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Induction of erythroferrone in healthy humans by micro-dose recombinant erythropoietin or high-altitude exposure

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Running heads: low dose erythropoietin induces erythroferrone

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Abstract

The erythropoietin (Epo)-erythroferrone (ERFE)-hepcidin axis coordinates erythropoiesis and iron homeostasis. While mouse studies have established that Epo-induced ERFE production represses hepcidin synthesis by inhibiting hepatic BMP/SMAD signaling, evidence for the role of ERFE in humans is limited. To investigate the role of ERFE as a physiological erythroid regulator in humans, we conducted two studies: first, 24 males received six injections of saline (placebo), recombinant Epo (rhEpo) 20 UI kg^{-1} (micro-dose) or 50 UI kg^{-1} (low-dose). Second, we quantified ERFE in 22 subjects exposed to high altitude (3800 m) for 15 hours. In the first study, total hemoglobin mass (Hb_{mass}) increased after low- but not after micro-dose injections, when compared to placebo. Serum ERFE levels were enhanced by rhEpo, remaining higher than after placebo for 48 (micro-dose) or 72 hours (low-dose) post-injections. Conversely, hepcidin levels decreased when Epo and ERFE arose, before any changes in serum iron parameters occurred. In the second study, serum Epo and ERFE increased at high altitude. The present results demonstrate that in healthy humans ERFE responds to slightly increased Epo levels not associated with Hb_{mass} expansion and down-regulates hepcidin in an apparently iron-independent way. Notably, ERFE flags micro-dose Epo, thus holding promise as novel anti-doping biomarker.

Introduction

Erythropoiesis and iron metabolism are tightly linked and inadequate iron supply to developing erythrocytes results in anemia, a condition affecting a large segment of the world's population. The coordination between erythropoietic activity and iron homeostasis is provided by hepcidin, which controls body iron balance by negatively regulating the activity of the iron exporter, ferroportin [1,2]. Hepcidin expression is inhibited by iron deficiency and high erythropoietic activity [1,2], a response that increases iron availability to meet iron needs for hemoglobin (Hb) synthesis. Accordingly, we and others have demonstrated that recombinant human erythropoietin (rhEpo) administration to healthy humans is followed by a prompt hepcidin down-regulation [3-5]. The identification and characterization of erythroferrone (ERFE), a hepcidin-inhibiting factor produced by erythroblasts in response to Epo, provided an additional link between erythropoietic activity and iron homeostasis [6]. Mouse studies established that ERFE synthesized in response to Epo impairs hepcidin transcription by inhibiting hepatic BMP/SMAD signaling [6,7]. The development of a validated assay for human ERFE allowed to show that ERFE is increased in patients with β-thalassemia and in response to blood donation or administration of high doses of rhEpo [8]. Moreover, serum ERFE levels are elevated in patients with chronic kidney disease (CKD) treated with rhEpo [9] and in children affected by iron deficiency anemia [10]. However, another study did not find increased ERFE levels in CKD patients [11]. In addition, ERFE did not change in patients with reduced erythropoiesis caused by androgen deprivation therapy [12] or athletes exposed to altitude [13] and, unexpectedly, decreased in parallel with hepcidin in subjects experiencing Hb_{mass} expansion due to altitude exposure [14]. Therefore, the effect of accelerated erythropoiesis on ERFE in humans remains uncertain. Here, we tested the hypothesis that in healthy humans, ERFE is a physiologic erythroid regulator, i.e. responds to moderate erythropoietic stimulation, such as very low rhEpo doses or high altitude exposure.

Methods

Details of the methods are available in Supplementary material.

Study design: for the rhEpo study, Hb_{mass}, indices of iron homeostasis and hematological parameters were repeatedly measured in 24 healthy males receiving six injections (every second/third day) of saline (placebo), rhEpo 20 $UI.kg^{-1}$ (micro-dose) or rhEpo 50 $UI.kg^{-1}$ (low-dose) Written informed consent to participation in the study was provided by all subjects. The study (NCT03276910) was approved by the French ethics committee (CPP Est-III, EudraCT 2017-

000375-82). For the high-altitude study, iron parameters were determined in 22 healthy subjects exposed to high altitude (3800 m) for 15 hours. Written informed consent to participation in the study was provided by all subjects. The study (NCT02778659) was approved by the French ethics committee (CPP Sud-Est-III, EudraCT 2015-004512-38).

Results

Three days after the last rhEpo injection, Hb_{mass} was higher in subjects treated with rhEpo low-dose than those receiving placebo (Fig 1A). In contrast, treatment with rhEpo micro-doses did not induce significantly higher Hb_{mass} levels in comparison to placebo treatment. Hb_{mass} was not significantly altered following placebo treatment but nonetheless tended to decrease, possibly because of frequent blood sampling. Short treatment duration may otherwise explain the marginal change of Hb_{mass} following rhEpo micro-doses. Unlike Hb_{mass}, Hb concentration and hematocrit progressively increased with both rhEpo doses (Fig 2A and 2B). As expected, circulating Epo levels increased after each injection, but rapidly declined, in particular after micro-dose treatment (Fig 1B). ERFE levels showed a significant dose-related increase after each injection, remaining above placebo levels up to 48 (micro-dose) or 72 hours (low-dose) and decreasing thereafter (Fig 1C). Following low-dose rhEpo, ERFE reached levels similar to those found in patients with anemia induced by bleeding (see Supplementary material, Analyses section). The ERFE variation pattern mirrored that of serum Epo (Fig 3A). Conversely, serum hepcidin decreased when Epo and ERFE rose, independently of the dose, and remained low as long as Epo and ERFE were above baseline values (Fig 1D). Both ERFE and hepcidin returned to placebo levels one week after the last injection. Interestingly, there was no cumulative effect of repeated rhEpo injections on ERFE levels (Fig. 1C). Consistent with previous studies [3-5], the decrease in serum ferritin with rhEpo was progressive (Fig. 2C). In contrast, transferrin saturation (Tfsat) was not significantly altered by rhEpo injections (Fig 2D).

We also found concomitant increases in Epo and ERFE in healthy subjects exposed to a high altitude condition associated with O_2 saturation of 85 ± 3% (Fig 4A and 4B). The correlation between ERFE and serum Epo found at high altitude (Fig 3B) and with rhEpo treatment (Fig 3A) suggests that hypoxia-related signaling is not directly involved in ERFE induction, as previously shown in mice [6]. ERFE tended to increase more with rhEpo micro-dose than with high altitude ($P = 0.22$), whereas Epo increased less with micro-dose than with high altitude ($P = 0.04$) (result not shown). We speculate that the shorter time of exposure to elevated Epo levels at high altitude (15 h) versus micro-dose (24 h) may account for the observed trend. Tfsat, which was close to the level defining iron deficiency (<20%), did not change (Fig 4C). Consistent with the low Tfsat, hepcidin

concentration at sea level was below the detection limit in 15 subjects and was unchanged after exposure to high altitude in the subjects with detectable baseline values (Fig 4D). Lack of correlation between ERFE and hepcidin was previously found in CKD patients, in which increased ERFE levels were not accompanied by lower hepcidin [9]. In line with a previous report showing unaltered iron availability and no signs of inflammation at high altitude,[15] the inflammatory marker IL-6 was not affected by high altitude (1.28 ± 1.04 $pg.mL^{-1}$, vs. 1.25 ± 0.9 $pg.mL^{-1}$ at sea level), whereas ferritin concentration showed a small increase (from 125 ± 8 to 132 ± 7 $ng.mL^{-1}$), nonetheless remaining within the normal range (Fig 4E).

Discussion

Recently, mouse studies have shown that acute rhEpo treatment down-regulates hepcidin in an ERFE-independent manner by decreasing serum iron and Tfsat [16,17]. Moreover, the demonstration that rhEpo administration down-regulates hepcidin also in mice lacking ERFE [18] suggests that prolonged erythropoietic stimulation inhibits hepcidin expression in mice by depleting iron stores, whereas ERFE represents an acute regulator of stress erythropoiesis [18]. Conversely, the present results show that in healthy humans ERFE responds even to low Epo levels which are not associated with Hb_{mass} expansion, a functional marker of erythropoietic response [19]. This conclusion is also supported by our findings in a physiological condition such as high-altitude hypoxia (Fig 4). Furthermore, our data showing no alterations in Tfsat and a progressive decrease in ferritin with repeated rhEpo injections are consistent with the view that ERFE may inhibit hepcidin transcription directly in the absence of changes in serum and liver iron [7]. In fact, the ERFE-hepcidin axis was affected early, i.e. 24 hours after the first rhEpo injection, while other serum iron parameters were unchanged at that time, as t test analysis showed no difference in ferritin between groups at 24 and 48 hours after the first injection (see Supplementary material, Statistical analysis section). However, it is well conceivable that under conditions of strong erythropoietic stimulation, such as in mice treated with high doses of rhEpo (8000 $UI.kg^{-1}$) [16-18], increased iron consumption for erythropoiesis leads to iron depletion and hepcidin repression.

The introduction of the Athlete's Blood Passport (ABP) [20] has improved blood doping detection, although also the ABP has several limitations [21], in particular detecting micro-dose rhEpo doping [22]. A study in which ERFE was measured in 6 subjects receiving intravenously or subcutaneously relatively high doses of rhEpo or analogs, using an assay different from the one used in this study did not suggest ERFE as a reliable marker for rhEpo doping [23], although (while our manuscript was under revision) the same group reported that a different ELISA assay was able to detect increased

ERFE levels in the same samples [24]. Conversely, increased erythropoiesis induced by training did not affect ERFE and hepcidin levels in runners [25]. Our results demonstrate that ERFE is sensitive enough to flag even micro-dose rhEpo, correlates with Epo levels (see Fig 3A and 3B) and has a detection window longer than that of Epo, thereby indicating that ERFE holds promise as a novel anti-doping biomarker for ABP implementation, although additional studies are required. In view of our results, ferritin or hepcidin could also be considered as potential micro-dose rhEpo biomarkers, however both factors may be confounded by iron supplementation, a legal practice commonly used by athletes.

In summary, the present results demonstrate that in healthy humans ERFE is promptly enhanced in response to moderately increased Epo levels and represses hepcidin in an iron-independent way. ERFE determination may provide additional analytical support for the fight against doping.

Authorship Contributions

PR designed and coordinated the project, planned and performed experiments and co-wrote the manuscript; EG provided collection and interpretation of data and co-wrote the manuscript. S.R. collected and analyzed data. DG and AC collected and analyzed data. MR analyzed data and performed statistical analysis, A-K L, PBa, PBo, SV, GS, MU collected data, CL analyzed data and revised the manuscript, CC provided clinical support, PS designed and coordinated the project, discussed the results and co-wrote the manuscript. GC conceived and coordinated the study, interpreted data and co-wrote the manuscript. All authors discussed the results and commented on the manuscript.

Conflict-of-interest disclosure

The authors declare no competing financial interests

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FIGURE LEGENDS

Figure 1. Effects of repeated administration of very low to low doses of recombinant human erythropoietin (rhEpo) on total hemoglobin mass, erythropoietin, erythroferrone and hepcidin. Panel A, total hemoglobin mass, determined at baseline (Pre) and 72 hours after the last of the six injections (Post) of placebo (n = 7), rhEpo 20 UI.kg^{-1} (micro-dose) (n = 7) or 50 UI.kg^{-1} (low-dose) (n = 8). ANOVA indicates a significant time × treatment interaction (P = 0.049). *indicates a significant difference between rhEpo low-dose and placebo. Data are means ± SD. Individual Hb_{mass} values are available in Supplementary Figure 1.

Serum concentrations of erythropoietin (Epo, panel B), erythroferrone (ERFE, ln transformed, panel C, n = 7 for the placebo condition) and hepcidin (ln transformed, panel D) before, during and after six injections of placebo (n = 8), micro-dose (n = 8) or low-dose (n = 8) rhEpo. Vertical dotted lines indicate injections. Baselines are means from duplicate blood samples collected on separated days. Blood sampling was repeatedly performed at 24 hours (two occurrences), 48 hours (four occurrences) and 72 hours (two occurrences) after an injection. Additional samples were obtained at seven (7 d) and fourteen days (14 d) after the last injection. Numbers above the abscissa line (i.e. 24, 48, 72, 7d and 14d) indicate these sampling times. Data are means ± SD. For each panel, inserted tables report estimated marginal means ± standard errors and P-values for the effect of the group (placebo, micro-dose, low-dose), the occurrence (i.e. the number of samples at 24 hours, 48 hours, 72 hours, 7 days and 14 days, from 1 to 4) and the group × occurrence (G × O) interaction. Multiple comparisons between groups at 24 hours, 48 hours or 72 hours are reported in each table, * denoting a difference with placebo and ‡ a difference with micro-dose (see Supplementary material, Statistical analysis section).

Figure 2. Effects of repeated administration of very low to low doses of recombinant human erythropoietin (rhEpo) on hematologic and iron parameters. Blood hemoglobin concentration (panel A), hematocrit (panel B), serum concentration of ferritin (ln transformed, panel C) and transferrin saturation (panel D) before, during and after six injections of placebo (n = 8), rhEpo 20 UI.kg^{-1} (micro-dose) (n = 8) or 50 UI.kg^{-1} (low-dose) (n = 8). Vertical dotted lines indicate injections. Baselines are means from duplicate blood samples collected on separated days. Blood sampling was repeatedly performed at 24 hours (two occurrences), 48 hours (four occurrences) and 72 hours (two occurrences) after an injection. Additional samples were obtained at seven (7 d) and fourteen days (14 d) after the last injection. Numbers above the abscissa line (i.e. 24, 48, 72, 7d and 14d) indicate these sampling times. Data are means ± SD. For each panel, inserted tables report

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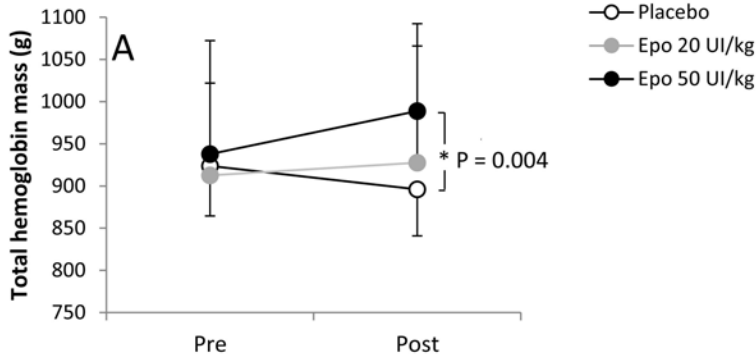
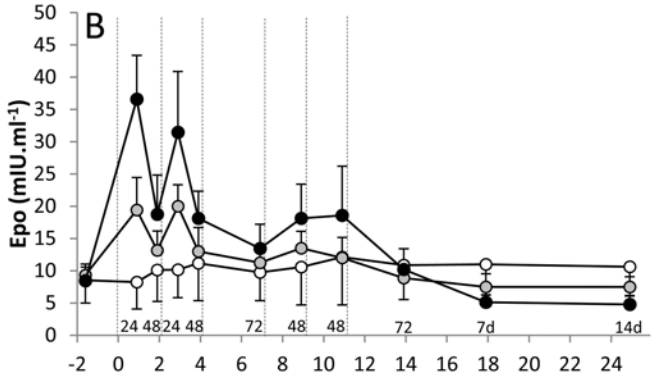
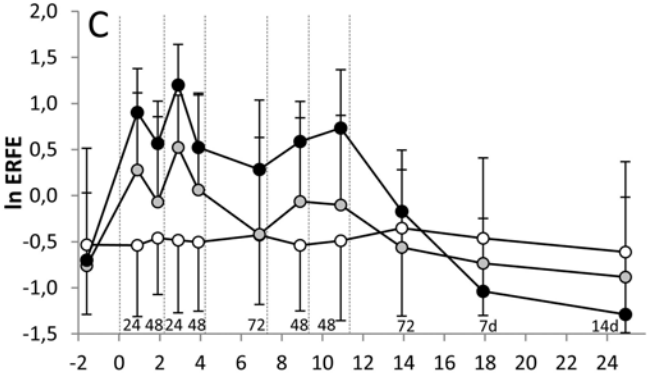
Figure 3. Relationships between erythroferrone and erythropoietin with rhEpo and high altitude. Relationship between individual serum concentrations (ln transformed) of ERFE and Epo, before, during and after six injections of placebo, micro-dose or low-dose rhEpo in healthy subjects (panel A), and at sea level and after 15 hours of exposure to high altitude (3800 m) in healthy subjects (panel B). Regression equations, coefficients of determination and P values are reported on each panel. Relationships between hepcidin, Epo and ERFE during rhEpo injections are available in Supplementary Figure 2.

Figure 4. Effects of acute exposure to high altitude. Individual serum concentrations and means ± SD of erythropoietin (Epo, panel A, n = 21), erythroferrone (ERFE, ln transformed, panel B, n = 21), transferrin saturation (panel C, n = 22), hepcidin (ln transformed, panel D, n = 7) and ferritin (panel E, n = 22), at sea level and after 15 hours of exposure to high altitude (3800 m). Of note, out of 22 subjects, 15 subjects had sea-level hepcidin concentrations which were below the detection limit, therefore hepcidin statistical analysis was performed on 7 subjects. P values denote differences between sea level and high altitude.

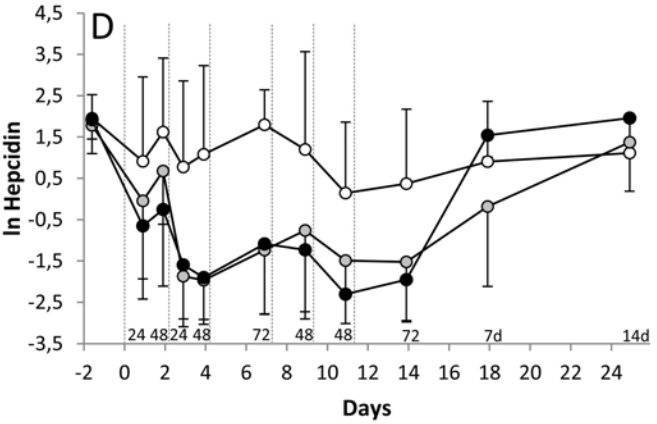




	PLA	EPO20	EPO50	Group	Occurrence	G × O
24 h	9.6 ± 1.5	19.2 ± 1.5*	34.2 ± 1.4*‡	P = 0.0001	P = 0.51	P = 0.25
48 h	11.2 ± 0.8	12.4 ± 0.9	18.7 ± 0.8*‡	P = 0.0001	P = 0.99	P = 0.97
72 h	10.5 ± 0.7	9.5 ± 0.7	12.1 ± 0.7*‡	P = 0.04	P = 0.07	P = 0.08
7 days	11.2 ± 0.7	7.0 ± 0.7*	5.4 ± 0.7*	P = 0.0001		
14 days	10.8 ± 0.8	7.1 ± 0.8*	5.0 ± 0.8*	P = 0.0001		

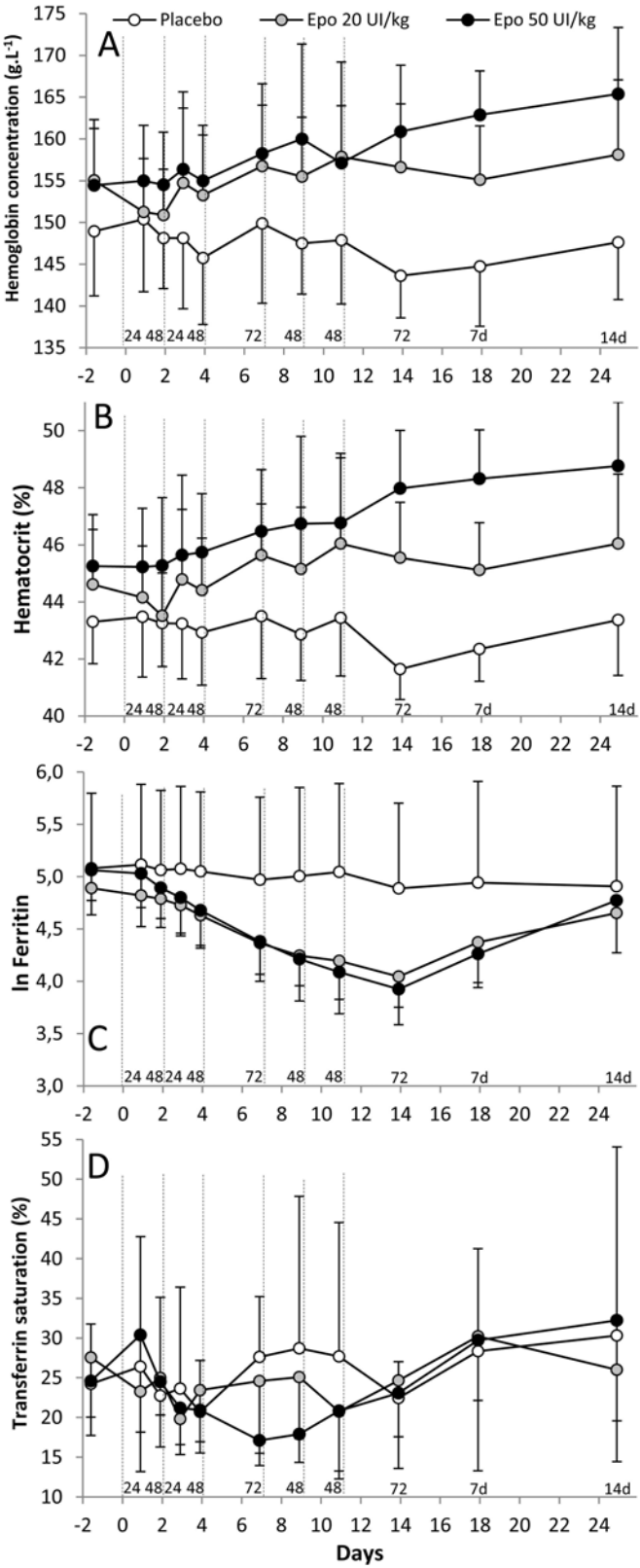


	PLA	EPO20	EPO50	Group	Occurrence	G × O
24 h	-0.59 ± 0.11	0.45 ± 0.10*	1.07 ± 0.10*‡	P = 0.0001	P = 0.10	P = 0.69
48 h	-0.59 ± 0.09	0.02 ± 0.08*	0.62 ± 0.08*‡	P = 0.0001	P = 0.98	P = 0.97
72 h	-0.49 ± 0.13	-0.42 ± 0.12	0.08 ± 0.12*‡	P = 0.004	P = 0.23	P = 0.33
7 days	-0.59 ± 0.15	-0.65 ± 0.14	-1.01 ± 0.14	P = 0.09		
14 days	-0.75 ± 0.25	-0.79 ± 0.23	-1.26 ± 0.23	P = 0.26		



	PLA	EPO20	EPO50	Group	Occurrence	G × O
24 h	0.78 ± 0.42	-0.85 ± 0.42*	-1.16 ± 0.42*	P = 0.005	P = 0.05	P = 0.37
48 h	0.95 ± 0.28	-0.78 ± 0.28*	-1.48 ± 0.29*	P = 0.0001	P = 0.0001	P = 0.70
72 h	1.04 ± 0.35	-1.31 ± 0.35*	-1.54 ± 0.35*	P = 0.0001	P = 0.04	P = 0.52
7 days	0.85 ± 0.50	-0.11 ± 0.50	1.52 ± 0.49	P = 0.09		
14 days	1.13 ± 0.33	1.34 ± 0.33	1.97 ± 0.33	P = 0.19		

Figure 1



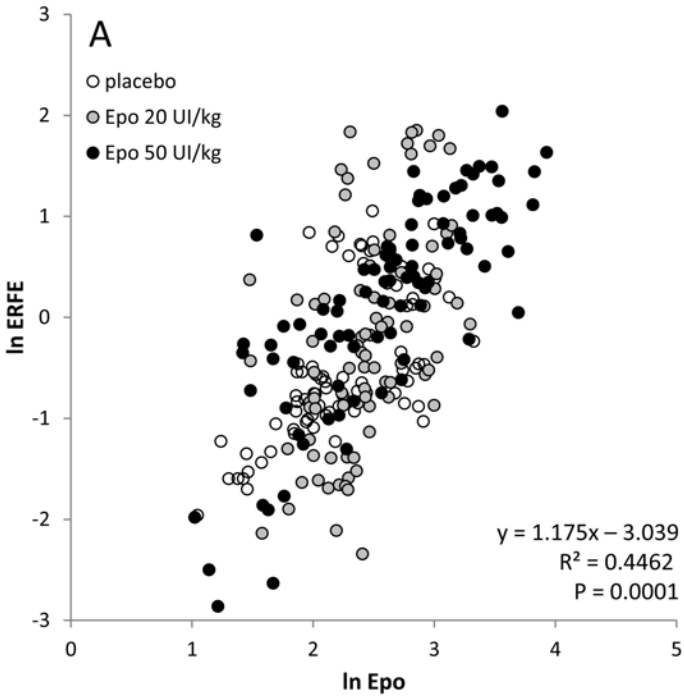
	PLA	EPO20	EPO50	Group	Occurrence	G × O
24 h	153 ± 1.2	151 ± 1.2	154 ± 1.2	P = 0.16	P = 0.52	P = 0.23
48 h	150 ± 0.9	153 ± 0.9	155 ± 0.9*	P = 0.002	P = 0.03	P = 0.35
72 h	150 ± 1.3	155 ± 1.3*	158 ± 1.3*	P = 0.0001	P = 0.39	P = 0.05
7 days	147 ± 1.6	154 ± 1.6*	162 ± 1.6*‡	P = 0.0001		
14 days	150 ± 2.3	157 ± 2.2	164 ± 2.2*	P = 0.002		

	PLA	EPO20	EPO50	Group	Occurrence	G × O
24 h	44.4 ± 0.4	44.3 ± 0.4	44.6 ± 0.4	P = 0.81	P = 0.52	P = 0.68
48 h	44.1 ± 0.3	44.6 ± 0.3	45.3 ± 0.3*	P = 0.02	P = 0.009	P = 0.38
72 h	43.4 ± 0.4	45.4 ± 0.3*	46.5 ± 0.3*	P = 0.0001	P = 0.70	P = 0.004
7 days	42.9 ± 0.5	45.0 ± 0.5*	47.9 ± 0.5*‡	P = 0.0001		
14 days	44.0 ± 0.8	45.9 ± 0.7	48.2 ± 0.7*	P = 0.005		

	PLA	EPO20	EPO50	Group	Occurrence	G × O
24 h	5.02 ± 0.03	4.90 ± 0.03*	4.86 ± 0.03*	P = 0.001	P = 0.001	P = 0.08
48 h	4.97 ± 0.03	4.60 ± 0.03*	4.41 ± 0.03*‡	P = 0.0001	P = 0.0001	P = 0.0001
72 h	4.86 ± 0.05	4.33 ± 0.05*	4.10 ± 0.05*‡	P = 0.0001	P = 0.0001	P = 0.06
7 days	4.86 ± 0.11	4.52 ± 0.11	4.20 ± 0.11*	P = 0.001		
14 days	4.83 ± 0.09	4.79 ± 0.09	4.71 ± 0.09	P = 0.66		

	PLA	EPO20	EPO50	Group	Occurrence	G × O
24 h	25.8 ± 2.6	20.2 ± 2.6	26.3 ± 2.5	P = 0.20	P = 0.09	P = 0.62
48 h	25.8 ± 1.6	22.1 ± 1.6	21.6 ± 1.6	P = 0.13	P = 0.78	P = 0.33
72 h	25.6 ± 1.6	23.7 ± 1.7	20.5 ± 1.6	P = 0.09	P = 0.88	P = 0.07
7 days	29.5 ± 4.1	28.3 ± 4.1	30.5 ± 4.0	P = 0.93		
14 days	31.1 ± 5.9	24.7 ± 6.0	32.8 ± 5.9	P = 0.62		

Figure 2


A
placebo
Epo 20 UI/kg
Epo 50 UI/kg
ln ERFE
ln Epo
y = 1.175x – 3.039
R² = 0.4462
P = 0.0001

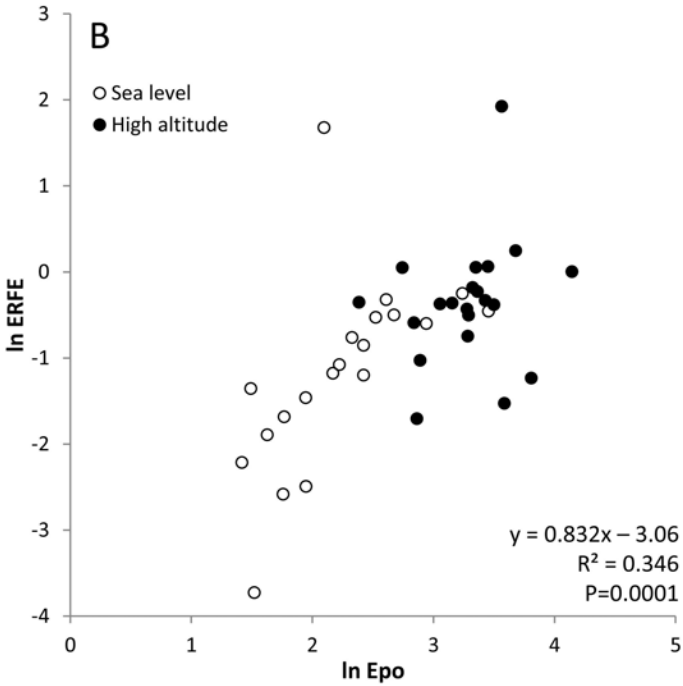

B
Sea level
High altitude
ln ERFE
ln Epo
y = 0.832x – 3.06
R² = 0.346
P=0.0001

Figure 3

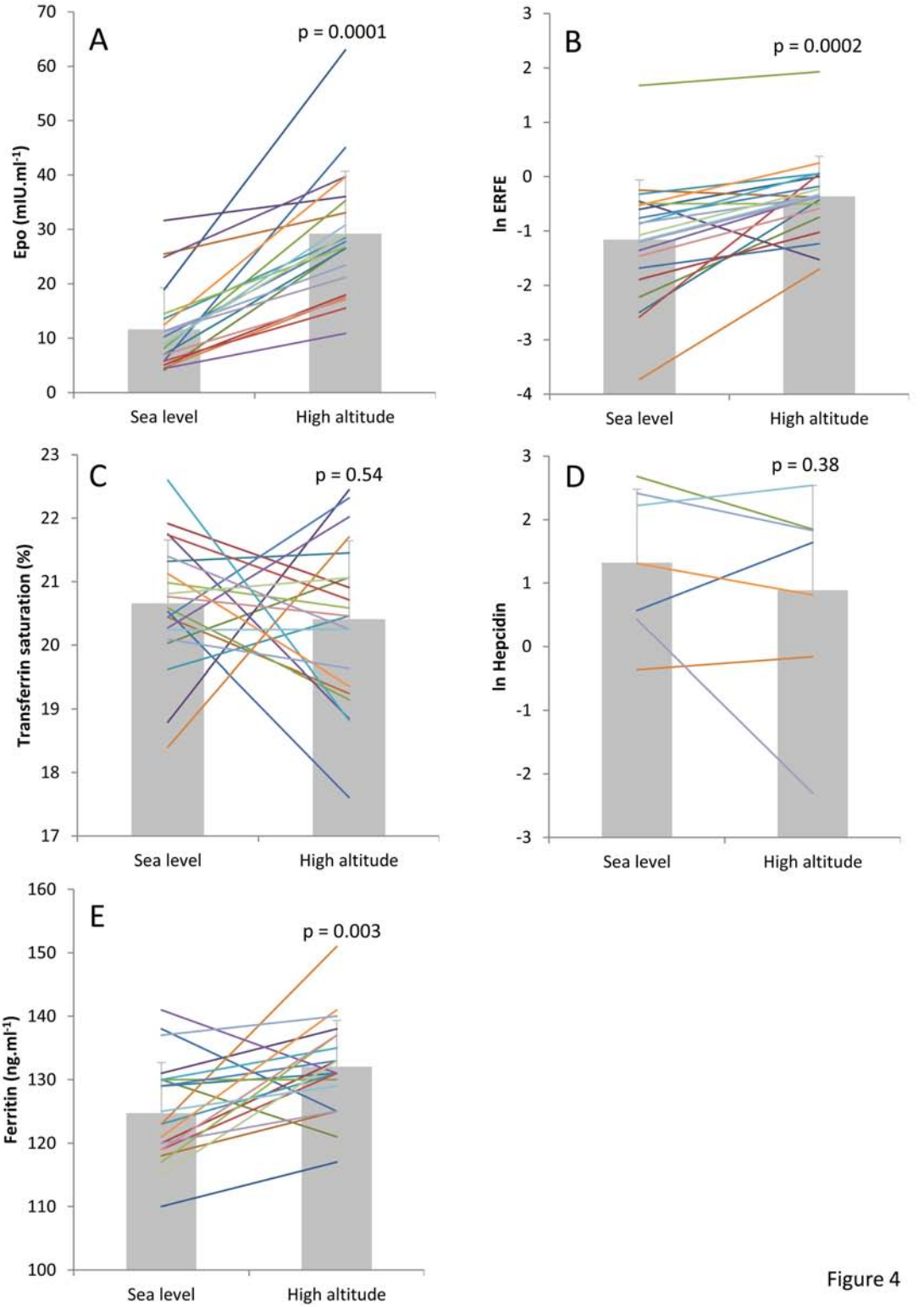

A
p = 0.0001
Epo (mIU.ml-1)
70
60
50
40
30
20
10
0
Sea level
High altitude
B
p = 0.0002
ln ERFE
3
2
1
0
-1
-2
-3
-4
Sea level
High altitude
C
p = 0.54
Transferrin saturation (%)
23
22
21
20
19
18
17
Sea level
High altitude
D
p = 0.38
ln Hepcidin
3
2
1
0
-1
-2
-3
Sea level
High altitude
E
p = 0.003
Ferritin (ng.ml-1)
160
150
140
130
120
110
100
Sea level
High altitude

Figure 4

Supplementary material

Experimental design

Study 1. Recombinant erythropoietin (rhEpo) treatment. 24 healthy non-athlete male subjects (age 35 ± 9 years, height 177 ± 5 cm, body mass 74 ± 7 kg) gave written informed consent to participate in a randomized, double-blind, placebo-controlled study (NCT03276910) approved by the ethics committee (CPP Est-III, EudraCT 2017-000375-82). Only male subjects were included in this preliminary study to avoid the confounding effects that monthly blood loss in premenopausal women may have on iron parameters. After duplicate baseline collection on days -3 and 0, the subjects received six subcutaneous injections of saline (placebo, n=8) or rhEpo (epoietin alpha, Eprex®, Janssen-Cilag) at two different doses, i.e. 20 UI.kg^{-1} (micro-dose, n=8) or 50 UI.kg^{-1} (low-dose, n=8) on days 0, 2, 4, 7, 9 and 11, according to doping protocols used by athletes. Venous blood (12 ml) was collected on days 1, 2, 3, 4, 7, 9, 11, 14, 18 and 25. These 10 samples were collected 24 hours (two occurrences), 48 hours (four occurrences), 72 hours (two occurrences), 7 days (one occurrence) and 14 days (one occurrence) after an injection. All sampling and subsequent injections were performed in the morning at the same time for each subject. Hb_{mass} was determined supine on the mornings on days -3 (Pre) and 14 (Post) via a carbon-monoxide rebreathing technique (OpCO, Detalo Instruments, Copenhagen), as described elsewhere [1]. Carboxyhemoglobin levels were assessed with a co-oximeter ABL80-COOX-OSM (Radiometer, Copenhagen).

Study 2. High-altitude exposure. 22 healthy subjects (eight women, age 36 ± 10 years, height 174 ± 9 cm, body mass 69 ± 11 kg) gave written informed consent to participate in a study (NCT02778659) approved by the ethics committee (CPP Sud-Est-III, EudraCT 2015-004512-38) [2]. Venous blood was collected supine upon wake up at 07:15 a.m. under the same temperature conditions at sea level and after 15 hours of exposure to hypobaric hypoxia (Aiguille du Midi, 3800 m).

Analyses

Hb concentration (Hb) and hematocrit (Hct) were assessed on whole blood with a XN Series analyzer (Sysmex).

Serum was stored at -80°C and ERFE levels were determined using an ELISA assay (Intrinsic LifeSci.) that detects ERFE in a standard range 0.16-10 ng.mL^{-1} and has been validated with clinically relevant human samples [3]. To further validate this assay, we measured serum ERFE

concentration in 3 patients with anemia induced by bleeding (Hb concentration 96 ± 0.6 $g.L^{-1}$). In line with previous findings showing a 3-4-fold increase of ERFE in blood donors [3], we found that ERFE levels were 3.7 ± 1.4 $ng.mL^{-1}$ during anemia induced by bleeding, as compared to the mean concentration of 0.7 ± 1.2 $ng.mL^{-1}$ determined at baseline in the healthy subjects examined in the present studies 1 and 2. Of note, ERFE after low-dose rhEpo reached levels of 3.6 ± 1.3 $ng.mL^{-1}$, similar to those observed during anemia induced by bleeding.

The other proteins were evaluated using commercially available ELISA for Epo (Quantikine, R&D Systems), IL-6 (high sensitivity, eBioscience), ferritin (Architect Ferritin, Abbott, IL). Serum iron was assessed by FerroZine colorimetry, transferrin was measured by nephelometry with N latex human transferrin and transferrin saturation (Tfsat) was calculated. Serum hepcidin was measured by SELDI-TOF mass spectrometry as described elsewhere [4].

Statistical analysis

Reported values are means ± SD. Statistics were performed with SPSS® (version 22.0, IBM Corp, Armonk, NY). In study 1, Hb_{mass} data were analyzed using a two-way repeated measure ANOVA (time × treatment) with the Bonferroni's method for pairwise comparisons. Mixed effects models were used to compare Epo, ERFE, hepcidin, Hb, Hct, ferritin, and Tfsat between groups at the different time points (study 1). Data were ln transformed to meet the conditions of applications of parametric tests, when needed. The group was included as a fixed factor, and baseline levels of these parameters were used as a covariate. We also tested the occurrence (one to four occurrences, depending on the time point considered) to assess the cumulative effect of repeated rhEpo injections, as well as the group × occurrence interaction. Since there was a strong cumulative effect on ferritin (see Fig 2C) preventing us to determine the onset of ferritin decline with the present statistical model, we also perform t tests on ferritin concentrations at 24 and 48 h after the first rhEpo injection. t tests indicated that ferritin levels in placebo subjects were similar to those observed in rhEpo-treated subjects with micro-dose (P values of 0.17 and 0.18 at 24 and 48 hours, respectively) or low-dose (P values of 0.39 and 0.29 at 24 and 48 hours, respectively). To evaluate the acute effect of rhEpo injection on ERFE, hepcidin and ferritin laboratory values, we calculated the area under the curve (AUC), by using the first three time points (i.e. baseline, 24 and 48 h after first injection). AUC were analyzed using a non-parametric Kruskal-Wallis test, indicating a significant rhEpo treatment effect for ERFE (placebo: 1.6 ± 1.4; Epo 20 $UI.kg^{-1}$: 3.1 ± 3.6; Epo 50 $UI.kg^{-1}$: 4.0 ± 1.6; P = 0.03), but no treatment effect for hepcidin (placebo: 15.3 ± 8.9; Epo 20 $UI.kg^{-1}$: 7.7 ± 5.6; Epo 50 $UI.kg^{-1}$: 6.3 ± 3.6; P = 0.11) or ferritin (placebo: 399 ± 242; Epo 20 $UI.kg^{-1}$: 259 ± 69; Epo 50 $UI.kg^{-1}$: 313 ± 105; P = 0.37). In study 2, data were analyzed using a one-

way repeated measure ANOVA. For both studies, the relationship between two quantitative parameters was examined by linear regression. Unpaired t tests were performed on ln-transformed data to compare the effects of single rhEpo micro-dose injection (at 24 h) versus high-altitude exposure on ERFE and Epo increases. A P value of $< .05$ was considered significant.

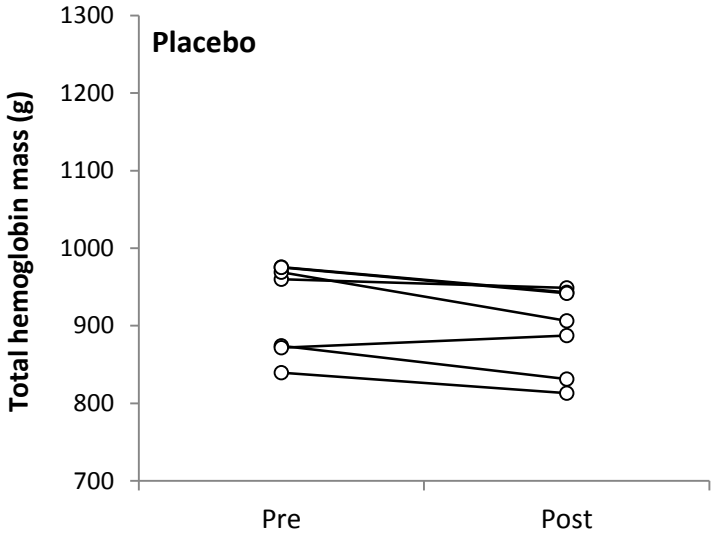
Supplementary references

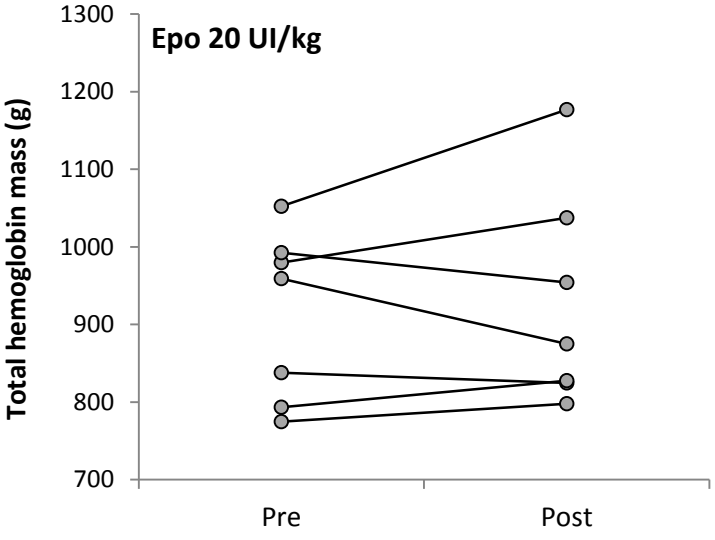
1. Siebenmann C, Keiser S, Robach P, Lundby C. CORP: The assessment of total hemoglobin mass by carbon monoxide rebreathing. J Appl Physiol (1985). 2017;123(3):645-654.
2. Bouzat P, Sechaud G, Banco P, et al. The effect of zolpidem on cognitive function and postural control at high altitude. Sleep. 2018;41(10).
3. Ganz T, Jung G, Naeim A, et al. Immunoassay for human serum erythroferrone. Blood. 2017;130(10):1243-1246.
4. Udali S, Castagna A, Corbella M, et al. Hepcidin and DNA promoter methylation in hepatocellular carcinoma. Eur J Clin Invest. 2018;48(2):e12870.

Supplementary figure legends

Supplementary Figure 1. Individual values of total hemoglobin mass, determined at baseline (Pre) and 72 hours after the last of the six injections (Post) of placebo (n = 7), rhEpo 20 $UI.kg^{-1}$ (micro-dose) (n = 7) or 50 $UI.kg^{-1}$ (low-dose) (n = 8). Means, standard deviations and statistics are reported on Figure 1A.

Supplementary Figure 2. Relationship between individual serum concentrations (ln transformed) of hepcidin plotted against Epo (panel A) and erythroferrone (ERFE, panel B) before, during and after six injections of placebo, micro-dose or low-dose rhEpo in healthy subjects. Regression equations, coefficients of determination and P values are $y = -1.138x + 2.782$, $R^2 = 0.1025$, $P=0.0001$(panel A), and $y = -0.538x – 0.052$, $R^2 = 0.0721$, $P = 0.0001$ (panel B).


Placebo
Total hemoglobin mass (g)
1300
1200
1100
1000
900
800
700
Pre
Post


Epo 20 UI/kg
Total hemoglobin mass (g)
1300
1200
1100
1000
900
800
700
Pre
Post

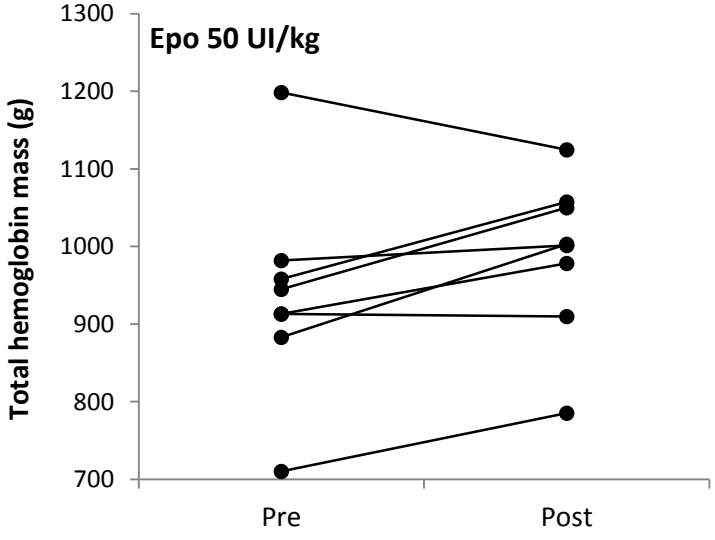
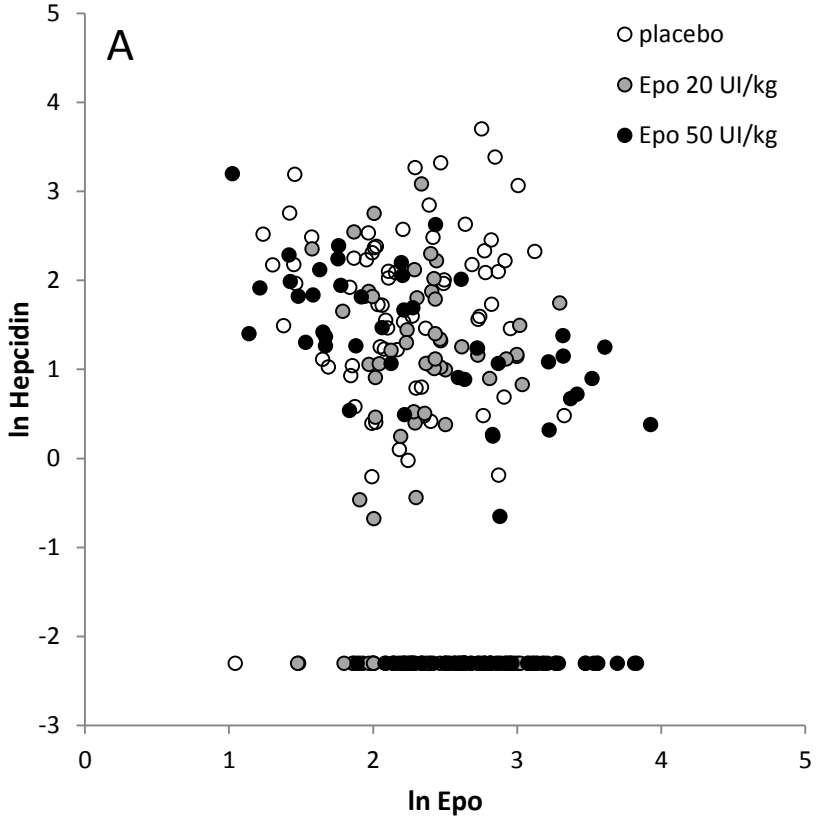

Epo 50 UI/kg
Total hemoglobin mass (g)
1300
1200
1100
1000
900
800
700
Pre
Post

Figure S1


A
placebo
Epo 20 UI/kg
Epo 50 UI/kg
ln Hepcidin
ln Epo
5
4
3
2
1
0
-1
-2
-3
0
1
2
3
4
5

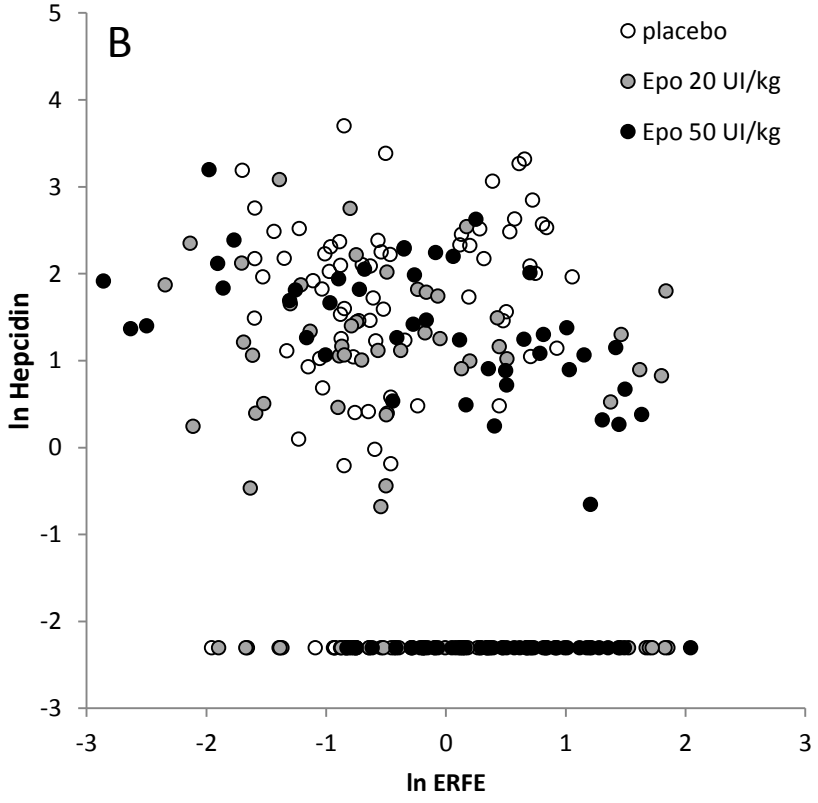

B
placebo
Epo 20 UI/kg
Epo 50 UI/kg
ln Hepcidin
ln ERFE
5
4
3
2
1
0
-1
-2
-3
-3
-2
-1
0
1
2
3

Figure S2