Clinical interpretation of serum hepcidin-25 in inflammation and renal dysfunction

Michael X. Chen a,b,c,*, Nathan Kuehne a, Andre Mattman d, Jun Liu b, Grace Van der Gugten d, Bruce Wright e

a Department of Laboratory Medicine, Pathology and Medical Genetics, Island Health, Victoria, British Columbia, Canada
b Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, British Columbia, Canada
c Division of Medical Sciences, Island Medical Program, University of Victoria, Victoria, British Columbia, Canada
d Department of Pathology and Laboratory Medicine, St Paul’s Hospital, Vancouver, British Columbia, Canada

1. Introduction

Hepcidin is a liver-derived peptide hormone that principally regulates systemic iron homeostasis. Serum hepcidin levels are under the influence of various stimuli, particularly inflammation and renal dysfunction. The measurement of hepcidin in circulation is a potentially useful clinical tool in the diagnosis, monitoring and treatment of iron metabolism disorder, although clinical interpretation of hepcidin level remains difficult. We evaluated the diagnostic potential and limitations of hepcidin-25 by investigating its relationship with iron and hematological indices, inflammation, and renal dysfunction.

Methods: This retrospective study included 220 adult patients not requiring dialysis. Variations of biologically active hepcidin-25 were examined using a mass spectrometry-based assay in various inflammatory and renal states. The log[hepcidin]:log[ferritin] ratio was calculated as an hepcidin index.

Results: In 220 adult patients not requiring dialysis, variation in hepcidin-25 level was significantly larger once CRP exceeded 10 mg/l (p < 0.001). Inflammation was not a determinant of hepcidin-25 in the setting of renal dysfunction. Hepcidin-25 median (7.37 nM) and variance were significantly higher (p < 0.001), once estimated glomerular filtration rate (eGFR) dropped below 30 ml/min/1.73 m². The log[hepcidin]:log[ferritin] index normalized hepcidin levels. Patients with iron deficiency have a notably lower index when compared to controls (-0.66 vs 0.3).

Conclusion: Severe renal dysfunction (eGFR < 30) affected hepcidin-25 expression and clearance to variable degree between individuals. Although, hepcidin-25 testing is not warranted in patients with infection, inflammatory autoimmune conditions (CRP > 10 mg/l) and/or severe renal dysfunction (eGFR < 30), the hepcidin index may serve as a potential biomarker for iron deficiency in complex cases.

Abbreviations: ACD, anemia of chronic disease; CBC, complete blood count; CKD, chronic kidney disease; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; Hb, hemoglobin; IDA, iron deficiency anemia; ID, iron deficiency; IRIDA, iron refractory iron deficiency anemia; LIS, lab information system; MCV, mean corpuscular volume; HPLC/MS/MS, high-performance liquid chromatography tandem mass spectrometry.

* Corresponding author at: Clinical Chemistry, University of British Columbia, 1 Hospital Way, Core Laboratory, Victoria, BC V8Z 6R5.

E-mail address: Michaelx.chen@ubc.ca (M.X. Chen).

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respond to oral iron therapy have high or non-suppressed serum hepcidin level [2,7]. The measurement of hepcidin in circulation is, therefore, a potentially useful clinical tool in the diagnosis, monitoring and treatment of iron metabolism disorders [8].

Analytical challenges continue to exist for antibody-based hepcidin measurement. Accurate quantification of bioactive hepcidin isoform, hepcidin-25 (25aminoacids in size) remains largely unavailable for clinical laboratories. Most immunoassays lack specificity for hepcidin-25 [9–11]. Measurement of total hepcidin levels is an overestimation and clinically not useful, as N-terminal degradation of hepcidin leads to smaller isoforms (hepcidin-24, –23, –22, and –20) of unknown clinical significance [12,13]. As a first step, we developed a high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) method for hepcidin-25 quantitation. This method provided an automatable platform for a simple and cost-effective assay suitable for clinical implementation [14]. Recent development of secondary reference material (sRM) for hepcidin-25 and ongoing international collaborations provide a framework for worldwide assay standardization [15–17].

Clinical interpretation of individual hepcidin-25 levels remains difficult. Production of hepcidin is under the influence of various, often opposing stimuli [7]. Daily excretion by the normal kidneys is approximatively proportional to serum hepcidin concentrations [9,18]. Many studies of healthy controls have revealed substantial inter-individual variation in circulating hepcidin levels, and, thus, wide reference intervals [9,10,19–21]. In a diseased state, serum hepcidin levels can be affected by iron status, anemia, hypoxia, renal insufficiency, and inflammation [22–25]. Due to this dynamic and multifactorial regulation, appropriate interpretation of hepcidin-25 levels requires additional biochemical and clinical context [7,26]. Iron deficiency can occur insidiously as a symptom or syndrome over a spectrum of severity [27].

In this study, we report the diagnostic potential and limitations of hepcidin-25 measurement in a broader context by investigating its relationship with iron and hematological indices, inflammation, and renal dysfunction. Relevant clinical states and biochemical cut-off points were identified to support clinical decision making. We also evaluated log[hepcidin]:log[ferritin] ratio as a potential index to discriminate high ferritin (due to interleukin-6 exposure) and low ferritin (due to iron deficiency) in renal dysfunction.

2. Materials and Methods

This retrospective study included 220 adult patients from Vancouver Island Health Authority, British Columbia, Canada. Patients < 19 years of age and hemodialysis patients were excluded. Patients with significant hyperferritinemia (ferritin > 600μg/L) were also excluded to avoid overlap with hemochromatosis. Study participants had fasting morning blood tests including Complete Blood Count (CBC) and iron studies, as a standard part of healthcare between May and July 2019. Lab Information System (LIS) were accessed to obtain demographic information, and relevant test results including ferritin, hemoglobin (Hb), mean corpuscular volume (MCV), creatinine, estimated glomerular filtration rate (eGFR), and C-reactive protein (CRP). Missing tests were added to original sample for analysis in VIHA clinical core laboratory. All add-on tests met clinical sample integrity and stability requirements.

Patient consent was waived due to the following reasons: (i) the study involves no more than minimal risk, (ii) waiving informed consent will not adversely affect the rights and/or welfare of the subjects, and (iii) it is not practicable to conduct the research without waiving consent. Whenever appropriate, participants will be provided with additional pertinent information after their participation.

Hb and MCV were determined on an automated hematology analyzer (Sysmex Corporation, Kobe, Japan). Iron studies were determined on an immunochemistry analyzer (Beckman Coulter, Fullerton, CA). CRP and creatinine determined on a clinical chemistry analyzer (Beckman Coulter, Fullerton, CA). The high-sensitivity CRP assay has a detection limit down to 0.3 mg/L. eGFR was derived for each sample from measured creatinine and calculated according to the CKD-EPI formula [26]. Hepcidin-25 quantitation was performed on all study participants using a high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) in-house method [14]. The assay has a linear analytical range of 0.1–100 nmol/L (r-squared > 0.99). Intra-day and inter-day imprecisions were < 3% and < 6%, respectively.

Clinical conditions and laboratory parameters that are currently in use in our health region are described in Table 1. Characteristics of participants included in the study are described in Table 2. For assessments of inflammation, samples were placed into one of four groups according to CRP values, guided by established cut-offs [28]. Clinically recommended CRP categories are low-average cardiovascular risk (<3 mg/l), high cardiovascular risk (>3 mg/l and < 10 mg/l), mild inflammation (CRP > 10 mg/l and < 40 mg/l), and bacterial infection (CRP ≥ 40 mg/l) [29–31]. Renal status was classified into CKD stages based on eGFR cut-offs defined by the National Kidney Foundation [32]. Patients with serum ferritin > 600 μg/L were excluded to ensure that hyperferritinemia were not associated with hemochromatosis. A serum ferritin > 600 μg/L provides a sensitive indicator of patients at risk for clinical manifestations of hemochromatosis warranting follow-up genetic testing.

2.1. Statistical analysis

Non-parametric Mann-Whitney and Kruskal-Wallis tests were used to assess differences in serum hepcidin-25 between sample groups. Whiskers depict the minimum and maximum non-outlier values. All statistical tests were two-sided using α = 0.05 as a cut-off to define statistical significance. Analyses of variance were conducted via unpaired Welch’s T test. Analyses were conducted in Prism version 8.4.2.

3. Results

3.1. Hepcidin-25 responses to pathophysiologic states

The relative differences between median hepcidin-25 concentrations observed for all clinical conditions are shown in Table 3 and Fig. 1. Control cohort (n = 36) were selected in absence of biochemical evidence of inflammation, renal insufficiency, iron disorder and anemia, as defined in Table 1. Overall, the median hepcidin-25 concentration was 3.18 nmol/l. The 2.5 percentile to 97.5 percentile was 0.560–13.0 nmol/l.

| Clinical conditions | Laboratory parameters |
|---------------------|-----------------------|
| Iron deficiency (ID) | Ferritin < 15 μg/l (female) or < 20 μg/l (male) |
| Hyperferritinemia    | Ferritin > 200 μg/l (female) and < 600 μg/l |
|                      | Ferritin > 300 μg/l (male) and < 600 μg/l |
| Non-ID Anemia        | Hemoglobin < 120 g/l (female) or < 130 g/l (male) |
| Inflammation status  | CRP categories: |
|                      | CRP < 3 mg/l |
|                      | CRP ≥ 3 mg/l and < 10 mg/l |
|                      | CRP ≥ 10 mg/l and < 40 mg/l |
|                      | CRP ≥ 40 mg/l |
| Renal status         | Chronic kidney disease (CKD) stages: |
|                      | Stage 1: eGFR ≥ 90 |
|                      | Stage 2: 60 ≤ eGFR < 90 |
|                      | Stage 3: 30 ≤ eGFR < 60 |
|                      | Stage 4: eGFR < 30 |
| Control              | All the following: |
|                      | Ferritin ≥ 20 μg/l (male) or ≥ 15 μg/l (female) |
|                      | Ferritin ≤ 200 μg/l |
|                      | Hemoglobin ≥ 120 g/l (male) or ≥ 130 g/l (female) |
|                      | CRP ≤ 10 mg/l |
|                      | eGFR ≥ 90 ml/min/1.73 m² |
Concentrations were also identified in higher CRP categories (CRP higher median hepcidin-25 concentrations of 6.90 nmol/l and 15.8 mg/l). Hepcidin-25 median was higher in female (3.92 nmol/l) than male (3.18 nmol/l), but failed to reach statistical significance (p = 0.56). Our data of the control group corroborated previously published studies reporting considerable variations in hepcidin-25 concentrations [20,21,52-54]. Although gender specific differences did not reach statistical significance. Our data clarified the degree of variation of hepcidin-25 from other isoforms [34,56] . Additionally, hepcidin-25 has established its usefulness in differentiating iron deficiency from anemia of chronic disease (ACD), and in guiding appropriate routes of iron therapy [7,26,44-48]. It has been well recognized that hepcidin-25 quantification needs to be isoform specific, harmonization is required to ensure assay quality, and results should be interpreted in the light of the renal and inflammatory status. However, since concomitant iron deficiency and inflammation are at interplay in renal insufficiency [23,49-51], practical recommendations are lacking.

Our data of the control group corroborated previously published studies reporting considerable variations in hepcidin-25 concentrations [20,21,52-54]. Although gender specific differences did not reach statistical significance. Our data clarified the degree of variation of hepcidin-25 from other isoforms [34,56]. Patients with hyperferritinemia due to iron deficiency anemia (IRIDA) [36,37] and iron overload disorders [38-43]. Additionally, hepcidin-25 has established its usefulness in differentiating iron deficiency from anemia of chronic disease (ACD), and in guiding appropriate routes of iron therapy [7,26,44-48]. It has been well recognized that hepcidin-25 quantification needs to be isoform specific, harmonization is required to ensure assay quality, and results should be interpreted in the light of the renal and inflammatory status. However, since concomitant iron deficiency and inflammation are at interplay in renal insufficiency [23,49-51], practical recommendations are lacking.

### Table 2

**Clinical and demographic characteristics (Mean ± SD).**

|                | Control (n = 36) | eGFR (n = 24) | Hyperferritinemia (n = 24) | Iron Deficiency (n = 24) | Anemia without ID (n = 25) |
|----------------|-----------------|---------------|---------------------------|-------------------------|--------------------------|
| Age (years)    | 42.5 ± 2.75     | 63.2 ± 2.17   | 64.8 ± 2.34               | 3.09 ± 0.40             | 3.18 ± 0.54              |
| Male (%)       | 33              | 43             | 44                        | 17                      | 19                       |
| Ferritin (μg/L)| 7.38 ± 17.4     | 127           | 113 ± 10.9                | 4.6 ± 0.1               | 1.96                     |
| Hb (g/L)       | 140 ± 2.41      | 127 ± 2.12    | 113 ± 10.9                | 4.6 ± 0.1               | 1.96                     |
| MCV            | 88.2 ± 0.90     | 93.5 ± 0.16   | 92.4 ± 0.18               | 2.18 ± 0.5              | 1.58                     |
| Creatinine (μmol/L) | 59.3 ± 123 | 73.9 ± 4.06   | 81.8 ± 5.1                | 5.1 ± 0.7               | 5.70                     |
| CRP (mg/L)     | 2.01 ± 3.02     | 2.12 ± 0.38   | 2.08 ± 0.32               | 0.392 ± 0.33            | 0.327 ± 0.37             |

ID = iron deficiency, eGFR = estimated glomerular filtration rate, Hb = hae moglobin, CRP = C-reactive protein, MCV = Mean Corpuscular Volume.

### Table 3

**Serum Heparidin-25 variations in different clinical states.**

| N      | Median (P2.5 - P97.5) | P-value* (median) | p-value* (variance) |
|--------|-----------------------|-------------------|---------------------|
| Control| 36                    | 3.18 (0.56-13.0)  | <0.05               |
| Inflammation|              |                   |                     |
| CRP < 9 | 129                  | 3.09 (0.10-13.9)  | <0.05               |
| CRP ≥ 9  | 36                    | 3.77 (0.14-20.3)  | <0.05               |
| CRP ≥ 10 < 40 | 18               | 6.90 (0.54-23.4)  | <0.001              |
| CRP ≥ 40 | 13                    | 15.8 (0.16-41.1)  | <0.001              |
| Renal function|                |                   |                     |
| eGFR ≥ 90 | 76                   | 2.50 (0.10-23.8)  | <0.05               |
| eGFR < 60 < 90 | 70                  | 3.92 (0.13-26.1)  | <0.05               |
| eGFR < 30 < 60 | 26                  | 3.96 (0.14-15.6)  | <0.05               |
| eGFR < 30 | 14                    | 7.47 (0.07-37.4)  | <0.001              |
| Iron Deficiency| 24                 | 0.26 (0.08-5.01)  | <0.001              |
| Hypoferritinemia| 24                 | 6.62 (0.73-13.7)  | <0.001              |
| Non-ID Anemia| 25                   | 4.54 (0.29-10.9)  | <0.05               |

*P values for comparisons between patients and controls.

### 3.2. log[heparidin]:log[ferritin] ratio as an index

The relative differences between log[heparidin]:log[ferritin] ratios observed for all clinical conditions are shown in Fig. 2 and Table 4. This log ratio normalized the hepcidin concentration to ferritin secretion. Median log ratios were comparable to the control group (0.30), except for the group with iron deficiency (-0.66), which was significantly reduced (p < 0.001). Small incremental increases in log ratio were observed as CRP increased and eGFR reduced, respectively. The changes did not reach statistical significance (p > 0.05).

### 4. Discussion

Iron-related disorders are common and clinically important. Hepcidin is the key regulator of iron balance and a promising companion diagnostic tool for iron disorders. Heparidin-25 is a mature bioactive peptide. N-terminal truncated hepcidin isoforms have shown to have little or no activity at the ferroportin (FP-1) receptor [33], and, therefore, are unlikely to have a significant effect on iron metabolism. Studies have identified promising applications of hepcidin-25 measurements in evaluations of iron deficiency anemia (IDA) [9,34,35], iron refractory iron deficiency anemia (IRIDA) [36,37] and iron overload disorders [38-43]. Additionally, hepcidin-25 has established its usefulness in differentiating iron deficiency from anemia of chronic disease (ACD), and in guiding appropriate routes of iron therapy [7,26,44-48]. It has been well recognized that hepcidin-25 quantification needs to be isoform specific, harmonization is required to ensure assay quality, and results should be interpreted in the light of the renal and inflammatory status. However, since concomitant iron deficiency and inflammation are at interplay in renal insufficiency [23,49-51], practical recommendations are lacking.

Our data of the control group corroborated previously published studies reporting considerable variations in hepcidin-25 concentrations [20,21,52-54]. Although gender specific differences did not reach statistical significance. Our mass spectrometry method measured relatively lower concentrations of hepcidin-25 than immunoassays [10,20,21], in agreement with evidence found in a recent round robin (interlaboratory) study [17,55]. The discrepancies were most probably due to improved selectivity of the mass spectrometry method in distinguishing hepcidin-25 from other isoforms [34,56]. Patients with hyperferritinemia due to metabolic derangement revealed a 2-fold increase in the hepcidin-25 median, indicating that hormone synthesis is responsive to iron storage. In iron deficiency (ID), hepcidin-25 levels were appropriately suppressed to allow maximal iron absorption.

There was also agreement that hepcidin-25 positively correlated with changes in inflammatory status measured by CRP levels [42,57-59]. However, our data clarified the degree of variation of hepcidin-25 in various clinically relevant inflammatory states. We noted that control group and patients with CRP < 10 mg/l had comparable
inter-individual variation in hepcidin-25 concentration. Once CRP rose above 10 mg/l, the inter-individual variation of hepcidin-25 were substantially larger and overlapped with the control group ($p < 0.001$). In such inflammatory states (CRP $> 10$ mg/l), single measurement of hepcidin-25 measurement will not be clinically meaningful in the evaluation of iron disorders.

We also found eGFR is not a major determinant of serum hepcidin-25 concentration in patients with eGFR $\geq 30$ ml/min/1.73 m$^2$ (CKD stage 3 or lower). In contrast, hepcidin-25 became significantly elevated, but more importantly, more variable ($p < 0.001$) once eGFR dropped below 30 ml/min/1.73 m$^2$. Admittedly, inflammation is regarded as a common comorbid condition in CKD, and inverse correlation between GFR and inflammation has been well recognized [60,61]. However, reproducible results were obtained when we corrected for variations attributable to inflammation (CRP $\geq 10$ mg/l). Therefore, CRP is not a relevant determinant of hepcidin-25 in the setting of renal insufficiency. Severe renal dysfunction (eGFR $< 30$ ml/min/1.73 m$^2$) affected hepcidin-25 expression and clearance to a significantly variable degree between individuals. This finding exposed an important caveat for hepcidin-25 interpretation as a companion diagnostic test for iron disorders.

Previous studies using immunoassays showed negative correlation of total hepcidin with eGFR in CKD [9,62,63]. A smaller study using MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry concluded that serum hepcidin-25 is independent of eGFR in CKD [58]. Our observation suggests renal handling of hepcidin-25 is dynamic and situational. Hepcidin-25 is a low molecular weight peptide that is filtered and reabsorbed similar to $\beta_2$-microglobulin or cystatin-C. But severe renal dysfunction (eGFR $< 30$ ml/min/1.73 m$^2$) might favor binding of hepcidin to other carrier proteins preventing it from being freely filtered.

Taken together, our findings clarify the usefulness and limitations of hepcidin-25 measurement and provide important caveats for clinical interpretation, although large inter-individual variations remain a concern during interpretation of hepcidin-25 measurement. Hepcidin-...
25 measurement is a promising tool to be added to the present battery of diagnostic tests for iron status, but it needs to be evaluated with CRP and eGFR cut-offs, or, alternatively, as an index adjusted for CRP and glomerular filtration rate.

In previous studies, an unadjusted hepcidin/ferritin ratio showed diagnostic potential in selected study populations. Ismail et al demonstrated that β-thalassemia children had lower hepcidin/ferritin ratio [64]. One recent meta-analysis concluded that the serum hepcidin/ferritin ratio was negatively associated with the risk of type 2 diabetes [65]. In the dialysis population, Nikura et al observed differences in hepcidin/ferritin ratio among patients on peritoneal dialysis and hemodialysis [66]. Our study compared hepcidin-25 levels in various renal, inflammatory, and iron states. We elucidated large degrees of intra- and interpersonal variation in hepcidin-25 level. Unadjusted hepcidin/ferritin ratio was calculated as an index, but it was inadequate to differentiate iron deficiency, especially in cohorts with high CRP or low eGFR (data not shown).

The proposed log[hepcidin]:log[ferritin] index normalizes the hepcidin-25 concentration to ferritin secretion whether the two proteins are co-produced by hepatocytes in response to increased hepatic iron stores or increased interleukin-6 exposure. This index demonstrated the ability to compensate for inflammation and for reduced eGFR. It allowed for comparison between groups with CKD varying from Stage 1 to Stage 4. Patients with iron deficiency have a notably lower index. This study has several limitations. First, it was a retrospective cross-sectional study, which cannot establish causality of the associations between hepcidin-25, ferritin, inflammation, and renal insufficiency. Secondly, urine collection and analysis were not part of standard of care, therefore, we did not measure hepcidin-25 in urine to evaluate and corroborate our hypothesis of renal handling of hepcidin-25. We also recognize that the number of patients in certain subgroups, especially those with significant inflammation (CRP > 10 mg/l) and renal insufficiency (eGFR < 30), were limited. Therefore, the resulting analysis should be more encompassing index, which normalizes hepcidin production to account for inflammation specifically via CRP, renal function via eGFR, anemia via hemoglobin, and iron stores via log ferritin, may prove useful in assessing hepcidin production for a given individual using readily available laboratory data. However, there was insufficient data in this retrospective study to systematically develop and assess such an index.

5. Conclusion

Hepcidin-25 testing is not warranted in patients with infection, inflammatory autoimmune conditions and/or severe renal dysfunction (CKD 3 +). The log[hepcidin]:log[ferritin] index may serve as a potential biomarker for iron deficiency in complex cases. Developing a more encompassing index, and assessing its diagnostic utility, will require a thorough understanding of hepcidin’s renal handling, larger studies, and likely use of the multiple of the median approach for reporting hepcidin-25 and ferritin measurements; both of which may vary according to the measuring laboratory in the absence of harmonized immunoassays for ferritin or mass spectrometry assays for hepcidin-25.

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Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the research ethics committee of Vancouver Island Health Authority (20191004QI).

Informed consent statement

Patient consent was waived due to the following reasons: (i) the study involves no more than minimal risk, (ii) waiving informed consent will not adversely affect the rights and/or welfare of the subjects, and (iii) it is not practicable to conduct the research without waiving consent. Whenever appropriate, participants will be provided with additional pertinent information after their participation.

Data availability statement

The data that have been used to support the findings of this study are available from the corresponding author upon request.

CRediT authorship contribution statement

Michael X. Chen: Conceptualization, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. Nathan Kuehne: Software, Formal analysis, Data curation, Writing – review & editing, Visualization. Andre Mattman: Investigation, Writing – review & editing. Jun Liu: Methodology, Validation. Grace Van der Gugten: Methodology. Bruce Wright: Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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