Effect of Nutritional Status on Human Paraoxonase-1 Activity in Patients with Chronic Kidney Disease

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Key Words
Paraoxonase • Obesity • Nutritional state • C-reactive protein • Chronic kidney disease • Reverse epidemiology • Hemodialysis

Abstract
Background/Methods: The association between nutritional status, antioxidant human paraoxonase-1 (PON1) activity and low grade inflammation in hemodialized (HD) patients with chronic kidney disease (CKD) is unclear. The aim of this study was to determine PON1 paraoxonase and lactonase activities, ADMA, adiponectin and leptin concentrations, and to clarify the relationship between paraoxonase activity and a set of cardiovascular risk factors in malnourished, normal weight and obese HD patients; 114 HD patients with end-stage renal failure were enrolled. Results: Leptin levels were significantly higher and PON1 paraoxonase activities were significantly lower in obese patients compared to the other groups. Plasma adiponectin concentration was significantly lower in obese subjects compared to malnourished patients. Paraoxonase activity was negatively correlated with CRP level in HD and malnourished patients. Furthermore, we found significant inverse correlation between paraoxonase activity and BMI in the whole patient group. In multiple regression analysis, PON1 lactonase activity, CRP level and leptin concentration proved to be independent predictors of paraoxonase activity. Conclusion: Despite the previous findings of reverse epidemiology for the mortality rate of HD patients, further studies are needed to clarify the effects of nutritional state on atherosclerosis in obese and malnourished patients with end-stage renal failure.
Cardiovascular diseases are the major cause of morbidity and mortality in chronic kidney disease (CKD) patients. Previous studies have suggested that traditional risk factors alone might not explain the higher prevalence and incidence of cardiovascular diseases in CKD patients on hemodialysis (HD) treatment [1, 2]. Non-traditional risk factors, such as inflammation, oxidative stress and malnutrition are gaining acceptance in CKD especially in HD patients [3]. Although traditional risk factors for cardiovascular diseases have been shown to correlate with an unfavorable outcome in the general population, these factors appear to be protective and are associated with an improved survival in patients with chronic heart disease. In the last decades, several studies have published this unexpected and paradoxical phenomenon in patients with chronic heart disease referred to as „reverse epidemiology”; both C-reactive protein (CRP) as a marker of inflammation and (pre)albumin as a marker of nutritional status have been shown to be important independent predictors of mortality. Therefore, it is conceivable that both malnutrition and inflammation – referred to as the malnutrition-inflammation complex syndrome [4] - may be related to the reverse epidemiology [5].

Most of the HD patients have some degree of malnutrition; protein and energy depletion have been associated with increased morbidity and mortality in this population in several previous studies [6, 7]. The available evidence suggests that low protein and energy intake in CKD along with the catabolic consequences of HD therapy may lead to the development of uremic malnutrition [6]. Numerous studies have shown that markers of malnutrition and inflammation, such as low body mass index (BMI), elevated CRP and increased plasma concentration of asymmetric dimethylarginine (ADMA), were strong independent predictors of cardiovascular mortality in CKD [3, 8].

Inflammation contributes to the development of cardiovascular disease by increasing vascular calcification and endothelial dysfunction in chronic kidney disease. On the other hand, various studies have concluded that CRP levels reflect well the degree of inflammation in hemodialyzed patients with metabolic syndrome and abdominal obesity [9-11].

Adipose tissue possesses various functions as energy storage and secretion of a number of adipocytokines including leptin, adiponectin with potential endocrine functions [12, 13]. Adiponectin have been shown to be inversely correlated with insulin resistance and cardiovascular diseases in obese or overweight patients. Adiponectin levels have been found to be decreased in obesity and increased during weight loss; however, CKD has been associated with markedly elevated levels of adiponectin probably due to its decreased renal excretion [13, 14]. Previously, it has been demonstrated that adiponectin has potential anti-inflammatory and anti-atherogenic properties due to its modulatory effect on endothelial adhesion molecules acting as protective factor against atherogenesis [15].

Another adipokine, leptin regulates the amount of body fat, food intake and energy homeostasis; leptin levels show positive correlation with body fat mass [16, 17]. Serum leptin levels have been previously reported to be elevated in patients with chronic renal failure and to correlate with C-reactive protein levels suggesting that inflammation may be an important factor in the development of hyperleptinemia in CKD [18].

Both obesity and chronic inflammation enhance oxidative stress that leads to the oxidative modification of lipoprotein particles resulting in accelerated atherosclerosis. Human serum paraoxonase (PON1) is an HDL-associated antioxidant enzyme which prevents low-density lipoprotein (LDL) from lipid peroxidation. In our previous work, we reported lower PON1 paraoxonase activity in HD patients [19, 20]. Previous studies have also demonstrated that PON1 paraoxonase activity was inversely associated with cardiovascular risk [21]; serum PON1 paraoxonase activity was shown to correlate negatively with the degree of oxidative stress [22, 23]. Several data suggest that PON1, which metabolizes a number of substrates such as paraoxon and phenylacetate, could also hydrolyze various esters, carbonates, thioesters and thiolactones via its lactonase activity [24]. A possible physiological substrate may be the homocysteine thiolactone known as a risk factor in atherosclerosis: metabolic
conversion of homocysteine to thiolactone and protein homocysteinylataion by thiolactone may play role in homocysteine-induced vascular damage [25, 26].

We hypothesized that the changes in PON1 paraoxonase and lactonase activity in HD patients are associated with their nutritional status and may contribute to the increased risk of accelerated atherosclerosis in CKD. Therefore, the aim of this study was to determine PON1 paraoxonase and lactonase activities, ADMA, adiponectin, leptin concentrations and to reveal the relationship between paraoxonase activity and cardiovascular risk factors in malnourished and obese HD patients.

Patients and Methods

Study population
One hundred fourteen patients receiving hemodialysis three times weekly between 2005 and 2009 were enrolled in the study. The mean duration of dialysis was 46 months and ranged from 4 to 140 months; each hemodialysis treatment lasted four hours. We excluded patients with alcoholism, liver disease, elevated liver enzymes, recent myocardial infarction (38.2% of patients had a previous history of angina pectoris or ischaemic heart disease), endocrine diseases (thyroid and parathyroid diseases, pituitary and adrenal gland disorders, etc.), pregnancy, lactation, patients on lipid-lowering therapy and smokers. We excluded the patients with myocardial infarction after the 3 months from the symptoms onset with Q wave on the ECG. According to their BMI, patients were divided into three groups: malnourished (BMI<20 kg/m2), normal weight (20 kg/m2≤BMI≤30 kg/m2) and obese (BMI>30 kg/m2) groups. Patients gave a written, informed consent to participate, and the study was performed according to the requirements of the Ethical Committee of the Medical and Health Science Center, University of Debrecen, as well as the Code of Ethics of the World Medical Association.

Blood sampling
After 12 hours of fasting, 10 ml venous blood sample was taken between 7.30 and 8.00 in the morning before dialysis. Lipid parameters and homocysteine concentrations were determined in fresh sera. The sera for enzyme activity measurements and for ELISA determinations were kept at – 70 oC before analysis.

Measurement of homocysteine and lipid parameters
Fasting plasma total Hcy concentrations were determined by enzyme-linked immunoassay and automated fluorescence polarization analyzer (FPIA, IMX System, Abbott Diagnostics, Rome, Italy). This assay is a fluorescence polarization immunoassay based on the highly selective enzymatic conversion of homocysteine to S-adenosyl-L-homocysteine, which is then recognized by a monoclonal antibody.

Serum cholesterol and triglyceride levels were measured by using enzymatic, colorimetric tests (GPO-PAP, Modular P-800 Analyzer, Roche/Hitachi), while high-density lipoprotein cholesterol (HDL-C) was assessed by a homogenous, enzymatic, colorimetric assay (Roche HDL-C plus 3rd generation). LDL-cholesterol was measured by homogenous, enzymatic, colorimetric assay (Roche LDL-C plus 2nd generation, Basel, Switzerland). The tests were performed according to the recommendation of the manufacturer.

Analysis of PON1 paraoxonase activity
PON1 paraoxonase activity was measured as previously described [19, 20]. Briefly, we used paraoxon (O,O-diethyl-O-p-nitrophenylphosphate, Sigma) as substrate, and the generation of 4-nitrophenol was measured spectrophotometrically. 50 µl serum was dissolved in 1 ml Tris/HCl buffer (100 mmol/l, pH=8.0) containing 2 mmol/l CaCl2 and 5.5 mmol/l paraoxon. We measured the absorbance at 412 nm (25 oC), using a Hewlett-Packard 8453 UV-visible spectrophotometer. Enzyme activity was calculated using the molar extinction coefficient 17100 M-1cm-1. One unit of paraoxonase activity is defined as 1 nmol of 4-nitrophenol formed per minute under the assay conditions mentioned above.

Analysis of PON1 arylesterase activity
Arylesterase activity was measured spectrophotometrically. The assay contained 1 mM phenylacetate in 20 mM Tris/HCl pH 8.0. The reaction was started by the addition of the serum and the increase in
absorbance was read at 270 nm as already described [20, 27]. Blanks were included to correct the spontaneous hydrolysis of phenylacetate. Enzyme activity was calculated using a molar extinction coefficient of 1310 M−1cm−1. 1 unit (U) is defined as 1 μmol phenylacetate hydrolyzed per minute.

**Analysis of PON1 lactonase activity**

PON1 lactonase activity was measured by a commercially available assay kit (Alfresa Auto HTLase; Alfresa Pharma Corporation, Japan). This kit utilizes gamma-thiobutyrolactone as substrate and Ellman’s procedure to monitor the accumulation of free sulphydryl groups via coupling with 5,5-dithiobis(2-nitrobenzoic acid) [28]. Intra-assay CV was less than 6%.

**Paraoxonase Genotyping**

PON1–55 and PON1–192 polymorphisms were determined using Light Cycler real-time technology based on fluorescence resonance energy transfer combined with melting point analysis as published previously [29]. Briefly, the regions of the PON1 gene surrounding the PON1–55 and PON1–192 polymorphisms resulting in 151- and 138-bp amplicon sizes were amplified. The sensor probe applied for PON1–55 genotyping was labeled with 5'-LC Red 640 dye, while to that for PON1–192 LC Red 705 fluorophore was attached. Both detection probe sets were located upstream at a distance of 2 nucleotides from each other. Both primers and fluorescence labeled probes were synthesized by TIB Molbiol Co. (Berlin, Germany). Polymerase chain reaction and melting curve analysis were performed in 20 µl-volume glass capillaries (Hoffmann-La Roche). During the melting point analysis followed by an ultrafast polymerase chain reaction, fluorescence intensity was monitored. Melting peaks were calculated from the melting curves by plotting the negative derivative of the fluorescence signal against temperature.

**ADMA measurement**

ADMA concentrations in serum of hemodialyzed patients were measured with commercially available ELISA kit (ADMA-ELISA, DLD Diagnostika GmbH, Hamburg, Germany). ADMA concentrations in samples were measured by a competitive enzyme immunoassay with intra-assay CVs ranging from 4.5 % to 7.5%, and inter-assay CVs ranging from 8.3% to 10.3%. Measurements of ADMA level in sera were performed according to the manufacturer’s instructions.

**Adiponectin and Leptin Measurement**

Total adiponectin and leptin concentrations in serum of hemodialyzed patients were measured with commercially available ELISA kits (Human Total Adiponectin/Acrp30 Quantikine and Human Leptin Quantikine Immunoassays, R&D Systems, Minneapolis, USA; ADMA-ELISA, DLD Diagnostika GmbH, Hamburg, Germany). Adiponectin assay employs the quantitative sandwich enzyme immunoassay technique with intra-assay CVs ranging from 2.5 % to 4.7%, and inter-assay CVs ranging from 6.8% to 6.9%. Precision of leptin measurement was intra-assay CVs ranging from 3.0% to 3.3%, inter-assay CV-s from 3.5% to 5.4%.

**Statistical methods**

SAS for Windows 6.12 (SAS Institute Inc.) computer program was used for the statistical analysis. Normality of data distribution was tested by Kolmogorov-Smirnov test. One-way analysis of variance was used to compare different groups of HD patients. Data were expressed as means ± SD in case of normal distribution, and medians and quartiles in case of non-normal distribution. Comparisons between groups were performed by analysis of variance (ANOVA with Tukey post test). In case of PON1 paraoxonase and lactonase activity we used median test and due to multiple testing Bonferroni-Holm correction was performed. Relationships between parameters were assessed by Pearson correlation analysis. We carried out multiple regression analysis (backward-stepwise method) to test which of the variables predicted best paraoxonase activity. PON1-192 and PON1-55 genotype distribution for 3 groups with different body weights were analysed by chi-square test.

**Results**

Studying the data of the 114 CKD patients treated with hemodialysis for more than six months, participants were divided into three groups according to their BMI as malnourished
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Leptin levels were significantly higher (p≤0.001) and PON1 paraoxonase activities were significantly lower (p=0.019) in obese patients compared to the malnourished group but this difference is on the border of significance with Bonferroni-Holm correction (Table 1). There were no significant differences among these groups in gender, age, duration of haemodialysis, smoking habits and diabetes.

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activity and CRP levels in obese and normal-weight HD patients. We found a significant positive correlation between BMI and CRP levels in all HD patients (Table 2) \( (r=0.586, p<0.01) \), in malnourished, \( r=0.402, p<0.01 \) and in obese subjects, \( r=0.485, p<0.01 \), respectively, not shown). Furthermore, we found a significant inverse correlation of PON1 paraoxonase activity and BMI in all patients on dialysis (Fig. 2) there is no figure 2, please check? \( (r=-0.314, p<0.05, \text{Table 2}) \) and in the whole patient population \( (r=-0.303, p<0.05, \text{not shown}) \). We could not detect significant correlation between serum PON1 paraoxonase activity and adiponectin levels in HD patients; however, a positive correlation was observed between these parameters in normal-weight HD patients \( (r=0.326, p<0.05, \text{not shown}) \). We found a positive correlation between adiponectin and PON1 lactonase activity \( (r=0.303, p<0.05, \text{not shown}) \) and a significant negative correlation between PON1 lactonase activity and CRP levels \( (r=-0.318, p<0.05, \text{not shown}) \) in all HD patients.

To test whether the association between PON1 paraoxonase activity and CRP levels in the univariate analysis was independent of age, BMI and other parameters, we carried out multiple regression analysis. Lactonase activity, CRP level and leptin concentration were independent predictors of PON1 paraoxonase activity adjusting age, BMI, ADMA and adiponectin to the model (Table 3).

The allelic frequencies and genotype distributions in the malnourished, normal-weight and obese HD patients are summarized in Table 4. The allelic frequencies were in accordance with the results of our previous studies \([30, 31]\) and the literature \([32, 33]\); they followed the Hardy-Weinberg equilibrium. There were no significant differences between the studied groups.

As shown in Table 1, no significant differences were observed in serum HDL-cholesterol concentrations, glucose and CRP levels in each groups. Malnourished subjects had significantly lower triglyceride levels and significantly lower mean total and LDL-cholesterol concentrations than the normal weight and obese patients. Creatinine concentrations were similar in the three groups of patients, and plasma levels of albumin were also similar in malnourished, obese and normal weight subjects.

**Discussion**

Our previous studies have shown that paraoxonase and lactonase activities of PON1 enzyme are significantly decreased in chronic renal failure \([19, 30]\). In the present work, we found higher PON1 paraoxonase activities in malnourished HD patients compared to
obese patients, and similar changes in lactonase activities; however, it did not prove to be statistically significant. We detected a positive correlation between adiponectin and PON1 lactonase activity and a significant negative correlation between lactonase activity and CRP levels in all HD patients. This result suggests an impaired antioxidant status in CKD patients with higher BMI and supports the initial hypothesis that the decrease in paraoxonase and lactonase activities through the reduction of their antiatherogenic effects may contribute to accelerated atherogenesis in CKD, especially in obese hemodialyzed patients.

Previous studies have demonstrated a direct relationship between BMI and CKD risk [34, 35] and have shown a correlation between BMI and the increasing prevalence of chronic renal failure in overweight and obese patients. In our study, a moderate increase in serum creatinine level was found parallel with BMI. It has been previously suggested that this increase may be linked to the progression of CKD in HD patients; however we could not demonstrate a significant difference between the obese and malnourished groups. Moreover, there was no connection between BMI and serum albumin levels in HD patients supporting the findings of other studies where albumin was an unreliable marker of the nutritional status in CKD and elderly patients [36, 37].

The role of leptin in kidney function has not been completely defined so far. It is thought to be a potential salt-regulating factor and may function pathophysiologically as a common link to obesity and hypertension [38]. It has been also recognized that hyperleptinemia was linked to renal structural changes associated with obesity. Although leptin is partly cleared by the kidneys and patients with either kidney disease or HD have been demonstrated to have higher leptin levels, our previous research showed that hyperleptinemia was not responsible for decreased paraoxonase activity in HD patients [39]. Therefore, hyperleptinemia may be an independent predictor for the progression of renal diseases and for the increased risk of cardiovascular diseases in HD patients with higher BMI.

Oxidative stress and inflammation have been implicated in albuminuria and renal dysfunction in uremic patients. It has been previously shown that low adiponectin levels are associated with inflammation and atherosclerosis in CKD; however, we could not find any relationship between adiponectin and PON1 paraoxonase activity in the present study.

This result suggests that PON1 paraoxonase activity may be a reliable indicator regarding the progression of renal failure in malnourished patients compared with the obese HD

Table 3: Multiple regression analysis for PON1 as a dependent variable (R²=0.211)

<table>
<thead>
<tr>
<th></th>
<th>Beta</th>
<th>t</th>
<th>p-level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>1.279</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.012</td>
<td>0.078</td>
<td>0.938</td>
</tr>
<tr>
<td>BMI</td>
<td>0.024</td>
<td>0.115</td>
<td>0.909</td>
</tr>
<tr>
<td>Lactonase</td>
<td>0.406</td>
<td>2.331</td>
<td>0.027</td>
</tr>
<tr>
<td>CRP</td>
<td>-0.488</td>
<td>-2.798</td>
<td>0.009</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>-0.247</td>
<td>-1.419</td>
<td>0.167</td>
</tr>
<tr>
<td>Leptin</td>
<td>-0.411</td>
<td>-2.069</td>
<td>0.047</td>
</tr>
<tr>
<td>ADMA</td>
<td>0.046</td>
<td>0.284</td>
<td>0.778</td>
</tr>
</tbody>
</table>

Table 4: The allelic frequencies and genotype distributions in the malnourished, normal-weight and obese hemodialized patients

<table>
<thead>
<tr>
<th></th>
<th>Malnourished</th>
<th>Normal-weight</th>
<th>Obese</th>
</tr>
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<tbody>
<tr>
<td>PON1-192</td>
<td>Q: 0.68</td>
<td>0.65</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>R: 0.32</td>
<td>0.35</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>QQ: 0.474</td>
<td>0.441</td>
<td>0.472</td>
</tr>
<tr>
<td></td>
<td>QR: 0.421</td>
<td>0.412</td>
<td>0.417</td>
</tr>
<tr>
<td></td>
<td>RR: 0.105</td>
<td>0.147</td>
<td>0.111</td>
</tr>
<tr>
<td>PON1-55</td>
<td>L: 0.32</td>
<td>0.3</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>M: 0.68</td>
<td>0.7</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>LL: 0.117</td>
<td>0.086</td>
<td>0.105</td>
</tr>
<tr>
<td></td>
<td>LM: 0.412</td>
<td>0.429</td>
<td>0.421</td>
</tr>
<tr>
<td></td>
<td>MM: 0.471</td>
<td>0.485</td>
<td>0.474</td>
</tr>
</tbody>
</table>

PON1-192 \( \chi^2 = 0.1955 \) p=0.9955;
PON1-55 \( \chi^2 = 1.5417 \) p=0.8192.
group. Investigating the relationship of different variables (age, BMI, HDL-C, CRP and ADMA levels) to paraoxonase activity in multiple regression analysis, only PON1 lactonase activity, leptin and CRP levels proved to be independent predictors of PON1 paraoxonase activity. Several authors have demonstrated that higher BMI is associated with improved survival in overweight and obese CKD patients on maintenance hemodialysis compared to the general population [4, 40, 41]. „Reverse epidemiology” in CKD is one of the most discussed and controversial topic regarding the mortality of HD patients. Reverse epidemiology suggests some beneficial effects of higher BMI in patients with CKD. Various studies have concluded that the presence of the malnutrition-inflammation complex syndrome may also explain the existence of reverse epidemiology in HD patients [10, 11, 40]. It seems that higher BMI itself is not beneficial but is a marker of lower catabolic rate. Thus, higher BMI and better prognosis are two independent consequences of a better metabolic status. In general, malnourished HD patients have worse prognosis than those with adequate body weight, whereas PON1 was higher in malnourished patients. These data indicate that PON1 is not involved in the mechanism of “reverse epidemiology”, and that protective effect of PON1 is either lost in malnourished patients or is outweighed by other detrimental mechanisms in this group.

**Conclusion** (ED: please approve, must stand in extra paragraph)

Our results show significantly lower activities of the antiatherogenic PON1 in obese HD patients compared to malnourished subjects. Despite our findings regarding the reverse epidemiology for the mortality of HD patients, further studies are needed to reveal the real effects of nutritional state on atherosclerosis in obese and malnourished CKD patients. There is growing evidence in the literature [42, 43] to support our initial hypothesis that the antioxidant properties of PON-1 enzyme are closely associated with PON1 paraoxonase activity and not with lactonase activity. Therefore, in the present study we have primarily investigated the relationship between PON1 paraoxonase activity and the antioxidant status in chronic kidney disease depending on nutritional status. Our goal was to evaluate the alteration of PON1 paraoxonase and lactonase activities and their correlations with nutrition levels in malnourished, normal-weight and obese hemodialyzed patients. Our result suggests that PON1 paraoxonase activity may be a reliable marker regarding the progression of renal failure in malnourished subjects compared with the obese hemodialyzed patients. To our best knowledge, there is no specific substance or enzyme, which can determine the lactonase activity of PON1 enzyme. Otherwise, we have shown in our previous study that the PON1 lactonase activity was not independent of PON1 paraoxonase activity in patients with chronic kidney disease [30].

**Conflict of Interests** (ED: please approve, must be added)

The authors of this manuscript state that they have no conflicts of interest.

**Aknowledgements**

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