Serum hepcidin-25 levels in patients with chronic kidney disease are independent of glomerular filtration rate

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Abstract

Background. Hepcidin is a key regulator of iron homeostasis and levels are elevated in patients with chronic kidney disease (CKD). Hepcidin may explain the often observed imbalance in iron metabolism in patients with CKD. We evaluated the influence of estimated glomerular filtration rate (eGFR) on serum levels of hepcidin-25 and its isoforms in patients with renal dysfunction.

Methods. Serum levels of the biologically active hepcidin-25 and its isoforms were determined in CKD and dialysis patients by a mass spectrometry-based assay.

Results. In 83 patients with CKD not requiring dialysis, serum hepcidin-25 levels were not significantly increased (5.1 nM versus 4.2 nM, P = 0.30) and positively correlated with ferritin (r = 0.74, P < 0.01). Multiple regression analysis showed ferritin to be the only significant predictor of hepcidin-25 levels. Serum hepcidin-25 levels were not dependent on eGFR. In contrast, hepcidin-20 and total hepcidin levels showed an independent significant inverse correlation with eGFR. In 48 haemodialysis patients, median hepcidin-25 levels were significantly higher than in CKD patients (9.4 nM versus 5.1 nM, P < 0.001) and again strongly correlated with ferritin (r = 0.79, P < 0.001).

Conclusions. eGFR is not a major determinant of serum hepcidin-25 levels. In contrast, the hepcidin isoforms hepcidin-20 and hepcidin-22 accumulate in patients with renal impairment.

Keywords: anaemia; CKD; dialysis; hepcidin; iron

Introduction

Hepcidin is a recently discovered low molecular weight (LMW) protein that plays an important role in iron homeostasis [1]. Hepcidin may explain the often observed imbalance in iron metabolism and the resistance to erythropoiesis-stimulating agents (ESA) in patients with impaired renal function. As such, hepcidin could become an important tool to predict ESA responsiveness and to guide treatment with ESA and intravenous iron. In addition, hepcidin has the potential to become a target of treatment [2,3].

Hepcidin is primarily produced by hepatocytes as an 84-amino-acid (84 aa) pre-prohepcidin. Subsequent posttranslational processing results in the biologically active 25 aa form (hepcidin-25) that is secreted in the plasma and excreted in the urine. Additional amino-terminal degradation results in two smaller isoforms (hepcidin-20 and 22) [4,5]. The biological significance of hepcidin-20 and 22 is unknown, although in vitro at supraphysiologic concentrations, hepcidin-20 shows broad spectrum antimicrobial activity [4]. Hepcidin inhibits the release of iron from macrophages and the absorption of dietary iron from the intestine. It does so by causing the internalization and degradation of the cellular iron exporter ferroportin, which is highly expressed in macrophages and duodenal enterocytes [6]. The net effect of hepcidin is to increase intracellular iron stores, decrease dietary iron absorption and decrease circulating iron concentrations.

Various physiologic and pathologic processes regulate the synthesis of hepcidin. An augmented demand for circulating iron due to iron deficiency, hypoxia [7], anaemia [7], conditions characterized by ineffective erythropoiesis or the use of ESA [8] leads to a decrease in hepcidin synthesis. On the other hand, hepcidin synthesis is increased by infection or inflammation [7]. This is believed to lead to the anaemia of chronic disease that is characterized by a decrease in circulating iron available for erythropoiesis, despite apparently normal iron stores. These latter features are similar to those observed in patients with impaired renal function.

Hepcidin measurements in serum and urine have long proven to be difficult. Recently, both reliable mass spectrometry (MS)-based assays and competitive immunoassays have become available [9,10]. MS assays have the advantage of distinguishing between the three hepcidin isoforms, hepcidin-25, 22 and 20. Immunoassays, however, will measure total hepcidin levels, with (depending on the specificity of the antibody) different contributions from each of the isoforms.

Our recent findings suggest that hepcidin-25, like other small peptides such as β2-microglobulin, is freely filtered and subsequently reabsorbed in the proximal tubules [11].
Accordingly, both serum hepcidin-25 and total hepcidin levels have been found to be elevated in a small series of patients with renal dysfunction [12,13]. Furthermore, Ashby et al., exploiting a radioimmunoassay, found total hepcidin levels to be elevated and positively correlated with ferritin and inversely correlated with estimated GFR (eGFR) [10]. Yet, it is unclear whether these results obtained for total hepcidin also hold for the bioactive hepcidin-25 isoform.

Here, we perform studies in patients with impaired renal function by exploiting our MS hepcidin assay and report measurements of all three hepcidin isoforms in patients with chronic kidney disease (CKD) and receiving haemodialysis (HD). To improve our understanding of the renal handling of hepcidin, we performed additional studies in patients treated by haemodialysis and peritoneal dialysis.

**Methods**

Blood samples of 24 healthy controls were collected randomly throughout the day. We obtained blood samples of 83 consecutive patients with CKD who visited the outpatient clinic of the Radboud University Nijmegen Medical Centre to ensure coverage of the full GFR range. Relevant clinical and biochemical data were retrieved from the patients' records.

Additional blood samples were collected from 48 consecutive patients on haemodialysis, using a biocompatible Fresenius polysulfone® dialysis membrane. In all patients, blood samples were collected at the start of haemodialysis. To determine whether the artificial kidney removed hepcidin, blood samples were drawn from both the arterial (blood flowing to the dialyzer) and the venous (blood flowing to the patient) segment of the dialysis circuit 5–15 min after start and 5–10 min before termination of the dialysis session in a subgroup of 15 patients. These patients did not receive intravenous iron or erythropoietin (EPO) during dialysis. In order to assess the presence of hepcidin in the dialysate, dialysate samples were collected within 30 min after the start of dialysis. After the haemodialysis session, several filters were flushed with a physiologic salt solution and acetoneitrile in order to determine the presence of hepcidin on the membrane.

We measured hepcidin-25 levels in blood and dialysate samples of three patients who were treated with peritoneal dialysis, using a pH-neutral, lactate-buffered, low glucose degradation products solution (Stay-Safe® Balance system, Fresenius Medical Care, Germany). The dialysate samples were taken after a 6–8-h dwell time.

In accordance with Dutch ethical regulations, all patients consented that blood and dialysate samples would be used for medical research. We measured the following parameters in the blood samples: serum creatinine, haemoglobin, serum iron, iron binding capacity, ferritin, C-reactive protein (CRP) and hepcidin isoforms.

**Results**

We included 83 patients with CKD not requiring dialysis and 48 patients on haemodialysis. Clinical and demographic characteristics of both groups and the control group are depicted in Table 1. Median eGFR of CKD patients was 36 ml/min/1.73 m² (range 6–93 ml/min/1.73 m²). Causes of CKD were glomerular disease (36%), vascular disease (21%), polycystic renal disease (12%), reflux nephropathy (9%), diabetic nephropathy (6%) and other (16%). Patients on haemodialysis were older and showed significantly higher values of ferritin and CRP. In HD patients, causes of renal disease were vascular disease (23%), glomerular disease (19%), diabetic nephropathy (17%), reflux nephropathy (13%), polycystic renal disease (4%) and other (24%).

**Chronic kidney disease**

Serum hepcidin-25 levels were not significantly increased in patients with CKD not requiring dialysis compared to 24 healthy controls (median 5.1 versus 4.2 nM, P = 0.30). Overall, in CKD patients, serum hepcidin-25 strongly correlated with ferritin (r = 0.74, P < 0.01; Figure 1A, Table 2), whereas no significant correlation was found between hepcidin-25 and serum creatinine or eGFR (r = 0.13, P = 0.26 and r = –0.12, P = 0.30, respectively; Figure 1B, Table 2). Multiple regression analysis—including the putative predictors age, ferritin, transferrin, haemoglobin, serum iron, CRP and eGFR—showed ferritin to be the only significant, independent predictor of hepcidin-25 levels (β = 0.736, P < 0.001, R² = 0.54). Of note, eGFR had no influence on hepcidin-25 levels.

We specifically questioned if the use of EPO influenced the results. Twenty-one patients (25%) were treated with EPO. As expected, EPO-treated patients had a lower eGFR (median 12 versus 42 ml/min/1.73 m², P < 0.001). There were no differences in serum ferritin levels (median 116 ver-
sus 106μg/l, \( P = 0.42 \). Serum hepcidin-25 levels were not significantly higher in EPO-treated patients (median 8.0 versus 4.8 nM, \( P = 0.31 \)). In multivariate regression analysis, EPO therapy was not a predictor of serum hepcidin-25 levels. Furthermore, limiting the analysis to EPO-naive patients did not alter the results observed for the whole group; again, serum hepcidin-25 was strongly correlated with ferritin (\( r = 0.74, P < 0.01 \)), and no significant correlation was found between hepcidin-25 and serum creatinine or eGFR (\( r = 0.16, P = 0.21 \) and \( r = -0.13, P = 0.32 \), respectively). Multiple regression analysis showed ferritin to be the only significant, independent predictor of hepcidin-25 levels (\( \beta = 0.861, P < 0.001 \), \( R^2 = 0.58 \)). Serum levels of hepcidin-20 and hepcidin-22 were below the lower limit of detection in 19% and 66% of patients, respectively. In patients with hepcidin isoforms above the LLCOD, hepcidin-20 and hepcidin-22 correlated with ferritin (\( r = 0.46, P < 0.01 \) and \( r = 0.41, P = 0.03 \), respectively; Figure 2) and eGFR (\( r = -0.49, P < 0.01 \) and \( r = -0.46, P = 0.01 \), respectively; Figure 2). Multiple regression analysis of the data, including patients with concentrations of the hepcidin isoforms above the detection limit, showed eGFR to be an independent, significant predictor of hepcidin-20 and total hepcidin levels (\( \beta = -0.467, P < 0.001 \) and \( \beta = -0.259, P = 0.001 \), respectively). Furthermore, the ratio hepcidin-25/total hepcidin was significantly influenced by eGFR: the ratio was 0.80 in patients in the highest tertile of eGFR and 0.72 in patients in the lowest tertile of eGFR (\( P = 0.044 \)). Thus, a decrease in eGFR was associated with an increase in hepcidin-20 and total hepcidin levels. Exclusion of the patients with levels below the detection limit could have biased the results. Therefore, we re-analysed the data. For this analysis, we attributed the lowest measurable value to the patients with levels below the detection limit. Also in this analysis, which thus included all patients, eGFR was an independent predictor of hepcidin-20 (\( \beta = -0.225, P = 0.016 \)). Of note, serum ferritin proved the strongest predictor of both hepcidin-20 and total hepcidin, similar to the results described above for serum hepcidin-25 levels.

**Haemodialysis**

We included 41 patients who received haemodialysis three times a week for ~4h and seven patients who were treated with nocturnal dialysis four times a week for ~8h. The majority of patients (n = 44) was treated with intravenous iron and all but one were treated with EPO; median time on dialysis was 26 months (range 1–64 months). A low-flux membrane was used in 36 patients, whereas 12 patients were dialysed using a high-flux membrane. Median blood flow was 300ml/min (range 130–400), median dialysis flow was 500ml/min (range 300–700) and median ultrafiltration volume was 2110ml/min (range 59–3800). Median hepcidin-25 levels were significantly higher compared to hepcidin-25 levels in patients with CKD (9.4 versus 5.1 nM, \( P < 0.001 \)). Again, hepcidin-25 was strongly correlated with ferritin (\( r = 0.79, P < 0.001 \)). Multiple regression analysis—including the putative predictors age, ferritin, transferrin, haemoglobin, serum iron and CRP—showed ferritin and serum iron to be significant, independent predictors of hepcidin-25 levels (\( R^2 = 0.70 \)).

A significant difference was observed between arterial and venous samples at the start of the dialysis procedure compatible with removal of hepcidin-25 by the artificial kidney (n = 15, 15.9 ± 15.1 versus 8.5 ± 7.5 nM, \( P = 0.001 \)). The median clearance of hepcidin-25 was 82ml/min. Surprisingly, despite a persistent arterial-venous difference at the end of dialysis (13.6 ± 11.5 versus 13.6 ± 9.8, \( P = 0.001 \)), only a very modest reduction in serum hepcidin-25 was observed at the end of haemodialysis (arterial values 15.9 ± 15.1 versus 13.6 ± 11.5 nM, \( P = 0.03 \)).

Hepcidin-25 and its isoforms were present in the ultrafiltrate, but levels were too low to allow precise calculation
of a sieving coefficient or to account fully for the observed arterial-venous difference. Hepcidin was also detected in significant amounts (range 0.4–2.0 nmol) after eluting the filter with acetonitrile, which strongly suggests binding of hepcidin to the artificial membrane.

**Table 2. Correlation of hepcidin-25 levels with biochemical and clinical variables in CKD (with and without EPO therapy) and haemodialysis patients**

<table>
<thead>
<tr>
<th>Variable</th>
<th>CKD (n = 83)</th>
<th>Haemodialysis (n = 48)</th>
<th>P-value</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Serum creatinine</td>
<td>0.13</td>
<td>0.25</td>
<td></td>
<td></td>
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<tr>
<td>eGFR</td>
<td>−0.12</td>
<td>0.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteinuria</td>
<td>−0.04</td>
<td>0.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transferrin</td>
<td>−0.27</td>
<td>0.01</td>
<td>−0.47</td>
<td>0.00</td>
</tr>
<tr>
<td>Transferrin saturation</td>
<td>0.14</td>
<td>0.19</td>
<td>−0.24</td>
<td>0.10</td>
</tr>
<tr>
<td>Ferritin</td>
<td>0.74</td>
<td>0.00</td>
<td>0.79</td>
<td>0.00</td>
</tr>
<tr>
<td>Iron</td>
<td>−0.05</td>
<td>0.64</td>
<td>−0.40</td>
<td>0.00</td>
</tr>
<tr>
<td>CRP</td>
<td>0.21</td>
<td>0.06</td>
<td>0.52</td>
<td>0.00</td>
</tr>
<tr>
<td>Hb</td>
<td>−0.04</td>
<td>0.75</td>
<td>−0.38</td>
<td>0.01</td>
</tr>
<tr>
<td>EPO-dose</td>
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<td>0.45</td>
<td>0.12</td>
<td>0.42</td>
</tr>
<tr>
<td>Iron-dose</td>
<td>0.14</td>
<td>0.21</td>
<td>−0.04</td>
<td>0.81</td>
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<td>Age</td>
<td>0.13</td>
<td>0.11</td>
<td>0.03</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Spearman’s rho was calculated because data were skewed, eGFR = estimated glomerular filtration rate, CRP = C-reactive protein, Hb = haemoglobin, n.a. = not available.

*\( n = 21, \hat{n} = 10 \).*

**Fig. 1.** (A) Relationship between serum hepcidin-25 and serum ferritin in patients with chronic kidney disease not requiring dialysis. There was a significant relationship (\( r = 0.74, P < 0.01 \)). Closed symbols represent patients treated with erythropoietin. (B) Relationship between serum hepcidin-25 and estimated glomerular filtration rate in patients with chronic kidney disease not requiring dialysis. There was no significant relationship (\( r = −0.12, P = 0.3 \)). Closed symbols represent patients treated with erythropoietin.

**Fig. 2.** Relationship between serum hepcidin-20 and estimated glomerular filtration rate in patients with chronic kidney disease not requiring dialysis categorized according to tertiles of ferritin. There was a significant relation between hepcidin-20 and eGFR (\( r = −0.39, P < 0.001 \)) and between hepcidin-20 and serum ferritin levels (\( r = 0.60, P < 0.001 \)).

**Peritoneal clearance of hepcidin**

We measured hepcidin-25 levels in three peritoneal dialysis patients. Hepcidin-25 was detected in the dialysate, with an average dialysate/plasma ratio of 0.22.

**Discussion**

We found that serum hepcidin-25 levels are not significantly increased in patients with renal dysfunction not
requiring dialysis. Haemodialysis patients, however, exhibited significantly higher hepcidin-25 levels than patients with CKD. Serum ferritin concentration was a significant predictor of hepcidin-25 levels in multiple regression analysis. In contrast, eGFR was not an independent predictor of hepcidin-25 levels in patients with CKD. Furthermore, serum hepcidin-25 levels decreased only slightly during haemodialysis (10–15%), despite an arterio-venous difference in hepcidin levels >40%. Admittedly, ultrafiltration may have resulted in more concentrated levels of hepcidin-25 and a minor underestimation of hepcidin clearance. Since blood samples were not drawn until after the initiation of haemodialysis, we cannot exclude an immediate effect of haemodialysis on serum hepcidin levels, yet it seems unlikely that hepcidin levels are substantially influenced by haemodialysis within such a short period of time.

Our data corroborate recently published studies reporting higher hepcidin levels in HD patients [10,12]. There is also agreement that serum ferritin is highly correlated with hepcidin levels in patients with CKD and HD [10,12,13]. However, the data seem to differ with respect to the role of the eGFR. In two studies, the independent effect of eGFR on hepcidin levels was not evaluated [12,13]. Ashby et al. reported that hepcidin levels in their patients were significantly correlated with eGFR, even when corrected for ferritin levels [10], whereas in the current study hepcidin-25 levels were not related to eGFR.

Instead, we found that hepcidin-20 and total hepcidin levels showed an independent significant negative relation with eGFR. The apparent discrepancies between our observations and those of Ashby et al.’s observations might therefore be explained by the use of different assays, and in particular the inability of the immunoassay used by Ashby to differentiate between the various isoforms. Alternatively, the conflicting results may be attributable to differences in the population studied. Also, the findings of Ashby et al. may reflect a type I error or we may have failed to detect a slight effect of eGFR on hepcidin levels due to a type II error. Yet, based on the data described above, we must conclude that serum concentrations of hepcidin-25, the bioactive hepcidin isoform, are mainly dependent on ferritin and not on eGFR.

Our observation that hepcidin-25 is not related to eGFR suggests that hepcidin-25 differs from other LMW proteins such as β2-microglobulin or cystatin-C. This might be explained by binding of hepcidin to a large carrier protein that precludes it to be freely filtered. However, our additional studies are inconsistent with such a hypothesis. First, hepcidin was present in the peritoneal dialysate, and when the peritoneal clearance of hepcidin was plotted on a curve depicting the relationship between the peritoneal clearance and the molecular mass of various other freely filtered marker proteins, the calculated clearance was compatible with the expected clearance of a freely filterable protein of similar molecular mass [17]. Second, the arterio-venous difference in haemodialysis patients suggests that hepcidin-25 is handled similar to other LMW proteins. Admittedly, in the latter experiments, we were unable to calculate the relative contribution of ultrafiltration and of sticking to the membrane of the artificial kidney.

Taken together, our findings suggest regulation of serum hepcidin-25 levels, thus attenuating any effect of eGFR. Removal of hepcidin-25 by the dialysis membrane is partly compensated for. Studies on the immediate effects of iron administration on the increase of serum hepcidin-25 levels are consistent with a fast hepcidin regulation [13,18].

In conclusion, serum hepcidin-25 levels are not dependent on eGFR. Moreover, serum levels of hepcidin-25 decrease only slightly during dialysis, despite considerable removal of hepcidin-25 by the artificial kidney. Hepcidin isoforms accumulate in patients with renal impairment. The biological relevance of the latter finding is unknown.

Conflict of interest statement. We have nothing to disclose. D.W.S. is a co-founder and Medical Director of the ‘Hepcidinanalysis.com’ initiative which aims to serve the scientific and medical community with high-quality hepcidin-25 measurements (www.hepcidinanalysis.com).

References
Dominance of traditional cardiovascular risk factors over renal function in predicting arterial stiffness in subjects with chronic kidney disease

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Abstract

Background. The predictors of arterial stiffness across the spectrum of renal function are unclear. These predictors were investigated across a wide range of estimated glomerular filtration rates (eGFR).

Methods. Carotid-femoral pulse wave velocity (PWV; an index of arterial stiffness) was measured in 264 subjects with chronic kidney disease (CKD) stages 3–5 from three nephrology clinics (‘lower GFR group’). PWV was also measured in 149 subjects without previously recognized CKD (‘higher GFR group’) including n = 26 with eGFR between 30 and 60 ml/min/1.73 m2 and n = 123 with eGFR between 60 and 100 ml/min/1.73 m2. The association between PWV and eGFR was investigated using linear regression.

Results. The 413 subjects had a mean age of 61.9 years, were 51% male, 28% diabetic and 79% hypertensive. In age-adjusted analyses within the ‘lower GFR group’, ‘higher GFR group’ and combined group, PWV correlated with higher systolic blood pressure (SBP), pulse pressure (PP), diabetes mellitus, body mass index (BMI) and resting heart rate (all p < 0.0008). In addition, PWV correlated inversely with eGFR in the ‘higher GFR group’ (p = 0.03) and combined group (p < 0.0001). In multivariable regression analyses of the combined group (n = 413), PWV was independently predicted by eGFR (p < 0.05). However, eGFR explained at most 4% of the variability in PWV in age-adjusted analyses (compared with 13–15% explained by SBP, PP or diabetes) and <1% of PWV variability in models adjusting for age, SBP, diabetes, heart rate and BMI (p < 0.0001).

Conclusion. Although eGFR may independently predict PWV, the contribution of GFR per se does not appear to be clinically meaningful when compared with traditional cardiovascular risk factors.

Keywords: arterial stiffness; arteriosclerosis; cardio-renal; kidney disease; pulse wave velocity

Introduction

Large ‘central’ artery stiffness, determined by the velocity of propagation of the percussion wave (pulse wave velocity, or PWV), predicts cardiovascular events in the general population and in end-stage renal disease [1–4]. Theoretical reasons support the hypothesis that chronic kidney disease (CKD) may modulate arterial stiffness. Progressive decrease in kidney function is associated with the same factors that predict increased arterial stiffness including aging, diabetes and hypertension [5,6]. Furthermore, other variables unique to CKD including vascular calcification