Modulation of hepcidin production during hypoxia-induced erythropoiesis in humans in vivo: data from the HIGHCARE project

Alberto Piperno1,2, Stefania Galimberti3, Raffaella Mariani1, Sara Pelucchi1, Giulia Ravasi1, Carolina Lombardi4, Grzegorz Bilo4, Miriam Reveda4, Andrea Giuliano1,4, Andrea Faini4, Veronica Mainini5, Mark Westerman6, Tomas Ganz7, Maria Grazia Valsecchi3, Giuseppe Mancia1, Gianfranco Parati1,4, on behalf of the HIGHCARE investigators.

1. Department of Clinical Medicine and Prevention, University of Milano-Bicocca and S.Gerardo Hospital, Monza, Italy.
2. Consortium for Human Molecular Genetics, Monza, Italy.
3. Centre of Biostatistics for Clinical Epidemiology, Department of Clinical Medicine and Prevention, University of Milano-Bicocca, Monza, Italy
4. Department of Cardiology, Istituto Auxologico Italiano, Milan, Italy
5. Department of Experimental Medicine, University of Milano-Bicocca. Monza, Italy.
6. Intrinsic LifeSciences LLC, La Jolla, California, USA
7. Department of Medicine and Pathology, David Geffen School of Medicine, UCLA, Los Angeles, CA, USA.

Scientific category: Red Cells, Iron and Erythropoiesis

Running title: Hepcidin modulation at high altitude

Corresponding Author:
Prof. Alberto Piperno
University of Milano-Bicocca
Clinical Medicine, San Gerardo Hospital
Via Pergolesi 33
20052, Monza (MI), Italy
Phone 039-2339555; Fax 039-322274
e-mail: alberto.piperno@unimib.it

For a complete list of HIGHCARE participants, please see the supplemental appendix.
Abstract

Iron is tightly connected to oxygen homeostasis and erythropoiesis. Our aim was to better understand how hypoxia regulates iron acquisition for erythropoiesis in humans, a topic relevant to common hypoxia-related disorders. Forty-seven healthy volunteers participated in the HIGHCARE project. Blood samples were collected at sea level and after acute and chronic exposure to high altitude (3400 to 5400 meters above sea level). We investigated the modifications in hematocrit, serum iron indices, erythropoietin, markers of erythropoietic activity, interleukin-6 and serum hepcidin. Hepcidin decreased within 40 hours after acute hypoxia exposure (p<0.05) at 3400 meters, reaching the lowest level at 5400 meters (80% reduction). Erythropoietin significantly increased (p<0.001) within 16 hours after hypoxia exposure followed by a marked erythropoietic response supported by the increased iron supply. Growth differentiation factor-15 progressively increased during the study period. Serum ferritin showed a very rapid decrease suggesting the existence of hypoxia-dependent mechanism(s) regulating storage iron mobilization. The strong correlation between serum ferritin and hepcidin at each point during the study indicates that iron itself or the kinetics of iron utilization in response to hypoxia may signal hepcidin downregulation. The combined and significant changes in other variables likely contribute to the suppression of hepcidin in this setting.
Introduction

Iron is an essential element required as a cofactor for proteins managing oxygen transport such as hemoglobin, myoglobin and cytochromes. Hypoxia, that occurs in a broad range of pathologic or environmental conditions, is primarily sensed in vertebrates by the hypoxia inducible family of transcription factors (HIFs) which activate or repress genes involved in adaptations to low oxygen pressure \(^1\). In the erythropoietic compartment, which contains approximately 70% of the body iron, hypoxia stimulates erythropoiesis and promotes hemoglobin synthesis which largely depends on iron availability \(^2\)-\(^6\). Accordingly, both hypoxia and anemia induce the synthesis of erythropoietin (EPO) and are the two main signals which increase iron absorption independently of iron stores \(^6\)-\(^9\). Thus, iron metabolism is tightly connected to oxygen homeostasis and erythropoiesis.

The liver peptide hepcidin, codified by \(HAMP\), is the key regulator of iron homeostasis. It acts by inhibiting intestinal iron absorption and release by macrophages and its production is regulated by different stimuli including inflammatory cytokines \(^10\), iron \(^11\), erythropoietic activity \(^12\)-\(^14\), anemia and hypoxia \(^15\)-\(^17\) mainly at the transcriptional level as described in recent reviews \(^18\),\(^19\). Anemia could mediate hepcidin suppression through multiple mechanisms including increased EPO or erythropoietic activity, increased iron demand or liver hypoxia \(^3\). The nature of the erythropoietic regulator of hepcidin is still uncharacterised, but may include one or more proteins released during active erythropoiesis. One of these regulators, GDF15, a divergent member of the transforming growth factor-beta super-family, has been identified also as an oxygen-regulated transcript responding to hypoxia \(^20\), suggesting that some homeostatic systems for iron and oxygen are responsive to both stimuli.

Previous studies reported a decrease of hepcidin expression in response to hypoxia \(^17\), however the physiological relevance and the mechanisms of hepcidin regulation by hypoxia are still uncertain and conflicting \(^12\),\(^15\),\(^16\),\(^21\),\(^22\). There is limited information on the hypoxia-induced modifications of mediators of iron homeostasis in vivo in humans. High altitude induces several physiological changes due to hypobaric hypoxia and healthy subjects exposed to high altitude represent a
convenient model to study these changes after acute or chronic exposure to hypoxia. The present study exploits a unique human resource: the availability of biological material from normal volunteers participating in the HIGHCARE (HIGH altitude Cardiovascular Research) 2008 project on Mount Everest south slopes in Himalaya. We planned to investigate the modifications induced by acute and chronic exposure to hypobaric hypoxia on serum iron indices, EPO, markers of increased erythropoietic activity and inflammation, and hepcidin itself. Our aim was to better understand how hypoxia regulates iron acquisition for the erythropoietic response, a topic relevant to several common hypoxia-related human disorders, that include both acute conditions and chronic disorders of aging, as heart failure, sleep-related breathing disorders, chronic bronchitis and anemia. To the best of our knowledge, this is the first study that examined in humans the temporal association between high altitude-induced changes in circulating hepcidin and serum iron indices, markers of increased erythropoietic demand and known soluble regulators of iron homeostasis in order to obtain information on how hypoxia modulates in vivo the biological response aimed to supply iron essential for erythropoiesis.

**Materials and Methods**

Participants. We enrolled 47 healthy volunteers (32 males and 15 females) in good general health, living permanently at less than 500 m above sea level (a.s.l.). Exclusion criteria were: known cardiovascular disease, any chronic cardiovascular therapy, repeated prolonged exposures to altitudes >3000 m a.s.l. in the 8 months preceding the expedition, history of severe mountain sickness, history of angioedema and pregnancy. Professional athletes were not included in the study. Subjects underwent a general health check-up including exercise test and echocardiography prior to the expedition. All subjects gave their written informed consent to the study procedures. The study protocol was approved by the Ethics Committee of Istituto Auxologico Italiano and the study was conducted in agreement with the Helsinki Declaration.
Six volunteers (five women and one man) were excluded from the study because they were iron deficient caused by menstrual blood losses (4 women) and blood donations (one man and one woman). They showed undetectable hepcidin levels at baseline and throughout the entire study.

Study design and procedures. Figure 1 summarizes the different steps of the study. The participants travelled by air from sea level (Milan, 140 m) to Kathmandu (Nepal, 1355 m) where they stayed for three days. Then they were brought by air transportation in a few hour time to Namche Bazaar (3400 m), where they stayed for another three days. From Namche Bazaar they trekked over 5 days to Mt. Everest south Base Camp (BC, 5400 m) where they remained for 11 days. They returned to sea level (Milan) over the next 6 days. During the study all volunteers were on the same standard diet. Samples were collected at five time points: sea level baseline (SL1), during acute (day 1-2) exposure to high altitude (3400 m) at Namche Bazaar (Namche), during acute (day 10-12) and prolonged (day 19-20) exposure to very high (5400 m) altitude at Mt. Everest Base Camp (BC1 and BC2, respectively) and again 3-4 months after returning to sea level (SL-return). In order to evaluate the different timing of EPO, hepcidin and hepcidin regulators response to acute hypoxia stimulation, volunteers were divided in two subgroups, matched for age and sex, which were tested at 16 (first morning after arrival at Namche) and 40 hours (next morning) after acute exposure at 3400 meter above the sea level. These two groups are classified as Namche group A (n= 20) and B (n= 21), respectively.

Experiments in Namche Bazaar were performed in comfortable hotel rooms while at BC they were performed in heated tents. Both in Namche Bazaar and in BC, laboratory temperature and barometric pressure were recorded by means of a microclimatic station. In the morning of the first and second day after arrival at Namche Bazaar as well as on days 10-12 and 19-20 at BC we measured several parameters including heart rate, systemic blood pressure, respiratory rate, body temperature and oxygen saturation. Blood samples were always obtained in the morning (between 7 and 9.30 a.m.) at fast, after the subject has been sitting for at least 15 minutes. Samples were immediately centrifuged and sera stored at -80° C at sea level, while during the expedition they
were stored in 2 ml cryogenic tubes and frozen in nitrogen vapor containers (Cryoshippers MVE Biological Systems, Cleveland, Ohio) for the whole period until the return in Italy, when they were definitively stored at -80° C.

Methods. We investigated the modifications induced by acute and chronic exposure to hypobaric hypoxia on serum iron indices, erythropoietin (EPO), soluble transferrin receptor (s-TFR) and GDF15 as markers of erythropoietic activation, interleukin-6 (IL-6) as a marker of inflammation and hepcidin. Serum iron, transferrin and ferritin were measured by standard methods, EPO was measured by Immulite EPO (Siemens Solutions Diagnostic Ltd, UK), s-TFR and GDF15 by Quantikine Human Immunoassay (R&D Systems, Inc. MN, USA). All these tests were performed at the laboratory of the Consortium for Human Molecular Genetics located in the University of Milano-Bicocca, Faculty of Medicine building, in Monza (Italy). Serum hepcidin was measured by the immunoassay developed by Intrinsic LifeSciences LLC, La Jolla, CA 23. Hematocrit was measured with a portable instrument (Radiometer ABL77, Copenhagen).

Statistical analysis. Due to the skewed nature of the parameters under evaluation, data were log-transformed. Results of the descriptive analysis are reported in terms of geometric means with 95% confidence intervals, and quartiles. We remark that at Namche, two volunteers, both belonging to Namche Group A, showed a marked increase of serum hepcidin (from 5.3 to 171 ng/ml and 72 to 184 ng/ml, respectively) which paralleled marked IL-6 increments (from 49.1 to 267.54 pg/mL and from 16.4 to 107.5 pg/mL, respectively). These alterations were probably due to an upper airway infection they developed in the first days after the arrival in Nepal. Accordingly, both hepcidin and IL-6 levels rapidly decreased at subsequent tests. This justified the exclusion of these values from all the longitudinal analyses, as explicitly indicated in the results and tables.

The evaluation of the changes in the log-measurements over altitude was done by fitting mean response profiles models 24, testing the effect of altitude, sex and the interaction altitude by sex. Results were reported for the final model including only significant effects (cut-point for significance = 0.01). An unstructured form of the variance-covariance matrix was used as it
minimized Akaike information criterion (AIC), compared to a set of competing forms (i.e. compound symmetry, heterogeneous autoregressive, exponential). When a significant overall effect of altitude was detected, contrasts between baseline (SL1) and the subsequent measurements (Namche, BC1 and BC2), and between the mean values at Namche and at BC1 were evaluated. Moreover, in case of a significant interaction term, the comparisons were performed separately for males and females. The same modelling approach was used for the analysis of changes in parameters after acute exposure to altitude at Namche as measured after 16 or 40 hours of exposure to hypoxia (group A and B).

In order to evaluate the influence of regulators of hepcidin, a multivariate linear regression model was considered. Regressors were screened in a univariate analysis for each parameter on log-hepcidin at each altitude. The multivariate model included those parameters which were significant in at least one univariate analysis (p-value<0.01), and were adjusted for age and gender.

Results

Table 1 shows volunteers’ characteristics at baseline (SL1) divided according to gender. As expected hematocrit, serum ferritin and hepcidin were lower in women than in men. Means and medians of the parameters studied at SL1, Namche, BC1 and BC2, and SL-return are reported in Table 2. All the changes that occurred at high altitude had disappeared by the time of measurement at sea level three months after return. Figure 2 shows the time course of each parameter at baseline and during the period of hypoxic exposure with geometric means and 95% confidence intervals. We observed overall a significant increase of hematocrit from baseline (42.1%; 95% CI 40.9-43.4) to BC2 (54.1%; 95% CI 52.7-55.6). This increase was evident both in men (from 43.5 to 55.5 %) and women (from 38.2 to 50.4 %) but women always maintained significantly lower values than men.

We observed a significant increase of hematocrit at Namche, but this was probably due to the rapid decrease of plasma volume associated with acute exposure to hypoxia not adequately compensated by increased water assumption. This is supported by the fact that the increase was
found in Namche group A (41.7% at baseline and 45.6% after 16 hours of high altitude exposure),
but not in volunteers belonging to Namche group B (42.5% at baseline and 42.9% after 40 hours of
altitude exposure) (Table 3).

As shown in Figure 2 and in Table 2, hepcidin levels significantly decreased after acute exposure to
hypoxia (Namche) reaching the nadir at BC1 and BC2, when its levels were reduced by more than
80%. While males and females started from different baseline values, their decreasing profile did
not differ significantly according to the longitudinal analysis (Suppl. Table 1). EPO rapidly
increased at Namche maintaining a plateau at BC1 and beginning to decrease at BC2. Transferrin
saturation augmented at Namche exclusively due to transient serum iron increase, because serum
transferrin showed a rapid and gradual increase likely related to iron depletion. It significantly
decreased at BC1, due to the increased iron utilization by the bone marrow, and remained fairly
similar at BC2. The decrease of transferrin saturation was more evident for females than males
(with a significant interaction term in the longitudinal model, see Suppl. Table 1). Serum ferritin
showed a rapid and progressive decrease that was already evident at Namche, indicating massive
mobilization of iron from stores. This progressive decrease was similar in males and females so that
a significant difference by gender was maintained in the longitudinal profile. The three remaining
parameters did not behave differently by gender but were affected by altitude. Specifically, sTFR
showed a marked increase from Namche to BC1 according to erythropoietic expansion, whereas
GDF15 showed a progressive, but slight increase from SL1 to Namche and BC1, both maintaining a
plateau at BC2. IL-6 levels increased at Namche and remained elevated at BC1 and BC2. As shown
by the results of the model on longitudinal profiles (Suppl. Table 1), changes from acute (Namche)
versus chronic (BC1) hypoxia were significant for all parameters, except for EPO, which showed
the major increase at Namche.

To better evaluate the time course of each parameter in response to acute hypoxia we examined the
results at 16 (Namche group A) and 40 (Namche group B) hours after acute hypoxia exposure
(Table 3). Groups A and B had comparable baseline values for all parameters (p-values>0.05).
EPO, GDF15, transferrin and ferritin significantly changed both in Namche group A and group B. After 40 hours, serum iron significantly augmented causing transferrin saturation increase. Hepcidin did decrease after 16 hours but not significantly so (p-value=0.0869), whereas it significantly decreased after 40 hours of exposure. Accordingly, only nine out of the 18 volunteers (50%) of group A showed a reduction of hepcidin levels as compared to 17 out of 21 (81%) in group B. Changes in IL-6 were more marked after a longer exposure, while sTFR did not show significant modifications in both groups.

Trying to evaluate the relations between hepcidin and its possible regulators (erythropoietic activity, iron stores, cytokine activation), we performed univariate and multivariate analyses at different times of exposure to hypoxia. Results of univariate analyses were used to screen for relevant factors to be included in the multivariate analysis. In the univariate analysis (data not shown), serum ferritin correlated with serum hepcidin at each point, while EPO inversely correlated with hepcidin only at BC1. Results of the multivariate model at different altitudes are reported in Suppl. Table 2. Serum ferritin seems to be the only independent predictor for hepcidin: a 10% decrease in serum ferritin is estimated to be related to a decrease in hepcidin of 6.5%, 7.3%, 7.6% and 8.2% at SL1, Namche, BC1 and BC2, respectively.

**Discussion**

During the 20 days period of high altitude exposure we observed a marked increase of hematocrit which required about 700 mg iron in men and 400 mg in women (based on approximate calculation of hemoglobin increase, hemoglobin iron content and subjects’ weight and blood volume). To compensate for this massive demand of iron by erythropoietic precursors, iron release from stores and intestinal absorption had to increase dramatically. Our results strongly suggest that hepcidin downregulation was central to these modifications.

Hepcidin suppression began some hours after acute hypoxia exposure reaching its nadir in the following week of exposure to high altitude. The timing of hepcidin suppression we observed is
similar to that recently reported in healthy subjects who received EPO injections, starting 24 hours after EPO administration.\textsuperscript{27,28} The delay and progression in hepcidin down-regulation contrasts with the hypothesis that hypoxia might directly be involved in hepcidin suppression and suggests that other factors are involved. Hypoxia generates a detectable increase in serum EPO within 90 minutes,\textsuperscript{9} which peaks within 2 days and thereafter declines over a period of 1-2 weeks in volunteers exposed to high altitude.\textsuperscript{6} Accordingly, we observed a rapid and marked increase of EPO which was maintained at BC1 and began to decline at BC2. It has been suggested that hypoxia could act on hepcidin down-regulation via EPO signaling,\textsuperscript{27,29} but animal studies indicated that the decrease of hepcidin expression by experimentally induced anemia was dependent on erythropoiesis and not directly mediated by EPO, anemia, or tissue hypoxia.\textsuperscript{12,14} The present study could not clarify this issue, but considering that hepcidin is a fast-responding hormone and that the timing of hepcidin suppression was gradual, we suggest that it could be mediated by circulating factor(s) released by cells in response to hypoxia-induced erythropoietic activation. Recent observations have indicated that GDF15 and twisted gastrulation 1 (TWSG1) could be erythropoietic regulators of hepcidin,\textsuperscript{13,30*} possibly opposing the effect of BMPs\textsuperscript{31}. GDF15 is expressed during erythroblast maturation mainly in more mature, hemoglobinized erythroblasts\textsuperscript{32} whereas TWSG1 is produced during the earlier stages of erythropoiesis.\textsuperscript{30} GDF15 is highly expressed in thalassemia,\textsuperscript{13} congenital diserythropoietic and sideroblastic anemias,\textsuperscript{32,33} and less in other forms of ineffective erythropoiesis.\textsuperscript{13,32-34} In addition, GDF15 was up-regulated in response to iron depletion in in vitro studies\textsuperscript{20} and human studies,\textsuperscript{20} but to a smaller extent (two-fold or less). A similar magnitude of increase was observed in our volunteers who showed a progressive GDF15 increase which peaks at BC2. This was likely the result of the combined action of erythropoiesis activation, iron depletion, but also of tissue hypoxia, inflammation or enhanced oxidative stress induced by high altitude exposure.\textsuperscript{20,35} However, GDF15 levels were below the threshold that down-regulated HAMP mRNA in primary hepatocytes in vitro,\textsuperscript{13} and did not show any correlation with hepcidin suggesting that GDF15 could cooperate to hepcidin suppression, but cannot be a main regulator in this setting.
Among all the parameters analysed, serum ferritin was the one showing the best correlation with hepcidin at each point during the study suggesting that iron itself, or the kinetics of iron utilization in response to hypoxia, may signal the suppression of hepcidin. At variance with that observed in patients with ineffective erythropoiesis in whom the hepcidin store regulator is overwhelmed by the erythroid signal\(^3\), our results indicate that in healthy individuals exposed to acute and chronic hypoxia, hepcidin production is modulated by the iron store regulator even in the presence of increased erythropoietic demand.

Unexpectedly, we observed a significant decrease of serum ferritin concentration in the very early stages of hypoxia exposure (16 hours) when there was no evident increase of erythropoiesis and hepcidin was not suppressed, yet. This finding contrasts with those induced by prolonged rhEpo treatment in healthy subjects showing a later decrease of serum ferritin secondary to erythropoiesis expansion and increased iron demand\(^{27,28}\). In animal models, stabilization of HIF in hepatocytes increases ferroportin expression possibly due to a HIF-dependent ferroportin mRNA up-regulation and/or to a post-translational stabilization of ferroportin\(^{16,36}\). Thus, we can hypothesise that acute hypoxia might induce a rapid mobilization of iron from storage cells to plasma in order to ensure enough iron to the mounting red blood cells hemoglobinization. The increase of serum iron and transferrin saturation observed at 40 hours might indicate that iron mobilisation was even higher than bone marrow request in this phase. Further studies are needed to confirm and extend all these findings.

At BC1 and BC2 the increased rate of erythropoiesis was paralleled by the marked decrease of tissue iron stores and increased iron utilization by the erythropoietic bone marrow, as shown by the marked reduction of serum ferritin and transferrin saturation which likely cooperated to further suppress hepcidin synthesis\(^{18}\). A recent study suggested that besides liver and spleen, skeletal muscle tissue can be another source of iron during high altitude-induced erythropoiesis\(^{37}\). This finding differs from that observed in healthy volunteers treated with rhEpo at sea level in whom skeletal muscle accumulated iron, despite systemic iron deficiency secondary to rhEpo-induced
erythropoiesis. It was hypothesized a role of hypoxia in triggering muscle iron mobilization by increasing ferroportin mRNA and decreasing transferrin receptor mRNA expression. These findings further emphasize the difference between accelerated erythropoiesis induced by rhEpo administration and hypoxia.

In agreement with previous studies we found an early and stable increase of IL-6 concentrations, which remained below the range expected in inflammatory diseases. The mild systemic inflammation induced by exposure to high altitude, however, did not substantially counteract hypoxia-induced hepcidin suppression. In fact, only the two volunteers who developed an upper respiratory infection at the very beginning of hypoxia exposure showed a marked increase of both IL-6 and hepcidin. The progressive decrease of hepcidin, despite the persistent slight increase of IL-6, and the absence of correlation between hepcidin and IL6 suggest that iron and erythroid signals prevailed on inflammation for hepcidin regulation in this setting as also shown in mouse models combining inflammation and erythropoiesis stimulation.

In conclusion, our results provide new insights into the interaction between hypoxia, erythropoiesis and iron metabolism in humans. In Figure 3 we summarised the physiological modifications induced by acute and chronic hypoxia exposure on iron homeostasis aimed at supplying enough iron to the bone marrow, mainly based on the present findings. Hypoxia induced a marked suppression of hepcidin which appeared to result from the combined action of hypoxia-induced increased erythropoiesis and iron depletion. Serum ferritin was a major determinant of serum hepcidin concentration whereas the role of EPO and GDF15 seemed less important. This entails new efforts aimed to discover other erythroid regulator(s) of hepcidin expression. Last, the very early decrease of serum ferritin suggests the existence of hypoxia-dependent and hepcidin-independent mechanisms regulating storage iron mobilization. These results could have implications for hypoxia-related acute and chronic disorders, contributing to explain the role of hepcidin in the pathophysiology of hypoxia adaptation. They represent a first innovative step and
should pave the way to future studies aimed at providing additional insights in this complex field and, hopefully, new markers of clinical utility.

Acknowledgment

We thank Viviana Mauri from Centro Analisi Monza (CAM) for technical support in measuring EPO, sTFR and serum iron indices, and Margherita Tamplenizza for technical support in collecting samples at high altitude.

Authorship Contributions

A.P. designed and performed research, analyzed data and wrote the paper; S.G. performed the statistical analysis and wrote the paper; R.M. collected data and wrote the paper; S.P. and G.R. collected data and performed the analysis of serum parameters; C.L., G.B., M.R., A.G., A.F. and V.M. performed research and collected samples and data; M.W. and T.G. contributed to the design of the study, measured serum hepcidin and reviewed data; M.G.V. reviewed statistical analysis and wrote the paper; G.M. reviewed the paper; G.P. designed the HIGHCARE project, performed research, reviewed data and reviewed the paper.

Disclosure of Conflicts of Interest

None declared

Funding

The HIGHCARE project was made possible by an unrestricted research grant from Boehringer Ingelheim, Germany, and Banca Intesa San Paolo, Italy. This project was supported also by A. DeMari, CutAway srl, Sport Specialist, EuroTech, Moccagatta Pogliani & Associati, DiaTecne, FMS, GE Healthcare, InterCure, Microlife, Omron, Oridion, Pollution, Rotem, Sapio life, Seda SpA, SensorMedics, Spacelabs Healthcare, srLabs, Tecnoel srl, TensioMed, Webbit srl companies. The study was also supported by Cariplo Foundation (project 2009-2483) and Ministry of Instruction, University and Research (MIUR) (PRIN 2008N73CJ5_004), to A.P.
References


Table 1: Baseline characteristics at sea level (SL1) by gender.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>mean#</td>
</tr>
<tr>
<td>Age (years)</td>
<td>10</td>
<td>35.5</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>10</td>
<td>56.6</td>
</tr>
<tr>
<td>BMI (kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>10</td>
<td>20.9</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>10</td>
<td>38.1</td>
</tr>
<tr>
<td>Hepcidin (ng/mL)</td>
<td>10</td>
<td>27.0</td>
</tr>
<tr>
<td>Erythropoietin (mU/mL)</td>
<td>10</td>
<td>13.0</td>
</tr>
<tr>
<td>Serum iron (μg/dl)</td>
<td>9</td>
<td>75.5</td>
</tr>
<tr>
<td>Transferrin (mg/dl)</td>
<td>8</td>
<td>262.5</td>
</tr>
<tr>
<td>Transferrin saturation (%)</td>
<td>8</td>
<td>21.8</td>
</tr>
<tr>
<td>Ferritin (ng/mL)</td>
<td>10</td>
<td>40.1</td>
</tr>
<tr>
<td>sTFR (pmol/mL)</td>
<td>10</td>
<td>20.5</td>
</tr>
<tr>
<td>GDF15 (pg/mL)</td>
<td>10</td>
<td>332.9</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>10</td>
<td>14.7</td>
</tr>
</tbody>
</table>

# geometric means are reported for all parameters except for age, weight and BMI
Table 2: Overall description of the relevant parameters as measured at different altitudes

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sea Level (140 m)</th>
<th>Namche (3400 m)</th>
<th>Base Camp 1 (5400 m)</th>
<th>Base Camp 2 (5400 m)</th>
<th>Sea Level return (140 m)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>month -1</td>
<td>day 1-2</td>
<td>day 10-12</td>
<td>day 19-20</td>
<td>month 3-4</td>
</tr>
<tr>
<td></td>
<td>n  mean# median</td>
<td>n  mean# median</td>
<td>n  mean# median</td>
<td>n  mean# median</td>
<td>n  mean# median</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>41 42.1 43.0</td>
<td>41 44.2 43.0</td>
<td>40 50.1 50.5</td>
<td>40 54.1 55.0</td>
<td>35 42.1 41.6</td>
</tr>
<tr>
<td>Hepcidin (ng/mL)*</td>
<td>38 49.4 60.7</td>
<td>39 32.7 35.9</td>
<td>38 7.6 5.1</td>
<td>37 9.2 8.4</td>
<td>29 42.9 53.4</td>
</tr>
<tr>
<td>Erythropoietin (mU/mL)</td>
<td>40 11.5 11.0</td>
<td>41 32.4 34.2</td>
<td>40 34.9 38.0</td>
<td>38 24.7 25.7</td>
<td>35 13.2 12.2</td>
</tr>
<tr>
<td>Serum iron (μg/dl)</td>
<td>39 75.8 74.0</td>
<td>41 102.8 106.0</td>
<td>38 55.9 58.0</td>
<td>26 78.23 83.5</td>
<td>32 92.8 925</td>
</tr>
<tr>
<td>Transferrin (mg/dl)</td>
<td>37 242.9 242.0</td>
<td>41 273.0 274.0</td>
<td>38 294.5 292.0</td>
<td>25 315.3 318.0</td>
<td>32 267.7 261.5</td>
</tr>
<tr>
<td>Transferrin saturation (%)</td>
<td>37 22.7 22.4</td>
<td>41 26.5 27.7</td>
<td>38 13.4 13.9</td>
<td>25 17.2 17.9</td>
<td>35 24.3 23.6</td>
</tr>
<tr>
<td>Ferritin (ng/mL)</td>
<td>40 102.4 115.0</td>
<td>41 80.6 88.0</td>
<td>39 42.2 42.0</td>
<td>38 42.1 41.5</td>
<td>31 91.8 97.0</td>
</tr>
<tr>
<td>sTFR (pmol/mL)</td>
<td>39 21.1 21.3</td>
<td>40 23.4 22.7</td>
<td>38 33.9 32.7</td>
<td>38 31.0 29.6</td>
<td>32 18.4 18.0</td>
</tr>
<tr>
<td>GDF15 (pg/mL)</td>
<td>38 338.7 386.8</td>
<td>41 500.7 488.6</td>
<td>39 601.6 588.3</td>
<td>39 551.6 577.4</td>
<td>28 383.8 401.8</td>
</tr>
<tr>
<td>IL-6 (pg/mL)*</td>
<td>38 17.9 16.2</td>
<td>39 25.8 26.7</td>
<td>36 34.3 31.7</td>
<td>38 29.5 28.4</td>
<td>28 19.0 17.2</td>
</tr>
</tbody>
</table>

# geometric mean
* 2 subjects were excluded from the analysis of Hepcidin and IL-6 due to upper airway infection
Table 3: Effect of acute hypoxia (Namche) on the relevant parameters by group A and B: 16 and 40 hours of exposure to altitude, respectively

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Sea Level mean#</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>20</td>
<td>41.7</td>
</tr>
<tr>
<td>Hepcidin (ng/mL)*</td>
<td>18</td>
<td>50.4</td>
</tr>
<tr>
<td>Erythropoietin (mU/mL)</td>
<td>20</td>
<td>11.8</td>
</tr>
<tr>
<td>Serum iron (μg/dl)</td>
<td>19</td>
<td>80.6</td>
</tr>
<tr>
<td>Transferrin (mg/dl)</td>
<td>18</td>
<td>244.7</td>
</tr>
<tr>
<td>Transferrin saturation (%)</td>
<td>18</td>
<td>24.1</td>
</tr>
<tr>
<td>Ferritin (ng/mL)</td>
<td>19</td>
<td>116.8</td>
</tr>
<tr>
<td>sTFR (pmol/mL)</td>
<td>19</td>
<td>20.3</td>
</tr>
<tr>
<td>GDF15 (pg/mL)</td>
<td>19</td>
<td>361.4</td>
</tr>
<tr>
<td>IL-6 (pg/mL)*</td>
<td>18</td>
<td>20.2</td>
</tr>
</tbody>
</table>

# geometric mean
* 2 subjects in group A were excluded from the analysis of Hepcidin and IL-6 due to upper airway infection
Figures legend:

**Figure 1.** Time course of the HIGHCaRe project: altitude, locations, means of transportation between locations, days of ascent, staying and descent, and of blood sampling.

**Figure 2.** Longitudinal profiles of the relevant parameters at different altitudes, expressed in terms of geometric means and 95% Confidence Intervals (vertical lines), by gender (—– females; —— males) and overall (——). Significant comparison with baseline are indicated by ** when p-value<0.0001 or * if p-value< 0.006. Two subjects were excluded from the analysis of Hepcidin and IL-6 due to upper airway infection.

**Figure 3.** Hypoxia-induced modifications of iron homeostasis (see also text). Erythropoietin activation is the first event occurring after acute hypoxia exposure inducing erythropoietic expansion which requires more iron for red blood cells hemoglobinization. Hepcidin downregulation is central for this function: it begins within 16-40 hours and peaks within few days of exposure, and thereafter persists for a long time. Iron and erythroid signals cooperate in hepcidin suppression increasing intestinal iron absorption and cellular iron release. Iron mobilization from stores occurs early, possibly involving an hypoxia-mediated event. Erythroid signal likely requires soluble mediators from bone marrow to the liver. Transferrin saturation decreased only in the phase of expansion of the erythropoietic mass contributing to hepcidin suppression.
Modulation of hepcidin production during hypoxia-induced erythropoiesis in humans in vivo: data from the HIGHCARE project

Alberto Piperno, Stefania Galimberti, Raffaella Mariani, Sara Pelucchi, Giulia Ravasi, Carolina Lombardi, Grzegorz Bilo, Miriam Revera, Andrea Giuliano, Andrea Faini, Veronica Mainini, Mark Westerman, Tomas Ganz, Maria Grazia Valsecchi, Giuseppe Mancia and Gianfranco Parati