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Alex H. Gifford
Dartmouth College

Lisa A. Moulton
Dartmouth College

Dana B. Dorman
Dartmouth College

Gordana Olbina
Intrinsic LifeSciences, LLC, La Jolla, California

Mark Westerman
Intrinsic LifeSciences, LLC, La Jolla, California

See next page for additional authors

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Alex H. Gifford, M.D.[1], Lisa A. Moulton, R.N.[1], Dana B. Dorman, R.N.[1], Gordana Olbina, Ph.D.[2], Mark Westerman, Ph.D.[2], H. Worth Parker, M.D.[1], Bruce A. Stanton, Ph.D.[3], and George A. O'Toole, Ph.D.[3]

Abstract

Background: Hypoferremia is a marker of disease severity in cystic fibrosis (CF). The effect of systemic antibiotics on iron homeostasis during CF pulmonary exacerbation (CFPE) is unknown. Our central hypotheses were that, by the completion of treatment, serum iron would increase, serum concentrations of interleukin-6 (IL-6) and hepcidin-25, two mediators of hypoferremia, would decrease, and sputum iron would decrease.

Methods: Blood and sputum samples were collected from 12 subjects with moderate-to-severe CF (median percentage-predicted forced expiratory volume in 1 second (FEV_1%) = 29%; median weight = 56 kg) within 24 hours of starting and completing a course of systemic antibiotics.

Results: After treatment, subjects showed median FEV_1% and body weight improvements of 4.5% and 2.0 kg, respectively ($p < 0.05$). Median serum iron rose by 2.4 μmol/L ($p < 0.05$), but 75% of patients remained hypoferremic. Median serum IL-6 and hepcidin-25 levels fell by 12.1 pg/mL and 37.5 ng/mL, respectively ($p < 0.05$). Median serum erythropoietin (EPO) and hemoglobin levels were unaffected by treatment. We observed a trend toward lower sputum iron content after treatment.

Conclusions: Hypoferremia is a salient characteristic of CFPE that improves with waning inflammation. Despite antibiotic treatment, many patients remain hypoferremic and anemic because of ineffective erythropoiesis. Clin Trans Sci 2012; Volume 5: 368–373

Keywords: iron, hepcidin, cystic fibrosis, interleukin-6, exacerbation

Introduction

Cystic fibrosis (CF) is a lethal genetic disease in which dysfunction of the CF transmembrane conductance regulator (CFTR) chloride channel in bronchial epithelial cells prevents hydration of airway surface liquid (ASL) in the lung. These thick secretions are a favorable environment for bacterial colonization, which incites chronic inflammation and eventual respiratory failure from bronchiectasis. The natural history of CF is characterized by acute exacerbations during which patients experience new or worsened respiratory and constitutional symptoms referable to infection.[1] Although no consensus definition exists for CF pulmonary exacerbation (CFPE),[2] the phenomenon is associated with accelerated lung function decline and premature death.[3,4]

At 3 months after intravenous antibiotics for CFPE, 25% of patients in a large cohort failed to regain their baseline percentage-predicted forced expiratory volume in 1 second (FEV_1%), a key spirometric endpoint in CF, as averaged over the 6 months before CFPE. Persistent infection by *Pseudomonas aeruginosa* (PA) was an independent risk factor for this irreversible impairment.[5] Growth within antibiotic-resistant biofilm communities is one means by which PA persists in the CF lung.[6] Polarized bronchial epithelial cells that express dysfunctional CFTR release iron into ASL, which augments the overlying growth of PA biofilms.[7] Hence, abnormal iron handling by airway epithelial cells likely contributes to PA lung infection in CF.

Clinical studies support the premise that iron homeostasis is uniquely aberrant in this disease. The iron content of CF sputum is approximately 2.5–5.5 times higher than sputum from patients with chronic obstructive pulmonary disease (COPD) and about 3.4–4.2 times higher than sputum from normal controls.[8–10] Reid et al.[11] reported a significant direct relationship between sputum iron and quantitative culture of PA, suggesting that its growth is related to iron availability. Three investigations[9–11] with a total of 34 subjects have revealed that sputum iron content does not change after CFPE therapy, but none of them addressed iron-related hematologic parameters.

Excess lung iron in CF presumably originates from host reserves despite complex regulatory mechanisms governing its uptake, utilization, and storage. Iron is necessary for oxygen transport by hemoglobin, mitochondrial energetics, and modulation of gene transcription.[12] Its absorption from dietary sources by duodenal enterocytes is markedly reduced during states of inflammation and systemic iron overload by the activity of hepcidin-25.[13] This polypeptide hormone of hepatic origin triggers the degradation of the iron-exporting protein ferroportin in mononuclear cells and enterocytes resulting in iron sequestration and attenuated uptake, respectively.[14,15] By these mechanisms, hepcidin-25 lowers blood iron, a condition called hypoferremia. Interleukin-6 (IL-6) potently stimulates hepcidin-25 production[16] thereby linking iron regulatory pathways to the inflammatory milieu. The hepcidin-IL-6 axis has heretofore not been investigated in CF.

The main goal of this study was to characterize how CFPE treatment influences iron handling in the circulatory and airway compartments given a paucity of data from concurrent assessment. Based on the available literature and our findings, we hypothesized that CFPE treatment would be associated with higher iron, and lower hepcidin-25 and IL-6 serum concentrations. Moreover, we postulated that sputum iron content would fall in response to treatment.

Methods

Definitions

CFPE was defined by new or acutely worsened sinopulmonary and constitutional signs and symptoms that included the following: increased cough frequency, increased daily sputum expectoration, wheezing, dyspnea, fevers, chills, sweating, anorexia, and weight loss.[17] An attending pulmonologist with longitudinal experience in caring for a given subject determined whether CFPE was apparent. Early CFPE was a point <24 hours after CFPE determination, and late CFPE was a point <24 hours

[1]Pulmonary Medicine, Dartmouth-Hitchcock Medical Center, Lebanon, New Hampshire, USA; [2]Intrinsic LifeSciences, LLC, La Jolla, California, USA; [3]Microbiology and Immunology, Geisel School of Medicine at Dartmouth, Hanover, New Hampshire, USA.

Correspondence: Alex H. Gifford (giffora@hitchcock.org)

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before finishing treatment. No subject received more than two scheduled doses of systemic antibiotics before drawing blood at the early CFPE time point. The duration of hospitalization was not specified *a priori* and was influenced by how long the subject wanted to remain in the hospital and the attending physician's appraisal of clinical progress. We defined hypoferremia according to other CF studies[17–19] as a serum iron ≤12 μmol/L, a threshold below which erythropoiesis becomes iron limited.[20] A hemoglobin level <13.7 g/dL, the lower limit of the reference range for our laboratory, established a diagnosis of anemia.

Enrollment

Blood and sputum samples were obtained from 12 adults with CF during early and late CFPE after provision of informed consent. The Committee for the Protection of Human Subjects (CPHS) at Dartmouth College approved the study protocol. Age ≥18 years and a history of PA airway colonization were inclusion criteria. Subjects were not eligible to participate if they were taking supplemental iron and if inclusion criteria were not satisfied. All subjects were treated with dual systemic antibiotics against PA based on susceptibility testing from the most recent sputum culture. No subject received iron supplementation or transfusion of packed red blood cells. Two subjects were using systemic corticosteroids or nonsteroidal antiinflammatory drugs before hospital admission, and these were continued without dose escalation. Sputum samples were spontaneously expectorated (i.e., not induced by inhalation of hypertonic saline) and were excluded from trace element analysis if any blood was visible. Routine chest physiotherapy and consultation with a nutritionist and a diabetes nurse specialist (if applicable) were offered to all subjects. The volume of blood drawn at each time point (i.e., early and late CFPE) was approximately 25 mL.

Measurement of complete blood counts and serum iron

Complete blood counts were done using a Sysmex XE-2100 autoanalyzer (Sysmex America, Inc., Mundelein, IL, USA). Serum iron was determined using a COBAS c311 autoanalyzer (Roche Diagnostics USA, Indianapolis, IN, USA).

Measurement of serum hepcidin-25

Serum hepcidin-25, the physiologically active polypeptide hormone of iron regulation, was quantified according to an established protocol[21] at Intrinsic LifeSciences, LLC (LaJolla, CA, USA). Briefly, the technique is a competitive enzyme-linked immunosorbant assay (C-ELISA) using biotinylation as a detection method. Samples were analyzed in duplicate, and mean values were used in subsequent calculations. The lower limit of detection was 5 ng/mL.

Measurement of serum erythropoietin

Serum erythropoietin (EPO) was measured using a commercially available double-sandwich ELISA (Quantikine® IVD®, R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer's instructions. Samples were analyzed singly. The lower limit of detection was 2.5 mU/mL.

Measurement of serum IL-6

Serum IL-6 was quantified using Bio-Plex human cytokine multiplex kits (Bio-Rad Inc., Hercules, CA, USA), according to the manufacturer's instructions. Samples were analyzed in triplicate, and mean values were used in subsequent calculations. For samples in which the concentration of IL-6 was undetectable, the lower limit of detection (1.9 pg/mL) was used in statistical analyses.

Measurement of sputum iron

Whole sputum plugs were weighed and digested with a standard volume of nitric acid. Total inorganic iron in each aliquot was quantified by inductively coupled plasma–mass spectroscopy (ICP–MS)[17] and is expressed as nanograms of iron per milligram of sputum.

Data analysis

Data are presented as medians and ranges unless otherwise noted. Biochemical parameters for each patient during early and late CFPE were compared using the Wilcoxon matched-pairs signed-rank test. The strength of association between nominal variables is given by Spearman rank correlation (ρ). A two-tailed p-value of <0.05 was considered statistical significant. GraphPad Prism® 5.0 (GraphPad Software, San Diego, CA, USA) was used for all analyses.

Results

Patients and treatment

Clinical attributes of the cohort at the time of CFPE determination are listed in *Table 1*. The median age of the cohort was 32 years. In general, patients had severe pulmonary function impairment, nutritional insufficiency, CF-related diabetes, hypoferremia, and mild anemia, a chronic illness phenotype that we described in a larger cross-sectional study.[17] Fifty-eight percent of patients possessed two F508-CFTR alleles, which is similar to the frequency of homozygosity for this mutation in Caucasians.[22] Patients received antibiotics for a median of 12 days (range: 5–19), which is congruent with pervasive practice patterns.[23]

CFPE Treatment is associated with weight gain and improved lung function

We compared clinical parameters for subjects in early versus late CFPE. In all cases, early CFPE was a point <24 hours after CFPE determination, and late CFPE was a point <24 hours before finishing treatment. Median increases in body weight and FEV_1% were 2.0 kg and 4.5%, respectively ($p < 0.01$ for late CFPE vs. early CFPE; *Figures 1A and B*). Body weight and FEV_1% were

	N = 12
Age (years)	32 (18–53)[a]
Gender (M/F)	9/3
FEV_1 (% predicted)	29 (21–48)[a]
Body weight (kg)	55.5 (40.2–75.1)[a]
ΔF508-CFTR homozygote	7 (58)[b]
CF-related diabetes	11 (92)[b]
Serum iron (μmol/L)	4.8 (1.6–16.1)[a]
Hemoglobin (g/dL)	12.9 (10.1–15.2)[a]

CFPE = CF pulmonary exacerbation. Data are presented as median and (ranges)[a] or number of subjects and (percentages)[b].

Table 1. Clinical characteristics of subjects at early CFPE.

significantly correlated at early CFPE (ρ = 0.76, $p < 0.01$) but not at late CFPE. FEV_1% directly correlated with serum iron at early CFPE (ρ = 0.58) and late CFPE (ρ = 0.65; $p < 0.05$ for both correlations).

CFPE treatment is associated with higher serum and lower sputum iron concentrations

Our previous work indicated a link between high serum iron, low sputum iron, and increased FEV_1%. Therefore, we assessed these parameters in CFPE subjects before and after treatment with IV antibiotics. According to our definition of hypoferremia (serum iron ≤12 µmol/L), 10 of 12 subjects (83%) were hypoferremic at early CFPE. Median serum iron rose by 2.42 µmol/L (13.5 mg/dL) by the end of therapy (*Figure 2A*). All but one of the subjects who were hypoferremic at early CFPE had serum iron readings <12 µmol/L at late CFPE and thus, remained iron-deficient. Only 9 of 12 subjects (75%) who furnished sputum samples at early CFPE were able to provide follow-up samples at late CFPE, which is not unusual for patients after completing a course of intravenous antibiotics. Among these subjects, median sputum iron fell by 0.92 ng/mg, a value that approached but did not attain significance at a 95% CI (p = 0.055; *Figure 2B*). Serum and sputum iron did not correlate significantly at early or late CFPE, which is a similar finding to our cross-sectional analysis of these parameters.[17]

CFPE treatment is associated with lower serum hepcidin-25 and IL-6 levels

We next quantified known mediators of iron homeostasis to explore possible mechanisms by which serum iron increased posttreatment. Median serum IL-6 fell by 12.1 pg/mL ($p < 0.05$ for late CFPE vs. early CFPE; *Figure 3A*). Median serum hepcidin-25 was 37.5 ng/mL lower at late CFPE ($p < 0.01$) (*Figure 3B*). Serum iron did not correlate with hepcidin-25 at either time point during CFPE; however, serum IL-6 was indirectly correlated with serum iron at late CFPE (ρ = –0.66, $p < 0.05$).

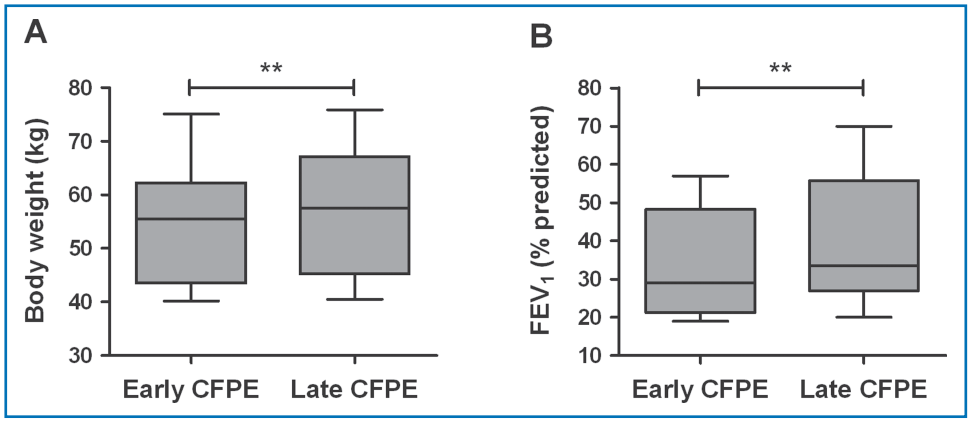

Figure 1. Changes in body weight and FEV_1% during treatment. Median values are denoted by the line within each shaded box. The upper and lower boundaries of the shaded box reflect the interquartile range of data. Whiskers signify minimum and maximum values. **$p < 0.01$ for comparison by Wilcoxon matched-pairs signed-rank test. CFPE = CF pulmonary exacerbation.

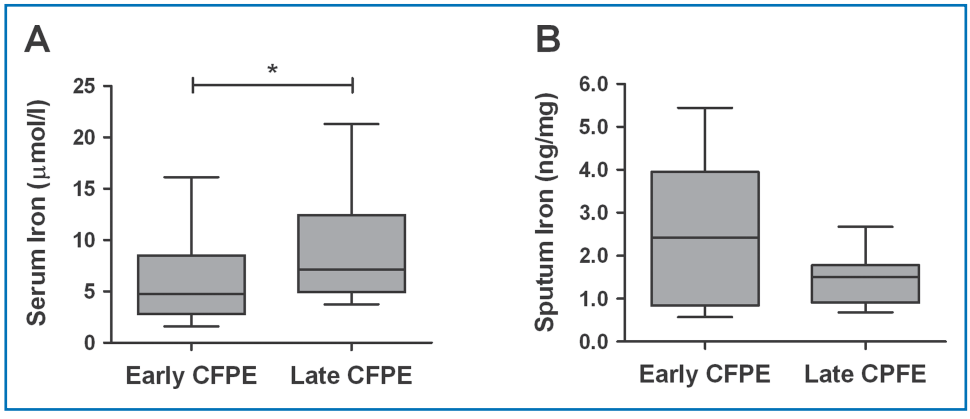

Figure 2. Changes in serum iron and sputum iron during treatment. Median values are denoted by the line within each shaded box. The upper and lower boundaries of the shaded box reflect the interquartile range of data. Whiskers signify minimum and maximum values. *$p < 0.05$ for comparison by Wilcoxon matched-pairs signed rank test. CFPE = CF pulmonary exacerbation.

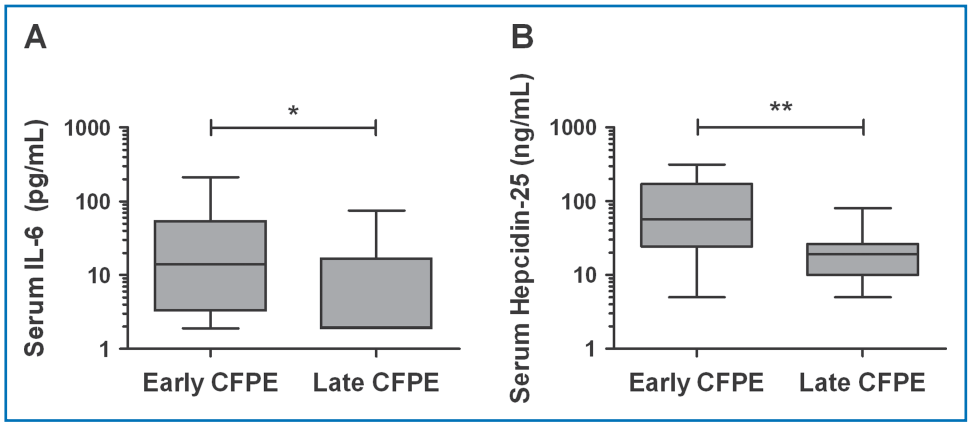

Figure 3. Changes in serum IL-6 and hepcidin-25 during treatment. IL-6 = interleukin-6. Median values are denoted by the line within each shaded box. The upper and lower boundaries of the shaded box reflect the interquartile range of data. Whiskers signify minimum and maximum values. *$p < 0.05$, **$p < 0.01$ for comparison by Wilcoxon matched-pairs signed-rank test. CFPE = CF pulmonary exacerbation. Note: logarithmic scale of ordinate.

CFPE treatment does not influence hemoglobin and serum erythropoietin

Given the observed changes in serum iron and regulators of iron homeostasis posttreatment, we next assessed the impact of

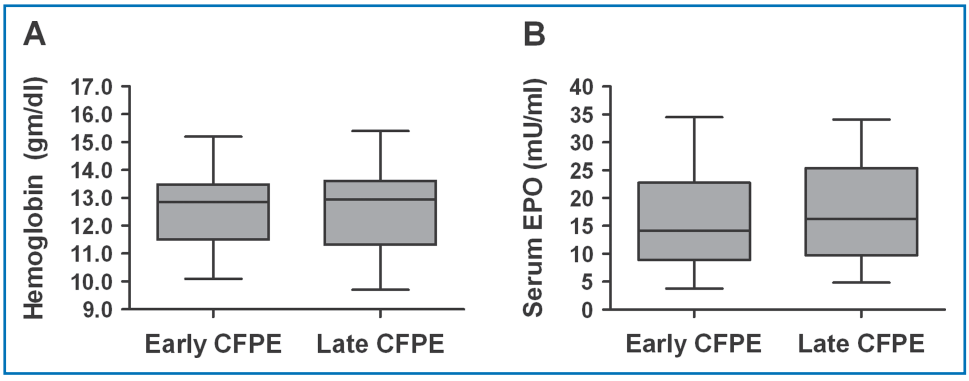

Figure 4. Changes in hemoglobin and serum erythropoietin during treatment. Median values are denoted by the line within each shaded box. The upper and lower boundaries of the shaded box reflect the interquartile range of data. Whiskers signify minimum and maximum values. CFPE = CF pulmonary exacerbation. EPO = erythropoietin.

these changes on anemia. During the treatment interval, median hemoglobin increased by only 0.1 gm/dL (*Figure 4A*). The median serum EPO concentration rose by 2.1 mU/mL (*Figure 4B*). Neither difference was statistically significant. Serum iron directly correlated with hemoglobin at early CFPE ($\rho = 0.87$, $p < 0.01$) and late ($\rho = 0.58$, $p < 0.05$) CFPE. Sputum iron did not correlate with hemoglobin. Thus, the observed changes in iron and mediators of iron homeostasis had no discernable effects on anemia.

Discussion

This prospective study was done to characterize iron homeostasis within the context of CFPE, periods of acutely compromised health that frequently occur in this disease and adversely affect prognosis. In a small cohort of adults treated with antibiotics and supportive care, we found that improved lung function and weight gain were associated with higher serum iron and lower circulating levels of mediators known to restrict iron bioavailability. Most of the subjects remained hypoferremic and mildly anemic despite statistically significant increases in serum iron. We observed a trend toward lower sputum iron content after treatment in 9 of the 12 subjects for whom samples were available from early and late CFPE.

Studies focusing on CFPE treatment responses are methodologically diverse; however, those in which intravenous antibiotics are used and changes in FEV_1% and body weight are reported, as in our study, demonstrate that these parameters improve modestly and commensurately.[24,25] Therefore, our subjects' treatment responses were not unusual. According to the 2010 CF Foundation patient registry, the median duration of intravenous antibiotic therapy for adults with CFPE in the United States was 14.7 days, with a range of 7.8–23.3 days.[26] The median in our study was 12 days with a range of 5–19 days, slightly shorter than nationwide medical practice. Treatment duration certainly could impact the extent to which biomarkers of iron homeostasis vary. Nonetheless, in our study, the subject with the shortest treatment interval (5 days) still experienced a 0.5 kg increase in body weight, a 6% improvement in FEV_1%, a 1.79 µmol/L increase in serum iron, and reductions in serum IL-6 and hepcidin-25 of 2 pg/mL and 104.9 ng/mL, respectively. These trends are similar to those observed among subjects treated for longer periods. Discerning how the length of therapy influences iron homeostasis during CFPE will require a study in which this parameter is controlled.

A significant limitation of our work is the inability to distinguish which interventions in a multifaceted regimen contributed more or less dramatically to the endpoints of better lung function and weight gain. Subjects not only received dual systemic antibiotic coverage for PA but also nutritional supplementation and daily airway clearance sessions to mitigate secretions. The majority of the cohort was diabetic, and as such, received insulin for glycemic control. Because these therapies represent standards-of-care in CF, it will be difficult to evaluate how a specific modality influences these metrics.[2,27]

To our knowledge, this is the first investigation to show that serum iron increases in response to CFPE therapy and the first to offer insights into possible mechanisms for this finding by examining trends in hepcidin-25 and IL-6. Cross-sectional analyses[18,28] of CF patients with varying disease severity report that hypoferremia has a prevalence of 62–74%. Applying a similar definition of iron deficiency to two adult CF cohorts, we found that subjects who recently experienced clinical deterioration had higher IL-6 and lower iron levels in plasma than those in good health,[17] which led us to suspect that IL-6 mediates hypoferremia in this disease. Nixon et al.[29] found that mean serum IL-6 in 12 patients with CFPE fell by 5.12 pg/mL ($p < 0.01$) after antibiotics, but they did not investigate how reductions in this cytokine related to changes in serum iron, hepcidin-25, and hemoglobin. Therefore, the median reduction in serum IL-6 that we observed (12.1 pg/mL) not only supports their observation but also highlights its clinical relevance by demonstrating how IL-6 relates to other biomarkers of iron homeostasis in CF.

We can find no published data supporting the contention that CFPE onset is heralded by increases in sputum iron. However, data from 34 subjects in 3 studies[9–11] suggest that sputum iron does not change significantly after antibiotics. Using a colorimetric assay to quantify total iron in denatured sputum samples from 14 patients, Reid et al.[9] found that median iron content was not different between the outset (47 µmol/L) and the end (33 µmol/L) of therapy ($p = 0.1$). The median duration of antibiotic administration in this study[9] was 12 days, which compares favorably with our median treatment interval (12 days). In six additional patients, Reid et al.[11] demonstrated that sputum iron was unaltered after a median of 8 antibiotic days (35.3 µmol/L vs. 35.6 µmol/L, $p > 0.05$). For a cohort of 14 patients, Gray et al.[10] used ICP-MS to show that sputum iron, unlike sputum zinc, did not fall significantly after CFPE therapy, although they do not provide the sputum iron data and do not comment on treatment modalities or their duration. Herein, we identified a trend toward lower sputum iron at the end of CFPE treatment in 9 of 12 subjects. A lack of paired samples for iron quantification in three instances is clearly a source of type II error that limits our ability to ascertain whether CFPE treatment reduces sputum iron. However, our observation that CFPE treatment does not change this parameter could also be consistent with the findings of the aforementioned authors.[9–11] Future investigations of sputum iron trends in CF should ensure uniformity of analytical techniques and treatment length and be adequately powered to detect a prespecified change in response to a specific intervention.

ICP–MS, the technique employed herein to measure total iron in each sputum aliquot, does not provide information about how the metal is distributed within the sample. The amount of iron in CF sputum could be proportional to the quantitative PA burden, as Reid et al.[11] advance, but it could also reflect iron bound to human airway proteins like ferritin and lactoferrin and to bacterial iron-scavenging molecules called siderophores.[30] Regardless of the metal's distribution, lower total sputum iron following CFPE treatment is theoretically advantageous because PA biofilm growth is augmented by iron.[7]

We provide evidence for ineffective erythropoiesis during CFPE, as hemoglobin levels were unaltered by treatment. Our data suggest two possible explanations for persistent anemia: serum iron was insufficient to support erythropoiesis and/or serum EPO did not adequately stimulate the growth and differentiation of erythrocyte precursors in the face of greater iron bioavailability. The former explanation is most likely given that erythropoiesis is impaired at serum iron levels $\leq$12 μmol/L,[20] and serum iron did not exceed this value in 75% of patients after treatment.

Our study design does not account for the potential effect of routine phlebotomy on lowering hemoglobin. Serial blood sampling for diagnostic tests can cause "iatrogenic" or "nosocomial" anemia during hospital admission, mainly because collected blood volumes often far exceed the minimum amount necessary for satisfactory assay performance.[31] For several reasons, this bloodletting phenomenon is unlikely to have contributed to our finding that median hemoglobin concentration did not increase by late CFPE. First, we obtained roughly 25 mL of blood at each of the two CFPE time points. A retrospective analysis of factors that significantly contributed to changes in hemoglobin values among general medical inpatients found that for every 50 mL of blood removed by phlebotomy, mean hemoglobin concentration would be expected to fall by 0.35 g/dL (95% CI, 0.24–0.46).[32] Median hemoglobin at late CFPE was actually a bit higher than at early CFPE (*Figure 4A*). Second, our subjects did not represent a hospitalized population at risk for large-volume phlebotomy. Wisser et al.[33] reported that iatrogenic blood loss of >200 mL is almost exclusively observed in patients with long-term mechanical ventilation, coagulation disorders, and repeated surgery. These features do not apply to the CF subjects in our study.

In conclusion, we found that recovery from CFPE is associated with higher serum iron levels, weight gain, and better lung function. The waning inflammatory milieu, as reflected by lower posttreatment serum hepcidin-25 and IL-6 concentrations, likely underlies the significant improvement in hypoferremia. Despite an increase in circulating iron reserves after treatment, a significant erythropoietic response was not identified. Our finding of no statistically significant reduction in sputum iron after antibiotics is consistent with several studies.[9–11] These findings raise the possibility that biochemical measures of iron homeostasis, particularly those from blood samples, could be used as surrogate endpoints in the design of CF treatment trials.

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