

Vanin-1 expression and methylation discriminate pediatric asthma corticosteroid treatment response

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Background: There is considerable heterogeneity in asthma treatment response.

Objective: We sought to identify biomarkers of corticosteroid treatment response in children with asthma and evaluate the utility and mechanistic basis of these biomarkers.

Methods: Children (5-18 years) presenting to the emergency department with an acute asthma exacerbation were recruited and followed during hospitalization. Nasal epithelial cells were collected on presentation to the emergency department (T₀) and 18 to 24 hours later (T₁), and T₁/T₀ gene expression ratios were analyzed to identify genes associated with good and poor corticosteroid treatment response phenotypes. The utility of these genes in discriminating between systemic corticosteroid treatment response groups was then tested prospectively in a new cohort of patients. A gene candidate (vanin-1 [*VNNI*]) that consistently distinguished good versus poor response phenotypes was further studied in an experimental asthma model, and *VNNI* promoter methylation was measured by means of bisulfite pyrosequencing in patients.

Results: *VNNI* mRNA expression changes were associated with systemic corticosteroid treatment response in children with acute asthma, and *VNNI* was required for optimal response to corticosteroid treatment in an experimental asthma model. A CpG site within the *VNNI* promoter was differentially methylated between good versus poor treatment response groups, and methylation at this site correlated with *VNNI* mRNA expression.

Conclusions: We have identified a biological basis for poor corticosteroid treatment response that can be used to distinguish a subgroup of asthmatic children who respond poorly to systemic corticosteroid treatment. *VNNI* contributes to corticosteroid responsiveness, and changes in *VNNI* nasal epithelial mRNA expression and *VNNI* promoter

methylation might be clinically useful biomarkers of treatment response in asthmatic children. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Childhood asthma, biomarker, vanin-1, treatment response, phenotype

Asthma affects 25.7 million persons in the United States, including 7 million children.¹ Although asthmatic patients share similar clinical symptoms, the disease is heterogeneous.² This heterogeneity contributes to the difficulty in both studying and treating asthma. Nearly two thirds of asthmatic children reported at least 1 attack in the past year,³ highlighting the suboptimal management of childhood asthma.⁴ The frequency of absent or incomplete efficacy in asthma treatment has been estimated to be 40% to 70%.⁵

Currently, systemic corticosteroid treatment is considered the most effective medication for control of chronic asthma and rescue of acute exacerbation. Transcriptional profiling of individual host responses is a necessary and fundamental next step to better understand this individual variation and identify biomarkers of systemic corticosteroid treatment response. This approach has been used successfully to classify subphenotypes of asthma, including treatment response phenotypes.⁶⁻⁹ Previous studies have often used samples requiring bronchoscopy or induced sputum collection, which is not always feasible in clinical practice, especially in children with an acute asthma exacerbation (AAE). In the present study we used genome-wide expression profiling of nasal epithelial cells to identify genes with temporal expression patterns (before and after treatment) that consistently and reliably discriminated between systemic corticosteroid treatment response groups among children hospitalized for asthma exacerbations. Nasal epithelial cells can be readily sampled safely during an asthma attack¹⁰ and reflect changes observed in the bronchial airways of asthmatic children.¹¹ We identified and replicated a gene, vanin-1 (*VNNI*), the mRNA expression of which consistently discriminated between good and poor responders to systemic corticosteroid treatment. We pursued mechanistic studies in an experimental asthma model and in human samples.

METHODS

Subjects

After institutional review board approval, children given a diagnosis of asthma at the age of 5 to 18 years who presented to the Cincinnati Children's Hospital Medical Center (CCHMC) emergency department (ED) with an AAE were recruited. Exclusion criteria are listed in the [Methods](#) section in this article's Online Repository at www.jacionline.org. Of the 57 subjects

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Abbreviations used

AAE:	Acute asthma exacerbation
AHR:	Airway hyperresponsiveness
BALF:	Bronchoalveolar lavage fluid
CCHMC:	Cincinnati Children's Hospital Medical Center
CTCF:	CCCTC-binding factor
DMSO:	Dimethyl sulfoxide
ED:	Emergency department
HCK:	Tyrosine protein kinase
HDM:	House dust mite
ICS:	Inhaled corticosteroid
LOS:	Length of stay
PPAR γ :	Peroxisome proliferator-activated receptor γ
qRT-PCR:	Quantitative real-time PCR
SOD2:	Superoxide dismutase 2
SRGN:	Serglycin
VNN1:	Vanin-1
WT:	Wild-type

consented, 21 were hospitalized for asthma exacerbation, and 15 had genome-wide mRNA expression data for both time points. These 15 patients were used as a discovery cohort to test the association between gene expression and systemic corticosteroid treatment response. A replication cohort of 25 children hospitalized for asthma were recruited to further validate the findings from the discovery cohort. Eighteen children hospitalized for AAEs were recruited for methylation studies, 5 of whom overlapped with the replication cohort.

Subjects provided demographic, environmental, asthma trigger, and personal and family allergy and asthma history data. Parental report of current inhaled corticosteroid (ICS) controller medication (eg, mometasone [Asmanex], Merck, Whitehouse Station, NJ; fluticasone [Flovent], GlaxoSmithKline, Research Triangle Park, NC; beclomethasone dipropionate [Qvar], Teva, Petah Tikva, Israel; budesonide [Pulmicort], AstraZeneca, London, United Kingdom; fluticasone/salmeterol [Advair], GlaxoSmithKline; mometasone/formoterol [Dulera], Merck; and budesonide/formoterol [Symbicort], AstraZeneca) was also collected. To assess baseline asthma symptom severity and control, a respiratory symptom score was calculated (based on frequency of wheeze, cough, shortness of breath, and chest tightness),¹² and the age-specific Asthma Control Test score was collected.¹³

Treatment protocol and treatment response definitions

Enrolled patients were treated according to the CCHMC evidence-based treatment protocol for inpatient asthma exacerbations.¹⁴⁻¹⁶ The admitting physician determined the initial interval of albuterol treatments, which were subsequently spaced based on physician or respiratory therapist assessments. Patients received 2 mg/kg/d prednisone while hospitalized, and ICSs were continued through a mouthpiece. Length of stay (LOS) was calculated as the number of hours from the time the admission decision was made to the time the subject met clinical discharge criteria (see the [Methods](#) section in this article's Online Repository). Good responders were defined as those with an LOS of 24 hours or less, and poor responders were defined as those with an LOS of greater than 24 hours.

Nasal epithelial cell sample collection and processing

Nasal epithelial samples were collected at 2 time points from each subject: (1) in the ED (T_0) and (2) on the inpatient floor 18 to 24 hours after receiving corticosteroids in the ED (T_1). The procedure, characterization of cell types, sample processing, and RNA isolation have been described previously.¹⁰ Nasal samples collected contained more than 90% epithelial cells, which is

similar to our previous findings.¹⁰ Expression profiles were generated on the Affymetrix Human Gene 1.0 ST platform (Affymetrix, Santa Clara, Calif). Quantitative real-time PCR (qRT-PCR) was used to validate and replicate candidate genes (see the [Methods](#) section and [Table E1](#) in this article's Online Repository at www.jacionline.org). DNA isolation, bisulfite treatment, and pyrosequencing analysis of methylation levels of 5 CpG sites within the *VNN1* promoter for the methylation cohort are detailed in the [Methods](#) section and [Table E2](#) in this article's Online Repository at www.jacionline.org.

Experimental asthma model

Vnn1^{-/-} mice and age- and sex-matched wild-type (WT) BALB/c mice (see the [Methods](#) section in this article's Online Repository) were exposed to intratracheal doses of house dust mite (HDM; 20 μ g in 50 μ L of saline) or saline 3 times a week for 3 weeks, as previously described.¹⁷ Mice were treated with intraperitoneal dexamethasone (3 mg/kg in dimethyl sulfoxide [DMSO]) or DMSO (100 μ L) for the last 5 days of the 3-week model. Twenty-four hours after the last HDM challenge, airway hyperresponsiveness (AHR) was assessed (see the [Methods](#) section in this article's Online Repository), bronchoalveolar lavage fluid (BALF) was collected and processed, and inflammatory cells were quantified, as previously described.¹⁷

Statistical analysis

Detection of differentially expressed genes in the discovery set. To identify candidate genes, we performed sequential filtering to balance concerns of type I and II errors ([Fig 1, A](#)). First, we sought to identify genes reliably expressed in nasal cells (raw signal >100 in at least 2 samples). Next, we sought to identify genes responsive to treatment ($T_1/T_0 \geq 1.5$ or $T_1/T_0 \leq 0.66$; T_1/T_0 is defined as gene expression at T_1 relative to that at T_0). Then we identified genes with significant differences in T_1/T_0 ratio between the good and poor responder groups. A *P* value threshold of .05 was used because independent replication samples and complementary biologic studies minimize the risk of false-positive discovery to minimize the risk of missing true associations. We then identified those genes with a high rate of prediction accuracy (≥ 0.80) through linear discriminant analysis. To validate these results, we performed qRT-PCR.

Microarray data analysis. Microarray cell image files were analyzed with GeneSpring GX software (Agilent Technologies, Santa Clara, Calif). Probe-level measurements were subject to initial background correction and normalization by using GC-robust multi-array average. Transcript levels were normalized per chip to the 50th percentile and per gene to median intensity.

Association testing. In the discovery phase we used *t* tests (with log transformation) to identify genes between good and poor responders. Linear discriminant analysis¹⁸ was applied to find genes that best discriminated between good and poor responders.

For replication, we first examined whether there were differences between the discovery and replication cohorts that might introduce bias. Time of admission was significantly different between the discovery and replication cohorts. Thus we matched our replication cohort to the discovery cohort based on month and T_0 and T_1 times by using propensity scores.¹⁹ Importantly, gene expression profiles were not considered in the matching process. After matching, we performed *t* tests comparing the quantitative PCR results from good and poor responders. A linear regression model was fitted to examine the association between the *VNN1* mRNA expression change (T_1/T_0) and the continuous length of hospital stay (in hours) in the combined discovery and replication cohorts.

For the experimental asthma model, individual AHR, total BALF cell counts, and eosinophil percentages in mice treated with HDM plus dexamethasone were compared with and normalized by the corresponding mean value in the HDM-treated group. The difference between the WT and *Vnn1*^{-/-} groups was determined by using the nonparametric Mann-Whitney test. A *P* value of less than .05 was considered significant. Percentage reduction was used to present the corticosteroid response results.

For methylation analysis, Pearson correlation was used to measure the correlation between changes in mRNA expression (T_1/T_0) and DNA methylation (mT_1-mT_0) of *VNN1*. The Fisher exact test was used to compare

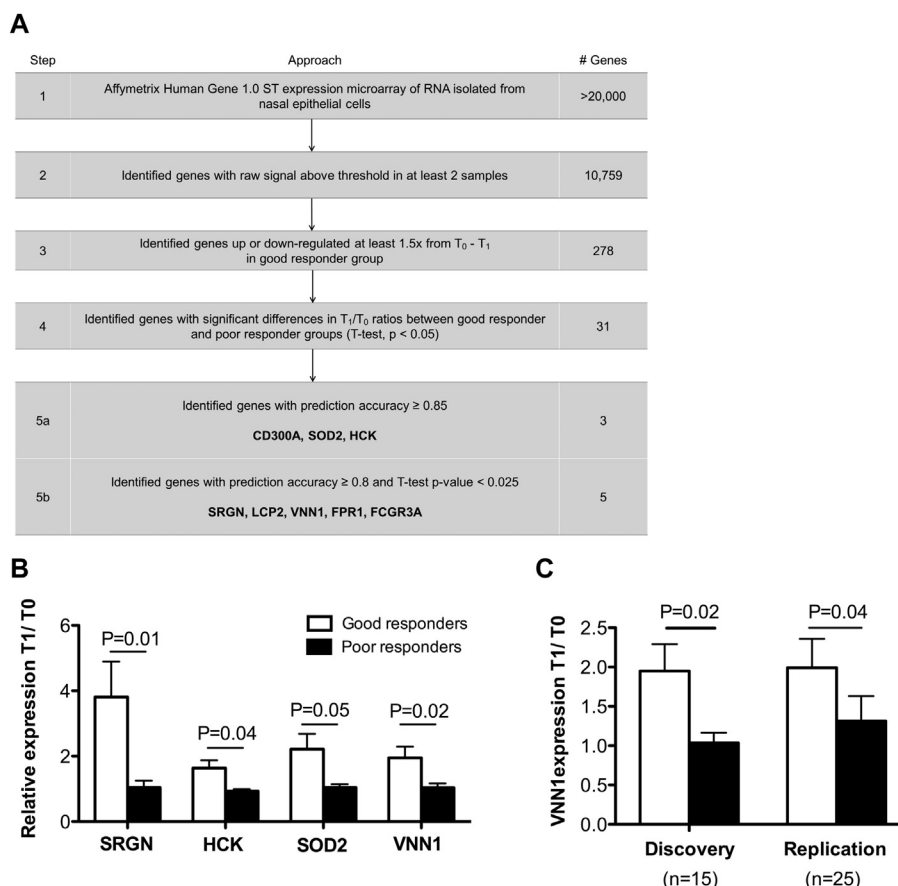


FIG 1. A, Overview of approach identifying genes with expressions in nasal epithelial cells that discriminate pediatric asthma corticosteroid treatment response. **B**, Expression of *SOD2*, *HCK*, *SRGN*, and *VNN1* was quantified by using qRT-PCR. **C**, *VNN1* expression was quantified in the discovery and replication cohorts. qRT-PCR results are expressed as average fold change at T_1 relative to T_0 , with glyceraldehyde-3-phosphate dehydrogenase as the internal control.

the difference in *VNN1* DNA methylation between patients with good and poor treatment response. A P value of less than .05 was considered significant.

RESULTS

Subjects

The discovery and replication cohorts were primarily male and nonwhite (Table I). The discovery cohort was older than the replication cohort, but within each cohort, there was no difference in age between the good and poor responders. There were no differences in individual parent-reported asthma triggers (data not shown), mean baseline respiratory symptom frequency scores, asthma control scores, or proportions of patients presenting to the ED while receiving a controller medicine between the discovery and replication cohorts (Table I). By design, the discovery and replication cohorts were similar with respect to month admitted, T_0 time, and T_1 time (Table I). Demographics and clinical features were also compared between the good and poor responders, and no differences were detected (Table II).

Identification of genes differentially expressed between good and poor responder groups in the discovery cohort

We used a multistep filtering process to identify genes (Fig 1, A). Starting with more than 20,000 genes, we identified 8 genes

that were nominally significant ($P \leq .05$) and had a prediction accuracy of 0.80 or greater. Of these 8 genes, qRT-PCR expression of superoxide dismutase 2 (*SOD2*), tyrosine protein kinase (*HCK*), serglycin (*SRGN*), and *VNN1* was significantly induced at T_1 in the good compared with the poor responder groups (Fig 1, B). *CD300A* was not detectable in most samples, and reliable results could not be achieved for lymphocyte cytosolic protein 2 (*LCP2*), formyl peptide receptor 1 (*FPR1*), and low-affinity immunoglobulin γ Fc region receptor III-A (*FCGR3A*) because of low copy numbers.

VNN1 mRNA expression change predicts corticosteroid treatment response in the replication cohort

To substantiate our findings, we recruited an independent prospective cohort to serve as a replication. *VNN1* mRNA expression was lower in the poor responder group compared with that seen in the good responder group ($P = .04$; Fig 1, C), replicating our findings from the discovery cohort ($P = .02$; Fig 1, C). Expression of *SOD2*, *HCK*, and *SRGN* was not significantly different between the treatment response groups (data not shown). We also evaluated LOS as a continuous outcome in the combined discovery and replication cohorts. We observed an inverse association between the *VNN1* mRNA

TABLE I. Description of the discovery, replication, and methylation cohorts

	Discovery cohort (n = 15)	Replication cohort (n = 25)	P value*	Methylation cohort (n = 18)
Age (y), mean (SD)	13.4 (3.8)	8.1 (2.8)	.0001†	8.7 (4.3)
Age range (y)	7.4-18.0	5.0-15.1		5.0-18.4
White (%)	33.3	8.0	.08§	11.1
Male sex (%)	73.3	64.0	.73§	66.7
Admission month, range	April-December	March-November	—	February-December
T ₀ sample time (24 h)	9.3-20.8	10.0-21.4	—	10.0-18.3
T ₁ sample time (24 h)	8.1-16.7	8.5-17.3	—	7.7-17.6
ACT score (SD)	16.0 (2.5)	16.0 (4.4)	.98‡	16.3 (4.0)
Baseline average respiratory symptom score (median) (SD)	1.5 (0.5)	1.8 (0.9)	.18‡	1.8 (0.8)
Presenting to ED on ICS controller medicine (%)	26.7	32.0	1.00§	55.6

The baseline average respiratory symptom score represents the average number of times per week the patient had coughing, wheezing, shortness of breath, or chest tightness, pain, or both. A score was assigned for each of the 4 symptoms, and then an average was taken. A higher score represents higher symptom frequency. The values of the scores for each symptom are as follows: 0, never; 1, less than 1 time per week; 2, 1 to 2 times per week; 3, 3 to 5 times per week; and 4, 6 to 7 times per week.

ACT, Asthma Control Test.

*Comparison between discovery and replication cohort populations.

†Student *t* test.

‡Mann-Whitney *U* test.

§Fisher exact test.

TABLE II. Demographics and clinical features of good and poor responders

	Good responders (n = 21)	Poor responders (n = 33)	P value*
Age (y), mean (SD)	10.2 (4.2)	9.9 (4.2)	.79‡
White (%)	20.0	15.2	.64†
Male sex (%)	70.0	69.7	.98†
Admission month range	March-December	February-November	—
T ₀ sample time (24 h)	10.0-20.5	9.3-21.4	—
T ₁ sample time (24 h)	9.8-14.8	7.7-17.6	—
ACT score (SD)	15.9 (4.0)	16.2 (3.3)	.95‡
Baseline average respiratory symptom score, median (SD)	1.7 (0.8)	1.7 (0.7)	.55‡
Presenting to ED on ICS controller medicine (%)	35.0	36.4	.92†

For a definition of the baseline average respiratory symptom score, see the footnote for Table I.

*Comparison between good and poor responders.

† χ^2 Test.

‡Mann-Whitney *U* test.

expression change (T_1/T_0) and the length of hospital stay in hours. The results from the linear regression model show that for every unit increase in *VNN1* expression, the stay length decreases by 7.7 hours ($P = .01$), further supporting our findings.

To evaluate whether the observed *VNN1* mRNA expression change was attributable to a baseline difference in *VNN1* mRNA expression at T_0 , we compared *VNN1* expression at T_0 of all patients; no significant difference was detected (data not shown). To test whether the baseline ICS exposure was a confounding factor for the corticosteroid treatment response, we compared the proportion of subjects who presented to the ED while receiving ICSs between the good and poor responders; no significant difference was detected (Table II).

Differential *VNN1* methylation in response to corticosteroid treatment in the good versus poor treatment response groups

Because changes in *VNN1* mRNA expression at T_1/T_0 were significantly associated with treatment response phenotypes in hospitalized asthmatic children and DNA methylation is an epigenetic mechanism regulating gene expression, we hypothesized that corticosteroid treatment might result in

differential *VNN1* methylation. To test this, we examined the methylation level of 5 CpG sites within the *VNN1* promoter (defined as 2 kb upstream from the transcription start site) in 20 patients (Table I), with simultaneous nasal epithelial RNA and DNA samples collected at both time points (T_0 and T_1). The methylation level at the CpG4 site (1380 bp upstream from the transcription start site) trended to decrease in the poor responders but increase in the good responders after treatment. There was a significant difference in the percentage of patients with increased versus decreased methylation at CpG4 at T_1 compared with T_0 among good versus poor responders ($P = .003$; Fig 2, A, lower panel). Furthermore, there was a positive correlation between the change in DNA methylation at CpG4 ($m_{T_1}-m_{T_0}$) and *VNN1* mRNA expression ($P = .02$, Pearson $r = 0.58$; Fig 2, B). These findings collectively suggest that methylation at the CpG4 site might be a crucial molecular event regulating *VNN1* gene expression and modulating the response to corticosteroid treatment.

Vnn1^{-/-} mice are less responsive to dexamethasone treatment in an experimental asthma model

To more directly examine the role of *VNN1* in the development of AHR and inflammation and corticosteroid treatment response,

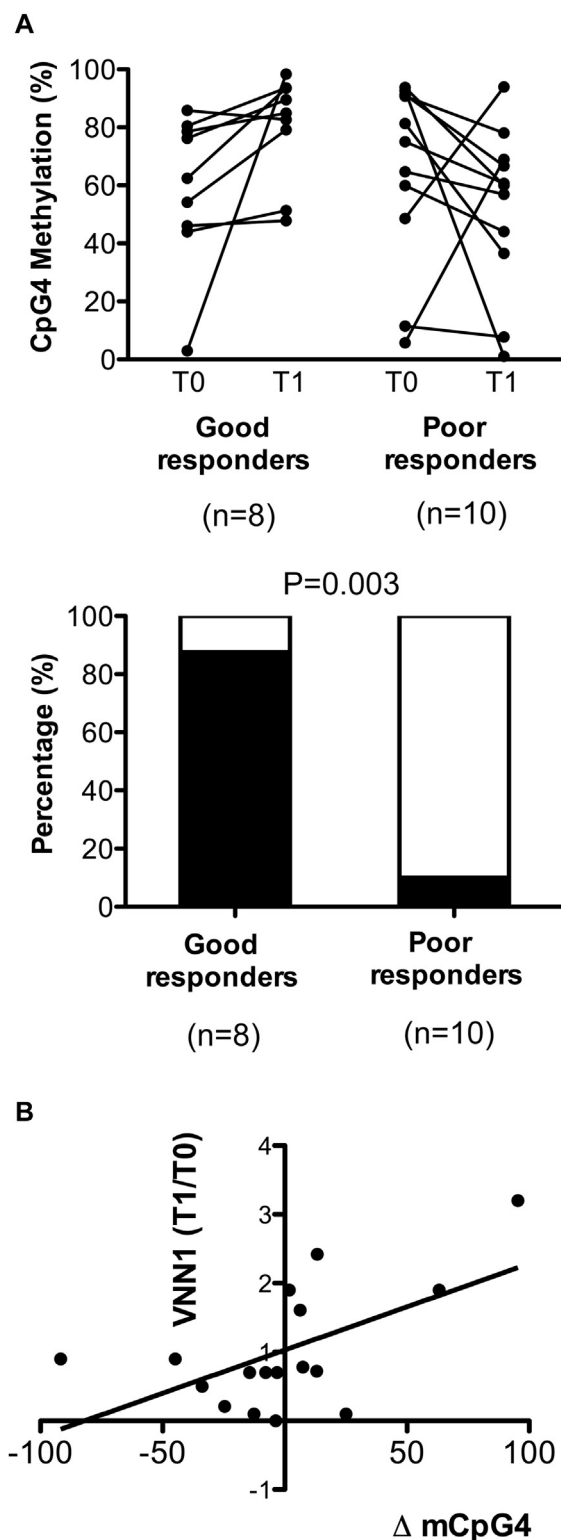


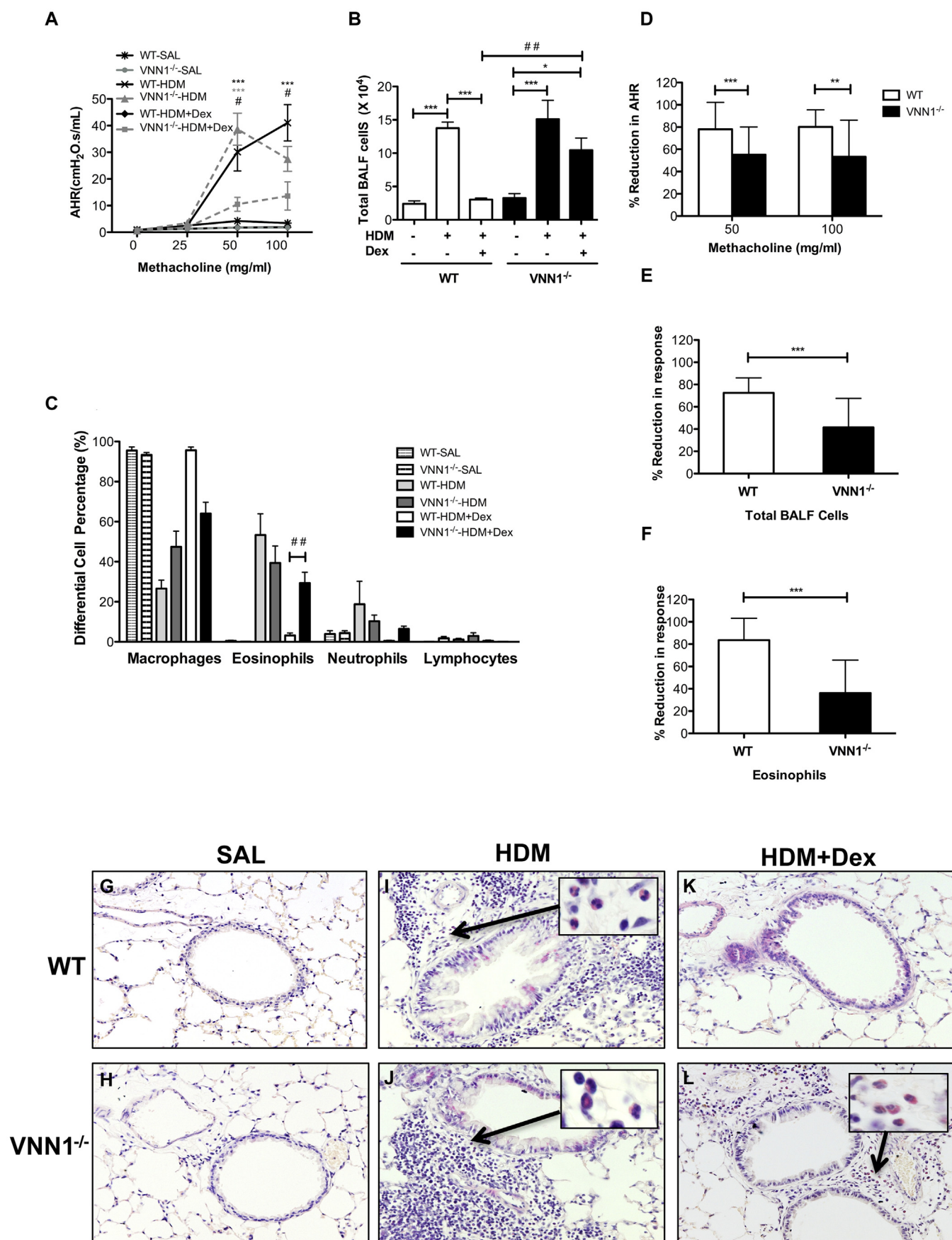
FIG 2. A, Upper panel, CpG4 percentage methylation at T₀ versus T₁ in good ($P = .07$) and poor ($P = .16$) responders. **Lower panel,** Percentage of patients with increased (black bars) and decreased (white bars) methylated CpG4 levels at T₁ versus T₀ in good and poor responders. $P = .003$. **B,** Positive correlation between the percentage change in VNN1 CpG4 methylation and fold change in VNN1 mRNA expression. $P = .02$, Pearson $r = 0.58$.

we studied *Vnn1*^{-/-} mice in an experimental asthma model. Repeated HDM exposure induced allergic airway inflammation and AHR in both WT and *Vnn1*^{-/-} mice, and the phenotype was comparable at higher doses of methacholine (50 and 100 mg/mL; Fig 3, A-C). At lower doses of methacholine (12.5 and 25 mg/mL), *Vnn1*^{-/-} mice exhibited significantly milder AHR compared with that seen in WT mice (data not shown), suggesting that VNN1 might contribute to the development of AHR. When we examined the response to dexamethasone treatment, VNN1 was required for optimal response to corticosteroid treatment. Dexamethasone significantly reduced AHR in WT mice, with an average percentage reduction of 78.1% (SD, 24.2%) and 80.2% (SD, 15.3%) at 50 and 100 mg/mL methacholine challenge, respectively (Fig 3, D). Dexamethasone also significantly alleviated airway inflammation in WT mice (Fig 3, G, I, and K), as reflected by a large reduction in total BALF cells (70.0% [SD, 13.5%]; Fig 3, E) and eosinophils (83.6% [SD, 19.6%]; Fig 3, F). In contrast, *Vnn1*^{-/-} mice were significantly less responsive to dexamethasone. AHR was reduced by an average percentage of 55.2% (SD, 24.9%) at 50 mg/mL methacholine and 53.3% (SD, 32.9%) at 100 mg/mL methacholine (Fig 3, D). Total BALF cells and eosinophils were only reduced by 40.9% (SD, 26.0%; Fig 3, E) and 36.1% (SD, 29.6%; Fig 3, F), respectively ($P < .001$). Importantly, substantial numbers of residual eosinophils remained in the lung tissue after dexamethasone treatment (Fig 3, H, J, and L) in *Vnn1*^{-/-} mice.

DISCUSSION

Children can have poorly controlled asthma for numerous reasons, including lack of compliance with medications, socioeconomic barriers, suboptimal environments with numerous asthma triggers, and biologic causes. It is important to identify the underlying causes that contribute to poorly controlled asthma in each patient so that management strategies can be personalized to achieve the best outcomes. We have identified a biological basis for poor corticosteroid treatment response that can be used to distinguish a subgroup of children with asthma who respond poorly to treatment. VNN1 mRNA expression and promoter methylation were induced after corticosteroid treatment in nasal epithelial cells of asthmatic children who respond well to corticosteroid treatment but not in those who were poorly responsive (ie, difficult to treat during an AAE). Thus these might be clinically useful biomarkers to identify children with a biologic cause for poor corticosteroid response who would benefit from a different treatment plan. Furthermore, based on animal studies, VNN1 contributes to corticosteroid responsiveness. Collectively, our studies suggest that targeting the VNN1 pathway might be a useful therapeutic strategy to enhance corticosteroid response among these difficult-to-treat patients.

Nasal epithelial expression of VNN1 discriminates between good and poor treatment responder phenotypes in children hospitalized for asthma exacerbations. Mechanistically, this is likely due to altered methylation at the CpG4 site of the VNN1 promoter in response to corticosteroids. We focused on children hospitalized for asthma because the inpatient setting provides a unique opportunity to characterize response to standardized treatment regimens for AAEs. Because nonadherence to medication was not an issue during hospitalization, differences



between patients could be largely attributed to variation in individual host response to treatment. Although our findings stem from the hospital environment, they might have broad implications for difficult-to-treat patients who are not fully responsive to corticosteroid treatment and, as a consequence, do not easily achieve asthma control.²⁰ This group of patients is the most challenging and accounts for more than 50% of health care use related to this disease.^{21,22} Whether adherence to corticosteroid medication affects *VNN1* mRNA expression and thus might affect asthma control in a chronic scenario is another interesting question worthy of further investigation.

A limitation of this study is that we did not have the data necessary to determine whether a given patient maintains his or her treatment response phenotype in subsequent exacerbations. Another limitation of our study is the lack of information regarding whether the protein level of VNN1 would be differentially expressed between the good and poor responders. The modest, yet reproducible alteration in mRNA expression might significantly affect the level or enzyme activity of VNN1 protein, which might subsequently cause variation in treatment response to corticosteroids.

Considerable advances have been made in recent years in identifying treatment response phenotypes of asthma that can help inform clinical decision making. One phenotype, which is associated with high IL-13 expression⁷ and increased circulating periostin levels, is associated with improved response to an IL-13 inhibitor²³ and anti-IL-4 receptor α therapy.²⁴ Furthermore, gene expression changes in response to ICSs in asthmatic adults identified that high baseline expression of 3 genes (calcium-activated chloride channel regulator 1 [*CLCA1*], periostin, and serpinB2) and 1 gene (FK506-binding protein [*FKBP51*]) were associated with good or poor clinical response to corticosteroids, respectively.⁶ In another study expression of 6 genes in induced sputum discriminated between eosinophilic and neutrophilic asthma and predicted response to treatment with ICSs.⁹ Our study adds to this small but expanding list of biomarkers that can inform clinical decision making. A major advantage of our study is the use of nasal epithelial cells, which can be readily and easily sampled at the point of clinical care, even in children experiencing an acute exacerbation.

Enhanced *VNN1* expression has been associated with multiple human diseases, including immune thrombocytopenia,²⁵ systemic lupus erythematosus,²⁶ and inflammatory bowel disease²⁷; however, dysregulated *VNN1* mRNA expression has not been reported in asthmatic patients. Consistent with this, the expression level of *VNN1* in mouse lungs was not altered by repeated allergen or IL-13 in experimental models of asthma.^{28,29} Our animal studies reveal that VNN1 might play dual roles in asthmatic patients. On the one hand, the absence of VNN1 prevents asthma development, as evidenced by lower AHR at lower methacholine doses.

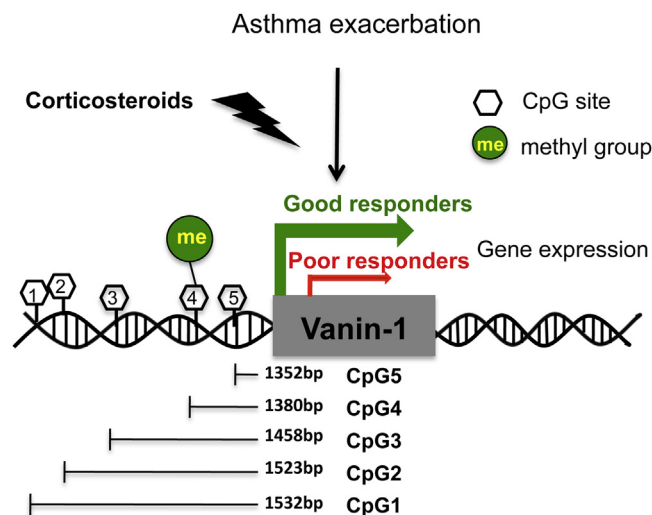


FIG 4. Proposed model. At baseline, the *VNN1* gene is modestly expressed, and this level of expression is not altered in patients with stable or acute asthma. During treatment for an AAE, corticosteroid treatment induces DNA methylation at the CpG4 site of the *VNN1* gene promoter, enhancing expression of the *VNN1* gene. Enhanced *VNN1* expression contributes to optimal response to corticosteroid treatment.

This is not surprising based on the biological function of *VNN1*. VNN1 is an epithelial ectoenzyme with pantetheinase activity that provides cysteamine/cystamine to tissues and is implicated in redox homeostasis.^{30,31} *Vnn1*^{-/-} mice have increased reduced glutathione stores and thus might be more resistant to asthma development. Consistent with this, *Vnn1*^{-/-} mice exhibit resistance to oxidative injury and reduced inflammatory responses to reactive oxygen species inducers in thymus.³¹ Nevertheless, even in the absence of VNN1, an asthma phenotype developed in the mice, including severe AHR and airway inflammation, indicating that VNN1 is not essential to asthma development. On the other hand, absence of the *VNN1* gene resulted in resistance to corticosteroid treatment that was reflected by persistent AHR and inflammatory cells in the lungs, despite treatment with dexamethasone in mice. Notably, eosinophils, a hallmark of pediatric severe therapy-resistant asthma,³² persisted in the BALF and lungs of the *Vnn1*^{-/-} mice after dexamethasone treatment. Together, our findings suggest that *VNN1* contributes to optimal host response to corticosteroid treatment.

Differential methylation of the *VNN1* promoter at CpG4 in response to corticosteroids is implicated as the mechanistic basis for the differential expression of *VNN1* in good versus poor responders. Our study supports a model whereby corticosteroid treatment induces methylation of *VNN1* at CpG4, and this leads to increased expression of *VNN1* in good responders but not poor responders (Fig 4). Methylation of CpG sites at promoter

FIG 3. A-C, Representative AHR (Fig 3, A), total BALF cell counts (Fig 3, B), and percentage differential BALF cells (Fig 3, C) in WT and *VNN1*^{-/-} mice treated with saline, HDM, or HDM plus dexamethasone (HDM+Dex; n = 6-8 per group). Fig 3, A: ****P* < .001, WT-HDM versus WT-HDM+Dex; ****P* < .001, *VNN1*^{-/-}-HDM versus *VNN1*^{-/-}-HDM+Dex; #*P* < .05, WT-HDM+Dex versus *VNN1*^{-/-}-HDM+Dex. Fig 3, B-F: **P* < .05, ****P* < .001, and ##*P* < .01. **D-F,** Percentage reduction in AHR (Fig 3, D, ***P* < .01), total BALF cell counts (Fig 3, E), and percentage BALF eosinophils (Fig 3, F) in mice treated with HDM+Dex (n = 20-28 per group). **G-L,** Data are from 4 independent experiments. Hematoxylin and eosin staining of lung tissues collected from WT (Fig 3, G, I, and K) and *VNN1*^{-/-} mice (Fig 3, H, J, and L) is shown. Also, mice treated with saline (Fig 3, G and H), HDM (Fig 3, I and J), and HDM plus dexamethasone (Fig 3, K and L) at a magnification of ×200 are shown. Insets show infiltrated eosinophils at a magnification of ×1000.

regions is generally believed to cause gene silencing.³³⁻³⁵ However, positive correlations between promoter methylation and increased gene expression have been reported.^{36,37} A recent study has verified numerous transcription factors with methylated CpG-dependent DNA-binding activity.³⁸ Furthermore, a zinc finger protein, CCCTC-binding factor (CTCF), was found to bind to the promoter region of the *VNN1* gene in small airway epithelial cells.³⁹ CTCF can function as a chromatin insulator, repressing gene expression by blocking the interaction between gene promoters and enhancers.⁴⁰ For example, CTCF binds to the imprinting control region of the *Igf2/H19* locus and silences *Igf2* expression through its enhancer-blocking activity. This activity is sensitive to DNA methylation because methylation of CpGs within the imprinting control region abolishes the binding of CTCF and thus allows *Igf2* expression.^{41,42} These findings provide possible explanations for our observation that the methylation level of the CpG4 motif was positively correlated with *VNN1* expression. The mechanism through which increased *VNN1* expression contributes to corticosteroid responsiveness remains to be elucidated. *VNN1* has been shown to promote inflammation in murine intestinal epithelium by inhibiting expression and ligand-induced activation of peroxisome proliferator-activated receptor γ (PPAR γ), an anti-inflammatory checkpoint, therefore upregulating the expression of downstream proinflammatory target genes.⁴³ We did not observe any change in PPAR γ expression in the lungs of *Vnn1*^{-/-} mice (data not shown). However, PPAR γ can be dysregulated in specific cell types, or an alternative downstream regulatory target of VNN1 other than PPAR γ might be relevant in this phenotype. *Vnn1* deficiency did not affect the development of airway inflammation in an experimental asthma model, but it remains possible that the resolution of the asthma phenotype might be affected.

In summary, nasal *VNN1* expression might be a clinically useful biomarker to identify a subset of children with difficult-to-treat asthma with a biologic cause for poor corticosteroid response. Targeting the *VNN1* pathway in this subset might be a useful therapeutic strategy to enhance corticosteroid response.

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Key messages

- *VNN1* mRNA expression is induced after systemic corticosteroid treatment in asthmatic children who respond well to treatment but not in those children who are poor treatment responders.
- *VNN1* is required for optimal response to corticosteroid treatment in experimental asthma.
- *VNN1* promoter methylation and expression are novel biomarkers of treatment response in asthmatic children.

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METHODS

Subjects

Exclusion criteria were as follows: (1) use of oral, nasal, or intravenous steroids, as well as nebulized or inhaled steroids, with a face mask within the past 14 days; (2) nasal malformations, tumors, or nasal obstruction that precludes sampling; (3) bleeding diathesis; (4) comorbid lung condition; (5) history of discharge home after birth from the neonatal intensive care unit or nursery on supplemental oxygen; (6) dependence on oral steroids or an immunosuppressive agent for a medical condition other than asthma; or (7) history of a congenital cardiac anomaly, heart lesion, or both requiring medication or surgery. Patients were discharged home when (1) oxygen saturation was greater than or equal to 91% on room air for at least 6 hours, (2) there was no evidence of respiratory distress, and (3) symptom control was achieved with albuterol nebulizer treatments spaced every 4 hours for 8 hours.

qRT-PCR analyses

Gene-specific primers were designed with Primer-BLAST software (National Center for Biotechnology Information), in which at least 1 intron was spanned in the genomic sequence to ensure that mRNA-derived products were amplified and the contamination of genomic products was minimized. The sequences of primers for the target genes are listed in Table E1. One to 2 µg of total RNA was used for cDNA synthesis per sample (SuperScript II cDNA synthesis kit; Invitrogen, Carlsbad, Calif). RT-PCR analysis was conducted with the iCycler (Bio-Rad Laboratories, Hercules, Calif) by using the iQ SYBR Green Supermix Taq polymerase mix (Bio-Rad Laboratories). The amount of double-stranded DNA product was indicated by the intensity of SYBR Green fluorescence and measured at the end of each extension cycle. The results were expressed as average fold changes in gene expression relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

DNA isolation, bisulfite treatment, and pyrosequencing analysis

Genomic DNA was extracted from nasal epithelial samples with the Allprep DNA/RNA Micro Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Two hundred nanograms of DNA from each sample

was bisulfite modified with the EZ DNA Methylation Kit (Zymo Research, Irvine, Calif). For pyrosequencing, a 2-step PCR reaction was performed with primer pairs designed to amplify the target region specifically, with the reverse primer biotinylated. Primer sequences used for the bisulfite pyrosequencing reactions are listed in Table E2. The chromosomal coordinates in the University of California at Santa Cruz February 2009 Human Genome Assembly for each CpG site were shown. The annealing temperature used for both PCR reactions was 50°C. Pyrosequencing analysis was conducted with a Pyromark Q96 MD (Qiagen) at the CCHMC Pyrosequencing Laboratory for Genetic and Epigenetic studies, and the DNA methylation percentage was determined by using Qiagen Pyromark CpG software, version 1.0.11.

Experimental asthma model

VNNI^{-/-} mice were kindly provided by Dr Ruslan Medzhitov at Yale School of Medicine with permission from Dr Franck Galland at Université de la Méditerranée, France.^{E1} Age- and sex-matched WT BALB/c mice were purchased from Harlan Laboratories (Indianapolis, Ind). All mice were housed in a specific pathogen-free environment in the animal facility at CCHMC. All animal procedures and protocols were approved by the Animal Care and Use Committee at the Cincinnati Children's Hospital Research Foundation.

Mice were exposed to intranasal doses of HDM (20 µg in 50 µL of saline) or saline (0.9% NaCl, 50 µL; control group) 3 times a week for 3 weeks, as previously described.^{E2} Mice were treated with intraperitoneal dexamethasone (3 mg/kg in DMSO) or DMSO (100 µL) for the last 5 days of the 3-week model. AHR was assessed 24 hours after the last HDM challenge by using a flexiVent system (SCIREQ, Montreal, Quebec, Canada), as previously described.^{E2} BALF was collected and processed, and inflammatory cells were quantified and analyzed, as previously described.^{E2}

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TABLE E1. Primer sequences used for qRT-PCR

Gene	Accession no.	Sense	Antisense
<i>SRGN</i>	NM_002727	CCTGGTTCTGGAATCCTCA	TCGAACATTGGTCCTTTTCTT
<i>SOD2</i>	NM_001024465	TTACAGCCCAGATAGCTCTT	ATGGCTTCCAGCAACTC
<i>HCK</i>	NM_001172133	TCTGCATCCCTGGTGTGTAA	AAGTTGATGGCTTCAGGAG
<i>VNN1</i>	NM_004666	CTCAGTGGCACTTTCGG	CAACCTCCCAAACAGAGTTAC
<i>GAPDH</i>	NM_002046	GGGGAAGGTGAAGGTCGGAGTCA	AGCCTTGACGGTGCCATGGAAT

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

TABLE E2. Primer sequences used for bisulfite pyrosequencing

Sense		Antisense
PCR		
Internal	AGGTGTTGTTTTTTTAATTATATTA	5Biosg/CTTAACTCCAAAAAATTCACTTCC*
External	TTTAAAGATGGTTTTTAATGTTTATTG	CCCAAAATCTCTTCACAAACTAC
Chromosomal coordinates†		Sequence
Sequencing		
CpG1, 2	chr6: 133036726 and 133036717	AGGTGTTGTTTTTTTAATTATATTA
CpG3	chr6: 133036652	GTTGTTTGGTTTTAAGGGAATTAG
CpG4, 5	chr6: 133036574 and 133036546	TTGGGTAATAATTAATGAGGTTTTG

*5'-Biotin.

†The chromosomal coordinates of each CpG site were retrieved from the University of California at Santa Cruz February 2009 Human Genome Assembly.