

# Cysteine oxidation impairs systemic glucocorticoid responsiveness in children with difficult-to-treat asthma

Susan T. Stephenson, PhD,<sup>a</sup> Lou Ann S. Brown, PhD,<sup>a,b</sup> My N. Helms, PhD,<sup>a,b</sup> Hongyan Qu, MS,<sup>a</sup> Sheena D. Brown, PhD,<sup>a</sup> Milton R. Brown, PhD,<sup>a,b</sup> and Anne M. Fitzpatrick, PhD<sup>a,b</sup> Atlanta, Ga

**Background:** The mechanisms underlying glucocorticoid responsiveness are largely unknown. Although redox regulation of the glucocorticoid receptor (GR) has been reported, it has not been studied in asthmatic patients.

**Objective:** We characterized systemic cysteine oxidation and its association with inflammatory and clinical features in healthy children and children with difficult-to-treat asthma. We hypothesized that cysteine oxidation would be associated with increased markers of oxidative stress and inflammation, increased features of asthma severity, decreased clinically defined glucocorticoid responsiveness, and impaired GR function.

**Methods:** PBMCs were collected from healthy children (n = 16) and children with asthma (n = 118) aged 6 to 17 years. Children with difficult-to-treat asthma underwent glucocorticoid responsiveness testing with intramuscular triamcinolone. Cysteine, cystine, and inflammatory chemokines and reactive oxygen species generation were quantified, and expression and activity of the GR were assessed.

**Results:** Cysteine oxidation was present in children with difficult-to-treat asthma and accompanied by increased reactive oxygen species generation and increased *CCL3* and *CXCL1* mRNA expression. Children with the greatest extent of cysteine oxidation had more features of asthma severity, including poorer symptom control, greater medication use, and less glucocorticoid responsiveness despite inhaled glucocorticoid therapy. Cysteine oxidation also modified the GR protein by decreasing available sulfhydryl groups and decreasing nuclear GR expression and activity.

**Conclusions:** A highly oxidized cysteine redox state promotes a posttranslational modification of the GR that might inhibit its function. Given that cysteine oxidation is prevalent in children with difficult-to-treat asthma, the cysteine redox state might represent a potential therapeutic target for restoration of glucocorticoid responsiveness in this population. (*J Allergy Clin Immunol* 2015;■■■:■■■-■■■.)

**Key words:** Biomarker, childhood asthma, treatment response, cysteine, inflammation, oxidative stress, refractory asthma, severe asthma

Glucocorticoids are the cornerstone of treatment for persistent asthma,<sup>1</sup> but the response to these medications is highly variable, particularly when administered at moderate-to-high dosages.<sup>2</sup> Although several large studies have shown that doubling the dose of inhaled glucocorticoids is of limited efficacy in asthmatic patients already receiving low-dose inhaled glucocorticoid therapy,<sup>3,4</sup> other studies suggest that quadrupling the dose might improve measures of asthma impairment and prevent exacerbations.<sup>5</sup> However, chronic inflammation might still persist in a subset of patients with more severe disease,<sup>6</sup> and the mechanisms underlying decreased glucocorticoid responsiveness in this population remain largely unknown.

Although the biology of the glucocorticoid receptor (GR) is complex, posttranslational modifications of the GR and associated downstream effects on glucocorticoid signaling have been described.<sup>7</sup> Although much of the literature has focused on serine phosphorylation, the function of the GR is also regulated by redox-dependent mechanisms,<sup>8,9</sup> which might be of relevance in asthmatic patients. Indeed, we have previously reported marked redox abnormalities in the airways and systemic circulation of asthmatic children that worsen with increased asthma severity.<sup>10-15</sup> Notably, in children with symptomatic asthma despite glucocorticoid therapy, the presence of reactive oxygen species (ROS) promotes oxidation of the amino acid thiol cysteine to its disulfide form (ie, cystine) and further depletes the pool of cysteine available for signal transduction and other cellular functions.<sup>12,13</sup> Given that cysteine oxidation also stimulates cytokine secretion<sup>16</sup> and promotes pathways associated with cellular stress and death,<sup>17</sup> we sought to (1) characterize systemic cysteine oxidation and its intracellular, inflammatory, and clinical features (including glucocorticoid responsiveness) in children with difficult-to-treat asthma and (2) explore the role of cysteine oxidation in GR function. We hypothesized that in children with difficult-to-treat asthma, greater cysteine oxidation would be associated with increased intracellular and extracellular oxidative stress and inflammation, increased features of asthma severity, and decreased glucocorticoid responsiveness. We further hypothesized that cysteine oxidation would impair nuclear GR expression and consensus binding.

## METHODS

### Participants

Children 6 to 17 years of age with physician-diagnosed asthma who were receiving current asthma treatment were enrolled from a difficult-to-treat asthma clinic at Emory University in Atlanta, Georgia. Asthmatic children had a history of 12% or greater FEV<sub>1</sub> reversibility after short-acting bronchodilator administration or airway hyperresponsiveness to methacholine evidenced by a

From <sup>a</sup>Emory University Department of Pediatrics and <sup>b</sup>Children's Healthcare of Atlanta Center for Cystic Fibrosis and Airways Disease Research.

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Corresponding author: Anne M. Fitzpatrick, PhD, 2015 Uppergate Dr, Atlanta, GA 30322. E-mail: [anne.fitzpatrick@emory.edu](mailto:anne.fitzpatrick@emory.edu).

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**Abbreviations used**

ACQ: Asthma Control Questionnaire  
 $E_h$ : Redox potential  
 GR: Glucocorticoid receptor  
 ROS: Reactive oxygen species

methacholine PC<sub>20</sub> value of 16 mg/mL or less. Healthy children without asthma were also enrolled for comparison. Exclusion criteria for all children included premature birth before 35 weeks' gestation, current smoking, immunodeficiency, pulmonary aspiration disorders, or vocal cord dysfunction.

The Emory University Institutional Review Board granted approval for this study. Written informed consent was obtained from parents or legal guardians. Children 12 to 17 years of age provided written informed assent, whereas children 6 to 11 years of age provided verbal assent.

**Participant characterization procedures**

Children were evaluated during 2 visits separated by 2 weeks. The baseline characterization visit was rescheduled if upper respiratory tract viral symptoms, acute worsening of asthma symptoms, antibiotic use, or systemic glucocorticoid use was reported within the preceding 2 weeks. Parents completed medical history questionnaires, and children completed the Asthma Control Questionnaire (ACQ) and the Pediatric Asthma Quality of Life Questionnaire.<sup>18,19</sup> Spirometry (KoKo PDS; Ferraris, Louisville, Colo) was performed at baseline and after receipt of up to 8 inhalations of albuterol sulfate (90 µg per actuation). The best of 3 forced vital capacity maneuvers was interpreted.<sup>20</sup> Exhaled nitric oxide concentrations were determined by using online methods (NIOX MINO; Aerocrine, Morrisville, NC).<sup>21</sup> Whole blood (up to 25 mL) obtained by means of venipuncture was collected into serum separation tubes and heparinized tubes containing a density gradient for PBMC isolation (Vacutainer CPT; BD, Franklin Lakes, NJ). Total serum IgE levels were quantified with an assay kit according to the manufacturer's instructions (Calbiotech, Spring Valley, Calif).

**Glucocorticoid responsiveness testing**

At completion of the baseline visit, a subset ( $n = 57$ ) of participants with symptomatic asthma despite moderate- to high-dose inhaled glucocorticoid therapy (ie, >200 µg fluticasone equivalent for children 6–11 years of age and >500 µg fluticasone equivalent for children ≥12 years old) received intramuscular triamcinolone (1 mg/kg, 60 mg maximum dose). Symptomatic asthma was defined according to available treatment guidelines<sup>1</sup> as self-reported asthma symptoms more than twice weekly or nocturnal awakenings from asthma at least 2 nights per month. Responsiveness to triamcinolone was assessed after 2 weeks and defined as an ACQ score of less than 0.75, which corresponds to "well-controlled asthma" with a positive predictive value and negative predictive value of 0.73 and 0.85, respectively.<sup>22</sup>

**Cysteine and cystine determination**

Aliquots were preserved in a 5% perchloric acid solution containing iodoacetic acid (6.7 µmol/L) and boric acid (0.1 mol/L) with 5 µmol/L γ-glutamyl-glutamate internal standard to prevent auto-oxidation before analysis.<sup>23</sup> Cysteine and cystine were quantified relative to γ-glutamyl-glutamate by means of reverse-phase HPLC, as described previously.<sup>12</sup> Samples were derivatized with dansyl chloride and separated on a 10-µm Ultrasil amino acid column (Waters Alliance 2690; Waters, Milford, Mass). Fluorescence was detected at 365 and recorded by 2 using detectors (Waters 474 [Waters] and Gilson 121 [Gilson, Middletown, Wis]). The redox potential ( $E_h$ ) of the cysteine/cystine thiol pair was calculated with the Nernst equation as follows:

$$E_h = E_o + RT/nF \ln[\text{disulfide}] / ([\text{thiol1}][\text{thiol2}]),$$

where  $E_o$  is the standard potential for the redox couple (−250 mV),  $R$  is the gas constant,  $T$  is the absolute temperature,  $n$  is the number of electrons transferred, and  $F$  is the Faraday constant.

**Cellular viability and ROS generation**

Cellular viability was determined with an automatic cell counter (Countess; Invitrogen, Grand Island, NY) after staining with 0.4% trypan blue. Intracellular ROS generation was assessed after incubation with 5 µmol/L 2',7'-dichlorodihydrofluorescein diacetate for 45 minutes. Relative fluorescent units were quantified in 5 to 10 separate visual fields with FluoView software (Olympus, Center Valley, Pa) after correction for background autofluorescence.

**RT-PCR**

Cells were added to 5 volumes of RNeasy lysis buffer (Life Technologies, Grand Island, NY), and RNA was extracted with a commercial kit (RNeasy Mini kit; Qiagen, Valencia, Calif). Ten nanograms of total RNA per sample was reverse transcribed with MultiScribe Reverse Transcriptase (62.5 U/50 µL reaction), RNase Inhibitor, oligoT primers, and MgCl<sub>2</sub> at a concentration of 5.5 mmol/L (Life Technologies). cDNA aliquots were preamplified for genes of interest by using the TaqMan PreAmp Master Mix Kit (Life Technologies). The preamplified cDNA was used to quantitate relative levels of *CCL3* (Hs00234142\_m1) and *CXCL1* (Hs00236937\_m1) in a 96-well assay system (StepOnePlus real-time PCR assay) with TaqMan primer pairs and probes (Life Technologies). Data were normalized to *B2M* (4333766F), *GAPDH* (4333764F), *ACTB* (4333762F), *PGK1* (4333765F), and *PPIA* (43337663F) housekeeping genes. Net cycle threshold values were used to calculate Δ cycle threshold values for each subject by using the average of the 5 housekeeping genes as a reference. mRNA gene expression was analyzed relative to the control group and expressed as fold change values.

**Cell culture**

Cell-culture experiments were performed with THP-1 monocytes (ATCC, Manassas, VA), MM.1S B lymphoblasts (ATCC), and primary PBMCs from healthy donors (AllCells, Alameda, Calif). Reduced and oxidized conditions were created by adding 300 µL of 10 mmol/L cysteine and 150 µL of 10 mmol/L cystine (Sigma-Aldrich, St Louis, Mo), respectively, to 15 mL of Dulbecco modified Eagle medium/F12 media without methionine, cysteine, cystine, and glutamine (HyClone, Life Technologies). Media was supplemented with 10% FBS, 1% penicillin/streptomycin (Cellgro; Corning Life Sciences, Corning, NY), and 50 mg/mL gentamicin sulfate (Cellgro). Cells were resuspended to a concentration of 1 million cells/mL and cultured for 4 hours at 37°C with 5% CO<sub>2</sub>. In selected experiments cells were also exposed to 100 nmol/L dexamethasone for 1 hour (Sigma-Aldrich), which was added at hour 3 of incubation.

**Western blotting**

Cells were suspended in 50 mmol/L Bis-Tris-HCL lysis buffer (pH 6.5) containing 0.5% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 150 mmol/L NaCl, 1 mmol/L EDTA, and 0.1 mmol/L phenylmethylsulfonyl fluoride. Protein sulfhydryl group (–SH) protein residues were labeled with biotinylated iodoacetamide by using the methods of Go et al<sup>24</sup> at a final concentration of 20 µmol/L for 15 minutes, after which iodoacetamide was added to a final concentration of 5 mmol/L. The GR was immunoprecipitated with an anti-GR receptor antibody (Santa Cruz Biotechnology, Dallas, Tex) and the Protein G Immunoprecipitation Kit (Sigma-Aldrich). Eluted sample was divided and run on two 10% SDS-PAGE gels. Proteins were transferred overnight to nitrocellulose membranes, and biotinylated iodoacetamine binding was visualized on an Odyssey Classic Infrared Imaging System (LI-COR, Lincoln, Neb) by incubating with streptavidin-conjugated IRDye 680RD (LI-COR) for 1 hour. Even loading of samples was determined by incubating the second membrane overnight at 4°C with human GR antibody raised in rabbit (Santa Cruz Biotechnology). Visualization was performed after secondary incubation with anti-rabbit IgG conjugated to IRDye 680RD (LI-COR).

**Nuclear isolation, total protein measurement, and nuclear GR expression and activation**

Nuclei were isolated with a commercial nuclear extract kit (Active Motif, Carlsbad, Calif). Protein concentrations were measured at 750 nm with BSA as

the standard (DC Protein Assay Kit; Bio-Rad Laboratories, Hercules, Calif). Equal nuclear protein volumes were added to a TransAM assay plate (Active Motif) coated with immobilized oligonucleotide containing the GR consensus binding site (5'-GGTACAnnnTGTCT-3'). GR activity was assessed per the manufacturer's instructions with an OD of 450 nm. SDS-PAGE was performed with 30  $\mu$ g of nuclear lysate loaded onto a precast gradient (4% to 20%) gel (Bio-Rad Laboratories). Proteins were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories) and incubated with human GR antibody raised in rabbit (Santa Cruz Biotechnology) and IRDye 800CW Donkey anti-Rabbit IgG and IRDye 680RD Donkey anti-Goat IgG (LI-COR). Membranes were visualized on an Odyssey Classic Infrared Imaging System and densitometry performed using Image Studio Lite version 4.0 (LI-COR).

### Statistical analyses

Statistical analyses were performed with IBM SPSS Statistics software (Version 22; SPSS, Chicago, Ill).  $\chi^2$  Tests were used for dichotomous variables. We used *t* tests and ANOVA with the Fisher least-significance-difference *post hoc* test for variables that were normally distributed. For skewed variables, statistical significance testing was performed with nonparametric Mann-Whitney *U* or Kruskal-Wallis tests, as appropriate. Bivariate Pearson correlations were used to examine associations between linear variables. Logistic regression analyses were performed to determine associations between tertiles of plasma cysteine/cystine  $E_h$  values and clinical outcomes. Univariate models were first used to narrow the list of covariates (statistically significant at  $P < .05$ ) to be incorporated into the final multivariate model. Significance was defined as an  $\alpha$  value of less than .05 by using 2-tailed tests.

## RESULTS

One hundred thirty-four children were enrolled for this study, but 20 were excluded because of an inadequate sample, leaving 114 children (healthy control subjects,  $n = 15$ ; asthmatic patients,  $n = 99$ ) in the final analysis (see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Features of the excluded children were not significantly different ( $12 \pm 4$  years of age, 15% white, 45% with body mass index percentile  $<85\%$ , and  $93\% \pm 13\%$  predicted FEV<sub>1</sub>;  $P > .30$  for each). Features of the included participants are shown in Table I. Children with asthma were more likely to be nonwhite and male and further characterized by greater allergic sensitization, more airflow limitation, and increased airway inflammation, as reflected by exhaled nitric oxide values, which is consistent with their asthma diagnosis. Children with asthma were also more obese, with higher body mass index percentiles.

### Characterization of systemic cysteine oxidation

Compared with healthy nonasthmatic children, children with asthma had lower baseline plasma concentrations of cysteine and higher plasma concentrations of the disulfide cystine, resulting in a more oxidized cysteine/cystine  $E_h$  (Fig 1). Extracellular cysteine oxidation in asthmatic participants was further accompanied by increased intracellular glutathione disulfide formation, increased ROS generation, and increased mRNA expression of the proinflammatory genes *CCL3* and *CXCL1* (Fig 2). Although all children with asthma had difficult-to-treat disease, children with severe asthma, which was defined by the need for high-dose inhaled glucocorticoids plus an additional controller medication to maintain asthma control,<sup>25</sup> had the lowest plasma cysteine concentrations and the most oxidized cysteine/cystine  $E_h$  (see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Children with severe asthma also had the greatest ROS generation and mRNA expression of *CXCL1* (see Fig E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

TABLE I. Features of the included participants

| Feature                                   | Healthy control subjects (n = 15) | Asthmatic patients (n = 99) | P value |
|-------------------------------------------|-----------------------------------|-----------------------------|---------|
| Age (y)                                   | 10 (9-13)                         | 12 (10-15)                  | .175    |
| Sex                                       |                                   |                             |         |
| Male                                      | 3 (20)                            | 66 (67)                     | .001    |
| Female                                    | 12 (80)                           | 33 (33)                     |         |
| Race                                      |                                   |                             |         |
| White                                     | 3 (20)                            | 16 (16)                     | .001    |
| Black                                     | 9 (60)                            | 69 (70)                     |         |
| Asian                                     | 3 (20)                            | 1 (1)                       |         |
| $\geq 1$ Race                             | 0                                 | 13 (11)                     |         |
| Body mass index                           |                                   |                             |         |
| $<85$ th percentile (normal weight)       | 9 (82)                            | 43 (43)                     | .036    |
| 85th to 95th percentile (overweight)      | 2 (18)                            | 26 (26)                     |         |
| $\geq 95$ th percentile (obese)           | 0                                 | 30 (30)                     |         |
| Self-reported allergic rhinitis           | 10 (67)                           | 91 (92)                     | .004    |
| Serum IgE (kU/L)                          | 61 (30-94)                        | 218 (40-503)                | .011    |
| Exhaled nitric oxide (ppb)                | 12 (9-14)                         | 29 (17-51)                  | $<.001$ |
| Baseline lung function                    |                                   |                             |         |
| FVC (% predicted)                         | 102 (88-110)                      | 105 (98-115)                | .652    |
| FEV <sub>1</sub> (% predicted)            | 98 (89-108)                       | 93 (81-105)                 | .046    |
| FEV <sub>1</sub> /FVC ratio               | 0.87 (0.83-0.93)                  | 0.78 (0.70-0.83)            | $<.001$ |
| FEV <sub>1</sub> /FVC ratio (% predicted) | 101 (95-109)                      | 90 (81-95)                  | $<.001$ |
| FEF <sub>25-75</sub> (% predicted)        | 92 (74-112)                       | 71 (56-86)                  | $<.001$ |
| Postbronchodilator lung function          |                                   |                             |         |
| FVC (% predicted)                         | 104 (90-121)                      | 110 (102-120)               | .294    |
| FEV <sub>1</sub> (% predicted)            | 108 (93-118)                      | 105 (93-115)                | .705    |
| FEV <sub>1</sub> /FVC ratio               | 0.90 (0.83-0.96)                  | 0.84 (0.78-0.88)            | .004    |
| FEV <sub>1</sub> /FVC ratio (% predicted) | 104 (94-110)                      | 97 (91-101)                 | .0100   |
| FEF <sub>25-75</sub> (% predicted)        | 108 (91-137)                      | 94 (81-110)                 | .161    |

Data represent medians (interquartile ranges) or numbers (percentages) of participants.

FEF<sub>25-75</sub>, Midexpiratory flow rate at 25% to 75% of vital capacity; FVC, forced vital capacity.

### Associations between systemic cysteine oxidation and clinical features of asthma

Given the heterogeneity in extracellular cysteine/cystine  $E_h$  observed within the group of asthmatic participants, associations between tertiles of plasma cysteine/cystine  $E_h$  values and asthma clinical features were explored. Asthmatic children with plasma cysteine/cystine  $E_h$  values in the highest (ie, most oxidized) tertile had more than 3-fold increased odds of high-dose inhaled glucocorticoid therapy and daily short-acting  $\beta$ -agonist therapy compared with children in the lowest (ie, more reduced) tertile. Moreover, children in the highest tertile also had evidence of poorer current and historical asthma control, as reflected by ACQ scores of greater than 1.5 at the characterization visit<sup>22</sup> and more frequent emergency department visits and intubations (see Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

### Associations between systemic cysteine oxidation and glucocorticoid responsiveness

To further understand associations between systemic cysteine oxidation and features of asthma severity in children,

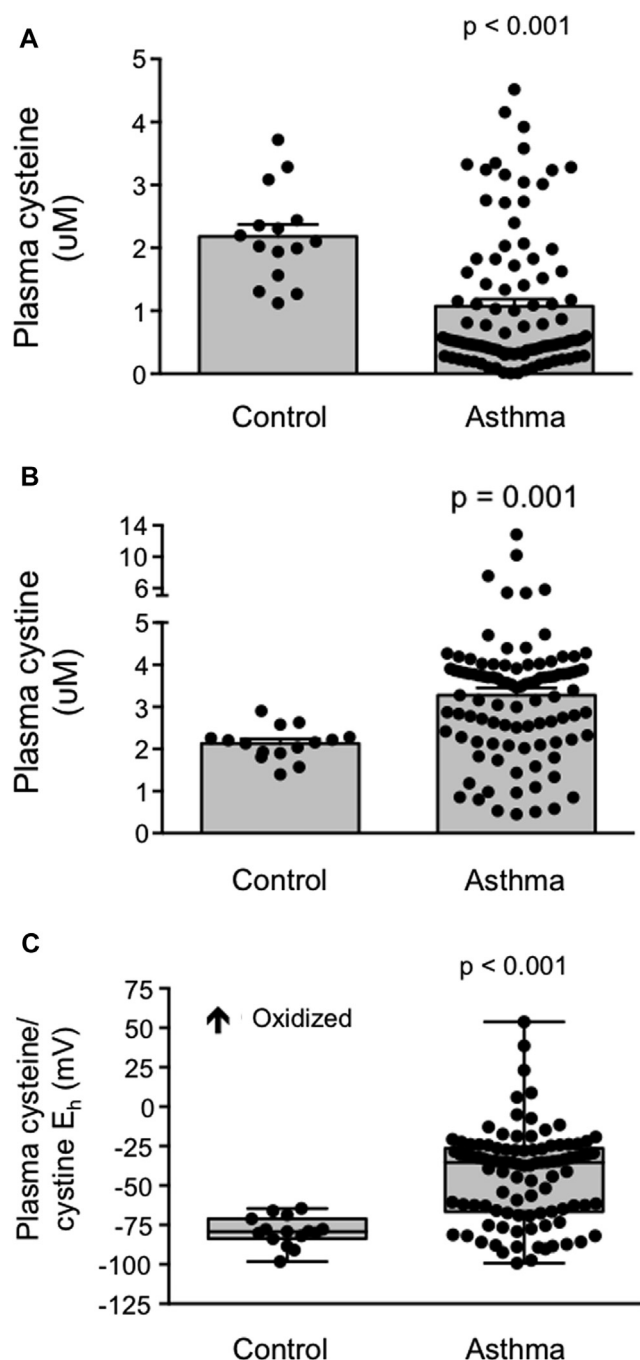


**FIG 1.** Plasma cysteine (A) and cystine (B) concentrations and cysteine/cystine  $E_h$  (C) in healthy control children and children with asthma. Cysteine and cystine data are presented as means  $\pm$  SEMs, and the cysteine/cystine  $E_h$  box plots are shown with minimum and maximum values. Dots represent individual participants: control subjects,  $n = 15$ ; asthmatic patients,  $n = 99$  for each panel.

we determined whether the baseline plasma cysteine/cystine  $E_h$  was also associated with clinical responsiveness to systemic glucocorticoids. For this analysis, a subset of asthmatic participants with poor asthma control despite treatment with moderate-to-high doses of inhaled glucocorticoids ( $n = 57$ ) received intramuscular triamcinolone and returned to the clinic for re-evaluation in 2 weeks. Features of the children who

received triamcinolone are presented in Table E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

At the 2-week visit, the prevalence of study-defined triamcinolone responsiveness and nonresponsiveness was 59% ( $n = 33$ ) and 41% ( $n = 23$ ), respectively. In addition to having poorer asthma control, asthmatic children who did not respond to triamcinolone also had more impaired asthma-related quality of life, increased exhaled nitric oxide concentrations, and greater prebronchodilator airflow limitation after the triamcinolone injection (Table II).<sup>22</sup> Furthermore, the plasma cysteine/cystine  $E_h$  was higher (ie, more oxidized) in triamcinolone nonresponders at both the baseline visit and the 2-week characterization visit (Fig 3)<sup>22</sup> because of lower cysteine concentrations in the nonresponders at both time points (responders vs nonresponders; baseline cysteine:  $1.32 \pm 1.50$  vs  $0.48 \pm 0.52$   $\mu\text{mol/L}$ ,  $P = .013$ ; 2-week cysteine:  $1.33 \pm 1.31$  vs  $0.58 \pm 0.79$   $\mu\text{mol/L}$ ,  $P = .40$ ). Plasma concentrations of cystine, mixed disulfides, glutathione, and glutathione disulfide did not differ between groups (data not shown). Stratification of participants by baseline tertiles of plasma cysteine/cystine  $E_h$  values also demonstrated increased odds of triamcinolone nonresponsiveness at the follow-up visit in the most oxidized group (odds ratio for highest vs lowest tertile, 5.83; 95% CI, 1.06-32.02;  $P = .042$ ). Overall, the plasma cysteine/cystine  $E_h$  for each participant, including healthy control subjects and children who did not receive triamcinolone, was relatively stable (see Fig E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). mRNA gene expression of *CCL3* and *CXCL1* was also increased in triamcinolone nonresponders compared with that in triamcinolone responders (see Fig E5 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)), which is consistent with the clinical observations of increased inflammation in these children (Table II).

### Proof-of-concept mechanistic studies

Because the volume of samples available from children was limited, proof-of-concept experimentation was also performed with THP-1 monocytes and primary PBMCs from healthy donors. Exposure of these cells to extracellular oxidizing versus reducing cysteine/cystine environments resulted in physiologically relevant  $E_h$  values and differences in intracellular glutathione disulfide and ROS generation (see Fig E6 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)) compared with what we previously observed in our pediatric participants. Oxidizing extracellular conditions were also associated with decreased availability of GR protein -SH groups for binding (see Fig E7, A and B, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Similar decreases in GR protein -SH group availability were observed in samples from children with difficult-to-treat asthma (see Fig E7, C and D). Furthermore, GR activity, as reflected by decreased GR consensus binding, was blunted in response to extracellular oxidizing conditions (Fig 4, A and B). Additional experiments with MM.1S cells, which naturally overexpress the GR, further revealed decreased expression of the GR in the nucleus with extracellular cysteine/cystine oxidation.

### DISCUSSION

To our knowledge, this is the first study to characterize cysteine oxidation and its association with glucocorticoid responsiveness

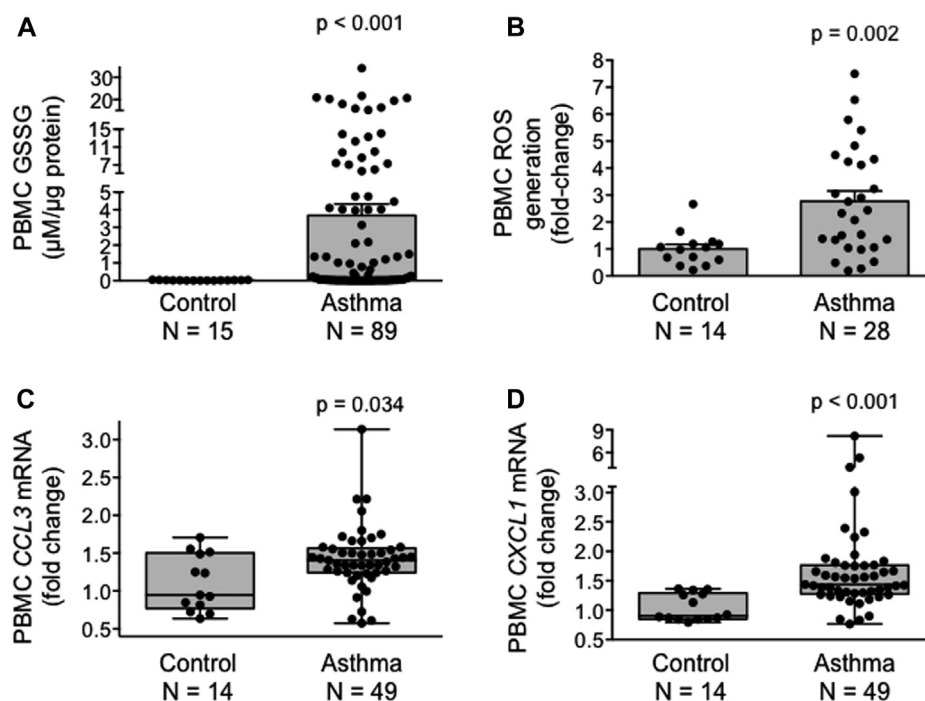


**FIG 2.** PBMC glutathione disulfide (GSSG) concentrations (A), ROS generation (B), and *CCL3* (C) and *CXCL1* (D) mRNA gene expression in healthy control children and children with asthma. GSSG and ROS data are presented as means  $\pm$  SEMs, and *CCL3* and *CXCL1* mRNA data are presented as box plots with minimum and maximum values. Dots represent individual values.

**TABLE II.** Features of triamcinolone responders versus nonresponders at the 2-week follow-up visit\*

| Feature                                                     | Triamcinolone responder (n = 33) | Triamcinolone nonresponder (n = 23) | P value |
|-------------------------------------------------------------|----------------------------------|-------------------------------------|---------|
| Pediatric Asthma Quality of Life Questionnaire total score† | 6.56 (6.25-6.83)                 | 5.41 (4.45-6.01)                    | <.001   |
| Exhaled nitric oxide (ppb)‡                                 | 14 (6-25)                        | 26 (12-45)                          | .040    |
| Baseline lung function                                      |                                  |                                     |         |
| FVC (% predicted)                                           | 105 (99-115)                     | 92 (84-117)                         | .038    |
| FEV <sub>1</sub> (% predicted)                              | 96 (87-102)                      | 80 (69-101)                         | .007    |
| FEV <sub>1</sub> /FVC ratio                                 | 0.78 (0.72-0.87)                 | 0.75 (0.67-0.80)                    | .207    |
| FEV <sub>1</sub> /FVC ratio (% predicted)                   | 90 (85-101)                      | 87 (78-92)                          | .093    |
| FEF <sub>25-75</sub> (% predicted)                          | 75 (61-91)                       | 56 (45-76)                          | .016    |
| Postbronchodilator lung function                            |                                  |                                     |         |
| FVC (% predicted)                                           | 109 (103-120)                    | 101 (93-117)                        | .111    |
| FEV <sub>1</sub> (% predicted)                              | 105 (98-114)                     | 96 (88-114)                         | .085    |
| FEV <sub>1</sub> /FVC ratio                                 | 0.85 (0.79-0.91)                 | 0.85 (0.81-0.87)                    | .692    |
| FEV <sub>1</sub> /FVC ratio (% predicted)                   | 98 (92-106)                      | 98 (95-100)                         | .523    |
| FEF <sub>25-75</sub> (% predicted)                          | 99 (83-113)                      | 94 (72-109)                         | .157    |

Triamcinolone response was defined as an ACQ score of less than 0.75.<sup>22</sup> Data represent medians (interquartile ranges).

FEF<sub>25-75</sub>, Midexpiratory flow rate at 25% to 75% of vital capacity; FVC, forced vital capacity.

\*One participant did not complete the follow-up visit and was excluded from this comparison.

†Pediatric Asthma Quality of Life Questionnaire scores range from 0 to 7, with higher scores indicating better quality of life.

‡Data were logarithmically transformed before statistical analysis.

in patients with difficult-to-treat asthma. Although heterogeneity in our results was noted, a significant degree of cysteine oxidation was noted in a subset of children with difficult-to-treat asthma and was accompanied by increased ROS generation and increased mRNA expression of *CCL3* and *CXCL1*. This heterogeneity was not clearly attributable to obesity or inhaled glucocorticoid exposure, although children with the greatest extent of cysteine oxidation had more features of asthma severity, including poorer symptom control; greater medication use, including use of high-dose inhaled glucocorticoids; and decreased clinically

defined glucocorticoid responsiveness. Although the precise mechanisms underlying these observations are unclear, our proof-of-concept experiments suggest that cysteine oxidation promotes a posttranslational modification of the GR that impairs its nuclear expression and binding to the glucocorticoid consensus.

Clinically defined impairments in glucocorticoid responsiveness have been previously reported in asthmatic patients, although there are no gold standard definitions for glucocorticoid response, and methodologies vary widely across studies.<sup>26</sup>

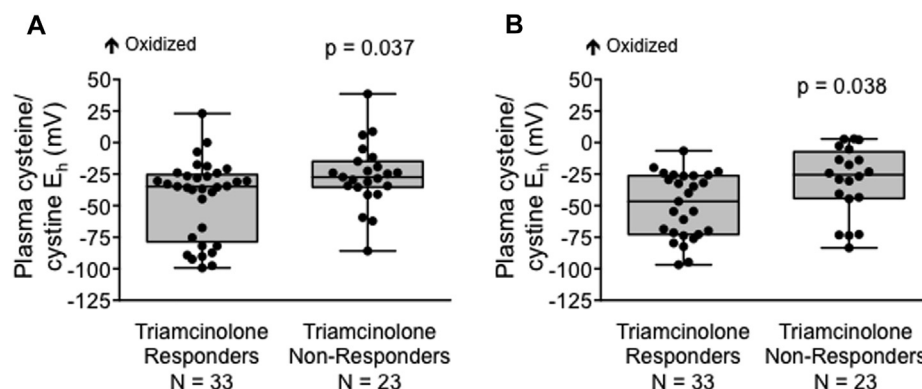


**FIG 3.** Plasma cysteine/cystine  $E_h$  in triamcinolone responders and triamcinolone nonresponders at the baseline visit before triamcinolone receipt (**A**) and 2 weeks after triamcinolone administration (**B**). Triamcinolone response was defined as an Asthma Questionnaire Control score of less than 0.75 at the 2-week visit.<sup>22</sup> Box plots are shown with minimum and maximum values. Dots represent individual values.

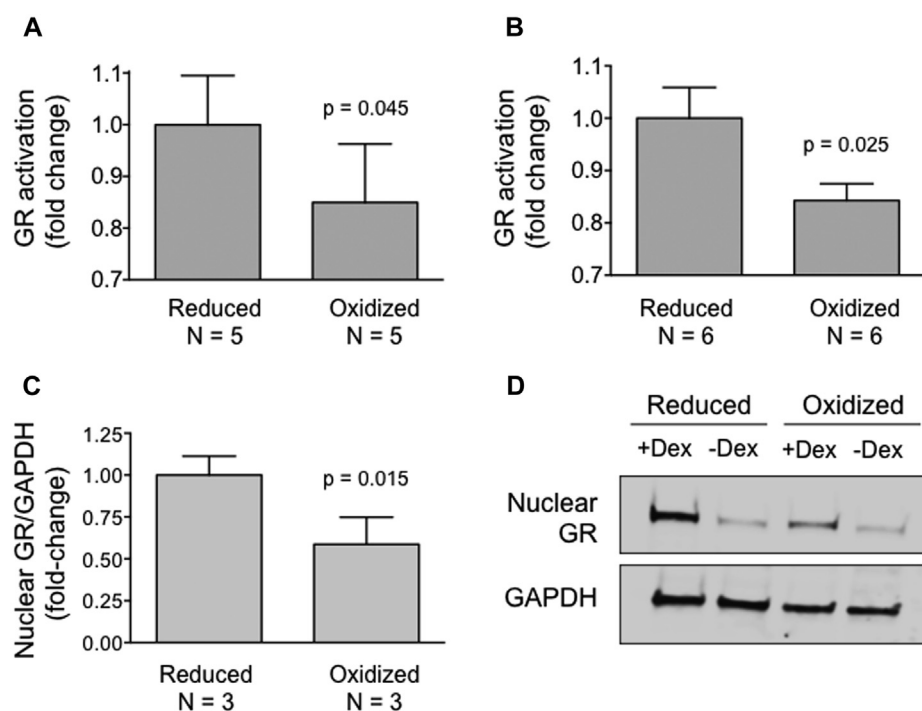


**FIG 4.** A-C, GR activation in THP-1 monocytes (Fig 4, A) and human primary PBMCs (Fig 4, B) and nuclear GR expression in MM.1S cells (Fig 4, C) after exposure to extracellular oxidizing versus reduced conditions. GR densitometry is normalized to glyceraldehyde-3-phosphate (*GAPDH*). D, Representative images of nuclear GR expression with (+) and without (–) dexamethasone (*Dex*) treatment.

A recent unbiased cluster analysis of children enrolled in the Childhood Asthma Management Research Program identified 2 clusters of childhood asthma with positive versus minimal responses to inhaled budesonide that differed significantly with regard to the rate of exacerbations, the number of controller medications for asthma, and longitudinal differences in pulmonary function.<sup>27</sup> A separate study of children with difficult-to-treat asthma similarly identified several groups of patients with varying degrees of responsiveness to intramuscular triamcinolone.<sup>28</sup> In that study only 11% of enrolled children had

“complete” responsiveness to triamcinolone, as determined by cut points of symptoms, FEV<sub>1</sub>, bronchodilator reversibility, and exhaled nitric oxide.<sup>28</sup> Rather, the majority of children were “partial” or “incomplete” responders,<sup>28</sup> highlighting the heterogeneity of the disorder as it is currently defined. It is also not clear how clinical end points, such as lung function or symptoms, associate with airway inflammation or other biological mechanisms that might underlie asthma phenotypes.

Although the biological mechanisms that regulate the response to glucocorticoids are not entirely clear, other studies have

described reduced binding of glucocorticoid to its receptor and reduced GR consensus binding in these patients<sup>29,30</sup> that might be related to persistent activation of p38 mitogen-activated protein kinase.<sup>31,32</sup> Other studies have reported increased expression of the GR $\beta$  isoform in airway cells,<sup>33</sup> immune cells,<sup>34</sup> and airway tissue<sup>35</sup> of patients with severe asthma that might act as a negative inhibitor and compete for binding of GR consensus. Abnormal histone acetylation has also been reported in patients with severe asthma<sup>36</sup> and chronic obstructive pulmonary disease<sup>37</sup> and is characterized by a reduction in histone deacetylase 2 expression and activity, which might be induced by oxidative and nitrosative stress.<sup>38,39</sup>

Like any protein, the GR contains a number of cysteine residues that maintain its structure and function. The hormone-binding domain of the GR (residues 529-777 in human subjects) contains 13 methionines but only 5 cysteines (Cys) at residues 622, 638, 643, 665, and 736 that are sensitive to changes in redox status.<sup>40</sup> Under oxidizing conditions, Cys-622, 638, and 643 form disulfide bonds<sup>41-43</sup> that might cause the GR to assume a folded conformation.<sup>44</sup> Additionally, disulfide bonds with Cys-481 in the DNA-binding domain of the GR have been shown to inhibit nuclear translocation, presumably through altered GR protein conformation.<sup>9</sup> Thus redox regulation of the GR is most likely due to functional alteration of the cysteine residues of the GR in the presence of ROS and not to a decrease in expression of the GR protein.<sup>45-47</sup>

This study has a number of limitations. Although adherence to inhaled glucocorticoids cannot be guaranteed in our participants with difficult-to-treat asthma, intramuscular triamcinolone removes some issues with inhaled glucocorticoid delivery given its depot effect.<sup>26</sup> Although previous studies with triamcinolone in children have demonstrated overall reductions in inflammatory biomarkers and asthma symptoms after the injection, variability in clinical response was noted.<sup>28,48</sup> Although a higher dose of triamcinolone might have conferred additional benefit,<sup>49</sup> these benefits are likely to be outweighed by the systemic side effects of the medication in school-age children who are still undergoing linear growth. Furthermore, although the use of PBMCs can be criticized, our previous work<sup>12</sup> and that of others<sup>34</sup> has shown similarities between PBMCs and airway cells in patients with severe asthma whose inflammation is not limited to the airways. The use of PBMCs also allowed for a control group that otherwise could not be obtained for ethical reasons.

In conclusion, we have shown that oxidation of the amino acid cysteine is associated with decreased responsiveness to systemic glucocorticoids in children with difficult-to-treat asthma. Although additional mechanistic studies are warranted, our findings also suggest that redox-related mechanisms can impair GR function in asthmatic patients. Although glucocorticoid responsiveness is likely associated with multiple mechanisms (including mechanisms independent of oxidative stress), the cysteine redox state might represent one of several potential therapeutic targets for the restoration of glucocorticoid responsiveness in this population.

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**Clinical implications: Systemic oxidative stress, as reflected by oxidation of the amino acid cysteine, is associated with poor asthma control and decreased responsiveness to systemic glucocorticoids in children with difficult-to-treat asthma.**

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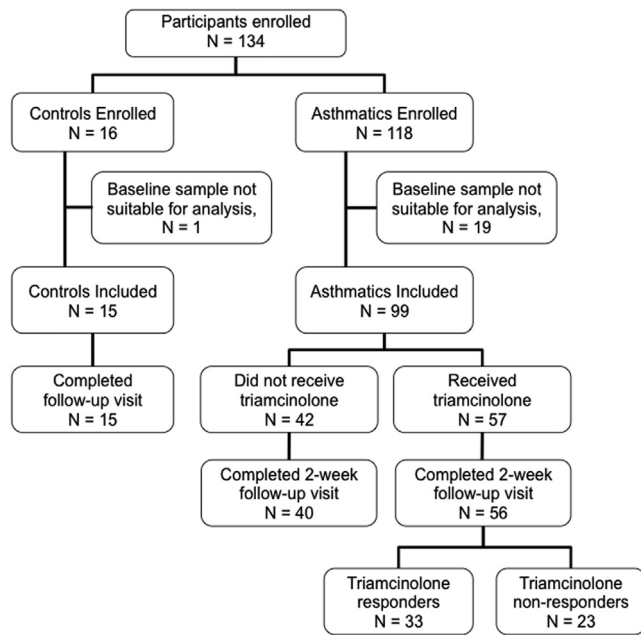


**FIG E1.** Flowchart of participant enrollment and inclusion in the study.

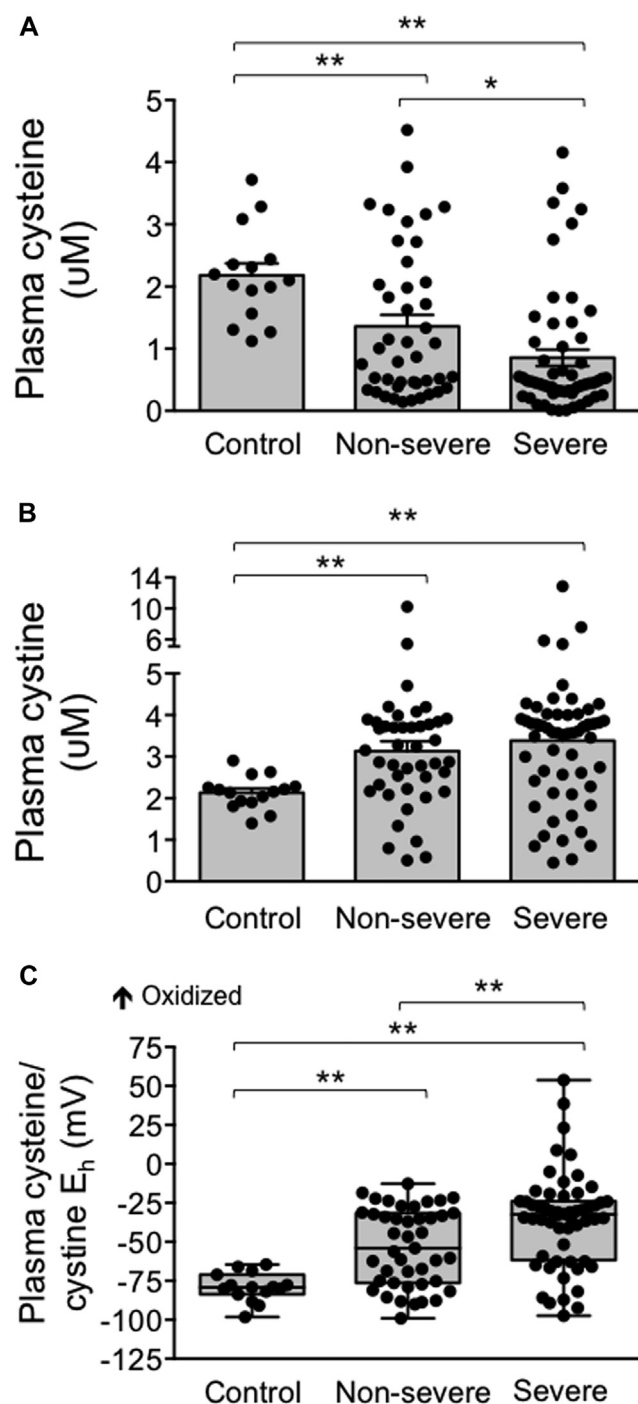


**FIG E2.** Plasma cysteine (A) and cystine (B) concentrations and the cysteine/cystine  $E_h$  (C) in healthy control children, children with nonsevere asthma, and children with severe asthma. Cysteine and cystine data are presented as means  $\pm$  SEMs, and the cysteine/cystine  $E_h$  box plots are shown with minimum and maximum values. Dots represent individual participants: control subjects,  $n = 15$ ; patients with nonsevere asthma,  $n = 43$ ; patients with severe asthma,  $n = 56$  for each panel. \* $P < .05$  and \*\* $P < .01$ .

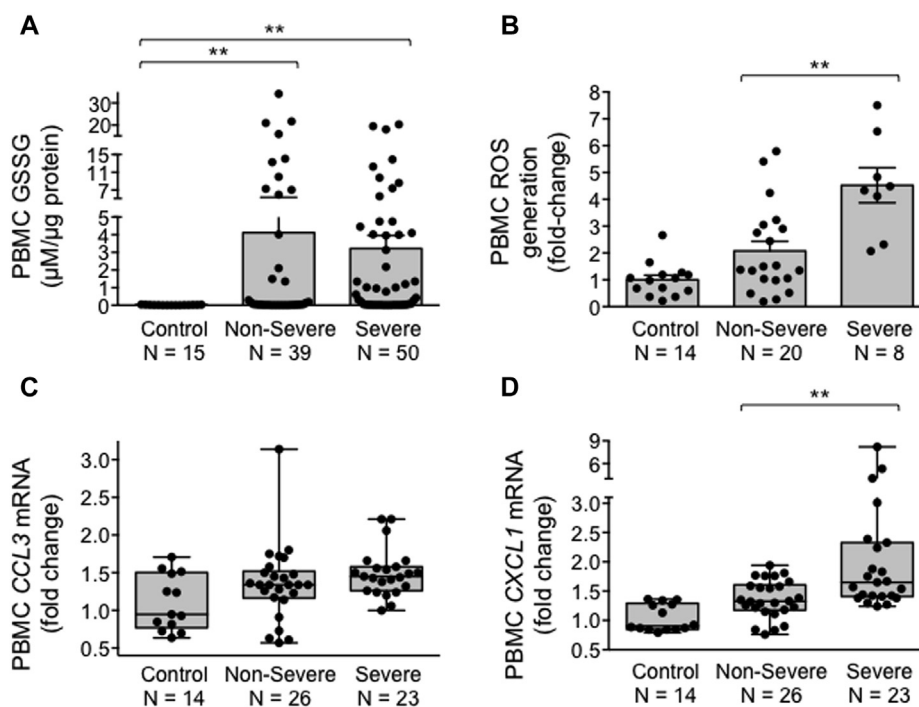


**FIG E3.** PBMC glutathione disulfide (GSSG) concentrations (A), ROS generation (B), and *CCL3* (C) and *CXCL1* (D) mRNA gene expression in healthy control children, children with nonsevere asthma, and children with severe asthma. GSSG and ROS data are presented as means  $\pm$  SEMs, and *CCL3* and *CXCL1* mRNA data are presented as box plots with minimum and maximum values. Dots represent individual values. \* $P < .05$  and \*\* $P < .01$ .

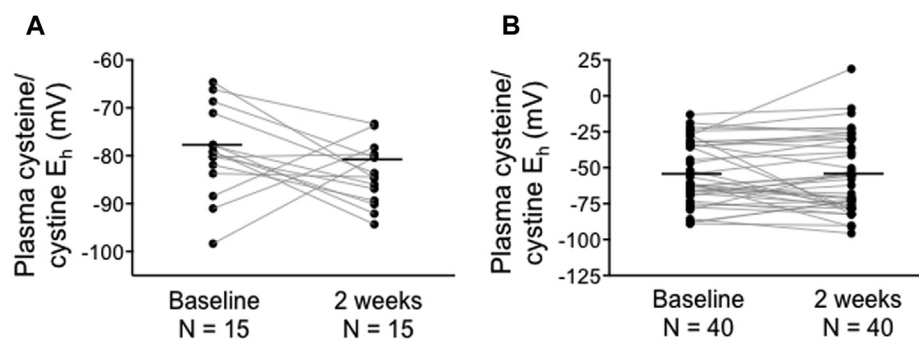


**FIG E4.** Stability of the plasma cysteine/cystine  $E_h$  between the baseline and 2-week follow-up visits in healthy control children (**A**) and children with asthma (**B**) who did not receive triamcinolone. Differences between the baseline and 2-week follow-up visits were not statistically significant.

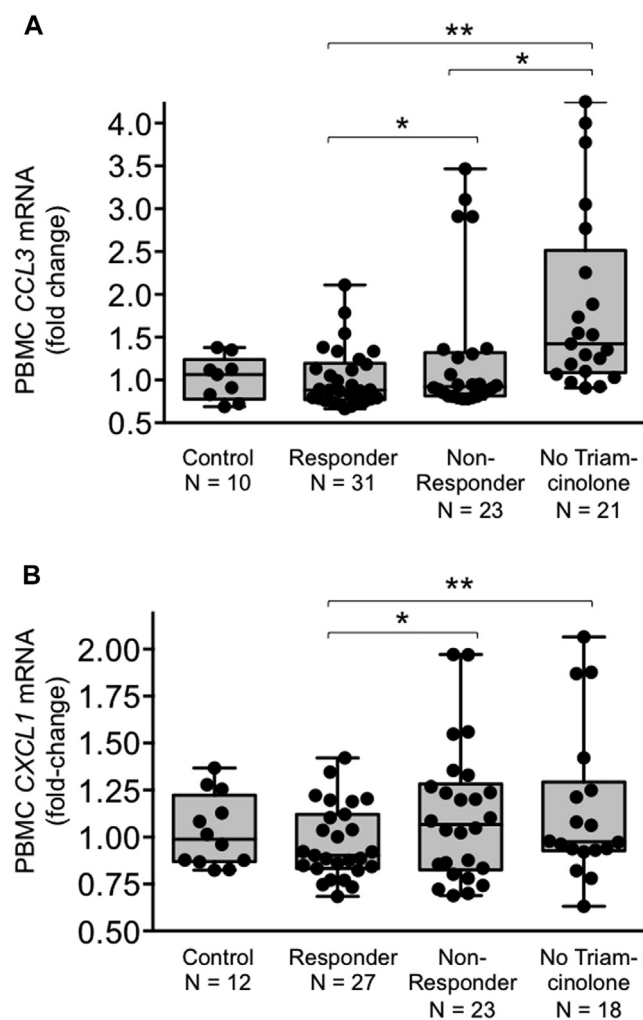


**FIG E5.** PBMC mRNA expression of *CCL3* (**A**) and *CXCL1* (**B**) in healthy control children, children with asthma who did and did not respond to triamcinolone, and children with asthma who did not receive triamcinolone. Box plots depict fold change values relative to control subjects and are shown with minimum and maximum values. Dots represent individual participants. \* $P < .05$  and \*\* $P < .01$ .

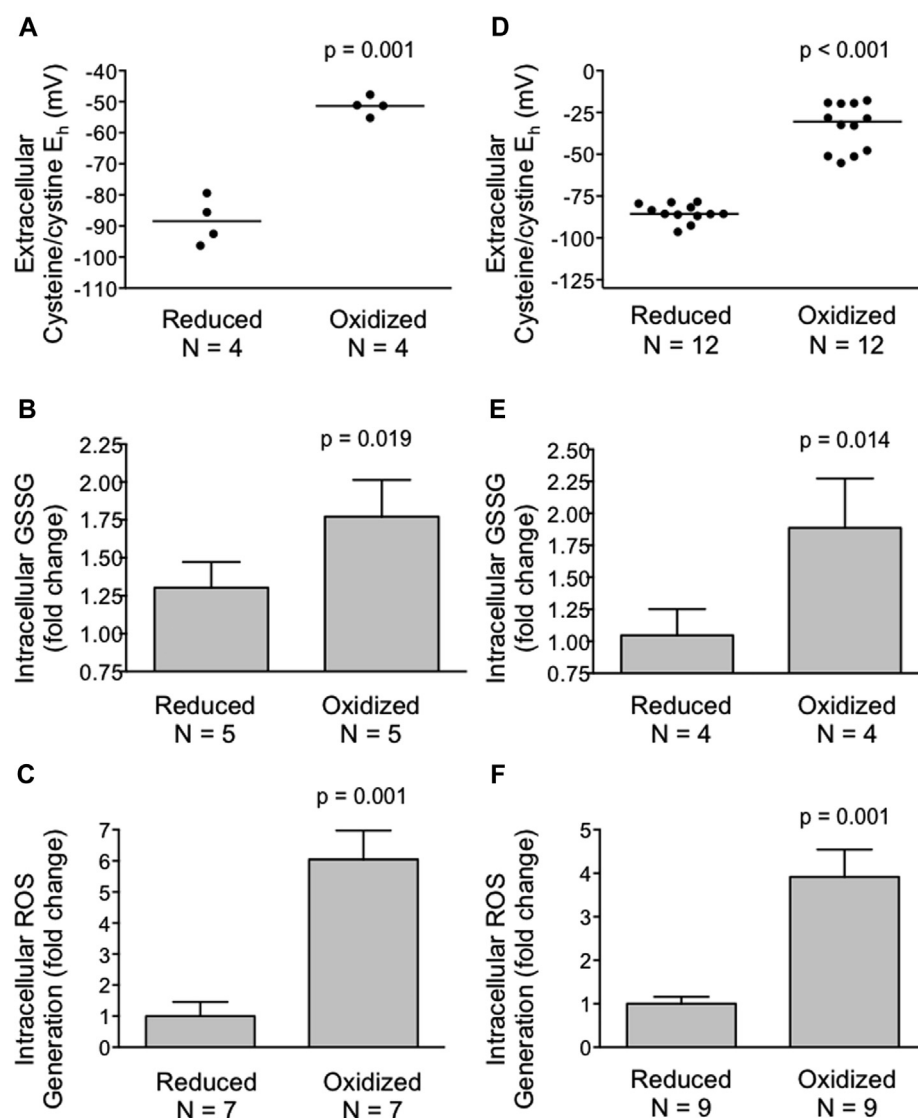


**FIG E6.** Extracellular cysteine/cystine  $E_h$ , intracellular glutathione disulfide (GSSG) concentrations, and intracellular ROS generation in THP-1 monocytes (A-C, respectively) and human primary PBMCs (D-F, respectively) after exposure to extracellular oxidizing versus reduced conditions. Horizontal lines in Fig E6, A and D, represent medians. Other data are shown as means  $\pm$  SEMs.

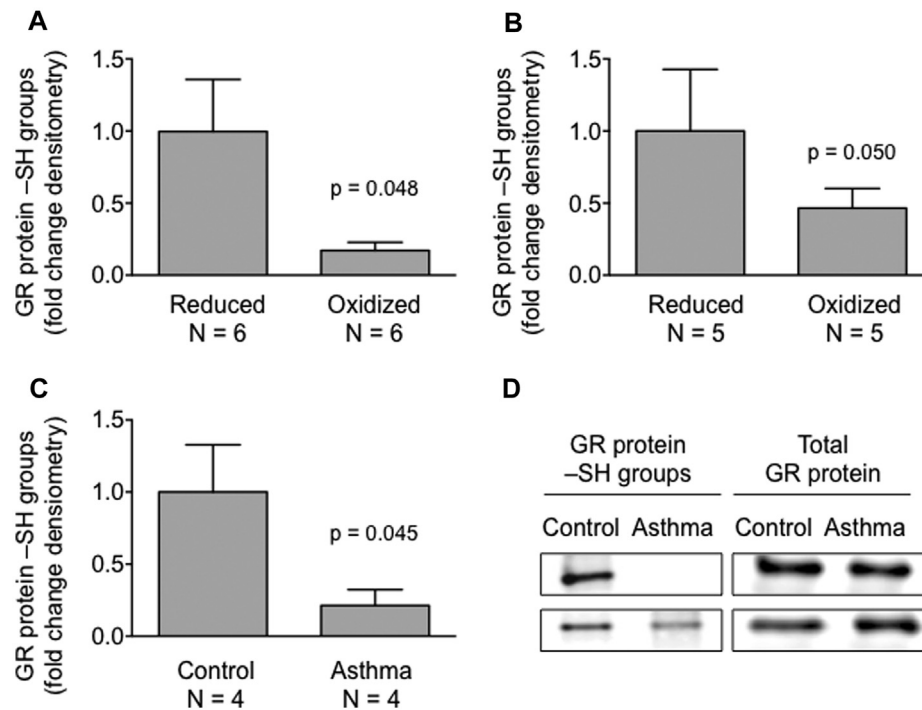


**FIG E7. A-C,** GR protein sulfhydryl (-SH) groups available for binding in THP-1 monocytes (Fig E7, A) and human primary PBMCs (Fig E7, B) after exposure to extracellular oxidizing versus reduced conditions and in healthy control children and children with asthma (Fig E7, C). Data are means  $\pm$  SEMs and normalized to total GR protein levels. **D,** Representative images from participants.

**TABLE E1.** Associations between tertiles of plasma cysteine/cystine  $E_h$  values\* and clinical features in participants with difficult-to-treat asthma

| Asthma feature                                       | Adjusted OR†<br>(95% CI) | P<br>value |
|------------------------------------------------------|--------------------------|------------|
| Middle tertile (−61.30 to −30.40 mV)                 |                          |            |
| High-dose inhaled glucocorticoids (previous 6 mo)    | 1.43 (0.46-4.42)         | .535       |
| Daily short-acting $\beta$ -agonists (previous 3 mo) | 2.26 (0.69-7.38)         | .177       |
| ACQ score >1.5‡                                      | 1.81 (0.46-7.05)         | .394       |
| ≥3 Oral corticosteroid bursts (previous year)        | 1.46 (0.49-4.38)         | .498       |
| Emergency department visit (previous year)           | 1.72 (0.55-5.43)         | .353       |
| Hospitalization for asthma (ever)                    | 1.27 (0.27-5.96)         | .761       |
| Intubation for asthma (ever)                         | 2.11 (0.53-8.32)         | .288       |
| Exhaled nitric oxide >35 ppb                         | 0.88 (0.28-2.749)        | .822       |
| FEV <sub>1</sub> /FVC ratio <80% of predicted value  | 0.79 (0.17-3.74)         | .768       |
| Highest tertile (−30.41 to 54 mV)                    |                          |            |
| High-dose inhaled glucocorticoids (previous 6 mo)    | 3.52 (1.05-11.79)        | .041       |
| Daily short-acting $\beta$ -agonists (previous 3 mo) | 3.87 (1.19-12.57)        | .024       |
| ACQ score >1.5‡                                      | 4.13 (1.10-15.46)        | .035       |
| ≥3 Oral corticosteroid bursts (previous year)        | 1.04 (0.34-3.15)         | .951       |
| Emergency department visit (previous year)           | 9.29 (2.31-37.35)        | .002       |
| Hospitalization for asthma (ever)                    | 2.17 (0.45-10.37)        | .333       |
| Intubation for asthma (ever)                         | 4.55 (1.25-16.60)        | .022       |
| Exhaled nitric oxide >35 ppb                         | 1.60 (0.53-4.80)         | .405       |
| FEV <sub>1</sub> /FVC ratio <80% of predicted value  | 1.98 (0.51-7.69)         | .324       |

FVC, Forced vital capacity; OR, odds ratio.

\*The lowest tertile of the plasma cysteine/cystine  $E_h$  (−99 to −61.29 mV) served as the reference group. A more positive  $E_h$  reflects greater oxidation of the sample.

†Analyses were adjusted for race, sex, and body mass index percentile.

‡ACQ scores range from 0 to 6, with lower scores indicating better asthma control.

**TABLE E2.** Features of the asthmatic participants at the baseline visit

| Feature                                                     | Did not receive triamcinolone (n = 42) | Received triamcinolone* (n = 57) | P value |
|-------------------------------------------------------------|----------------------------------------|----------------------------------|---------|
| Age (y)                                                     | 12 (10-15)                             | 12 (10-15)                       | .773    |
| Sex                                                         |                                        |                                  |         |
| Male                                                        | 22 (52)                                | 44 (77)                          | .010    |
| Female                                                      | 20 (48)                                | 13 (23)                          |         |
| Race                                                        |                                        |                                  |         |
| White                                                       | 14 (33)                                | 2 (4)                            | <.001   |
| Black                                                       | 26 (62)                                | 43 (75)                          |         |
| Asian                                                       | 1 (2)                                  | 0                                |         |
| ≥1 Race                                                     | 1 (2)                                  | 12 (21)                          |         |
| Body mass index                                             |                                        |                                  |         |
| <85th percentile (normal weight)                            | 17 (41)                                | 26 (46)                          | .660    |
| 85th to 95th percentile (overweight)                        | 13 (21)                                | 13 (23)                          |         |
| ≥95th percentile (obese)                                    | 12 (29)                                | 18 (32)                          |         |
| Asthma medications                                          |                                        |                                  |         |
| Inhaled corticosteroids                                     | 28 (67)                                | 57 (100)                         | <.001   |
| Montelukast                                                 | 14 (25)                                | 43 (75)                          | .009    |
| Self-reported allergic rhinitis                             | 36 (86)                                | 55 (97)                          | .052    |
| ACQ score†                                                  | 0.36 (0.14-0.61)                       | 1.29 (0.57-2.14)                 | <.001   |
| Pediatric Asthma Quality of Life Questionnaire total score‡ | 6.36 (5.71-6.85)                       | 5.30 (4.28-6.00)                 | <.001   |
| Serum IgE (kU/L)§                                           | 44 (20-254)                            | 365 (126-646)                    | <.001   |
| Exhaled nitric oxide (ppb)§                                 | 23 (12-43)                             | 28 (19-56)                       | .121    |
| Baseline lung function                                      |                                        |                                  |         |
| FVC (% predicted)                                           | 107 (103-117)                          | 100 (92-114)                     | .182    |
| FEV <sub>1</sub> (% predicted)                              | 97 (88-107)                            | 90 (75-101)                      | .044    |
| FEV <sub>1</sub> /FVC ratio                                 | 0.81 (0.77-0.84)                       | 0.77 (0.69-0.82)                 | .060    |
| FEV <sub>1</sub> /FVC ratio (% predicted)                   | 92 (88-96)                             | 90 (80-94)                       | .124    |
| FEF <sub>25-75</sub> (% predicted)                          | 79 (67-91)                             | 67 (49-77)                       | .118    |
| Postbronchodilator lung function                            |                                        |                                  |         |
| FVC (% predicted)                                           | 110 (101-118)                          | 109 (99-122)                     | .930    |
| FEV <sub>1</sub> (% predicted)                              | 108 (93-114)                           | 101 (93-115)                     | .770    |
| FEV <sub>1</sub> /FVC ratio                                 | 0.87 (0.82-0.88)                       | 0.83 (0.77-0.87)                 | .339    |
| FEV <sub>1</sub> /FVC ratio (% predicted)                   | 98 (93-102)                            | 96 (90-100)                      | .726    |
| FEF <sub>25-75</sub> (% predicted)                          | 103 (87-112)                           | 90 (73-107)                      | .421    |

Data represent medians (interquartile ranges) or numbers (percentages) of participants.

FEF<sub>25-75</sub>, Midexpiratory flow rate at 25% to 75% of vital capacity; FVC, forced vital capacity.

\*Triamcinolone was administered at the completion of the baseline characterization visit to participants with symptomatic asthma despite moderate- to high-dose inhaled corticosteroid therapy.

†ACQ scores range from 0 to 6, with lower scores indicating better asthma control.

‡Pediatric Asthma Quality of Life Questionnaire scores range from 0 to 7, with higher scores indicating better quality of life.

§Data were logarithmically transformed before statistical analyses.