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Review Article

Hydrogen sulfide as a novel biomarker of asthma and chronic obstructive pulmonary disease

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Abbreviations:

H₂S, Hydrogen sulfide; NO, Nitric oxide;
CO, Carbon monoxide; COPD, Chronic
obstructive pulmonary disease;
CBS, Cystathionine β-synthase;
CSE, Cystathionine γ-lyase; 3-MST, 3-
mercaptopyruvate sulfurtransferase;
CAT, Cysteine aminotransferase;
ROS, Reactive oxygen species; NF-
κB, Nuclear factor-kappa B; K_{ATP},
channels, sarcolemmal ATP sensitive K⁺
channels; NaHS, Sodium hydrosulfide;
GR, Glutathione reductase; IL-8, Interleukin-
8; TNF-α, Tumour necrosis factor-α;
NLRP3, Nod-like receptor pyrin domain
containing 3; LPS, Lipopolysaccharide;
PPG, dl-propargylglycine; iNOS, Inducible
nitric oxide synthase; NO, nitric oxide;
MBB, monobromobimane; ROC, Receiver
operating characteristic curve;
FeNO, Fractional exhaled nitric oxide;
FEV₁, Forced expiratory volume in one
second; ACO, Asthma COPD overlap

ABSTRACT

Hydrogen sulfide (H₂S) has recently been recognised as the third important gas-signalling molecule, besides nitric oxide and carbon monoxide. H₂S has been reported to be produced by many cell types in mammalian tissues and organs throughout the actions of H₂S-generating enzymes or redox reactions between the oxidation of glucose and element of sulfur. Although the pathological role of H₂S has not yet been fully elucidated, accumulative data suggest that H₂S may have biphasic effects. Briefly, it mainly has anti-inflammatory and antioxidant roles, although it can also have pro-inflammatory effects under certain conditions where rapid release of H₂S in tissues occur, such as sepsis. To date, there have been several clinical studies published on H₂S in respiratory disorders, including asthma and chronic obstructive pulmonary disease (COPD). According to previous studies, H₂S is detectable in serum, sputum, and exhaled breath, although a gold standard method for detection has not yet been established. In asthma and COPD, H₂S levels in serum and sputum can vary depending on the underlying conditions such as an acute exacerbation. Furthermore, sputum H₂S in particular correlates with sputum neutrophils and the degree of airflow limitation, indicating that H₂S has potential as a novel promising biomarker for neutrophilic airway inflammation for predicting current control state as well as future risks of asthma. In the future, concurrent measures of H₂S with conventional inflammatory biomarkers (fractional exhaled nitric oxide, eosinophils etc) may provide more useful information regarding the identification of inflammatory phenotypes of asthma and COPD for personalised treatment.

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Introduction

Hydrogen sulfide (H₂S) is a well-known toxic gas with a typical malodorous smell. In recent years, however, it has been recognised as the third gasotransmitter along with nitric oxide (NO) and carbon monoxide (CO),¹ and has been reported to be produced by many cell types in the mammals, including human.^{2–5} In the lung,

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in particular, the production of H₂S is thought to occur mainly in pulmonary arterial cells, airway smooth muscle cells, lung primary fibroblasts, and endothelial cells.^{1,6–8} However, the pathological and pathophysiological roles of H₂S in the lung are less well understood. Furthermore, there have been several clinical studies published on H₂S in respiratory disorders, including asthma, chronic obstructive pulmonary disease (COPD), pneumonia, and cystic fibrosis.^{9–18}

We here reviewed previously-identified pathophysiological roles of H₂S, especially in the lung. Furthermore, we also described the utility of serum and sputum H₂S as a potentially novel biomarker of neutrophil predominant airway inflammation in asthma and COPD.

Production of H₂S

The majority of endogenous H₂S is synthesized through the action of three H₂S synthetics: cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST)^{6,7,19,20} (Fig. 1A). CBS and CSE are present in the cytosol, whereas 3-MST is present in mitochondria as well as the cytosol.^{21,22} In human tissues, CBS and CSE are the dominant synthetases which are responsible for the production of endogenous H₂S via the metabolism of homocysteine, cystathionine and cysteine.^{19,23–25} CBS and CSE generate the majority of endogenous H₂S using homocysteine and cystathionine as a formation of cysteine. CSE is also responsible for the production of endogenous H₂S by degrading of cystathionine or eliminating disulfide on cysteine.^{19,23,25} 3-MST acts in combination with cysteine aminotransferase (CAT) to produce endogenous H₂S from cysteine as the third pathway.^{19,20,24,25} There is already some information on the expression of H₂S producing enzymes in the lung tissues.^{1,6–8,19,20,26} In mouse, CBS is predominantly expressed in airway vessels and epithelial cells, whereas CSE is expressed in lung parenchyma.^{19,26} In the human respiratory system, airway smooth muscle cells and lung primary fibroblast cells represent both CBS and CSE.^{1,6–8}

Another source of endogenous H₂S is known as the non-enzymatic pathway, caused by redox reaction between reducing equivalents from the oxidation of glucose and element of sulfur (Fig. 1B).^{23,27} H₂S is hydrolyzed to hydrosulfide and sulfide ions in the following sequential reactions: $\text{H}_2\text{S} \rightleftharpoons \text{H}^+ + \text{HS}^- \rightleftharpoons 2\text{H}^+ + \text{S}^{2-}$. About one-third of H₂S remains undissociated at pH 7.40 in an aqueous solution. H₂S easily passes through the cell plasma membranes since its solubility in lipophilic solvents is five fold greater than in water, and it is involved in various signal transductions related to inflammation.²³

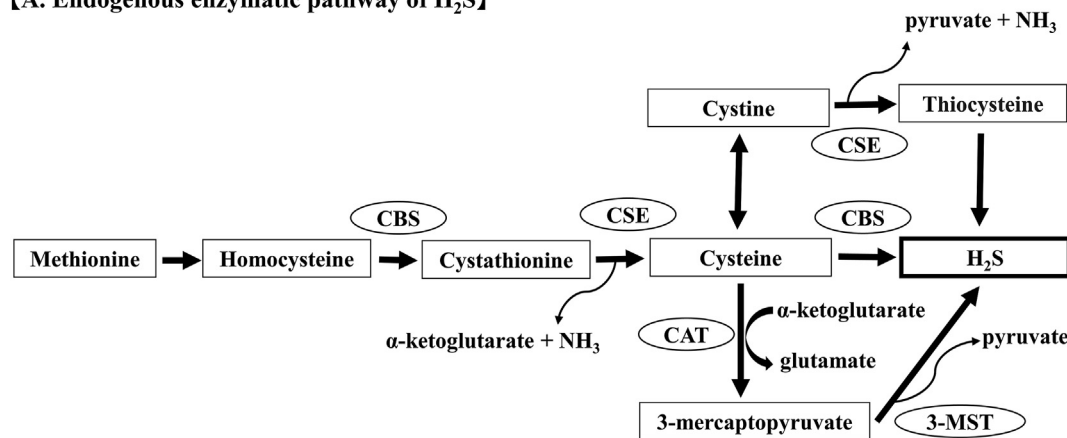
Role of H₂S

Anti-inflammatory effects of H₂S

The role of H₂S in the lung, as well as in the blood circulation, remains unclear. However, as the proverb 'poison drives out poison' says, many studies have suggested that H₂S may have antioxidant and anti-inflammatory effects in accelerating the resolution of inflammation.^{28–36}

Except for the lungs, H₂S scavenges various oxidants such as superoxide and peroxynitrite as an antioxidant role. For example, H₂S exhibits antioxidant effects against oxidative stress in Alzheimer's disease and Parkinson's disease, which are characterized by mitochondrial dysfunction due to reactive oxygen species (ROS) formation.³⁷ Regarding anti-inflammatory effects, administration of H₂S donors in murine model with colitis attenuates circulating pro-inflammatory cytokines and chemokines by inhibiting nuclear factor-kappa B (NF-κB) activation.²⁸ As similarly seen in the rat model with carrageenan-induced knee joint synovitis, exogenous H₂S reduces inflammatory cell infiltrations including neutrophils and lymphocytes in the knee joints.²⁹ These anti-inflammatory effects of H₂S can be partly explained by inhibiting adherence of leukocytes to the vascular endothelial cells via activation of sarcolemmal ATP sensitive K⁺ channels (K_{ATP} channels), resulting in the reduction of systemic inflammation.³⁰

[A. Endogenous enzymatic pathway of H₂S]



[B. Endogenous non-enzymatic pathway of H₂S]

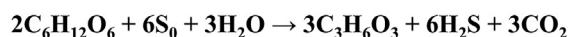


Fig. 1. (A) Synthesis of endogenous H₂S, enzymatic pathway and (B) non-enzymatic pathway. H₂S is synthesized from CBS and CSE via the metabolism of homocysteine, cystathionine and cysteine as substrates. 3-MST produces H₂S and pyruvate from 3-mercaptopyruvate, which is formed from cysteine and α-ketoglutarate produced by CAT. H₂S is also synthesized from the non-enzymatic pathway of elemental sulfur to H₂S using reducing equivalents obtained from the oxidation of glucose. H₂S, hydrogen sulfide; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; 3-MST, 3-mercaptopyruvate sulfurtransferase; CAT, cysteine aminotransferase.

In terms of resolution of inflammation in the lung, decreased levels of H₂S in pulmonary tissues were observed in the ovalbumin-sensitized and challenged mouse model.³¹ Then, administration of sodium hydrosulfide (NaHS) as an exogenous H₂S donor intraperitoneally reduced eosinophils and neutrophils with decreasing type 2 inflammatory cytokines (interleukin-5 and interleukin-13), and eotaxin-1 in bronchoalveolar lavage, resulting in the improvement of airway hyperresponsiveness.³¹ This anti-inflammatory effect of exogenous H₂S was reproduced through the action of inhaling NaHS in ovalbumin-sensitized mice where inhalation of NaHS improves lung function and inhibits airway hyperresponsiveness by modulating mast cells and in turn fibroblast activation.³² Exogenous H₂S may also prevent lung oxidative stress by decreasing levels of ROS and increasing activity of glutathione reductase (GR), resulting in reduction of airway inflammation.³³ In cigarette smoke exposed rat, serum H₂S levels were increased than control mice. Endogenous H₂S protects against cigarette smoke-induced lung oxidative stress, airway inflammation, airway hyperresponsiveness, and inhibits production of interleukin-8 (IL-8) and tumour necrosis factor- α (TNF- α) in the lung.³⁴ In addition, in vitro, administration of H₂S donor induces human airway smooth muscle and vascular smooth muscle relaxation through the K_{ATP} channels.³⁵ Moreover, intraperitoneal administration of NaHS prevents ozone-induced features of lung inflammation and emphysema in mice through regulation of the nod-like receptor pyrin domain containing 3 (NLRP3)-caspase-1 and p38 mitogen-activated protein kinase.³⁶ All these observations mentioned above indicate potential anti-inflammatory and antioxidant effects of H₂S in airway inflammatory disorders such as asthma and COPD (Table 1). Furthermore, in vitro and vivo, cellular H₂S has been reported to have broad-spectrum antiviral activity and play a role in controlling viral replication in response to respiratory syncytial virus infection.¹⁹ Recently, it has been pointed that H₂S not only plays a role as a regulator of signal transduction, but also forms reactive sulfur containing compounds, such as L-cysteine hydropersulfide (Cys-S-SH), which has critical regulatory functions in redox cell signalling.³⁸ Cysteine persulfide derivatives have nucleophilic and antioxidant activity, providing a potent antioxidant defence in cells, which may be associated with redox balance in the lungs.^{39,40} Therefore, it may be possible to use H₂S in clinical therapeutic applications for respiratory diseases.

Pro-inflammatory effects of H₂S

In contrast to antioxidant and anti-inflammatory effects of H₂S, other studies have suggested that H₂S has a proinflammatory effect (Table 1) in controlling lipopolysaccharide (LPS)-induced sepsis,⁴¹ acute pancreatitis,⁴² and burn injury.⁴³ This contrary effect may be due to a rapidly increasing H₂S concentration in the tissue. In LPS-induced sepsis mouse model, DL-propargylglycine (PPG), an inhibitor of CSE, reduced neutrophil infiltration and protected organ injury in endotoxemia. In contrast, the administration of NaHS (a rapid-releasing donor of H₂S) made a septic shock condition worse.⁴¹ Conversely, GYY4137 (a slow-releasing donor of H₂S) improved the histopathological changes in the endotoxemia-associated lung injury in mice. GYY4137 inhibited inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production in the endotoxemia lung, indicating that GYY4137 may contribute to the anti-oxidant, anti-nitrative, and anti-inflammatory properties.⁴⁴ As described above, rapid release of H₂S in tissue provided by the addition of NaHS may contribute to the proinflammatory effect, while the slow release of H₂S may be involved in the anti-inflammatory effect. This possibility can be partly supported by the findings of previous studies that evaluated the effects of

Table 1
Pathophysiological roles of H₂S.

Effects	Experimental system	Findings	Ref. No.
Anti-inflammatory effects	Asthma model (mouse)	Reduction of airway inflammatory cells, cytokines, and chemokines (IL-5, IL-13 and eotaxin-1)	31
		Inhibition of airway hyperresponsiveness	32
		Improvement of lung function	32
		Prevention of oxidative stress	33
		Reduction of airway inflammatory cells	34
		Inhibition of airway hyperresponsiveness	34
		Prevention of oxidative stress and emphysema	34,36
		Inhibition of IL-8 and TNF- α production	34
		Inhibition of RS viral replication	19
		Relaxation of airway smooth muscle through the K _{ATP} channels	35
Pro-inflammatory effects	Sepsis model (mouse)	NaHS (a rapid-releasing donor of H ₂ S) made a septic shock condition worse	41
		GYY4137 (a slow-releasing donor of H ₂ S) improved the endotoxemia-associated lung injury	44
		CSE inhibitor reduced the systemic inflammation associated with pancreatitis and lung injury	42
		NaHS (a rapid-releasing donor of H ₂ S) aggravated burn-associated systemic inflammation	43

COPD, chronic obstructive pulmonary disease; RSV, respiratory syncytial virus; RS, respiratory syncytial; IL-5, interleukin-5; IL-13, interleukin-13; IL-8, interleukin-8; TNF- α , tumour necrosis factor- α ; NaHS, sodium hydrosulfide; CSE, cystathionine γ -lyase; Ref. No., reference number.

different types of H₂S donors on cancer cells.^{45–47} GYY4137 caused an intracellular acidification, as well as cell death, in human breast adenocarcinoma cell (MCF7) as anti-cancer effect,⁴⁵ whereas high NaHS concentrations did not cause anti-cancer activity.⁴⁶ Further investigations are needed in order to elucidate why the effect of H₂S differs depending on the release rate of H₂S.

Measurement of H₂S

Accurate and reliable measurements of H₂S levels in the body fluids can provide critical information for understanding pathophysiological conditions as well as predicting clinical outcomes, and can also be of value in clinical applications for disease diagnosis and management. In recent years, due to the widespread interest in the field of H₂S chemical biology, detection methods of H₂S have been explored including high pressure liquid chromatography with monobromobimane (MBB), gas chromatography, methylene-blue method, fluorescence probe, and electrochemical sensors (ion sensitive electrodes and polarographic H₂S sensors, etc).⁴⁸ There is

currently no consensus on the measurement along with the type of assay that would most accurately measure H₂S levels, because although each technique is sensitive, highly precise, and useful, they also have limitations (Table 2). In addition, there have been no validation studies using human samples.

At present, the sulfide ion sensitive electrode method is the most commonly used methods to measure H₂S levels in the body fluid samples, with a detection range of 1–500 μM.^{12,48,49} The sulfide ion electrode consists of a sensing element bonded to an epoxy body. When the sensing element is in contact with a solution containing sulfide ions, an electrode potential, which depends on the level of free sulfide ions, develops across the sensing element. This potential is measured against a constant reference potential with a digital mV meter or concentration meter.^{48,50} When using this method, the calibration standard curve is prepared in advance. Briefly, standard solutions of some different concentrations of H₂S are made and sulfide antioxidant buffer was added in a ratio of 1:1. The electrode is placed into the standard solutions for the measurement of H₂S levels, and a calibration standard curve is constructed. Then, the electrode is put into the samples and the H₂S concentration is calculated using the calibration standard curve. We previously validated and reported several issues with respect to the reliability of this method for measuring H₂S levels in human samples in our preliminary study.¹² Firstly, the impact of storage effects on samples needs to be strongly considered before measuring long-term stored samples. Briefly, in our preliminary study, six serum samples were prepared and stored at –80 °C freezer. At Day 0, Day 1, Day 3, Day 7, Day 14, and Day 28 after preservation, the same amount of sulfide antioxidant buffer was added, and then H₂S levels were measured. As shown in Supplementary Figure 1A, the serum H₂S levels of the sample without buffer addition decreased over three days to half of those assayed at Day 0: this was not observed in the samples with buffer addition. This observation is consistent with the results of previous studies, and can convincingly explain the discrepancy of H₂S levels between our reports and other previous reports, described below in the sections of ‘Serum H₂S levels in asthma’^{10,12,49,51} and ‘H₂S levels in COPD’.^{13,15,16} Secondly, in terms of accuracy of H₂S measurement, we spiked five different concentrations (from 10 to 100 μM) of H₂S solutions to serum samples, and found a tight correlation between the measured and predicted H₂S levels seen in Supplementary Figure 1B(i). In addition, the Bland–Altman plot showed that the differences between measured and predicted H₂S levels were within a range of ±20%, indicating that this approach is accurate (Supplementary Fig. 1B(ii)). Finally, when evaluating the reproducibility of H₂S levels, serum from nine healthy volunteers were taken twice over a period of one week, and serum H₂S levels were

measured at the same time. We found that the mean coefficient of variation of H₂S levels over the week was 6.34%, which was acceptable for reproducible measurements (Supplementary Fig. 1C).

It would be more convenient to measure H₂S levels in exhaled breath. To date, there have been very few reports that measured H₂S levels in exhaled breath using different types of methods, such as electrochemical analyses, gas chromatography, or mass spectrometry analyses.^{9,52,53} In addition, there is no standardized method for measuring H₂S levels in exhaled air that has been collected in a bag. Further exploration is needed to accurately measure H₂S levels in exhaled breath.

H₂S as a biomarker of asthma

The roles of endogenous H₂S in the pathophysiology of several respiratory disorders, including asthma,^{31–33,35,54,55} COPD,^{34,36,56} cystic fibrosis,^{18,57} and respiratory viral infections,^{58,59} have been explored in vitro and in vivo for many years. However, there remain several clinical studies published on H₂S levels. Most previous studies have focused on serum H₂S levels measured using the selective ion electrode method, whereas there have been few studies which measured H₂S levels either in sputum or exhaled breath.

Serum H₂S levels in asthma

Endogenous H₂S in the systemic circulation (serum H₂S) is thought to be synthesized through the action of three H₂S generating-enzymes (CES, CBS, and 3-MST) in many cell types of human tissues, including vascular endothelial cells, vascular smooth muscle cells, and others. As shown in Table 3, Wu *et al.* has demonstrated for the first time that serum H₂S levels in adult asthmatic patients with stable state (55.8 ± 13.6 μM) were slightly reduced compared to those in healthy subjects (75.2 ± 13.0 μM). Furthermore, there was a further reduction in the levels of H₂S among adult asthmatic patients with acute exacerbation depending on the severity (mild 57.8 ± 6.30 μM; moderate 40.8 ± 5.10 μM; severe 31.3 ± 2.90 μM).⁵¹ These results were consistent with the results reported by Tian *et al.* Serum H₂S levels in asthmatic children with exacerbation (44.2 ± 11.0 μM) were significantly decreased compared with those in non-asthmatic children (52.6 ± 5.56 μM), and were correlated with lung function indices.¹⁰ These two studies have suggested that the levels of endogenous H₂S were reduced in asthma. However, in our recent study,¹² serum H₂S levels were found to be elevated in severe and non-severe asthmatic patients (283 ± 81.3 μM and 280 ± 179 μM, respectively) compared to healthy subjects (152 ± 84.0 μM), regardless of

Table 2
Measurement methods of H₂S.

Methods	Advantages	Limitations
Chromatography	High sensitivity	Asynchronous measure Need for hypoxic or anoxic conditions
Methylene-blue method	Relatively high cost performance Ease of handling	Asynchronous measure Need for acid chemical pre-treatment Lack of sensitivity at low concentration
Fluorescence probe	High sensitivity Real-time measurement	Sample damage due to photoexcitation Slow response time
Mass spectrometry	High sensitivity Realtime measurement Rapid response time	Easy to decompose in the collecting bag Difficult to measure quantitatively
Electrochemical sensor	High sensitivity Real-time measure in different kinds of samples Rapid response time Reproducibility Simple operation	Need for calibration every time to measure Need for anti-oxidant pre-treatment Ease of liquid leakage from electrolyte sensors

Created based upon the data from Reference.⁴⁸

Table 3Summary of serum H₂S levels (μM) in asthmatic patients.

Subjects	Healthy	Stable state	Exacerbation state	References	Ref. No.
Children	52.6 ± 5.56 μM	N/A	44.2 ± 11.0 μM**	Tian M <i>et al.</i>	10
Adult	75.2 ± 13.0 μM	55.8 ± 13.6 μM**	Mild: 57.8 ± 6.30 μM** Moderate: 40.8 ± 5.10 μM** Severe: 31.3 ± 2.90 μM**	Wu R <i>et al.</i>	51
Adult	152 ± 84.0 μM	Non-severe: 280 ± 179 μM* Severe: 283 ± 81.3 μM**	N/A	Saito J <i>et al.</i>	12
Adult	60.3 ± 16.6 μM	Controlled: 146 ± 82.1 μM*** Uncontrolled: 88.7 ± 57.5 μM [†]	75.1 ± 41.5 μM ^{††}	Suzuki Y <i>et al.</i>	49

Serum H₂S levels were measured using sulfide ion sensitive electrodes in all studies. Data are shown as means ± standard deviations.

N/A, not applicable; Ref. No., reference number.

*p < .05 vs healthy, **p < .01 vs healthy, ***p < .001 vs healthy, [†]p < .01 vs controlled state, ^{††}p < .001 vs controlled state.

asthma severity, and the levels were five times as high as those in the previous reports.^{10,51} The reason for the discrepancy between the former two studies and ours was not clear. However, some possible reasons can be speculated. One reason may be the influence of methodological issues on serum H₂S levels. As mentioned in the 'Measurement of H₂S in the body fluids' section, our preliminary data importantly suggest that serum H₂S levels of stored samples will decrease over three days to half of those assayed at Day 0 unless sulfide antioxidant buffer is added. This oxidation of stored samples can lower H₂S levels. The other reason may be the influence of degree of airway inflammation on serum H₂S levels, because H₂S levels in asthmatic patients with exacerbation were further decreased when compared to those in asthmatic patients with stable state.⁵¹ Our recent study supports this speculation.⁴⁹ Serum H₂S levels were higher in the stable asthmatic patients (146 ± 82.1 μM) than in the healthy subjects (60.3 ± 16.6 μM), and the patients with either uncontrolled (88.7 ± 57.5 μM) or exacerbation states (75.1 ± 41.5 μM) had similar serum H₂S levels to those in the healthy subjects (Fig. 2A). This observation was further confirmed in the same study by the comparison of H₂S levels in stable state and during exacerbation in the same asthmatic patients. In brief, serum H₂S levels in stable state (131 ± 81.9 μM) significantly dropped during exacerbation (69.6 ± 44.4 μM). These results are consistent with those of a previous study by Chen *et al.*, which reported serum H₂S levels in ovalbumin-sensitized rats decreased compared to those without sensitisation,⁵⁴ indicating that serum H₂S may be reduced in case of acute and greater inflammation. Taking the abovementioned results into

consideration, changes in serum H₂S levels may provide useful information for the prediction of asthma control status as well as asthma exacerbation. Further large prospective studies will be needed to confirm this observation.

Sputum H₂S levels in asthma

The main sources of endogenous H₂S in the lungs are thought to be airway structural cells, including airway and vascular smooth muscle cells with high expression levels of H₂S generating-enzymes (CES, CBS, and 3-MST). There have been only two studies which evaluated sputum H₂S levels in asthmatic patients, as shown in Table 4.^{12,49} Sputum H₂S levels both with severe (27.7 ± 14.6 μM) and non-severe asthmatic patients (26.7 ± 8.50 μM) were significantly higher than those in healthy subjects (11.4 ± 8.40 μM). These levels were not associated with salivary H₂S levels, which therefore may not have to be considered when measuring sputum H₂S levels.¹² In another cohort, sputum H₂S levels were measured in healthy subjects and asthmatic patients under different conditions (stable controlled state, uncontrolled state, and exacerbation state). Sputum H₂S levels in asthmatic patients with exacerbation (35.8 ± 18.4 μM) were the highest compared to those with stable controlled (21.8 ± 17.8 μM) and uncontrolled states (17.7 ± 10.5 μM) whose H₂S levels were similar to those in the healthy subjects (12.8 ± 3.30 μM) (Fig. 2B). These results indicate that sputum H₂S in addition to the change of serum H₂S can be a useful biomarker for predicting asthma exacerbation.

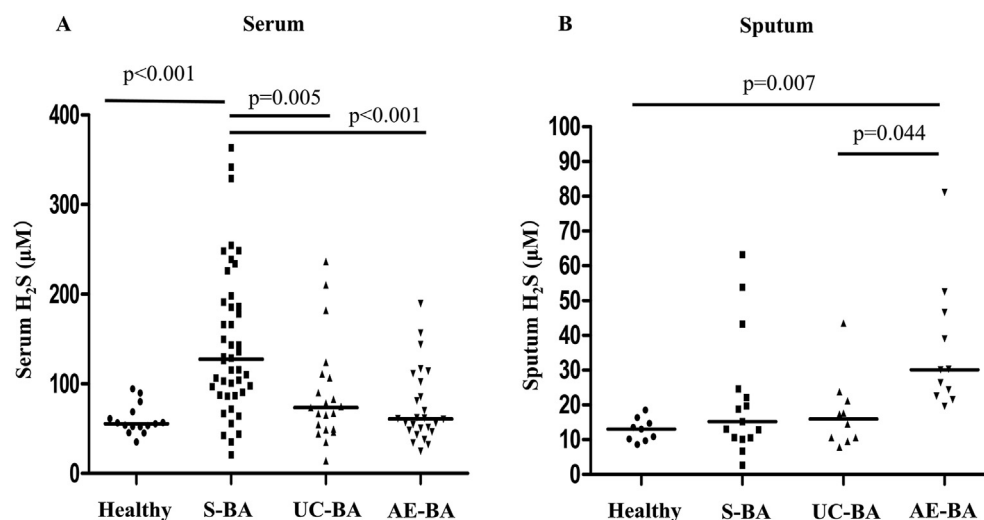


Fig. 2. (A) H₂S levels in serum and (B) sputum from stable controlled asthmatic subjects (S-BA: ■), uncontrolled asthmatic subjects (UC-BA: ▲), asthmatic subjects with acute exacerbation (AE-BA: ▼) and healthy subjects (Healthy: ●). H₂S, hydrogen sulfide. Modified from Suzuki *et al.*, 2018⁴⁹ with permission.

Table 4
Summary of sputum H₂S levels (μM) in asthmatic patients.

Subjects	Healthy	Stable state	Exacerbation state	References	Ref No.
Adult	11.4 ± 8.40 μM	Non-severe: 26.7 ± 8.50 μM** Severe: 27.7 ± 14.6 μM**	N/A	Saito J et al.	12
Adult	12.8 ± 3.30 μM	Controlled: 21.8 ± 17.8 μM Uncontrolled: 17.7 ± 10.5 μM	35.8 ± 18.4 μM*,†	Suzuki Y et al.	49

Sputum H₂S levels were measured using sulfide ion sensitive electrodes in all studies. Data are shown as means ± standard deviations.

N/A, not applicable; Ref No., reference number.

*p < .01 vs healthy, **p < .001 vs healthy, †p < .05 vs uncontrolled state.

Sputum to serum H₂S ratio as a biomarker for predicting future risks of asthma exacerbations

Very interestingly, the systemic (serum) and airway (sputum) H₂S levels showed opposite alterations during exacerbation as mentioned above. Therefore, the sputum-to-serum H₂S ratio (H₂S ratio) may be expected to be more useful as a unique biomarker for predicting asthma exacerbation. We assessed the ability of H₂S ratio to predict an ongoing asthma exacerbation by constructing a receiver operating characteristic (ROC) curve. The optimal cut-off level of H₂S ratio was 0.34 (area under the curve, 0.88; sensitivity, 81.8%; and specificity, 72.7%; p < 0.001) (Fig. 3). When 30 asthmatic patients were prospectively observed over three months, six asthmatic patients had H₂S ratio ≥ 0.34 and experienced at least one or more asthma exacerbations, while only one asthmatic patient with a H₂S ratio < 0.34 experienced exacerbation over the next three months after enrolment, showing a statistically significant difference. At the same time, we further identified the cut-off level of fractional exhaled nitric oxide (FeNO) for predicting an ongoing asthma exacerbation in the same cohort, which consisted of 60 asthmatic patients whose FeNO levels could be measured over the course of the study. The optimal cut-off level of FeNO for predicting an ongoing asthma exacerbation was 80.2 ppb (area under the curve, 0.69; sensitivity, 83.3%; and specificity, 68.5%, p = 0.136) (Fig. 3). When prospectively observing the same patients over a three month period, the exacerbation rate did not differ between asthmatic patients whose FeNO levels were ≥ 80 ppb and those whose levels were < 80 ppb. These findings indicate that H₂S ratio is more predictive of asthma exacerbation compared to FeNO.

Exhaled H₂S levels in asthma

H₂S levels in exhaled breath can provide more useful information non-invasively compared to serum and sputum H₂S. To

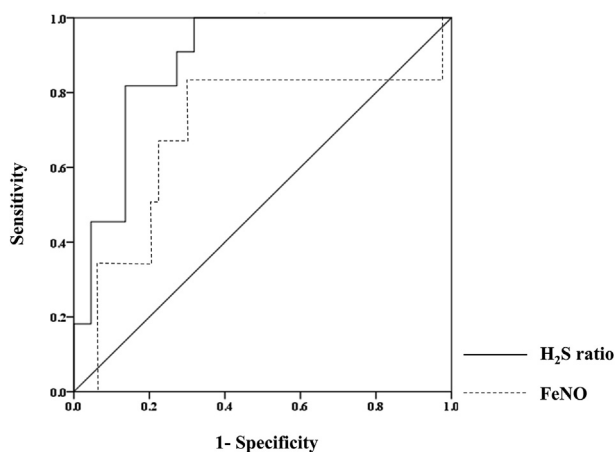


Fig. 3. The receiver operator characteristics curve for the sputum to serum H₂S ratio (solid line) and FeNO (dotted line) to predict acute exacerbation of asthma. H₂S, hydrogen sulfide; FeNO, Fractional exhaled nitric oxide.

date, there has only been one study that evaluated exhaled H₂S in asthma. Zhang *et al.* collected exhaled breath from 148 asthmatic patients into fluoropolymer gas sampling bags and then measured H₂S levels using an electrochemical sensor.⁹ They found that exhaled H₂S levels differ according to airway inflammatory patterns (eosinophilic, neutrophilic, mixed granulocytic, and paucigranulocytic). In brief, eosinophilic asthma showed the lowest exhaled H₂S levels (7.70 ± 4.20 ppb), while paucigranulocytic asthma had the highest exhaled H₂S levels (11.1 ± 4.60 ppb). However, the concentrations of H₂S seen in their study were very low and the differences between these two groups were very small, although statistically significant. It is not certain whether the method used in their study was accurate as they did not describe the detail. H₂S is easy to evaporate and be oxidised, therefore exhaled H₂S should be measured directly through the analysers or soon after exhaled breath is collected in the bags, otherwise its concentration can drop rapidly. In support of this theory, Morselli-Labate *et al.* measured exhaled H₂S levels in healthy subjects and patients with pancreatitis using mass spectrometry.⁵² They found that exhaled H₂S levels in patients with chronic pancreatitis (55.2 ± 19.1 ppb) were significantly higher than those in healthy subjects (47.8 ± 13.0 ppb). In another study, Toombs *et al.* measured exhaled H₂S levels using ion chromatography, and found that a level of 6.40 ± 2.40 ppb in normal volunteers increased transiently to 26.9 ± 4.60 ppb after sodium sulfide was injected intravenously.⁵³ Further prospective and explorative studies are warranted to standardise an accurate method for measuring H₂S in exhaled air.

Correlation between H₂S and asthma related parameters

It is desirable that a clinically applicable biomarker will not only differentiate asthma from other respiratory disorders, but also have associations with clinico-pathophysiological characteristics of asthma. In previous reports, serum H₂S was positively correlated with forced expiratory volume in one second (FEV₁) % predicted and negatively correlated with sputum neutrophils in patients with asthma.^{10,51} Meanwhile, sputum H₂S was negatively correlated with FEV₁% predicted and with reversibility to salbutamol.¹² In addition, there was a positive correlation between sputum H₂S and sputum % neutrophils.^{12,49} These opposite correlations between asthma-related parameters and H₂S levels in serum and sputum can be partly explained by the fact that serum and sputum H₂S levels show different trends depending on the degree of airway inflammation. However, it remains uncertain why the levels of serum H₂S decrease in a poor controlled state as well as exacerbation state of asthma. There are several possibilities for this. Firstly, serum H₂S in the systemic circulation can be consumed for suppressing airway inflammation. Secondly, serum H₂S may be representative of 'spillover' to the airways, which can lead to increased sputum H₂S levels and decreased serum H₂S levels. Further studies are needed to clarify the kinetics of H₂S in the circulation as well as in the lungs. There appear to be other issues we need to consider when interpreting H₂S levels in serum

and sputum. At first, serum H₂S levels may not be influenced only by asthma but also non-respiratory disorders such as heart, liver, renal and pancreatic disease.^{42,60–63} Thus, serum H₂S may not be specific to asthma. On the other hand, sputum H₂S levels may also be influenced by several factors. H₂S produced by bacteria might sustain bacterial growth and suppress their sensitivity to antibiotics.⁶⁴ Asthma medication can also influence H₂S levels, although previous studies have shown that sputum and serum H₂S levels were not associated with inhaled corticosteroid or theophylline treatment.^{12,16,49} However, even when all the above mentioned findings were taken into consideration, multiple linear regression analysis including asthma related parameters as well as serum and sputum H₂S revealed that sputum H₂S, but not serum H₂S, was independently associated with increased sputum neutrophils and decreased FEV₁% predicted.^{12,49} It is therefore interesting that sputum, rather than serum H₂S levels, could represent a novel promising biomarker of asthma, particularly as a marker of neutrophilic inflammation and chronic airflow obstruction and also reflection of β_2 agonist bronchodilator response.

H₂S levels in COPD

COPD is characterised by persistent progressive airflow limitation and chronic pulmonary inflammation associated with neutrophils and macrophages.⁶⁵ Thus, COPD is usually considered to be a different disorder from asthma, and H₂S can be expected as a more useful biomarker since neutrophilic airway inflammation is more predominant in COPD compared to asthma. Nevertheless, similar to H₂S studies in asthma, there have been few clinical studies discussing H₂S levels in COPD^{13,15,16} (Supplementary Table 1). Chen *et al.* reported for the first time that serum H₂S levels in COPD patients were found to be higher in stable state (smokers, $51.1 \pm 3.00 \mu\text{M}$ vs non-smokers, $49.8 \pm 3.80 \mu\text{M}$) and lower during acute exacerbation (smokers, $28.1 \pm 1.30 \mu\text{M}$ vs non-smokers, $39.4 \pm 3.90 \mu\text{M}$) than those in healthy subjects (smokers, $33.0 \pm 0.70 \mu\text{M}$ vs non-smokers, $37.9 \pm 0.90 \mu\text{M}$).¹³ Although they did not measure sputum H₂S levels, the results in terms of serum H₂S were consistent with those in our previous study, in which we concurrently measured sputum H₂S levels in addition to serum H₂S levels.¹⁵ Serum H₂S levels in stable COPD patients ($149 \pm 77.6 \mu\text{M}$) were higher than those in healthy subjects (smokers, $90.6 \pm 52.7 \mu\text{M}$ vs non-smokers, $91.0 \pm 62.2 \mu\text{M}$), but the levels in COPD patients with exacerbation state ($48.9 \pm 25.5 \mu\text{M}$) were lower than those in healthy subjects. On the other hand, sputum H₂S levels were elevated in COPD patients in both stable ($31.9 \pm 15.0 \mu\text{M}$) and exacerbation states ($50.4 \pm 24.4 \mu\text{M}$) compared to healthy subjects (smokers, $19.7 \pm 7.98 \mu\text{M}$ vs non-smokers, $12.1 \pm 6.64 \mu\text{M}$). This opposite alteration in terms of serum and sputum H₂S levels during exacerbation state was further confirmed by simultaneously measuring sputum and serum H₂S levels at baseline and during exacerbation.¹⁵ As a result, sputum H₂S levels increased and serum H₂S levels dropped during acute exacerbations of COPD, which was consistent with the results of our another previous asthma study.⁴⁹ Furthermore, sputum and serum H₂S levels correlated inversely with the degree of airflow obstruction and positively with sputum neutrophils, suggesting that H₂S levels are closely related to airway inflammation, as well as physiological characteristics of COPD.¹⁵ The effect of treatment on serum H₂S levels in COPD patients has also been reported. Although theophylline has been reported to reduce sputum neutrophils, it had no effect on serum H₂S levels.¹⁶ These studies discussed above suggest that H₂S may be influenced by the disease activities and

systemic inflammation in COPD. Recently, Asthma COPD overlap (ACO) has been proposed. Clinically, ACO is characterized by persistent airflow limitation with several asthma-associated features and COPD-associated features.⁶⁶ Therefore, H₂S in sputum as well as in serum may be a potentially useful biomarker for ACO, since H₂S, as mentioned above, may be associated with the pathophysiology of asthma as well as that of COPD. This should be explored further.

Conclusion

Significant progress has been made in the research field of sulfur species, and now H₂S is expected to be a third important gasotransmitter along with NO and CO. However, its action, regulation, and pathophysiological function in the lung has not yet been fully elucidated. At present, H₂S appears to have both pro-inflammatory and anti-inflammatory effects, depending on the underlying disease conditions. When it comes to being an inflammatory biomarker, H₂S in serum and sputum seems to vary with different conditions. In short, the differences in sputum and serum H₂S levels between patients in a stable state and those in an exacerbated state were entirely opposite, with an increase in sputum H₂S levels and concomitant decrease in serum H₂S levels during exacerbation. Furthermore, H₂S in sputum in particular may be associated with sputum neutrophils as well as degree of airflow obstruction. Therefore, H₂S has potential to become a novel promising biomarker for predicting not only neutrophilic airway inflammation but also current control status as well as future worsening risks of obstructive airway disorders, such as asthma and COPD. Hereafter, a standardized method, such as exhaled breath, which is simpler, more sensitive and noninvasive, should be established. Measuring H₂S with conventional inflammatory biomarkers (FeNO, eosinophils in blood and sputum) concurrently may provide more accurate and useful information on identifying inflammatory phenotypes of asthma, which can lead to a decision of personalized medicine. This needs further confirmation by future studies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2020.10.003>.

Conflict of interest

JS reports grants from Novartis Pharma and JMS Corporation during the conduct of the study. The rest of the authors have no conflict of interest.

Authors' contributions

Conception and design: YSu, JS and MM; drafting the manuscript: YSu, JS; final approval of the manuscript: YSu, JS, MM, and YSH.

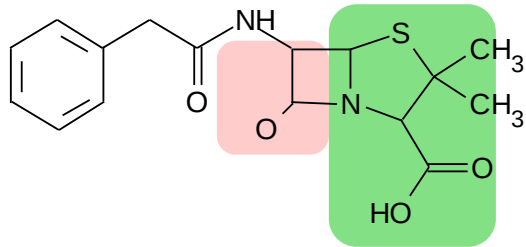
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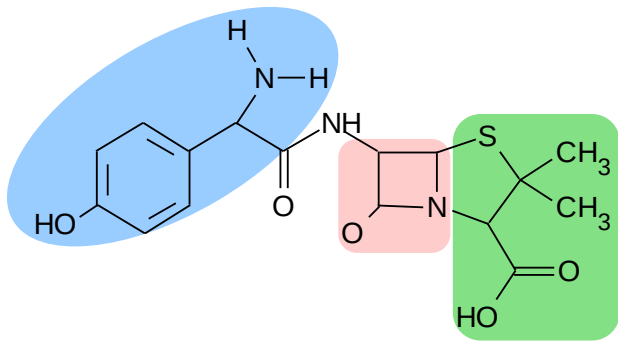
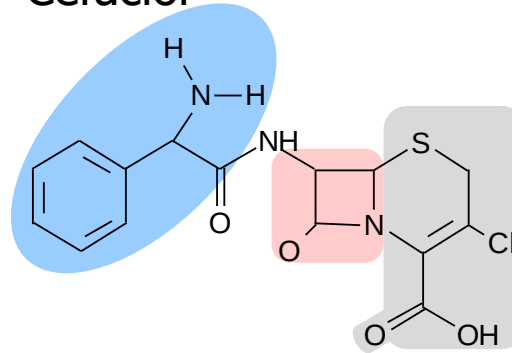
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Supplementary Fig. 1. Chemical structure of β -lactam and cephalosporin antibiotics

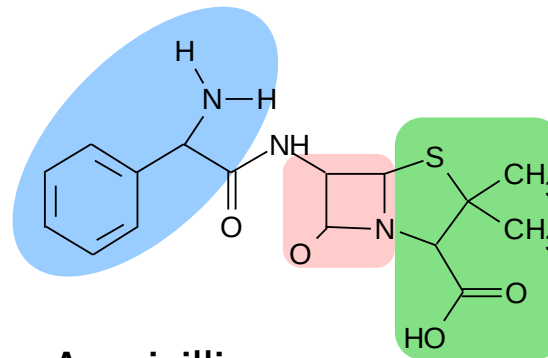
Penicillin G



Cefaclor



Amoxicillin



Ampicillin

Cross-reactivity in penicillins and cephalosporins is caused by similarities in the chemical structure of the drugs, which share their common β -lactam ring structure (● light pink). That is why they are known as “ β -lactam” antibiotics. The thiazolidine ring of penicillins (● light green) differentiates them from the dihydrothiazine ring of cephalosporins (● light grey). Cefaclor and ampicillin share their acyl side chain, and the one of amoxicillin is also rather similar (● light blue).

Pictures were drawn according to Blumenthal KG et al. Antibiotic allergy. Lancet. 2019;393(10167):183-98, and Zagursky RJ et al. Cross-reactivity in beta-lactam allergy. J Allergy Clin Immunol Pract. 2018;6(1):72-81. e1.