

ORIGINAL ARTICLE

Asthma and Rhinitis

Sputum-to-serum hydrogen sulphide ratio as a novel biomarker of predicting future risks of asthma exacerbation

Y. Suzuki | J. Saito  | M. Kikuchi | M. Uematsu | A. Fukuhara | S. Sato | M. Munakata

Department of Pulmonary Medicine, School of Medicine, Fukushima Medical University, Fukushima, Japan

Correspondence

Junpei Saito, Department of Pulmonary Medicine, School of Medicine, Fukushima Medical University, Fukushima, Japan.
Email: junpei@fmu.ac.jp

Funding information

Research grant 2017; Research grant; JSPS KAKENHI, Grant/Award Number: 15K09182

Summary

Background: Increased level of hydrogen sulphide (H_2S) in sputum is reported to be a new biomarker of neutrophilic airway inflammation in chronic airway disorders. However, the relationship between H_2S and disease activity remains unclear.

Objective: We investigated whether H_2S levels could vary during different conditions in asthma.

Method: H_2S levels in sputum and serum were measured using a sulphide-sensitive electrode in 47 stable asthmatic subjects (S-BA), 21 uncontrolled asthmatic subjects (UC-BA), 26 asthmatic subjects with acute exacerbation (AE-BA) and 15 healthy subjects. Of these, H_2S levels during stable, as well as exacerbation states, were obtained in 13 asthmatic subjects.

Results: Sputum H_2S levels were significantly higher in the AE-BA subjects compared to the UC-BA and healthy subjects ($P < .05$). However, serum H_2S levels in the AE-BA subjects were lower than in the S-BA subjects ($P < .001$) and similar to those in healthy subjects. Thus, the sputum-to-serum ratio of H_2S (H_2S ratio) in the AE-BA subjects was significantly higher than in the S-BA, UC-BA and healthy subjects ($P < .05$). Among all subjects, sputum H_2S levels showed a trend to decrease with FEV_1 %predicted and significantly positive correlations with sputum neutrophils (%), sputum IL-8 and serum IL-8. A multiple linear regression analysis showed that sputum H_2S was independently associated with increased sputum neutrophils (%) and decreased FEV_1 %predicted ($P < .05$). The cut-off level of H_2S ratio to indicate an exacerbation was ≥ 0.34 (area under the curve; 0.88, with a sensitivity of 81.8% and specificity of 72.7%, $P < .001$). Furthermore, half of the asthmatic subjects with H_2S ratios higher than the cut-off level experienced asthma exacerbations over the following 3 months after enrolment.

Conclusions: The H_2S ratio may provide useful information on predicting future risks of asthma exacerbation, as well as on obstructive neutrophilic airway inflammation as one of the non-Th2 biomarkers, in asthma.

KEYWORDS

airway inflammation, asthma, exacerbation, hydrogen sulphide, neutrophils

1 | INTRODUCTION

Asthma is characterized by a chronic airway inflammatory process that leads to bronchial hyperresponsiveness and bronchodilator response to β -adrenergic agonists.¹ This inflammatory profile can be varied on the basis of the predominant inflammatory cell types involved: eosinophilic, neutrophilic and paucigranulocytic.^{2,3} It has been understood that these inflammatory phenotypes are strongly linked with distinct clinical features, such as response to treatment. The presence of eosinophilic inflammation may be indicative of good therapeutic response to corticosteroid therapy, while that of neutrophilic inflammation may be the most frequent indication of corticosteroid insensitivity.^{4,5} Asthma severity can also be categorized according to these inflammatory phenotypes. Moore et al indicated that subjects with mild-to-moderate allergic asthma were likely to have minimal or eosinophilic predominant sputum inflammation, whereas those who had moderate-to-severe asthma showed neutrophilic-predominant sputum inflammation either alone or with eosinophilic sputum inflammation. In addition, sputum neutrophil percentages were the second most influential variable for detecting severe asthma.³ Therefore, if there were several biomarkers simply identifying these inflammatory phenotypes, it would be possible to accurately recognize the different clinicopathophysiological characteristics of asthma. In recent years, fractional exhaled nitric oxide (FeNO)⁶⁻⁹ and periostin^{10,11} have been recognized as biomarkers of eosinophilic predominant asthma and are suggested to be markers of therapeutic response to corticosteroids, as well as biologic agents such as omalizumab, mepolizumab and lebrikizumab.¹²⁻¹⁶ On the other hand, there has yet to be a useful biomarker of non-eosinophilic predominant asthma that is concurrently associated with the pathophysiological features of asthma.

Hydrogen sulphide (H_2S), a gas with a typical malodorous smell, is produced by many cell types in the lungs including pulmonary arterial cells, airway smooth muscle cells, lung primary fibroblasts and endothelial cells¹⁷⁻²⁰ through the action of three H_2S synthetics: cystathionine- β -synthase (CBS), cystathionine- γ -lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST).^{21,22} H_2S is now considered to be the third gasotransmitter along with nitric oxide (NO) and carbon monoxide (CO).²⁰

There have been few clinical studies published on H_2S in respiratory diseases. We previously reported that serum and sputum H_2S levels in asthmatic subjects were higher compared to healthy subjects and positively correlated with sputum neutrophils,²³ indicating that H_2S could be a marker of neutrophil dominant asthma. However, relationships between H_2S and disease activities in asthma remain unclear.

The aims of this study were, firstly, to compare serum and sputum H_2S levels in asthmatic subjects during different conditions defined as controlled, uncontrolled and in exacerbation state, and secondly, to evaluate whether H_2S could be an independent biomarker of neutrophilic airway inflammation in asthma.

2 | METHODS

2.1 | Subjects

In total, 68 stable asthmatic subjects and 26 asthmatic subjects with acute exacerbation were recruited from a clinic belonging to Fukushima Medical University Hospital. Fifteen healthy subjects were also recruited from the staff of Fukushima Medical University. The diagnosis of asthma was made by respiratory physicians according to a clinical history of characteristic symptoms (ie, recurrent coughing, wheezing, chest tightness and breathlessness), as well as a history of either forced expiratory volume in 1 second (FEV₁) reversibility $\geq 12\%$, positive bronchial hyperresponsiveness or sputum eosinophilia of $\geq 3\%$.¹ Subjects with any concomitant respiratory diseases other than asthma were excluded. Current smokers were also excluded. Healthy volunteers had normal lung function, a smoking history of < 5 pack-years and no history of pulmonary disorders within 4 weeks prior to the study recruitment. This study was approved by the Ethics Committee of Fukushima Medical University and registered to UMIN (UMIN000010509). All participants provided written informed consent.

2.2 | Definition of asthma control and exacerbation

Asthmatic subjects were categorized as having controlled, partly controlled or uncontrolled asthma according to the Global Initiative for Asthma (GINA) guidelines.¹ Uncontrolled asthmatic subjects (UC-BA) were defined as having three or more of following features over 4 weeks before recruitment: (1) daily symptoms more than twice a week; (2) any limitation of activities; (3) any nocturnal symptoms or awakening; (4) need for reliever/rescue treatment more than twice a week; and (5) spirometry (peak expiratory flow [PEF] or FEV₁) $< 80\%$ predicted or personal best if known. Subjects who had two or fewer of these features were classified as having stable asthma (S-BA), which includes controlled and partly controlled asthma.¹ Asthmatic subjects either with moderate or severe acute exacerbations (AE-BA) were included as having asthma exacerbation in line with the American Thoracic Society/European Respiratory Society statement.²⁴ Severe exacerbations were defined as requiring treatment with oral prednisolone, an increase in the maintenance dose of prednisolone for at least ≥ 3 days, hospitalization or an emergency room visit because of asthma, requiring systemic corticosteroids. Moderate exacerbations were defined as having one or more of the following for ≥ 2 days consecutively: deterioration in symptoms, deterioration in PEF levels or increased rescue bronchodilator use that is not severe enough to warrant systemic corticosteroid use.²⁴

2.3 | Study design

This was a cross-sectional observational study. At study entry, asthma control and exacerbation were assessed using the above-mentioned methods. Spirometry, FeNO measurements and sputum

and peripheral blood samplings for the measurements of H₂S, as well as interleukin (IL)-8 and IL-5, were performed. An asthma control test (ACT) questionnaire was also administered to all asthmatic subjects.²⁵ After the enrolment, all asthmatic subjects were asked to keep a record of exacerbations over the course of a year and were reviewed every 3 months. In 13 asthmatics, H₂S levels in sputum and serum were obtained during exacerbation and during stable state.

2.4 | Sputum induction and processing

Sputum induction was performed with inhaled nebulized 5.0% NaCl solution.²⁶ Sputum plugs were harvested and processed with 0.1% dithiothreitol (DTT) and phosphate-buffered saline (PBS). The sputum supernatant of 0.8–1.0 mL was aliquoted into the serum tube. Then, the same amount of sulphide antioxidant buffer composed of 0.5 mol/L sodium salicylate, 0.12 mol/L ascorbic acid and 2.2 mol/L sodium hydroxide (SAOB II; Thermo Fisher Scientific, Beverly, MA, USA) was added and stored at -80°C for later analysis of H₂S. The rest of the sputum supernatant was also stored at -80°C for IL-8 and IL-5 measurements. Cytospins (Auto Smear CF-120; Sakura Finetech Japan Corporation, Tokyo, Japan) were prepared for differential cell counts.

2.5 | Blood processing

Next, 10 mL of blood was obtained and centrifuged at 800 *g* for 10 minutes. Then, 1 mL serum was aliquoted into the serum tube and 1 mL of SAOB II was added in the same way as the sputum processing and the mixture was stored at -80°C for later measurements of H₂S. The rest of the serum samples were also stored as they were at -80°C for later analysis of cytokine and chemokine.

2.6 | Measurement of H₂S

H₂S levels in the serum and sputum were measured using a sulfide-sensitive electrode (Model 9616; Orion Research; Beverly, MA, USA). Briefly, standard solutions were made using 0.1% DTT plus saline or saline only for sputum and serum H₂S measurements, respectively, to minimize any effect of DTT. The electrode was placed into the standard solutions for measurement of H₂S levels, and a calibration standard curve was constructed. The electrode was then put into the samples, and the H₂S concentrations were calculated using the calibration standard curve. Validation of the H₂S measurements, including accuracy, reproducibility and storage effects of samples, has been reported elsewhere.²³

2.7 | Measurement of IL-5 and IL-8

IL-5 and IL-8 levels in serum and sputum supernatants were measured as one of the Th2 and Th1 type of inflammatory markers, respectively, using a commercially available sandwich ELISA kit (R&D Systems, Minneapolis, Minnesota, USA).

2.8 | Statistical analysis

Statistical analyses were performed with SPSS for Windows (version 21, IBM, IL, USA) and Graph Pad Prism (version 5, GraphPad Software, USA). At least 14 subjects were needed per group for 70% power to detect a 20% difference in H₂S levels between each group with a two-sided α of 0.05.²⁷ Data were shown as median and interquartile range (IQR). Comparisons of continuous variables between groups were made using Kruskal-Wallis and Mann-Whitney U test with Bonferroni correction. Chi-square or Fisher's exact test was used for comparisons of categorical data. Differences in paired samples in the same subjects were analysed by Wilcoxon matched-pairs signed rank test. Correlation of continuous variables was evaluated using Spearman's rank correlation coefficient analysis. Then, multiple linear regression analysis was conducted to determine the independent association of H₂S levels with parameters whose *P* value was $<.1$ in the univariate correlation analysis. Finally, a receiver operating characteristic (ROC) curve was constructed to determine the predictive value for asthma exacerbations. A two-tailed *P* value of $<.05$ was considered statistically significant.

3 | RESULTS

3.1 | Characteristics of participants

Table 1 shows the clinical characteristics of the subjects. Age and FeNO levels were significantly higher, and height, sputum macrophages and FEV₁/FVC were significantly lower in all asthmatic subjects than in the healthy subjects ($P < .01$). Sputum neutrophils in all asthmatic subjects were higher than in the healthy subjects, although those in the UC-BA subjects did not reach a level of significance. Sputum eosinophils were significantly higher in the UC-BA subjects than in the healthy subjects ($P < .05$).

3.2 | H₂S levels in serum and sputum

Sputum H₂S levels in the AE-BA subjects (median 30.1 $\mu\text{mol/L}$; IQR 22.5–46.6 $\mu\text{mol/L}$) were significantly higher compared to the healthy subjects (median 13.0 $\mu\text{mol/L}$; IQR 9.9–15.6 $\mu\text{mol/L}$) ($P = .007$), as well as to the UC-BA subjects (median 16.0 $\mu\text{mol/L}$; IQR 10.3–21.9 $\mu\text{mol/L}$) ($P = .044$) (Figure 1A). The concomitant serum H₂S levels in the S-BA subjects (median 128 $\mu\text{mol/L}$; IQR 88.2–190 $\mu\text{mol/L}$) were significantly higher compared to those in the healthy subjects (median 55.4 $\mu\text{mol/L}$; IQR 52.3–68.8 $\mu\text{mol/L}$) ($P < .001$), whereas serum H₂S levels in the UC-BA subjects (median 73.6 $\mu\text{mol/L}$; IQR 49.0–109 $\mu\text{mol/L}$) and the AE-BA subjects (median 60.9 $\mu\text{mol/L}$; IQR 47.8–104 $\mu\text{mol/L}$) were significantly lower than in the S-BA subjects ($P = .005$ and $P < .001$, respectively) and were similar to those in the healthy subjects (Figure 1B).

For further confirmation of the difference in serum H₂S levels based on asthma conditions, paired samples of serum during exacerbation, as well as stable state were obtained from the 13 asthmatic subjects. Serum H₂S levels during exacerbation (median 60.6 $\mu\text{mol/L}$;

TABLE 1 Clinical characteristics

	Healthy (n = 15)	Stable controlled asthma (n = 47)	Uncontrolled asthma (n = 21)	Asthma with exacerbation (n = 26)
Age (y)	36.0 (31.0, 46.0)	66.0 (54.0, 72.0)***	58.0 (43.5, 70.0)**	62.5 (47.0, 68.5)**
Sex (male/female)	12/3	23/24	10/11	11/15
Height (cm)	170 (163, 174)	158 (150, 165)**	158 (150, 168)**	158 (150, 165)**
Weight (kg)	68.0 (56.0, 72.0)	59.5 (51.8, 68.0)	59.6 (53.8, 65.5)	62.5 (55.2, 69.5)
Smoking history (never/former/current)	14/1/0	30/17/0	13/8/0	18/8/0
Smoking (pack-years)	0.0 (0.0, 0.0)	0.0 (0.0, 17.0)	0.0 (0.0, 22.0)	0.0 (0.0, 0.0)
Sputum macrophages (%)	82.6 (70.8, 85.9)	15.3 (2.6, 28.1)***	23.0 (9.5, 33.7)***	11.1 (6.3, 32.9)***
Sputum neutrophils (%)	9.5 (6.8, 21.3)	76.5 (62.7, 84.1)***	42.6 (6.1, 74.0)	66.3 (18.0, 72.1)*
Sputum eosinophils (%)	2.0 (0.3, 2.4)	3.4 (1.3, 7.2)	23.7 (10.9, 54.0)*	7.9 (1.0, 53.3)
Sputum lymphocytes (%)	5.5 (3.5, 6.5)	2.0 (1.4, 4.1)	1.9 (1.1, 3.9)	2.9 (1.4, 3.6)
FVC (% predicted)	105 (97.2, 113)	99.5 (88.4, 107)	96.6 (82.6, 117)	85.9 (71.4, 106)
FEV ₁ (% predicted)	97.1 (90.5, 105)	89.6 (76.5, 102)	89.4 (57.1, 105)	80.4 (48.3, 93.1)*
FEV ₁ /FVC ratio (%)	83.7 (80.1, 89.3)	74.6 (68.7, 79.9)**	64.7 (60.0, 78.9)***	64.6 (53.1, 80.1)**
FeNO point (ppb)	17.5 (13.7, 22.4)	44.4 (32.2, 86.3)**	57.9 (27.0, 116)**	98.2 (58.6, 146)***
ACT score (pts)	N/A	22.0 (17.5, 23.0)	15.0 (11.5, 19.5)	12.5 (11/0, 16.3)†
ICS +/-	N/A	38/9	13/8	21/5
ICS (fluticasone equivalent) (µg)	N/A	500 (200, 1000)	320 (0.0, 900)	500 (290.1000)
LABA +/-	N/A	29/18	14/7	18/8

ACT, asthma control test; FeNO, fractional exhaled nitric oxide; FEV₁, forced expiratory volume in one-second; FVC, forced vital capacity; ICS, inhaled corticosteroid; LABA, long-acting β -agonist; N/A, not applicable.

Data are shown as median (interquartile range).

* $P < .05$ vs healthy, ** $P < .01$ vs healthy, *** $P < .001$ vs healthy, † $P < .05$ vs stable controlled asthma.

IQR 42.7–86.2 $\mu\text{mol/L}$) were significantly lower compared with those during stable state (median 124 $\mu\text{mol/L}$; IQR 59.7–180 $\mu\text{mol/L}$) ($P = .035$) (Figure 2).

3.3 | Sputum-to-serum H₂S ratio

We examined the ratio of H₂S in sputum to that in serum (H₂S ratio). This H₂S ratio in the AE-BA group (median 0.40; IQR 0.39–0.76) was significantly higher than that in the healthy subjects (median 0.23; IQR 0.18–0.26) ($P = .012$), S-BA subjects (median 0.18; IQR 0.09–0.35) ($P = .003$) and UC-BA subjects (median 0.17; IQR 0.07–0.41) ($P = .022$) (Figure 3).

3.4 | Relationships between H₂S levels and asthma parameters

Sputum H₂S levels showed a negative correlation with sputum macrophages (%) ($r = -.523$, $P = .001$) and a positive correlation with sputum neutrophils (%) ($r = .371$, $P = .014$). There were no correlations between sputum H₂S levels, FEV₁/FVC, FeNO levels and ACT scores, although there was a non-significant trend between sputum H₂S and FEV₁ %predicted (Table 2).

Serum H₂S levels did not correlate with any parameters related to asthma, such as inflammatory cell differentials in blood and sputum, FeNO levels, ACT scores and lung function (Table 2).

According to the multiple linear regression analysis, sputum H₂S was independently associated with increased sputum neutrophil percentage and decreased FEV₁ %predicted ($P < .05$) (Table 3). There was no parameter independently associated with serum H₂S levels. Sputum and serum H₂S levels were not influenced by age, sex, height or weight.

3.5 | IL-8 and IL-5 levels in serum and sputum

IL-8 and IL-5 levels only in serum, but not in sputum, were higher in the AE-BA subjects compared to the healthy subjects. We investigated whether the sputum-to-serum ratios of each IL-8 and IL-5 levels were also altered during exacerbations. There were no significant differences in the sputum-to-serum ratios of each IL-8 and IL-5 between the groups (Figure 4).

3.6 | Relationships between H₂S levels and Th1 and Th2 markers

Sputum H₂S levels showed significant positive correlations with IL-8 levels in serum ($r = .525$, $P = .001$), as well as in sputum ($r = .359$, $P = .031$), but not with IL-5 levels in either serum or sputum. On the other hand, serum H₂S levels correlated with IL-5 levels ($r = .263$, $P = .029$) and IL-8 levels ($r = .232$, $P = .041$) only in serum, but not in sputum (Table S1).

FIGURE 1 H₂S levels in sputum (A) and serum (B) from stable controlled asthmatic subjects (S-BA:■), uncontrolled asthmatic subjects (UC-BA:▲), asthmatic subjects with acute exacerbation (AE-BA:▼) and healthy subjects (Healthy:●). Horizontal bars indicate the median values

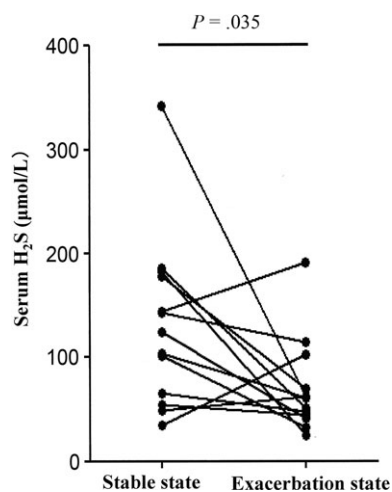
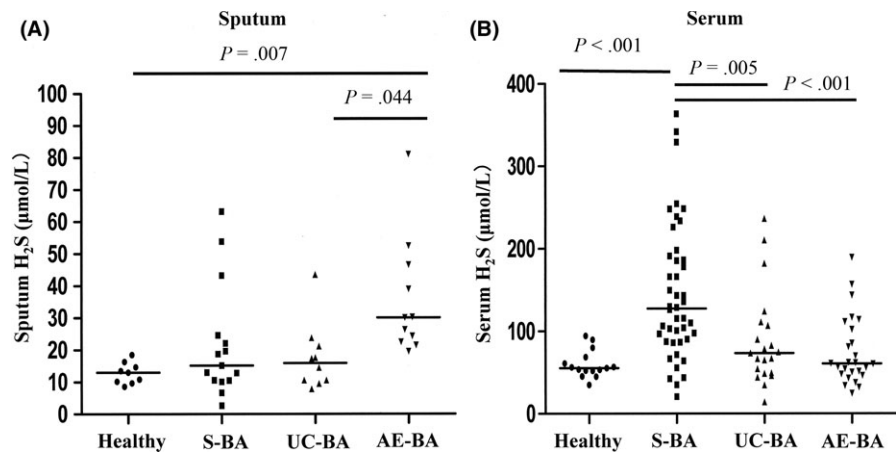


FIGURE 2 Difference in serum H₂S levels between stable and exacerbation states in the same asthmatic subjects. During the exacerbation state, serum H₂S levels mostly decreased from those in the stable state

3.7 | H₂S ratio as onset of asthma exacerbations

A ROC curve was constructed to determine the cut-off level of H₂S ratio that indicates an ongoing exacerbation from stable asthma. The optimal cut-off level of H₂S ratio was ≥ 0.34 (area under the curve; 0.88, sensitivity of 81.8% and specificity of 72.7%, $P < .001$) (Figure 5).

3.8 | Predicting future risks of asthma exacerbation using H₂S ratio

Thirty asthmatic subjects (11 S-BA, 10 UC-BA and nine AE-BA), who simultaneously yielded enough sputum and serum samples for the measurement of H₂S could be followed over the course of a year. Over the next 3 months after enrolment, six (46.2%) asthmatic subjects with a H₂S ratio of 0.34 or more experienced at least one or more asthma exacerbations, while only one (5.9%) asthmatic subject with a H₂S ratio of <0.34 suffered from an exacerbation ($P = .01$)

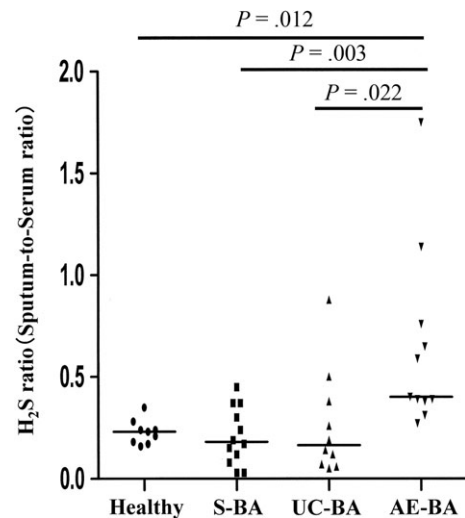


FIGURE 3 Sputum-to-serum H₂S ratio in stable controlled asthmatic subjects (S-BA:■), uncontrolled asthmatic subjects (UC-BA:▲), asthmatic subjects with acute exacerbation (AE-BA:▼) and healthy subjects (Healthy:●). Horizontal bars indicate median values

(Table 4). This difference was not observed over the next 6, 9 and 12 months. Furthermore, H₂S ratio showed a significant negative correlation with the time interval from asthma exacerbation onset to a hospital visit, that is, the greater the H₂S ratio was, the shorter the time of presentation to hospital ($r = -.443$, $P = .014$).

Additionally, there were no significant differences between the subjects who provided sputum and serum samples simultaneously and those who did not in terms of asthma control status and clinical characteristics, including lung function parameters, sputum cell differentials and H₂S levels in sputum and serum.

4 | DISCUSSION

In patients with asthma, sputum H₂S levels were significantly higher in the AE-BA subjects compared to the UC-BA and healthy subjects. On the contrary, serum H₂S levels were higher in the S-BA subjects than in the healthy subjects, and the UC-BA and AE-BA

TABLE 2 Correlation analysis between clinical parameters and H₂S levels in sputum and serum

	Sputum H ₂ S		Serum H ₂ S	
	<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value
Age (y)	.240	.113	−.192	.049
Height (cm)	−.099	.524	−.236	.016
Weight (kg)	.121	.432	−.113	.255
Smoking (pack-years)	−.025	.869	.073	.455
FVC (% predicted)	−.203	.215	−.007	.950
FEV ₁ (% predicted)	−.276	.088	−.072	.528
FEV ₁ /FVC ratio (%)	−.199	.225	−.160	.157
FeNO point (ppb)	.229	.068	.163	.148
Sputum macrophages (%)	−.523	.001	−.029	.851
Sputum neutrophils (%)	.371	.014	.065	.675
Sputum eosinophils (%)	.251	.105	.086	.579
Sputum lymphocytes (%)	−.181	.245	−.011	.945

ACT, asthma control test; FeNO, fractional exhaled nitric oxide; FEV₁, forced expiratory volume in one-second; FVC, forced vital capacity; H₂S, hydrogen sulphide.

subjects had similar serum H₂S levels to the healthy subjects. Serum H₂S levels significantly dropped during exacerbation in asthmatics that had H₂S levels previously measured while their asthma was stable. Thus, the H₂S ratio in the AE-BA subjects was higher than in the S-BA, UC-BA and healthy subjects. Furthermore, half of the asthmatic subjects with a H₂S ratio higher than the cut-off level experienced at least one or more asthma exacerbations over the next 3 months after the study enrolment. Finally, sputum H₂S levels showed a trend to decrease with FEV₁ %predicted and positive correlations with sputum neutrophils (%), sputum IL-8 and serum IL-8 levels, indicating that sputum H₂S levels may be associated with obstructive neutrophilic-predominant airway inflammation in asthma.

This is the first study to examine the levels of H₂S in serum and sputum during different conditions such as controlled, uncontrolled and acute exacerbation status in asthma. Our previous study reported that serum and sputum H₂S levels in asthmatic subjects were higher compared to healthy subjects, although there was no significant difference between asthma severities.²³ These results were in concordance with those of the current study to a larger extent, but our new finding is that H₂S levels in serum were lower in the UC-BA and AE-BA subjects than in the S-BA subjects. In addition, more interestingly, sputum H₂S levels were higher in AE-BA subjects than in the S-BA and UC-BA, which were entirely opposite to serum H₂S levels. These observations were further verified by the changes in serum H₂S levels between the stable and the exacerbation state obtained from the same asthmatic subjects. There have only been two reports discussing the relationship between H₂S levels and disease activity, although those investigations were all conducted in chronic obstructive pulmonary disease (COPD) subjects and not in asthmatic subjects.^{27,28} Chen et al²⁸ found that serum H₂S levels in stable COPD patients were higher than in healthy non-smokers and were lower than in COPD subjects with acute

TABLE 3 Independent parameters associated with H₂S in sputum using multiple linear regression analysis

Risk factor	β value	95% CI	<i>P</i> value
Sputum H ₂ S			
Sputum neutrophils (%)	0.353	0.016 to 0.336	.032
FEV ₁ (% predicted)	−0.441	−0.587 to −0.085	.010

FEV₁, forced expiratory volume in one-second; H₂S, hydrogen sulphide. Overall $R^2 = 0.292$.

exacerbation. In addition, they also revealed that serum H₂S levels were decreased among stable COPD patients depending on the stage of airway obstruction.²⁸ Similarly, we previously revealed that sputum H₂S levels in COPD subjects with acute exacerbation were higher than in stable COPD and healthy subjects, but serum H₂S levels in COPD subjects with acute exacerbation were lower than in stable COPD subjects.²⁷ These findings are quite comparable to our results and support our investigation suggesting that looking at changes in sputum and serum H₂S levels could be used as a marker of identifying disease states, as well as ongoing exacerbation in asthma.

Furthermore, taking the results mentioned above into consideration, the H₂S ratio may be expected to be more useful as a unique biomarker of a warning system for predicting the risk of exacerbations in asthma. This is because the systemic (serum) and the airway (sputum) levels of H₂S showed an opposite alteration during an exacerbation. In fact, the H₂S ratio increased to double the amount during acute exacerbation, and a ratio of ≥ 0.34 was indicative of an ongoing exacerbation. Moreover, half of the asthmatic subjects with a H₂S ratio ≥ 0.34 experienced asthma exacerbations in 3 months period. In contrast, sputum-to-serum IL-8 and IL-5 ratios did not differentiate the AE-BA subjects from the healthy or S-BA subjects. This difference between H₂S ratio, IL-8 and IL-5 ratio for detecting asthma exacerbation can be explained by the finding that serum IL-8 and IL-5 levels increased in contrast to the reduction in serum H₂S levels during exacerbations. Similar results have been reported in COPD patients, which revealed that H₂S ratio increased during acute exacerbation in COPD patients.²⁷ These findings, taken together with our results, may suggest that H₂S ratio, rather than single measurements of H₂S levels either in serum or sputum, could be a more sensitive marker for not only reflecting ongoing exacerbation but also predicting future risks of asthma exacerbations.

The source of H₂S in sputum and in serum in asthmatic subjects is currently being investigated. In blood circulation, it is likely that vascular endothelial cells and vascular smooth muscle cells produce H₂S,²⁹ while in the airway, airway smooth muscle cells are likely to be the major source.^{18,20} In mouse lung, both H₂S producing enzymes, CSE and CBS, are mainly expressed in pulmonary vascular smooth muscle cells and endothelial cells, as well as in airway smooth muscle cells.²⁰ Furthermore, increased expression of CBS was observed in airway smooth muscle cells from COPD patients.¹⁸ These findings may underlie the increased H₂S in sputum and serum, which is in agreement with previous studies.^{23,27,28} However, the mechanisms by which H₂S levels are decreased in serum and

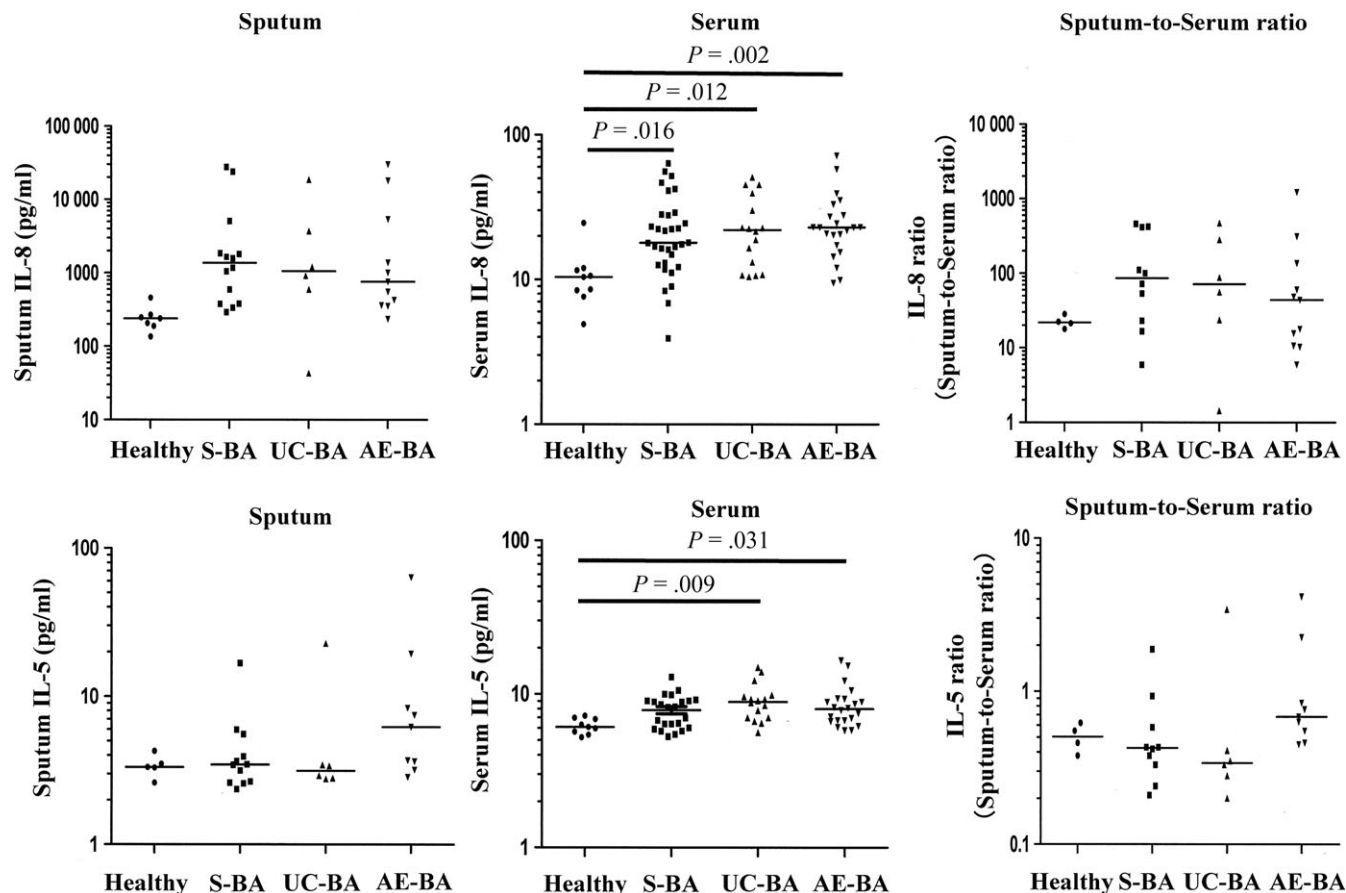


FIGURE 4 IL-8 and IL-5 in sputum and serum, and their sputum-to-serum ratio in stable controlled asthmatic subjects (S-BA:■), uncontrolled asthmatic subjects (UC-BA:▲), asthmatic subjects with acute exacerbation (AE-BA:▼) and healthy subjects (Healthy:●). Horizontal bars indicate median values

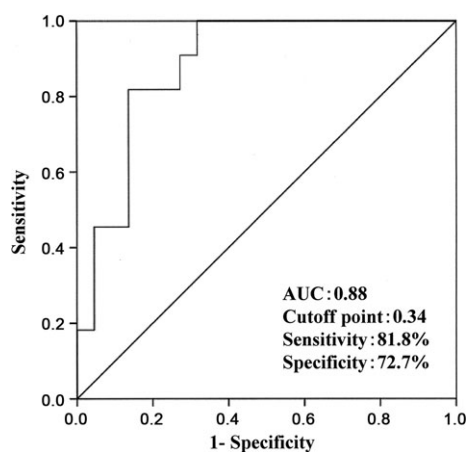


FIGURE 5 The receiver operator characteristics curve for the sputum-to-serum H_2S ratio to predict acute exacerbation of asthma

increased in sputum during exacerbation are unclear. They may be a reflection of increased sequestration of H_2S from the systemic circulation into the airways.

The role of H_2S in asthma is also debatable. There is evidence to indicate that H_2S may have anti-inflammatory and antioxidant

TABLE 4 H_2S ratio as a predictor of asthma exacerbation

	H_2S ratio < 0.34	H_2S ratio $\geq$ 0.34	Total
Exacerbation	1 (5.9)	6 (46.2)	7
No exacerbation	16 (94.1)	7 (53.8)	23
Total	17 (100)	13 (100)	30

H_2S , hydrogen sulphide.

Data are presented as n (%) or n. Chi-squared test: $P = .010$.

effects in the lung. In vitro, H_2S can induce vascular smooth muscle relaxation³⁰ and also suppress airway smooth muscle proliferation and IL-8 release.¹⁸ In support of this effect, aggravated airway hyperresponsiveness and increased airway inflammation occurred in mouse models of asthma with deficiency of H_2S -producing enzymes.³¹ Therefore, H_2S may have a protective role in asthma. However, other studies have suggested that H_2S has a proinflammatory role in controlling LPS-induced sepsis,³² acute pancreatitis³³ and burn injury³⁴ and that inhibiting H_2S was protective of organ injury in endotoxaemia.³² Thus, systemic production of H_2S might be down-regulated during an acute exacerbation, resulting in the decrease in serum H_2S levels. Further investigations will be needed to investigate the interrelationships between systemic and airway production of H_2S .

It is important to detect neutrophilic airway inflammation in asthma because the presence of neutrophilic-predominant airway inflammation may be associated with more severe asthma and possibly, corticosteroid insensitivity.^{4,5} At present, a more convenient biomarker for neutrophilic asthma does not exist. In the present study, there was a positive correlation between sputum H₂S levels and sputum neutrophils (%), which is in agreement with previous reports.^{23,27} The mechanism of the relationship between H₂S and neutrophils in sputum is unclear. However, Dal-Secco et al³⁵ previously reported that the incubation of neutrophils with IL-8 enhanced H₂S production in vitro study. We also found that sputum H₂S levels showed positive correlations with IL-8 levels in sputum, as well as in serum. These findings, including our results, indicate that sputum H₂S could be expected as a new non-Th2 biomarker for detecting neutrophilic airway inflammation.

In our study, a relationship was observed between sputum H₂S and the degree of airflow obstruction obtained from the multiple linear regression analysis although this relationship was weak. A similar result was observed in our previous cohort in asthma and COPD where the higher levels of sputum H₂S, the higher the degree of airflow obstruction^{23,27}. This indicates that H₂S can be considered as a biomarker not only of airway inflammation but also of airflow obstruction. Supporting the above-mentioned relationship, only FEV₁ %predicted and sputum neutrophilia were independently associated with sputum H₂S when using a multiple linear regression analysis. Therefore, sputum H₂S could be a surrogate biomarker for detecting obstructive neutrophilic airway inflammation in asthma.

There are several limitations in this study. Firstly, sputum and serum samples were obtained simultaneously from only 30 asthmatic subjects because many asthmatic subjects were not able to produce adequate amount of sputum for measuring H₂S levels. However, we believe the influence of this selection bias on H₂S levels would be small because a proportion of the subjects included for the ROC analysis were balanced in regard to asthma status. In addition, there were no significant differences in subjects' characteristics between those who provided sputum and serum samples concurrently and those who did not. Secondly, serum H₂S levels may be influenced by systemic non-respiratory diseases such as heart, hepatic, renal and pancreatic disease.³⁶ However, there was no significant difference in serum H₂S levels between the absence and presence of these diseases (data not shown). Thirdly, the effect of asthma medication on H₂S is not known. However, there was no significant difference in serum and sputum H₂S levels between subjects with or without inhaled corticosteroid in this study (data not shown). In addition, a previous study revealed that inhaled corticosteroids and theophylline had no effect on H₂S levels in COPD patients.³⁷ Therefore, we believe that the influence of asthma medication on H₂S was negligible. Finally, bacteria in the upper and lower airways may contribute to H₂S production.²⁷ H₂S produced by bacteria may sustain bacterial growth and suppress its sensitivity to antibiotics.³⁸ Thus, H₂S could partly reflect the presence of bacterial infection in the airways and lungs.

In conclusion, concomitant measurements of H₂S in sputum and serum may provide useful information for evaluating asthma conditions, especially predicting future risks of asthma exacerbations. Furthermore, sputum H₂S levels, rather than serum H₂S levels, may represent a novel promising biomarker of neutrophilic-predominant airway inflammation in asthma. In the future, further prospective studies will be required to investigate whether H₂S ratio could estimate the therapeutic response to poorly controlled asthma in a large asthma cohort.

ACKNOWLEDGEMENT

This project was supported by grants from the Novartis Pharma Industry, JMS Corporation and JSPS KAKENHI Grant Number 15K09182. We would like to thank Dr. Ryuichi Togawa, Dr Yuki Sato, Dr Takefumi Nikaido, Dr Xintao Wang and Dr Yoshinori Tanino for constructive comments. We would also like to thank the Scientific English Editing Section of Fukushima Medical University for their linguistic assistance in proofreading the manuscript.

CONFLICT OF INTEREST

Dr. JS reports grants from Novartis Pharma Industry, grants from JMS Corporation, grants from JSPS KAKENHI, during the conduct of the study. Other authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

JS conceived and designed the study. YS, JS, KM and MM analysed and interpreted the data. YS, JS, MK, MU, AF and SS involved in execution and recruitment. YS and JS drafted the manuscript. YS, JS and MM gave final approval of the manuscript.

ORCID

J. Saito  <http://orcid.org/0000-0002-9876-1160>

REFERENCES

1. Global Initiative for Asthma. Global strategy for asthma management and prevention. (updated 2016; cited December 25, 2016). Guideline. Available from: www.ginasthma.org.
2. Wenzel SE. Asthma: defining of the persistent adult phenotypes. *Lancet*. 2006;368:804-813.
3. Moore WC, Hastie AT, Li X, et al. Sputum neutrophil counts are associated with more severe asthma phenotypes using cluster analysis. *J Allergy Clin Immunol*. 2014;133:1557-1563.e5.
4. Haldar P, Pavord ID, Shaw DE, et al. Cluster analysis and clinical asthma phenotypes. *Am J Respir Crit Care Med*. 2008;178:218-224.
5. Jatakanon A, Uasuf C, Maziak W, et al. Neutrophilic inflammation in severe persistent asthma. *Am J Respir Crit Care Med*. 1999;160(5 Pt 1):1532-1539.
6. Saito J, Inoue K, Sugawara A, et al. Exhaled nitric oxide as a marker of airway inflammation for an epidemiologic study in schoolchildren. *J Allergy Clin Immunol*. 2004;114:512-516.

7. Sato S, Saito J, Sato Y, et al. Clinical usefulness of fractional exhaled nitric oxide for diagnosing prolonged cough. *Respir Med*. 2008;102:1452-1459.
8. Fukuhara A, Saito J, Sato S, et al. Validation study of asthma screening criteria based on subjective symptoms and fractional exhaled nitric oxide. *Ann Allergy Asthma Immunol*. 2011;107:480-486.
9. Dweik RA, Boggs PB, Erzurum SC, et al. An official ATS clinical practice guideline: interpretation of exhaled nitric oxide levels (FENO) for clinical applications. *Am J Respir Crit Care Med*. 2011;184:602-615.
10. Jia G, Erickson RW, Choy DF, et al. Periostin is a systemic biomarker of eosinophilic airway inflammation in asthmatic patients. *J Allergy Clin Immunol*. 2012;130:647-654.e10.
11. Izuohara K, Arima K, Ohta S, et al. Periostin in allergic inflammation. *Allergol Int*. 2014;63:143-151.
12. Corren J, Lemanske RF, Hanania NA, et al. Lebrikizumab treatment in adults with asthma. *N Engl J Med*. 2011;365:1088-1098.
13. Smith AD, Cowan JO, Brassett KP, et al. Exhaled nitric oxide: a predictor of steroid response. *Am J Respir Crit Care Med*. 2005;172:453-459.
14. Hanania NA, Wenzel S, Rosen K, et al. Exploring the effects of omalizumab in allergic asthma: an analysis of biomarkers in the EXTRA study. *Am J Respir Crit Care Med*. 2013;187:804-811.
15. Pavord ID, Korn S, Howarth P, et al. Mepolizumab for severe eosinophilic asthma (DREAM): a multicentre, double-blind, placebo-controlled trial. *Lancet*. 2012;380:651-659.
16. Ortega HG, Liu MC, Pavord ID, et al. Mepolizumab treatment in patients with severe eosinophilic asthma. *N Engl J Med*. 2014;371:1198-1207.
17. Baskar R, Li L, Moore PK. Hydrogen sulfide induces DNA damage and changes in apoptotic gene expression in human lung fibroblast cells. *FASEB J*. 2007;21:247-255.
18. Perry MM, Hui CK, Whiteman M, et al. Hydrogen sulfide inhibits proliferation and release of IL-8 from human airway smooth muscle cells. *Am J Respir Cell Mol Biol*. 2011;45:746-752.
19. Olson KR, Whitfield NL, Bearden SE, et al. Hypoxic pulmonary vasodilation: a paradigm shift with a hydrogen sulfide mechanism. *Am J Physiol Regul Integr Comp Physiol*. 2010;298:R51-R60.
20. Wang P, Zhang G, Wondimu T, et al. Hydrogen sulfide and asthma. *Exp Physiol*. 2011;96:847-852.
21. Wang R. Two's company, three's a crowd: can H₂S be the third endogenous gaseous transmitter? *FASEB J*. 2002;16:1792-1798.
22. Whiteman M, Le Trionnaire S, Chopra M, et al. Emerging role of hydrogen sulfide in health and disease: critical appraisal of biomarkers and pharmacological tools. *Clin Sci (Lond)*. 2011;121:459-488.
23. Saito J, Zhang Q, Hui C, et al. Sputum hydrogen sulfide as a novel biomarker of obstructive neutrophilic asthma. *J Allergy Clin Immunol*. 2013;131:232-234. e1-3.
24. Reddel HK, Taylor DR, Bateman ED, et al. An official American Thoracic Society/European Respiratory Society statement: asthma control and exacerbations: standardizing endpoints for clinical asthma trials and clinical practice. *Am J Respir Crit Care Med*. 2009;180:59-99.
25. Nathan RA, Sorkness CA, Kosinski M, et al. Development of the asthma control test: a survey for assessing asthma control. *J Allergy Clin Immunol*. 2004;113:59-65.
26. Pizzichini E, Pizzichini MM, Efthimiadis A, et al. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. *Am J Respir Crit Care Med*. 1996;154 (2 Pt 1):308-317.
27. Saito J, Mackay AJ, Rossios C, et al. Sputum-to-serum hydrogen sulfide ratio in COPD. *Thorax*. 2014;69:903-909.
28. Chen YH, Yao WZ, Geng B, et al. Endogenous hydrogen sulfide in patients with COPD. *Chest*. 2005;128:3205-3211.
29. Skovgaard N, Goulliaev A, Aalling M, et al. The role of endogenous H₂S in cardiovascular physiology. *Curr Pharm Biotechnol*. 2011;12:1385-1393.
30. Wang R. Signaling pathways for the vascular effects of hydrogen sulfide. *Curr Opin Nephrol Hypertens*. 2011;20:107-112.
31. Zhang G, Wang P, Yang G, et al. The inhibitory role of hydrogen sulfide in airway hyperresponsiveness and inflammation in a mouse model of asthma. *Am J Pathol*. 2013;182:1188-1195.
32. Collin M, Anuar FB, Murch O, et al. Inhibition of endogenous hydrogen sulfide formation reduces the organ injury caused by endotoxemia. *Br J Pharmacol*. 2005;146:498-505.
33. Bhatia M, Wong FL, Fu D, et al. Role of hydrogen sulfide in acute pancreatitis and associated lung injury. *FASEB J*. 2005;19:623-625.
34. Zhang J, Sio SW, Mochhala S, et al. Role of hydrogen sulfide in severe burn injury-induced inflammation in mice. *Mol Med*. 2010;16:417-424.
35. Dal-Secco D, Cunha TM, Freitas A, et al. Hydrogen sulfide augments neutrophil migration through enhancement of adhesion molecule expression and prevention of CXCR2 internalization: role of ATP-sensitive potassium channels. *J Immunol*. 2008;181:4287-4298.
36. Li L, Moore PK. Putative biological roles of hydrogen sulfide in health and disease: a breath of not so fresh air? *Trends Pharmacol Sci*. 2008;29:84-90.
37. Kirkham PA, Whiteman M, Winyard PG, et al. Impact of theophylline/corticosteroid combination therapy on sputum hydrogen sulfide levels in patients with COPD. *Eur Respir J*. 2014;43:1504-1506.
38. Shatalin K, Shatalina E, Mironov A, et al. H₂S: a universal defense against antibiotics in bacteria. *Science*. 2011;334:986-990.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Suzuki Y, Saito J, Kikuchi M, et al. Sputum-to-serum hydrogen sulphide ratio as a novel biomarker of predicting future risks of asthma exacerbation. *Clin Exp Allergy*. 2018;00:1-9. <https://doi.org/10.1111/cea.13173>

Online Data Supplement

**Sputum-to-serum hydrogen sulfide ratio as a novel biomarker of predicting
future risks of asthma exacerbation**

Yasuhito Suzuki, MD, PhD, Junpei Saito, MD, PhD, Masami Kikuchi, MT,
Manabu Uematsu, MD, PhD, Atsuro Fukuhara, MD, PhD, Suguru Sato, MD,
PhD, and Mitsuru Munakata, MD, PhD.

Department of Pulmonary Medicine, Fukushima Medical University, School of
Medicine, Fukushima, Japan.

Correspondence;

All correspondence should be addressed to Junpei Saito, MD, PhD

Address: Department of Pulmonary Medicine, Fukushima Medical University,
School of Medicine, 1 Hikarigaoka, Fukushima City, Fukushima Prefecture,
960-1295, Japan.

Email: junpei@fmu.ac.jp

Tel: +81-24-547-1360

Fax: +81-24-548-9366

Table S1. Relationships between H₂S levels and Th1 and Th2 markers

	Sputum H ₂ S		Serum H ₂ S	
	r	p value	r	p value
Serum IL-8 (pg/ml)	0.525	0.001	0.232	0.041
Serum IL-5 (pg/ml)	0.036	0.832	0.263	0.029
Sputum IL-8 (pg/ml)	0.359	0.031	0.050	0.771
Sputum IL-5 (pg/ml)	0.338	0.063	0.038	0.844

H₂S, hydrogen sulfide;

IL-8, Interleukin-8; IL-5, Interleukin-5