SUPPLEMENTARY APPENDIX

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

Paul Robach,[1*] Elena Gammella,[2*] Stefania Recalcati,[2] Domenico Girelli,[3] Annalisa Castagna,[3] Matthieu Roustit,[4] Carsten Lundby,[5] Anne-Kristine Lundby,[5] Pierre Bouzat,[4] Samuel Vergès,[6] Guillaume Séchaud,[4] Pierluigi Banco,[4] Mario Uhr,[7] Catherine Cornu,[8] Pierre Sallet[9] and Gaetano Cairo[2]

[1]National School for Mountain Sports, Chamonix, France; [2]Department of Biomedical Sciences for Health, University of Milan, Italy; [3]Department of Medicine, University of Verona, Italy; [4]Grenoble Alpes University Hospital, Grenoble, France; [5]Center for Physical Activity Research, University Hospital, Copenhagen, Denmark; [6]HP2 Laboratory, U1042, Grenoble Alpes University, INSERM, Grenoble, France; [7]Department of Hematology Synlab-Suisse, Lugano, Switzerland; [8]Hospices Civils de Lyon INSERM CIC1407/UMR5558, Hôpital Louis Pradel, Bron, France and [9]"Athletes for Transparency" Association, Lyon, France

**PR and EG contributed equally as co-first authors.*

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Correspondence: *GAETANO CAIRO* - gaetano.cairo@unimi.it

PAUL ROBACH - paul.robach@ensm.sports.gouv.fr

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

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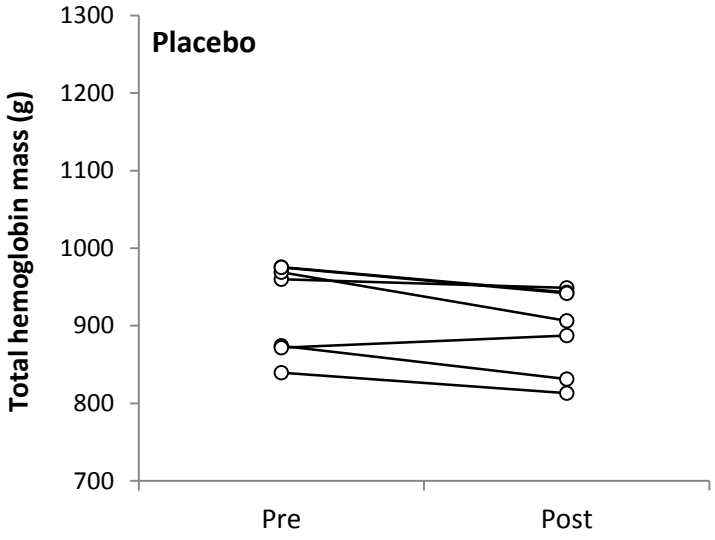
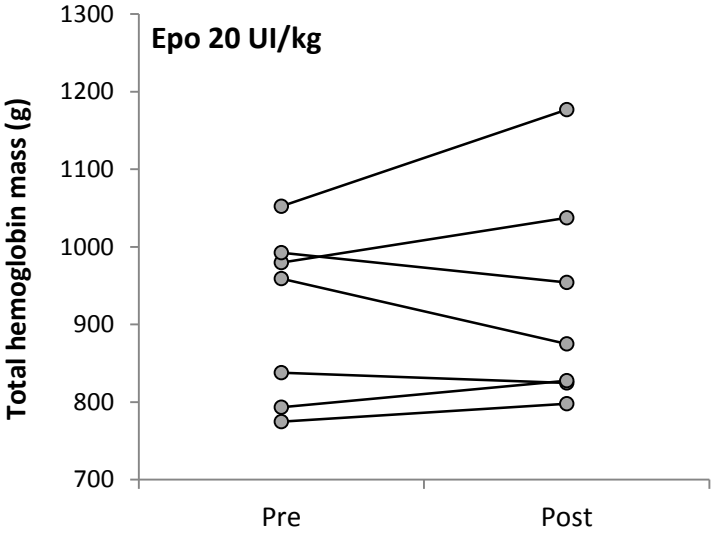
Supplementary figure legends

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

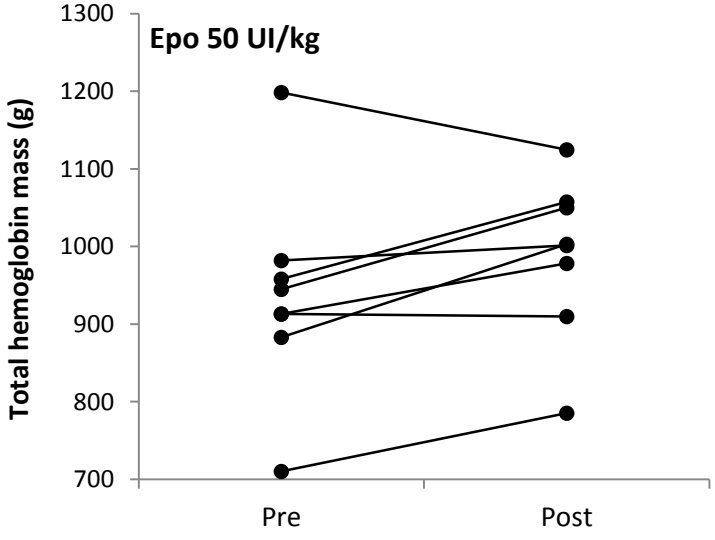
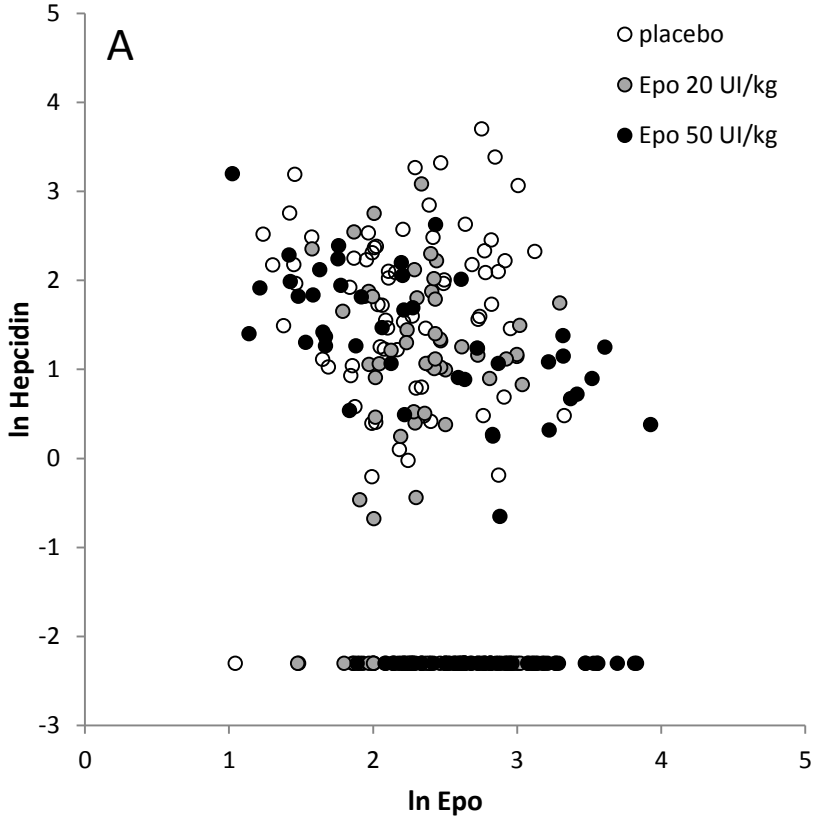

Epo 50 UI/kg
Total hemoglobin mass (g)
1300
1200
1100
1000
900
800
700
Pre
Post

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

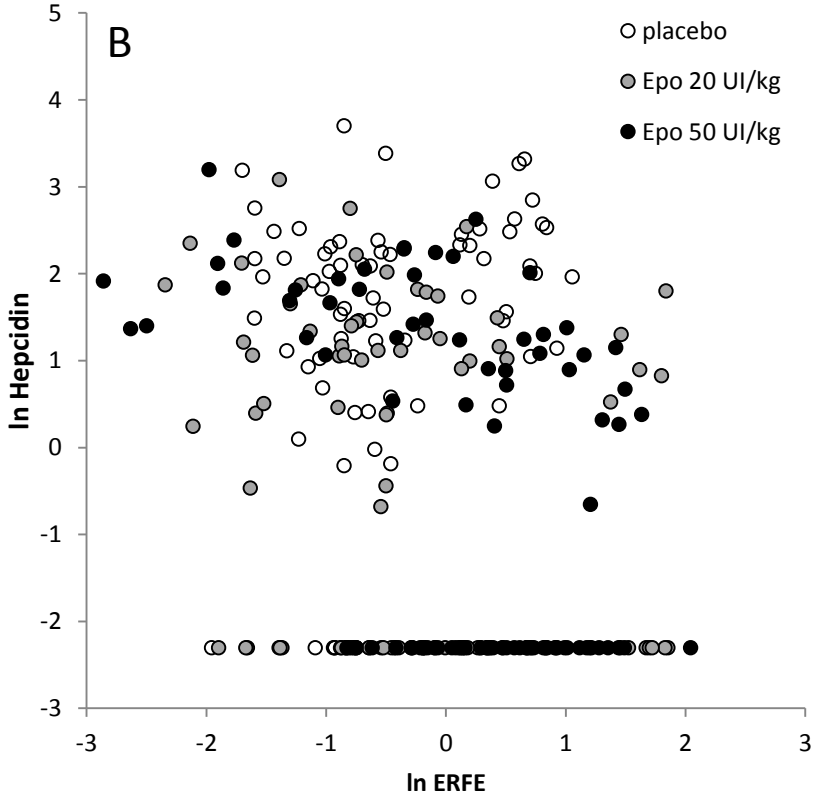

B
placebo
Epo 20 UI/kg
Epo 50 UI/kg
ln Hepcidin
ln ERFE
5
4
3
2
1
0
-1
-2
-3
-3
-2
-1
0
1
2
3

Figure S2