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Research Article

Evaluation of Hepcidin Isoforms in Hemodialysis Patients by a Proteomic Approach Based on SELDI-TOF MS

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The hepatic iron regulator hormone hepcidin consists, in its mature form, of 25 amino acids, but two other isoforms, hepcidin-20 and hepcidin-22, have been reported, whose biological meaning remains poorly understood. We evaluated hepcidin isoforms in sera from 57 control and 54 chronic haemodialysis patients using a quantitative proteomic approach based on SELDI-TOF-MS. Patients had elevated serum levels of both hepcidin-25 and hepcidin-20 as compared to controls (geometric means: 7.52 versus 4.69 nM, and 4.06 versus 1.76 nM, resp., $P < .05$ for both). The clearance effects of a single dialysis session by different dialysis techniques and membranes were also investigated, showing an average reduction by 51.3% ± 29.2% for hepcidin-25 and 34.2% ± 28.4% for hepcidin-20 but only minor differences among the different dialysis modalities. Measurement of hepcidin isoforms through MS-based techniques can be a useful tool for better understanding of their biological role in hemodialysis patients and other clinical conditions.

1. Introduction

Regulation of iron metabolism is crucial in different types of cells, especially in hepatocytes, enterocytes, and macrophages [1]. The major player of this regulation has been recognized in the small peptide hepcidin, that inhibits iron export from the cell membrane through internalization and degradation of ferroportin [2]. Hepcidin is a defensin-like peptide prevalently produced by the liver as an 84-amino acid precursor, that undergoes sequential proteolytic cleavage to form the 25-amino acid bioactive hormone (hepcidin-25). Additional N-terminal cleavage results in the production of two smaller isoforms, hepcidin-22 and hepcidin-20, whose physiological role is still unclear. Hepcidin-22 has been found only in urine, where it may merely represent a degradation product. On the other hand, hepcidin-20 is also present in blood and appears to possess greater antimicrobial activity than hepcidin-25 [3] but no capability to bind ferroportin [4]. Moreover, it has been recently indicated as a possible serum marker for the acute phase of myocardial infarction [5], but systematic investigations on this isoform in human diseases are lacking.

Until recently, studies on hepcidin have been limited by technical difficulties in establishing reliable assays in biological fluids [6]. Proteomic approaches including Surface-Enhanced Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (SELDI-TOF MS) are increasingly used for the evaluation of biomarkers of clinical interest [7, 8]. Indeed, we and others have contributed to the development and validation of an accurate assay of hepcidin in serum and urine by means of SELDI-TOF MS [9–12]. At variance with traditional immunochemical methods, the SELDI-TOF MS technique has the potential to evaluate not only the predominant 25-amino acid form, but also the shorter isoforms.

Hepcidin has been recently object of intense investigation as a potential biomarker of iron status in patients with chronic kidney diseases (CKD) [13]. Indeed, an adequate iron availability is critical in hemodialysis (HD) patients, since it appears to influence the response to recombinant erythropoietin (EPO), the mainstay of treatment of anemia in this condition. In HD patients, various factors may modulate serum hepcidin levels with opposing influence (reviewed in [14]). For example, hepcidin may increase because of reduced glomerular filtration, iron therapy, and inflammation, with interleukin-6 as a well-known stimulus for its production [13, 15]. On the other hand, hepcidin may be reduced by hypoxia, iron deficiency, and EPO therapy by itself [11]. Recent studies using different hepcidin assays have established that serum hepcidin-25 levels are generally increased in HD patients [16–19], but whether or not it can represent a useful marker to predict resistance to EPO remains controversial.

Moreover, little is known about the levels of the hepcidin isoforms in HD patients, as well as about the effect of a single dialysis session on the clearance of hepcidins from the blood.

The aims of this study were to evaluate serum levels of hepcidin-25 and hepcidin-20 by quantitative SELDI-TOF MS [9] in chronic HD patients, their relationship with markers of iron status and/or inflammation, and the effects of a single dialysis using different protocols on the clearance of hepcidin isoforms.

2. Materials and Methods

Fifty-four stable HD patients treated at the Hemodialysis Service of our hospital (Ospedale Policlinico, Verona, Italy) during the period June 2008–July 2009 were included in the study. Patients were on maintenance with intravenous erythropoiesis-stimulating agents (ESAs) epoetin or darbepoetin and chronically treated with intravenous iron, which was stopped 10 weeks before the study. All subjects were dialyzed against standard, not ultrapure, dialysate. Different protocols were used. Thirty-nine patients were on bicarbonate HD (BHD), 23 with low-flux synthetic (11 polysulfone, Fresenius, Germany and 12 polyamide, Gambro, Sweden), and 16 with high-flux membranes (6 cellulose triacetate, Nipro, Japan, 5 each polysulfone, Fresenius, Germany, and polymethylmethacrylate, Toray, Japan). Eleven patients were on acetate-free biofiltration (AFB), a low-volume hemodiafiltration technique based on buffer-free dialysate, a biocompatible high-flux AN69 membrane, and sterile hypertonic bicarbonate infusion in post-dilution mode (Hospal, France) [20]. Finally 4 patients were on HFR (double-chamber hemodiafiltration with reinfusion of regenerate ultrafiltrate), a technique that utilizes convection, diffusion, and adsorption, using a 0.7 m^2 high permeability polyphenylene membrane as a convective dialyzer, a 1.70 m^2 low-flux polyphenylene membrane as a diffusive dialyzer, and a regenerating adsorbent cartridge containing undissolvable macroporous-structured styrenic resin as adsorbent material (Bellco, Italy) [21]. Dialyzers were not reused. In all patients the length of the dialysis session was set at 240 minutes, and the dialysate flow rate at 500 mL/min, the dialysis blood pump flow rate at 300 mL/min, and in the patients on AFB, the 164 mM bicarbonate reinfusion fluid rate was at 2 L/h.

Table 1: Clinical features and baseline laboratory data.

	HD patients	Controls
Number of patients	54	57
Gender (Male/Female)	29/25	38/21
Age (years)	67 ± 14	35 ± 15
HFE genotype		
wt/wt (n)	35	—
H63D/wt (n)	14	—
C282Y/wt (n)	2	—
Hemoglobin (g/dL)	11.4 ± 1.0	14.4 ± 1.2
Iron (μg/dL)	45.2 (43.1−60.0)	96.4 (89.1–104.2)
Transferrin Saturation (%)	20.5 ± 8.3	28.9 ± 9.0
Ferritin (ng/mL)	174 (156−258)	90 (47−149)
CRP (mg/dL)	4.7 (5.0−8.9)	0.8 (0.4−1.4)
Interleukin-6 (pg/mL)	6.8 ± 5.8*	—

*Reference range in normal subjects = 0.2–3.2 pg/mL.

Blood samples for laboratory testing were obtained prior to the first-of-the-week hemodialysis sessions. Blood samples were collected for detection of C-reactive protein (CRP), ferritin, and interleukin-6 (IL-6) before the dialysis session. Serum ferritin was measured by routine laboratory methods, IL-6 by enzyme-linked immunoadsorbent assay (by Human IL-6 ELISA BMS213/2CE Bender MedSystems GmbH, Vienna, Austria), and CRP by commercially available automated PENIA assays (Dade Behring, Germany).

The effect of the dialysis session on serum hepcidin-25 and hepcidin-20 was evaluated in 39 patients (14 on low-flux BHD, 12 on high-flux BHD, 9 on AFB, and 4 on HFR) by measuring hepcidins also at the end of the dialysis sessions and by calculating the reduction ratio (RR) as follows: RR = (Cpre − Cpost/Cpre) × 100, where Cpre is the concentration at the start of dialysis and Cpost the concentration at the end of dialysis. Cpost was corrected for hemoconcentration due to ultrafiltration by multiplying the uncorrected Cpost for a correction factor computed as the ratio predialysis Hb/postdialysis Hb.

Fifty-seven controls were enrolled among healthy volunteers participating in a phase II trial at the Centre for Clinical Research of the Azienda Ospedaliera-Universitaria di Verona, as described previously in detail elsewhere [12]. Briefly, at enrollment they completed a questionnaire with specific items relevant to iron metabolism (i.e., any history of blood donations, previous pregnancy, menstrual losses, etc.) and were evaluated by laboratory studies including ferritin, CRP, liver function tests, and creatinine. To be considered as appropriate "normal controls" for the serum hepcidin assay, all these parameters were required to be normal.

The main clinical features of the subjects included in the study are shown in Table 1.

Each patient gave written informed consent. The study was conducted according to the principles contained in

the Declaration of Helsinki. The protocol was approved by the Institutional Review Board of the Azienda Ospedaliera-Universitaria di Verona.

2.1. Serum Hepcidin-25 and Hepcidin-20 Assay. We used a protocol based on PBSCIIc mass spectrometer and copper-loaded immobilized metal-affinity capture ProteinChip arrays (IMAC30-Cu^{2+}). Five μL of serum were applied to an IMAC30- Cu^{2+} surface that binds hepcidin based on its affinity to Cu^{2+} ions. The binding surface was equilibrated and washed with appropriate buffers according to the manufacturs's instrunctions (Bio-rad, Hercules, CA). Subsequent work-up, SELDI-TOF MS instrumental settings, read-out, and data analysis are described elsewhere [12], with the addition that protein chip handling was performed in a nitrogen atmosphere to prevent methionine oxidation [9].

Briefly, we used synthetic hepcidin-25 (Peptides International, Louisville, KY) for external calibration and a synthetic hepcidin analogue (Hepdicin-24, Peptides International, Louisville, KY) as an internal standard [9]. Spectra were collected in duplicate for each sample, with or without the internal standard spiked in at a concentration of 10 nM. Concentrations of both serum hepcidin-25 and hepcidin-20 were expressed as nM and were the results of the following equations.

(1) Hepcidin 25 concentration: (sample 2789 m/z peak intensity) $\times$ 10 nM/(hepc24 spiked sample 2673 m/z peak intensity—nonspiked sample 2673 m/z peak intensity).

(2) Hepcidin 20 concentration: (sample 2192 m/z peak intensity) $\times$ 10 nM/(hepc24 spiked sample 2673 m/z peak intensity—nonspiked sample 2673 m/z peak intensity).

Standard curves of the internal standard were constructed by serial dilutions of hepcidin-24 (0–20 nM) in tubes with blank serum to an end volume of 500 μL. These were immediately applied to IMAC-Cu^{2+} Chips, and processed according to protocol and measured by MS. Linear standard curves were obtained for hepcidin-24 blank serum ($y = 4.90\times + 3.97$; $R^2 = 0.994$). Based on the measured background noise in each MS spectrum for serum samples, the lower limit of detection (LLOD) ranged from 0.55 to 1.55 nM. Since this method is based on the level of hepcidin-25 peak intensity relative to that of hepcidin-24, we determined hepcidin-24/hepcidin-25 intensity ratios in blank serum samples spiked with both synthetic compounds in duplicate of 8 different concentrations. As compared to our original description of the method [9], recent technical improvements allowed us to increase the mean peak ratios hepcidin-24/hepcidin-25 from 0.71 to 0.93.

Protocols for identification of peaks by immunological and mass spectrometry approaches have been described in detail elsewhere [22]. Hepcidin-25 and hepcidin-20 concentrations of 0.55 nM were arbitrarily assigned to samples with undetectable serum levels of hepcidin isoforms. The intra- and inter-assay coefficient of variations of this method ranged from 6.1 to 7.3 percent, and from 5.7 to 11.7 (mean 7.7) percent, respectively, [9].

Table 2: Correlations between serum hepcidin-25 levels, inflammation indices, and serum hepcidin-20 in HD patients.

	Correlation coefficient	P
Ferritin	0.478	.003
IL-6	0.096	.572
CRP	0.016	.910
Hepcidin-20	0.575	<.001

Table 3: Correlations between serum hepcidin-20 levels, inflammation indices, and serum hepcidin-25 in HD patients.

	Correlation coefficient	P
Ferritin	0.240	.153
IL-6	0.163	.335
CRP	0.093	.505
Hepcdin-25	0.575	<.001

2.2. Statistical Analysis. All calculations were performed using the SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). As some of the continuous variables of interest, including serum hepcidin and ferritin, showed a non-Gaussian distribution, their values were log-transformed and expressed as geometric means with 95% Confidence Intervals (CIs). For other variables, results were expressed also as means ± SD unless otherwise indicated. Correlations between quantitative variables were calculated by Pearson r test. Results were considered significant when P was <.05 (two tailed).

3. Results

Figure 1 shows the typical spectra obtained for the region where hepcidin peptides are detectable. The corresponding peaks of both hepcidin-20 and hepcidin-25 are visible, along with the peak of the added standard. Concentrations of hepcidin-20 and hepcidin-25 were calculated for each patient (before and after dialysis, when available) and control. Both hepcidin-25 and hepcidin-20 were significantly higher in HD patients as compared to controls (Figures 2 and 3): geometric means with 95% confidence intervals were 7.52 nM (5.39–10.48) in HD patients versus 4.69 nM (3.79–5.81) in controls for hepcidin-25, and 4.06 nM (3.45–4.77) in HD patients versus 1.76 nM (1.32–2.35) in controls for hepcidin-20, $P <$ .05 for both.

Of note, hepcidin-20 was undetectable in 22 out of 57 healthy subjects, while it was always detectable in HD patients.

Tables 2 and 3 show the correlation coefficients of hepcidin-25 and hepcidin-20 with serum ferritin and markers of inflammation. Hepcidin-25 was positively correlated with serum ferritin, but not with inflammatory markers. On the other hand, hepcidin-20 was not significantly correlated

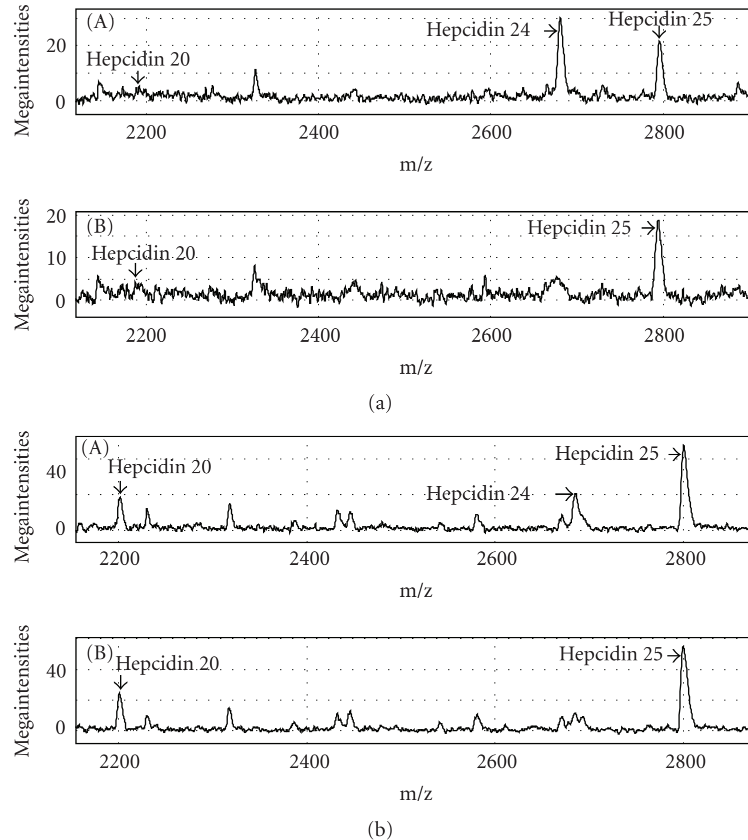

Figure 1: SELDI-TOF MS profile of hepcidin-24-spiked serum samples of hemodialysis patient (a) and control (b). The hepcidin isoforms hepcidin-20, hepcidin-24 (synthetic analogue, panel (B)), and hepcidin-25 are indicated by arrows.

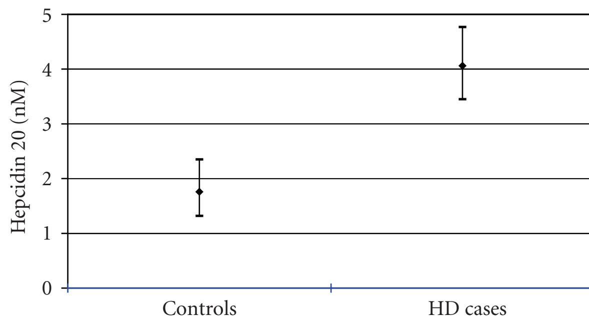

Figure 2: Hepcidin-20 in controls and HD patients. Data presented as geometric mean with 95% confidence interval.

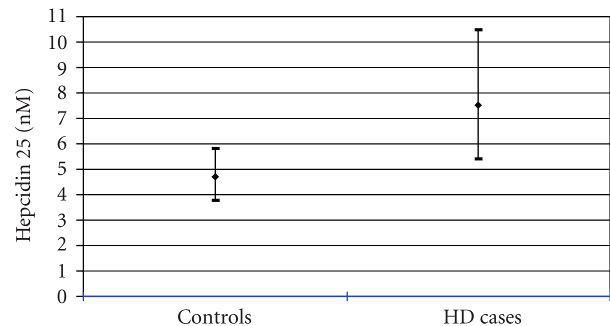

Figure 3: Hepcidin-25 in controls and HD patients. Data presented as geometric mean with 95% confidence interval.

with either ferritin or inflammatory markers, but was correlated positively with serum hepcidin-25.

The effects of a single dialysis session by the different dialysis techniques and membranes on serum hepcidin isoforms are shown in Figure 4. On average, we observed a reduction of 51.3%± 29.2% for hepcidin-25, and of 34.2%± 28.4% for hepcidin-20. The hepcidin-25 reduction ratio was similar by low- and high-flux BHD; removal by BHD was also similar to AFB and HFR. On the other hand, hepcidin-25 removal by the AFB was significantly lower than by HFR ($P = .033$) (Figure 4(a)). With respect to hepcidin-20 removal, no significant difference was noted using the different dialysis techniques (Figure 4(b)). Of note, there was no difference in predialysis serum levels of both isoforms between the different dialysis modalities (Table 4).

4. Discussion

In this study, SELDI-TOF MS was confirmed as a valuable approach for the quantification of hepcidin levels in serum, not only with respect to the main isoform, hepcidin-25, but

Table 4: Predialysis serum hepcidin-25 and hepcidin-20 levels and the different HD techniques.

	Low-flux BHD ($n = 23$)	High-flux BHD ($n = 16$)	AFB ($n = 11$)	HFR ($n = 4$)
Hepcidin-25 (nM)	8.0 (7.7–16.2)	7.4 (6.3–16.0)	7.4 (6.0–22.9)	5.7 (0–19.5)
Hepcidin-20 (nM)	4.4 (3.7–6.8)	3.8 (3.1–5.9)	4.2 (2.9–7.4)	2.7 (1.3–4.3)

Data are presented as geometric mean (95% Confidence Interval).

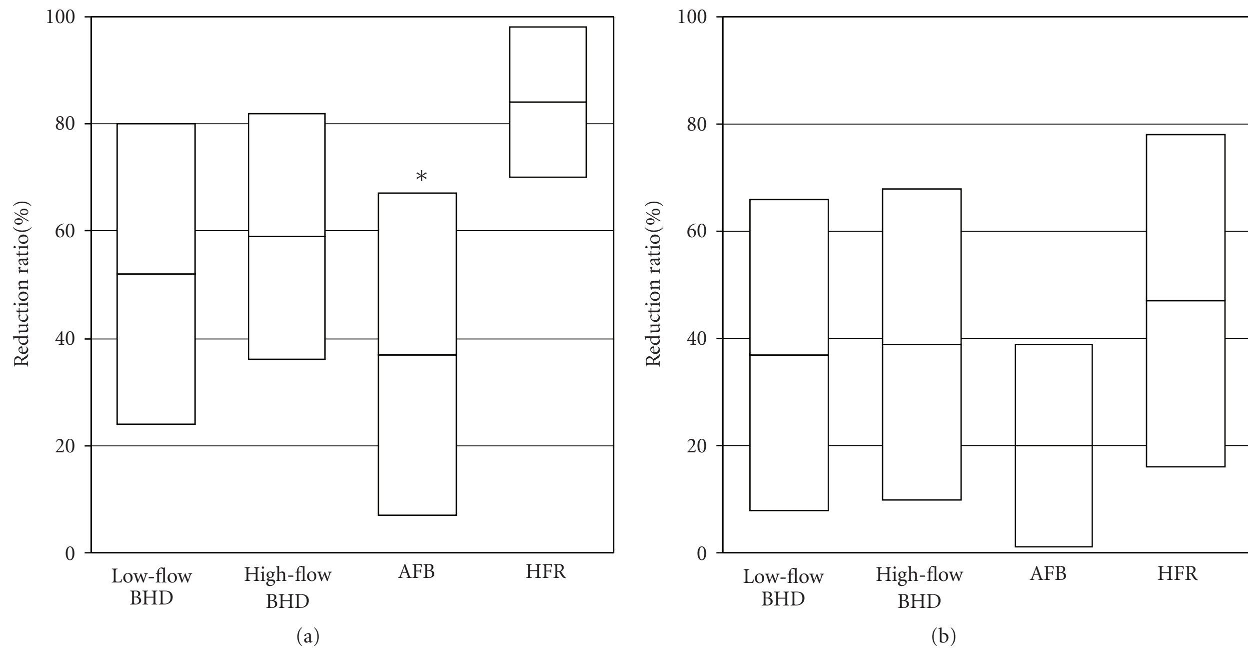

Figure 4: Reduction Ratio (%) of serum hepcidin-25 (a) and hepcidin-20 (b) by the different hemodialysis techniques. (*Statistically significant $P < .05$).

also to hepcidin-20. According to recent data obtained with various methodological approaches, including competitive ELISA [19], radioimmunoassay [17], micro-HPLC-tandem-MS [18], as well as this method [18], hepcidin-25 was proven to be increased in HD patients as compared to control subjects. Two studies [17, 19] also observed a gradual increase of hepcidin-25 across the spectrum of predialysis CKD, suggesting that diminished renal clearance is likely the main cause of elevated serum hepcidin-25 in these pathological conditions. The positive correlation with serum ferritin, confirmed in this study, indicates that the physiological regulation of hepcidin-25 by iron stores is maintained in CKD, though at an upper level as compared to controls subjects. Besides these confirmatory results, this study focused on two relatively unexplored aspects of hepcidin pathophysiology in HD, for example, the evaluation of serum levels of hepcidin-20 and the effects of different dialysis procedures on blood levels of hepcidin isoforms.

4.1. Baseline Serum Hepcidin-20 Levels in HD Patients. We evaluated for the first time serum hepcidin-20 levels in both normal subjects and HD patients. On average, hepcidin-20 was significantly higher in HD patients as compared to controls. More precisely, hepcidin-20 was undetectable in a substantial fraction (near 38%) of healthy subjects but was always detectable in HD patients. While hepcidin-20 was significantly and positively correlated with hepcidin-25, it was not correlated with either serum ferritin or markers of inflammation, such as CRP and IL-6. No obvious pathophysiological role of hepcidin-20 can be inferred by these results. The lack of correlation with iron stores and/or inflammation, for example, the classical stimuli for the iron bioactive hepcidin-25 isoform, argues against a role of hepcidin-20 in these conditions. For example, if we had noted a positive relationship with CRP and/or IL-6, one could speculate about a selective, inflammatory-driven, posttranslational modification of hepcidin to increase the relative fraction of the isoform with the highest reminiscent antimicrobial activity. Rather, our results may indicate two contributing mechanisms as responsible of the increased hepcidin-20 levels in HD: an altered proteolytic processing and/or a reduced renal excretion of a catabolic product normally produced in small amount and nearly completely eliminated. In line with this interpretation is the positive correlation we observed between hepcidin-20 and hepcidin-25, suggesting a "tracking effect" of the main isoform. While the pathophysiological role of hepcidin-20 in HD, if any exists, remains to be elucidated, the significant increase we noted is not without practical relevance. Indeed, as pointed out in a recent review [23], while different methods uniformly found

high hepcidin levels in HD, absolute values varied as much as 10-fold depending on the hepcidin assay used. In particular, the immunological methods generally reported the highest levels of “hepcidin-25” as compared to MS-based methods, including the present SELDI-TOF MS. This may be due to, at least in part, cross-reaction of antibodies with hepcidin-20. Since in terms of iron homeostasis hepcidin-25 is the sole bioactive form, MS-based methods may be more accurate for clinical investigations, notwithstanding being more complex than traditional immunological methods.

4.2. Effects of Different Dialysis Procedures on Blood Levels of Hepcidin Isoforms. The dialysis session appeared to significantly reduce average hepcidin-25 levels, as also reported by others using MS-based methods [18], but not immunological methods [17]. We also observed that the extent of the reduction by dialysis showed a high interindividual variability (see below) [24]. Of note, the lack of hepcidin-25 reduction after dialysis reported with [17] an immunological method was based on results in only 6 HD patients, suggesting the large individual variability as a possible confounder, beyond the effect of the different methodology employed.

All HD techniques evaluated in our study showed a comparable removal of hepcidin-25 by dialysis, suggesting that the removal of this molecule is largely due to diffusion. The most striking result of our study is indeed the large individual variability of the reduction ratio in patients treated by the same dialysis modality (ranging from 0 to 95%), suggesting that postdialysis levels may be influenced by factors other than removal by dialysis. One such factor may be an increased production of hepcidin-25 during the dialysis procedure, which is known to activate an inflammatory response due to the interaction of blood with the dialysis membranes and/or with a less-than-sterile dialysis fluid [25]. This possibility is supported by the finding that the greatest removal was observed by HFR, a dialysis technique associated with more efficient removal of cytokines and reduced levels of markers of inflammation (such as CRP and IL-6) when compared to standard hemodialysis [21]. Alternatively, the improved reduction ratio by HFR may be due to adsorption, which may provide an important additional modality for hepcidin-25 removal by dialysis. In addition, mean predialysis serum hepcidin-25 levels were lower in patients on long-term HFR (although this difference was not statistically significant), again suggesting that dialysis modalities with lower activation of the inflammatory response may contribute to reduce the hepcidin-25 burden.

In our study, removal of hepcidin-25 by BHD with low- and high-flux membranes was similar, at variance with what reported by Weiss and coworkers [18], who showed that postdialysis hepcidin-25 levels were lower with high-flux than low-flux BHD, suggesting an improved removal by high-flux membranes. These findings, however, may not be necessarily conflicting, since the effects of dialysis were evaluated differently in the two studies. Weiss and coworkers reported average postdialysis serum levels, while we computed the reduction ratio by dialysis. In addition, the differences between the two studies may be explained by the large intraindividual variability of the effect of dialysis reported by both and their small sample size.

Although the removal of hepcidin-20 by the different dialysis modalities was slightly lower than hepcidin-25, this difference was not statistically significant, suggesting that the different hemodialysis modalities remove the two isoforms by the same extent and through common mechanisms.

We are aware that the results of our study on the effect of the different dialysis techniques on circulating levels of hepcidin-25 and hepcidin-20 are preliminary and should be considered with caution. Clearly, additional studies with larger sample size and more adequate design are needed to fully understand the role of the dialysis procedure on serum levels of the two hepcidin isoforms.

5. Conclusions

Proteomics can be very useful in molecular medicine nowadays, as precious tools for biomarker discovery and quantitation. SELDI-TOF MS proteomic analysis enabled us to evaluate hepcidin isoforms in sera from HD patients before and after dialysis, giving new insights into the complex pathophysiology of hepcidin in chronic kidney disease.

Abbreviations

SELDI-TOF MS:	Surface-Enhanced Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry
CKD:	Chronic kidney disease
HD:	Hemodialysis
EPO:	Erythropoietin
ESA:	Erythropoiesis-stimulating agents
BHD:	Bicarbonate HD
AFB:	Acetate-free biofiltration
HFR:	Double chamber hemodiafiltration with reinfusion of regenerate ultrafiltrate
CRP:	C-reactive protein
IL-6:	Interleukin-6
CIs:	Confidence intervals.

Acknowledgments

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