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Hepcidin response to acute iron intake and chronic iron loading in dysmetabolic iron overload syndrome

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Abstract

Background—The pathogenesis of dysmetabolic iron overload syndrome (DIOS) is still unclear. Hepcidin is the key regulator of iron homeostasis controlling iron absorption and macrophage release.

Aim—To investigate hepcidin regulation by iron in DIOS.

Methods—We analysed urinary hepcidin at baseline and 24 h after a 65 mg oral iron dose in 24 patients at diagnosis and after iron depletion ($n=13$) and compared data with those previously observed in 23 healthy controls. Serum iron indices, liver histology and metabolic data were available for all patients.

Results—At diagnosis, hepcidin values were significantly higher than in controls ($P<0.001$). After iron depletion, hepcidin levels decreased to normal values in all patients. At baseline, a significant response of hepcidin to iron challenge was observed only in the subgroup with lower basal hepcidin concentration ($P=0.007$). In iron-depleted patients, urinary hepcidin significantly increased after oral iron test ($P=0.006$).

Conclusions—Our findings suggest that in DIOS, the progression of iron accumulation is counteracted by the increase in hepcidin production and progressive reduction of iron absorption, explaining why these patients develop a mild–moderate iron overload that tends to a plateau.

A specific form of iron overload associated with components of metabolic syndrome has been described and variably named insulin resistance hepatic iron overload syndrome or dysmetabolic iron overload syndrome (DIOS) (1, 2). Most of these patients have non-alcoholic fatty liver disease (NAFLD) or non-alcoholic steato-hepatitis (NASH), one of the most common forms of chronic liver disease (3), which is now considered the hepatic manifestation of the metabolic syndrome (4). Although no epidemiological study is available, the association between mild–moderate iron overload and metabolic alterations is

common in developed countries. However, pathogenetic mechanisms that link metabolic abnormalities, insulin resistance and/or NAFLD to iron accumulation are still unclear.

Hepcidin is the key regulator of systemic iron homeostasis (5). It is secreted primarily by hepatocytes and in little amounts also by adipocytes, macrophages and other tissues (6). Hepcidin binds to the iron exporter ferroportin, causes its internalization and intracellular degradation and thereby inhibits the flow of iron from duodenal enterocytes and macrophages into plasma (7). Hepatic hepcidin production is under the control of several stimuli, some (iron excess and inflammation) inducing and some (erythropoietic activity, iron deficiency and hypoxia) decreasing its synthesis through different pathways (6).

HFE-related haemochromatosis is associated with insufficient hepcidin production (8, 9) resulting in increased transferrin saturation (TS) and parenchymal iron accumulation. Compared with haemochromatosis, TS is frequently normal in DIOS and hepatic iron accumulation is lower and involves both parenchymal and reticuloendothelial cells (1, 10), suggesting a different pathogenetic mechanism. Only few studies measured hepcidin expression in DIOS. Hepatic mRNA, urinary (11) and serum (12) hepcidin levels were found to be significantly higher than in controls, but were lower when corrected for serum ferritin (SF) levels (hepcidin/ferritin ratio) (11). Although high hepcidin levels seem to suggest that DIOS patients may retain the ability to increase hepcidin production in response to chronic iron load, the low hepcidin/ferritin ratio might indicate that hepcidin response is inadequate to the amount of iron overload.

In the present study, we evaluated hepcidin peptide expression in patients with DIOS at diagnosis and after iron depletion to assess the influence of iron accumulation on hepcidin production and compared these results with those previously observed in healthy subjects (8). In addition, as a simple test to evaluate the homeostatic capacity of the iron regulatory system in these subjects, we measured hepcidin in basal conditions and 24 h after a single dose of oral iron both at diagnosis and after iron depletion. This test is based on previous observations showing that healthy subjects (13), but not HFE-haemochromatosis patients (8), acutely respond to a small dose (65 mg) of oral iron by increasing their hepcidin production and urinary excretion.

Patients and methods

Subjects

The study included 24 adult consecutive Italian patients (21 men and 3 women) affected by DIOS enrolled from February 2005 to January 2007. DIOS was defined according to Mendler *et al.* (1): histological hepatic iron overload associated with at least one feature of metabolic syndrome, with or without NAFLD/NASH. All patients had a total iron score (TIS), according to Deugnier *et al.* (14) (see 'Methods'), at least equal or higher than 12.

Exclusion criteria were (i) other known causes of hepatic iron overload (15) and (ii) conditions able to influence hepcidin production (6). Thus, we excluded (i) hereditary haemochromatosis, ferroportin disease, aceruloplasminaemia and hypotransferrinaemia; iron loading anaemias, history of blood transfusions and prolonged parenteral iron treatment;

chronic viral hepatitis, porphyria cutanea tarda, alcohol intake (>40 g/day in men and >20 g/day in women), end-stage cirrhosis; (ii) anaemias, neoplasia, acute or chronic inflammatory disorders, pregnancy and chronic renal failure. Because proton pump inhibitor therapy may influence iron absorption, thus interfering with the oral iron test, it was also considered among exclusion criteria.

All patients with DIOS were studied before starting phlebotomy and 13 of them (11 men and 2 women) also after iron depletion (see 'Methods'). Patients with DIOS were invited not to change their lifestyle (diet or physical activity) during phlebotomy treatment and their weight was regularly checked.

Control group included 23 healthy subjects who were studied in the same period and whose data have been already published (8).

Methods

Iron and metabolic indices

Haemoglobin, TS, SF, C-reactive protein, glucose and insulin, total and high-density lipoprotein cholesterol, triglycerides, serum alanine aminotransferases and γ -glutamyl transferase were measured by commercial kits. Body mass index (BMI), abdominal waist and blood pressure were also collected at baseline and at iron depletion. The features of metabolic syndrome were defined according to the NCEP-ATPIII criteria (16). HOMA index was chosen to evaluate insulin sensitivity (17).

Liver histology

Liver specimens were stained with haematoxylin and eosin, trichrome for collagen and Perls for iron grading. Hepatic steatosis and fibrosis were graded according to Kleiner's score (18), and liver iron overload by Deugnier's semiquantitative score, with hepatocyte iron score (HIS) ranging from 0 to 36, sinusoidal iron score (SIS) from 0 to 12 and portal iron score (PIS) from 0 to 12. The sum of HIS, SIS and PIS is the TIS that ranges from 0 to 60 (14).

Genetic analysis

All patients were tested for *HFE* p.Cys282Tyr and p.Hys63Asp mutations by real-time PCR (Celbio, Milano, Italy). *HFE* and *TfR2* sequencing were performed only in patients with TS >45%; *HAMP* and *HFE2* sequence analyses were performed only in severe iron overload (TIS>27) and *SLC40A1* sequencing was performed in all. Sequence analysis were performed by ABI Prism 3100 Avant DNA sequencer (PE Applied Biosystems, Foster City, CA, USA) and compared with GenBank reference sequences.

Oral iron test

Oral iron challenge [65 mg oral iron as ferrous sulphate (CVS Pharmacy, CVS Corporation, Woonsocket, RI, USA)] was performed before and after phlebotomy therapy, as previously reported (8). Patients collected first-morning urines on day 1 when they also ingested iron and after 24 h. The protocol was approved by the Ethical Committee of Human

Experimentation and is in accordance with the Helsinki Declaration of 1975. Informed consent was obtained from all patients and controls.

Iron depletion therapy

Phlebotomies (400 ml in men and 350 ml in women) were performed every 2 weeks until iron depletion was achieved, as defined by SF values below 100 µg/l with normal TS (16–45%). Because patients with DIOS may frequently develop an iron-deficient erythropoiesis (TS<16%) (19) with or without anaemia during phlebotomy treatment (20), serum iron indices were tested every four phlebotomies and, if needed, the frequency of phlebotomies was reduced (every 3, 4 or more weeks) in order to reach iron depletion. At iron depletion, the amount of iron removed by phlebotomy was calculated as previously reported (21). After iron depletion, iron challenge was carried out at a time delay of at least 30 days from the last phlebotomy to exclude the inhibitory effect of iron deficiency or anaemia on hepcidin production. If iron-deficient erythropoiesis or anaemia was still present, iron challenge was delayed until needed. The median after the last phlebotomy was 86 days (range 42–231).

Urinary hepcidin measurement

After collection, urines were preserved with 0.01% sodium azide and stored in a 50 ml polypropylene tube frozen at –20 °C until shipping on dry ice to UCLA. Urinary creatinine concentrations were measured by UCLA Clinical Laboratories. Urinary hepcidin was determined by an immunodot assay as described previously and expressed as ng hepcidin/mg creatinine (22). Briefly, cationic peptides were extracted from urine by cation exchange chromatography and subsequently measured by chemiluminescence using rabbit anti-human hepcidin primary antibodies.

Statistical analysis

Data were expressed as median and range. All comparisons involving quantitative variables were performed by non-parametric tests: Mann–Whitney was used to compare patients and controls and Wilcoxon signed-rank test to compare hepcidin levels before and after phlebotomy and hepcidin response to oral iron test. Fisher's exact test was used to compare frequencies between groups. Correlations between quantitative variables were evaluated by Spearman's test. All tests were two sided and with a significance level of α equal to 0.05. Analyses were carried out by the GRAPHPAD PRISM statistical analysis software (version 3.02) (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Table 1 shows hepatic histological data of patients with DIOS at diagnosis. Fourteen patients (58.3%) had the metabolic syndrome, seven (29.2%) and three (12.5%) had two and only one component of the metabolic syndrome respectively. Two (8.3%) patients were heterozygotes for p.Cys282Tyr, six (25%) were heterozygotes for p.Hys63Asp and 16 (66.7%) carried none.

Table 2 shows some demographical data, iron indices and urinary hepcidin concentration in patients with DIOS before and after phlebotomy treatment and in healthy controls. Before phlebotomy therapy, patients with DIOS had basal hepcidin levels significantly higher than healthy controls ($P<0.0001$) but lower hepcidin/ferritin ratio [median: 0.18 ng/mg creatinine (range 0.07–0.7) vs 0.45 ng/mg creatinine (range 0.02–2.7)] ($P=0.0018$). In DIOS patients, log ferritin positively correlated with TIS and SIS ($r=0.49$, $P=0.0157$, and $r=0.50$, $P=0.012$ respectively), but not with HIS. After phlebotomy treatment, urinary hepcidin levels decreased in all DIOS patients ($P=0.0003$) (Fig. 1). Urinary hepcidin and iron indices did not significantly differ between phlebotomized DIOS patients and controls (Table 2).

Metabolic indices of patients studied before and after phlebotomy treatment are reported in Table 3. No significant change was observed between groups.

Hepcidin response to iron challenge

Before phlebotomy treatment, hepcidin did not increase significantly 24 h after iron challenge in patients with DIOS, considered all together (Fig. 2A). As hepcidin levels may influence iron absorption, then we divided DIOS patients in two subgroups below and above the median value (163 ng/mg creatinine) to evaluate whether hepcidin response might differ according to the basal hepcidin concentration. We found a significant increase of hepcidin only in the subgroup with lower basal hepcidin concentration ($P=0.007$) (Table 4). No other difference was found in the two subgroups regarding age, iron and metabolic indices, C-reactive protein, liver function tests and histology (percentage of steatosis, Kleiner's and Deugnier's scores) and *HFE* genotypes (data not shown).

After iron depletion, we observed a significant increase of hepcidin 24 h after oral iron test ($P=0.006$) (Fig. 2B).

Correlations of hepcidin

Because DIOS patients were significantly older than healthy controls, we evaluated whether the lack of hepcidin response might be because of an age-related reduction of intestinal iron absorption, but we could not find any relationship between basal hepcidin, hepcidin response to oral iron test and age in patients at diagnosis and in controls. Basal hepcidin and hepcidin response in DIOS patients did not correlate with serum and hepatic iron indices, liver function tests, degree of steatosis, metabolic indices and C-reactive protein.

At iron depletion, hepcidin did not correlate with time delay from the last phlebotomy, indicating it was not influenced by a late erythropoietic drive.

Discussion

The present study confirms that in DIOS patients, urinary hepcidin is higher than in healthy controls and demonstrates that after iron depletion hepcidin decreases to normal value. This differs from that of the previously observed in iron-depleted patients with *HFE*-haemochromatosis in whom urinary hepcidin markedly decreased below normal value, revealing the *HFE*-dependent defect of hepcidin production when the stimulus induced by increased iron stores disappeared (8). These findings suggest that (a) the increased hepcidin

level in DIOS at diagnosis depends mainly on the response of hepcidin to chronic iron overload; (b) iron overload in DIOS does not depend on defective hepcidin production. Urinary hepcidin of DIOS patients was markedly higher than in C282Y homozygotes (median: 163 vs 43 ng/mg creatinine) and also slightly higher than in C282Y/H63D compound heterozygotes (163 vs 134 ng/mg creatinine) (8). This indicates that hepcidin production in response to iron accumulation in DIOS is more adequate than in HFE haemochromatosis. Although our and other studies show a lower hepcidin-to-ferritin ratio in DIOS than in healthy controls, it should be considered that SF overestimates hepatic iron overload in DIOS because of hepatocellular necrosis, local inflammation and high iron accumulation in Kupffer cells (1, 10, 23). Accordingly, in our series SF correlated with sinusoidal but not with hepatocytic iron. Thus, the hepcidin-to-ferritin ratio seems not to be as reliable in DIOS as in haemochromatosis patients. In theory, the normalization of hepcidin concentration for the amount of iron overload by directly measuring liver iron concentration could be a better option, but there are no studies that verified this issue, which would require quantification of iron stores also in healthy controls.

When evaluating the response of hepcidin to an oral iron test, we observed that DIOS patients at diagnosis showed a variable response to oral iron test that seemed to be related to basal hepcidin level. Patients with 'higher' basal hepcidin did not respond, whereas those with 'lower' basal hepcidin showed a significant response to iron challenge. Very recently, Ruivard *et al.* (12) showed that intestinal iron absorption was significantly lower and serum hepcidin was higher in DIOS patients than in non-iron-overloaded overweight patients and in healthy controls. They hypothesized that in DIOS patients, the decrease of iron absorption was related to an increased production of hepcidin secondary to both insulin resistance and iron excess. Thus, we suggest that the lack of response to acute iron test in DIOS patients with 'higher' hepcidin might be caused by the inhibitory action of hepcidin on intestinal iron absorption. This is different to that observed in haemochromatosis patients in whom the lack of response to iron ingestion is because of a defect of iron sensing at hepatocyte level (8, 24). We have no clear demonstration of our hypothesis in DIOS because we measured TS at 24 h after oral iron, which is unlikely to reflect the absorption of the test dose. Documenting an early increase in serum iron or TS after oral iron in patients would increase confidence that the test dose is absorbed. In addition, it is possible that measuring also hepcidin in serum at different times after oral iron test would help in the interpretation of the results because serum assay measures hepcidin concentration at a single time point, whereas urinary hepcidin at 24 h reflects cumulative overnight hepcidin synthesis.

Despite these limitations, our findings indicate that in DIOS patients, the progression of iron accumulation is counteracted by the increase of hepcidin production and progressive reduction of iron absorption, thus explaining why patients with DIOS develop mild–moderate iron overload that tends to a plateau (20).

After iron depletion, we observed a significant positive response of hepcidin to oral iron test. Although this finding requires confirmation because of the small number of patients studied, it suggests that removing iron load and reducing hepcidin level improves iron absorption and, in turn, the acute response to iron challenge.

Overall, our results suggest that metabolic alterations have less important role than iron in determining hepcidin levels in our patients. This is also supported by the lack of relationship between urinary hepcidin and metabolic indices. Also, the French study (12) supports the idea that iron stores have a more major impact than metabolic alterations on hepcidin synthesis in DIOS. In fact, they showed that only DIOS, but not overweight (without iron overload) patients had significantly higher serum and urinary hepcidin than lean controls. They found significant correlations between serum (but not urinary) hepcidin and markers of insulin resistance and BMI in the whole group of subjects studied (which included lean controls, overweight patients without iron overload and DIOS), but not in the group of DIOS patients alone. Differently to us, Ruivard and colleagues selected patients according to the BMI ($>27 \text{ kg/m}^2$) and most of them were obese, a condition often associated with other alterations of the metabolic syndrome, increased cytokine and hepcidin production by adipose tissue (25, 26). However, the influence of adipose tissue in modulating blood hepcidin concentrations is controversial and limited to massively obese patients at most (26).

Our study does not clarify the mechanisms responsible for iron overload in DIOS. Because only a subset of patients with metabolic abnormalities develop iron overload (27), we can speculate that DIOS might be a multifactorial disease that results from a genetic background propitious for developing mild–moderate iron overload. The mild dysregulation of iron homeostasis in DIOS seems not to depend on defective hepcidin production and requires further studies. It is possible that metabolic alterations and/or hepatic steatosis can make more manifest the mild iron phenotype by increasing SF at a level disproportionate to the amount of liver iron overload. Our study indicates that DIOS patients retain some ability to increase hepcidin production in response to iron load, thus limiting iron absorption and the development of more marked iron overload.

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Figure 1. Basal urinary hepcidin in patients with dysmetabolic iron overload syndrome studied before and after phlebotomy treatment.

Figure 2.

Urinary hepcidin before and after oral iron test in patients with dysmetabolic iron overload syndrome before phlebotomy (A) and after iron depletion (B).

Table 1

Main histological findings of patients with dysmetabolic iron overload syndrome at diagnosis

	DIOS patients (n=24)
Total iron score	15 (12–31)
Hepatocyte iron score	11 (9–24)
Sinusoidal iron score	4 (3–7)
Steatosis	20 (83.3)
Steato-hepatitis	15 (62.5)
Hepatic fibrosis without cirrhosis	15 (62.5)
Cirrhosis	1 (4.2)

1. Data are reported as median (range) or number (percentage).
2. DIOS defined according to Mendler *et al.* (1).
3. Iron scores according to Deugnier *et al.* (14).
4. Hepatic steatosis: patients with $\geq 5\%$ of hepatocytes involved by steatosis.
5. Steato-hepatitis: patients with NAFLD activity score (NAS) ≥ 5 according to Kleiner *et al.* (18).
6. Hepatic fibrosis: patients with fibrosis ≥ 1 according to Kleiner *et al.* (18)
7. DIOS, dysmetabolic iron overload syndrome; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score.

Table 2

Demographical data, iron status and hepcidin concentration in patients with dysmetabolic iron overload syndrome before phlebotomy and after iron depletion compared with healthy controls

	DIOS patients		Healthy controls (n=23)
	Before phlebotomy (n=24)	After iron depletion (n=13)	
Males/females	21/3	11/2	19/4
Age (years)	56 (30–76) *	53 (33–68) *	34 (21–59)
Haemoglobin (g/dl)	15.3 (12–17.3)	14.2 (12.4–16.3)	14.9 (12.1–17.3)
Transferrin saturation (%)	38 (18–59) *, [†]	24 (16–32)	26 (18–40)
Serum ferritin (µg/l)	935 (362–1693) *, [†]	56 (38–169)	89 (15–267)
Iron removed (g)	–	2.2 (1.0–5.0)	–
Hepcidin (ng)/creatinine (mg)	163 (72–449) *, [†]	33 (4–110)	41 (3–225)

DIOS defined according to Mendler *et al.* (1).

Data are reported as median (range).

* *P*-value<0.0005 vs healthy controls.

[†] *P*-value<0.0005 vs DIOS patients after iron depletion.

DIOS, dysmetabolic iron overload syndrome.

Table 3

Metabolic data of patients with dysmetabolic iron overload syndrome before phlebotomy and after iron depletion

	DIOS patients		<i>P</i> -value
	Before phlebotomy (<i>n</i> =24)	After iron depletion (<i>n</i> =13)	
BMI (kg/m ²)	25.1 (21.5–33.6)	26.0 (23.4–31.2)	0.861
Abdominal waist (cm)	95 (73–119)	97 (88–114)	0.808
Triglycerides (mg/dl)	114 (42–505)	145 (38–285)	0.874
HDL cholesterol (mg/dl)	49 (36–76)	50 (39–112)	0.484
SBP (mmHg)	130 (120–150)	125 (110–140)	0.572
Glucose (mg/dl)	98 (82–146)	101 (78–157)	0.567
Insulin (mU/ml)	11.6 (2.8–41.4)	14.5 (6.2–52.4)	0.712
HOMA index	2.55 (0.68–9.91)	3.83 (1.34–15.2)	0.557

1. DIOS defined according to Mendler *et al.* (1).

2. Data are reported as median (range).

3. BMI, body mass index; DIOS, dysmetabolic iron overload syndrome; HDL, high-density lipoprotein; HOMA, homeostasis model assessment; SBP, systolic blood pressure.

Table 4

Urinary hepcidin before and after oral iron test in patients with dysmetabolic iron overload syndrome divided according to the median urinary hepcidin at diagnosis

	Hepcidin (ng)/creatinine (mg)		<i>P</i> -value
	Before test	After test	
Patients with low hepcidin (<i>n</i> =11)	149 (72–156)	172 (107–257)	0.007
Patients with high hepcidin (<i>n</i> =13)	203 (163–449)	208 (144–564)	0.626

1. Low–normal hepcidin: <163 ng/mg creatinine; high hepcidin: 163 ng/mg creatinine.

2. Data are reported as median (range).