

Immunochemical and Mass-Spectrometry–Based Serum Heparin Assays for Iron Metabolism Disorders

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BACKGROUND: Heparin is an iron-regulatory peptide hormone that consists of 3 isoforms: bioactive heparin-25, and inactive heparin-22 and heparin-20. Heparin is instrumental in the diagnosis and monitoring of iron metabolism disorders, but reliable methods for its quantification in serum are sparse, as is knowledge of their relative analytical strengths and clinical utility.

METHODS: We developed a competitive (c)-ELISA and an immunocapture TOF mass-spectrometry (IC-TOF-MS) assay. Exploiting these 2 methods and our previously described weak cation exchange (WCX)-TOF-MS assay, we measured serum heparin concentrations in 186 patients with various disorders of iron metabolism and in 23 healthy controls.

RESULTS: We found that (a) the relative differences in median heparin concentrations in various diseases to be similar, although the absolute concentrations measured with c-ELISA and WCX-TOF-MS differed; (b) heparin isoforms contributed to differences in heparin concentrations between methods, which were most prominent in patients with chronic kidney disease; and (c) heparin concentrations measured by both the c-ELISA and IC-TOF-MS correlated with ferritin concentrations <60 µg/L, and were suitable for distinguishing between iron deficiency anemia (IDA) and the combination of IDA and anemia of chronic disease.

CONCLUSIONS: c-ELISA is the method of choice for the large-scale quantification of serum heparin concentrations, because of its low limit of detection, low cost, and high-throughput. Because of its specificity for bioactive heparin-25, WCX-TOF-MS can be regarded as a valuable special-purpose assay for disorders with

variable concentrations of heparin isoforms, such as chronic kidney disease.

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Heparin is a hepatocyte-produced peptide hormone that regulates systemic iron homeostasis (1, 2). The mature bioactive form of heparin is a 25 amino acid peptide. Other isoforms in human blood and urine are the N-terminal truncated heparin-20 and -22 peptides, which are without apparent biological function (3). By modulating heparin production, an organism controls intestinal iron absorption, iron uptake, and mobilization from stores to meet the body iron need (1, 2). Heparin concentrations are decreased in conditions that demand increased serum iron concentrations (i.e., increased erythropoietic activity and iron deficiency), whereas concentrations are increased in infection and inflammation (4, 5).

Since the discovery of heparin and its crucial role in iron homeostasis, there has been substantial interest in developing reliable assays to measure heparin concentrations in body fluids. Accurate assessment of heparin concentrations in serum would improve our understanding of iron metabolism disorders and allow heparin to become a useful tool in the differential diagnosis and clinical management of these diseases.

Few investigative tools have been available for measuring heparin in biological fluids (6–9). We and others have reported related serum heparin quantification methods (10–18). Assays based on mass spectrometry (MS)⁶ require relatively expensive equipment, but these methods are advantageous because they can be used to distinguish between heparin-25, -22, and -20. Immunoassays, on the other hand, are

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⁶ Nonstandard abbreviations: MS, mass spectrometry; c-ELISA competitive ELISA, IC, immunocapture; WCX, weak cation exchange; IDA, iron deficiency anemia; ACD, anemia of chronic diseases; MM, multiple myeloma; HH, hereditary hemochromatosis; CKD, chronic kidney disease; RA, rheumatoid arthritis; LLOD, lower limit of detection; pl, isoelectric point.

more accessible for clinical laboratories, but measure only total hepcidin concentrations. Differences in methods and their analytical performance hinder the comparability of hepcidin data (19).

Here we report the development and validation of a competitive (c)-ELISA as a replacement for our previously described RIA (17), and a novel immunocapture (IC)-TOF-MS method, which combines antibody-based capture of hepcidin by IC and detection by TOF-MS. We compared hepcidin concentrations measured by using these novel methods with concentrations measured with our previously described weak cation exchange (WCX)-TOF-MS assay (10, 18) in 186 samples from patients with different iron metabolism disorders and 23 healthy controls. We determined the differences in absolute concentrations, and the degree to which the presence of hepcidin isoforms influenced the concentrations. We assessed the extent to which these methods reflect physiological responses to low iron stores, and whether the methods were able to differentiate between samples from patients with iron-deficiency anemia (IDA) and those with the combined presence of IDA and anemia of chronic disease (ACD).

Materials and Methods

SAMPLES

For our study of 3 hepcidin assays we used serum samples from 186 patients with a variety of iron disorders and 23 healthy volunteers. Samples were collected from all study participants from February 2006 to May 2009. Blood was drawn into BD Vacutainer® SST™ II Advance collection tubes and centrifuged for 10 min at 3600g. Serum was then divided into aliquots that were placed in polypropylene tubes, stored at -80°C , and thawed immediately before use. The samples were collected in accordance with protocols approved by the relevant institutional review boards, and informed consent was obtained in accordance with the Declaration of Helsinki. The definition of sample selection is described in detail in Supplemental Data 1 in the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol56/issue10>. Briefly, for the overall comparison of the 3 hepcidin assays, a total of 209 samples were collected from (a) healthy controls ($n = 23$) (20), (b) IDA patients ($n = 10$), (c) ACD patients ($n = 10$), (d) multiple myeloma patients ($n = 6$), (e) HFE-hereditary hemochromatosis (HH) patients at presentation ($n = 9$), (f) iron-depleted HFE-HH patients ($n = 8$) (21), (g) C282Y/H63D HFE compound-heterozygous HH patients at presentation ($n = 5$), (h) iron-depleted hemojuvelin-mutated HH patients ($n = 3$), (22) (i) chronic kidney disease (CKD) patients ($n = 84$), (18)

(j) coronary artery bypass graft surgery patients ($n = 22$), (23) (k) septic shock (sepsis) patients ($n = 19$), (l) healthy volunteers who were injected with lipopolysaccharide ($n = 5$), (7) and (m) metabolic syndrome patients ($n = 5$).

To evaluate the clinical utility of the 3 hepcidin assays, we selected samples from 27 anemic patients with rheumatoid arthritis (RA), and 19 samples from patients with ferritin concentrations $<60 \mu\text{g/L}$, all of whom had laboratory results that excluded the presence of inflammation, HH, or matriptase-2 defects (24).

LABORATORY MEASUREMENTS

Using an Abbott Aeroset analyzer, we measured total serum iron by the ascorbate/FerroZine colorimetric method, serum creatinine by enzymatic/colorimetric detection (Roche Diagnostics B.V.), and C-reactive protein by immunologic agglutination detection with latex-coupled polyclonal anti-C-reactive protein antibodies (Abbott B.V. Diagnostics Division). We quantified serum ferritin with an Immulite 2500 immunometric assay (Siemens). Routine hematologic characteristics were assessed by a Sysmex XE-2100 analyzer (Goffin Meyvis).

STUDY DESIGN

Hepcidin concentrations were measured in a blinded fashion by 3 different in-house-developed methods: our previously described WCX-TOF-MS assay (10, 18), a c-ELISA, and IC-TOF-MS. The latter method was developed to measure samples with concentrations below the lower limit of detection (LLOD) ($<0.5 \text{ nmol/L}$) of the WCX-TOF-MS assay.

WCX-TOF-MS and c-ELISA were used to quantify hepcidin concentrations in all 209 samples, whereas IC-TOF-MS was used to measure hepcidin in 25 samples with hepcidin concentrations below 0.5 nmol/L as assessed by WCX-TOF-MS. These samples were obtained from patients with IDA ($n = 10$), iron-depleted juvenile HH ($n = 3$), HFE-related HH ($n = 9$), and multiple myeloma ($n = 1$), and from healthy female controls ($n = 2$).

We further explored the clinical utility of the 3 assays by using samples from 27 anemic RA patients, categorized by conventional parameters into 3 groups (see online Supplemental Data 1), i.e., IDA ($n = 8$), combined IDA and ACD ($n = 8$) and ACD ($n = 11$), and samples from 19 patients with ferritin $<60 \mu\text{g/L}$. The samples from these 19 patients with ferritin $<60 \mu\text{g/L}$ included all 10 samples from patients with IDA included in the total group of 209 samples described above, and samples from 9 additional patients.

In this study, we expressed hepcidin concentrations in nanomoles per liter ($1 \text{ nmol/L} = 2.789 \mu\text{g/L}$).

BLANK AND REFERENCE SERUM, ANTIBODIES, AND HEPCIDIN ISOFORMS

The blank serum used in this study was obtained from an iron-depleted patient with juvenile hemochromatosis (10, 22). Hecpidin concentrations in this patient were below the LLOD as assessed by WCX-TOF-MS (10, 18).

The reference sample used in the immunochemical assays (denoted 140799) was generated by pooling 474 routine patient serum samples collected at the Department of Laboratory Medicine of the Radboud University Nijmegen Medical Center. The pool was divided into aliquots that were placed in glass vials (500 $\mu\text{L}/\text{vial}$), lyophilized, sealed, and stored at 4 °C.

For the immunochemical assays we used a previously described in-house prepared polyclonal rabbit-antihepcidin-25 antibody (17).

Hepcidin-20 [isoelectric point (pI), 8.53], hepcidin-22 (pI, 8.53), hepcidin-24 (pI, 8.51), and hepcidin-25 (pI, 8.22) were used to determine cross-reactivity in the various methods (ExpASY Proteomics Server, http://www.expasy.ch/tools/pi_tool.html). Isolated urinary hepcidin-20 and -22 were kindly provided by E. Nemeth, University of California, Los Angeles. Synthetic hepcidin-24 and -25 were obtained from Peptide International (www.pepnet.com).

We assessed the concentration of all hepcidin isoforms by direct MALDI-TOF-MS analysis, using hepcidin-24 as an internal standard. The final assigned concentration of hepcidin-25 was identical to that provided by Peptide International (assessed by amino acid sequence analysis).

PREVIOUSLY DESCRIBED HEPCIDIN ASSAY

We performed WCX-TOF-MS as described previously by a combination of WCX bead-based hepcidin enrichment followed by TOF-MS (10, 18). An internal standard (synthetic hepcidin-24; Peptide International) was used for quantification. Mass-to-charge (m/z) spectra were generated by using MALDI-TOF-MS (Microflex LT, BrukerDaltonics). Spectra were analyzed by using Bruker Daltonics FlexAnalysis software. A detailed protocol of this method is described in online Supplemental Data 2. Total hepcidin concentration was defined as the sum of hepcidin-25, -22, and -20 concentrations.

The LLOD of this method for serum was 0.5 nmol/L, with an intraassay CV of 3.7% at 7.9 nmol/L, 2.3% at 13.4 nmol/L, and 2.2% at 3.1 nmol/L. The interassay CV was 9.1% at 7.8 nmol/L and 3.9% at 12.9 nmol/L. The median reference concentration of serum hepcidin-25 has previously been determined to be 4.2 nmol/L (range 0.5–13.9 nmol/L) (18).

NOVEL HEPCIDIN ASSAYS

Competitive ELISA. 96-Well plates (Nunc Maxisorb™ flat bottomed) were coated overnight with goat-antirabbit IgG (Fc) antibody, blocked with BSA for 2 h, and then incubated with rabbit-antihuman hepcidin antibody for 2 h (17). Next, the study samples, the reference sample, and a biotinylated hepcidin-25 calibrator were added to the wells and incubated overnight at 4 °C. The plates were then incubated for 1 h with conjugate, and substrate was added for 15 min. The color reaction was stopped and absorbance measured at 492 nm. A more detailed protocol is described in online Supplemental Data 3.

Immunocapture TOF-MS. Rabbit-antihuman hepcidin antibody was first coupled to protein A sepharose beads (17). Next, serum and the internal standard, hepcidin-24, were incubated for 1 h with the beads-antibody complex. Hecpidin was then eluted from the beads with 50% acetonitrile and 0.5% trifluoroacetic acid, and 1 μL was applied to a MicroScout Plate 96 polished steel plate, and mass-to-charge (m/z) spectra were generated by using TOF-MS as described for the WCX-TOF-MS assay. A detailed protocol is described in online Supplemental Data 4.

Validation of c-ELISA. We used hepcidin-25 as a calibrator to generate the dose–response curve for the c-ELISA. The analytical LLOD was estimated as the minimum hepcidin concentration evoking a response 2.5-fold the SD of the zero calibrator. To assess the functional LLOD, we constructed plots of CVs of duplicate measurements of different serum samples measured in several dilutions vs concentration. Duplicate CVs of the reference preparations in all hepcidin assays were used to assess the intra- ($n = 12$) and interassay ($n = 40$) imprecision. Assay specificity was tested by constructing dose–response curves for hepcidin-20, hepcidin-22, and hepcidin-25. The analytical sensitivity was calculated at the 95% level of confidence on the basis of general variance of experimental data around the calibration curves. The functional sensitivity was calculated from the precision profile (at 20%) constructed from the CVs of duplicate measurements of 953 routine samples.

All measurements were performed in duplicate. The dose–response curve was approximated in a sigmoid 4 parameter logistic model: $y = [d + (a)d]/[1 + (x/c)^b]$, in which x stands for the analyte concentration and y for the intensity measured.

Validation of IC-TOF-MS. The functional LLOD was determined as a peak intensity of a signal/noise ratio = 3. The intraassay CV for hepcidin-25 was determined with 3 human serum samples with concentrations of

Table 1. Analytical characteristics of hepcidin assays.

	WCX-TOF-MS	c-ELISA	IC-TOF-MS
Analytical LLOD, pmol/L	NA ^a	20.8	NA
Functional LLOD, pmol/L	500	26.5	100
Intraassay CV, range, %	2.2–3.7	4.8	3.9–13.1
Interassay CV, range, %	3.9–9.1	11.2	—
Recovery, range; mean, %	96–102; 99	86–114; 98	95–105; 100
Linearity	Yes	Yes	Yes
Parallelism	—	Yes	—
Cross-reactivity hepcidin-20, %	0	68	0 ^b
Cross-reactivity hepcidin-22, %	0	47	0 ^b

^a NA, not applicable; —, not determined; analytical LLOD, lowest level that can be detected based on a protein standard; functional LLOD, lowest level that can be detected based on human serum samples.
^b No cross-reactivity was indicated because MS results are specific for hepcidin-25.

0.1, 0.2, and 1.3 nmol/L, respectively (n = 8 for all samples).

Cross-reactivity of the antibody with hepcidin-24 was tested by the addition of both peptides (hepcidin-24 and -25) to a blank serum in a similar concentration. After the immunocapture procedure, the hepcidin-24 concentration relative to that of hepcidin-25 was assessed.

We calculated recovery of hepcidin-25 as described for the WCX-TOF-MS method (n = 4).

STATISTICAL ANALYSIS

The Spearman correlation, the *t*-test of the independent samples, and the Bland–Altman analysis were used to determine relationships and differences between the various variables, as indicated in the results section and Figure legends. A 2-sided *P* < 0.05 was considered statistically significant. All statistical analyses were performed with Statistical Package for the Social Sciences (SPSS) 16.0 statistical software, except for the Bland–Altman plots, which were obtained by using GraphPad Prism 4.0 software (GraphPad Software). When hepcidin concentrations were determined to be <LLOD, a value of 0.5 × LLOD was assigned for statistical analysis, which implicates a value of 0.25 nmol/L for the WCX-TOF-MS results and 0.05 nmol/L for the IC-TOF-MS results.

Results

POPULATION CHARACTERISTICS

The population characteristics for the 209 samples from patients with various iron disorders are depicted in online Supplemental Table 1.

CHARACTERISTICS OF HEPCIDIN ASSAYS

The analytical characteristics for the WCX-TOF-MS, c-ELISA, and IC-TOF-MS methods were determined in parallel and are summarized in Table 1 and described in detail in online Supplemental Data files 3 and 4.

Next, hepcidin concentrations in clinical samples (see online Supplemental Table 1) were measured by these 3 methods: WCX-TOF-MS (n = 209), c-ELISA (n = 209), and IC-TOF-MS (n = 25, all samples that measured <0.5 nmol/L in the WCX-TOF-MS). Hepcidin medians and ranges obtained by these 3 methods for the various diseases are shown in online Supplemental Table 2. Overall, median total serum hepcidin concentrations measured by the c-ELISA were moderately, but significantly, higher (*P* < 0.0001) compared with hepcidin-25 concentrations measured by WCX-TOF-MS (median 6.6 nmol/L, range 0.17–77.5 vs 4.6 nmol/L, range <0.5–82.5 nmol/L, n = 209; online Supplemental Table 2). We found significant positive Spearman correlations (*P* < 0.001) between hepcidin-25 as well as total hepcidin measured by WCX-TOF-MS and c-ELISA total hepcidin (*R* = 0.912, Fig. 1A; *R* = 0.920, Fig. 1B). In addition, IC-TOF-MS hepcidin-25 concentrations correlated significantly (*P* < 0.0001) with total hepcidin measured by c-ELISA (*R* = 0.743, Fig. 1C).

Bland–Altman plots illustrated these overall higher concentrations measured by c-ELISA compared with WCX-TOF-MS (Fig. 1, D and E). The relative differences between c-ELISA and WCX-TOF-MS measurements were similar for WCX-TOF-MS-assessed hepcidin-25 (Fig. 1D) and total hepcidin concentrations (Fig. 1E). The hepcidin concentrations obtained

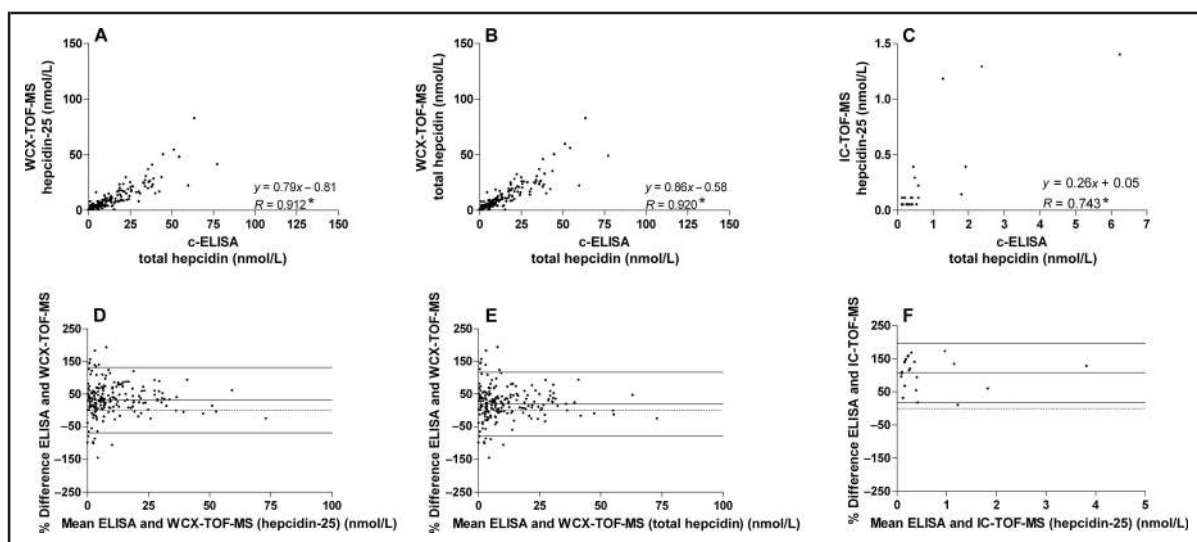


Fig. 1. Relation between the serum hepcidin methods.

Correlation of c-ELISA hepcidin concentrations with: (A), WCX-TOF-MS hepcidin-25 in the whole hepcidin range ($n = 209$); (B), WCX-TOF-MS total hepcidin in the whole hepcidin range ($n = 209$); and (C), IC-TOF-MS hepcidin-25 for samples measured as <0.5 nmol/L by WCX-TOF-MS ($n = 25$). Bland–Altman plots for (D), c-ELISA and WCX-TOF-MS (hepcidin-25) (bias = 31.9%, SD = 51.0%); (E), c-ELISA and WCX-TOF-MS (total hepcidin) (bias = 19.7%, SD = 49.7%); and (F), c-ELISA and IC-TOF-MS (hepcidin-25) (bias = 107.6%, SD = 46.1%). Total hepcidin of the MS assay is the sum of hepcidin-25, -22, and -20. Spearman R and the regression formula are shown. *, Significant with $P < 0.001$. The solid lines in D–F represent the mean percent differences with 95% limits of agreement of the samples. The limits of agreement are obtained by using the SDs of the percent differences between the methods. The dotted line represents the zero difference between methods depicted on the y axis.

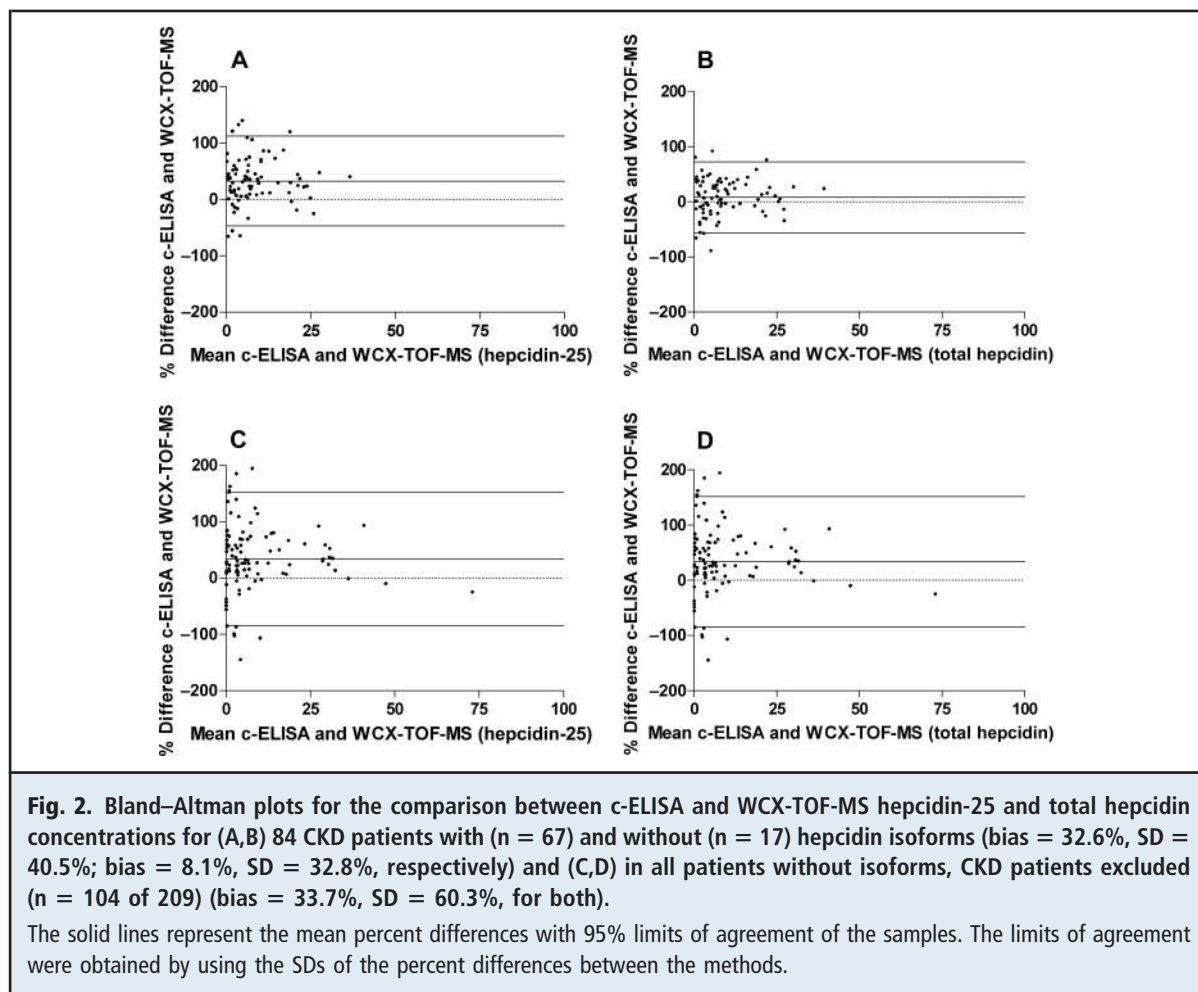
by c-ELISA were substantially higher than those measured by IC-TOF-MS (Fig. 1, C and F).

HEPCIDIN CONCENTRATIONS IN DISEASES WITH MEASURABLE ISOFORMS

The observed differences in absolute concentrations may be due to the inability of the c-ELISA to distinguish between the hepcidin isoforms, because it cross-reacts 47% and 68% with the respective hepcidin-22 and -20 isoforms in a given sample. This contrasts with TOF-MS assays, which are able to separately detect and quantify the hepcidin-25, -22, and -20 isoforms by their different masses. In serum samples from healthy controls and patients with low hepcidin concentrations, no isoforms were detected. However, exploiting this feature of WCX-TOF-MS, we detected hepcidin-22 and -20 isoforms in 67 of 84 patients with CKD, 2 of 22 patients after coronary artery bypass graft, 12 of 19 sepsis patients, and 7 of 10 patients with ACD. For patients with these disorders, the median percent hepcidin-25 concentrations of total hepcidin were 80.8% (range 31.6%–100.0%), 100.0% (83.5%–100.0%), 86.5% (55.9%–100.0%), and 85.3% (69.9%–100.0%), respectively. These wide ranges show that the contribution of

hepcidin-25 to total hepcidin is not fixed and differs between diseases and patients.

The prevalence and median contribution of isoforms to total hepcidin was highest for the CKD patients. More specifically, in the lower range (hepcidin-25 <5 nmol/L) 17.6% of the CKD patients had isoforms that contributed $>50\%$ to the concentration of total hepcidin, whereas for the whole range this was the case only for 8.3% of the patients. Therefore, we used this patient group ($n = 84$) to evaluate the contribution of hepcidin isoforms to total hepcidin concentrations in more detail. The mean CKD hepcidin-25 concentrations measured by WCX-TOF-MS were 1.4 times lower than the mean c-ELISA (total) hepcidin concentrations. This difference substantially decreased toward 1.1 times lower when we compared mean WCX-TOF-MS total hepcidin concentrations with c-ELISA hepcidin concentrations. The Bland–Altman plots (Fig. 2) illustrate these observations by showing that, for patients with isoforms, the relative difference between the c-ELISA and WCX-TOF-MS was higher when plotted for WCX-TOF-MS hepcidin-25 (Fig. 2A) than for WCX-TOF-MS total hepcidin (Fig. 2B). These differences between WCX-TOF-MS total hepcidin and hepcidin-25 plots were not



observed in patients without isoforms (Fig. 2, C and D).

CLINICAL UTILITY OF HEPCIDIN ASSAYS

To specifically address the potential clinical utility, we evaluated the extent to which our assays had the ability to distinguish between patient groups and to measure hepcidin concentrations reflecting a physiological response. For this evaluation we used the hepcidin concentrations that were determined in the 209 samples from patients with different iron metabolism disorders and healthy controls.

As shown in Fig. 3, the relative differences between median hepcidin concentrations observed for all diseases were similar for the WCX-TOF-MS and the c-ELISA. We also found that both methods reflected physiological responses, i.e., the median hepcidin concentrations were (a) higher for the inflammatory diseases (septic shock, lipopolysaccharide, ACD, and metabolic syndrome), (b) lower for IDA

compared to controls, and (c) similar for iron-overloaded HH patients and controls, but lower in iron-depleted HH patients. Moreover, wide concentration ranges for heterogeneous diseases such as CKD and the metabolic syndrome were observed (Fig. 3).

To assess if WCX-TOF-MS, c-ELISA, and IC-TOF-MS reflected the physiological response to low iron stores in the very low hepcidin range, we selected patients with IDA or ferritin concentrations $<60 \mu\text{g/L}$, in the absence of iron disorders other than iron deficiency. As expected, ferritin did not correlate with hepcidin concentrations measured by WCX-TOF-MS, because the majority of these samples had hepcidin concentrations below the LLOD of this method (Fig. 4A). In contrast, ferritin correlated significantly with hepcidin concentrations measured by c-ELISA and IC-TOF-MS ($R = 0.773$, $P < 0.001$ and $R = 0.789$, $P < 0.001$, respectively; Fig. 4, B and C). Occasionally, however, the c-ELISA provided relatively high hepcidin

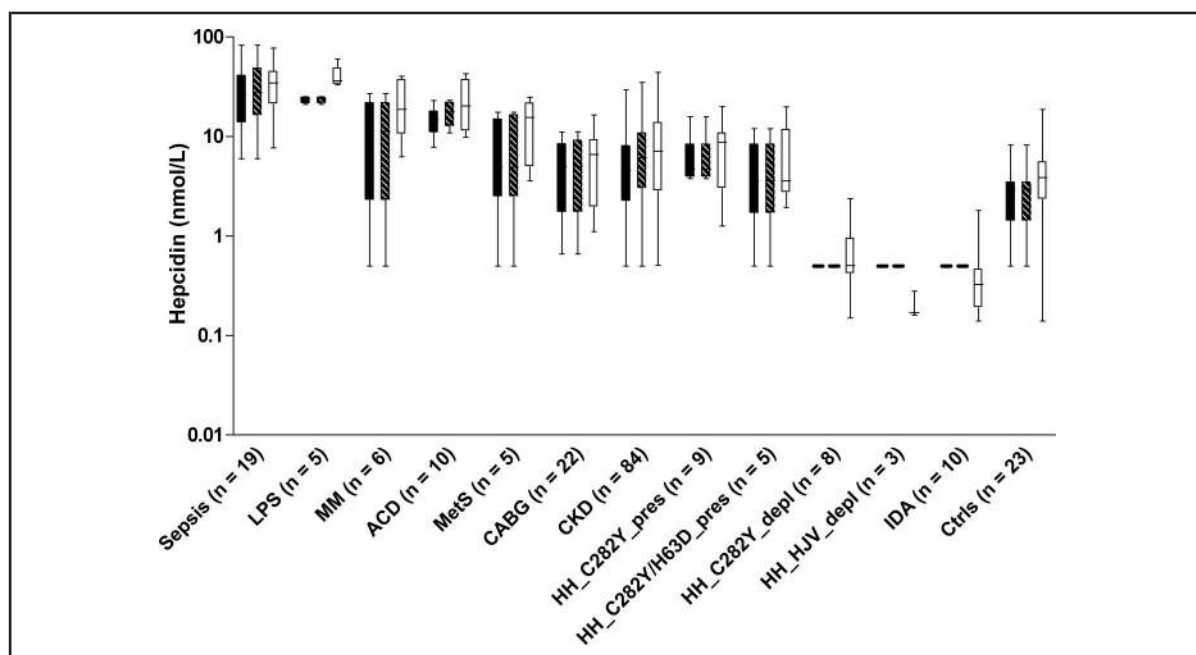


Fig. 3. Hepcidin concentrations in the various iron disorders as measured by the WCX-TOF-MS (hepcidin-25, closed bars; total hepcidin, striped bars) and c-ELISA (total hepcidin, open bars) hepcidin assays.

The boxes represent the 25th–75th percentiles and the error bars represent the minimum and maximum values with the median. LPS, healthy volunteers injected with lipopolysaccharide; MM, multiple myeloma; ACD, anemia of chronic disease; MetS, metabolic syndrome; CABG, coronary artery bypass surgery; CKD, chronic kidney disease; HH_C282Y_pres, homozygous hemochromatosis at presentation, before iron depletion therapy; HH_C282Y/H63D_pres, HFE C282Y/H63D compound heterozygotes before iron depletion therapy; HH_C282Y_depl, C282Y homozygous HFE hemochromatosis after iron depletion therapy; HH_HJV_depl, juvenile HH after iron depletion therapy; IDA, iron deficiency anemia; Ctrl, healthy controls.

concentrations in samples for which, based on low ferritin concentrations, a low hepcidin response was anticipated (Fig. 4B).

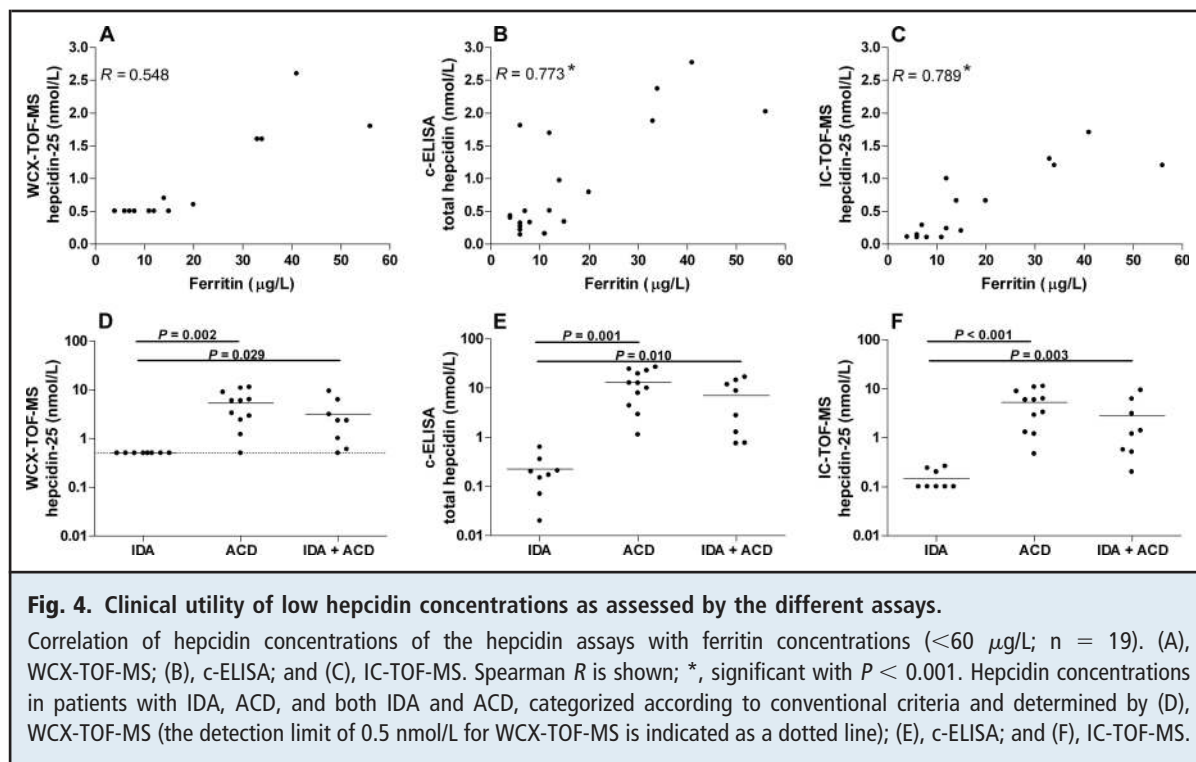
Finally, we assessed the ability of all 3 methods to differentiate between 3 forms of anemia in RA patients. These analyses indicated that both the c-ELISA and IC-TOF-MS were more suitable than WCX-TOF-MS (Fig. 4, D–F) for distinguishing patients with IDA from those with ACD and IDA.

Discussion

Our c-ELISA and IC-TOF-MS methods for quantifying serum hepcidin concentrations allowed us to perform a comparison of immunochemical and MS serum hepcidin assays, including our previously developed WCX-TOF-MS-assay. We assessed the pros and cons of these different methodologies for the quantification of serum hepcidin concentrations for a broad variety of patients with iron metabolism disorders.

Absolute hepcidin concentrations differed between c-ELISA and the WCX-TOF-MS, corroborating

previous findings (17). These differences might be attributed to different standards used, and the absence of a validated calibrator. However, this cause is unlikely in our case, because we used hepcidin-25 as an external standard for the c-ELISA and hepcidin-24 as an internal standard for the WCX-TOF-MS methods. These 2 standards were found to have identical binding characteristics to the surface of the WCX beads. Other factors that may have contributed to different concentrations measured by the different methodologies include: (a) differential aggregation of the synthetic or native hepcidin during workup (25); (b) serum matrix effects that differentially influence the recognition of the antibody for biotinylated synthetic hepcidin-25 or native hepcidin or differentially affect enzyme-linked coloration reactions in the c-ELISA assay; and (c) differences between the methods in measuring free or hepcidin bound to α -2-macroglobuline or albumin (26). Unfortunately, it is currently unknown if WCX-TOF or c-ELISA measures free or protein-bound hepcidin. This important question is a subject of our ongoing investigations.



Hepcidin isoforms were shown to be present in serum of patients with diseases characterized by increased hepcidin concentrations, such as ACD, sepsis, and CKD. The presence of hepcidin isoforms has been reported previously for patients with CKD and hemodialysis (6, 18) and these isoforms likely contributed to the here described differences in reported WCX-TOF-MS (hepcidin-25) and c-ELISA (total) hepcidin concentrations. To date, the role of hepcidin-20 and -22 isoforms in iron homeostasis is unclear. We therefore believe, especially in individual patient care, that it is important to be able to selectively quantify hepcidin-25, particularly in patients with diseases for which a high occurrence of hepcidin isoforms has been reported. Alternatively, immunochemical methods exploiting monoclonal antibodies that selectively recognize hepcidin-25 may prove to be useful.

Comparison of c-ELISA and IC-TOF-MS methodology, which is also referred to as mass spectrometric immunoassay, is not novel but has provided valuable insights as to the isoform specificity of assays (27). Here, we found that hepcidin concentrations in both assays—using the same antibodies—showed an increasing physiological response with increasing ferritin concentrations in the iron-deficiency range. Occasionally, however, the c-ELISA provided relatively high hepcidin concentrations in samples for which, based on low ferritin concentrations, a low hep-

cidin response was anticipated. Apparently, in contrast to IC-TOF-MS, the c-ELISA is responsive to matrix differences in these very low hepcidin ranges, which affects the interaction of the hepcidin antibody with either the native or labeled synthetic hepcidin. In these cases the internal standard used in the MS assays might better correct for matrix differences than the external standard in the c-ELISA. These subtle differences in assay performance may become important when their outcomes are used to guide individual patient treatment.

We found both the c-ELISA and the IC-TOF-MS to better differentiate anemic RA patients with IDA from those with IDA and ACD than WCX-TOF-MS. Notably, Theurl et al., using liquid chromatography tandem mass spectrometry in somewhat differently defined patients with chronic infections, autoimmune diseases, and malignancies, were not able to distinguish IDA patients from those with the combined presence of IDA and ACD (28). Therefore, both of our novel methods might prove to be valuable in the guidance of iron supplementation in anemic patients, among whom are patients with rheumatic diseases and patients in malaria endemic regions (29).

The results of our study revealed the pros and cons of the various hepcidin platforms. We found our c-ELISA to be more sensitive and less specific for hepcidin-25 compared to our WCX-TOF-MS assay. In our hands, sample throughput was higher for c-ELISA

than for MS assays. In theory MS methods, however, can also be high throughput by automation (30, 31). This technique requires robust protocols, because hepcidin readily sticks to laboratory plastics, especially with the use of small sample volumes. Although MS is an increasingly used technology, ELISA is still more common in clinical laboratories because it precludes the need for expensive equipment. Altogether, we believe that these observations and thoughts on the analytical performance and the suitability of the methods for routine clinical use are representative for these assays in general. However, it should be realized that the relative performance of c-ELISA and IC-TOF-MS assays (to be) developed in other laboratories is highly dependent on the affinity and specificity (cross-reactivity) of the used hepcidin antibodies.

We conclude that c-ELISA is the current method of first choice for the quantification of serum hepcidin, because of its low limit of detection, low costs, and high throughput. MS is preferred in disorders with increased concentrations of hepcidin isoforms, such as CKD, because of its specificity for bioactive hepcidin-25.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 re-

quirements: (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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