A Dual-Monoclonal Sandwich ELISA Specific for Hepcidin-25

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BACKGROUND: Hepcidin, a key regulator of iron metabolism, binds to the iron transporter ferroportin to cause its degradation. In humans, hepcidin deficiency has been linked to hemochromatosis and iron overload, whereas increased concentrations have been reported in anemia of cancer and chronic disease. There is currently an unmet clinical need for a specific immunoassay with a low limit of quantification to measure serum concentrations of hepcidin-25, the active form of the protein.

METHODS: We generated 2 antihepcidin-25 monoclonal antibodies and used them to build a sandwich ELISA. We correlated ELISA results to hepcidin-25 measurements by LC-MS and used ELISA to measure serum hepcidin-25 concentrations in normal individuals, cancer patients, and patients with rheumatoid arthritis.

RESULTS: The sandwich ELISA was highly specific for hepcidin-25, having a limit of quantification of 0.01 μg/L (10 pg/mL). Serum concentrations of hepcidin-25 measured by ELISA correlated with hepcidin-25 concentrations measured by using an independent LC-MS assay (r = 0.98, P < 0.001). Hepcidin-25 concentrations were increased in patients with cancer (median 54.8 μg/L, 25%–75% range 23.2–93.5 μg/L, n = 34) and rheumatoid arthritis (median 10.6 μg/L, 25%–75% range 5.9–18.4 μg/L, n = 76) compared with healthy individuals (median 1.2 μg/L, 25%–75% range 0.42–3.07 μg/L, n = 100).

CONCLUSIONS: The use of 2 monoclonal antibodies in a sandwich ELISA format provides a robust and convenient method for measuring concentrations of the active form of hepcidin. This ELISA should help to improve our understanding of the role of hepcidin in regulating iron metabolism.
haptoglobin (24). Prohaptoglobin is further processed via removal of its N-terminal 39 amino acids to yield haptoglobin-25 (the active form of the hormone), which can then undergo further N-terminal processing to yield haptoglobin-20 and haptoglobin-22, both of which are inactive (24). Hence, early competitive immunoassays struggled to differentiate physiologically relevant haptoglobin-25 from the inactive, less relevant haptoglobin-22, haptoglobin-20, and prohaptoglobin species (25, 26).

As a result, many of the described assays specific for haptoglobin-25 have been LC-MS–type assays (27–29). Although such assays are accurate and precise, their complexity, expense, and high level of operator expertise prevent their implementation into most routine clinical laboratories. To address the need for a highly specific and robust immunoassay, we have developed a sandwich ELISA by using 2 monoclonal antibodies for the measurement of haptoglobin-25 in human serum. In this study, we show that our sandwich ELISA specifically measures haptoglobin-25 and has a high correlation with LC-MS (28).

In addition, we demonstrate that haptoglobin-25 concentrations are increased in patients with cancer and rheumatoid arthritis compared with healthy individuals.

**Materials and Methods**

**HUMAN SPECIMENS**

One hundred serum samples from healthy volunteers (ages 18–64 years, mean age 37 years) were purchased from Valley Biomedical. The samples spanned a broad ethnic distribution (24 African American, 24 Hispanic, and 52 Caucasian), with each group consisting of 50% women and 50% men. Among women, 40 of 50 individuals were age ≤45 years. We also obtained 34 serum samples from cancer patients. These specimens included 17 samples from patients with hematological malignancies (8 acute myelocytic leukemia, 3 lymphoma, 2 multiple myeloma, 2 myelodysplastic syndrome, 1 chronic lymphocytic leukemia, and 1 chronic myelocytic leukemia) and 17 samples from patients with nonhematological tumors (3 renal, 2 head and neck, 2 melanoma, 2 lung, 2 squamous cell carcinoma, 1 ameloblastoma, 1 prostate, 1 thyroid, 1 colon, 1 hepatic, and 1 urethral). Samples from 76 patients with rheumatoid arthritis were also obtained from patients who gave their permission for serum samples to be banked for future exploratory analysis. After obtaining protocol approval from an institutional review board and proper informed consent, all samples were collected, stored, and deidentified to protect patient privacy. Samples were received on dry ice and stored at −70 °C before analysis of haptoglobin levels. Ferritin analysis was performed by using a Beckman Dxi 800 chemiluminescent immunoassay.

HEPCIDIN-25 GENERATION, PRODUCTION OF ANTIHEPCIDIN ANTIBODIES, AND LABELING OF ANTIBODIES

Synthesized human hepcidin-25, hepcidin-22, and hepcidin-20 proteins were obtained from Peptides International or produced recombinantly. Proteins were divided into aliquots and stored at −70 °C before use. Two different monoclonal antihepcidin antibodies (antihepcidin-1 and antihepcidin-2) were produced by immunizing mice with either human hepcidin-25 protein or an N-terminal hepcidin peptide (amino acids 1–7) conjugated with a 5–amino acid peptide linker to an ovalbumin peptide (amino acids 232–336) sequence. Of each antibody, 1 mg was biotinylated by using a Pierce kit, and 1 mg was labeled with ruthenium by using a MesoScale Discovery (MSD)2 kit for electrochemiluminescent detection. Afterward, the labeled antibodies were diluted in 50% glycerol and stored at −20 °C.

HEPCIDIN ELISA

A human hepcidin-25 MSD ELISA was constructed by using the reagents described above. Streptavidin-coated and blocked wells were incubated for 1 h with biotinylated antihepcidin-2 antibody (2 mg/L). Afterward, the wells were aspirated and washed 3 times with Tris-buffered saline with Tween (TBST) containing 10 mmol/L Tris, pH 7.40, 150 mmol/L NaCl, with 1 mL Tween 20 per liter. Next, 100 μL hepcidin standards (varying concentrations of hepcidin-25 protein in assay buffer consisting of 50 mmol/L HEPES, pH 7.40, 150 mmol/L NaCl, 10 mL/L Triton X-100, 5 mmol/L EDTA, and 5 mmol/L EGTA) was added to the wells to generate a calibration curve. Serum samples were diluted 1:20 in an assay buffer and added to their respective wells, and the ELISA plate was incubated for 1 h at room temperature. After aspiration, the wells were washed 3 times with TBST, and 100 μL of a 1:1000 dilution of conjugate antibody (ruthenium-labeled antihepcidin-1 antibody, 1 mg/L) was added to the wells for a 1-h incubation at room temperature. After aspiration, the wells were washed 3 times with TBST, and the plate was developed by using an MSD reader, which recorded ruthenium electrochemiluminescence.

MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT (MALDI-TOF) ANALYSIS

Antibodies were evaluated for their ability to immunoprecipitate endogenous hepcidin from human serum via matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer on antibody-
bound hepcidin performed after sample reduction. The different antihepcidin antibodies were coated onto wells of a 96-well Nunc standard ELISA plate in carbonate-bicarbonate (pH 9.4) buffer for 1 h at room temperature at a concentration of 2 mg/L. The wells were aspirated and washed 3 times with TBST. Human serum samples containing a known amount of hepcidin (diluted in assay buffer) were added at 100 µL/well for 1 h at room temperature. The wells were aspirated and washed 3 times with TBST. Captured hepcidin was eluted by adding 40 µL/well of 0.1% formic acid for 5 min at room temperature. Eluted samples were collected and concentrated with a C4 ZipTip (Millipore). A 0.5-µL volume of sample was spotted onto a MALDI target, and an equal volume of matrix solution was added (50% acetonitrile, 0.1% trifluoroacetic acid saturated with α-cyan-4-hydroxycinnamic acid). The sample was dried and analyzed with a 4700 TOF-TOF Mass Spectrometer (Applied Biosystems) operated in linear mode.

DATA ANALYSIS

MSD software and SigmaPlot version 8.0 were used for fitting ELISA calibration curves. Data were plotted by using version 2.98 of the program FigP (Biosoft). For each group of individuals or patients studied, the median, 25th percentile, 75th percentile, and interquartile range were determined. Comparisons of hepcidin-25 concentrations between respective groups were performed by using the Wilcoxon nonparametric rank sum test. In each case, a P value of < 0.05 was considered to indicate statistical significance.

Results

In the first series of experiments, MALDI-TOF spectrometry was used to determine which hepcidin species were bound to antihepcidin antibodies in human serum. Antihepcidin-1 antibody bound mainly to hepcidin-25 (Fig. 1A and B) and to a lesser extent to hepcidin-20. There was no indication that antihepcidin-1 antibody bound to prohepcidin (predicted molecular weight of 7.7 kDa).

Similar experiments were performed to determine which hepcidin species were bound by antihepcidin-2 antibody. We observed that the antihepcidin-2 antibody was only bound to hepcidin-25 in human serum (Fig. 1C and D). No hepcidin-20, hepcidin-22, prohepcidin, or other hepcidin species were bound, indicating that the antihepcidin-2 antibody was highly specific for hepcidin-25.

The antibodies were then investigated for pairing in a sandwich ELISA. The optimal pairing was found to be antihepcidin-2 antibody as the capture antibody and antihepcidin-1 antibody as the conjugate antibody. Fig. 2A shows a typical calibration curve obtained with the final ELISA orientation described above, in which hepcidin-25 protein was prepared at a concentration of 10 µg/L and serially diluted to create a calibration curve. Based on a 3-SD evaluation from the zero calibrator, the limit of quantification of the ELISA was determined to be 0.01 µg/L, and therefore indicated that the ELISA was adequate for measuring serum hepcidin-25 concentrations, on the basis of previous estimates of human serum hepcidin-25 concentrations measured by LC-MS assays (28). The sandwich ELISA was confirmed to be specific for hepcidin-25 (Fig. 2A) and did not recognize hepcidin-20 or hepcidin-22. To ensure that hepcidin-20 and hepcidin-22 were not being generated from hepcidin-25 during the activation of proteolytic enzymes associated with the clotting process, we collected matching serum and EDTA plasma samples from 6 healthy donors at 2 different time points (12 serum samples and 12 matching EDTA plasma samples). We compared hepcidin-25 results obtained from each pair of matched samples by using the ELISA. All serum results were within 15% of the EDTA plasma values, suggesting that hepcidin-20 and hepcidin-22 were not generated to any appreciable degree during the clotting process. Finally, ELISA dilution curves for the recombinant standard and actual human serum samples were determined to be parallel, and ELISA demonstrated dilutional linearity.

On the basis of these results, we compared the sandwich ELISA method to a previously described gold standard LC-MS assay shown to be specific for hepcidin-25 (28) by using 52 human serum samples from a mixture of healthy individuals and cancer patients. The hepcidin-25 ELISA values showed a correlation of r = 0.98 (P < 0.001) with the LC-MS values, confirming that the sandwich ELISA specifically measured hepcidin-25.

Freeze-thaw stability was evaluated by testing 4 different serum samples. The results showed a consistent 80%–120% recovery, even after 5 freeze-thaw cycles (sample A: 0.16, 0.16, 0.17, 0.17, and 0.17 µg/L, respectively; sample B: 4.5, 4.4, 4.6, 4.6, and 4.4 µg/L, respectively; sample C: 8.9, 9.6, 9.7, 9.9, and 9.6 µg/L, respectively; sample D: 15.1, 15.1, 15.4, 15.7, 15.4, and 15.4 µg/L, respectively). The imprecision of the ELISA was assessed by using human serum samples containing 0.16, 4.5, and 15.1 µg/L of endogenous hepcidin-25. Intraassay (n = 20) imprecision results (CVs) were 3.4%, 4.5%, and 3.5%, respectively.

To assess the recovery of hepcidin-25 added into human serum, hepcidin-25 protein was added to 3 different human serum samples, each containing very low concentrations of endogenous hepcidin-25, at concentrations of 250, 25, 2.5, and 0.25 µg/L. These samples were analyzed by using the sandwich ELISA. Mean
(SD) results were 287 (6) μg/L, 24.2 (0.2) μg/L, 2.0 (0.1) μg/L, and 0.23 (0.01) μg/L, respectively.

Next, 100 serum samples from healthy individuals (50 men and 50 women) were analyzed by ELISA. The concentrations of hepcidin-25 ranged from <0.02 to 25 μg/L (median 1.20 μg/L, 25%–75% range 0.42–3.07 μg/L) (Fig. 2A), consistent with the concentrations previously reported in healthy individuals by using an LC-MS assay (28). Hepcidin-25 concentrations in healthy individuals were lower in women (median 0.84 μg/L, 25%–75% range 0.42–2.35 μg/L) (Fig. 3B) than in men (median 1.70 μg/L, 25%–75% range 0.57–4.76 μg/L). Hepcidin-25 concentrations in these 100 individuals were also compared with serum ferritin concentrations (Fig. 3C) and were found to correlate with serum ferritin ($r = 0.71$, $P < 0.001$). Not surprisingly, hepcidin-25 concentrations were only modestly correlated with hemoglobin concentrations ($r = 0.22$, $P = 0.03$). We observed that 4 of 50 men and 27 of 50 women had ferritin concentrations <10 μg/L (Fig. 3C), suggesting that many individuals were iron deficient. Iron-deficient individuals would be expected to have very low hepcidin concentrations, which is what we observed. All but 4 of these individuals had a hepcidin-25 concentration of <1 μg/L, and the remaining 4 individuals had hepcidin-25 concentrations between 1 and 2 μg/L. Because the data in Fig. 3A did not appear to be evenly distributed, we also plotted the data as a frequency distribution of log hepcidin. A much more even distribution of data was observed (Fig. 3D), with a log hepcidin mean of 0.01 and a 95% CI of $-0.14$ to $0.16$.

Finally, we compared sandwich ELISA hepcidin-25 results in patients with cancer ($n = 34$) and rheuma-
tients with cancer (median 54.8 μg/L, 25%–75% range 23.2–85.8 μg/L, n = 21) and women (median 54.8 μg/L, 25%–75% range 22.1–93.5 μg/L, n = 13) who had cancer. Interestingly, cancer patients with hematological (median 76.3 μg/L, 25%–75% range 54.8–112.0 μg/L, n = 17) and nonhematological malignancies (median 38.9 μg/L, 25%–75% range 16.2–44.2 μg/L, n = 17) each demonstrated markedly increased hepcidin-25 concentrations compared with healthy individuals.

Because of the log-normal distribution of hepcidin-25 concentrations for healthy individuals (Fig. 3D), group distributions were not assumed to be normal. Therefore, statistical analyses were performed that did not assume normality. For each group of individuals or patients studied, the median, 25th percentile, 75th percentile, and interquartile range were summarized (Table 1). Next, comparisons were performed between respective groups by using the Wilcoxon non-parametric rank sum test. The results from these comparisons (Table 2) indicated that for each comparison performed, the differences in hepcidin-25 concentrations between respective groups were statistically significant.

**Discussion**

Our results demonstrate that the sandwich ELISA described here is capable of measuring hepcidin-25 concentrations in human serum. This work builds upon previously reported assays for hepcidin (25, 26, 30–33). Compared with previous reported immunoassays, however, which are single antibody and competitive (25, 26, 30–33), this assay uses 2 independent monoclonal antibodies in a sandwich format to specifically measure hepcidin-25.

Compared with existing assays that use a competitive format (either ELISA or RIA), this method has advantages inherent in the sandwich assay format. In particular, responses are directly correlated with increasing hepcidin concentrations. In contrast, in competitive ELISA methods, absorbance values are inversely correlated with hepcidin concentrations (25, 26, 30–33). Also, because competitive ELISA methods rely on a single antibody, specificity may be an issue, and indeed, some competitive assays may cross-react with prohepcidin or other inactive species, making results difficult to interpret (25, 26). Another advantage of our assay is its improved limit of quantification of 0.01 μg/L compared with existing assays (25, 26, 30–33). This may be especially important, since we observed that 44% of healthy volunteers had serum hepcidin-25 concentrations of <0.9 μg/L. Interestingly, 31 of 100 healthy individuals (4 men and 27 women) also had very low ferritin concentrations (<10 μg/L), suggesting that some healthy donors, particu-

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**Fig. 2. Development of the hepcidin-25 sandwich ELISA method.**

(A), Calibration curve (synthesized hepcidin-25) starting at a concentration of 10 μg/L with serial dilutions. Anti-hepcidin antibodies were oriented as anti-hepcidin-2 capture and anti-hepcidin-1 conjugate. (B), Comparison of hepcidin-25 ELISA to a previously described LC-MS method for hepcidin-25 (28).
larly premenopausal women, may be iron deficient, perhaps because of menstrual blood loss and/or frequent blood donations. As expected, all individuals with ferritin concentrations below 10 µg/L had very low hepcidin-25 concentrations.

As a result of the 2 antibodies used, our sandwich ELISA is specific for hepcidin-25 and correlates extremely well with a previously described gold standard method LC-MS assay for hepcidin-25 (28). From a practical standpoint, the advantage of an ELISA over an LC-MS is that the ELISA can be implemented in clinical laboratories that do not have the complex equipment or the highly specialized operator expertise required to perform LC-MS type assays. In addition, ELISA has the potential for higher throughput than an LC-MS assay and therefore provides the basis for first dual monoclonal sandwich immunoassay, which can be used to measure hepcidin-25.

Fig. 3. Further characterization of the hepcidin-25 ELISA.
(A), Distribution of serum hepcidin-25 concentrations for 50 healthy men and 50 healthy women spanning a broad ethnic distribution as described in Materials and Methods. (B), Comparison of serum hepcidin-25 concentrations for men and women. (C), Hepcidin-25 ELISA concentrations for 50 men and 50 women compared with serum ferritin concentrations for the same individuals. (D), Hepcidin-25 ELISA concentrations plotted on a frequency distribution graph versus log hepcidin.

Fig. 4. Comparison of ELISA hepcidin-25 concentrations in healthy individuals with the concentrations in patients with cancer and rheumatoid arthritis.
This higher throughput is particularly relevant for a clinical assay that measures human serum hepcidin-25 concentrations (1, 21). The sandwich ELISA developed and described here can be used clinically to further increase our understanding of the role of hepcidin-25 in regulating iron metabolism. Using ELISA, we were able to demonstrate that circulating hepcidin-25 concentrations were increased in patients with cancer and rheumatoid arthritis (34) compared with healthy individuals. Data obtained from cancer patients, however, must be interpreted with caution. We studied a limited number of patients, and some of the most common cancer types were relatively underrepresented. It will be important to investigate additional factors such as the effect of radiation (which may induce cytokines) and coexisting infections on hepcidin-25 concentrations in patients with different tumor types.

In addition to these observations, there are several other uses for the sandwich ELISA that we developed. In cases of suspected hereditary hemochromatosis, the low limit of quantification of ELISA should be able to indicate whether abnormally low concentrations of hepcidin are responsible for the disease, thus obviating the need for a complex genetic workup. In suspected cases of secondary iron overload, measurement of serum hepcidin-25 concentrations would provide useful information as well (35, 36). In patients with anemia of chronic disease that is unresponsive to erythropoietin, this hepcidin ELISA may be used to verify that increased serum hepcidin is at least partly responsible for the erythropoietin-resistant anemia. Likewise, in the setting of chronic renal disease, this ELISA could be used to determine serum hepcidin concentrations to better direct therapy.

This sandwich ELISA could also be used to help diagnose iron deficiency anemia in difficult cases in which it may coexist with anemia of chronic disease. In uncomplicated iron deficiency, hepcidin concentrations would be expected to be quite low, whereas in anemia of chronic disease without coexisting iron deficiency, hepcidin concentrations would be expected to be increased (1, 7–10). Thus, in patients with anemia of chronic disease, a relatively low serum hepcidin concentration might also indicate the presence of coexisting iron deficiency.

In summary, our hepcidin sandwich ELISA should help to improve our understanding of the role of hepcidin in regulating iron metabolism. Unlike recently described immunoassays (25, 26, 30–33), the use of the 2 antibodies in the sandwich format provides specificity for the active form of the protein, a limit of quantification of 0.01 μg/L, and a broad dynamic range. It is thus possible that this assay or one similar to it could become part of the routine iron profile panel performed on automated clinical instruments. As a result, patient workups that currently include serum iron, ferritin, and percent transferrin saturation may someday also include hepcidin-25.

### Table 1. Hepcidin-25 concentrations for groups of healthy individuals or patients.\(^a\)

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<th>n</th>
<th>25th percentile</th>
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<th>75th percentile</th>
<th>Interquartile range</th>
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<td>10.6</td>
<td>18.4</td>
<td>12.5</td>
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</table>

\(^a\) For each group of individuals or patients studied, the median, 25th percentile, 75th percentile, and interquartile range were determined.

### Table 2. Comparisons of hepcidin-25 concentrations between respective groups of healthy individuals and patients.\(^a\)

<table>
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<td>Healthy men (n = 50) vs healthy women (n = 50)</td>
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<td>Non-hematological cancer patients (n = 17) vs healthy individuals (n = 100)</td>
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<tr>
<td>Hematological cancer patients (n = 17) vs healthy individuals (n = 100)</td>
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<tr>
<td>Rheumatoid arthritis patients (n = 76) vs healthy individuals (n = 100)</td>
<td>&lt;0.001</td>
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</table>

\(^a\) Comparisons of hepcidin-25 concentrations between healthy men and women and between healthy individuals and patients with cancer or rheumatoid arthritis were performed by using a Wilcoxon nonparametric rank sum test.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data, (b) drafting.
or revising the article for intellectual content, and (c) final approval of the published article.

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