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Hepcidin as a potential biomarker for blood doping

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The concentration of hepcidin, a key regulator of iron metabolism, is suppressed during periods of increased erythropoietic activity. The present study obtained blood samples from 109 elite athletes and examined the correlations between hepcidin and markers of erythropoiesis and iron metabolism (i.e., haemoglobin, erythropoietin (EPO), ferritin, erythroferrone (ERFE), and iron concentration). Furthermore, an administration study was undertaken to examine the effect of recombinant human EPO (rhEPO) delta (Dynepo™) on hepcidin concentrations in healthy male volunteers. The effects on hepcidin were then compared with those on reticulocyte percentage (Ret%) and ferritin concentration. There was a significant positive correlation between hepcidin and ferritin, iron, and haemoglobin levels in athletes, whereas hepcidin showed an inverse correlation with ERFE. Administration of rhEPO delta reduced hepcidin levels, suggesting that monitoring hepcidin may increase the sensitivity of the Athlete Biological Passport (ABP) for detecting rhEPO abuse. Copyright © 2016 John Wiley & Sons, Ltd.

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Keywords: hepcidin; blood doping; EPO

Introduction

The greatest deterrent to blood doping using 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 the reticulocyte percentage (Ret%), to detect abnormal absolute and/or relative changes in individual profiles that may indicate doping with ESAs; however, the shift towards blood transfusions and 'micro-dose' injections of ESAs means that new markers, which can be integrated into the ABP haematological module to increase its sensitivity, are required to detect ESA abuse.[2,3]

Previous studies show that proteins involved in erythropoiesis and iron metabolism have potential utility as biomarkers to detect blood doping.[4–6] Ferritin is an iron storage protein in the liver. It is detectable in serum and is a potential biomarker for ESA abuse and blood transfusions; in particular, it is indicative of blood withdrawal.[5,6] Leuenberger *et al.* suggested that measuring iron levels in ethylenediaminetetraacetic acid (EDTA)-plasma may be a cost-effective method of screening for blood transfusions,[4,7] thereby providing evidence of blood manipulation. In 2014, erythroferrone (ERFE) was identified as a novel erythroid regulator of iron metabolism in a mouse model.[8] ERFE is thought to act by suppressing hepcidin levels during periods of increased erythropoietic activity.[8] Hepcidin is a key player in iron metabolism (Figure 1).[9] This recently discovered peptide hormone is produced by hepatocytes and induces internalization and degradation of ferroportin.[10] By inhibiting ferroportin, hepcidin prevents gut cells from allowing iron into the hepatic portal system, thereby reducing the absorption of dietary iron. Degradation of ferroportin also reduces iron release from macrophages. Recent reports suggest that hepcidin levels are altered by blood transfusions. For example, the mean hepcidin concentration in the blood increases significantly (by 7-fold) at 12 h post-transfusion, and remains 4-fold higher at 1 day post-transfusion in a human clinical study.[5,11] Thus, hepcidin may be a novel biomarker for detecting blood transfusion in an anti-doping context. In addition, Honda *et al.* showed that long-term administration of ESAs such as darboepoetin-α, or continuous administration of erythropoietin receptor activator, to patients on haemodialysis reduces both hepcidin and ferritin levels.[12] As a follow-up to our earlier work, the aim of this study was to examine the correlation between hepcidin levels and markers of erythropoiesis in elite athletes. To simulate a doping control context, we also examined the effect of recombinant human erythropoietin (rhEPO) delta (Dynepo™) administration on hepcidin levels in healthy male volunteers.

Material and methods

Analytical methods

Hepcidin levels were measured by liquid-chromatography high resolution mass spectrometry (LC-HRMS) as previously described.[5] EPO, ferritin, and iron levels were measured using Dimension EXL 2000 and Immulite 2000 XPi technology (Siemens Healthcare Diagnostic SA, Zürich, Switzerland), and haemoglobin (Hb) concentrations were measured using a fully automated haematology

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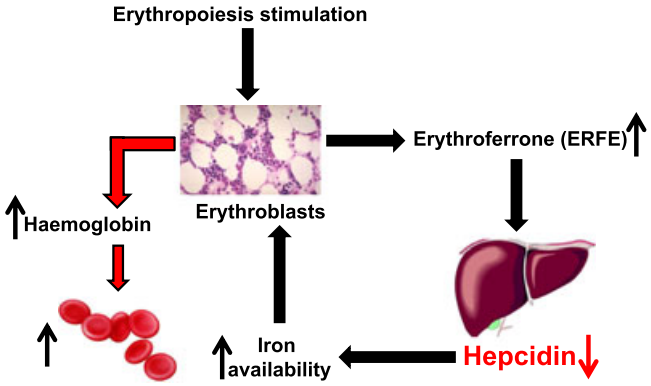

Figure 1. Effect of erythropoiesis on regulators of iron metabolism. After stimulation of erythropoiesis, erythroblasts increase production of erythroferrone (ERFE), which is then secreted into the circulation where it represses hepcidin production by the liver. Suppressing hepcidin increases the amount of iron available for synthesis of haemoglobin and new red blood cells.

analyzer (Sysmex XN 2000, Sysmex, Norderstedt, Germany), as described by Leuenberger *et al.*[5] The OFF-score was calculated as follows, as previously described[6]:

$$OFF - score = [Hb] - 60\,x\,\sqrt{Ret\%} \quad (1)$$

ERFE concentrations were measured using commercial human ELISA kits (Aviscera Bioscience, Santa Clara, CA, USA), according to the manufacturer's instructions. The following assay characteristics were specified by the manufacturer: limit of quantification, 5 ng/mL; antibody specificity for two different ERFE epitopes, 100%. The specificity of human antibodies for their target protein was validated by western-blotting. The concentration of the ERFE standards supplied by the manufacturer ranged from 31.25 to 2000 ng/mL. Intra-assay and inter-assay precision was 6–8% and 8–12%, respectively. Linearity and recovery were tested. A recovery of 118% was obtained using a 4-fold dilution of ERFE standards.

Correlation between hepcidin levels and markers of erythropoiesis and iron metabolism in elite athletes

Anonymized routine doping control in-competition EDTA-blood samples (n = 109) were used to investigate the correlation between hepcidin levels and markers of erythropoiesis and iron metabolism in high-level male athletes. In all cases, the athlete provided written informed consent. After reception, whole blood was analyzed for haematological parameters and then centrifuged for 15 min at 1500 g. The resulting plasma was aliquoted and frozen at -20 °C.

Administration of rhEPO delta

Details regarding the participants and the time of plasma sample collection are described elsewhere.[13] Briefly, 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 UI; Dynepo™, Dynepo Shire Pharmaceuticals, Basingstoke, UK) on Days 1, 3, and 5 of the study. Samples were collected in the morning (07:00–09:00 a.m.). A complete red blood cell count was performed, and the reticulocyte cell population was measured using a Sysmex analyzer (XT-2000i analyzer, Sysmex, Norderstedt, Germany).

All subjects provided written informed consent, and the protocol was authorized by the Ethical Commission for the Clinical Research of the Faculty of Biology and Medicine (University of Lausanne, Switzerland) (Protocol no. 02/09).

Statistical analysis

Unless specified otherwise, data are expressed as the mean ± SEM versus baseline. Statistical comparisons were performed using a two-tailed Student's t-test or a non-parametric Wilcoxon signed-rank test. The Shapiro-Wilk test was used to test the normality of the distribution. $P < 0.05$ was considered statistically significant. Conditions were compared using one-way ANOVA (the *aov* function in R), and post-hoc pairwise comparisons were performed with Tukey's Honestly Significant Difference test (the *Tukey HSD* function in R). All statistical comparisons and Spearman's correlation calculations were performed using standard software (StataIC, StataCorp).

Results

Spearman's correlation analysis was performed to examine the correlation between hepcidin, ferritin, iron in EDTA-plasma, EPO, Hb, and ERFE (Table 1). The results revealed a significant positive correlation between hepcidin and Hb, iron and ferritin. In contrast, a significant negative correlation between ERFE and hepcidin was observed.

As expected, erythropoietic activity following injection of rhEPO led to an increase in Ret% (Figure 2). Administration of rhEPO also affected hepcidin and ferritin levels (Figure 2 and Table 2). Ferritin was decreased 2-fold at Day 7 when compared with the day before treatment. By contrast, hepcidin levels fell by 13.7-fold after three injections.

Ret%, Hb levels, and the OFF-score are all considered indirect biomarkers with respect to the ABP strategy.[1] A mean 2.3-fold increase in Ret% was observed after three rhEPO injections. By contrast, there was no change in Hb levels (Table 2). The OFF-score decreased after rhEPO injection, mainly due to the change in Ret% (Table 2).

Discussion

We found a significant positive correlation between hepcidin levels and Hb levels and EDTA-plasma iron. Hb is a key parameter in the

Table 1. Correlation between erythropoiesis, iron levels, and other blood parameters.

Correlation (n = 109)	ERFE	Hb	Hepcidin	EPO
Hb	**-0.23			
Hepcidin	*-0.20	*0.25		
EPO	**0.30	-0.12	-0.09	
Iron	*-0.20	**0.29	**0.29	0.06
Ferritin	-0.01	0.17	**0.49	-0.11

*$P < 0.05$ and

**$P < 0.01$ indicate statistically significant correlations. Significant values are highlighted in bold.

EPO, erythropoietin; ERFE, erythroferrone; Hb, haemoglobin; iron, EDTA-plasma iron.

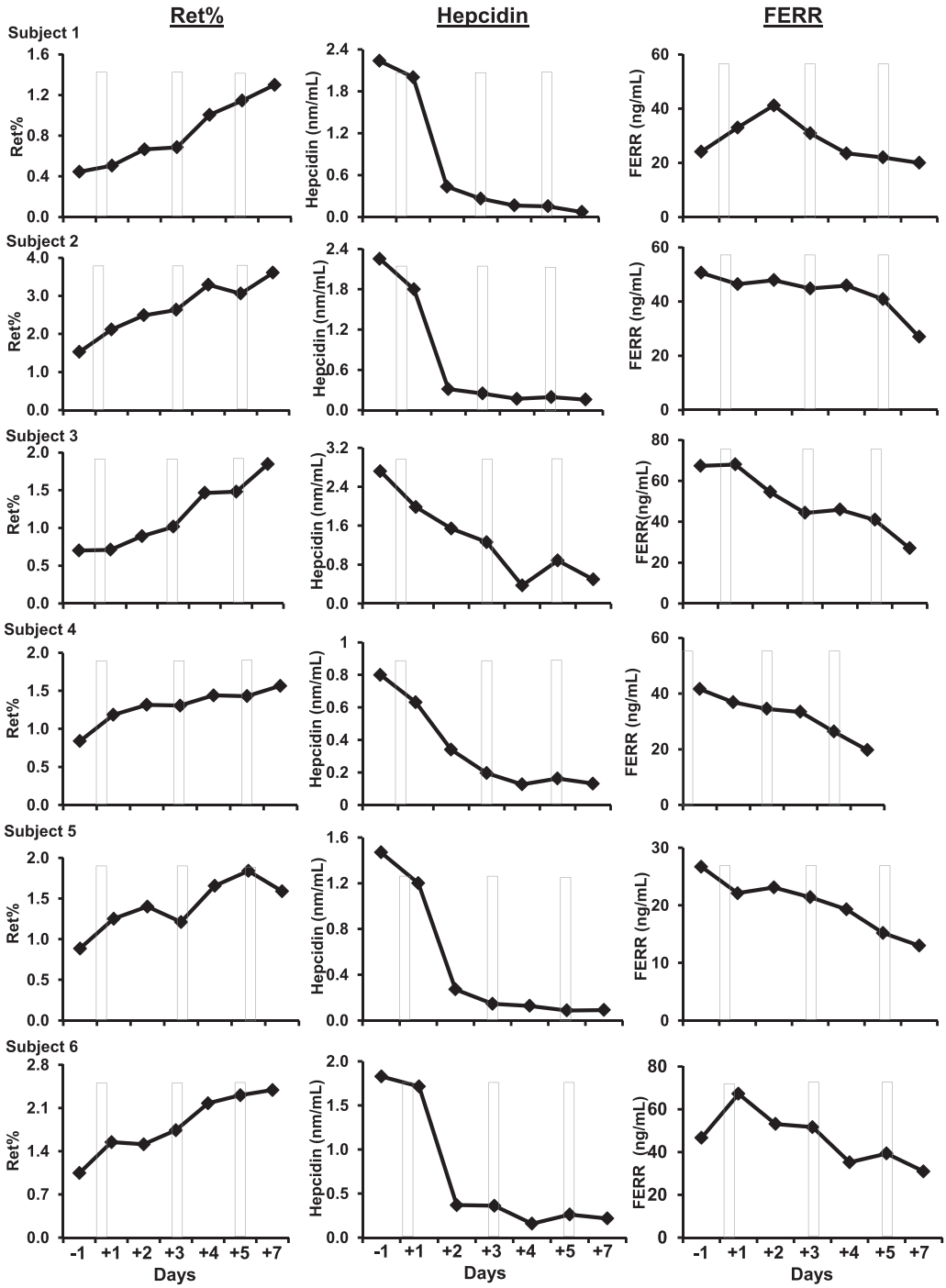

Figure 2. Effect of recombinant human erythropoietin (rhEPO) delta administration on the reticulocyte percentage (Ret%) and hepcidin and serum ferritin concentrations in six subjects. White rectangles indicate the times of rhEPO administration. FERR, ferritin.

ABP strategy.[1] That suggests that hepcidin could complement the follow-up of Hb level to detect ESA abuse.

Previously, both hepcidin and EDTA-plasma iron were identified as biomarkers for blood transfusion.[4,5] Measurement of iron levels in plasma samples obtained using EDTA as an anti-coagulant is not recommended due to the strong chelating effect of EDTA; however, the relationship between iron concentrations in paired serum and EDTA-plasma samples demonstrates a strong linear correlation.[7,14] The correlation between hepcidin levels and Hb and EDTA-plasma iron levels in athletes described herein provides additional information supporting the use of these parameters as potential biomarkers for blood doping.

There was also a significant positive correlation between hepcidin and ferritin. This correlation has been demonstrated in previous studies,[12,15,16] and strengthens the significant findings obtained using Spearman's correlation analysis herein. There was a significant positive correlation between ERFE and EPO, and a significant negative correlation between ERFE and Hb (Table 1); similar findings were reported in mice.[8] The correlation between erythropoiesis and factors that play a role in iron metabolism are

Table 2. Hematological variables and iron metabolism after injection of rhEPO delta (n = 6).

	Day-1	Day + 1	Day + 2	Day + 3	Day + 4	Day + 5	Day + 7
Ret%	**0.91 ± 0.12**	**1.22 ± 0.19**	**1.38 ± 0.21**	**1.43 ± 0.23**	**1.84 ± 0.27***	**1.88 ± 0.23***	**2.05 ± 0.28***
Hb	**15.23 ± 0.11**	**15.62 ± 0.25**	**15.80 ± 0.19**	**15.60 ± 0.22**	**15.53 ± 0.23**	**15.43 ± 0.22**	**15.47 ± 0.19**
Off-score	**125.0 ± 0.3.18**	**119.52 ± 3.84**	**116.6 ± 4.53**	**113.0 ± 5.79**	**100.08 ± 7.0***	**97.98 ± 5.87***	**93.12 ± 5.87***
He pci din	**1.88 ± 0.23**	**1.55 ± 0.18**	**0.54 ± 0.16****	**0.41 ± 0.14****	**0.19 ± 0.03****	**0.29 ± 0.10****	**0.20 ± 0.05****
Ferritin	**43.06 ± 6.00**	**46.38 ± 6.13**	**42.78 ± 3.93**	**37.92 ± 3.68**	**33.03 ± 3.37**	**28.8 ± 3.37**	**21.5 ± 3.37***

***P ≤ 0.05 and**

****P ≤ 0.01**; **compared with the day before injection of rhEPO delta. significant values are highlighted in bold.**

in line with the demonstrated link between ERFE, EPO, ferritin, and hepcidin (Figure 1).

A previous study revealed that administration of long-term ESAs to patients on haemodialysis reduces hepcidin and ferritin levels.[12] Our study examined the regulation of hepcidin, ferritin, and Ret% by rhEPO in healthy human subjects following administration of EPO delta, a human recombinant form of EPO that is very similar to endogenous EPO.[13] The data presented herein suggest that hepcidin is more sensitive than Ret%, and ferritin as potential indirect marker of rhEPO abuse. Hepcidin is a peptide hormone comprising 25 amino acids and is easily measured in human plasma or serum by LC-HRMS, a technique available in most accredited laboratories. Thus, hepcidin could be the first biomarker of ESA abuse that can be routinely detected by LC-HRMS.

Athletes show inter-individual variations in hepcidin levels.[5] Therefore, individual follow-up (as occurs in the ABP strategy) is appropriate for detecting this biomarker. As is the case for many variables in an anti-doping context, hepcidin levels are influenced by inflammation.[17] For example, interleukin-6 (IL-6) is both necessary and sufficient for hepcidin induction. Therefore, measuring inflammatory markers such as IL-6 could easily be incorporated into a test to rule out the effects of inflammation (as described by Leuenberger *et al.*[5]).

By contrast, we found that ERFE responses following rEPO administration were highly variable (Supplemental Figure). Induction of high levels of ERFE following administration of rEPO was observed in a mouse model of β thalassemia, which is associated with a chronic inflammatory state.[18] Recently, Honda *et al.* observed clear induction of ERFE in patients on haemodialysis.[12] The healthy subjects included in the present study did not have thalassemia, were not on haemodialysis, and had normal Hb levels (Table 2). Therefore, our data suggest that ERFE is not a reliable indirect biomarker that can be used to identify ESA abuse in healthy athletes.

This study has some limitations. First, the influence of confounding factors such as physical exercise on the proposed biomarkers was not explored. The effects of iron administration (injection or oral absorption) on the levels of hepcidin and other markers of iron metabolism are currently under investigation. Moreover, it would be interesting to examine the impact of micro-doses of rhEPO delta on hepcidin levels over a longer period.

In conclusion, the data presented herein suggest that rhEPO delta, a first-generation recombinant EPO, regulates hepcidin levels. In 2014, World Anti-Doping Agency statistics revealed that more than 82% of adverse analytical findings in rEPO cases were first generation.[19] The data presented herein suggest that hepcidin has utility as a potential indirect biomarker for detecting first generation rEPO with previously observed with blood transfusion. Thus, hepcidin is of general interest in the field of blood doping.

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