



Epigenome-wide analysis links SMAD3 methylation at birth to asthma in children of asthmatic mothers

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TITLE: Epigenome-wide Analysis Links *SMAD3* Methylation at Birth to Asthma in Children of Asthmatic Mothers

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KEY MESSAGES

- The trajectory to childhood asthma begins at birth and involves epigenetic modifications in innate pro-inflammatory and immunoregulatory pathways.
- Neonatal immune cells collected at birth harbor differentially methylated regions that distinguish children who will and will not develop asthma by age 9.
- In three independent birth cohorts, DNA methylation at the *SMAD3* promoter was selectively increased in asthmatic children of asthmatic mothers and was associated with risk of childhood asthma.

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47 **CAPSULE SUMMARY:**

48 The trajectory to childhood asthma begins at birth and involves epigenetic (DNA
49 methylation) modifications in innate pro-inflammatory and immunoregulatory pathways.
50 Maternal asthma appears to strongly influence this trajectory.

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52 **AUTHOR CONTRIBUTIONS:** ADV and GW measured and analyzed DNA methylation.
53 SJM, AB, DAS, JR, JNJ, ACJ and MMV performed statistical and network analyses. ICL
54 generated ELISA data. JAC, AS and AC designed and provided samples from the
55 MAAS study. DJJ, JEG, and RFL designed and provided data from the COAST study.
56 ALW and MH designed and provided samples from the IIS study. CO, SG, and MH
57 contributed to data interpretation. DV designed the study, oversaw data analyses and
58 interpretation, and wrote the paper with ADV. All authors reviewed the final manuscript.

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ABSTRACT

Background The timing and mechanisms of asthma inception remain imprecisely defined. Although epigenetic mechanisms likely contribute to asthma pathogenesis, little is known about their role in asthma inception.

Objective To assess whether the trajectory to asthma begins already at birth and epigenetic mechanisms, specifically DNA methylation, contribute to asthma inception.

Methods We used Methylated CpG Island Recovery Assay (MIRA)-chip to survey DNA methylation in cord blood mononuclear cells (CBMC) from 36 children (18 non-asthmatic, 18 asthmatic by age 9) from the Infant Immune Study (IIS), an unselected birth cohort closely monitored for asthma for a decade. *SMAD3* methylation in IIS (n=60) and in two replication cohorts (The Manchester Asthma and Allergy Study, n=30, and the Childhood Origins of ASThma Study, n=28) was analyzed by bisulfite sequencing or Illumina 450K arrays. CBMC-derived IL-1 β was measured by ELISA.

Results Neonatal immune cells harbored 589 differentially methylated regions (DMRs) that distinguished IIS children who did and did not develop asthma by age 9. In all three cohorts, methylation in *SMAD3*, the most connected node within the network of asthma-associated DMRs, was selectively increased in asthmatic children of asthmatic mothers and was associated with childhood asthma risk. Moreover, *SMAD3* methylation in IIS neonates with maternal asthma was strongly and positively associated with neonatal production of IL-1 β , an innate inflammatory mediator.

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86 **Conclusions** The trajectory to childhood asthma begins at birth and involves
87 epigenetic modifications in immunoregulatory and pro-inflammatory pathways. Maternal
88 asthma influences epigenetic mechanisms that contribute to the inception of this
89 trajectory.

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91 **ABSTRACT WORD COUNT:** 246

92 **KEY WORDS:** epigenetics, DNA methylation, childhood asthma, *SMAD3*

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94 **ABBREVIATIONS:**

95 CBMC: cord blood mononuclear cells

96 COAST: Childhood Origins of ASThma

97 DMR: differentially methylated region

98 GWAS: genome-wide association study

99 IIS: Infant Immune Study

100 MAAS: Manchester Asthma and Allergy Study

INTRODUCTION

Asthma is the most prevalent chronic disease of childhood (1). Epidemiological evidence suggests that the disease often begins during the pre-school years even when chronic symptoms appear much later in life (2). However, firm criteria to pinpoint how early a child's trajectory to asthma truly begins are currently lacking. The mechanisms underlying asthma inception also remain largely unknown. Subtle modifications of both innate and adaptive immune responses accompany and often precede the diagnosis of childhood asthma (3, 4), consistent with the notion that immune and respiratory alterations at an early window of susceptibility converge to place the child on a path to the disease. Genome-wide association studies (GWAS) have identified multiple genetic variants that influence asthma susceptibility (5) but have accounted for only a modest proportion of the total phenotypic variance, providing a compelling rationale for seeking additional risk factors for asthma. In this context, epigenetic mechanisms are especially worth investigating because environmental and developmental influences are essential for asthma pathogenesis (1), and epigenetic processes ensure the timed unfolding of developmental programs and plastic responses to environmental cues, including those delivered in utero by the maternal milieu (6).

Little is known about the role of epigenetic mechanisms in childhood asthma (7). A recent epigenome-wide study compared DNA methylation patterns in peripheral blood mononuclear cells from 6-12 year old inner-city children with persistent atopic asthma and healthy controls, and found that several immune genes involved in T cell maturation, Th2 immunity and oxidative stress were hypomethylated in asthmatic

children (8). While these results are novel, their significance remains unclear because it is difficult to determine whether epigenetic alterations concurrent with asthma are a cause or a consequence of the disease. Moreover, a cross-sectional study cannot provide insights into the timing and mechanisms of asthma inception. A recent candidate gene study pointed to an association between *IL2* promoter methylation at birth and asthma exacerbations during childhood (9), but relevant pathways were not further interrogated.

In an attempt to define when the trajectory to asthma begins and which pathways are involved, we performed an epigenome-wide search for DNA methylation signatures associated with childhood asthma in cord blood mononuclear cells (CBMC) from children enrolled in the Infant Immune Study (IIS). In this unselected birth cohort, the development of asthma and immune responses was monitored at multiple times from birth to age 9 (4, 10). We reasoned that the detection at birth of differentially methylated regions (DMRs) associated with asthma during childhood would both support a perinatal origin of the disease and highlight epigenetic mechanisms potentially contributing to asthma inception. We show herein that DNA methylation signatures associated with asthma during childhood were indeed present in neonatal blood immune cells, and clustered in immunoregulatory and pro-inflammatory pathways. Moreover, hypermethylation of the *SMAD3* promoter was selectively detected in asthmatic children of asthmatic mothers and was associated with risk of childhood asthma in the IIS population and in two comparable birth cohorts.

METHODS

Study design and participants The IIS unselected birth cohort includes 482 children and was designed to assess patterns of immune maturation in early life and their impact on asthma risk (4, 10). At enrollment, parents completed a respiratory health history questionnaire, a cord blood sample was obtained, and their child's health was followed prospectively. Childhood asthma was defined as physician-diagnosed, with symptoms or medication use for asthma in the past year reported at least once on the age 2, 3, 5 or 9-year questionnaires.

Figure 1 shows an overview of our study design. We performed a nested case-control, epigenome-wide study of DNA methylation in CBMC ($2\text{-}8 \times 10^6$ cells per sample) from a discovery population of 36 children from the IIS. This population was randomly selected among available samples but balanced for asthma status in the child (18 non-asthmatic, 18 asthmatic by age 9) and an effort was made to balance cases and controls by maternal asthma (Table E1). Additional targeted analysis of DNA methylation was performed in the 31 samples available from the IIS discovery population and in samples from 29 additional IIS children of comparable characteristics (Table E2). The distribution of cases and controls is provided in Figure 1. Availability of adequate samples was the only inclusion criterion besides asthma status of the child and her/his mother. Overall, the study population did not differ from the rest of the IIS population except for a greater proportion of asthma and maternal asthma, as per study design (Table E1).

The results of targeted DNA methylation analysis performed in the IIS were replicated in

CBMC from 30 children with asthmatic mothers from the Manchester Asthma and Allergy Study (MAAS) and 28 children from the Childhood Origins of ASThma (COAST) study (Figure 1). The characteristics of the MAAS and COAST study populations are described in Table E2. The MAAS unselected birth cohort includes 1085 children monitored for asthma and allergy from birth to age 11 years. An asthma diagnosis required at least one of the following criteria reported on age 5 or 8 questionnaires: 1) physician diagnosis of asthma, 2) the use of asthma medications during the previous 12 months. Controls required a negative report on both of these criteria and no report of wheezing in the previous 12 months (11). The COAST birth cohort enrolled 289 neonates at risk for asthma and allergy, i.e., having at least one parent with asthma and/or allergies. Asthma was diagnosed at age 6 years based on the documented presence of one or more of the following characteristics in the previous year: 1) physician diagnosis of asthma, 2) use of physician-prescribed albuterol for coughing or wheezing episodes, 3) use of a daily controller medication, 4) step-up plan including use of albuterol or short-term use of inhaled corticosteroids during illness, and 5) use of prednisone for asthma exacerbations (12). All three studies were approved by the appropriate Institutional Review Boards. Informed consent was obtained from the parents of all research participants.

DNA methylation profiling and DMR identification DNA methylation was profiled by Methylated CpG Island Recovery Assay (MIRA)-chip (Roche-NimbleGen), as detailed in the Online Repository, Methods section, and Figure E1. DNA methylation microarray

data from this publication were submitted to the NCBI Gene Expression Omnibus (GEO) database and assigned the identifier GSE85228.

Regions that were differentially methylated between asthmatics and non-asthmatics (DMRs) were identified using a Probe Sliding Window-ANOVA [Roche-NimbleGen (13)] that detects maximal inter-group differences in signal intensities relative to a user-defined threshold. Probability scores (P -values) are then assigned to each probe on the array using a repeated measure ANOVA model. In this study, DMRs were defined by region length ≥ 300 bp, magnitude (between-group mean \log_2 ratio difference) ≥ 0.2 , significance threshold (adjusted using the Benjamini-Hochberg False Discovery Rate method to account for multiple testing) = 0.01, using a sliding window size = 750 bp (Table E3). A positive magnitude difference indicated hypermethylation in asthmatics relative to non-asthmatics. DMRs containing ≤ 5 CpG sites within a 750 bp window centered on the DMR [a configuration likely to result in sub-optimal capture of methylated DNA (14)] were excluded from further analyses.

Results of this analysis were technically validated by bisulfite sequencing (Online Repository, Methods section and Table E4). Microarray-derived estimates of DNA methylation intensity strongly correlated with DNA methylation levels measured by bisulfite sequencing over the entire DNA methylation range [Spearman correlation coefficient (ρ)=0.48, $P=1.2 \times 10^{-13}$] and at intermediate DNA methylation levels (8-92%, $P=0.006$; Figure E2). *SMAD3* methylation estimates from bisulfite sequencing and microarrays were also highly correlated ($\rho=0.46$, $P=0.009$).

Functional DMR annotation is fully described in the Online Repository, Methods section. In brief, DMRs were annotated to genes based on the closest transcription start site. For pathway analysis, multiple genes were allowed to be associated with a single DMR (RefSeq genes +/- 5 kb).

DNA methylation analysis in the COAST cohort was performed using the Infinium HumanMethylation450 BeadChip array (Illumina) (Online Repository, Methods section).

Network analysis A molecular interaction network was constructed by uploading the list of DMR-containing genes into the Ingenuity Pathway Analysis software (Qiagen) and using all available interaction data in the Ingenuity Systems Knowledge Base. Genes with no interactions were removed from the analysis to maximize the signal-to-noise ratio. The gene interaction network was then interrogated to detect enrichment for biological functions, canonical pathways and upstream regulators (defined as an enrichment for known targets of a given gene in a given gene list) using Ingenuity Pathway Analysis tools.

Cytokine measurements IL-1 β concentrations were measured by ELISA (Quantikine, R&D Systems) in the supernatants of LPS-stimulated IIS CBMC (n=57) also tested for *SMAD3* promoter methylation.

Statistical analyses DMRs were detected using a Probe Sliding Window-ANOVA which uses a repeated measure ANOVA model for the probes in each sliding window

(13). Fisher's Exact Test and one sample test of proportions were used to compare proportions for categorical variables, and Student's t-test or Wilcoxon two-sample test were used to compare mean levels for continuous variables. Variables with skewed distributions were log-transformed prior to assessment by t-test. Two-sided *P*-values less than 0.05 were considered significant. Spearman correlation was used to test for association between median DNA methylation intensity (microarray) and percentage DNA methylation (bisulfite sequencing). When analyzing the genomic locations of DMRs and the co-localization of DMRs and DNase I hypersensitive sites, permutations were performed by randomly selecting 589 independent probes (the number of DMRs) and calculating the χ^2 statistic for each sampling. We recorded the number of times (out of 50,000) that the permuted χ^2 statistic was larger than the observed χ^2 statistic and divided by the number of permutations to get the empirical *P*-value. Linear regression was used to test for an interaction between child asthma and maternal asthma on *SMAD3* methylation. Pearson's χ^2 test was used to identify significant associations between child and parental characteristics at birth and asthma during childhood. Meta-analysis of the association between *SMAD3* CpG7 methylation and childhood asthma risk in neonates born to asthmatic mothers was performed using estimates from each study cohort to compute the combined estimate of risk.

RESULTS

Cord blood cells from IIS children harbor asthma-associated DMRs Our multi-step study of the contribution of epigenetic mechanisms to the development of asthma in early life included: 1) a discovery phase in which an epigenome-wide approach was used to identify candidate regions that were differentially methylated in cord blood samples from 36 IIS children (18 non-asthmatic, 18 asthmatic by age 9); 2) a targeted analysis phase in which DNA methylation of the most compelling candidate region was measured in CBMC from 29 IIS neonates who did, and 31 IIS neonates who did not develop asthma by age 9; and 3) a final phase in which the results of the targeted analysis performed in IIS were replicated in two comparable birth cohorts, MAAS and COAST (Figure 1).

Relying on the longitudinal design of the IIS birth cohort, we initially searched for neonatal epigenetic signatures of childhood asthma by profiling DNA methylation in CBMC from a discovery cohort of 36 children (Figure 1, Figure E1 and Table E1). Five hundred and eighty-nine independent regions were differentially methylated in asthmatic and non-asthmatic children (Table E3). Among these, 199 were hypermethylated and 390 were hypomethylated at birth in children who became asthmatic.

Asthma-associated DMRs were distributed across all chromosomes but were non-randomly distributed with respect to genome location ($\chi^2= 19.49$, $df = 7$, $P=0.007$), with an overrepresentation at intergenic regions (standardized residual: 2.91) and an underrepresentation at transcription start sites (standardized residual: -2.23, with

absolute values > 1.96 significantly contributing to the overall χ^2 test statistics) (Figure E3). Of the DMRs with the most significant methylation differences, some mapped to biologically plausible genes such as *ATG9A* (involved in autophagy and innate immune responses (15); $-\log_{10}$ adjusted *P*-value 5.569), *OR6K6* (an olfactory receptor expressed in sputum during asthma exacerbations (16); $-\log_{10}$ adjusted *P*-value 4.408), and *GALNT2* (an N-acetylgalactosaminyltransferase carrying polymorphisms associated with lung function (17); $-\log_{10}$ adjusted *P*-value 4.895). Moreover, several asthma-associated DMRs mapped to loci (\pm 5kb from transcription start sites) identified in GWAS for asthma (*RORA*, *SMAD3*) and asthma-related traits such as atopic dermatitis (*FLG*), allergic rhinitis (*TMEM232*), lung function (*ANK1*, *DLEU7*, *SNRPN*, *RORA*, *CFDP1*) and airflow obstruction (*SPATA13*) (<http://www.ebi.ac.uk/gwas>). Functional annotation using Ingenuity Pathway Analysis linked DMR-associated genes to biological processes including immune function and immune and lung development (Table E5). We also mapped the locations of neonatal asthma-associated DMRs to DNase I hypersensitive sites, regions of increased chromatin accessibility typically endowed with regulatory activity (18). Overall, 72 asthma-associated DMRs mapped to DNase I hypersensitive sites (29 monocyte-specific, 15 T cell-specific, 28 in both cell types: Table E6), which represented a significant enrichment (Table E7, permutation *P*-value < 2×10^{-5} for both cell types). The co-localization of asthma-associated differential DNA methylation and enhanced chromatin accessibility supports the potential biological significance of asthma-associated DMRs.

Asthma-associated DMRs cluster in regulatory and pro-inflammatory gene

networks To elucidate the functional implications of our findings and gain a pathway-based view of asthma-associated methylome alterations at birth, Ingenuity Pathway Analysis tools were used to construct a molecular interaction network of DMR-associated genes on the basis of prior knowledge of the physical and functional connections between the molecules encoded by those genes. The network included 146 genes organized around several nodes, the most connected of which was the transcription factor *SMAD3* (Figure 2). When the network was interrogated to detect enrichment for known targets of upstream regulators, *IL1B*, an innate pro-inflammatory cytokine overexpressed in asthmatics (19-21), emerged as the top regulator of a gene subset that included *RORA* and *RELB*, transcription factors essential for innate and adaptive responses, *UBD*, an innate immunity gene regulated by the NF-kB inhibitor A20/TNFAIP3 (22), and the asthma-associated neurotrophin *BDNF* (19) (overlap $P=2.26 \times 10^{-5}$, Table 1).

The SMAD3 promoter is significantly hypermethylated in asthmatic children of

asthmatic mothers *SMAD3* is not only the most connected node in the network of asthma-associated DMRs (Figure 2), but is also a well-replicated asthma-associated gene from GWAS (23-25). Moreover, *SMAD3* acts as a master regulator of TGF- β signaling, thereby controlling the differentiation of Treg and Th17 cells that play critical and opposite roles in asthma (1, 26). Finally, the *SMAD3* DMR lies within the distal promoter of the gene, a location that provides ample opportunity for DNA methylation to influence *SMAD3* gene expression (Figure E4). Therefore, subsequent analyses of the

nexus between neonatal DNA methylation and trajectory to childhood asthma specifically targeted *SMAD3*. We used bisulfite sequencing to precisely quantify methylation at the *SMAD3* promoter DMR (321 bp, 8 consecutive CpG sites) in a total of 60 IIS neonates (31 non-asthmatics, 29 asthmatics: Figure 1). Mean *SMAD3* methylation levels at birth were 41.3% (95% CI 35.5-47.5) in non-asthmatics and 47.1% (95% CI 40.6-53.1) in asthmatics, a difference that did not reach statistical significance ($P=0.2$ by Wilcoxon two-sample test: Figure E5). Because these results may have reflected heterogeneity within the study population, we next examined the entire IIS population for associations between childhood asthma and potential risk factors measurable at birth in the child (sex, ethnicity, mode of delivery, total cord IgE, 17q21 rs8076131 genotype) and parents (maternal asthma, maternal allergy, paternal asthma, paternal allergy, maternal smoking during pregnancy). As shown in Table E8, maternal asthma exhibited a distinctive association with childhood asthma in IIS ($P=0.003$ by χ^2 test). When the relation between *SMAD3* methylation at birth and childhood asthma was examined separately in children with and without maternal asthma, *SMAD3* methylation was found to be significantly increased in asthmatic compared to non-asthmatic children of asthmatic mothers ($P=0.005$ by Wilcoxon two-sample test). In contrast, asthmatic and non-asthmatic children of non-asthmatic mothers did not differ in their *SMAD3* methylation levels [P for interaction (by linear regression) = 0.001; Figure 3A].

Although associations between *SMAD3* variants [rs17228058 (25), rs744910 (23), rs17294280 (24)] and asthma have been reported in GWAS, asthma-related *SMAD3* methylation differences were unlikely to be influenced by *SMAD3* genotype. Indeed,

sequencing identified no polymorphisms within the *SMAD3* DMR (data not shown). Moreover, asthma-associated *SMAD3* variants are located at least 80 kb away from the *SMAD3* DMR, whereas the relationship between DNA methylation and genetic variation appears to decay rapidly beyond 5 kbs (27).

The association between SMAD3 promoter hypermethylation and childhood

asthma replicates in two independent birth cohorts The association between neonatal *SMAD3* hypermethylation and childhood asthma found in IIS neonates born to asthmatic mothers was assessed for replication in two birth cohorts, MAAS and COAST (Table E2). Bisulfite sequencing of the *SMAD3* DMR in 30 MAAS children born to asthmatic mothers revealed significantly higher DNA methylation in the asthmatic group ($P=0.049$ by Wilcoxon two-sample test; Figure 3B). Comparable results were obtained by assessing *SMAD3* methylation in 28 COAST neonates using the Illumina Infinium HumanMethylation450 BeadChip array. Figure 3C shows that methylation at cg02486855, the seventh CpG in the *SMAD3* DMR and the only *SMAD3* DMR CpG interrogated on the Illumina platform, did not significantly differ between asthmatic and non-asthmatic children of non-asthmatic mothers ($P=0.4$ by Wilcoxon two-sample test). In contrast, significant cg02486855 hypermethylation was detected in neonates born to asthmatic mothers who developed asthma during childhood, compared to neonates who did not ($P=0.04$ by Wilcoxon two-sample test; Figure 3C). The data generated by bisulfite sequencing and on the Illumina platform were comparable because when percent methylation values for *SMAD3* CpG7 were extracted from the bisulfite sequencing data for IIS and MAAS, again we found significant hypermethylation in

asthmatic compared to non-asthmatic children of asthmatic mothers in IIS ($P=0.004$ by Wilcoxon two-sample test; Figure E6), and a difference approaching significance in MAAS ($P=0.09$ by Wilcoxon two-sample test; Figure E6).

Cord blood contains a mixture of cell types with potentially distinct DNA methylation profiles. The availability of COAST DNA methylation data generated on the Illumina450 BeadChip array allowed us to estimate cord blood cell proportions using an algorithm recently developed that integrates Illumina DNA methylation data with information from a cord blood reference panel (28). Figure E7 shows that cell proportions thus estimated did not significantly differ between asthmatic and non-asthmatic children, regardless of maternal asthma history. Most importantly, the *SMAD3* methylation differences detected between asthmatic and non-asthmatic children of asthmatic mothers remained significant ($P=0.04$ by Wilcoxon two-sample test) even after adjusting for CBMC composition (Figure E8).

Finally, we asked whether the *SMAD3* methylation levels measured at birth in children of asthmatic mothers are associated with risk for childhood asthma in our three cohorts. A meta-analysis revealed that for each 10% increase in *SMAD3* CpG7 methylation there is nearly a two-fold increased risk of childhood asthma (meta-analysis Odds Ratio=1.95, [95%CI: 1.23, 3.10], $P=0.005$; heterogeneity $P=0.5$).

Maternal asthma modifies the relation between neonatal SMAD3 methylation and IL-1 β producing capacity *SMAD3* knock-out mice exhibit increased expression of *Il1b*

(29), which is also the top upstream regulator of genes containing asthma-associated DMRs in this study and a critical pro-inflammatory mediator in human asthma (19-21). Therefore, we relied again on the IIS to explore the relationship between neonatal LPS-induced IL-1 β production, *SMAD3* promoter methylation and childhood asthma. The distributions of *SMAD3* methylation and log IL-1 β production in non-asthmatic and asthmatic children without and with a maternal history of asthma were compared by dividing mean percentage *SMAD3* methylation and log IL-1 β secretion at the median, thereby creating four groups (low/low, low/high, high/low and high/high). No distribution differences were observed among children of non-asthmatic mothers ($P=0.79$ by Fisher's Exact Test; Figure 4A). In contrast, the children of asthmatic mothers who developed asthma were almost entirely found among those with high *SMAD3* promoter methylation and high IL-1 β secretion ($P=0.009$ by Fisher's Exact Test; Figure 4B). Moreover, asthmatic children of asthmatic mothers had higher LPS-induced IL-1 β than non-asthmatic children ($P=0.03$ by Student's t test), whereas comparable IL-1 β levels were measured in children without a maternal history of asthma ($P=0.65$ by Student's t test; Table 2). These results suggest that a strong relationship exists between neonatal *SMAD3* methylation, production of the innate cytokine IL-1 β , and childhood asthma. Furthermore, our data suggest that this relationship is powerfully influenced by maternal asthma.

DISCUSSION

Although asthma is the most common chronic complex disease of childhood (1), the timing and mechanisms of its inception remain largely unknown. This gap in knowledge severely hinders efforts aimed at preventing this disease, which no therapeutic regimen can currently cure. Our study, the first epigenome-wide search for asthma-associated methylome signatures at birth, sheds new light on these critical but still open questions. The finding that almost 600 genomic regions were differentially methylated at birth between children who did and did not develop asthma later in life strongly suggests that the trajectory to asthma begins at birth if not prenatally and involves epigenetic mechanisms. As importantly, data from three birth cohorts showed that *SMAD3* promoter hypermethylation was associated with childhood asthma selectively in neonates with a maternal history of asthma - one of the strongest, and mechanistically one of the most elusive, risk factors for asthma in the child (30, 31). These findings are especially noteworthy in the context of the current dearth of characteristics measurable at birth that effectively predict asthma during childhood. Of note, the link between epigenetic *SMAD3* dysregulation at birth and childhood asthma appeared to be selective for neonates born to asthmatic mothers, a finding that suggests that heterogeneity is deeply embedded in the pathogenesis of, and the trajectory to, childhood asthma (1, 32). More generally, to our knowledge this is the first time that a neonatal epigenetic characteristic linked to asthma during childhood is robust enough to replicate across three independent birth cohorts.

Our study highlighted *SMAD3* and IL-1 β as main players in the trajectory to childhood asthma. *SMAD3*, the most connected node in the network of asthma-associated DMRs, is known to be critical for the regulation of both the asthma-protective Treg and the asthma-promoting Th17 cell differentiation programs. Altered Treg and Th17 activities have been reported in childhood asthma (1, 26) and conversely, maternal exposure to asthma-protective environments such as farming has been shown to activate the Treg compartment (33) and influence the expression of Th17 markers (34). On the other hand, IL-1 β , which emerged as the primary upstream regulator of genes harboring asthma-associated DMRs, is increasingly recognized as a key asthma mediator in both children (20) and adults, especially in neutrophilic asthma (19, 21). Our data emphasize the functional connection between *SMAD3* and IL-1 β . Indeed, in neonates who became asthmatic by age 9, *SMAD3* promoter hypermethylation, an epigenetic configuration consistent with low *SMAD3* expression, was strongly associated with high IL-1 β production. This convergence is likely to destabilize the Treg program, enhance inflammation, and promote Th17 differentiation (35), ultimately favoring the development of asthma. While it is unclear whether these mechanisms operate pre- and/or perinatally, detection of the relationship between *SMAD3* methylation and IL-1 β production selectively among children of asthmatic mothers implies that the *in utero* environment is critical for directing the epigenetic trajectory towards childhood asthma.

Our results should be interpreted with caution because our discovery population was small, albeit longitudinally phenotyped for asthma in great detail, and environmental exposures were not comprehensively assessed. Moreover, DNA methylation did not

distinguish between 5-methylcytosine and other cytosine modifications and was assessed in mixed rather than isolated cell populations. However, our data from the COAST population suggest that differential methylation by asthma status did not reflect asthma-associated differences in CBMC proportions. We also acknowledge that the DNA methylation differences we detected were not extreme, albeit more substantial than those recently reported in other studies (8, 36). This is a recurring theme in the literature (37), and systematic studies are needed to comprehensively assess how relatively modest DNA methylation differences modify disease trajectories. In general, the functional impact of such differences will likely depend on the regulatory properties of the locus in which they reside, and the extent to which additional epigenetic processes, such as post-translational histone modifications, influence those properties.

Gene expression analyses are often used to complement epigenetic studies, but samples for such analyses were not collected at birth in our study populations. On the other hand, samples for cytokine protein assessments were available and proved essential to integrate our epigenetic findings. Finally, our search for asthma-associated differential methylation returned almost 600 regions, only some of which mapped to genes involved in immunoregulation and inflammation. DMRs that reside in functionally interesting genes but lack a link to immune regulation or inflammation may also contribute to asthma pathogenesis, albeit through different mechanisms. With these caveats, we propose that in a proportion of children with childhood-onset asthma, a distinctive methylome is in place already at birth, particularly within innate immunoregulatory and pro-inflammatory pathways, and promotes a trajectory that may

ultimately lead to clinical disease. Some of the epigenetic mechanisms that contribute to the inception of this trajectory are strongly influenced by the milieu associated with maternal asthma. A scenario in which epigenetic modifications at an early window of susceptibility promote a long-term developmental trajectory to asthma is consistent with the emerging paradigm that chronic non-communicable diseases have their origins in early life through an epigenetic calibration of set points for later responsiveness and function (38, 39).

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FIGURE LEGENDS

Figure 1. Overview of study design. IIS: Infant Immune Study, MAAS: Manchester Asthma and Allergy Study, COAST: Child Origins of ASThma study. NN: Non-asthmatic child with a Non-asthmatic mother, NA: Non-asthmatic child with an Asthmatic mother, AN: Asthmatic child with a Non-asthmatic mother, AA: Asthmatic child with an Asthmatic mother.

Figure 2. Molecular interaction network of asthma-associated DMRs. The network was constructed using all available interaction data in the Ingenuity Systems Knowledge Base. The most highly connected gene (*SMAD3*, 17 connections) is highlighted in black. Targets of *IL1B*, the top upstream regulator of network genes, are highlighted in gray. Types of interactive molecules are defined in the Legend.

Figure 3. Effects of maternal asthma on the association between neonatal *SMAD3* methylation and childhood asthma in IIS (panel A), MAAS (panel B) and COAST (panel C). *SMAD3* methylation In IIS and MAAS was assessed by bisulfite sequencing and expressed as mean percent DNA methylation across 8 consecutive CpG sites in the *SMAD3* DMR. In COAST, percent *SMAD3* methylation at cg02486855, the seventh CpG in the *SMAD3* DMR, was assessed by the Infinium HumanMethylation450 BeadChip array. N: non-asthmatic, A: asthmatic. *P*-values by Wilcoxon two-sample test. The results presented in panel A were unaffected after adjusting for ethnicity.

Figure 4. *Effects of maternal asthma on the relation between neonatal SMAD3 methylation and IL-1 β protein production.* The distributions of SMAD3 methylation and log IL-1 β producing capacity in non-asthmatic and asthmatic children without and with a maternal history of asthma were compared by dividing mean percentage SMAD3 methylation and log IL-1 β secretion at the median, thereby creating four groups (low/low, low/high, high/low and high/high). Distributions for asthmatics and non-asthmatics were compared across all four quadrants (panel A) or focusing the analysis on the high/high and low/low quadrants (panel B) (*P* by Fisher's Exact Test). N: non-asthmatic, A: asthmatic.

654 **Table 1.** *Upstream regulators of genes containing asthma-associated DMRs*

Upstream Regulator	Molecule Type	Overlap P^*	Target molecules in dataset
IL1B	cytokine	2.26E-05	ACAN, BDNF, CFTR, COL10A1, CXCL10, CYP7A1, FGFR3, GNAS, LDHA, NCOA2, NEUROD1, PFKP, RELB, RORA, SCX, SNCA, UBD
HSPG2	enzyme	8.60E-05	ACAN, COL10A1, FGFR3
BDNF	growth factor	1.43E-04	BDNF, DUSP6, NRXN1, PRKCZ, PTBP1, RPL35A, SLC17A8, TMEM45A, TTC3
SNCA	enzyme	1.56E-04	BDNF, CFL1, DNM1, SNCA, SYN3
PRMT5	enzyme	3.51E-04	CXCL10, CYP7A1, FASN

655 * Enrichment for known targets of a given gene in a given gene list. The Table shows the top hits provided by this analysis.

656

Table 2. *LPS-stimulated CBMC-production of IL-1 β in IIS children with or without a history of maternal asthma.*

	Children with Asthmatic Mothers		Children with Non-asthmatic Mothers	
	Asthmatic* (n=7)	Non-asthmatic (n=12)	Asthmatic* (n=19)	Non-asthmatic (n=16)
Geometric Mean (ng/ml)	16.5	6.0	6.7	7.7
95% CI (ng/ml)	6.9-39.4	4.8-7.6	4.1-10.9	4.7-12.5
P^{\dagger}	0.03		0.65	

* Physician-diagnosed with symptoms or medication use for asthma in the past year reported at least once on the age 2, 3, 5 or 9-year questionnaires

\dagger by Student's t-test after \log_{10} transformation.