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**Effects of inhaled corticosteroids on DNA methylation in peripheral blood cells in children with asthma.**

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1 **Effects of inhaled corticosteroids on DNA methylation in peripheral blood cells in children**  
2 **with asthma**

3  
4 **Running title:**

5 Childhood asthma, corticosteroids and DNA methylation

6  
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13  
14 **Key words:**

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16  
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25  
26 **Abbreviations:**

27 AP-1: Activating Protein 1

28 NF-κB: Nuclear Factor Kappa B

29 CpGs: Cytosine-phosphate-Guanine (CpG) sites

30 DNA: deoxyribonucleic acid

31 COPD: Chronic Obstructive Pulmonary Disease

32 EWAS: Epigenome-Wide Association Study

33 FDR: False Discovery Rate

34 QQ plot: Quantile-Quantile plot

35 **To the Editor,**

36

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

42

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

48

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

57

58 BAMSE is a Swedish prospective birth cohort study [5]. A total of 4089 children born 1994-1996  
59 enrolled and information was collected in repeated questionnaires. Blood samples were taken at the  
60 8- and 16-year follow-ups (n=2480; 61 % and n=2547; 62 %, respectively) [6]. For the present  
61 study, we included all subjects with a doctor's diagnosis of asthma ever up to 8 years and with  
62 DNA methylation data available for analyses (n=215) [3]. Subjects were grouped based on  
63 exposure established in the questionnaires: any medication for breathing difficulties (n=130), any  
64 inhaled corticosteroids or combination medication for any period of time (n=107), and inhaled  
65 corticosteroids continuously (at least 2 consecutive months) (n=39), all in the last 12 months.  
66 STOPPA (Swedish Twin Study on Prediction and Prevention of Asthma), a cohort study of twins  
67 aged 9-14 years [7] was used for replication analyses, and a subset of BAMSE 16-year cohort

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

70

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

76

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

83

84 **Table 1.** Distribution of background characteristics of BAMSE subjects with DNA methylation data  
 85 measured at 8 years of age in relation to type of asthma treatment. Results shown as n (%), compared to the  
 86 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 <sup>†</sup>	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 <sup>‡</sup>	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 <sup>¶</sup>	101 (47 %)	65 (50 %)	55 (51 %)	21 (54 %)
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87 † Sensitization is defined as an IgE antibody level of 0.35 kUA/L or greater against any inhalant allergen at age 8.

88 ‡ 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  
89 questionnaire 0 (median age of 2 months).

90 § Socioeconomic status for the household at the time of questionnaire 0, according to dominance order in two classes.

91 ¶ Mother and/or father with doctor's diagnosis of asthma and asthma medication and/or doctor's diagnosis of hay fever  
92 in combination with furred pets- and/or pollen allergy at the time of questionnaire 0.

93

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

101

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

108

CpG site	Gene <sup>†</sup>	Distance (bp) <sup>‡</sup>	Coefficient, BAMSE 8 <sup>§</sup>	p value, BAMSE 8	p value, STOPPA	p value, Meta analysis*	p value, BAMSE 16	Coefficient BAMSE 8: no cell adjustment <sup>¶</sup>	p value, BAMSE 8: no cell adjustment
<i>Any asthma medication exposure, n=130</i>									
cg25214924	<i>AK058177</i>	-42380	-0.016	2.5E-08	0.29	2.6E-03	0.62	-0.013	1.0E-05
cg03877376	<i>TBX5</i>	85	0.008	2.0E-07	-	-	0.64	0.007	7.3E-06
cg20423602	<i>ADARB2-AS1</i>	-8232	-0.014	5.5E-07	0.13	1.5E-06	0.21	-0.012	1.2E-05
cg15954046	<i>LMNA</i>	303	-0.012	5.5E-07	0.60	1.9E-04	0.51	-0.006	6.8E-02
cg23966329	<i>UBE2G1</i>	-162	-0.003	1.3E-06	0.34	7.4E-03	0.89	-0.003	1.1E-06
cg14063914	<i>SERAC1</i>	349	-0.007	1.7E-06	0.45	4.3E-04	0.72	-0.008	7.3E-08
cg21731304	<i>NMNA T3</i>	-212	-0.021	2.0E-06	0.49	3.3E-05	0.15	-0.021	4.3E-06
<i>Any corticosteroid exposure, n=107</i>									

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

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

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

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

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

120

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

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

129

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

145

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

152

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

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

159

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

163

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165

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212 EM reports personal fees from Novartis (advisory board reimbursement) during the conduct of the  
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