The Haptoglobin 2-2 Phenotype Affects Serum Markers of Iron Status in Healthy Males


Background: Human iron status is influenced by environmental and genetic factors. We hypothesized that the genetic polymorphism of haptoglobin (Hp), a hemoglobin-binding plasma protein, could affect iron status.

Methods: Reference values of serum iron status markers were compared according to Hp phenotypes (Hp 1-1, Hp 2-1, Hp 2-2; determined by starch gel electrophoresis) in 717 healthy adults. Iron storage was investigated in peripheral blood monocyte-macrophages by measuring cytosolic L- and H-ferritins and by in vitro uptake of radiolabeled (125I) hemoglobin-haptoglobin complexes.

Results: In males but not in females, the Hp 2-2 phenotype was associated with higher serum iron ($P < 0.05$), transferrin saturation ($P < 0.05$), and ferritin ($P < 0.01$) concentrations than Hp 1-1 and 2-1, whereas soluble transferrin receptor concentrations were lower ($P < 0.05$). Moreover, serum ferritin correlated with monocyte L-ferritin content ($r = 0.699$), which was also highest in the male Hp 2-2 subgroup ($P < 0.01$). In vitro, monocyte-macrophages took up a small fraction of 125I-labeled hemoglobin complexed to Hp 2-2 but not to Hp 1-1 or 2-1.

Conclusions: The Hp 2-2 phenotype affects serum iron status markers in healthy males and is associated with higher L-ferritin concentrations in monocyte-macrophages because of a yet undescribed iron delocalization pathway, selectively occurring in Hp 2-2 subjects.

Distribution of iron between the different body compartments is performed mostly by transferrin, a plasma protein that donates iron to the cells through a receptor-mediated pathway (1, 2). Hemoglobin (Hb) iron is also recycled to the plasma after destruction of senescent erythrocytes by macrophages that release iron back to plasma transferrin (2). In addition to these well-described routes of iron recycling, other proteins can contribute to iron turnover, but their importance has not been evaluated fully. Among them, haptoglobin (Hp) is a plasma protein responsible for the removal of free Hb from the circulation (3). After hemolysis, stable Hp-Hb complexes are formed that are delivered to the hepatic parenchymal cells by receptor-mediated endocytosis (4, 5). In this way, Hp reduces the loss of Hb through the glomeruli, protects against peroxidative kidney injury, and allows the recycling of heme iron (3).

In humans, Hp is characterized by a genetic polymorphism with three structurally different phenotypes (Hp 1-1, Hp 2-1, and Hp 2-2), which result from the expression of two different alleles (Hp1 and Hp2) of the Hp gene located on chromosome 16q22. The phenotypes show an important molecular heterogeneity; Hp 1-1 is a small molecule (86 kDa) of well-defined structure, whereas Hp 2-1 is characterized by heteropolymers (86–300 kDa), and Hp 2-2 forms large macromolecular complexes (170–1000 kDa) (3).

The physiological importance of Hp-Hb complex formation has become evident by the increased susceptibility to Hb-driven lipid peroxidation demonstrated in conditions of hypo- or anhaptoglobinemia (Hp0 phenotype).
were investigated. Functional differences between the various Hp phenotypes have important biological consequences (3). The hepatic "clearance" of free Hb in plasma appears to be less efficient for Hp 2-2 than for the other Hp phenotypes, producing some degree of iron-driven oxidative stress that is reflected by a lower stability of serum vitamin C in healthy Hp 2-2 subjects (11, 12).

From these observations, we postulated that functional differences between the Hp phenotypes might affect human iron status. Body iron stores are found associated with ferritin mainly in the hepatocytes and the mononuclear phagocytic system (13). We derived a working hypothesis that a less efficient transport of free plasma Hb to the liver could result in "delocalization" of iron into monocyte-macrophages.

In this study, we wanted to investigate the influence of Hp polymorphism on iron status in healthy subjects. For this purpose, we measured serum indicators of body iron compartments: iron and transferrin saturation (iron transport compartment), ferritin (iron storage compartment), and soluble transferrin receptors (sTfR; functional iron compartment) (14–16). Intracellular iron status in human monocyte-macrophages was studied by measuring cytosolic L- and H-ferritin concentrations (13). In vitro, interactions of Hp-Hb complexes with monocyte-macrophages were investigated.

Materials and Methods

Subjects

The study population consisted of 717 healthy Caucasian individuals (344 males, ages 18–50 years; 373 nonpregnant females, ages 19–47 years) from the same region (Flanders, Belgium). Exclusion criteria were anemia (defined as Hb <130 g/L for males and <120 g/L for females), recent blood transfusion or blood donation (within 3 months before the study), intake of iron and/or vitamin preparations, alcohol intake >20 g/day and within 1 week before blood sampling, and serum C-reactive protein (CRP) concentration >10 mg/L. All subjects gave oral informed consent to participate in this study, which was approved by the ethical committee of the University Hospital of Gent.

Analysis of Serum and Plasma Samples

Venous blood was drawn between 0900 and 1000, allowed to clot, and centrifuged (1000g for 10 min at room temperature). The supernatant serum was collected for analysis. The Hp phenotype was determined using starch gel electrophoresis of Hp-supplemented serum, followed by visualization of Hp-Hb bands by staining the gel with metal-enhanced peroxidase reagents (Pierce Corp.) as described previously (17). Serum Hp, CRP, ferritin, and sTfR were assayed by fixed-time immunonephelometry using commercial rabbit anti-human antisera on a BN II nephelometer (Dade Behring), calibrated against the CRM 470 reference material (18). Transferrin saturation was calculated from measurements of serum iron concentration by spectrophotometry (ferrozine method) and serum transferrin concentration by immunoturbidimetry using commercial reagents on a Hitachi 747 analyzer (Roche Diagnostics). Assuming that two iron atoms bind to one molecule of transferrin, serum transferrin saturation (%) was calculated according to the equation (19):

\[
\text{Serum iron (\mu mol/L)} \times 3.98
\]

Blood was simultaneously collected in tubes containing EDTA for determination of plasma Hb and hematocrit (Sysmex SE-9500; Toa Medical Electronics).

L- and H-Ferritin in Monocyte-Macrophages

Heparinized venous blood (50 mL) was obtained from 44 healthy males (ages 25–46 years) with known Hp phenotype. Peripheral blood mononuclear cells were isolated by centrifugation (400g for 30 min at 20 °C) through Ficoll-Isoopaque (Nycomed), followed by purification of the monocytes by density gradient centrifugation using Percoll (Pharmacia; 600g for 30 min at 20 °C) (20). Monocytes (purity, 85–90%) were harvested from the interface, and aliquots of 1–2 × 10^6 cells were washed in phosphate-buffered saline (PBS), pH 7.4 (Unipath). The cells were lysed by sonication in cold hypotonic buffer (20 mmol/L Tris, 40 mmol/L KCl, 10 mL/L Triton, 1 mmol/L phenylmethylsulfonyl fluoride) containing a protease inhibitor mixture (Roche). The resulting lysates were then centrifuged (14 000g for 30 min at 4 °C), and the total protein content of the supernatants was determined spectrophotometrically by the Bradford method (Bio-Rad) in triplicate, calibrated using bovine serum albumin.

Cytosolic L-ferritin and H-ferritin contents were measured by enzyme-linked immunosorbent assays based on monoclonal antibodies specific for L-ferritin (LFO3) and H-ferritin (HIO2) and calibrated using the corresponding recombinant homopolymers expressed in Escherichia coli (21). The bound ferritin was detected with the same antibodies conjugated to horseradish peroxidase (colorimetric reaction with o-phenylenediamine dihydrochloride). All measurements were performed in triplicate, and results are expressed as µg of ferritin/g of total proteins.

Preparation of Radiolabeled Hp-Hb Complexes

Radioactive iodine (from Na^125I) was incorporated into human Hb (Sigma) by the chloramine-T method making use of the radioiodination method (ICN) according to the manufacturer’s protocol. Afterward, free ^125I was removed by gel filtration over a Sephadex G10 column. Specific activity of ^125I-labeled Hb, measured in a gamma counter (LKB Wallac), ranged between 0.9 and 1.6 × 10^6 cpm/mg protein. Before use in a cellular uptake assay, ^125I-labeled Hb was diluted in 1 mL of PBS containing 50 mL/L fetal calf serum to a final concentration of 1.0 g/L. Hp-Hb complexes were prepared by identical dilution of...
125I-labeled Hb in 1 mL of PBS containing 50 mL/L fetal calf serum and purified human Hp 1-1, Hp 2-1, and Hp 2-2 phenotypes (Sigma; final Hp concentrations, 0.5 and 1.0 g/L). Binding of 125I-labeled Hb to Hp phenotypes was checked electrophoretically by comparison with the typical migration patterns of unlabeled Hp-Hb complexes on starch gel electrophoresis and by detection of radioactivity in Hp-Hb bands on the gel.

125I-Hb uptake in monocyte-macrophages
Peripheral blood monocytes (2 \times 10^7 cells), isolated by two-step density gradient centrifugation as described above, were suspended in 1 mL of prewarmed (37 °C) 125I-labeled Hb preparations or phenotype-matched 125I-labeled Hb-Hp complexes. After 20 min preincubation at 37 °C (to account for Hp binding to adhesion molecules), the cell suspensions were centrifuged (1000 g for 10 min at 20 °C), the supernatants were transferred to another tube, the cells were washed in PBS, and baseline radioactivity in the cell pellets was measured (LKB Wallac). The cells were then resuspended in the supernatants, and measurements of cell-associated radioactivity were repeated at 1-h intervals using the same procedure, during an incubation period of 3 h at 37 °C under gentle shaking. The rate of cellular accumulation of radioactivity was calculated as:

\[
\frac{cpm(t) - cpm(t_0)}{t - t_0}
\]

where \( t_0 = 20 \) min. The values were corrected for radioisotope decay. Identical experiments were performed in presence of the macrophage-activator zymosan A (Sigma; final concentration, 0.1 g/mL) (24). To distinguish intracellular uptake from cell surface binding, monocytes were incubated at 4 °C (25). 125I-Labeled Hb uptake was also studied in monocytes pretreated with 0.1 g/L pronase E (protease type XIV from Streptomyces griseus; Sigma) for 30 min at 37 °C and washed with PBS. As a negative control, additional experiments were carried out in presence of a 10-fold excess of unlabeled Hb (added to 125I-labeled Hb before preparation of Hp-Hb complexes).

Statistics
Results are given as mean ± SD or median and interquartile range, where appropriate. The Wilcoxon test was used for evaluation of differences between subgroups. Correlations between data were examined using regression analysis. Statistical significance was considered at the level of \( P < 0.05 \).

Results
The distribution of Hp phenotypes in the study population (16% Hp 1-1, 48% Hp 2-1, and 36% Hp 2-2) was in Hardy-Weinberg equilibrium and yielded a relative Hp1 allele frequency of 0.404, which corresponds to the expected frequency in European populations (3). The Hp phenotype distribution was comparable between sex groups and is shown in Table 1. Serum Hp concentrations corresponded with the Hp phenotype-adjusted reference values in Caucasians and were ~30% lower in Hp 2-2 than in Hp 1-1 individuals (3). Serum CRP, plasma Hb, and hematocrit were comparable between Hp phenotypes in both sexes (Table 1).

Serum markers of iron status
Table 2 compares the serum iron status according to gender and Hp phenotype. In all subgroups, serum concentrations of the various iron status markers were within the expected reference intervals. However, reference values of serum iron and transferrin saturation among males differed significantly among Hp phenotypes, showing highest concentrations in the Hp 2-2 subgroup (\( P < 0.05 \)), whereas serum transferrin concentrations were comparable. More significantly, serum ferritin concentrations in males were higher in the Hp 2-2 subgroup than in the Hp 1-1 and 2-1 subgroups (\( P < 0.01 \)). Fig. 1 illustrates this Hp phenotype-dependent variation in ferritin concentrations within the expected reference interval. In contrast, serum sTfR concentrations were significantly (\( P < 0.05 \)) lower in Hp 2-2 males than in those carrying another Hp phenotype. The observed differences were not statistically significant among females.

| Table 1. Serum Hp, serum CRP, plasma Hb, and hematocrit according to Hp phenotypes in healthy subjects.\(^a\) |
|---|---|---|---|---|
| \( n \) | \( Hp \), g/L | \( CRP \), mg/L | \( Hb \), g/L | Hematocrit, % |
| Males | | | | |
| Hp 1-1 | 56 | 1.19 (0.87–1.42) | 0.69 (0.30–1.39) | 155 (142–166) | 45.0 (41.2–46.8) |
| Hp 2-1 | 164 | 0.99 (0.74–1.24) | 0.72 (0.38–1.46) | 154 (144–167) | 45.1 (40.7–46.8) |
| Hp 2-2 | 124 | 0.74 (0.52–1.01)\(^b\) | 0.68 (0.35–1.43) | 156 (143–165) | 45.1 (41.0–47.0) |
| Females | | | | |
| Hp 1-1 | 62 | 1.16 (0.91–1.60) | 0.71 (0.29–1.40) | 141 (132–151) | 40.4 (37.6–42.4) |
| Hp 2-1 | 180 | 1.05 (0.82–1.23) | 0.69 (0.35–1.42) | 140 (134–152) | 40.8 (37.4–42.5) |
| Hp 2-2 | 131 | 0.75 (0.58–0.99)\(^b\) | 0.70 (0.30–1.38) | 142 (132–154) | 40.7 (37.8–42.3) |

\( a \) Data are median (interquartile range).

\( b \) \( P < 0.01 \) (comparison between Hp 2-2 and Hp 1-1/2-1, Wilcoxon test).
FERRITIN CONTENT IN MONOCYTE-MACROPHAGES

In peripheral blood monocytes from 44 healthy males, L- and H-ferritin contents (measured in triplicate) were compared between the Hp phenotypes (Table 3). L-Ferritin concentrations in monocyte cell lysates from Hp 2-2 subjects (n = 17) were approximately twofold higher than in monocytes from subjects carrying Hp 1-1 (n = 9) or Hp 2-1 (n = 18; P, 0.01). In contrast, cytosolic H-ferritin contents in monocytes did not differ between the Hp phenotypes.

There was a positive correlation between monocyte L-ferritin concentrations and serum ferritin concentrations in the 44 subjects (r = 0.699; P, 0.001; Fig. 2). This correlation was better in the Hp 2-2 subgroup (r = 0.589) than in the Hp 1-1 and 2-1 subgroups (r = 0.357 and r = 0.362, respectively).

HB UPTAKE IN MONOCYTE-MACROPHAGES

Peripheral blood monocytes were incubated (37 °C) with 125I-labeled Hb and phenotype-matched 125I-labeled Hb-Hp complexes, and cell-associated radioactivity was measured at 1-h intervals during 3 h starting from an initial baseline measurement after 20 min (t0). The rate of cellular accumulation of radioactivity in the interval t – t0 = 3 h was used for comparison (Fig. 3). Incubation of monocytes with 125I-labeled Hb (1.0 g/L) produced a higher baseline radioactivity in the presence of Hp than in the absence of Hp, which can be attributed to earlier reported Hp binding to surface membrane molecules on mononuclear cells (22, 23). In the absence of Hp and in the presence of Hp 1-1 or Hp 2-1 (0.5 and 1.0 g/L), cell-associated (baseline) radioactivity changed very slowly during the next 3 h. In contrast, loading of monocytes with 125I-labeled Hb complexed to Hp 2-2 (1.0 g/L) produced higher baseline concentrations of cell-associated radioactivity, which continued to increase at a constant rate during the full incubation period. However, this cellular accumulation of radioactivity represented only a small fraction of the total amount of Hb-Hp 2-2 complexes added to monocytes (~0.2% after 3 h). The cellular uptake of 125I-labeled Hb was linear and occurred at a lower rate when less Hp 2-2 (0.5 g/L) was added (Fig. 3A).

Activation of monocytes with zymosan A produced a moderate cellular accumulation of 125I-labeled Hb in the absence of Hp and in the presence of Hp 1-1 and 2-1 because of fluid phase uptake, but this occurred at a higher rate in the presence of Hp 2-2 (Fig. 3B). Approximately 0.4% of the Hp-Hb 2-2 complexes added to zymosan-activated monocytes was internalized after 3 h. On the other hand, incubation experiments performed at 4 °C did not demonstrate cellular uptake of Hb-Hp 2-2 complexes, whereas baseline radioactivity attributable to cell

Table 2. Serum iron, transferrin saturation, ferritin, and sTfR concentrations according to Hp phenotypes in healthy subjects.a

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
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<tr>
<td>n</td>
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<tr>
<td>Hp 1-1</td>
<td>56</td>
<td>62</td>
</tr>
<tr>
<td>Hp 2-1</td>
<td>164</td>
<td>180</td>
</tr>
<tr>
<td>Hp 2-2</td>
<td>124</td>
<td>131</td>
</tr>
<tr>
<td>Iron, μmol/L</td>
<td>18.6 (12.9–22.0)</td>
<td>16.6 (13.1–22.7)</td>
</tr>
<tr>
<td>Transferrin saturation, %</td>
<td>26.6 (18.5–31.5)</td>
<td>22.9 (18.1–31.4)</td>
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<tr>
<td>Ferritin, μg/L</td>
<td>66 (41–104)</td>
<td>24 (15–55)</td>
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<tr>
<td>sTfR, mg/L</td>
<td>1.42 (1.15–1.70)</td>
<td>1.53 (1.19–1.78)</td>
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<td>n</td>
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<tr>
<td>n</td>
<td>9</td>
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<tr>
<td>n</td>
<td>17</td>
<td>18</td>
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<tr>
<td>Mean ± SD</td>
<td>326 ± 83</td>
<td>366 ± 109</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>82 ± 26</td>
<td>84 ± 22</td>
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a Data are median (interquartile range).
b,c Comparison between Hp 2-2 and Hp 1-1/2-1 (Wilcoxon test): b P < 0.05; c P < 0.01.

Table 3. Intracellular L- and H-ferritin concentrations in peripheral blood monocyte cell lysates from 44 healthy males with different Hp phenotypes.a

<table>
<thead>
<tr>
<th></th>
<th>Hp 1-1 (n = 9)</th>
<th>Hp 2-1 (n = 18)</th>
<th>Hp 2-2 (n = 17)</th>
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</thead>
<tbody>
<tr>
<td>L-Ferritin, μg/g protein</td>
<td>326 ± 83</td>
<td>366 ± 109</td>
<td>687 ± 152b</td>
</tr>
<tr>
<td>H-Ferritin, μg/g protein</td>
<td>82 ± 26</td>
<td>84 ± 22</td>
<td>79 ± 28</td>
</tr>
</tbody>
</table>

a Data are mean ± SD.
b P < 0.01 (comparison between Hp 2-2 and Hp 1-1/2-1, Wilcoxon test).
surface binding was comparable between the three Hp phenotypes (data not shown). The effect of Hp 2-2 on cellular accumulation of $^{125}$I-labeled Hb was also not detected when a 10-fold excess of unlabeled Hb was added or after pronase pretreatment of the monocytes (data not shown).

**Discussion**

This is the first report showing the effect of Hp polymorphism on iron turnover in healthy individuals. In male Caucasians but not in females, we demonstrated an influence of Hp polymorphism on serum iron status markers, although these values remained within the expected reference interval. Hp 2-2 is associated with higher serum iron concentrations and transferrin saturation compared with the other Hp phenotypes, reflecting a relative increase of the iron transport compartment (14). More significantly, Hp 2-2 males are characterized by higher iron stores, as evidenced by higher serum ferritin concentrations (14). The sTfR concentrations in serum are lower in this Hp 2-2 subgroup, suggesting a lower cellular expression of membrane transferrin receptors and thus a relative increase of the functional iron compartment (14–16).

Serum ferritin is a positive acute phase reactant, and inflammatory cytokines and immune activation are known to affect iron metabolism (26,27). However, serum CRP concentrations were not different between Hp phenotypes, indicating that the influence of Hp 2-2 on the serum iron status is not biased by some degree of subclinical inflammation. The observed effect of Hp polymorphism on circulating sTfR concentrations cannot be attributed to different degrees of erythropoiesis because plasma Hb and hematocrit values were comparable between Hp phenotypes (14). Among females, the lack of correlation between Hp polymorphism and iron status could be explained by menstrual bleeding.

The observed association of Hp 2-2 with higher iron stores, at least in males, can be explained by a degree of iron delocalization in monocyte-macrophages. Indeed, we found a more pronounced iron storage in monocytomacrophages of Hp 2-2 subjects, as evidenced by higher cytosolic L-ferritin concentrations in peripheral blood monocytes. Remarkably, cytosolic H-ferritin contents in monocytes were not different among the three Hp phenotypes, similarly to what is observed in iron-loaded livers where only the L-ferritin is up-regulated (28). The positive correlation we found between serum and mono-
cyte ferritin concentrations suggests that increased L-ferritin synthesis in monocyte-macrophages produces higher ferritin concentrations in the circulation (14).

Earlier studies suggested that, in intravascular hemolysis, parts of Hb-derived iron are incorporated ("delocalized") into slowly turned over, poorly accessible iron storage compartments of the mononuclear phagocytic system, largely bypassed in the normal endogenous cycling of iron (29). In vitro experiments in our study showed that human monocytes take up a small fraction of Hb-Hp 2-2 complexes at 37°C but not at 4°C. In contrast, free Hb and Hb bound to Hp 1-1 or 2-1 are not internalized in monocytes. However, because even in zymosan-activated monocytes (24) only a small fraction of Hb-Hp 2-2 is internalized, these experiments only explain in part the iron delocalization observed in Hp 2-2 subjects.

In vivo, monocyte-macrophages can take up Hb via phagocytosis of senescent erythrocytes, after which iron is released from heme intracellularly and stimulates ferritin mRNA translation (30, 31). Hepatocytes usually take up ~2 mg of iron/day (the typical plasma iron turnover is ~30 mg/day) in the form of Hb-Hp complexes, present in the circulation after intravascular disintegration of ~10% of erythrocytes, which occurs even in healthy subjects (29). High-affinity receptors (Kd ~7.4 nmol/L) expressed on hepatocytes are involved in binding and subsequent internalization of Hb-Hp complexes (5). In contrast, only a low affinity (Kd ~6.5 nmol/L) Hp binding on monocytes has been reported in the literature (22). If hepatocytes, in vivo, represent the major target cells for the Hb-Hp complexes, it remains unclear how monocyte-macrophages (without receptors or with only low-affinity receptors) could acquire Hb-Hp 2-2 complexes. However, nothing is known on the relative affinities of these receptors toward Hp 2-2 vs other Hp phenotypes. The Hp 2-2 molecule differs from the other phenotypes by its larger size because of polymerization (up to 1000 kDa), and our present findings suggest that it is taken up more efficiently than Hp 1-1 or 2-1 complexes, presumably by a pinocytosis-like phenomenon (24). Although only a small proportion of the added Hb-Hp 2-2 complexes is taken up in our experiments, this does not necessarily reflect the in vivo situation where tissue macrophages might have a more active pinocytotic activity. To support our hypothesis, further basic research is necessary to demonstrate the iron delocalization pathway.

The Hp polymorphism has been associated with the prevalence and the clinical outcome of many pathologies with altered iron metabolism such as cancer, infections, atherosclerosis, and neurological disorders (3). Males carrying the Hp 2-2 phenotype are at risk for premature atherosclerotic disease, a condition where iron-driven lipid oxidation is known to play a role (32, 33). The data presented in this report support the observation made by our group in a large cohort of HIV-infected patients, where Hp 2-2 was associated with iron-driven oxidative stress (vitamin C depletion), enhanced HIV-1 replication, and higher mortality (34–36). However, the involvement of Hp in pathological processes might be even more complex because the protein participates in various immunoinflammatory functions of lymphocytes, granulocytes, and monocytes (37–39).

Important sources of variability in the laboratory-based assessment of iron status include differences between subjects (e.g., age, sex, race, ethnicity, geographical region, or altitude of residence) and within-subject variations (e.g., circadian change and inflammation). In the present study, we identified a genetic variability arising from the Hp polymorphism in healthy males. In interpreting serum markers of iron status, this Hp phenotype-dependent variation in reference values may increase the risk of either unnecessary additional investigations (e.g., bone marrow biopsy) or failure to detect subclinical disease accurately, particularly in some cases of (pre-)latent iron deficiency or iron overload.

In conclusion, delocalization of iron into poorly exchangeable storage compartments of the mononuclear phagocytic system affects the iron status in males carrying the Hp 2-2 phenotype. This effect is a potential cause of over- or underestimation of serum iron status markers.

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