Diurnal Rhythm rather than Dietary Iron Mediates Daily Hepcidin Variations


BACKGROUND: The iron-regulating hormone hepcidin is a promising biomarker in the diagnosis of iron disorders. Concentrations of hepcidin have been shown to increase during the day in individuals who are following a regular diet. It is currently unknown whether these increases are determined by an innate rhythm or by other factors. We aimed to assess the effect of dietary iron on hepcidin concentrations during the day.

METHODS: Within a 7-day interval, 32 volunteers received an iron-deficient diet on 1 day and the same diet supplemented with 65 mg ferrous fumarate at 0815 and 1145 on another day. Blood was drawn to assess ferritin, hepcidin-25, and transferrin saturation (TS) throughout both days at 4 time points between 0800 (fasted) and 1600. A linear mixed model for repeated data was used to analyze the effect of iron intake on TS and hepcidin concentrations.

RESULTS: Baseline values of hepcidin at 0800 correlated significantly with ferritin ($r = 0.61$). During the day of an iron-deficient diet the mean TS was similar both in men and in women, whereas hepcidin increased. During the day with iron supplementation the mean TS was significantly higher both in men and in women, and the mean hepcidin was moderately but significantly higher in women (1.0 nmol/L, 95% CI, 0.2–1.8) but not in men (0.0 nmol/L, 95% CI, −0.8 to 0.8).

CONCLUSIONS: Our data demonstrate that ferritin sets the basal hepcidin concentrations and suggest that innate diurnal rhythm rather than dietary iron mediates the daily hepcidin variations. These findings will be useful for optimizing sampling protocols and will facilitate the interpretation of hepcidin as an iron biomarker.

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The iron-regulating peptide hormone hepcidin is produced by hepatocytes in the liver and is excreted in the circulation (1). Hepcidin is the primary regulator of iron homeostasis: it modulates iron availability for erythropoiesis by promoting the internalization and degradation of ferroportin, a key cellular iron exporter, which is essential for both iron absorption in the duodenum and recycling of iron by macrophages. Hepcidin is a negative regulator of iron absorption and mobilization. High levels inhibit both duodenal iron absorption and release of iron from macrophages, whereas low levels promote these processes. Hepcidin production is increased by inflammation, which explains the relatively low serum iron levels in the anemia of chronic disease. In contrast, anemia and hypoxia have been shown to increase iron absorption and mobilization by decreasing hepcidin production (1). Discovery of the central role of hepcidin has shed new light on the pathophysiology of iron disorders. In addition, serum hepcidin is a promising biomarker in the assessment of disorders of iron homeostasis (2). It has been shown to increase during the day in individuals who are following a regular diet (3–6). Previous findings suggest that the transcriptional regulators upstream stimulatory factor 1 (USF1)5 and USF2, which are related to genes involved in diurnal rhythm, are potentially significant modulators of hepcidin expression (7). However, other factors might also affect the daily pattern of serum hepcidin. Serum hepcidin concentrations have been found to markedly increase with acute

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5 Nonstandard abbreviations: USF1, upstream stimulatory factor 1; TS, transferrin saturation; CRP, C-reactive protein; Hb, hemoglobin; BMI, body mass index; ALT, alanine aminotransferase; MCV, mean corpuscular volume.
changes in serum iron induced by oral challenge with iron sulfate (6, 8–11), suggesting that the daily increase might be secondary to diet.

Here we studied the effects of oral iron challenge on serum hepcidin profiles during the day. To this end we assessed the differences of serum hepcidin and transferrin saturation (TS) in 32 healthy volunteers during 2 days of an iron-deficient diet, 1 day with and 1 without iron supplementation. This study provides more insight in the mechanism of the daily regulation of hepcidin and is instrumental to optimize sampling protocols and to facilitate interpretations of hepcidin as novel biomarker in clinical practice, nutritional population surveys, and interventions.

Materials and Methods

STUDY POPULATION, DIET, AND SAMPLING PROTOCOL

Approval for this study was obtained from the Radboud University Medical Centre institutional review board according to the declaration of Helsinki. The study was performed in February 2011 on 44 apparently healthy volunteers. Written informed consent was obtained from all study participants.

Healthy study participants (n = 44) were screened 1 week before the start of the study and on 2 test days at 0800 after an overnight fast. In total 32 study participants were included. Twelve individuals were excluded after an overnight fast. In total 32 study participants were randomized among 2 groups of 16 (test groups I and II, respectively) based on age, sex, and body mass index (BMI) (<28 kg/m² for men or <80 kg/m² for women) or <13.1 g/dL (men) or <11.8 g/dL (women) (n = 3), ferritin concentrations <15 µg/L or >280 µg/L for men or >80 µg/L for premenopausal women or >180 µg/L for postmenopausal women (n = 3), body mass index (BMI) >30 kg/m² (n = 1), alanine aminotransferase (ALT) >45 U/L (n = 0). The volunteers were randomized among 2 groups of 16 (test groups I and II, respectively) based on age, sex, and ferritin.

On test day 1 the 32 volunteers received an iron-deficient diet (no-iron diet, <0.0 mg total iron per 100 g) consisting of a breakfast and lunch comprising a choice among the following products: low-fat yogurt, half-fat vanilla yogurt, low-fat fromage frais, full-fat vanilla custard, buttermilk, semiskimmed milk, breakfast cereal cornflakes (without enrichment with iron, from Euroshopper), white bastard sugar (Dutch product), sweetener, crisps maize Bugles, black tea, and water (12). Breakfast and lunch were served at 0830 and 1200, respectively. For test group I this iron-deficient diet was supplemented with 2 tablets of 65 mg ferrous fumarate (iron diet). To decrease enhancement or inhibition of iron absorption by the nature of the food matrix (e.g., inhibition by milk products and black tea), both tablets were taken 15 min before food intake: at 15 min before breakfast (0815) and at 15 min before lunch (1145), respectively.

On test day 2, 7 days after test day 1, test group I received the no-iron diet, while test group II received the iron diet.

In the crossover study design the interval of 7 days between test day 1 and test day 2 was considered to be long enough to wash out the effect of oral iron administration on test day 1 on the serum iron parameters on test day 2. The time points of oral iron supplementation were chosen to best reflect regular food intake, consisting of an (iron-containing) breakfast in the morning and lunch at noon.

Blood samples in both groups were taken at consecutive time points at both days. The first sample was taken at 0800 after a minimum overnight fasting period of 8 h. Subsequent samples were taken after breakfast at 1100, after lunch at 1300, and at 1600.

Serum samples for hepcidin measurements were processed, divided into aliquots, and stored in polypropylene tubes at −80 °C. Routine laboratory parameters and hepcidin concentrations were measured within 8 hours and 4 weeks after collection, respectively. TS and serum hepcidin were determined in the samples of all time points, whereas other parameters [serum ferritin, Hb, erythrocyte mean corpuscular volume (MCV), CRP, and ALT] were assessed only for the samples collected at 0800.

LABORATORY MEASUREMENTS

Serum concentrations of iron, transferrin, CRP, and ALT were determined on the Abbott Aeroset analyzer (Abbott Diagnostics Division). The total iron-binding capacity (µmol/L) was calculated from transferrin (g/L) multiplied by 25. The percentage TS was calculated as 100 times the plasma iron concentration divided by the total iron-binding capacity. We quantified serum ferritin by the use of a solid-phase, 2-site chemiluminescent immunometric assay (Immulite 2000 and 2500, Siemens) and routine hematology characteristics on a Sysmex XE-2100 analyzer. Serum hepcidin-25 concentrations were measured using a combination of weak cation exchange and TOF-MS (13).

STATISTICAL METHODS

A Mann–Whitney test was used to test the statistical significance of differences between men and women in the characteristics studied.

A linear mixed model for repeated data was used to study the individual profiles of both TS and serum hepcidin, separately and in particular, whether these profiles depended on iron diet and on sex. The dependent variable was the marker in question. The independent class variables were time point, sex, and diet. The inde-
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Results

Characteristics of the study population
The characteristics of the study population are depicted in Table 1. A total of 16 men and 16 women were included in this study. Men and women were of similar ages, BMI, and MCV. However, ferritin, Hb, and ALT differed significantly between sexes. All biomarker values were found to be within local reference intervals.

Serum Hepcidin Concentrations
Baseline values of hepcidin at 0800 correlated significantly with ferritin both on the day with and without oral iron (r = 0.61, P < 0.01 and r = 0.61, P < 0.01, respectively).

The observed means and SDs of serum hepcidin at each time point by diet for all volunteers and for men and women, separately, are shown in Table 2. On the day of no-iron ingestion, the mean hepcidin values increased from 0800 to 1600, with the larger portion of increase before 1100, both in men and women. The mean increase in men was 1.8 nmol/L (95% CI, 0.7–3.0) and in women 1.3 nmol/L (95% CI, 0.3–2.2).

The mean differences of the hepcidin values between the day with oral supplementation and the day without as estimated with a linear mixed model are shown in Table 3. First, we found that the effect of oral iron supplementation was statistically significant different between men and women. The second column of Table 3 shows that the mean hepcidin concentration in women, after correction for baseline values, was significantly higher on the day with oral supplementation up to 1600, being 1.0 nmol/L (95% CI, 0.2–1.8) higher at each time point of measurement compared to the day with no supplementation. By contrast, no significant difference in mean hepcidin concentrations between the 2 days was found in men (0.0 nmol/L, 95% CI, −0.8 to 0.8). This difference of the effect of oral iron on mean serum hepcidin concentrations between men and women (1.0 nmol/L, 95% CI, −0.1 to 2.1) is only partly explained by differences in ferritin values, e.g., when hepcidin concentrations were corrected for ferritin at 0800 this difference was reduced to 0.7 nmol/L (95% CI, −6.1 to 2.1). The agreement of the observed data with the estimated mean profiles on both days can be seen in Fig. 1. More specifically, the observed means of the hepcidin concentrations were in agreement with the estimated means using a model of equal differences at each point of measurement up to 1600 (i.e., the parallel-line model).

Second, we found that, irrespective of sex and supplemental iron use, the mean hepcidin concentration was statistically significantly lower at 1100 compared to 1600 (−0.8 nmol/L, 95% CI, −1.4 to −0.2) and that at 1300 the mean concentration was also lower compared to 1600, but did not reach the level of statistical significance (−0.4 nmol/L, 95% CI, −0.8 to 0.1) (Table 3, columns 3–5). More specifically, between 1100 and 1600 serum hepcidin showed the same pattern of increase on days with and without oral iron supplementation in both men and women.

Table 1. Characteristics of the study population.a

<table>
<thead>
<tr>
<th></th>
<th>Men (n = 16)</th>
<th>Women (n = 16)</th>
<th>All (n = 32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>Median (range)</td>
<td>Median (range)</td>
<td>Median (range)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23 (19–28)</td>
<td>23 (19–28)</td>
<td>23 (19–28)</td>
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<tr>
<td>Ferritin, µg/L</td>
<td>15.0 (13.5–16.3)</td>
<td>13.4 (12.1–14.7)</td>
<td>14.2 (12.1–16.3)</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>&lt;5 (&lt;5)</td>
<td>&lt;5 (&lt;5–8)</td>
<td>&lt;5 (&lt;5–8)</td>
</tr>
<tr>
<td>ALT, U/Lb</td>
<td>26 (18–36)</td>
<td>20 (10–43)</td>
<td>21 (10–43)</td>
</tr>
<tr>
<td>Hb, g/dLb,c</td>
<td>88 (81–94)</td>
<td>90 (84–96)</td>
<td>89 (81–96)</td>
</tr>
</tbody>
</table>

*Results obtained at 0800 during screening (1 week before test day 1). For all study participants the laboratory results are within reference ranges.
†Significantly different between men and women (independent samples t-test, P < 0.05).
‡To convert Hb (g/dL) into Hb (mmol/L) multiply by 0.621.
Finally, we observed that, independent from sex and oral iron intake, the profile of individual hepcidin concentrations during the day was higher when the baseline concentration at 0800 was higher [1.6 nmol/L per unit increase at 0800 (95% CI, 1.3–1.8)]. In other words, at the various time points of the day the hepcidin concentration of an individual in relation to that of other individuals remained relatively constant.

**TS Profiles**

The observed mean and SD of TS at each point of measurement by diet of men and women separately are shown in Table 2. On the day of no-iron intake, the mean TS values showed a rather constant pattern (regarding the SD) from 0800 to 1600, both in men and women.

The mean differences of the TS values between the day with oral supplementation and the day without, as estimated with a linear mixed model, are shown in Table 3. First, as expected (Table 2), we found no statistically significant differences between men and women, and the mean TS profiles were similar for men and women. As seen in the second column of Table 3, on average the (overall) TS value, after correction for baseline values, was significantly higher on the day with oral supplementation up to 1600. The mean TS value adjusted for baseline was estimated to be 12.2% (95% CI, 9.6–14.8) higher at each time point of measurement compared with the day when no supplementation was given. The agreement of the observed data with the estimated mean profiles on both days is displayed in Fig. 2. The observed means of the TS values were in agreement with the estimated means when using a model of equal differences at each point of measurement up to 1600 (i.e., the parallel-line model).

Second, irrespective of sex and supplemental iron use, the mean TS level (with TS of 28.1% at 0800) was nearly identical at 1100 in comparison with 1600 (2.8%, 95% CI, 2.0–3.6), whereas the mean level at 1300 was statistically significantly higher in comparison with 1600 (2.6%, 95% CI, 1.7–3.6).

Finally the estimated profile of TS levels through the day was higher when the baseline level at 0800 was higher, but not equally higher at each time point of measurement. The increase became smaller as time progressed during the day. At 1100 the increase per unit increase at 0800 was 0.9% (95% CI, 0.8–1.0), at 1300 0.5% (95% CI, 0.3–0.7), and at 1600 0.3% (95% CI, 0.2–0.5), respectively.

**Relationship of Hepcidin with TS**

When serum hepcidin was corrected for TS, the effect of oral iron administration on the mean concentration of serum hepcidin decreased [adjusted results: women 0.5 nmol/L (95% CI, −0.3 to 1.4), men, −0.5 nmol/L (95% CI, −1.2 to 0.3); unadjusted: women 1.0 nmol/L (95% CI, 0.2 to −1.8), men 0.0 nmol/L (95% CI, −0.8 to 0.8)]. However, when TS was corrected for hepcidin

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**Table 2. Observed mean (SD) of serum hepcidin and transferrin saturation at different time points on 1 day of an iron-deficient diet and on 1 day of an iron-deficient diet supplemented with 2 tablets of 65-mg ferrous fumarate.**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Sex</th>
<th>0800a</th>
<th>1100</th>
<th>1300</th>
<th>1600</th>
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<tr>
<td></td>
<td></td>
<td>1.9 (1.7)</td>
<td>2.9 (2.9)</td>
<td>3.2 (2.8)</td>
<td>3.4 (3.1)</td>
</tr>
<tr>
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<td>2.3 (2.0)</td>
<td>3.4 (3.6)</td>
<td>3.8 (3.2)</td>
<td>4.2 (4.0)</td>
</tr>
<tr>
<td></td>
<td>Women (n = 16)</td>
<td>1.5 (1.2)</td>
<td>2.4 (1.9)</td>
<td>2.6 (2.3)</td>
<td>2.7 (1.9)</td>
</tr>
<tr>
<td>Ironc</td>
<td>All (n = 32)</td>
<td>2.2 (2.1)</td>
<td>3.7 (4.1)</td>
<td>4.2 (4.2)</td>
<td>4.7 (3.9)</td>
</tr>
<tr>
<td></td>
<td>Men (n = 16)</td>
<td>2.7 (2.3)</td>
<td>3.7 (3.3)</td>
<td>4.3 (3.7)</td>
<td>5.2 (4.2)</td>
</tr>
<tr>
<td></td>
<td>Women (n = 16)</td>
<td>1.7 (2.0)</td>
<td>3.7 (5.0)</td>
<td>4.1 (4.8)</td>
<td>4.2 (3.6)</td>
</tr>
<tr>
<td></td>
<td>All (n = 32)</td>
<td>27.4 (10.7)</td>
<td>29.2 (10.7)</td>
<td>29.4 (9.3)</td>
<td>28.2 (8.3)</td>
</tr>
<tr>
<td></td>
<td>Men (n = 16)</td>
<td>27.3 (10.9)</td>
<td>28.8 (11.4)</td>
<td>29.8 (10.8)</td>
<td>30.0 (9.9)</td>
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<tr>
<td></td>
<td>Women (n = 16)</td>
<td>27.5 (10.8)</td>
<td>29.6 (10.4)</td>
<td>28.9 (7.8)</td>
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<td>Ironc</td>
<td>All (n = 32)</td>
<td>28.8 (14.2)</td>
<td>39.4 (15.5)</td>
<td>45.1 (17.6)</td>
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<td>Men (n = 16)</td>
<td>29.4 (14.6)</td>
<td>39.6 (15.7)</td>
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<td>40.7 (14.0)</td>
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<tr>
<td></td>
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<td>28.1 (14.3)</td>
<td>39.1 (15.8)</td>
<td>45.1 (19.2)</td>
<td>41.6 (18.1)</td>
</tr>
</tbody>
</table>

* Fasted.

b No iron, 1 day of iron-deficient diet.

c Iron, 1 day of iron-deficient diet supplemented with 2 tablets of 65 mg ferrous fumarate (at 0815 and 1145).
the effect of oral iron administration on TS was similar to that observed in the unadjusted data [adjusted: 12.6% (95% CI, 9.9–15.2), unadjusted: 12.2% (95% CI, 9.6–14.8)].

Discussion

We investigated the effects of oral iron challenge on serum hepcidin and TS profiles during the day. To this end, in healthy volunteers we compared serum hepcidin and TS profiles obtained during a day of an iron-deficient diet supplemented with iron (iron diet) with those obtained during a day of an iron-deficient diet without iron supplementation (no-iron diet). For supplementation in the iron diet, we chose supraphysiological iron doses to increase the chances of finding an effect of ingested iron on hepcidin concentrations during the day. In fact, iron dosing consisted of 2 doses of 65 mg ferrous fumarate, which is around 7 times more than that of a regular Western diet (14), but lower than the recommended 65 mg, 3-times-per-day dose for treatment of iron deficiency. Moreover, for the no-iron diet we chose to provide volunteers with an iron-deficient diet instead of requesting them to go without food during the day, to prevent the previously reported increase of serum hepcidin concentrations upon prolonged fasting (15).

We found that serum ferritin at baseline was an important correlate of the serum hepcidin concentration, confirming earlier studies (4–6, 9, 16–18). Furthermore, hepcidin increased during the days both with and without oral iron administration in both men and women. This suggests that iron intake is not essential for the increases in serum hepcidin concentrations during the day. These data are in agreement with recent observations of similar hepcidin diurnal patterns for individuals with and without food intake and corroborate previous findings that transcriptional regulators related to genes involved in diurnal rhythm are potentially significant modulators of hepcidin expression (7, 15). However, daily variation might also be secondary to innate diurnal rhythm of one of the many biological determinants of serum hepcidin concentrations (1, 2).

We observed mean hepcidin concentrations in women (but not in men) to be significantly higher on a day with oral iron supplementation compared with a day without oral iron supplementation. Several reported studies have shown that hepcidin increases in serum and urine upon oral iron dosing, although non-responders have also been observed (6, 8, 9, 11, 16). Our study, however, cannot be compared easily with these previous studies on hepcidin response to oral iron, because its study design differed from the earlier
Fig. 1. The estimated mean profiles of hepcidin from 1100 to 1600 of men (A) and of women (B), both on 1 day of an iron-deficient diet (thick broken line) and on 1 day of an iron-deficient diet supplemented with 2 doses of 65 mg of ferrous fumarate (thick solid line).

The estimated profiles were determined by using a linear mixed model with adjustment for baseline values. The thin dashed lines indicate the appropriate 95% confidence bands. The vertical bars indicate 1 SD of the observed mean (dot, with supplements; dash, without supplements). Note that the observed data are in line with the model that used equal differences at each point of measurement (i.e., the parallel-line model).
studies by its comparison of hepcidin changes over time on days with and without oral iron ingestion.

The differences that we observed in hepcidin increase by oral iron loading between men and women, which were partly determined by ferritin levels, was interesting. We hypothesize that low body iron stores in women suppress hepcidin production to assure sufficient iron availability for erythropoiesis. In case of an increase in serum iron availability by oral iron administration, the necessity of hepcidin suppression in individuals with low ferritin is temporarily missing, and hepcidin concentrations increase despite low body iron stores. Because ferritin concentrations only partly explain differences between the sexes in daily increases of hepcidin upon oral iron supplementation, other factors including testosterone might be involved. Testosterone has been reported to suppress hepcidin synthesis and might therefore contribute to blunting the increase of daily hepcidin concentrations in men when supplemented with iron (19).

Our data showed that TS remained similar during a day of no iron, but was higher on a day with iron supplementation for both men and women. These data are not in full agreement with previous studies, e.g., we and others previously found that TS decreases during a day of a regular diet, especially after noon (4, 20). These inconsistencies between studies in the variation in daily TS may be due to relatively small sample sizes, and differences in time of blood sampling and diet.

When we corrected serum hepcidin concentrations for TS, the effect of oral iron administration on the mean concentrations of serum hepcidin decreased. However, when TS was corrected for hepcidin, the effect of oral iron administration on TS was similar to that observed in the unadjusted data. This result suggests that TS reflects the differences of the iron-diet on serum hepcidin concentrations more than hepcidin alone, that oral iron influences hepcidin via TS and, in addition, that other (unknown) factors besides dietary iron affected hepcidin concentrations in the current study. These factors are likely to be responsible for our observations that iron intake is not crucial for the increases in serum hepcidin concentrations during the day.
We also measured serum iron during these studies and explored the relationship between serum iron and hepcidin concentrations. First, we found that TS and serum iron were highly correlated ($r = 0.91$) and second, as would be expected with such a high correlation, we found that the results using serum iron were nearly identical to the results using TS (both with respect to the diet as well as to the relationship with hepcidin concentrations). Because TS is considered more useful for clinical interpretation in most clinical situations (21), we presented the detailed results using TS, in line with our previous work on this subject (4).

Interestingly, on a day when ferrous fumarate was administered, increased concentrations of hepcidin and TS were attributable to a more pronounced increase of these parameters between 0800 and 1100, whereas after 1100 changes in both hepcidin and TS were similar for both the iron-deficient and iron groups. It appears that the second dose of oral iron at 1145 did not affect hepcidin or TS. These observations suggest an inhibitory effect of the first iron-dose-induced hepcidin concentrations on intestinal iron uptake and corroborate previous reports on the mucosal block phenomenon on iron absorption. This mucosal block describes the ability of an initial dose of ingested iron to block iron absorption of a second dose given 2–4 h later. The exact mechanism of this mucosal block is unknown, but in vitro and animal studies suggest the involvement of inhibition of enterocyte import of ingested iron by the ferrous iron transporter divalent metal transporter 1 at the brush border secondary to an gested iron by the ferrous iron transporter divalent metal transporter 1.

Importantly, the latter mechanism is supported by recent human studies showing an inverse relation between intestinal iron uptake and circulating hepcidin concentrations (9, 16).

To conclude, our data clearly show that serum hepcidin concentrations increase during a day of an iron-deficient diet, and that these concentrations are moderately higher in women but not in men following oral iron intake. This sex difference may be attributed to lower ferritin concentrations in women but other factors also could be involved, including the effects of testosterone. Our data moreover suggest that ferritin sets the basal concentrations and that TS in conjuction with the innate daily rhythm contributes to the daily variations in hepcidin concentrations. These findings may reflect the distinct mechanism of hepcidin regulation by ferritin (associated with the chronic “iron store” regulator), TS (or ferri-transferrin as the acute “circulating iron” regulator) and clock genes (responsible for diurnal variations) (7, 27, 28). Our data demonstrate that an innate daily rhythm rather than dietary iron mediates daily hepcidin regulation, which will facilitate interpretations of hepcidin as a novel iron biomarker.

References

10. Girelli D, Trombini P, Busti F, Campostirini N.

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