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RESEARCH ARTICLE

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Is iron deficiency caused by *BMPR2* mutations or dysfunction in pulmonary arterial hypertension patients?

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Abstract

Iron deficiency is common in idiopathic and heritable pulmonary arterial hypertension patients (I/HPAH). A previous report suggested a dysregulation of the iron hormone hepcidin, which is controlled by BMP/SMAD signaling involving the bone morphogenetic protein receptor 2 (BMPR-II). Pathogenic variants in the *BMPR2* gene are the most common cause of HPAH. Their effect on patients' hepcidin levels has not been investigated. The aim of this study was to assess whether iron metabolism and regulation of the iron regulatory hormone hepcidin was disturbed in I/HPAH patients with and without a pathogenic variant in the gene *BMPR2* compared to healthy controls. In this explorative, cross-sectional study hepcidin serum levels were quantified by

enzyme-linked immunosorbent assay. We measured iron status, inflammatory parameters and hepcidin modifying proteins such as IL6, erythropoietin, and BMP2, BMP6 in addition to BMPR-II protein and mRNA levels. Clinical routine parameters were correlated with hepcidin levels. In total 109 I/HPAH patients and controls, separated into three groups, 23 *BMPR2* variant-carriers, 56 *BMPR2* noncarriers and 30 healthy controls were enrolled. Of these, 84% had iron deficiency requiring iron supplementation. Hepcidin levels were not different between groups and corresponded to the degree of iron deficiency. The levels of IL6, erythropoietin, BMP2, or BMP6 showed no correlation with hepcidin expression. Hence, iron homeostasis and hepcidin regulation was largely independent from these parameters. I/HPAH patients had a physiologically normal iron regulation and no false elevation of hepcidin levels. Iron deficiency was prevalent albeit independent of pathogenic variants in the *BMPR2* gene.

KEYWORDS
genetics, hepcidin, iron homeostasis, pulmonary vascular disease

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a rare and severe form of pulmonary hypertension (PH) with high morbidity and mortality. It leads to increased pulmonary vascular resistance, right ventricular hypertrophy and right heart failure caused by a vascular remodeling of lung microcirculation.[1] In particular, heritable PAH (HPAH) patients with a pathogenic variant in the *bone morphogenetic protein receptor type II* (*BMPR2*) gene are characterized by an earlier age at diagnosis, worse hemodynamics and higher rate of lung transplantation as well as all-cause mortality,[2,3] Thus, it is an unmet need to improve targeted therapies and supportive treatments. Iron supplementation to correct for anemia or iron status is part of the general measures in the PH guidelines.[1] While anemia or even iron deficiency independent of anemia were reported to worsen the outcome of PAH patients,[4,5] three smaller pilot studies investigating iron supplementation in PAH patients provided inconclusive results.[6–8] Two randomized, placebo-controlled trials of patients with PAH who presented with iron deficiency in the absence of anemia showed no clinical benefit of i.v. iron supplementation after 12 weeks neither when data were analyzed separately nor when data were combined by the British and Chinese research groups.[9] While iron status improved in patients, exercise capacity, hemodynamics and NT-proBNP or BNP levels remained unchanged.[9] In contrast, in a rat model of PH and in cultured human pulmonary artery endothelial cells iron deficiency promoted oxidative stress and apoptosis which may contribute to the worse clinical outcome in iron deficient patients.[10] Overall, iron supplementation should be considered as a general measure in anemic patients and may be considered in PH patients with iron deficiency according to the new PH guidelines.[1] Thus, it remains essential to understand iron deficiency in PAH patients and to identify the subset of patients who may benefit from iron supplementation.

The underlying mechanism of iron deficiency or anemia development in PAH patients is not well established. One hypothesis for a disturbed iron status is that elevated levels of the iron-regulatory hormone hepcidin in PAH patients lead to impaired iron uptake in the gut.[4] Another explanation could be related to the reduced BMPR-II signaling in PAH patients.[11,12] BMPR-II is expressed in hepatocytes, where it plays a critical role in activating hepcidin expression in response to bone morphogenetic protein 2 (BMP2) and BMP6 ligands. In healthy subjects binding of the ligand BMP6 to the BMP type one receptor ALK-2 and the BMP type 2 receptor BMPR-II leads to an increase in hepcidin gene expression.[13,14] Similarly, the binding of BMP2 or a BMP2/BMP6 heterodimer to the BMP type one receptor ALK-3 and BMPR-II also result in an increase of hepcidin levels.[14,15] Thus, a reduced expression of BMPR-II, for example due to pathogenic variants, would be expected to reduce hepcidin expression. However, the opposite was shown by Rhodes et al.[4] who demonstrated a paradoxical increase of hepcidin expression triggered by a knockdown of *BMPR2* in human hepatoma cells. The authors speculated that this hepcidin increase was

mediated via alternative BMP type 2 receptor (ActR-IIA) signaling which could be responsible for iron deficiency in PAH patients with downregulated BMPR-II signaling. This theory was supported by the observation of *BMPR2* variant-carriers showing higher rates of iron deficiency in comparison to *BMPR2* noncarriers.[5] Hence, the objective of this study was first, to measure the prevalence of iron deficiency at the time of study and in the past medical history of our I/HPAH patients, second, to quantify hepcidin and BMP pathway regulators in *BMPR2* variant-carriers, *BMPR2* noncarriers and healthy controls and thirdly, to investigate putative links between hepcidin, the BMPR-II pathway and iron deficiency in our patients.

METHODS

Study design

This was an explorative, cross-sectional study analyzing the iron status and hepcidin metabolism in PAH patients. Patients with HPAH were included at the PH centers in Dresden, Heidelberg, and Leipzig. All IPAH patients and age and gender matched healthy controls were enrolled in Heidelberg. Some information on this cohort has been published previously.[12]

The ethics committees of the Medical Faculty of Heidelberg University Hospital, the Technical University of Dresden and the University of Leipzig had no objection against the conduct of the study (internal numbers: S-226/2019, EK 495112019, 414/19-lk, respectively). All participants provided written informed consent. The study complied with the Declaration of Helsinki in its current version.

Study population

Inclusion criteria were patients aged ≥18 years with I/HPAH invasively diagnosed by right heart catheterization (RHC) with a mean pulmonary arterial pressure ≥25 mmHg, pulmonary arterial wedge pressure ≤15 mmHg and pulmonary vascular resistance >3 Wood Units according to PH guidelines at the time of the study.[16] Patients had to be under stable, optimized medical therapy for PAH for at least 2 months before entering the study. Healthy controls did not have an acute infection nor any cardiovascular or lung disease or any condition influencing iron metabolism. Exclusion criteria for all participants included pregnancy, lactation, intravenous iron supplementation within the preceding 2 months, acute infection or comorbidities affecting iron metabolism such as hemolytic anemias, genetic disorders of hemoglobin, diabetes, systemic cardiovascular disease, sickle cell disease, and thalassemia.

Clinical investigations

Routine clinical parameters for patient characterization included all parameters to confirm the correct diagnosis including hemodynamics measured by RHC, spirometry and lung function which were performed during the inclusion visit, as well as laboratory parameters for the assessment of renal function (creatinine, urea, glomerular filtration rate) or further parameters which might influence iron status.

Routine laboratory assessment of patients for the iron status included ferritin, serum iron, transferrin, transferrin saturation, soluble transferrin receptor, ferritin index calculated as soluble transferrin receptor/log of ferritin, hemoglobin, hematocrit, erythrocyte count, erythrocyte distribution width, mean erythrocyte individual volume, mean erythrocyte individual weight, mean corpuscular hemoglobin concentration, percentage of hypochromic erythrocytes, and erythropoietin. Inflammation status was assessed by measuring C-reactive protein (CRP) and interleukin-6 (IL6). For healthy controls ferritin, serum iron, transferrin, and transferrin saturation were measured.

Expression analysis

In addition to routine laboratory tests serum protein expression values were measured by enzyme-linked immunosorbent assay (ELISA) for hepcidin (Hepcidin 25 bioactive HS ELISA Kit; DRG), BMP2 (Human BMP-2 ELISA Kit; Elabscience) and BMP6 (Human BMP-6; RayBiotech). *BMPR2* expression in whole blood was measured by quantitative polymerase chain reaction (qPCR) and ELISA as reported previously.[12] Serum was frozen at −80°C before analysis. QPCR measurements were performed as technical triplicates, ELISA measurements were carried out as technical duplicates.

Genetic characterization

EDTA-blood samples were used to extract DNA by an automated procedure (Autopure or QIAsymphony; Qiagen). Next generation sequencing data was obtained with a patented (EP3507380) PAH-specific gene panel including all currently known PAH genes (customized SureSelect QXT kit; Agilent) as described previously.[2]

Multiplex ligation-dependent probe amplification (MLPA) was used to identify exon deletions and duplications in the genes *ACVRL1, BMPR2*, and *ENG* (P093-C2; MRC-Holland). Familial variants were sought by Sanger sequencing (ABI Genetic Analyzer 3130xl; Applied Biosystems) or MLPA. Variants were classified following the American College of Medical Genetics and Genomics guidelines.[17] Disease-causing pathogenic variants and likely pathogenic variants were defined for this work as "mutations."

Statistical analyses

Statistical analyses were carried out by two medical statisticians (N. B. and S. L.). Descriptive statistics with mean ± standard deviation and frequency tables were used to present data including patient characteristics, clinical parameters and laboratory data of iron metabolism. Frequency data were presented as *n* and % and were analyzed by χ^2 tests. Clinical characteristics and laboratory data of the three groups (*BMPR2* mutation-carriers, *BMPR2* noncarriers, and healthy controls) were compared with Mann–Whitney *U* test for independent samples. Differences between patients with and without iron deficiency were analyzed by Mann–Whitney *U* test and students' *t* test. Association between clinical characteristics and laboratory data was investigated by Pearson correlation. $p < 0.05$ were considered statistically significant. Due to the exploratory nature of the study, *p* values were not corrected for multiple testing. All analyses were performed with SPSS V 25.0 (IBM Corp) or SAS 9.4 for Windows (SAS Corp).

RESULTS

Patient characteristics

For this study 79 patients with an invasively confirmed diagnosis of IPAH or HPAH and 30 healthy controls were prospectively recruited between May 2019 and January 2020.[12] All patients received an extended iron status assessment in addition to the routine diagnostic testing. The *BMPR2* expression analysis and the cohort's clinical characteristics were described previously.[12] Briefly, of 79 patients, 58 were female (73%) with a mean age of 51.4 ± 16 years. Genetic testing revealed 23 (29.1%) PAH patients to be *BMPR2* mutation-carriers and 56 (70.9%) to be *BMPR2* noncarriers. Pulmonary pressure and pulmonary vascular resistance showed significantly higher values in *BMPR2* mutation-carriers versus *BMPR2* noncarriers at the time of the study (Table 1). Healthy controls were age and gender matched to IPAH patients. Out of 30 healthy controls, 22 were female (73%) with a mean age of 46.8 ± 13.9 years. Table 1 shows patients' characteristics of *BMPR2* noncarriers and *BMPR2* mutation-carriers including data of the extended iron status screening.

Iron storage status

In the past recorded medical history iron deficiency was identified at least once in 84% of patients (63 of 75 patients), defined as ferritin ≤50 μg/L (*BMPR2* mutation-carriers: 89% (17 of 19 patients), *BMPR2* non-carriers: 82% (46 of 56 patients, p = n.s.). Most of them were treated with intravenous ferric carboxy-maltose (Ferinject; Vifor Pharma) after iron deficiency was detected. On average *BMPR2* noncarriers received intravenous ferric carboxy maltose 103 ± 100 weeks before study enrollment ($n = 34$, 61%) and *BMPR2* mutation-carriers 110 ± 91 weeks before study enrollment ($n = 13$, 57%). At the timepoint of study inclusion 31 of 77 (40%) patients presented with iron deficiency. For two patients, ferritin values could not be determined. Thirty-nine percent of *BMPR2* noncarriers and 42% of *BMPR2* mutation-carriers were iron deficient (p = n.s.), Figure 1. Healthy controls were age and gender matched to *BMPR2* noncarriers and were, therefore, mostly young women for whom iron deficiency is not uncommon. Hence, also of "healthy controls," 43% were also iron deficient.

On average PAH patients showed an iron storage status with the cohort means within the lower third of the respective reference ranges. Mean ferritin of all patients was of 92.8 ± 91.9 μg/L (reference range 35–230 μg/L for women, 30–300 μg/L for men), serum iron 14.6 ± 6.8 μmol/L (reference ranges 12–27 μmol/L in women, 14–32 μmol/L in men), transferrin 2.4 ± 0.4 g/L (reference range 2.0–3.6 g/L), transferrin saturation 25.9 ± 16.3% (reference range 16%–45%), ferritin index 2.0 ± 1.75 (reference range <2.0 when CRP > 5 mg/L) and soluble transferrin receptor 3.2 ± 1.8 mg/L (reference range 1.9–4.4 mg/L for women, 2.2–5.0 mg/L for men). Respective values for *BMPR2* noncarriers and *BMPR2* mutation-carriers are listed in Table 1. Healthy controls showed higher or similar iron status parameters. Ferritin levels were 101.4 ± 99.6 μg/L, serum iron 17.0 ± 4.0 μmol/L, transferrin 2.7 ± 0.4 g/L, transferrin saturation 25.3 ± 7.1%, ferritin index 1.61 ± 0.79 and soluble transferrin receptor 2.66 ± 0.67 mg/L. Transferrin levels ($p < 0.001$) and serum iron levels ($p = 0.007$) were significantly higher in healthy controls compared to PAH patients (Figure 2).

TABLE 1 Clinical characteristics of *BMPR2* non-carriers and *BMPR2* mutation-carriers.

		***BMPR2* noncarriers ($n = 56$) Mean ± SD or *n* and (%)**	***n*°**	***BMPR2* mutation-carriers ($n = 23$) Mean ± SD or *n* and (%)**	***n*[a]**	***p* Value**
Characteristics	Age, years	51.67 ± 16.59		50.87 ± 14.70		0.38
	Sex, female	41 (73.2)		17 (73.9)		1.0
	Age at diagnosis, years	45.91 ± 17.77		30.95 ± 33.07	20	0.09
	$PaO_2 < 60$, mmHg	12 (21.4)		4 (18.2)		0.51
Iron status	Hepcidin, ng/mL	19.61 ± 18.75		19.16 ± 16.62		0.65
	Ferritin, µg/L	90.96 ± 91.26		97.62 ± 95.66	21	0.79
	Transferrin, g/L	2.31 ± 0.45		2.50 ± 0.3	20	0.001*
	Iron, µmol/L	14.16 ± 6.83		15.72 ± 6.86	20	0.019*
	Transferrin saturation, %	26.17 ± 17.30	54	24.64 ± 11.91	14	0.50
	Soluble transferrin receptor, mg/L	3.30 ± 1.90	55	2.94 ± 1.04	16	0.20
	Ferritin index	2.11 ± 1.97	54	1.62 ± 0.67	16	0.42
	Erythrocytes,/pL	4.75 ± 0.49		4.92 ± 0.48		0.13
	Hemoglobin, g/dL	13.96 ± 1.35		14.60 ± 1.64		0.16
	Hematocrit, L/L	0.42 ± 0.04		0.44 ± 0.04		0.11
	MCV, fL	88.38 ± 5.08		89.06 ± 6.09		0.62
	MCH, pg	29.54 ± 1.92		29.78 ± 2.48		0.59
	MCHC, g/dL	33.43 ± 1.01		33.23 ± 1.00		0.40
	Hypochromic erythrocytes, %	1.49 ± 3.58		0.62 ± 0.94	16	0.32
	RDW, %	14.02 ± 0.94		14.24 ± 1.1		0.20
	Erythropoietin, mU/mL	15.76 ± 12.70	55	13.09 ± 6.61	17	0.49
Laboratory	CRP, mg/L	6.61 ± 7.25		8.15 ± 13.11	20	0.43
	Interleukin-6, pg/mL	2.76 ± 3.39	55	2.01 ± 0.05	16	0.05
	Creatinine, mg/dL	1.23 ± 2.97		0.84 ± 0.21		0.80
	Urea, mg/dL	31.98 ± 14.98		30.97 ± 5.86	22	0.73
	Glomerular filtration rate, mL/min/1.73 cm^2	92.87 ± 26.95		88.92 ± 23.70		0.37
	NT-proBNP, ng/L	541.18 ± 961.64		725.90 ± 1114.58	22	0.48
BMP pathway	BMP2, pg/mL	396.3 ± 462.0		371.8 ± 292.9		0.52
	BMP6, pg/mL	773.3 ± 1888.6		652.0 ± 1029.9	18	0.41
	BMPR2, pg/mL	477.2 ± 428.5	38	513.0 ± 489.0	15	0.52
Most recent right heart catheterization	Mean pulmonary arterial pressure, mmHg	41.71 ± 14.44		48.41 ± 10.34	17	0.029*
	Cardiac output, L/min	4.22 ± 1.65	44	3.11 ± 1.92	8	0.003*
	Pulmonary arterial wedge pressure, mmHg	9.32 ± 3.38		9.40 ± 2.61	16	0.65
	Pulmonary vascular resistance, WU	9.2 ± 6.79	44	15.06 ± 6.49	14	<0.001*

Abbreviations: BMP(R), bone morphogenetic protein (receptor); CRP, C-reactive protein; PAH, pulmonary arterial hypertension; PaO_2, arterial blood oxygen pressure; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; RDW, red cell distribution width; WU, wood units.

[a]*n* is provided in case of missing values.

*Statistically significant with $p < 0.05$.

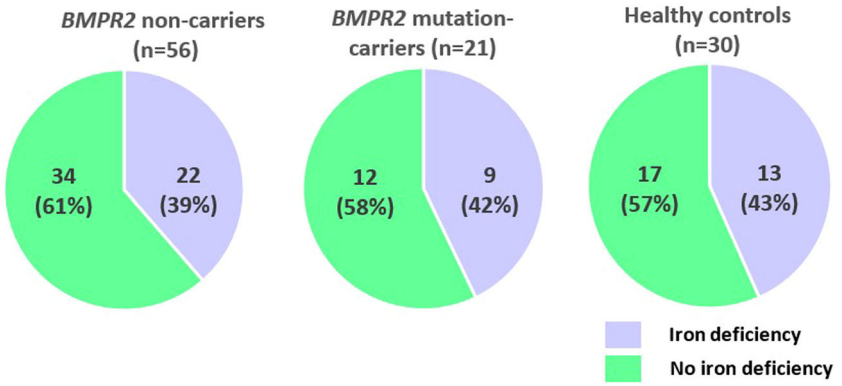

FIGURE 1 Frequency of iron deficiency in *BMPR2* noncarriers, *BMPR2* mutation-carriers and healthy controls at timepoint of study. Iron deficiency was defined as ferritin ≤50 μg/L. The prevalence of iron deficiency was similar between *BMPR2* mutation-carriers, *BMPR2* noncarriers and age and gender matched controls.

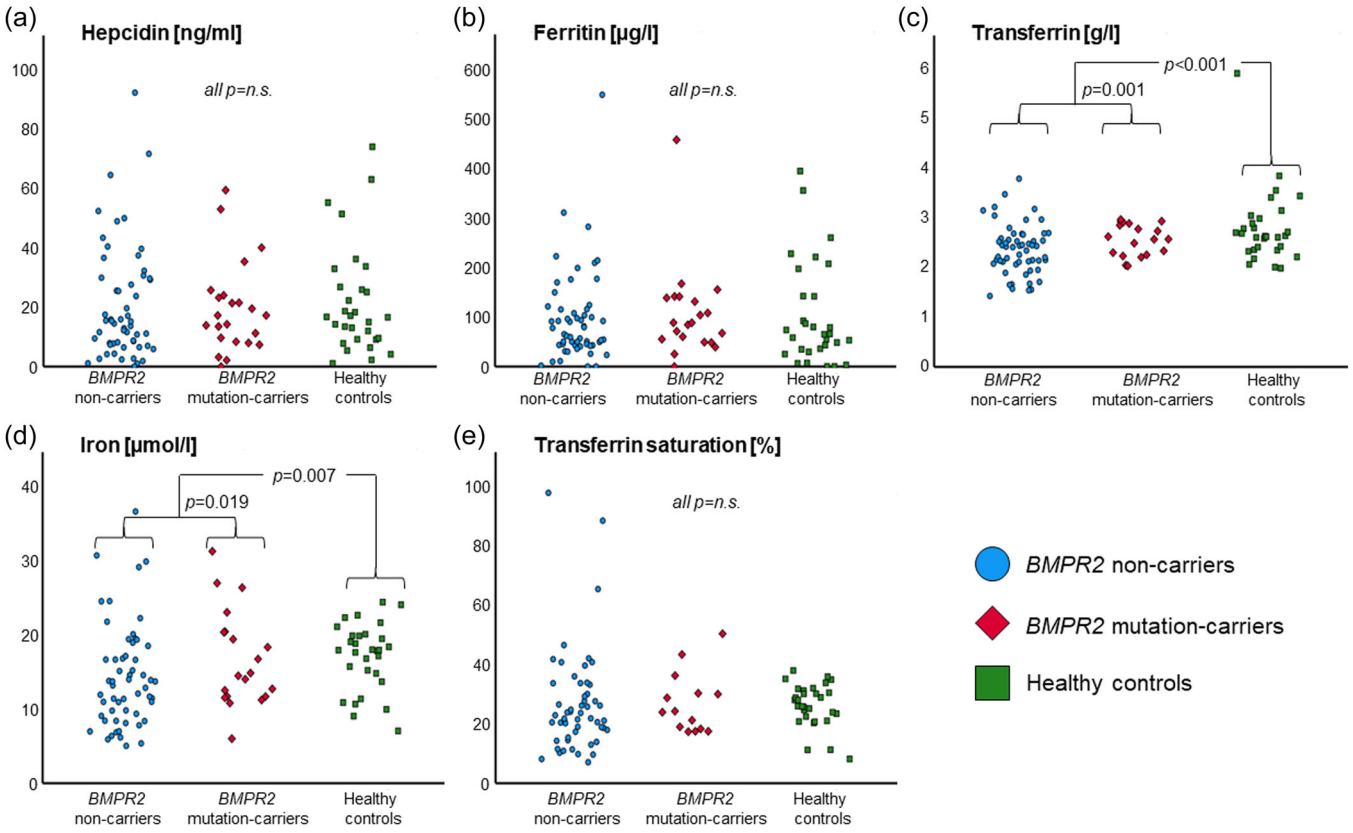

FIGURE 2 Scatterplots of iron parameters in *BMPR2* noncarriers, *BMPR2* mutation-carriers, and healthy controls. (a, b) Hepcidin and ferritin levels showed no difference between groups. (c) Transferrin was significantly higher in *BMPR2* mutation-carriers versus noncarriers and in healthy controls versus PAH patients. (d) Serum iron was significantly higher in *BMPR2* mutation-carriers versus noncarriers and in healthy controls versus PAH patients. (e) Transferrin saturation showed no difference between groups. PAH, pulmonary arterial hypertension.

Only six PAH patients (8%) presented with anemia defined as hemoglobin levels <12 g/dL for women and <13 g/dL for men. While only a small number of iron deficient patients were anemic, mean corpuscular volume ($p = 0.008$) and mean corpuscular hemoglobin ($p = 0.015$) were significantly lower in patients with ferritin levels ≤50 μg/L (Table 2). Also, the percentage of hypochromic erythrocytes was significantly higher in this cohort ($p < 0.001$). This is consistent with an empty iron store and first signs of insufficient erythropoiesis. This is also supported by significantly higher erythropoietin ($p = 0.017$), higher transferrin levels ($p < 0.001$), higher soluble transferrin receptor levels ($p < 0.001$) and lower serum iron ($p < 0.001$) and transferrin saturation in iron deficient patients ($p < 0.001$), Table 2.

TABLE 2 Difference in iron storage status in patients with and without iron deficiency.

Iron storage status	PAH patients with ferritin > 50 µg/L ($n = 46$) Mean ± SD or n and (%)	n[a]	PAH patients with ferritin ≤ 50 µg/L ($n = 31$) Mean ± SD or n and (%)	n[a]	p Value*
Hepcidin, ng/mL	28.0 ± 19.2		7.4 ± 5.0		<0.001
Ferritin, µg/L	134.9 ± 98.2		30.3 ± 13.1		<0.001
Transferrin, g/L	2.21 ± 0.36		2.60 ± 0.40	30	<0.001
Iron, µmol/L	16.3 ± 6.7		11.9 ± 6.2	30	<0.001
Transferrin saturation, %	30.7 ± 17.6		18.5 ± 10.6	30	<0.001
Soluble transferrin receptor, mg/L	2.86 ± 0.90	44	3.81 ± 2.51	27	0.031
Ferritin Index	1.42 ± 0.48	44	2.98 ± 2.57	27	<0.001
Erythrocytes,/pL	4.74 ± 0.47		4.88 ± 0.54		0.489
Hemoglobin, g/dL	14.3 ± 1.34		13.9 ± 1.59		0.117
Hematocrit, L/L	0.43 ± 0.04		0.42 ± 0.05		0.098
MCV, fL	90.0 ± 4.4		86.4 ± 6.0		0.008
MCH, pg	30.1 ± 1.4		28.8 ± 2.7		0.015
MCHC, g/dL	33.5 ± 0.9		33.2 ± 1.1		0.325
Hypochromic erythrocytes, %	0.57 ± 0.98	44	2.43 ± 4.82	28	<0.001
RDW, %	13.8 ± 0.7		14.5 ± 1.1		0.006
Erythropoietin, mU/mL	14.1 ± 12.7	44	26.2 ± 36.2	28	0.017
$PaO_2 < 60$, mmHg	7 (15.2)		9 (30)		0.105

Abbreviations: MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PAH, pulmonary arterial hypertension; PaO2, arterial blood oxygen pressure; RDW, red blood cell distribution width.

[a]n is provided in case of missing values;

*p-values of Mann-Whitney test

Hepcidin levels and pathway related parameters

Hepcidin values were significantly lower ($p < 0.001$, Table 2) in iron deficient patients, a physiological response that promotes iron absorption. No significant difference in hepcidin protein levels could be identified between *BMPR2* noncarriers (19.61 ± 18.75 ng/mL), *BMPR2* mutation-carriers (19.16 ± 16.62 ng/mL) and healthy controls (21.7 ± 18.0 ng/mL). Even when hepcidin values were analyzed in subgroups of patients with no signs of inflammation (CRP < 10 mg/L), no increased markers for hypoxia (erythropoietin <21.5 mU/mL) and with an adequate excretion via the kidney (glomerular filtration rate >50 mL/min/1.73 cm^2) no differences could be identified. Of the 31 (25.8%) iron deficient patients eight showed increased erythropoietin levels ≥21.5 mU/mL and very low hepcidin values (≤4 ng/mL), suggesting that mechanisms triggered by both, iron deficiency and the erythropoietin-induced blood hormone erythroferrone may be involved in reducing hepcidin levels. Hepcidin values more than double of the mean value (>40 ng/mL) of the cohort were identified in 10 patients. None of them showed iron deficiency. Of note, the reference values of hepcidin described for healthy pre-, postmenopausal women and men measured with the same experimental set-up,[18] were not significantly different to the hepcidin values of our cohort, when it was grouped in pre-, postmenopausal women and men.

Another interesting finding was that no correlation could be identified between serum hepcidin levels and BMPR-II protein expression or *BMPR2* mRNA levels or the ligands BMP2, BMP6. Values showed no differences between the three groups. Values of control patients were 17.0 ± 16.7 ng/mL for hepcidin, 283.8 ± 206.5 pg/mL for BMP2 and 617.1 ± 1175.9 pg/mL for BMP6. As previously reported, only *BMPR2* mRNA in whole blood but not protein levels in serum were statistically significant reduced in mutation-carriers versus *BMPR2* noncarriers.[12] Similar to the BMP ligands, inflammatory markers, such as IL6-levels or the C-reactive protein or

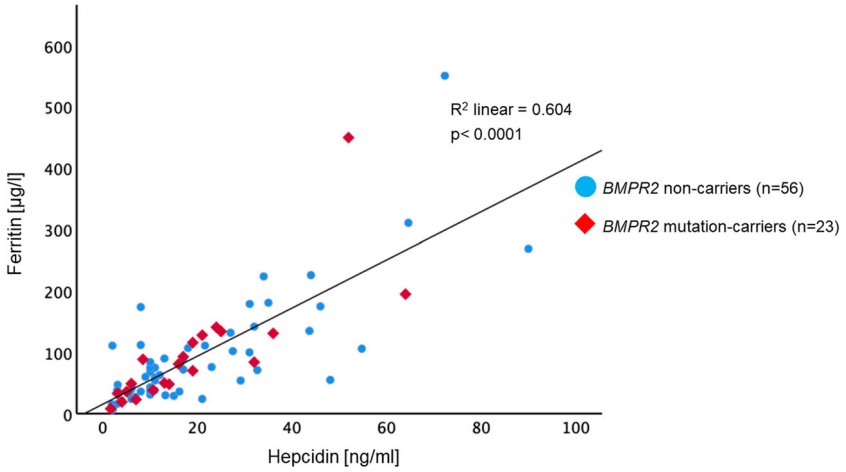

FIGURE 3 Scatterplot of the correlation of hepcidin and ferritin for *BMPR2* mutation-carriers and *BMPR2* noncarriers.
Data of *BMPR2* mutation-carriers is visualized using red dots, data of *BMPR2* noncarriers with blue squares.

TABLE 3 Correlation of hepcidin with further iron metabolism related parameters.

Iron parameters	Pearson's correlation	Sig. (2-tailed)	*n*[a]
Ferritin, µg/L	0.777	<0.001	77
Transferrin, g/L	−0.319	0.005	76
Iron, µmol/L	0.034	0.768	76
Transferrin saturation, %	0.074	0.550	68
Soluble transferrin receptor, mg/L	−0.247	0.038	71
Ferritin Index	−0.361	0.002	70
Erythrocytes,/pL	−0.245	0.030	
Hemoglobin, g/dL	−0.066	0.561	
Hematocrit, L/L	−0.094	0.412	
Mean corpuscular volume, fL	0.239	0.034	
Mean corpuscular hemoglobin, pg	0.219	0.052	
MCHC, g/dL	0.115	0.315	
Hypochromic erythrocytes, %	−0.225	0.057	72
Red cell distribution width, %	−0.206	0.068	
Erythropoietin, mU/mL	−0.064	0.594	72

Abbreviation: MCHC, mean corpuscular hemoglobin concentration.
[a]*n* is provided in case of missing values.

the hypoxia-indicating marker erythropoietin did not correlate with serum hepcidin levels.

By contrast, a strong positive correlation was observed in patients between serum hepcidin and ferritin (Pearson's correlation: 0.777, $p < 0.0001$; Figure 3), and a negative correlation with transferrin ($p = 0.005$), soluble transferrin receptor ($p = 0.038$), ferritin index ($p = 0.002$), and erythrocyte count (0.030) (Table 3).

DISCUSSION

In this study we comprehensively analyzed iron-related biomarkers in PAH patients and demonstrated that the iron deficiency observed is sensed correctly and reacted upon by a decrease in serum hepcidin levels. Strong correlations were observed between serum hepcidin and markers for iron availability in storage compartments (e.g., ferritin), the circulation (e.g., transferrin) and for the iron demand of erythropoiesis (e.g., soluble transferrin receptor). By contrast, signaling mediators such as BMP2 and BMP6 (secreted from liver sinusoidal endothelial cells), erythropoietin or IL6 did not correlate with hepcidin levels. We therefore propose that iron deficiency is sensed in hepatocytes directly. This finding is of clinical importance as this suggests that in principle also oral iron supplementation in PAH patients should be able to counteract iron deficiency as it should not hampered by falsely elevated hepcidin levels.

HEPCIDIN REGULATION IN PAH PATIENTS

The aim of this study was to investigate whether our PAH patient cohort showed elevated serum hepcidin levels as previously reported for PAH patients[4] and to clarify the potential impact of a disturbed BMPR-II pathway on the hepcidin regulation by comparing iron markers in *BMPR2* mutation-carriers and *BMPR2* non-carriers. Our data revealed that hepcidin values did not differ between *BMPR2* mutation-carriers, *BMPR2* non-carriers, and healthy controls. Differences between the data shown here and those by Rhodes et al.[4] are the median hepcidin levels, which were lower in our patient cohort (13 ng/mL) compared to 34 ng/mL reported by Rhodes and colleagues.

In the subgroup of iron deficient patients, all patients had hepcidin levels below or close to the mean value (19 ng/mL) of the cohort. This was even the case in patients presenting with elevated IL6 and/or CRP levels suggesting that hepcidin levels in these patients were not increased by inflammation. This agrees with previous studies in PAH patients reporting hepcidin values to be independent of the inflammatory markers IL6 and CRP.[4,7]

In the iron deficient patient subgroup, we selected those with additionally increased erythropoietin levels and could identify very low hepcidin values. Erythropoietin levels are increased in conditions of severe iron deficiency and anemia. In addition, erythropoietin stimulates the expression of the blood hormone erythroferrone, which sequesters BMP6 and thus decreases hepcidin mRNA transcription. As a consequence, dietary iron absorption and iron release from iron stores is increased.[13,19] Overall, these data suggest, that in principle PAH patients should be able to compensate their iron deficiency by increasing iron absorption. However, in clinical practice, PAH patients often do not respond adequately to oral iron substitution.

Hepatic hepcidin mRNA expression is controlled by BMP2/6 expressed in liver sinusoidal endothelial cells in response to iron signals.[20,21] BMP2/6 bind heterodimeric BMP type I and type II receptor complexes (including BMPR-II) to signal to hepcidin via SMAD proteins. As BMP2 and BMP6 levels in serum were unaltered when PAH patients were compared to the control cohort, iron deficiency appears to be adequately sensed by hepatocytes to reduce hepcidin levels.

Even though 23 patients in our cohort carried a heterozygous, pathogenic variant (mutation) in the gene *BMPR2*, no difference in hepcidin levels or any other iron biomarker was observed in comparison to *BMPR2* non-carriers. Interestingly, the same was recently shown in mice with a heterozygous *Bmpr2* mutation on an iron deficient diet.[22] Reduction of hepcidin mRNA levels were independent from the *Bmpr2* genotype of mice. Thus, the pathogenic variants in *BMPR2* leading to worse hemodynamics and even higher mortality in PAH patients[3] appear to be unrelated to the iron status of PAH patients. One explanation for the intact hepcidin regulation upon BMP2 or BMP6 binding could be the use of the alternative type 2 BMP receptor ActR-IIA.[15] The receptor BMPR-II and the ActR-IIA receptor can be used interchangeably to initiate the hepcidin activation cascade.[14]

IRON METABOLISM STATUS

More than 80% of our patients were iron deficient at least once according to their medical records. Even though they were regularly screened for iron deficiency and treated with intravenous iron supplementation therapy according to recommended supportive therapies in the PH guidelines,[16] the percentage of iron deficiency at the time of study enrollment remained high with about 40%. This percentage is in concert with previous studies measuring iron deficiency in IPAH or HPAH patients,[5,23,24] even though different definitions of iron deficiency were applied.

Iron supplementation had not been part of the general measures for PH treatment when Rhodes and colleagues undertook their study in 2011. Iron deficient PAH patients in their study presented with elevated hepcidin levels as well as serum iron and ferritin levels around half the values of our patient cohort. Thus, iron supplementation may have influenced long term iron storage levels of PAH patients.

Since our study now contradicts the previous hypothesis by Rhodes and colleagues, who attributed iron deficiency in PAH to inappropriately raised hepcidin levels, there is currently no clear explanation for the high prevalence of iron deficiency in our cohort despite regular iron supplementations. Additional mechanisms may be operational in PAH patients that restrict iron availability. For example, iron may be redistributed into macrophages by hepcidin-independent mechanisms involving ferroportin transcriptional repression.[25,26] Similar mechanisms may be operational in the gut.[27] Also, hepcidin levels in the systemic circulation may not be the same as in specific cells. For example, a downregulation of hepcidin via a *BMPR2* mutation was shown in murine pulmonary artery smooth muscle cells.[28] These and other hypotheses remain to be tested.

Of note is, however, a similar rate of iron deficiency in age and gender matched controls. In contrast to PAH patients, the control group has not received regular, high dose iron supplementation in the past. Thus, despite similar levels of iron deficiency at the study timepoint in patients and controls, there may be a more severe or more recurrent iron deficiency in PAH patients as one would have expected iron deficiency to be lastingly counteracted by regular, high dose iron supplementation in PAH patients.

LIMITATIONS

Only the most recent PH guidelines provided a definition of iron deficiency for PH patients based on established cut-offs from heart failure patients, namely, ferritin <100 μg/L or 100–299 μg/L and transferrin saturation <20%.[1] While our definition (ferritin ≤50 μg/L) defers from the current definition it should have identified patients with an even more severe iron deficiency. Our

healthy controls were age and gender matched to *BMPR2* noncarriers. Since they were no patients, no full laboratory assessment nor clinical measurements could be obtained. Similarly, we were restricted to the standard laboratory measurements obtained for patients. In this regard, ferroportin would have been another interesting iron pathway member to investigate. The employed methods could have also influenced identified protein levels. The ELISA to measure hepcidin levels in this study was not the same approach as used by Rhodes and colleagues who employed a competitive radioimmunoassay.[29] While the spread of hepcidin values was similar between studies, Rhodes and colleagues measured higher maximum and median levels. Also, the patient cohort itself may not be comparable between this study and Rhodes and colleagues. Due to the adapted PH guidelines in 2015 recommending i.v. iron supplementation in iron deficient patients, it was not possible to recruit a sufficient number of patients who had never received intravenous iron supplementation in the past. Nevertheless, our study reflects iron metabolism, investigated with state-of-the-art methods in a representative, current PAH population.

In conclusion, I/HPAH patients showed high prevalence of iron deficiency with an adequate hepcidin response. BMPR-II and hepcidin expression were unrelated and *BMPR2* mutation status had no influence on iron status. Thus, further studies are necessary to investigate the reasons for iron deficiency in PAH patients.

AUTHOR CONTRIBUTIONS

Christina A. Eichstaedt: Conceptualization; methodology; resources; data curation; writing—original draft preparation; writing—review and editing; visualization; supervision; project administration; funding acquisition. **VivienneTheobald**: Conceptualization; methodology; validation; formal analysis; investigation; data curation; writing—original draft preparation. **Ekkehard Grünig**: Conceptualization; methodology; resources; writing—original draft preparation; supervision; funding acquisition. **Nicola Benjamin**: Methodology; validation; formal analysis; data curation; visualization; writing—original draft preparation. **Hans-Jürgen Seyfarth**: Investigation; writing—review and editing. **Michael Halank**: Investigation; writing—review and editing. **Daniel Kazdal**: Investigation; writing- review and editing. **Marc A. Schneider**: Investigation; writing review and editing. **Sarah Richtmann**: Investigation; writing—review and editing. **Katrin Hinderhofer**: Investigation; resources; writing—review and editing. **Panagiota Xanthouli**: Investigation; writing—review and editing. **Benjamin Egenlauf**: Investigation; writing—review and editing. **Satenik Harutyunova**: Investigation; writing—review and editing. **Richard Sparla**: Investigation; writing—review and editing. **Marius M.Hoeper**: Investigation; writing—review and editing. **Danny Jonigk**: Investigation; writing—review and editing. All authors have read and agreed to the published version of the manuscript. **Martina Muckenthaler**: Conceptualization; methodology; resources; writing—review and editing; supervision.

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CONFLICTS OF INTEREST STATEMENT

B. J. received consulting, travel and speaking fees from Actelion, MSD, Bayer and OMT. C. A. E. received lecture fees from MSD and has an issued patent: “Gene panel specific for pulmonary hypertension and its uses” European Patent ID: EP3507380. D. K. received lecture fees from Agilent, AstraZeneca, BMS, Illumina, Incyte, Lilly, Pfizer, and Takeda. E. G. received research grants outside the submitted work from Actelion, Bayer, GSK, United Therapeutics, Novartis, Bellerophon, OMT, Pfizer, REATA and speaker honoraria outside the submitted work from Actelion, GSK, MSD. He has an issued patent: “Gene panel specific for pulmonary hypertension and its uses” European Patent ID: EP3507380. H. -J. S. received consulting fees from Actelion and speaker fees from Actelion, Bayer, GSK, Janssen and MSD. K. H. has an issued patent: “Gene panel specific for pulmonary hypertension and its uses” European Patent ID: EP3507380. M. H. received consulting and lecture fees from Acceleron, Actelion, AstraZeneca, Bayer, BerlinChemie, GSK, Janssen-Cilag, MSD, Novartis. M. M. H. received consulting and lecture fees from Acceleron, Actelion, Bayer, GSK, Janssen, MSD, Pfizer. N. B. received speaker fess from Actelion, Bayer and MSD. P. X. received personal fees from OMT and MSD. S. H. received personal fees from OMT and Janssen. The remaining authors declare no conflict of interest.

ETHICS STATEMENT

The ethics statement for this work is the study was conducted in accordance with the Declaration of Helsinki, and approved by the ethics committee of the Medical Faculty of Heidelberg University Hospital, Germany (protocol code S-226/2019, 25.04.2019).

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