SERUM HYDROGEN SULFIDE AND OUTCOME ASSOCIATION IN PNEUMONIA BY THE SARS-CoV-2 CORONA VIRUS

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Sources of funding

G. Renieris is funded from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement grant European Sepsis Academy (agreement No 676129).
The study is funded by the Hellenic Sepsis Study Group.

Competing interests

E.J. Giamarellos-Bourboulis has received honoraria from AbbVie USA, Abbott CH, InflaRx GmbH, MSD Greece, XBiotech Inc. and Angelini Italy; independent educational grants from AbbVie, Abbott, Astellas Pharma Europe, AxisShield, bioMérieuxInc, InflaRx GmbH, and XBiotech Inc.; and funding from the FrameWork 7 program HemoSpec (granted to the National and Kapodistrian University of Athens), the Horizon2020 Marie-Curie Project European Sepsis Academy (granted to the National and Kapodistrian University of Athens), and the Horizon 2020 European Grant ImmunoSep (granted to the Hellenic Institute for the Study of Sepsis).
ABSTRACT

Background: The pneumonia of COVID-19 illness has often a subtle initial presentation making mandatory the use of biomarkers for evaluation of severity and prediction of final patient disposition. We evaluated the use of hydrogen sulfide (H2S) for the outcome of COVID-19 pneumonia.

Materials & Methods: We studied 74 patients with COVID-19. Clinical data were collected, and survival predictors were calculated. Blood was collected within 24 hours after admission (day 1) and on day 7. H2S was measured in sera by monobromobimane derivation (MBB) followed by high performance liquid chromatography and correlated to other markers like procalcitonin (PCT) and C- reactive protein (CRP). Tumor necrosis factor alpha (TNFα) and interleukin (IL)-6 were also measured in serum.

Results: Survivors had significantly higher H2S levels on day 1 and 7 after admission. A cut-off point of 150.44 μM could discriminate survivors from non-survivors with 80% sensitivity, 73.4% specificity and negative predictive value 95.9%. Mortality after 28 days was 32% with admission levels lower or equal to 150.44μM and 4.1% with levels above 150.44μM (p: 0.0008). Mortality was significantly greater among patients with a decrease of H2S levels from day 1 to day 7 greater or equal to 36% (p: 0.0005). Serum H2S on day 1 was negatively correlated with IL-6 and CRP and positively correlated with the absolute lymphocyte count in peripheral blood.

Conclusion: It is concluded that H2S is a potential marker for severity and final outcome of pneumonia by the SARS-CoV-2 coronavirus. Its correlation with IL-6 suggests anti-inflammatory properties.

Key-words: hydrogen sulfide; SARS- CoV-2; interleukin-6; mortality; biomarker;
INTRODUCTION

Since December 2019, humanity is experiencing a novel pandemic by the novel SARS-CoV-2 coronavirus-19 that is causing the disease known as Covid-19 (1). As of April 17th 2020, 2,265, 271 confirmed cases were reported worldwide, causing 154,900 deaths (https://www.who.int/emergencies/diseases/novel-coronavirus-2019). The main reason for death is severe respiratory failure (SRF) developing in the field of community-acquired pneumonia (CAP). It seems that derangement of the lung endothelium is the hallmark of disease pathogenesis. This is hypothesized by published evidence showing elevated levels of d-dimers and vascular endothelial growth factor in these patients(2,3).

Hydrogen sulfide (H$_2$S), long thought of solely as an environmental toxicant, is now known to be an endothelial product that drives angiogenesis, promotes vasorelaxation, reduces atherosclerosis and prevents ischemia-reperfusion injury (4,5). In spite of being considered having anti-inflammatory properties through inhibition of nuclear factor-kB (6), a recent animal model showed complex interaction with angiotensin-2 that is the receptor SARS-CoV-2 is using to invade host epithelia (7). In light of these observations suggesting a pivotal role of H$_2$S in the pathogenesis of Covid-19, we studied the serum levels of H$_2$S and its association with final outcome in a cohort of patients with Covid-19 pneumonia. Due to the described anti-inflammatory properties, we hypothesize that elevated levels of H$_2$S in serum are associated with a favourable outcome of Covid-19 pneumonia.

PATIENTS & METHODS

This study was conducted in eight departments participating in the Hellenic Sepsis Study Group from beginning of March 2020. The study protocol was approved by the Ethics Committees of the participating hospitals. Patients were included after written informed consent was provided by themselves or by first-degree relatives in case of patients unable to consent. We enrolled patients admitted with lower respiratory infection as diagnosed by the presence of infiltrates in chest X-ray or in computed tomography of the lung and who were tested positive upon admission by molecular testing of respiratory secretions for SARS-CoV-2. Blood was sampled within the first 24 hours of hospital admission and repeated after seven days. Exclusion criteria were: a) HIV-1 infection and b) neutropenia defined as less than 1000 neutrophils/mm$^3$. SRF was defined as any ratio of partial oxygen pressure to fraction of inspired oxygen below 200 necessitating mechanical ventilation. The clinical study flow chart is shown in Figure 1.
The following variables were recorded: i) demographics; ii) vital signs; iii) admission Acute Physiology and Chronic Health Evaluation (APACHE) II score, Charlson’s Comorbidity Index (CCI), Sequential Organ Failure Assessment (SOFA) score (8) and Pneumonia Severity Index (PSI) (9); (iii) absolute blood cell counts and biochemistry on admission and follow-up; and 28-day survival.

Immediately after sampling, blood was collected into sterile and pyrogen-free tubes and transported ice-cold for centrifugation within less than 10 minutes. H$_2$S was measured in patient serum by using monobromobimane (MBB) derivation followed by high performance liquid chromatography as it has previously been described (10,11). MBB, monosodium phosphate (NaH$_2$PO$_4$), disodium phosphate (Na$_2$HPO$_4$) and 5-sulfosalicylic acid (SSA) were purchased from Sigma-Aldrich (St. Lewis MO, USA). Sodium sulfide (Na$_2$S), diethylenetriaminepentaacetic acid (DTPA) and trifluoroacetic acid (TFA) were purchased from Alfa Aesar (Erlenbachweg, Germany). Tris-HCl buffer (0.1 M pH 9.5) was purchased from AlterChem (Athens, Greece). For the preparation of the derivatization buffer (Tris-HCl 0.1 M pH 9.5, 0.1 mM DTPA) DTPA was dissolved in tris-HCl. All solvents and buffers, as well as the tubes used for the derivatization reaction, were deoxygenated by using nitrogen gas flow (10 secs and 30 secs respectively). The solutions for the sulfidestandard curve were prepared by dissolving Na$_2$S in phosphate buffer, to final concentrations of 4-250 μM and the quantification limit was 10 μM. The MBB 10 mM derivatization solution was prepared by dissolving MBB in acetonitrile was then aliquoted in dark containers and kept at -20°C. The SSA 200 mM stop solution was freshly prepared before each measurement by dissolving SSA in distilled water. All sample preparations were conducted under dim room lighting. After deoxygenation, 30 μl of serum sample or standard, 70 μl Tris-HCl 0.1 M pH 9.5 0.1 mM DTPA and 50 μl MBB 10 mM were added in the tubes. The mixture was incubated at hypoxic conditions (1% O$_2$) at 37°C for 60min. The derivatization reaction was stopped by adding 50 μl of 200 mM SSA, followed by vortexing for 10 seconds. The vials were then left on ice for 10 minutes and centrifuged at 12000 rpm, 4°C for another 10 minutes. Finally, 100 μl of the supernatant were transferred at darkened HPLC vials and kept at 4°C. Analysis was done by an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) using one LiChroCART Reverse-Phase (RP) C18 4.6 x 250 mm, 5 μm analytical column, with a Purospher RP-18E 4 x 4 mm, 5μM guard column (Merck, Darmstadt, Germany). Analysis was performed at 25°C, using gradient elution. The mobile phases consisted of acetonitrile (0.1% TFA, v/v) and water (0.1% TFA), v/v), at a 0.6 ml/min flow rate. All measurements
were carried out at excitation and emission wavelengths of 390 nm and 475 nm respectively. The retention time of the derivatization product was 12.7 minutes.

Serum concentrations of tumor necrosis factor alpha (TNFα) and interleukin (IL)-6 were measured in duplicate by an enzyme immunoassay (R&D, Minneapolis, USA). The lowest detections limits were 40 pg/ml TNFα 40 pg/ml and 10 pg/ml for IL-6. Procalcitonin (PCT) was measured by a time-resolved amplified cryptate emission technology assay according to the manufacturer's instructions (Kryptor, Brahms, Hennigsdorf, Germany). The lower detection limit was 0.06 ng/ml. C-reactive protein (CRP) was estimated in duplicate by a nephelometric assay (Behring, Berlin, Germany). The lowest limit of detection was 0.2 mg/dl.

The association between H2S levels on day 1 and 28-day survival was the primary study endpoint. The association between change in H2S levels between day 1 and 7 and 28-day survival was the secondary study endpoint.

Statistics

categorical data were presented as frequencies and quantitative variables as mean ± SE. Comparisons between groups were done using the Fisher exact test for categorical data, the two-sided Student’s t test or Mann-Whitney U test for quantitative data. Correlations were performed using the Spearman’s rank of order. Survival was compared by the log-rank test. Odds ratios (OR) and 95% confidence intervals (CIs) for were calculated by the Mantel and Haenszel’s statistics. Receiver operating characteristic (ROC) curves were analyzed for outcome prediction; the best cut-off was selected using the Youden index. Stepwise forwards Cox regression analysis with hazard ratios (HRs) and confidence intervals (CIs) was used to investigate independent variable associated with 28-day outcome. Any p value below 0.05 was considered statistically significant.

RESULTS

Seventy-four patients were enrolled. Their demographics in association to 28-day outcome are shown in Table 1 and in supplementary Table 1 http://links.lww.com/SHK/B52. Two patients died before day; five patients were discharged before day 7 and four patients denied blood sampling on day 7; therefore measurements on day 7 were run in 63 patients.

Serum H2S of days 1 and 7 was significantly higher among 28-day survivors. IL-6 and PCT of days 1 and 7 were higher among non-survivors whereas CRP of day 7 was higher among non-survivors. H2S was negatively associated with IL-6, PCTand CRP (Figure 1).
Non-survivors had higher absolute neutrophil counts and significantly lower lymphocytes which are consistent with already reported characteristics of COVID-19 patients (12). Serum H$_2$S was negatively correlated with the absolute neutrophil count; a positive correlation with the absolute lymphocyte count was found (Figure 2).

The above-mentioned results led to further evaluation of admission H$_2$S as a marker of survival. Following ROC curve analysis, it was found that serum levels of H$_2$S on day 1 lower than 150.44 μM had the best trade-off for sensitivity and specificity for death (Figure 3A and B). In total, 49 patients had less or equal and 25 patients had more than 150.44 μM H$_2$S in serum on day 1. Mortality after 28 days was 32% and 4.1% respectively (Figure 3C). The OR for death was 11.11 (95% CI: 2.13-5.88, p: 0.001).

ROC curve analysis revealed the following baseline values to be associated with unfavourable outcome: CCI greater or equal to 3, APACHE II score greater or equal to 10, PSI greater or equal to 113 and SOFA score greater or equal to 4. Forward stepwise Cox regression analysis showed that serum H$_2$S on day 1 above 150.44 μM is an independent protective factor for unfavourable outcome of COVID-19 even in the presence of severity scores (Table 1).

We further investigated the association between over-time change of serum H$_2$S and outcome. ROC curve analysis showed a cutoff decrease of 36% of H$_2$S by day 7 as the best discriminator for death (Figures 3D and E). Survival of these patients was prolonged (Figure 3F). Moreover, the change of serum H$_2$S between day 1 and 7 was negatively associated with the duration of hospitalization (Figure 3G).

**DISCUSSION**

This study suggests the gasotransmitter H$_2$S as a potentially predictive variable of the outcome of pneumonia by SARS-CoV-2 mainly due to the high negative predictive value.

Although one interpretation of the presented findings is for the use of serum H$_2$S as biomarker, we do feel that the intrinsic value is for H$_2$S as a reflection of the endothelial function of the body vasculature. The kinetics in the circulation reveal that 28-day survivors are those who consume less of this gas. Disturbed bioavailability of H$_2$S has been suggested as an indicator of enhanced pro-inflammatory responses and of endothelial dysfunction (13, 14). Both these conditions often accompany severe COVID-19.

The negative association between serum H$_2$S and IL-6 is interesting. IL-6 has been proposed as the principle pro-inflammatory cytokine involved in the cytokine storm that leads to severe lung injury, respiratory failure and death by COVID-19 (15). This has even
led to the evaluation of therapeutic strategies of modulation of IL-6 production. Tocilizumab, a blocker of IL-6R, which can effectively block IL-6 signal transduction pathway, is currently being evaluated in several clinical studies in COVID-19 patients worldwide (16). Our results postulate that H₂S is an endogenous down-regulator of IL-6, the consumption of which is a driver to unfavorable outcome. It may also lead to considerations for exogenous H₂S supplementation as treatment strategy. Indeed, inhaled H₂S has been shown to reduce pro-inflammatory cytokines, among which IL-6, and increase the survival of mice after experimental endotoxemia (17).

Additionally, serum H₂S was positively correlated with the lymphocyte count. Lymphopenia is a key characteristic of COVID-19 patients (12) and is considered a predictor of mortality (15). The negative association between endogenous H₂S and the lymphocyte count may be consistent with in vitro data supporting a role of H₂S as T cell activator (18).

The significant role of H₂S for evaluating COVID-19 patients further relies on serial measurements. This study has shown that all patients who sustain elevated levels after 7 days had no risk of an unfavourable outcome. This finding could suggest that serial measurements of serum H₂S could be utilized as an adjunctive criterion, together with other established biomarkers such as CRP and PCT, for decision making. However, these are data coming from a small cohort and mandate validation in larger cohorts of patients.

**Acknowledgements**
The study has received part funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement grant European Sepsis Academy (agreement No 676129) and in part funding by the Hellenic Institute for the Study of Sepsis.
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FIGURE LEGENDS

A.

B.

C.

D.

E.

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K.

L.

M.

N.

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Figure 1
Hydrogen sulfide (H$_2$S) as predictor for outcome of pneumonia by the SARS-CoV-2 coronavirus

Serum levels of H$_2$S in serum of patients on day 1 (A) and 7 (B) after hospital admission. Comparison by the Mann Whitney U test; ** p < 0.01; **** p < 0.0001.

Levels of interleukin (IL)-6 (C, E), tumor necrosis factor alpha (TNFα) (G, I), procalcitonin (PCT) (K, M) and C-reactive protein (CRP) (O, Q) in serum of patients on day 1 and 7 after hospital admission. Comparison by the Mann Whitney U test; ** p < 0.01; *** p < 0.001; **** p < 0.0001; ns: non-significant

Correlation of IL-6 (D, F), TNFα (H, J), PCT (L, N) and CRP (P, R) with H$_2$S on day 1 and 7 after hospital admission. Spearman rank correlation coefficients (r$_s$), interpolation lines and p-values are provided.
Figure 2 Association of hydrogen sulfide (H₂S) on day 1 and 7 and white blood cell counts

Absolute neutrophil and lymphocytes counts on days 1 and 7 between survivors and non-survivors are shown on panels A, C, E and G; comparison by the Mann Whitney U test are shown; * p< 0.05; **** p< 0.0001; ns: non-significant

Correlations between absolute neutrophil and lymphocytes counts on days 1 and 7 and serum with H₂S are shown on panels B, D, F and H. Spearman rank correlation coefficients (rₛ), interpolation lines and p-values are provided.
Figure 3 Hydrogen sulfide (H$_2$S) as prognostic biomarker for pneumonia by the SARS-CoV-2 coronavirus

A) ROC curve of serum H$_2$S on day 1 for 28-day survival of pneumonia by SARS-CoV-2, AUC area under the curve, 95% confidence intervals (CIs) and p value are given.

B) Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of serum H$_2$S on day 1 for 28-day mortality

C) Kaplan-Meier analysis of 28-day survival in association with serum H$_2$S admission levels; the log-rank test and p-value are given.

D) Fold-changes of H$_2$S between day 1 and 7 according to outcome; the dotted line refers to the 36% cut-off. Comparison by Mann Whitney U test; **** p< 0.0001.

E) ROC curve of serum H$_2$S fold change for 28-day outcome

F) Kaplan-Meier analysis for 28-day outcome in association with over-time change of serum H$_2$S; log-rank test and the p-value are given.

G) Correlation of fold-change of H$_2$S between days 1 and 7 after hospital admission with the duration of hospitalization; r$_s$, interpolation lines and p-values are provided.
Table 1. Baseline clinical and laboratory characteristics of patients with pneumonia by SARS-CoV-2 coronavirus and step- wise forward Cox regression analysis of parameters associated with unfavorable outcome

|                                | Univariate analysis |                      |                      | Multivariate analysis |
|--------------------------------|---------------------|----------------------|----------------------|-----------------------|
|                                | Survivors (n=64)    | Non-survivors (n=10) | p-value              | HR                    |
| Age ≥ 64 years (n, %) #         | 27 (42.2)           | 8 (80)               | 0.005                | 19.00                 |
|                                |                     |                      |                      | 0.92 – 391.00         | 0.056                |
| Male gender (n, %)              | 45 (70.3)           | 9 (90.0)             | 0.269                |                       |
| CCI ≥ 3 (n, %) #                | 14 (21.9)           | 7 (70.0)             | 0.004                | 2.08                  |
|                                |                     |                      |                      | 0.17 – 25.26          | 0.566                |
| APACHE II score ≥ 10 (n, %) #   | 11 (17.2)           | 5 (50.0)             | 0.033                | 16.35                 |
|                                |                     |                      |                      | 0.44 – 612.87         | 0.131                |
| PSI ≥ 113 (n, %) #              | 13 (20.3)           | 7 (70.0)             | 0.0002               | 4.88                  |
|                                |                     |                      |                      | 0.27 – 88.17          | 0.283                |
| SOFA ≥ 4 (n, %) #               | 7 (10.9)            | 2 (20.0)             | 0.005                | 1.76                  |
|                                |                     |                      |                      | 0.15 – 20.65          | 0.652                |
| serum H_2S on day 1 ≥ 150.44 μM# | 47 (73.4)           | 2 (20.0)             | 0.011                | 0.01                  |
|                                |                     |                      |                      | 0.01 - 0.519          | 0.022                |

Main comorbidities (n, %)

|                                | Survivors (n=64)    | Non-survivors (n=10) | p-value              | HR                    |
|                                |                     |                      |                      | 95% CIs               |
| Type 2 diabetes mellitus       | 4 (6.3)             | 2 (20.0)             | 0.184                |                       |
| Chronic heart failure          | 1 (1.6)             | 0 (0)                | 0.865                |                       |
| Coronary heart disease         | 17 (26.6)           | 3 (30.0)             | 0.543                |                       |
| Chronic renal disease          | 5 (7.8)             | 3 (30.0)             | 0.070                |                       |
| COPD                           | 3 (4.7)             | 0 (0)                | 0.642                |                       |
| SRF                            | 16 (25)             | 9 (90)               | 0.0001               | 41.95                 |
|                                |                     |                      |                      | 1.49 – 1183.15        | 0.028                |

Abbreviations: HR: Hazard ratio; CI: Confidence intervals; SD: standard deviation; H_2S: hydrogen sulfide; APACHE: acute physiology and chronic health evaluation; CCI: Charlson’s comorbidity index; COPD: chronic obstructive pulmonary disease; PSI: pneumonia severity index; SOFA: sequential organ failure; SRF: severe respiratory failure

The p-values of comparisons by the Fischer exact test are provided

#Cut- off point of each variable was determined based on the coordinate point with the maximum value of the Youden index