RESEARCH ARTICLE

Refinement of metabolite detection in cystic fibrosis sputum reveals heme correlates with lung function decline

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Abstract

The bacterial growth environment within cystic fibrosis (CF) sputum is complex, dynamic, and shaped by both host and microbial processes. Characterization of the chemical parameters within sputum that stimulate the *in vivo* growth of airway pathogens (*e.g. Pseudomonas aeruginosa*) and their associated virulence factors may lead to improved CF treatment strategies. Motivated by conflicting reports of the prevalence and abundance of *P. aeruginosa*-derived metabolites known as phenazines within CF airway secretions, we sought to quantify these metabolites in sputum using quadrupole time-of-flight mass spectrometry. In contrast to our previous work, all phenazines tested (pyocyanin (PYO), phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide, and 1-hydroxyphenazine) were below detection limits of the instrument (0.1 μM). Instead, we identified a late-eluting compound that shared retention time and absorbance characteristics with PCA, yet generated mass spectra and a fragmentation pattern consistent with ferriprotoporphyrin IX, otherwise known as heme B. These data suggested that UV-vis chromatographic peaks previously attributed to PCA and PYO in sputum were mis-assigned. Indeed, retrospective analysis of raw data from our prior study found that the heme B peak closely matched the peaks assigned to PCA, indicating that the previous study likely uncovered a positive correlation between pulmonary function (percent predicted forced expiratory volume in 1 second, or ppFEV1) and heme B, not PCA or any other phenazine. To independently test this observation, we performed a new tandem mass-spectrometry analysis of 71 additional samples provided by the Mountain West CF Consortium Sputum Biomarker study and revealed a positive correlation (ρ = −0.47, p<0.001) between sputum heme concentrations and ppFEV1. Given that hemoptysis is strongly associated with airway inflammation, pulmonary exacerbations and impaired lung function, these new data suggest that heme B may be a useful biomarker of CF pathophysiology.

Introduction

Pseudomonas aeruginosa lung infections are a leading cause of morbidity and mortality in cystic fibrosis (CF) patients [1, 2]. Our laboratory has studied a class of small molecules produced by *P. aeruginosa* known as phenazines, including pyocyanin and its biogenic precursor phenazine-1-carboxylic acid (PCA). As phenazines are known virulence factors [3], we and others have explored the possibility of using phenazine concentrations as a marker for disease progression [4–6]. Previously, we reported that sputum concentrations of pyocyanin and PCA negatively correlate with lung function in CF patients [6]. Our study used high performance liquid chromatography (HPLC) to quantify phenazines by UV–vis absorbance after extraction from sputum.

Since our initial study, methods for metabolite analysis have advanced considerably, aided in large part by usage of mass spectrometry (LC-MS) and tandem mass spectrometry (LC-MS/MS). Because a more recent study employing LC-MS/MS revealed a surprising decoupling of *P. aeruginosa* metabolites in sputum and the detection of *P. aeruginosa* through culturing or microbiome profiles [4], we decided to check whether we could reproduce our previous findings by analyzing sputum samples from a different patient cohort with a new LC-MS instrument in our laboratory. New samples were provided by the Mountain West CF Consortium Sputum Biomarker study [7]. In the course of performing our new analyses, comparison of our old HPLC data to our new LC-MS data led us to realize that the peak previously assigned to PCA instead originates from heme, and the peak assigned to pyocyanin originates from an as-yet unknown compound. Here we present new data showing that heme concentration positively correlates with lung function decline in CF patients.

Results

Identification of heme in sputum

Motivated by a recent LC-MS/MS study that reported phenazines in only a fraction of samples that were sequence-positive for *P. aeruginosa* [4], we sought to determine whether we could repeat our previously reported correlation between phenazines and disease progression (measured by percent predicted forced expiratory volume in 1 second, or ppFEV1). We obtained fresh sputum samples from the Mountain West CF Consortium (MWCFC) sputum biomarker study and analyzed 71 sputum supernatants using HPLC coupled to UV–vis detection and a single quadrupole mass spectrometer (Waters Acquity QDa). Patients with confirmed CF enrolled in the MWCFC study were randomly selected from all sputum producers older than twelve years of age and enrolled during clinical stability (Table 1). Selected patients had a mean age of 27 years (SD = 13), ppFEV1 of 68% (SD = 23), and weight-for-age *z*-score of −0.17 (SD = 0.96) at the time of enrollment. Patients had a median of 2.0 pulmonary exacerbations in the year prior to enrollment (range 0–6), diabetes in 24%, pancreatic sufficiency in 9.9% and 5-year survival predictions [8] ranging from 9.4% to >99% (median 95%). There were no significant differences in clinical characteristics, microbiology or treatment profiles between patients with and without heme measurement results from the greater MWCFC patient cohort (Table 1), strongly suggesting that missing heme measurements were missing at random [9] and that inferences based on the measurements made are not biased due to a specific pattern of patient selection. As previously reported, there were no differences between the characteristics of the 114 patients in the MWCFC study group and the other 14,394 patients twelve years of age and able to produce sputum found in the 2014 CF Foundation Patient Registry [7].

Table 1. Patient cohort characteristics.

Patient Characteristics, Infections and Treatments[a]	Heme Result Obtained n = 71	Heme Measurement Not Performed n = 43	p-value
Male sex[b]	30 (0.42)	23 (0.53)	0.25
Age, Years (SD)[c]	27 (13)	30 (11)	0.21
ppFEV$_1$, Percent Predicted (SD)[c]	68 (23)	74 (21)	0.17
Weight-for-age z-score (SD)[c]	-0.17 (0.96)	-0.18 (1)	0.96
Pulmonary exacerbations in year prior to enrollment[d] median (range)	2 (0–6)	2 (0–7)	0.056
Height, cm[c]	167 (10.2)	167 (9.62)	0.64
5-Year Predicted Survival[d]	0.95 (0.094->0.99)	0.97 (0.34->0.99)	0.14
Diabetes[b]	17 (0.24)	8 (0.19)	0.66
Pancreatic Sufficiency[b]	7 (0.099)	2 (0.047)	0.52
CF related arthropathy[b]	4 (0.056)	4 (0.093)	0.72
Methicillin Sensitive *S. aureus*[b]	34 (0.48)	17 (0.40)	0.5
Methicillin Resistant *S. aureus*[b]	14 (0.2)	7 (0.16)	0.83
P. aeruginosa[b]	45 (0.63)	25 (0.58)	0.72
B. cepacia complex[b]	2 (0.028)	1 (0.023)	>0.99
S. maltophilia[b]	4 (0.056)	3 (0.07)	>0.99
Achromobacter spp[b]	3 (0.042)	2 (0.047)	>0.99
Candida spp[b]	14 (0.2)	3 (0.07)	0.11
Aspergillus spp[b]	10 (0.14)	2 (0.047)	0.2
MAI complex[b]	0 (0)	1 (0.023)	0.8
M. abscessus[b]	0 (0)	3 (0.07)	0.8
Patients on inhaled tobramycin[b]	21 (0.30)	17 (0.40)	0.37
Patients on inhaled aztreonam[b]	27 (0.38)	13 (0.30)	0.52
Patients on daily oral azithromycin[b]	39 (0.55)	23 (0.53)	>0.99
Patients on inhaled hypertonic saline[b]	41 (0.58)	30 (0.70)	0.28
Patients on inhaled DNase[b]	64 (0.90)	37 (0.86)	0.72

[a] Data are presented as counts (fraction affected), mean (SD) or median (range) as appropriate for the variable and distribution of values. See other footnotes for specific annotations.

[b] Number of patients affected (fraction affected).

[c] Mean (SD) for variables with normal distribution.

[d] Median (Range) for variables with non-normal distribution.

To our surprise, and in contrast to our prior study, we were unable to detect the phenazines pyocyanin, PCA, phenazine-1-carboxamide, or phenazine-1-hydroxide in any of the samples within our detection limit (approximately 0.1 µM) in either the UV–vis or mass channels. Instead, we observed a late-eluting compound in all 71 samples which had an absorbance spectrum that overlaps with the phenazine spectrum but is distinguished by a maximum at 398 nm. This compound produced positive ions at an *m/z* of 616.2, further distinguishing it from any known phenazine, and it was present even in patients who were culture-negative for *P. aeruginosa*. We noted a striking similarity between the UV–vis spectrum of this compound with published spectra for the porphyrin ring of heme-like compounds [10]. We therefore wondered whether this peak represented a compound related to heme, and whether we might have mis-assigned it previously in sputum samples, which are more chemically complex than bacteriological media.

To further interrogate the peak, we transferred our method to an instrument coupled to a high-resolution quadrupole time-of-flight mass spectrometer (Waters Xevo). Again we
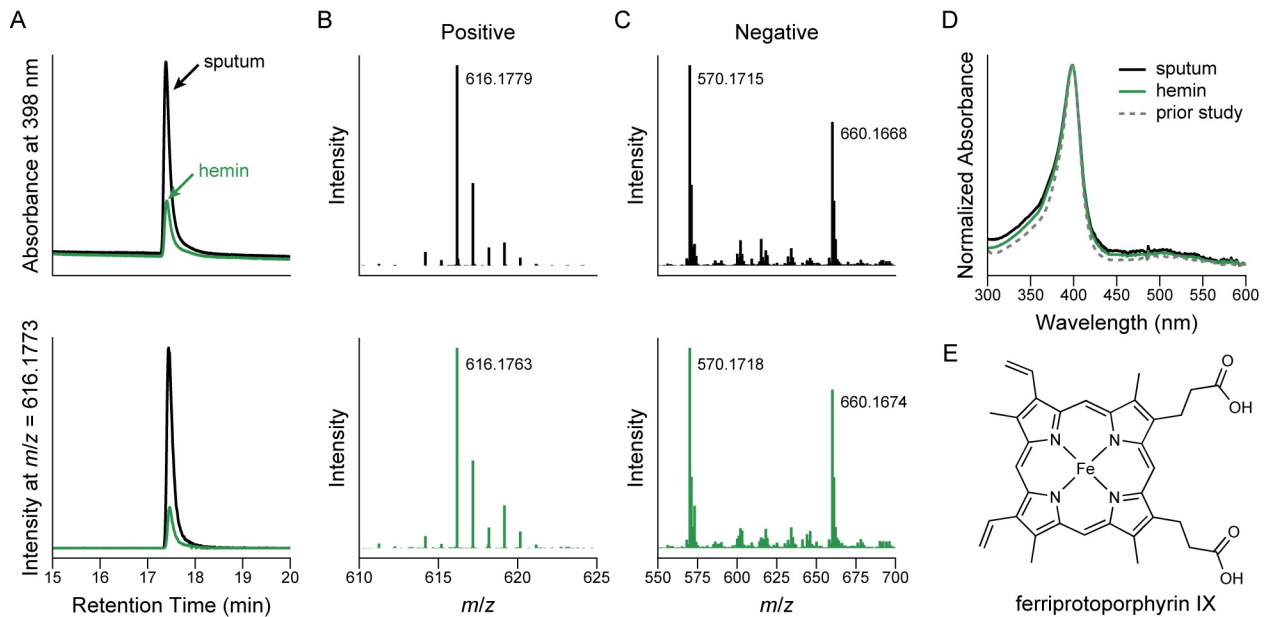
Fig 1. Identification of heme in sputum samples by comparing sputum (black) to a hemin standard (green). (A) UV–vis chromatogram (top) and extracted ion chromatogram (616.1773 ± 0.01 Da) from the positive mass channel (bottom) demonstrating identical retention times. (B) The associated positive ions detected from the peak shown in A, comparing the sputum peak (top) to the hemin standard (bottom). (C) The associated negative ions detected from the peak shown in B, comparing the sputum peak (top) to the hemin standard (bottom). A collision-energy ramp of 10 to 14 eV was applied to generate a fragmentation pattern. (D) Comparison of the UV–vis spectra for the peaks in A (black and green) compared to the same peak identified in our prior study (gray, dashed). For clarity, the spectra are normalized to their maximum value. (E) The chemical structure assigned to the peak in A, ferriprotoporphyrin IX.

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consistently observed a UV–vis chromatographic peak at 398 nm (Fig 1A). Consistent with its identity as a heme, this peak had a retention time identical to that of a hemin standard (Fig 1A). Both the sputum samples and the hemin standard gave an equivalent peak in the extracted ion chromatogram for an m/z of 616.1773 ± 0.01 (Fig 1A), the m/z expected for hemin having lost its associated chloride ion to produce ferriprotoporphyrin IX. We note that the hemin standard was dissolved in base, a condition known to replace the chloride ion with hydroxide [11] which is subsequently removed in the acidic conditions of our chromatography, therefore explaining the origin of ferriprotoporphyrin IX from our hemin standard. In the positive ion channel (Fig 1B), sputum and hemin produced equivalent signals that were indistinguishable from the expected mass of ferriprotoporphyrin IX within the resolution of the instrument (Δppm = 0.97 and −1.6, respectively). Similarly, in the negative channel (Fig 1C), both sputum and hemin produced ions consistent with the formic acid adduct of ferriprotoporphyrin IX (Δppm = −0.45 and 0.45, respectively), an additive used in our separation method. Application of collision energy produced equivalent fragmentation patterns in the negative ion mode (Fig 1C) with the major daughter ion appearing around 570.1718, consistent with two decarboxylations or a single decarboxylation and loss of formic acid. The sputum peak and the hemin standard also produced indistinguishable UV–vis spectra (Fig 1D) which closely matched previous reports for heme [10]. Based on identical retention times, mass and UV–vis spectra, and mass fragmentation patterns, we assign this peak to ferriprotoporphyrin IX, also known as heme B (Fig 1E).

Re-analysis of the original data

Though the MWCFC cohort differed in inclusion criteria from that used in our prior Children's Hospital Boston (CHB) sputum study (*i.e.* a positive diagnosis of CF based on genotyping or sweat test and positive *P. aeruginosa* culture), we were nevertheless surprised that we were unable to detect phenazines in the MWCFC samples with our newer instrument. At the time of our previous analysis, we were using an older HPLC instrument equipped with UV–vis detection but not mass detection, and so we became concerned that we may have mis-identified other compounds as phenazines using this older instrument and decided to revisit our old raw data. In the CHB study, we employed a standard protocol, used by our group and others for routine phenazine analysis in bacteriological cultures, which detects phenazines by their retention times in UV–vis chromatograms (387 nm for pyocyanin and 364 nm for PCA). We used this method to quantify phenazine production from 779 clinical isolates of *P. aeruginosa* from CF sputum from 47 CHB patients [6]. Re-examination of the raw data confirmed appropriate phenazine assignment in samples from pure cultures of *P. aeruginosa*. To quantify phenazines in CF sputum, we co-injected pyocyanin or PCA into sputum from which we could not culture *P. aeruginosa* (a major source of phenazines in CF patients) and ran standard curves. In these control co-injection samples, we observed distinct HPLC peaks at 387 nm and 364, as would be expected for pyocyanin or PCA, respectively. Accordingly, we used absorption at these wavelengths to quantify phenazines in all sputum samples. With the benefit of our new awareness that heme B shares retention and absorption characteristics with PCA, we revisited the complete spectral data for each peak from the raw data on our old HPLC, suspecting that we might have unwittingly mis-assigned phenazines to other compounds in sputum. Indeed, analysis of the full UV–vis spectra revealed that the sputum peak we had tracked at 364 nm and assigned to PCA had an absorbance maximum at 398 nm (Fig 2A), suggesting it is unlikely to be PCA despite similar retention times and overlapping absorbance spectra. Instead, this peak's absorbance spectrum closely matched the spectrum of ferriprotoporphyrin IX identified in our new study (Fig 1D), suggesting we had mis-assigned the heme peak as PCA. Similarly, the sputum peak previously assigned to pyocyanin has an absorbance maximum around 360 nm (Fig 2B) instead of 387 nm, suggesting that pyocyanin is not the major component of this peak. The early-eluting portion of the chromatogram that contains pyocyanin is crowded by multiple overlapping peaks with similar spectra, and so we have been unable to characterize it further.

Despite our best efforts to perform appropriate controls, including co-injections, the UV–vis spectra reveal that the peaks for pyocyanin and PCA were incorrectly assigned in the original CHB study. This error stemmed from the co-elution of phenazines with sputum compounds that have similar absorbance spectra and the lack of a MS system on the older HPLC instrument. In addition, the older instrument produced significant retention time variability between HPLC samples (up to ±1 minute for PCA), further masking the incorrect assignment. While we cannot rule out the possibility that phenazines were present in our old samples, the distinct UV–vis absorbance spectra indicate that phenazines were not the major component of the assigned peaks, and so our original report overestimated the phenazine concentration in CF sputum samples.

Heme concentrations correlate negatively with lung function

As the re-analysis suggests, the interpretation of our original HPLC data was confounded by co-eluting compounds that are present in sputum. The similarity between the peak we previously analyzed as PCA and what we have now identified as ferriprotoporphyrin IX (Fig 1D) suggests that, instead of a correlation between phenazines and ppFEV1, the original study
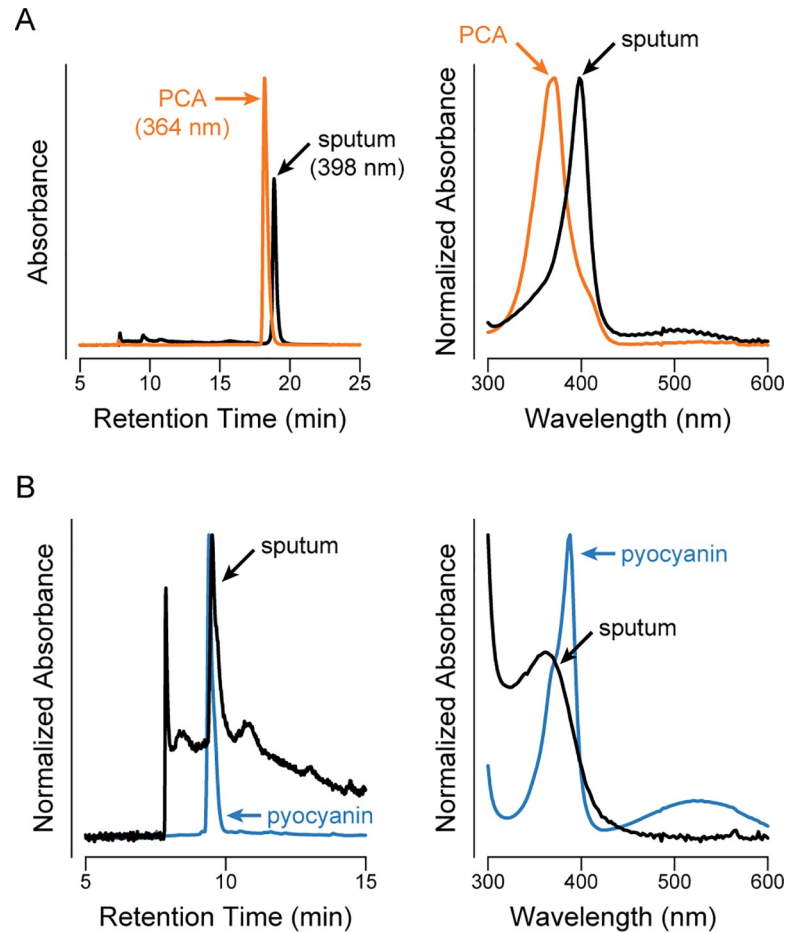
Fig 2. Re-analysis of HPLC peaks previously assigned as phenazines from original data collected on an older HPLC. UV–vis chromatograms are shown on the left, and the full UV–vis spectra of the peaks indicated with an arrow are shown on the right. Where indicated on the y-axis, the data have been normalized to bring the data into the same scale for clarity. (A) Analysis of the PCA peak, comparing a pure PCA standard (chromatogram at 364 nm, orange) to a sputum sample (chromatogram at 398 nm, black). (B) Analysis of the pyocyanin peak, comparing pyocyanin in *P. aeruginosa*-free sputum (blue) to a *P. aeruginosa*-positive sputum sample (black) (both chromatograms at 387 nm).

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measured a correlation between heme and ppFEV1. To evaluate this possibility, we quantified the concentration of ferriprotoporphyrin IX in our MWCFC samples from our new dataset. Heme concentrations in sputum were log-normal in distribution (Anderson-Darling test statistic 0.61, $p = 0.11$). Further, a plot of heme concentration versus ppFEV1 reveals a statistically significant (Spearman's $\rho = -0.47$, $p < 0.001$) correlation between sputum heme levels and ppFEV1 (Fig 3). Although the concentration of ferriprotoporphyrin IX in the original samples is unknown, it is likely that the original PCA concentration data instead represents heme concentration with an unknown conversion factor, and so both studies have independently confirmed a correlation between the heme signal in HPLC analysis and lung function in CF patients.

Discussion

Several independent analyses have detected phenazines in sputum from CF patients [4, 5]. Our new data collection effort and the correction reported here to our previous results [6] was partially motivated by a more recent report of detectable phenazine concentrations in only 3 of 27
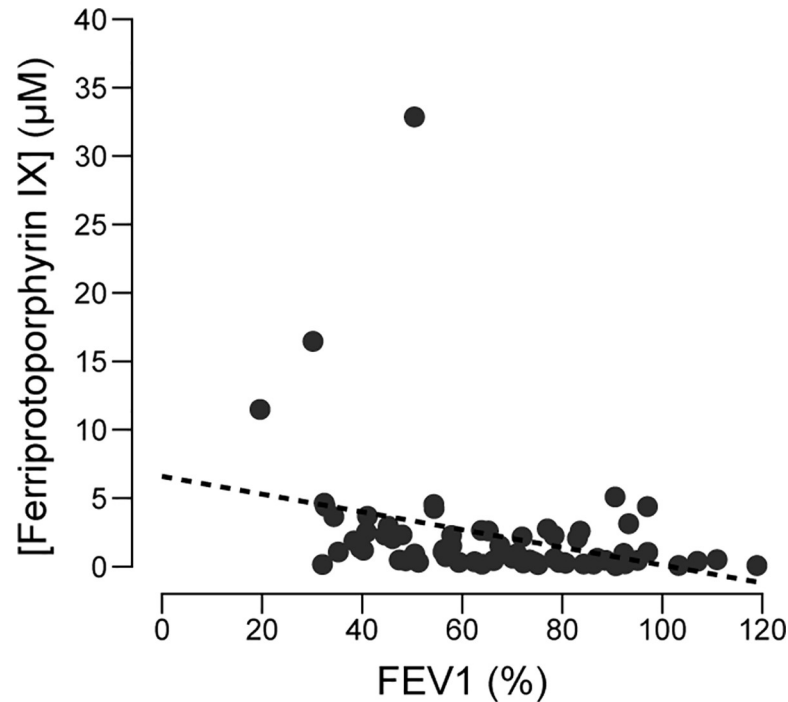
Fig 3. Correlation between ferriprotoporphyrin IX concentration and disease status as measured by percent predicted FEV1 (Spearman's ρ = −0.47, p = 3.6 × 10^{-5}). Each data point represents a single sputum sample from a unique patient. The dashed line illustrates a linear regression through the data.

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patients [4]. Interestingly, extensive isolation of several hundred clinical *P. aeruginosa* strains in our lab has verified that phenazine production is a nearly universal capability of *P. aeruginosa* strains that inhabit CF patients, a result that is still valid from our original study [6]. All phenazine analyses in the original paper that were performed in bacteriological growth medium were accurate. We therefore find it interesting that phenazines and other *P. aeruginosa* metabolites are detected only sporadically in sputum samples, often from patients that are culture-negative for *P. aeruginosa* [4]. Together these findings are suggestive of significant macro- and micro-scale heterogeneity within CF lungs. Consistent with regional heterogeneity, *P. aeruginosa* strains within different regions are known to undergo independent evolution with minimal cross-colonization [12]. It is difficult to verify that independent samples originate from the same region of the lung, and so heterogeneity between sputum samples may account for differences between metabolite and microbial analyses within the same patient. Furthermore, sputum samples are extremely viscous and poorly mixed, leading to high spatial heterogeneity within individual samples [13]. Sputum viscosity originates from extracellular polymers including DNA [14], and because pyocyanin and other phenazines are known DNA intercalators [15], it is likely that cells within the sputum matrix encounter a micro-scale phenazine concentration that is considerably higher than that determined by bulk measurements. We anticipate that new methods to image metabolites at large and small spatial scales will provide insights into the CF lung environment and clarify the role of pathogen-derived metabolites in disease progression. Although it remains possible that phenazine concentration negatively correlates with lung function, our original study overestimated the total phenazine concentration and the true bulk concentrations were likely below our limit of detection.

The form of heme we measured in this study, ferriprotoporphyrin IX or heme B, is the most abundant heme found in humans and the oxygen-carrying component of hemoglobin in

red blood cells. Given that the majority of components in lung sputum are host-derived [4], the heme likely originated from blood rather than from microbial sources. Consistent with this assumption, hemoptysis, or coughing up blood, is observed in up to 60% of CF patients [16–18]. While minor blood streaking in sputum does not usually prompt treatment, massive hemoptysis is a marker of pulmonary exacerbations and correlates with impaired lung function and mortality in adults [16, 17], and so the correlation between heme and lung function decline may identify heme as a potentially useful biomarker of clinical disease in CF.

Previously we also reported a correlation between sputum iron and lung function decline [19]. This correlation arose from the abundance of Fe(II) which predominated, rather than from Fe(III) [19]. We also reported a correlation between Fe(II) and what we believed was PCA [18], which we now recognize was heme. As heme is a major carrier of iron, this correlation suggests blood-derived heme may be a significant source of sputum iron. In the context of lung infections, iron is an important nutritional regulator of pathogenicity and biofilm formation in bacteria, including *P. aeruginosa* [20], and pathogenic bacteria can extract iron from heme [10]. Given that iron chelation is now being explored as a potential therapy for lung infections [21], there is a need to better understand the sources of sputum iron and its influx rate, of which our results suggest host-derived heme could be a significant contributing factor.

The prognosis for CF patients has improved remarkably in recent decades, owing not only to advances in treatments but also in diagnostics and preventative care [22]. While the role of pathogen-derived metabolites in disease state is still uncertain, host-derived factors are clearly abundant [4] and may provide new markers to guide treatment. The prevalence of heme in every sputum sample we measured, and the simplicity of its detection by HPLC owing to its characteristic 398 nm absorbance peak, suggest it can be a useful diagnostic component for monitoring CF disease activity. Additional research may uncover a causative link between sputum heme and lung disease through the interplay between iron and pathogenic microbes.

Materials and methods

HPLC method from the original study

The original study used a Beckman Gold HPLC equipped with preparative-scale pumps on a model 126P solvent module. The separation used a Waters Symmetry C18 column with dimensions 4.6 mm × 250 mm and a particle size of 5 μm. The injection volume was 100 μl, supplied via a model 508 autosampler, and the flow rate was 1.0 mL/min. Solvent A was water with 0.1% trifluoroacetic acid (TFA) and Solvent B was acetonitrile with 0.1% TFA. Compounds were eluted using a linear gradient of 15% B from 0 to 2 minutes, 15% to 83% B from 2 to 22 minutes, and 0% B from 22 to 30 minutes. UV–vis data were collected from 200 to 600 nm using a model 168 photodiode array detector.

Sample preparation

Sputum samples were obtained after informed consent from patients with confirmed CF aged 12 and older able to expectorate sputum during clinical stability. The study protocol was reviewed and approved at each of the 9 participating centers in the Mountain West CF Consortium (MWCFC). To derive a sample of patients representative of all patients in the United States who are 12 years of age and older and also produce sputum, we used the pseudo-random number generator method [23] to randomly assign selection priorities to every eligible patient in the MWCFC. After accounting for previously observed enrollment rates for observational studies and rates of attendance at CF clinics in the region, we recruited patients according to the randomly assigned priorities for the study. While maintaining randomization, we controlled the rates of enrollment to reduce seasonal and center-specific biases by setting a

threshold selection priority for recruitment by center. Detailed methods are provided in Liou et al. [7].

Samples were processed within 4 hours of expectoration by dilution 1:1 with Hanks buffered saline solution (Sigma, St Louis, MO), vigorous vortex mixing for 1 minute followed by centrifugation at 2,800 g and 4˚C for 20 minutes to obtain lipid and aqueous fractions and pellets. For this study, we used aqueous fractions that were mixed 1:1 with protease inhibitor cocktail (Sigma). Fractions were frozen and stored at −80˚C until use. The samples were thawed at room temperature and centrifuged at 13,000 *g* for 15 minutes. The supernatant was transferred to a Waters Total Recovery Vial and injected directly into the HPLC without further processing. Hemin was purchased from Sigma-Aldrich and was dissolved to a stock concentration of 10 mM using 100 mM aqueous ammonia. A standard curve from 0.01 to 100 μM was created by diluting the stock solution into water.

Spirometry

Clinical data including spirometry data were collected at the same time as sputum samples. The forced expiratory volume in 1 second (FEV1) was measured according to American Thoracic Society standards [24]. FEV1 was normalized to percent predicted FEV1 (ppFEV1) for age, sex, height, race and ethnicity using NHANES III equations [25] as was done clinically at all participating centers at the time of study enrollment.

High resolution LC-MS

HPLC separations for high resolution mass spectrometry were performed on a Waters Acquity I-Class UPLC connected to a Waters Acquity PDA detector and a Waters Xevo G2-XS quadrupole time-of-flight mass spectrometer. The sample chamber was maintained at 4˚C. The separation used a Waters XBridge BEH C18 XP column with dimensions 3.0 × 100 mm and a particle size of 2.5 μm. The column was equipped with a VanGuard guard column comprising the same resin. The injection volume was 5 μl and the flow rate was 0.5 mL/min. The column was maintained at 40˚C. Solvent A was water with 0.1% formic acid and solvent B was acetonitrile with 0.1% formic acid. Compounds were eluted using a linear gradient of 0% to 90% from 0 to 24 minutes, 90% B from 24 to 25 minutes, and 0% B from 25 to 35 minutes. Mass scans from 150 to 1000 Da were collected in separate chromatographic runs for positive and negative ionization modes with a scan time of 0.3 s. For both modes, the mass spectrometry parameters were: capillary voltage, 1.5 kV; sampling cone, 30 V; source offset, 80 V; source, 120˚C; desolvation, 550˚C; cone gas, 50 L/h; desolvation gas, 800 L/h. The analyser mode was set to resolution and the dynamic range was set to normal. In the negative ion mode, fragmentation data were collected in a second data channel using a collision-ramp energy of 10 to 14 eV. A solution of 20 pg/μL leucine enkephalin was used as the LockSpray solution with a flow rate of 20 μl/min. The instrument was controlled using the software MassLynx.

Routine LC-MS for ferriprotoporphyrin IX quantification

For routine analysis, HPLC separations were performed on a Waters Alliance HPLC connected to a Waters 2998 PDA detector and a Waters Acquity QDa single quadrupole mass spectrometer. The sample chamber was maintained at 10˚C. Separations were performed the same as for the high-resolution method, except that the gradient included a constant 2% methanol supplied via solvent C, which was found to greatly improve the reproducibility of pyocyanin measurements. Mass scans were collected simultaneously from 100 to 900 Da in the positive mode and 100 to 600 Da in the negative mode. In addition, a selected ion recording channel was collected in the positive mode for 211.09, 224.08, and 225.07 Da, the masses

expected for pyocyanin, phenazine-1-carboxamide, phenazine-1-carboxylic acid, respectively. The probe temperature was 600˚C and the capillary voltage was 0.8 kV. UV–vis data were collected from 200 to 800 nm at 20 Hz with a resolution of 1.2 nm. The instrument was controlled using the software Empower. Peaks representing ferriprotoporphyrin IX were integrated using the Apex Track algorithm within Empower and compared against a linear calibration curve of samples derived from hemin. The measured concentrations were multiplied four-fold to account for the dilution that occurred during sample processing.

Data and statistical analysis

Observations of anticipated phenazine detections demonstrated non-detectability through the first 71 samples that we analyzed, yet heme was detectable in each of those samples. Comparison of characteristics of the patients providing the tested samples with the remaining samples showed that further measurements were unlikely to detect phenazines and change any interpretations. Accordingly, no further samples were tested, as the tested group of patients was representative of the whole MWCFC cohort and of the US population of sputum producers (Table 1).

To ensure uniformity, data from the three instruments were exported and compared together within a series of Python scripts. Plots were generated using the Matplotlib package and labeled with Adobe Illustrator. The Spearman correlation coefficient was calculated using the spearmanr function in the SciPy package. Assessments of normality were performed in R.

Human subjects

MWCFC work was reviewed and approved by the Investigational Review Board (IRB) of University of Utah (IRB_00011571), St. Luke's Health System (13_0547), Billings (Study 14.07), Western (for the Las Vegas CF Center) (1146159, WIRB PRO NUM: 20140721, INVEST NUM: 112264, WO NUM: 1–837046–1, Protocol Number 20130530), National Jewish Health (HS-2797), Colorado Multiple IRB (for Children's Hospital Colorado) (13–3172), Phoenix Children's Hospital (13–087), University of Arizona (1407394040), and the University of New Mexico (14–085). No patients were enrolled prior to IRB approval. All patients younger than 18 provided assent with informed written consent provided by parents or other legal guardians. Each adult patient gave informed written consent on his or her own behalf. A detailed description of patient recruitment is provided in Liou et al. [7].

Author Contributions

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Data curation: Nathaniel R. Glasser, Ryan C. Hunter, Theodore G. Liou.

Formal analysis: Nathaniel R. Glasser, Ryan C. Hunter, Theodore G. Liou, Dianne K. Newman.

Funding acquisition: Theodore G. Liou, Dianne K. Newman.

Investigation: Nathaniel R. Glasser, Theodore G. Liou, Dianne K. Newman.

Methodology: Nathaniel R. Glasser.

Project administration: Ryan C. Hunter, Theodore G. Liou, Dianne K. Newman.

Supervision: Dianne K. Newman.

Validation: Nathaniel R. Glasser.

Writing – original draft: Nathaniel R. Glasser, Ryan C. Hunter, Theodore G. Liou, Dianne K. Newman.

Writing – review & editing: Nathaniel R. Glasser, Ryan C. Hunter, Theodore G. Liou, Dianne K. Newman.

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