Iron Deficiency and Raised Hepcidin in Idiopathic Pulmonary Arterial Hypertension: Clinical Prevalence, Outcomes, and Mechanistic Insights

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Objectives

This study sought to understand the prevalence and clinical relevance of iron deficiency in patients with idiopathic pulmonary arterial hypertension (IPAH).

Background

Iron availability influences the pulmonary vascular response to hypoxia in humans and may be significant in the pathogenesis of IPAH.

Methods

Iron deficiency, defined by raised levels of soluble transferrin receptor (sTfR), was investigated in 98 patients with IPAH. Hepcidin and erythropoietin (EPO) levels were also measured. The effect of bone morphogenetic protein (BMP) receptor knockdown on BMP-6–stimulated hepcidin production was assessed in human hepatoma HepG2 cells. Relationships between sTfR and exercise capacity, functional class, and all-cause mortality were analyzed.

Results

Circulating sTfR levels were raised in 63% of IPAH patients, indicating significant iron deficiency. Consistent with this, iron, ferritin, and transferrin saturation levels were reduced and red cell distribution width increased, without overt anemia. Hepcidin correlated inversely with sTfR and positively with increasing ferritin. Hepcidin was inappropriately raised in IPAH independent of the inflammatory marker interleukin-6. EPO levels were also raised and correlated inversely with hepcidin. BMP receptor-type 2 (BMPR2) knockdown in HepG2 cells increased BMP-6–stimulated hepcidin expression. sTfR increased with World Health Organization functional class (p < 0.05), correlated negatively with exercise capacity (p = 0.027), and values >28.1 nmol/l independently predicted survival (p = 0.011).

Conclusions

Iron deficiency is common in IPAH patients and associated with disease severity and poor clinical outcome. Inappropriately raised hepcidin levels, which impair iron absorption from the gut, may be a factor.

Pulmonary arterial hypertension (PAH) is characterized by increased pulmonary vascular resistance due to vasoconstriction, thrombosis, and structural remodeling of pulmonary arterioles (1). Pressure overload of the right ventricle leads to progressive hypertrophy and dilation, followed by end-stage heart failure and death. Therapeutic options have increased in recent years, but the prognosis remains poor.

There is considerable interest in the role of iron status in PAH. Iron availability affects pulmonary hemodynamics; the availability of iron modifies basal pulmonary artery pressure and the pulmonary vasoconstrictor response to hypoxia in humans (2–4). Increased red cell distribution width (RDW), a clinical marker of iron-deficiency anemia, or the presence of anemia itself, is associated with a poor prognosis in pulmonary hypertension (5,6). Recently, iron deficiency, defined by reduced serum iron and transferrin saturations, was reported in 30 of 70 patients with idiopathic pulmonary arterial hypertension (IPAH), which correlated negatively with exercise capacity, a surrogate of prognosis. Interestingly, oral iron therapy was ineffective at restoring normal ferritin levels suggesting a problem with iron absorption (7).
A major regulator of body iron homeostasis is hepcidin, which acts to reduce dietary iron absorption and increase cellular iron storage (8). A number of factors may influence hepcidin levels in PAH. Of particular interest is bone morphogenetic protein (BMP) signaling. BMP receptor-type II (BMPR2) expression is reportedly reduced in all IPAH patients (9), and loss-of-function mutations in BMPR2 have been linked to >70% of familial PAH and 10% to 20% of IPAH cases (10). BMP signaling regulates hepatic expression of hepcidin (11–13). BMP-6 has emerged as the principal BMP regulating hepatic hepcidin expression in vivo, and its levels are regulated by iron in the liver and intestine (11–14). Iron stimulates BMP-6 expression (14), leading to increased hepcidin expression; conversely, BMP-6 knockout mice develop severe iron overload due to markedly reduced hepcidin expression (11). Reduced BMP signaling would predict raised hepcidin levels in PAH. Other factors that may affect hepcidin expression in PAH include inflammatory cytokines, hypoxia, erythropoietic activity, soluble transferrin receptor (sTfR), and growth differentiation factor (GDF)-15 (8).

We hypothesized that iron deficiency is prevalent and clinically important in IPAH and that increased hepcidin expression, possibly related to dysfunctional BMP signaling, might contribute to this. We investigated the relationship of iron deficiency to circulating hepcidin levels and survival in IPAH, and explored potential mechanisms of hepcidin dysregulation.

Methods

Patient samples. Plasma samples with sufficient volume for the required analyses were obtained from the Imperial College Pulmonary Hypertension biorepository. This repository comprises blood samples from consenting patients with IPAH attending Hammersmith Hospital, London, between 2002 and 2009. The diagnosis of IPAH was based on standard criteria with confirmation by right heart catheterization and exclusion of other forms of pulmonary hypertension (15). Hemodynamic data were acquired at catheterization. Patients with comorbidities that might affect iron homeostasis, including hemolytic anemias, genetic disorders of hemoglobin, diabetes, and systemic cardiovascular disease, were excluded. Survival status was censored on January 11, 2010. Healthy controls were recruited from Imperial College London, United Kingdom. Ethical approval was received from local research ethics committees. All samples were obtained with informed and written consent. Blood samples were coincident with measurements of World Health Organization (WHO) functional class, 6-min walk distance, and oxygen saturation. Plasma samples were collected into ethylenediaminetetraacetic acid (EDTA) tubes (chilled on ice) and stored at −80°C. Human liver tissue samples were used to demonstrate hepatic BMPR2 expression. Samples were obtained from unused transplant donor tissues, kept on ice, and then stored frozen at −80°C until use.

Circulating iron parameters. sTfR, GDF-15, and interleukin (IL)-6 levels were determined by enzyme–linked immunosorbent assay (R&D Systems Europe, Abingdon, Oxfordshire, United Kingdom). Hepcidin levels were determined by a competitive radioimmunoassay, as previously described (16). Erythropoietin (EPO) was measured using an Immulite-2000 analyzer (Siemens, Frimley, Surrey, United Kingdom). All other measures were determined by standard clinical pathology accredited hospital assays. Iron deficiency was defined by high levels of sTfR (normal range: 8.7 to 28.1 nmol/l), which are largely unaffected by inflammation and thus provide a more robust peripheral marker of body iron stores (17). Serum iron (normal range: male: 9 to 29 μmol/l; female: 7 to 27 μmol/l), transferrin saturation (<20%), and ferritin (<100 μg/l) were also measured. The normal range of hepcidin was previously determined at 2 to 55 ng/ml and independently validated in a second group of healthy volunteers (18). EPO had a normal range of 3.3 to 16.6 mIU/ml. Anemia was assessed by hemoglobin measurements (lower limits: females: 12 g/dl, males: 13 g/dl). RDW indexes were measured on the Sysmex-XE-2100 auto-analyzer (Sysmex, Wymbush, Milton Keynes, United Kingdom), a method based upon electrical impedance using hydrodynamic focusing.

Cell culture. HepG2 (human hepatocellular carcinoma) cells (LGC Standards, Teddington, Middlesex, United Kingdom) were seeded at 5 × 105 cells per well in 6-well plates in minimum essential medium (MEM, Sigma-Aldrich, Gillingham, Kent, United Kingdom) containing 10% fetal bovine serum and antibiotics/antimicrotics. Short interfering RNA (siRNA) transfections were performed the following day in OPTI-MEM medium, using Oligofectamine (Invitrogen, Paisley, United Kingdom) and siRNA duplexes that targeted a scrambled sequence or the type II BMP receptors, BMPR2, ActRIIA and ActRIIB (Invitrogen). Three days post-transfection, cells were treated with vehicle or 10 ng/well rhBMP-6 (R&D Systems). Protein was extracted in RIPA (radio-immunoprecipitation assay) buffer (Sigma-Aldrich). RNA was extracted using the Trizol method (Invitrogen).

Immunoblotting. Immunoblotting was performed using a mouse monoclonal antibody for human BMPR2 at 1:400 dilution overnight at 4°C (BD Biosciences, Oxford, United Kingdom) followed by anti-mouse IgG (GE Healthcare, Amersham, Buckinghamshire, United Kingdom) at 1:2,000 for 1 h at room temperature.
Reverse-transcription PCR. RNA was reverse transcribed using Thermoscript reverse-transcription PCR (RT-PCR) kits (Invitrogen). Twenty nanograms of cDNA product was used with 10 μl of PCR MasterMix (Promega, Southampton, Hampshire, United Kingdom). Primers (annealing temperatures and cycle number) were as follows: hepcidin (60°C, 33 cycles) forward primer: 5’-GGCTCTGGTTT-TCCACACACAG-3’, reverse: 5’-TCTTTCGCTCT-TGGAAACATGG-3’; Actin (60°C, 27 cycles) forward: 5’-CATGCCATCCTGGCTTGA-3’, reverse: 5’-CCGGGCGCATCCTCTTGT-3’; BMPR2 (53°C, 33 cycles) forward: 5’-CTTGGGGGCTTCCGCGAGA-3’, reverse: 5’-TGGTGGTGTGTGCAGAGGTGG-3’.

Data presentation and statistical analysis. Protein measurements are displayed as dot-plots, bars indicate medians. Clinical and hemodynamic data are presented as group mean ± SD, or as median values with interquartile ranges (IQRs). Several variables were not normally distributed, as shown by the Kolmogorov-Smirnov test, and were therefore transformed to their natural logarithm (ln) or square root, as appropriate to best normalize the data, before further analysis. Continuous data across categorical variables were compared by Kruskal-Wallis analysis of variance (ANOVA). Correlations were assessed by the Spearman rank test. Cox regression analyses identified prognostic predictors. Significant predictors from univariate analysis (p < 0.05) were then entered into a multivariable model. These variables were compared by stepwise backward hazard ratios Cox analysis; variables removed at p > 0.10. RT-PCR results were compared by ANOVA, with Tukey post-hoc analysis performed if p < 0.05. All calculations were performed with SPSS version 17.0 (SPSS, Inc., Chicago, Illinois) or GraphPad Prism4 (GraphPad Software Inc, La Jolla, California).

Results

Iron deficiency is prevalent in IPAH. Subject characteristics are detailed in Table 1. Circulating sTfR levels were raised (median: 33.0 nmol/l, IQR: 23.0 to 43.0 nmol/l) in IPAH, with 62 of 98 (63%) patients having levels above the normal range (>28.1 nmol/l) compared with 16.7% of healthy controls. sTfR levels were raised irrespective of warfarin therapy (Online Fig. 1). Higher sTfR levels were observed in IPAH versus controls across all age groups (Online Fig. 2A) and irrespective of gender (Online Fig. 2B). Seventy of the patients were British, but elevated levels could also be observed in some patients in other ethnic groups (Online Fig. 3).

Low serum iron (8.7 μmol/l, IQR: 5.0 to 14.0 μmol/l) and ferritin (50.0 μg/l, IQR: 27.5 to 142.0 μg/l) levels were present in the IPAH patients and transferrin saturations (19.0%, IQR: 12.0% to 29.0%) were <20% in over one-half of the patients assessed (Fig. 1). Levels in healthy volunteers are detailed in Online Table 1. Although a large proportion of the IPAH patients displayed features of iron deficiency, they were not overtly anemic, with mean hematocrit and hemoglobin levels of 0.47 ± 0.06 and 14.7 ± 2.5 g/dl (Fig. 1), and mean cell volume/hemoglobin values of 88.9 ± 9.1 fl and 29.8 ± 3.1 pg, respectively. Median red cell distribution width was 14.9% (IQR: 13.7% to 16.4%), towards the upper end of the normal range (8.3% to 17.5%). sTfR and ferritin levels did not differ between patients on different PAH therapies (Online Table 2).

Hepcidin levels are abnormal in IPAH and relate to iron status. Hepcidin levels were significantly higher in patients with IPAH, compared with healthy controls (34.5 ng/ml, IQR: 3.6 to 83.0 ng/ml; n = 98 versus 25.5 ng/ml, IQR: 11.7 to 50.0 ng/ml; n = 106; p = 0.001), and 33 (34%) patients had concentrations above the previously defined upper limit of 55 ng/ml (Fig. 2A) (18). Hepcidin levels did not correlate with IL-6 values before (Online Fig. 4) or after adjusting for iron status (Fig. 2B), suggesting raised hepcidin levels were not due to inflammation. In agreement with this, no correlation was observed with C-reactive protein in a subgroup of patients where C-reactive protein measurements were available (rho = 0.02, p = 0.92, n = 45). In contrast to healthy controls, where all hepcidin levels were >2 ng/ml, there was also a cohort of 21 (21%) IPAH patients with hepcidin levels <2 ng/ml (Fig. 2A) that exhibited marked iron deficiency (Figs. 2B and 2C). Whereas hepcidin levels were lowest in these patients, correlating inversely with sTfR (Fig. 2B) and positively with...
increasing ferritin levels (Fig. 2C), a significant proportion of patients with iron deficiency displayed inappropriately normal or even high hepcidin levels. Linear regression modeling confirmed that IPAH patients had significantly higher hepcidin levels than controls after adjusting for sTfR levels (Fig. 2B) or ferritin and EPO levels (Online Table 3). Hepcidin levels did not differ between subgroups on different PAH-targeted therapies (Online Table 2).

Hepcidin stimulation by BMP-6 is increased by BMPR2 knockdown. Human hepatoma HepG2 cells express both BMPR2 and hepcidin, and in response to BMP-6 stimulation, hepcidin mRNA expression and protein secretion are significantly increased (Fig. 3). Knockdown of BMPR2 by siRNA 3 days prior to treatment (Online Fig. 5) augmented the response to BMP-6 (Fig. 3). This augmentation was not seen when either activin receptor type II A or B (ActRIIA and ActRIIB) were knocked down with siRNA. Basal hepcidin expression was unchanged. Significant BMPR2 expression was also demonstrated in human liver tissue samples obtained from unused donors (Fig. 3).

Erythropoietin is raised in IPAH and relates to lower hepcidin levels. EPO levels were raised in the IPAH population (17.3 IU/l, IQR: 12.1 to 43.0 IU/l vs. 9.7 IU/l, IQR: 7.4 to 12.6 IU/l in controls, p < 0.001) and correlated inversely with hepcidin (Figs. 4A and 4B). EPO levels did not correlate with red blood cell counts, hematocrit, nor hemoglobin levels, but did correlate with RDW (rho = 0.527, p < 0.001). EPO levels did not correlate with peripheral oxygen saturations or plasma hemoglobin content, but did correlate inversely with the product of these 2 measures (rho = −0.357, p = 0.006).

We found raised GDF-15 levels in patients with high sTfR (Table 2), which also correlated with increased RDW (rho = 0.332, p < 0.001). GDF-15 did not correlate with EPO (p = 0.1) or hepcidin (p = 0.8).

sTfR levels relate to functional class, exercise capacity and survival in IPAH. sTfR levels were related to other iron indexes, increased with WHO functional class (p < 0.0001) (Fig. 5A), and correlated negatively with 6-min walk distance (Table 2). During the follow-up period, 39 patients died. Patients with raised sTfR (>28.1 nmol/l) had higher mortality (p = 0.022) (Fig. 5B). sTfR levels predicted mortality in IPAH, and this was independent of WHO functional class, 6-min walk distance, and age (p = 0.011) (Table 3). Whether patients were sampled before or after treatment was commenced did not predict survival in this group (p = 0.09) (Table 3).
Discussion

The importance of iron regulation in cardiovascular disease (19) and in the control of pulmonary vascular tone is now recognized (2–4). We have identified iron deficiency without overt anemia in a large proportion (approximately two-thirds) of patients with IPAH, characterized primarily by raised plasma sTfR, but also by reduced serum iron, transferrin saturation, and ferritin levels and increased RDW.

Circulating soluble transferrin receptor levels are a sensitive marker of tissue iron availability, which, unlike ferritin and serum iron measurements, are unaffected by inflammation (17). When iron deficiency is present, sTfR levels are increased disproportionately to erythropoietic activity (20), as the bone marrow attempts to maximize iron uptake. For these reasons, sTfR is the best available circulating indicator of iron deficiency in diseases with an inflammatory component, such as IPAH.

The cause of iron deficiency in IPAH is not immediately clear. Three possibilities to be considered are chronic blood loss, polycythemia due to the development of hypoxemia, and insufficient iron absorption from the gut. The majority of IPAH patients receive warfarin and are female, many of whom are pre-menopausal, and this might implicate blood loss. However, this is not a complete explanation. sTfR levels were also raised in patients not taking warfarin and did not significantly differ with gender nor age; women above age 60 years and men with IPAH also exhibited raised sTfR levels.

This agrees with the data of Ruiter et al. (7), who observed an equal prevalence of iron deficiency in pre- and
post-menopausal women with IPAH and no reported abnormal gastrointestinal or gynecological blood loss. Furthermore, the distribution of plasma hepcidin levels in the IPAH patient population is not consistent with blood loss as a single cause; iron deficiency from chronic blood loss would normally be associated with reduced circulating hepcidin (21). The iron-deficient patients had lower peripheral oxygen saturations, but in the absence of an associated
increase in hemoglobin or hematocrit, it is unlikely that reactive polycythemia could explain the deficiency seen. Hepcidin inhibits dietary uptake and mobilization of iron via degradation of the unique iron exporter, ferroportin, on enterocytes and reticuloendothelial cells, respectively (8). The low or undetectable hepcidin levels observed in some of the profoundly iron-deficient IPAH patients may represent an attempt to increase dietary iron absorption. 

The low or undetectable hepcidin levels observed in some of the profoundly iron-deficient IPAH patients may represent an attempt to increase dietary iron absorption. Hepcidin levels raised or maintained at normal levels in the presence of raised sTfR in a significant proportion of IPAH patients.

### Table 2: Patient Characteristics Stratified by Normal/High sTfR Levels

<table>
<thead>
<tr>
<th>Parameter</th>
<th>sTfR Levels</th>
<th>Spearman Rank Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal (8.7–28.1 nmol/l)</td>
<td>High (&gt;28.1 nmol/l)</td>
</tr>
<tr>
<td>Age, yrs</td>
<td>(n = 36)</td>
<td>(n = 62)</td>
</tr>
<tr>
<td></td>
<td>45.5 ± 16.0</td>
<td>48.1 ± 15.7</td>
</tr>
<tr>
<td>WHO class</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I/II</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>III</td>
<td>19</td>
<td>35</td>
</tr>
<tr>
<td>IV</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>Peripheral O₂ sat., %</td>
<td>95.0 (93.0–98.0)*</td>
<td>93.5 (88.0–95.5)*</td>
</tr>
<tr>
<td>O₂ sat. post-6-min walk, %</td>
<td>95.0 (88.0–97.0)*</td>
<td>88.5 (80.0–95.0)*</td>
</tr>
<tr>
<td>Iron, µmol/l</td>
<td>13.0 (7.4–16.6)*</td>
<td>7.7 (3.3–12.1)*</td>
</tr>
<tr>
<td>TIBC, µmol/l</td>
<td>59.0 (55.0–67.0)*</td>
<td>61.9 (47.3–68.0)*</td>
</tr>
<tr>
<td>Transferrin saturation, %</td>
<td>27.0 (18.8–35.1)*</td>
<td>15.6 (10.7–22.0)*</td>
</tr>
<tr>
<td>Ferritin, µg/l</td>
<td>66.5 (38.0–170.0)*</td>
<td>43.0 (20.5–91.5)*</td>
</tr>
<tr>
<td>sTfR/log ferritin</td>
<td>0.8 (0.7–1.0)*</td>
<td>1.9 (1.4–2.8)*</td>
</tr>
<tr>
<td>Hepcidin, ng/ml</td>
<td>53.0 (31.5–102.5)*</td>
<td>14.4 (0.9–56.0)*</td>
</tr>
<tr>
<td>EPO, mIU/ml</td>
<td>12.5 (10.0–19.5)*</td>
<td>32.5 (14.8–52.0)*</td>
</tr>
<tr>
<td>RBC, x10^12/l</td>
<td>4.8 ± 0.6</td>
<td>5.3 ± 0.6</td>
</tr>
<tr>
<td>Hemoglobin, g/dl</td>
<td>15.3 ± 1.7</td>
<td>14.4 ± 2.8</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.43 ± 0.05</td>
<td>0.45 ± 0.04</td>
</tr>
<tr>
<td>Red cell distribution width, %</td>
<td>13.9 (13.5–14.9)*</td>
<td>15.7 (14.4–17.6)*</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>2.1 (1.5–4.7)*</td>
<td>5.6 (2.9–10.8)*</td>
</tr>
<tr>
<td>GDF-15, pg/ml</td>
<td>549 (350–875)*</td>
<td>915 (359–1,860)*</td>
</tr>
<tr>
<td>NT-proBNP, ng/l</td>
<td>532 (186–1,031)*</td>
<td>838 (410–1,448)*</td>
</tr>
</tbody>
</table>

Clinical and biochemical measures are stratified by the upper limit of the normal range (28.1 nmol/l). Statistics shown are from Kruskal-Wallis analysis of variance for World Health Organization class and Spearman rank correlations for all other parameters. Bold type indicates significant relationship. *Variable is not normally distributed and therefore given as median (interquartile range).

NT-proBNP = N-terminal pro-B-type natriuretic peptide; RBC = red blood cells; sTfR = soluble transferrin receptor; TIBC = total iron binding capacity.
suggests that inappropriate hepcidin production may contribute to the iron deficiency by inhibiting dietary iron uptake. This hypothesis is supported by the observation that only a small proportion of IPAH patients respond to oral iron therapy (7).

Regulation of hepcidin production in IPAH. In the investigation of the factors underlying elevated hepcidin levels in IPAH, 3 mechanisms were considered: inflammation, BMP signaling, and erythropoietic activity.

Inflammation can raise hepcidin levels, primarily through IL-6 induction (22), and raised hepcidin production underlies anemia of chronic disease (23). IL-6 levels were raised in IPAH versus controls, and despite low serum iron and transferrin saturations, most patients had ferritin levels within the normal range, indicating that inflammation was present in these patients to a certain degree. However, sTfR levels were raised, and plasma hepcidin did not correlate with IL-6 in IPAH patients whether or not iron status was taken into account by considering sTfR or ferritin levels in parallel. This is inconsistent with anemia of chronic disease, where ferritin levels are normal or increased and sTfR levels repressed (23). Combined, our data suggest that inflammation and IL-6 stimulation, though present, are not the main cause of raised hepcidin levels in IPAH.

BMP signaling may link IPAH and dysregulated hepcidin production. BMPR2 protein expression is reduced in all patients with IPAH (9), and loss-of-function mutations in BMPR2 increase susceptibility to the development of PAH (10). In human liver, BMP-6 signaling is postulated to involve ActRIIA rather than BMPR2 receptors (24), but down-regulation of BMPR2 can increase BMP-6 and -7 signaling through ActRIIA (25). Indeed, using hepatocellular carcinoma cells, we have shown that BMPR2, but not ActRIIA or ActRIIB, knockdown leads to a concomitant increase in hepcidin production in response to BMP-6 stimulation. We also demonstrated BMPR2 mRNA expression in 5 of 5 of the human liver samples we examined, which has not previously been shown (24). Down-

![Figure 5](image_url) **Figure 5** sTfR Levels in IPAH

**A** Distribution of soluble transferrin receptor (sTfR) levels according to World Health Organization (WHO) functional class in patients with IPAH and in healthy controls. p value relates to Kruskal-Wallis ANOVA. **B** Kaplan-Meier survival estimates stratified by sTfR level below (blue solid line) or above (green dashed line) the pre-defined upper limit of the normal range, 28.1 nmol/l.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Simple Model Hazard Ratio (95% CI)</th>
<th>Sig.</th>
<th>Multiple Covariate Model Hazard Ratio (95% CI)</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (per 10 yrs)</td>
<td>1.387 (1.128–1.707)</td>
<td>0.002</td>
<td>0.493</td>
<td></td>
</tr>
<tr>
<td>WHO functional class</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I/II vs. IV</td>
<td>0.133 (0.038–0.468)</td>
<td>0.005</td>
<td>0.105</td>
<td></td>
</tr>
<tr>
<td>III vs. IV</td>
<td>0.535 (0.277–1.033)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-min walk distance (per 100 m)</td>
<td>0.618 (0.499–0.764)</td>
<td>&lt;0.001</td>
<td>0.653 (0.515–0.829)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ln[Soluble transferrin receptor]</td>
<td>2.449 (1.292–4.642)</td>
<td>0.006</td>
<td>2.569 (1.240–5.320)</td>
<td>0.011</td>
</tr>
<tr>
<td>Peripheral artery O2 saturation, %</td>
<td>0.955 (0.901–1.012)</td>
<td>0.118</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated versus treatment naive</td>
<td>1.289 (0.961–1.728)</td>
<td>0.090</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Simple model indicates predictive power of individual variables. Significant predictors from univariate analysis were then entered into a multiple covariate model, which was developed by the backwards stepwise hazard ratios analysis method. Soluble transferrin receptor was normalized by In-transformation before entry into the model. **Bold type** indicates significant relationship.

CI = confidence interval; WHO = World Health Organization.
regulation of BMPR2 may augment plasma hepcidin levels in PAH, predicting that familial PAH may be associated with more severe iron deficiency and increased hepcidin expression.

Erythropoietic activity inhibits hepcidin expression under anemic conditions (26) and is impaired in anemia of chronic disease (23). Serum from patients with overactive erythropoiesis inhibits hepcidin expression in vitro (27) and recombinant human EPO administration lowers hepcidin levels in healthy volunteers (28). Dysfunctional erythropoiesis or erythropoietic signaling could contribute to inappropriately increased hepcidin levels. EPO levels correlated inversely with hepcidin in IPAH patients, consistent with a model where EPO directly or indirectly inhibits hepcidin production. EPO does not suppress hepatocyte hepcidin levels in vitro (29), and other circulating factors implicated as possible mediators include GDF-15 (27), a TGF-β superfamily member that is elevated in IPAH (30). GDF-15 was raised in iron-deficient patients, but did not correlate with EPO or hepcidin levels, suggesting GDF-15 is not important in regulating hepcidin in IPAH.

**Iron status and pulmonary hypertension.** sTfR levels increased significantly with disease severity, correlated inversely with exercise capacity, and independently predicted survival after adjustments for standard clinical measures including 6-min walk distance and WHO class. These data, together with the recent observation that RDW and anemia predict mortality in pulmonary hypertension (5,6), suggest that iron deficiency is clinically relevant in IPAH.

Iron status influences pulmonary vascular responses to hypoxia. Iron chelation elevates pulmonary vascular resistance in humans (2) and increases sensitivity to acute hypoxic exposure (3). Iron deficiency induced by venesection also exacerbates pulmonary vasoconstriction at high altitude (4), whereas iron infusion reduces these pulmonary vascular responses (3,4). The underlying mechanism may involve normoxic stabilization of hypoxia inducible factors (HIF) as HIF hydroxylases require iron as a cofactor (31,32). HIF-α-deficient mice develop less severe pulmonary hypertension and vascular remodeling when exposed to chronic hypoxia (33,34), and pulmonary endothelial HIF-1α expression is increased in PAH (35). By preventing HIF activation, iron replacement therapy may potentially reduce pulmonary endothelial dysfunction, HIF-dependent gene activation, and vascular remodeling in pulmonary hypertension.

Iron repletion increases exercise capacity and reduces New York Heart Association functional class in left heart failure (19,36,37). Contrary to previous observations (36), improvements were similar in patients irrespective of anemia (19). Coupled with our findings, this paves the way for a study investigating the role of iron-replacement therapy in IPAH.

**Study limitations.** One limitation of this study is the lack of longitudinal data; it would be of interest to investigate the onset and time course of iron deficiency in IPAH.

The control population in this study was younger and contained a more equal distribution of sexes; however, gender differences in hepcidin are related to lower ferritin levels in younger women, hence no gender-dependent differences in iron parameters were observed. The lack of occult blood stool measurements and gynecological questionnaire limits our ability to rule out bleeding as a cause of iron deficiency in IPAH. There is no reason to believe that our patients were receiving a poor diet. Furthermore, Ruiter et al. (7) found that more than one-half of IPAH patients who received oral iron therapy were unresponsive, suggesting that absorption, rather than dietary content, is a significant problem in IPAH. The survival analysis included patients who had commenced therapy. When this was analyzed as a factor, survival did not significantly differ between treated and treatment-naive groups. We would also like to emphasize that we are not suggesting sTfR levels should be utilized as a prognostic biomarker in IPAH, but that it illustrates the clinical relevance of iron deficiency. Although we elected to study only those patients with IPAH, it would be of interest for further studies to investigate the prevalence and clinical significance of iron deficiency in other forms of PAH to determine the potential for therapeutic intervention.

**Conclusions**

We have characterized iron deficiency in a significant proportion of patients with IPAH and demonstrate a relationship between sTfR levels and disease severity and outcome. Inappropriately raised hepcidin levels may contribute to the development of iron deficiency in some patients with IPAH. Importantly, iron status represents an accessible target for novel therapeutic intervention.

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APPENDIX

For supplemental figures and tables, please see the online version of this article.