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Erythroferrone as a sensitive biomarker to detect stimulation of erythropoiesis

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Running Title : Erythroferrone as potential blood doping biomarker

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Abstract

Erythroferrone (ERFE) is a glycoprotein hormone secreted by erythroblasts in response to erythropoietin stimulation. ERFE suppresses hepatic synthesis of the master iron-regulatory hormone, hepcidin. The impact of erythropoiesis stimulation on ERFE secretion in humans is poorly understood. This paucity of information is due in part to the lack of available means for ERFE quantification in serum samples. In the present study, we tested a new sensitive sandwich immunoassay for human ERFE. We used this assay to demonstrate that injection of various erythropoiesis stimulating agents (ESAs) increased blood ERFE levels in healthy volunteers. After exogenous stimulation of erythropoiesis, ERFE increased up to 8-fold with a detection window of 13 days. The impact of one unit of blood withdrawal on erythropoiesis stimulation of ERFE was also tested. ERFE significantly increased after blood withdrawal in subjects injected with both iron and saline solution, suggesting that iron supplementation did not mask ERFE increase after blood withdrawal. We assessed the effects of exercise-induced muscle damage on ERFE by comparing ERFE levels with creatine kinase levels in samples from subjects with heavy exercise loads, and determined that this was not a confounder. The ERFE assay is a sensitive means to investigate the connection between iron metabolism and erythropoiesis in humans, and to detect ESA abuse in the anti-doping field.

Introduction

The greatest deterrent to blood doping with erythropoiesis stimulating agents (ESAs) is the haematological module of the Athlete Biological Passport (ABP) [1]. The haematological module focuses on long-term monitoring of specific blood parameters, such as haemoglobin concentration (Hb) and reticulocyte percentage (Ret%), to detect abnormal absolute and/or relative changes in individual profiles that may indicate doping with ESAs. Stimulation of erythropoiesis can be also observed after the blood withdrawal step necessary for autologous blood transfusion, which is an alternative means for blood doping [1]. However, the shift towards blood transfusions and micro-dose injections of ESAs necessitates the development of new markers that can be integrated into the ABP haematological module to increase its sensitivity [2, 3]. Proteins involved in erythropoiesis and iron metabolism have been suggested as potential biomarkers for ESA abuse [4]. An example for this is hepcidin which is downregulated by recombinant human erythropoietin (rhEPO) administration [5]. In 2014, erythroferrone (ERFE) was discovered as a novel erythroid regulator of iron metabolism in a mouse model [6]. However, ERFE has been described as having the same or a similar structure as myonectin [7]. In its role as an iron metabolism regulator, ERFE mediates hepcidin suppression during increased erythropoietic activity stimulated by endogenous and exogenous EPO, and facilitates compensatory iron acquisition during recovery from haemorrhage-induced anaemia [8]. These observations suggested that ERFE could be a viable biomarker for detection of blood doping. However, because these experiments were almost exclusively performed in mice, comparative studies in humans are necessary, yet quantification of ERFE with a validated immunoassay remains challenging.

Previously, different ERFE immunoassays were tested to investigate the link between iron metabolism and erythropoiesis in human [9, 10]. However, the data presented suggested that these assays were not valuable for detection of ESA abuse in anti-doping context, mainly due

to sensitivity. Furthermore, the influence of various confounding factors were not explored in these different studies.

In the present study, testing and validation of a new commercial available sensitive sandwich immunoassay for human ERFE were performed. The impact of ESA administration and blood withdrawal on ERFE levels in human subjects, as well as potential confounding factors such as iron supplementation and exercise-induced muscle damage, were investigated.

Material and methods

Human ERFE ELISA

The commercial ERFE (human) Matched Pair Detection Set (AG-46B-0012-KI01; AdipoGen Life Sciences, Epalinges, Switzerland) was used per the manufacturer's instructions (https://adipogen.com/ag-46b-0012-erythroferrone-human-matched-pair-detection-set.html). Briefly, 100 µL of standard and serum samples was added to the ELISA plate and detected by absorbance. The limit of detection was determined by adding three standard deviations to the mean value of 50 background level (zero concentration of ERFE) standards. The standard of the ELISA kit is the full-length Erythroferrone recombinant protein with a His-tag expressed in mammalian cells. This protein has been validated by AdipoGen Life Sciences and is characterized to be stable for at least one year when stored at 4°C. To assess intra-assay precision, four samples of known human recombinant ERFE concentration were assayed in replicate six times. To test inter-assay precision, three samples of known human ERFE concentration were assayed in four separate assays. The limit of detection of the immunoassay was calculated as an apparent concentration of 40 pg/mL. The intra- and inter-day variation ranges were 2–6% and 5–9%, respectively.

Clinical study samples

Clinical studies: administration of erythropoietic agents

Details regarding the participants and the time of serum sample collection of the different clinical studies are described in previous studies which are summarized below.

For the first generation rhEPO clinical study, six healthy Caucasian males (mean age, 27.0 years (SD, 4.1); mean body mass index (BMI), 23.9 kg/m^2 (SD, 2.66)) received a single intravenous injection of rhEPO delta (5000 IU; Dynepo™, Dynepo Shire Pharmaceuticals, Basingstoke, UK) on days 1, 3 and 5 of the study as previously described [11].

For the third generation rhEPO clinical study, six healthy Caucasian men with a mean age of 23.0 years (SD, 2.97) and a mean BMI of 23.3 kg/m^2 (SD, 1.48) received a single subcutaneous or intravenous injection of 200 mg of C.E.R.A. (MIRCERA; Roche Pharma AG, Reinach, Switzerland) as in [12].

In another study, six healthy Caucasian males received a single intravenous injection of the EPO analog Hematide/peginesatide (Affymax, Inc. Palo Alto, CA) at 50 µg/kg as described previously [13].

Clinical study: blood withdrawal and iron injection

A randomized, single-blind, placebo-controlled trial was approved by the Human Research Ethics Committee of the Canton of Vaud in Switzerland (Protocol: 2016-00324). Participants were randomized to receive an IV injection of either iron or placebo at a ratio of 1:1 using R studio software (Version 1.0.44). Volunteers were blinded to the study treatment by covering blood bags with opaque bags. The ClinicalTrials.gov identifier is NCT03014921, and the study is entitled “Impact of Iron Injection on Blood Donation: a Randomized and Controlled Clinical Trial.” The study design is shown in Supplementary Figure 1. Written informed consent was obtained from each subject prior to enrolment in the study.

Male Caucasian volunteers aged 20–35 years with a ferritin concentration ≤ 50 μg/L ± 10% and a BMI of 18–30 who were eligible for blood donation according to national regulations were invited to participate.

The iron supplement used was a 250 mL perfusion of 0.9% NaCl (B. Braun Medical AG, Crissier, Switerland) combined with 10 mL of a 500 mg ferric carboxymaltose complex (Ferinject®, Vifor Pharma, Villars-sur-Glâne, Switzerland). The placebo used in the control group was a 250 mL perfusion of 0.9% NaCl. (Supplementary Figure 1).

Clinical study: impact of exercise-induced muscle damage on serum ERFE levels

Ten healthy, Caucasian middle- and long-distance runners took part in this study. The subjects provided written informed consent, and the study was approved by the institutional review board (E2015000073) of ADL Qatar. Serum samples were collected at sea level at various time points over a period of 2 weeks. To assess the level of muscle damage, serum samples were analysed for creatine kinase (CK), a circulating protein that is considered an indirect marker for muscle damage. To reflect individual responses to exercise-induced muscle damage, three samples per subject were included in this study: the sample with the lowest and the two samples with the highest CK values. The highest concentration of CK obtained in this study was 1330 IU/L.

Clinical chemistry, immunology and haematology

All venous blood samples were obtained from an antecubital vein according to standard procedures. Blood was drawn into 8.5 mL serum tubes (SST II Advance, BD Vacutainer, ref. 366644). Serum tubes were centrifuged for 15 min at 1500 rcf after collection and stored at -20°C or -80°C until further analysis. Transferrin saturation (TSAT) was measured using an automated Dimension EXL 2000 technology system (Siemens Healthcare Diagnostic SA Zurich, Switzerland) according to the manufacturer's instructions.

Ferritin concentration was measured with a Centaur instrument. Erythropoietin (EPO) was measured in serum using an Immulite system (Siemens Healthcare Diagnostic SA, Zurich, Switzerland). CK was measured on a fully automated Cobas Integra 400 plus analyser (Roche Diagnostics, Rotkreuz, Switzerland). Reticulocyte percentage (Ret%) was measured using a fully automated haematology analyser (Sysmex XN 2000, Sysmex AG, Yverdon-les-Bains, Switzerland). Serum hepcidin concentration was measured by liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) according to a method described by Leuenberger et al. [5].

Statistical analyses

A Shapiro test was used to determine the normality of the data. For normally distributed data, comparisons between and within groups were performed using an ANOVA followed by post-hoc pairwise comparisons (t-test adjusted by the Bonferroni correction and Tukey HSD). Non-parametric comparisons were performed using the Kruskal-Wallis and Wilcoxon tests. Correlations were assessed using a Spearman's correlation test. A p-value < 0.05 was considered significant. All tests were conducted using R studio software (Version 1.0.44). Data are presented as mean ± SEM, unless otherwise noted.

Results

After injection of recombinant EPOs and the analog peginesatide, ERFE substantially increased in minimum of 50 % in all subjects (Figure 1A–C). In the Dynepo study, ERFE reached the highest concentrations on day 3 after the second injection. Interestingly, the responses were dependent on the individual, with two of the six subjects showing only small changes. Additional injections did not further increase ERFE levels. For peginesatide, ERFE was increased by an average of 8-fold on day 6 from 0.9 to 6.3 ng/mL, with concentrations

decreasing back to baseline on day 13. In the C.E.R.A. group, subjects had an average 6-fold increase from 0.64 to 3.9 ng/mL, which returned to baseline on day 10. A positive significant Spearman's correlation (rho=0.73; p=1.34e-08) was observed between ERFE concentration and Ret% after stimulation by C.E.R.A. injection (Supplementary Figure 2).

Table 1 summarizes the iron (ferritin, TSAT and hepcidin) and erythropoiesis variables at baseline and after blood withdrawal preceded by saline (saline group) or iron (iron group) injection. The clinical study design is shown in the supporting information (Supplementary Figure 1). Baseline concentration in iron markers was significantly higher in the iron group than in the saline group, presumably due to the 14 day preceding iron injection. In both groups, ferritin and TSAT were deceased after blood withdrawal. As expected, EPO was significantly induced from day +1 up to day +15 to compensate for blood loss due to withdrawal. By contrast, hepcidin was significantly decreased in the saline group, but no change was observed in the iron-treated group, after blood withdrawal.

In the saline group, the ERFE concentration rose on day +1 (Figure 2A). This elevation lasted until day +9, after which the ERFE concentration returned close to the initial value of the baseline. For the iron group, a significant ERFE increase was observed from day +3 until day +6 (Figure 2B).

Healthy blood donors (30 females and 40 males) were subjected to ERFE measurements (Supplementary Figure 3). The median ERFE concentration in males and females was 0.57 ng/mL (95% CI, 0.53–0.65 ng/mL) and 0.48 ng/mL (95% CI, 0.45–0.55 ng/mL), respectively. The maximum and minimum concentration in males and females were 0.17 and 1.11 and 0.16 and 1.1 ng/mL, respectively. No significant sex difference in ERFE concentrations was observed (Supplementary Figure 3).

To assess the impact of exercise-induced muscle damage, CK values from human subjects with a high exercise load were compared with ERFE concentrations (Figure 3A). No significant correlation (Spearman's rho = 0.30, p=0.1) was observed between ERFE and CK concentrations. Also, ERFE concentration was unchanged in iron-injected subjects (Figure 3B). By contrast, other haematological and iron biomarkers, such as Ret%, TSAT, ferritin and hepcidin, were impacted by iron injection (Supplementary Table 1).

Discussion

Impact of ESAs injection and blood withdrawal on ERFE level

This study investigated whether serum ERFE levels could be used to detect small changes in erythropoiesis. The impacts of different ESAs and the withdrawal of one bag of blood (450 mL) on ERFE concentration were investigated. The goal was to test and validate a new specific and sensitive sandwich immunoassay. Previously, the association between ERFE and biomarkers of erythropoiesis and iron metabolism has been evaluated using a commercially available sandwich ELISA kit constituting mouse monoclonal antibodies [10]. We previously evaluated this commercially available kit, and found that ERFE quantification by this method was not reproducible [14]. ERFE responses following rhEPO administration were highly variable, and not coherent [14]. Other commercial ELISAs produced with mouse antibodies were also tested unsuccessfully.

Previously, Ganz et al. developed a rabbit monoclonal antibody-based sandwich immunoassay for detection of human ERFE [9]. A 14 ng/mL limit of quantification was reported for this assay. Contrastingly, the limit of quantitation for our validated ELISA was 0.04 ng/mL. However, both assays and standards should be directly compared to draw conclusions.

The response to blood withdrawal was examined in subjects injected previously with iron or saline solution (placebo). ERFE was increased in both contexts, reaching a maximum concentration after 6 days. In placebo patients, this ERFE increase was coincident with a decline in serum hepcidin concentrations, but this correlation was not present when iron was supplemented before blood withdrawal. Previously, Mirciov et al. demonstrated that high circulating iron levels overcome ERFE inhibition of hepcidin in mice [15]. This effect did not alter the expression of ERFE itself, and seemed linked to the interference of di-ferric transferrin. This explanation could be also extrapolated to the present study, due to the high TSAT in our iron injection group. Our data suggest that, in humans, the inhibitory effect on hepcidin is not needed when enough circulating iron is available, similar to mice.

The personalized follow-up of Ret% is the most common indirect biomarker for detection of erythropoiesis stimulation in the context of the ABP. Due to the cellular feature of Ret%, analyses should be performed within approximately 48 hours after collection. By contrast, many serum biomarkers can be analysed up to 10 years after storage at -20°C. In fact, some samples used in the present study were collected 10 years ago in previous clinical studies (Figure 1) [11-13]. Although ERFE concentration significantly correlates with Ret%, the relative increase observed in ERFE concentration was higher than that of Ret%. Indeed, after ESA injection (peginesatide) an increase in ERFE up to 8-fold was observed.

Assessment of different confounding factors on ERFE concentration

ERFE was identified as an erythroid regulator of iron metabolism [6, 8]. However, ERFE had earlier been described as myonectin, or C1q tumour necrosis factor α-related protein isoform 15" (CTRP15), and in mice, was highly expressed in muscle tissue [7]. In this context, muscle ERFE levels were increased after physical activity in rodents. Because myonectin is produced also in the muscle of humans, serum samples from a previously conducted exercise study were

used to determine if muscle damage could cause myonectin to potentially leak into the blood, affecting circulating ERFE. In the present study, no increase of circulating ERFE was detectable after muscle damage, as indicated by CK levels (Figure 3A). These data suggest that this approach is a specific measurement of circulating ERFE secreted from erythroid origin after erythropoietic stimulation, rather than regulation of ERFE by myocyte metabolism. The apparent specificity of our sandwich immunoassay is currently under investigation in our laboratory. However, a difference of ERFE isoforms caused by specific glycosylation in erythroid cells or a different secretion of the quaternary structure (monomeric vs multimeric proteins) in muscle versus erythroid cells is a theoretically possible explanation.

A single infusion of iron was reported to improve fatigue, mental health, cognitive function and erythropoiesis in iron-deficient women with normal or borderline Hb concentrations [16]. Some endurance athletes inject iron to maintain a high serum ferritin concentration [17]. IV iron supplementation improves fatigue and overall mood in runners without a clinical iron deficiency [18]. Iron injection is not considered to be a doping method, but could potentially interfere with anti-doping biomarkers involved in iron metabolism. In the present study, we identified that iron injection did not affect ERFE concentration (Figure 3B). As expected, iron injection dramatically affected ferritin, TSAT and hepcidin (Supplementary Table 1). Interestingly, increased Ret%, an actual haematological maker of the ABP, was observed after iron injection, as previously demonstrated in females [16]. Together, these data reinforce the specificity of the presented ERFE immunoassay for anti-doping purposes.

Regarding the assessment of other confounding factors, no influence of sex on ERFE concentrations was observed (Supplementary Figure 3). To ensure the specificity of ERFE' response to ESAs administration, the impact of altitude exposure as a confounding factor on ERFE should be characterized. Because ERFE and reticulocytes correlate closely

(Supplementary Figure 2), the influence of altitude exposure on Ret% measurement should also be valuable for ERFE as biomarker.

In conclusion, we tested a new immunological assay for human ERFE that can sensitively detect the stimulation of erythropoiesis, including ESA abuse and blood withdrawal. In an anti-doping context, this assay could be a useful tool for retrospective investigations in which analysis of Ret% is no longer possible due to sample degradation. Moreover, this assay could be useful to investigate the interactions between erythropoiesis and iron metabolism in human clinical studies.

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Table 1: Iron and erythropoiesis biomarker analyses

Saline (n=8)	Baseline	Day 0	Day +1	Day +2	Day +3	Day +6	Day +9	Day +15	Day +30
Ferritin (µg/mL)	50.39 ± 9.42	48.29 ± 8.48	44.51 ± 8.57	41.46 ± 7.86	**40.28 ± 7.99***	**31.13 ± 5.30***	**26.79 ± 4.71****	**19.93 ± 3.05****	**22.76 ± 4.12****
TSAT (%)	26.98 ± 3.97	27.01 ± 3.02	27.28 ± 3.58	28.71 ± 4.26	25.11 ± 3.07	21.04 ± 3.69	**15.70 ± 1.95***	**15.31 ± 1.65***	19.08 ± 4.22
Hepcidin (nM)	1.50 ± 0.30	1.45 ± 0.40	**1.12 ± 0.39***	**0.70 ± 0.20****	**0.56 ± 0.14****	**0.55 ± 0.11****	**0.43 ± 0.10****	**0.39 ± 0.15****	**0.43 ± 0.10****
EPO (mUI/mL)	10.01 ± 0.93	11.68 ± 0.97	**16.16 ± 2.08****	**16.38 ± 2.08****	**15.69 ± 1.86****	**16.15 ± 1.76****	**15.75 ± 1.80****	**13.71 ± 1.07****	11.16 ± 0.92
Iron (n=8)	**Baseline**	**Day 0**	**Day +1**	**Day +2**	**Day +3**	**Day +6**	**Day +9**	**Day +15**	**Day +30**
Ferritin (µg/mL)	224.51 ±22.27##	220.29 ± 20.41	**176.56 ± 18.26***	**158.99 ± 17.69***	**154.29 ± 18.99***	**121.50 ± 13.79***	**102.01 ± 13.29****	**80.69 ± 12.97****	**64.69 ± 12.66****
TSAT (%)	34.84 ± 5.64#	34.81 ± 5.19	38.81 ± 5.43	34.53 ± 4.15	30.12 ± 3.64	34.93 ± 3.32	32.35 ± 5.42	27.66 ± 5.11	**23.30 ± 4.06***
Hepcidin (nM)	3.69 ± 1.90#	3.49 ± 1.62	3.61 ± 1.27	3.40 ± 2.14	1.95 ± 0.99	2.59 ± 1.09	1.99 ± 1.05	3.07 ± 1.49	3.36 ± 2.01
EPO (mUI/mL)	11.81 ± 1.36	10.01 ± 1.81	**19.46 ± 1.74****	**16.36 ± 1.60****	**18.24 ± 1.10****	**14.70 ± 1.93***	**14.14 ± 1.43***	**16.60 ± 2.62***	10.75 ± 1.67

Data are expressed as mean ± SEM. Significant changes relative to baseline (the mean of day -4 and day -1) are indicated by bold text with grey highlighting. Day 0 corresponds to the mean of hours +3, +6 and +12 after saline or iron injection. Saline: saline-supplemented group; Iron: iron-supplemented group. TSAT: transferrin saturation; EPO: erythropoietin. *p<0.05, **p<0.01. Variance was calculated within groups compared with baseline. #p<0.05, ##p<0.01. Variance was calculated between groups compared with baseline.

Figure 1

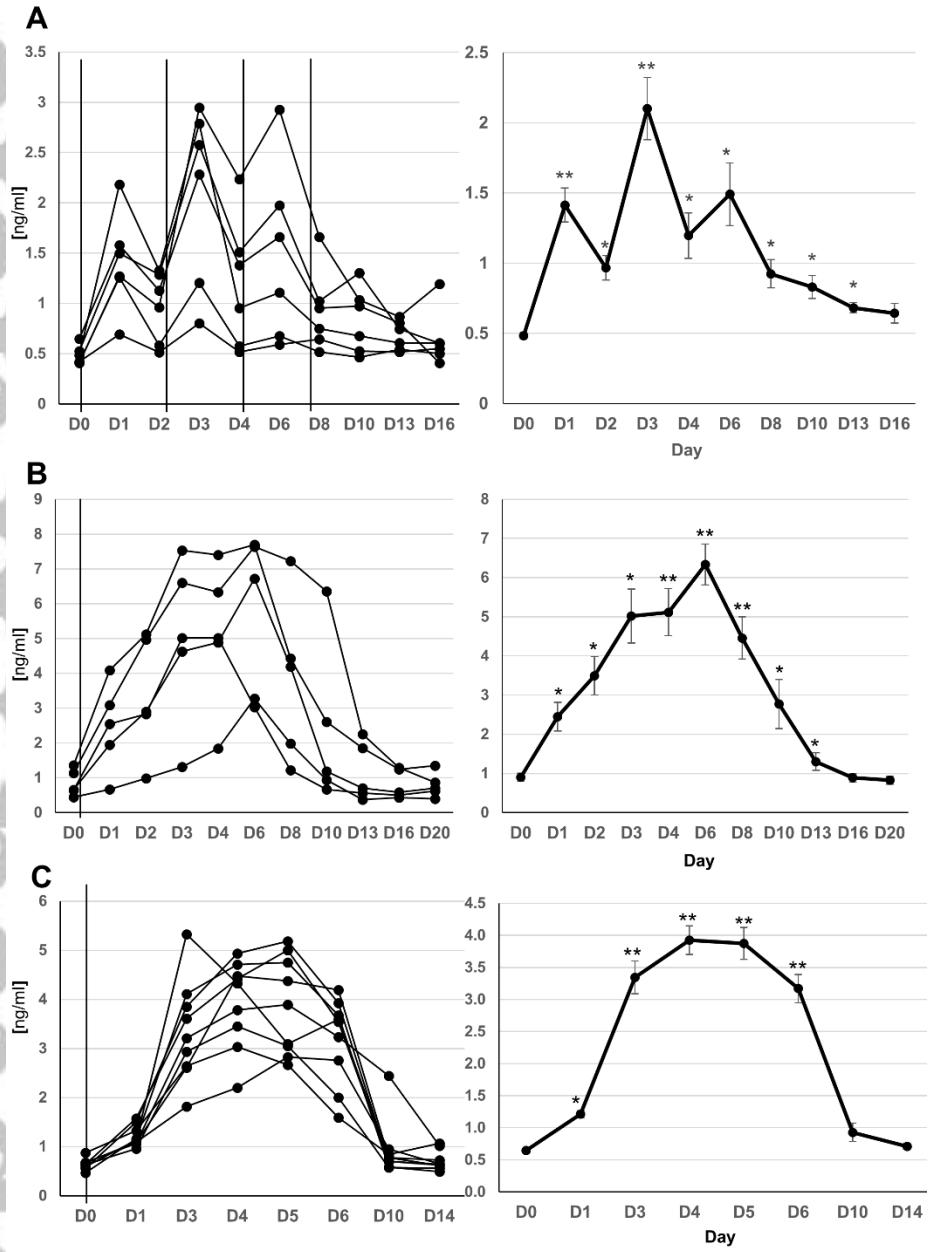


Figure 1: ERFE levels after injection of different ESAs.

(A) Effect of recombinant erythropoietin (rEPO) delta administration on individual (left panel) and mean (right panel) ERFE concentrations. **(B)** Effect of peginesatide administration on individual (left panel) and mean (right panel) ERFE concentrations. **(C)** Effect of C.E.R.A. administration on individual (left panel) and mean (right panel) ERFE concentrations. Vertical black lines represent ESA administration times. D: Days. *$p<0.05$, **$p<0.01$. Variance was calculated within groups compared with D0.

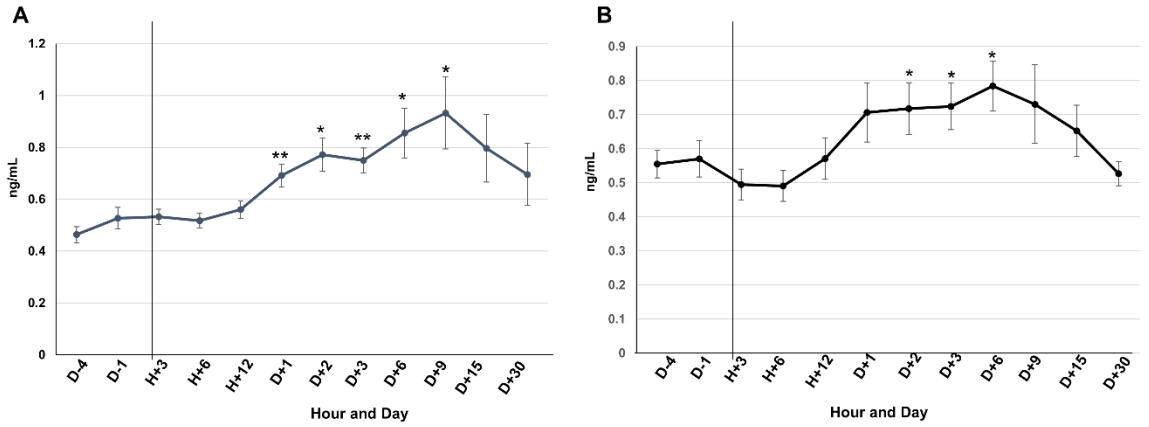


Figure 2: ERFE levels after blood withdrawal.

Effect of blood withdrawal on ERFE concentration in **(A)** saline **(B)** and iron groups. Vertical red lines represent blood withdrawal times. D: Days; H: Hours. *p<0.05, **p<0.01. Variance was calculated within groups compared with baseline (D-1 and D-4).

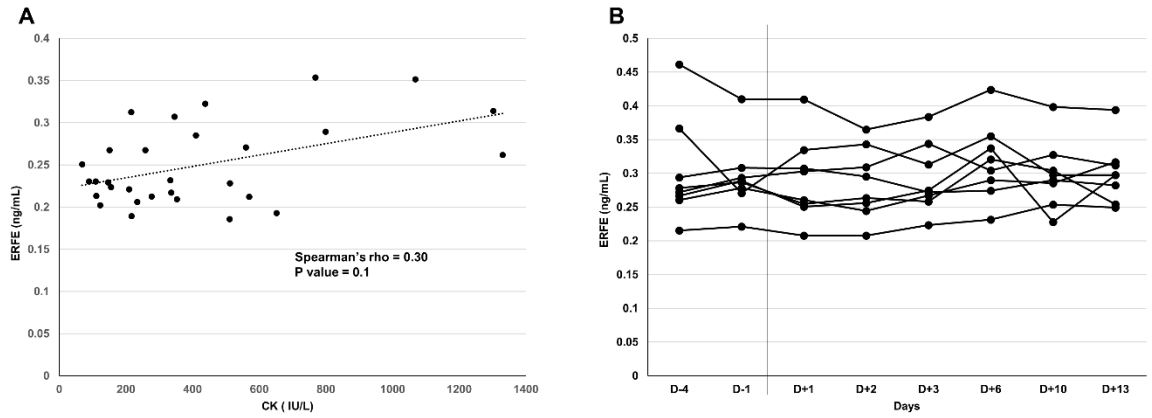
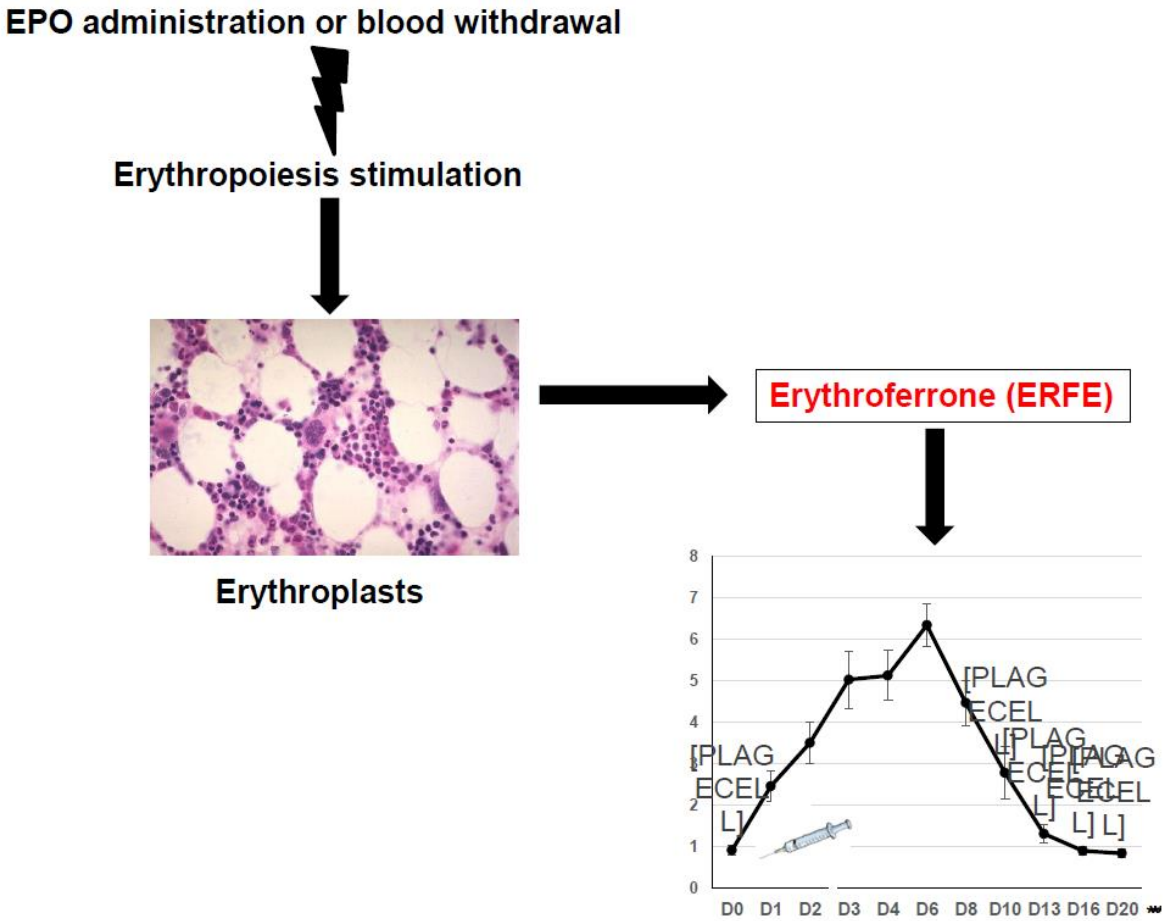


Figure 3. Muscle damage and iron injection did not affect ERFE levels.

(A) Effect of muscle damage (creatine kinase: CK) on ERFE concentration. **(B)** ERFE levels in eight individuals after iron injection (500 mg of Ferinject®).



Summary :

We tested a new immunological assay for human ERFE that can sensitively detect the stimulation of erythropoiesis, including ESA abuse and blood withdrawal. In an anti-doping context, this assay could be a useful tool for retrospective investigations in which analysis of Ret% is no longer possible due to sample degradation. Moreover, this assay could be useful to investigate the interactions between erythropoiesis and iron metabolism in human clinical studies.