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Uremic toxins, inflammation biomarkers and hepcidin in older CKD patients switched from high flux HD to OL-HDF

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Abstract: Anemia is a common complication that is associated with mortality in patients with chronic kidney disease (CKD). Despite the use of erythropoiesis-stimulating agents (ESAs) to correct anemia, some patients are hyporesponsive due to the state of micro-inflammation caused by uremic toxins and hepcidin, a hormone that plays a central role in iron homeostasis. Hemodiafiltration (OL-HDF) has been associated with better clearance of uremic toxins, such as indoxyl sulfate (IS), p-cresyl sulfate (PCS) and indole acetic ascorbic (IAA) than conventional hemodialysis (HD). The aim of the study was to evaluate the effect of OL-HDF treatment on the concentration of IS, PCS, IAA, hepcidin and inflammatory biomarkers in CKD patients. Thirty-one patients (> 65 years old) incident in OL-HDF were followed for 6 months. IS, PCS, IAA, biochemical parameters, hepcidin and inflammatory cytokines were evaluated at baseline and after 6 months. We observed a significant decrease in IS and CRP plasma concentration, an increase in hemoglobin and hematocrit after 6 months of treatment with OL-HDF (p <0.05). This prospective observational study demonstrated that OL-HDF was capable to reduce IS and CRP in older patients. Whether this reduction may have an impact on clinical outcomes must be investigated in a future study.

Anemia is a common complication in patients with chronic kidney disease (CKD) and is associated with poor quality of life, increased risk of cardiovascular disease, and overall mortality1,2. The cause of anemia in CKD, mainly in end-stage renal disease (ESRD/dialysis) includes shortening red blood cell (RBC) survival, erythropoietin deficiency, functional iron deficiency due to poor dietary iron absorption, higher iron requirements during erythropoiesis-stimulating agents (ESAs) supplementation, and overproduction of hepcidin3,4.

Despite the use of ESAs, 20% of patients are hyporesponsive5. ESA hyporesponsiveness portrays worsened prognosis6 and scaling ESA doses to achieve hemoglobin (Hb) targets may elevate cardiovascular, thrombotic, and mortality risks7. According to the RISCADIV study, IL-6 was shown to be a strong predictor of ESA hyporesponsiveness and C-reactive protein (CRP) levels were higher in patients with the highest quartile of ESA hyporesponsiveness, i.e., quartile IV (p < 0.001), and predicted all-cause mortality and cardiovascular events8. Accordingly, inflammation is
an essential factor in the management of anemia and in regulation of the response of 
ESA\(^9,10\).

In CKD patients, causes of inflammation tend to be multifactorial. However, the 
activation of pro-inflammatory cytokines in uremia is mainly triggered by the 
accumulation of uremic toxins (UTs), due to the progressive loss of kidney filtration 
capacity\(^11,12\). UTs are associated with elevated CRP and IL-6 levels in patients on 
HD\(^13,14\). Moreover, impaired renal clearance and increased inflammatory status, 
contribute to elevated serum hepcidin levels, which are involved in the pathogenesis of 
anemia in this population\(^15\).

Hepcidin is synthesized in the liver and regulates iron homeostasis by binding to 
ferroportin, an iron exporter that transfers iron into plasma, which is abundant in 
duodenal enterocytes, macrophages of the reticuloendothelial system and hepatocytes. 
Hepcidin blocks ferroportin-dependent iron efflux via the internalization and 
degradation of ferroportin in the above cells in response to intracellular or extracellular 
iron concentration and inflammatory mediators that decrease serum iron levels\(^15\). 
The elevated IL-6 serum levels increase hepcidin synthesis mainly in hepatocytes which 
contribute to elevated hepcidin in the circulation\(^16\). Hepcidin levels are increased in 
CKD patients\(^17\) as well as in experimental animal models of CKD\(^18,19\). Hepcidin has 
been a focus of investigation on the management of anemia in CKD patients\(^20,21\).

Hemodialysis (HD) has been the most common method of renal replacement 
therapy worldwide for patients with end-stage renal disease (ESRD)\(^22\). However, HD is 
not very effective in removing uremic toxins with medium molecular weight or those 
binding to proteins, such as indoxyl sulfate (IS), p-Cresylsulfate (PCS), and indole 
acetic acid (IAA)\(^23,24\) which are associated with the signaling of the inflammatory 
response, decreasing erythropoiesis and compromising the growth and differentiation of 
red blood cells in the bone marrow\(^25,26\). These UTs also induce reactive oxygen species 
(ROS) as well as inflammation and exert adverse effects on various organs\(^27\). IS 
accumulation inhibits the hypoxia-inducible factor and thus reduces EPO production\(^28\).
It has been shown that IS removal improves the effect of ESA on anemia in late-stage 
CKD patients\(^29\), indicating that IS mediates anemia via EPO regulation. In addition, IS 
participates in the induction of hepcidin in hepatocytes and high serum levels\(^30\).

Online hemodiafiltration (OL-HDF), a modality that combines diffusion and 
convection, removes greater clearance of medium molecular weight or protein-bound 
uremic toxins than HD\(^31\). OL-HDF may potentially reduce the adverse effects of 
inflammation\(^32\). In fact, den Hoedt CH et al. have reported that OL-HDF was able to 
decrease systemic inflammation compared to HD\(^33\). OL-HDF has been described to 
 improve RBC lifespan in ESRD patients\(^34\) and decreased hepcidin serum levels\(^35\).
However, the effect of OL-HDF on serum concentration of IS, PCS, IAA, biomarkers of 
inflammation and hepcidin associated with anemia still deserves confirmation\(^4\). 
Therefore, the present study aimed to explore the effect of OL-HDF on the serum 
concentration of protein-bound uremic toxins, biomarkers of inflammation and hepcidin 
in older patients who switched from high flux HD to OL-HDF.

**Results**

Out of 40 patients initially included, 9 dropped out due to COVID-19 (N=2), 
kidney transplantation (N=1) or changed to another dialysis center (N=6). The final 
analysis was performed with 31 patients. As detailed in Table 1 most patients were male 
and the mean age was 77.4 ± 7.1 years. Hypertensive nephrosclerosis (45.2%) and 
diabetes (35.5%) accounted for most of the underlying kidney disease.
Table 1. Characteristics of patients.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>n=31</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>77.4 ± 7.1</td>
</tr>
<tr>
<td>Male gender, n (%)</td>
<td>20 (64.5)</td>
</tr>
<tr>
<td>Body Mass Index (Kg/m²)</td>
<td>25.8 ± 5.8</td>
</tr>
<tr>
<td>Dialysis vintage, days, mean ± SD</td>
<td>45 ±20</td>
</tr>
<tr>
<td><strong>Cause of kidney disease, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Hypertensive Nephrosclerosis</td>
<td>14 (45.2)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>11 (35.5)</td>
</tr>
<tr>
<td>Chronic Glomerulonephritis</td>
<td>1 (3.2)</td>
</tr>
<tr>
<td>Other/unknown</td>
<td>5 (16.1)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD or median (25,75).

After 6 months of OL-HDF follow-up, we observed a significant decrease in IS and CRP serum concentrations (Table 2, Figure 1 and Figure 2b), but we did not observe differences for hepcidin and other inflammatory biomarkers (Table 2, Figure 2a, 2c, 2d).

Table 2. Uremic toxins, hepcidin and inflammatory biomarkers at baseline (Pre-OL-HDF) and after 6 months on online hemodiafiltration (Post-OL-HDF).

<table>
<thead>
<tr>
<th></th>
<th>Pre-OL-HDF (T0)</th>
<th>Post-OL-HDF (T6)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indoxyl sulfate</td>
<td>69.9 (36.3 – 86.0)</td>
<td>67.9 (52.7 – 114)</td>
<td>0.01</td>
</tr>
<tr>
<td>p-cresyl sulfate</td>
<td>187 (131-266)</td>
<td>183 (128-258)</td>
<td>1.0</td>
</tr>
<tr>
<td>Indole acetic acid</td>
<td>7.87 (5.64-11.1)</td>
<td>8.10 (5.01-10.2)</td>
<td>0.7</td>
</tr>
<tr>
<td>Hepcidin (pg/mL)</td>
<td>64141 (1506-133835)</td>
<td>97835 (2729-142118)</td>
<td>0.1</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>6.31 (0.52-10)</td>
<td>3.43 (0.31-31)</td>
<td>0.004</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>7.38 (3.4-14.0)</td>
<td>6.35 (2.6-13.8)</td>
<td>0.3</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>2.65 (0.1-20.0)</td>
<td>2.32 (0.1-58.7)</td>
<td>0.4</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>2.25 (1.3-4.0)</td>
<td>2.01 (1.4-3.7)</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Values are expressed as median (25,75). (CRP - C-reactive protein; IL-6 - interleukin 6; IL-10 - interleukin 10 and TNF-α - α tumoral necrosis factor.)
Figure 1. Serum concentration of uremic toxins (IS, PCS and IAA) at baseline (T0) and after 6 months (T6) of online hemodiafiltration (OL-HDF).

Figure 2. Serum concentration of hepcidin (a), CRP (b), IL-6 (c) and TNF-α (d) at baseline (T0) and after 6 months (T6) of online hemodiafiltration (OL-HDF).
During the follow-up, 87.5% (28/31) of patients were supplemented with ESAs and 100% (31/31) were supplemented with iron (Table 3). We observed that hemoglobin, hematocrit and transferrin, increased after 6 months of OL-HDF (Table 3, Figure 3a, 3b, 3e). We did not observe significant differences between these parameters with a reduction of IS (data no shown). There is no relevant changes for the concentrations of serum iron, ferritin and transferrin saturation after 6 months of treatment with OL-HDF (Figure 3c, 3d e 3f).

### Table 3. Iron Metabolism and Anemia biomarkers at baseline (Pre-OL-HDF) and after 6 months on online hemodiafiltration (Post-OL-HDF).

<table>
<thead>
<tr>
<th></th>
<th>Pre-OL-HDF (T0)</th>
<th>Post-OL-HDF (T6)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>10.3 (8-13)</td>
<td>11.7 (8.8-13)</td>
<td>0.003</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>30 (24-40)</td>
<td>34 (25-40)</td>
<td>0.002</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>16 (12-33)</td>
<td>16 (12-31)</td>
<td>0.15</td>
</tr>
<tr>
<td>Serum iron (µg/dL)</td>
<td>58 (27-101)</td>
<td>64 (20-213)</td>
<td>0.32</td>
</tr>
<tr>
<td>Ferritin (ng/mL)</td>
<td>212 (43-1602)</td>
<td>339 (16-1650)</td>
<td>0.11</td>
</tr>
<tr>
<td>Transferrin (mg/dL)</td>
<td>169 (109-227)</td>
<td>200 (118-277)</td>
<td>0.01</td>
</tr>
<tr>
<td>Transferrin Saturation (%)</td>
<td>30 (16-67)</td>
<td>35 (8-124)</td>
<td>0.68</td>
</tr>
</tbody>
</table>

**Drugs administered at baseline**

<p>| | |</p>
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>ESAs supplementation (%)</td>
<td>87.5</td>
</tr>
<tr>
<td>ESA dosage (UI/week)</td>
<td>8000 (4000 – 12000)</td>
</tr>
<tr>
<td>Iron supplementation (%)</td>
<td>100</td>
</tr>
<tr>
<td>Iron dosage (mg/week)</td>
<td>100 (50 – 100)</td>
</tr>
</tbody>
</table>

*Values are expressed as median (25,75); RDW = Red cell distribution width.*
We observed a positive correlation between IL-6 and hepcidin ($r = 0.32; p = 0.01$) (Figure 4). We did not observe a significant correlation between UTs, hepcidin and inflammation biomarkers (data not shown) and hepcidin and other inflammation biomarkers (data not shown).
Discussion

The aim of the present study was to evaluate whether toxin removal by OL-HDF in older patients would reduce inflammation and improve anemia. We observed a reduction of IS in association with a reduction of CRP and an improvement of anemia. Levels of PCS, IAA, other inflammatory biomarkers and hepcidin did not change.

Locham S et al reported that a total of 28,000 patients undergoing HD with > 60 years old had 42% of normal/mild, 49% of moderate, and 9% severe anemia, according to hemoglobin levels (normal/mild = >10 g/dL in females, >12 g/dL in males; moderate = 7 - 9.9 g/dL in females, 9 - 11.9 g/dL in males; and severe anemia <7 g/dL in females; <9 g/dL). The lower Hb levels is prevalent in HD, and it is associated with mortality, mainly in older patients. Anemia among patients on HD is usually managed by iron supplementation and ESAs. This approach was capable to improve anemia status in the current study. Since there was no increase in iron storage parameters, the improvement of inflammation (reduction of CRP) and the reduction of IS is likely to have contributed to our results.

The reduction of IS has also been reported in a previous study that followed 36 younger patients for six months in OL-HDF. Krieter et al., in a study comparing HD and OL-HDF, observed a significant reduction in plasma concentrations of free and total IS and total PCS. The reduction in uremic toxins was not observed for p-cresyl sulfate or Indole acetic acid, which is in agreement with previous study. Regarding inflammatory markers, we found a reduction in CRP but not in IL-6 or TNF-α. IL-6 is one of the most studied cytokines due to its extensive proinflammatory activity and usually presents high levels in younger CKD patients. However, conflicting results can be observed in the literature regarding of IL-6 in adults aged 60 years or older CKD patients. In our study, these cytokines were already high in baseline and no differences were observed in plasma levels of IL-6, TNF-α and IL-10 from patients after 6 months of OL-HDF. According to
our results, Ojeda R et al. did also no observe changes in these cytokines in older CKD patients after OL-HDF treatment\textsuperscript{44}. In contrast, some authors have reported a diminished in serum levels of IL-6 after OL-HDF\textsuperscript{32,45}. OL-HDF dialysis treatments are considered better than HD to remove middle molecule size. However, some studies have reported that inflammatory markers tend to behave differently in the elderly, regardless of the treatment applied\textsuperscript{46,47}.

Hepcidin was higher at initial and did not diminish after 6 months of OL-HDF and correlated with positively with IL-6. It has been described that inflammation contributes to increasing hepcidin in CKD patients\textsuperscript{16,48}. However, a possible explanation for the absence of reduced hepcidin concentration in the current study could be the specific population studies based on age and presence of residual renal function, unsimilar to previous studies. In contrast with our results, the REDERT study observed a lower serum level of hepcidin after 6 months of OL-HDF\textsuperscript{49}. However, in this study, the population was younger (66.7 ± 15.4 years), had low-grade inflammation and the initial serum concentration of hepcidin was half of that obtained in our patients.

In summary, our study observed that in older individuals with CKD on maintenance OL-HDF was capable to decrease IS and CRP but not serum levels of hepcidin and inflammatory cytokines after 6 months of follow-up. The improvement of anemia might reflect the action of ESAs and iron administration. Although not a unanimous finding among inflammatory and uremic toxins evaluated, the reduction of CRP and IS could have contributed to the improvement of anemia.

The current study should be interpreted considering some limitations such as the small sample size, the lack of information on cardiovascular endpoints and the lack of a more detailed information on ESAs use during each month of follow-up. However, our study has also the strength of have included older patients, a population that has an increased prevalence on dialysis and is scarcely represented in previous studies. In conclusion, this prospective observational study demonstrated that OL-HDF was capable to reduce IS and CRP in older patients. Whether this reduction may have an impact on clinical outcomes must be investigated in a future study.

**Patients and Methods**

**Study design**

This is a prospective observational study that evaluated older stable patients (65 years old or more) incidents on OL-HDF at baseline and after 6 months. The STROBE checklist\textsuperscript{50} was used for this prospective observational study. This study was approved by the Ethical Advisory Committee of the Universidade Nove de Julho/UNINOVE: C.A.A.E# 97475918.5.0000.5511. All patients provided written informed consent.

**Patients**

Thirty-one clinically stable patients (n = 31) were continually included. They were recruited at the Hospital Sancta Maggiore, in the period between June 2020 and November 2021. Inclusion criteria were patients with CKD aged > 65 years, on OL-HDF, within the first 90 days of renal replacement therapy. Patients were not included in the presence of chronic liver disease, auto-immune disease (i.e. systemic lupus erythematosus, rheumatoid arthritis), stages III or IV congestive heart failure, chronic degenerative neurological disease, chronic use of corticosteroids, use of topical or
systemic hormonal therapy, morbid obesity, severe peripheral vascular insufficiency, history of infection and/or inflammation within the latest 1 month, hepatitis B, HIV and/or C virus infection, current COVID-19, current malignancy, and use of antibiotics or anti-inflammatory drugs for less than 1 month before study entry.

Before starting the first OL-HDF session (Pre-OL-HDF) and after 6 months on OL-HDF (Pos-OL-HDF), 20 mL of blood was collected for evaluation of bound protein uremic toxins (IS, PCS, IAA); inflammation markers (C-reactive protein - PCR, interleukin 6 and 10 - IL-6 and IL-10, respectively, and tumoral necrosis factor - TNF-α) and parameters for evaluation of anemia as total serum iron, ferritin, transferrin saturation index, hemoglobin, hematocrit and ESAs dosage.

**OL-HDF treatment**

OL-HDF was post-dilution, aiming whenever possible convection volume ≥ 22 L/treatment, using high-flux and high-efficiency dialyzer (polysulfone model FX100, Fresenius Medical Care®). The blood flow was set at 350ml/min and dialysate flow at 500 mL/min, adjusted according to the adapt flow sensor of the dialysis machines (Model 5008 Fresenius Medical Care®). Dialysis duration and frequency were adjusted according to the presence of residual renal function. All patients received continuous heparin as an anticoagulant during the procedure.

The composition of the dialysis solution used was CPHD with Glucose 23G/44 – (Fresenius Medical Care®) with the following composition: pH: 5.2; Glucose: 1.5%, 2.5% and 4.5%; Calcium: 3.5 mEq/L or 2.5 mEq/L; Potassium: ZERO mEq/L; Sodium: 132 mEq/L or 134 mEq/L; Magnesium: 0.5 mEq/L; Chloride: 96 mEq/L or 101 mEq/L; Lactate: 40 mEq/L.

**Laboratory Methods**

**Iron Metabolism and Anemia biomarkers**

The iron metabolism biomarkers (serum iron, ferritin, transferrin and transferrin saturation index) were performed by COBAS 8000 automated analyzer (Roche Diagnostics) and hematimetric parameters for evaluation of anemia (hemoglobin, hematocrit) were performed by hematology analyzer Advia 2120 (Siemens Diagnostics) at pre-OL-HDF and pos-OL-HDF.

**Inflammatory biomarkers**

After blood collection according to the study design, a 10 mL aliquot was centrifuged, and the serum was separated and stored in a -80°C freezer for biomarkers measurement at pre-OL-HDF and pos-OL-HDF.

IL-6 was measured by ELISA (QuantiKine Human hsIL-6, catalog HS600C, R&D Systems, Minneapolis, MN), and the intra- and inter-assay CVs were <5% and <7%, respectively. The TNF-α was determined by ELISA (QuantiKine Human hsTNF-α, catalog HSTA00E, R&D Systems, Minneapolis, MN), and the intra- and inter-assay CVs were <5% and <7%, respectively. The IL-10 was measured by ELISA (QuantiKine Human hs IL-10, catalog HS100C, R&D Systems, Minneapolis, MN), and the intra- and inter-assay CVs were <5% and <7%, respectively. The high-sensitive C reactive protein (hsCRP) was measured by immunoturbidimetry (reference/sensitivity 04628918190/0.30 mg/L) on the Cobas® 6000 modular platform (Roche Diagnostics)
and hepcidin was measured by ELISA (Quantikine Human hsHepcidin, catalog DHP250, detection limit 1.70 pg/mL, R&D Systems, Minneapolis, MN). All serum levels of biomarkers were measured according to the manufacturer’s instructions.

**Uremic toxins**

Blood samples were centrifuged at 2500 rpm for 15 min and stored at -80°C. Serum (total and free fractions) IS, PCS, and IAA were quantified by high-performance liquid chromatography with fluorescent detection, as described by Borges et al.\(^5^1\) at pre-OL-HDF and pos-OL-HDF.

**Statistical methods**

Data normality was verified by the Shapiro-Wilk test. Continuous numeric variables were expressed according to their parametric or nonparametric distribution, as mean and standard deviation or median and percentiles (25-75%), respectively. Categorical data is described as absolute values and percentages of the total sample. Analyses between baseline and 6 months were performed using the paired T-test or Wilcoxon test, as appropriate. The correlation between independent variables was analyzed using the Pearson or Spearman test, when appropriate. A significance level of 5% (p < 0.05) was established for the statistical tests. Analyzes were performed using IBM SPSS STATISTICS software for Windows (IBM Corp., Armonk, N.Y., U.S.A.) version 25.

**Statement of Ethics**

The study was performed in accordance with the Declaration of Helsinki and approved by the Ethical Advisory Committee of the Universidade Nove de Julho/UNINOVE: C.A.A.E# 97475918.5.0000.5511. Informed consent was obtained from all subjects involved in the study or by their legal representative.

**Funding Sources**

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**Data Availability Statement**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**References**


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**Author Contributions**


**Disclosure Statement**

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.