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Risk of childhood asthma is associated with CpG-site polymorphisms, regional DNA methylation and mRNA levels at the *GSDMB*/*ORMDL3* locus

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Single-nucleotide polymorphisms (SNPs) in *GSDMB* (Gasdermin B) and *ORMDL3* (ORMDL sphingolipid biosynthesis regulator 3) are strongly associated with childhood asthma, but the molecular alterations contributing to disease remain unknown. We investigated the effects of asthma-associated SNPs on DNA methylation and mRNA levels of *GSDMB* and *ORMDL3*. Genetic association between *GSDMB*/*ORMDL3* and physician-diagnosed childhood asthma was confirmed in the Swedish birth-cohort BAMSE. CpG-site SNPs (rs7216389 and rs4065275) showed differences in DNA methylation depending on carrier status of the risk alleles, and were significantly associated with methylation levels in two CpG sites in the 5′ UTR (untranslated region) of *ORMDL3*. In the Swedish Search study, we found significant differences in DNA methylation between asthmatics and controls in five CpG sites; after adjusting for lymphocyte and neutrophil cell counts, three remained significant: one in *IKZF3* [IKAROS family zinc finger 3 (Aiolos); cg16293631] and two in the CpG island (CGI) of *ORMDL3* (cg02305874 and cg16638648). Also, cg16293631 and cg02305874 correlated with mRNA levels of *ORMDL3*. The association between methylation and asthma was independent of the genotype in rs7216389, rs4065275 and rs12603332. Both SNPs and CpG sites showed significant associations with *ORMDL3* mRNA levels. SNPs influenced expression independently of methylation, and the residual association between methylation and expression was not mediated by these SNPs. We found a differentially methylated region in the CGI shore of *ORMDL3* with six CpG sites less methylated in CD8$^+$ T-cells. In summary, this study supports that there are differences in DNA methylation at this locus between asthmatics and controls; and both SNPs and CpG sites are independently associated with *ORMDL3* expression.

INTRODUCTION

Asthma is characterized by an abnormal airway inflammation leading to reduced airflow and symptomatic wheezing and dyspnea. The risk of developing asthma has a strong genetic component, with estimated heritability ranging from 35 to 85% (1). A genome-wide association study (GWAS) identified a previously unknown asthma-susceptibility locus on chromosome

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17q21, harboring the adjacent genes *ORMDL3* (ORMDL sphingolipid biosynthesis regulator 3) and *GSDMB* (Gasdermin B). This genetic association has been confirmed in ethnically diverse populations (2–5), and gene–environment interactions have been detected between susceptibility alleles and exposure to cigarette smoke (6–9) and furred pets (10). The best associated single-nucleotide polymorphism (SNP) was rs7216389 in *GSDMB*, which strongly correlated with mRNA levels of *ORMDL3* in lymphoblastoid cell lines from asthmatic children (11). Further studies have shown that several of the alleles conferring asthma risk correlate with high mRNA levels of *GSDMB* and *ORMDL3* not only in cell lines (12), but also in primary blood leukocytes (13). Since multiple transcripts were affected, the mechanism was anticipated to be complex and involves alterations in a domain-wide manner (12,13). However, the molecular alterations contributing to asthma remain unknown.

Recent *in vitro* experiments have suggested that epigenetic modifications may also contribute to the pathogenic mechanism underlying the 17q21 locus. The relationship between genotype and epigenotype has been suggested by the fact that SNPs associated with asthma affect genotype-dependent DNA–protein interactions, nucleosome positioning and insulator binding (12). Methylation of cytosines in CpG sites is an important epigenetic modification that influences the binding of nuclear proteins (14), regulates gene expression and might be affected by environmental exposures such as pollutants and pesticides (15,16). Indeed, altered DNA methylation might be part of the explanation for the increased prevalence of asthma and allergy during the last decades (17). Experiments in cell lines have shown that DNA methylation levels in *GSDMB* differ depending on the haplotypes (18). In addition, there are preliminary data in cord blood samples suggesting differences in DNA methylation levels of *ORMDL3* between asthmatic children non-exposed to farm environments and healthy children (19). Since genetic variants associated with increased mRNA expression of *GSDMB* and *ORMDL3* are more frequent in asthmatics (11,13), our initial hypothesis was that asthma predisposing alleles contribute to decreased DNA methylation in asthmatic patients compared with controls. Nevertheless, recent studies have shown that methylation patterns regulating the genome may be complex, and increased methylation in certain gene regions like CpG island (CGI) shores and gene bodies can also lead to increased mRNA expression (20).

Some of the most replicated asthma-associated SNPs in the 17q21 locus coincide with CpG sites [e.g. rs7216389 (C/T), rs4065275 (A/G) and rs12603332 (T/C)]; and we therefore defined them as *CpG-site SNPs*. As the nucleotide substitution either creates (rs7216389-C) or removes (rs4065275-A or rs12603332-T) a CpG site and thus the possibility of methylation in that position, these SNPs are of interest to study. For example, rs7216389-C creates a CpG site (CAAACA*CG*CATG), while rs7216389-T removes it (CAAACA*TG*CATG). We have previously found that removal of a CpG site by a SNP may affect the binding of nuclear proteins (21). Methylated cytosines are recognized by methyl-binding proteins and other factors that can recruit protein complexes and affect regional methylation patterns. In this way, CpG-site SNPs may affect DNA methylation of other CpG sites in their genomic surroundings. Recently, Li *et al*. (22) described a *cis*-methQTL effect of haplotypes on regional DNA methylation levels in the 17q21.31 locus. However, the methylation levels of the asthma-associated SNPs rs7216389, rs4065275 and rs12603332 are unknown, and a comprehensive evaluation on their effects as quantitative trait loci for the levels of DNA methylation (methQTL) in other CpG sites spanning the 17q21 region has not yet been reported. The DNA methylation landscape of *GSDMB/ORMDL3* in primary human leukocytes is also largely unknown. It is possible that CpG sites that do not coincide with SNPs (*non-polymorphic CpG sites*) may also influence the susceptibility to asthma and/or mRNA levels of *GSDMB* and *ORMDL3*.

The aims of this study were (1) to analyze the genetic association between CpG-site SNPs in *GSDMB/ORMDL3* and childhood asthma; (2) to analyze the DNA methylation levels of CpG-site SNPs associated with asthma; (3) to compare the DNA methylation levels of *GSDMB/ORMDL3* between asthmatic children and healthy controls; (4) to evaluate the relationship between CpG-site SNPs, DNA methylation and mRNA expression in the 17q21 locus; and (5) to elucidate the DNA methylation landscape of *ORMDL3* in sorted blood leukocytes. A summary of the research questions, samples and methods is presented in Figure 1.

RESULTS

CpG-site SNPs were associated with asthma under recessive model

In total, 16 SNPs were genotyped in children from the BAMSE study (292 cases and 1661 controls). They included 2 GWAS SNPs in *GSDMB* (rs2305480 and rs7216389) previously associated with asthma (2,11) and 14 SNPs in *ORMDL3* from dbSNP (build 131) (Fig. 2A). The focus was on SNPs located within CpG sites (CpG-site SNPs) in the CGI and CGI shores of *ORMDL3*. We found that most of these putative SNPs were either monomorphic or had very low minor allele frequencies (MAFs <0.001) in this population and were therefore not studied further. Thus, seven SNPs were tested for genetic association with asthma and related traits, whereof three were CpG-site SNPs (rs7216389, rs4065275 and rs12603332). We confirmed the association between five of the SNPs (rs2305480, rs7216389, rs4065275, rs8076131 and rs12603332) and physician-diagnosed asthma up to age 4 and 8 years (Supplementary Material, Table S1). No association was found with atopic sensitization (data not shown). The linkage disequilibrium (LD) between these five SNPs was very high and formed an LD block of 20.6 kilobases (kb) spanning *GSDMB* and *ORMDL3* (Fig. 2B). At this stage of the study, we demonstrated genetic association between three CpG-site SNPs (rs7216389, rs4065275 and rs12603332) in the *GSDMB/ORMDL3* locus and the risk of childhood asthma in the Swedish population (Supplementary Material, Table S1).

Due to strong LD in this region, the three CpG-site SNPs formed a diplotype combination (rs7216389-TT, rs4065275-GG, and rs12603332-CC) that was more frequently observed in asthmatic patients compared with controls (Fig. 2C). Next, the three asthma-associated SNPs coinciding with a CpG site were analyzed further to determine the DNA methylation levels at each locus. The allele rs7216389-T (C/T) removed a CpG site in the first intron of *GSDMB* rendering this site unmethylated, whereas the rs4065275-G and rs12603332-C created two CpG sites. Therefore, the allele carrier status of an individual determines the possibility of methylation. We measured the DNA methylation levels of these SNPs by pyrosequencing individuals

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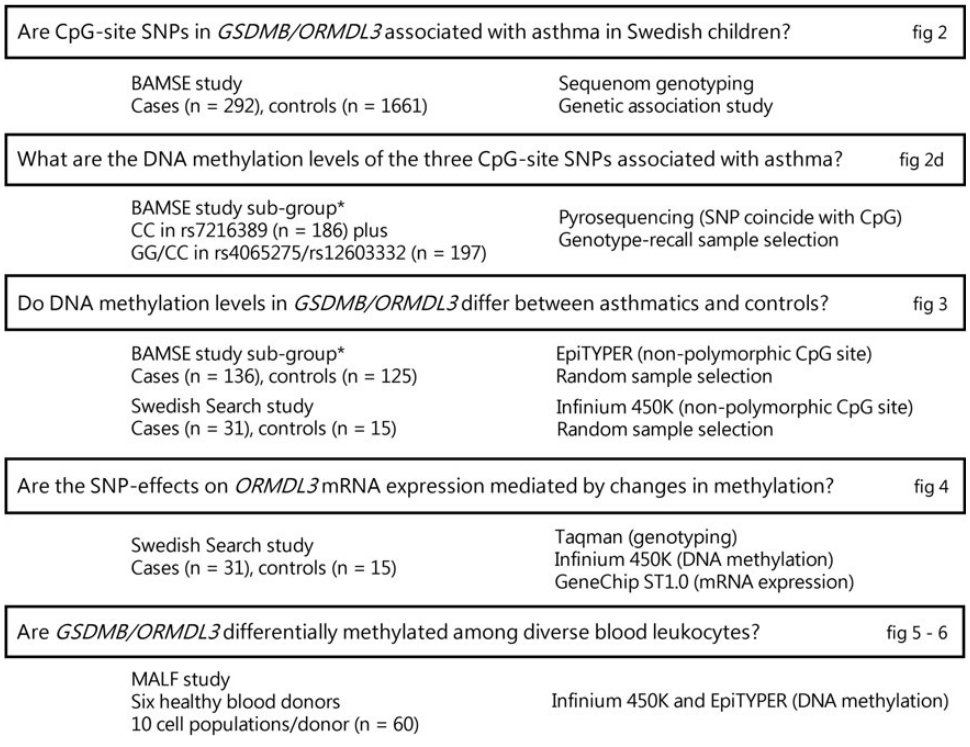

Figure 1. Flow chart summarizing the research questions and samples analyzed in this study. *CpG-site SNP*: SNP that coincides with a CpG site. Depending on the nucleotide substitution, the SNP can either create or remove the CpG site. Non-polymorphic CpG site: sequence where a cytosine nucleotide occurs next to a guanine in the linear sequence of bases and that is not affected by SNP. Asterisks mean that 156 children overlap between the subgroups analyzed by pyrosequencing and EpiTYPER®.

from the BAMSE cohort homozygous for the diplotype TT-GG-CC ($n = 197$). Pyrosequencing was chosen because it is a method that can analyze polymorphisms and DNA methylation at the same time due to the sequential sequencing according to a predefined order of nucleotide addition. DNA was extracted from peripheral blood leukocytes. The rs4065275-GG, which created a CpG site and CTCF-binding region (chr17:38089855–38080935) in the 5′ UTR (untranslated region) of *ORMDL3*, was found hemi-methylated and the rs12603332-CC located in the CGI shore of *ORMDL3* was unmethylated (Fig. 2D).

The analysis of methylation levels in an independent group of individuals in the BAMSE study harboring the opposite combination (diplotype CC-AA-TT, $n = 186$) revealed that rs7216389-CC was highly methylated, whereas rs4065275 and rs12603332 were completely unmethylated (Fig. 2D) in peripheral blood leukocytes. These data suggest that the carrier status of polymorphic variants in *GSDMB/ORMDL3* configures different methylation patterns (Fig. 2D). Based on the methylation results concerning the CpG-site SNP data, we were interested in studying the DNA methylation levels in other non-polymorphic CpG sites located in their surroundings and the association with asthma in a random subgroup of children from the BAMSE study (Supplementary Material, Figs S1 and S2).

CpG sites in the surroundings of rs7216389 in *GSDMB* were highly methylated and levels did not differ between asthmatic children and controls

A subgroup of children from the BAMSE study were randomly selected (136 cases and 125 controls) to investigate the DNA methylation levels in the intronic region of *GSDMB* surrounding the asthma-associated CpG-site SNP rs7216389 (chr17: 38069519–38070149, 631 bp). DNA was extracted from peripheral blood leukocytes at 8 years of age and analyzed by EpiTYPER® (Supplementary Material, Table S10). Ten non-polymorphic CpG sites were measured, of which nine were highly methylated (mean ± SD, 94.1% ± 1.74) and one was hemi-methylated (gCpG4, mean ± SD, 59% ± 9.3; Supplementary Material, Fig. S1). We found no differences in DNA methylation levels for any of these sites between asthmatic children and controls (Supplementary Material, Table S2).

CpG-site SNPs are significant predictors of DNA methylation levels in the 5′ UTR of *ORMDL3*

In the same subgroup of BAMSE children ($n = 261$), we also measured the DNA methylation levels in the CGI shore of *ORMDL3* surrounding the CpG-site SNP rs12603332 (chr17: 38081927–38083226, 1300 bp). Ten non-polymorphic CpG sites were measured by EpiTYPER® in peripheral blood leukocytes, of which seven were successfully analyzed. The intragenic edge of the CGI shore was found to be methylated (CpG1, CpG2.3, CpG4 and CpG7), while the CpG sites adjacent to the CGI were completely unmethylated (CpG16, CpG17 and CpG18; Supplementary Material, Fig. S2). We found that methylation at age 8 years was significantly lower in CpG2.3 and CpG7 in children with a diagnosis of asthma by age 4 years, compared with healthy controls (CpG2.3, $\beta = -0.018$, $P = 0.04$; CpG7, $\beta = -0.021$, $P = 0.04$). These CpG sites were located in the CGI shore of *ORMDL3* and separated by

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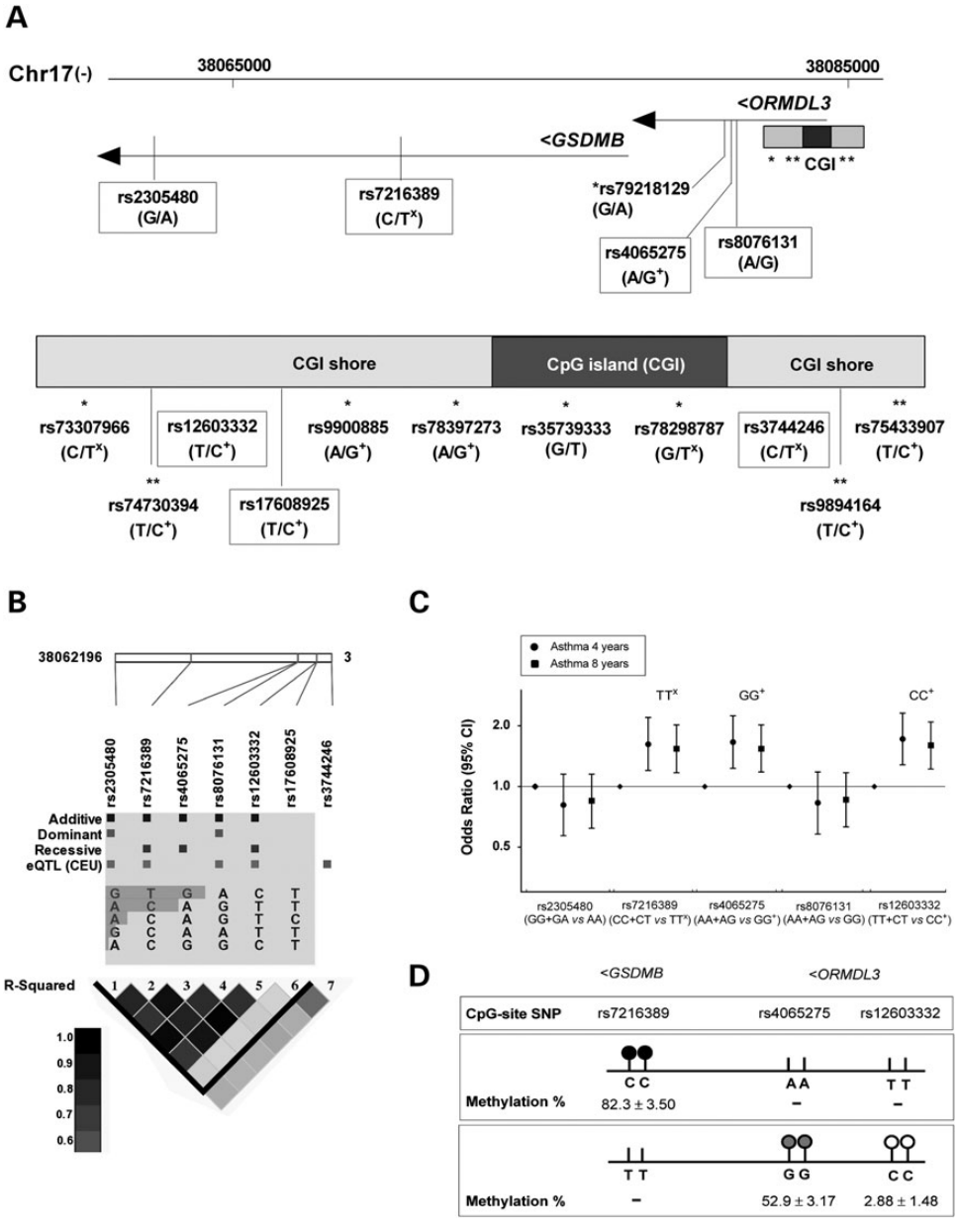

Figure 2. (**A**) The *GSDMB/ORMDL3* locus and location of genotyped SNPs in the BAMSE study ($n = 292$ cases and 1661 controls) with focus on CpG-site SNPs within the CGI and CGI shore of *ORMDL3*. SNPs within boxes were included in the genetic association analysis. Monomorphic (asterisks) or with MAF <0.001 (double asterisks), (multiplication) SNP removes a CpG site, (plus) SNP creates a CpG site; CGI: CpG island. (**B**) LD plot of seven informative polymorphisms whereof five confirmed genetic association. The presence of additive, dominant or recessive effects is indicated by filled squares. eQTL: described as quantitative trait locus in the CEU population. (**C**) Effects of *GSDMB/ORMDL3* polymorphisms on the risk of asthma at 4 and 8 years in children in the BAMSE study. Odds ratio (OR) and confidence interval (CI) under recessive model. (**D**) DNA methylation levels of CpG-site SNPs rs7216389, rs4065275 and rs12603332 and patterns of methylation in homozygotes measured in peripheral blood leukocytes. Filled lollipops represent methylated CpG sites; gray lollipop, hemi-methylated CpG sites and white, unmethylated CpG sites. "–" means unmethylated.

211 bp. However, when the genotype was included in the model, and the exact same subjects were analyzed ($n = 258$), the association between methylation and asthma become statistically non-significant. Interestingly, the risk genotype TT in rs7216389 was associated with decreased methylation in CpG2.3 ($\beta = -0.047$, $P < 0.0001$) and CpG7 ($\beta = -0.061$, $P < 0.0001$). Similar results were observed for the risk genotype GG in rs4065275 and CC in rs12603332: CpG2.3 ($\beta = -0.050$, $P < 0.0001$) and CpG7 ($\beta = -0.065$, $P < 0.0001$). These data indicate for the first time that SNPs associated with asthma (rs7216389, rs4065275 and rs12603332) are also associated with DNA methylation levels of CpG sites located in other positions outside the CpG-site SNP, such as those in the 5′ UTR of *ORMDL3* (i.e. CpG2.3 and CpG7). Since this region is part of a large LD block, the causal polymorphisms responsible for asthma and differences in DNA methylation remain to be elucidated. Our EpiTYPER® analyses revealed that the CGI of *ORMDL3* was unmethylated in peripheral blood leukocytes,

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and the SNP rs12603332 was located in an unmethylated region. We decided to further analyze DNA methylation levels in the 17q21 region using an independent dataset of asthmatic children and controls from the Swedish Search study. In contrast to BAMSE, both gene expression data and cell counts at the time of blood sampling were available in this material.

The DNA methylation landscape of the 17q21 locus between asthmatics and non-asthmatic children in the Swedish Search study

To obtain a broader view of the DNA methylation landscape in the 17q21 locus, we studied DNA methylation in combination with genotypes and gene expression in the Swedish Search study ($n = 46$). Sixty-one non-polymorphic CpG sites distributed in *IKZF3* [IKAROS family zinc finger 3 (Aiolos)], *ZPBP2* (zona pellucida binding protein 2), *GSDMB*, *ORMDL3* and *GSDMA* (Gasdermin A) were included in the analyses (Supplementary Material, Table S4). Both genotypes of the asthma-associated CpG-site SNPs (Supplementary Material, Table S5) and mRNA levels were available for these samples. The CpG sites close to and within the CGI of *ORMDL3* were unmethylated in peripheral blood leukocytes verifying the EpiTYPER® data from the BAMSE study. When DNA methylation levels were compared as a continuous variable stratifying the children according to disease severity (healthy versus controlled asthma versus severe asthma), we found significant DNA methylation differences in six CpG sites in peripheral blood leukocytes (Fig. 3A, upper panel). Of these, one CpG site was excluded (cg18711369, black dot) as the probe sequence included the SNP rs73307962. The most statistically significant CpG site (cg16638648, $P = 0.0001$) was located in the 5′ UTR of *ORMDL3* and showed higher methylation levels in children with controlled persistent asthma, compared with healthy controls, and children with severe asthma (Fig. 3A, lower panel). The same trend was observed for the difference in mRNA levels of *ORMDL3* in peripheral blood leukocytes between children with controlled persistent asthma and healthy controls (Fig. 3B). The observed differences in DNA methylation between asthmatics and controls were significant after adjusting the regression analyses for the number of lymphocytes and neutrophils (Table 1) as well as monocytes (data not shown) at the time of sampling. In addition, when the genotypes in rs7216389, rs4065275 and rs12603332 were taken into account in the model, the differences between the five CpG sites and asthma remained significant (Table 1).

The potential functional effects of the asthma-associated CpG sites were suggested by the significant correlation between cg16293631, cg12360886 and cg02305874 and the mRNA levels of *ORMDL3* in peripheral blood leukocytes (Table 2). Interestingly, this relationship was found with *ORMDL3* mRNA levels, but not with *GSDMB* mRNA levels (Table 2). Further analyses revealed that the relation between these CpG sites and *ORMDL3* mRNA levels was independent of disease status and genotype (except for cg13432737, see below). Also, there was a strong correlation between DNA methylation levels of the studied CpG sites located within the 17q21 locus. This correlation was observed even though the maximum physical distance between asthma-associated CpG sites was 97.9 kb (Fig. 3C). These findings indicated that individual CpG sites cannot be considered as independent events and therefore correction for multiple testing would be too conservative.

Causal inference test revealed that both SNPs and CpG sites showed significant and independent associations with *ORMDL3* mRNA levels

Six polymorphisms in the 17q21 region (rs9303277, rs4795397, rs12936231, rs7216389, rs4065275 and rs12603332) were confirmed as a quantitative trait locus for expression (eQTL) of *ORMDL3* and *GSDMB*. The carriers of the risk genotypes rs7216389-TT (*GSDMB*), rs4065275-GG and rs12603332-CC (*ORMDL3*) had increased mRNA levels of *GSDMB* and *ORMDL3* in peripheral blood leukocytes (Supplementary Material, Fig. S3). These results are in line with the fact that the children with asthma showed increased expression of *ORMDL3* (Fig. 3B). The association between the CpG-site SNPs and the expression of *GSDMB* and *ORMDL3* in peripheral blood leukocytes was independent of disease status and specific for *GSDMB/ORMDL3* since none of the SNPs had any effect on the expression of the nearby gene *IKZF3* (Supplementary Material, Fig. S3). We also evaluated the relationship between the allele doses of CpG-site SNPs (rs7216389, rs4065275 and rs12603332) and the DNA methylation levels in peripheral blood leukocytes in the 17q21 locus (methQTL). We found significant methylation differences in two CpG sites separated by 222 bp in *IKZF3*: cg20709984 ($F = 4.79$, $P = 0.01$) and cg13432737 ($F = 5.18$, $P = 0.009$). The association between genotype and methylation in cg13432737 was significant after adjusting for asthma, age and gender; however, the magnitude of the difference was small ($\beta = 0.003$, $P = 0.02$) (Supplementary Material, Fig. S3). Interestingly, the effect of cg13432737 on *ORMDL3* mRNA levels (Table 2) was no longer significant after adjusting for genotype, and the effect of the genotype on the DNA methylation of cg13432737 was not significant after adjusting for mRNA levels (Supplementary Material, Fig. S3).

We then used the causal inference test to evaluate whether DNA methylation changes in three CpG sites that showed association with asthma (cg16293631, cg12360886 and cg02305874) and correlation with *ORMDL3* mRNA levels (Table 2) were mediating the genotype effect on gene expression (23–25). This approach is based on a series of conditional association analyses for the evaluation of the following effects: (1) the SNP combination (genotype) on the *ORMDL3* mRNA levels; (2) the SNP combination on DNA methylation levels adjusted by *ORMDL3* mRNA levels; (3) DNA methylation levels on *ORMDL3* mRNA levels adjusted by the SNP combination and (4) SNP combination on the *ORMDL3* mRNA levels adjusted by methylation. If methylation is in the causal pathway between genotype and mRNA levels, it is expected that a significant association between genotype and mRNA levels disappears when adjusting for the methylation levels.

In the three CpG sites associated with asthma, we discovered that (1) the SNP combination had significant effects on the *ORMDL3* mRNA levels; (2) there was no effect of the SNP combination on the DNA methylation levels of these sites after adjusting by mRNA levels and (3) DNA methylation levels were significantly associated with *ORMDL3* mRNA levels even after adjustment for genotype. The relationships between genotype and methylation as well as methylation and *ORMDL3*

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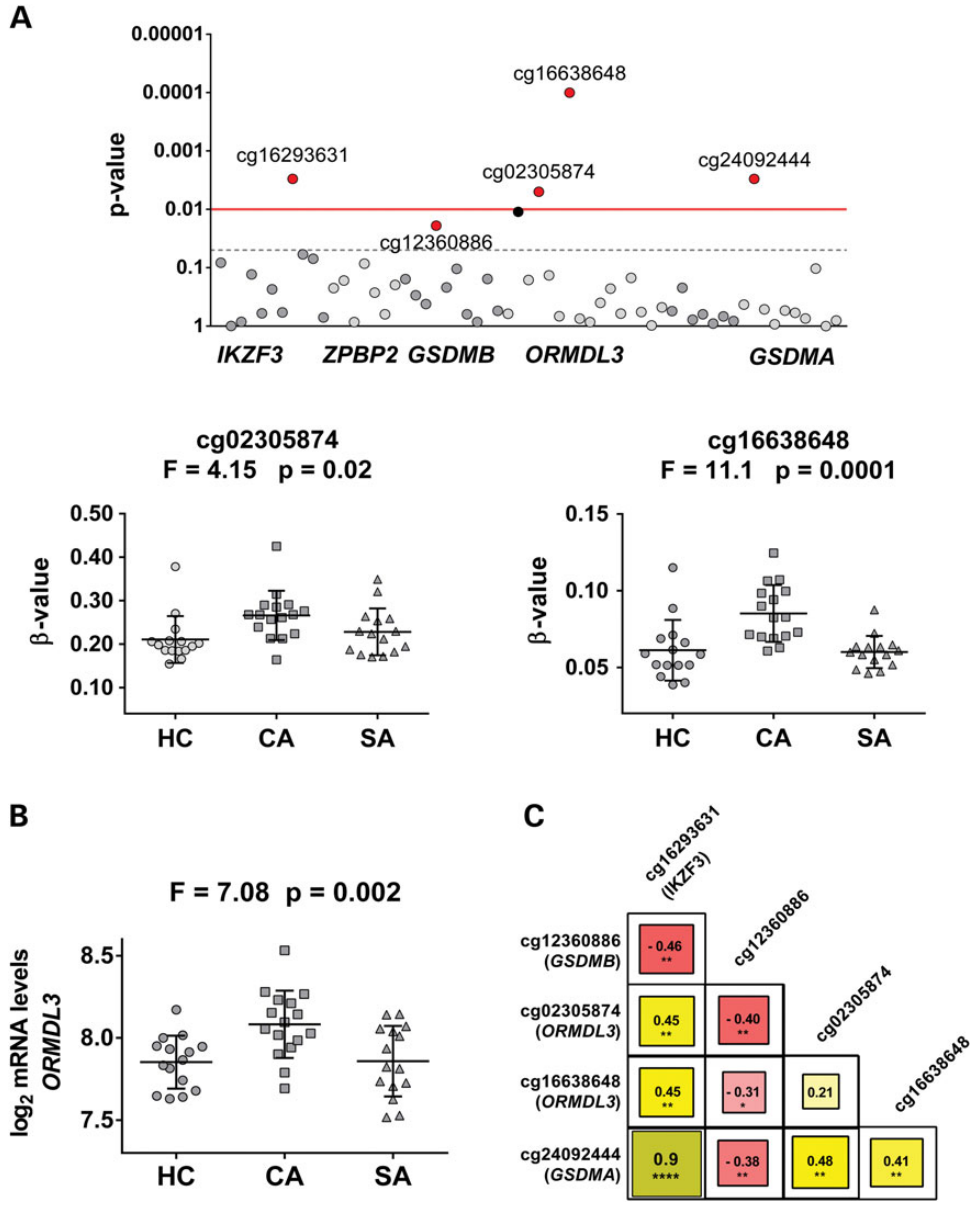

Figure 3. (**A**, upper panel) Dot-plot showing the distribution of the 61 CpG sites analyzed in the Swedish Search study ($n = 46$) and the *P*-value after 10 000 permutations of comparing the three groups (healthy controls versus controlled asthma versus severe asthma, Kruskal–Wallis test). Dotted line: significance level at $P = 0.05$; red line: significance level at $P = 0.01$; red circles: differentially methylated probes according to asthma; black circle: CpG site excluded as the probe sequence is affected by a SNP; lower panel: DNA methylation levels (β-values) for the two CpG sites located in *ORMDL3* (cg02305874 and cg16638648). HC: healthy controls ($n = 15$); CA: controlled asthma ($n = 16$); SA: severe asthma ($n = 15$). Error bars represent mean and standard deviation. All CpG sites were analyzed in peripheral blood leukocytes using the Infinium 450K assay. (**B**) mRNA expression of *ORMDL3* according to disease status in children of the Swedish Search study. HC: healthy controls ($n = 15$); CA: controlled asthma ($n = 16$); SA: severe asthma ($n = 15$). Error bars represent mean and standard deviation. Gene expression was analyzed in peripheral blood leukocytes using the GeneChip Human ST1.0 array from Affymetrix. (**C**) Spearman correlation coefficient for the relation between DNA methylation levels within the asthma-associated CpG sites. Correlation significant at $^{*}P = 0.05$, $^{**}P = 0.01$ level and $^{***}P < 0.00001$ level (two-tailed). Positive and negative correlations are colored in yellow and red gradients, respectively.

expression for the CpG sites associated with asthma (cg16293631, cg 12360886 and cg02305874) are presented in Fig. 4A and B, respectively. Because the effects of the SNPs on mRNA levels remained significant after adjustment for methylation (Fig. 4C), we can conclude that methylation at the asthma-associated sites cg16293631, cg12360886 and cg02305874 is not a mediator between the SNPs and *ORMDL3* mRNA levels. We here show for the first time that both non-polymorphic CpG sites (i.e. cg16293631, cg12360886 and cg02305874) and asthma-associated SNPs (rs7216389, rs4065275 and rs12603332) had a significant and independent association with the mRNA levels of *ORMDL3* (Fig. 4C). However, since changes in expression or cellular stage could cause changes in methylation, with this data set, we cannot conclude whether the association between methylation and *ORMDL3* mRNA levels is causal or due to reverse causation; or that both (methylation and gene expression) are being influenced by an unmeasured confounding factor.

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Table 1. Differentially methylated CpG sites between asthmatics and controls in blood leukocytes (Swedish Search study, $n = 46$)

CpG site Illumina ID	Locus	Coordinate	Gene region	Methylation levels (Illumina β-value, median IQR) Healthy ($n = 15$)	Controlled asthma (n = 16)	Severe asthma (n = 15)	Healthy versus controlled asthma versus severe asthma (ANOVA, P-value)*	Healthy versus controlled asthma after adjustment for cell counts‡ A->M	Healthy versus controlled asthma after adjustment for the genotype combination** A->M
cg16293631	*IKZF3*	chr17:38020606	TSS200 (CGI)	0.53 (0.48–0.58)	0.61 (0.56–0.64)	0.53 (0.51–0.55)	$F = 7.55$ $P = 0.002$	Crude $\beta = 0.068$, $P = 0.004$ Adj_lym $\beta = 0.043$, $P = 0.04$ Adj_neu $\beta = 0.049$, $P = 0.01$	Adj $\beta = 0.07$, $P = 0.001$
cg12360886	*GSDMB*	chr17:38074070	5′ UTR	0.85 (0.83–0.88)	0.80 (0.78–0.85)	0.85 (0.83–0.88)	$F = 3.64$ $P = 0.034$	Crude $\beta = -0.044$, $P = 0.016$ Adj_lym $\beta = -0.032$, $P = 0.09$ Adj_neu $\beta = -0.040$, $P = 0.03$	Adj $\beta = -0.04$, $P = 0.01$
cg02305874	*ORMDL3*	chr17:38083354	5′ UTR (CGI)	0.19 (0.18–0.20)	0.26 (0.22–0.28)	0.22 (0.18–0.25)	$F = 4.15$ $P = 0.02$	Crude $\beta = 0.055$, $P = 0.009$ Adj_lym $\beta = 0.056$, $P = 0.01$ Adj_neu $\beta = 0.048$, $P = 0.02$	Adj $\beta = 0.059$, $P = 0.007$
cg16638648	*ORMDL3*	chr17:38083781	5′ UTR (CGI)	0.05 (0.05–0.06)	0.08 (0.07–0.09)	0.06 (0.05–0.06)	$F = 11.1$ $P = 0.0001$	Crude $\beta = 0.023$, $P = 0.002$ Adj_lym $\beta = 0.024$, $P = 0.005$ Adj_neu $\beta = 0.022$, $P = 0.004$	Adj $\beta = 0.026$, $P = 0.01$
cg24092444	*GSDMA*	chr17:38118574	TSS1500	0.53 (0.51–0.58)	0.58 (0.56–0.62)	0.54 (0.51–0.56)	$F = 6.42$ $P = 0.004$	Crude $\beta = 0.042$, $P = 0.007$ Adj_lym $\beta = 0.024$, $P = 0.1$ Adj_neu $\beta = 0.033$, $P = 0.02$	Adj $\beta = 0.05$, $P = 0.001$

IKZF3: Ikaros family zinc finger protein 3; *ORMDL3*: ORMDL sphingolipid biosynthesis regulator 3; *GSDMA*: Gasdermin A; TSS: transcriptional start site; CGI: CpG Island; IQR: interquartile range.
*DNA methylation levels in all asthma-associated CpG sites were normally distributed; global test for difference between any of the groups.
‡Regression coefficient for the effect of asthma on DNA methylation levels (dependent variable) adjusted by counts of lymphocytes (lym) and neutrophils (neu) (10^9/l).
**Regression coefficient for the effect of asthma on DNA methylation levels (dependent variable) adjusted by genotype combination (rs7216389, rs4065275 and rs12603332).

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Table 2. Relationship between DNA methylation levels and *ORMDL3* mRNA expression in blood leukocytes (Swedish Search study, $n = 46$)

CpG site Illumina ID	Locus	Coordinate	Gene region	Methylation level (median, IQR)	Correlation *ORMDL3* mRNA		Correlation *GSDMB* mRNA	
					Spearman (rho)	*P*-value	Spearman (rho)	*P*-value
cg13432737	*IKZF3*	chr17:37922420	Intragenic	0.05 (0.04–0.05)	0.46	0.001	0.35	0.01
cg16293631[a]	*IKZF3*	chr17:38020606	TSS200 (CGI)	0.54 (0.51–0.60)	0.39	0.007	0.28	0.053
cg12360886[a]	*GSDMB*	chr17:38074070	5′ UTR	0.84 (0.80–0.87)	−0.37	0.01	−0.21	0.15
cg02305874[a]	*ORMDL3*	chr17:38083354	5′ UTR (CGI)	0.21 (0.18–0.26)	0.36	0.01	0.10	0.5
cg14647739	*ORMDL3*	chr17:38084012	TSS200 (CGI)	0.93 (0.92–0.94)	−0.34	0.01	−0.06	0.6

CGI: CpG island; IQR: interquartile range.
[a]Differentially methylated between children with controlled persistent asthma and healthy controls.

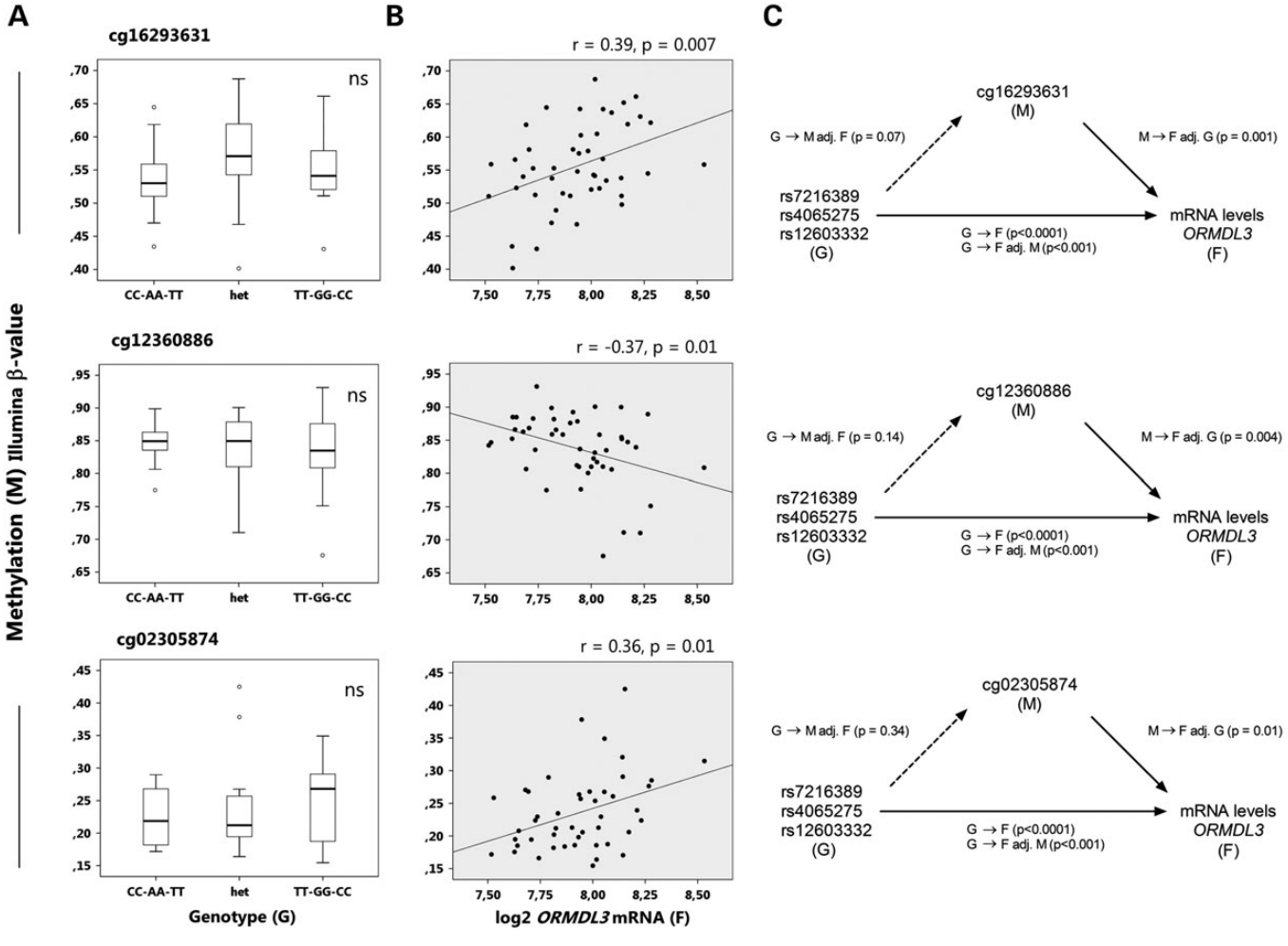

Figure 4. (**A**) DNA methylation levels (*y*-axis) of the CpG sites cg16293631, cg12360886 and cg02305874 according to the allele doses of the CpG-site SNPs rs7216389, rs4065275 and rs12603332 in the Swedish Search study ($n = 45$). Children were grouped based on the carrier status for the genotype combination in the asthma-associated SNPs: CC-AA-TT ($n = 10$), heterozygotes ($n = 22$) and TT-GG-CC ($n = 13$); ns: non-significant. (**B**) Correlation between DNA methylation levels in asthma-associated CpG sites (cg16293631, cg12360886 and cg02305874) and *ORMDL3* mRNA levels. (**C**) A causal inference test between the effects of the asthma-associated SNPs, methylation and mRNA expression in the Swedish Search study ($n = 45$). G: genotype combination; M: methylation level; F: phenotype (mRNA levels for *ORMDL3* in peripheral blood leukocytes). Solid arrows indicate the direction of significant effects. Adj.: adjusted. The DNA methylation levels in asthma-associated CpG sites (cg16293631, cg12360886 and cg02305874) were significantly associated with *ORMDL3* mRNA levels even after adjusting by asthma-associated SNPs (rs7216389, rs4065275 and rs12603332). In addition, the known effects of asthma-associated SNPs on *ORMDL3* expression remained significant after adjusting by DNA methylation, which indicates that methylation changes in cg16293631, cg12360886 and cg02305874 were not a mediator for the effect of the genotype on gene expression. Thus, the residual association between methylation and expression was not influenced by those asthma-associated SNPs. Altogether, these results support that both, genotype and methylation, are associated with *ORMDL3* expression but independently of each other.

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The 5′ UTR of *ORMDL3* harbors a differentially methylated region in CD8$^+$ T-cells

Since mRNA expression is an important readout of underlying genetic and epigenetic signatures, we attempted to identify which leukocyte population might be a relevant cell target for *ORMDL3*. To further explore the landscape of DNA methylation in the 17q12-21 asthma-susceptibility locus, we measured DNA methylation in 64 CpG sites spanning from *IKZF3* to *GSDMA* in sorted leukocytes from six healthy male blood donors ($n = 60$) using the Infinium 450K assay (Supplementary Material, Table S6) (26). Of these, 11 CpG sites were located in the intragenic and regulatory regions of *GSDMB* (Fig. 5A). In addition, we found that most of the CpG sites in *GSDMB* were methylated, with the exception of one CpG site (cg05725940), located in the proximity of the putative transcriptional start site (TSS) of

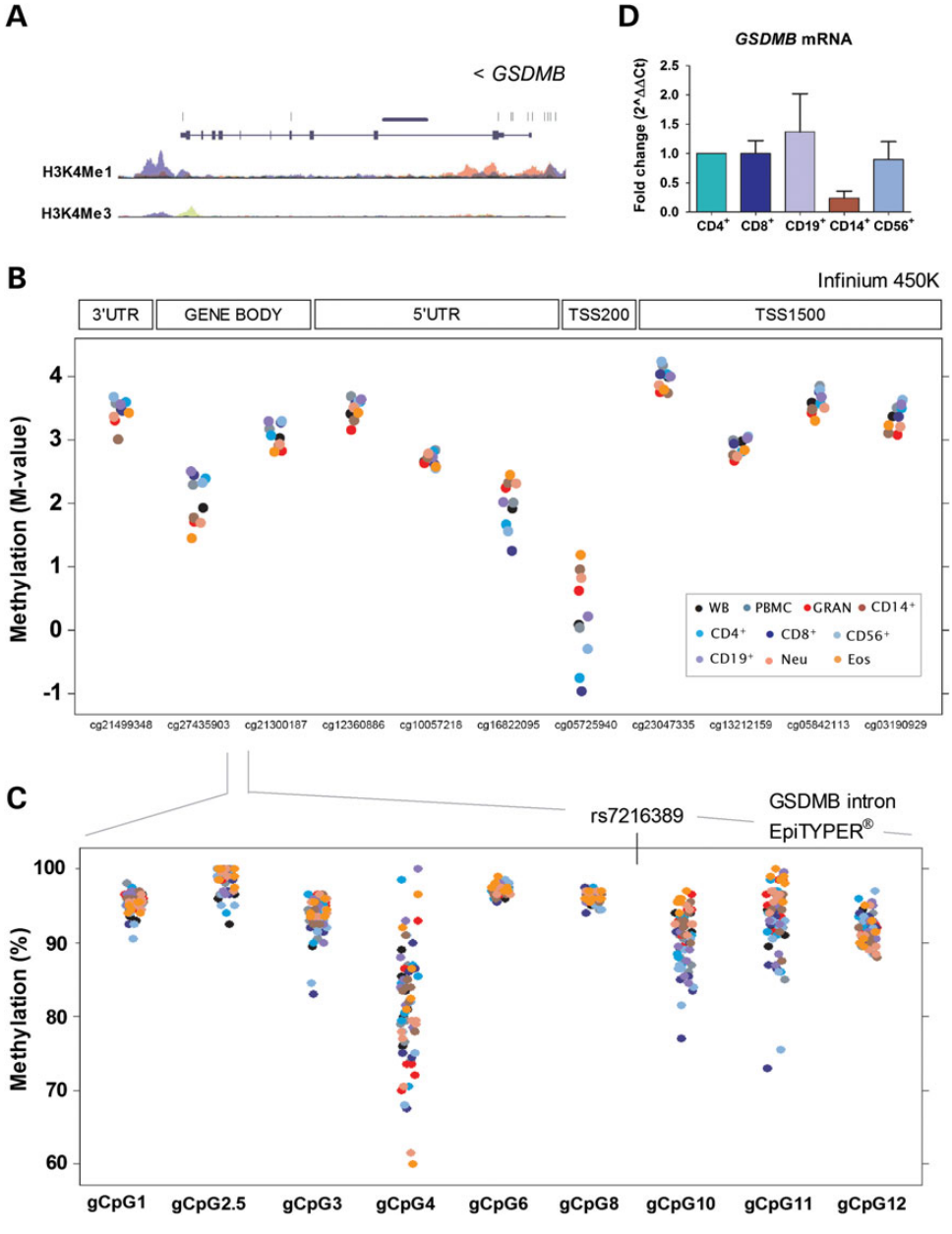

Figure 5. (**A**) Schematic overview of *GSDMB* with underlying chromatin signatures and the location of the 11 CpG sites analyzed for DNA methylation by the Infinium 450K assay in nine sorted blood leukocyte populations and whole blood from six healthy adult donors ($n = 60$). H3K4Me1: monomethylated histone 3 lysine 4; H3K4Me3: trimethylated histone 3 lysine 4. (**B**) DNA methylation levels for *GSDMB* are expressed as *M*-values. Positive values (>1) indicate methylated. Negative values (less than -1) indicate unmethylated. A value of 0 indicates hemi-methylated. TSS: transcription start site; UTR: untranslated region; WB: whole blood; PBMC: peripheral blood mononuclear cells; GRAN: granulocytes; CD14$^+$: monocytes; CD4$^+$: CD4$^+$ T-cells; CD8$^+$: CD8$^+$ T-cells; CD56$^+$: NK cells; CD19$^+$: B-cells; Neu: neutrophils; Eos: eosinophils. (**C**) DNA methylation levels of 10 additional CpG sites in *GSDMB* surrounding the CpG site. SNP rs7216389 in sorted blood leukocytes and whole blood from the six healthy adult donors ($n = 60$) as measured by EpiTYPER®. Each dot represents a sample. (**D**) *GSDMB* mRNA expression in sorted CD4$^+$ T-cells, CD8$^+$ T-cells, CD19$^+$ B-cells, CD14$^+$ monocytes and CD56$^+$ NK cells. The samples used for measuring gene expression were from the same cells used for measuring DNA methylation and presented in B and C. Bars represent mean and standard deviation.

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GSDMB. This CpG site had less methylation in cells of the lymphoid lineage (mainly $CD4^+$ and $CD8^+$ T-cells; Fig. 5B). We also found that 10 additional CpG sites surrounding rs7216389 previously studied in BAMSE (Supplementary Material, Fig. S1) were highly methylated in peripheral blood leukocytes from healthy donors (Fig. 5C).

The analysis of 25 CpG sites across *ORMDL3* confirmed that the CGI of *ORMDL3* was unmethylated in all blood leukocytes (Fig. 6A). Interestingly, the CGI shore in the 5′ UTR region harbored three CpG sites (cg22144450, cg18711369 and cg10909506) with reduced DNA methylation levels in $CD8^+$ T-cells (Fig. 6A). This differentially methylated region (DMR) in the 5′ UTR of *ORMDL3* covered at least 1617 bp. The CpG site cg18711369 was annotated as coinciding with an enhancer. In contrast, the DNA methylation levels of the CGI shore in the upstream region of the promoter and in the distal promoter were very homogeneous between the different leukocyte populations (Fig. 6A). The decreased DNA methylation levels in the 5′ UTR of *ORMDL3* in $CD8^+$ T-cells might explain the increased mRNA expression of *ORMDL3* in these cells compared with other blood leukocytes (Fig. 6C and Supplementary Material, Fig. S4) and suggested that $CD8^+$ T-cells may be an important cell type in the connection between *ORMDL3* and asthma susceptibility.

To obtain higher resolution of the DMR, the seven CpG sites located in the CGI shore of *ORMDL3* and previously studied in peripheral blood leukocytes in BAMSE (Supplementary Material, Fig. S2) were also analyzed in sorted blood leukocytes. The EpiTYPER® measurements revealed that the three sites CpG2.3, CpG4 and CpG7 were significantly less methylated in $CD4^+$ and $CD8^+$ T- cells, compared with other leukocytes (Fig. 6B). Taken together with the CpG sites discovered by the Infinium 450K assay (cg22144450, cg18711369 and cg10909506), the results confirmed the existence of a DMR for $CD8^+$ T-cells in the 5′ UTR of *ORMDL3* in very close proximity to rs12603332, one of the CpG-site SNPs conferring risk to

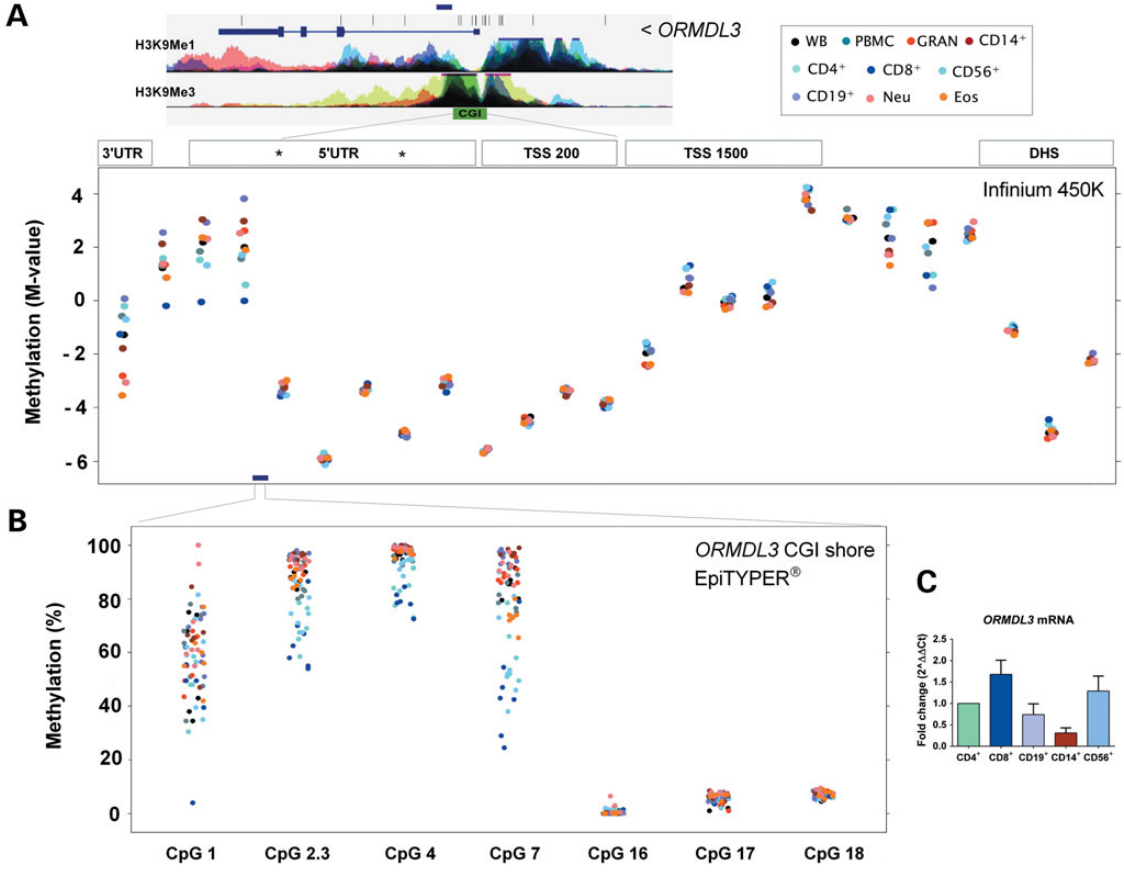

Figure 6. (**A**) A schematic overview of *ORMDL3* with underlying chromatin signatures and the location of the 25 CpG sites analyzed for DNA methylation levels by the Infinium 450K assay in nine sorted blood leukocyte populations and whole blood from six healthy adult donors ($n = 60$). WB: whole blood; PBMC: peripheral blood mononuclear cells; GRAN: granulocytes; $CD14^+$: monocytes; $CD4^+$: $CD4^+$ T-cells; $CD8^+$: $CD8^+$ T-cells; $CD56^+$: NK cells; $CD19^+$: B-cells; Neu: neutrophils; Eos: eosinophils. DNA methylation levels are expressed as *M*-values. Positive values (>1) indicate methylated. Negative values (less than -1) indicate unmethylated. A value of 0 indicates hemi- methylated. CGI: CpG island; TSS: transcription start site; DHS: DNAse hypersensitivity site; asterisks: asthma-associated CpG sites. The Illumina ID of the studied CpG sites starting from the left side: cg12655416, cg22144450, cg18711369, cg10909506, cg02305874, cg13476672, cg04145193, cg16638648, cg01482279, cg05271360, cg02152119, cg14647739, cg09155575, cg19511844, cg04308185, cg10444806, cg08932654, cg26971420, cg15575533, cg22833065, cg13200575, cg22624070, cg02965290, cg02642223, cg09772097. The blue bar indicates the position of the EpiTYPER assays in the CGI shore of ORMDL3, which is not covered by Illumina 450K. (**B**) DNA methylation levels of seven additional CpG sites in the CGI shore of *ORMDL3* in sorted blood leukocytes and whole blood from the six healthy adult donors ($n = 60$) as measured by EpiTYPER®. Each dot represents a sample. (**C**) *ORMDL3* mRNA expression in sorted $CD4^+$ T-cells, $CD8^+$ T-cells, $CD19^+$ B-cells, $CD14^+$ monocytes and $CD56^+$ NK cells. The samples used for measuring gene expression were from the same cells used for measuring DNA methylation and presented in A and B. Bars represent mean and standard deviation.

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asthma. The sites CpG2.3 and CpG7 were also less methylated in peripheral blood leukocytes in children with asthma (Supplementary Material, Table S3).

DISCUSSION

We here confirmed the genetic association between *GSDMB/ORMDL3* and childhood asthma. A recessive genetic effect driven by three CpG-site SNPs—rs7216389-TT (*GSDMB*), rs4065275-GG and rs12603332-CC (*ORMDL3*)—was detected (Fig. 2C). The risk allele either removes [rs7216389 (T)] or creates [rs4065275(G) and rs12603332(C)] a CpG site. Asthmatic children are more frequently carriers of the combination TT-GG-CC leading to elimination of a methylation site in *GSDMB* and hemi-methylation in the 5′ UTR of *ORMDL3* (Fig. 2D). These CpG-site SNPs not only affect DNA methylation levels in the position they coincide with, but were also associated with the DNA methylation levels of two other CpG sites in the 17q21 locus located in the cell type-specific DMR in the 5′ UTR of *ORMDL3* (CpG2.3 and CpG7). We also found that non-polymorphic CpG sites as well as asthma-associated SNPs had significant and independent association with the mRNA levels of *ORMDL3* in peripheral blood leukocytes (Fig. 4C).

By modeling the mRNA levels of *ORMDL3* and *GSDMB* according to allele doses of the CpG-site SNPs, we found that the risk genotypes TT-GG-CC significantly increased the mRNA levels of *ORMDL3* and *GSDMB* in peripheral blood leukocytes (Supplementary Material, Fig. S3). These results replicate previous findings in T lymphocytes (27) and cord blood lymphocytes (28). Since the mRNA expression of *ORMDL3* and *GSDMB* is co-regulated (12,13) and the LD in the region is strong, it is still unclear which of the genes that is contributing to asthma susceptibility. In agreement with previous studies using lymphoblastoid cell lines (18), our methylation analysis confirmed that *GSDMB* is highly methylated in primary blood leukocytes (Fig. 5). *GSDMB* has been considered a candidate gene for asthma, but our results suggest that the observed contribution of *GSDMB* might be due to its strong co-regulation with *ORMDL3* (12,13). Indeed, there were no differences in DNA methylation levels in *GSDMB* in peripheral blood leukocytes between asthmatic children and controls in the surroundings of the GWAS SNP rs7216389 (Supplementary Material, Table S2). As found in whole blood, most of the CpG sites were homogeneously methylated in sorted blood leukocytes of healthy individuals (Fig. 5). Altogether, the methylation and lower expression of *GSDMB* compared with *ORMDL3* in peripheral blood leukocytes (Supplementary Material, Fig. S5) suggest that it might not be a functionally important asthma gene in the 17q21 locus.

The DNA methylation landscape of *ORMDL3* in sorted blood leukocytes showed that *ORMDL3* was significantly less methylated in T lymphocytes, especially $CD8^+$ T-cells (Fig. 6 and Supplementary Material, Table S6). The decreased DNA methylation levels of T lymphocytes spanned over several CpG sites in the 5′ UTR region, CGI shore and gene body of *ORMDL3* (Fig. 6). This suggests a role for this gene in T-cell function and potentially in the pathogenesis of asthma. Indeed, among peripheral blood leukocytes, the B-cells, $CD4^+$ and $CD8^+$ T-cells are the main sources of *ORMDL3* (Fig. 6C). Similar results were found by the FANTOM5 consortium (Supplementary Material, Fig. S4) (29). This is to our knowledge the first study describing a DMR in *ORMDL3* for $CD8^+$ T-cells. In addition to asthma, the relevance of *ORMDL3* in inflammatory pathways has been suggested by both experimental data and genetic association in other inflammatory diseases such as Crohn's disease, ankylosing spondylitis and type I diabetes (30–32). In *Saccharomyces cerevisiae* and mammals, the ORMDL proteins act as negative regulators of the sphingolipid synthesis (33,34). *ORMDL3* encodes a transmembrane protein in the endoplasmic reticulum (ER) that mediates protein folding (35). Overexpression of *ORMDL3* alters the calcium levels in the ER and induces cellular stress by the unfolded-protein response (36). This is an important mechanism for T-cell activation, since ER stress can initiate intrinsic inflammation through induction of cytokine production or activation of inflammatory genes (37,38). Further studies are needed to evaluate whether alterations in the genetic and epigenetic mechanisms controlling *ORMDL3* expression affect these processes, especially in T-cells, and whether dysregulation of the inflammatory responses related to *ORMDL3* originates in T-cells.

Since *ORMDL3* is strongly associated with early-onset asthma, we hypothesize that differences in DNA methylation in the CGI of *ORMDL3* might predispose to altered inflammatory responses particularly in $CD8^+$ T-cells. The effect of bronchiolitis before the age of 2 years on the risk of early-onset asthma is higher in carriers of the risk genotypes in the 17q21 locus (39), and subpopulations of IL-13-producing $CD8^+$ T-cells have been shown to accumulate in the airways of asthmatics (40). A recent study reported that the associations of 17q21 variants with asthma were restricted to children who had had human rhinovirus wheezing illness (41). In predisposed children, ORMDL3 might mediate an enhanced inflammatory reactivity to early stimuli such as respiratory viruses. Further studies are needed to elucidate whether the differences in DNA methylation found in asthmatic children affect the regulation of *ORMDL3* in $CD8^+$ T cells and their mechanistic connection to the disease.

Furthermore, DNA methylation analysis in children in the Swedish Search dataset confirmed significant differential methylation of the CGI of *ORMDL3* between children with controlled persistent asthma and healthy controls in peripheral blood leukocytes. Since differential leukocyte cell counts at the time of sampling were included in the analyses, it was confirmed that asthma-associated CpG sites within the CGI of *ORMDL3* did not coincide with cell lineage-affected CpG sites, and as shown in Figure 6A the CGI of *ORMDL3* was homogeneously unmethylated in all leukocytes. As this was a cross-sectional analysis, it was not possible to determine whether the differences in DNA methylation levels found in the *ORMDL3* promoter of asthmatics, and the consequent up-regulation of mRNA levels is an intrinsic pathogenic defect predisposing to asthma or is secondary to ongoing inflammatory processes in the patients. The reason why differences in methylation levels were observed between healthy controls and children with controlled persistent asthma but not with children having severe asthma is not clear. We hypothesize that severe asthma is a different molecular (and clinical) phenotype than controlled persistent asthma, and that the effect of the 17q21 locus may be more mechanistically relevant for those with controlled persistent asthma. We cannot exclude that the higher doses of inhaled corticosteroids

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received by the children with severe asthma influence methylation and expression levels of *ORMDL3* (42).

In summary, this study presents the DNA methylation landscape of *GSDMB* and *ORMDL3* in primary blood leukocytes and the connection with genetics and gene expression. In peripheral blood leukocytes, DNA methylation levels in CpG sites within the 17q21 locus correlated with mRNA expression of *ORMDL3*. Under physiological conditions, cell-specific DNA methylation in the CGI shore of *ORMDL3* might regulate differential mRNA expression, especially in $CD4^+$ and $CD8^+$ T-cells. We found significant differences in the DNA methylation levels of the *ORMDL3* promoter of asthmatic children, independent of age, gender, genotype and differential leukocyte cell counts, which might partially explain the increased *ORMDL3* expression observed in cases. Our results strongly support the role of both genetic and epigenetic factors contributing to asthma susceptibility in the 17q21 locus. Methylation differences in *ORMDL3* might have an important impact on T-cells and promote lymphocyte-driven inflammation.

MATERIALS AND METHODS

Ethics statement

This study was conducted following the ethical principles stated in the Declaration of Helsinki and approved by the local Ethics Committees. Written informed consent was obtained from all participating individuals or parents and/or legal guardians.

Subjects

The BAMSE study

BAMSE is a prospective population-based Swedish birth-cohort, where newborn infants were recruited 1994–1996 and questionnaire data about baseline study characteristics were obtained from 4089 children (43,44). Parents answered questionnaires on the children's symptoms related to allergy and lifestyle factors at approximately age 1, 2, 4 and 8 years. This study includes DNA extracted from whole blood samples of 2033 children at 8 years of age (1051 boys and 982 girls) for genetic association analyses, of which 1953 were included in this study (Fig. 1). DNA methylation analysis was performed in subgroups including a total of 383 children with pyrosequencing and 261 with EpiTYPER (Fig. 1). Of these, 156 children overlap between the subgroups analyzed by pyrosequencing and EpiTYPER®. Analyzed phenotypes were: *Asthma ever up to 4 or 8 years* defined as physician-diagnosed asthma after 3 months of age and up to 4 or 8 years of age. *Atopic sensitization* was defined as having allergen-specific serum IgE ($\geq$0.35 kU/l) against a mixture of common airborne allergens (Phadiatop®) and/or common food allergens (fx5®) (ImmunoCAP™, Phadia AB, Uppsala, Sweden) at 8 years of age.

The Swedish Search study

The Swedish Search study is a multicenter investigation in which school-aged children (mean 12.6 $\pm$ 3.9 years) with problematic uncontrolled severe asthma were compared with age-matched peers with mild controlled persistent asthma (45). Among children who fulfilled the definition of problematic severe asthma, we identified children who were severely resistant to therapy (called severe asthma) based on detailed clinical characterization [insufficient asthma control despite level 4 treatment, according to Global Initiative for Asthma (GINA) criteria (45)]. All patients had been without airway infections or asthma exacerbations during a 2-week period prior to inclusion. Details of the recruitment process are presented elsewhere (45). A subset of 46 children of this observational cohort was included in this study (Supplementary Material, Table S7). Global gene expression data have been presented previously (46). We have included 15 children with problematic uncontrolled severe asthma 'severe asthma' (with an Asthma Control Test, ACT score $<$19), 16 with controlled persistent asthma 'controlled asthma' (selected from the controlled, persistent asthma group, with an ACT score of $>$20 but $<$24) and 15 healthy controls recruited at Astrid Lindgren Children's Hospital, Stockholm. The controls had been admitted for elective surgical procedures such as scrotal hernia. DNA and RNA were extracted from whole blood sampled at the same time point; and white blood cell counts were obtained for each individual (Supplementary Material, Table S7). DNA was extracted using standard methods and RNA with the RiboPure™ Blood extraction kit (Applied Biosystems/Ambion, Austin, TX, USA).

The MALF study (sorted leukocytes)

To evaluate the methylation landscape in the 17q21 region, a whole blood sample and nine purified blood cell populations (PBMC, granulocytes, $CD4^+$, $CD8^+$, $CD56^+$, $CD19^+$, $CD14^+$, $CD16^+$ cells and eosinophils) were obtained from six healthy male blood donors (mean age 38 $\pm$ 13.6 years) previously recruited within the MALF study (47). The procedure has been described previously in detail (26). The selection criteria, besides that the individuals met the criteria to be regarded as healthy blood donors by the Karolinska University Hospital, Stockholm, included no clinical symptoms or history of eczema, asthma or any allergic disease, low total serum IgE level ($<$122 kU/l) and a negative Phadiatop® (ImmunoCAP™). About 450 ml of blood were collected between July and October 2010 from the median cubital vein into transfusion bags containing citrate phosphate dextrose (Terumo Corporation, Japan), stored at room temperature and processed within 24 h as described previously (26). Genomic DNA was isolated from cell pellets using the QIAmp DNA Micro Kit (QIAGEN, Germany) according to the manufacturer's instructions (26). The study was approved by the regional ethics committee, and all participants gave their written informed consent.

SNP genotyping

MALDI-TOF mass spectrometry

A total of 17 putative SNPs were genotyped in BAMSE by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (SEQUENOM®, Inc.): 3 SNPs in *GSDMB* including the GWAS hits (rs2305480, rs7216389 and rs77749396) and 14 putative SNPs spanning the CGI shore, CGI and 5′ UTR region of *ORMDL3* (rs79218129 rs4065275, rs8076131, rs73307966, rs74730394, rs12603332, rs17608925, rs9900885, rs78397273, rs35739333, rs78298787, rs3744246, rs9894164 and rs75433907). The variants were selected based on (1) previous evidence of genetic association with asthma, (2) if they were predicted to affect transcription factor-binding sites

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and/or (3) if they coincide with CpG sites within the promoter of *ORMDL3* including the CGI or the CGI shores (1000 bp downstream and 1000 bp upstream of the CGI). Sixteen SNPs passed the quality criteria and were further analyzed (rs77749396 was excluded). Information on the SNPs genotyped is presented in Supplementary Material, Table S8. Primers for multiplex PCR and extension reactions were designed by the SpectroDesigner software (Sequenom GmbH, San Diego, CA, USA, available on request). PCR and extension reactions were performed according to manufacturer's standard protocols. The analysis was done by MALDI-TOF mass spectrometry (Sequenom GmbH). Each assay was validated using a set of 14 trios families, in total 42 individuals. Concordance analyses with the HapMap data as well as analysis of the parent–offspring compatibility with the produced genotypes were performed. No significant deviation from Hardy–Weinberg equilibrium ($P > 0.05$ using the χ^2 test) was seen for any of the SNPs and the average genotyping concordance rate was 98%.

TaqMan allelic discrimination

In the Swedish Search study, seven SNPs were genotyped across *IKZF3*, *ZPBP2*, *GSDMB*, *ORMDL3* and *GSDMA* (rs9303277, rs12936231, rs4795397, rs7216389, rs4065275, rs12603332 and rs3859192) using TaqMan allelic discrimination according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Genotype calls were achieved with a maximum of one undetermined genotype per assay, with the exception of rs4795397 where two individuals failed (Supplementary Material, Table S5).

DNA methylation

Targeted analyses using pyrosequencing and EpiTYPER in BAMSE and sorted leukocytes

DNA methylation levels on the asthma-associated CpG-site SNPs (rs7216389, rs4065275 and rs12603332) were measured by pyrosequencing (PyroMark Q24 system, QIAGEN). This analysis included three subgroups of children who were homozygous for the rare genotypes of rs7216389 ($n = 186$), rs4065275 and rs12603332 ($n = 197$). Primers were designed in the PyroMark Assay Design Software v2.0 and specificity validated by gradient PCRs. Methylation levels were exported as percentages from the PyroMark Q24 software (v2.0.6). Detailed information on pyrosequencing assays is presented in Supplementary Material, Table S9.

In addition, 20 CpG sites in *GSDMB/ORMDL3* were analyzed in a subgroup of children from BAMSE ($n = 261$) and in the sorted blood leukocytes from six healthy donors using EpiTYPER® (Fig. 1). Genomic DNA was bisulfite converted with the EZ-96 DNA Methylation Kit (Zymo Research Corporation, USA) according to the manufacturer's instructions. Five validated assays targeted 10 non-polymorphic CpG sites surrounding the CpG-site SNP rs7216389 in *GSDMB* and 10 non-polymorphic CpG sites surrounding the CpG-site SNP rs12603332 in the CGI shore of *ORMDL3* (Supplementary Material, Table S10). DNA methylation results were manually checked in the epigrams, and the raw peak data from EpiTYPER® were analyzed using the MassArray package (v 2.0.1) implemented in R (48). DNA methylation levels at every CpG site were analyzed as a continuous variable ranging from 0 (unmethylated) to 1 (methylated) and expressed as a percentage (0–100%). Mean methylation level of duplicates was used.

Illumina 450K in the Swedish Search study and sorted leukocytes

DNA methylation levels in the Swedish Search study ($n = 46$) were measured using the Infinium® Human Methylation 450K Bead chip technology (Illumina, USA). Genomic DNA was bisulfite converted with the EZ-96 DNA Methylation Kit (Zymo Research Corporation) according to the manufacturer's instructions. Data were extracted for the CpG sites spanning the region including the five genes along the 17q21 locus (*IKZF3*, *ZPBP2*, *GSDMB*, *ORMDL3* and *GSDMA*). In the Swedish Search study, DNA methylation values were analyzed as a β-value (AVB) after peak height correction using the IMA package implemented in R (49). Sixty-one CpG sites passed the quality control in GenomeStudio and the IMA package. Detailed information on the 61 CpG sites analyzed in Swedish Search is presented in Supplementary Material, Table S4.

DNA methylation levels of blood and sorted leukocytes (PBMCs, granulocytes, $CD4^+$, $CD8^+$, $CD56^+$, $CD19^+$, $CD14^+$, $CD16^+$ cells and eosinophils) from six healthy male donors have been previously measured using the Infinium® Human Methylation 450K Bead Chip (Illumina, Inc.) (26). Sixty samples of genomic DNA were bisulfite converted with the EZ-96 DNA Methylation Kit (Zymo Research Corporation) according to the manufacturer's instructions, as previously described (26). Data were extracted for the CpG sites ($n = 64$) spanning the asthma-susceptibility region along the 17q21 locus (*IKZF3*, *ZPBP2*, *GSDMB*, *ORMDL3* and *GSDMA*). Detailed information on the 64 CpG sites analyzed is presented in Supplementary Material, Table S6.

Gene expression

In the Swedish Search study, mRNA levels of *IKZF3*, *ZPBP2*, *GSDMB*, *ORMDL3* and *GSDMA* in peripheral blood leukocytes were measured by the GeneChip® Human Gene ST1.0 Array (Affymetrix, Santa Clara, CA, USA) as previously described (46), Gene Expression Omnibus repository; accession no. GSE27011. Robust Multi-Array normalized values were used for correlations of DNA methylation levels with gene expression.

In sorted blood leukocytes, RNA was extracted from a total of 40 samples (including $CD4^+$, $CD8^+$, $CD56^+$, $CD19^+$ and $CD14^+$ cells), isolated as described previously (26). Granulocyte fractions did not produce enough amounts of good quality RNA and were not analyzed. mRNA was transcribed by using the QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's recommendations. Gene expression was analyzed using a multiplexed TaqMan assay on demand on the 7500 Fast Real-Time PCR system (Applied Biosystems). Reactions were multiplexed in order to analyze each target and the endogenous control (cyclophilin A [PPIA]: Hs99999904_m1) in the same well using the TaqMan Gene Expression master mix (Applied Biosystems) according to the manufacturer's recommendations. Five separate genes were analyzed: *IKZF3*: Hs00232635_m1, *ZPBP2*: Hs00418432_m1, *GSDMB*: Hs00218565_m1, *ORMDL3*: Hs00918021_m1, and *GSDMA*: Hs00937853_m1. Data were analyzed using the comparative C_t method ($2^{-\Delta\Delta C_t}$) with expression in $CD4^+$ cells as a calibrator (50).

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Statistical analysis

Analyses were performed using IBM SPSS v.20 (IBM Corp.), STATA 12 statistical software (Stata Corporation, College Station, TX, USA) and R (R Foundation for Statistical Computing). Allelic and genotype frequencies were calculated for each SNP. After excluding monomorphic or polymorphisms with MAF <0.001 in this population, seven SNPs were included in the genetic association study. LD among these SNPs was calculated using the r^2 coefficient implemented in Haploview (51) and LD plus (https://chgr.mc.vanderbilt.edu/ldplus/). Allele and genotype association tests were done using the SVS 7.6.9 software. The effect of covariates was evaluated using multivariate models in STATA 12 (Stata Corporation).

For methylation analyses, we computed the normality of the methylation levels in each CpG site by the Kolmogorov–Smirnov test. In BAMSE, the association between DNA methylation and asthma was evaluated by linear regression models adjusted for covariates (Supplementary Material, Tables S2 and S3). The association between genotypes (rs7216389 and rs12603332) and the DNA methylation levels in BAMSE was evaluated by linear regression adjusted by the presence of asthma. In the Swedish Search study, the comparisons of DNA methylation levels between patients with controlled persistent asthma, severe asthma and healthy controls were performed by parametric (ANOVA) and non-parametric (Kruskal–Wallis test) methods according to the normality. The CpG sites that showed significance (Fig. 3A and B) were further adjusted for age, differential cell counts and genotypes (Table 2) using linear regression analysis. We performed these adjusted comparisons between healthy controls and patients with controlled asthma ($n = 31$), since there was no difference in methylation levels between healthy controls and severe asthmatics (Fig. 3A, lower panel). A Spearman correlation test was used to analyze the relationship between DNA methylation levels across all the CpG sites and between DNA methylation levels and gene expression [cor.test (stats) R tool].

For eQTL analyses, the mRNA levels of *IKZF3*, *GSDMB* and *ORMDL3* were modeled by linear regression on the allele copies of each SNP assuming additive and recessive effects. These analyses were adjusted by age and disease as covariates. Since there was strong LD between the SNPs and therefore the regression coefficients and *P*-values were very similar, we grouped the children into three groups (i.e. CC-AA-TT, heterozygotes and TT-GG-CC) based on the carrier status for the genotype combination in the asthma-associated SNPs (Fig. 2D). This variable was used as genotype (G) in the causal inference tests. For methQTL analysis, we performed a global test for differences in DNA methylation according to the allele copies of each SNP by ANOVA. In the CpG sites that resulted significant, the association was verified by linear regression models adjusted for age, disease and gender as covariates. For causal inference tests, we evaluated the effects of: (1) SNP combination on *ORMDL3* mRNA levels; (2) SNP combination on DNA methylation adjusted by *ORMDL3* mRNA levels; (3) DNA methylation on *ORMDL3* mRNA levels adjusted by the SNP combination and (4) SNP combination on *ORMDL3* mRNA levels adjusted by methylation. None of the CpG sites that were associated with asthma and/or mRNA expression of *ORMDL3* coincided with those CpG sites harboring a polymorphism in the probe sequence (Supplementary Material, Table S4). The significance level was set at $P < 0.05$.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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