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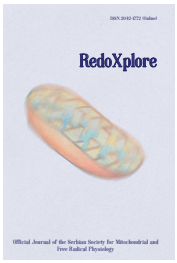

Research Article

Association of Redox Status and Inflammatory Markers with Clinical Outcomes in Haemodialysis Patients

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Keywords

Cardiovascular risk
Chronic kidney disease
Haemodialysis
Inflammation
Oxidative stress
PON1 phenotypes
TNF-α

Abstract

Purpose: This study aimed to investigate the association between paraoxonase 1 (PON1) activity phenotypes and redox status parameters, while evaluating the role of the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) as a predictor of mortality and cardiovascular risk in patients undergoing chronic haemodialysis (HD).

Methods: This study cohort comprised patients with end-stage renal disease (ESRD). PON1 paraoxonase (POase) and arylesterase (ARE) activities, alongside a comprehensive redox profile, were determined spectrophotometrically. TNF-α concentrations were quantified via ELISA. PON1 phenotypes (QQ, QR, RR) were assigned using the POase/ARE ratio and the antimodal method. Survival analysis was performed using the Kaplan-Meier method.

Results: The RR phenotype was the most prevalent among HD patients (43.2%) and was significantly associated with the highest levels of oxidative stress, specifically elevated total oxidative status (TOS) and advanced oxidation protein products (AOPP) ($p < 0.001$). TNF-α concentrations were significantly higher in patients who died during the study compared to survivors (5.22 vs. 3.45 pg/mL, $p = 0.050$). Kaplan-Meier analysis confirmed that TNF-α levels > 5 pg/mL are a significant predictor of shorter survival (log-rank = 4.21, $p = 0.040$). Elevated TNF-α directly correlated with dyslipidemia progression and a higher atherosclerosis index.

Conclusions: The PON1 RR phenotype and elevated TNF-α concentrations (> 5 pg/mL) serve as critical biomarkers of oxidative and inflammatory status in HD patients. Their synergistic interaction contributes to accelerated atherogenesis and increased mortality, highlighting their utility in clinical risk stratification.

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Introduction

Chronic kidney disease (CKD), according to the Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines, is defined as structural or functional damage of the kidneys and/or a decrease in the glomerular filtration rate (GFR) below 60 ml/min/1.73 m^2 lasting longer than three months [1]. The National Guideline of Good Clinical Practice, in accordance with KDOQI guidelines, classified CKD into five stages according to GFR, with the fifth stage representing the terminal stage of the disease (end-stage renal disease, ESRD), when patients require kidney replacement therapy [1,2]. The main causes of CKD are diabetes and hypertension, but obesity, older age, and cardiovascular diseases also play a significant role [2,3]. Haemodialysis (HD) is a key method for the treatment of patients in the terminal stage of the disease because it enables extracorporeal blood purification, removal of toxins and excess fluid, as well as correction of electrolyte and acid–base status [4]. Although it has prolonged the life expectancy of patients, the haemodialysis process itself carries certain risks. The interaction of blood with dialysis membranes can stimulate the synthesis of pro-inflammatory cytokines and activate pro-oxidative mechanisms [5].

Central to the systemic complications of ESRD is oxidative stress, a disturbance in the balance between pro-oxidants (such as reactive oxygen species – ROS) and antioxidants, in which pro-oxidants overpower defensive mechanisms, leading to damage to proteins, lipids, and nucleic acids [6,7]. In patients undergoing dialysis, this process is further intensified because the treatment itself leads to the loss of antioxidants and the activation of pro-oxidative factors [5]. Within the antioxidant defense system, a key role is played by paraoxonase 1 (PON1), a calcium-dependent enzyme associated with high-density lipoprotein (HDL) particles that prevents the oxidation of low-density lipoprotein (LDL) cholesterol and cellular membranes [8]. The gene responsible for the synthesis of PON1 is highly polymorphic, and the most significant is the PON1 Q192R polymorphism, which determines the phenotype of enzyme activity [9]. In patients with CKD, PON1 activity is significantly reduced due to the accumulation of uremic toxins, chronic inflammation, and increased oxidative stress, which directly contributes to the accelerated development of atherosclerosis [10,11].

CKD is also characterized by a chronic low-grade inflammatory state that contributes to the progression of damage to renal structures. Inflammation in CKD stimulates the release of pro-inflammatory cytokines that activate immune cells and contribute to the fibrotic process [12]. One of the most important pro-inflammatory markers is tumor necrosis factor-alpha (TNF-α), a cytokine produced by macrophages during acute and chronic inflammation, whose concentration increases in patients undergoing haemodialysis. Increased levels of TNF-α are directly associated with a decrease in glomerular filtration and enhanced systemic inflammation. Studies indicate that high concentrations of TNF-α may be a strong predictor of mortality and shorter survival in patients on a haemodialysis program [13–15].

Oxidative stress and chronic inflammation do not operate in isolation but act synergistically, creating a vicious cycle that increases cardiovascular risk, which is the leading cause of death in this population of patients [16–19]. A decrease in oxidative potential (through reduced PON1 function) and an increase in pro-inflammatory burden (through elevated TNF-α) lead to endothelial dysfunction and vascular calcification [12]. Understanding the relationship between specific PON1 phenotypes, redox status parameters, and TNF-α levels is essential for the early identification of the most vulnerable patients and for the prevention of cardiovascular complications.

The primary aim of this study was a comprehensive evaluation of biomarkers of oxidative stress and inflammation in patients with end-stage renal disease undergoing a chronic haemodialysis program. The study was focused on determining the distribution of PON1 activity phenotypes (QQ, QR, and RR) based on the ratio of paraoxonase and arylesterase activity, as well as analyzing their direct impact on redox status parameters. At the same time, the clinical significance of TNF-α as a potential predictor of mortality and shorter survival in this patient population was also examined, using Kaplan–Meier survival analysis.

Materials and Methods

The study was planned and conducted in accordance with applicable national regulations and the ethical principles of the Declaration of Helsinki, with prior approval from the Ethics Committee of the Clinical Hospital Center Zvezdara. All participants provided informed consent to participate in the study.

Participants, sampling, and sample storage

The study included 125 patients diagnosed with chronic kidney disease who were undergoing a regular chronic haemodialysis program at the Clinical Hospital Center Zvezdara. The majority of patients underwent standard bicarbonate haemodialysis using biocompatible polysulfone membranes. This modality was selected as it represents the clinical standard at the Clinical Hospital Center Zvezdara, allowing for a consistent evaluation of inflammatory responses. The primary diseases and main causes of CKD development in these patients were identified as arterial hypertension, diabetes mellitus, glomerulonephritis, and polycystic kidney disease, while in a portion of the participants, the cause of CKD was unknown. The control group consisted of 22 healthy subjects without known kidney disease or other chronic illnesses. Within the total study population, TNF-α concentrations were also determined in a subgroup of 45 patients. Peripheral blood samples were collected from all participants immediately before the haemodialysis procedure and transported to the Faculty of Pharmacy, where serum was separated by centrifugation at 3000x g for 15 minutes and stored at a temperature of −80 °C until the time of analysis.

Analysis of PON1 activity and determination of phenotypes

To assess the functional status of the PON1 enzyme, spectrophotometric methods were applied to measure two activities: paraoxonase activity (POase, expressed in U/L), which is based on the rate of paraoxon hydrolysis with the formation of the colored p-nitrophenoxide anion, and arylesterase activity (ARE, expressed in kU/L), which is measured based on the degradation of phenylacetate [20,21]. Based on the ratio of these two activities (POase/ARE), using the antimodal method, patients were classified into three specific phenotypes: QQ (low POase/high ARE activity), RR (high POase/low ARE activity), and QR (intermediate values).

Assessment of redox status and pro-inflammatory markers

Total oxidative status (TOS, in μmol/L), total antioxidant status (TAS, in mmol/L of Trolox equivalents), and the level of advanced oxidation protein products (AOPP, in μmol/L of chloramine T equivalents) were measured using automated methods on the ILAB 300+ biochemical analyzer [22-24]. The pro-oxidant–antioxidant balance (PAB, in U/L) and total sulfhydryl groups (tSHG, in mmol/L) were determined by a spectrophotometric method using the SPECTROstar® nano UV/VIS spectrophotometer (BMG LABTECH, Ortenberg, Germany) [25,26]. The concentration of TNF-α (expressed in pg/mL) was determined using a non-competitive sandwich ELISA method, employing a commercial TNF-α DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA).

ARE and POase activities of the PON1 enzyme, as well as redox status parameters, were determined in the laboratory of the Department for Medical Biochemistry at the Faculty of Pharmacy, University of Belgrade.

Determination of clinical–biochemical parameters

In all patients, biochemical and hematological parameters relevant to this study were determined, including C-reactive protein (CRP, mg/L), triglyceride concentration (mmol/L), erythrocyte count ($x10^{12}$/L), and haemoglobin concentration (g/L). Biochemical parameters (CRP and triglycerides) were determined using routine methods on the automated biochemical analyzer Roche/Hitachi Cobas c501, and the data were obtained from the information system of the Laboratory Diagnostics Service of the Clinical Hospital Center Zvezdara. Hematological parameters (erythrocyte count and haemoglobin concentration) were determined on the automated hematology analyzer Sysmex XN-1000 using the method of flow fluorescent cytometry. Cardiovascular risk was assessed using the total cholesterol/HDL-C ratio, while the atherosclerosis index was calculated as the LDL-C/HDL-C ratio.

Statistical analysis

All statistical analyses were performed using the software package PASW Statistics 18 (IBM SPSS Statistics). The distribution of data was examined using the Kolmogorov–Smirnov test. Continuous data with a normal distribution were presented as mean ± standard deviation, while data that did not follow a normal distribution were presented as median and interquartile range (25th–75th percentile). Categorical variables were presented as numbers and/or percentages. Differences between categorical variables were analyzed using the χ^2 test. Comparisons of continuous variables that did not follow a normal distribution were performed using the Mann–Whitney U test or Kruskal–Wallis test. Correlations between

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the examined parameters were assessed using Spearman's correlation analysis. Patient survival in relation to TNF-α levels was analyzed using the Kaplan–Meier method. Statistical significance was defined as $p \leq 0.05$.

Results

Sociodemographic characteristics and clinical profile of patients on haemodialysis

Demographic data and the main causes of ESRD in 125 patients undergoing haemodialysis are presented in **Table 1**. The leading causes of ESRD identified were arterial hypertension (43.1%) and diabetes mellitus (23.1%), while glomerulonephritis and polycystic kidney disease were present to a lesser extent.

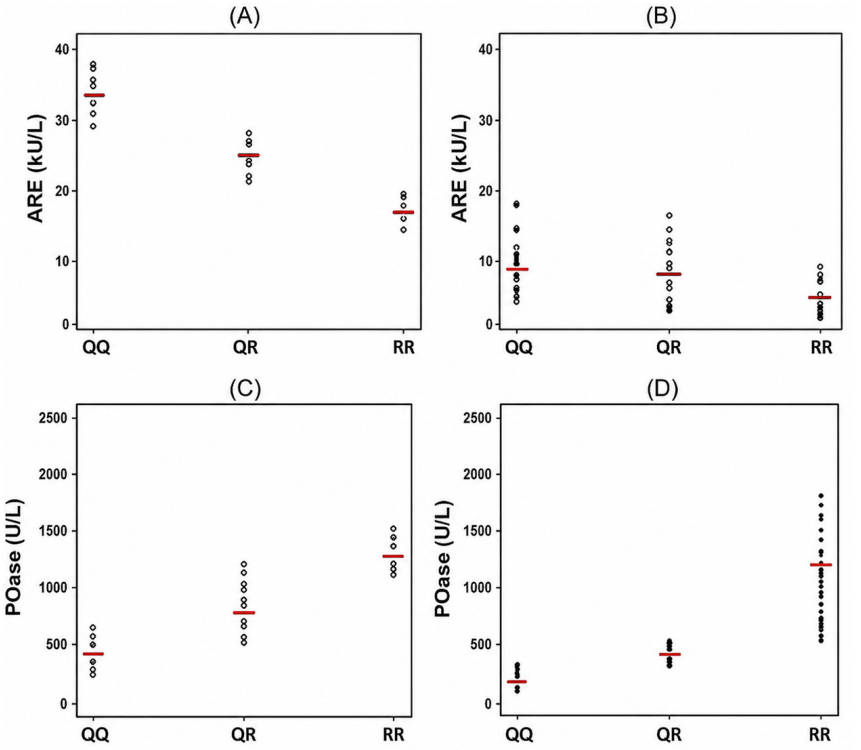

Figure 1. Paraoxonase and arylesterase activity ratio (POase/ARE) for all three phenotypes (QQ, QR, and RR): (A) ARE activity in the control group, (B) ARE activity in patients with CKD undergoing haemodialysis, (C) POase activity in the control group, and (D) POase activity in patients with CKD undergoing haemodialysis.

Table 1. Sociodemographic data and main causes of ESRD in patients on haemodialysis.

Parameters (units)	Patients on haemodialysis (N=125)
Age (years)	66 (54-72)
Sex – m/f (%)	75.4/24.6
BMI* (kg/m^2)	25.7 ± 4.7
Systolic blood pressure (mm Hg)	130 (120-142)
Diastolic blood pressure (mm Hg)	80 (65-80)
Dialysis duration	40 (20-91)
Primary cause of end-stage renal disease	
Hypertension (%)	43.1
Diabetes mellitus (%)	23.1
Glomerulonephritis (%)	10.8
Polycystic kidney disease (%)	10.8
Other (%)	12.3

*BMI – body mass index; continuous data with a normal distribution are presented as mean ± standard deviation; data that do not follow a normal distribution are presented as median and interquartile range, while categorical data are presented as percentages.

Distribution of PON1 phenotypes and enzyme activity

The results showed that patients undergoing haemodialysis have significantly lower POase and ARE activity compared with the control group ($p < 0.001$) (**Figure 1**). Based on the ratio of these activities, it was determined that the RR phenotype was the most prevalent among patients with kidney disease (43.2%), while the QR phenotype predominated in the control group (68.2%) (**Table 2**).

Table 2. Distribution of phenotypes and values of POase and ARE activity of PON1 in both study groups.

PON1 phenotype	CKD, N=125	Control group, N=22	P
QQ, n (%)	39 (31.2%)	4 (18.2%)	$\chi^2 = 15.9$ ($p < 0.001$)
QR, n (%)	32 (25.6%)	15 (68.2%)	
RR, n (%)	54 (43.2%)	3 (13.6%)	
Paraoxonase and arylesterase PON1 activity			
POase activity	278 (170-469)	744 (671-922)	< 0.001
ARE activity	38 (26-52)	173 (138-227)	< 0.001

Categorical data are presented as absolute frequencies (percentages); data that do not follow a normal distribution are presented as median and interquartile range.

Association of PON1 phenotypes with redox status and inflammation

The values of POase and ARE activity of PON1, redox status parameters, as well as biochemical and hematological parameters determined in patients are presented according to phenotypes in **Table 3**.

type and lowest in those with the QQ phenotype ($p < 0.001$) (**Figure 2B**).

The values of oxidative stress parameters (TOS and AOPP) were highest in patients with the RR phenotype and lowest in those with the QQ phenotype ($p < 0.001$ for both parameters) (**Figure 3**), while no statistically significant difference was

Table 3. Changes in POase and ARE activity of PON1, redox status parameters, and biochemical and hematological parameters in patients with CKD according to PON1 phenotypes.

Parameter	QQ	QR	RR	P
POase (U/L)	147 (129-190)	261 (158-562)*	426 (221-653)$^{***,\#}$	< 0.001
ARE (kU/L)	68 (52-78)	34 (17-60)*	12 (8-20)$^{***,\#\#}$	< 0.001
TOS (μmol/L)	7.2 (4.9-15.2)	85.3 (11-132)***	128.9 (10.6-133.5)$^{***,\#\#\#}$	< 0.001
AOPP (μmol/L)	30.4 (24.6-36.6)	33.0 (24.5-44.9)	48.6 (42.4-51.7)$^{***,\#\#}$	< 0.001
PAB (U/L)	48 (39-54)	46 (41-59)	46 (37-58)	0.329
TAS (mmol/L)	1334 (1261-1493)	1344 (1279-1471)	1326 (1225-1460)	0.707
tSHG (mmol/L)	0.254 (0.206-0.320)	0.277 (0.211-0.414)	0.257 (0.219-0.319)	0.784
CRP (mg/L)	4.6 (1.6-10.6)	8.4 (2.0-15.0)	3.4 (1.4-8.5)*	0.050
Triglycerides (mmol/L)	1.60 (1.00-2.25)	1.50 (1.30-2.15)	1.35 (0.90-2.00)$^{*,\#}$	0.048
Erythrocyte count (x10^{12}/L)	3.15 (2.96-3.49)	3.48 (3.21-3.74)*	3.35 (2.97-3.64)	0.049
Haemoglobin (g/L)	98 (91-107)	106 (101-114)*	102 (91-112)	0.043

POase – paraoxonase activity, ARE – arylesterase activity, TOS – total oxidative status, AOPP – advanced oxidation protein products, PAB – pro-oxidant–antioxidant balance, TAS – total antioxidant status, SHG – sulfhydryl groups; the data do not follow a normal distribution and are presented as median and interquartile ranges.
p from the Kruskal–Wallis test; $^{*}p < 0.05$, $^{***}p < 0.001$ vs. QQ phenotype; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ according to the Mann–Whitney U test.

POase activity values were highest in patients with the RR phenotype and lowest in those with the QQ phenotype ($p < 0.001$) (**Figure 2A**). ARE activity values were highest in patients with the RR pheno-

found between these groups for PAB values. For the values of antioxidant defense parameters (TAS and SHG), no statistically significant difference was also found between these groups.

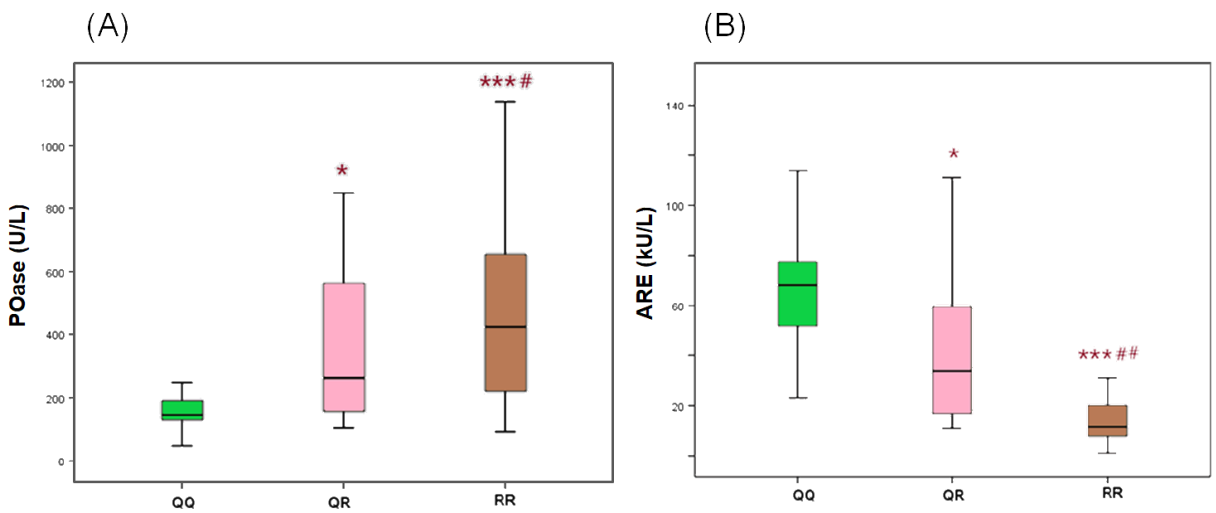

Figure 2. Values of POase (A) and ARE (B) activity in the three PON1 phenotypes ($^{*}p < 0.05$, $^{***}p < 0.001$ vs. QQ; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. QR).

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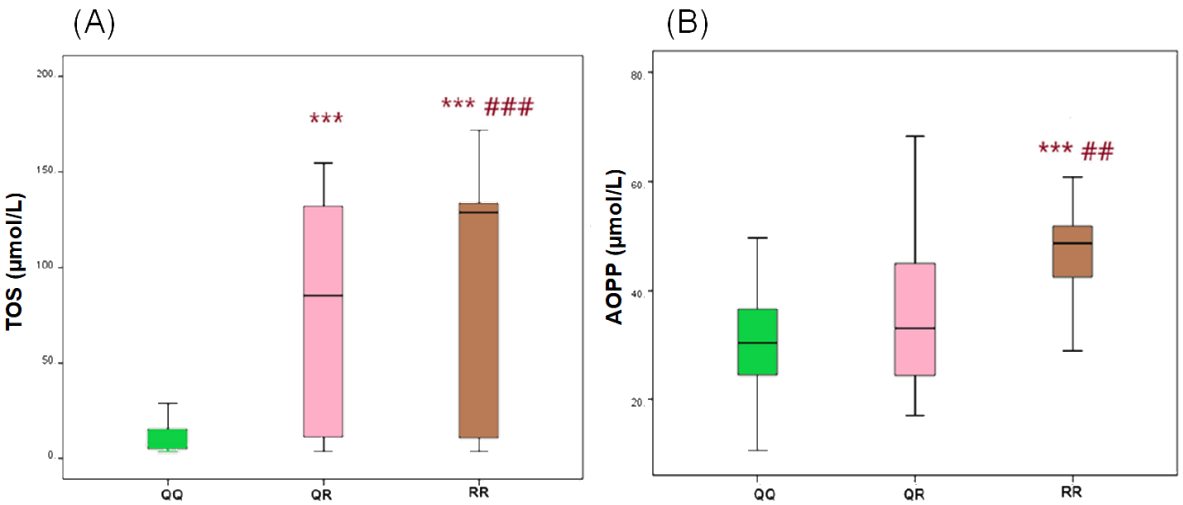

Figure 3. TOS (A) and AOPP (B) values in the three PON1 phenotypes (***p < 0.001 vs. QQ; ##p < 0.01, ###p < 0.001 vs. QR).

Regarding biochemical and hematological parameters, patients with the RR phenotype showed statistically significantly elevated values of CRP (p = 0.050) and triglycerides (p = 0.048), while patients with the QR phenotype showed statistically significantly elevated values of erythrocyte count (p = 0.049) and haemoglobin concentration (p = 0.043).

Furthermore, a statistically significant negative correlation was found between ARE activity and oxidative stress parameters (AOPP: ρ = −0.378, p < 0.001; TOS: ρ = −0.265, p < 0.005), while POase activity showed a positive correlation (AOPP: ρ = 0.311, p < 0.005; TOS: ρ = 0.356, p < 0.001) (**Figure 4**).

TNF-α as a predictor of mortality and clinical outcome

Assessment of pro-inflammatory status showed that TNF-α concentrations were significantly higher in patients who died during the course of the study

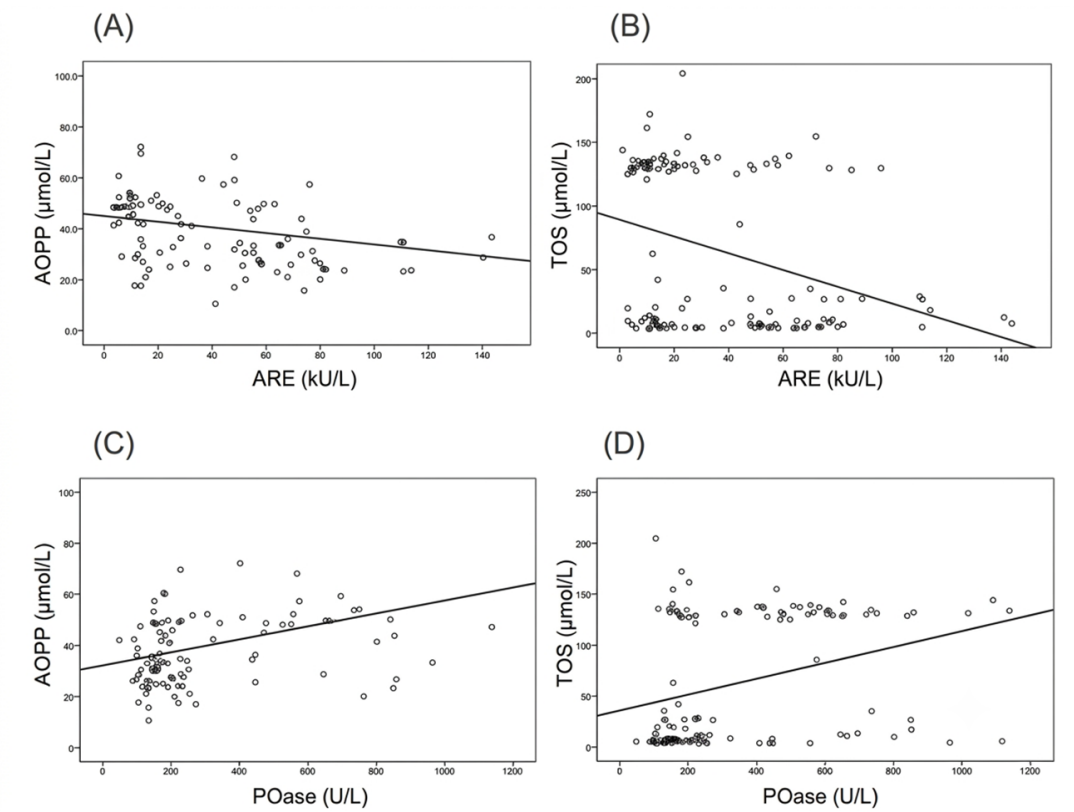

Figure 4. Significant negative correlation of ARE activity with AOPP concentration (A) and TOS (B); significant positive correlation of POase activity with AOPP concentration (C) and TOS (D).

compared with survivors undergoing haemodialysis (5.22 vs. 3.45 pg/mL, p = 0.050). Kaplan–Meier survival analysis was performed and confirmed that high TNF-α concentrations (> 5 pg/mL) are a strong predictor of shorter survival in patients on haemodialysis (log-rank = 4.21, p = 0.040) (**Figure 5**).

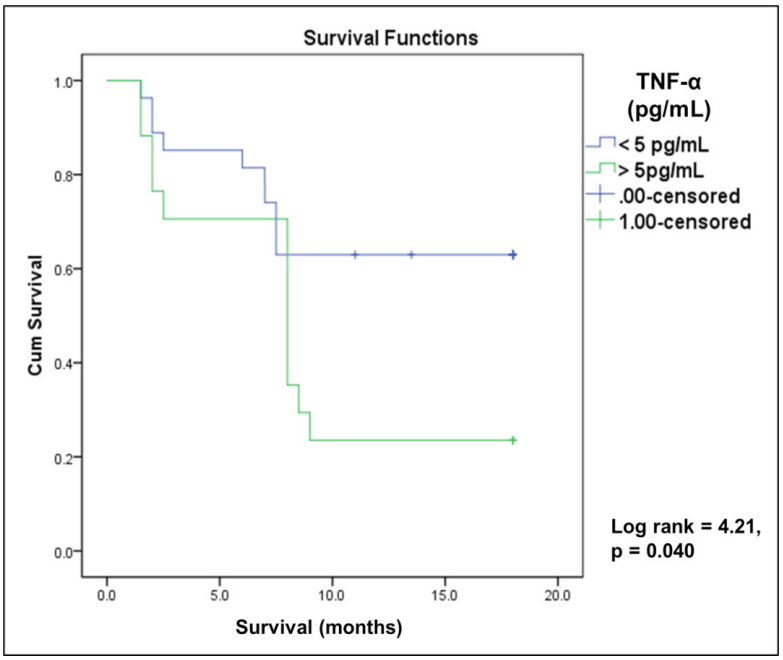

Figure 5. Kaplan–Meier survival analysis.

Correlation of TNF-α with lipid status and atherosclerosis

Patients were divided into tertiles based on their TNF-α levels (tertile I: < 3.0 pg/mL; tertile II: 3.1-5.6 pg/mL; tertile III: > 5.6 pg/mL). A statistically significant increase in cardiovascular disease (CVD) risk factors (p = 0.033) and the atherosclerosis index (p = 0.018) was observed across the tertiles (**Figure 6**). Post-hoc analysis (Mann-Whitney U test) revealed that both the second and third tertiles had significantly higher values compared to the first tertile (p < 0.05), as indicated by the statistical markers in Figure 6. Specifically, the cardiovascular disease (CVD) risk factor increased from a median of 3.02 (IQR 2.62-3.76) in the first tertile to 3.79 (IQR 3.46-5.39) and 3.66 (IQR 3.49-4.02) in the second and third tertiles, respectively. Similarly, the atherosclerosis index was higher in the second (median 2.13; IQR 1.71-3.02) and third tertiles (median 2.06; IQR 1.82-2.41) compared to the first tertile (median 1.39; IQR 1.27-1.86).

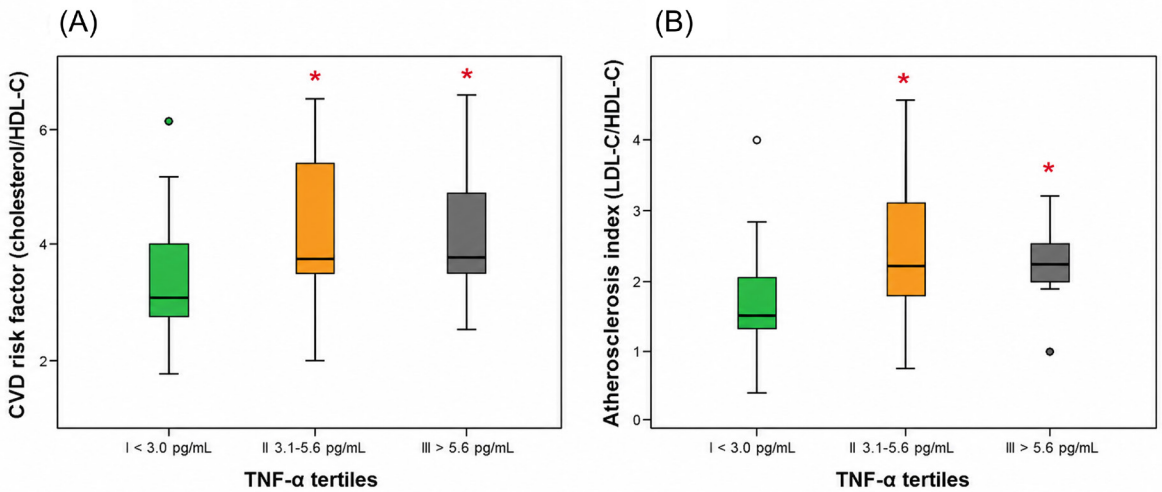

Figure 6. CVD risk factor (A) and the atherosclerosis index (B) comparison according to TNF-α tertiles.
*p < 0.05 vs. the first tertile according to the Mann–Whitney U test.

Using Spearman correlation analysis, TNF-α concentration showed a positive correlation with haemoglobin concentration ($\rho = 0.307$, $p = 0.043$) and the atherosclerosis index ($\rho = 0.387$, $p = 0.010$), while it showed a negative correlation with HDL cholesterol ($\rho = -0.343$, $p = 0.023$) in the entire group of patients.

Discussion

The results of this study indicate that patients on a chronic haemodialysis program function in a state of persistent biochemical imbalance, characterized by reduced antioxidant capacity and increased inflammatory burden. In this patient population, arterial hypertension and diabetes mellitus are the most common traditional risk factors, which is consistent with global epidemiological data on ESRD [2,3]. Within this environment of persistent oxidative and inflammatory stress, the antioxidant defence system is critically compromised, specifically regarding the functional status of PON1 enzyme. As a calcium-dependent esterase associated with HDL particles, PON1 plays a vital role in preventing the oxidation of LDL cholesterol and cellular membranes [8]. In our study, both POase and ARE activities were found to be significantly lower in patients compared to healthy controls ($p < 0.001$), indicating a pervasive loss of antiatherogenic protection in the renal cohort.

A significant finding in this study pertains to the shift in the phenotypic distribution of PON1. Using the antimodal method to analyse the POase/ARE ratio, it was determined that the RR phenotype was the most prevalent among patients (43.2%), whereas the QR phenotype predominated in the control group. This phenotypic distribution is particularly relevant to the patient's redox status, as patients with the RR phenotype exhibited the highest levels of oxidative stress, i.e., elevated values of TOS and AOPP ($p < 0.001$). The exhaustion of the enzymatic defence was further evidenced by the negative correlation found between ARE activity and pro-oxidants, specifically AOPP ($\rho = -0.378$) and TOS ($\rho = -0.265$). Interestingly, a positive correlation was observed between POase activity and these pro-oxidants in the RR phenotype. In the context of the extreme redox burden typical of ESRD, this relationship is best interpreted as an unsuccessful compensatory mechanism where the body attempts to upregulate POase activity in response to a rising oxidative tide, which ultimately fails to restore biochemical homeostasis as renal pathology progresses [10].

To provide a more comprehensive clinical assessment, the study also evaluated the pro-inflammatory status of the participants, focusing on TNF-α. Produced by macrophages, this cytokine is known to intensify systemic inflammation and accelerate the decline of glomerular filtration [13]. Within a clinically representative subgroup of the larger cohort where TNF-α was determined, concentrations were significantly higher in patients with a fatal outcome during the study compared to survivors, with median values of 5.22 pg/mL and 3.45 pg/mL, respectively. Kaplan-Meier survival analysis confirmed the prognostic weight of this marker, demonstrating that TNF-α exceeding 5 pg/mL serves as a strong predictor of shorter patient survival ($p = 0.040$). These findings highlight a dangerous synergy between inflammation and metabolic disturbances, where elevated TNF-α directly corresponds with the progression of dyslipidaemia and increased cardiovascular risk [15]. Specifically, TNF-α showed a significant positive correlation with the atherosclerosis index ($\rho = 0.387$) and a negative correlation with HDL cholesterol ($\rho = -0.343$), suggesting that systemic inflammation and lipid imbalances are inextricably linked in the progression of vascular damage.

The interaction between oxidative stress and chronic inflammation creates a vicious cycle: the pro-oxidative environment associated with the RR phenotype and the high inflammatory burden of elevated TNF-α act synergistically to drive endothelial dysfunction and vascular calcification. This suggests that the combination of high oxidative stress and systemic inflammation is the primary driver of accelerated cardiovascular ageing and the resulting high mortality rates in this population [7,12]. While these synergistic interactions provide a compelling framework for understanding cardiovascular risk, the interpretation of these findings must be tempered by specific methodological constraints. Although the limitations of the study included a relatively small number of participants, it is also important to acknowledge the limitations of causal inference inherent to the observational design of our research. While our findings demonstrate strong associations between the PON1 RR phenotype, elevated TNF-α, and patient outcomes, they do not definitively establish a direct cause-and-effect relationship. Nevertheless, these results provide clear evidence that routine monitoring of PON1 phenotypes and TNF-α levels could contribute to better risk stratification in patients undergoing haemodialysis. While the measurements performed (such as the automated

biochemical analysis of TOS and AOPP) provide valuable prognostic insights for risk stratification, they remain associations that require further longitudinal validation within an observational framework.

Conclusion

Chronic kidney disease and the process of haemodialysis lead to the PON1 RR phenotype becoming the most prevalent among these patients, which directly correlates with elevated levels of oxidative stress and reduced antioxidant potential. Alongside these disturbances, the pro-inflammatory cytokine TNF-α (particularly levels above 5 pg/mL) serves as a strong predictor of mortality and shorter life expectancy, accompanying the development of dyslipidemia and an increase in the atherosclerosis index. The synergy between the low antioxidant capacity of the RR phenotype and high inflammation identifies patients with the highest cardiovascular risk, making these biomarkers crucial for timely clinical risk stratification and the prevention of complications.

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