A time course of hepcidin response to iron challenge in patients with *HFE* and *TFR2* hemochromatosis

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ABSTRACT

Background

Inadequate hepcidin production leads to iron overload in nearly all types of hemochromatosis. We explored the acute response of hepcidin to iron challenge in 25 patients with *HFE*-hemochromatosis, in two with *TFR2*-hemochromatosis and in 13 controls. Sixteen patients (10 C282Y/C282Y homozygotes, 6 C282Y/H63D compound heterozygotes) had increased iron stores, while nine (6 C282Y/C282Y homozygotes, 3 C282Y/H63D compound heterozygotes) were studied after phlebotomy-induced normalization of iron stores.

Design and Methods

We analyzed serum iron, transferrin saturation, and serum hepcidin by both enzyme-linked immunosorbent assay and mass-spectrometry at baseline, and 4, 8, 12 and 24 hours after a single 65-mg dose of oral iron.

Results

Serum iron and transferrin saturation significantly increased at 4 hours and returned to baseline values at 8-12 hours in all groups, except in the iron-normalized patients who showed the highest and longest increase of both parameters. The level of hepcidin increased significantly at 4 hours and returned to baseline at 24 hours in controls and in the C282Y/H63D compound heterozygotes at diagnosis. The hepcidin response was smaller in C282Y-homozygotes than in controls, barely detectable in the patients with iron-depleted *HFE*-hemochromatosis and absent in those with *TFR2*-hemochromatosis.

Conclusions

Our results are consistent with a scenario in which *TFR2* plays a prominent and *HFE* a contributory role in the hepcidin response to a dose of oral iron. In iron-normalized patients with *HFE* hemochromatosis, both the low baseline hepcidin level and the weak response to iron contribute to hyperabsorption of iron.

Key words: hemochromatosis, hepcidin, iron challenge, phlebotomy, transferrin receptor 2.

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The online version of this article has a Supplementary Appendix.

Introduction

Impaired production of the iron regulatory hormone hepcidin is the common pathogenic mechanism leading to iron overload in nearly all cases of hereditary hemochromatosis, and the degree of hepcidin deficiency roughly correlates with the severity of the disease.[1] Hepcidin deficiency leads to increased ferroportin expression on the basolateral surfaces of absorptive enterocytes and on iron recycling macrophages and increases the release of iron into the circulation.[2] Hepcidin is undetectable or present at an extremely low level in the severe, juvenile form of hemochromatosis due to mutations of hepcidin[3] or hemojuvelin,[4] and is decreased/inadequate in *HFE* C282Y homozygotes[5-7] and in the few studied patients with *TFR2*-hemochromatosis.[8,9] Hepcidin levels inappropriately low for the severity of iron loading have also been found in animal models of the different types of hemochromatosis.[10-13]

The molecular mechanism of iron sensing that maintains systemic iron homeostasis in mammals is unclear, but both HFE and transferrin receptor 2 (TFR2) have been implicated in this function due to their interaction and the ability of TFR2 to bind diferric transferrin.[14,15] The HFE-TFR2 complex is proposed to trigger hepcidin activation in response to increased holotransferrin concentration.[16,17] Loss of both HFE and TFR2 activity was reported to cause the phenotype of severe juvenile hemochromatosis in a single patient.[18] Therefore, when HFE or TFR2 is defective the loss of iron sensing could contribute to inadequate hepcidin production.

In a previous study[5] we measured urinary hepcidin excretion and iron parameters in basal conditions and 24 h after a single dose of oral iron and showed that the hepcidin response is blunted in *HFE*-hemochromatosis at diagnosis and that the defect is even more evident in patients whose iron stores have been normalized by phlebotomy. However, since the urinary hepcidin measurements and the associated serum iron measurements were done 24 h after iron intake, we did not detect the transient increase of transferrin saturation that occurs as the result of absorption of the dose of iron. Transferrin saturation measured 24 h after oral ingestion of iron is likely influenced by the hepcidin response rather than by the absorbed iron. In addition the study did not consider the possibility that the hepcidin response might occur earlier than 24 h, as was later shown to be the case by Lin *et al.*[19] in normal subjects.

Here we extended the previous study by performing a time course analysis of serum iron, transferrin saturation and hepcidin response over 24 h after a single dose of oral iron, in *HFE*-hemochromatosis patients both at diagnosis and after phlebotomy-induced normalization of iron stores , in normal controls and in two siblings with *TFR2*-hemochromatosis.

Design and Methods

Patients and controls

We studied 25 Italian patients with *HFE*-hemochromatosis and two with *TFR2*-hemochromatosis followed at our institutions in Monza, Verona and Milan and 13 healthy volunteers. Sixteen patients (10 C282Y/C282Y homozygotes and 6 C282Y/H63D compound heterozygotes) were studied at diagnosis and had increased iron stores, while nine patients (6 C282Y/C282Y homozygotes and 3 C282Y/H63D compound heterozygotes) had their iron stores normalized by phlebotomy and are subsequently referred as to "iron-depleted". The latter patients were studied at least 30 days after the last phlebotomy. After medical evaluation and routine laboratory tests (complete blood count, transferrin saturation, serum ferritin) on the test day patients ingested 65 mg oral iron in the form of ferrous sulfate (Iron 65 mg CVS Pharmacy) at 8 a.m. after an overnight fast. Blood samples for measurements of serum iron, transferrin saturation and serum hepcidin were obtained at time 0 (basal, before iron ingestion), and then 4, 8, 12 and 24 h after the assumption of iron. Participants were instructed to avoid meat and alcoholic drinks during the study period. Blood samples at 4 and 12 h were obtained before lunch and dinner, respectively, while the samples at 24 h were obtained after another overnight fast. We included consenting male adult patients who had a diagnosis of hemochromatosis based on iron parameters and genetic testing showing either C282Y homozygous or C282Y/H63D compound heterozygous genotypes. For patients enrolled at diagnosis, transferrin saturation was at least 45%, and serum ferritin greater than 300 μg/L.

Exclusion criteria were coexistence of anemia, β-thalassemia trait, inflammatory diseases, infections, chronic B or C viral hepatitis, heavy alcohol intake and other known iron loading disorders or intake of medications known to affect iron absorption.

The two siblings with *TFR2*-hemochromatosis were studied after phlebotomy-induced normalization of iron stores. Clinical and molecular data of this family, carrying the AVAQ 594-597 deletion, have been described in detail elsewhere.[20]

The healthy controls were volunteer males with normal iron and hematologic parameters, and without any of the previously indicated conditions.

The study protocol was approved by the ethical committees of all institutions. Informed consent was obtained from all participants according to the Declaration of Helsinki.

Methods

Serum iron, transferrin saturation and serum ferritin were measured by standard methods. Liver iron concentration was measured by magnetic resonance imaging using the Rennes University algorithm, as previously described.[21] Serum hepcidin concentration was measured by two methods, enzyme-linked immunosorbent assay (ELISA) and surface-enhanced laser desorption/ionization time of flight (SELDI-TOF) mass spectrometry, at Intrinsic LifeSciences (La Jolla, CA, USA) and Verona laboratories, respectively. The ELISA was a competitive assay using synthetic hepcidin (Bachem Biosciences, King of Prussia, PA, USA) for standardization, whose methodology and performance characteristics were previously described.[7] The mass spectrometry method was based on SELDI-TOF, using a synthetic hepcidin analog (Hepdicin-24, Peptides International, Louisville, KY, USA) as an internal standard, as previously described,[22] with recent technical improvements allowing us to increase the mean peak ratio of hepcidin-24/hepcidin-25 from 0.71 to 0.93.[23]

Statistical analysis

All calculations were performed with the Stata 11.0 statistical package (StataCorp). Because of their skewed distribution, serum hepcidin levels at different time points were expressed as medians with interquartile ranges. Hypothesis tests about different distributions of serum hepcidin levels at different time points were conducted using the Wilcoxon signed-ranks test. To properly evaluate total hepcidin response after stimulation by oral iron, areas under the serum hepcidin curves were estimated by the classical trapezoidal rule, commonly used in numerical integration.[24] Areas

under the curves were calculated considering only areas above the hepcidin baseline value. Areas below baseline were not considered. Square root transformation of areas under the curves was applied to obtain symmetrical, approximately Gaussian distributions. Comparisons of areas under the curves in subgroups were performed using the two-tailed Student t-test. Correlations between quantitative variables were assessed using Spearman's rho test. *P* values less than 0.05 were considered statistically significant.

Results

Between November 2007 and July 2009 25 patients and 13 controls were enrolled in the study. Table 1 shows the clinical data and iron parameters of the *HFE* patients and controls, including their pre-test serum hepcidin levels. The two *TFR2* homozygous siblings, whose data are not reported in Table 1, were aged 40 and 48 years. Their serum ferritin concentrations were 41 and 34 μg/L and transferrin saturation 97% and 93% at 2 and 3 months after the last maintenance phlebotomy, respectively.

Hepcidin levels at baseline correlated positively with serum ferritin (Spearman's rho = 0.87 and 0.65 with the ELISA and mass spectrometric method, respectively; $P<0.05$ for both) in controls, but not in patients with *HFE*-hemochromatosis at diagnosis (Spearman's rho = -0.19 and -0.16 with the ELISA and mass spectrometric method, respectively; *P*= NS for both). No correlation was found between hepcidin levels and transferrin saturation in either controls or *HFE* patients. The time courses of median serum iron, transferrin saturation and serum hepcidin, as measured by the two methods in all groups of patients and controls, are shown in Figure 1A-D. Data are shown separately for C282Y homozygotes and C282Y/H63D compound heterozygotes at diagnosis and are pooled together for *HFE* patients after iron depletion because they behaved homogeneously. Details including the median and interquartile range of serum iron, transferrin saturation, and hepcidin levels at all time points in the different groups are reported in *Online Supplementary Table S1*. In healthy controls, serum iron and transferrin saturation increased at 4 h ($P=0.0024$ for iron and $P=0.0191$ for transferrin saturation *versus* basal levels), returned to baseline values at 8 h and reached the lowest value at 12 h, likely as an effect of the increased hepcidin. The serum iron and transferrin saturation time courses of patients at diagnosis (both C282Y homozygotes and C282Y/H63D compound heterozygotes) were similar to those of controls (Figure 1A-B and *Online Supplementary Table S1*), indicating that the patients were still able to absorb iron, despite their high basal serum iron and transferrin saturation levels. Patients studied after normalization of iron stores had the highest increase of serum iron ($P=0.0077$ *versus* basal levels) and transferrin saturation of all groups, peaking at 4 h and followed by a slow but significant reduction that reached baseline values at 24 h (Figure 1A-B and *Online Supplementary Table S1*). The striking additional increase of serum iron and transferrin saturation in iron-depleted patients in comparison with other groups in terms of square roots of area under the curve is depicted in *Online Supplementary Figure S1A,B*. In the two iron-depleted *TFR2* patients, the incremental increase in serum iron concentration was similar to that in the other groups, but from a much higher baseline level, while their transferrin saturation was near 100% throughout the time-course (Figure 1A,B).

The two methods used to measure serum hepcidin gave concordant results. *Online Supplementary Figure S2* shows the correlation between hepcidin levels measured by ELISA and those measured by mass spectrometry (Spearman's rho 0.85, $P<0.0001$ in a total of 185 paired measurements). For technical reasons (thawing of samples during shipment to the laboratory in Verona), serum hepcidin could not be measured by SELDI-TOF mass spectrometry in three out of the 13 controls. The difference in the hepcidin levels detected by the two methods might be due in part to the fact that ELISA can detect total hepcidin including minor hepcidin isoforms,[25] whereas mass spectrometry measures only hepcidin-25.

Hepcidin levels in controls were significantly increased at 4 h ($P=0.0037$ and $P=0.0051$ *versus* basal levels with the ELISA and mass spectrometry method, respectively) and reached a maximum at 8-12 h, returning to the baseline level at 24 h (Figure 1C,D and *Online Supplementary Table S1*). The hepcidin response of C282Y/H63D subjects was

Table 1. Characteristics of patients with *HFE* hemachromatosis and controls at the time of testing.

	Cases (n=25)				Controls (n=13)
	At diagnosis (n=16)		After iron depletion (n=9)		
	C282Y/C282Y (n=10)	C282Y/H63D (n=6)	C282Y/C282Y (n=6)	C282Y/H63D (n=3)	
Age (years)	47±12	42±12	38±9	51±5	35±12*
Transferrin saturation (%)	70.5 (46-83)°	45 (37-57)	50.5 (46-58)	26 (23-48)	34 (30-41)
Serum ferritin (μg/L)	920±310	705±326	47.5±23.2	53.3±12.1	120±62^
Hemoglobin (g/dL)	14.7±0.7	15.8±1.1	14.8±0.7	15.3±0.2	15.1±0.9
Liver iron content‡ (μmol/g)	259±54	170±51	-	-	-
Iron removed (g)	-	-	4.2±2.9	3.1±1.1	-
Serum hepcidin (ELISA) (ng/mL)	41.9 (24-65.6)	65.1 (50.8-71.1)	12.8 (1-20)	7 (6.6-33.8)	59.6 (41.6-71.6)
Serum hepcidin (MS) (ng/mL)	12.2 (7.9-19.2)	22.4 (13.1-35.4)	1.5 (1.5-1.5)	1.5 (1.5-6.4)	10.3 (7-18)

*Data are presented as mean ± standard deviation or median with interquartile ranges (in brackets). *P=0.02,* versus *C282Y/C282Y at diagnosis. °P=0.04,* versus *C282Y/H63D at diagnosis. ^P=0.004* versus *all patients after iron depletion. ‡measured by magnetic resonance imaging (normal value < 36 μmol/g); ELISA: enzyme-linked immunosorbent assay; MS: mass spectrometry.*

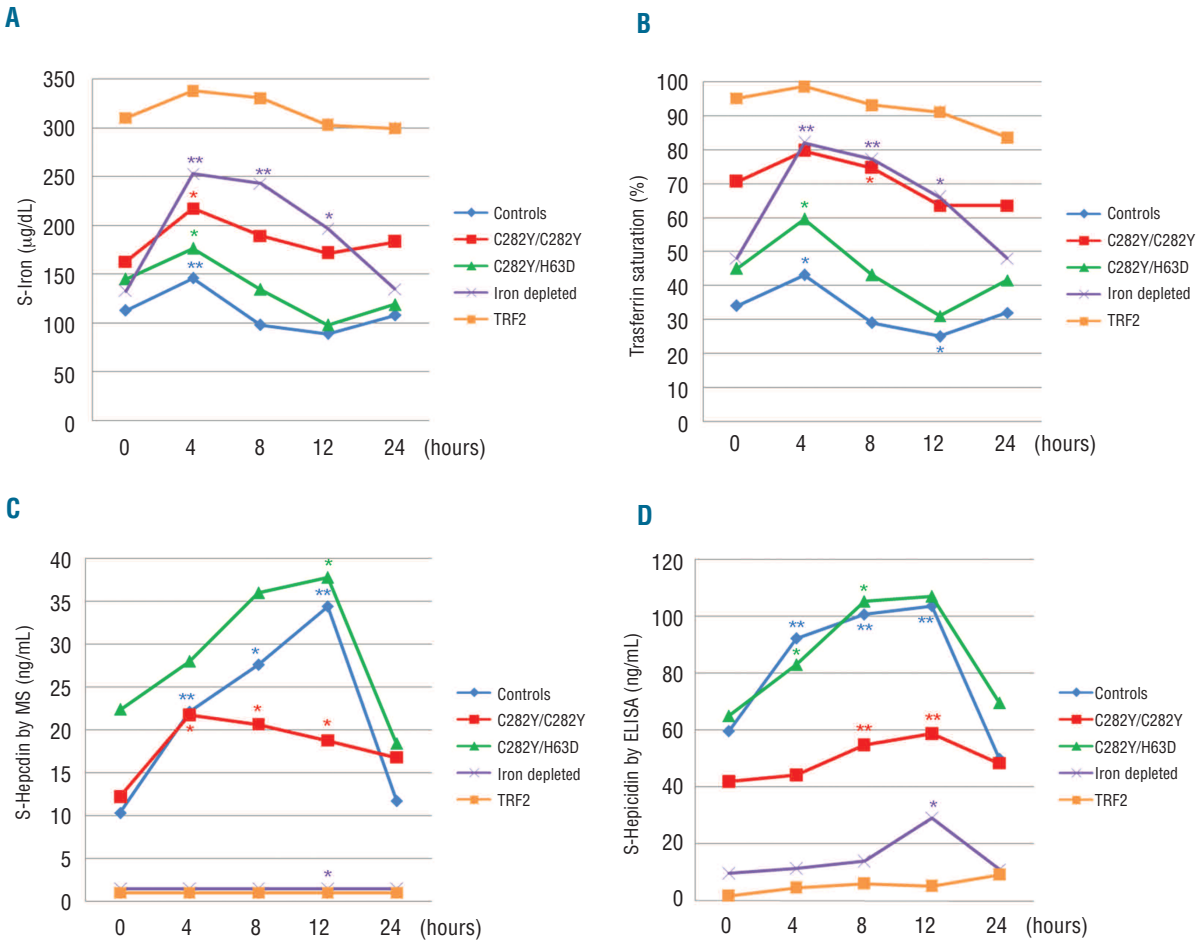

Figure 1. Results of the oral iron test. Time course of serum iron (A), transferrin saturation (B) and serum hepcidin by mass spectrometry (C) and ELISA (D) methods in patients and controls (blue lines). Red and green lines represent C282Y homozygotes and C282Y/H63D compound heterozygotes studied at diagnosis (i.e. with increased iron stores), respectively. Purple lines represent *HFE*-hemochromatosis patients studied after iron depletion, including both genotypes. Orange lines represent the two *TFR2*-hemochromatosis patients, who were also studied after phlebotomy-induced normalization of iron stores. The asterisks indicate statistical significance: * $P<0.05$ ** $P<0.01$ *versus* baseline values. For each parameter studied median levels are reported, while bars reflecting variations are not shown here because of partial overlapping. Details about variations (e.g. interquartile ranges) at each time point and statistics are given in *Online Supplementary Table S1*. "0" on the x-axis represents the baseline, before ingestion of the iron pill.

similar to that of controls. Hepcidin levels of C282Y homozygotes at diagnosis showed a smaller increase (at 8-12 h) than in controls (Figure 1C,D and *Online Supplementary Table S1*). Serum hepcidin changed little in iron-depleted patients. In the few responsive patients the small increase was delayed to 12 h and the maximum level reached was near the lower limit of detection of the methods. In the two *TFR2*-hemochromatosis siblings hepcidin levels were barely detectable and the time course curve was flat (Figure 1C,D and *Online Supplementary Table S1*).

We quantified the total increment in hepcidin after oral ingestion of iron using area under the curve values. In healthy subjects, substantial within-group differences in areas under the curves were observed with both hepcidin assays, likely related to variations in both iron absorption and in the hepcidin response (Figure 2A,B). In contrast, as shown in Figure 2, patients had a more uniform response. C282Y homozygous patients at diagnosis produced significantly less hepcidin in response to iron than controls (P=0.023) when the hepcidin level was measured by ELISA. Hepcidin measured by the mass spectrometry method showed a similar but statistically not significant decrease, likely because hepcidin was measured with this method in fewer controls. Patients studied after normalization of iron stores showed lower hepcidin area under the curve levels than controls, with both methods (P=0.0039 for ELISA and P=0.0053 for mass spectrometry).

Discussion

This report extends and elaborates on a previous study on hepcidin response after an oral iron challenge in patients with *HFE*-hemochromatosis, in whom we measured urinary hepcidin 24 h after iron intake.[5] In the present study a time course was performed to analyze serum hepcidin levels in parallel with serum iron and transferrin saturation changes. As previously observed in several studies,[5-7] basal hepcidin levels of C282Y/C282Y homozygotes and C282Y/H63D compound heterozygotes at diagnosis were similar to and even higher than the levels in controls;

however, as previously pointed out, they were relatively deficient considering the patients' iron load. Over the time course of 24 h, the median hepcidin response to iron challenge was blunted in C282Y homozygous patients both at diagnosis and after iron depletion by phlebotomy when compared to controls.

A possible limitation of this study is the theoretical overlap of a circadian rhythm of hepcidin[7,26] with our serial measurements. Although we cannot exclude a contribution of diurnal variations to the changes in hepcidin levels, such an effect is likely to have influenced all individuals evenly without affecting the differences observed at the group level. Of note, a recent study in healthy individuals receiving 60 mg of radiolabeled iron orally showed an increase of serum hepcidin at 6 h only in subjects who effectively absorbed iron.[27] Given the similar time-points and doses of iron used in our study, this supports the belief that the variations of hepcidin we observed were stimulated by the exogenous iron.

HFE and TFR2 are thought to have a role in sensing circulating iron concentrations, most likely in the form of holotransferrin.[16,17] The hepcidin increase at 4 h in controls, in whom HFE and TFR2 are functional, is a response to increased transferrin saturation after iron ingestion. In C282Y/C282Y patients, the increases in serum iron and transferrin saturation after the iron challenge indicate that the iron was absorbed even when basal levels of circulating iron were high. However, the hepcidin response to the increases in serum iron and transferrin saturation was blunted, clearly indicating that HFE is required for a full hepcidin response to the acute increase of circulating iron and transferrin saturation.

C282Y/H63D compound heterozygotes at diagnosis, although having increased iron stores, had a response similar to that of normal controls, both in time course and in terms of total (area under the curve) amount of hepcidin. We previously observed that the level of urinary hepcidin at 24 h did not significantly increase in C282Y/H63D compound heterozygotes after the oral iron test and proposed that such patients have a defective acute hepcidin response to oral iron,[5] a result not confirmed by the present study. This discrepancy is likely due to the different protocols and assays (serum *versus* urine) used in the two studies, with multiple serum measurements giving a more accurate view than a single time point analysis of urinary hepcidin 24 h after the iron challenge.

In both C282Y/C282Y homozygotes and C282Y/H63D compound heterozygotes, iron depletion further blunted the hepcidin response despite a greater and more prolonged increase in serum iron and transferrin saturation than in the other groups of patients. It is unlikely that the low hepcidin response after iron depletion is dependent on increased erythropoietic activity: the study was performed at a time remote from phlebotomy, and hemoglobin, serum ferritin and basal transferrin saturation levels in this group of "iron-depleted" patients were normal (Table 1) and certainly not in the range of iron deficiency. Depletion of iron stores by phlebotomy appears to reset hepcidin at the original basal levels that are lower than normal. Low hepcidin explains the persistent elevation of serum iron after the iron challenge, likely resulting from a continuous flux from duodenal enterocytes and, in particular, from macrophages, which exceeds the iron requirements of the bone marrow. After iron depletion, C282Y homozygotes and C282Y/H63D compound heterozygotes responded similarly, whereas, when iron overloaded they seem to have different hepcidin responses to acute iron administration. Although the sample sizes were small, this may suggest a difference in iron sensing and hepcidin modulation depending on genotype severity and baseline iron load.

Patients with *TFR2*-hemochromatosis are extremely rare and we could not recruit any patients at diagnosis. However, the two *TFR2* patients studied after "iron depletion" maintained extremely high serum iron and transferrin saturation levels, in contrast to the *HFE* "iron-depleted" patients. The hepcidin levels of these latter patients were the lowest of those in all groups. Although serum iron curves in the two *TFR2* patients showed a time-dependent increment similar to those of *HFE* patients, transferrin saturation changed less, probably because near the saturation

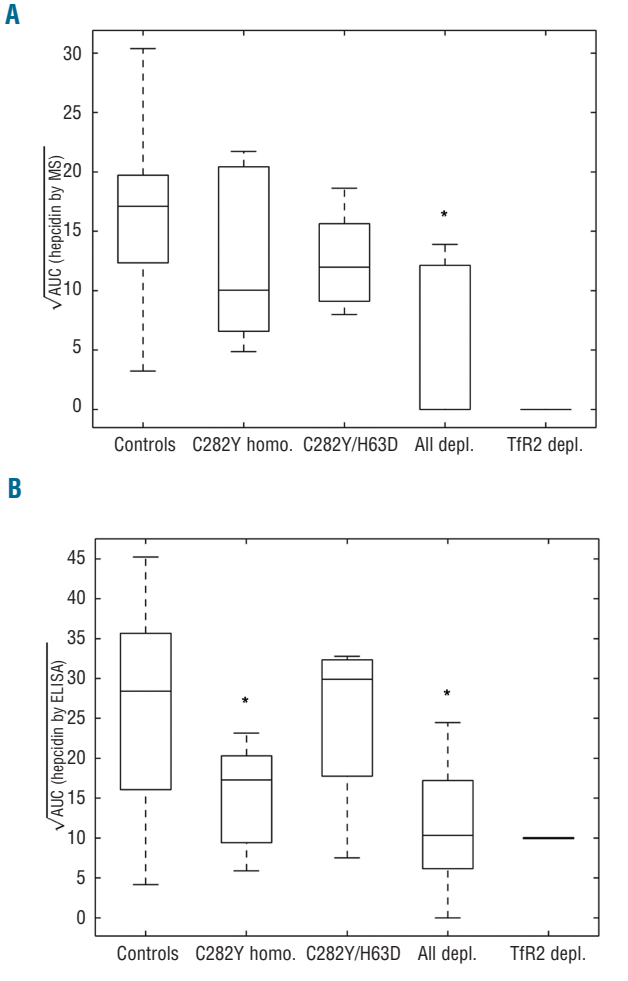

Figure 2. Box plots of square root area under the curve (AUC) of serum hepcidin in the different groups. C282Y homozygotes and C282Y/H63D compound heterozygotes at iron depletion are grouped. (A) Serum hepcidin as measured by mass spectrometry (MS). *Significantly different *versus* controls (P=0.0053). Other significant differences: all patients at diagnosis *versus* all iron-depleted patients (P=0.0085); C282Y homozygotes at diagnosis *versus* C282Y homozygotes after iron depletion (P=0.0194). (B) Serum hepcidin as measured by ELISA. *Significantly different *versus* controls (P=0.023 for C282Y homozygotes, and P=0.0039 for iron-depleted patients). Other significant differences: all patients at diagnosis *versus* all iron-depleted patients (P=0.0294); C282Y homozygotes at diagnosis *versus* C282Y/H63D at diagnosis (P=0.0375).

point it no longer reflects serum iron. We hypothesized that when transferrin is nearly saturated, iron administration increases mostly the non-transferrin bound iron compartment that is not sensed by TFR2. Hepcidin did not increase in the two patients with *TFR2* hemochromatosis when measured by either method. A previously described third patient with *TFR2* hemochromatosis whose urinary hepcidin was measured 24 h after an iron challenge also showed a lack of hepcidin response.[9] We propose that TFR2 may be involved in iron-sensing and may mediate the increase in hepcidin in response to the acute iron stimulus. However, detailed testing of this hypothesis would require lowering the transferrin saturation which, in our experience, is difficult even with weekly phlebotomy. Similar differences in hepcidin response to 1-day iron loading were observed in mouse models of hemochromatosis.[28] While *Hfe*-null mice had a blunted increase in hepcidin mRNA, the response was absent in *Tfr2*-mutant mice. Together, these results are consistent with a scenario in which TfR2 has a more prominent role than HFE in iron sensing. This is in keeping with the more severe iron overload detected in *Tfr2*-null mice than in *Hfe*-deficient ones[29] and with the earlier appearance of iron overload in patients with *TFR2*-hemochromatosis[18,30-32] than in those with *HFE*-hemochromatosis.

Since the discovery of hepcidin, its measurement has represented a considerable challenge.[25,33] Both the methods used in the present work were previously demonstrated to be sufficiently accurate for detecting physiological and pathological changes in serum hepcidin in healthy volunteers and in patients with various disorders of iron homeostasis.[22,7,34,35] Indeed, results with the two methods were essentially concordant from a qualitative point of view, although quantitative differences were observed, e.g. higher absolute levels with the ELISA method. Similar results have been recently reported by others.[36] As discussed in detail elsewhere,[33,36] such differences may be mainly due to heterogeneous calibrations and/or lack of absolute specificity of the antibody used in the ELISA for the hepcidin-25 isoform, at variance with the direct detection of the hepcidin-25 peak by mass spectrometry. Since we still lack a "gold standard" reference method for the hepcidin assay, the qualitative concordance of results with the two methods can be viewed as reassuring for the general validity of this study. Nevertheless, further work is needed to improve and standardize the current methods for hepcidin assays.[33]

The low hepcidin levels in patients studied after normalization of iron stores again emphasize the need to revise the current phlebotomy protocols, as previously proposed:[5] a less stringent protocol permitting somewhat higher iron stores would likely reduce the amount of absorbed iron and decrease the patient's burden of maintenance phlebotomies. Animal and human studies indicate that hepcidin concentrations are the principal determinant of iron absorption. A pilot study with serial measurements of serum hepcidin might be useful for establishing the appropriate degree of iron depletion that prevents iron toxicity but does not massively increase iron absorption.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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