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ORIGINAL ARTICLE

Global hypomethylation in childhood asthma identified by genome-wide DNA-methylation sequencing preferentially affects enhancer regions

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Abbreviations: DMR, differentially methylated region; EPX, eosinophil peroxidase; IL5RA, interleukin 5 receptor subunit alpha; LINA, Lifestyle and environmental factors and their influence on newborns allergy risk; LISA, Lifestyle-related factors on the immune system and the development of allergies in childhood; PASTURE, Protection against allergy: study in rural environments.

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Abstract

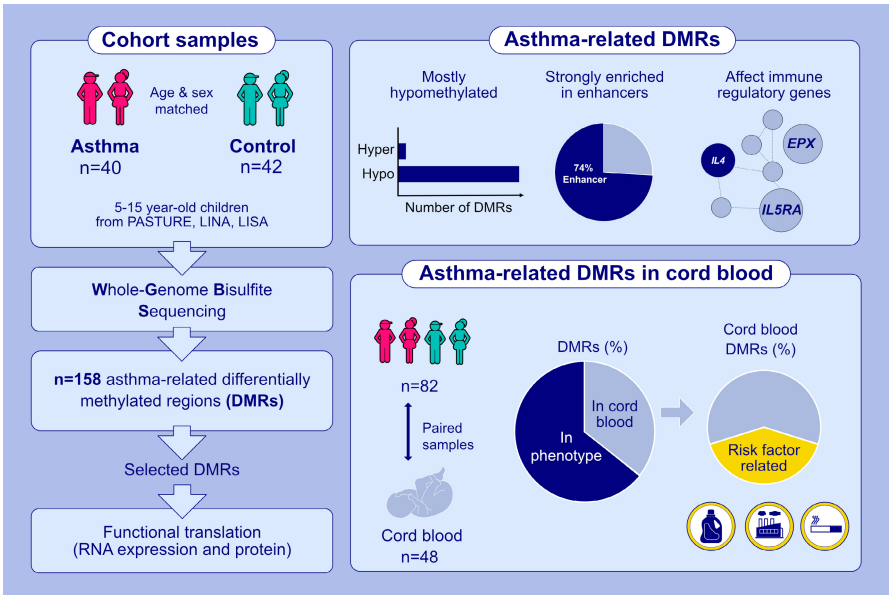
Background: Childhood asthma is a result of a complex interaction of genetic and environmental components causing epigenetic and immune dysregulation, airway inflammation and impaired lung function. Although different microarray based EWAS studies have been conducted, the impact of epigenetic regulation in asthma development is still widely unknown. We have therefore applied unbiased whole genome bisulfite sequencing (WGBS) to characterize global DNA-methylation profiles of asthmatic children compared to healthy controls.

Methods: Peripheral blood samples of 40 asthmatic and 42 control children aged 5–15 years from three birth cohorts were sequenced together with paired cord blood samples. Identified differentially methylated regions (DMRs) were categorized in genotype-associated, cell-type-dependent, or prenatally primed. Network analysis and subsequent natural language processing of DMR-associated genes was complemented by targeted analysis of functional translation of epigenetic regulation on the transcriptional and protein level.

Results: In total, 158 DMRs were identified in asthmatic children compared to controls of which 37% were related to the eosinophil content. A global hypomethylation was identified affecting predominantly enhancer regions and regulating key immune genes such as *IL4*, *IL5RA*, and *EPX*. These DMRs were confirmed in $n = 267$ samples and could be linked to aberrant gene expression. Out of the 158 DMRs identified in the established phenotype, 56 were perturbed already at birth and linked, at least in part, to prenatal influences such as tobacco smoke exposure or phthalate exposure.

Conclusion: This is the first epigenetic study based on whole genome sequencing to identify marked dysregulation of enhancer regions as a hallmark of childhood asthma.

KEYWORDS
asthma, cord blood, DNA-methylation, prenatal exposure



GRAPHICAL ABSTRACT

Whole-genome bisulfite sequencing was applied to blood samples from 40 asthmatic and 42 control subjects aged 5-15 years, as well as to paired cord blood samples. 158 predominantly hypomethylated DMRs were identified, preferentially affecting enhancers of key immune genes such as *IL4*, *IL5RA*, and *EPX*. Of the DMRs identified in the established phenotype, 56 were already perturbed at birth and partially linked to known asthma risk factors (e.g. tobacco smoke or phthalate exposure).

1 | INTRODUCTION

Asthma is the most common chronic inflammatory disease in childhood. With an estimated prevalence of asthma ranging from 2.6% to 30.5%[1] varying according to the age and origin of the children, childhood asthma is a major health concern worldwide. Over the last decades, the prevalence of childhood asthma increased in a majority of countries worldwide, which has been mainly attributed to an interaction of genetic predisposition with a changing environment and a Westernized lifestyle.[1,2] Although the etiology of pediatric asthma remains incompletely understood, its origin is thought to be found early in life.[3] There is a larger number of studies supporting the notion that asthma-related immune alterations are already established during the prenatal development phase when the maturation of the immune system begins.[4] Although the molecular mechanisms initiating and maintaining these aberrant immune functions are largely unknown, epigenetic mechanisms are thought to play a central role in not only mediating the adverse effects of an intrauterine environment but also in preserving the established asthma-promoting phenotype.[4] However, the knowledge of asthma-related epigenetic modifications is limited and no genome-wide studies at a single base-pair resolution are available. So far, DNA-methylation changes in asthma, have been described based on target-specific analyses or on DNA-methylation microarrays[5-9] covering 27,000–850,000 CpG sites of the approximately 28 million CpGs of the human genome.

To date, several childhood asthma-associated DNA-methylation changes at single CpG sites located in immune regulatory genes such as *ALOX12*, *IL13*, and *RUNX3*, or genes involved in arachidonic acid metabolism, T cell differentiation, and IgE production, have been described in whole blood samples.[7,10] In addition, more than 100 differentially methylated sites were identified by array-based epigenome-wide association studies (EWAS) on respiratory cells, such as buccal cells or epithelial cells of the nasopharynx, amongst others CpGs in the close vicinity of established asthma-associated genes, such as *ZFPM1*, *NLRP3*, *IFNGR2*, *NTRK1*, or *ALOX15*.[11-13] However, all of the current EWAS on asthma are biased by the preselection of CpG sites covered by the commercially available DNA-methylation arrays.

The genomic localization of DNA-methylation changes is critical for their functional impact on gene expression and associated relevance to the disease phenotype. Perturbations in regulatory regions, and in particular enhancers regulating multiple genes, are assumed to drive disease progression.[14] Enhancers are not commonly in close vicinity of their target gene, but rather may be located several thousands of base pairs away.[15] Although previous studies of asthma-associated DNA-methylation changes provided valuable information on CpG sites potentially contributing to disease etiology and suggested an enhancer-centric epigenetic dysregulation,[9] a plethora of enhancer elements have since been identified that are not covered by DNA-methylation arrays and thus have previously escaped analysis. Even with the advanced EPIC array only 7% of distal and 27% of proximal ENCODE regulatory elements, and less than 4% of all CpGs of the genome are represented.[16]

As a consequence of this limited genomic coverage of previous methylation array studies only little is known about enhancer dysregulation in childhood asthma. To overcome this knowledge gap, this study used a different approach and determined the unbiased global DNA-methylation profile at a single-base pair resolution by applying

whole-genome bisulfite sequencing (WGBS). Whole blood samples of 40 asthmatic children from three independent prospective birth cohorts were compared to 42 sex- and age-matched controls. It is well known that the methylation of adjacent CpG sites is mutually dependent[17] and regional changes in DNA-methylation are assumed to be functionally more relevant than single CpG positions.[18] Thus, we determined differentially methylated regions (DMRs) rather than reporting methylation changes at single CpG positions and subsequently confirmed our findings by targeted methylation analyses in larger number of cases that included subjects from two of the three cohorts. The comprehensive assessment of the genomic distribution of the DMRs was complemented by elucidating the functional consequences of aberrant DNA-methylation associated with key immune modulating genes. To this end, cord blood—available for a subset of the children—provided the opportunity to assess potential prenatal priming of the DNA-methylation changes identified in asthmatic children.

2 | METHODS

Detailed information can be found in the Data S1.

2.1 | Study characteristics

This study comprises data and samples derived from the three different birth cohorts LINA,[19] LISA,[20] and PASTURE.[21] A detailed cohort description can be found in the Data S1. Participation in all three cohort studies was voluntary and written informed consent was given by the parents or children if applicable. The studies were approved by their respective ethics committees (LINA: 046–2006, 160–2008, 160b/2008, EK-BR-02/13–1, 169/13ff, 150/14ff, LISA: 398–12-05112012, PASTURE: 02046, 9/11-E1/651–2002, 415-E401/4–2007).

2.2 | Asthma outcome

Asthma was defined based on the confirmative answer to the question: "Has a physician-diagnosed your child with asthma during the last 12 months (=current asthma)?" asked in the parent-reported questionnaires at the time-point when blood samples were obtained for DNA-methylation analysis.

2.3 | Sample selection

From each of the three cohorts, cases and controls were randomly selected to derive a balanced selection of children diagnosed with asthma and of age- and sex-matched controls. As a prerequisite a sufficient quantitative and qualitative amount of genomic DNA had to be available. For the asthma group only children with a physician-made asthma diagnosis at the time of WGBS analysis were selected. For the control group, children were chosen who never reported wheezing symptoms, obstructive bronchitis, asthma, rhinitis or atopic dermatitis. A total of 40 children aged five to 15 years of age with a current asthma diagnosis and 42 age- and sex-matched controls were selected for WGBS analysis. An overview of the selected samples is provided in Table S1.

For 48 children investigated at the time of an established asthma phenotype paired cord blood DNA samples were available (n = 23 asthma, n = 25 controls; Table S1) and also subjected to whole genome bisulfite sequencing.

2.4 | Whole-genome bisulfite sequencing (WGBS)

To assess quantitative DNA-methylation information at single base pair resolution, whole blood genomic DNA samples from 82 children of the three cohorts and 48 matched cord blood samples available from LINA and PASTURE (Table S1, Table S2) were subjected to WGBS (see Data S1 for details) as previously described.[22] All samples showed bisulfite conversion rates >99%.

2.5 | Pre-processing of WBGS data

Sequencing data for each sample were input to the one touch pipeline[23] and processed using bwa v0.6.1.[24] and methylCtools v1.0.0[25] resulting in tab-separated output files containing CpG position, number of reads with a methylated cytosine at this position, total number of reads covering the CpG and a *snp score*,[26] which is the estimated probability of the CpG to be a SNP. CpGs were removed from the whole cohort if at least one of the 82 samples had a *snp score* of 0.25 or greater.

2.6 | Determination of asthma-associated DMRs

Asthma-related DMRs were determined by a three-step procedure (i–iii). (i) DMRs were defined as at least three consecutive differentially methylated CpG sites between asthmatics (n = 40) and controls (n = 42). DMRs were called by two independent algorithms, a DMR calling strategy, which was applied in the latest meta-analysis on childhood asthma using 450k array data.[8] For our WGBS data we used DSS version v2.12.0,[27] and metilene version v0.2–6[28] as DMR calling tools. For DSS we used a Wald-test p-value threshold of 0.01 to mark a CpG as differentially methylated. The minimum DMR length was set to 50 bp, the maximum distance between two CpGs was set to 100 bp and the fraction of differentially methylated CpGs was set to minimum 0.3. Metilene uses circular binary segmentation followed by two dimensional Kolmogorov–Smirnov test (2D-KS test) and a DMR was considered significant if the obtained q-value

was less than 0.05. Only chromosomes 1–22 were included in the analysis, while sex chromosomes were omitted. DSS adopts a highly appropriate beta binomial model for modelling DNA-methylation from WGBS count data but does not provide significance testing nor multiple testing correction of the identified DMRs. On the other hand, metilene offers the ability to perform multiple testing correction for the identified DMRs. Given the different approaches and features adopted by these two tools, we deemed their overlap to be highly conservative, thereby reducing potential false positives. (ii) To reduce the likelihood of false-positive DMR calls, we kept only the metilene DMRs that overlapped at least by 1 bp with the DMRs from DSS. The overlap was determined by using *intersectBed* from Bedtools version 2.24.0.[29] (iii) Concordant DMRs were tested for significance in each of the three cohorts LINA, LISA, and PASTURE by a factorial ANOVA using R version 4.0.2.[30] Log transformed β-values with a pseudo count of 0.006 of all differentially methylated CpGs within a DMR were modelled by using the disease condition asthma/control and the CpG position within a DMR. If the Bonferroni adjusted p-values in each of the three cohorts were $p < 0.05$ then a DMR was considered as significantly differentially methylated and retained for further analysis.

2.7 | Overlap with previous asthma-associated EWAS

Previous asthma-associated EWAS studies in the PubMed database were identified by the search term: ("asthma" OR "wheeze") AND ("WGBS" OR "EWAS" OR "450k" OR "850k" OR "27k" OR "epigenome-wide" OR "HumanMethylation450K BeadChip") AND "blood" (query data 27.10.2022). This search retrieved 68 publications, from which two reviews, one RCT and one systematic review were excluded. After manual curation 22 EWAS studies (including meta-analyses) remained that reported DNA-methylation changes in blood related to asthma or lung function (Figure S1A). DNA-methylation changes described in these manuscripts were related to the DMRs observed in our study.

2.8 | Gene annotation and definition of enhancer and promoter DMRs

Genomic annotation of DMRs to the nearest transcription start site (TSS) from Gencode v19 gene models in human genome version hg19 was obtained by using the 'closest' module from Bedtools. Promoter regions were defined as 2 kb up- and downstream of the TSS. DMRs overlapping with at least 1 bp were categorized as promoter DMRs. DMRs were defined as enhancer DMRs, if their genomic location intersected at least 1 bp with GeneHancer,[31] ENCODE,[32] or ROADMAP[33] enhancer regions, or with an active histone mark as previously identified in LINA children according to Bauer et al.[22] (Table S3). Predicted target genes of enhancer DMRs were identified by using GeneHancer.

2.9 | DMR classification

All asthma-related DMRs were classified into different categories: (i) genotype−/non-genotype-associated, (ii) cell-type-dependent, (iii) already present in cord blood. Asthma-related DMRs already present in cord blood were overlapped with previous EWAS studies investigating prenatal factors that affect DNA-methylation (see Data S1 for details and Figure S1B).

According to previous works,[19,22] a DMR was categorized as genotype-associated (gDMR) whenever a significant correlation between the methylation value of the DMR and any SNP in a +/−5 kb window around the DMR was determined (see Data S1 for details). Likewise, DMRs with no significant association to methylation quantitative trait loci (meQTLs) were classified as a non-genotype associated DMR (ngDMR). All meQTL SNPs were checked against the EMBL GWAS catalogue[34] (Query date: 01.11.2022) for previous associations to any phenotypic outcomes including asthma.

To determine whether the asthma-related DMRs were already differentially methylated at the time of birth, WGBS-based DNA-methylation data of matched cord blood samples were analysed ($n = 48$, Table S1, Table S2). Whenever a DMR was significantly differentially methylated at the time of birth as determined by factorial ANOVA followed by a multiple test correction (Bonferroni-corrected $p < 0.05$, corresponding to a nominal $p < 0.00032$ separately in all three cohorts), the corresponding DMR was classified as a cord blood asthma-DMR already present at the time of birth.

To identify which cord blood DMRs were associated with a prenatal influencing factor, previously published array- or WGBS-based EWAS conducted with cord blood samples were evaluated (see Figure S1B and Data S1 for details). This included studies on maternal smoking during pregnancy, maternal mental health, maternal disease such as diabetes and atopy, maternal BMI and diet, or environmental exposures. Whenever a CpG or region previously associated with a prenatal influencing factor overlapped with at least 1 bp with a cord blood DMR in our data set, this DMR was considered to be associated with this prenatal influencing factor.

2.10 | Cell-type dependency

The frequency of the main blood cell types (T cells, B cells, NK cells, monocytes, neutrophils, eosinophils) was estimated by deconvolution of the WGBS data using *EpiDish*.[35]

Next, the cell-type dependency of DMRs was determined using adjusted multiple regression models with the mean DNA-methylation of the DMR as the dependent variable and the main blood cell-type estimates as the independent variables (confounder: child's sex, cohort, prenatal tobacco smoke exposure, family history of atopy, parental school education, maternal age at birth, growing up on a farm). DMRs significantly (Bonferroni-corrected $p < 0.05$, corresponding to a nominal $p < 0.00032$) associated to a specific blood cell type were classified as cell-type-dependent (see Data S1 for details).

2.11 | Enhancer-, pathway- and TFBS motifenrichment

We used Fisher's exact test in R to test if asthma-related DMRs were enriched for enhancer elements (Table S3) when comparing them with all other methylated regions in the genome that have similar characteristics as our DMRs but are not called as such (see Data S1 for details).

For gene enrichment analysis the genomic positions of asthma-related DMRs were subjected to GREAT (Genomic Regions Enrichment of Annotations Tool) version 3.3.0 analysis tool[36] setting "whole genome" as background and a significance level of $\alpha < 0.05$.

The MEME-ChIP tool implemented in the MEME Suite version 5.4.1 (Motif-based sequence analysis tools)[37] was used to identify transcription factor-binding site (TFBS) based on the HOCOMOCOv11 core HUMAN database including de novo motifs within the asthma-related DMRs. DMRs were elongated by 20 bp at the start and at the end to ensure an intersection with motif sequences. Only motifs with a length of four to fifteen nucleotides were considered. Motif enrichment with an *E*-value <0.05 (estimate of the statistical significance of each motif) was considered significant.

2.12 | Network analysis and natural language processing

For network and module analysis of DMR-associated genes including all enhancer DMR target genes or genes closest to the next TSS ($n = 435$ genes) were subjected to Cytoscape analysis version 3.8.2.[38] The Reactome Functional Interaction (FI) plugin version 8.0.4 (released Feb 2022) was used to determine network patterns of common and predicted interactions as estimated via Naïve Bayes Classifier excluding linker genes. Cluster FI network was applied to identify cluster of genes (=modules).[39] Subsequently, a pathway enrichment analysis (significance cut-off: FDR<0.01) was performed using the databases CellMap, Reactome, KEGG, NCI PID, Panther and BioCarta for each module.

To identify genes in the network, previously associated with asthma-related outcomes, natural language processing (NLP, see Data S1 for details) was applied. In brief, mentions of genes and gene products were searched in the PubMed and PubMed Central open access literature databases and additionally filtered by the following terms "asthma", "asthmatic", "asthmatics", "wheeze", "bronchial hyperreactivity", "airway hyperreactivity", "bronchial hyperresponsiveness", or "hyperreactive airway disease".

2.13 | Targeted analyses: DNA-methylation, transcription, and protein measurement

Targeted analyses were performed in a larger sample set obtained from the 6–8 years old LINA children and the 15-years old LISA children from the Leipzig study centre. No further PASTURE samples were available for these analyses. All available samples from LINA and LISA fulfilling these two criteria were included: (i) samples from children diagnosed with asthma by a physician and (ii) control samples that never reported wheezing symptoms, obstructive bronchitis, or asthma, however they could have developed atopic dermatitis or rhinitis. An overview of the selected samples for these analyses is provided in Table S1 and Table 2.

Targeted DNA-methylation analysis was performed for a set of selected DMRs in $n = 127$ LINA and $n = 140$ LISA samples using the Sequenom's MassARRAY platform (San Diego, CA, USA, Table S4 for primer sequences, Figure S2) as previously described.[22]

Functional translation of methylation changes for selected genomic regions was determined by RNA and protein expression analyses of the associated genes. Whole blood samples for transcriptional analyses were collected at the same time as blood samples for DNA-methylation analyses. RNA expression data were obtained for *EPX*, *IL4*, and *IL5RA* for $n = 126$ LINA and $n = 140$ LISA samples by qPCR on the Biomark HD system as previously described[22] (see Table S5 for primer sequences).

Within the LINA study phytohemagglutinin (PHA)-stimulated IL-4 concentrations obtained from a whole blood assay were available. IL-4 concentrations were measured by cytometric bead array (BD CBA Human Soluble Flex Set system, Becton Dickinson, Heidelberg, Germany) as previously described.[40]

Detailed information can be found in the Data S1.

2.14 | Statistics

2.14.1 | WGBS samples

To determine potential differences in the study characteristics between asthmatic and control children a Fisher's exact- test or Mann–Whitney *U*-test were applied. As confounding factors in the models analyzing WGBS-data the child's sex, cohort, prenatal tobacco smoke exposure, family history of atopy, parental school education, maternal age at birth, growing up on a farm and cell composition were included.

2.14.2 | Targeted analyses

To test whether there were differences between asthmatic and control children of the LINA and LISA cohorts with respect to the child's age and sex, prenatal tobacco smoke exposure, family history of atopy, parental school education, maternal age at birth, growing up on a farm, or the presence of rhinitis or atopic dermatitis in the child, Fisher's exact- test or Mann–Whitney-*U* test were applied.

A Mann–Whitney-*U* test was used to determine if there were significant differences in DNA-methylation and transcription between groups. Spearman correlation was used to determine the association between DNA-methylation, relative gene expression, or protein concentration. Correlation coefficients are reported as effect size

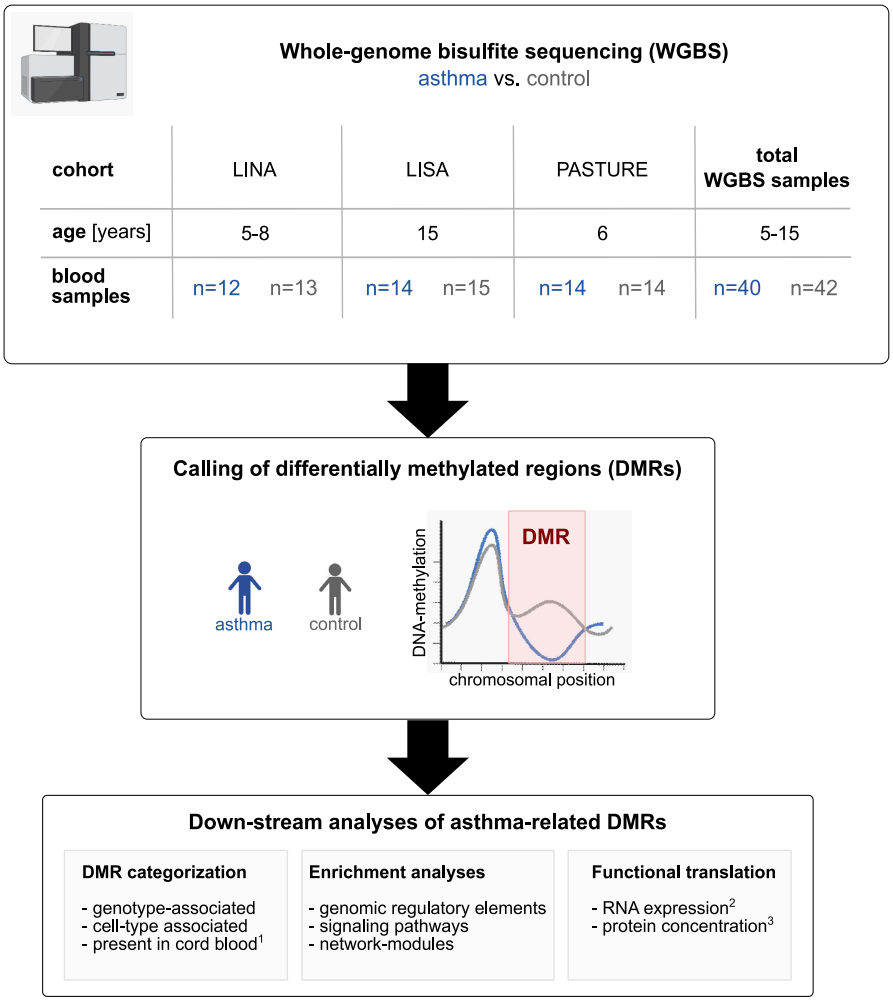


FIGURE 1 Study design. Blood samples derived from asthmatics or control children of the three cohorts were subjected to WGBS to determine asthma-related DMRs. DMRs comparing asthmatic and control children were determined by the two independent DMR-calling algorithms DSS and metilene. Asthma-related DMRs were subsequently analyzed. DMR = differentially methylated region, WGBS = whole-genome bisulfite sequencing, LINA = lifestyle and environmental factors and their influence on newborns allergy risk, LISA = influences of lifestyle-related factors on the immune system and the development of allergies in childhood, PASTURE = Protection Against Allergy: Study in Rural Environments. [1]based on available cord blood sample of LINA and PASTURE. [2]based on available whole blood samples of LINA and LISA. [3]based on available plasma samples of LINA.

measures (point biserial (r_{pb}) for Mann–Whitney U and Spearman's rho ρ). The selection of confounders associated with asthma or affecting DNA-methylation patterns was based on a priori knowledge. The child's sex, cohort, prenatal tobacco smoke exposure, family history of atopy, parental school education and maternal age at birth were introduced as confounding factors in all models.

Confounder-adjusted logistic regression analyses were applied to compare the DNA-methylation and relative gene expression of asthmatic and control children. Confounder-adjusted mediation analyses were performed using the *PROCESS* macro version v3.4[41] for SPSS. Statistical significance of the indirect effect was determined by bootstrapping as implemented in the *PROCESS* macro version 3.4.[41] Bias-corrected 95% confidence intervals were derived from the distribution of bootstrap estimates of the indirect effect from random resampling of 5000 samples. Only for non-dichotomous independent variables a standardized indirect effect was calculated. Effect sizes of regression analyses are either provided as unstandardized *b*, standardized β, or as odds ratio (OR).

Statistical analyses were performed using STATISTICA for Windows Version 12.0/13.0 (Statsoft Inc. Europe, Hamburg, Germany), IBM SPSS Statistics for Windows Version 25 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) or R version 4.0.2.[30] *P*-values ≤0.05 were considered significant.

3 | RESULTS

3.1 | Genome-wide DNA-hypomethylation in childhood asthma

To evaluate epigenetic alteration in the global DNA-methylation pattern of asthmatic children at single base-pair-resolution, we performed WGBS and subsequent DMR calling of whole blood samples from $n = 82$ children participating in the LINA, LISA, or PASTURE cohort (Figure 1, Table S1). In total, samples from $n = 40$ asthmatic children were compared to $n = 42$ age-matched controls without an asthma history or other respiratory symptoms (Table 1). High quality WGBS data were derived with a mean genome coverage of 56.3x (Table S2A). To retain highly confident asthma-related DMRs for downstream analyses, a multiple-step DMR-calling approach was utilized (Figure S3). Using these two independent DMR-calling algorithms, DSS and metilene, 1021 and 758 DMRs were determined, respectively. DMRs overlapping

TABLE 1 Characteristics of LINA, LISA and PASTURE subcohorts used for whole-genome bisulfite sequencing (WGBS)

	control $n = 42$ n (%)	asthma $n = 40$ n (%)	p-value	LINA $n = 25$ n (%)	LISA $n = 29$ n (%)	PASTURE $n = 28$ n (%)
age [years]						
median	6.0	6.0	0.778[c]	6.0	15.0	6.0
LQ/UQ	6.0/15.0	6.0/15.0		5.0/6.0	15.0/15.0	6.0/6.0
child's sex						
female	21 (50.0)	15 (37.5)	0.275[a]	13 (48)	18 (62.1)	5 (17.9)
male	21 (50.0)	25 (62.5)		12 (52)	11 (37.9)	23 (82.1)
growing up on a farm						
no	38 (90.5)	34 (85.0)	0.514[a]	25 (100)	29 (100)	18 (64.3)
yes	4 (9.5)	6 (15.0)		0 (0.0)	0 (0)	10 (35.7)
prenatal tobacco smoke exposure						
no	40 (95.2)	35 (87.5)	0.259[a]	25 (100)	27 (93.1)	23 (82.1)
yes	2 (4.8)	5 (12.5)		0 (0.0)	2 (6.9)	5 (17.9)
maternal age at birth						
median	31.0	31.6	0.414[c]	30.8	30.0	31.5
LQ/UQ	29.0/33.0	28.9/35.0		28.3/37.5	28.0/32.0	29.6/34.4
parental education level						
low	2 (4.8)	1 (2.5)	0.029[a]	0 (0.0)	0 (0.0)	3 (10.7)
middle	9 (21.4)	19 (47.5)		4 (16.0)	13 (44.8)	11 (39.3)
high	31 (73.8)	20 (50.0)		21 (84.0)	16 (55.2)	14 (50.0)
family history of atopy						
no	22 (52.4)	8 (20)	0.003[a]	5 (20.0)	15 (51.7)	10 (35.7)
yes	20 (47.6)	32 (80)		20 (80.0)	14 (48.3)	18 (64.3)
phenotype						
control	n.a.	n.a.	n.a.[b]	13 (48)	15 (51.7)	14 (50.0)
asthma	n.a.	n.a.		12 (52)	14 (48.3)	14 (50.0)
rhinitis	0 (0.0)	15 (37.5)		5 (20.0)	5 (17.2)	5 (6.1)
atopic dermatitis	0 (0.0)	13 (32.5)		3 (12.0)	7 (24.1)	3 (3.7)

Abbreviations: LQ = lower quartile, UQ = upper quartile.

[a]from Fisher's exact test.

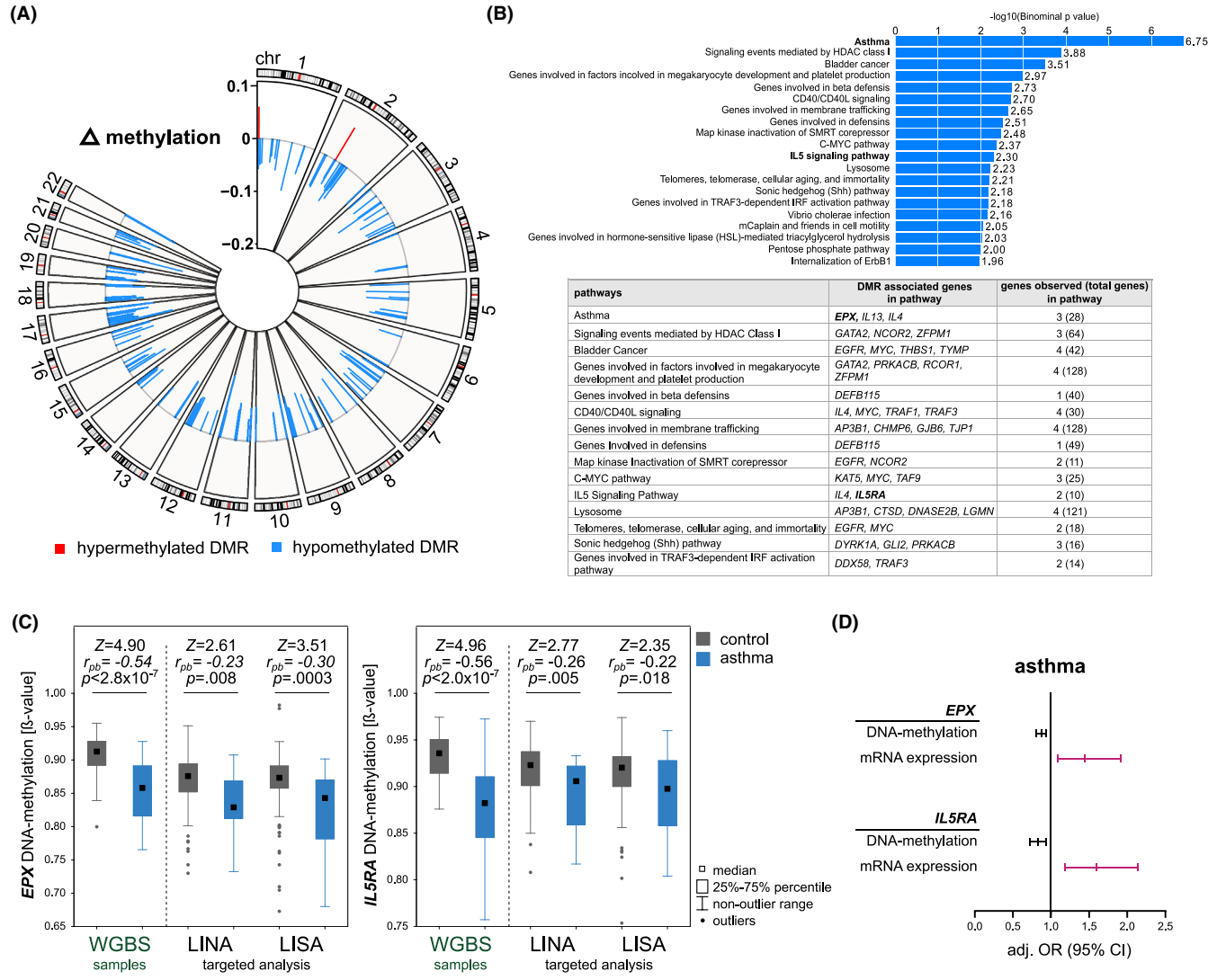
[b]note that controls were selected from non-atopic children only.

[c]from Mann–Whitney U-test.

between these two approaches (n = 385) were subjected to factorial ANOVA analysis to assess whether significant DNA-methylation differences could be observed separately in each of the three cohorts and were in the same direction. Only these concordant DMRs (n = 158 out of n = 385) were retained for further assessment (Figure S3, see Table S6A for asthma-related DMR list). These 158 asthma-related DMRs were distributed over all autosomes (Figure 2A) and had a read coverage of 31.5x in average (Table S2B). Unsupervised cluster analysis of these derived 158 DMRs resulted in a clear separation between asthmatics and control children (Figure S4A). The vast majority of the asthma-related DMRs were hypomethylated in asthmatic children (Figure 2A), while only two hypermethylated DMRs located in the *TET3* (*ten-eleven translocation 3 or tet methylcytosine dioxygenase 3*) gene and the long coding RNA *AL645608.1* were identified. In line with previous asthma EWAS studies our DMRs overlapped with several CpG sites or DMRs identified based on array approaches (see Table S7 for overlap and references and Figure S1A for evaluated EWAS studies).

3.2 | Genetic and cell type composition influences on asthma-related DMRs

Since the level of DNA-methylation can be strongly dependent on the genotype or the cell type composition, asthma-related DMRs were categorized according to cell type-dependency and genotype-association (gDMRs). Based on this categorization, 38 out of the 158 DMRs were associated with the genetic background (24.1%), while the remaining 120 DMRs (75.9%) were classified as non-genotype



pathways	DMR associated genes in pathway	genes observed (total genes) in pathway
Asthma	***EPX***, *IL13, IL4*	3 (28)
Signaling events mediated by HDAC Class I	*GATA2, NCOR2, ZFPM1*	3 (64)
Bladder Cancer	*EGFR, MYC, THBS1, TYMP*	4 (42)
Genes involved in factors involved in megakaryocyte development and platelet production	*GATA2, PRKACB, RCOR1, ZFPM1*	4 (128)
Genes involved in beta defensins	*DEFB115*	1 (40)
CD40/CD40L signaling	*IL4, MYC, TRAF1, TRAF3*	4 (30)
Genes involved in membrane trafficking	*AP3B1, CHMP6, GJB6, TJP1*	4 (128)
Genes Involved in defensins	*DEFB115*	1 (49)
Map kinase Inactivation of SMRT corepressor	*EGFR, NCOR2*	2 (11)
C-MYC pathway	*KAT5, MYC, TAF9*	3 (25)
IL5 Signaling Pathway	*IL4,* ***IL5RA***	2 (10)
Lysosome	*AP3B1, CTSD, DNASE2B, LGMN*	4 (121)
Telomeres, telomerase, cellular aging, and immortality	*EGFR, MYC*	2 (18)
Sonic hedgehog (Shh) pathway	*DYRK1A, GLI2, PRKACB*	3 (16)
Genes involved in TRAF3-dependent IRF activation pathway	*DDX58, TRAF3*	2 (14)

FIGURE 2 DMR distribution and down-stream analyses. (A) Circos plot represents the distribution of the 158 differentially methylated regions (DMRs) identified in asthmatic children vs. controls across all autosomes. The outer circle shows the 22 autosomes. The bars in the inner circle represent the DMRs and their chromosomal location. Hypermethylated DMRs are indicated as red bars, hypomethylated DMRs in blue. The height of each bar indicates the DNA-methylation differences between asthmatics and controls. (B) KEGG pathway enrichment for all asthma-DMRs based on their genomic location. (C) DNA-methylation difference between asthmatic children and controls of the WGBS samples (asthma $n = 40$, controls $n = 42$), LINA study (asthma $n = 19$, controls $n = 108$) and LISA study (asthma $n = 25$, controls $n = 115$) for DMRs related to *EPX* and *IL5RA* as determined by sequencing or MassARRAY, respectively (p-value from Mann–Whitney U-test, r_{pb}: point biserial correlation coefficient). (D) Association of *EPX* and *IL5RA* DNA-methylation (black whiskers) and transcription (magenta whiskers) to asthma outcome in meta-analysis combining LINA and the LISA study (DNA-methylation: asthma $n = 44$, controls $n = 223$, *EPX* transcription: asthma $n = 44$, controls $n = 220$, *IL5RA* transcription: $n = 44$ asthma $n = 222$ controls). Given are ORs with +/−95% CIs from logistic regression adjusted for child's sex, cohort, prenatal tobacco smoke exposure, family history of atopy, parental school education and maternal age at birth using ln-transformed DNA-methylation values.

associated DMRs (ngDMRs). A total of 465 meQTLs were identified in relation to the 38 gDMRs, of which none has been previously described as an asthma risk factor in genome-wide association studies (Table S6B). However, including all phenotypic traits of the GWAS catalogue, we found 14 DMRs associated with at least one trait. For eight of these DMRs, the trait showed a loose phenotypic association with asthma (Table S6B) including lung function (rs645601 and rs7700998). Five SNPs were associated to counts of different blood cell types with SNPs rs4328821 and rs7646596 upstream of the *RPN1*-DMR associating to the eosinophil count. Additionally, rs12699415 related to the *MAD1L1*-DMR was linked to idiopathic pulmonary fibrosis.[34]

We observed an enhanced eosinophil frequency in the blood of asthmatic children (Mann–Whitney *U* test: Z = 3.42, $r_{pb} = 0.32$, $p = 0.017$, Table S8), but not for the remaining cell types, that is, B cells, T cells, monocytes, NK cells or neutrophils. We applied adjusted multiple regression analyses to test whether different cell type frequencies have an impact on the DNA-methylation level of

TABLE 2 Characteristics of the LINA and LISA cohort considered in the targeted analysis

	LINA ($n = 127$)			LISA ($n = 140$)		
	control $n = 108$ n (%)	asthma $n = 19$ n (%)	p-value	control $n = 115$ n (%)	asthma $n = 25$ n (%)	p-value
age [years]						
median	7.0	6.9	0.553[b]	15.0	15.0	n.a.
LQ/UQ	7.0/7.0	7.0/7.0		15.0/15.0	15.0/15.0	
Child's sex						
female	54 (50.0)	7 (36.8)	0.328[a]	73 (62.4)	11 (44.0)	0.117[a]
male	54 (50.0)	12 (63.2)		44 (37.6)	14 (56.0)	
growing up on a farm						
no	108 (100)	19 (100)	n.a.	117 (100)	25 (100)	n.a.
yes	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
prenatal tobacco smoke exposure						
no	95 (88.0)	19 (100)	0.214[a]	101 (87.8)	23 (92.0)	0.737[a]
yes	13 (12.0)	0 (0.0)		14 (12.2)	2 (8.0)	
maternal age at birth						
median	30.7	32.6	0.126[b]	29.5	29.0	0.746[b]
LQ/UQ	27.8/34.0	28.9/36.3		27.0/32.0	27.0/31.0	
parental education level						
low	1 (0.9)	0 (0.0)	0.522[a]	1 (0.9)	0 (0.0)	0.392[a]
middle	21 (19.4))	2 (10.5)		43 (37.4)	13 (52.0)	
high	86 (79.6)	17 (89.5)		71 (61.7)	12 (48.0)	
family history of atopy						
yes	72 (66.7)	15 (78.9)	0.423[a]	49 (42.6)	16 (64.0)	0.076[a]
no	36 (33.3)	4 (21.1)		66 (57.4)	9 (36.0)	
atopic phenotype						
rhinitis	7 (6.5)	7 (36.8)	0.170[a]	18 (15.7)	6 (24.0)	0.573[a]
atopic dermatitis	24 (22.2)	8 (42.1)		24 (20.9)	12 (48.0)	

Abbreviations: LQ = lower quartile, UQ = upper quartile.

[a]from Fisher's exact test.

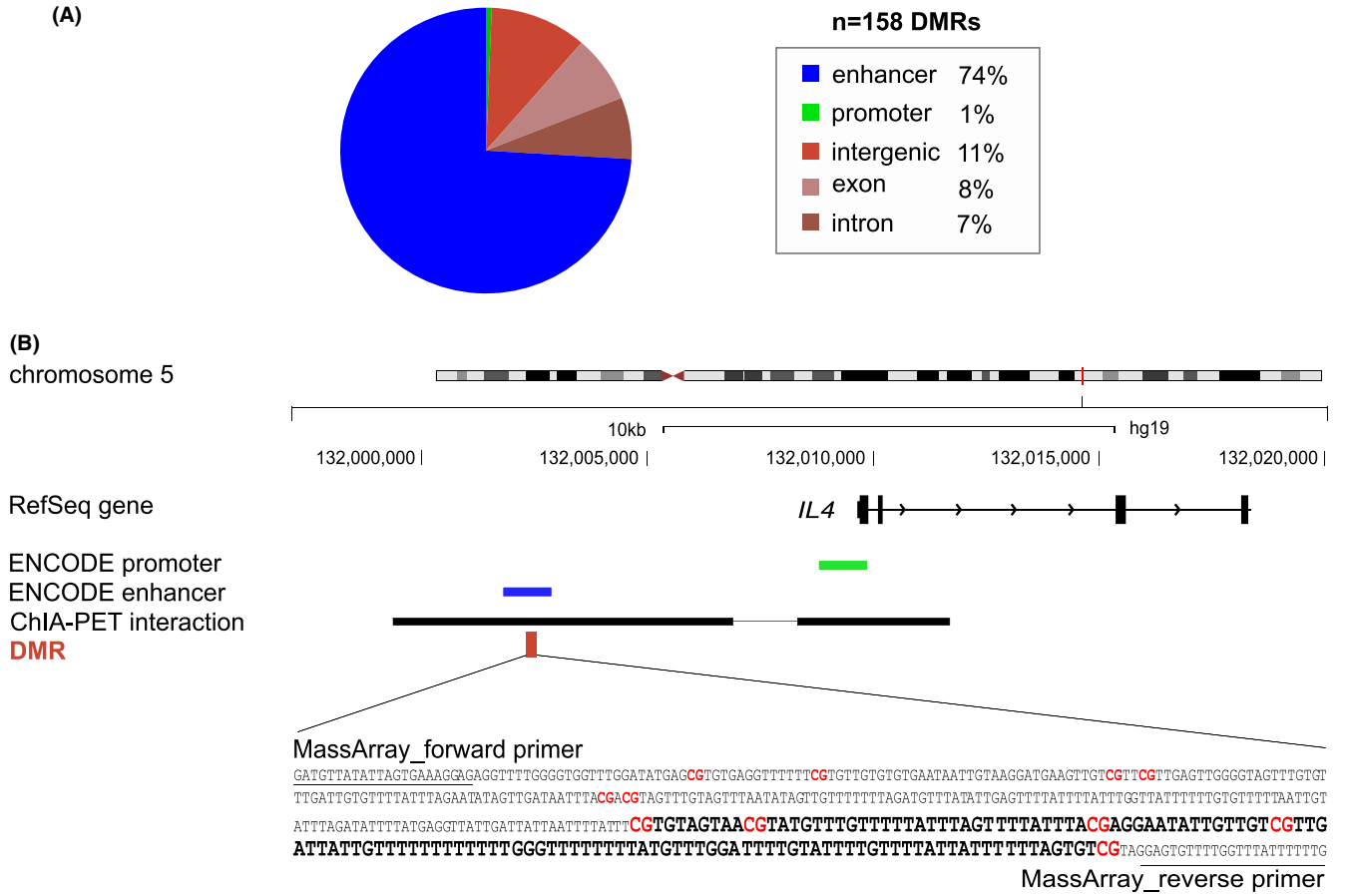
[b]from Mann–Whitney U-test.

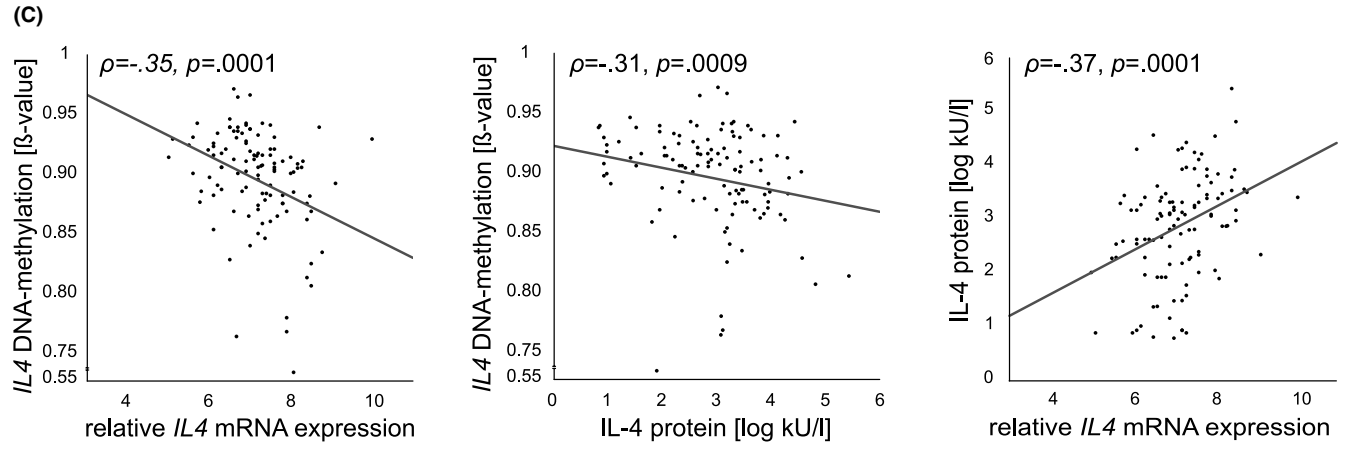
the determined DMRs. To this end, 37% of the asthma-DMRs (58 DMRs) were associated with the eosinophil proportion and only three DMRs in total to B cells, T cells, monocytes, NK cells or neutrophils (Table S6A). However, even after accounting for these cell types in the adjusted multiple regression models, asthma was still a significant contributor of the DNA-methylation status for all cell-type-dependent DMRs (Table S9).

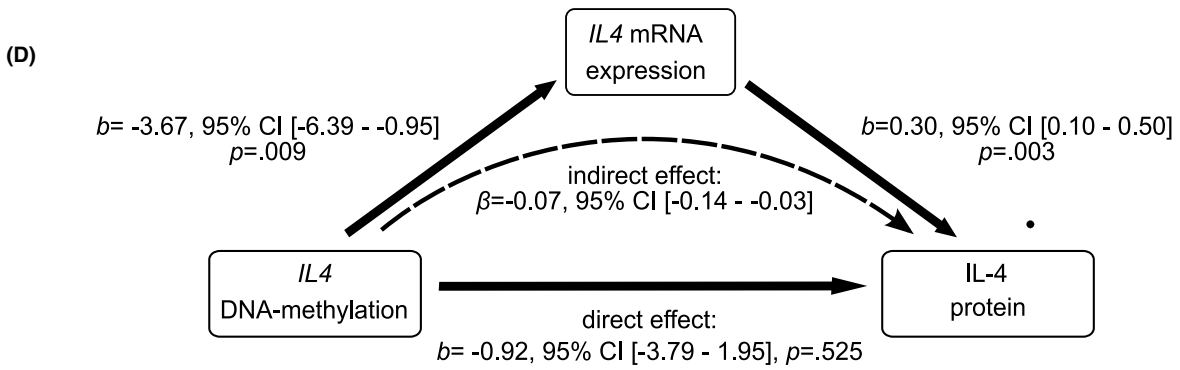
3.3 | Altered DNA-methylation pattern associates with perturbed immune regulation

To elucidate the relevance of the asthma-related aberrant DNA-methylation profile, a pathway enrichment analysis was performed. Besides a strong enrichment in the asthma pathway, we found classical immune system-related pathways enriched, such as IL-5- known

FIGURE 3 Genomic location of asthma-related DMRs and functional translation of *IL4* enhancer hypomethylation. (A) Pie chart represents the proportional distribution of the genomic regions affected by asthma-related DMRs. (B) Genomic location of the *IL4* DMR and the genomic region analysed by MassARRAY in the UCSC genome browser.[64] (C) Scatterplots show the association of *IL4* DNA-methylation to *IL4* transcription ($n = 112$) and IL-4 protein concentration ($n = 115$) and the association of IL-4 protein concentration to *IL4* transcription ($n = 111$) in children of six years of the LINA study. Correlation coefficient (ρ) and p-value from Spearman correlation. (D) Mediation analysis for the relationship of *IL4* enhancer DNA-methylation, *IL4* transcription, and IL-4 protein concentration of children of six years of LINA ($n = 111$). Model was adjusted for child's sex, prenatal tobacco smoke exposure, family history of atopy, parental school education and maternal age at birth. IL-4 protein concentrations were determined after PHA-stimulation. Protein and DNA-methylation data were ln-transformed before analysis. Effect sizes for indirect path are given as standardized β-values with +/−95% CIs. Significance determined by bias-corrected bootstrapping. MA = MassARRAY, DMR = differentially methylated region.







to be crucial for asthma pathophysiology[42,43] (Figure 2B, Table S10). To ensure that the DNA-methylation differences observed in the small number of sequenced samples can be reproduced in larger sample numbers, targeted analyses were performed in further samples ($n = 267$) including six to eight-year-old LINA children ($n = 127$) and 15-years-old LISA adolescents ($n = 140$, Table S1, Table 2). Here, we focused on DMRs that are likely to influence aberrant immune gene expression driving asthma onset. Therefore, the DNA-methylation of two prototypical DMRs (Figure S4B) linked to genes of the asthma pathway (eosinophil peroxidase, *EPX*)—the pathway with the strongest enrichment—and the IL-5 signalling pathway (*IL5RA*) (Figure 2B) known to promote severe atopic asthma associated with eosinophilia,[42] was measured in the larger sample set using a targeted DNA-methylation assay. Significant hypomethylation of these DMRs located in the sixth exon of *EPX*, and in the *IL5RA* promoter, could be confirmed in meta-analysis combining samples of the LINA and LISA cohort (adj. OR/95% CI *EPX*: 0.87/0.81–0.94, $p = 0.0004$; *IL5RA*: 0.83/0.73–0.94, $p = 0.003$, $n = 223$ controls vs. $n = 44$ asthmatics, Figure 2C,D) using logistic regression adjusted for the child's sex, cohort, prenatal tobacco smoke exposure, family history of atopy, parental school education and maternal age at birth.

Furthermore, for both DMRs a negative correlation with the relative gene expression of the associated genes *EPX* was observed ($\rho = -0.40$, $p = 1.4\times10^{-11}$, $n = 264$) and *IL5RA* ($\rho = -0.32$, $p = 1.4\times10^{-7}$, $n = 266$, Figure S5A). In line, expression of *EPX* and *IL5RA* is not only increased in asthmatic children (Figure S5B) but is also associated with an increased risk for asthma during childhood (relative expression *EPX*: adj. OR/95% CI: 1.44/1.09–1.91, $p = 0.010$, $n = 220$ controls vs. $n = 44$ asthmatics, *IL5RA*: adj. OR/95% CI: 1.59/1.19–2.13, $p = 0.002$, $n = 222$ controls vs. $n = 44$ asthmatics, Figure 2D).

3.4 | DNA-methylation changes in asthma affect regulatory hubs

The identified DMRs showed enrichment for 20 binding motifs related to different transcription factors previously associated with asthma including the Th2 master regulator GATA3[44] (Table S11). Additionally, two third of the DMRs were located in genomic regulatory elements, 74% of the DMRs intersecting with enhancers, and 1% with promoters (Figure 3A). In particular, the DMR enrichment in enhancer regions was highly significant (OR/95% CI: 5.83/4.05–8.53, $p < 4.0\times10^{-26}$). Among the DMRs overlapping with a ROADMAP enhancer active in specific blood cells (Table S3), 17 DMRs overlapped with a T helper cell-type specific enhancer including a hypomethylated enhancer DMR associated with the mTORC1 scaffolding protein coding gene *RPTOR* (Table S6A).

One of those hypomethylated enhancer regions showed an enhancer specific ENCODE histone modification profile and a ChiA-PET interaction to the *IL4* promoter (Figure 3B). Although IL4 is one of the key regulators in allergic diseases including asthma, the relevance of this particular enhancer region associated to IL-4 expression has not been addressed so far. We confirmed the asthma-related DNA-hypomethylation of this *IL4* enhancer in the meta-analysis combining the two cohorts LINA and LISA (adj. OR/95% CI: 0.83/0.74–0.94, $p = 0.002$, $n = 223$ controls vs. $n = 44$ asthmatics). In addition, in the LINA cohort, where IL-4 protein concentration measurements were available (Table S1), the *IL4* enhancer DNA-methylation was associated with *IL4* transcription ($\rho = -0.35$, $p = 0.0001$) and PHA-stimulated IL-4 protein concentrations ($\rho = -0.31$, $p = 0.0009$, Figure 3C). In line, two confounder-adjusted mediation models were applied to evaluate the relevance of this hypomethylated *IL4* enhancer region in asthma: The first model showed a significant indirect effect of *IL4* enhancer DNA-methylation on IL-4 protein concentration via *IL4* transcription as a mediator (β/95% CI: −0.07/−0.14–−0.03, Figure 3D), whereas the direct effect was not significant (b/95% CI: −0.92/−3.79–1.95, $p = 0.525$). Second, the asthma phenotype contributed to an increase in IL-4 protein concentration in asthmatics again solely indirectly via the DNA-methylation changes of this *IL4* enhancer and *IL4* transcription as mediators (Figure S6, indirect effect: b/95% CI: 0.05/0.01–0.13; direct effect b/95% CI: 0.23/−0.26–0.73, $p = 0.352$).

3.5 | Genes affected by DNA-methylation changes are functionally connected

To elucidate whether DMR associated genes ($n = 435$ genes, Table S6A) were functionally connected, these genes were subjected to network analysis based on established protein–protein interactions with a subsequent pathway enrichment of the derived network modules. The resulting network consisted of 102 genes in thirteen distinct modules. These modules were related, among others, to immune response and inflammation, cilium assembly and general gene regulation, and to Jak–STAT signalling (Figure 4, Table S12). The vast majority of the network genes (97 out of 102 genes) were targets of differentially methylated enhancers. Our NLP analysis revealed that 33.3% of these enhancer target genes such as the central transcription factors of the immune system *RELA* (NFκB subunit encoding gene), *GATA2*, and *ZFPM1*, the Th2 cytokine *IL4*, or the mTOR complex 1 scaffold protein *RPTOR* have previously been described in the literature in association with asthma (red genes in Figure 4, Table S13A). In addition, we identified novel genes not yet associated to asthma, such as the A-kinase anchoring protein-9 (*AKAP9*). *ANKAP9* is prominently expressed in T cells and involved in immune synapse formation.[45] Among the proteins interacting with ANKAP9 for its proper function are TUBGCP2/TUBGCP6, for which we also observed an enhancer DMR.[46]

3.6 | Prenatal priming for asthma

To discriminate between DMRs that are a consequence of the disease from those predisposing an individual, we subjected matched cord blood samples ($n = 23$ asthmatics vs. $n = 25$ controls) to WGBS

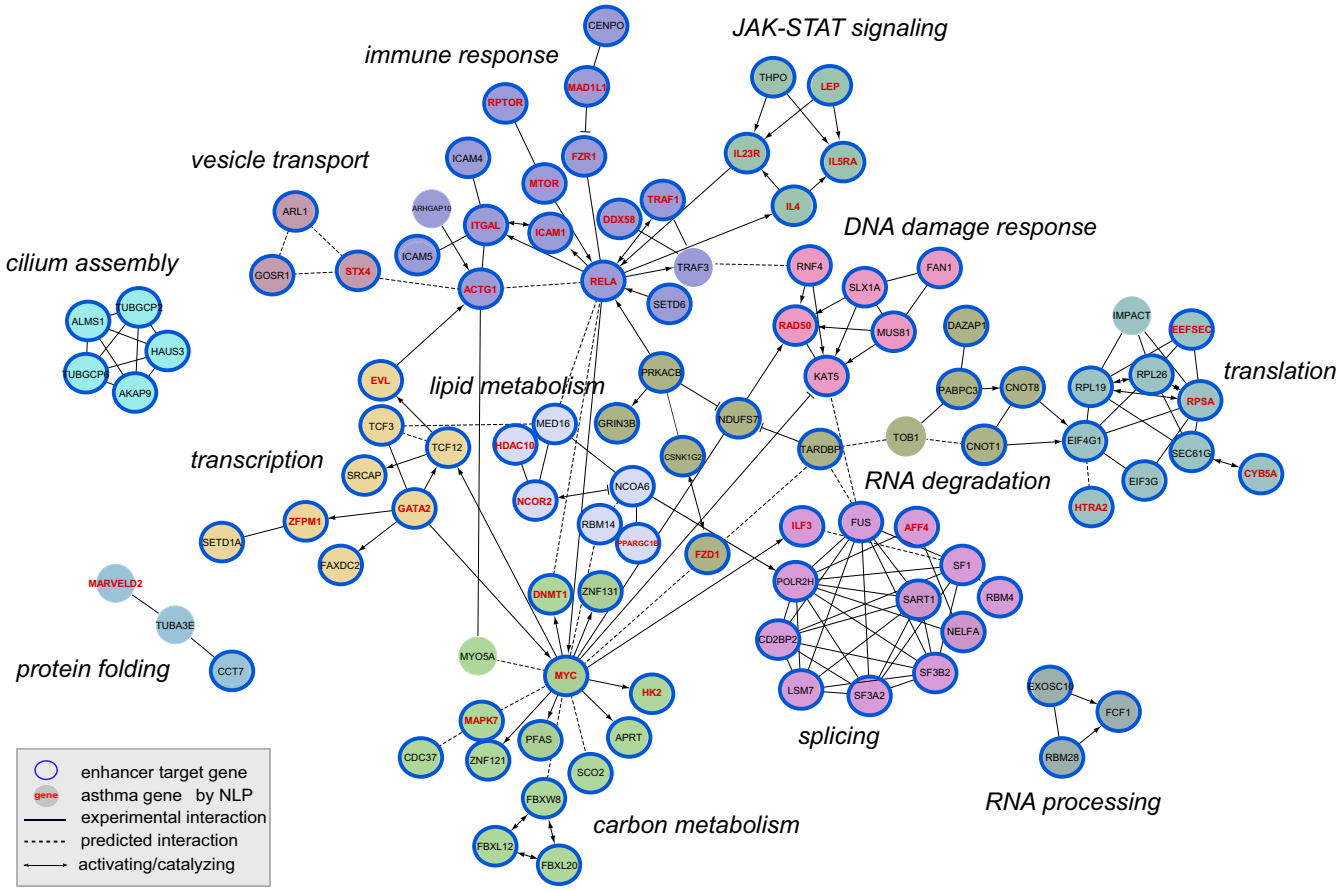
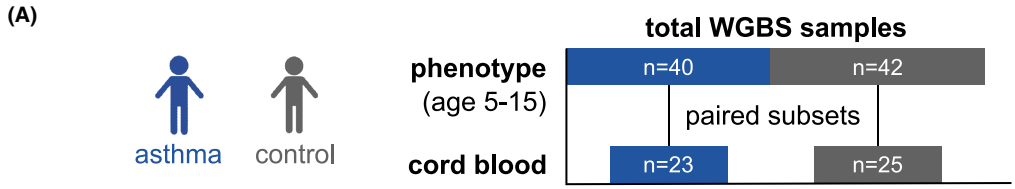


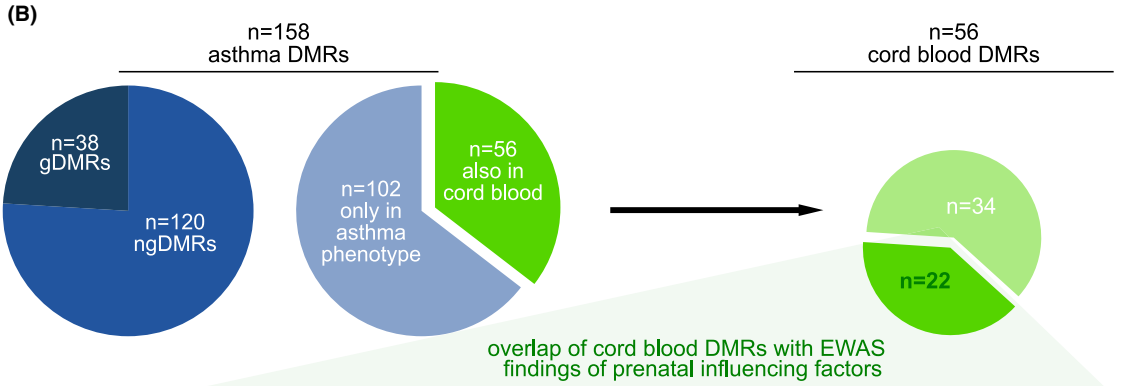
FIGURE 4 Network module analysis of asthma-DMR-associated genes. Shown are all asthma-DMR-associated genes, which show a predicted or experimentally based interaction. Only modules with more than one connection are shown. Target genes of enhancers affected by a DMR are highlighted by blue outline circles. Genes related to asthma or similar terms as determined by the natural language processing tool are indicated in red font. Module nomenclature is based on subsequent pathway enrichment analysis (Table S12).

and assessed whether the methylation changes of the 158 asthma-related DMRs were already present at time of birth (Figure 5A). Of the DMRs identified in the established asthma phenotype 35% (56/158 DMRs) were already significantly differentially methylated in cord blood samples (Table S6C). Most of the cord blood DMRs were again located in enhancers (43 out of 56), 39% (n = 22/56 DMRs) were gDMRs including those already identified in GWAS as a risk factor for lung dysfunction and idiopathic pulmonary fibrosis[34] (Table S6C). For 22 out of the 56 cord blood DMRs, we found an overlap with previous EWAS studies investigating the impact of a variety of different prenatal factors on DNA-methylation (Figure S1B). These factors included exposure to tobacco smoke, to air pollution or to environmental chemicals such as phthalates or lead, maternal diet-related metabolites as well as factors related to maternal health like gestational diabetes or preeclampsia (Figure 5B, Table S13B). When focusing our network analysis on cord blood DMR associated genes the network was comprised of several members of the LFA-1 signaling pathway (Figure 5C). Next to *ITGAL* coding for one of the subunits of LFA-1 (=CD11a), also the LFA-1 ligand ICAM-1, and the co-chaperones *ANKAP9*, *TUBGC2/6* were among the target genes of DMRs already observed in cord blood.

4 | DISCUSSION

To characterize the complete genome-wide DNA-methylation pattern in childhood asthma, this study determined the DNA-methylation profile of 40 asthmatic and 42 control children by utilizing WGBS followed by calling of differentially methylated regions (DMRs) and discriminating between genotype-, and non-genotype-associated as well as cell-type-dependent, or -independent DNA-methylation changes. In total, 158 regions were found to be differentially methylated in childhood asthma, all hypomethylated except for two, which includes a hypermethylated enhancer region for *TET*. Since TET proteins initiate DNA-demethylation, this DMR might be directly related to the global DNA-methylation aberrations observed in asthma. Whether this DMR in asthma is an initiating event or a compensatory mechanism remains to be elucidated in follow-up studies. The predominant global hypomethylation suggests a pronounced epigenetic activation affecting a variety of immune-related genes associated with asthma development and exacerbation. Here, with this first EWAS using a genome-wide sequencing approach and thus not relying on pre-selected CpGs as performed in previous asthma EWASs, we show that this epigenetic activation





	DMR#	associated genes*	prenatal influence
no enhancer	1	*AL645608.1*	lead
	2	*TFCP2L1*	maternal diabetis
	3	*WDR37*	maternal diabetis
	4	*IMPAD1*	S-adenosylhomocysteine
	5	*PDE1C*	tobacco smoke, maternal diabetis
enhancer	6	*AL359736.1*	lead
	7	*RP11-478C1.7;ZFYVE28;RNF4;NOP14-AS1;NELFA; POLN;HAUS3;RP11-317B7.3;C4orf48;MXD4*	air pollution
	8	*AC073046.25;TET3;MGC10955;ALMS1;CCT7;MOB1A; SLC4A5;HTRA2;DGUOK-AS1*	lead
	9	*KIF25;KIF25-AS1;FRMD1*	lead
	10	*GJB2;MIR4499*	lead, tobacco smoke
	11	*MIR4458HG;RP11-480D4.2;MTRR*	maternal diabetis
	12	*ACSF3;CBFA2T3;ZC3H18;GALNS;AC137932.1;PIEZO1; RP5-1142A6.9;ANKRD11;BANP;ZFPM1;MIR5189*	nPFOS
	13	*CTD-2542L18.1;GSE1*	preeclampsia, lead
	14	*RP11-62J1.4;RBM28;LEP*	tobacco smoke
	15	*OPCML*	tobacco smoke, folate
	16	*RP11-443B20.1;CENPO;ATAD2B;MFSD2B;UBXN2A*	maternal diabetis
	17	*ZNF426;ZNF846;ICAM1;ZNF121;DOCK6;ILF3;KRI1;CTC-325H20.4; DNMT1;ZNF561;ZNF699;FBXL12;ZNF559;CDC37;ZNF266;S1PR2; CTD-2369P2.8;ICAM4;ICAM5;EIF3G;RAVER1;PPAN;ZNF562*	lead
	18	*PANX2*	maternal diabetis, lead
	19	*CTD-2201E18.5;ZNF131*	nPFOS
	20	*TRMU*	phthalate, lead
	21	*GPC1;MIR149;ANKMY1*	tobacco smoke
	22	*LINC00960;RP11-803B1.3;LSP1P2*	tobacco smoke

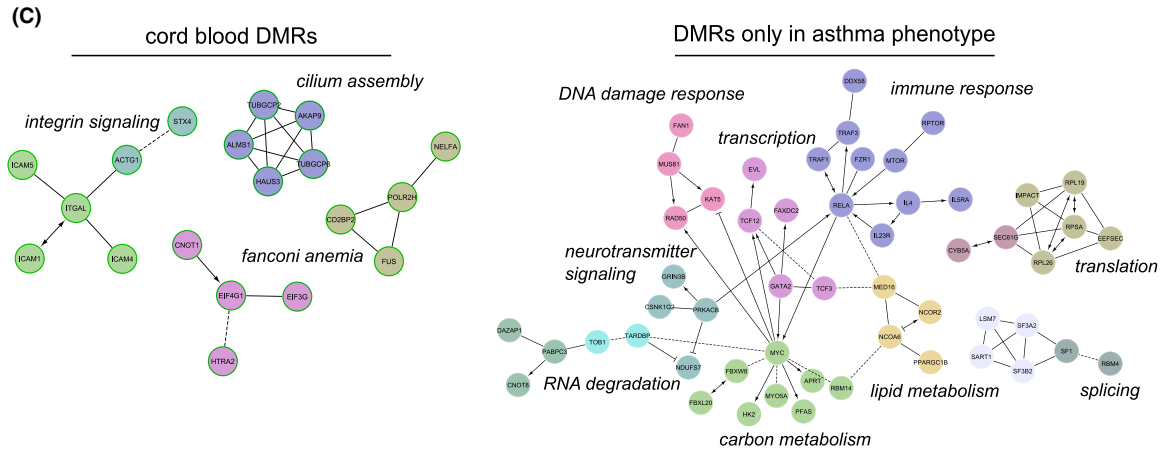


FIGURE 5 Cord blood asthma-DMRs. (A) Matched cord blood samples were subjected to WGBS to determine the DNA-methylation level of the asthma-related DMRs at time of birth in control children compared to those children who later developed asthma. (B) Pie charts represent the portion of genotype-, and non-genotype-associated DMRs (g/ngDMRs) and those DMRs, which were already differentially methylated in cord blood samples (=cord blood DMRs). The table lists all prenatal influencing factors that have previously been associated with CpGs included in the $n = 56$ cord blood asthma-related DMRs (see Table S6C for EWAS references and cord blood DMR list). Genes highlighted in red font were described with asthma as identified by natural language processing (see Table S13B for references). #ngDMRs indicated with light blue and gDMR with dark blue background. *Enhancer target genes were derived from GeneHancer, in cases where no GeneHancer annotation was available the closest TSS gene is given. (C) Network module analysis for cord blood DMR associated genes (left panel) and for genes associated to DMRs only present in asthma phenotype (right panel). Only modules with more than one connection are shown. Module nomenclature is based on network of Figure 4. PFOS = perfluorooctane sulfonic acid, NLP = natural language processing.

primarily affects enhancer elements indicating that a predominant enhancer activation underlies the exacerbated immune response characteristic of childhood asthma.[47] The tight connectivity of these epigenetically dysregulated asthma genes is evident in our inferred interaction network. A comprehensive search of the current scientific literature by NLP analytics revealed that while almost 34% of the enhancer target genes have already been associated with asthma or asthma-related terms, several of the enhancer-DMRs have not yet been discussed in the context of asthma. Most of the asthma-DMRs were enriched for multiple TFBS indicating multiple regulatory effects of the epigenetically perturbed regions. Most of the transcription factors binding to these DMR-enriched TFBS motifs are known to be associated with asthma, such as GATA3,[44] NFACT1,[48] IRF-1,[49] GATA-6,[50] STAT2,[51] THB,[52] or EGR1,[53] and even possess a master regulatory capacity of Th2 differentiation.[54,55]

LFA-1 is mainly known for its role in T cell adhesion and Th1 effector polarization. However, a recent report shows that LFA-1 and its ligand ICAM-1 are expressed on group 2 innate immune cells (ILC2). ILC2 are able to induce eosinophilic lung injury and are elevated in the blood of asthmatics compared to healthy controls.[56] Knock-down of LFA-1 or ICAM-1 both attenuated airway hyperresponsiveness, reduced airway inflammation and decreased lung ILC2 accumulation in mouse models of allergic asthma.[57] As such the observed cord blood DNA-hypomethylation of several regions involved in the LFA-1 signalling cascade might predispose children to a higher risk of allergic asthma.

The vast majority of DMRs was not associated with a meQTL indicating that mainly other than genetic factors contribute to the observed aberrant DNA-methylation in childhood asthma. About one third (35%) of the asthma-related DMRs were already found in cord blood. A variety of environmental insults experienced during the highly susceptible prenatal developmental phase—mostly related to maternal lifestyle factors during pregnancy—have been associated with an increased asthma risk of the child. A comparison to previous EWAS studies revealed that 22 of the asthma-related DMRs already identified in cord blood, including 17 differentially methylated enhancers, overlapped with DNA-methylation changes described in association to prenatal asthma risk factors (for references refer to Table S13B). Among others, these factors included maternal exposure to tobacco smoke or environmental chemicals as well as maternal health (e.g. gestational diabetes, preeclampsia). Although more studies are necessary to investigate whether these regions of persistent differential DNA-methylation are missing links between an adverse intrauterine environment and childhood asthma development, it is prudent to reduce these adverse exposures during vulnerable periods.

This study has to be seen in the light of some limitations. The sample size of whole-genome sequencing approaches seems to be low when compared to previous EWAS using less cost-intensive array based epigenetic profiling methods,[5,8,58] however, in comparison to previous WGBS studies[59–62] we included a considerable higher number of samples. In addition, the enrichment of the DMRs in the asthma pathway, the overlap between the DMR-associated genes with known asthma genes such as *IL4*, *EPX*, *IL5RA* and *ZFPM1* as identified by NLP, in conjunction with the overlap of previously reported CpG sites (e.g. *ACOT7*, *DEGS2*, *EPX* and *GATA2*) of asthma EWAS support the validity of the applied strategy to determine asthma-related DMRs. Although we confirmed the differential DNA-methylation of selected DMRs and their influence on associated target gene expression that are likely to contribute to asthma pathology in a larger sample set, further studies are needed to show whether the DMRs observed in our study can be replicated in independent cohorts and to determine the effect of the identified DMRs on the transcriptome. In addition, since more than half of the asthmatic children reported rhinitis or atopic dermatitis in their life, we cannot exclude that the observed asthma-related DMRs might also be influenced by other atopic diseases such as rhinitis or atopic dermatitis.

The whole blood-based sequencing of DNA-methylation might be seen as a further limitation. To overcome this problem, the proportion of the different cell populations was determined by a deconvolution approach and the DMRs annotated with respect to their cell-type dependency. The deconvolution approach might have led to misclassification or underrepresentation of minor cell types. However, we were able to annotate the small population of eosinophils and to show a significant difference in the eosinophil count between children with asthma and controls without respiratory disease. For a global overview of aberrant DNA-methylation changes and an unbiased interpretation of EWAS,[63] we deem the here utilized approach more appropriate, i.e., not to adjust for cell-type composition beforehand, but rather to determine all DMRs and subsequently annotate them as cell-type-dependent or genotype-associated.

To our best knowledge, this is the first study evaluating the children's methylome at single base-pair resolution—including the comprehensive information on the genetic background—using

repeatedly collected samples of the same individual. We were able to confirm our findings in a larger sample set of two cohorts and showed functional translation to the transcriptional and protein level for selected DMRs. We identified global DNA-methylation changes particularly affecting enhancers, which likely contribute to an altered gene expression of key immune genes involved in asthma pathology. Most of the immune system-related epigenetic alterations including the hypomethylated *IL-4* enhancer, or the *IL5RA* promoter are not present in cord blood, supporting the notion that they are developed during the shift of the immune response toward a Th2 reactivity contributing to the development of an atopic asthma phenotype. Although most of the cord blood DMRs are not directly related to the immune dysfunction characterizing the asthmatic phenotype, these regions related to genes involved in LFA-1 signaling. In light of the emerging role of LFA-1 in ILC2-modulated allergic asthma, these cord blood DNA-methylation changes might be involved in predisposing children to a higher risk for asthma development. Future studies will show if these regions have the ability to predict high-risk children.

AUTHOR CONTRIBUTIONS

IL, RE, MKa, and ST provided project leadership. AvB, BS, JH, MB, SR, EvM, JR, ADC, RL, MKa, AMK, IL, GH were involved in the recruitment and field work of the cohorts. GH provided cytokine data. MB provided the RNA transcription data. SDM, MK, MB, TB, and CH performed the DMR calling and DMR annotation. MK, LT, DW, OM, and CP performed or guided targeted methylation analyses. MK, SDM, MM, MB, NI, TB, CH, ST, GS, and LT performed or supervised data analysis. SS, EF, UH, MK and ST performed or evaluated NLP analysis. LT, ST, MK, and IL wrote the manuscript. All authors were involved in the discussion and contributed to the final manuscript.

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CONFLICT OF INTEREST STATEMENT

All other authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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