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Abbreviations:

AP-1: Activating Protein 1

NF-κB: Nuclear Factor Kappa B

CpGs: Cytosine-phosphate-Guanine (CpG) sites

DNA: deoxyribonucleic acid

COPD: Chronic Obstructive Pulmonary Disease

EWAS: Epigenome-Wide Association Study

FDR: False Discovery Rate

QQ plot: Quantile-Quantile plot

To the Editor,

Asthma is a chronic heterogeneous inflammatory airway disease. Its treatment includes bronchodilators and anti-inflammatory medication such as corticosteroids. Corticosteroids reduce transcription of AP-1 and NF-κB and hence may affect DNA methylation. Epigenetics refers to changes in DNA that can affect transcription, such as methylation of a cytosine nucleotide beside a guanine nucleotide (CpGs).

Asthma is associated with differentially methylated CpGs in specific genes [1, 2]. In the largest study to date, asthmatic children had significantly lower blood methylation levels at 14 CpGs compared to controls [3]. One previous study found 19 CpGs that were differentially methylated in blood during systemic corticosteroid exposure in patients with COPD [4]. Possible effects of inhaled asthma medication on peripheral blood methylation profiles are currently unknown.

Our aim was to study the association between inhaled corticosteroids and DNA methylation in peripheral blood cells in children with asthma. First, we performed an epigenome-wide association study (EWAS) investigating the effects of variable inhaled corticosteroid exposure on DNA methylation in 8-year-olds with diagnosed asthma in the BAMSE (Barn/Child, Allergy, Milieu, Stockholm, Epidemiology) cohort followed by replication attempts. Second, using a candidate gene approach, we evaluated if identified CpGs from the systemic steroid study [4] and the largest asthma study to date [3], in total 33 CpGs, were differentially methylated in relation to inhaled asthma treatment.

BAMSE is a Swedish prospective birth cohort study [5]. A total of 4089 children born 1994-1996 enrolled and information was collected in repeated questionnaires. Blood samples were taken at the 8- and 16-year follow-ups (n=2480; 61 % and n=2547; 62 %, respectively) [6]. For the present study, we included all subjects with a doctor's diagnosis of asthma ever up to 8 years and with DNA methylation data available for analyses (n=215) [3]. Subjects were grouped based on exposure established in the questionnaires: any medication for breathing difficulties (n=130), any inhaled corticosteroids or combination medication for any period of time (n=107), and inhaled corticosteroids continuously (at least 2 consecutive months) (n=39), all in the last 12 months. STOPPA (Swedish Twin Study on Prediction and Prevention of Asthma), a cohort study of twins aged 9-14 years [7] was used for replication analyses, and a subset of BAMSE 16-year cohort

(n=96 cases) was used for additional look-up (Tables E1-2). The regional ethics committee in Stockholm approved the studies, and written consent was obtained from all parents.

Robust linear regression was used for the analysis. The reference group comprised subjects diagnosed with asthma without any asthma medication in the last 12 months (n=85). We applied the Benjamini-Hochberg method to control the false discovery rate (FDR) at 5 %. P values below FDR were considered statistically significant in EWAS. Analyses were performed separately in BAMSE and STOPPA followed by fixed-effects meta-analysis using METAL.

Beta value was a dependent variable and each mode of asthma medication was a binary independent variable. Each model was adjusted for sex, age, sensitization to airborne allergens at 8 years, wheezing in the last 12 months, mother's smoking at least 1 cigarette per day at baseline and/or during pregnancy, bisulfite treatment date, and estimated cell types according to the Houseman method [6] (Table 1). Similar subject groupings and identical models were applied in STOPPA and BAMSE 16-year analyses.

Table 1. *Distribution of background characteristics of BAMSE subjects with DNA methylation data measured at 8 years of age in relation to type of asthma treatment. Results shown as n (%), compared to the total number of subjects in each group.*

	Diagnosed asthma (n=215)		Asthma treatment					
			Any medication for diagnosed asthma (n=130)		Any inhaled corticosteroid treatment (n=107)		Continuous inhaled corticosteroid treatment (n=39)	
Male	134 (62 %)		83 (64 %)		68 (64 %)		28 (72 %)	
Age in years (mean, SD)	8.1, 0.4		8.1, 0.4		8.1, 0.4		8.1, 0.4	
Sensitization†	106 (49 %)		79 (61 %)		66 (62 %)		28 (72 %)	
At least 1 episode of wheezing in the past 12 months	104 (48 %)		96 (74 %)		83 (78 %)		31 (79 %)	
Either parent smoked at the time of the 8-year questionnaire	46 (21 %)		22 (17 %)		20 (19 %)		6 (15 %)	
Mother's smoking‡	34 (16 %)		14 (11 %)		12 (11 %)		3 (8 %)	
Socioeconomic status at baseline, § blue collar worker compared to white collar worker	35 (16 %)	180 (84 %)	23 (18 %)	107 (82 %)	20 (19 %)	87 (81 %)	9 (23 %)	30 (77 %)

One or both parents' asthma/hay fever/ allergy¶	101 (47 %)	65 (50 %)	55 (51 %)	21 (54 %)

† Sensitization is defined as an IgE antibody level of 0.35 kUA/L or greater against any inhalant allergen at age 8.
‡ The child's mother smoked at least 1 cigarette per day at any point of time during the pregnancy and/or at the time of questionnaire 0 (median age of 2 months).
§Socioeconomic status for the household at the time of questionnaire 0, according to dominance order in two classes.
¶ Mother and/or father with doctor's diagnosis of asthma and asthma medication and/or doctor's diagnosis of hay fever in combination with furred pets- and/or pollen allergy at the time of questionnaire 0.

In total, methylation at 24 individual CpGs was significantly associated at FDR level with asthma treatment in BAMSE (Table 2; Figures E1-3). However, none of these EWAS hits was nominally significant in the replication study STOPPA or in BAMSE 16-year-olds, and in the meta-analysis, none of the CpGs reached genome-wide significance (FDR). As a sensitivity analysis, we repeated regression analyses in BAMSE 8-year-olds, not adjusting for cell types, and found overall very consistent results comparing the regression coefficients in the models with and without cell type adjustment (Table 2).

Table 2. *Statistically significant CpGs (defined as p value below respective FDR) from epigenome-wide association study analyses of* ***any asthma medication, any corticosteroid medication, and continuous corticosteroid medication exposure*** *in the last 12 months and DNA methylation change in peripheral blood cells from Swedish 8-year-olds. Total sum of subjects included in each group is stated after the exposure type (n). FDR for all is 2,2E-06. "-"indicates missing value as these CpGs were not included in the STOPPA DNA methylation data after normalization. See appendix for further description.*

CpG site	Gene†	Distance (bp) ‡	Coefficient, BAMSE 8§	p value, BAMSE 8	p value, STOPPA	p value, Meta analysis*	p value, BAMSE 16	Coefficient BAMSE 8: no cell adjustment ¶	p value, BAMSE 8: no cell adjustment
Any asthma medication exposure, n=130									
cg25214924	*AK058177*	-42380	-0.016	2.5E-08	0.29	2.6E-03	0.62	-0.013	1.0E-05
cg03877376	*TBX5*	85	0.008	2.0E-07	-	-	0.64	0.007	7.3E-06
cg20423602	*ADARB2-AS1*	-8232	-0.014	5.5E-07	0.13	1.5E-06	0.21	-0.012	1.2E-05
cg15954046	*LMNA*	303	-0.012	5.5E-07	0.60	1.9E-04	0.51	-0.006	6.8E-02
cg23966329	*UBE2G1*	-162	-0.003	1.3E-06	0.34	7.4E-03	0.89	-0.003	1.1E-06
cg14063914	*SERAC1*	349	-0.007	1.7E-06	0.45	4.3E-04	0.72	-0.008	7.3E-08
cg21731304	*NMNAT3*	-212	-0.021	2.0E-06	0.49	3.3E-05	0.15	-0.021	4.3E-06
Any corticosteroid exposure, n=107									

cg16048421	*LOC338579*	0	0.014	3.9E-07	0.80	7.2E-04	0.29	0,015	7,2E-07
cg15115986	*C1orf112*	-20	-0.004	4.9E-07	-	-	0.48	-0,004	2,7E-07
cg03877376	*TBX5*	85	0.008	5.5E-07	-	-	0.68	0,007	1,2E-05
cg03146079	*ADD1*	0	-0.005	5.9E-07	0.32	1.6E-06	0.99	-0,005	7,4E-07
cg17629264	*MAPK8IP2*	-390	-0.021	6.1E-07	0.34	1.5E-05	0.18	-0,014	4,3E-03
cg24144651	*BC043227*	-560	-0.009	8.8E-07	-	-	0.18	-0,006	2,8E-03
cg00025044	*ERCC6*	-1952	-0.011	1.0E-06	0.19	3.7E-05	0.42	-0,015	1,2E-08
cg25745861	*TMEM54*	-2782	0.012	1.1E-06	0.46	1.5E-05	0.85	0,016	5,8E-08
cg14136328	*SYT1*	-69884	-0.013	1.1E-06	0.32	1.4E-03	0.43	-0,010	5,8E-05
cg18046087	*KLC2*	0	-0.006	1.1E-06	0.76	4.9E-05	0.87	-0,007	3,9E-08
cg03043078	*MMP17*	2420	-0.006	2.0E-06	0.99	4.0E-05	0.23	-0,006	1,3E-06
Continuous corticosteroid exposure, n=39									
cg07665222	*ACRV1*	-1393	-0.022	3.3E-07	0.28	3.8E-06	0.75	-0,018	2,3E-04
cg03877376	*TBX5*	85	0.010	9.4E-07	-	-	0.94	0,009	2,2E-05
cg22997262	*LOC100128531*	-2329	0.017	1.1E-06	0.43	9.5E-03	0.54	0,018	9,2E-09
cg15074789	*EPHA2*	0	-0.008	1.2E-06	-	-	0.96	-0,008	9,4E-06
cg13688889	*FOXE1*	-6829	-0.048	1.4E-06	0.72	3.1E-04	0.20	-0,049	1,6E-06
cg00947413	*MIR3679*	-99303	-0.044	1.8E-06	0.22	1.4E-05	0.26	-0,046	1,0E-07
cg26281051	*DEFB129*	-95	-0.018	2.0E-06	0.46	2.9E-04	0.30	-0,017	1,3E-04
cg25745861	*TMEM54*	-2782	0.016	2.6E-06	0.71	1.4E-03	0.54	0,018	9,8E-06
cg13492223	*FUT6*	-159	0.017	2.9E-06	0.81	6.0E-04	0.98	0,019	4,6E-04

†, ‡ Gene annotation according to Illumina450K. CpGs were annotated using the IlluminaHumanMethylation450k.db R package, with enhanced annotation for nearest genes within 10Mb of each site, as previously described [8].

§ Regression coefficient, adjusted for sex, age, sensitization to airborne allergens at 8 years, wheezing in the last 12 months, mother's smoking at least 1 cigarette per day at baseline and/or during pregnancy, bisulfite treatment date, and estimated cell types. Reference group includes subjects without any asthma medication.

*Meta-analysis of results in BAMSE 8-year-old cohort and STOPPA using a fixed-effects model weighted by the inverse of the variance using METAL. BAMSE 16-year-olds were not included in the meta-analysis due to overlap with BAMSE 8 data.

¶ Regression coefficient, adjusted for sex, age, sensitization to airborne allergens at 8 years, wheezing in the last 12 months, mother's smoking at least 1 cigarette per day at baseline and/or during pregnancy, and bisulfite treatment date. Reference group includes subjects without any asthma medication.

Next, we investigated possible DNA methylation changes in the 33 selected CpGs from the literature [3], [4]. Three CpGs were nominally significant in BAMSE 8-year-olds when comparing any corticosteroid exposure to no medication and six CpGs showed nominally significant

methylation increases in relation to continuous corticosteroid exposure with three CpGs increasing ≥1%. However, none of the CpGs survived multiple testing adjustment (Tables E3-5). We investigated the 33 candidate CpGs in a subset of BAMSE 16-year-olds with an asthma diagnosis through identical analyses and congruently found no FDR-significant associations with asthma treatment.

In summary, several CpGs in EWAS were found differentially methylated in BAMSE at the FDR genome-wide significance level and results were very similar in models with and without cell-type adjustment. However, none of these CpGs replicated even at a nominal significance level in STOPPA or BAMSE 16-year cohort, and after meta-analyses, none of the CpGs survived multiple test adjustment. Thus, our study does not find evidence for DNA methylation changes in relation to inhaled asthma treatment, although changes through other epigenetic mechanisms cannot be ruled out. Our results are based on an observational study and hence do not produce intention-to-treat results. There are some limitations: firstly, we could not completely adjust for severity of asthma as the severity is reflected in the medication mode itself. Secondly, in the 8-year follow-up we did not enquire about systemic steroid use and hence there may be subjects that have used systemic corticosteroids in the "any medication" group. Thirdly, heterogeneity between the BAMSE and STOPPA cohorts unlikely explains the lack of replication as both cohorts are from areas with similar lifestyle factors, ethnic background and sensitization patterns, although more mothers smoked during pregnancy in BAMSE and more parents in STOPPA had asthma, hay fever or allergies.

Furthermore, we explored potential treatment–methylation associations using a candidate gene approach. We selected CpGs that were found robustly associated with asthma (per se) in the large study by Xu et al [3], where the authors did not specifically investigate potential influence from medication. We found a handful nominally associated CpGs with increased methylation in peripheral blood cells, whereas for asthma, Xu et al reported consistently lower methylation levels. However, none survived multiple test adjustment in our study.

There are well-known side effects from long-term systemic corticosteroid treatment, and the study by Wan et al found DNA methylation differences in COPD patients associated with systemic steroid use [4]. By exploring the top CpGs from Wan et al, we found no significant methylation differences in children and adolescents with asthma associated with inhaled corticosteroid

treatment. However, it should be noted that Wan et al studied adult COPD patients and we included children and adolescents with asthma in our study.

In conclusion, we found no evidence that inhaled corticosteroids or other asthma medications affect peripheral blood cell DNA methylation levels to any major extent, although smaller effects cannot be excluded.

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Conflicts of interest

EM reports personal fees from Novartis (advisory board reimbursement) during the conduct of the study; MK, OG, VU, CS, DG, IK, AB, GP and CA declare that they have no conflicts of interest.