

Exhaled Hydrogen Sulfide Predicts Airway Inflammation Phenotype in COPD

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BACKGROUND: The role of exhaled H₂S as a marker of airway inflammation and its relationship with COPD severity remain to be determined. **METHODS:** Airway inflammation was classified in 77 COPD subjects based on the presence of inflammatory cells in induced sputum. We investigated the association between disease phenotype and exhaled H₂S, lung function, and plasma levels of several inflammatory factors, including tumor necrosis factor alpha, interleukin-8, and leukotriene B₄. **RESULTS:** In total, 33.77% of enrolled COPD subjects were diagnosed with eosinophilia. These subjects had a longer disease course, smoked fewer cigarettes, and experienced more frequent exacerbation events before study enrollment. However, they also had worse lung function and larger residual volume, they demonstrated greater changes in FEV₁ following bronchodilator inhalation. Although levels of plasma inflammatory factors did not significantly differ between subjects with and without eosinophilia, subjects without eosinophilia had significantly higher levels of exhaled H₂S (9.19 ± 2.74 vs 7.24 ± 1.68 parts per billion, $P = .01$). Furthermore, exhaled H₂S levels were negatively correlated with induced sputum eosinophils ($r = -0.45$, $P = .05$), and positively correlated with inspiratory capacity in COPD subjects ($r = 0.51$, $P = .026$), but did not correlate significantly with plasma inflammatory factors. A cut-off value of 7.10 parts per billion of exhaled H₂S predicted a non-eosinophilic phenotype with 68.6% sensitivity and 77.9% specificity. **CONCLUSIONS:** Exhaled levels of H₂S were lower in subjects with eosinophilia. Increased levels of exhaled H₂S predicted a non-eosinophilic phenotype in our study population. *Key words:* chronic obstructive pulmonary disease; airway inflammation phenotype; eosinophilia; hydrogen sulfide. [Respir Care 2015;60(2):251–258. © 2015 Daedalus Enterprises]

Introduction

COPD is a preventable but incurable disease characterized by persistent air flow limitation and chronic inflammation of the airways, lung parenchyma, and pulmonary vasculature.¹ Smoking is a major risk factor for COPD

onset, but additional risk factors may contribute to COPD in smokers and non-smokers, such as indoor/outdoor air pollution, occupational exposures, tuberculosis, lower respiratory tract infections during childhood, chronic asthma, poor socioeconomic status, and poor nutrition,^{1,2} leading to progressive air flow limitation associated with enhanced chronic inflammation of the airways and lungs.¹ Both airway and systemic inflammation contribute to exacerbations of COPD and to declining lung function. COPD is now recognized as a heterogeneous disease with multiple clinical phenotypes, and, importantly, disease progression and prognosis differ significantly among patients.

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Induced sputum analysis is used to assess airway inflammatory diseases.³ Previous studies have suggested that the bronchial tissues of COPD subjects contain higher levels of neutrophils compared with healthy controls.⁴ Airway neutrophil counts are significantly associated with COPD severity. Moreover, COPD is often referred to as a neutrophilic pulmonary disease, because neutrophils induce pathophysiological changes by secreting proteases and pro-inflammatory cytokines that cause tissue damage.^{5,6} In healthy non-smokers, the cells present in induced sputum are 60–70% macrophages, 30–40% neutrophils, and < 2% eosinophils. In contrast, the proportion of eosinophils in the sputum of COPD patients can reach 20–40%.^{7,8} Despite this, the use of induced sputum markers of airway inflammation as indicators of COPD remains controversial.

Previous studies have suggested that, compared with healthy controls, subjects with stable COPD have significantly increased serum levels of hydrogen sulfide (H₂S). In contrast, patients with exacerbations of COPD have significantly decreased levels of serum H₂S. Thus, levels of endogenous H₂S may represent a potential indicator of COPD severity.^{9–11} However, the relationship between levels of exhaled H₂S and airway or systemic inflammation has not yet been determined. Recently, the analysis of signaling molecules present in exhaled air has become a popular technique for noninvasive assessment of airway inflammation.^{12,13} To gain a more complete understanding of the relationship between airway inflammation and COPD, we compared clinical phenotypes, exhaled H₂S levels, and systemic inflammation levels among subjects who were grouped according to the type of inflammatory cells present in induced sputum.

Methods

Study Participants

Between March 2009 and April 2010, 77 subjects (60 male and 17 female, average age 67.09 ± 8.29 y) with COPD were recruited at the Peking University Third Hospital. COPD was diagnosed according to the GOLD 2007 criteria for COPD⁴ and to the COPD treatment guidelines of the Chinese Medical Association (2007 revision).¹⁴ A positive COPD diagnosis required that the ratio of FEV₁ to FVC following bronchodilator inhalation was < 70%; additionally, the post-bronchodilator (salbutamol: 400 μg) FEV₁ improvement rate had to be < 12%. Inclusion criteria were: (1) age 40–80 y, with stable or declining symptoms of cough, sputum, dyspnea, and wheezing; (2) no antibiotic or glucocorticoid treatment within the previous 2 weeks, and no upper respiratory tract infection within the previous 4 weeks; and (3) no previous diagnosis of bronchiectasis, tuberculosis, asthma, lung cancer, or other re-

QUICK LOOK

Current knowledge

COPD is the fourth leading cause of death in the world and an important cause of chronic disability, representing a major economic and social burden worldwide. Airway inflammation is an important pathologic component and target of pharmacotherapy. To date, monitoring airway inflammation has been difficult.

What this paper contributes to our knowledge

Plasma inflammatory factors were not significantly different between COPD subjects with and without eosinophilia. Subjects without eosinophilia had significantly higher levels of exhaled hydrogen sulfide (H₂S), which was negatively correlated with induced sputum eosinophils and positively correlated with inspiratory capacity. Exhaled H₂S predicted a non-eosinophilic phenotype.

spiratory diseases. Subjects with confirmed ongoing eosinophilic bronchitis were excluded. All subjects underwent a bronchodilation reversibility test to rule out chronic asthma in patients with symptoms like child/adolescent onset of asthma; variation of symptoms from day to day, symptoms at night or early morning; allergies, rhinitis, and/or eczema; family history of asthma; or largely reversible air flow limitation.⁴ Patients with COPD that developed from asthma (based on the patient's medical history) were excluded.

Subjects with > 3% eosinophils in the induced sputum were assigned to the eosinophilia group,³ and all other subjects were assigned to the non-eosinophilia group. This study was approved by the ethics committee of Peking University Third Hospital (IRB00006761-2012029), and all participants provided written informed consent.

Health Assessment

Subjects' clinical characteristics, including age, gender, height, weight, smoking history, and disease history were recorded. Dyspnea was scored according to the Medical Research Council scale questionnaire,¹⁵ and quality of life was assessed according to a Chinese translation of the St George's Respiratory Questionnaire.¹⁶

Lung Function Tests

Pulmonary function was measured using a large spirometer (Elite Series DL, Medgraphics, Saint Paul, Minnesota). Measured parameters included FVC, FEV₁, FEV₁/FVC,

and inspiratory capacity. Diffusing capacity of the lung for carbon monoxide and total lung capacity were also assessed using the single-breath method and plethysmography, respectively. Reference values for diffusing capacity and total lung capacity were based on those previously published for a Chinese population.¹⁷

Sampling and Processing of Induced Sputum

Airway inflammation was assessed by analysis of induced sputum. Participants rinsed their mouths and gargled with water before sputum collection. Subjects inhaled 200 µg of salbutamol aerosol, followed by 4% aerosolized sodium chloride solution 15 min later; 3–5 mL of sputum was collected, mixed with 4 volumes of 0.1% dithiothreitol, and then allowed to oscillate at 37°C for 15 min.¹⁸ The mixture was homogenized by continuous pipetting and passed through a nylon mesh (48-µm pore size) to remove mucus and debris. The filtrate was weighed, and 10 µL was reserved for cell counting using a hemocytometer. The filtrate was then centrifuged for 10 min at 1,500 rpm, and the supernatant was cryopreserved at –80°C. The cell pellet was resuspended in phosphate-buffered saline to a concentration of 1×10^7 cells/mL. Cells were smeared onto a glass slide and stained with Wright-Giemsa. A total of 400 non-squamous cells were counted, and the proportion of neutrophils, macrophages, lymphocytes, and eosinophils were calculated. Specimens with more than 20% of squamous cells were not used for the study.

Detection of Exhaled Hydrogen Sulfide

Participants performed a slow vital capacity maneuver into 3-L Tedlar reservoir bags (Dalian Haide Technology, Dalian, People's Republic of China) without holding their breath. The reservoir was sealed at the end of the maneuver and then analyzed for exhaled H₂S and NO using an Interscan 4170-1999b portable hydrogen sulfide gas detector and a 4540-1999b portable nitric oxide gas detector (Interscan Corp, Simi Valley, California), respectively.

Approximately 30 min before each test, a C-12 type filter was connected to the gas inlet to ensure that the reading was stable before adjusting to zero; after this was confirmed, the C-12 filter was removed. Exhaled air was directed to the sampling ports of the detectors. The range for the detector was 0–1,999 parts per billion. The lower limit of quantitation was 1% of the full range; repeated readings fell within 0.5% of the full range. The sample pump flow was controlled by an adjustable voltage regulator and was set at approximately 1 L/min. The reproducibility of 3 consecutive measurements of exhaled H₂S and NO was high (coefficient of variation of < 15%).

Detection of Cytokines in Plasma

A sample of venous blood (8 mL) was collected in ethylenediaminetetraacetic acid-containing tubes from fasting subjects. Plasma was collected after centrifugation and was cryopreserved at –80°C. Plasma levels of tumor necrosis factor alpha, interleukin-8 (IL-8), and leukotriene B₄ were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. Tumor necrosis factor alpha and IL-8 ELISA kits were purchased from Wuhan Boster Biological Technology (Wuhan, People's Republic of China; items EK0525 and EK0413, respectively), and leukotriene B₄ ELISA kit was purchased from R&D Systems (Minneapolis, Minnesota; item 1R610).

Statistical Analysis

Data that are not normally distributed are presented as median (interquartile range), and inter-group comparisons were made using the rank-sum test. Normally distributed data are presented as mean ± SD, and inter-group comparisons were made using an independent samples *t* test. Correlation analysis was performed using the Spearman test. The cut-off concentration of exhaled hydrogen sulfide was selected to yield the highest possible Youden index score (sensitivity + specificity – 1). The area under the receiver operating characteristic (ROC) curve for exhaled hydrogen sulfide was calculated. The model was considered acceptable when area under the curve > 0.70. All statistical analyses were performed using SPSS 13.0 (SPSS, Chicago, Illinois). Statistical significance was defined at *P* < .05.

Results

Subjects' Characteristics

Seventy-seven COPD subjects (60 male and 17 female) were enrolled in the study; of these subjects, 71 were smokers and 6 had never been smokers. Induced sputum was sampled, and cells were counted. Based on this analysis, 26 (33.77%) subjects were assigned to the eosinophilia group, and 51 participants were assigned to the non-eosinophilia group. Neither age nor BMI significantly differed between the 2 groups (Table 1). Before enrollment in this study, subjects with eosinophilia consumed fewer cigarettes, had a longer disease course, and experienced more frequent exacerbation events requiring hospitalization. The lung function of individuals diagnosed with eosinophilia was also worse, showing a higher residual volume. They also displayed a larger change in FEV₁ after bronchodilator inhalation.

Table 1. Characteristics and Lung Function of COPD Subjects With or Without Eosinophilia

	Eosinophilia Group (n = 26)	Non-Eosinophilia Group (n = 51)	P
Gender (male/female)	19/7	41/10	.47
Age (y)	69.3 ± 9.7	67.7 ± 7.5	.43
BMI	25.0 ± 4.6	23.2 ± 1.4	.09
Cigarette consumption (pack-years)	34.1 ± 11.2	51.6 ± 17.7	.02
Smoker/never-smoker	23/3	48/3	.40
Disease course before inclusion (y)	7.3 ± 3.8	4.5 ± 2.8	.007
Instances of exacerbation before inclusion	4.0 ± 1.3	2.8 ± 1.6	.003
MRC scores	3.1 ± 1.0	2.7 ± 1.2	.19
SGRQ symptom scores	69.8 ± 17.0	61.5 ± 22.2	.13
SGRQ activity scores	66.4 ± 19.7	56.6 ± 25.8	.12
SGRQ impact scores	38.3 ± 28.6	30.6 ± 19.2	.16
SGRQ total scores	52.2 ± 19.7	43.4 ± 19.0	.08

BMI = body mass index
MRC = Medical Research Council
SGRQ = St George's Respiratory Questionnaire

Classification of Induced Sputum Samples

The total number of cells in induced sputum samples obtained from subjects with eosinophilia (median: 4.57×10^6 cells/mL; range: $2.81\text{--}5.63 \times 10^6$ cells/mL) did not significantly differ from the number detected in samples from subjects without eosinophilia (median: 4.98×10^6 cells/mL; range: $2.44\text{--}6.02 \times 10^6$ cells/mL). Subjects with eosinophilia had elevated relative levels of eosinophils (5.70%) and macrophages (16.40%) compared with those without eosinophilia (1.00% and 7.10%, $P < .001$ and $P = .004$, respectively). In contrast, subjects without eosinophilia had higher relative levels of neutrophils (89.00%) compared with those with eosinophilia (70.25%) ($P < .001$) (Table 2).

Measurement of Exhaled H₂S and Levels of Plasma Inflammatory Mediators

Subjects without eosinophilia had significantly higher levels of exhaled H₂S (9.19 ± 2.74 parts per billion) compared with subjects with eosinophilia (7.24 ± 1.68 parts per billion, $P = .01$). However, plasma levels of tumor necrosis factor alpha, IL-8, and leukotriene B₄ did not significantly differ between the 2 groups (Fig. 1).

Correlation Analysis of Exhaled H₂S With Lung Function and Levels of Inflammatory Factors

Exhaled H₂S levels positively correlated with inspiratory capacity ($r = 0.51$, $P = .03$), an indicator of lung

Table 2. Induced Sputum Cell Classification and Lung Function in COPD Subjects With or Without Eosinophilia

	Eosinophilia Group (n = 26)	Non-Eosinophilia Group (n = 51)	P
Macrophages (%)	16.40 (9.40–28.50)	7.10 (4.00–18.80)	.004
Lymphocytes (%)	2.55 (1.40–3.85)	1.70 (0.80–3.00)	.02
Neutrophils (%)	70.25 (48.20–80.00)	89.00 (79.00–93.80)	< .001
Eosinophils (%)	5.70 (3.18–12.35)	1.00 (0–1.90)	< .001
FEV ₁ /FVC (%)	48.4 ± 9.6	50.8 ± 7.7	.27
FVC% (%)	67.6 ± 12.6	68.9 ± 16.0	.73
FEV ₁ % (%)	38.4 ± 16.2	43.5 ± 15.7	.03
ΔFEV ₁ (%)	8.7 ± 2.7	6.2 ± 2.5	.009
TLC% (%)	109.5 ± 27.2	107.4 ± 21.5	.74
RV% increase (%)	133.1 ± 47.1	123.6 ± 38.1	.68
D _{LCO} % (%)	14.1 ± 4.3	12.1 ± 6.4	.16

TLC = total lung capacity
RV = residual volume
D_{LCO} = diffusing capacity of the lung for carbon monoxide

function. Exhaled H₂S levels tended to be associated negatively with St George's Respiratory Questionnaire activity scores ($r = -0.43$, $P = .05$). Exhaled H₂S levels negatively correlated with the percentage of sputum eosinophils in COPD subjects ($r = -0.45$, $P = .05$) (Fig. 2).

Receiver Operating Characteristic Curves

ROC curves were used to predict exhaled H₂S levels corresponding to the non-eosinophilic phenotype (Fig. 3). The area under the curve for exhaled H₂S used to predict the non-eosinophilic phenotype was 0.732 ($P < .01$). Using a cut-off value of exhaled H₂S of 7.10 parts per billion, a sensitivity of 68.6%, and a specificity of 77.9% were achieved.

Discussion

The goals of the present study were to describe the clinical features of eosinophilic airway inflammation in COPD subjects and to investigate the relationship among levels of eosinophils, H₂S, and systemic inflammation. In this study, subjects with eosinophilia accounted for 33.77% of all participants. In contrast to COPD subjects without eosinophilia, those with eosinophilia had a longer disease course, lower quality of life scores, worse lung function and health status, and experienced more frequent exacerbation events before enrollment in the study. These subjects also consumed fewer cigarettes and exhibited a better response to bronchodilators. Levels of exhaled H₂S were reduced in individuals with eosinophilia and significantly correlated with inspiratory capacity.

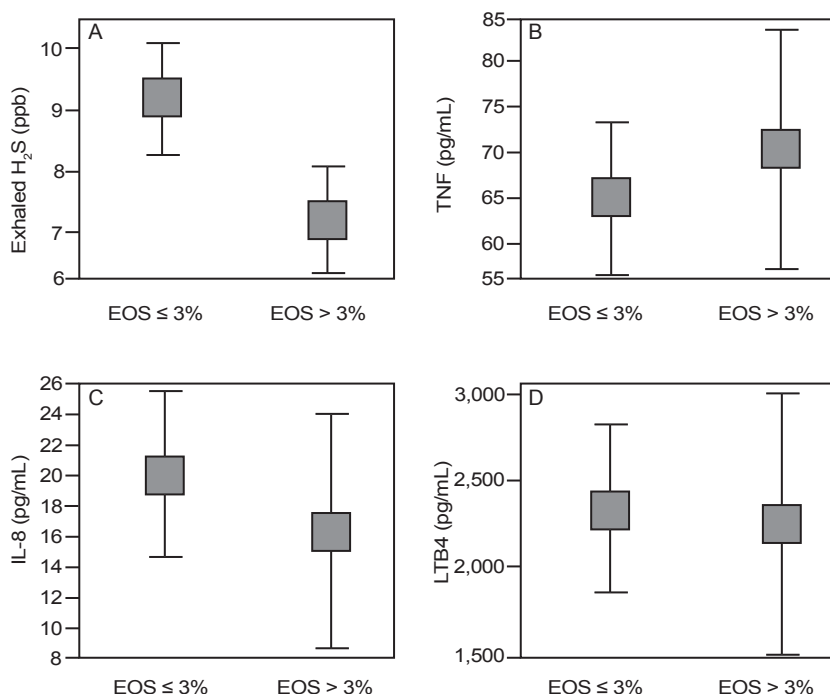


Fig. 1. Exhaled H₂S and plasma inflammatory mediators tumor necrosis factor alpha (TNF), interleukin-8 (IL8), and leukotriene B₄ (LTB₄) in COPD subjects with eosinophilia (EOS > 3%) or without eosinophilia (EOS ≤ 3%). A: $P = .01$, B: $P = .51$, C: $P = .44$, and D: $P = .82$. Data are shown as mean \pm SD. ppb = parts per billion.

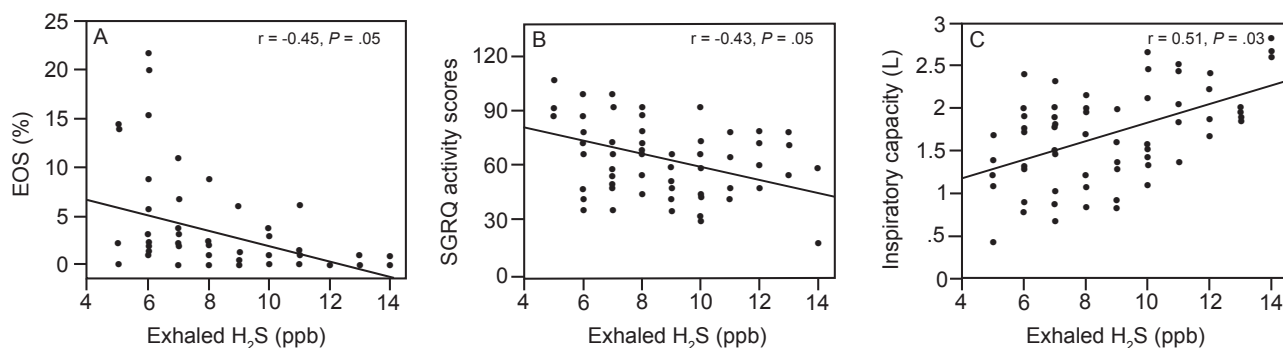


Fig. 2. Correlation analysis of exhaled H₂S with eosinophilia (EOS), St George's Respiratory Questionnaire (SGRQ) activity scores, and inspiratory capacity. ppb = parts per billion.

Previous studies have reported that activated eosinophils negatively correlate with FEV₁ in bronchial biopsy specimens.¹⁹ Moreover, the levels of eosinophils in mucosal bronchial biopsies from patients with acute exacerbations of COPD were 30-fold greater than the levels in patients with stable COPD.²⁰ These findings indicate that eosinophils play a key role, independent of neutrophils, in the development of COPD and exacerbation of airway inflammation. When COPD is complicated by chronic bronchitis, neutrophils can be detected in the airways.²¹ Smoking is thought to induce the neutrophil-associated flow of eosinophils into the airway, but eosinophilic COPD may also develop from eosinophilic bronchitis. Eosinophilic

bronchitis is a common cause of chronic, persistent cough in middle-aged adults that is characterized by increased sputum eosinophils; however, these patients do not experience any symptoms of air flow obstruction, airway hyper-responsiveness, or changes in lung function. Although patients exhibit normal lung function at the time of eosinophilic bronchitis diagnosis, they may experience a subsequent dramatic decline in lung function (FEV₁) coupled with progression to COPD.²² Unfortunately, due to the cross-sectional nature of our study, we were unable to determine whether the onset of COPD in study participants was actually due to past events of eosinophilic bronchitis. Additionally, because of the features associated with

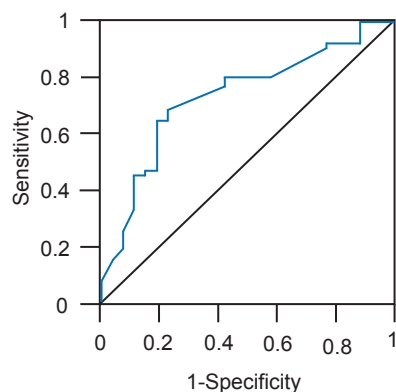


Fig. 3. Receiver operating characteristic (ROC) curve showing the predictive impact of exhaled H₂S on non-eosinophilic COPD. The area under the curve was 0.732, and the cut-off value of exhaled H₂S was 7.10 parts per billion with a sensitivity of 68.6% and a specificity of 77.9%.

eosinophilia (ie, longer disease course and less cigarette smoking), we could not exclusively attribute induced sputum eosinophilia to cigarette smoking. Even though subjects with eosinophilia displayed more severe clinical symptoms and poorer lung function, they exhibited better response to bronchodilators, which is supported by previous findings.²⁰ Additionally, subjects with eosinophilia responded well to systemic corticosteroid therapy.^{23,24} Using a noninvasive method, we found that bronchodilator therapy achieved a satisfactory outcome in COPD subjects with induced sputum eosinophilia. However, whether this outcome could be achieved with glucocorticoid therapy requires further study.

H₂S functions to regulate the inflammatory response. Levels of H₂S in the serum of subjects with stable COPD were significantly higher than those in controls.^{9,25} Importantly, serum H₂S levels positively correlate with FEV₁%, induced sputum lymphocyte count, and macrophage count; however, levels negatively correlate with induced sputum neutrophil count. In a smoking-induced chronic COPD rat model, endogenous H₂S exerted a protective function, reducing inflammation and bronchodilation.²⁶ Intraperitoneal injection of NaHS has been shown to alleviate rat airway hyper-responsiveness, as well as to reduce IL-8 and tumor necrosis factor alpha levels in rat lung tissues. Another study found that, in a mouse model of COPD, cystathionine γ -lyase protein levels and the capacity for H₂S synthesis in the lungs were significantly reduced.²⁷ We examined the potential relationship between serum and exhaled H₂S, but no significant correlation was found (data not shown). Serum H₂S is distributed in all tissues and organs in levels that are affected by different local and systemic conditions. Only a part of this H₂S is exhaled, and the amount being exhaled is dependent upon airway conditions. Compared with serum H₂S levels, exhaled H₂S appears to reflect changes in the airways. ROC analysis

indicated that exhaled H₂S could predict the non-eosinophilic phenotype in our study; a cut-off of 7.10 parts per billion of exhaled H₂S achieved a sensitivity of 68.6% and a specificity of 77.9%. Based on our findings, we conclude that levels of exhaled H₂S could be used to predict airway inflammation in COPD. Measurement of exhaled H₂S is simple and noninvasive. Although analysis of induced sputum is also a noninvasive procedure, it may cause wheezing exacerbation and has a certain failure rate. Furthermore, the induced sputum specimen requires processing before it can be analyzed for cell type and count.

COPD subjects with eosinophilia have lower levels of H₂S in exhaled gases; exhaled H₂S negatively correlates with eosinophil count in induced sputum but positively correlates with lung function (inspiratory capacity) in COPD patients. We previously found that exhaled H₂S levels positively correlate with exhaled NO in patients with COPD.^{9,13,25} This close relationship between exhaled NO and H₂S highlights the possibility that H₂S may be useful as a COPD biomarker. Similar to previous studies, we observed no correlation between exhaled H₂S and predicted FEV₁. However, exhaled H₂S did correlate with inspiratory capacity, another indicator of lung function. Inspiratory capacity is well correlated with maximum oxygen intake and exercise tolerance, and is more sensitive to obstructive airway changes than FEV₁ during the stable phase.^{28,29} COPD is a complex disease with multiple clinical manifestations, and patients with comparable air flow limitation (FEV₁) may present with different phenotypes.

COPD pathogenesis is not merely a result of airway inflammation, it is also significantly influenced by systemic inflammation.¹ We specifically focused on the inflammatory cytokines and chemokines tumor necrosis factor alpha, IL-8, and leukotriene B₄, as these factors have been previously shown to play an important role in the development and severity of COPD.¹ In many diseased states, levels of inflammatory cytokines are increased. However, the relationship between COPD airway inflammation and systemic inflammation remains undefined. Because COPD is characterized by chronic inflammation of the airways, lung parenchyma, and pulmonary vasculature, it is generally believed that COPD-induced pulmonary inflammation is the source of systemic inflammation. Activated inflammatory cells and secreted inflammatory mediators may enter the systemic circulation and/or pulmonary circulation, and activate inflammatory cells, triggering a generalized inflammatory response. However, Vernooij et al³⁰ performed a well-controlled experiment in which they collected both sputum and blood from the same group of COPD patients. They found that sTNF-R55 expression was increased in sputum but not in blood, and that sTNF-R75 expression increased in blood but not in sputum. Importantly, neither measurement reliably correlated with FEV₁.³⁰ Thus, the authors suggested that systemic inflam-

mation is not the result of residual local inflammation.³⁰ We found that tumor necrosis factor alpha, IL-8, and leukotriene B₄ levels did not differ significantly between subjects with and without eosinophilia, nor did levels significantly correlate with exhaled H₂S.

However, it is important to keep in mind that we measured only a limited number of cytokines and chemokines, and we cannot rule out the possibility that other aspects of the systemic inflammatory response may be significantly altered between these groups. Additionally, our study sample size was small. Furthermore, the present study was cross-sectional, and we did not enroll controls. Finally, patients with ongoing eosinophilic bronchitis were excluded, but we had no mean to assess undiagnosed past events of eosinophilic bronchitis. Future studies, either cohort or longitudinal studies, with a larger sample size and control subjects will allow us to better characterize the inflammatory state of COPD and its relationship with H₂S.

Conclusions

In conclusion, subjects with eosinophilia had a longer disease course, poorer lung function, and worse health status. Despite this, they exhibited a better response to bronchodilators, suggesting that airway inflammation could be a useful indicator for COPD phenotypic analysis. Levels of exhaled H₂S were lower in subjects with eosinophilia and showed predictive impact on non-eosinophilic phenotype in our study population. ROC curves showed a good sensitivity and specificity of exhaled H₂S in predicting COPD without eosinophilia. Whether endogenous H₂S participates in the development or progression of COPD, whether it is important in the diagnosis and monitoring of the disease, and whether it is useful as a guide for COPD treatment require further investigation.

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