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Epigenome-wide association study identifies DNA methylation markers for asthma remission in whole blood and nasal epithelium

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Abstract

Background: Asthma is a chronic respiratory disease which is not curable, yet some patients experience spontaneous remission. We hypothesized that epigenetic mechanisms may be involved in asthma remission.

Methods: Clinical remission (ClinR) was defined as the absence of asthma symptoms and medication for at least 12 months, and complete remission (ComR) was defined as ClinR with normal lung function and absence of airway hyperresponsiveness. We analyzed differential DNA methylation of ClinR and ComR comparing to persistent asthma (PersA) in whole blood samples ($n = 72$) and nasal brushing samples ($n = 97$) in a longitudinal cohort of well characterized asthma patients. Significant findings of whole blood DNA methylation were tested for replication in two independent cohorts, Lifelines and Epidemiological study on the Genetics and Environment of Asthma (EGEA).

Results: We identified differentially methylated CpG sites associated with ClinR (7 CpG sites) and ComR (129 CpG sites) in whole blood. One CpG (cg13378519, Chr1) associated with ClinR was replicated and annotated to *PEX11* (Peroxisomal Biogenesis Factor 11 Beta). The whole blood DNA methylation levels of this CpG were also different between ClinR and healthy subjects. One ComR-associated CpG (cg24788483, Chr10) that annotated to *TCF7L2* (Transcription Factor 7 Like 2) was replicated and associated with expression of *TCF7L2* gene. One out of seven ClinR-associated CpG sites and 8 out of 129 ComR-associated CpG sites identified from whole blood samples showed nominal significance ($P < 0.05$) and the same direction of effect in nasal brushes.

Conclusion: We identified DNA methylation markers possibly associated with clinical and complete asthma remission in nasal brushes and whole blood, and two CpG sites identified from whole blood can be replicated in independent cohorts and may play a role in peroxisome proliferation and Wnt signaling pathway.

Keywords: Asthma remission, DNA methylation, Whole blood, Nasal brushes

Introduction

Asthma is a chronic airway disorder that affects more than 300 million people around the world. Asthma cannot be completely cured with the available treatment up

to now. However, some asthma patients may grow out of this disease, which is called asthma remission.

Asthma remission is more common in patients with childhood onset asthma, and the proportions of remission differ in different age groups at follow-up (33–53% in adolescence, 6–33% in young adulthood and 11–52% in adulthood) [1, 2]. There are two types of asthma remission, one is defined by absence of asthma symptoms and medication for at least one year, which is called “clinical remission” (ClinR). Some ClinR subjects may still have

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airway hyperresponsiveness (AHR) or a low lung function. Therefore, “complete remission” (ComR) was put forward [3]. In addition to criteria of ClinR, ComR has to meet additional criteria of normal lung function and absence of AHR. This is a more rare phenomenon, that takes place in 5–22% of asthmatics [2].

Asthma remission is associated with both genetic and environmental factors. Environmental factors including breast feeding and having pets in childhood were reported to be positively associated with asthma remission [4]. A recent genome-wide association study (GWAS) identified one SNP (single nucleotide polymorphism) that was associated with ClinR and three SNPs with ComR. One of these SNPs was also associated with expression of known asthma genes including *ILRL1* and *IL13* in lung tissue [5].

Epigenetic mechanisms such as DNA methylation may help to build a link between genetic factors and the environment. DNA methylation can be regulated by SNPs, and can reflect environmental exposures, ageing, cell type constitution and activation [6]. DNA methylation refers to the addition of a methyl-group to cytosine, which usually happens when a cytosine is located next to a guanine in the 5' to 3' direction (CpG site), and this may relate to the regulation of gene expression [7]. Epigenome-wide association studies (EWAS) have provided insights into the development of asthma and its remission. CpG sites associated with asthma in both blood and nasal epithelial cells have been identified, such as CpG sites located in *DICER1*, *STX3*, and *LIPIN1* in blood cells, and *CDHR3*, *FBXL7* and *NTRK1* in nasal epithelial cells [8, 9]. Vermeulen et al. [10] identified 4 CpG sites and 42 regions that were differentially methylated between remission and persistent asthma (PersA) in bronchial biopsies, and top CpG sites were annotated to genes including *ACKR2* and *DGKQ* by gene expression.

Although this latter paper provided a proof of concept of the relation between asthma remission and DNA methylation, bronchial biopsies are not easy to obtain for further studies. DNA methylation in whole blood and nasal epithelium is a good proxy for bronchial epithelium and can therefore also help to understand the mechanism of asthma remission. Here, we hypothesize that epigenetic mechanisms may be involved in asthma remission, reflected in different DNA methylation patterns in whole blood and nasal epithelium. To test this hypothesis, we performed an EWAS of whole blood and nasal DNA in subjects with PersA, ClinR and ComR. We investigated a longitudinal cohort in which asthma was initially carefully defined and the remission status was assessed during follow-up (median 39 years). We subsequently replicated the whole blood DNA results

in two independent cohorts, and also verified the top results from whole blood DNA in cells obtained by nasal brushing.

Methods

A full description of methods is provided in the online supplement.

Study populations

Subjects included in this study were from long-term follow-up studies in the University Medical Center Groningen (UMCG). This study consists of two sources of subjects, (1) subjects from the third visit (2013–2014) of a longitudinal study described previously by Carpaij et al [4], and (2) additional asthma remission subjects that were followed up from previous genetic studies [11] and were re-invited in 2013–2014 to take nasal brushes and blood samples, during which the remission status was assessed again. The participants included in this cohort were different from those of the cohort described by Vermeulen et al. [10] from our research institute. All participants were diagnosed with asthma at baseline with a doctor diagnosis of asthma and AHR. Then, subjects had at least one follow-up medical examination during adulthood in which their asthma status was evaluated by questionnaires and in most subjects additionally with spirometry and an AHR test [5]. The medical ethical board of the UMCG approved the studies and all participants gave written informed consent.

We replicated findings in whole blood in two independent cohorts: the Lifelines population-based cohort in The Netherlands (where we could replicate our results on ClinR using Illumina 450 K array) and the Epidemiological study on the Genetics and Environment of Asthma (EGEA) cohort in France, a case control and family study on asthma, where we could replicate our results on ClinR and ComR with methylC-capture sequencing [12]. No cohort was available to replicate our nasal findings. Extensive information on these cohorts is described in the Additional file 1.

Phenotype definition

The presence of PersA, ClinR and ComR was determined at the most recent visit. ClinR was defined according to the following criteria: (1) No use of any asthma medication, and (2) no symptoms (asthma attacks and/ or wheezing) in the past year. ComR was defined as ClinR combined with (3) no AHR (PC_{20} (provocative concentration causing a 20% fall in FEV_1 (forced expiratory volume in 1 s)) methacholine ≤ 39.3 mg mL⁻¹), and (4) $FEV_1\%$ predicted pre-bronchodilator $>80\%$. PersA was defined as the presence of asthma symptom and/or the

use of asthma medication. Detailed phenotype definitions of the replication cohorts are described in the Additional file 1.

DNA methylation measurements and statistical analyses

DNA was extracted from 72 whole blood and 103 nasal brushing samples taken at the most recent visit. Genome-wide DNA methylation was determined using Illumina Infinium HumanMethylation450 BeadChips. After quality control, 72 whole blood samples and 97 nasal epithelium samples (of in total 103 unique subjects) with 436,824 CpG sites remained for following steps.

We used robust linear regression to determine the differential methylation between persistent asthma and asthma remission: (1) PersA versus ClinR, and (2) PersA versus ComR, in whole blood and nasal brushing samples, with adjustment for covariates that are known to affect DNA methylation (age, sex, smoking status, pack years, and batch). For whole blood samples, we performed adjustment on the percentage of monocytes, B cells, NK cells, CD4+T cells, CD8+T cells, and granulocytes, which were predicted by the Houseman [13] algorithm using minfi [14] package. For nasal brushing samples, we applied the R package sva [15] to estimate significant surrogate variables (SVs), representing unknown latent factors that capture heterogeneity in data, such as cell type composition. One SV for each analysis (ClinR and ComR) was generated and added to the model respectively. Differentially methylated regions (DMR) were identified using comb-p v0.48 [16] and DMRcate [17].

Replication and meta-analyses

Genome-wide significant CpG sites that passed Bonferroni correction ($P < 1.14 \times 10^{-7}$, which is $0.05 / 436,824$) in whole blood DNA were selected for replication in two independent cohorts, Lifelines and EGEA. Lifelines did not include an assessment of AHR, so only included a ClinR phenotype available for replication. The weighted Z-score method was used to meta-analyze the results of discovery and replication cohorts, considering EGEA used methylC-capture sequencing method, which was different from discovery and Lifelines (450 K array), to investigate DNA methylation. CpG sites that passed the epigenome-wide significance threshold of $P < 1.14 \times 10^{-7}$ (Bonferroni correction) in the meta-analysis of all studies were considered to be replicated. Finally, top sites identified in whole blood were verified in nasal brushes.

Annotation and functional relevance

Significant CpG sites were annotated by GREAT v3.0.0 [18]. We correlated the significant CpG sites we identified to the expression level of gene nearby (with a region

of ± 250 kb) by *cis* expression quantitative trait DNA methylation (*cis*-eQTM) analysis. Blood eQTM was assessed in the BIOS consortium dataset [19]. Matched nasal RNA sequencing data and DNA methylation data was used to perform eQTM analysis for significant nasal CpG sites and these results were additionally replicated in the larger collection of nasal brushes obtained in the PIAMA study [20]. The genes that were identified in eQTM analysis were used for pathway analysis by ConsensusPathDB [21]. To check if the results of replicated CpG sites were affected by inhaled corticosteroids (ICS), we stratified asthma subjects by ICS usage, and compared the DNA-methylation in the remission group to asthma with ICS and asthma without ICS group, respectively, by t-test. To evaluate the impact of BMI and allergic rhinitis on the results, we performed sensitivity analyses on the replicated blood CpG sites by additionally adjusting for allergic rhinitis and BMI respectively. We also performed sensitivity analysis for all significant nasal CpG sites.

Results

Subject characteristics

Subject characteristics of the discovery and replication cohorts are shown in Table 1. Regarding the discovery cohort, the median [range] duration of follow-up of the entire population was 39 [4–49] years. Most of the subjects (group 1, $n = 76$) were from the study described by Carpaij et al. [4] at the third visit; the remaining (group 2, $n = 27$) were remission subjects added to this study to further enrich remission cases. Characteristics of subjects from the two groups are shown in Additional file 2: Table S1. By definition, group 2 had a higher proportion of remission subjects, and showed better lung function. Of the 103 subjects included in this study, 54 (52.4% of total subjects) had ClinR, and 20 of the ClinR subjects had ComR (19.4% of total subjects). At baseline, subjects in remission later had higher FEV₁ and FVC level compared to PersA, but no difference regarding FEV₁%predicted and FVC %predicted values was identified among the groups (Additional file 3: Figure S1). Within the whole dataset, 72 subjects (44 PersA, 28 ClinR and 10 of ClinR subjects are ComR) had whole blood methylation data and 97 (44 PersA, 53 ClinR and 19 of ClinR subjects are ComR) had nasal methylation data, with 66 subjects providing both (Fig. 1). White blood cell composition was estimated, and we did not observe significant cell composition differences among PersA, ClinR and ComR group in six cell types. (Additional file 3: Figure S2).

Differential methylation in the discovery cohort of ClinR

Whole blood DNA methylation levels at seven individual CpG sites and 19 differentially methylated regions (DMR)

Table 1 Characteristics of study participants in the discovery and replication cohorts

	Discovery cohort			Lifelines			EGEA		
	PersA (n = 49)	ClinR (n = 54)	ComR (n = 20)	PersA (n = 99)	ClinR (n = 25)	Healthy (n = 636)	PersA (n = 106)	ClinR (n = 15)	ComR (n = 3)
Characteristics at last visit									
Age at remission status, years	49.8 (48.5; 51.3)	50.5 (48.3; 57.6)	50.5 (48.4; 59.8)	46.9 (40.7;50.7)	43.2 (35.7;50.3)	46.3 (39.2;52.9)	52.6 (36.7; 61.4)	49.2 (36.7; 57.6)	30.5 (28.2; 59.1)
Male, n (%)	31 (63.3%)	37 (68.5%)	15 (75.0%)	58 (58.6%)	10 (40.0%)	384 (60.4%)	49 (46.7%)	7 (46.7%)	1 (33.3%)
Duration of follow-up, years	39 (38;40.5)	39 (37; 42)	39 (37;41)				11.3 (10.7; 12.1)	11.6 (10.7; 12.2)	11.6 (10.0; 12.7)
Current-smoking, n (%)	4 (8.2%)	8 (14.8%)	2 (10.0%)	27 (27.3%)	14 (56.0%)	207 (32.5%)	10 (9.5%)	2 (13.3%)	1 (33.3%)
Ex-smoking, n (%)	13 (26.5%)	18 (33.3%)	7 (35.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	35 (33.3%)	5 (33.3%)	0
Never-smoking, n (%)	32 (65.3%)	28 (51.9%)	11 (55.0%)	72 (72.7%)	11 (44.0%)	429 (67.5%)	60 (57.1%)	8 (53.3%)	2 (66.7%)
ICS, n (%)	31 (63.3%)	0	0	48 (48.5%)	0 (0.0%)	0 (0.0%)	72 (70.6%)	0	0
Allergic rhinitis, n (%)	33 (67.3%)	30 (55.6%)	9 (45.0%)	59 (59.6%)	13 (52.0%)	123 (19.3%)	70 (67.3%)	11 (73.3%)	2 (66.7%)
BMI	25.7 (23.7; 28.5)	26.8 (23.7; 28.3)	26.0 (22.8; 28.6)	25.7 (22.9;28.0)	26.0 (23.7;29.6)	25.8 (23.6;28.1)	23.9 (22.4; 27.5)	22.5 (19.9; 28.0)	22.0 (19.9; 22.4)
FEV1% pred	80.6 (71.1; 91.9)	90.2 (82.9; 104.7)	101.8 (85.9; 110.0)	81.2 (70.9;91.6)	87.1 (74.0;93.4)	101.5 (96.1;108.7)	87.0 (71.7; 98.4)	95.2 (87.5; 107.2)	97.5 (90.7; 121.5)
FVC% pred	94.2 (88.4; 103.7)	101.7 (95.2; 111.5)	106.6 (101.2; 115.9)	94.1 (85.8;102.2)	100.3 (90.4;109.2)	104.0 (97.5;111.4)	98.5 (84.8; 108.9)	95.2 (86.9; 106.1)	96.3 (86.9; 119.2)
FEV1/FVC	0.67 (0.60; 0.72)	0.72 (0.68; 0.75)	0.72 (0.68; 0.76)	0.67 (0.62;0.76)	0.67 (0.62;0.76)	0.79 (0.75;0.83)	0.69 (0.61; 0.77)	0.81 (0.78; 0.82)	0.80 (0.80; 0.91)
Characteristics at baseline									
Age, years	10.0 (9.0; 11.0)	11.0 (9.0; 17.5)	11.5 (9.8; 15.8)				40.6 (25.7; 48.7)	37.0 (26.0; 46.0)	18.9 (18.8; 46.4)
FEV1% pred	76.6 (67.4; 84.0)	77.1 (67.5; 83.8)	78.1 (73.5; 83.7)				81.7 (66.2; 95.9)	90.6 (82.0; 103.0)	82.0 (80.1; 122.9)
FVC% pred	88.8 (84.0; 96.1)	88.3 (80.9; 95.3)	88.9 (79.8; 96.5)				95.5 (83.7; 104.3)	86.4 (81.9; 100.6)	84.5 (70.1; 118.8)
FEV1/FVC	0.73 (0.64; 0.78)	0.73 (0.66; 0.79)	0.77(0.66; 0.80)				0.70 (0.63; 0.80)	0.86 (0.82; 0.89)	0.86 (0.83; 0.96)
Start asthma before age 16, n (%)	48 (98.0%)	50 (92.6%)	19 (95.0%)	59 (62.1%)	18 (81.8%)		52 (49.5%)	8 (53.3%)	2 (66.7%)

Continuous data are presented as median (25th percentile; 75th percentile), category data are presented as number (percentage)

Italic values represent significant differences compared with persistent asthma with $P < 0.05$ in the discovery cohort

were significantly associated with ClinR (Fig. 2a, Table 2, Additional file 2: Table S2). The Q-Q plot (Additional file 3: Figure S3a) and the inflation factor λ of 1.064 indicated no obvious inflation of the results. Three out of the seven CpG sites were significantly correlated with gene-expression level in *cis* (FDR < 0.05), resulting in four CpG-gene pairs that showed negative correlation (Additional

file 2: Table S3). These four eQTM genes were enriched in two pathways: G Protein Signaling Pathways and Hemostasis ($P < 0.01$, Additional file 2: Table S4).

We also identified four epigenome-wide significant CpG sites and 24 DMRs that associated with ClinR in nasal brushes (Table S5-S6). The Q-Q plot (Additional file 3: Figure S4a) and the λ of 1.125 indicated

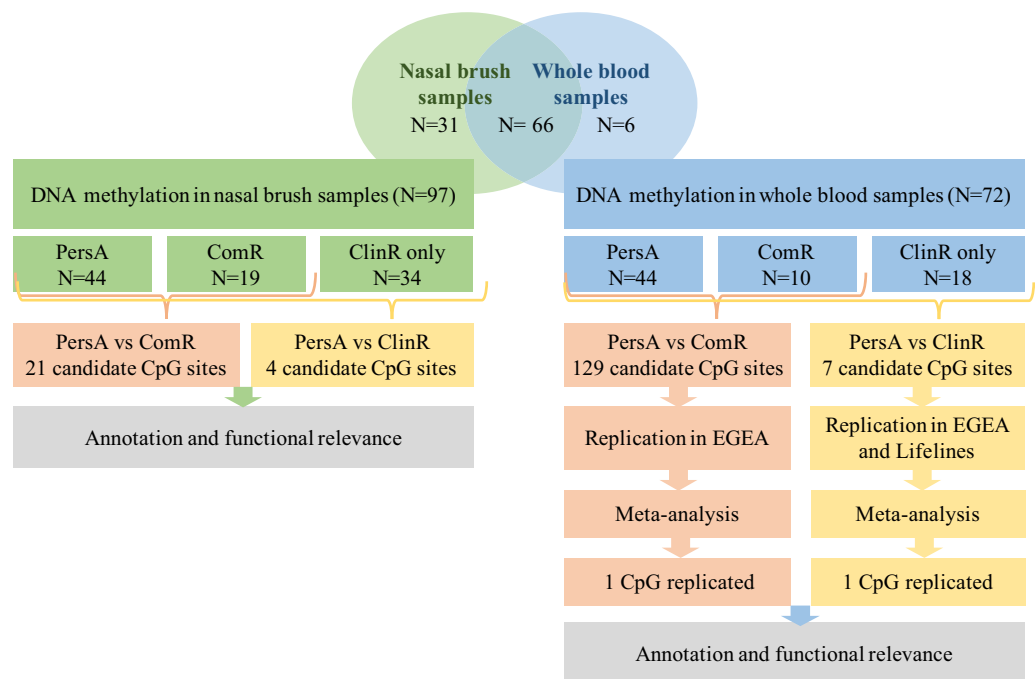


Fig. 1 Study design. In the discovery panel, 103 samples were assessed in this study, 72 subjects had whole blood DNA methylation data, 97 subjects had nasal DNA methylation data and 66 subjects have both. Epigenome-wide association studies were performed on clinical remission (ClinR) and complete remission (ComR) respectively in both whole blood and nasal samples. Significant CpG sites identified from whole blood were further replicated in two independent cohorts. Results of ComR were replicated in EGEA study (93 out of 129 probes available in EGEA); results of ClinR were replicated in EGEA study (4 out of 7 probes available) and Lifelines (all 7 probes available)

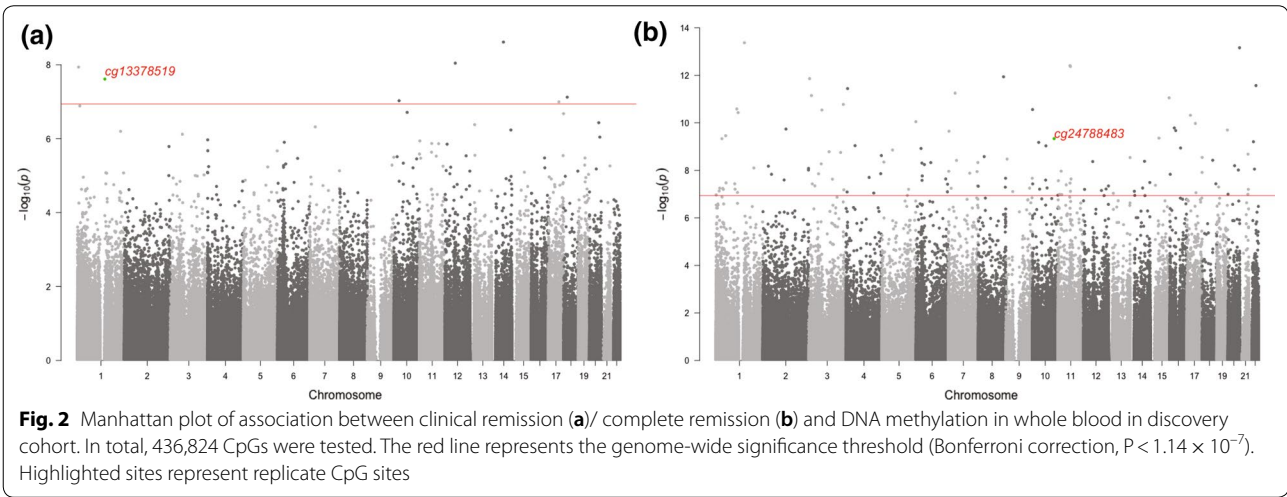


Fig. 2 Manhattan plot of association between clinical remission (a)/ complete remission (b) and DNA methylation in whole blood in discovery cohort. In total, 436,824 CpGs were tested. The red line represents the genome-wide significance threshold (Bonferroni correction, $P < 1.14 \times 10^{-7}$). Highlighted sites represent replicate CpG sites

no obvious inflation of the results. We verified the CpG sites identified from whole blood samples in nasal brushes, and found that one CpG (cg15404785) associated with ClinR showed nominal significance ($P < 0.05$) and the same direction of effect in nasal brushes (Table S7). Four ClinR-associated CpG sites

were correlated to expression level of four genes in *cis* (nominal significant $P < 0.05$) in nasal brushes, with one pair (cg07673230-*RPL30* (Ribosomal Protein L30)) also being nominal significant and in the same direction in the PIAMA dataset (Additional file 2: Table S8).

Table 2 Seven genome-wide significant CpG sites (P value < 1.14 × 10⁻⁷) of ClinR in relation to methylation in whole blood

CpG	CHR	Great annotation (distance to TSS)	Discovery			Replication-EGEA			Replication-Lifelines			Meta-analysis		
			Coef	SE	P	N	Coef	SE	P	N ^b	Coef	SE	P	Direction
cg10125195	12	LACRT (+133)	1.32E-02	2.30E-03	9.03E-09	72	1.098	0.748	0.142	80 (69/11)	2.73E-04	3.56E-03	0.939	124 1.58E-04 + + +
cg12713893	18	SNRPD1 (+44)	4.64E-03	8.63E-04	7.52E-08	72	7.127	5.359	0.184	120 (106/14)	4.19E-04	1.11E-03	0.705	124 2.90E-04 + + +
cg15404785	14	TRMT5 (-279,717); TMEM30B (+20,765)	-3.09E-02	5.17E-03	2.43E-09	72	0.423	0.150	0.005	105 (93/12)	-4.93E-03	6.11E-03	0.420	124 7.58E-02 - + -
cg27252019	17	MKS1 (+550)	-3.86E-02	7.24E-03	1.01E-07	72	-0.033	0.269	0.902	91 (80/11)	5.71E-03	1.01E-02	0.572	124 1.80E-02 - +
cg06185924	10	ACBD5 (-42)	1.10E-02	2.06E-03	9.36E-08	72	NA	NA	NA	106 (92/14)	-4.72E-03	2.33E-03	0.043	124 1.05E-01 + NA -
cg13378519 ^a	1	PEX11B (+18)	-5.99E-03	1.07E-03	2.43E-08	72	NA	NA	NA	119 (105/15)	-1.08E-02	2.61E-03	3.57E-05	124 2.58E-11 - NA -
cg17900884	1	ICMT (-91)	3.58E-03	6.28E-04	1.15E-08	72	NA	NA	NA	114 (101/13)	-1.78E-04	9.86E-04	0.857	124 9.16E-04 + NA -

Pmeta: the P value of meta-analysis of results from discovery, EGEA and Lifelines by using weighted Z-score method

Direction: the direction of regression coefficient in discovery, EGEA and Lifelines

NA: the result for this probe was not available in EGEA

^a The CpG site was replicated with meta P value < 1.14 × 10⁻⁷

^b N (persA/ClinR), N varies from one CpG to another because of the methyl/C-capture sequencing method used and QC criteria in EGEA

Table 3 Top ten CpG sites in meta-analysis of ComR in relation to methylation in whole blood showing the same direction of effect in discovery and replication cohort

CpG	CHR	Great annotation (distance to TSS)	Discovery			Replication-EGEA			Meta-analysis	
			Coef	SE	P	N	Coef	SE	P	N ^b
cg24788483 ^a	10	HABP2 (− 401132), TCF7L2 (+ 201,644)	− 1.31E− 02	2.11E− 02	4.62E− 10	54	− 1.348	0.544	0.013	56 (53/3)
cg15223066	10	ITGB1 (− 23,896), NRP1 (+ 354472)	− 8.56E− 03	1.39E− 03	6.65E− 10	54	− 0.658	0.470	0.161	92 (89/3)
cg26909813	17	RPRML (− 605)	− 2.68E− 02	4.15E− 03	1.06E− 10	54	− 0.621	0.539	0.249	95 (92/3)
cg02341571	15	PATL2 (+ 150)	− 1.69E− 02	2.71E− 03	4.39E− 10	54	− 0.253	0.388	0.515	64 (62/2)
cg00269245	16	SLC12A3 (− 41116), NUP93 (+ 93986)	1.13E− 02	1.86E− 03	1.14E− 09	54	0.832	1.109	0.453	70 (68/2)
cg24833566	4	MTNR1A (− 152680), FAT1 (+ 15608)	1.02E− 02	1.71E− 03	2.38E− 09	54	0.658	0.758	0.386	77 (74/3)
cg25881850	11	KCNK4 (− 1426)	− 1.23E− 02	2.23E− 03	3.43E− 08	54	− 0.838	0.816	0.304	68 (67/1)
cg24730612	14	FOS (− 95503), TMED10 (− 6640)	− 1.47E− 02	2.50E− 03	4.15E− 09	54	− 0.260	0.489	0.595	62 (61/1)
cg23250019	11	SLC29A2 (+ 818)	2.15E− 02	4.00E− 03	7.62E− 08	54	0.645	0.541	0.234	82 (79/3)

Pmeta: the P value of meta-analysis of results from discovery and EGEA by using weighted Z-scores method
Direction: the direction of regression coefficient in discovery and EGEA
A list of all 129 significant CpG sites identified in discovery cohort and the information of replication in EGEA is shown in Additional file 2: Table S9
^a The CpG site was replicated with meta P value < 1.14 × 10^{− 7}
^b N (persA/ComR), N varies from one CpG to another because of the methylC-capture sequencing method used and different QC criteria in EGEA

Differential methylation in the discovery cohort of ComR

We identified 129 individual CpG sites and 53 regions that were significantly associated with ComR in whole blood DNA (Fig. 2b, Table 3, Additional file 2: Table S9, 10). Q-Q plot is shown in Additional file 3: Figure S3b and λ was 1.178. The eQTM analysis identified 99 CpG-gene pairs for 45 out of the 129 CpG sites ($FDR < 0.05$, Additional file 2: Table S3). These eQTM genes were enriched in eight pathways ($P < 0.01$, Additional file 2: Table S4), and the top pathways were Activation of SMO (Smoothen), Platelet Adhesion to exposed collagen, and Cilium Assembly.

In nasal brushes, we identified 21 CpG sites and 62 DMRs that were associated with ComR (Additional file 2: Table S11, 12). Q-Q plot is shown in Additional file 3: Figure S4b and λ was 1.318. Eight of the ComR-associated CpG sites identified from whole blood also showed nominal significance in nasal brushes with the same direction of effect (Additional file 2: Table S13). Seven ComR-associated CpG sites were correlated to expression level of nine genes in *cis* (nominal significant $P < 0.05$) in nasal brushes, and four pairs also showed nominal significant and in the same direction in PIAMA dataset (Additional file 2: Table S8).

Replication study

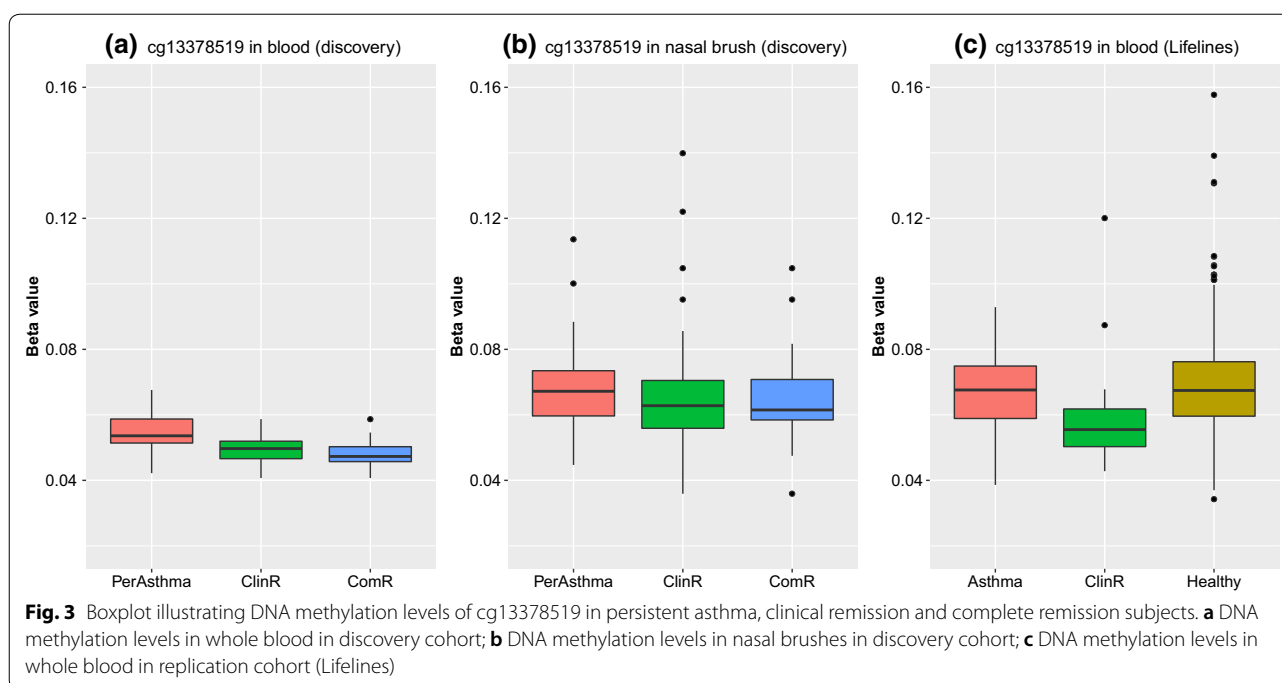
We replicated the top findings in whole blood in two independent cohorts. In replication cohort EGEA, four ClinR-associated CpG sites and 93 ComR-associated CpG sites were available, with one CpG site for ClinR and one for ComR showing nominal significance ($P < 0.05$) and the same direction as the discovery cohort. In the replication cohort Lifelines, which only had the ClinR phenotype, seven ClinR-associated CpG sites were all available with one CpG with $P < 0.05$ and the same direction. After meta-analysis, one CpG associated with ClinR and another one CpG associated with ComR were replicated (meta $P < 1.14 \times 10^{-7}$). The ClinR-associated CpG (cg13378519) was located on Chr 1 and close to gene *PEX11B* (Peroxisomal Biogenesis Factor 11 Beta). The CpG site was not available in the EGEA study and was only replicated in Lifelines. This CpG was lower methylated in ClinR subjects compared to PersA (Fig. 3a). A similar trend was also found in nasal brushes although the association was not statistically significant (Fig. 3b). The methylation levels at this CpG also differed between ClinR and healthy subjects in Lifelines cohort (Fig. 3c), indicating that DNA methylation status of asthma remission subjects is not equal to that of healthy subjects. The ComR-associated CpG (cg24788483) was located on chr10 and close to gene *HABP2* (Hyaluronan Binding Protein 2) and *TCF7L2* (Transcription Factor 7 Like 2). This CpG was lower methylated in ComR subjects

compared to PersA in whole blood, but showed no obvious difference in nasal brushes (Additional file 3: Figure S5). In whole blood *cis*-eQTM analysis, this CpG was significantly negatively correlated to the expression level of *TCF7L2* gene ($P = 9.12 \times 10^{-7}$).

To evaluate potential SNP effects within the probe for the two replicated CpG sites, the β value distributions were visually assessed in the discovery cohorts (Additional file 3: Figure S6), and no bimodal distribution was detected indicating no underlying SNP effect within the probe. The regional co-methylation plots of genes annotated to replicated CpG sites are shown in the Additional file 3: Figure S7, which showed that cg13378519 is located in the promoter region of *PEX11B* gene, and cg24788483 is located in heterochromatin region of *TCF7L2* gene. We did not identify any SNP that was associated with the two CpG sites using an online database [19].

We verified if the results of the two replicated CpG sites were affected by inhaled corticosteroids (ICS), known to affect DNA methylation at multiple sites in the genome (Additional file 3: Figure S8). The boxplots showed that the methylation levels in the remission groups (both ClinR and ComR) were significantly lower than both persistent asthma with ICS usage group and persistent asthma without ICS usage group, which indicated that the results were not affected by the use of ICS. We evaluated the impact of rhinitis on our results, as rhinitis shows comorbidity with asthma. The replicated ClinR-associated CpG site showed similar effect size and P value before and after adjusting for rhinitis, while the ComR-associated CpG showed similar effect size yet the P value changed from $4.62E-10$ to $4.07E-05$ after correction for rhinitis (Additional file 2: Table S14). Additional adjustment for BMI did not change the results of the two replicated CpG sites, indicating that BMI was not a confounder (Additional file 2: Table S14). The significant CpG sites identified from nasal samples showed similar effect size and P value before and after adjusting for BMI and for rhinitis (Additional file 2: Table S15).

We also compared our results from the discovery cohort with the published DNA methylation study of asthma remission in bronchial biopsies (Vermeulen et al. [10]). One CpG (cg13525448), located close to *LBX1* (Ladybird Homeobox 1) gene and *TLX1* (T Cell Leukemia Homeobox 1) gene, in their top list of remission vs persistent asthma reached nominal significance ($P < 0.05$) and showed the same direction of effect in our results of ComR in whole blood (Additional file 2: Table S16). Two genes of their DMRs associated with ClinR, at the gene *PTCHD3* (patched domain-containing protein 3) and *LOC100507389*, were also present in our results of DMRs associated with ClinR in nasal brushes (Additional file 2: Table S6).



Discussion

In this study, we identified one CpG site associated with ClinR and a different CpG site associated with ComR from whole blood, that were replicated in independent cohorts. We also revealed that 25 CpG sites were associated with asthma remission phenotypes in nasal brushes. In the nasal dataset, two differentially methylated regions were previously observed in another DNA methylation study of asthma remission using bronchial biopsies. This is the first epigenome-wide association study of asthma remission in both whole blood and nasal epithelial cells. This study is important since understanding asthma remission may provide new leads for future asthma treatments, and epigenetic studies may help to reveal these cellular mechanisms.

The two replicated CpG sites were annotated to genes which were not known to be involved in asthma (remission) before. ClinR-associated CpG cg13378519 was located in the promoter region of *PEX11B* gene. *PEX11B* encodes a protein of the PEX11 family [22]. Overexpression of the *PEX11B* gene in human cells induces peroxisome proliferation [23, 24]. Our results could indicate a possible role of peroxisomal proliferation as being involved in asthma remission. ComR-associated CpG cg24788483 was located in the *TCF7L2* gene and also correlated with the expression level of this gene. The *TCF7L2* gene product is a transcription factor that plays a role in the Wnt signaling pathway. SNPs in *TCF7L2* were previously reported to be strongly associated with type-2 diabetes and this gene plays a role in

the pancreatic insulin secretory response to incretins [25, 26]. Previously, the risk of asthma was reported to be higher in type 2 diabetes patients [27] and it has been suggested that treatment targeting insulin resistance may have a positive effect on asthma patients [28]. However, it has never been studied whether this treatment could contribute to asthma remission. In addition, we could not infer from the current results of association analysis whether the DNA methylation signal we identified is the cause or consequence of asthma remission.

Although remission subjects do not have asthma symptoms any more, they still may be different from healthy people. Airway abnormalities such as basement membrane thickening still exist in clinical and complete remission subjects [29]. Vermeulen et al. [10] reported that the DNA methylation profile in remission subjects is different from that in healthy subjects. Our data in whole blood for our significantly replicated CpG confirmed this observation. The methylation levels of the ClinR associated CpG (cg13378519) were different between ClinR and healthy subjects, which indicated that the methylation status of subjects with asthma remission may not simply return to that of healthy people.

DNA methylation is cell-type specific. Both of the studied cells or tissues are highly heterogeneous and this may confound the association between DNA methylation and disease. Thus, we applied the widely used Houseman's cell type correction method in blood samples, and for nasal brushes, we used SVA which showed good performance in cell type correction when reference data is

lacking [30]. DNA methylation is also tissue specific. It is yet unknown if mechanisms of remission include local, tissue specific airway (epithelial) effects, immune cell effects, or both. Matched data from whole blood and nasal brush samples enable us to compare DNA methylation profiles associated with asthma remission in two different tissues, as proxies of both immunological and airway specific mechanisms. Although we identified several interesting CpG sites related to remission in nasal epithelium, our results of nasal brushes still need further replication. In previous studies, several DNA methylation sites were significantly associated with asthma in both whole blood and nasal cells [9, 31]. In our study, although no significant enriched cross-tissue effect of DNA methylation in asthma remission was shown, we did identify nine CpG sites in blood DNA that could be replicated in DNA of nasal brushed cells. This indicated that there may be shared DNA methylation signals in blood and nasal cells. We interpret these findings as either indicative of the presence of cross-tissue epigenetic mechanisms in blood and epithelial cells, or the possibility that the DNA methylation signal in nasal brushed cells is partly driven by immune cells in the nose [20], that are also present in blood.

DNA methylation might be related to the regulation of gene expression, and eQTM analysis may help to understand the function of CpG sites. Among the eQTM genes that correlated with ClinR-associated CpG sites in whole blood, the protein encoded by *PRKCH* (Protein Kinase C Eta) plays a key role in epithelial tight junction regulation which is important in maintaining the integrity and function of the airway epithelial barrier [32]; *PDE1B* (Phosphodiesterase 1B) encoded a protein belonging to phosphodiesterases (PDEs) family, and various PDE inhibitors showed anti-inflammatory, anti-remodelling and bronchodilator effect and are potential treatment of asthma [33]. In whole blood, 99 eQTM genes that were associated with ComR were enriched in eight pathways, among which three pathways were related to airway epithelial function: activation of SMO (Smoothen), cilium assembly and focal adhesion. The activation of SMO activity in bronchial epithelia enhanced the allergen-induced goblet cell metaplasia, which is defined as a reversible transformation of airway epithelial cells to mucous cells such as goblet cells and may occur in asthma [34]. Airway cilia are important for clearance of inhaled particles and pathogens. One study showed asthma patients had less ciliated cells in airway samples than healthy people [35], and changes in ciliary function may be relevant for the development of asthma in children [36]. Focal adhesion between the cell membrane and matrix are essential elements of airway smooth muscle cells migration which may play an important role

during the airway remodeling of persistent asthma [37]. Blood is considered as an easily accessible surrogate tissue for asthma study, and previous studies showed some shared gene expression pattern and pathways between blood and airway/ lung [38, 39]. Our results suggest that genes related to asthma remission associated CpG sites may reflect a role for airway epithelial barrier and airway remodeling in asthma remission.

Corticosteroids treatment is usually used to control inflammation and improve control of asthma. One previous study identified differences in DNA methylation of CpG sites in blood associated with systemic corticosteroid treatment in patients with COPD, suggesting the potential effect of corticosteroids on the DNA methylation profile [40]. In our study, we therefore assessed the effect of ICS on the methylation levels of the two replicated CpG sites and found that the results were not affected by ICS usage. However, the effect of corticosteroid treatment on DNA methylation in nasal and blood samples of patients with asthma and remission is largely unknown and should be further investigated in the future.

Rhinitis and asthma often co-exist and show shared genetic origins [41]. A previous study of our group also showed a shared DNA methylation signal of asthma and rhinitis, suggesting the importance of considering the presence of rhinitis when doing EWAS of asthma in nasal epithelium [20]. In this study, we showed that additional adjustment for rhinitis changed the results on one of the replicated CpG sites (cg24788483) identified from blood. This remission associated CpG site was associated with active rhinitis in the absence of asthma, suggesting that blood DNA methylation profiles of persistent asthma and rhinitis overlap. This indicates that we should control for allergic rhinitis in future studies of asthma remission.

When comparing our results with those of Vermeulen et al. [10] in bronchial biopsies, one CpG (cg13525448) in their top list of remission vs persistent asthma reached nominal significance ($P < 0.05$) in our data and showed the same direction in our results of ComR in whole blood (Additional file 2: Table S16). This CpG site is annotated to *LBX1* gene and *TLX1* gene by position. Besides, among their DMRs associated with ClinR, regions at the gene *PTCHD3* and *LOC100507389* also showed up in our results of ClinR in nasal brushes. SNPs in *PTCHD3* gene were previously associated with asthma in African American children [42].

There are strengths and limitations in this study. To the best of our knowledge, this is the only cohort worldwide to study asthma remission in nasal brushes, which enable us to compare the DNA methylation profile in relation to asthma remission in whole blood and nasal epithelium. However, because of the uniqueness

of this data, we could not replicate our results in nasal brushes in another independent cohort. Notably, we had matched DNA methylation and gene expression data in nasal brushes, which help us to get a better understanding of the function of CpG sites associated with asthma remission in nasal brushes. Regarding the limitations of this study, firstly, this study was performed in a relatively small sample with limited number of ComR cases, and the results were replicated in EGEA which is also with few ComR cases, which lead to low power of replication of ComR results. Secondly, although we studied over 450,000 DNA methylation sites, this only represents 1.6% of the human DNA methylome. Thirdly, we studied mixed contributions of cells in whole blood and nasal brushes; and although we corrected for cell types using established methods, potentially stronger results may be anticipated when studying pure cell types, as was previously shown for DNA methylation sites associated with asthma in purified blood eosinophils [8]. Finally, we used a cross sectional analysis at follow up to investigate asthma remission. Future studies, with prospective designs, should be performed to investigate if these DNA methylation sites can predict future asthma remission.

In conclusion, we identified replicable DNA methylation signals associated with clinical and complete asthma remission, which may play a role in peroxisome proliferation and Wnt signaling pathway. This could help in identifying the underlying mechanisms of asthma remission and also of the chronicity of the disease.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s13601-020-00365-4>.

Additional file 1. Supplementary materials.

Additional file 2: Table S1. Characteristics of subjects from two groups. **Table S2.** Differentially methylated regions associated with ClinR in whole blood identified by both comb-p (Sidak p-value) and DMRcate (FDR) methods. **Table S3** Correlation analysis of DNA methylation and gene expression levels (in cis, +/- 250 kb) for asthma remission in blood. **Table S4.** Pathway analysis of eQTM genes correlated with ClinR and ComR in blood. **Table S5.** Genomewide significant (pvalue < 1.14E-07) CpGs associated with ClinR in nasal brushes. **Table S6.** Differentially methylated regions associated with ClinR in nasal brushes identified by both comb-p (Sidak p-value) and DMRcate (FDR) methods. **Table S7.** Look up of ClinR-associated CpGs (identified from blood) in the results of nasal brushes. **Table S8.** Correlation analysis of DNA methylation and gene expression levels (in cis, +/- 250 kb) for asthma remission in nasal brushes. **Table S9.** 129 genome-wide significant (pvalue < 1.14E-07) ComR-associated CpGs in whole blood in discovery cohort. **Table S10.** Differentially methylated regions associated with ComR in whole blood identified by both comb-p (Sidak p-value) and DMRcate (FDR) methods. **Table S11.** Genome-wide significant (p value < 1.14E-07) CpGs associated with ComR in nasal brushes. **Table S12.** Differentially methylated regions associated with ComR in nasal brushes identified by both comb-p (Sidak p-value) and DMRcate (FDR) methods. **Table S13.** Look up of ComR-associated CpG sites (identified from blood) in the results of nasal brushes. **Table S14.** Summary statistics of replicated CpGs (identified from blood) before

and after adjusting for BMI and rhinitis. **Table S15.** Summary statistics of significant CpGs (identified from nasal samples) before and after adjusting for BMI and rhinitis. **Table S16.** Look up of CpGs associated with ClinR identified by Vermeulen et al. in this study.

Additional file 3: Figure S1. Lung function of different groups at baseline and last visit. In this figure, t-test was used in comparing means of any two groups (ns: P>0.05, *: P<0.05, **: P<0.01, ***: P<0.001, ****: P<0.0001). **Figure S2.** Estimated cell proportions among different groups. In this figure, t-test was used in comparing means of any two groups (ns: P>0.05). **Figure S3.** Quantile–quantile plot for epigenome-wide meta-analysis of the association between asthma remission and blood DNA methylation (n = 72). (a) ClinR, (b) ComR. **Figure S4.** Quantile–quantile plot for epigenome-wide meta-analysis of the association between asthma remission and nasal DNA methylation (n = 97). (a) ClinR, (b) ComR. **Figure S5.** Boxplot illustrating DNA methylation levels of cg24788483 in persistent asthma, clinical remission and complete remission subjects in discovery cohort. (a) DNA methylation levels in blood, (b) DNA methylation in nasal brushes. **Figure S6.** Density distributions of DNA methylation levels of two replicated CpGs in 72 blood samples from discovery cohorts. **Figure S7.** Regional association plot of two replicated CpGs. For each plot from top to bottom the tracks included are: 1) Log10(P values) from the discovery 4 years model with CpGs indicated by dots. 2) Annotation tracks for the plotted genomic region taken from UCSC Genome Browser. 3) Pairwise correlation matrix across the displayed CpGs. **Figure S8.** DNA methylation levels of two replicated CpGs in remission subjects and asthma patients stratified by ICS usage. In this figure, t-test was used in comparing means of any two groups (*: P<0.05, ***: P<0.001, ****: P<0.0001).

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Authors' contributions

CQ, JMV, HMB, CJX and GHK contributed to the design of the study. CQ, CJX, DA van der P and DA contributed to data analysis. JMV, AMEN, FND and VS contributed to study procedures, participant recruitment or laboratory procedures. All authors contributed to results interpretation. CQ, CJX and GHK contributed to the initial manuscript draft. All authors revised the manuscript draft for important intellectual content. All authors read and approved the final manuscript.

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Availability of data

DNA methylation data has been deposited at the European Genome-phenome Archive (EGA), which is hosted by the EBI and the CRG, under accession number EGAS00001004766.

Ethics approval and consent to participate

This study was approved by the medical ethical board of University Medical Center Groningen, and all participants gave written informed consent.

Competing interests

GHK reports grants from Lung Foundation of the Netherlands, TEVA the Netherlands, Vertex, GSK, Ubbo Emmius Foundation, TETRI foundation, outside the submitted work. The rest of the authors declare that they have no relevant conflict of interests.

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Additional file 1: Supplementary materials

Supplementary Methods

Sample collection

Nasal epithelial cells were collected by brushing. Briefly, the right nostril of the subjects was examined and the inferior turbinate was located using a speculum and penlight. Brushing was performed with Cytosoft brush CP-5B (Cyto-Pak) after local anesthesia with 1% lidocaine spray. The lateral area underneath the inferior turbinate was then brushed for 3 seconds and the brush was placed in a 2ml screw-cap Eppendorf tube and put into a freezer at -80°C until further processing. In total 4 brushes (2 for DNA isolation and 2 for RNA isolation) were collected.

DNA methylation measurement and quality control

DNA was extracted from 72 whole blood and 103 nasal brushing samples. DNA from whole blood was extracted using QIAamp blood kit (Qiagen, Benelux BV, Venlo, the Netherlands) and nasal epithelium samples was extracted using DNA investigator kit (Qiagen, Benelux BV, Venlo, the Netherlands). DNA concentration was determined by Nanodrop measurement and Picogreen quantification. 500 ng of DNA was bisulphite-converted using the EZ 96-DNA methylation kit (Zymo Research), following the manufacturer's standard protocol. After verification of the bisulphite conversion step using Sanger Sequencing, Genome-wide DNA methylation was measured using the Illumina Infinium HumanMethylation450 beadchip (450K array; Illumina, Inc., San Diego, CA, USA). DNA concentration was normalized and the samples were randomized to avoid batch effects, and all paired (blood and epithelium) samples were hybridized on the same chip. One standard female DNA sample was included in this step for control reasons, to verify chip to chip variation.

DNA methylation data were pre-processed in R with the Bioconductor package Minfi¹, using the original IDAT files extracted from the HiScanSQ scanner. We implemented sample filtering to remove bad quality samples (call rate <99%). Furthermore, we used both the 65 SNP probes to check for concordances between paired DNA brush and blood samples from the same individuals. Paired samples that showed a SNP signal with a Pearson correlation coefficient <0.9 were regarded as sample mix-ups and were excluded from the study. We also verified the methylation distribution of the X-chromosome to verify gender. We subsequently removed six samples due to call rate < 99%, none of samples were removed due to sample mix-up or gender mismatch. During processing, the low-quality probes (>10% of samples with detection p-value > 0.01; 1,842 probes), the probes on sex chromosomes (11,648 probes), the probes that mapped to multiple loci (29,233 probes), 65 SNP-probes and the probes containing SNPs at the target CpG sites with a MAF>5% (6,818 probes) were excluded². We implemented “DASEN”³ to perform signal correction and normalization. After quality control, 72 blood samples and 97 nasal epithelium samples with 436,824 probes remained for following steps.

Methylation levels (beta values, β) at a given CpG site were derived from the ratio of the methylated probe intensity to overall fluorescence intensity (sum of methylated and unmethylated probe intensities): β is equal to $M / (U + M + \alpha)$, where M is intensity of the methylated probe, U is the intensity of the unmethylated probe, and α is the constant offset with the default value of 100. After association analysis, the inflation factor for each model was calculated using the function “estlambda” with method of “median” in R package GenABEL⁴.

Nasal RNA sequencing and quality control

Total RNA was extracted using AllPrep DNA/RNA Mini kit (Qiagen, Venlo, the Netherlands) from nasal brushing samples. Samples were lysed in 600 µl RLT-plus buffer using an IKA Ultra Turrax T10 Homogenizer, and RNA was purified according to the manufacturer's instructions. RNA samples were dissolved in 30 µl RNase free water. Concentrations and quality of RNA were checked using a Nanodrop ND-1000 and run on a Labchip GX (PerkinElmer, Waltham, MA).

Initial quality check of and RNA quantification of the samples was performed by capillary electrophoresis using the LabChip GX (PerkinElmer, Waltham, MA). Non-degraded RNA-samples with integrity scores RIN > 6 were selected for subsequent sequencing analysis. Sequence libraries were generated by Poly (A) enrichment using the TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA) using the Sciclone NGS Liquid Handler (PerkinElmer, Waltham, MA). In case of contamination of adapter-duplexes, an extra purification of the libraries was performed with the automated agarose gel separation system Labchip XT (PerkinElmer, Waltham, MA). Whenever sample preparation failed, either a new sample preparation was attempted or the sample was replaced by a spare nose brush sample from the same individual. The obtained cDNA fragment libraries were loaded in pools of multiple samples onto Illumina HiSeq2500 sequencer using default parameters for paired-end sequencing (2 × 100 bp). If sequencing of a library gave insufficient reads, a second sequencing run was performed to generate additional reads, target was 15 million read pairs per sample.

The trimmed fastQ files were aligned to build b37 of the human reference genome using HISAT (version 0.1.5) allowing for 2 mismatches⁴. Reads from separate runs of the same library were merged. Before gene quantification, SAMtools (version 1.2) was used to sort the aligned reads⁵. The gene level quantification was performed by HTSeq (version 0.6.1p1)

using `--mode=union --stranded=no` and using Ensembl version 75 as gene annotation database⁶.

Quality control (QC) metrics were calculated for the raw sequencing data, using the FastQC tool (version 0.11.3). Alignments of RNA of 86 subjects were obtained. QC metrics were calculated for the aligned reads using Picard-tools (version 1.130) (<http://picard.sourceforge.net>) CollectRnaSeqMetrics, MarkDuplicates, CollectInsertSizeMetrics and SAMtools flagstat. We discarded 17 samples due to poor alignment metrics. In addition, we checked for concordance between sex-linked (XIST and Y-chromosomal genes) gene expression and reported sex. Two more samples were discarded for lack of concordance. This resulted in high quality RNAseq data from 67 subjects.

Expressed features were excluded from analysis if less than half of the samples had counts per million mapped reads (CPM) of at least 5/M, where M is the median library size in millions. This left 17,479 expressed features for analysis. Raw count data were transformed to log2CPM using voom and analysed in the limma package⁷.

Differentially methylated regions (DMR)

Differentially methylated regions (DMR) were identified using comb-p v0.48⁸ and DMRcate⁹. For comb-p, we set the seed P value as 0.05, the adjusted region-level P value cutoff to determine DMR (Sidak-corrected P) as 0.05 and the maximum distance to search for adjacent peaks as 1000 bp. For DMRcate, we set the width of estimation as 1000 bp, and P value cutoff to determine DMR as 0.01. Comb-p and DMRcate use different algorithms to identify significantly DMRs. Comb-p uses a one-step Šidák correction method for multiple comparisons¹⁰, while DMRcate uses FDR method. To reduce false positives, we only

considered a DMR to be statistically significant if it was significant by both methods, according to the definition used in each¹¹.

Replication and meta-analysis

Genome-wide significant CpG sites that passed Bonferroni correction ($P < 1.14 \times 10^{-7}$, which is $0.05 / 436,824$) in whole blood DNA were selected for replication in two cohorts Lifelines and EGEA (see below for cohort description). We replicated results of ClinR in both cohorts and replicated results of ComR only in EGEA cohort since Lifelines did not have a ComR phenotype. The meta-analysis was performed using weighted Z-score method. For results of ClinR, results of three CpG probes were not available in EGEA cohort, and we only meta-analyze the results of these three CpG sites in discovery and Lifelines.

Cohort description

1) Epidemiological study on the Genetics and Environment of Asthma (EGEA) cohort

Population

The Epidemiological Study on the Genetics and Environment of Asthma (EGEA: <https://egeanet.vjf.inserm.fr>) is a French 20-year follow-up cohort that included at baseline (early 1990s) a group of asthmatic patients with their first-degree relatives and a group of control subjects. 2047 adults and children were recruited from 1991 to 1995 (EGEA1). A first follow-up of the EGEA population was conducted from 2003 to 2007 (EGEA2; 1845 subjects), and a second follow-up was conducted from 2011 to 2013 (EGEA3; 1558 subjects). At each survey a detailed respiratory questionnaire (self-completed in EGEA3) was included, and in the 2 first surveys a clinical examination including lung function testing, measure of bronchial responsiveness, skin prick tests, and total IgE measurement was

performed. No follow-up bias related to asthma status and asthma-related phenotypes was observed. A rich biobank, including blood samples, has been constituted (BB-0033-00043). The EGEA study was approved by the appropriate ethics committees. The current analysis was conducted among asthma participants with available data to define asthma remission at EGEA2, and with available methylome data (n=124, including 106, 15 and 3 individuals with PersA, ClinR and CompR).

Phenotype definition

Asthma: subjects who used asthma medications in the past 12 months or who had asthma symptoms (asthma attacks or wheezing in the chest) in the past 12 months.

Clinical remission: subjects who did not use asthma medication and did not have asthma symptoms (asthma attacks or wheeze).

Complete remission: no asthma medication, no asthma symptoms (asthma attacks or wheeze), no BHR and FEV1 % predict >80% (GLI 2012).

Methylome data

The DNA was extracted from buffy coat and high-resolution methylome data in regulatory elements of immune cells was generated by MethylC-capture sequencing (MCC-Seq)¹². Overall, the panel designed covered 4,861,805 CpGs.

Regarding quality control, DNA methylation of each CpG was measured by the number of methylated reads over the total number of sequenced reads. We filtered CpGs with the number of total sequenced reads are less than 5X. We further filtered the CpGs if the difference of methylation level between forward strand and reverse strand is great than 20% for CpGs with $\geq 2X$ of both forward strand and reverse strand. In addition, CpGs overlapping SNPs

from dbSNPs (137) with minor allele frequency (MAF) >0.01 and CpGs located within ENCODE DAC blacklisted regions or Duke excluded regions were discarded.

Statistical analysis

For each CpG site, we fitted a generalized linear model assuming a binomial distribution of methylation levels (considering the proportion of methylated reads weighted for sequence read coverage as the dependent variable), and asthma remission outcome as independent variable. Models were adjusted for age, sex, smoking status (never smokers versus ever smokers) and measured blood proportions of monocytes, lymphocytes, neutrophils, eosinophils and basophils. The R¹³ function `glmer` in the `lme4` package¹⁴ was used. The R code for the association was: `glmer (cbind (Methylated_Read_Counts, Unmethylated_Read_Counts) ~ Asthma_Remission_Status + age + sex + smoking + neutro + lympho + mono + eosino + baso)`. The convergence of the model was performed by the BOBYQA algorithm¹⁵ which was found the most efficient on our data in terms of convergence errors and warnings. All continuous variables used in the model were individually centered (subtracting the mean of the variable) and scaled (dividing by the standard deviation of the variable) in order to increase the stability of the model and to decrease the computational time. P-values were calculated using the Wald test.

2) Asthma remission methylation project (Lifelines)

Population and measurements

In total, 1,656 subjects were selected of the Dutch population-based cohort study Lifelines¹⁶. All subjects provided written informed consent and the study was approved by the Medical Ethics Committee of the University Medical Center Groningen, Groningen, the Netherlands. Subjects were selected from the larger cohort based on having complete data on sex, age, height,

smoking history (pack-years, never- or current-smoker), airway obstruction ($FEV_1/FVC < 70\%$), and occupational related exposures. Self-reported never-smokers with 0 pack-years of smoking and current-smokers with a smoking history greater than 5 pack-years were selected. Ex-smokers were not included.

Genome-wide methylation assay

The Illumina Infinium Human Methylation 450K arrays were used to define genome-wide DNA methylation levels at >450,000 specific CpG sites. We randomized 1,656 subjects based on sex, exposure, and airway obstruction over the chips. Using 500 ng DNA for each sample, we first performed a bisulphite conversion using the EZ- 96 DNA methylation kit (Zymo research Corporation, Irvine, USA), which was validated using commercially available bisulphite conversion 1 control samples (Zymo Research Corporation, Irvine, USA). After this step the samples were processed according to the Illumina 450K protocol (Illumina Inc., San Diego, USA). Quality-control (QC) steps were performed using the Minfi package in R¹, and included the removal of samples with >1% of all probes having a detection p-value >0.01, and samples with an incorrect sex or SNP prediction. We removed single probes with a detection p-value >0.01, sex chromosome probes, cross-reactive probes, probes measuring SNPs, and probes where the CpG itself or the single base extension (SBE) site is a SNP². The data were normalized using DASEN implemented in the watermelon package in R³. The final data set contained data for 1,561 subjects and 420,938 CpG probes. Beta-values were used to represent DNA methylation levels, which is the ratio between the intensities of methylated versus unmethylated probes, ranging from 0 to 1.

Phenotype definition

Healthy: 1) no doctor's diagnosed asthma, 2) no symptoms, 3) no use of asthma medication, 4) FEV1%pred > 90% (based on GLI-2012), 5) FEV1/FVC > lower limit of normal (based on GLI-2012). Based on this definition we have in total 636 healthy subjects in Lifelines.

Active asthma: doctor's diagnosed asthma AND (2 or more symptoms OR use of asthma medication). Based on this definition we have in total 99 subjects with active asthma in Lifelines, of whom 48 use inhaled corticosteroids (combination beta-agonists/inhaled corticosteroids OR inhaled corticosteroids). Symptoms included: wheeze (Did you have this wheezing while you did not have a cold? Have you ever suffered from wheezing on the chest?), shortness of breath at rest (Have you ever had an attack of shortness of breath at rest during the day?), and woken by an attack (Have you ever woken up with an attack of shortness of breath?).

Clinical Remission: doctor's diagnosed asthma AND no symptoms AND no use of asthma medication. Based on this definition we have in total 25 subjects with clinical remission in Lifelines.

Statistical analysis

Robust linear regression model was used to determine differential methylation between asthma and clinical remission. Model was adjusted for age, gender, smoking status, pack years, first 7 PC of methylation data and the percentage of monocytes, B cells, NK cells, CD4⁺ T cells, CD8⁺ T cells, neutrophils and eosinophils, which were predicted by the Houseman¹⁷ algorithm.

Functional annotation and pathway analysis

Annotation by position

Significant CpG sites were firstly annotated by GREAT 3.0.0¹⁸. Top sites identified in whole blood were further looked up in nasal brushes. Regional association plots of replicated CpG sites were generated using R package coMET¹⁹. In the association plot we included 15 CpG sites upstream to the target CpG site and 15 CpG sites downstream to the target CpG site. The association of replicated CpG sites identified from whole blood with SNP nearby ($\pm$ 250kb) were checked in BIOS qtl browser (<https://genenetwork.nl/biosqtlbrowser/>), which is called *cis*-MeQTL²⁰.

cis-eQTM analysis

After annotating each significant CpG to its nearest genes, we also annotated the CpG by the expression of nearby genes, which is called *cis* expression quantitative trait methylation (*cis*-eQTM). For blood *cis*-eQTM, we used DNA methylation and gene expression data from 3,075 samples in the Biobank-based integrative omics study (BIOS) consortium from the following cohorts: Leiden Longevity Study, Lifelines Study, Rotterdam Study, and Netherland Twin Study²⁰. *cis*-eQTMs for significant CpG sites identified in blood were conducted for gene expression transcripts within $\pm$ 250 kb of each CpG site. In each cohort, the methylation M value (log2 ratio of methylated versus unmethylated probe intensities) was regressed on gene expression and adjusted for age, sex, lymphocyte proportion, monocyte proportion, and RNA flow cell number. The results from each cohort were meta-analyzed using an inverse variance-weighted fixed-effects model in METAL²¹. We accounted for multiple testing by controlling for the false discovery rate (FDR) at 0.05.

For nasal brushes we have matched DNA methylation and RNAseq data in the discovery cohort. We then assessed the association of DNA methylation with gene expression *cis* ($\pm$ 250kb from the CpG) for all significant CpGs identified in nasal EWAS. In total 63 samples

had matched nasal DNA methylation and nasal RNAseq data. We performed linear regression analysis, and the model is: gene expression level $\sim$ DNA methylation M values + age + gender + smoking + pack years. Because of the small sample size in discovery cohort, we select the CpG-gene pairs at a nominal significant level ($P < 0.05$), and then replicated these pairs in PIAMA dataset²² which consists of 244 samples with matched DNA methylation and gene expression data.

The eQTM genes were used for pathway analysis by ConsensusPathDB²³.

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Additional file 3:

Figure S1. Lung function of different groups at baseline and last visit. In this figure, t-test was used in comparing means of any two groups (ns: $P > 0.05$, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$)

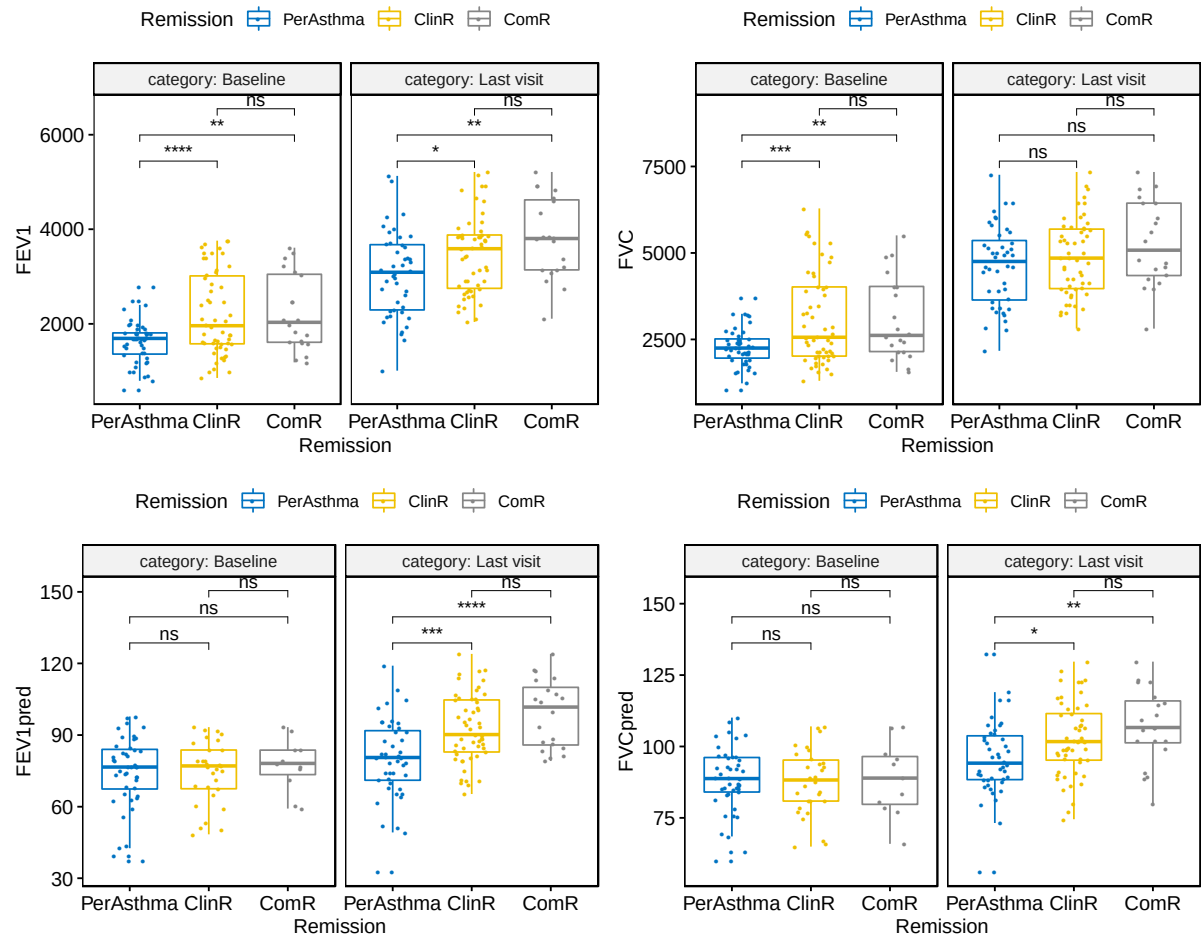


Figure S2. Estimated cell proportions among different groups. In this figure, t-test was used in comparing means of any two groups (ns: $P > 0.05$)

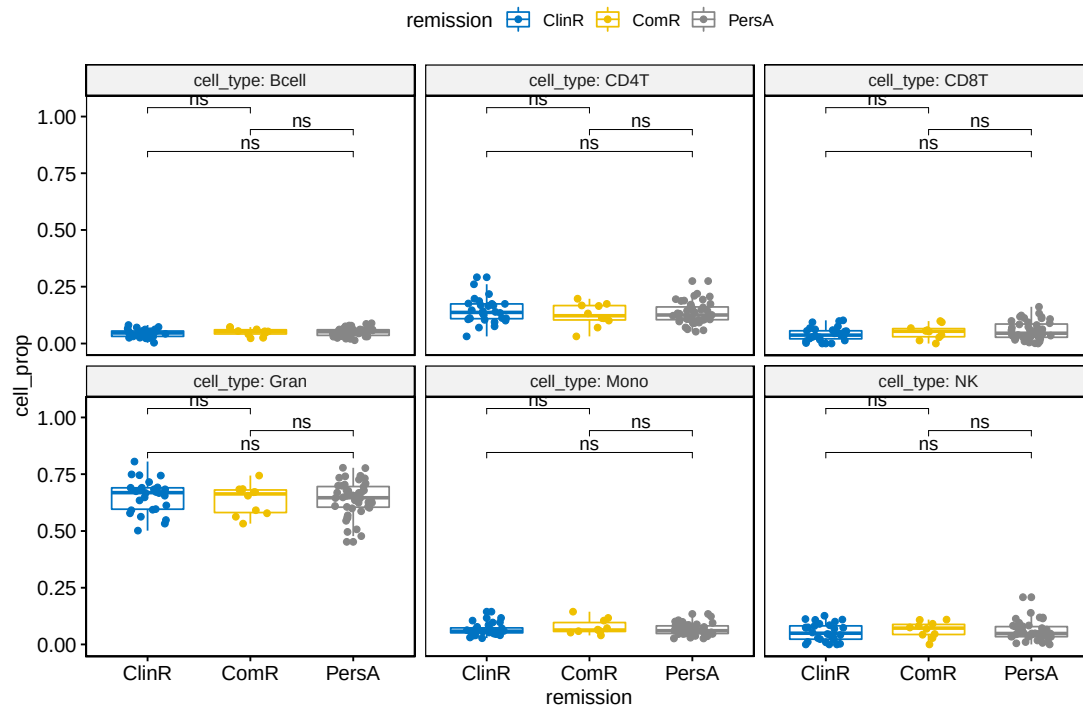


Figure S3. Quantile–quantile plot for epigenome-wide meta-analysis of the association between asthma remission and blood DNA methylation (n = 72). (a) ClinR, (b) ComR.

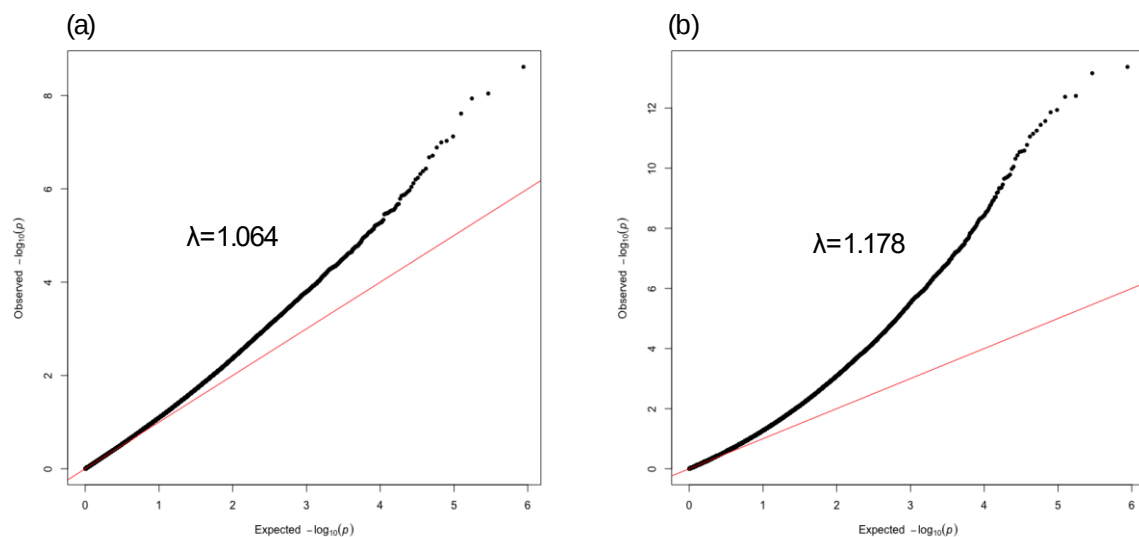


Figure S4. Quantile–quantile plot for epigenome-wide meta-analysis of the association between asthma remission and nasal DNA methylation (n = 97). (a) ClinR, (b) ComR.

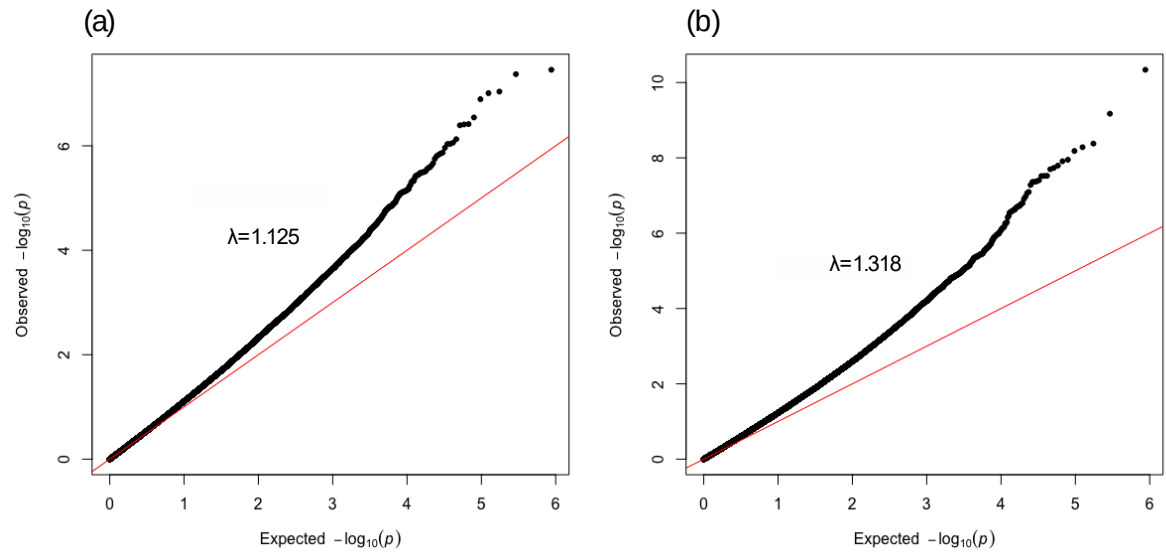


Figure S5. Boxplot illustrating DNA methylation levels of cg24788483 in persistent asthma, clinical remission and complete remission subjects in discovery cohort. (a) DNA methylation levels in blood, (b) DNA methylation in nasal brushes.

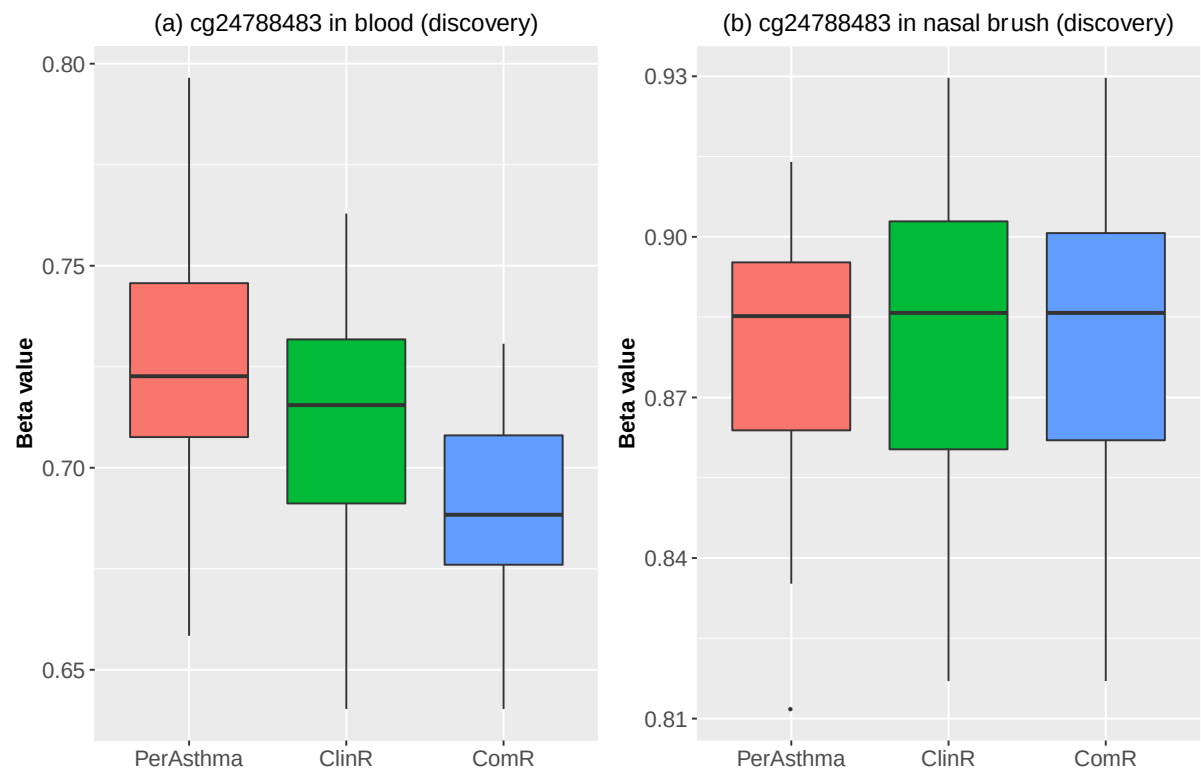


Figure S6. Density distributions of DNA methylation levels of two replicated CpGs in 72 blood samples from discovery cohorts.

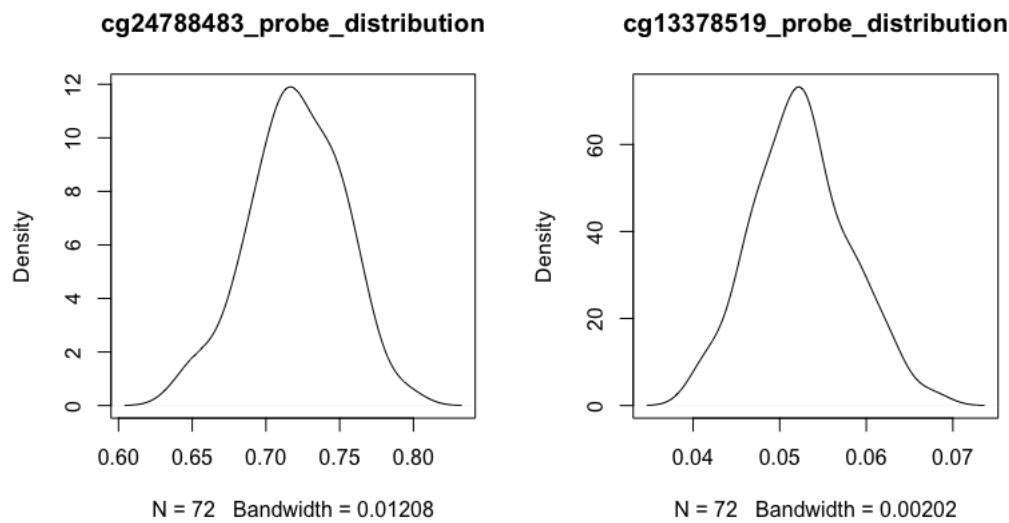


Figure S7. Regional association plot of two replicated CpGs. For each plot from top to bottom the tracks included are: 1) Log10(P values) from the discovery 4 years model with CpGs indicated by dots. 2) Annotation tracks for the plotted genomic region taken from UCSC Genome Browser. 3) Pairwise correlation matrix across the displayed CpGs.

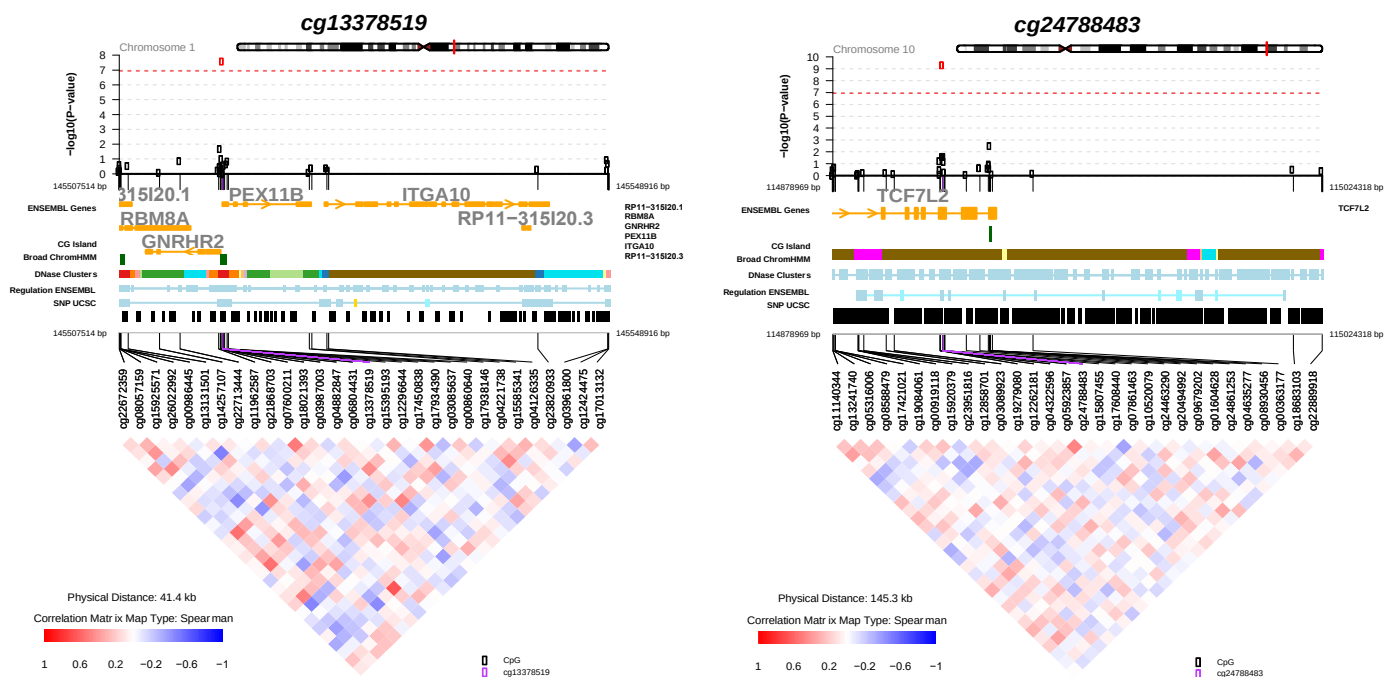


Figure S8. DNA methylation levels of two replicated CpGs in remission subjects and asthma patients stratified by ICS usage. In this figure, t-test was used in comparing means of any two groups (*: $P < 0.05$, ***: $P < 0.001$, ****: $P < 0.0001$)

