Host cystathionine-γ lyase derived hydrogen sulfide protects against *Pseudomonas aeruginosa* sepsis

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Abstract

Hydrogen sulfide (H\(_2\)S) has recently been recognized as a novel gaseous transmitter with several anti-inflammatory properties. The role of host-derived H\(_2\)S in infections by *Pseudomonas aeruginosa* was investigated in clinical and mouse models. H\(_2\)S concentrations and survival was assessed in septic patients with lung infection. Animal experiments using a model of severe systemic multidrug-resistant *P. aeruginosa* infection were performed using mice with a constitutive knock-out of cystathionine-γ lyase (Cse) gene (Cse\(^{-/-}\)) and wild-type mice with a physiological expression (Cse\(^{+/+}\)). Experiments were repeated in mice after a) treatment with cyclophosphamide; b) bone marrow transplantation (BMT) from a Cse\(^{+/+}\) donor; c) treatment with H\(_2\)S synthesis inhibitor aminoxyacetic acid (AOAA) or propargylglycine (PAG) and d) H\(_2\)S donor sodium thiosulfate (STS) or GYY3147. Bacterial loads and myeloperoxidase activity were measured in tissue samples. The expression of quorum sensing genes (QS) was determined in vivo and in vitro. Cytokine concentration was measured in serum and incubated splenocytes. Patients survivors at day 28 had significantly higher serum H\(_2\)S compared to non-survivors. A cut-off point of 5.3 μM discriminated survivors with sensitivity 92.3%. Mortality after 28 days was 30.9% and 93.7% in patients with H\(_2\)S higher and less than 5.3 μM (\(p = 7 \times 10^{-6}\)). In mice expression of Cse and application of STS afforded protection against infection with multidrug-resistant *P. aeruginosa*. Cyclophosphamide pretreatment eliminated the survival benefit of Cse\(^{-/-}\) mice, whereas BMT increased the survival of Cse\(^{+/+}\) mice. Cse\(^{-/-}\) mice had increased pathogen loads compared...
Hydrogen sulfide and Pseudomonas aeruginosa infections

Author summary

Multidrug- resistant *Pseudomonas (P.) aeruginosa* is one of the most common causes of ventilator-associated pneumonia (VAP), which consist one of the most common causes of sepsis in hospitalized patients. The increasing antimicrobial resistance of *P. aeruginosa* necessitates the investigation of new treatment strategies. In this study we highlight host derived hydrogen sulfide (H$_2$S), produced by cystathionine-γ-lyase (CSE), as a new protective mechanism against *Pseudomonas aeruginosa* infection. We have shown that serum H$_2$S levels above 5.3μM can discriminate survivors of *P.aeruginosa* related sepsis with high sensitivity and is correlated with reduced mortality. We verified this finding in an animal model of CSE knockout mice. The protective role of endogenous H$_2$S resides in the enhancement of neutrophil recruitment and the phagocytic activity of neutrophils, as well as by inhibiting the cell-to-cell communication system of *P. aeruginosa* (quorum sensing system), rendering it more vulnerable to phagocytosis.

Introduction

Ventilator-associated pneumonia (VAP) is a common and life-threatening complication of mechanical ventilation and one of the most common causes of sepsis in hospitalized patients. *Klebsiella (K.) pneumoniae, Acinetobacter (A.) baumannii* and *Pseudomonas (P.) aeruginosa* are responsible for more than 50% of VAP cases in Greece and are characterized by high rates of antimicrobial resistance [1]. This mandates the investigation of new strategies, which can tackle the problem of antimicrobial resistance and lead to resolution of VAP.

Hydrogen sulfide (H$_2$S) is the third gaseous transmitter -after nitric oxide and carbon monoxide- that is produced mainly through the action of three distinct enzymes cystathionine γ-lyase (CSE), cystathionine β- synthase (CBS) and 3- mercaptopyruvate sulfurtransferase (3MST) [2]. In mammals, Cse is constitutively expressed in many peripheral organs and is upregulated in response to cellular stress. CSE-derived H$_2$S promotes angiogenesis, drives vasorelaxation, reduces atherosclerosis and prevents ischemia-reperfusion injury [2,3]. Genetic ablation or pharmacological inhibition of CSE promotes leukocyte adherence to the vessel wall and increases cytokine production [4,5]. The pro-inflammatory effects observed after inhibition of CSE led us study the levels of host H$_2$S in cases of lower respiratory tract infections (LRTIs) including ventilator associated pneumonia (VAP) and pneumonia caused by the novel SARS-CoV-2 virus (COVID-19 disease). Results showed that plasma H$_2$S levels are higher among survivors of LRTI by COVID-19; non-survivors were characterized by disability to increase plasma H$_2$S over the first seven days of follow-up [6].

In this study we focused on the selective role of host H$_2$S in the pathogenesis of infections by *P. aeruginosa*. Using a two-step approach, we demonstrate that host-H$_2$S is associated with the outcome of VAP caused by *P. aeruginosa* but not by other pathogens. We then investigated the underlying mechanism of action of host derived H$_2$S in an animal model of infection by MDR *Pseudomonas aeruginosa*.

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**Materials and methods**

**Ethics statement**

The Hellenic Sepsis Study Group (HSSG) is collecting clinical data and biosamples from patients with sepsis since 2006 from 65 study sites across Greece (intensive care units, emergency departments and departments of internal medicine or surgery; www.sepsis.gr). The patients are enrolled after written consent provided by themselves or by their first-degree relatives (for patients unable to consent). The protocol is approved by the Ethics Committees of the participating hospitals.

Animal experiments were conducted in the unit of animals for medical scientific purposes of ATTIKON University General Hospital (Athens, Greece) according to EU Directive 2010/63/EU and to the Greek law 2015/2001, which incorporates the Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes of the Council of Europe (code of the facility EL 25BIO014, approval no. 1853/2015). All experiments were licensed from the Greek veterinary directorate under the protocol number 5591/19-10-2017.

**Clinical study**

Adults with clinically or microbiologically documented infections and at least two signs of the systemic inflammatory response syndrome (SIRS) are enrolled. Since March 2016 all patients in the database were re-classified into non-sepsis, sepsis and septic shock using the Sepsis-3 classification criteria [7]. Exclusion criteria were: a) HIV-1 infection; b) neutropenia defined as less than 1,000 neutrophils/mm$^3$; and c) chronic use of corticosteroids defined as more than 1 mg/kg/day of prednisone equivalent for more than 15 days. Infections and organ dysfunction are defined according to already-published international criteria [8]. Blood sampling is done within the first 24 hours from enrollment.

The following variables are recorded: i) demographics; ii) Acute Physiology and Chronic Health Evaluation (APACHE) II score, Charlson Comorbidity Index (CCI) and Sequential Organ Failure Assessment (SOFA) score; (iii) biochemistry, absolute blood cell counts and arterial gases; iv) appropriateness of the administered antimicrobial treatment defined as the susceptibility of the isolated pathogen to at least one of the empirically prescribed antimicrobials according to the antibiotic susceptibility testing; (v) quantitative cultures of blood, urine and tracheobronchial secretions (TBS) performed on baseline day 1.

Among patients entered in the database, patients with microbiologically confirmed ventilator associated pneumonia (VAP) due to monomicrobial infection by *P. aeruginosa* (group A); *K. pneumoniae* (group B) and *A. baumannii* (group C), were selected. Pathogens grew at $\geq 10^5$ cfu/ml in tracheobronchial secretions. VAP was defined according to already-published international criteria [8]. Survival was recorded for 28 days. 10 ml of blood was collected after peripheral venipuncture within the first 24 h of enrollment. Serum was prepared by centrifugation at 900 g, and samples were transported within the same day to the central lab and stored at – 80˚C until processing. Blood samples were also isolated from healthy volunteers, who were matched with patients with VAP for age and gender.

**H$_2$S measurements**

H$_2$S was measured in the serum samples of healthy volunteers and patients by using monobromobimane (MBB) derivation followed by high performance liquid chromatography as previously described [6,9,10]. MBB, monosodium phosphate (Na$_2$HPO$_4$), disodium phosphate (Na$_3$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). HPLC grade acetonitrile (ACN) and water were purchased from Scharlab (Sentmenat, Barcelona, Spain). Phosphate buffer pH 8.5 20 mM was prepared by dissolving NaH$_2$PO$_4$ and Na$_2$HPO$_4$ in distilled water. 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 (10mins and 30secs respectively). The solutions for the sulfide standard curve were prepared by dissolving Na$_2$S in phosphate buffer, to final concentrations of 4–250 μM and the quantification limit was 1 μM. The MBB 10 mM derivatization solution was prepared by dissolving MBB in ACN, 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 60 sec. The derivatization reaction was stopped by adding 50 μl of 200 mM SSA, followed by vortexing for 10 secs. The vials were then left on ice for 10min and centrifuged at 12000 rpm, 4°C for another 10 mins. Finally, 100 μl of the supernatant were transferred to darkened HPLC vials and kept at 4°C. The chromatographic experiments were conducted on an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany). The separation was performed on a 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, both obtained from Merck (Darmstadt, Germany). Analysis was performed at 25°C, using gradient elution (Table B in S1 Text). The two mobile phases consisted of ACN (A, 0.1% TFA, v/v) and water (B, 0.1% TFA), v/v), at a 0.6 ml/min flow rate. The sample injection volume was 20 μl. All solvents were filtered through a 0.45 μm membrane filter (Agilent). 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.

Animal studies

Pathogens. Three different P. aeruginosa isolates from tracheobronchial secretions of patients with sepsis due to VAP were used; a) the P. aeruginosa 6–11–19 isolate with minimal inhibitory concentration (MIC) of amikacin, ceftazidime, ciprofloxacin and meropenem >256, >512, >16 and 8 μg/ml respectively; b) the P. aeruginosa 19–2–45 isolate 2 with MIC of amikacin, ceftazidime, ciprofloxacin and meropenem >256, >512, >128 and >256 μg/ml respectively; and c) the P. aeruginosa 20–1–30 isolate with MIC of amikacin, ceftazidime, ciprofloxacin and meropenem >256, >512, >64 and >64 μg/ml respectively. Isolates were genetically distinct as defined by pulsed-field gel electrophoresis (PFGE) of their DNA to exclude similar isolates coming from horizontal spread (S3A Fig). Additionally, a K. pneumoniae 87B producing carbapenemase KPC-2 isolate with MIC of amikacin, meropenem, aztreonam and colistin >512, >512, 128 and 32 μg/ml respectively. The MIC was measured with the microdilution method and the production of KPC-2 by polymerase chain reaction.

Animals. We used 406 male and female mice (7–8 weeks old). Mice were allowed to acclimate for seven days before beginning the experiments. They were housed in individually ventilated cages, up to 5 mice per cage on 12-h dark/ light cycle and allowed free access to standard dry rodent diet and water, supplemented with 1g/l L- cysteine (AppliChem, Darmstadt, Germany) [11]. Analgesia was achieved with paracetamol suppositories, in order to avoid interactions with the immune system.
H₂S synthesis is catalyzed by three different enzymatic systems, cystathionine γ-lyase (CSE), which is the key enzyme for H₂S synthesis in the liver and kidney, cystathionine β-synthase (CBS), which is overexpressed in brain tissue and 3-mercaptopropionate sulfurtransferase (3MST) which is ubiquitously expressed [12]. We investigated the role only of CSE and 3MST, due to the fact that homozygotic CBS deficiency is related to a neonatal mortality of over 90% [13,14]. Cse⁺/⁻ mice were created after breeding of Cse⁺/⁺ mice (Friedrich Shiller University Hospital, Jena, Germany). The Cse⁺/⁻ mice were generated as previously described [13]. In short chimeric male Cse⁺/⁻ mice were produced after injection of Cth⁺/⁺ stem cells into C57BL/6 blastocysts. These were then crossed with C57BL/6 female mice to obtain Cse⁺/+ pups. Cse⁺/+ pups were backcrossed multiple times to achieve high genetic homogeneity on C57BL/6 background. The Cse⁺/+ males and females produced were finally bred to obtain wild-type (Cse⁺/+), Cse⁻/⁻, and Cse⁺/- littersmates. 3Mst⁺/+ and 3Mst⁻/⁻ mice were created, as described previously [15].

**Genotyping.** 4 weeks after birth genomic DNA for genotyping mice was prepared from the tail tips by the rapid, hot sodium hydroxide and Tris (HotSHOT) [16]. A small tail punch (<2mm) was prepared using a sharp sterile surgical scissor and the tail biopsy was placed immediately into sterile polypropylene microfuge tube and stored at -80°C until DNA isolation and purification. The CSE null and wild-type alleles were detected by a three-primer PCR mild modified protocol [17] of tail-tip DNA: N1 (5’-TGC GAG GCC AGA GGC CAG TTG TGT AGC-3’), F1 (5’- TGT TCA TGG TAG GTT TGG CC-3’) and R1 (5’-TCA GAA CTC GCA GGG TAG AA-3’). These primers amplify a ~500 bp wild type band (from primers F1 and R1) and a ~450 bp targeted (null) band (from primers N1 and R1). All primers were purchased from IDT (IDT, San Jose, California, USA). The samples were spun down and 2 μl of the supernatant was used as a template in a 25-μl reaction using 12 μl BioMix (Bioline GmbH, Luckenwalde, Germany). 3.33 pmol/ml of each primer and 1.5–2.0 mM MgCl₂. qRT-PCR was performed by the iQ5 cycler system (BioRad, Hercules, CA, USA). The thermal cycling conditions consisted of a 7 min initial denaturation cycle at 95°C; followed by 30 cycles of denaturation at 60°C for 75 s; annealing at 72°C for 2 min; extension at 95°C for 1 min. Finally, the reaction ended with 1 cycle at 60°C for 75 s and with a final extension at 72°C for 10 min. After this, samples were kept at 4°C. Reactions containing Molecular Grade Water (AppliChem, GmbH, Germany) were used as negative controls to evaluate for potential contamination. Specificity was confirmed by electrophoretic analysis of the reaction products by 2% w/v agarose electrophoresis gel in 0.5 x Tris–Borate EDTA (TBE) buffer (5V/cm) and 1 μg/mL ethidium bromide staining (AppliChem).

**Animal studies.** Cse⁺/+ and Cse⁻/⁻ mice were infected by intraperitoneally injecting 1x10⁸ cfu/mouse of MDR P. aeruginosa isolate 6–11–19, concentration in which P. aeruginosa is characterized by a lethality of up to 60% in infection models in rodents [18]. Mice were then randomized via a typical randomization table in treatment groups receiving 200 μl subcutaneously of: a) 120 mg/kg of the H₂S biosynthesis inhibitor aminooxyacetic acid AOAA (Sigma-Aldrich), which inhibits both CBS and CSE catalytic activity [19] diluted in 0.9% NaCl once per day for 4 days or 0.9% NaCl once daily for 4 days; b) 200 μmol/kg/mouse PAG (Sigma-Aldrich), which is a specific CSE inhibitor [20], diluted in 0.9% NaCl administrated intraperitoneally once per day for 4 days; c) 2 g/kg/mouse [21] sodium thiosulfate 10% (STS, Dr. Franz Köhler Chemie GmbH, Bensheim, Germany) diluted in WFI once per day for 4 days or WFI once per day for 4 days and d) 25mg/kg/mouse [22] GYY3147 (Sigma-Aldrich). Some experiments were repeated in CSE⁺/+ and CSE⁻/⁻ mice after induction of neutropenia through i.p. injection of 150mg/kg and 100mg/kg of cyclophosphamide (Baxter, Chicago, Illinois, USA) 4 and 1 days before the MDR P. aeruginosa injection. Additional survival experiments were done using 1 x 10⁸ cfu/mouse of the MDR P. aeruginosa isolates 19–2–45 and 20–1–30 and 1 x
$10^8$ cfu/mouse of the carbapenemase-producing *Klebsiella pneumoniae* isolate (Lee et al., 2015) as well as challenge with 10 or 30 mg/kg/mouse bacterial endotoxin (LPS) of *E. coli* O55:B5 (Sigma-Aldrich). Some experiments were repeated in 3Mst$^{+/+}$ and 3Mst$^{-/-}$ mice. On each day of experimentation, we infected up to 3 mice from each group. Survival was recorded every 12 hours for 7 days. Analgesia was achieved by subcutaneous administration of meloxicam 5 mg/kg.

Cse$^{+/+}$ and Cse$^{-/-}$ mice were sacrificed at certain timepoints after infection with MDR *P. aeruginosa* isolate 6–11–19 by s.c. injection of 300 mg/kg ketamine, followed by cervical dislocation. Under sterile conditions a midline abdominal incision was performed, intestines were displaced to the left and the lower vena cava was punctured by a 20-gauge needle. 500 μl of whole blood was aspirated, collected into sterile and pyrogen-free tubes (Vacutainer, Cockeysville, MD, USA) and centrifuged. Serum was stored at -80˚C. Then, segments of the liver and of the lower lobe of the right lung were excised and collected into sterile tubes with 1 ml NaCl 0.9%. The samples were weighted and homogenized. Additionally, segments of the spleen were stored in RPMI 1640 (Biochrom, Berlin, Germany) and of the lower lobe of the right lung in RNAlater (Qiagen, Hilden, Germany).

**Bone marrow transplantation (BMT).** Survival experiments were repeated in Cse$^{-/-}$ and Cse$^{+/+}$ mice after bone marrow transplantation [23]. Recipient Cse$^{-/-}$ and Cse$^{+/+}$ mice were placed in an acrylic container (22 cm diameter, 12 cm depth) and irradiated with a single dose of 9.5 Gy at a dose rate pf 300Gy/min (Energy 6MV photons) in a VitalBeam irradiator (Varian, CA, USA). One day after the irradiation donor BMT was done using bone marrow from Cse$^{+/+}$ mice. More precisely, mice were killed and both hind legs were removed under aseptic conditions Bone marrow cells (BMCs) were collected by flushing the femurs with PBS using one 21G needle. Bone marrow was gently homogenized using a pipette through a 40μm cell strainer. BMCs were counted with a Neubauer plate and suspended in PBS to $1\times10^7$ BMCs/ml. The irradiated recipient Cse$^{-/-}$ and Cse$^{+/+}$ mice were then injected $1\times10^6$ BMCs in 100 μl volume intravenously via a tail vein. Mice were allowed to recover for 1 month post-BMT. Reconstitution was confirmed by analysis of complete blood counts. Then, mice were infected by intraperitoneally injecting $1\times10^8$ cfu/mouse of MDR *P. aeruginosa* isolate 6–11–19. Survival was recorded for 7 days.

**Blood and serum analysis.** Complete blood count was performed with the ADVIA 2120i system (SIEMENS, Munich, Germany). Creatinine was measured via the Jaffe method, with lowest detection limit 0.03 mg/dl. Aspartate transaminase (AST) and alanine transaminase (ALT) were measured by the IFCC method with the ADVIA 1800 system (SIEMENS). Lowest detection limits were 0.8 U/l and 0.6 UI/l respectively.

**Cytokine stimulation and measurements.** Segments of mice spleens stored in RPMI 1640 were gently squeezed and passed through a sterile filter (250 mm, 12–13 cm, AlterChem Co, Athens, Greece) for the collection of splenocytes. After three serial washings, cells were counted on Neubauer plates with trypan blue for exclusion of dead cells. A total of $5\times10^6$ cells/ml were then incubated into sterile 24-well plates in RPMI-1640, supplemented with 2 mM glutamine, 10% fetal bovine serum, 100 U/ml of penicillin G and 0.1 mg/ml of streptomycin in the absence or presence of 10 ng/ml LPS of *E. coli* O55:B5 or $5\times10^5$ cfu/ml heat killed *Candida albicans*. After 24 hours or 5 days of incubation at 37˚C in 5% CO$_2$, the plates were centrifuged, and the supernatants were collected. Concentrations of TNFα was measured in duplicate in supernatants from stimulated splenocytes, in tissue supernatants and in serum via enzyme-linked immunosorbet assays (ThermoFisher Scientific, California, USA). The lower detection limit was 39 pg/ml. Additionally, IL-1α, IL-1β, IL-6, IL-10, IL-12, IFNγ and IFNβ in serum were determined by the LEGENDplex mouse TH 1/2 cytokine panel (13-plex) (Biolegend, California, USA) according to the manufacturer’s instructions.
Determination of myeloperoxidase (MPO) activity. Tissue segments were homogenized with T-PER (ThermoFisher Scientific, Massachusetts, USA) and centrifuged at 10,000 rpm at 4°C. Then the homogenates were incubated in wells of a 96-well plate at 37°C with 4.2 mM tetramethylbenzidine (Serva, Heidelberg, Germany), 2.5 mM citrate, 5 mM NaH₂PO₄ and 1.18 mM H₂O₂ pH 5.0 at a final volume of 150 μl. After 5 minutes the reaction was terminated by adding 50 μl 0.18M H₂SO₄. Absorbance was read at 450 nm against blank wells. Results were adjusted for tissue sample protein content on Bradford assay (Sigma-Aldrich) and they were expressed as MPO units/mg protein/g.

Expression quorum sensing bacterial genes. RNA was isolated from mice lung segments stored in RNAlater (Qiagen, Hilden, Germany), following the RNeasy mini kit protocol (QIA-GEN) according to the manufacturer’s recommendations. RNA concentration was measured spectrophotometrically using the absorbance ratio of 260/280 nm. Gel electrophoresis was used to check the purity and integrity of the total RNA. Additionally, the fragmented bacterial RNA was analyzed using the Agilent 2100 Bioanalyzer System (Agilent Technologies, Waldbronn, Germany) following the manufacturer’s protocol. 1 μg isolated RNA from *P. aeruginosa* was used as template, with the first strand iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) for the reverse transcription for cDNA synthesis; conditions were 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. Quantitative real-time PCR (qRT-PCR) analysis of the expression of quorum sensing (QS) genes *rhl*, *rhlR*, *las*, *lasR*, *pqs* and *pqsR* was carried out using the *P. aeruginosa* specific primers described [24] (Table C in S1 Text). All primers were purchased from IDT (IDT, San Jose, California, U.S.A). qRT-PCR was performed with the iQ5 cycling system (BioRad, Hercules, CA, USA) using 2 μl cDNA, 10 μl iTaq Universal SYBR Green Supermix, (BioRad), 6 μl Molecular Grade Water (AppliChem) and 0.1 pmol/ml sense and antisense primers to a final volume of 20-μl on duplicate samples in 96-well plate. The thermal cycler profile involved a preliminary denaturation at 95°C for 1 min; followed by 40 cycles of denaturation at 95°C for 30 s; annealing at 52°C for 30 s; and extension for at 72°C 1 min.; followed by cooling at 4°C. Amplification was followed by a melting curve from 55°C to 95°C increasing of 0.5 each time for verification of the amplified product. The threshold was adjusted according to the amplification curves of all evaluated genes. No reverse transcriptase controls were prepared from total RNA and no template controls were prepared with Molecular Grade Water (AppliChem) in place of total RNA to indicate potential genomic DNA contamination in isolated bacterial RNA and contamination of reagents, respectively. Specificity was confirmed by electrophoretic analysis in 3% w/v agarose electrophoresis gel (5V/cm) and ethidium bromide staining (AppliChem). Analysis of relative gene expression was achieved according to the ΔΔCT method [25]. The number of transcripts of QS genes in the lung was normalized to the respective number of bacteria in the lung at the time of sacrifice [26].

Additionally, each MDR *P. aeruginosa* isolate was incubated at a starting inoculum of 1 x 10⁷ cfu/ml in tubes with Mueller-Hinton broth with and without 1mM of the H₂S donor GYY4137 (Sigma-Aldrich) and with and without 1mM of the AOAA (Sigma-Aldrich). After 2 and 6 hours of incubation RNA was isolated from 1ml of each dilution, using the same methodology as described above. RNA was then used for measurement of expression of quorum sensing genes. An aliquot of 0.1ml of each dilution was diluted 1:10 into Mueller-Hinton broth (Becton Dickinson) six consecutive times; 0.1 ml of each dilution was plated onto MacConkey agar (Becton Dickinson). After incubation for 24 hours at 37°C, the number of viable colonies was counted. The results were expressed as log₁₀ of colony forming units per ml (cfu/ml). The number of transcripts of QS genes was normalized to the respective bacterial load. The experiment was performed in duplicate.

Determination of splenic leukocyte subpopulations by flow cytometry. Isolated spleen cells were incubated for 15 min in the dark with the monoclonal antibodies anti-CD45 PC5
(ThermoFisher Scientific), anti-CD11b PE (phycoerythrin, emission 575 nm, ThermoFisher Scientific), anti-CD11c FITC (fluorescein isothiocyanate, emission 525 nm, ThermoFisher Scientific). Cells were analyzed after running through the CYTOMICS FC500 flow cytometer (Beckman Coulter Co, Miami, Florida). Results were expressed as percentages.

**Bacterial outgrowth assays.** i) **in vitro clearance.** A log-phase culture ($1 \times 10^5$ cfu/mL) of MDR *P. aeruginosa* was opsonized with 5% mouse serum (Sigma-Aldrich) at room temperature for 15 min. $5 \times 10^6$ leukocytes/ml were isolated from mice spleens as described above, and were incubated at 37˚C in 5% CO$_2$ for 30 min into sterile 24-well plates in RPMI 1640 supplemented with 10% FBS with and without the H$_2$S donor 1mM GYY3147 (Sigma-Aldrich) [27]. Then, $1 \times 10^4$ cfu/mL bacteria were added in each well and the plates were incubated at 37˚C. ii) **in vivo.** One aliquot of 0.1 ml of the tissue homogenates was diluted 1:10 into Mueller-Hinton broth six consecutive times; 0.1 ml of each dilution was plated onto MacConkey agar (Becton Dickinson). After incubation for 24 hours at 37˚C, the number of viable colonies was counted. The results were expressed as log$_{10}$ of colony forming units per gram tissue (cfu/g).

**Quantification and statistical analysis**

Categorical data were presented as frequencies and quantitative variables as mean ± 95% confidence intervals (CIs) or median + interquartile range (IQR). Comparisons between groups were done using the Fisher exact test (categorical data), and the Mann-Whitney U test for two group comparisons, the Wilcoxon signed rank test for related samples comparison and one-way ANOVA with the Bonferroni correction for multiple group comparison (quantitative data). Correlations between variables were performed using the Spearman’s rank of order. Resolution of VAP in patients and survival in mice was compared between groups by the log-rank test. Odds ratios (OR) and 95% confidence intervals (CIs) for were calculated by the Mantel and Haenszel’s statistics. The receiver operating characteristic (ROC) curve was analyzed for the efficiency of serum hydrogen sulfide as a biomarker for the resolution of VAP. Other demographic variables associated with unfavorable outcome were transformed into dichotomous variables after ROC curve analysis. For each parameter the coordinate point with the maximum value of the Youden index was used as a cut-off. Step-wise Logistic regression analysis with odds ratios (ORs) and confidence intervals (CIs) was used to investigate if serum hydrogen sulfide is an independent variable for resolution of VAP. Any $p$ value below 0.05 was considered statistically significant.

**Results**

**Clinical study**

The clinical study flow chart is shown in S1 Fig. We used a dataset of 6814 patients with sepsis from Greek hospitals. The multi-step selection process involved the following filters: a) selection of patients with VAP-related sepsis; b) selection of patients with microbiological confirmation of VAP by a single pathogen (pathogen growth in TBS $\geq 10^5$ cfu/ml and exclusion of patients growing more than one microorganism); and c) selection of patients with monomicrobial infection by *P. aeruginosa*, *K. pneumoniae* or *A. baumannii* as defined by the isolation of these species in tracheobronchial secretions. Following this approach, we ended-up with 225 patients who developed sepsis due to microbiologically confirmed VAP by *P. aeruginosa* (71 patients; 31.5%), *K. pneumoniae* 60 patients, 26.7%) and *A. baumannii* (94 patients,41.8%), respectively. Demographics of groups were similar (Table A in S1 Text).

Bacterial VAP led to reduced serum H$_2$S levels compared to healthy volunteers (S2 Fig). When serum levels of H$_2$S on day 1 of enrollment were compared between 28-day survivors
and non-survivors, they were higher only among patients with infection by *P. aeruginosa* (p < 0.0001) (Fig 1A). Compared to healthy volunteers, serum H$_2$S levels of survivors of infection by *P. aeruginosa* did not differ significantly, whereas non-survivors had significantly lower H$_2$S levels. These findings suggest that defective host-derived H$_2$S responses may be specific for unfavorable outcome of *P. aeruginosa* infections and prompted further study on patients with *P. aeruginosa* infection.

Following ROC curve analysis, it was found that serum levels of H$_2$S on day 1 above 5.3μM had the best trade-off for sensitivity and specificity for survival from sepsis due to VAP by *P. aeruginosa* (Fig 1B and 1C). In total, 55 patients had higher and 16 patients had less than or equal to 5.3 μM H$_2$S in serum on day 1. After 28 days 38 (69.1%) patients with levels > 5.3 μM and 1 (6.3%) patient with levels ≤ 5.3 μM survived (Fig 1D). The odds ratio (OR) for survival from sepsis due to VAP by *P. aeruginosa* was 33.33 (95% Confidence Interval (CI): 4.10–250.00, p = 7 x 10$^{-6}$).

ROC curve analysis revealed the following baseline values to be associated with unfavourable outcome: age > 61 years, Acute Physiology and Chronic Health Evaluation (APACHE) II score > 23, Charlson Comorbidity Index (CCI) > 4, Sequential Organ Failure Assessment (SOFA) score on day 1 > 10 and bacteremia by *P. aeruginosa*. All above variables entered into logistic regression analysis (Table 1). Analysis showed that serum H$_2$S on day 1 above 5.3 μM was an independent protective factor for favorable outcome.

**Animal studies**

Following the results of the human study, we aimed to assess the potential protective role of host-derived H$_2$S and investigated the underlying mechanisms in an animal model of severe MDR *P. aeruginosa* infection. 7-day survival of Cse$^+/+$ mice was significantly lower (11.8%) as compared to Cse$^{+/+}$ mice (47.1%) (Fig 2A). This finding could be replicated after experimental infection with two additional MDR *P. aeruginosa* strains (S3B and S3C Fig). Application of STS and Gyr4137, two clinically used H$_2$S donors, restored the survival of Cse$^{+/+}$ mice to a level comparable to wild-type mice (43.3% and 46.7% respectively compared to 11.8% of untreated Cse$^{−/−}$ mice) (Fig 2B). This was further confirmed using a pharmacological approach. In Cse$^{+/+}$ mice infected by *P. aeruginosa*, the H$_2$S biosynthesis inhibitor AOAA significantly decreased survival (11.8% compared to 47.1% of untreated Cse$^{+/+}$ mice) (Fig 2C). The selective CSE inhibitor PAG also significantly decreased survival (11.8% compared to 47.1% of untreated Cse$^{+/+}$ mice) (Fig 2C). Treatment with STS in Cse$^{+/+}$ mice led to prolongation of survival (64.7% compared to 47.1%). MDR *P. aeruginosa* infection led more rapidly to the development of multorgan failure (MOF) in the Cse$^{−/−}$ mice compared with the Cse$^{+/+}$ mice, as shown by the greater increase of serum aminotransferases ALT and AST and serum creatinine (Fig 2D–2F).

We next investigated if the protective role of host-derived H$_2$S is enzyme- and pathogen-specific. First, we demonstrated that H$_2$S levels measured in serum and tissue samples from non-infected and infected by MDR *P. aeruginosa* mice with a physiological expression of Cse (Cse$^{+/+}$) were significantly higher compared to non-infected and infected mice with a constitutive knock-out of the gene encoding for Cse (Cse$^{−/−}$) respectively (Fig 3A–3C), showing that CSE deletion leads to deficient H$_2$S levels in vivo. Moreover, H$_2$S levels in serum increased after infection by MDR *P. aeruginosa*, only in Cse$^{+/+}$ mice. In order to study the importance of the enzymatic system of host H$_2$S production, we performed another set of experiments using mice with homozygous deficiency for 3Mst. 3Mst deletion does not lead to a significant alteration of serum H$_2$S levels in mice [28]. Indeed, survival from infection by MDR *P. aeruginosa* was similar between 3Mst$^{+/+}$ (42.9%) and 3Mst$^{−/−}$ (38.5%) mice (Fig 3D). These findings...
Fig 1. Circulating H$_2$S and outcome of patients with sepsis due to ventilator associated pneumonia (VAP) by P. aeruginosa. A) Serum levels of H$_2$S in healthy volunteers and on day 1 among survivors and non survivors of sepsis due to VAP by three major pathogens were measured by HPLC. Only statistically significant comparisons are provided (comparisons by the Mann Whitney U test); * p < 0.05, ** p < 0.001, *** p < 0.0001. B) ROC curve of H$_2$S to predict survival of sepsis due to VAP by P. aeruginosa, AUC area under the curve. C) Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of serum H$_2$S level >5.3 µM for survival of patients with sepsis due to VAP by P. aeruginosa. D) Kaplan- Meier analysis for survival after sepsis due to VAP by P. aeruginosa, among patients with serum H$_2$S levels > and ≤ 5.3 µM. Results of the log-rank test and the p- value are given.

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Hydrogen sulfide and Pseudomonas aeruginosa infections

demonstrate that the susceptibility of the host for infection by P. aeruginosa is selectively driven by H$_2$S produced through the catalytic activity of CSE.

Similar to prior findings [29], the difference in MDR P. aeruginosa virulence in Cse$^{-/-}$ and Cse$^{+/+}$ mice is not related to LPS, as there was no difference in survival of Cse$^{-/-}$ and Cse$^{+/+}$ mice challenged either with 10 mg/kg/mouse LPS or 30 mg/kg/mouse LPS (Fig 3E and 3F). The protective role of H$_2$S could not be reproduced after KPC- K. pneumoniae infection (47.6% survival of both Cse$^{-/-}$ and Cse$^{+/+}$ mice) (Fig 3G), indicating a pathogen-specific effect.

We then sought to investigate the mechanisms that lead to death in the case of host H$_2$S deficiency. Cytokine analysis did not show significant differences between Cse$^{-/-}$ and Cse$^{+/+}$ mice in TNFα levels in serum and tissue supernatants and in stimulated splenocytes (S4 Fig). Interleukin (IL)-1α, IL-1β, IL-6, IL-10, IL-12, IL-27, Interferon (IFN)γ and IFNβ levels of serum were also similar between groups (S5 Fig). This suggests that the protective role is not directly associated with a change in the concentration in the blood of circulating cytokines.

We next investigated if the protective effect of H$_2$S stems from favorable phagocytosis. To investigate this, we followed a multistep approach. At first, we verified that absolute counts of

Table 1. Baseline and clinical characteristics of patients with sepsis due to P. aeruginosa associated VAP, univariate and step- wise forward logistic regression analysis of parameters associated with unfavorable outcome of sepsis due to VAP by P. aeruginosa.

| Survivors (n = 39) | Non Survivors (n = 32) | Univariate analysis | Step- wise logistic regression analysis |
|-------------------|------------------------|---------------------|---------------------------------------|
| Male gender (n, %) | 29 (74.4) 18 (56.3)   | 0.44 (0.16–1.21) 0.134 |
| Age > 61 (n, %)   | 20 (51.3) 24 (75)     | 2.85 (1.03–7.88) 0.035 |
| APACHE II 1 > 23 (n, %) * | 14 (43.8) | 6.81 (1.95–23.71) 0.002 |
| CCI > 4 (n, %) * | 8 (20.5) 17 (53.1) | 4.39 (1.53–12.45) 0.006 |
| SOFA day 1 > 10 (n, %) * | 18 (56.3) | 8.74 (2.71–28.17) 0.0001 |
| H$_2$S in serum on day 1 > 5.3 µM * | 17 (53.1) | 0.03 (0.01–0.24) 7x10⁻⁶ |
| Septic shock (n, %) | 22 (56.4) 25 (78.1) | 2.76 (0.92–7.89) 0.078 |
| Pathogen isolation in a blood sample (n%) | 6 (15.4) 12 (37.5) | 3.5 (1.07–10.18) 0.032 |
| Appropriateness of antimicrobial therapy (n%) | 25 (64.1) 21 (65.6) | 1.07 (0.40–2.85) 0.547 |
| Intake of corticosteroids (n%) | 4 (10.3) 7 (21.9) | 2.45 (0.65–9.28) 0.204 |
| **Main comorbidities (n%)** | | | |
| Diabetes mellitus Typ 2 | 4 (10.3) 2 (6.3) | 0.58 (0.10–3.41) 0.683 |
| Chronic heart failure | 2 (5.1) 5 (15.6) | 3.43 (0.62–19.00) 0.231 |
| Coronary heart disease | 8 (20.5) 2 (6.3) | 0.26 (0.51–1.32) 0.102 |
| COPD | 3 (7.7) 3 (9.4) | 1.24 (0.23–6.62) 0.564 |
| Chronic renal failure | 1 (2.6) 0 (0) | 0.97 (0.93–1.03) 0.549 |
| Solid tumor | 1 (2.6) 1 (3.1) | 1.23 (0.74–20.40) 0.702 |

**Abbreviations VAP: Ventilator associated pneumonia; APACHE: Acute physiology and chronic health evaluation; CCI: Charlson’s Comorbidity Index; SOFA: Sequential organ failure assessment; H$_2$S: hydrogen sulfide; OR: Odds ratio; CI: Confidence intervals; COPD: Chronic obstructive pulmonary disorder

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

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all blood cell lines, including neutrophils and lymphocytes, did not differ between healthy \( \text{Cse}^{+/+} \) and \( \text{Cse}^{-/-} \) mice (S6 Fig). Then, we studied mice rendered neutropenic by cyclophosphamide and found that the survival offered by \( \text{Cse}^{+/+} \) was lost: neutropenic \( \text{Cse}^{-/-} \) mice and neutrophilic \( \text{Cse}^{+/+} \) mice exhibited similarly high mortality rates (Fig 4A). In a different set of experiments, survival was assessed in \( \text{Cse}^{+/+} \) and \( \text{Cse}^{-/-} \) mice after BMT from a \( \text{Cse}^{-/-} \) donor. Although BMT did not significantly alter serum hydrogen levels of \( \text{Cse}^{-/-} \) and \( \text{Cse}^{+/+} \) mice (S7 Fig), transplanted \( \text{Cse}^{-/-} \) mice had prolonged survival compared to naïve \( \text{Cse}^{-/-} \) mice, which was similar to the survival of naïve \( \text{Cse}^{+/+} \) mice and \( \text{Cse}^{-/-} \) mice after BMT (Fig 4B). Taken together, these findings suggest that the survival benefit of \( \text{Cse}^{-/-} \) over \( \text{Cse}^{+/+} \) mice is not owed to a baseline excess of immune cells, but potentially to an intrinsic dysfunction of \( \text{Cse}^{+/+} \) neutrophils, which is reverted after BMT. Additionally, inflammatory mediators produced by neutrophils, namely IL-23, MCP-1 and GM-CSF were lower in \( \text{Cse}^{-/-} \) mice compared to \( \text{Cse}^{+/+} \) mice (Fig 4C). Moreover, the expression of these mediators increased after infection in \( \text{Cse}^{-/-} \) mice. In addition, the bacterial load of all three MDR \( \text{P. aeruginosa} \) isolates 6 h after infection was greater in the liver, in the lung and in the spleen of \( \text{Cse}^{-/-} \) mice (Figs 4D, S2D and S2E). Myeloperoxidase (MPO) activity, which is a marker for the presence of neutrophils in tissue homogenates, was lower in the liver and in the lung of \( \text{Cse}^{-/-} \) mice compared with \( \text{Cse}^{+/+} \) mice 6h after \( \text{P. aeruginosa} \) infection (Fig 4E). The ratio of CD11b+CD11c+CD45 of the spleen, which mostly represent the ratio of the granulocyte to the macrophage population [30], was significantly greater in \( \text{Cse}^{+/+} \) mice compared to \( \text{Cse}^{-/-} \) mice 6h after \( \text{P. aeruginosa} \) infection (Fig 4F). This was accompanied by a reduced bacterial load in the spleen of \( \text{Cse}^{+/+} \) mice compared to \( \text{Cse}^{-/-} \) mice (Fig 4C). A negative correlation between neutrophil to macrophages ratio and bacterial load could be shown, suggesting that greater counts of neutrophils are associated with an effective clearance of MDR \( \text{P. aeruginosa} \) (Fig 4G). We then sought to investigate the influence of \( \text{H}_{2}\text{~S} \) on the in vitro phagocytic activity of leukocytes. Indeed, we found that phagocytic activity of leukocytes was lower in \( \text{Cse}^{-/-} \) mice than in \( \text{Cse}^{+/+} \) mice. Phagocytic activity of leukocytes from \( \text{Cse}^{-/-} \) mice was normalized after preincubation with the \( \text{H}_{2}\text{~S} \) donor GYY4137. GYY4137 enhanced also the phagocytic ability of \( \text{Cse}^{-/-} \) derived leukocytes after 2 hours but not after 4 hours from bacterial challenge (Fig 4H and 4I). All the above findings suggest a deficient mechanism of neutrophil recruitment and neutrophil mediated phagocytosis in \( \text{Cse}^{-/-} \) mice. This is further proved by the positive correlation between bacterial load and tissue MPO activity in tissues of \( \text{Cse}^{+/+} \) mice, which was not seen in \( \text{Cse}^{-/-} \) mice (S8 Fig).

The fact that endogenous \( \text{H}_{2}\text{~S} \), produced from the CSE enzyme, exerts a protective mechanism against MDR \( \text{P. aeruginosa} \) infection but not against infection elicited by other gram-negative bacteria, such as \( \text{K. pneumoniae} \), indicates that host-derived \( \text{H}_{2}\text{~S} \) interacts with a unique system that is crucial for the pathogenesis of infections by \( \text{P. aeruginosa} \). Quorum sensing (QS) is such a unique system, which controls the virulence of \( \text{P. aeruginosa} \) [24,31]. Modulation of \( \text{H}_{2}\text{~S} \) levels, by the addition of an \( \text{H}_{2}\text{~S} \) donor or an \( \text{H}_{2}\text{~S} \) biosynthesis inhibitor, did not affect in vitro bacterial growth (Fig 5A). Then, we studied the expression of QS genes of the three selected isolates under different concentrations of \( \text{H}_{2}\text{~S} \). Increased \( \text{H}_{2}\text{~S} \) levels, through the addition of an \( \text{H}_{2}\text{~S} \) donor, led to a reduction of expression of core QS genes over time.
compared to normal or reduced H$_2$S levels (Fig 5B–5F). Then, we investigated the effect of tissue H$_2$S levels on QS expression. Indeed, the expression of QS genes rhlI, lasR and pqsA in the lung was decreased over time in Cse$^{+/+}$ but not in Cse$^{-/-}$ mice (Fig 6A–6F), and after 24 hours expression of QS genes rhlI, las and lasR in the lung was significantly greater in Cse$^{-/-}$ mice compared to Cse$^{+/+}$ mice. These findings suggest a H$_2$S mediated modulation of QS activation. Moreover, we found a negative correlation between the expression of QS gene rhlI, rhlR, las, lasR and pqsA in the lung and the H$_2$S levels in the lung of Cse$^{+/+}$ mice. This was not seen in Cse$^{-/-}$ mice (S9 Fig). These results imply a central role of H$_2$S produced specifically through CSE in the modulation of the QS system, which regulates the tissue outgrowth of MDR P. aeruginosa.

**Discussion**

Our study adds novel clinical data about the importance of host-derived H$_2$S for the pathogenesis of infection by *P. aeruginosa*. Our conclusions are supported both by clinical observations in humans and by mechanistic data in mice. Non-survivors from severe infection by *P. aeruginosa* fail to produce as high levels of H$_2$S as survivors. This finding is fully corroborated in animal findings where the presence of H$_2$S protects against *P. aeruginosa* induced mortality through a dual mechanism of action: it primes phagocytosis by host neutrophils; and it is a negative regulator of QS that is crucial in the pathogenesis of *P. aeruginosa* infection in the lung.

Hydrogen sulfide production through the CSE enzyme has been observed both in pro- and eucaryotic organisms. This evolutionary conservation of CSE derived H$_2$S, together with findings from several studies, which have demonstrated H$_2$S as a mediator of several human physiological functions, longevity and stress resistance, have shifted the point of view of the research community away from considering H$_2$S as a toxic gas and recognizing its place as a third gaseous transmitter [3,5,32].

Neutrophils, by intracellular killing following phagocytosis, are considered to be the major mediators of the innate immune response in the case of an acute *P. aeruginosa* infection [33,34]. But also, in the case of chronic infection, such as in cystic fibrosis (CF), neutrophilic activity is a key mediator for clearance of *P. aeruginosa*, mainly by the formation of neutrophil extracellular traps (NETs) [35,36]. The key role of neutrophils for the survival advantage of mice with a physiological expression of Cse$^{+/+}$ over Cse$^{-/-}$ mice, is supported by the fact that this advantage is eliminated when neutrophils are depleted. Additionally, hematopoietic reconstitution of the bone marrow of Cse$^{-/-}$ mice through transplantation of bone marrow cells from Cse$^{+/+}$ mice led to a significant increase of survival after *P. aeruginosa* infection. Moreover, Cse$^{+/+}$ mice have higher levels of MPO which is a surrogate marker of neutrophil recruitment and neutrophil activity, Production of MCP-1, IL-23 and GM-CSF, inflammatory mediators which regulate the chemotaxis and activity of neutrophils chemotaxis, increased after infection but only in Cse$^{+/+}$ mice suggesting a defect in chemotaxis of neutrophils induce by the lack of H$_2$S. The a) decreased efficiency of leukocytes of Cse$^{-/-}$ mice in killing *P. aeruginosa* in vitro; b) the reversal of this phenomenon after restoration of H$_2$S levels through pre-
incubation with an H₂S donor; and c) the increased bacterial loads in infected Cse⁻/⁻ mice suggest that host H₂S is significant for the efficient phagocytosis of *P. aeruginosa*.

*P. aeruginosa* consists one of the most usual causes for nosocomial infections and is characterized by very high resistance rates against 3rd and 4th generation antibiotics in many countries including Greece [31]. The high virulence of *P. aeruginosa* in CF patients relates to its ability to avoid phagocytosis by forming complex biofilms, which help *P. aeruginosa* grow in the patients airway [35]. The biofilm formation remains under control of the QS system, which is unique cell to cell communication mechanism of *P. aeruginosa*. The function of the QS system is regulated by three systems, controlled by the *rhl* / *rhlR*, *lasI / lasR* and *pqsA / pqsR* from which *rhlII*, *lasI* and *pqsA* genes are responsible for the synthesis of acyl homoserine lactones (AHLs) and *rhlR*, *lasR* and *pqsR* are transcriptional regulators [24]. AHLs are diffusible signaling molecules. An increase in the concentration of extracellular AHLs, most often as an answer to the increasing number of *P. aeruginosa* colonies, leads to an increase of intracellular AHLs, which boost the expression of genes that regulate the growth of *P. aeruginosa* and the biofilm formation [37]. *P. aeruginosa* cells remain in the biofilm, protected from phagocytosis from tissue macrophages [38]. In vivo data show that the inhibition of the expression of one or two of the core Qs genes *rhlII* and *lasI* decreases the ability of *P. aeruginosa* to induce an acute pulmonary infection in a murine model [39]. In a prospective clinical study of 60 patients with exacerbation of chronic cystic fibrosis, the concentration of eight different AHLs in the sputum, in plasma and in urine samples was measured. The patients suffered from chronic CF and the measurements were made in the phase of acute exacerbation and also after the administration of antibiotic treatment. The concentrations of the eight AHL molecules were significantly higher at the initial phase of CF exacerbation and were positively correlated with an increase in the *P. aeruginosa* load in sputum. AHL concentration was significantly decreased in the case of successful antibiotic treatment [40]. Moreover, an analysis of sputum samples from 22 children with CF showed that higher concentrations of H₂S in sputum were inversely correlated with the amount of sputum produced and was negatively correlated with the likelihood for inpatient hospitalization or oxygen supplementation, suggesting that sulfide could be a marker of good health in CF patients [41]. This effect of hydrogen sulfide was attributed to an enhancement of the reduction capacity, possibly rendering the airway microenvironment inhospitable for aerobes. The findings of our study are consistent with published data since expression of QS genes *rhlII*, *lasR* and *pqsA* was decreased over time in Cse⁺/⁺ but not Cse⁻/⁻ mice. Moreover, we demonstrated that modulation of H₂S levels in vitro also influences QS expression of *P. aeruginosa* isolates. Thus, under the presence of H₂S *P. aeruginosa* becomes over time less efficient in
Fig 5. Modulation of growth and expression of the quorum sensing (QS) genes of multi-drug resistant (MDR) Pseudomonas aeruginosa in vitro. MDR P. aeruginosa isolates 6–11–19, 19–2–45 and 20–1–30 were incubated for 2 and 6 hours with/ without H₂S donor GYY4137 and H₂S biosynthesis inhibitor aminooxycetic acid (AOAA). Experiment was performed in duplicate. A) Bacterial growth at 0, 2 and 6 hours of incubation; B-G) Fold change of number of transcripts of QS genes rhlI, rhlR, lasI, lasR and pqsA per log₂ of MDR P. aeruginosa for each isolate is shown. Comparison by the ANOVA test with Bonferroni correction for multiple comparisons; Only statistically significant values are shown; * p< 0.05.

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AhR/Pnic-1 cell- to cell communication and evasion of the host innate immune response. Additionally, a negative correlation was found between lung H₂S levels and the expression of QS gene rhlI, rhlR, lasI, lasR and pqsA in Cse⁺/⁺ mice but not in Cse⁻/⁻ mice. Subsequently, H₂S produced specifically through CSE acts as a restrain of QS activation, rendering MDR P. aeruginosa susceptible to phagocytosis.

Previous studies show that garlic extract, which contains several polysulfides, which in vivo stimulate the synthesis of hydrogen sulfide, inhibits the quorum sensing system of P. aeruginosa rendering it vulnerable to elimination through polymorphonuclear cells [42–44]. However, the exact mechanism by which H₂S regulates the expression of the quorum sensing genes of P. aeruginosa still remains unclear. AHLs have been shown to reduce the activation of the nuclear factor-kappa β (NF-κB) pathway, which in term leads to a deficient innate immune response [45,46]. On the contrary, H₂S produced by CSE has been found to up-regulate the expression and actions of NF-κB, through sulfhydration [47]. Thus, the up-regulation of the NF-κB pathway mediated by H₂S is a potential mechanism for the limitation of the activation of the quorum sensing system of P. aeruginosa.

The importance of H₂S is even more complex, when one considers that bacteria are themselves capable of producing H₂S, through the same enzymatic systems as eukaryotic organisms. In vitro data show that Escherichia coli can use the H₂S they produce, as an autoinducer of bacterial growth [48]. It seems that H₂S is also necessary for the maintenance of bacterial resistance against antimicrobial treatment. Staphylococcus aureus, Escherichia coli, Bacillus anthracis and P. aeruginosa strains, in which the genes that express the H₂S producing enzymes Cse, Cbs and 3Mst were genetically silenced, were rendered susceptible to previously ineffective antibiotics in vitro [49,50] as well as to elimination by the host immune system in spleen co-culture experiments in vitro and in mouse models of wound infection in vivo [27]. Yet other H₂S-related mechanisms apply to the elimination of Mycobacteria by the immune system: M. tuberculosis appears to coopt the host H₂S production and use it to drive its own metabolism and proliferation: in M. tuberculosis infected mice, while also upregulating host macrophage CSE expression to a level where the H₂S produced begins to suppress the immune function of the macrophage: under these conditions, deletion of host CBS or CSE suppresses bacterial growth and improves the survival of the infected mice [51,52]. The above examples illustrate that the role of host and bacterial H₂S in infection is pathogen and context-dependent, and this must be kept in mind when considering future translational efforts centered on the pharmacological modulation of H₂S homeostasis.

The translation into the clinical setting of findings of pre-clinical studies on the role of H₂S in infection poses a great challenge, mainly because of the great variance of measured H₂S levels in the plasma, which ranges from 2μM to over 500μM [6,53–55]. The measured levels seem to be disease- and pathogen- specific and also depend on the measuring method. This variance renders the use of a general threshold of H₂S plasma levels difficult and necessitates a more personalized approach.

In conclusion, we can show that Cse-derived host H₂S is a defense mechanism that acts via reduction of host pathogen load, affording resistance to infection. H₂S is also a possible biomarker for the severity and outcome of MDR P. aeruginosa infections and could be utilized as
an adjunctive criterion, together with other established biomarkers like the SOFA score, CRP and PCT for decision making. The mechanism resides on the inhibition of the expression of the QS system of \( P. \) aeruginosa, rendering it susceptible to an efficient neutrophil guided phagocytosis.

**Supporting information**

S1 Fig. Patient selection process. Description of the selection steps for the three groups of comparison. Abbreviations: VAP: ventilator associated pneumonia; TBS: tracheobronchial secretions.

(TIF)

S2 Fig. Serum H\( _2 \)S decreases in bacterial ventilator associated pneumonia. Serum levels of H\( _2 \)S in healthy volunteers and on day 1 among patients with sepsis due to VAP were measured by HPLC. Comparisons by the Mann Whitney U test; \( ** \) \( p < 0.01 \).

(TIF)

S3 Fig. Effect of H\( _2 \)S production deficiency on the outcome of infection by \( P. \) aeruginosa.

A) DNA genomic patterns of \( P. \) aeruginosa isolates 6–11–19 (P1); 19–2–45 (P2) and 20–1–30 (P3) determined by pulse field gel electrophoresis. L: DNA Ladder. Survival analysis between B) \( Cse^{+/+} \) and \( Cse^{-/-} \) mice after infection with MDR \( P. \) aeruginosa isolate 19–2–45; C) \( Cse^{+/+} \) and \( Cse^{-/-} \) mice after infection with MDR \( P. \) aeruginosa isolate 20–1–30; Results of the log-rank test and the relevant \( p \)-values are given. D) Bacterial load (cfu/g) in the liver and in the lung of \( Cse^{+/+} \) and \( Cse^{-/-} \) mice after infection with MDR \( P. \) aeruginosa isolate 19–2–45; E) Bacterial load (cfu/g) in the liver and in the lung of \( Cse^{+/+} \) and \( Cse^{-/-} \) mice after infection with MDR \( P. \) aeruginosa isolate 20–1–30. Comparisons by the Mann Whitney U test. \( \ast \) \( p < 0.05 \), \( ** \) \( p < 0.01 \).

(TIF)

S4 Fig. Lack of effect of host H\( _2 \)S on production of tumor necrosis factor alpha (TNF\( \alpha \)) after multi-drug resistant (MDR) \( Pseudomonas aeruginosa \) infection. \( Cse^{+/+} \) and \( Cse^{-/-} \) mice (\( n = 6 \) per group per timepoint) were sacrificed 6 and 24 hours after experimental infection by MDR \( P. \) aeruginosa isolate 6–11–19. Concentration of TNF\( \alpha \) A) in serum, B) tissue supernatants and C) supernatants of stimulated splenocytes (stimuli medium, LPS from Escherichia coli O55:B5 for 24h and \( C. \) albicans for 5 days). Comparison by the Mann Whitney U test; ns non-significant.

(TIF)

S5 Fig. Lack of effect of endogenous H\( _2 \)S on the production of pro- and anti-inflammatory cytokines after multi-drug resistant (MDR) \( Pseudomonas aeruginosa \) infection. \( Cse^{+/+} \) and \( Cse^{-/-} \) mice (\( n = 6 \)) were sacrificed 6 hours after experimental infection by MDR \( P. \) aeruginosa isolate 6–11–19. Concentration of IL-1\( \alpha \), IL-1\( \beta \), IL-6, IL-10, IL-12, IL-27, IFN\( \gamma \), IFN\( \beta \) in serum. Comparison by the Mann Whitney U test; ns non-significant.

(TIF)

S6 Fig. Absence of correlation of the CSE enzyme with baseline blood cell counts. Healthy \( Cse^{+/+} \) and \( Cse^{-/-} \) mice (\( n = 8 \) per group) were sacrificed. Absolute counts of A) red blood cells (RBCs); B) platelets; C) white blood cells (WBCs); D) neutrophils and E) lymphocytes were
determined. Comparison by the Mann Whitney U test; ns non-significant.

(TIF)

S7 Fig. Absence of effect of bone marrow transplantation on serum H2S level. H2S levels in serum of naïve Cse+/+ and Cse−/− mice before and after bone marrow transplantation (BMT) were measured by high-performance liquid chromatography (HPLC). Comparison by the ANOVA test with Bonferroni correction for multiple comparisons; ns non-significant, *p < 0.05, **p < 0.01.

(TIF)

S8 Fig. Neutrophil activity against multi-drug resistant (MDR) Pseudomonas aeruginosa is mediated by the CSE enzyme. Cse+/+ and Cse−/− mice (n = 6 per group per timepoint) were sacrificed 6, 12 and 24 hours after experimental infection by MDR P. aeruginosa isolate 6–11–19. Correlations between bacterial outgrowth and MPO in the liver and in the lung for each group. Spearman rank correlation coefficient (r_s), relevant p-value and interpolation line for each group are given.

(TIF)

S9 Fig. Modulation through CSE derived H2S of the quorum sensing (QS) system of multi-drug resistant (MDR) Pseudomonas aeruginosa. A-F) Correlation between Transcripts of QS genes rhII, rhIR, lasI, lasR, pqsA, pqsR per log_{10} of MDR P. aeruginosa in the lung and H2S levels in the lung in Cse+/+ and Cse−/− mice, sacrificed 6 after experimental infection by MDR P. aeruginosa isolate 6–11–19. (6 mice per group). Spearman rank correlation coefficient (r_s), interpolation line for each group and relevant p-value are given.

(TIF)

S1 Text. Table A in S1 Text. Baseline and clinical characteristics of patients with sepsis due to VAP grouped by pathogen Table B in S1 Text: Mobile phase gradient of HPLC Table C in S1 Text: Quorum sensing (QS) genes and primers of P. aeruginosa isolates used for qRT-PCR.

(DOC)

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