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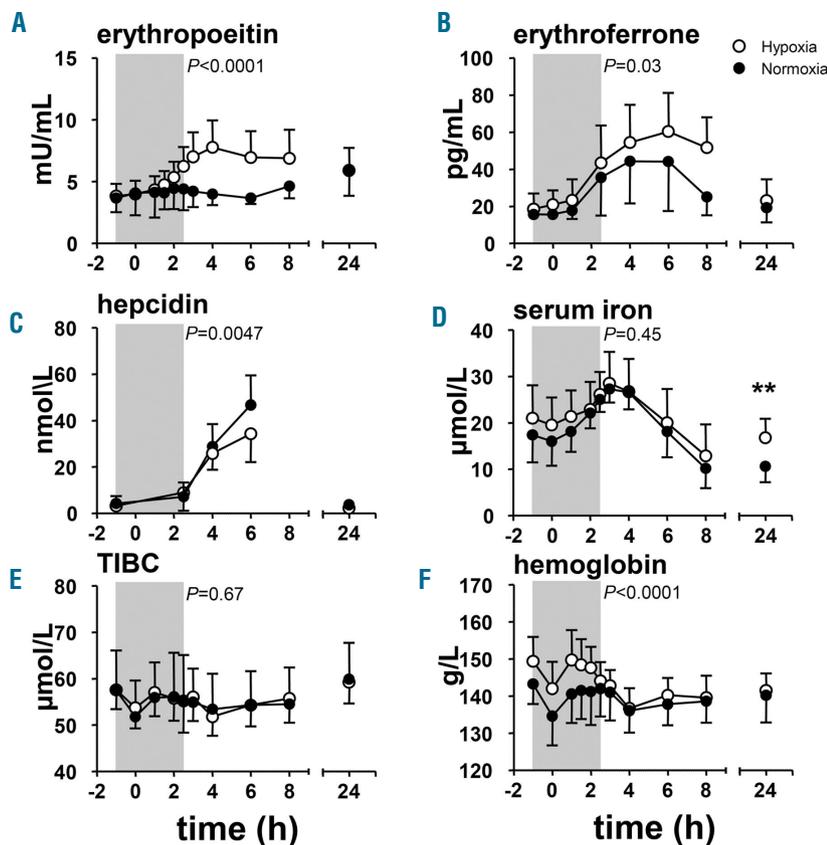
### Hypoxia attenuates inflammation-induced hepcidin synthesis during experimental human endotoxemia

Hepcidin is the central mediator of iron homeostasis that limits iron availability in the extracellular compartment and causes iron-restricted erythropoiesis. Inflammation is a strong hepcidin inducer, which can lead to anemia of inflammation, whereas hypoxia can suppress hepcidin synthesis. Inflammation, hypoxia and anemia are often encountered in critically ill patients. In the present study, we determined the effects of hypoxia on parameters of iron regulation and homeostasis in humans *in vivo* during systemic inflammation induced by endotoxin administration. We report that systemic inflammation results in increased plasma hepcidin levels and a subsequent decrease in serum iron levels. Hypoxia attenuates these endotoxemia-induced effects on hepcidin and iron parameters, likely through increased synthesis of erythropoietin and erythroferrone.

Hepcidin binds to the iron exporter ferroportin expressed on macrophages and duodenal enterocytes, leading to its internalization and degradation, thereby reducing iron export into the extracellular compartment.<sup>1</sup> Hepcidin regulation is mainly driven by body iron status and systemic inflammation, which is a major hepcidin inducer. The redistribution of iron into iron-storing cells as a result of increased hepcidin synthesis during infection is considered a part of the innate immune response against extracellular proliferating pathogens, as iron is a vital nutrient for microbial growth and replication. In contrast to inflammation, hypoxia is a strong hepcidin

suppressor.<sup>2</sup> Hypoxia promotes increased transport of iron from storage sites to facilitate erythropoiesis in the bone marrow.<sup>3,4</sup> Erythropoiesis is induced by erythropoietin, which increases the production of erythroferrone, a known suppressor of hepcidin synthesis.<sup>5</sup> The great majority of critically ill patients suffer from inflammatory conditions, such as sepsis, major surgery and trauma, and also develop anemia.<sup>6</sup> This so-called “anemia of inflammation” is associated with worse outcome.<sup>7,8</sup> As such, efforts have been made to develop therapies that inhibit hepcidin synthesis or mitigate its downstream effects.<sup>9</sup> Besides causing anemia, pronounced systemic inflammation often leads to concurrent tissue hypoxia in critically ill patients due to respiratory dysfunction, hemodynamic instability, microthrombi, and tissue edema. Therefore, there may be some interplay between both phenomena with regard to hepcidin synthesis and iron homeostasis, although this has not yet been studied in humans. Here, we investigated the effect of hypoxia on erythropoietin, erythroferrone and hepcidin synthesis, and iron homeostasis parameters in healthy volunteers during experimental endotoxemia, a standardized controlled model of systemic inflammation in humans *in vivo*.

Data were obtained from subjects participating in an open-label, randomized, parallel group intervention study ([clinicaltrials.gov identifier: 01978158](https://clinicaltrials.gov/ct2/show/NCT01978158)) aimed at investigating the effects of modulation of oxygen status during systemic inflammation on immunological (primary outcome) and many other parameters, including hemodynamic effects, coagulation, the endocrine response, and renal function (complete list provided at <https://clinicaltrials.gov/ct2/show/NCT01978158>). The pri-



**Figure 1. Parameters of iron regulation and homeostasis.** (A) Plasma erythropoietin. (B) Plasma erythroferrone. (C) Plasma hepcidin. (D) Serum iron. (E) Total iron binding capacity (TIBC). (F) Hemoglobin. The gray box indicates the period of hypoxic exposure; endotoxin was administered at 0 hours. Data are expressed as Mean ± Standard Deviation. Between-group differences over time (baseline to 8 hours post-endotoxin administration) were analyzed with repeated measures two-way ANOVA (interaction term displayed in each panel). \*\*P < 0.01, unpaired Student t-test.

mary outcomes have been published elsewhere and are freely accessible.<sup>10</sup> The trial was approved by the local ethics committee and carried out according to Good Clinical Practice standards and the Declaration of Helsinki, including current revisions. The Methods used have been published in detail elsewhere.<sup>10</sup> Briefly, after obtaining written informed consent, subjects were declared eligible based on a screening procedure that included a full medical history, physical examination, electrocardiogram and routine laboratory tests. Twenty male non-smoking subjects, aged between 18 and 35 years, were randomized to receive either hypoxia (n=10) or normoxia (n=10) during experimental endotoxemia. Hypoxic exposure [peripheral oxygen saturation (SaO<sub>2</sub>) 80-85%] started one hour (h) before, and continued until 2.5 h after endotoxin administration, and was achieved by feeding a nitrogen/medical air mixture into an airtight respiratory helmet (CaStar, StarMed, Italy). Normoxic subjects were fitted with the same respiratory helmet fed with medical air (FiO<sub>2</sub> 21%). Purified standard reference *Escherichia coli* O:113 LPS (Clinical Center Reference Endotoxin, National Institutes of Health, Bethesda, MD, USA) was administered intravenously at a dose of 2 ng/kg body weight to elicit a systemic inflammatory response. Blood samples were obtained from an arterial catheter (up to 8 h after endotoxin administration) or venipuncture (at 24 h post endotoxin). Plasma erythropoietin and erythroferrone concentrations were determined by ELISA (eBioscience, Vienna, Austria, and MyBioSource, San Diego, CA, USA, respectively). Plasma hepcidin levels were measured using a specifically designed competitive ELISA<sup>11</sup> with a lower limit of detection of 0.42 nmol/L; none of the measured concentrations fell below this lower limit. Serum iron and transferrin were determined using routine analytical methods (Architect c16000, Abbott Diagnostics, Lake Forest, USA), as was hemoglobin (Sysmex XE 5000, Sysmex Corp., Kobe, Japan). Total iron binding capacity (TIBC) was calculated as: TIBC (μmol/L)=25 (μmol/g) x transferrin (g/L). Transferrin saturation (TSAT) was calculated as: TSAT (%)=serum iron [(μmol/L)/TIBC (μmol/L)] x 100.

Plasma cytokine levels were determined by Luminox assay (Merck Millipore, Billerica, MA, USA).

Data are expressed as Mean±Standard Deviation, and between-group differences over time (baseline to eight hours after endotoxin administration) were analyzed with repeated measures two-way analysis of variance (ANOVA, interaction term). Differences between groups at baseline and 24 h after endotoxin administration were evaluated using unpaired Student *t*-tests. *P*<0.05 was considered statistically significant.

As detailed elsewhere,<sup>10</sup> an SaO<sub>2</sub> of 80-85% was achieved using a mean FiO<sub>2</sub> of 11.4±0.7% in the hypoxia group. All subjects experienced typical flu-like symptoms that peaked 90 minutes (min) after endotoxin administration and subsided within a few hours. Hypoxia resulted in increased plasma erythropoietin concentrations from 90 min after endotoxin administration onwards (Figure 1A). In normoxic subjects, erythropoietin levels were increased only at 24 hours (h) post endotoxin administration, and at that time point, concentrations were similar to those observed in subjects who had previously been exposed to hypoxia. In both groups, circulating erythroferrone and hepcidin levels increased over time (Figure 1B and C). Hypoxia augmented the endotoxemia-induced increase in erythroferrone and attenuated the increase in hepcidin levels, of which the latter effect manifested later (6 h after endotoxin administration). Baseline serum iron concentrations were comparable in both groups (21±7.1

vs. 17.4±5.9 μmol/L in the hypoxia and normoxia groups, respectively; *P*=0.23). In line with previous work,<sup>12,13</sup> endotoxemia resulted in an initial increase in serum iron, peaking three hours after endotoxin administration, followed by a decrease to below baseline values that was most pronounced eight hours post-endotoxin administrations (Figure 1D). Transferrin saturation showed almost the exact same profile as serum iron concentrations with no differences between the treatment groups up till eight hours post-endotoxin administration (*data not shown*). However, 24 hours after endotoxin administration, significantly higher levels of serum iron (16.8±4.1 vs. 10.6±3.4 μmol/L; *P*=0.002) and transferrin saturation (28.8±8.5 vs. 17.8±5.5; *P*=0.003) were observed in the hypoxia group compared with the normoxia group. No clear effects of either endotoxemia or hypoxia were observed on TIBC (Figure 1E). Baseline hemoglobin levels were higher in the hypoxia group (149.4±6.6 vs. 143.3±5.4 g/L; *P*=0.04), and an initial decrease was observed in both groups due to our prehydration protocol<sup>14</sup> (Figure 1F). Furthermore, there was a significant difference in hemoglobin concentrations over time between groups. Baseline hemoglobin levels did not correlate with baseline or peak hepcidin levels, or with serum iron or TSAT at baseline or *t*=24 hours in either group (all *P*>0.10). Plasma cytokine levels markedly increased in all subjects after endotoxin administration.<sup>10</sup> Hypoxia resulted in a 2-fold increase in concentrations of the anti-inflammatory cytokine interleukin (IL)-10, and a 30-50% reduction in levels of pro-inflammatory cytokines tumor necrosis factor alpha, IL-6 and IL-8.<sup>10</sup> There were no significant correlations between cytokine responses [area under the concentration-time curve (AUC)] and peak or AUC hepcidin concentrations (all *P*>0.10).

Taken together, our data demonstrate that exposure of healthy volunteers to hypoxia during experimental endotoxemia enhances erythropoietin and erythroferrone concentrations, and partially suppresses inflammation-induced hepcidin synthesis. Furthermore, hypoxia mitigates the inflammation-induced decrease in serum iron levels and transferrin saturation at later time points.

Since its discovery in 2000, many regulatory pathways concerning hepcidin synthesis have been discovered. The main hepcidin-enhancing pathways are the BMP-SMAD pathway, activated by high iron stores, and the JAK-STAT3 pathway induced by inflammatory mediators such as IL-6,<sup>15</sup> which accounts for the phenomenon known as anemia of inflammation.<sup>16,17</sup> This occurs in both chronic (e.g. autoimmune diseases) and acute (e.g. sepsis) inflammatory conditions.<sup>18</sup> Previous studies revealed that hepcidin levels are increased in septic patients, and that this is associated with the development of anemia.<sup>18</sup> Our data show that hypoxia can partially reverse the effects of acute inflammation on hepcidin synthesis and its downstream effects on serum iron levels and transferrin saturation.

Hypoxia has been described to suppress hepcidin synthesis through various mechanisms, many of which involve a group of transcription factors called hypoxia inducible factors (HIF). Although HIF do not directly affect hepcidin levels,<sup>19</sup> HIF induction results in increased erythropoietin production, in turn leading to increased synthesis of erythroferrone, a known hepcidin suppressor;<sup>5</sup> this cascade was also observed in this study. An alternative explanation for the observed change in hepcidin is that HIF induction also increases levels of furin, a protease that cleaves hemojuvelin and thereby produces soluble hemojuvelin, an inhibitor of the BMP-SMAD pathway.<sup>20</sup> The relevance of HIF for hepcidin regulation

and for iron mobilization and utilization *in vivo* is supported by the notion that patients who use pharmacological HIF-inducers (called prolylhydroxylase inhibitors) for renal anemia present decreased hepcidin and ferritin levels, and an increase in total iron binding capacity and hemoglobin (e.g. for vadadustat<sup>21</sup>). Finally, hypoxia may decrease hepatic hepcidin transcription *via* induction of platelet derived growth factor-BB.<sup>22</sup> Due to the nature of this study, we cannot ascertain a cause-effect relationship between hepcidin levels at six hours and iron levels at 24 hours. Nevertheless, a murine study has shown that exogenously administered hepcidin affects iron levels for up to 48 hours, long after hepcidin levels had returned to baseline.<sup>23</sup> Therefore, such a cause-effect relationship may be present in humans as well.

Interestingly, erythroferrone levels also increased in normoxic subjects after endotoxin administration. This increase was not preceded by enhanced concentrations of erythropoietin, which is, to the best of our knowledge, the only known inducer of erythroferrone synthesis.<sup>5</sup> Therefore, it might be speculated that other inflammation-related mechanisms also induce erythroferrone synthesis.

Worthy of mention is the fact that experimental human endotoxemia is a relatively mild model of systemic inflammation. As such, our results may not be directly applicable to critically ill patients. In addition, long-term effects of hypoxia on iron parameters remain to be determined, as we did not obtain blood samples from the study subjects later than 24 hours after endotoxin administration. Finally, there were significant between-group differences in baseline hemoglobin levels and in the change in hemoglobin concentrations over time, which can only be explained by an unbalanced randomization. Although no relationships between baseline hemoglobin level and peak levels of, or changes in hepcidin or iron levels were identified, we cannot exclude this as a confounding factor.

In conclusion, we demonstrate that hypoxia augments erythropoietin and erythroferrone levels, partially attenuates the inflammation-induced increase in hepcidin concentrations, and mitigates the decrease in serum iron levels and transferrin saturation. These results may be of special relevance to critically ill patients, in whom inflammation and hypoxia often coincide.

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