

Usefulness of PBMCs to predict clinical response to corticosteroids in asthmatic patients

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Background: Blood tests are needed to identify steroid-resistant (SR) asthmatic patients early so that they can be managed with alternative anti-inflammatory therapy.

Objective: We sought to assess the usefulness of peripheral blood to predict steroid response in asthmatic patients.

Methods: Nineteen asthmatic patients with FEV₁ of less than 80% of predicted value were classified as SR or steroid sensitive (SS) based on change in lung FEV₁ percentage after 7 days of oral prednisone. Blood was collected at baseline (visit 1) and 30 days after prednisone administration (visit 3). PBMCs were cultured for 4 hours with or without 10⁻⁷ mol/L

dexamethasone, and cellular response to dexamethasone was determined by using real-time PCR based on expression analysis of steroid-regulated genes. Suppression of PHA-induced T-cell proliferation by dexamethasone was assessed.

Results: Prednisone significantly improved FEV₁ percentages in SS asthmatic patients (mean $\pm$ SE: 17.5% $\pm$ 2.4%) but not SR asthmatic patients (0.8% $\pm$ 2.0%, $P < .001$). Before prednisone treatment, mitogen-induced kinase phosphatase 1 ($P = .01$) and IL-8 mRNA ($P < .05$) levels were significantly higher in PBMCs from SR asthmatic patients. TNF- α ($P < .05$) and IL-8 fold suppression by dexamethasone ($P < .05$) were significantly reduced in PBMCs from SR asthmatic patients. The expression of glucocorticoid receptor (GCR) β , but not GCR- α , was significantly increased in PBMCs of SR asthmatic patients ($P = .01$). The dexamethasone inhibitory concentration of 50% for PBMC proliferation was significantly higher for SR asthmatic patients ($P < .05$). These markers no longer differed between groups in PBMCs 30 days after prednisone administration. The composite score of assays at baseline before prednisone was significantly different between SR and SS asthmatic patients ($P < .001$).

Conclusions: PBMCs from SR asthmatic patients have higher baseline mitogen-induced kinase phosphatase 1, IL-8,

and GCR- β mRNA levels; have a lower GCR- α /GCR- β mRNA ratio; are less responsive to suppression of TNF- α and IL-8 by dexamethasone; and require more dexamethasone to suppress T-cell proliferation compared with SS asthmatic patients. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Asthma, corticosteroids, glucocorticoid receptor

Corticosteroids are recommended for the treatment of persistent asthma.^{1,2} However, steroid resistance is increasingly being recognized in the treatment of asthma.³⁻⁶ Biomarkers are needed to identify steroid-resistant (SR) asthmatic patients before prolonged courses of systemic steroids, which can lead to serious adverse events, and allow the early introduction of alternative anti-inflammatory therapy before progression of airways disease occurs.⁷

Blood sampling, rather than bronchoscopy, is a more practical approach for identification of SR asthmatic patients in clinical practice. Gene expression profiling is a simple and sensitive method by which patients' PBMCs can serve as markers that can parallel clinical corticosteroid responsiveness. On a cellular level, corticosteroids interact with the glucocorticoid receptor (GCR) α , which acts as a ligand-dependent transcription factor.^{8,9} Transcription of a number of steroid-regulated targets, such as mitogen-induced kinase phosphatase 1 (MKP-1), is directly induced by GCR- α (transactivation).⁸ Transcription of proinflammatory cytokines, such as IL-8 and TNF- α , is inhibited by GCR- α through interaction with other transcriptional factors (transrepression).^{8,10} GCR- β is a GCR isoform that does not bind steroids, has a distinct set of targets regulated through transcription,^{11,12} and has been shown to be increased under SR conditions.^{11,13-15}

In the current study we examined whether PBMC response to corticosteroids *in vitro* can be used to predict patients' clinical responses to corticosteroids. Although some of these assays have been previously used in mechanistic reports,^{10,13-20} these markers have not been previously used to prospectively examine whether they can predict patients' clinical responses to corticosteroids before treatment with oral prednisone.

METHODS

Subjects

Nineteen adult asthmatic patients with a baseline FEV₁ percent predicted value of less than 80% were recruited. All patients selected had lack of asthma control, as demonstrated based on the number of nocturnal events per month, rescue short-acting β -agonist use, and controller medication use. None of the subjects had received systemic glucocorticoid therapy for at least 6 months

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Abbreviations used

GCR: Glucocorticoid receptor
 IC₅₀: Inhibitory concentration of 50%
 ICS: Inhaled corticosteroid
 MKP-1: Mitogen-induced kinase phosphatase
 SR: Steroid resistant
 SS: Steroid sensitive

before the study. Some of the study participants were not taking inhaled corticosteroids (ICSs) at the time of enrollment but had used ICSs in the past. None of the study participants were smokers. The study outline is shown in Fig 1. At visit 1, spirometric testing was done. Patients were then subjected to a 1-week course of 40 mg/d oral prednisone. At visit 2 (7 days after the initial visit), the corticosteroid response of asthmatic patients was classified clinically based on change in prebronchodilator morning FEV₁ percent predicted after prednisone burst. Asthmatic patients were defined as SR if they had less than 10% improvement in FEV₁ and as steroid sensitive (SS) if they had more than 12% improvement in FEV₁.

At visit 2, all asthmatic patients who were found to be SR underwent a prednisone pharmacokinetics study. Patients' blood samples were obtained at baseline and 2 and 6 hours after oral prednisone administration (40 mg/1.73 m² body surface area) and analyzed by using HPLC for plasma prednisolone concentrations. The prednisolone concentration at 2 hours was classified as abnormal if less than 400 ng/mL was detected. Prednisolone clearance (estimated log clearance = $2.66 + [6 \text{ h after dose concentration}] [-0.00167]$) of greater than 255 mL/min/1.73 m² was considered rapid.²¹ Only subjects with normal prednisone pharmacokinetics profile were included in the study (n = 19 asthmatic patients). Patients returned 30 days after the prednisone burst (visit 3). In this study blood was collected from study subjects at the initial visit before the oral prednisone burst (visit 1) and 30 days after the prednisone burst (visit 3). Eleven SR and 8 SS asthmatic patients were recruited. Patients' ICS doses were calculated in budesonide equivalents. This study was approved by the Institutional Review Board at National Jewish Health, Denver, Colorado.

Specimen collection and culture

PBMCs were isolated from heparinized blood by means of Ficoll-Hypaque gradient centrifugation.²⁰ Cells were lysed immediately in RNA lysis buffer for the analysis of gene expression at baseline or cultured for 4 hours with or without 10⁻⁷ mol/L dexamethasone. The cells were then harvested and stored in the RNA lysis buffer at -80°C.

Real-time PCR

Total RNA was extracted by using the RNeasy Mini Kit (Qiagen, Valencia, Calif), reverse transcribed into cDNA, and analyzed by means of real-time

TABLE I. Patients' characteristics*

	SR asthma (n = 11)	SS asthma (n = 8)†
Age (y), mean ± SE	33.1 ± 4.7	31.5 ± 5.2
Sex (male/female)	3/8	2/6
Race (C/AA/other)	2/6/3	5/1/2
Baseline FEV ₁ (% predicted), mean ± SE	67.0 ± 3.3	65.8 ± 2.2
FEV ₁ (% reversal with albuterol), mean ± SE	17.3 ± 2.8‡	30.3 ± 3.8
FEV ₁ (% change after prednisone burst), mean ± SE	0.8 ± 2.0§	17.5 ± 2.4
Corticosteroid medications		
ICS/LABA	6	4
ICS	3	0
None	2	4

LABA, Long-acting β-agonist.

*In this study blood was collected at visits 1 and 3 for the *in vitro* steroid response assays.

†One SS asthmatic patient gave blood only at visit 1.

‡P < .05.

§P < .001 compared with SS asthmatic patients.

||For the SR and SS asthmatic patients who received an ICS/long-acting β-agonist or an ICS, the mean ± SD value of the ICS dose in budesonide equivalents was 408 ± 107 and 840 ± 188 μg, respectively.

PCR with the dual-labeled fluorogenic probe method (ABI Prism 7000 sequence detector; Applied Biosystems, Foster City, Calif). The expression of MKP-1, IL-8, TNF-α, GCR-α, and GCR-β was analyzed. Relative gene expression levels were calculated and normalized to the corresponding levels of the housekeeping gene (18sRNA). Standard curves for all targets were generated by using the fluorescent data from 2-fold serial dilutions of 500 ng of total RNA of the target sample. GCR-α and GCR-β standard curves were generated from 10-fold serial dilutions of the respective GCR plasmids, as previously described.²²

Proliferation assay

PBMCs were stimulated with 1 μg/mL PHA and cultured with or without 10⁻¹⁰ to 10⁻⁶ mol/L dexamethasone, and cell proliferation was assessed at 72 hours based on tritiated thymidine incorporation. The degree of cellular sensitivity to corticosteroids was expressed as the inhibitory concentration of 50% (IC₅₀), which is herein defined as the molar concentration of dexamethasone that inhibited the proliferation of PHA-stimulated lymphocytes to 50% of the level seen in media alone.²⁰

Statistical analysis

Analysis was conducted with GraphPad Prism, version 4.03 (GraphPad Software, Inc, La Jolla, Calif). Data were analyzed by using unpaired *t* tests

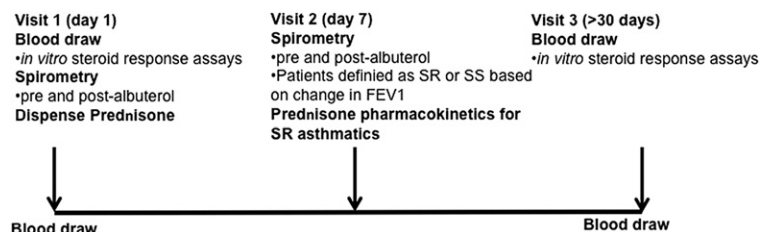


FIG 1. Outline of study design. Asthmatic patients with an FEV₁ percent predicted value of less than 80% were recruited into the study. Blood was drawn at the time of initial oral prednisone administration (visit 1) and 30 days after oral prednisone treatment (visit 3). The patients returned 7 days after oral prednisone treatment for spirometry (visit 2). At this visit, patients were defined as SR if less than 10% improvement in FEV₁ percent predicted occurred and as SS if greater than 12% improvement in lung volume occurred. Glucocorticoid pharmacokinetics screening was done for all SR asthmatic patients. Only patients with a normal prednisone pharmacokinetics profile were included in the study.

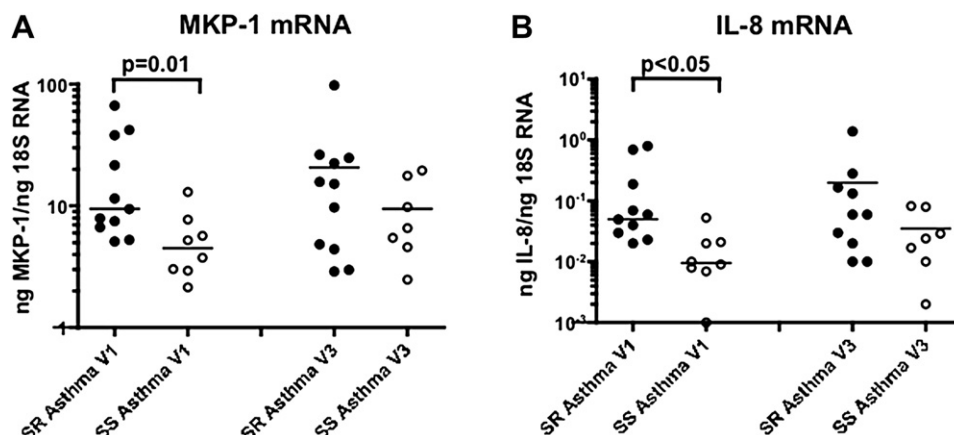


FIG 2. MKP-1 and IL-8 gene expression by PBMCs of SR and SS asthmatic patients, as detected by means of real-time PCR before (visit 1 [V1]) and 30 days after (visit 3 [V3]) prednisone burst. MKP-1 (A) and IL-8 (B) mRNA expression is significantly higher in freshly isolated PBMCs from SR than SS asthmatic patients at visit 1 but not visit 3. The difference between the SR and SS asthma groups was analyzed by using the non-parametric Mann-Whitney test.

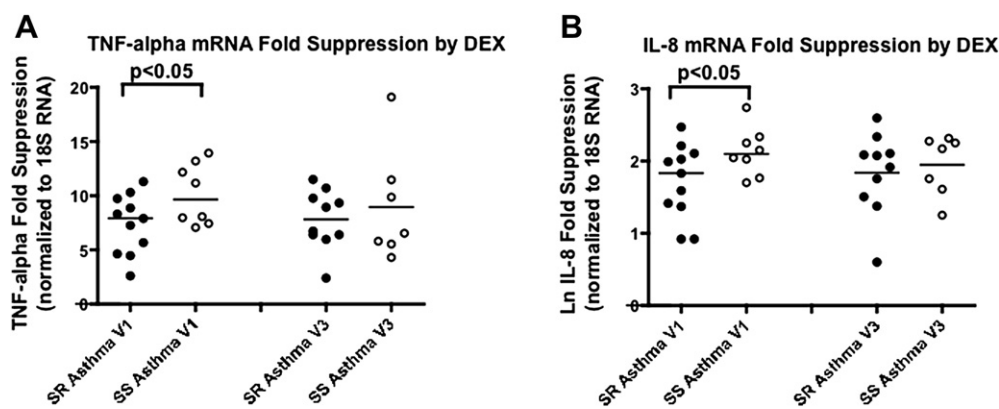


FIG 3. Changes in the expression of steroid-regulated targets in PBMCs from SR and SS asthmatic patients after *in vitro* treatment with dexamethasone (DEX) before (visit 1 [V1]) and 30 days after (visit 3 [V3]) prednisone burst. Significantly reduced suppression of TNF- α (A) and IL-8 (B) mRNA by dexamethasone is observed in PBMCs from SR asthmatic patients than in PBMCs from SS asthmatic patients, as detected by means of real-time PCR at visit 1 but not visit 3. The difference between the SR and SS asthma groups was analyzed by using the unpaired *t* test.

(2-group comparisons), and the Wilcoxon nonparametric test was applied to data with a non-Gaussian distribution. The difference in composite score between the study groups was calculated by using the exact permutation test.²³ Differences were considered significant at a *P* value of less than .05. The effect of ICS treatment on the responses in the SR and SS asthma groups was assessed by using general linear (regression) models in which terms for ICS use and an interaction between ICS use and type of asthma (SR/SS asthma groups) were included.

RESULTS

Study subjects

Patients' characteristics for the study participants are provided in Table I. After 7 days of oral prednisone treatment, SS asthmatic patients had a substantial improvement in lung function, with a Δ FEV₁ (mean $\pm$ SE) of $17.5\% \pm 2.4\%$. However, almost no change in FEV₁ percent predicted was noted in the SR asthma group, with a Δ FEV₁ (mean $\pm$ SE) of $0.8\% \pm 2.0\%$ ($P < .001$ compared to SS asthmatic patients). Both SR and SS asthmatic patients demonstrated greater than 12% improvement in FEV₁ percent

predicted after albuterol administration, which is consistent with the American Thoracic Society definition of asthma (Table I).²⁴

Markers of *in vitro* responsiveness to corticosteroid treatment before prednisone burst

PBMCs were isolated from patients' blood before their prednisone burst (visit 1) and 30 days after their prednisone burst (visit 3). The expression of selected gene targets was analyzed by means of real-time PCR at baseline, as well as after treating cells with 10^{-7} mol/L dexamethasone for 4 hours. MKP-1 was selected as a well-known glucocorticoid transactivation target.^{25,26} IL-8 and TNF- α were selected as targets because they are regulated by glucocorticoids through transrepression.^{4,27} The relative expression of GCR- α and GCR- β , the 2 major GCR isoforms, was analyzed in freshly isolated PBMCs as well. GCR- β is a GCR isoform that antagonizes GCR- α and is therefore another likely biomarker candidate.²⁸

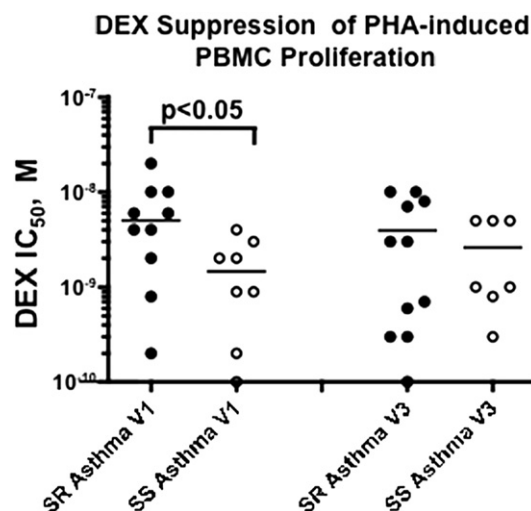


FIG 4. PBMCs from SR asthmatic patients compared with SS asthmatic patients require significantly more dexamethasone (DEX) to suppress PHA-induced T-cell proliferation at visit 1 (V1) but not visit 3 (V3). The difference between the SR and SS asthma groups was analyzed by using the nonparametric Mann-Whitney test.

It was found that at baseline, MKP-1 (Fig 2, A) and IL-8 (Fig 2, B) mRNA expressions were significantly higher in PBMCs from SR asthmatic patients than SS asthmatic patients ($P = .01$ and $P < .05$, respectively). Significant differences between PBMCs from SR and SS asthmatic patients in response to 4 hours of dexamethasone treatment were observed. TNF- α and IL-8 mRNA were both less suppressed by dexamethasone in PBMCs from SR patients than in PBMCs from SS patients ($P < .05$ for each of these cytokines. Fig 3).

As shown by using mitogen-induced proliferation assays, in the presence of different concentrations of dexamethasone, T cells from SR asthmatic patients required a significantly higher concentration of dexamethasone to suppress proliferation than T cells from SS patients ($P < .05$, Fig 4). GCR- β ($P = .01$; Fig 5, B), but not GCR- α (Fig 5, A), expression was significantly increased in PBMCs from SR asthmatic patients, resulting in a significantly lower molar ratio of GCR- α to GCR- β ($P < .01$; Fig 5, C).

Markers of *in vitro* responsiveness to corticosteroid treatment 30 days after prednisone burst

The same panel of *in vitro* assays was run for the blood samples from the same patients collected 30 days after their prednisone burst. The PBMC markers that were significantly different between SR and SS asthmatic subjects at visit 1, before oral prednisone treatment, could no longer distinguish steroid responders from nonresponders at visit 3 (Figs 2-5, visit 3 data).

Composite score of peripheral blood markers to distinguish SR versus SS asthma

The expression levels of the above targets (ie, MKP-1, IL-8, TNF- α fold suppression, IL-8 fold suppression, dexamethasone IC₅₀ to suppress PHA-induced T-cell proliferation, GCR- β , and GCR- α /GCR- β ratio) were ranked into quartiles, and each value for the selected target was assigned a score of 1 through 4

depending on the quartile in which their value fit. The score of 1 was assigned for the least responsive quartile (ie, highest MKP-1, IL-8 baseline, GCR- β expression, dexamethasone IC₅₀ for the suppression of the PHA-induced T-cell proliferation, lowest TNF- α and IL-8 fold suppression by dexamethasone, and lowest GCR- α /GCR- β ratio), and the score of 4 was given for the most responsive quartile. The scores for each target were summed up, and a composite score was calculated. Because 7 targets were scored, 7 and 28 were considered the least and the most steroid-responsive scores, respectively. With this scoring strategy, it was found that the score for the SR asthma group was (mean $\pm$ SD) 14.4 ± 4.1 and the score for the SS asthma group was 22.3 ± 3.0 at visit 1. By using exact the permutation test (rearranging observations between the SR and SS asthma groups), it was found that composite scores differed at visit 1 ($P < .001$) but not at visit 3 ($P = .27$).

DISCUSSION

It has been demonstrated that a subset of asthmatic patients do not respond to treatment with corticosteroids.^{3,5,6,29} National¹ and international² guidelines do not adequately address this issue, except to "step up" therapy. It is estimated that 15% to 25% of asthmatic patients do not respond to oral corticosteroid treatment.⁵ Currently, there is a void in our understanding of the basic mechanisms of corticosteroid resistance, leading to uncertainty on how best to improve therapeutic response in patients with SR asthma.^{4-6,29,30} It is important to identify patients whose symptoms do not improve with oral steroid treatment early because these patients can be subjected to unwanted side effects from systemic steroids administered long-term.^{4,5,8} Ironically, these SR asthmatic patients do not have therapeutic benefit because corticosteroids do not shut off their ongoing airway inflammation. Our recent work has demonstrated that under SR conditions (defined clinically by lack of change in lung function after oral prednisone treatment), there is decreased GCR- α -mediated transactivation, transrepression, or both and increased abundance of the GCR- β isoform that antagonizes GCR- α function.^{7,11,13-19,22} In the current study we examined the expression and dexamethasone regulation of an array of markers that measure the spectrum of branch points involved in transactivation and transrepression to determine whether they can predict clinical steroid responsiveness.

Patients' clinical responses to corticosteroids in this study were defined based on change in lung function after a 1-week course of oral prednisone. The expression of the selected markers was examined in the PBMCs of asthmatic patients before prednisone burst and 30 days after prednisone burst. The following markers were significantly different between the SR and SS asthma groups before prednisone burst: at baseline, PBMCs from SR asthmatic patients had higher MKP-1, IL-8, and GCR- β mRNA levels and a lower GCR- α /GCR- α mRNA ratio; TNF- α and IL-8 fold suppression by dexamethasone was significantly lower in PBMCs from SR than SS asthmatic patients. Significantly more dexamethasone was required to suppress T-cell proliferation in PBMCs from SR than from SS asthmatic patients. As emphasized, the goal of the current project is to predict patients' responsiveness to the oral prednisone treatment without the actual need for patients to undergo an oral prednisone burst. Our experience with recruitment of patients for this study indicates that many patients are opposed to oral prednisone bursts. Although peripheral

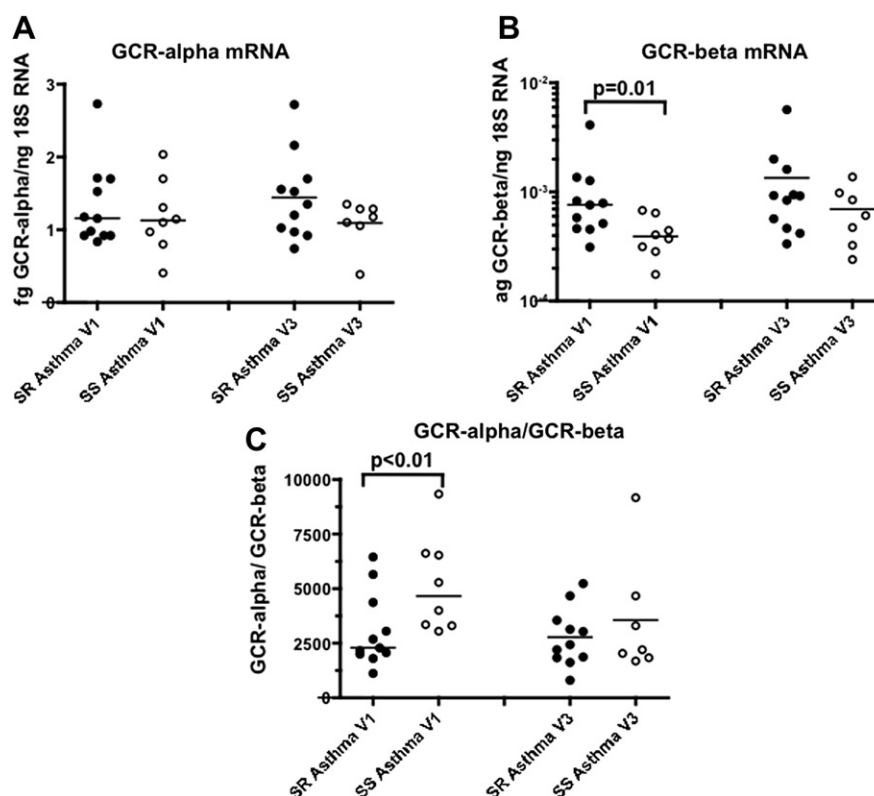


FIG 5. The expression of GCR isoforms in PBMCs from SR and SS asthmatic patients before (visit 1 [V1]) and 30 days after (visit 3 [V3]) prednisone burst, as detected by using real-time PCR. GCR- β (**B**), but not GCR- α (**A**), mRNA expression is significantly increased in freshly isolated PBMCs of SR asthmatic patients, resulting in a significantly lower molar ratio of GCR- α /GCR- β mRNA copies in the cells from SR asthmatic patients compared with that seen in SS asthmatic patients (**C**) at visit 1 but not visit 3. The difference between the SR and SS asthma groups was analyzed by using the nonparametric Mann-Whitney test.

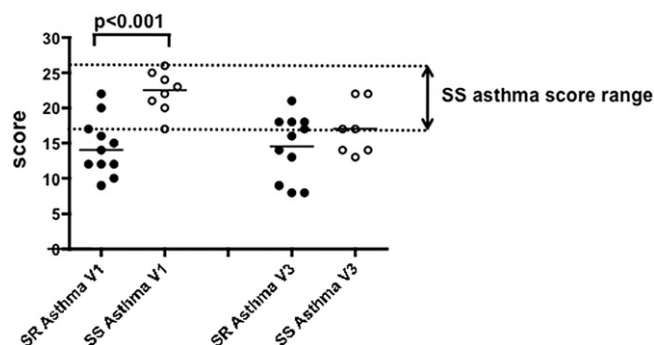


FIG 6. The composite score for steroid response assays in the SR and SS asthma groups before (visit 1 [V1]) and 30 days after (visit 3 [V3]) prednisone burst. The expression levels of the various individual targets used in this study (ie, MKP-1, IL-8, TNF- α fold suppression, IL-8 fold suppression, dexamethasone inhibitory concentration of 50% to suppress PHA-induced T-cell proliferation, GCR- β , and GCR- α /GCR- β ratio) was rank ordered into quartiles, and each value for the selected target was assigned a score of 1 through 4 depending on the quartile in which the value fit. A score of 1 was assigned for the least responsive quartile (ie, highest MKP-1, IL-8 baseline, GCR- β expression, dexamethasone inhibitory concentration of 50% for the suppression of the PHA-induced T-cell proliferation, lowest TNF- α , IL-8 fold suppression by dexamethasone, and lowest GCR- α /GCR- β ratio), and the score of 4 was given for the most responsive quartile. The scores for each target were summed, and a composite score was calculated. Because 7 targets were scored, 7 and 28 were considered the least and most steroid-responsive scores, respectively. The difference in composite score between the SR and SS asthma groups was calculated by using the exact permutation test.

blood biomarkers are often questioned for their relevance while assessing lung disease, we are not aware of any lung-specific markers that can predict patients' steroid responsiveness either. This study was done in a small group of patients. The results of the study need to be validated in a larger independent study to assess their variability and validity.

To our knowledge, there have only been 2 previous reports attempting to develop biomarkers that can be used as predictors for the clinical response to corticosteroids in asthmatic patients. In contrast to our current study, these 2 studies used ICS treatment to determine patients' clinical responses to corticosteroids. One study performed gene expression profiling in inflammatory cells of SR and SS asthmatic patients by using PBMCs in search of genes that accurately predict responders and nonresponders to inhaled steroids.³¹ The latter study focused on genes that were up-regulated or downregulated by *in vitro* IL-1 β /TNF- α stimulation and reversed with steroid treatment in the SS asthma group. Through analysis of the most extreme phenotypes of patients, 15 genes were selected that predicted steroid responses of SR and SS asthmatic patients with 84% accuracy. There was no follow-up to this study. We previously attempted to use the identified set of genes in the study by Wenzel³⁰ and were not able to predict patients' responses from the gene expression profile (data unpublished).

The other study²⁹ using gene array expression profiling of epithelial brushings from subjects with asthma before and after treatment with either an ICS (fluticasone) or placebo in a

double-blind randomized controlled trial identified 2 evenly sized and distinct subgroups, “T_H2-high” and “T_H2-low” asthma, which were no longer distinguishable for the T_H2 expression profile after treatment with ICSs. These subgroups differed significantly in expression of IL-5 and IL-13 in bronchial biopsy specimens and in airway hyperresponsiveness, serum IgE levels, blood and airway eosinophilia, subepithelial fibrosis, and airway mucin gene expression before ICS treatment. The lung function improvements expected with ICSs were restricted to the T_H2-high asthma group only. Because the T_H2-high profile was no longer distinguishable after ICS treatment, the potential markers identified can only be used for steroid-naïve patients.

A recent gene expression profiling study³² examined the expression of steroid-regulated genes in PHA-stimulated PBMCs in healthy control subjects. Steroid responders and nonresponders were defined based on the degree of morning cortisol suppression in response to overnight oral prednisone treatment. BMPRII was found as one of the most differentially regulated transcripts in response to 6 hours of dexamethasone treatment in 72-hour stimulated PBMCs from steroid responders and nonresponders. No significant difference in bone morphogenetic protein receptor type II (BMPRII) expression was observed in our study groups at visits 1 and 3, and therefore measurement of BMPRII would not improve the sensitivity of detection of SR asthmatic patients (see Fig E1 in this article's Online Repository at www.jacionline.org).

In our study it was found that selected targets tested could be used only in the subjects before oral prednisone exposure because 30 days after oral prednisone burst, no differences in the expression and dexamethasone regulation of these targets was observed. Therefore the data demonstrate that this assay would not work for patients with severe asthma who are taking chronic oral steroids because they likely have other reasons beyond immune steroid resistance for their lack of well-controlled symptoms (eg, airway remodeling). Nevertheless, our current study is important in that a composite score was developed to predict SR asthma before use of oral prednisone and might therefore be clinically useful. Given the small sample size, we cannot speculate about the reasons for the difference in the proposed markers before and 30 days after. There appears to be more variability in the 30-day assay for the markers evaluated. No difference in the expression of the selected set of markers in subjects taking or not taking ICSs was noted. Given the small sample sizes, future studies are required to assess the contribution of ICS treatment on the steroid response variables analyzed.

In this study we developed a composite score for the markers tested. The expression of each marker was given a score of 1 through 4 based on the quartile in which the expression value for the marker fit from a data distribution. Then the scores for the 7 markers used were summed. The composite score was found to be significantly lower in the SR asthma group compared with that in the SS asthma group ($P < .001$); 8 of 11 SR asthmatic patients fell outside the SS asthma score range (Fig 6). Therefore SR asthma could be predicted in patients who have scores outside the SS asthma scoring range.

Our scoring of steroid response assays across the spectrum of transactivation and transrepression actions of corticosteroids reveal heterogeneity of SR asthma because in different subjects from 1 to 5 markers scored in the lowest responsive quartile, suggesting that various components contribute to the

development of steroid resistance. Overall, the results of our study provide evidence that, in the future, the analysis of a set of peripheral blood markers before a course of prednisone might have usefulness in predicting the clinical response to prednisone in a majority of asthmatic patients with clinical resistance to corticosteroids. Because the current sample size is small, the markers suggested in this work should be further explored for their variability and validity in a larger independent study.

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Clinical implications: A set of potential peripheral blood markers to predict patients' clinical responses to oral corticosteroids is suggested. Proposed assays require validation in a larger independent study to assess their variability/validity.

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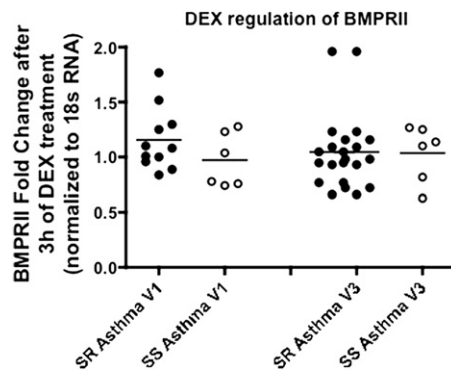


FIG E1. Dexamethasone (*DEX*) induction of BMPRII is not different in PBMCs of SR and SS asthmatic patients at visit 1 (*V1*) and visit 3 (*V3*). The difference between the SR and SS asthma groups was analyzed by using the unpaired *t* test).