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## EPO and hepcidin plasma concentrations in blood donors and $\beta$ -thalassemia intermedia are not related to commercially tested plasma ERFE concentrations

#### To the Editor:

The recently discovered hormone erythroferrone (ERFE) is produced by erythroblasts in response to erythropoietin (EPO) and mediates hepcidin production during stress erythropoiesis. ERFE has been previously described as FAM132B, myonectin, or c1q-tumor-necrosis factor

a-related protein isoform 15 (CTRP15), a skeletal muscle derived myokine that links skeletal muscle to lipid homeostasis in liver and adipose tissue in response to alterations in energy state.<sup>1</sup>

In a mouse model, erythroblasts produce ERFE in response to EPO, leading to an increase in plasma ERFE concentration.<sup>2</sup> Elevations in ERFE levels suppress hepcidin-25 synthesis of the liver to allow iron acquisition from absorption and storage sites, favoring recovery from anemia secondary to blood loss. ERFE knockout mice have normal hematological parameters, but are unable to suppress hepcidin after phlebotomy or EPO injection.<sup>1</sup> As a result, ERFE deficient mice have a slower recovery after blood loss. In a mouse model for  $\beta$ -thalassemia intermedia, plasma ERFE levels were massively increased and ERFE messenger RNA levels elevated in the marrow and spleen. Ablation of ERFE in these mice restored hepcidin levels to normal and reduced liver iron content and serum iron concentration, demonstrating that ERFE could be a pathological suppressor of hepcidin in ineffective erythropoiesis.<sup>2</sup> However, there is no assay available yet for human plasma. Quantification of ERFE concentrations in human plasma may provide new insights in the pathophysiology of hematological diseases. Counteracting elevations in ERFE may result in less iron accumulation and improvement of anemia in patients with iron loading anemias, such as  $\beta$ -thalassemia intermedia. Analysis of the gene encoding for ERFE might identify subjects with variants who are less suitable for blood donation.

Recently, associations between ERFE and biomarkers of erythropoiesis and iron metabolism have been evaluated for the first time in patients with chronic kidney disease on hemodialysis exploiting a commercially available sandwich ELISA kit for the quantification of ERFE (FAM132B) in human serum samples.<sup>3</sup> Correlations were found between levels of ERFE and hepcidin, ferritin, and soluble transferrin receptor (sTfR).<sup>3</sup> In the present study we aim to validate this kit by measurement of ERFE in  $\beta$ -thalassemia intermedia patients and in healthy blood donors before and after blood donation and to correlate findings with other markers of erythropoiesis and iron metabolism.

Two populations were studied: patients diagnosed with  $\beta$ -thalassemia intermedia, as described previously<sup>4</sup> and Dutch male whole blood donors, as part of a study on changes in Hb and iron parameters in time after blood donation.<sup>5</sup> The study on blood donors was approved by the Medical Ethical Committee Arnhem-Nijmegen in the Netherlands and the Ethical Advisory Council of Sanquin Blood Supply. All participants gave their written, informed consent. Use of plasma from  $\beta$ -thalassemia intermedia patients for this study conforms to the code for proper secondary use of human tissue in the Netherlands and the declaration of Helsinki.

For the  $\beta$ -thalassemia intermedia patients, we selected 10 leftover heparin plasma samples from a total of 38 samples collected for our previous study,<sup>4</sup> so as to ensure a wide range of EPO levels. For the

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TABLE 1 Donor and patient characteristics, and heparin plasma ERFE levels at different dilutions

Study ID	Age (years)	Hb (g/dL)	Hepcidin (nM)	sTfR (mg/L)	EPO (mU/mL)	ERFE (pg/mL)					
						2× dil <sup>a</sup>	10× dil <sup>a</sup>	100× dil <sup>a</sup>	1000× dil	10 000× dil	
Blood donors											
1	−0 <sup>b</sup>	43	13.6	9.2	1.08	5.5	531	4296	39 120	ND	ND
	−4		12.8	4.4	1.08	12.7	592	4225	37 838	28 236	ND
14	−0	31	14.4	2.7	1.22	7.9	862	3788	9318	ND	ND
	−4		NA	1.5	1.22	12.6	940	3950	10 081	<LLOD	ND
24	−0	47	15.4	6.5	1.29	5.0	512	4002	32 454	ND	ND
	−4		14.2	3.2	1.27	10.6	495	4129	17 072	<LLOD	ND
26	−0	46	15.8	2.4	1.45	8.2	772	4908	17 658	ND	ND
	−4		14.6	1.0	1.32	12.4	813	4455	28 463	22 689	ND
34	−0	50	15.5	2.8	1.05	14.5	583	5103	15 997	ND	ND
	−4		15.4	1.7	1.13	17.6	669	4497	8761	<LLOD	ND
39	−0	43	15.5	0.4	0.97	9.1	674	4057	3857	ND	ND
	−4		13.6	2.3	0.86	20.4	850	3436	4546	<LLOD	ND
52	−0	50	13.4	5.1	1.27	7.1	737	3864	5964	ND	ND
	−4		12.0	0.5	1.08	14.7	709	3270	4562	<LLOD	ND
β-thalassemia intermedia											
42		43	7.2	<0.5	7.78	1740	ND	ND	24 068	35 880	<LLOD
6		33	8.3	<0.5	10.00	1750	ND	ND	18 376	13 611	<LLOD
119		32	9.6	<0.5	12.70	324	ND	ND	21 075	45 013	<LLOD
16		35	8.6	<0.5	14.70	577	ND	ND	22 927	86 325	<LLOD
96		47	6.3	3.8	7.56	162	ND	ND	26 962	72 305	<LLOD
106		9	8.8	1.9	5.05	101	ND	ND	25 320	19 341	<LLOD
17		30	6.6	<0.5	15.40	189	ND	ND	27 280	42 948	<LLOD
5		38	8.2	<0.5	19.30	167	ND	ND	24 415	52 463	<LLOD
30		40	9.7	4.4	4.49	29.3	ND	ND	10 243	<LLOD	<LLOD
99		17	8.8	1.6	10.00	31.4	ND	ND	20 656	26 926	<LLOD

<sup>a</sup>For samples of β-thalassemia intermedia patients, we used 100-fold diluted samples as the lowest dilution to measure ERFE. We anticipated this 100-fold dilution would allow the measurement of ERFE levels since the measurement range of the kit is given as 15.6–1000 pg/mL and concentrations observed in a mouse model of β-thalassemia intermedia were 10–25 ng/mL.<sup>1</sup> For blood donors, we also included dilutions of 2- to 10-fold based on the assumption that pre-donation values would be <100 pg/mL as observed for wild-type mice,<sup>1</sup> with a reported 30-fold increase upon phlebotomy.<sup>2</sup>

<sup>b</sup>−0 = measurement at baseline, before donation, −4 = measurement at day 4.

dil, dilutions; NA, not available; ND, not determined; LLOD, lower limit of detection as reported in kit manual: 3.9 pg/mL; sTfR, soluble transferrin receptor.

blood donors, we selected 7 heparin plasma samples from a total of 49 samples that were collected both before and at day 4 after donation of 500 mL blood in the context of our earlier study on changes in iron parameters following whole blood donation.<sup>5</sup> At day 4, EPO levels were found to be highest and hepcidin levels to be lowest in time after blood donation.<sup>5</sup> In addition, to cover a wide range of changes in ERFE levels, donor samples were selected so as to assure a variety in both baseline EPO levels and relative changes in EPO levels between baseline and day 4 after donation.

ERFE concentrations were determined in December 2015 with the Human Protein FAM132B (FAM132B) ELISA Kit, MyBioSource,

San Diego, Ca, USA (cat. No. MBS940905; lot no.: R09144170). This ELISA reports a detection range of 15.6–1000 pg/mL, a lower limit of detection (LLOD) of 3.9 pg/mL and an intra-assay and inter-assay CV <8% and <10%, respectively. EPO, hepcidin, and sTfR levels were measured previously as described,<sup>4</sup> except for hepcidin levels in blood donors, which were measured by competitive ELISA.<sup>5</sup>

In general, donors had Hb and hepcidin levels lower and EPO levels higher 4 days after donation (Table 1), compared to baseline levels. In patients diagnosed with β-thalassemia intermedia, hepcidin levels were generally lower and EPO and sTfR levels were higher than in blood donors.

ERFE levels increased with higher dilutions indicating a low dilution linearity (Table 1). At a 100× dilution for ERFE levels, we did not observe a correlation between ERFE and EPO, or ERFE and hepcidin (Supporting Information Figure 1A,B). Furthermore, in blood donors there was no correlation between delta-(day 0-4) hepcidin and delta-(day 0-4) ERFE levels (hepcidin (nM) =  $8 \times 10^{-5}$  ERFE (pg/mL) + 1.93, Pearson  $R^2 = 0.06$ ), neither between delta (day 0-4) EPO and delta (day 0-4) ERFE levels (EPO (mU/mL) =  $3 \times 10^{-5}$  ERFE (pg/mL) - 6.29, Pearson  $R^2 = 0.005$ ).

We cannot fully exclude that the absence of correlations between ERFE and EPO, and ERFE and hepcidin in these samples may be attributed to ERFE instability, since our samples of  $\beta$ -thalassemia intermedia patients were stored at  $-80^\circ\text{C}$  for 6-7 years, and thawed 2-5 times before ERFE measurement, and not much is known on the effects of prolonged storage and freeze-thawing on ERFE integrity. However, ERFE measurements in blood donors were performed in aliquots stored at  $-80^\circ\text{C}$  for only 1-2 years, and are therefore less likely to be affected by ERFE degradation.

The absence of a correlation between ERFE and hepcidin plasma levels in our study differs from a previous report on associations between both analytes that were observed using the same FAM132B ELISA in a study among hemodialysed patients.<sup>3</sup> However, our observations on lack of correlations of ERFE plasma levels and plasma hepcidin and EPO levels are in agreement with those obtained in a study among patients with chronic mountain sickness (as defined by excessive erythrocytosis, hemoglobin  $\geq 21$  g/dL, and hypoxemia with no other medical explanation), who underwent isovolemic venesection of 500 mL on four consecutive days (days 1-4).<sup>6</sup> Using two other plasma FAM132B ELISA kits, the authors found no significant rise in plasma FAM132B at three different time points up till day 20 after venesection, whereas, comparable to our observations in blood donors after blood donation,<sup>5</sup> hematocrit and plasma hepcidin decreased, and EPO levels increased.<sup>5,6</sup>

Taken together, our data obtained in whole blood donors and patients diagnosed with  $\beta$ -thalassemia intermedia with the commercially available kit for human ERFE measurements of MyBiosource does not corroborate the concept of the increased EPO–increased ERFE–lower hepcidin axis as observed in mouse models. The full comprehension of the role of this axis in men therefore awaits the development of an analytically and biologically validated assay for human plasma ERFE levels.

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#### CONFLICT OF INTEREST

Nothing to report.

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#### SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

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## Thrombospondin-1 gene polymorphism is associated with estimated pulmonary artery pressure in patients with sickle cell anemia

To the Editor:

Sickle cell disease (SCD) is known to promote end-organ damage over time. Patients with especially high rates of intravascular hemolysis in SCD have decreased nitric oxide (NO) bioavailability, which is associated with