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Letter

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A Comprehensive Analysis of the Erythropoietin-erythroferrone-hepcidin Pathway in Hereditary Hemolytic Anemias

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Iron overload is an important complication in both transfusion-dependent and transfusion-independent patients with hereditary hemolytic anemias. Hereditary hemolytic anemias encompass a heterogeneous group of anemias with varying levels of hemolysis and ineffective erythropoiesis, also varying within disease entities.

Generally, anemia induces production of erythropoietin (EPO), which activates early erythroid progenitors and their production of erythroferrone (ERFE).[1] ERFE is known as a negative regulator of hepcidin, the latter is regarded as the master regulator of systemic iron availability.[2] Hepatic hepcidin expression is upregulated by iron levels; and ERFE inhibits hepcidin expression.[3] Effectively, this EPO-ERFE-hepcidin pathway secures iron availability for erythropoiesis.

In addition, soluble transferrin receptor (sTfR) and growth differentiation factor 15 (GDF15) may play a role in the interplay between erythropoiesis and iron. sTFR is derived from cleavage of the membrane-bound transferrin receptor and plasma levels correlate with erythroid precursor mass. GDF15 is expressed and secreted during erythroblast maturation and has been associated positively with EPO. Uncertainty remains on the role of GDF-15 in hepcidin regulation in human, in whom it does not seem to be a physiologic regulator of hepcidin. Plasma levels are high in nontransfusion-dependent β-thalassemia and have been suggested to suppress hepcidin expression[4]; however, studies in GDF15-knockout mice indicate otherwise.[5]

In hereditary hemolytic anemias, the bone marrow is dominated by an expanded pool of erythroid progenitors displaying varying degrees of stress erythropoiesis and inefficient erythropoiesis.[6] Currently, no studies are available that report on a combination of the values of the individual components of the EPO-ERFE-hepcidin pathway in hereditary hemolytic anemias other than hemoglobinopathies. To characterize the interplay between erythropoiesis and iron levels, the parameters of the EPO-ERFE-hepcidin pathway were measured and assessed in patients with various forms of hereditary hemolytic anemias, with varying levels of disease severity and iron burden.

Plasma levels of ERFE and sTFR were quantified by Intrinsic ERFE IE ELISA Kit (Intrinsic Lifescience, CA) and human sTfR ELISA kit (RD194011100, Biovendor, Czech Republic), respectively. A custom-built Luminex Screening assay (R&D Systems, MN) was used in combination with the Bio-Plex Multiplex system (Bio-Rad, CA) to perform the analysis of plasma GDF15. Hepcidin in plasma was quantified by liquid chromatography tandem mass spectrometry validated for routine practice.

Calculations were performed using IBM SPSS Statistics version 25.0.0.2 (SPSS Inc., Chicago, IL). Nonparametric data were reported as median values with interquartile range (IQR). Mann-Whitney *U* test was used for comparisons between groups. EPO-ERFE-hepcidin pathway and GDF15 and sTFR correlation analyses were performed using Spearman's rho correlation coefficient. Quantile normalized, log2-transformed data was used as input for the heatmaps, generated to visualize disease-specific patterns and individual variation. Hierarchical clustering of the columns was performed by the complete linkage method.

Hereditary xerocytosis (HX) patients and sickle cell disease (SCD) patients were excluded from the correlation analysis and prediction model, based on known extrinsic factors influencing hepcidin regulation independent of the EPO-ERFE-hepcidin pathway. Data on EPO and sTFR were not available for the Danish cohort.

Parameters of the EPO-ERFE-hepcidin pathway were measured in 2 cohorts consisting of 115 patients and 20 healthy volunteers in total. The first (Dutch) cohort consisted of 82 patients and 10 healthy controls from the ZEbRA trial, a cross-sectional observational study on rare congenital hemolytic diseases

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conducted at the University Medical Center Utrecht (NL5189). The second (Danish) cohort included 33 patients and 10 healthy controls included from the Copenhagen General Population Study (H-KF-01–144/01).

The cohorts consisted of patients diagnosed with hereditary spherocytosis (HS), pyruvate kinase deficiency (PKD), β-thalassemia, HX (HX due to *PIEZO1* gain-of-function mutations), and SCD. The cohort thereby encompassed a heterogeneous population of patients with regard to transfusion requirements, degree of anemia, iron load, iron-reducing treatments, and spleen status. An overview of the main clinical characteristics of the patients (per diagnosis) and individual components of the EPO-ERFE-hepcidin pathway is provided in Table 1. In general, median EPO values and ERFE values were increased in patients when compared to healthy controls (EPO: 25.2 IU/L, IQR: 15.3–40.0 versus 3.4 IU/L, IQR: 2.8–4.4, $P < 0.001$, and ERFE: 14.5 ng/mL, IQR: 6.1–33.3 versus 0.2 ng/mL, IQR: 0.0–1.5, $P < 0.001$). Additionally, levels of sTFR in patients were significantly higher than in controls (8.9 μg/mL, IQR: 5.9–13.3 versus 1.3 μg/mL, IQR: 1.1–1.7, $P < 0.001$).

Hepcidin:ferritin ratios (×1000; to correct hepcidin values for iron load)[7] were considerably lower in patients when compared with healthy volunteers (20.8, IQR: 8.6–46.9 versus 114.6, IQR: 90.3–195.8; $P < 0.001$), statistical significance persisted when each disease was compared to the control population (all comparisons $P < 0.001$). Notably, hepcidin:ferritin ratios were moderately higher in non–transfusion-dependent patients than in transfusion-dependent patients (resp. 32.9, IQR: 15.4–60.1 versus 8.5, IQR: 3.9–13.2; $P < 0.001$). GDF15 levels were extremely high in β-thalassemia patients compared with all other diagnoses (all interdisease comparisons $P < 0.001$). Notably, median GDF15 values in non–transfusion-dependent thalassemia and transfusion-dependent thalassemia patients were comparable (resp. 2534 pg/mL, IQR: 979–4588 and 3145 pg/mL, IQR: 2281–3449; $P = 0.56$). Similarly, high GDF15 and suppressed hepcidin levels were previously reported in 3 forms of hereditary anemias with pronounced ineffective erythropoiesis: β-thalassemia,[4] congenital dyserythropoietic anemia type I and type II.[8,9] In β-thalassemia, high GDF15 levels are suggested to stem from increased oxidative stress in erythroblasts.[10] Moreover, our observation of high ERFE in β-thalassemic patients is in line with other studies.[1,11] Compared with the other disease entities, hepcidin values in HX were low in relation to EPO, ERFE, and sTFR. Our findings are in line with reports linking *PIEZO1* gain-of-function mutations to hepcidin suppression.[12]

To explore the relation of the individual components of the EPO-ERFE-hepcidin pathway, correlation analyses were performed between EPO, ERFE, hepcidin:ferritin ratio, sTFR, and GDF15 (Table 2 and Supplemental Digital Content, Figure S1, http://links.lww.com/HS/A181). The hepcidin:ferritin ratio was negatively correlated with EPO, ERFE, sTFR, and GDF15. Meanwhile EPO, ERFE, sTFR, and GDF15 were clearly positively correlated: in particular worth mentioning is the close and strong relationship between EPO and ERFE, EPO and sTFR, and ERFE and sTFR as specified in Table 2.

As a sensitivity analysis, we performed the correlation analyses in all subgroups (HS, PKD, and β-thalassemia), the presence of a positive relation between EPO, ERFE, and sTFR, and negative relation between the hepcidin:ferritin ratio, and either EPO, ERFE, or sTFR was confirmed in all subgroups. The presence of a positive relation between GDF15 and either EPO, ERFE, or sTFR, and negative relation between GDF15 and the hepcidin:ferritin ratio was not consistently present in all disease entities: in contrast to the other disease entities, in HS patients there

Table 1.

Demographic, Clinical Characteristics, Parameters of Hemolysis, Erythropoiesis, and Iron Metabolism per Disease.

	Healthy Controls[a]	HS	PKD	β-thalassemia	HX	SCD
General characteristics						
N	20	33	30	29	8	15
Median age	NA	47 (37–59)	43 (27–49)	30 (27–36)	48 (32–59)	30 (27–35)
Male (fraction)	NA	0.52	0.47	0.45	0.5	0.4
Splenectomy (fraction)	0	0.33	0.7	0.52	0.13	0.07
Transfusion dependency (fraction)	0	0	0.23	0.83	0	0.33
Iron chelation[b] or phlebotomy[c] (fraction)	0	0.12	0.33	0.86	0.5	0.33
Hemolysis						
LDH (IU/L)	NA	193 (181–231)	189 (164–326)	187 (146–259)	NA	313 (263–393)
Total bilirubin (μmol/L)	NA	27 (19–51)	56 (41–78)	44 (31–60)	37 (23–71)	31 (22–47)
Erythropoiesis						
Hb (g/dL)	14.2 (13.7–15.1)	13.2 (11.6–15.2)	9.6 (8.5–12.0)	9.2 (8.5–9.5)	13.9 (13.3–14.2)	9.8 (8.7–12.2)
Reticulocytes ($\times 10^9$)	59 (44–72)	235 (135–346)	691 (266–978)	110 (49–205)	482 (307–614)	178 (107–260)
EPO (IU/L)[d]	3.4 (2.8–4.4)	12.8 (5.7–30.6)	24.7 (17.0–31.5)	37.5 (18.7–63.4)	26.9 (18.8–31.4)	32.0 (20.9–67.7)
ERFE (ng/mL)	0.24 (0.24–1.53)	5.7 (2.1–24.4)	15.2 (7.8–31.5)	13.2 (9.2–16.7)	14.7 (10.8–19.3)	7.4 (5.1–11.8)
sTFR (μg/mL)[e]	1.3 (1.1–1.7)	5.5 (2.5–8.0)	10.6 (7.4–15.1)	13.2 (9.3–16.7)	6.9 (5.1–11.3)	9.7 (6.4–14.3)
GDF15 (pg/mL)	168 (115–298)	261 (184–409)	253 (230–401)	2761 (1629–3430)	346 (210–697)	682 (274–836)
Iron metabolism						
Hepcidin (μg/L)	14.9 (5.6–21.8)	10.8 (6.3–21.8)	9.6 (5.1–12.8)	7.5 (2.6–12.3)	4.6 (1.0–17.0)	6.2 (2.0–16.6)
TSAT (fraction)	0.26 (0.18–0.33)	0.36 (0.27–0.50)	0.50 (0.33–0.74)	0.93 (0.86–1.21)	0.48 (0.19–0.56)	0.43 (0.25–0.74)
Ferritin (μg/L)	62[f] (25–135)	247 (109–374)	496 (251–833)	774 (475–1325)	142 (61–362)	562 (62–744)
Hepcidin:ferritin ratio (×1000)	114.6[f] (90.3–195.8)	50.0 (32.2–68.0)	15.0 (8.7–27.0)	8.5 (3.8–14.0)	36.1 (10.5–47.0)	18.4 (6.4–25.2)

Unless otherwise indicated, data are median values (interquartile range). The Dutch cohort included 82 patients diagnosed with HS (N = 24), PKD (N = 23), β-thalassemia (N = 12), HX (N = 8), and SCD (N = 15); the Danish cohort included 33 patients diagnosed with HS (N = 9), PKD (N = 7), and β-thalassemia (N = 17).

[a]Not reported values were NA.

[b]Iron chelator use was defined as prescription of an iron chelating agent during the last 12 mo.

[c]Phlebotomies apply to a regular phlebotomy scheme in the past.

[d]No EPO data available in the Danish cohort.

[e]No sTFR data available in the Danish cohort.

[f]No ferritin data available in the Danish healthy controls.

EPO = erythropoietin; ERFE = erythroferrone; GDF15 = growth differentiation factor 15; Hb = hemoglobin; HS = hereditary spherocytosis; HX = hereditary xerocytosis; LDH = lactate dehydrogenase; NA = not available; PKD = pyruvate kinase deficiency; SCD = sickle cell disease; sTFR = soluble transferrin receptor; TSAT = transferrin saturation.

Table 2.

Correlation Table EPO-ERFE-hepcidin Pathway.

	Hepcidin:Ferritin Ratio (×1000)	ERFE (ng/mL)	EPO (IU/L)[a]	sTFR (μg/mL)[b]	GDF15 (pg/mL)
Hepcidin:ferritin ratio (×1000)		−0.74 (−0.85 to −0.57)	−0.69 (−0.83 to −0.50)	−0.68 (−0.81 to −0.52)	−0.43 (−0.65 to −0.16)
ERFE (ng/mL)	−0.74 (−0.85 to −0.57)		0.86 (0.74–0.91)	0.80 (0.64–0.89)	0.55 (0.32–0.72)
EPO (IU/L)[a]	−0.69 (−0.83 to −0.50)	0.86 (0.74–0.91)		0.80 (0.65–0.88)	0.59 (0.35–0.75)
sTFR (μg/mL)[b]	−0.68 (−0.81 to −0.52)	0.80 (0.64–0.89)	0.80 (0.65–0.88)		0.39 (0.12–0.60)
GDF15 (pg/mL)	−0.43 (−0.65 to −0.16)	0.55 (0.32–0.72)	0.59 (0.35–0.75)	0.39 (0.12–0.60)	

Values represent the correlation coefficient (*R*) and 95% confidence interval. All presented values were statistically significant.

[a]No EPO data available in the Danish cohort.

[b]No sTFR data available in the Danish cohort.

EPO = erythropoietin; ERFE = erythroferrone; GDF15 = growth differentiation factor 15; sTFR = soluble transferrin receptor.

was no (negative) relation between GDF15 and hepcidin:ferritin ratio, $r = 0.04$, IQR: −0.31 to 0.39). Hence, we confirm a robust correlation between in the EPO-ERFE-hepcidin pathway in various hereditary hemolytic anemias.

As expected, hepcidin values were generally suppressed in all patients with hereditary hemolytic anemias compared with healthy controls. Noteworthy is the observation that hepcidin values were comparable in transfusion-dependent and transfusion-independent patients. This observation is not in line with the theory that regular transfusions suppress erythropoiesis, reduce erythron iron requirements and thus reduce hepcidin suppression.[13,14] We observed a strong positive correlation between the 3 parameters involved in hepcidin regulation, all representing the size of the erythron: EPO, ERFE, and sTFR. However, our data are limited in size and may represent a biased selection of more severe diseased patients in the transfusion-dependent thalassemia subgroup. Luspatercept is approved for treatment of ineffective erythropoiesis in β-thalassemia. It would be of great interest to investigate its effect on the erythron characterized to an expanded erythroid progenitor pool.

To analyze interindividual variation in the EPO-ERFE-hepcidin pathway and to identify the presence of disease-specific patterns, we depicted individual patient data in a heatmap (Supplemental Digital Content, Figure S2, http://links.lww.com/HS/A182), the influence of disease severity, spleen-status, iron load, and transfusion requirements is visualized in Supplemental Digital Content, Figure S3a-e, http://links.lww.com/HS/A183; http://links.lww.com/HS/A184; http://links.lww.com/HS/A185; http://links.lww.com/HS/A186; http://links.lww.com/HS/A187. Hierarchical clustering of the individual patients based on EPO, ERFE, sTFR, GDF15, the hepcidin:ferritin ratio, and reticulocyte count was not able to completely separate disease entities, including transfusion dependency. However, several clusters of patients of 1 disease entity could be discriminated (eg, β-thalassemia and SCD patients). The anemias with the most divergent patterns were PKD and HX. The EPO-ERFE-hepcidin pathways of the included SCD patients clustered in 3 subgroups differing in hepcidin:ferritin ratios and EPO values. We assume that the variability in EPO-ERFE-hepcidin regulation in the SCD patients represents the complex interplay between iron, inflammation, hypoxia, and erythropoiesis.[15] Hence, although anemia, hemolysis, ineffective erythropoiesis, and iron overload are features shared among the hemolytic anemias, marked differences in patterns of EPO, ERFE, sTFR, GDF15 and hepcidin expression exist between diseases as well as between patients diagnosed with 1 disease entity.

Altogether, this report provides insight in the balance of the EPO-ERFE-hepcidin pathway in hereditary hemolytic anemias. This first report confirming a uniform regulation of the EPO-ERFE-hepcidin pathway in hereditary hemolytic anemias raises questions on the individual contribution of factors, such as transfusion requirements, iron load, chelation therapy, or spleen status, that may explain observed differences between disease entities and between patients. In particular, worth mentioning is the role of (adequate) iron chelation in β-thalassemia patients as it may reduce the level of ineffective erythropoiesis and thereby may alter all parameters of the EPO-ERFE-hepcidin pathway.[6]

However, variability among disease entities and among individuals illustrates the complexity of the communication between erythropoiesis and iron.

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Disclosures

The authors have no conflicts of interest to disclose.

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