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Airway Glutathione Homeostasis is Altered in Children with Severe Asthma:

Evidence for Oxidant Stress

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By

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An abstract of
A thesis submitted to the Faculty of the Graduate School of Emory University
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Abstract

Airway Glutathione Homeostasis is Altered in Children with Severe Asthma: Evidence for Oxidant Stress

By Anne M. Fitzpatrick, Ph.D.

Severe asthma in children is a complex disorder characterized by persistent airway inflammation and increased formation of reactive oxygen species. Because glutathione (GSH) is an important antioxidant in the epithelial lining fluid (ELF), we hypothesized that airway GSH homeostasis was altered in children with severe asthma and was characterized by decreased GSH and increased glutathione disulfide (GSSG) concentrations. Bronchoalveolar lavage was obtained from 65 children with severe asthma, including 35 children with baseline airway obstruction evidenced by $FEV_1 < 80\%$. Control data was obtained from 6 children with psychogenic (habit) cough or vocal cord dysfunction undergoing diagnostic bronchoscopy and 35 healthy adult controls. GSH, GSSG, and other determinants of airway oxidative stress including glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx), malondialdehyde (MDA), 8-isoprostane, and hydrogen peroxide (H_2O_2) were measured in the ELF. The ELF redox potential was calculated from GSH and GSSG using the Nernst equation. Compared to controls, severe asthmatics had lower airway GSH with increased GSSG despite no differences in GST, GR, and GPx activities between groups. This was accompanied by increased MDA, 8-isoprostane, and H_2O_2 concentrations in the ELF. GSH oxidation was most apparent in severe asthmatics with airway obstruction and was supported by an upward shift in the ELF GSH redox potential. We conclude that children with severe asthma have increased biomarkers of oxidant stress in the ELF which are associated with increased formation of GSSG and a shift in the GSH redox potential toward the more oxidized state.

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ABBREVIATIONS

BAL:	Bronchoalveolar lavage
ELF:	Epithelial lining fluid
F_{ENO}:	Fraction of exhaled nitric oxide
FEF_{25-75}:	Forced expiratory flow
FEV_1:	Forced expiratory volume in one second
FVC:	Forced vital capacity
GPx:	Glutathione peroxidase
GSH:	Reduced glutathione
GSSG:	Oxidized glutathione
GR:	Glutathione reductase
GST:	Glutathione S-transferase
H_2O_2:	Hydrogen peroxide
ICS:	Inhaled corticosteroid
MDA:	Malondialdehyde

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INTRODUCTION

Severe refractory asthma is a complex disorder characterized by airway hyper-responsiveness, obstructive changes in pulmonary function, and persistent airway inflammation despite high-dose inhaled corticosteroid (ICS) treatment (1-3). Although the inflammatory response is important for the initiation of tissue repair, the exaggerated responses associated with severe asthma result in excessive reactive oxygen species (ROS) formation and tissue destruction (4). This resulting imbalance between pro-oxidant and antioxidant forces leads to an ongoing cycle of inflammation in the asthmatic airway which ultimately contributes to irreversible airway injury (5).

Glutathione (GSH), a tripeptide thiol, is an abundant airway antioxidant (6) which reduces organic hydroperoxides and protects the airway from lipid peroxidation (7). In response to hydroperoxides, GSH is released by glutathione S-transferase (GST) and becomes oxidized through a reaction involving glutathione peroxidase (GPx). This process forms glutathione disulfide (GSSG), which can be reduced to GSH by an NADPH-dependent glutathione reductase (GR) reaction (Figure 1). The relationship between GSH and GSSG is a critical regulator of cellular processes and antioxidant defense. With excessive GSSG accumulation, airway GSH homeostasis is altered, resulting in impaired cellular signaling and increased susceptibility to lung injury (8-10).

Although GSH homeostasis has been previously assessed in the blood (11), sputum (12) and bronchoalveolar lavage (BAL) (13) of mild asthmatics, no study to date has directly examined airway GSH concentrations in patients with severe asthma. The purpose of this study was to quantify epithelial lining fluid (ELF) GSH homeostasis in children with severe asthma. We hypothesized that children with severe asthma would have increased airway

GSSG and greater generalized airway oxidation as measured by increased hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and 8-isoprostanes.

BACKGROUND

Although the inflammatory response is an essential component of tissue repair, this response is exaggerated in severe asthmatics and is associated with an ongoing cycle of increased reactive oxygen species (ROS) formation and tissue destruction (14,15). This cycle is often referred to as "oxidant stress" and reflects an imbalance between pro-oxidant and anti-oxidant forces.

The primary ROS in the airways include superoxide anion (O_2^-), hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2) (Figure 2). Superoxide ion is derived from external environmental sources as well as internal cellular processes, and in the presence of H_2O_2, is quickly converted to OH^-. Free OH^- oxidizes cell membrane phospholipids and generates bioactive molecules such as malondialdehyde (MDA) and 8-isoprostanes, which impair cellular function while activating pro-inflammatory transcription pathways (16).

A number of studies have reported increased ROS generation and lipid peroxidation byproducts in patients with asthma. Compared to controls, asthmatics have decreased superoxide dismutase and catalase activity in the bronchial epithelium (17, 18). This inactivation is attributable to oxidation of essential proteins (18) and ultimately results in apoptosis and cell death (196). Furthermore, both steroid-naïve and ICS-treated asthmatics have higher concentrations of airway 8-isoprostanes (20-21). These concentrations are highest in severe asthmatics (19) and correlate with the degree of bronchial hyperreactivity (21). Likewise, airway MDA and H_2O_2 concentrations are also elevated in asthmatics and are associated with disease severity (23, 24), measures of pulmonary function such as FEV_1 (24, 25), and bronchial hyperresponsiveness to histamine (26). Although the bulk of the oxidative burden occurs in the airways of asthmatic patients, oxidant stress may also be

reflected in the systemic circulation. Several investigators have reported increased total reactive oxygen metabolite levels (27) and decreased total antioxidant capacity (23) in the serum of stable asthmatics, which worsen with acute exacerbations (28). Others have shown lower circulating superoxide dismutase activity and increased MDA concentrations which are associated with asthma severity (29-31). These data suggest that oxidative stress and lipid peroxidation are important contributors to the pathophysiology of severe asthma. Given the relationships between oxidative biomarkers and clinical asthma indicators such as pulmonary function, oxidant stress may account for many of the features of children with severe asthma which persist despite corticosteroid treatment.

GSH, a tri-peptide thiol, is the primary airway antioxidant defense against lipid peroxidation (6). Although research on GSH in asthma is limited, a few studies have reported lower GSH and increased GSSG concentrations in the ELF (13) and exhaled breath (32, 33) of asthmatics as compared to controls. Similar findings have also been reported in the plasma (31, 34). Whereas one study noted normalization of GSH and GSSG after corticosteroid treatment (32) in mild asthmatics, the homeostasis between GSH and GSSG in severe asthmatics is unclear but warrant further study. In both humans (35) and mice (36), GSH depletion is associated with significant bronchial hyper-responsiveness, particularly in the presence of the Th2 cytokine IL-13 (37). Although airway hyper-responsiveness is attenuated *in vivo* with exogenous GSH (38, 39), a clinical trial of aerosolized GSH resulted in significant bronchoconstriction with acute symptom worsening, presumably due to increased GSSG formation (40). While more studies on GSH in asthma are needed, these data suggest that oxidation of GSH to GSSG may have profound implications in the asthmatic airway, including increased susceptibility to lung injury.

METHODS

Null hypothesis. The primary null hypothesis was that GSH and GSSG concentrations were not different between children with severe asthma and controls. The secondary null hypothesis was that H_2O_2, MDA, and 8-isoprostanes were not different between groups.

Study design. This study assumed a cross-sectional, four-group descriptive design. Children with severe asthma were stratified according to the presence or absence of airflow obstruction (AO). Children with severe asthma with (AO+) and without (AO-) airflow obstruction were compared to non-asthmatic pediatric controls and healthy, non-smoking adult controls.

Sample. A convenience sample of severe asthmatic children 5-17 years of age attending a difficult asthma clinic at Emory University were recruited for this study. Participants were clinically stable and were under the care of a pediatric pulmonary specialist. Participants underwent flexible bronchoscopy as indicated for persistent asthma symptoms despite appropriate treatment with high-dose inhaled and systemic corticosteroids (41). The subsequent BAL sample was divided between the research and clinical laboratories according to a protocol approved by the local Institutional Review Board. Informed consent was obtained from all caregivers. Children also provided verbal and written assent.

Severe asthmatic children met published criteria for persistent asthma (42) and had at least a 12% change in the forced expiratory volume in one second (FEV_1) after short-acting bronchodilator administration. Severe asthma was diagnosed according to criteria developed by the NIH/NHLBI Severe Asthma Research Program (1, 2) based on an American Thoracic

Society consensus panel report (Table I) (43). Thresholds for high-dose ICS were adjusted for children and defined as ≥ 440 mcg of fluticasone equivalent per day for children less than 12 years and ≥ 880 mcg for children 12-17 years (2). All asthmatic children were treated with a stable dose of ICS or oral corticosteroids for at least eight weeks prior to bronchoscopy. Children with immunodeficiency, history of premature birth or other pulmonary morbidities were excluded. Asthmatics were screened for corticosteroid adherence. Known co-morbid conditions associated with asthma including sinus infection, sleep disorders, and gastroesophageal reflux were addressed and treated before bronchoscopy.

Controls for this study were recruited from two populations: 1) children with psychogenic (habit) cough or vocal cord dysfunction, and 2) healthy, non-smoking adult volunteers. Control children had no family history of asthma, a negative bronchodilator response, no evidence of aeroallergen sensitivity, and normal exhaled nitric oxide (F_{ENO}) concentrations. Adults serving as controls were non-smokers with no known pulmonary disorders and no respiratory symptoms.

Outcome variable. The primary outcome variable was the percentage of GSSG in the ELF. Secondary outcome variables included the redox potential (E_h) of the GSH:GSSG couple and concentrations of GSH, total GSH + GSSG, H_2O_2, MDA, and 8-isoprostanes in the ELF.

Predictor variables. To further test the relationship between GSH oxidation and asthma severity, regression analyses were performed using the percentage of GSSG as the predictor and measures of asthma severity, including hospitalization within the previous year, ICS dose, daily prednisone use, F_{ENO}, FEV_1, and FEV_1 bronchodilator reversibility, as

dependent variables. To evaluate factors that might affect GSH oxidation in the ELF, the percentage of GSSG was treated as the dependent variable and age, gender, ethnicity, ICS dose, prednisone dose, and the percentage of airway eosinophils and neutrophils were used as predictors.

Procedures. Spirometry was performed with a portable spirometer (KoKo® Legend, Ferraris, Louisville, CO) according to ATS criteria for reproducibility (44) and was interpreted according to population reference standards (45). F_{ENO} was collected with a reservoir bag at a fixed exhaled flow rate of 0.35 L/s and analyzed offline by chemiluminescence (Sievers NOA^{TM} 280-I, Ionic Instruments, Boulder, CO) (46). Nicotine exposure was verified in healthy adult controls using a urinary cassette test (Accutest®, Jant Pharmacal, Encino, CA) with a cotinine cut-off of 200 ng/mL. Venipuncture was performed in all participants immediately prior to bronchoscopy for plasma urea determination.

Bronchoscopy in pediatric participants was performed by pediatric pulmonologists using a laryngeal mask airway or endotracheal tube and inhaled sevoflurane. BAL fluid was collected from the right middle lobe with three 1 mL/kg (50 mL maximum) saline lavages flushed through the suction channel of a flexible bronchoscope (Olympus BF-3C160 [3.7 mm] or BF-P160 [4.9 mm], Olympus America Inc., Melville, NY). Bronchoscopy was performed in adults by physicians trained in pulmonary and critical care medicine using a flexible bronchoscope (Olympus BF-1T20D) passed trans-nasally into the right middle lobe. Subjects received intravenous midazolam and fentanyl for the procedure. Three 50 mL saline aliquots were instilled and immediately aspirated. The BAL from all participants was pooled under continuous low pressure suction. In children, the BAL return volume was divided between the research and clinical laboratories. The samples submitted to the clinical

laboratories were subjected to standard culture and sensitivity testing, viral respiratory panels, and cytopathological stains for bacteria and fungi.

BAL fluid was centrifuged at 1200 rpm for 7 minutes at 4° C to separate the supernatant and cellular fractions. The supernatant was removed and divided into 250 μl aliquots. To prevent auto-oxidation of the samples during storage, one sample aliquot was preserved immediately after collection for GSH and GSSG analysis in a 5% perchloric acid solution containing iodoacetic acid (6.7 μM) and boric acid (0.1 M) with 5 μM of γ-glutamyl-glutamate internal standard (47). A second sample aliquot was preserved in 5 μL of 2.5 mg/mL butylated hydroxytoluene for 8-isoprostane measurement. Aliquots were stored at -80°C prior to analysis. The cell pellet was resuspended in 1 mL Dulbecco's Modified Eagles Medium containing 10% fetal calf serum. Total cell counts were performed manually with a hemocytometer. Cellular differentials were assessed after Wright staining.

The protein content of the BAL supernatant was assessed using a Coomassie (Bradford) protein assay (Pierce Biotechnology, Rockford, IL) with a detection limit of 1 μg/mL at an absorbance of 595 nm. Urea nitrogen was measured in plasma and BAL supernatant using a quantitative colorimetric assay (Pointe Scientific, Canton, MI) with sensitivity of 0.05 to 150 mg/dL. The dilution of the BAL was calculated from $[urea]_{plasma}/[urea]_{BAL}$ (48).

GSH and GSSG concentrations were measured in BAL supernatant by reverse phase high performance liquid chromatography after derivatization of the samples with dansyl chloride (49). Derivatives were separated on a 10 micron Ultrasil amino-column with detection at 365 nm (Waters 2690, Milford, MA). Fluorescence detection was recorded by

two detectors (Waters 474 and Gibson model 121). GSH and GSSG were quantified relative to γ-glutamyl-glutamate by integration.

The redox potential (E_h) of the GSH/GSSG thiol pair in ELF was calculated with the Nernst equation, $E_h = E_o + RT/nF \ln [disulfide]/([thiol1][thiol2])$ (50). The E_o is the standard potential for the redox couple, R is the gas constant, T is the absolute temperature, n is 2 for the number of electrons transferred, and F is Faraday's constant. The standard potential E_o for the two GSH/GSSG couple was -264 mV at pH = 7.4. Adjustment for pH was made by a +5.9 mV change in E_o with every 0.1 decrease in pH.

Enzymatic activities of GST, GR, and GPx were quantified in the BAL supernatant with commercially available assay kits (Cayman Chemical, Ann Arbor, MI). For GST, samples were concentrated to molecular weight cut-off of 3,000. GST activity was determined after conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH. GST activity was analyzed over five minutes at 340 nm with a CDNB extinction coefficient of 0.0096 $\mu M^{-1}cm^{-1}$. GR and GPx samples were concentrated to a 10,000 molecular weight cut-off. GR activity was determined by the rate of NADPH oxidation and GPx activity was determined through a coupled reaction with GR. GR and GPx activities were analyzed over five minutes at 340 nm absorbance with an NADPH extinction coefficient of 0.00622 0.00622 $\mu M^{-1}cm^{-1}$. Each assay had a wavelength accuracy of ± 2 nm with repeatability of ± 0.2 nm.

Malondialdehyde (MDA) concentrations were determined in the BAL supernatant with a spectrophotometric assay (Bioxytech® MDA-586, Oxis International, Foster City, CA) at 586 nm absorbance with a detection limit A_{586} of 0.0088. This assay is based on the reaction of N-methyl-2-phenylindole with MDA at 25ºC. 8-Isoprostane concentrations were

obtained by ELISA (Cayman Chemical, Ann Arbor, MI) with absorbance at 420 nm and detection limit of approximately 2.7 pg/mL. H_2O_2 concentrations were determined spectrophotometrically from a microplate assay kit (Amplex® Red, Molecular Probes, Eugene, OR) with an excitation of 560 nm, fluorescence emission detection at 590 nm, and sensitivity of 50 nM.

Sample size calculations. Sample size calculations were performed using an effect size of 0.75 generated from our preliminary data on ELF GSSG in severe asthmatics versus non-asthmatic controls. Using a two-tailed α of 0.05 and β of 0.20 and assuming unequal sample sizes, a sample of 30 children with severe asthma and 10 non-asthmatic pediatric controls was needed to achieve power of 0.814. Although attrition was unlikely due to the cross-sectional design, we attempted to over-sample by at least 10% to ensure adequate power to address our study hypotheses. This resulted in a target sample size of 33 severe asthmatic children with airflow obstruction (AO+), 33 severe asthmatic children without airflow obstruction (AO-), and 13 pediatric controls. Although we had no preliminary data on GSSG in adult controls, we also aimed to recruit 33 healthy, non-smoking adults for comparison.

Statistical analyses. Data were analyzed with SPSS® software (Version 15, SPSS Inc., Chicago, IL). Children with severe asthma were stratified according to the baseline airway obstruction, defined as an $FEV_1 < 80\%$ predicted or an FEV_1:FVC < 0.80 (45). GSH, GSSG, MDA, 8-isoprostane, and H_2O_2 concentrations were expressed per mL of ELF according to the urea dilution (48) and were logarithmically transformed prior to statistical analysis given their non-normal distribution. GST, GR, and GPx enzymatic activities were also expressed per mL of ELF and logarithmically transformed prior to analysis. Differences

between groups were assessed by analysis of variance with Tukey's post-hoc testing with significance defined as $\alpha \leq 0.05$. Bivariate Pearson correlations were used to examine associations between variables. To test the relationship between GSH oxidation and asthma severity, univariate logistic and linear regression analyses were performed using the percentage of GSSG as the independent variable and measures of asthma severity, including hospitalization within the previous year, ICS dose, daily prednisone use, F_{ENO}, FEV_1, and FEV_1 bronchodilator reversibility, as dependent variables. To evaluate factors that might affect GSH oxidation in the ELF, multivariate backward elimination linear regression was performed using the percentage of GSSG as the dependent variable and age, gender, ethnicity, ICS dose, prednisone dose, and the percentage of airway eosinophils and neutrophils as predictors. Multicollinearity between predictors was assessed with tolerance statistics. Entry and removal probabilities were set at 0.05 and 0.10, respectively.

RESULTS

Seventy four children with severe asthma, 13 pediatric controls, and 35 healthy adult controls were recruited for this study. After recruitment, 7 of the 13 pediatric controls were diagnosed with BAL colonization (n = 3) or chronic aspiration (n = 4) and were excluded from data analysis due to the confounding effects of colonization and aspiration on oxidant stress. Nine severe asthmatics also had BAL colonization with *Streptococcus pneumoniae* (n = 4), *Haemophilus influenzae* (n = 2), or *Moraxella catarrhalis* (n = 3) and were similarly excluded. Features of the excluded children appear in Tables II-IV. Although the clinical features of excluded children did not differ from those included in the final analysis, the excluded children did have greater evidence of ELF oxidant stress. Compared to pediatric controls, the non-asthmatic children excluded from data analysis had lower GSH, lower total GSH + GSSG concentrations, a higher percentage of GSSG, and a more oxidized redox potential (E_h) in the ELF (Table III). Likewise, the severe asthmatics excluded from the final analysis also had lower GSH, higher GSSG, and a more oxidized redox potential when compared to the severe asthmatics included in the final data analysis (Table IV).

The resulting pediatric control group contained six children. Post-bronchoscopy diagnoses in this group included psychogenic (habit) cough (n = 4) and vocal cord dysfunction (n = 3). None of the children serving as controls were receiving ICS at the time of bronchoscopy. Given the symptomatic nature of these children, 35 healthy adults were also recruited for comparison. Adults in this control group were non-smokers free of respiratory symptoms and medication use; however, they were significantly older (Table V).

Features of the groups appear in Tables V and VI. Children with severe asthma were stratified according to the baseline airway obstruction, defined as an FEV_1 < 80% predicted

or an FEV_1 to FVC ratio < 0.80 (45). Severe asthmatics with airway obstruction (AO+) had increased FEV_1 bronchodilator reversibility with albuterol despite higher ICS doses and a higher prevalence of hospitalization within the previous year (Table V).

Flexible bronchoscopy with BAL was well tolerated in all participants. Bronchospasm greater than 15 seconds was observed in three severe asthmatic children and responded immediately to bronchodilators and positive airway pressure. No subject required overnight hospitalization or prolonged observation.

The composition of the BAL is presented in Table VII. BAL samples from adult controls were characterized by larger return volumes and higher cell counts. Adult and pediatric controls also had significantly less protein and fewer neutrophils and eosinophils than severe asthmatics with (AO+) and without (AO-) airway obstruction ($p < 0.05$, Table VII).

GSH, GSSG, and GSH/GSSG redox potential in ELF. Compared to adult and pediatric control subjects, children with severe asthma had significantly lower GSH (Figure 3) and lower total GSH + GSSG concentrations in the ELF (adult control: 436 ± 249 μM; pediatric control: 260 ± 230 μM; severe asthma (AO-): 134 ± 150; severe asthma (AO+): 129 ± 134 μM; $p < 0.001$ for severe asthma AO-/+ versus adult controls, $p = 0.07$ for severe asthma AO+/- versus pediatric controls). In severe asthmatics with airway obstruction, the majority (~60%) of the total pool was in the oxidized (GSSG) form (Figures 4, 5). Using the Nernst equation, the oxidative redox potential (E_h) for the GSH/GSSG pair was significantly more reduced in the adult controls as compared to the other groups, with the greatest oxidation apparent in severe asthmatics with airway obstruction (Figure 6).

Airway GST, GR, and GPx activities. To determine whether altered GSH-dependent enzymatic activities might account for increased GSH oxidation in severe asthma, GST, GR, and GPx were quantified in the ELF. GST activities (expressed per mL of ELF) were similar between groups (adult control: 1.25 ± 0.94; pediatric control: 1.00 ± 0.99; severe asthma (AO-): 1.02 ± 1.14; severe asthma (AO+): 1.19 ± 1.65 nmol/min/mL, p = 0.305). GR activities (adult control: 0.87 ± 1.00; pediatric control: 0.83 ± 1.18; severe asthma (AO-): 0.99 ± 0.68; severe asthma (AO+): 0.89 ± 0.82 nmol/min/mL, p = 0.406) and GPx activities (adult control: 1.40 ± 1.25; pediatric control: 2.04 ± 1.33; severe asthma (AO-): 1.83 ± 1.10; severe asthma (AO+): 2.06 ± 1.23 nmol/min/mL, p = 0.719) were also not different between groups.

Associations between GSH and other oxidative biomarkers. MDA, 8-isoprostanes, and H_2O_2 were significantly elevated in the ELF of severe asthmatics as compared to adult and pediatric controls (Figures 7-9). In severe asthmatics with and without airway obstruction, MDA, 8-isoprostanes and H_2O_2 were positively correlated with the percentage of airway GSSG and the oxidative redox potential (E_h) of the GSH:GSSG couple ($p \leq 0.05$ for each association, Table VIII).

Clinical predictors of GSH oxidation in severe asthmatics. Within the group of severe asthmatics, total GSH + GSSG concentrations were not associated with baseline FEV_1 or the presence of airway eosinophils or neutrophils. Total GSH + GSSG concentrations, the percentage of GSSG and the redox potential (E_h) of the GSH:GSSG couple were significantly associated with baseline F_{ENO} (GSH + GSSG: r = -0.60, p = 0.003; % GSSG: r = 0.480, p = 0.032; E_h GSH:GSSG: r = 0.43, p = 0.047). To test the association between altered GSH homeostasis and clinical markers of asthma severity, univariate logistic and

linear regression analyses were performed using the percentage of GSSG as the predictor and hospitalization within the previous year, prednisone use, ICS dose, F_{ENO}, baseline FEV_1, and FEV_1 bronchodilator reversibility as dependent variables. Using this approach, hospitalization within the previous year (-2 log L = 61.49, p = 0.001) and baseline F_{ENO} (R^2 = 0.23, p = 0.032) were significantly predicted by the percentage of GSSG (Tables IX, X).

In order to evaluate factors that might affect airway GSSG formation, stepwise linear regression analysis was performed on children with severe asthma with the percentage of GSSG as the dependent variable and age, gender, ethnicity, ICS dose, prednisone dose, and the percentage of airway eosinophils and neutrophils as predictors. With the multivariate model, only gender (p = 0.038) and airway eosinophils (p = 0.031) were predictive of airway GSH oxidation (final model R^2 = 0.32, p = .045, Table XI).

DISCUSSION

This is the first study to provide a detailed assessment of airway GSH homeostasis in children with severe asthma. Total ELF GSH + GSSG concentrations were significantly lower in symptomatic children than in adult controls and were accompanied by greater GSH oxidation as measured by GSSG. GSSG was increased nearly twofold in severe asthmatics with airway obstruction and was further associated with increased MDA, 8-isoprostane, and H_2O_2 concentrations. Enzymatic activities of GST, GR, and GPx were similar between groups and were not associated with ELF GSH levels. These results suggest that GSH homeostasis is altered in children with severe asthma as a function of increased ELF oxidation, which favors the conversion of GSH to GSSG and signals compromised antioxidant function.

GSH was first characterized in the ELF of healthy, non-smoking adults nearly twenty years ago. In the classic study by Cantin and associates (6), total GSH + GSSG concentrations in the alveolar ELF of healthy adults were approximately 430 μM, with less than 5% of the total pool in the oxidized (GSSG) form. While smoking initially increases GSH concentrations (6), long-term smoking has been associated with increased GSSG formation (51). Similar increases in GSSG with resulting declines in GSH have been noted in adults with chronic alcoholism (52, 53), human immunodeficiency virus (54), pulmonary fibrosis (55, 56), and acute respiratory distress syndrome (57, 58). These studies suggest that ELF GSH homeostasis is disturbed in a variety of pulmonary disorders and may account for some of the respiratory morbidity associated with pulmonary disease.

Few studies have examined GSH homeostasis in asthma, and fewer still have measured GSH in the ELF. In an earlier study, Smith et al. (59) noted that total GSH +

GSSG concentrations in the ELF were higher in mild asthmatic adults than controls. However, GSH concentrations were inversely correlated with bronchial hyperresponsiveness, suggesting a relationship between antioxidant defense and pulmonary function (59). Although similar studies of steroid-naïve mild asthmatics have shown no differences in baseline GSH concentrations as compared to controls (13, 60), baseline GSSG is elevated (13) and increases further with segmental antigen challenge (60). These data suggest that airway oxidant stress is a defining feature of asthma which may be present in spite of normal clinical features.

The redox potential is a measure of the capacity to reduce reactive oxygen species where the more negative the number, the greater the reduction potential. We observed redox potentials in symptomatic children that were significantly lower than those of healthy adults. Children with severe asthma had ELF redox potentials that, on average, were approximately 75 mV more oxidized than adult controls. Other studies of plasma have suggested that the GSH redox potential increases in an age-dependent manner by 0.7 mV per year (61, 62). If a similar trend is present in ELF, then the oxidant stress observed in our sample of children with severe asthma is more similar to that of an older adult. Furthermore, a 75 mV shift in the GSH redox potential is more than sufficient to cause a 17-fold change in the ratios of reduced to oxidized forms of proteins such as thioredoxins (50).

This study has a number of limitations. Because bronchoscopy cannot be performed on asymptomatic children solely for research purposes, our pediatric control group was limited to symptomatic children with psychogenic cough or vocal cord dysfunction undergoing bronchoscopy for diagnostic purposes. It is therefore possible that the GSH concentrations we observed are not representative of those from healthy asymptomatic

children. Similarly, because we were limited to a convenience sample of severe asthmatic children, it is unclear if ELF GSH concentrations differ in severe asthmatics who are well controlled on high doses of ICS and do not undergo bronchoscopy. The fact that our adult control group was significantly older also raises the question as to whether age is a determinant of antioxidant defense across the lifespan. Additional studies are needed before these questions can be adequately addressed.

It is also possible the differences in GSH homeostasis that we observed could be attributed to the confounding effects of asthma treatment or other clinical features. Although ICS dose was highly associated with GSH and oxidative biomarkers, ICS dose failed to predict GSH oxidation in multivariate analysis. The fact that ICS dose is a surrogate of severe asthma and the major criteria for diagnosis (43) likely accounts for this relationship. Alternatively, these data may indicate that corticosteroids alone are insufficient to counteract airway oxidant stress in this population. The elevated MDA, 8-isoprostane, and H_2O_2 concentrations observed in severe asthmatics with airway obstruction also question the steroid sensitivity of this population. Additional studies are needed to thoroughly examine the relationship between GSH homeostasis, corticosteroid treatment, and clinical indicators of severe asthma in children.

For this study, we asked pediatric participants and their caregivers to list all current medications. We then questioned children and their parents as to the specific dosages and frequency of administration. While this method of medication assessment likely yielded accurate information regarding current asthma treatment, it may also have underestimated the use of over-the-counter medications such as acetaminophen. In a recent report from the International Study of Asthma and Allergies in Childhood (ISAAC), acetaminophen

(paracetamol) use was associated with a dose-dependent increased risk of asthma symptoms in children 6 to 7 years of age (63). This finding warrants further study. Acetaminophen is metabolized by cytochrome P450 to form the toxic species N-acetyl-p-benzoquinone imine, which is detoxified by GSH. With repeated acetaminophen use or excessive dosages, liver GSH stores are depleted, resulting in liver damage (64). Future studies should consider the effects of acetaminophen use on altered airway GSH homoestasis, particularly in patients with severe asthma.

In summary, we have demonstrated significant alterations of GSH homeostasis in children with severe refractory asthma characterized by decreased GSH, increased GSSG, and greater oxidation as measured by the GSH redox potential, MDA, 8-isoprostane and H_2O_2 concentrations in the ELF. Given the antioxidant properties of GSH, further studies are needed to understand the impact of altered GSH homeostasis on asthma severity. While previous studies have provided some support for the role of dietary antioxidants in allergic airway disease, the results of antioxidant supplementation studies have been largely disappointing (65). However, these previous studies focused on clinical outcomes such as pulmonary function and did not take into account the effects of antioxidant therapy on extracellular antioxidant balance or intracellular signaling (65). Given our findings of altered GSH homeostasis in severe asthma, additional studies are warranted to better understand the effects of airway GSH on respiratory morbidity. Ultimately, these data argue for interventions to increase ELF GSH concentrations in this population of children who are otherwise very difficult to treat.

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Table I. Criteria for severe asthma in children adopted by the NIH/NHLBI Severe Asthma Research Program and based on the American Thoracic Society consensus panel report.

Major criteria (must have at least one)
• Daily high-dose ICS Children < 12 years: ≥ 440 μg fluticsone equivalent/day Children ≥ 12 years: ≥ 880 μg fluticasone equivalent/day • Daily oral corticosteroid use
Minor criteria (must have at least two)
• Treatment with a daily controller medication in addition to ICS • Daily short-acting bronchodilator use (at least 5 of 7 days) • Airway obstruction with FEV_1 < 80% predicted at baseline • One or more emergency room visits in the previous 12 months • Three or more oral corticosteroid bursts in the previous 12 months • History of worsening symptoms with a reduction in corticosteroid dose • History of intubation

Table II. Characteristics of pediatric subjects excluded from data analysis.

Subject	Asthma status	Gender	Age	FEV_1 (%)	Reason for Exclusion
1	Non-asthmatic	Female	14	77	Chronic aspiration[1]
2	Non-asthmatic	Male	9	82	*H. influenzae*
3	Non-asthmatic	Female	13	105	*S. pneumoniae*
4	Non-asthmatic	Female	15	95	Chronic aspiration
5	Non-asthmatic	Female	16	94	Chronic aspiration
6	Non-asthmatic	Male	15	78	Chronic aspiration
7	Non-asthmatic	Female	14	92	*S. pneumoniae*
8	Asthmatic	Male	8	104	*H. influenzae*
9	Asthmatic	Male	9	90	*M. catarrhalis*
10	Asthmatic	Male	16	106	*S. pneumoniae*
11	Asthmatic	Male	10	64	*S. pneumoniae*
12	Asthmatic	Male	19	100	*H. influenzae*
13	Asthmatic	Female	8	68	*M. catarrhalis*
14	Asthmatic	Female	7	92	*S. pneumoniae*
15	Asthmatic	Female	11	82	*S. pneumoniae*
16	Asthmatic	Male	9	85	*M. catarrhalis*

[1] Chronic aspiration was diagnosed according to the presence of lipid-laden macrophages and a positive barium swallow or esophageal pH monitoring study.

Table III. Biomarkers of oxidant stress in non-asthmatic children excluded from data analysis, compared to those of pediatric controls included in data analysis. Data are expressed per mL of epithelial lining fluid (ELF) and represent the mean ± SD, with [a]$p < 0.05$ versus pediatric controls included in data analysis. Differences between groups were analyzed with non-parametric Mann-Whitney U statistics.

	Pediatric controls Included in analysis (n = 6)	Pediatric subjects excluded from analysis (n = 7)
GSH (μM)[a]	231 ± 218	23 ± 16[a]
GSSG (μM)	29 ± 30	48 ± 47
GSH + GSSG (μM)	260 ± 219	71 ± 55[a]
% GSSG	16 ± 14	61 ± 23[a]
E_h GSH:GSSG (mV)	-147 ±30	-82 ± 21[a]
H_2O_2 (μM)[a]	6 ±7	11 ± 6[a]
MDA (μM)[a]	20 ± 10	41 ± 12[a]
8-Isoprostanes (μM)	205 ± 100	273 ± 17

Table IV. Biomarkers of oxidant stress in severe asthmatic children excluded from data analysis, compared to those from severe asthmatics without airflow obstruction (SA AO-) and severe asthmatics with airflow obstruction (SA AO+) included in data analyses. Data are expressed per mL of epithelial lining fluid (ELF) and represent the mean ± SD, with [a]$p < 0.05$ versus SA AO-, [b]$p < 0.05$ versus SA AO+. Differences between groups were analyzed with non-parametric Mann-Whitney U statistics. Post-hoc testing was performed on significant variables with ANOVA and Tukey tests.

	SA AO- Included in analysis (n = 30)	SA AO+ Included in analysis (n = 25)	Severe asthmatics Excluded (n = 9)
GSH (μM)	94 ±107	57 ± 95	34 ± 51[a]
GSSG (μM)	39 ±61	72 ± 76	16 ± 15[b]
GSH + GSSG (μM)	134 ±150	129 ± 134	50 ± 48[a,b]
% GSSG	36 ±27	62 ± 24	55 ±38[a]
E_h GSH:GSSG (mV)	-119 ±44	-96 ± 30	-82 ± 45[a]
H_2O_2 (μM)	12 ± 13	15 ± 18	31 ± 46
MDA (μM)	38 ± 12	51 ± 39	95 ± 93
8-Isoprostanes (μM)	405 ± 274	1073 ± 1929	827 ± 1228

Table V. Baseline characteristics of adult controls (AC), pediatric controls (PC), severe asthmatics without airflow obstruction (SA AO-), and severe asthmatics with airflow obstruction (SA AO+). Data represent the mean ± SD or the frequency (%), where [a]$p < 0.05$ vs. AC; [b]$p < 0.05$ vs. PC; [c]$p < 0.05$ vs. SA AO-

	AC (n = 35)	PC (n = 6)	SA AO- (n = 35)	SA AO+ (n = 30)
Age (years)	39 ± 11	10 ± 6[a]	8 ± 5[a]	10 ± 5[a]
Male	14 (40%)	4 (67%)	21 (60%)	14 (47%)
Ethnicity:				
Caucasian	16 (46%)	5 (83%)	26 (74%)	7 (23%)[a,b,c]
African American	18 (51%)	1 (17%)	9 (26%)	22 (73%)[a,b,c]
Other	1 (3%)	0	0	1 (3%)
Daily ICS dose[1]	0	0	596 ± 228[a,b]	905 ± 280[a,b]
Daily medications:				
Budesonide	0	0	12 (34%)[a,b]	7 (23%)[a,b]
Fluticasone	0	0	2 (6%)	2 (7%)
Fluticasone/salmeterol	0	0	20 (57%)[a,b]	21 (70%)[a,b]
Montelukast	0	0	23 (66%)[a,b]	30 (100%)[a,b]
Prednisone	0	0	0	16 (53%)[a,b,c]
Hospitalization[2]	0	0	8 (23%)[a,b]	40 (100%)[a,b,c]

[1] In micrograms of fluticasone equivalents per day.

[2] Refers to an asthma-related hospitalization within the previous year.

Table VI. Baseline pulmonary function measures and exhaled nitric oxide (F_{ENO}) concentrations in adult controls (AC), pediatric controls (PC), severe asthmatics without airflow obstruction (SA AO-), and severe asthmatics with airflow obstruction (SA AO+). Data represent the mean ± SD or the frequency (%), where [a]$p < 0.05$ vs. AC; [b]$p < 0.05$ vs. PC; [c]$p < 0.05$ vs. SA AO-

	AC (n = 35)	PC (n = 6)	SA AO- (n = 35)	SA AO+ (n = 30)
Pulmonary function:[1]				
FVC (%)	96 ± 16	92 ± 12	100 ± 16	83 ± 21[a,c]
FEV_1 (%)	102 ± 16	93 ± 9	96 ± 17	68 ± 20[a,b,c]
FEV_1: FVC	0.87 ± 0.06	0.88 ± 0.05	0.84 ± 0.11	0.72 ± 0.14[a,b,c]
FEV_1:FVC (%)	105 ± 8	98 ± 4	98 ± 9	82 ± 14[a,b,c]
FEF_{25-75} (%)	125 ± 33	93 ± 11	93 ± 28	48 ± 26[a,b,c]
FEV_1 reversibility (%)[2]	3 ± 6	1 ± 2	7 ± 4	14 ± 9[a,b]
F_{ENO} (offline, ppb)	6 ± 4	6 ± 1	14 ± 12[a,b]	14 ± 11[a,b]

[1] To account for differences in height and lung growth, pulmonary function data are expressed as percentages of predicted values. FEV_1:FVC is expressed both as an absolute ratio and as the percentage of predicted values.

[2] Calculated by: (FEV_1 post-bronchodilator – FEV_1 pre-bronchodilator)/predicted FEV_1*100

Table VII. Composition of the BAL fluid in adult controls (AC), pediatric controls (PC), severe asthmatics without airflow obstruction (SA AO-), and severe asthmatics with airflow obstruction (SA AO+). Data represent the mean ± SD, with [a]$p < 0.05$ vs. AC and [b]$p < 0.05$ vs. PC.

	AC (n = 35)	PC (n = 6)	SA AO- (n = 35)	SA AO+ (n = 30)
BAL recovery (%)	44 ± 13	38 ± 16	30 ± 15[a]	31 ± 19[a]
Leukocyte count (x 10^6)	9.30 ± 5.52	4.25 ± 3.55[a]	3.26 ± 2.75[a]	3.38 ± 3.06[a]
Cellular differential (%)				
Macrophages	90.6 ± 3.6	91.8 ± 5.5	89.6 ± 6.6	86.9 ± 7.0
Neutrophils	2.4 ± 1.5	2.4 ± 2.1	5.0 ± 4.9[a,b]	4.9 ± 4.3[a,b]
Eosinophils	0.6 ± 0.6	0.2 ± 0.5	1.2 ± 1.6[a,b]	1.9 ± 3.7[a,b]
Lymphocytes	5.6 ± 2.8	5.4 ± 6.3	5.9 ± 4.6	6.4 ± 6.0
Protein (μg/dL)	121 ± 70	173 ± 98	229 ± 86[a,b]	224 ± 178[a,b]
Urea (mg/dL)	0.41 ± 0.50	0.35 ± 0.48	0.49 ± 0.41	0.37 ± 0.39
Plasma urea (mg/dL)	10.5 ± 3.7	11.7 ± 3.7	13.6 ± 3.2	14.2 ± 2.7
Urea dilution factor[1]	74 ± 47	75 ± 57	72 ± 94	73 ± 40
ELF recovered (mL)[2]	0.64 ± 0.31	0.20 ± 0.08	0.31 ± 0.40	0.41 ± 0.71
pH (log [H+])	6.82 ± 0.39	6.90 ± 0.27	7.01 ± 0.44	6.92 ± 0.60

[1] Calculated from $[urea_{plasma}] / [urea_{BAL}]$

[2] Calculated from mL of BAL return / urea dilution factor

Table VIII. Pearson correlation coefficients (*r*) for biomarkers of airway oxidant stress in the epithelial lining fluid (ELF) of children with severe asthma.[1,2]

	GSH:GSSG E_h	% GSSG	H_2O_2	8-Isoprostanes	MDA
GSH:GSSG E_h	1	0.854	0.299	0.315	0.509
		$p < 0.001$	$p = 0.049$	$p = 0.027$	$p = 0.031$
% GSSG		1	0.351	0.485	0.343
			$p < 0.020$	$p = 0.005$	$p = 0.024$
H_2O_2			1	0.338	0.343
				$p = 0.050$	$p = 0.049$
8-Isoprostanes				1	0.698
					$p < 0.001$
MDA					1

[1] Data are from the combined sample of severe asthmatics with (AO+) and without (AO-) airflow obstruction. Adult and pediatric controls were excluded from this analysis.

[2] Coefficients for H_2O_2, 8-isoprostanes and MDA were obtained after logarithmic transformation due to a non-normal distribution.

Table IX. Results of univariate logistic regression of the percentage of oxidized glutathione (% GSSG) in the epithelial lining fluid (ELF) on clinical features of severe asthma in children.[1]

Dependent variable	(β)	SE	Exp (β)	p-value	95% C.I.
Hospitalization[2]					
% GSSG	0.043	0.012	1.044	0.001	(1.019, 1.070)
Constant	-2.267	0.706	0.104	0.001	
Daily prednisone use[2]					
% GSSG	0.007	0.015	1.007	0.615	(0.979, 1.037)
Constant	-1.215	0.935	0.297	0.194	

[1] Data are from the combined sample of severe asthmatics with (AO+) and without (AO-) airflow obstruction, excluding adult and pediatric controls.

[2]Modeled as yes = 1, no = 0

Table X. Results of univariate linear regression of the percentage of oxidized glutathione (% GSSG) in the epithelial lining fluid (ELF) on clinical features of severe asthma in children.[1]

Dependent variable	(β)	SE	t	p-value	95% C.I.
F_{ENO}[2]					
% GSSG	0.012	0.005	2.320	0.032	(0.001, 0.022)
Constant	1.810	0.286	6.323	<0.001	
ICS dose (μg/day)					
% GSSG	1.795	2.484	0.723	0.477	(-3.332, 6,922)
Constant	671.034	151.425	4.431	<0.001	
Baseline FEV_1					
% GSSG	-0.148	0.092	-1.596	0.116	(-0.333, 0.038)
Constant	91.548	5.147	17.787	<0.001	
FEV_1 reversibility (%)					
% GSSG	0.010	0.096	0.106	0.917	(-0.194, 0.214)
Constant	11.000	5.686	1.934	0.072	

[1] Data are from the combined sample of severe asthmatics with (AO+) and without (AO-) airflow obstruction, excluding adult and pediatric controls.

[2]Data were logarithmically transformed for analysis due to a non-normal distribution.

Table XI. Results of backward multivariate linear regression of selected clinical features of severe asthma in children on the percentage of glutathione disulfide (GSSG) in the epithelial lining fluid (ELF).[1]

Model	β	SE	t	p-value	95% C.I.
Included variables[2]					
Constant	76.604	10.850	7.060	< 0.001	(53.603, 99.604)
Gender	-27.726	12.254	-2.263	0.038	(-53.703, -1.749)
BAL eosinophils (%)[3]	-16.469	6.957	-2.367	0.031	(-31.218, -1.720)
Excluded variables					
Ethnicity	0.114		0.491	0.631	
Prednisone dose (mg)	0.064		0.234	0.818	
Age (years)	-0.017		-0.061	0.952	
BAL neutrophils (%)[3]	-0.169		-0.717	0.484	
ICS dose	0.355		1.515	0.151	

[1] Data are from the combined sample of severe asthmatics with (AO+) and without (AO-) airway obstruction, excluding adult and pediatric controls.

[2]Sum of squares (model/total) = 4170.2 / 12,966.1, R^2 = 0.322, p = 0.045

[3]Data were logarithmically transformed for analysis due to a non-normal distribution.

Figure 1. Diagram of airway glutathione homeostasis. ROOH and ROH represent organic hydroperoxides.

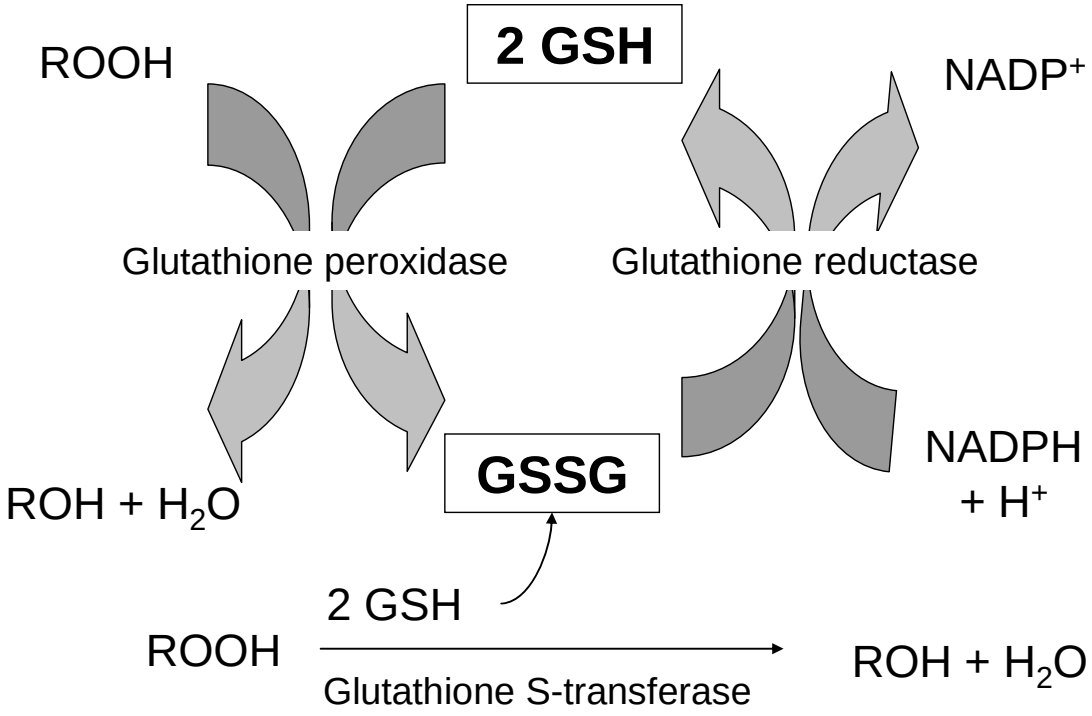

Figure 2. Diagram of lipid peroxidation in the airways resulting from exposure to superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-).

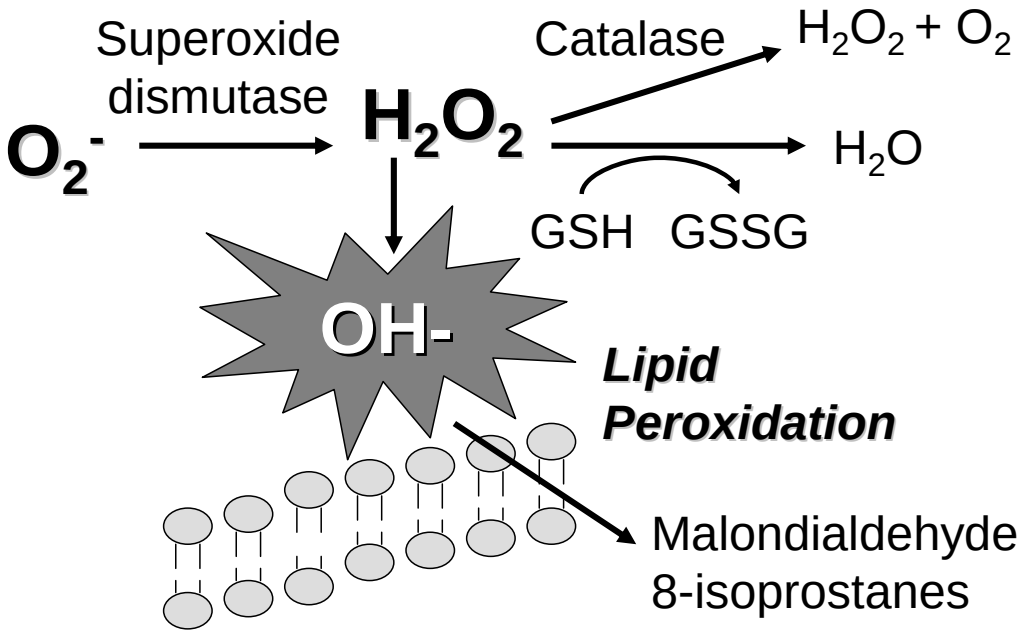

Figure 3. Glutathione (GSH) concentrations in the epithelial lining fluid (ELF) of children with severe asthma. Data represent the mean ± SEM per mL of ELF with AC = adult control (n = 31), PC = pediatric control (n = 6), AO- = severe asthma without airway obstruction (n = 31), and AO+ = severe asthma with airway obstruction (n = 25). [a]$p < 0.01$ versus AC, [b]$p < 0.05$ versus PC.

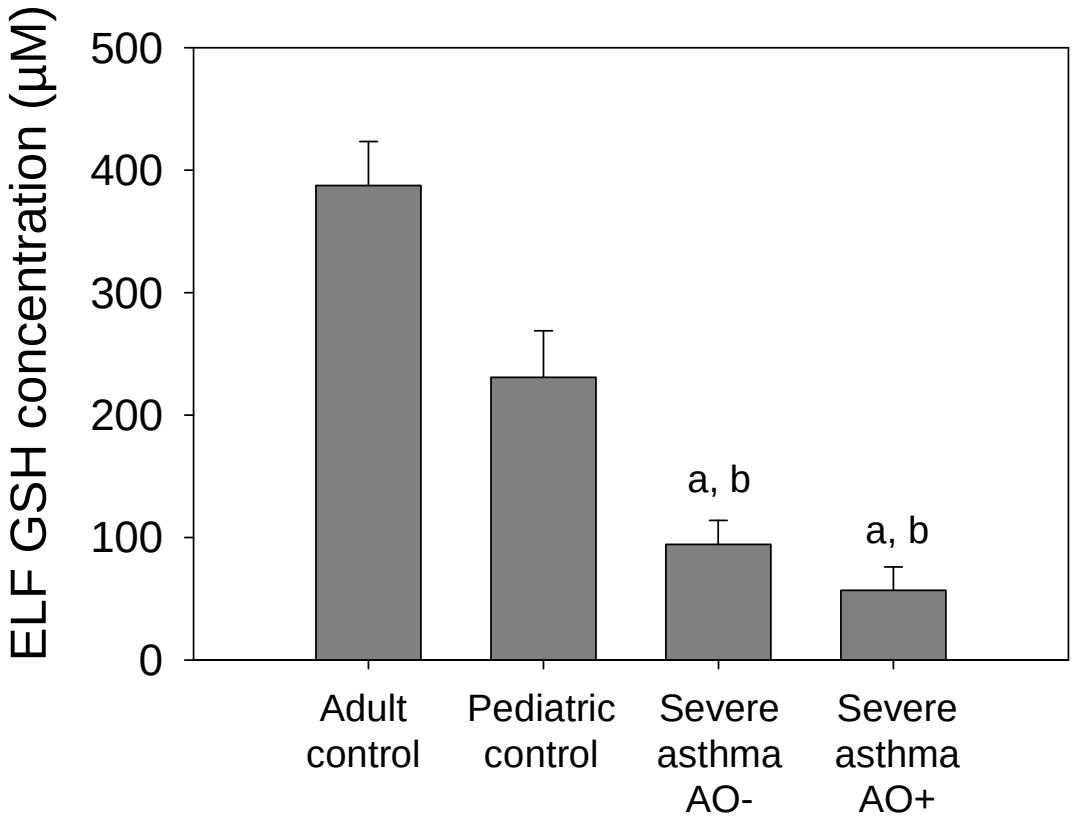

Figure 4. Glutathione disulfide (GSSG) concentrations in the epithelial lining fluid (ELF) of children with severe asthma. Data represent the mean ± SEM per mL of ELF with AC = adult control (n = 31), PC = pediatric control (n = 6), AO- = severe asthma without airway obstruction (n = 31), and AO+ = severe asthma with airway obstruction (n = 25). [a]$p < 0.01$ versus AC, [b]$p < 0.05$ versus PC.

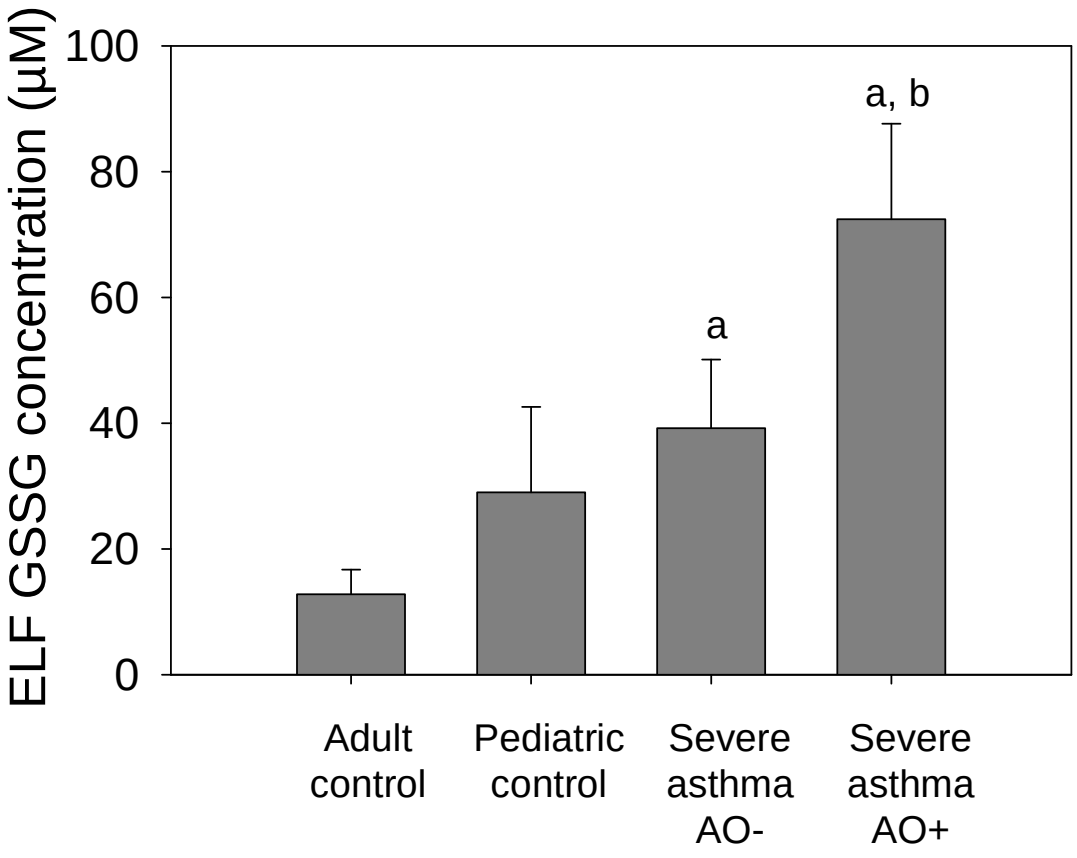

Figure 5. The percentage of glutathione disulfide (% GSSG) in the epithelial lining fluid (ELF) of children with severe asthma. Data represent the mean ± SEM per mL of ELF with AC = adult control (n = 31), PC = pediatric control (n = 6), AO- = severe asthma without airway obstruction (n = 31), and AO+ = severe asthma with airway obstruction (n = 25). [a]$p < 0.01$ versus AC, [b]$p < 0.05$ versus PC.

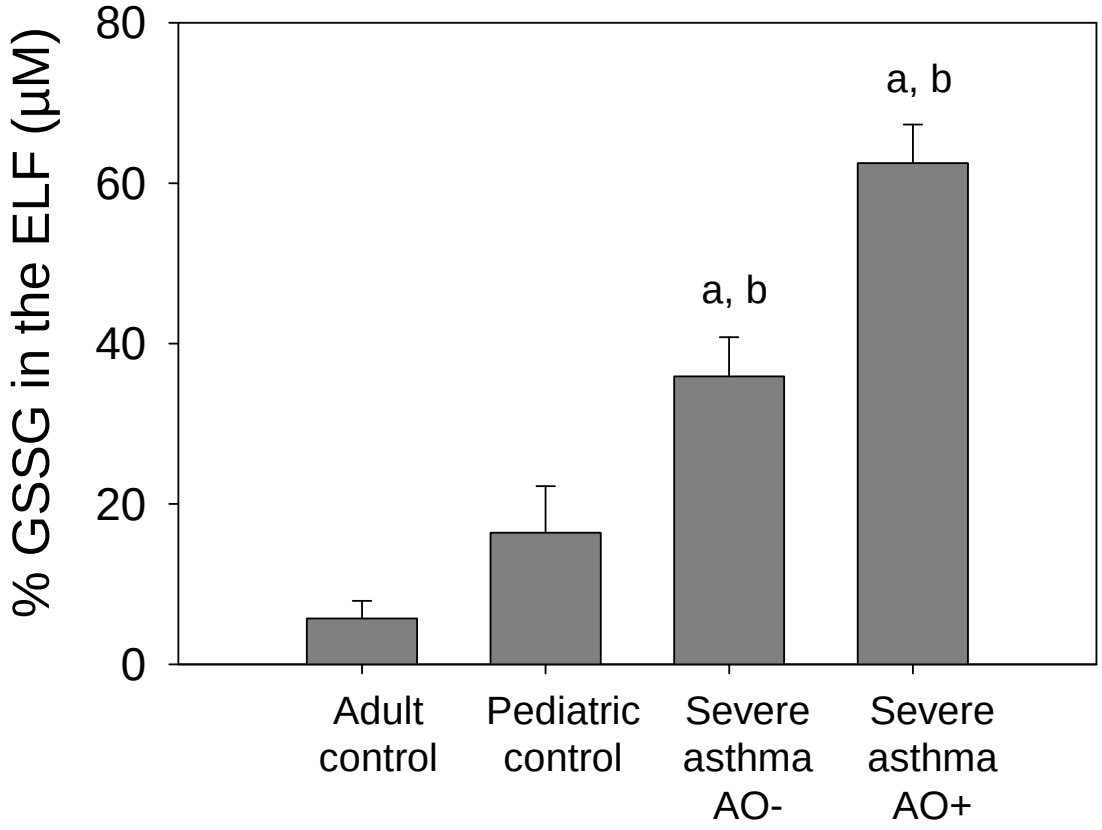

Figure 6. The redox potential (E_h) of the GSH/GSSG pair in the epithelial lining fluid (ELF) of children with severe asthma. Data represent the mean ± SEM per mL of ELF with AC = adult control (n = 31), PC = pediatric control (n = 6), AO- = severe asthma without airway obstruction (n = 31), and AO+ = severe asthma with airway obstruction (n = 25). [a]$p < 0.01$ versus AC, [b]$p < 0.05$ versus PC.

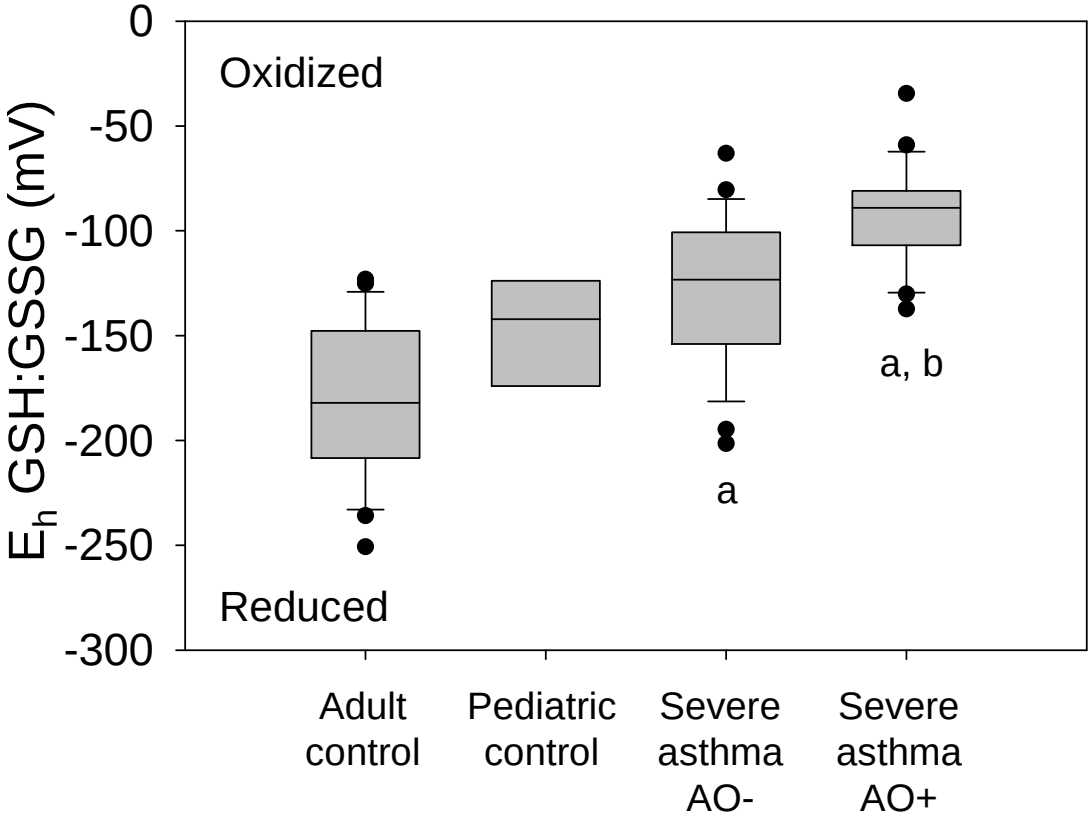

Figure 7. Malondialdehyde (MDA) concentrations in the epithelial lining fluid (ELF) of children with severe asthma. Data represent the mean ± SEM per mL of ELF with $^{a}p < 0.01$ versus AC, $^{b}p < 0.05$ versus PC, and $^{c}p < 0.05$ versus severe asthma AO-.

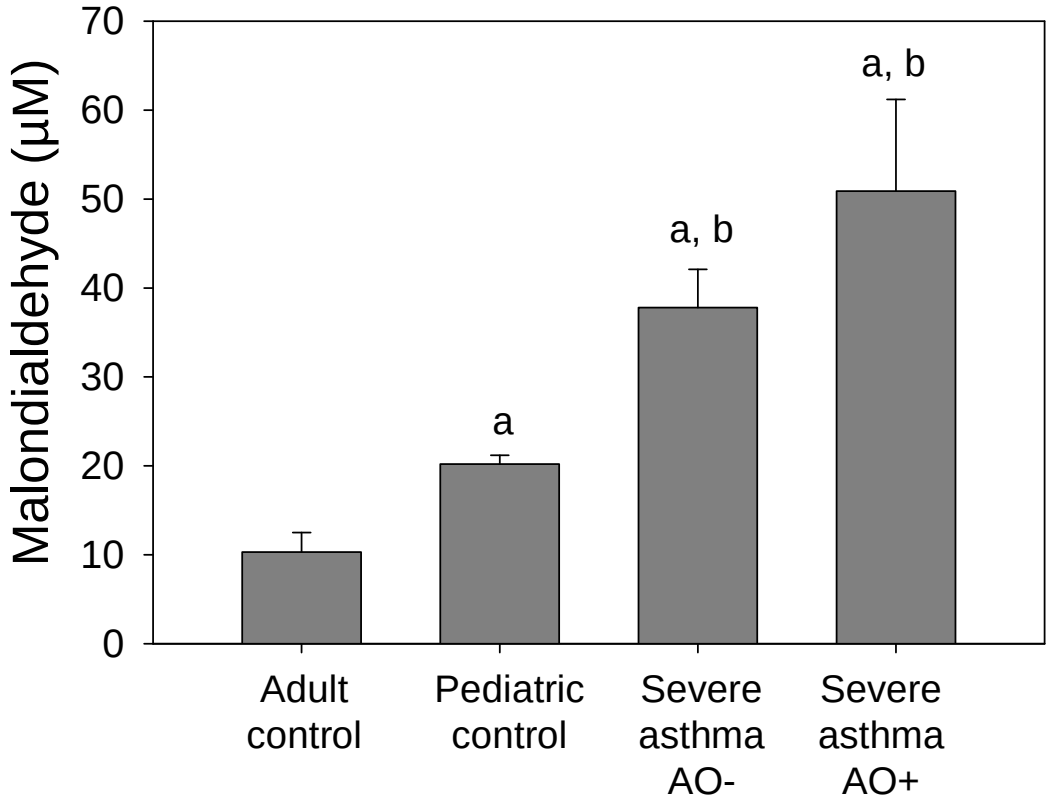

Figure 8. 8-isoprostane concentrations in the epithelial lining fluid (ELF) of children with severe asthma. Data represent the mean ± SEM per mL of ELF with [a]$p < 0.01$ versus AC, [b]$p < 0.05$ versus PC, and [c]$p < 0.05$ versus severe asthma AO-.

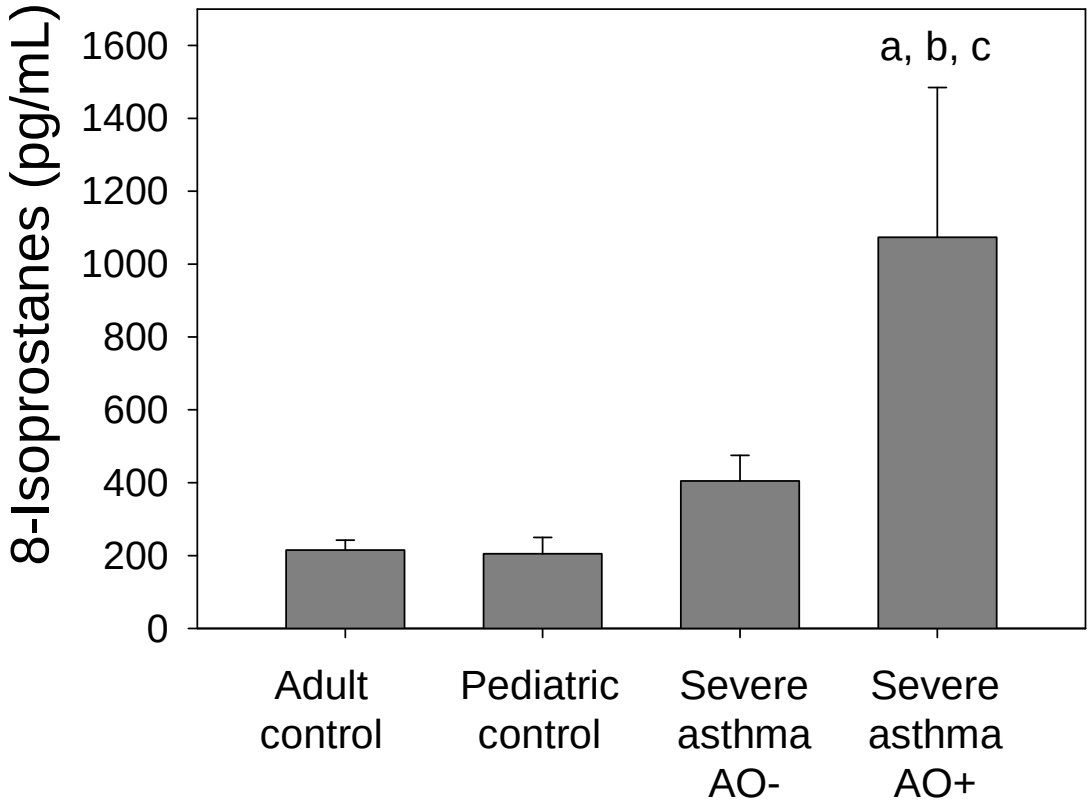

Figure 9. Hydrogen peroxide (H_2O_2) concentrations in the epithelial lining fluid (ELF) of children with severe asthma. Data represent the mean ± SEM per mL of ELF with $^{a}p < 0.01$ versus AC, $^{b}p < 0.05$ versus PC, and $^{c}p < 0.05$ versus severe asthma AO-.