Systemic Steroid Exposure Is Associated with Differential Methylation in Chronic Obstructive Pulmonary Disease

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***Rationale*: Systemic glucocorticoids are used therapeutically to treat a variety of medical conditions. Epigenetic processes such as DNA methylation may reflect exposure to glucocorticoids and may be involved in mediating the responses and side effects associated with these medications.**

***Objectives*: To test the hypothesis that differences in DNA methylation are associated with current systemic steroid use.**

***Methods*: We obtained DNA methylation data at 27,578 CpG sites in 14,475 genes throughout the genome in two large, independent cohorts: the International COPD Genetics Network ($n_{discovery}$ = 1,085) and the Boston Early Onset COPD study ($n_{replication}$ = 369). Sites were tested for association with current systemic steroid use using generalized linear mixed models.**

***Measurements and Main Results*: A total of 511 sites demonstrated significant differential methylation by systemic corticosteroid use in all three of our primary models. Pyrosequencing validation confirmed robust differential methylation at CpG sites annotated to genes such as *SLC22A18*, *LRP3*, *HIPK3*, *SCNN1A*, *FXYD1*, *IRF7*, *AZU1*, *SIT1*, *GPR97*, *ABHD16B*, and *RABGEF1*. Functional annotation clustering demonstrated significant enrichment in intrinsic membrane components, hemostasis and coagulation, cellular ion homeostasis, leukocyte and lymphocyte activation and chemotaxis, protein transport, and responses to nutrients.**

***Conclusions*: Our analyses suggest that systemic steroid use is associated with site-specific differential methylation throughout the genome. Differentially methylated CpG sites were found in biologically plausible and previously unsuspected pathways; these genes and pathways may be relevant in the development of novel targeted therapies.**

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Systemic corticosteroids are associated with a wide range of therapeutic responses and side effects that may persist after drug withdrawal. These characteristics suggest a possible role for epigenetic modifications. Previous epigenetic studies on steroid exposure have examined histone modifications or DNA methylation of candidate genes involved in steroid biosynthesis, metabolism, or receptors.

What This Study Adds to the Field

Our study explores the association of differential DNA methylation at sites throughout the entire genome. We report robustly associated sites in biologically plausible and previously unsuspected pathways.

Keywords: DNA methylation; glucocorticoids; chronic obstructive pulmonary disease

Systemic glucocorticoids possess potent antiinflammatory, immunomodulatory, and antineoplastic properties and are integral in the treatment of chronic obstructive pulmonary disease (COPD) and asthma exacerbations, autoimmune diseases, allergic reactions, and certain malignancies. However, despite their clinical utility, systemic glucocorticoid use is often complicated by multiple side effects, including weight gain and redistribution of body fat, osteoporosis, cardiovascular disease, impaired immune response and wound healing, and alterations in glucose and lipid metabolism. The responses and side effects experienced by individuals exposed to glucocorticoids are highly variable and may persist for extended periods after drug withdrawal; these phenomena may be mediated through epigenetic processes.

Epigenetic changes, such as histone modifications (1, 2), DNA methylation, and alterations in chromatin structure, vary between individuals (3) and can affect glucocorticoid receptor expression and binding (3, 4) as well as the subsequent responses to endogenous (5) or exogenously administered (1, 2, 6) steroids. Conversely, glucocorticoid exposure can cause epigenetic changes and may be mediated in part through changes in the expression or activity of DNA methyltransferases (7–9). Dynamic, site-specific changes in DNA methylation have been observed after glucocorticoid exposure *in vitro* (4) and *in vivo* (10). However, investigations into the associations between glucocorticoids and DNA methylation remain limited to a small number of CpG sites within selected candidate genes (10–13). We hypothesized that, given the extensive systemic effects of therapeutic glucocorticoids, exposure to these medications would

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be associated with differential methylation in peripheral blood DNA at multiple sites throughout the genome. Some of these results have been previously reported in the form of an abstract (14).

METHODS

Subjects and Cohorts

The discovery cohort consisted of 1,085 subjects participating in the International COPD Genetics Network (15, 16) (ICGN), a family-based study with probands between the ages of 45 and 65 years, 5 or more pack-years of cigarette smoking, FEV_1 less than 60% predicted, and a FEV_1/FVC ratio of less than 90% predicted. Siblings of probands with at least 5 pack-years of cigarette smoking were also enrolled. The replication cohort consisted of 369 subjects from the Boston Severe Early-Onset COPD Study (17) (EOCOPD), a family-based study where probands have an FEV_1 less than 40% predicted by age 53 in the absence of severe α-1 antitrypsin deficiency. Although extended pedigrees were recruited, DNA methylation data were obtained only on probands and their siblings. The protocols were approved by the appropriate Internal Review Boards; all subjects provided written informed consent.

Variable Definitions

Subjects in ICGN were asked to list all current medications. Current systemic steroid use was present if they answered affirmatively to the following questions: "Are any of the above listed medications steroid/prednisone pills?" and "Currently taking?" Subjects in EOCOPD were considered to have current systemic steroid use if they responded affirmatively to the question "Do you take prednisone?" For both cohorts, current smoking was defined as a positive response to the question "Do you currently smoke cigarettes (as of 1 month ago)?" Obstructive lung disease was defined as an FEV_1/FVC ratio of less than 0.7.

DNA Extraction, Bisulfite Treatment, and Methylation Array

DNA was extracted from whole blood. Quantitative assessment of methylation was performed on bisulfite-converted DNA using the Illumina (San Diego, CA) Infinium HumanMethylation27K BeadChip. Methylation at each site is expressed as the Illumina "β value" (β), a variable that ranges from zero (no methylation) to 1 (complete methylation). CpG sites are discussed using the industry-assigned identifier ("cg") as outlined in the Illumina HumanMethylation27K manifest. The protocol is outlined in the online supplement (18).

Pyrosequencing

Technical validation of selected sites was performed in the ICGN cohort using the Pyromark96MD (Qiagen Inc., Valencia, CA). Details are provided in the online supplement.

Statistical Analysis

The ComBat (19) function as implemented in the *sva* package (20, 21) in R (release 2.12) was used to account for batch effects. To account for familial correlations in the data, generalized linear mixed models (22) were used to analyze the methylation data. The Illumina "β" value was modeled as the dependent variable, with oral steroid use included as the independent variable. Models were adjusted for age, sex, and FEV_1 % predicted (Model 1); pack-years of smoking (Model 2); and current smoking status (Model 3). CpG sites with a P value below the Bonferroni-adjusted threshold for significance ($<1.89 \times 10^{-6}$) in ICGN and a P value of less than 0.05 with the same direction of effect in EOCOPD were considered significant.

Subgroup Analysis: Matched Cohort

Subjects in ICGN who reported current systemic steroid use were matched (1:1) by sex, age (±2 yr), and FEV_1 % predicted (±10%) with subjects who did not report current systemic steroid use ($n_{total} = 160$). Differences in the mean Illumina β values were tested using an unpaired Student's t test.

RESULTS

The characteristics of the subjects in each cohort are listed in Table 1, and the ranges for continuous variables by current systemic steroid use are shown in Table E1 in the online supplement. Eighteen subjects were missing a response for oral steroid use in the EOCOPD study and were excluded from analysis. Approximately 7.4 and 14.2% of subjects reported current systemic steroid use in the ICGN and EOCOPD cohorts, respectively. By design, subjects in the EOCOPD cohort had a lower mean age; the higher prevalence of female subjects in the EOCOPD cohort has also been previously described (17, 23). Subjects who reported current systemic steroid use had significantly lower FEV_1 % predicted, lower rates of current smoking, higher rates of inhaled corticosteroid use, and greater pack-years of cumulative smoke exposure than subjects who were not on steroids in both cohorts. Although the majority of subjects reporting current systemic steroid use had obstructive lung disease, four subjects in ICGN and two subjects in EOCOPD reporting systemic steroid use did not have obstruction as measured by spirometry.

In Model 1, 614 sites demonstrated significant differential methylation with respect to systemic steroid use; the top 20 sites by Liptak-combined P values (24, 25) are illustrated in Table 2, and the complete list of significant sites is reported in Table E2. In Model 2, 599 CpG sites were significantly associated with current steroid use (Table E3). In Model 3, 514 sites were significantly associated with current systemic steroid use (Table E4). A total of 511 sites were significant in Models 1, 2, and 3;

TABLE 1. COHORT CHARACTERISTICS

	ICGN		EOCOPD*	
Current Systemic Steroid Use	Yes ($n = 80$)	No ($n = 1,005$)	Yes ($n = 50$)	No ($n = 301$)
Age	57.7 (6.7)	57.2 (8.2)	49.0 (3.9)†	47.2 (7.6)
Male gender	43 (53.8%)	547 (54.4%)	11 (22.0%)†	114 (37.9%)
Current smokers	23 (28.8%)	373 (37.1%)	5 (10.0%)†	90 (29.9%)
Pack-years	48.7 (26.7)†	41.1 (26.1)	37.6 (19.3)†	27.2 (23.1)
Inhaled steroid use	58 (72.5%)†	324 (32.2%)	39 (78%)†	85 (28.2%)
FEV_1% predicted	36.3 (18.6)†	71.4 (33.1)	18.5 (10.0)†,‡	62.3 (33.7)‡

Definition of abbreviations: EOCOPD = Early Onset COPD Study; ICGN = International COPD Genetics Network.

Data are presented as n (%) or mean (SD).

* Eighteen subjects in EOCOPD cohort were missing response to systemic and inhaled steroid data and were excluded from analysis.

† Univariate P value < 0.05 when compared with subjects who are not currently taking systemic steroids.

‡ Prebronchodilator values.

TABLE 2. TOP 20 CPG SITES WITH SIGNIFICANT DIFFERENTIAL METHYLATION BY CURRENT SYSTEMIC STEROID USE (MODEL 1)

		ICGN		EOCOPD		
CpG names	Gene Symbol	Difference in Mean Methylation*	*P* Value†	Difference in Mean Methylation*	*P* Value‡	Liptak Combined *P* Value
cg19906550	*SLC22A18*	−0.10	5.96×10^{-35}	−0.05	3.56×10^{-4}	4.71×10^{-37}
cg27461196	*FXYD1*	−0.15	1.80×10^{-33}	−0.08	3.40×10^{-4}	1.27×10^{-35}
cg25600606	*HIPK3*	−0.15	9.29×10^{-34}	−0.08	7.95×10^{-4}	1.76×10^{-35}
cg08700306	*LRP3*	−0.09	3.60×10^{-33}	−0.05	2.85×10^{-4}	2.07×10^{-35}
cg09868035	*ABHD16B*	−0.09	7.93×10^{-33}	−0.05	1.47×10^{-3}	2.97×10^{-34}
cg17709873	*LTA*	0.10	3.36×10^{-32}	0.06	7.32×10^{-4}	5.32×10^{-34}
cg01526089	*P2RX1*	−0.13	5.38×10^{-33}	−0.06	3.52×10^{-3}	6.29×10^{-34}
cg00795812	*PDCD1*	0.09	7.21×10^{-33}	0.05	4.05×10^{-3}	1.01×10^{-33}
cg18884741	*RABGEF1*	−0.17	1.98×10^{-32}	−0.07	7.69×10^{-3}	6.61×10^{-33}
cg07730301	*ALDH3B1*	−0.10	3.29×10^{-31}	−0.05	1.06×10^{-3}	7.58×10^{-33}
cg26215727	*SCNN1A*	−0.09	2.33×10^{-29}	−0.05	4.82×10^{-5}	2.13×10^{-32}
cg21019522	*SLC22A18*	−0.08	2.50×10^{-29}	−0.04	5.59×10^{-4}	2.60×10^{-31}
cg06270401	*DYRK4*	−0.12	1.10×10^{-29}	−0.06	2.69×10^{-3}	7.14×10^{-31}
cg25634666	*FOLR3*	−0.11	6.60×10^{-29}	−0.06	8.15×10^{-4}	1.02×10^{-30}
cg00974864	*FCGR3B*	−0.11	2.50×10^{-28}	−0.06	5.92×10^{-4}	2.68×10^{-30}
cg08368934	*GPR97*	−0.10	5.13×10^{-29}	−0.06	2.36×10^{-3}	2.71×10^{-30}
cg17823175	*AZU1*	−0.12	1.41×10^{-27}	−0.07	1.62×10^{-4}	4.00×10^{-30}
cg00645579	*IRF7*	−0.09	2.08×10^{-27}	−0.05	6.08×10^{-4}	2.23×10^{-29}
cg15518883	*SIT1*	0.08	4.44×10^{-27}	0.05	3.03×10^{-4}	2.32×10^{-29}
cg24211388	*AIF1*	−0.12	3.02×10^{-28}	−0.06	5.40×10^{-3}	4.29×10^{-29}

Definition of abbreviations: EOCOPD = Early Onset COPD Study; ICGN = International COPD Genetics Network.

* Difference in mean methylation = (mean methylation subjects on steroids) − (mean methylation subjects not on steroids). Methylation values may range from 0 to 1.

† *P* value from regression Model 1 (adjusted for age, sex, and FEV_1 % predicted)

‡ One-sided *P* value from regression Model 1.

these are listed in Table E5. To explore the possibility of overadjustment due to incorporating age and sex as covariates simultaneously with FEV_1 % predicted values, we repeated the above analyses substituting unadjusted FEV_1 (in liters) for FEV_1 % predicted. As expected, the number of significant sites was increased in the unadjusted FEV_1 analysis (data not shown). The overlap between the results obtained using unadjusted FEV_1 and FEV_1 % predicted was excellent, with nearly identical ranking of the most highly associated sites.

Functional annotation clustering performed using the official HUGO gene symbol annotated to each of the 511 overlapping sites with a high classification stringency in the database for Annotation, Visualization, and Integrated Discovery Bioinformatics Resources 6.7 (26, 27) showed significant enrichment in genes annotated to the following processes: intrinsic membrane components, hemostasis and coagulation, cellular ion homeostasis, leukocyte and lymphocyte activation and chemotaxis, protein transport, and responses to nutrients (Table 3).

Pyrosequencing validation was performed for selected highly associated CpG sites chosen on the basis of a preliminary analysis in the ICGN cohort. Robust differences in mean methylation by current systemic steroid use were confirmed for all sites examined (Table 4) and are illustrated in Figure E1.

A recent publication by Du and colleagues (28) raised the issue of heteroscedasticity at CpG sites with extremely low (<0.2) or high (>0.8) Illumina β values and suggested the use of M values (defined as the log2 of Cy5/Cy3 ratios) in lieu of β values for association testing. We repeated our analyses using the same covariates and criteria for significance as our β-value analyses and obtained similar overall results. In Model 1, 724 sites were significant in the M-value analysis, 585 of which overlapped with the 614 sites reported in the β-value analysis. For Model 2, 707 sites were significant in the M-value analysis, 567 of which overlapped with the 599 sites reported in the β-value analysis. In Model 3, 616 CpG sites were significant in the M-value analysis, 483 of which overlapped with the 514 sites reported in the β-value analysis. A total of 604 sites were significant in Models 1, 2, and 3 in the M-value analysis, 479 of which overlap with the 511 significant sites in Models 1, 2, and 3 of the β-value analysis. All 12 pyrosequenced sites were among the 604 overlapping sites.

Because our cohorts included subjects with and without COPD, we performed a sensitivity analysis examining differential methylation in subjects with obstructive lung disease (n_{ICGN} = 643; n_{EOCOPD} = 223). The characteristics of this subgroup are illustrated in Table E6. The difference in mean FEV_1 % predicted by current systemic steroid use was attenuated, whereas the difference in pack-years between the groups is no longer significant. Using the same criteria for significance as used in our primary analyses, 484 CpG sites demonstrated significant differential methylation with respect to current systemic steroid use in Model 1; the top 20 most highly associated sites are reported in Table 5, and the full list is reported in Table E7. A total of 461 of these sites overlap with the 614 sites identified in the corresponding analysis in the full cohort. For Model 2, 468 CpG sites were differentially methylated in the COPD-only analysis; 448 of these sites overlap with the 599 sites identified using the full cohort (Table E8). For Model 3, 397 CpG sites were differentially methylated; 384 of these overlap with the 524 sites from the full cohort analysis (Table E9). A total of 392 CpG sites were significant in all three models in the COPD-only analyses; 371 of these sites overlap with the 511 CpG sites identified in the full-cohort analyses (Table E10).

Acute exacerbations, which are the most common indication for systemic steroid use in our study populations, are known to increase as lung function declines (29) and to decrease with inhaled corticosteroid use (30). We examined the impact of these two important confounders in separate exploratory analyses. First, we performed an epigenome-wide association analysis using Model 1 and replaced current systemic steroid use with inhaled corticosteroid use as the independent predictor; no significantly differentially methylated sites were found. Second, although our primary analyses adjusted for differences in lung function by including FEV_1 % predicted as a covariate, we sought to further mitigate the differences in disease severity by performing a matched analysis in a subgroup of subjects in

TABLE 3. SIGNIFICANTLY ENRICHED TERMS FROM FUNCTIONAL ANNOTATION CLUSTERING* PERFORMED ON GENES DEMONSTRATING DIFFERENTIAL METHYLATION BY CURRENT SYSTEMIC STEROID USE

GO Term	Process	Benjamini-Hochberg *P* Value
GO:0031224	Intrinsic to membrane	2.5×10^{-2}
GO:0007599	Hemostasis	8.7×10^{-4}
GO:0050878	Regulation of body fluid levels	2.6×10^{-3}
GO:0050817	Coagulation	4.1×10^{-3}
GO:0007596	Blood coagulation	4.1×10^{-3}
GO:0048878	Chemical hemostasis	2.2×10^{-3}
GO:0006873	Cellular ion homeostasis	3.2×10^{-2}
GO:0055082	Cellular chemical homeostasis	3.5×10^{-2}
GO:0050801	Ion homeostasis	4.0×10^{-2}
GO:0042592	Homeostatic process	2.8×10^{-2}
GO:0050865	Regulation of cell activation	4.1×10^{-3}
GO:0002694	Regulation of leukocyte activation	7.9×10^{-3}
GO:0051249	Regulation of lymphocyte activation	8.0×10^{-3}
GO:0030595	Leukocyte chemotaxis	7.5×10^{-3}
GO:0060326	Cell chemotaxis	9.7×10^{-3}
GO:0050900	Leukocyte migration	1.4×10^{-2}
GO:0055074	Calcium ion homeostasis	7.7×10^{-3}
GO:0055066	Di-, trivalent inorganic cation homeostasis	1.1×10^{-2}
GO:0006874	Cellular calcium ion homeostasis	1.4×10^{-4}
GO:0055065	Metal ion homeostasis	1.5×10^{-2}
GO:0030005	Cellular di-, trivalent inorganic cation homeostasis	1.5×10^{-2}
GO:0007204	Elevation of cytosolic calcium ion concentration	1.7×10^{-2}
GO:0006875	Cellular metal ion homeostasis	2.6×10^{-2}
GO:0051480	Cytosolic calcium ion homeostasis	2.8×10^{-2}
GO:0030003	Cellular cation homeostasis	3.9×10^{-2}
GO:0051222	Positive regulation of protein transport	9.6×10^{-3}
GO:0042330	Taxis	3.1×10^{-2}
GO:0006935	Chemotaxis	3.1×10^{-2}
GO:0002683	Negative regulation of immune system process	3.0×10^{-2}
GO:0051250	Negative regulation of lymphocyte activation	4.0×10^{-2}
GO:000758	Response to nutrient	3.0×10^{-2}

* Functional annotation clustering performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatics Resources 6.7.

ICGN matched on age, gender, and FEV_1 % predicted. We examined the differences in mean methylation at the 511 overlapping significant CpG sites from our primary analyses; all 511 sites continued to demonstrate a significant ($P < 0.05$) difference in mean methylation by current systemic steroid use, with a consistent direction of effect as that observed in the full cohort analysis (Table E11).

DISCUSSION

The most well studied epigenetic processes investigating systemic steroid exposure are histone modifications, and considerable evidence supports a link between histone acetylation and steroid resistance (2, 12). Investigations into the role of DNA methylation have been limited to the study of various candidate genes, such as the cytochrome P450 enzymes (31–35), transporter proteins (36–38), or the glucocorticoid receptor (4, 39) itself. Given the broad effects of systemic steroids, including evidence supporting a dose-dependent effect on DNA methyltransferase expression (7), we hypothesized that exposure to systemic steroids may be associated with differential methylation at multiple loci throughout the genome. We now report the first genome-wide analysis of the association between glucocorticoids and DNA methylation in peripheral blood. Our analyses suggest differential methylation at numerous, specific CpG dinucleotides throughout the genome located in established as well as previously unsuspected pathways.

Because systemic corticosteroids are often administered for their antiinflammatory and immune-modulating properties, significant associations with CpG sites annotated to genes located within these pathways can be viewed as biologically plausible. Examples include members of the tumor necrosis factor superfamily, such as lymphotoxin α (*LTA*), tumor necrosis factor ligands (*TNFSF12*, *TNFSF13B*, and *TNFSF12-TNFSF13*),IL-1β and IL-2, and various receptors such as *CD27*, Fc fragment of IgG, low-affinity IIIb receptor (*FCGR3B*), and chemokine receptor 2 (*CXCR2*). Several of the CpG sites validated through pyrosequencing are also involved in immune responses. Azurocidin 1 (*AZU1*), also known as heparin binding protein, is a peptide with antimicrobial, chemotactic, and inflammatory mediator properties contained within the azurophilic granules of neutrophils (40–42). The signaling threshold regulating transmembrane adaptor 1 (*SIT1*) has been shown to have a role in T-cell receptor–mediated signaling (43, 44). Interferon regulatory factor 7 (*IRF7*) is involved in mediating the expression of interferon α and the response to viral infection (45, 46); interestingly, relative hypermethylation at the *IRF7* promoter has been correlated with decreased expression in the nasal epithelium of smokers (47).

CpG sites annotated to processes associated with well accepted, clinically relevant side effects of therapeutic glucocorticoids, such as hyperglycemia/insulin resistance, weight gain, and osteoporosis, can also be viewed as biologically plausible associations. Examples of genes implicated in such processes include the insulin receptor (*INSR*), leptin (*LEP*), and the cytochrome P450, family 27, subfamily B, polypeptide 1 (*CYP27B1*), a protein better known as 25-OH vitamin D-1α-hydroxylase, which is involved in calcium homeostasis through the conversion of vitamin D into its active form (48). Associations with genes involved in the synthesis and regulation of steroid hormones also appear logical. An example is cg25600606, a highly associated and validated site, located within the homeodomain-interacting protein kinase 3 (*HIPK3*) gene, a serine–threonine protein kinase that participates in cAMP-mediated steroidogenesis (49).

Although associations with genes annotated to biologically plausible pathways can be viewed as proof-of-concept associations, the greater value of a genome-wide approach may lie in exploring genes and pathways that do not have an obvious biological relevance. Three strongly associated and validated examples include the abhydrolase domain containing 16B (*ABHD16B*), the lipoprotein receptor–related protein (*LRP3*), and G protein–coupled receptor 97 (*GPR97*). Very little is known about the structure and function of *ABHD16B*. *GPR97* was discovered through a search of human genome databases and, based on the general function of G protein–coupled receptors, may be involved in signal transduction (50). *LRP3* displays significant structural homology to the low-density lipoprotein receptors; however, functional studies demonstrated no significant binding of LRP3 to receptor-associated protein (RAP). Thus, the biological function of LRP3 may be distinct from lipoprotein metabolism (51).

Cg19906550 and cg21019522 map to the solute carrier family 22, member 18 (*SLC22A18*) gene. This gene encodes a poly-specific

TABLE 4. PYROSEQUENCING VALIDATION OF SELECTED CpG SITES IN THE INTERNATIONAL CHRONIC OBSTRUCTIVE PULMONARY DISEASE GENETICS NETWORK COHORT

CpG	Gene Symbol	Completion Rate	Difference in Mean Methylation*	*P* Value†
cg19906550	*SLC22A18*	97.4%	−0.08	5.78×10^{-13}
cg21019522	*SLC22A18*	97.3%	−0.06	2.14×10^{-11}
cg08700306	*LRP3*	97.5%	−0.11	2.78×10^{-12}
cg25600606	*HIPK3*	94.2%	−0.15	6.29×10^{-14}
cg26215727	*SCNN1A*	96.8%	−0.12	2.20×10^{-16}
cg27461196	*FXYD1*	96.8%	−0.13	2.33×10^{-6}
cg00645579	*IRF7*	98.0%	−0.10	2.20×10^{-16}
cg17823175	*AZU1*	95.6%	−0.07	5.11×10^{-8}
cg15518883	*SIT1*	97.9%	0.08	1.77×10^{-12}
cg08368934	*GPR97*	97.1%	−0.09	3.73×10^{-12}
cg09868035	*ABHD16B*	98.2%	−0.07	1.45×10^{-8}
cg18884741	*RABGEF1*	85.3%	−0.09	4.94×10^{-6}

* Difference in mean methylation = (mean methylation in subjects on current steroids) – (mean methylation in subjects NOT on steroids). Methylation values range from 0 to 1.

† *P* value for unpaired Student's *t* test.

cation transporter and lies within the 11p15.5 region known to contain multiple well characterized imprinted genes such as insulin-like growth factor 2 (*IGF2*) and *H19* (52, 53). *SLC22A18* appears to be subject to imprinting as well, though the strength of the resulting allelic expression bias appears to vary by tissue (52, 53), developmental stage (53), and individual (52–54). The degree to which DNA methylation contributes to differences in allelic expression is unclear (55); although increased methylation at the *SLC22A18* promoter has been described in the context of several different tumors, the correlation with expression changes remains imperfect and varies by tissue and tumor type (55–57).

Another validated site, cg26215727, is located within the sodium channel, non–voltage-gated 1 α subunit (*SCNN1A*) gene. This gene encodes the α subunit of an amiloride-sensitive epithelial sodium channel that is critical in regulating fluid transport in various organs such as the lung, kidney, liver, and pancreas (58, 59). The promoter of the *SCNN1A* gene is known to contain a glucocorticoid response element (GRE) (60), and several studies have demonstrated increased expression of *SCNN1A* associated with increased endogenous (61) or exogenously administered (60, 62) glucocorticoids.

We therefore explored whether additional putative GREs were in close proximity to the differentially methylated sites identified in our analyses. Because GREs are defined through a combination of relatively short (15 base pair), often variable consensus sequences and, more importantly, functional validation by demonstrating direct glucocorticoid receptor (GR) binding or reduced inducibility after deletion or mutation of the putative GRE, exhaustive studies examining putative GREs on a genome-wide scale have been limited. In a study by Reddy and colleagues (63), chromatin immunoprecipitation followed by sequencing (CHiP-seq) was used to assess GR binding throughout the genome in A549 human lung epithelial carcinoma cell

TABLE 5. TOP 20 CpG SITES WITH DIFFERENTIAL METHYLATION BY CURRENT SYSTEMIC STEROID USE IN SUBJECTS WITH OBSTRUCTIVE LUNG DISEASE (MODEL 1)

		ICGN		EOCOPD			
CpG	Gene Symbol	Difference in Mean Methylation*	*P* Value†	Difference in Mean Methylation*	*P* Value‡	Liptak Combined *P* Value	Overlaps with Model 1 (full cohort)
cg25600606	*HIPK3*	−0.15	5.93×10^{-27}	−0.07	2.90×10^{-3}	4.47×10^{-28}	Yes
cg19906550	*SLC22A18*	−0.10	3.15×10^{-26}	−0.05	1.79×10^{-3}	1.24×10^{-27}	Yes
cg27461196	*FXYD1*	−0.15	3.45×10^{-26}	−0.07	1.92×10^{-3}	1.48×10^{-27}	Yes
cg08700306	*LRP3*	−0.09	1.16×10^{-25}	−0.05	1.60×10^{-3}	3.86×10^{-27}	Yes
cg18884741	*RABGEF1*	−0.16	1.90×10^{-26}	−0.06	2.46×10^{-2}	2.99×10^{-26}	Yes
cg17709873	*LTA*	0.10	3.03×10^{-25}	0.06	4.43×10^{-3}	3.41×10^{-26}	Yes
cg09868035	*ABHD16B*	−0.09	3.19×10^{-25}	−0.04	6.52×10^{-3}	5.96×10^{-26}	Yes
cg01526089	*P2RX1*	−0.13	2.71×10^{-25}	−0.05	8.29×10^{-3}	7.08×10^{-26}	Yes
cg07730301	*ALDH3B1*	−0.09	5.47×10^{-25}	−0.04	5.41×10^{-3}	7.82×10^{-26}	Yes
cg26215727	*SCNN1A*	−0.09	4.52×10^{-23}	−0.05	1.09×10^{-4}	8.78×10^{-26}	Yes
cg00795812	*PDCD1*	0.09	2.11×10^{-25}	0.04	1.30×10^{-2}	1.06×10^{-25}	Yes
cg17823175	*AZU1*	−0.12	2.63×10^{-23}	−0.06	1.28×10^{-3}	6.02×10^{-25}	Yes
cg25634666	*FOLR3*	−0.10	1.14×10^{-23}	−0.05	4.40×10^{-3}	1.13×10^{-24}	Yes
cg06270401	*DYRK4*	−0.12	5.48×10^{-24}	−0.05	1.05×10^{-2}	1.75×10^{-24}	Yes
cg00974864	*FCGR3B*	−0.11	3.60×10^{-23}	−0.05	2.87×10^{-3}	2.03×10^{-24}	Yes
cg21019522	*SLC22A18*	−0.08	9.62×10^{-23}	−0.04	3.12×10^{-3}	5.84×10^{-24}	Yes
cg08368934	*GPR97*	−0.10	2.30×10^{-23}	−0.04	1.10×10^{-2}	7.35×10^{-24}	Yes
cg24211388	*AIF1*	−0.12	2.48×10^{-23}	−0.05	1.14×10^{-2}	8.30×10^{-24}	Yes
cg10758292	*DEFA1*	−0.13	2.66×10^{-22}	−0.06	2.85×10^{-3}	1.41×10^{-23}	Yes
cg05681757	*FGD4*	−0.09	6.46×10^{-21}	−0.05	2.30×10^{-4}	2.51×10^{-23}	Yes

Definition of abbreviations: EOCOPD = Early Onset COPD Study; ICGN = International COPD Genetics Network.

* Difference in mean methylation = (mean methylation subjects on steroids) − (mean methylation subjects not on steroids). Methylation values range from 0 to 1.

† *P* value from regression Model 1 (adjusted for age, sex, and FEV_1 % predicted).

‡ One-sided *P* value from regression model.

lines 1 hour after exposure to dexamethasone. The median distance of GR binding to the transcription start site of a gene was 11 kb for up-regulated genes and 146 kb for down-regulated genes. We therefore examined whether any GR binding sites (as described by Reddy and colleagues [63]) were located within ±11 kb and ±146 kb of the 511 CpG sites significant in all three models of our primary analysis. Thirty-nine sites had at least one putative GR binding site within 11 kb, whereas 230 CpG sites had at least one GR binding site within 146 kb (Table E12).

Extensive differential gene expression in blood after the administration of exogenous glucocorticoids has been described *in vivo* and *in vitro*. In additional exploratory analyses, we examined the overlap between genes with differential methylation from our study (475 unique genes from the 511 overlapping significant sites from our primary analysis) and loci with differential expression reported in the literature. Menke and colleagues (8) examined differential gene expression in blood 3 hours after a single dose of dexamethasone in healthy control subjects and in subjects with depression. Of 2,760 differentially expressed transcripts reported in their healthy control subjects, 97 transcripts (in 78 unique genes) overlapped with differentially methylated loci from our study (Table E13); 76 of these transcripts (in 60 unique genes), including transcripts for *SLC22A18*, *HIPK3*, and *LRP3*, demonstrated an anticorrelated direction of effect relative to the methylation changes in our cohort. In depressed subjects, 1,151 transcripts were differentially expressed in Menke and colleagues (8). Forty transcripts (in 36 unique genes) overlapped with our loci (Table E14); 29 of these transcripts (in 25 unique genes) demonstrated an anticorrelated direction of effect. In contrast to the acute exposure studied by Menke and colleagues (8), Lit and colleagues examined differential expression in children and adolescents with Duchenne's muscular dystrophy exposed to chronic glucocorticoids (64). Of the 524 differentially expressed probes reported, 40 transcripts (in 31 unique genes) were annotated to genes that overlap with our differentially methylated sites (Table E15); 36 of the transcripts (in 28 unique genes) demonstrated an anticorrelated direction of effect relative to the changes in methylation in our cohort. Finally, we examined publicly available gene expression data (Gene Expression Omnibus accession number GSE29342) obtained in lymphocyte cell lines exposed to dexamethasone for 1 hour, as reported by Maranville and colleagues (9). A total of 467 transcripts (in 327 unique genes) were annotated to genes identified as containing a differentially methylated CpG site from our analyses. Of these, 206 transcripts (in 155 unique genes) demonstrated nominal differential methylation (unadjusted Student's t test, $P < 0.05$). The results are summarized in Table E16. Additional studies in populations with contemporaneous functional or experimental data are needed.

Although our study used two large, independent cohorts and rigorous statistical thresholds for significance, we acknowledge the following limitations that may affect the interpretation or generalizability of the results reported herein. First, we rely upon subjects' self-report to define current steroid use and do not have details regarding the dosage, timing, or duration of exposure to therapeutic steroids. Second, we do not know the degree to which cell type heterogeneity affects our results. Third, although we assessed the methylation at over 26,000 autosomal sites in our analysis, coverage of the genome is relatively sparse, with an average of only two CpG sites per gene. Genes that have previously been implicated in the response to steroids, such as the glucocorticoid-induced transcript 1 (*GLCCI1*) (65), were not assayed on our platform. An additional concern regarding the use of an array-based platform is the unknown impact of sequence and structural variations on these platforms; this is an area of active controversy in the field and underscores the importance of technical validation through a second technology. Although it was not feasible for us to evaluate all of the statistically significant sites through pyrosequencing, all of the sites we pyrosequenced remained robustly associated with differences in mean methylation comparable to the estimates obtained through the array-based data. Finally, we acknowledge difficulty in delineating the degree to which the reported associations are confounded by the indication for steroid use (i.e., to what degree the reported epigenetic changes are associated with decreased lung function or COPD exacerbations rather than with systemic steroid use). Several highly associated sites from our analysis, including *FXYD1* and *LRP3*, were previously reported to be associated with FEV_1 % predicted and with the FEV_1/FVC ratio (66). *LRP3* was noted to be differentially expressed after systemic glucocorticoid exposure among the healthy control subjects studied by Menke and colleagues (8). Additional studies of DNA methylation in populations with longitudinal data and in populations not ascertained on lung disease are needed. Despite these limitations, we contend that our analyses support the hypothesis that systemic steroid use is associated with site-specific differential methylation throughout the genome. The CpG sites identified in our study are located in biologically plausible and previously unsuspected pathways, some of which may be relevant to the development of novel targeted therapies in the future.

Author disclosures are available with the text of this article at www.atsjournals.org.

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