found enriched pathways mainly engaged in antigen processing, antigen presentation and pro-

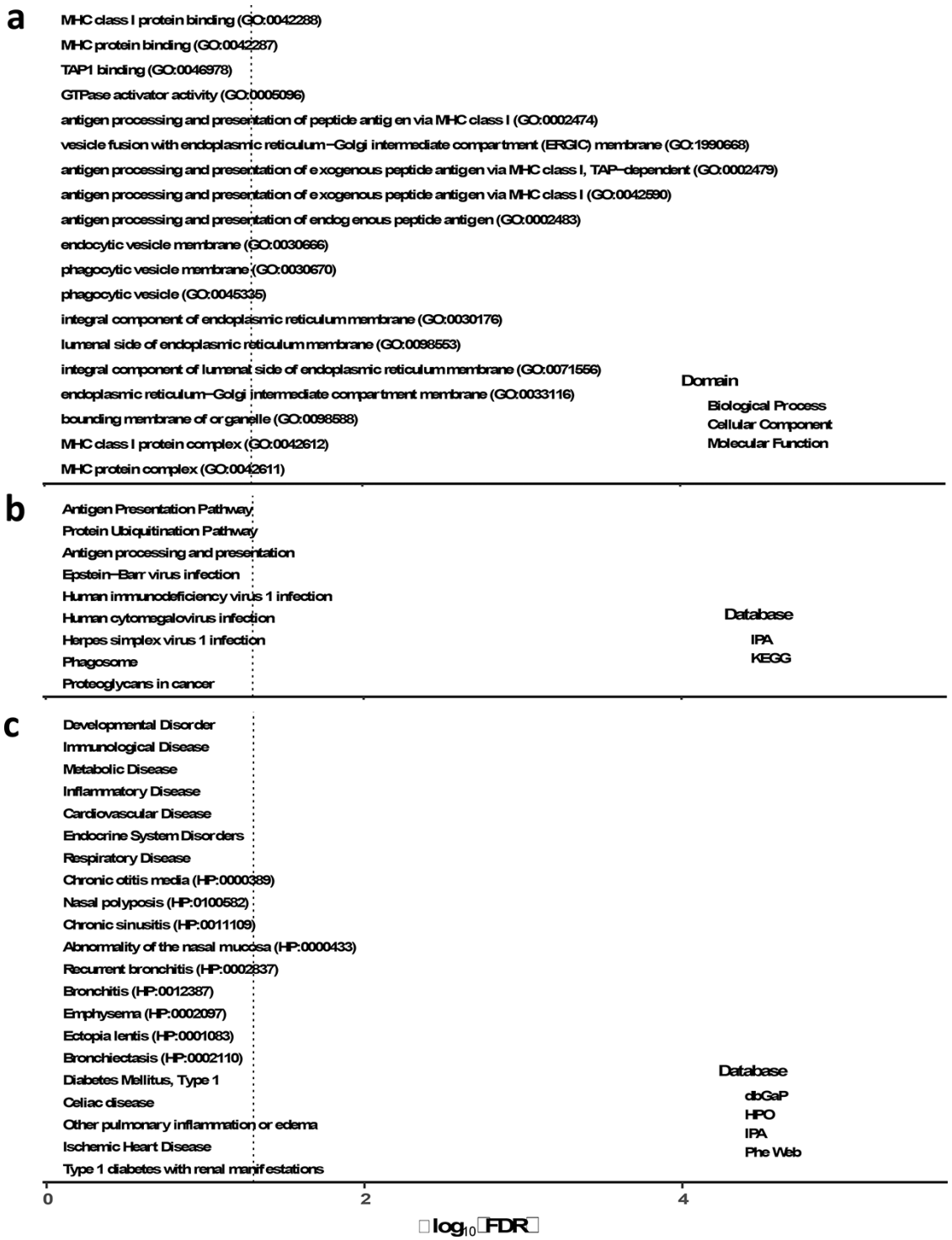


**Figure 3:** Enrichment analysis of the 21 genes mapped by the 20 CpGs showing the strongest mediation effect in the NHB group. (a) GO terms, (b) IPA canonical pathways and KEGG pathways and (c) diseases and HPO terms. The vertical dotted line indicates the position with FDR equal to 0.05

tein ubiquitination (Fig. 3b). Using different sources of human phenotype database, we discovered multiple associated diseases, including immunological disease, inflammatory disease, chronic sinusitis, bronchitis, bronchiectasis and type 1 diabetes mellitus (Fig. 3c). Regulatory networks associated with the mapped genes are shown in Fig. 4a, in which the MHC class I complex and the *NFkB* complex were identified as regulatory hubs. Using the Genotype-Tissue Expression (GTEx) database, we found abundant expression of *Major Histocompatibility Complex, Class I, E* (*HLA-E*), *TAP Binding Protein* (*TAPBP*) and *Transporter 2 ATP Binding Cassette Subfamily B Member* (*TAP2*) genes in lung tissue (see Supplementary Fig. S3a).

For the 11 genes mapped by the top 20 CpGs associated with the duration of exposure to epidural anesthesia in the NHB group, we found enriched pathways, including basal cell carcinoma signaling and epithelial adherens junction signaling, and associated

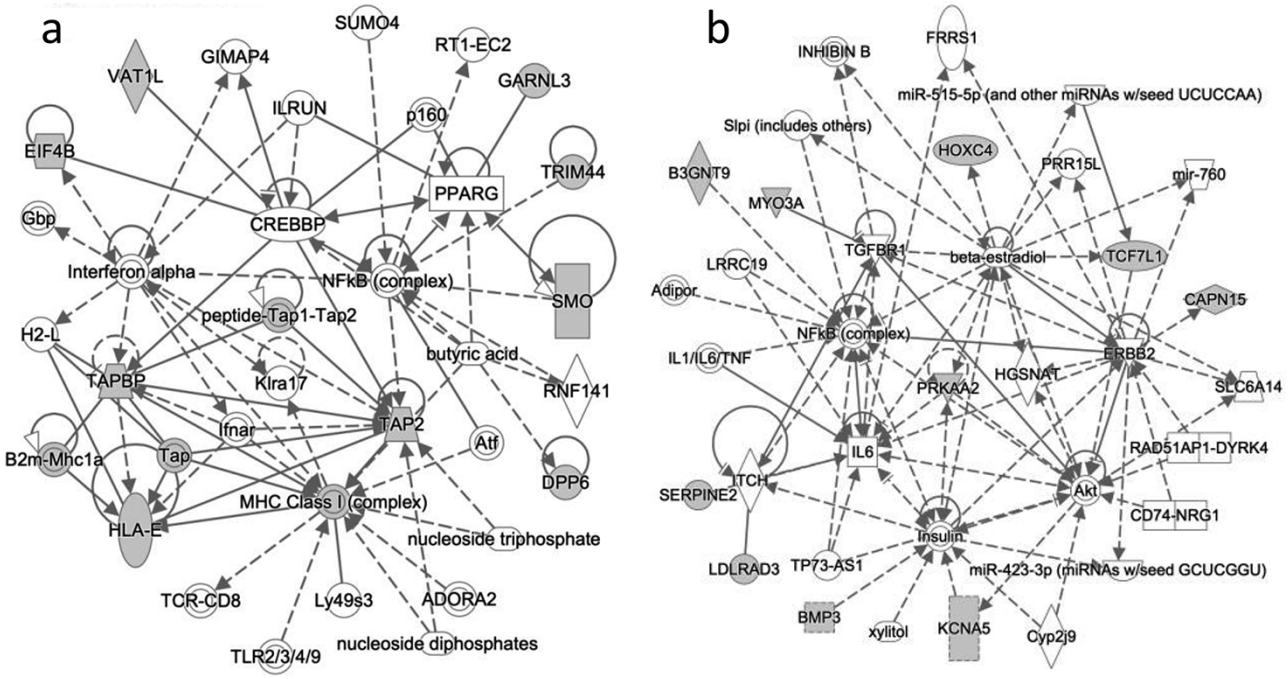


**Figure 4:** Regulatory network generated by IPA. Nodes are molecule names with shapes representing the type of molecule and interaction. Shaded shapes are mapped genes in our analysis, and open shapes represent molecules that are not present in our dataset but placed by IPA due to their interactions with our mapped genes. (a) The IPA network associated with the genes mapped by the top 20 mediating CpGs. (b) The IPA network identified by the genes mapped by the top 20 CpGs associated with the duration of exposure to epidural anesthesia

disease and human phenotypes, including Palpitations, Atrial Fibrillation, Arrhythmia and Hypothyroidism (see Supplementary Table S5). The hubs of associated regulatory networks included insulin and *NFkB* complex (Fig. 4b). Using the GTEx database, abundant expression of *Potassium Voltage–Gated Channel Subfamily A Member* 5 and *Small Optic Lobes Homolog* was identified in the aorta, atrial appendage and lung tissue (see Supplementary Fig. S3b).

## Discussion

In spite of the widespread use of neuraxial anesthesia for intrapartum pain relief during labor, limited studies have investigated its long-term effects on childhood outcomes. The molecular mechanisms linking maternal anesthesia exposure during delivery and childhood outcomes also remain unclear although epigenetics is hypothesized to contribute. Our key findings were (i) the duration of exposure to epidural anesthesia was longer in NHW than in NHB, but this was not statistically significant; (ii) the duration of exposure to epidural anesthesia at delivery marginally decreased childhood asthma risk in NHB; and (iii) this association was in part mediated by DNA methylation detectable in umbilical cord blood mixed leukocytes.

In a previous study, we reported a negative association between the duration of epidural anesthesia and the development of asthma in children at 5 years of age, with the magnitude of risk showing a sex-specific manner [8]. This study in turn revealed that the duration of exposure to epidural anesthesia was associated with a marginally lower risk of asthma in the NHB group, while no significant association was observed in the NHW group, consistent with previous research showing ethnic disparities between the NHB and NHW population in terms of the development of childhood asthma [11, 32–35]. Our study also highlights the shorter duration of exposure to epidural anesthesia in NHB women, which, although not significant, is in keeping with disparities in epidural analgesia use in NHB women previously reported in the USA [3, 4]. These health-care disparities may in part be due to widely held racial stereotypes about labor pain intensity where NHW women are thought to experience more severe labor pain than women of color when in reality women of color experience more severe labor pains [36]. This results in a double discounting of labor pain in NHB women and other women of color who have their more severe labor pain both under-perceived and undertreated when compared with NHW women [36]. Interestingly, we did not observe any significant association between the duration of epidural anesthesia and childhood asthma in the NHW group, which may be due to low statistical power given our limited sample size in the NHW group ($N = 52$ and 7 cases of asthma). However, the estimated OR of 1.38 in the NHW group suggested a possible opposite effect of the duration of exposure to epidural anesthesia on the development of childhood asthma in the NHW population. Taken together our study highlights the potential beneficial effects of epidural anesthesia in NHB women in reducing asthma in their offspring, yet a threshold effect for the duration of exposure remains unknown.

Epidural analgesia effectively relieves labor pain and is widely chosen by many parturient women [37]. Even though studies have shown that epidural analgesia for labor may be associated with neonatal outcomes, the underlying molecular mechanisms are still unclear [38, 39]. In this study, we hypothesized that the duration of epidural anesthesia during delivery may induce epigenetic alternations in umbilical cord blood, which may further affect the development of childhood asthma. We identified several differentially methylated CpGs associated with the duration of epidural anesthesia at delivery which mapped to genes involved in regulatory networks harboring NFkB complex, Akt complex and $\beta$-estradiol. These pathways may indirectly point to the role of epidural anesthesia in attenuating or modulating nociceptive and

stress-related pathways associated with maternal labor pain and the associated maternal and fetal stress responses [14, 16, 21]. The pathways identified are also biologically plausible. NFkB, for example, is a key transcription factor in stress regulation and stress adaptation [40, 41]. Traumatic stress, which may be associated with labor, may enhance PI3-Akt signaling in the basolateral amygdala in the brain, leading to the persistence of fear memory and posttraumatic stress disorder like symptoms [42]. NFkB and Akt pathways have been implicated in both inflammatory and opioid-induced neuropathic pain pathways [43–46]. Estradiol can modulate nociception and both the hypothalamic-pituitary adrenal axis and sympathetic nervous system reactivity, which are key components of the acute neurohumoral stress response to labor that are also influenced by prenatal psychosocial stressors and trauma [47, 48]. In summary, our results suggest that the association between the duration of exposure to epidural anesthesia and umbilical cord blood DNA methylation may influence the expression of genes involved in pathways that affect the stress reactivity, neurohumoral stress response and nociception; however, these effects on long-term childhood outcomes remain unclear.

Among the 21 genes directly mapping to the top 20 CpGs showing the mediation effect in the NHB group, 12 of them were reported to be involved in asthma or acute respiratory disease in previous studies. Interestingly, one of the 20 mediating CpGs (cg08354908) identified in our study is also located in the previously reported differential DNA methylation regions associated with asthma [13]. Abbasi *et al.* detected over-expression of *Cytidine/Uridine Monophosphate Kinase 2*(*CMPK2*) in clinical samples of children with acute respiratory infection [49]. A family-based case-control study discovered that the *tripartite motif containing 44* (*TRIM44*) gene may be associated with susceptibility to asthma in the NHB population [50]. *TAP2*, *TAPBP*, *HLA-E* and *Proline Rich Coiled-Coil 2A* (*PRRC2A*) (also known as *BAT2*) genes are located in the human leukocyte antigen super-locus, which has been associated with diseases such as asthma, diabetes and various other autoimmune disorders [51]. Interestingly, a recent study reported that DNA methylation in the *TBC1D8* gene also mediated the association between body mass index (BMI) trajectory in childhood and asthma in young adulthood, even though the CpG site was different from the one in our analysis [52]. In recent studies using remnant bronchoalveolar lavage fluids of children with asthma and mouse models, inhibition of the *Smoothened, Frizzled Class Receptor* (*SMO*) gene reduced inflammation in the airway by blocking the sonic hedgehog signaling [53, 54]. In addition, differential expression of *eukaryotic translation initiation factor 4B* (*EIF4B*) was detected in the blood tissue of children diagnosed with allergic asthma and nonallergic asthma [55]. Both genome-wide association and gene expression analysis of single-cell RNA sequencing have identified a significant association of *RASGEF1A* with asthma in a cohort of both children and adults [56, 57]. Both *SYTL3* and *Ribosomal Protein L13* (*RPL13*) genes have also been differentially expressed between patients with asthma and healthy controls [58, 59]. The central nodes of the regulatory network identified by our analysis, i.e. MHC class I complex and NFkB complex, regulate immune-related genes and are well-known participants in the pathophysiology of asthma [60–62]. The overlap in the regulatory networks for genes associated with the duration of exposure to epidural anesthesia and those that have a significant mediation effect on the development of childhood asthma (e.g. NFkB) demonstrates a possible link between the effect of the duration of exposure to epidural anesthesia on the maternal stress response to labor, nociception pathways, epigenetic regulation and development of immunity in the newborn and the development of childhood asthma potentially initiated during the late antepartum and intrapartum periods in NHB.

There is mounting evidence, suggesting that exposure to pre- and postnatal psychosocial maternal stress plays a role in immune system programming or fine-tuning in utero to adapt the offspring to postnatal life with maladaptation implicated in the development of asthma, obesity, diabetes, heart disease, cancer and mental health disorders [63–65]. NHB women are more frequently exposed to adverse childhood events and other chronic psychosocial stressors throughout their life course than NHW women, and these differences in exposure might in part explain some of the disparities in pain perception, the prevalence of childhood asthma, wheezing and other chronic medical childhood diseases [66–68]. However, changes in maternal physiology that protect the fetus from the direct adverse effects of stress-related glucocorticoid hormones' alternative pathways may play a role in fetal programming [69]. Our findings provide preliminary evidence for the role of epigenetic mechanisms regulating the expression of genes involved in immune system programming, but how gestational and stress hormones, catecholamines and immunoregulatory cytokines initiate these epigenetic changes still needs to be clarified. Furthermore, both acute and chronic stressors can program immunity in the offspring and determine the duration of its effects. The latter stages of pregnancy also coincide with the immunocompetence window of vulnerability, where the adaptive immune system continues to develop its functional capabilities with ongoing maturation of the expression of MHC class II molecules on the surface of monocytes [70, 71]. With our preliminary findings that the duration of exposure to epidural anesthesia may reduce the risk of asthma in the offspring of NHB women through DNA methylation, it is still unclear how these effects are modified by exposure to psychosocial stressors which in pregnancy may affect maternal stress reactivity and independently predict pain perception during labor [72]. It is also unclear if there is a threshold level of exposure to epidural anesthesia at which this reduction in asthma risk is observed. Previous studies examining the effect of epidural anesthesia on maternal hyperthermia and autism spectrum disorders in the offspring have suggested that a duration of exposure of ~4–6 h is a threshold above which adverse effects are observed, suggesting that a hormetic dose–response phenomenon may exist [1, 73–75].

This study has several limitations. First, due to the small sample size, we are likely underpowered to achieve family-wise significance from epigenome-wide association analysis. Instead, the top CpGs with the smallest *P*-values were selected to conduct mediation analysis. However, functional analysis of the mapped genes showed consistent results with previous studies, adding preliminary evidence of the mediating role of umbilical cord blood DNA methylation on the association between the duration of exposure to epidural anesthesia and the development of childhood asthma. The limited sample size also makes it infeasible to investigate the partial mediation effect by fitting all mediator CpGs together, but the low correlation between the top CpGs implied independence of individual mediation effect. Secondly, our findings were based only on umbilical cord blood samples. Nevertheless, studies have shown that DNA methylation changes are largely concordant in a broad variety of tissues [76, 77], and abundant expression of *HLA-E*, *TAPBP*, *EIF4B*, *TAP2* and *TRIM44* genes in lung, heart and artery tissue (Supplementary Fig. S3a) was identified by searching the GTEx database, suggesting that our findings in umbilical cord blood samples might be generalized to

a wide range of tissues. Thirdly, in 10 of the 20 CpGs with significant mediation effects identified, the direct and indirect effects were in opposite directions, implying a complex role of DNA methylation with the association between the duration of exposure to epidural anesthesia and childhood asthma development. However, inconsistent mediation should be interpreted with caution. Future analysis addressing the detailed relationship between the DNA methylation level of individual CpG and the expression of mapped genes would be helpful in interpreting any observed mediation effects. Finally, multiple factors, including genetic predisposition psychosocial factors and environmental exposures, have been implicated in the pathogenesis of childhood asthma [78]. Our data set lacked data on measures of maternal psychosocial stressors perinatally and even though we carefully adjusted for the potential confounders available within our dataset, sibling studies that control for shared environment, psychosocial factors and genetic predisposition will be needed to further validate the observed epigenetic mediation effect.

In summary, our findings suggest that the duration of exposure to epidural anesthesia at delivery was associated with childhood asthma in the NHB group and umbilical cord blood DNA methylation mediated the association. The molecular mechanisms underlying the mediation involved regulatory pathways related to the MHC class I complex, NFkB complex and Akt signaling. Further systematic studies with large sample sizes and multiple tissue types are needed to elucidate the complete perspective of the underlying mechanism of the observed association and mediation effects with a view to designing and developing interventions and policies to mitigate as early as possible the risk of asthma development in a high-risk patient population.

# Materials and Methods

## Study Participants

Participants for this study derived from the NEST cohort, a prebirth cohort study of mother–child pairs recruited between 2005 and 2011. Details on recruitment and enrollment strategies have been described previously [79, 80]. In brief, to be eligible for inclusion, participants had to be 18 years or older, English speaking and plan to deliver the index offspring at the Duke University Medical Center. Women intending to move before the birth of offspring, relinquish custody of the index offspring, or who had been known to be positive for human immunodeficiency virus were excluded. A total of 3690 eligible women attending Duke Medicine prenatal clinics were approached, and 2681 consented and were enrolled. Among the 2681 women enrolled, we measured the umbilical cord blood CpG methylation profile for 248 offspring using the Infinium 450K Human Methylation Beadchip. Within the 248 mother–child pairs, we first excluded participants with extreme preterm births (defined as gestational age <28 weeks) ($n = 0$), then we sequentially excluded participants with missing maternal obesity ($n = 26$), parity ($n = 1$), maternal smoking status ($n = 2$), offspring asthma diagnosis ($n = 42$), maternal asthma diagnosis ($n = 9$), breastfeeding status ($n = 39$), infant birth weight ($n = 1$) and missing infant gender data ($n = 1$), resulting in 127 mother–child pairs for further analysis. These variables have been previously reported to be associated with umbilical cord blood CpG methylation or the development of asthma [24–26, 81]. The remaining patients had complete data on anesthesia exposure populated from the Duke University Hospital Electronic Innovian® Anesthesia record. They were further subdivided into NHW ($n = 75$) or NHB ($n = 52$). This study was approved by the Duke University Institutional Review Board.

## DNA Methylation

Cord blood was collected at delivery and genomic DNA was purified from the buffy coat and 500-ng aliquots were submitted to the Duke Genome Sciences Core Laboratory for processing using procedures previously described [82]. Purified genomic DNA specimens were bisulfite-converted using the EZ-96 DNAm kit (Zymo Research Corporation, Irvine, CA, USA) according to manufacturer instructions. Methylation was measured by Infinium 450K Arrays.

Raw intensity files were loaded by the *minfi* package to calculate the methylation level at each CpG as the $\beta$-value, and the data were exported for quality control and processing [83]. Beta Mixture Quantile dilation strategy was used to adjust for intra-array differences between Illumina Type I and Type II probes [84]. The batch effect (HumanMethylation450 BeadChip plate number) was corrected using the *ComBat* function in the R package *SVA* [85]. We excluded four samples with gender mismatch based on principal component analysis of the whole genome DNA methylation profile. Using the annotations for Illumina's 450K array data [86], we also removed 16 998 probes containing single nucleotide polymorphisms (SNPs), 543 probes with an SNP in the single-base extension site and 11 458 probes mapping to the sex chromosomes. $\beta$-Values were considered as outliers and were removed if they were below $Q_1 - 3\times$IQR or above $Q_3 + 3\times IQR$, where $Q_1$, $Q_3$ and IQRs stand for the first quantile, third quantile and the IQR between $Q_3$ and $Q_1$, respectively; a total of 383 235 $\beta$-values were excluded as outliers. $\beta$-Values with detection $P$-value >0.05 were also removed to filter out probes with poor signal. We further excluded 42 224 probes that were missing in >10% of the samples, leaving a total of 414 289 CpG probes for further analysis.

## Anesthesia Exposure

The data on anesthesia drugs and exposure extracted and used to populate the NEST cohort have been previously described [8]. Briefly, we extracted dosage data of local anesthetics, opioids, antiemetics, antibiotics and vasopressors administered to the mother during delivery. The duration of anesthesia exposure was defined as the time from the placement of neuraxial anesthesia (epidural, spinal or combined spinal–epidural) to the time of the delivery of the baby. Both time points were extracted from the electronic anesthesia records. For this analysis, we limited the anesthesia exposure to the duration of exposure to epidural anesthesia based on the results of our prior work which demonstrated that the duration of epidural anesthesia exposure was associated with a significant reduction in the odds of developing childhood asthma in males [8]. We also adjusted for the spinal administration of local anesthetics and opioids since this was also associated with a reduction of asthma in both sexes.

## Assessment of Covariates

Questionnaire data were used to assess maternal sociodemographic data which included educational level, race/ethnicity, gestational age at delivery, history of asthma, history of smoking and prepregnancy obesity. Medical records were used to obtain data on weight gain during gestation, delivery route and birthweight. Questionnaires administered during the postnatal period were used to obtain information on the duration of breastfeeding and asthma diagnosis—these were verified using medical records.

### Assessment of Asthma

Childhood asthma was categorized as a dichotomous variable, and the diagnosis of childhood asthma was based on an algorithm developed using a combination of billing and prescription records, and two questions from a follow-up questionnaire, i.e. (i) "What was the outcome of your child's doctor visits? Normal/Concerns, and if there are Concerns, specify?" and (ii) "Was the child diagnosed with any condition by his/her doctor? Yes/No." Supporting the validity of this algorithm, the accuracy compared with a full medical records review was >99%, and the prevalence was consistent with the known prevalence of asthma in 5–9-year-old children [13].

### Statistical Analysis

To account for the cell-type heterogeneity in DNA methylation, we estimated the cell-type proportions using the method of Houseman *et al.* [87], implemented by the *estimateCellCounts* function of the *minfi* package using the cord blood reference panel of Bakulski *et al.* [88, 89]. Then for each CpG site, we obtained the residualized $\beta$-values adjusting for cell-type proportion using linear regressions for downstream analyses.

To evaluate the association between childhood asthma and the epidural anesthesia duration, we used the following logistic regression model:

$$logit\,(P(Y=1\mid X,Z)) = \theta_0 + \theta_1 X + \theta_2^T Z + \in_\theta \quad (1)$$

where $Y$ is an indicator variable of the occurrence of childhood asthma, $logit\,(P) = \log\left(\frac{P}{1-P}\right)$ is the logit function, $X$ is the epidural anesthesia duration in hours, $Z$ is the confounders, $\theta$'s are the corresponding regression coefficients and superscript $^T$ represents transpose operation. In race-stratified analysis, confounder $Z$ included spinal anesthesia administration, gender, maternal asthma, maternal smoking, maternal obesity, maternal age at delivery, maternal gestational weight gain, maternal education, birth weight, breastfeeding status, delivery route and gestational age at delivery. In race-combined analysis, $Z$ included the above-mentioned confounders plus maternal race and the interaction term between race and the duration of exposure to epidural anesthesia.

To identify CpGs that mediate the association between childhood asthma and the duration of exposure to epidural anesthesia, we considered the following models:

$$M = \beta_0 + \beta_1 X + \beta_2^T Z + \epsilon_\beta \quad (2)$$

$$logit\,(P(Y=1)) = \gamma_0 + \gamma_1 M + \gamma_2 X + \gamma_3^T Z + \epsilon_\gamma \quad (3)$$

where $M$ is the $\beta$-values of a CpG; $Y$ and $Z$ are the same as in Model (1); $\beta$'s and $\gamma$'s are the corresponding regression coefficients in Models (2) and (3), respectively. First, we conducted epigenome-wide mediation analysis for all 414 289 CpGs using Models (2) and (3) and identified promising CpGs for further follow-up analyses. For a given CpG, we obtained the mediation *P*-value using the joint significant method [30], i.e. $P_{\text{med}} = \max\left(P_{\beta_1}, P_{\gamma_1}\right)$, with $P_{\beta_1}$ and $P_{\gamma_1}$ being the *P*-values for coefficients $\beta_1$ and $\gamma_1$, respectively. The joint significant method has been shown to have improvements in power [30, 90]. Given our small sample size, we would have limited power with the Bonferroni multiple testing correction; we instead considered the top 20 CpGs with the smallest mediation *P*-values as "promising" and focused on them for further follow-up as previously recommended by others [30, 90]. Next, for the top 20 promising CpGs, we estimated the mediation effects, direct effects and total effects using the R package *mediation* via the bootstrap method with 5000 random draws [91]. In the NHB group, we also examined the results of Model (2) to explore how the epidural anesthesia duration may affect the DNA methylation profile in umbilical cord blood. All statistical analysis were conducted in R version 4.2.0 [92].

### Functional Analysis

For each of the top CpGs mediating the association between epidural duration and asthma in the NHB group, we identified the gene(s) that harbored or were within 50 kb near the CpG and formed the "mediation" gene set. We repeated the same steps for the top CpGs associated with epidural duration in the NHB group and generated the "association" gene set. Given a gene set of interest, we identified canonical pathways and disease phenotypes enriched by these genes using IPA. We replicated the enrichment analysis via Enrichr, using libraries including KEGG 2021 Human, GO 2021, Human Phenotype Ontology (HPO), PheWeb 2019 and dbGaP database [93]. Tissue-specific expressions of the mapped genes were examined using the GTEx database (https://gtexportal.org/home/).

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## Data availability

The data underlying this article will be shared upon reasonable request to the corresponding author.

## Supplementary data

Supplementary data are available at *EnvEpig* online.

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*Conflict of interest statement.* The authors declare that they have no competing interests.

## Declarations

Ethics Approval and Consent to Participate. This study was approved by the Institutional Review Board at the Duke University Medical Center. Informed consent was obtained from all participants.

## Author contributions

C.H., J.Y.T. and T.K.A. initiated the research question. C.H., J.Y.T., T.K.A. and Y.W. conceived and developed the study design. Y.W. conducted the DNA methylation analysis and statistical analysis. Y.W., Y.H. and R.M. prepared and extracted the clinical data. T.K.A. and Y.W. wrote the first draft of the manuscript. C.H. and J.Y.T. reviewed and edited the manuscript. All authors helped shape the research, discussed the results and contributed to the final manuscript. All authors read and approved the final manuscript.

## Abbreviations

| BMI | Body mass index |
|---|---|
| *CMPK2* | *Cytidine/Uridine Monophosphate Kinase 2* |
| *EIF4B* | Eukaryotic translation initiation factor 4B |
| GO | Gene Ontology |
| GTEx | Genotype-Tissue Expression |
| *HLA-E* | *Major Histocompatibility Complex, Class I, E* |
| HPO | Human Phenotype Ontology |
| IPA | Ingenuity Pathway Analysis |
| IQR | Interquartile range |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| NEST | Newborn Epigenetics STudy |
| NHB | Non-Hispanic Blacks |
| NHW | Non-Hispanic Whites |
| OR | Odds ratio |
| *RASGEF1A* | *RasGEF Domain Family Member 1A* |
| *RPL13* | *Ribosomal Protein L13* |
| *SMO* | *Smoothened, Frizzled Class Receptor* |
| *SYTL3* | *Synaptotagmin Like 3* |
| *TAP2* | *Transporter 2 ATP Binding Cassette Subfamily B Member* |
| *TAPBP TAP* | *Binding Protein* |
| *TRIM44* | *Tripartite motif containing 44* |

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