1

Immunoassay for Human Serum Hepcidin

Tomas Ganz[1,2], Gordana Olbina[1], Domenico Girelli[3], Elizabeta Nemeth[1,2] and Mark Westerman[1].

[1]Intrinsic LifeSciences, LLC, La Jolla, California, United States, 92037, [2]Medicine and Pathology, David Geffen School of Medicine, University of California, Los Angeles, California, United States, 90095, and [3]Department of Clinical and Experimental Medicine, University of Verona, Italy.

Scientific Section: Red Cells

1

Abstract

We developed and validated the first serum ELISA for hepcidin, the principal iron-regulatory hormone that has been very difficult to measure. In healthy volunteers, the 5-95% range of hepcidin concentrations was 29-254 ng/ml in men (n=65) and 17-286 ng/ml in women (n=49), with median concentrations 112 vs. 65, p<0.001. The lower limit of detection was 5 ng/ml. Serum hepcidin concentrations in 24 healthy subjects correlated well with their urinary hepcidin (r=0.82). Serum hepcidin appropriately correlated with serum ferritin (r=0.63) reflecting the regulation of both proteins by iron stores. Healthy volunteers showed a diurnal increase of serum hepcidin at noon and 8pm compared to 8am, and a transient rise of serum hepcidin in response to iron ingestion. Expected alterations in hepcidin levels were observed in a variety of clinical conditions associated with iron disturbances. Serum hepcidin concentrations were undetectable or low in patients with iron deficiency anemia (ferritin<10 ng/ml), iron-depleted HFE hemochromatosis, and juvenile hemochromatosis. Serum hepcidin concentrations were high in patients with inflammation (CRP>10 mg/dl), multiple myeloma, or chronic kidney disease. The new serum hepcidin ELISA yields accurate and reproducible measurements that appropriately reflect physiologic, pathologic and genetic influences, and is informative about the etiology of iron disorders.

Introduction

Hepcidin is the principal iron-regulatory hormone that mediates the homeostasis of extracellular iron concentrations[1;2]. Although the peptide is initially synthesized as an 84-amino acid preprohepcidin, it is processed in hepatocytes by a signal peptidase and the prohormone convertase furin[3] to its bioactive form, a 25-amino acid peptide circulating in plasma[4;5] and excreted in urine[5]. Hepcidin acts by regulating iron influx into plasma from tissues engaged in iron storage or transport: duodenal enterocytes that absorb dietary iron, hepatocytes that store iron and macrophages that recycle iron from senescent erythrocytes. At the molecular level, hepcidin binds to the sole known cellular iron efflux channel, ferroportin, and induces its internalization and lysosomal degradation[6] by mechanisms similar to those that inactivate other more conventional membrane receptors. N-terminally truncated breakdown products of hepcidin are detectable in plasma[7] and urine[5;8] but show impaired ability to internalize ferroportin[9].

Hepcidin synthesis is physiologically increased by elevated plasma iron concentration[10;11], decreased by erythropoietic activity[12], and pathologically increased by inflammation[10;13]. Hepcidin excess plays the major role in anemia of inflammation[14-16] and iron-resistant iron-deficiency anemia[17-20]. At the opposite extreme, hepcidin deficiency is the cause of iron overload in most hereditary hemochromatoses[21] and contributes to iron overload in β-thalassemia and other iron-loading anemias[22].

Despite the importance of hepcidin for systemic iron homeostasis and iron–related pathologies, the methodologies for measuring physiologically relevant hepcidin concentrations have been cumbersome and their availability to the clinical research community has been very limited. Most of the studies of hepcidin in humans have relied

on a human urinary hepcidin assay[23], based on selective extraction of hepcidin from urine by cation-exchange chromatography, and its detection in an immunodot format by anti-hepcidin antibody and chemiluminescence. To account for the variable dilution of urine samples, hepcidin concentration is normalized to urinary creatinine. This assay is laborious and its utility depends on the expected but as yet unverified relationship between normalized urinary hepcidin levels and plasma hepcidin concentrations. More recently, hepcidin assays based on mass spectroscopic techniques[7;8;24;25] have been introduced but most are semiquantitative and depend on expensive equipment that is not widely available. An immunoassay reported to detect human prohepcidin[26] does not reflect the expected physiological or pathological changes[27] in mature hepcidin concentrations seen with other hepcidin assays. We now describe the performance of a competitive enzyme-linked immunoassay (C-ELISA) for human hepcidin. This assay accurately and reproducibly detects physiologic and pathologic changes in serum or urine hepcidin levels, and can be readily performed in a high-throughput format.

Materials and Methods

Hepcidin C-ELISA

Antibody to human hepcidin was prepared as described previously[13] and purified on staphylococcal protein A columns (Thermo Fisher Scientific, Rockford, IL), according to the manufacturer's protocol. 96-well plates were coated with the antibody and incubated with 100 μl (standard samples) or 200 μl (samples with very low concentration of hepcidin) of 1:20 dilution of serum or 1:10 dilution of urine in Tris-buffered saline containing 0.05% Tween-20 (TBS-Tween 20), with 10 ng/ml of biotinylated hepcidin-25 (Intrinsic LifeSciences, La Jolla, CA) added as the tracer. Standard curves were prepared by serial two-fold dilution of synthetic hepcidin (Bachem, King of Prussia, PA) 4000 ng/ml in TBS-Tween 20 buffer containing the tracer. The integrity and bioactivity of synthetic hepcidin and biotinylated hepcidin were verified by mass spectrometry and by bioassay with ferroportin-GFP expressing HEK-293 cells[9]. After washing, the assay was developed with streptavidin-peroxidase and tetramethyl benzidine. The enzymatic reaction was stopped by sulfuric acid and the plate was read at 450 nm on a DTX 880 microplate reader (Beckman Coulter, Inc.). Standard curves were fitted with 12-point fit using GraphPad Prism software (GraphPad Software, San Diego, CA). The fitted curve was then used to convert sample absorbance readings to hepcidin concentrations. Urinary creatinine concentrations were measured by the Jaffe reaction using the Creatinine Parameter Assay (R&D Systems, Minneapolis, MN).

Urine and serum collection

All samples were collected in accordance with protocols approved by the relevant Institutional Review Boards and the Helsinki Protocol. Serum and urine samples were stored frozen at -80C.

Serum and Urine Donors

Sera from healthy volunteers were obtained in Verona, Italy and Salt Lake City, UT and included in this study based on health history, and normal (based on local standards) laboratory values of Hgb, serum Fe/TIBC, ferritin, sTfR, and CRP. Patients with inherited iron disorders were recruited by cooperating physicians in the US, Canada, Italy, Greece and France. The specific diagnoses were established by family history, genetic testing and the presence of appropriate biochemical abnormalities. Serum samples of patients with iron deficiency or inflammation were obtained from the UCLA Clinical Laboratory as discards of sera that contained less than 10 ng/ml of ferritin or more than 10 mg/dl of C-reactive protein respectively. Sera of patients with multiple myeloma were obtained from the Veteran's Administration myeloma study group. Sera of patients with chronic renal disease were obtained at UCLA, selecting patients without any known underlying inflammatory disorders.

Physiologic Studies

The time course of response to the ingestion of 65 mg or 130 mg of iron in the form of one or two iron sulfate tablets were determined in healthy volunteers by serial serum sampling starting 24h before and ending 24h after iron ingestion.

Statistical analysis

Data were analyzed using Sigmastat (Systat Software Inc., San Jose, CA).

Results
Assay performance

Selectivity—The competitive ELISA standard curve (Figure 1) was not affected by the addition of any of the hepcidin-poor sera obtained from 6 patients with serum ferritin<10 ng/ml, indicating that there was little interference from non-hepcidin serum components. In addition, no competition was detected when an antimicrobial peptide structurally related to hepcidin, protegrin-1[28], was added at concentrations 16 to 500 ng/ml (data not shown). The lower limit of hepcidin detection as defined by two standard deviation above the zero standard was 5.5 ng/ml.

Intra-assay precision—Eleven serum samples from healthy volunteers were assayed in replicates (n=5-12, Supplemental Figure 1A), yielding a coefficient of variation (CV) 5-19% with higher variations at lower hepcidin concentrations.

Inter-assay reproducibility—Serum samples from 24 healthy volunteers were measured in three independent assays (Supplemental Figure 1B). The average CV was 12% and the median CV was 7%. The range was 0-44%, with the higher variations observed in serum samples with hepcidin concentrations below 30 ng/ml.

Hepcidin stability during sample storage—15 serum and 15 urine samples from healthy subjects were assayed before and after storage for 6 months at -80C. The median (interquartile range) change in hepcidin concentrations in serum was -5% (+6% , -11%) and in urine -6% (+7%, -14%).

Normal range—We determined the normal range of hepcidin in serum using samples from healthy volunteers (65 men, 49 women). The subjects' hematological and iron parameters are described in Table 1. After adjustments for multiple comparisons, there were no significant differences between US and Italian sites in age, hemoglobin, transferrin saturation or ferritin, and the data from both sites were therefore combined by gender. The 5-95% range of hepcidin concentrations (Table 2) was 29-254 ng/ml in men and 17-286 ng/ml in women, and the medians differed significantly, 112 vs. 65, p<0.001 by Mann-Whitney rank sum test. Although there was a trend for age-related increase in serum hepcidin in both genders, it did not reach statistical significance (Supplemental Figure 2). A much larger number of older men and women will be required to verify this trend.

Correlation with urinary hepcidin—Hepcidin is cleared through the kidneys, and for technical reasons, most of the initial studies of the role of hepcidin in iron pathophysiology used urinary hepcidin assays. This is an indirect measurement of the circulating hormone and has never been correlated with the more physiologically relevant serum hepcidin levels. We measured urinary hepcidin concentrations by C-ELISA in the 24 healthy volunteers and normalized the values to urinary creatinine. Median urinary hepcidin (ng/mg creatinine) was 502 with a range of 71 to 1762. As was the case for serum hepcidin, urinary hepcidin concentrations in women were lower than in men, with median 394 vs 861 ng/mg creatinine, p=0.004 by Mann-Whitney test. In this group of healthy subjects, normalized urinary hepcidin concentrations showed good correlation with serum hepcidin concentrations (Figure 2). However, this correlation would be expected to deteriorate when renal function declines.

Serum hepcidin responses to physiologic variation and pathophysiologic perturbations

Correlation with ferritin and with estimated iron stores—We next asked whether serum hepcidin concentrations measured by C-ELISA reflected iron stores similar to other laboratory markers commonly used for estimating iron stores (Figure 3). Indeed, serum hepcidin concentrations of healthy subjects correlated well with serum ferritin (r =0.63, p<10^{-13} for log(hepcidin) vs log(ferritin)).
.

Response to acute iron load and diurnal variation—To determine whether the assay detects the expected hepcidin response to iron loading, we examined the time course of hepcidin before and after the administration of oral iron (Figure 4). In subjects with detectable iron absorption, indicated by a rise of serum iron 4h after challenge as compared to the same time of day during the control period, a corresponding but more prolonged rise in serum hepcidin was seen. Serum iron showed the expected diurnal increase at noon and in the afternoon[29] through the control period and after iron challenge in those subjects who did not detectably absorb iron. During the control

period, the mean noon, 4 pm and 8 pm hepcidin concentrations were higher than 8am values, and this reached statistical significance for the noon and 8pm values (one way repeated measures ANOVA, Holm-Sidak method, overall $p<0.05$). Overall, the 8am fasting hepcidin concentrations appeared to be more consistent than concentrations at other times of the day.

Serum hepcidin in iron disorders— Hepcidin concentrations were measured in the sera of patients with various iron disorders (Figure 5). In agreement with previous urinary hepcidin measurements[30], serum hepcidin was inappropriately normal in iron-overloaded and low in iron-depleted patients with the adult form of hereditary hemochromatosis due to mutations in the gene HFE. Serum hepcidin was also low in patients with juvenile hemochromatosis due to mutations in hemojuvelin. As would be expected from the physiology of hepcidin regulation, serum hepcidin was undetectable in 18/19 patients with iron deficiency (ferritin < 10 ng/ml). Serum hepcidin was abnormally increased in patients with inflammation (CRP > 10 mg/dl), in patients with multiple myeloma, a condition associated with excessive production of IL-6[31], a known stimulus for hepcidin synthesis[23], and in patients with chronic kidney disease without associated inflammatory disorders.

Discussion

We developed a serum C-ELISA for human hepcidin that correctly detects the expected physiologic and pathologic variations in hepcidin concentrations. The selectivity of the C-ELISA is indicated by the very low serum hepcidin levels that were found in iron deficiency and in severe forms of hereditary hemochromatosis. Consistent with the proposed mechanism of action of hepcidin, the range of measured serum hepcidin concentrations in normal and pathological samples (<5 ng/ml to >4000 ng/ml, i.e. 0-1.5 µM) is in the range known to internalize the iron export channel ferroportin[6]. Our C-ELISA revealed for the first time a significant gender difference in serum hepcidin. This difference is most likely due to the lower iron stores in women, also reflected in lower serum ferritin concentrations (Table 1 and Figure 3). Serum hepcidin concentrations showed diurnal changes similar to serum iron concentrations, and also increased markedly with acute changes in serum iron induced by oral challenge with iron sulfate (Figure 4). The kinetics of the hepcidin response to iron may be complex because hepatocytes are exposed to boluses of iron in portal blood as well as to changing iron concentrations in systemic blood, and hepcidin changes have a feedback effect on iron concentrations by affecting the release of iron from enterocytes and from macrophage and hepatocyte stores.

Hepcidin and serum ferritin respond similarly to inflammation and changes in iron stores, and this is reflected in the strong correlation between hepcidin and ferritin in healthy volunteers. However, hepcidin responses take place on the time scale of a few hours, whereas changes in ferritin concentrations are much slower[32]. In several pathological conditions, the correlation between serum ferritin and hepcidin is dramatically altered. In various forms of hereditary hemochromatosis, hepcidin is deficient as a result of mutations in hepcidin regulators or in the hepcidin gene itself[33]. In untreated or partially treated patients with hereditary hemochromatosis, ferritin is high

but hepcidin is inappropriately normal, low or absent. While erythropoietic activity has no known direct effect on ferritin, it strongly suppresses hepcidin synthesis[12]. Pathological suppression of hepcidin (in the face of greatly increased serum ferritin) is also seen in β-thalassemia[22;34-37] where it results in increased intestinal iron absorption and contributes to systemic iron overload, the main cause of morbidity and mortality in this condition.

Assuming a normal serum creatinine concentration of 1 mg/dl and serum hepcidin of 100 ng/ml, the normal ratio of serum hepcidin to serum creatinine is about 10,000 ng/mg creatinine. In our healthy volunteers, the urine contained a median of 500 ng hepcidin/mg creatinine. Thus 95% of hepcidin is retained in the kidney either because it is not freely filtered through the glomerular membrane and/or because it is reabsorbed and degraded in the proximal tubules like other small peptides[38;39]. Although we found good correlations between urinary and serum hepcidin concentrations in our healthy donors, urinary hepcidin concentrations may not accurately reflect the serum concentrations of hepcidin in kidney diseases.

The C-ELISA for human serum hepcidin should be useful in improving our understanding of the pathogenic role of hepcidin in various iron disorders, and in the development of appropriate therapeutic interventions. In contrast to ferritin, a useful marker of iron stores, changes in hepcidin concentrations are frequently the cause of iron disorders. Thus, diagnostic measurements of hepcidin concentrations should be more informative about the etiology of iron-related disease. Large scale human studies will be required to establish the utility of serum hepcidin measurements in the diagnosis and clinical management of iron disorders.

Contribution: T.G. designed the research, analyzed data, and wrote the manuscript, G.O., E.N., and M.W. designed the research, performed experiments, analyzed data and edited the manuscript, D.G. designed the research, analyzed data and edited the manuscript.

Conflict-of-interest disclosure: T.G., G.O., E.N. and M.W. are officers or employees of Intrinsic Lifesciences LLC and have ownership interest in the company. Intrinsic

Correspondence: Tomas Ganz, e-mail: tganz@mednet.ucla.edu or Mark Westerman, mwesterman@intrinsiclifesciences.com

Reference List

1. Nicolas G, Bennoun M, Devaux I et al. Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice. P.N.A.S. 2001;98:8780-8785.

2. Ganz T, Nemeth E. Iron imports. IV. Hepcidin and regulation of body iron metabolism. Am J Physiol Gastrointest Liver Physiol 2006;290:G199-G203.

3. Valore EV, Ganz T. Posttranslational processing of hepcidin in human hepatocytes is mediated by the prohormone convertase furin. Blood Cells, Molecules, and Diseases 2008;40:132-138.

4. Krause A, Neitz S, Magert HJ et al. LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS Lett. 2000;480:147-150.

5. Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J.Biol.Chem. 2001;276:7806-7810.

6. Nemeth E, Tuttle MS, Powelson J et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 2004;306:2090-2093.

7. Tomosugi N, Kawabata H, Wakatabe R et al. Detection of serum hepcidin in renal failure and inflammation by using ProteinChip System. Blood 2006;108:1381-1387.

8. Kemna E, Tjalsma H, Laarakkers C et al. Novel urine hepcidin assay by mass spectrometry. Blood 2005;106:3268-3270.

9. Nemeth E, Preza GC, Jung CL et al. The N-terminus of hepcidin is essential for its interaction with ferroportin: structure-function study. Blood 2006;107:328-333.

10. Pigeon C, Ilyin G, Courselaud B et al. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. J.Biol.Chem. 2001;276:7811-7819.

11. Lin L, Valore EV, Nemeth E et al. Iron transferrin regulates hepcidin synthesis in primary hepatocyte culture through hemojuvelin and BMP2/4. Blood 2007;110:2182-2189.

12. Pak M, Lopez MA, Gabayan V, Ganz T, Rivera S. Suppression of hepcidin during anemia requires erythropoietic activity. Blood 2006;108:3730-3735.

13. Nemeth E, Valore EV, Territo M et al. Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. Blood 2003;101:2461-2463.

14. Nicolas G, Bennoun M, Porteu A et al. Severe iron deficiency anemia in transgenic mice expressing liver hepcidin. P.N.A.S. 2002;99:4596-4601.

15. Rivera S, Liu L, Nemeth E et al. Hepcidin excess induces the sequestration of iron and exacerbates tumor-associated anemia. Blood 2005;105:1797-1802.

16. Roy CN, Mak HH, Akpan I et al. Hepcidin antimicrobial peptide transgenic mice exhibit features of the anemia of inflammation. Blood 2007;109:4038-4044.

17. Du X, She E, Gelbart T et al. The Serine Protease TMPRSS6 Is Required to Sense Iron Deficiency. Science 2008;320:1088-1092.

18. Finberg KE, Heeney MM, Campagna DR et al. Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA). Nat.Genet. 2008;40:569-571.

19. Melis MA, Cau M, Congiu R et al. A mutation in the TMPRSS6 gene, encoding a transmembrane serine protease that suppresses hepcidin production, in familial iron deficiency anemia refractory to oral iron. Haematologica 2008

20. Guillem F, Lawson S, Kannengiesser C et al. Two nonsense mutations in the TMPRSS6 gene in a patient with microcytic anemia and iron deficiency. Blood 2008

21. Pietrangelo A. Hereditary hemochromatosis. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 2006;1763:700-710.

22. Nemeth E, Ganz T. Hepcidin and iron-loading anemias. Haematologica 2006;91:727-732.

23. Nemeth E, Rivera S, Gabayan V et al. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. J.Clin.Invest. 2004;113:1271-1276.

24. Murphy AT, Witcher DR, Luan P, Wroblewski VJ. Quantitation of hepcidin from human and mouse serum using liquid chromatography tandem mass spectrometry. Blood 2007;110:1048-1054.

25. Kemna EH, Tjalsma H, Podust VN, Swinkels DW. Mass spectrometry-based hepcidin measurements in serum and urine: analytical aspects and clinical implications. Clin.Chem. 2007;53:620-628.

26. Kulaksiz H, Gehrke SG, Janetzko A et al. Pro-hepcidin: expression and cell specific localisation in the liver and its regulation in hereditary haemochromatosis, chronic renal insufficiency, and renal anaemia. Gut 2004;53:735-743.

27. Roe MA, Spinks C, Heath AL et al. Serum prohepcidin concentration: no association with iron absorption in healthy men; and no relationship with iron status in men carrying HFE mutations, hereditary haemochromatosis patients undergoing phlebotomy treatment, or pregnant women. Br.J.Nutr. 2007;97:544-549.

28. Kokryakov VN, Harwig SS, Panyutich EA et al. Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins. FEBS Lett. 1993;327:231-236.

29. Wiltink WF, Kruithof J, Mol C, Gré Bos M, van Eijk HG. Diurnal and nocturnal variations of the serum iron in normal subjects. Clinica Chimica Acta 1973;49:99-104.

30. Piperno A, Girelli D, Nemeth E et al. Blunted hepcidin response to oral iron challenge in HFE-related hemochromatosis. Blood 2007;110:4096-4100.

31. Lauta VM. A review of the cytokine network in multiple myeloma: diagnostic, prognostic, and therapeutic implications. Cancer 2003;97:2440-2452.

32. Kemna E, Pickkers P, Nemeth E, van der HH, Swinkels D. Time-course analysis of hepcidin, serum iron, and plasma cytokine levels in humans injected with LPS. Blood 2005;106:1864-1866.

33. Ganz T, Nemeth E. Regulation of iron acquisition and iron distribution in mammals. Biochim.Biophys.Acta 2006;1763:690-699.

34. Kattamis A, Papassotiriou I, Palaiologou D et al. The effects of erythropoetic activity and iron burden on hepcidin expression in patients with thalassemia major. Haematologica 2006;91:809-812.

35. Kearney SL, Nemeth E, Neufeld EJ et al. Urinary hepcidin in congenital chronic anemias. Pediatr.Blood Cancer 2007;48:57-63.

36. Origa R, Galanello R, Ganz T et al. Liver iron concentrations and urinary hepcidin in beta-thalassemia. Haematologica 2007;92:583-588.

37. Gardenghi S, Marongiu MF, Ramos P et al. Ineffective erythropoiesis in β-thalassemia is characterized by increased iron absorption mediated by down-regulation of hepcidin and up-regulation of ferroportin. Blood 2007;109:5027-5035.

38. Carone FA, Peterson DR, Flouret G. Renal tubular processing of small peptide hormones. J.Lab.Clin.Med. 1982;100:1-14.

39. Christensen EI, Birn H. Hormone, growth factor, and vitamin handling by proximal tubule cells. Curr.Opin.Nephrol.Hypertens. 1997;6:20-27.

Table 1. Characteristics of healthy subjects

Variable	USA, Men	USA, Women	Italy, Men	Italy, Women
	Median (range)			
N	30	28	35	21
Age	32.7 (19-60)	32.6 (20-58)	31 (18-63)	28 (18-81)
Hgb	15.7 (13.8-17)	13.8 (12.8-15.4)	14.9 (13.6-17.3)	13.4 (11.7-16.2)
Tf sat%	31.8 (15.5-64.3)	26.3 (12.1-49.0)	27.0 (16.0-59.0)	25.7 (12.1-46.0)
Ferritin	109 (42-398)	39 (13-85)	118 (15-288)	46 (18-140)

Table 2. Serum hepcidin concentrations in healthy subjects

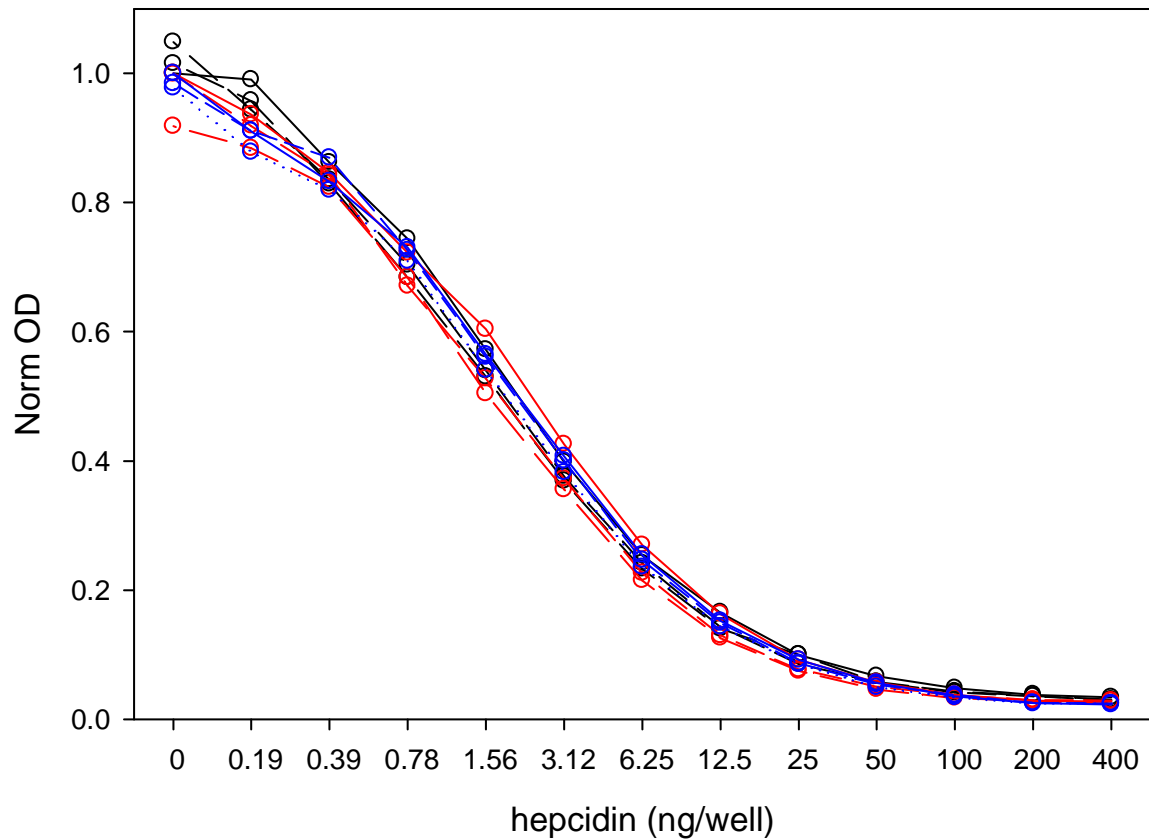| Sample	Unit	min	5%	10%	median	mean	90%	95%	max
serum, men	ng/ml	10	29	38	112	121	224	254	298
serum, women		5	17	25	65	87	157	286	352

Figure 1. Comparison of standard curves performed in serum-free buffer or in 1:20 dilution of sera from six iron-deficient patients. On three different days (red, blue and black lines), standard curves were performed in buffer (solid line) and in sera from two of the six patients (dotted and dashed lines). The OD values for all plots were normalized by dividing with the "no hepcidin" buffer OD for that day. The differences between serum and paired buffer OD ranged from -1%±4% to -13%±8%. The points were fitted with a sigmoidal logistic 4-parameter curve optimized through 200 iterations (Sigmaplot, Systat Software, San Jose, CA).
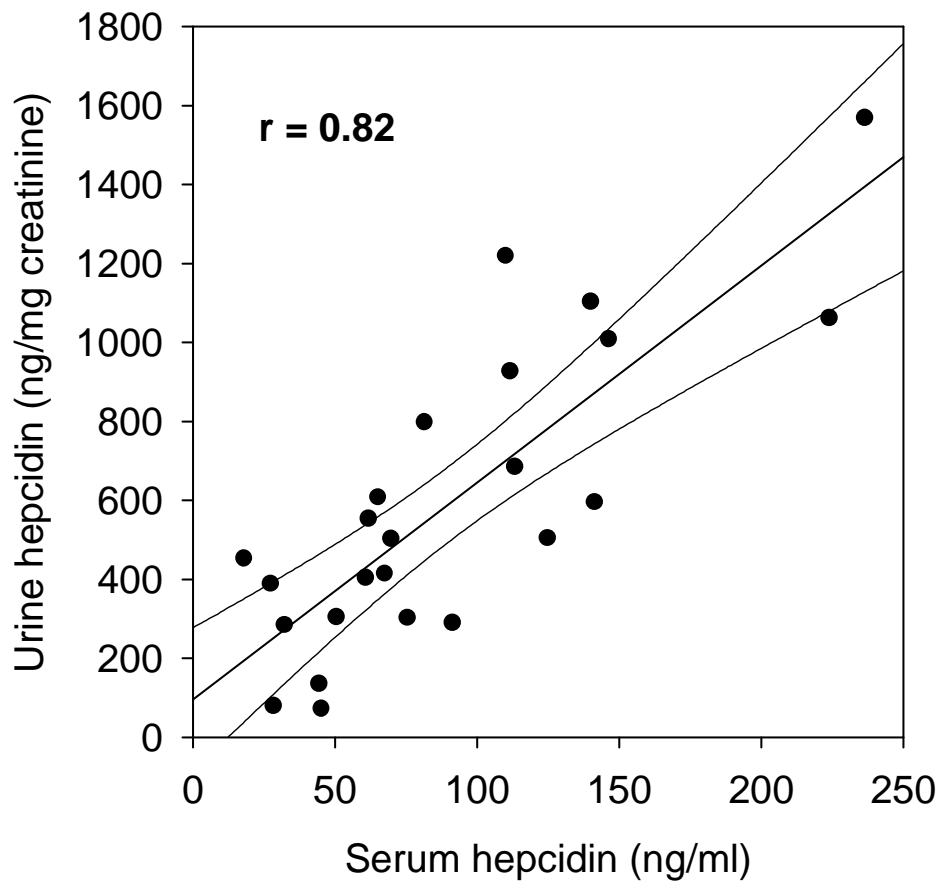
Figure 2. Correlation between serum and urine hepcidin concentrations in 24 healthy subjects measured by Hepcidin C-ELISA. Linear regression and 95% confidence limits are shown.
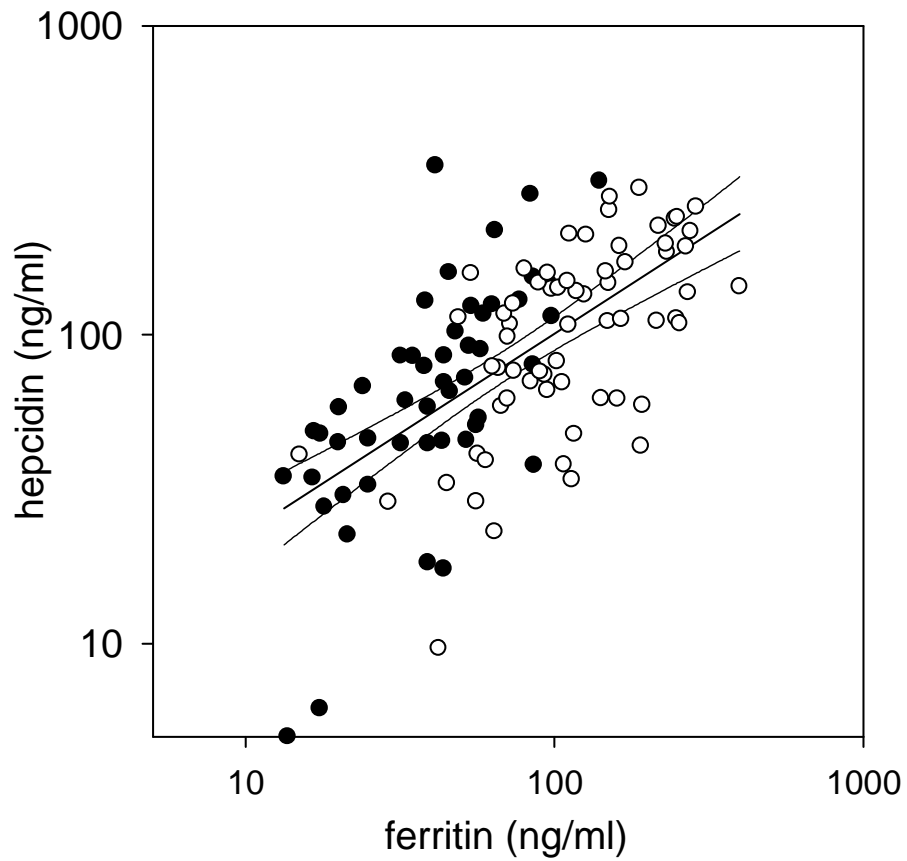
Figure 3. Correlation of serum hepcidin with serum ferritin in healthy subjects. Values for men (empty) and women (solid) with a common regression line (r=0.63) and 95% confidence intervals are shown.
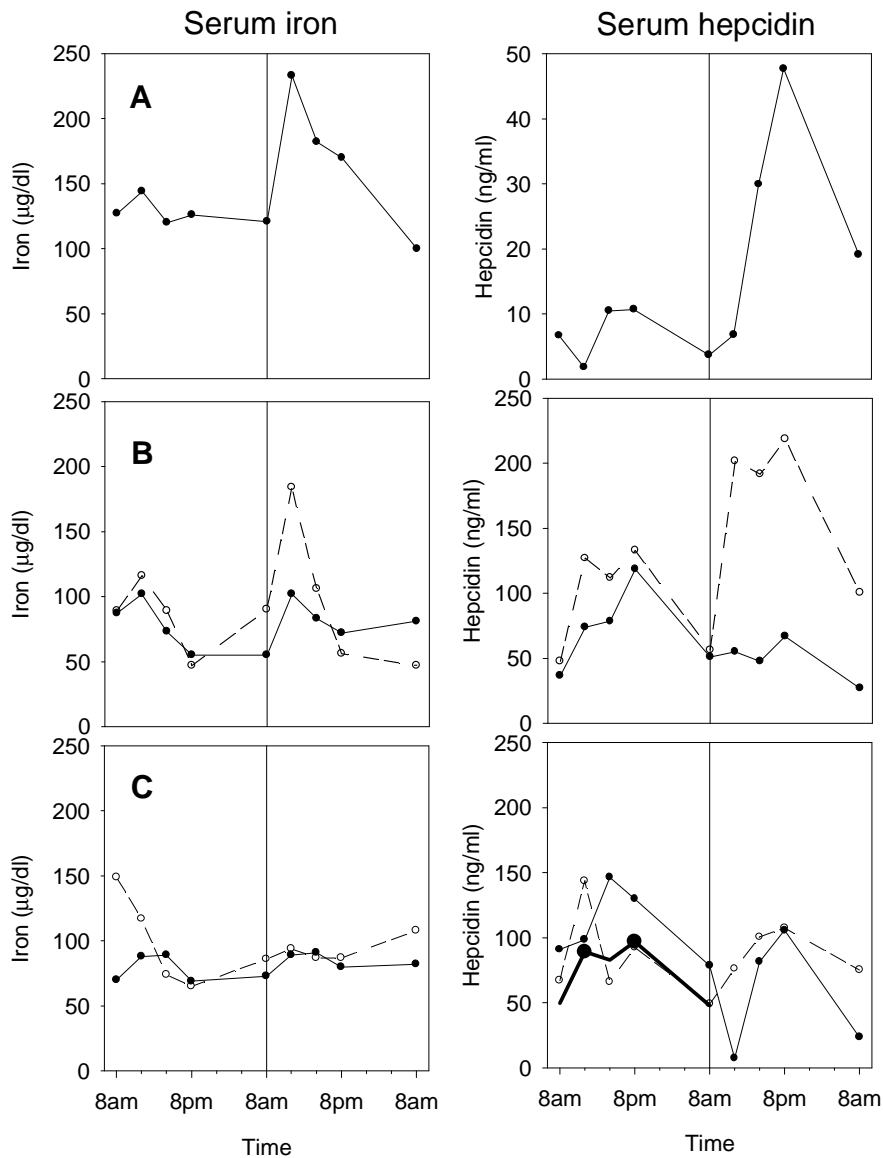
Figure 4. Serum iron and serum hepcidin before and after a small iron challenge. Serum samples were obtained from three subjects (A, B, C) over 48 hours and an iron sulfate dose was given at 8am of the second day as indicated by the vertical line in all panels. Subject A responded to 65 mg of iron by increasing both serum iron and hepcidin. Subject B was tested on two separate occasions, first with 65 mg (solid line), then with 130 mg of iron (dashed line), and only responded to the larger dose by increasing serum iron and hepcidin. Subject C was tested on two separate occasions with 65 mg of iron (solid and dashed line) and did not increase either serum iron or hepcidin indicating that the iron dose was not well absorbed. The average time dependence of the five serial pre-challenge hepcidin measurements in all subjects is also shown in panel C as a bold black line. Hepcidin concentration showed diurnal variation, with noon and 8pm values significantly higher (●) than baseline 8am values (one way repeated measures ANOVA, Holm-Sidak method).
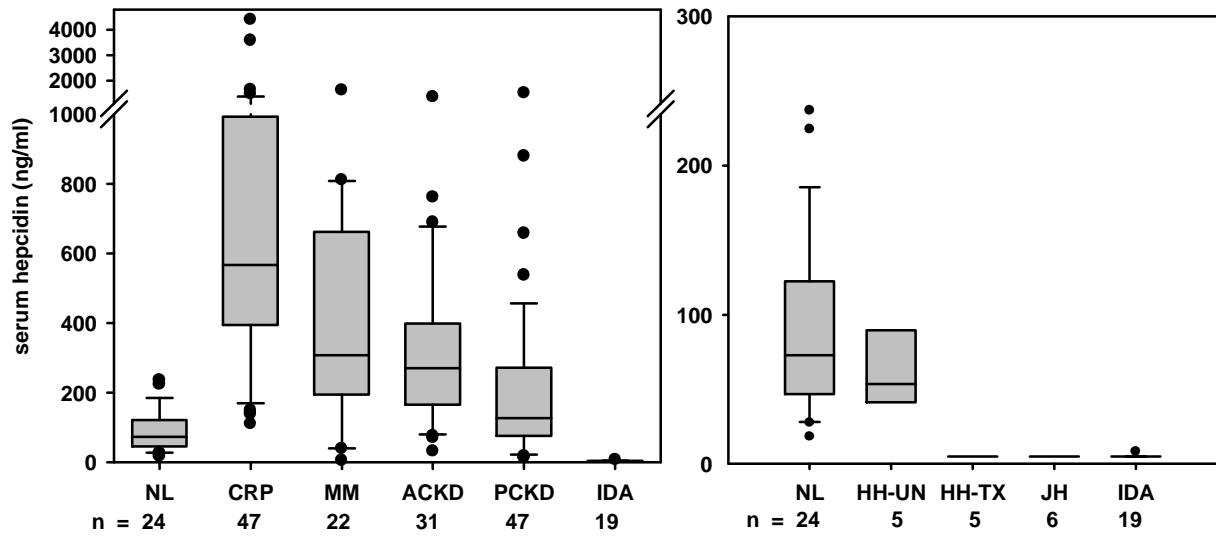
16

Figure 5. Serum hepcidin in iron disorders. Boxplots show 25%, median and 75% with whiskers showing 10% and 90% and outliers as circles. Groups include healthy volunteers (NL), pretreatment HFE hemochromatosis (HH-UN), iron-depleted HFE hemochromatosis (HH-TX), juvenile hemochromatosis due to mutations in hemojuvelin (JH), iron deficiency (ID), patients with inflammation (INF, CRP >10 mg/dl), multiple myeloma (MM), and adult or pediatric chronic kidney disease (not requiring dialysis, ACKD and PCKD respectively). All disease groups except for HH-UN differed significantly from normal (p<0.001 except PKCD p<0.01, Mann-Whitney test).