DISSERTATION

A Comparative Assessment of Oxidative Stress Biomarkers in Hemodialysis Patients for Predicting All-Cause Mortality
Eine vergleichende Bewertung von Biomarkern für oxidativen Stress bei Hämodialysepatienten zur Vorhersage der Gesamtmortalität

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AOPP	Advanced oxidation protein products
CKD	Chronic kidney disease
CVD	Cardiovascular disease
DM	Diabetes mellitus
ESRD	End-stage renal disease
HD	Hemodialysis
MHD	Maintenance hemodialysis
MPO	Myeloperoxidase
nox-PTH	Non-oxidized parathyroid hormone
OS	Oxidative stress
oxLDL	Oxidized low-density lipoprotein
RRT	Renal replacement therapy
PD	Peritoneal Dialysis
ROS	Reactive oxygen species

Abstract

Introduction: The presence of oxidative stress (OS) is evident in the early stages of chronic kidney disease (CKD), maintenance hemodialysis (MHD) patients with end-stage renal disease (ESRD) are particularly at risk for this condition. While there is an ongoing debate about the association between OS and mortality, it is generally acknowledged that OS plays a role in the progression of CKD and ESRD.

Methods: In this prospective study, a total of 347 patients undergoing HD were included, and four biomarkers related to OS were assessed. These biomarkers included oxidized low-density lipoprotein (ox-LDL), myeloperoxidase (MPO), advanced oxidation protein products (AOPPs), and carbonyl proteins. During the follow-up period lasting 60 months, 9 patients were lost to follow-up, and the mortality rate of the remaining 347 patients was 48.4%, meaning 168 deaths.

Results: Concerning OS byproducts, carbonyl protein levels were lower in survivors (105.40 ng/mL (interquartile range (IQR) 81.30–147.85) versus 129.65 ng/mL (IQR 93.20–180.33); $p<0.001$), also shown in subgroup analysis for male patients (103.70 ng/mL (IQR 76.90–153.33) versus 134.55 ng/mL (IQR 93.95–178.68); $p=0.0014$). However, no significant differences were found in MPO, AOPP, and ox-LDL in either of the two sex subgroups. The results of the Kaplan-Meier survival analysis revealed that patients with high-level carbonyl protein patients (>117.85 ng/mL) had a significantly lower survival rate ($p<0.001$, as determined by the log-rank test). A positive correlation was found between carbonyl proteins and all-cause mortality in both halves of the study according to Univariate Cox regression analysis. In patients with MHD, carbonyl proteins continued to be a statistically significant predictor of mortality even after adjusting for conventional risk factors. According to the univariate Cox regression analysis, continuous MPOs and log MPOs had statistically significant correlations with all-cause mortality, while binary MPO (divided by the median of MPO) did not. Multivariate Cox analysis showed that MPO still significant associated with mortality prediction, even after adjusting for multiple factors.

Conclusion: The findings of this study suggest that the effectiveness of ox-stress biomarkers in predicting all-cause mortality in HD patients differs. Specifically, the study indicates that carbonyl proteins and MPO can independently predict all-cause mortality

in HD patients, while AOPPs and oxLDL are not significantly associated with all-cause mortality in HD patients.

Zusammenfassung

Einleitung: Die Präsenz von oxidativem Stress (OS) ist in den frühen Stadien der chronischen Nierenerkrankung (CKD) deutlich erkennbar und seine Schwere nimmt bei Patienten mit terminaler Niereninsuffizienz (ESRD), die sich einer langfristigen Hämodialyse (MHD) unterziehen, zu. Während die Debatte über den Zusammenhang zwischen OS und Mortalität fortgesetzt wird, wird allgemein anerkannt, dass OS eine Rolle bei der Progression von CKD und ESRD spielt.

Methoden: In dieser prospektiven Studie wurden insgesamt 347 Patienten, die sich einer Hämodialyse unterzogen, eingeschlossen und vier Biomarker für OS untersucht. Zu diesen Biomarkern gehörten Carbonylproteine, Myeloperoxidase (MPO), fortgeschrittene Oxidationsproteinprodukte (AOPPs) und oxidiertes Low-Density-Lipoprotein (ox-LDL). Während des 60-monatigen Nachbeobachtungszeitraums konnten 9 Patienten nicht weiterverfolgt werden, und bei den übrigen Patienten (insgesamt 347) lag die Sterblichkeitsrate bei 48.4 %, was 168 Todesfällen entspricht.

Ergebnisse: Was die Nebenprodukte des OS betrifft, so waren die Carbonylproteine bei den Überlebenden niedriger (105,40 ng/ml (Interquartilsbereich (IQR) 81,30-147,85) gegenüber 129,65 ng/ml (IQR 93,20-180,33); $p < 0,001$), mit ähnlichen Ergebnissen für die männlichen Patienten (103,70 ng/ml (IQR 76,90-153,33) gegenüber 134,55 ng/ml (IQR 93,95-178,68); $p = 0,0014$). Bei MPO, AOPP und ox-LDL wurden jedoch keine signifikanten Unterschiede zwischen den beiden Geschlechtsuntergruppen festgestellt. Die Ergebnisse der Kaplan-Meier-Überlebensanalyse zeigten, dass Patienten mit höheren Carbonylproteinkonzentrationen (Gruppe >117,85 ng/mL) eine signifikant niedrigere Überlebensrate hatten ($p < 0,001$, bestimmt durch den Log-Rank-Test). Die univariate Cox-Regressionsanalyse zeigte eine positive Korrelation zwischen Carbonylproteinen und der Gesamtmortalität in der oberen und unteren Hälfte. Auch nach Adjustierung für konventionelle Risikofaktoren blieben die Carbonylproteine ein statistisch signifikanter Prädiktor für ein erhöhtes Sterberisiko bei MHD. Die univariate Cox-Regressionsanalyse zeigte, dass die kontinuierliche MPO und die logarithmische MPO signifikant mit der Gesamtmortalität verbunden waren, während die binäre MPO

(basierend auf dem Median der MPO) keine signifikante Korrelation aufwies. Die multivariate Cox-Analyse zeigte, dass die mit der MPO assoziierte Mortalitätsvorhersage auch nach Anpassung an mehrere Faktoren signifikant blieb.

Schlussfolgerung: Die Ergebnisse der Studie deuten darauf hin, dass die Wirksamkeit von Ox-Stress-Biomarkern bei der Vorhersage der Gesamtmortalität bei HD-Patienten unterschiedlich ist. Insbesondere zeigt die Studie, dass Carbonylproteine und MPO unabhängig voneinander die Gesamtmortalität bei HD-Patienten vorhersagen können, während AOPPs und oxLDL nicht signifikant mit der Gesamtmortalität bei HD-Patienten assoziiert sind.

1 Introduction

1.1 Oxidative Stress

Pro and antioxidants are necessary for the normal functioning of cells. As reactive oxygen species decompose, oxidative stress (OS) occurs when pro-oxidant production is not balanced with antioxidant defense mechanisms. A system of antioxidants cannot scavenge reactive oxygen species (ROS) produced under OS (1). As the most important free radicals in biological systems, ROS are characterized as chemically active species containing oxygen and possessing an unpaired electron. ROS include superoxide anion (O2-), hydroxyl radical (HO-), hydrogen peroxide (H2O2), peroxynitrite (ONOO-), as well as lipid radicals (2). ROS are generated by several cell types, including leukocytes, macrophages, endothelial cells, and vascular smooth muscle cells. In mitochondria, ROS levels are estimated to be five to ten times higher than in the cytosol and nucleus (3). Cellular dysfunction can be caused by ROS through multiple mechanisms, including cell proliferation, hypertrophy, and apoptosis (4). Several disorders, including cardiovascular disease and chronic kidney disease, are associated with the pathomechanisms of heightened OS.

1.2 Oxidative Stress in Kidneys

The intricate vascularization of the kidneys enables them to receive approximately 25% of the cardiac output, while their multifaceted functions, including regulating body fluids and blood pressure, eliminating waste products, and generating red blood cells, require a significant amount of energy expenditure, accounting for approximately 7% of daily energy consumption (5). The kidneys have abundant mitochondria, and their heightened metabolic activity, diverse cell types, including endothelial cells, vascular smooth muscle cells, mesangial cells, and tubular epithelial cells, are exceptionally susceptible to oxidative stress-induced damage, proteinuria, inflammation, and fibrosis. As one of the primary sources of antioxidant enzymes, kidney dysfunction in chronic kidney disease (CKD) is linked to reduced levels of these enzymes and elevated levels of pro-oxidants. There has already been evidence of higher OS in early stages of CKD. Research has revealed that individuals with early-stage CKD (1-2) exhibit an increased OS status in their red blood cells (RBCs) compared to healthy individuals (6). Impaired mitochondrial respiration has been observed in CKD (stages 2-3) patients. This finding indicated

significant dysregulation of the mitochondrial respiratory system in CKD patients, which was strongly linked to increased OS (7). And with CKD deterioration, plasma level of oxidized albumin gradually increased (8). Patients with end-stage renal disease (ESRD) exhibit elevated levels of pro-oxidant activity and decreased levels of antioxidant activity.

1.3 Oxidative Stress in Hemodialysis

As CKD progresses, renal replacement therapy (RRT) is inevitable, especially in the end-stage. Since the introduction of hemodialysis (HD) in the United States in 1962, the number of patients with kidney failure has significantly increased (9). But an increasing amount of evidence indicates that HD is associated with an elevated state of oxidative stress, which has been attributed to the loss of antioxidants during dialysis as well as the accumulation of oxidative products. Within minutes of initiating HD, exposure of blood to the dialyzer membrane and dialysate leads to the activation of complement factors, platelets, and polymorphonuclear white blood cells (PMNs), resulting in the production of ROS. One significant biomarker of OS is PMN stimulation, which is known to progressively increase with the stages of CKD and is particularly pronounced in HD patients (10). Red blood cell oxidized glutathione levels were found to increase during HD, indicating that both reduced renal function and dialysis contribute to impaired cellular redox homeostasis in RBCs (11).The mitochondrial respiration of patients with CKD and individuals undergoing hemodialysis is impaired (7). Chen et al. suggested that the plasma levels of $O2^-$ in HD patients were significantly higher compared to healthy individuals, which is a potent reactive oxygen species with pro-oxidant properties (12). A direct elevation in ROS plasma levels after each HD session was demonstrated in another study (13). Maher et al. reported an increase in lipid peroxidation products within 30 minutes of HD initiation (14), which was also confirmed in a separate study (15).

1.4 Oxidative Stress Biomarkers

Plasma proteins and amino acids that are oxidatively modified can be used as biomarkers for oxidative stress in vivo (16). Oxidants possess an extremely short half-life measured in seconds, rendering them compounds of exceptional reactivity. Due to this fact, measuring them in vivo is typically not achievable. In most cases, specific end-products of the reactions are used to measure oxidative stress. In contrast to proteins, carbohydrates, and nucleic acids, oxidized lipids exhibit lifespans spanning from hours to

weeks, positioning them as excellent markers for oxidative stress. Advanced oxidation protein products (AOPP) primarily originate from serum albumin through the attack of hypochlorous acid free radicals, and it serves as a useful indicator of protein damage caused by oxidation (17). Oxidized low-density lipoprotein (oxLDL) promotes endothelial cell apoptosis and exacerbates dysfunction, while also contributing to cardiomyocyte remodeling after ischemia or inflammation and fibrosis in the kidney (18). Myeloperoxidase (MPO), as a major component of leukocytes' bactericidal arsenal, is a heme enzyme synthesized and secreted by neutrophils and monocytic cells, and an important source of ROS (19). Carbonyl proteins, recognized as a prevalent and stable biomarker for identifying extensive oxidative damage to proteins, have been observed to maintain heightened levels in the bloodstream for a duration of up to 18 hours (20).

2 Methods

2.1 Study Population

We recruited 347 patients from two dialysis centers who were on stable hemodialysis associated with our inpatient facility at Campus Charité Mitte (KfH Dialysezentrum-Neukölln, Berlin, and KfH Dialysezentrum-Moabit, Berlin) (21). This study received approval from local ethics committees, and informed consent was acquired from all individuals participating in the study. Every patient underwent hemodialysis utilizing standard bicarbonate dialysis and biocompatible membranes, occurring on a frequency of three to four times weekly. Dialysate flow rates were 500 mL/min and blood flow rates were 250-300 mL/min, sufficient to provide a urea kinetic on dialysis (KT/V) of at least 1.3. Patients with malignancies, ongoing infections, pregnancy, or a lack of willingness to engage were not included in the study. Each patient was equipped with a fully operational permanent access device. Patients who underwent transplantation were excluded from the analysis starting from the transplantation date.

2. 2 Data Collection

Baseline demographics and medical data, i.e., age, sex, body mass index (BMI), dialysis vintage, systolic and diastolic blood pressure (systolic blood pressure (SBP) and diastolic blood pressure (DBP)), comorbidity (presence of diabetes, hypertension, or cardiovascular diseases), smoking and drinking status, medication (use of renin-angiotensin-aldosterone system (RAAS) inhibitors, beta-blockers, calcium channel blockers, erythropoietin, and diuretics) were obtained. Blood samples were collected from patients before one HD session at study entrance. The following parameters were assessed in the clinical laboratory using standardized methods: hemoglobin (Hb), ferritin, transferrin, fasting blood glucose, creatinine, potassium, calcium, phosphorus, intact parathyroid hormone (iPTH), non-oxidized parathyroid hormone (n-ox PTH), serum albumin, blood urea nitrogen (BUN), low density lipoprotein (LDL), high density lipoprotein (HDL), and high sensitive C-reactive protein (hsCRP) (21).

2. 3 Measurement of Myeloperoxidase, Advanced Oxidation Protein Products, Oxidized Low-Density Lipoprotein, and Carbonyl proteins Concentrations.

The measurements of MPO were conducted using enzyme immunoassay from Immundiagnostik AG, Germany, following the instructions provided by the manufacturer

(Immundiagnostik AG instructions: For the in vitro determination of MPO in serum and plasma: https://www.immundiagnostik.com/media/pages/testkits/k-6631b/1c8a7c2444-1679409720/k6631b_2022-01-05_mpo_plasma_2std.pdf) (21).

Before conducting the assay, it is required to dilute the serum samples at a ratio of 1:40. Subsequently, 100µl of the diluted solution is utilized in the test. 1. Before employing, cleanse the wells through five rounds of washing using 250µl of wash buffer each time. Following the ultimate wash, remove any lingering wash buffer by firmly tapping the plate onto absorbent paper. 2. Dispense 100µl of standards, controls, or diluted samples individually into their corresponding wells. 3. Securely seal the plate and allow it to incubate for one hour at room temperature (15-30°C) on a horizontal shaker set to 550rpm with a 2mm orbit for agitation. 4. Remove the contents from each well and perform five wash cycles using 250µl of wash buffer. Following the last washing step, eliminate any remaining residual wash buffer. 5. Dispense 100µl of the diluted conjugate (CONJ) into each respective well. 6. Securely seal the plate and allow it to incubate for one hour at room temperature on a horizontal shaker. 7. Remove the contents from each well and perform five wash cycles using 250µl of wash buffer. Following the final washing step, eliminate any remaining residual wash buffer. 8. Dispense 100µl of the substrate (SUB) into each respective well. 9. Allow incubation to take place for a duration of 10 to 20 minutes at room temperature while keeping the environment dark. 10. Dispense 100µl of the stop solution (STOP) into each well and ensure thorough mixing. 11. Measure the absorbance promptly using an ELISA reader at 450nm relative to 620nm (or 690nm) as a reference. If the extinction of the highest standard exceeds the range of the photometer, absorption must be measured immediately at 405nm against 620nm as a reference.

The quantitative determination of AOPPs were conducted utilizing the AOPP Kit from Immundiagnostik AG, Germany, following the instructions provided by the manufacturer: (Immundiagnostik AG instructions: For the in vitro determination of AOPP in EDTA plasma: https://www.immundiagnostik.com/media/pages/testkits/kr7811w/c44b60fb8a-1679409720/kr7811w_2019-04-02_aopp.pdf) (21).

Before analysis, centrifuge freshly collected EDTA plasma in 1.5ml reaction tubes at 3000g for 30s • Mix 125µl centrifugated EDTA plasma with 25µl delipidation reagent (DELIP), vortex dilution 1:1.2 • Incubate for 10min at room temperature (15–30°C) • Afterwards, centrifuge at 3000g for 5min • Mix 100µl delipidated EDTA plasma with 400µl assay buffer (ASYBUF) in a 1.5 ml reaction tube, vortex, final dilution 1:6. 1.Add

each 200µl standards/controls/diluted samples into the respective wells. 2.Directly determine the absorption of standards, control, and samples at 340nm.

The measurement of oxLDL was conducted utilizing enzyme immunoassay from Immundiagnostik AG, Germany, following the instructions provided by the manufacturer: (Immundiagnostik AG instructions: For the in vitro determination of ox-LDL/MDA adducts in EDTA plasma, serum and dried blood spots: https://www.immundiagnostik.com/media/pages/testkits/k-7810/32d60c5fac-1679409720/k7810_2022-06-22_ox-ldl_mda_addukt.pdf) (21).
Serum samples must be diluted 1:10 before performing the assay. The rest of the ELISA procedure is the same as for the MPO test described above.

The measurement of carbonyl protein was conducted with enzyme immunoassay from Immundiagnostik AG, Germany, following the instructions provided by the manufacturer: (Immundiagnostik AG instructions: For the in vitro determination of protein-bound carbonyls in human serum and plasma: https://www.immundiagnostik.com/media/pages/testkits/k-7870/095e244d0b-1679409720/k7870_2022-08-22_carbonylproteine.pdf) (21).
The derivatized samples must be diluted 1:20000 in assay buffer before use in the test: • 30µl derivatized sample + 570µl assay buffer, mix well = 1:20 (dilution I) • 30µl dilution I + 570µl assay buffer, mix well = 1:20 (dilution II) • 20µl dilution II + 980µl assay buffer, mix well = 1:50 (dilution III). This results in a final dilution of 1:20000. For analysis, pipet 100µl of dilution III per well. Bring all reagents and samples to room temperature (15–30°C) and mix well. The rest of the ELISA procedure can be found in the MPO test.

2.4 Statistical Analysis

A significance level of $p < 0.05$ was used to determine statistical significance. All analytical procedures were conducted utilizing SPSS version 25.0 (Chicago, IL, USA). Descriptive variables are presented as median (interquartile range) or frequency (percentage). The Mann-Whitney U test was executed to assess variations between individuals who survived and those who did not. Kaplan-Meier cumulative survival curves were generated and stratified based on the median (values below and above), with a subsequent comparison of group survival conducted using the log-rank test. Concurrently examining

the connections between risk factors and survival time, a multivariate Cox regression analysis was conducted to account for potential confounding variables. Hazard ratios (HR) and their corresponding 95% confidence intervals (CI) were computed. Based on findings from the univariate Cox regression, three models were formulated for the purpose of multivariate Cox regression analysis. Model A was adjusted for demographics (age, hypertension, and cardiovascular disease (CVD)); Model B was adjusted for clinical parameters (serum creatinine, transferrin, phosphorus, n-oxPTH, albumin); Model C was adjusted for all risk factors in both models A and B (21).

3 Results

3.1 Study Cohort and Descriptive Data (Entire Cohort)

In this study, a cohort of 347 HD patients were involved, with a median age of 66 years (IQR 56–75). The gender distribution consisted of 229 male patients, 117 female patients, and one patient with unspecified gender. Overall, 130 patients were diagnosed with diabetes mellitus (DM), while 161 had a previous history of cardiovascular disease (CVD). Additionally, over three-quarters of the patients exhibited hypertension (77.5%) (Table 1).

Table 1: Clinical and biochemical characteristics of dialysis patients

Characteristics	All (n=347)	Survivors (n=170)	Non-Survivors (n=168)	P-value
Age (years)	66.0 (56.0-75.0)	60.50 (49.00-69.00)	71.00 (66.00-78.00)	<0.001
Gender (M/F)	229/117	114/56	110/58	0.759
Body mass index, kg/m^2	24.40 (22.01-27.60)	24.20 (22.12-28.30)	24.57 (21.71-26.99)	0.541
Drinker, n (%)	62 (17.90%)	30 (17.60%)	32 (9.10%)	0.740
Smoker, n (%)	108 (31.10%)	54 (31.80%)	52 (14.80%)	0.872
Diabetes mellitus, n (%)	130 (37.50%)	55 (32.40%)	74 (21.10%)	0.027
Hypertension, n (%)	269 (77.50%)	134 (78.80%)	135 (38.50%)	0.727
Cardiovascular disease, n (%)	161 (46.40%)	82 (48.20%)	101 (28.80%)	<0.001
Dialysis vintage (days)	263.00 (31.00-1219.25)	221.00 (31.00-939.25)	351.00 (31.00-1461.00)	0.004
Dialysis dose (Kt/V)	1.04 (0.91-1.16)	1.03 (0.91-1.16)	1.04 (0.91-1.17)	0.749
Medication, n (%)				
RAAS inhibitors	88 (25.40%)	46 (27.1%)	41 (11.70%)	0.577
Beta-blockers	204 (58.8%)	116 (68.2%)	86 (24.50%)	0.001
Calcium channel blockers	104 (30.00%)	60 (35.3%)	43 (12.30%)	0.053
Erythropoietin	171 (49.30%)	82 (48.2%)	89 (25.40%)	0.414
Diuretics	194 (55.90%)	98 (57.6%)	95 (27.10%)	0.838
Hemoglobin (g/dL)	10.20 (9.10-11.63)	10.25 (9.00-11.67)	10.20 (9.20-11.70)	0.865
Ferritin (ng/mL)	532.00 (253.25-1125.88)	527.50 (225.00-1065.75)	532.00 (281.00-1235.00)	0.540
Transferrin (µg/mL)	138.00(106.00-173.00)	145.00 (121.00-173.50)	128.50 (99.00-172.25)	0.003
Fasting blood glucose (mg/dL)	108.00 (90.00-134.00)	114.50 (94.50-143.60)	104.00 (87.00-123.60)	0.006
Creatinine (mg/dL)	6.62 (4.23-8.34)	6.67 (4.15-8.53)	6.60 (4.23-7.96)	0.007
Potassium (mmol/L)	4.70 (4.10-5.28)	4.60 (4.00-5.30)	4.77 (4.21-5.26)	0.734
Calcium (mmol/L)	2.24 (2.10-2.40)	2.20 (2.09-2.40)	2.27 (2.10-2.47)	0.414
Phosphorus (mmol/L)	1.61 (1.19-2.10)	1.70 (1.22-2.12)	1.54 (1.11-2.06)	0.051
iPTH (ng/L)	49.90 (18.68-124.60)	68.19 (21.75-171.05)	39.76 (14.47-101.90)	0.003
n-ox PTH	5.86 (2.38-14.01)	7.18 (3.05-16.26)	4.99 (1.98-11.11)	0.003
Albumin (g/dL)	3.30 (2.90-3.70)	3.40 (3.05-3.80)	3.10 (2.80-3.60)	0.001
BUN (mg/dL)	195.12 (146.70-267.67)	201.05 (152.64-267.67)	189.63 (131.73-279.50)	0.822
LDL (mg/dL)	92.70 (72.20-121.20)	100.80 (75.05-127.40)	89.00 (70.70-112.00)	0.013
HDL (mg/dL)	39.90 (32.20-50.80)	38.60 (31.00-50.20)	42.30 (34.30-54.00)	0.435
hsCRP (mg/L)	2.60 (1.00-5.20)	2.30 (0.70-4.50)	2.80 (1.20-6.63)	0.006
MPO (ng/mL)	106.84 (67.71-188.38)	102.27 (67.37-176.37)	118.90 (69.46-199.24)	0.176
AOPPs (µmol/L)	107.79 (78.79-149.94)	109.99 (80.59-156.72)	107.57 (79.53-146.80)	0.588
ox-LDL (mg/dL)	84.90 (44.80-180.55)	87.55 (45.85-197.63)	83.10 (44.53-176.35)	0.779
Carbonyl proteins (ng/mL)	117.85 (84.73-163.18)	105.40 (81.30-147.85)	129.65 (93.20-180.33)	<0.001

The median value of each variable is shown (IQR). For continuous variables, Mann-Whitney U tests were used for comparisons between survivors and non-survivors, and Chi2-tests were used for categorical variables.

Abbreviations: RAAS: Renin-Angiotensin-Aldosterone-System; iPTH: intact parathyroid hormone; n-oxPTH: non-oxidized parathyroid hormone; BUN: Blood urea nitrogen; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; hsCRP: High sensitivity C-reactive protein; MPO: Myeloperoxidase; AOPPs: Advanced oxidation protein products; ox-LDL: Oxidized low-density lipoprotein. This table was modified from our published paper: Zuo J, et al.2022 (21).

3.2 Oxidative Stress Biomarkers and All-Cause Mortality (Entire Cohort)

3.2.1 Comparison between Survivors and Non-Survivors

Based on the outcome, the HD patients were categorized into two groups: survivors and non-survivors. Table 1 shows the demographic and clinical information for each respective group. Throughout the 60-month observation period, 9 patients (including 1 individual with undisclosed gender) were lost to follow-up, and 168 patients (48.4%) experienced mortality. Among the cohort of 347 HD patients, those who survived exhibited a younger age, lower occurrences of DM and CVD, along with notably reduced hsCRP levels. Additionally, survivors demonstrated higher levels of transferrin, fasting blood glucose, iPTH, n-oxPTH, serum albumin, and LDL in comparison to non-survivors.

3.2.2 Mann–Whitney U Test

Concerning the ox-stress byproducts, carbonyl proteins were lower in survivors (105.40 ng/mL (IQR 81.30–147.85) versus 129.65 ng/mL (IQR 93.20–180.33); $p < 0.001$) (Figure 1), and among male survivors, this trend continues (103.70 ng/mL (IQR 76.90–153.33) versus 134.55 ng/mL (IQR 93.95–178.68); $p = 0.0014$) (Figure 1). However, there are no significant differences in MPO, AOPPs, and ox-LDL between the two groups (Table 1; Figure 2).

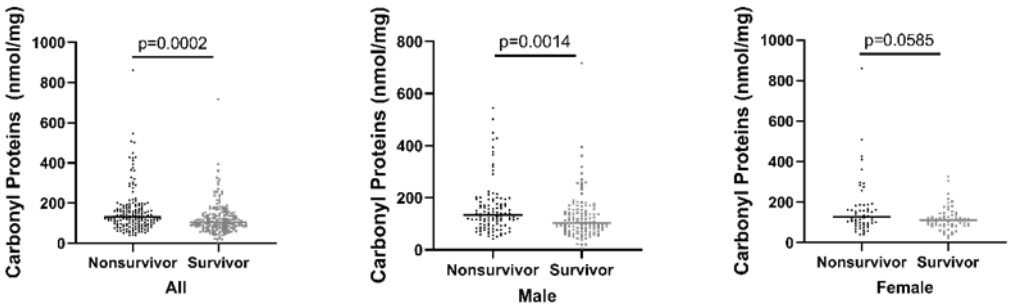


Figure 1: Plots of serum Carbonyl proteins concentrations.

In survivors, the median serum carbonyl proteins were significantly lower than in non-survivors, demonstrated by the Mann-Whitney U test [105.40 (IQR 81.30-147.85) versus 129.65 (IQR 93.20-180.33); $p<0.001$]. This figure was modified from our published paper: Zuo J, et al.2022 (21).

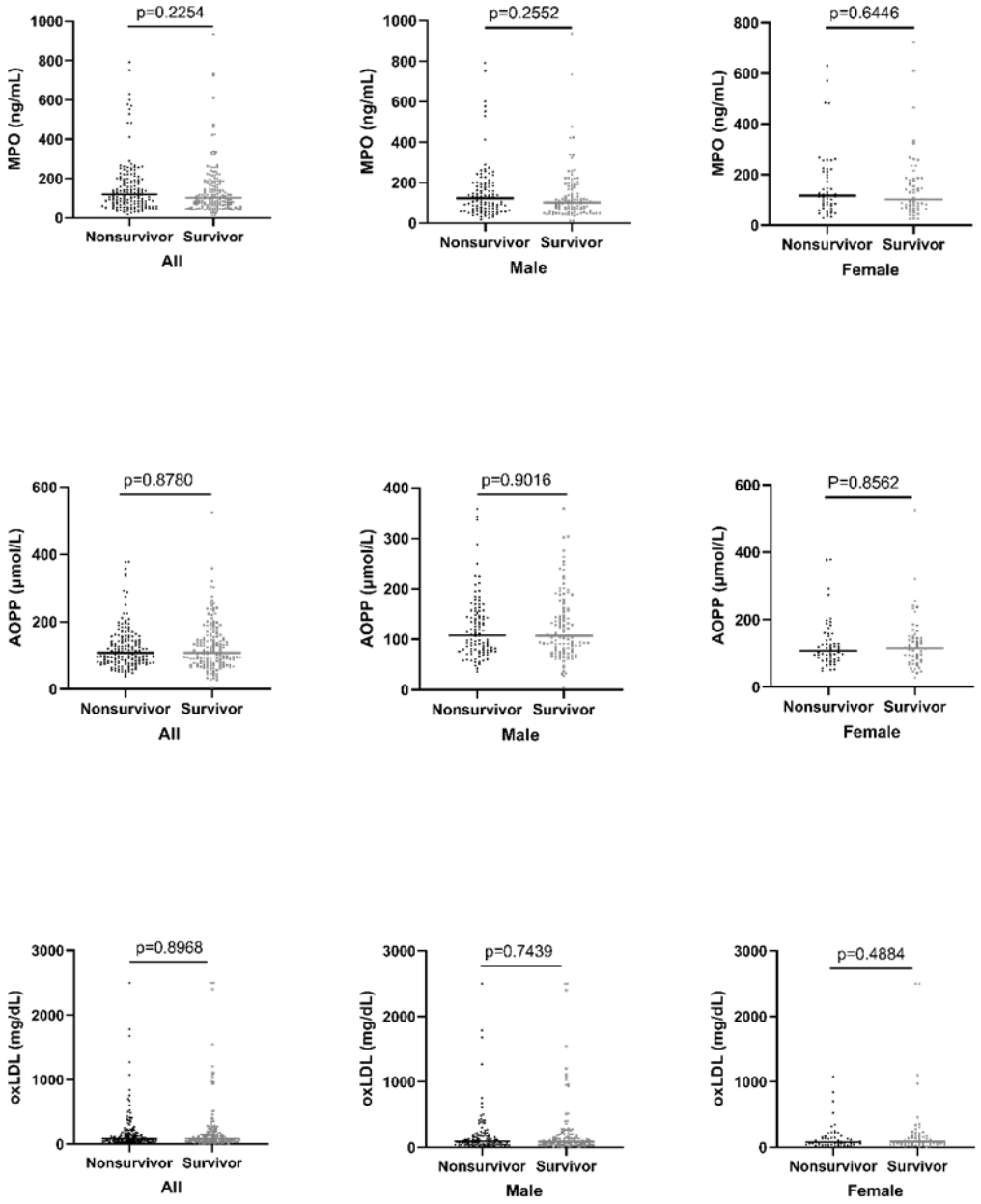


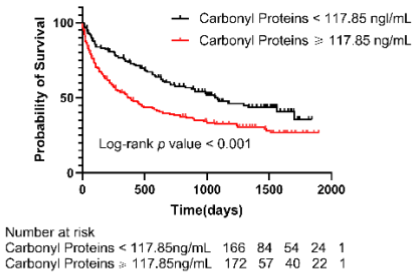
Figure 2. Plots of serum MPO, AOPPs, oxLDL.

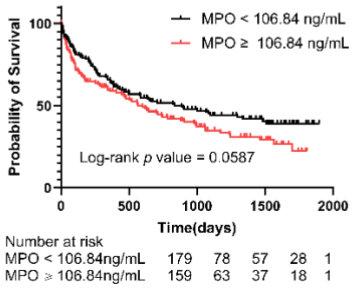
The Mann-Whitney U test found no significant differences between survivors and non-survivors in median MPO, AOPPs, and oxLDL.

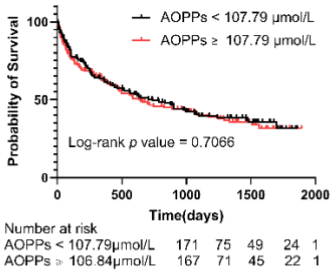
Abbreviations: MPO: Myeloperoxidase; AOPPs: Advanced oxidation protein products; ox-LDL: Oxidized low-density lipoprotein. This figure was modified from our published paper: Zuo J, et al.2022 (21).

3.2.3 Kaplan-Meier survival analysis

Figure 3 shows the Kaplan–Meier curves depicting all-cause mortality based on the median concentrations of each of the four oxidative stress byproducts at the baseline. The analysis demonstrated that the group with lower carbonyl protein concentrations (carbonyl proteins < 117.85 ng/mL) exhibited a higher possibility of survival within this study cohort (p < 0.001). AOPPs, MPO, and oxLDL did not show this statistical significance.







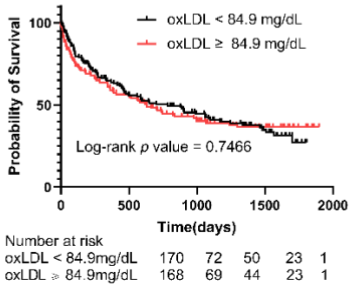


Figure 3. Kaplan-Meier survival curves for all-cause mortality.

Patients were divided according to the median values of variables.

Abbreviations: MPO: Myeloperoxidase; AOPPs: Advanced oxidation protein products; ox-LDL: Oxidized low-density lipoprotein. This figure was modified from our published paper: Zuo J, et al.2022 (21).

3.2.4 Cox regression analysis

Next, we conducted both univariate and multivariate Cox regression analyses. Univariate Cox's proportional hazards regression analysis showed that age (HR = 1.062 CI 95% (1.047–1.077) $p < 0.001$), CVD (HR = 1.440 CI 95% (1.056–1.963) $p = 0.021$), transferrin (HR = 0.995 CI 95% (0.992–0.998) $p = 0.002$), creatinine (HR = 0.875 CI 95% (0.819–0.935) $p < 0.001$), phosphorus (HR = 0.771 CI 95% (0.595–1.000) $p = 0.05$), iPTH (HR = 0.998 CI 95% (0.997–1.000) $p = 0.012$), n-oxPTH (HR = 0.986 CI 95% (0.973–1.000) $p = 0.043$), albumin (HR = 0.663 CI 95% (0.515–0.855) $p = 0.001$), MPO (HR = 1.000 CI 95% (1.000–1.000) $p < 0.001$) and carbonyl proteins (HR = 1.002 CI 95% (1.001–1.003) $p = 0.001$) had a significant association with survival (Table 2). Even after accounting for conventional risk factors among HD patients using various models (as detailed in the "Methods" section), the initial levels of carbonyl proteins continued to stand as a statistically significant predictor of an elevated risk of mortality (Table 3). Continuous MPO and logarithmically transformed MPO exhibited significant associations with all-cause mortality, except for the categorical binary MPO (divided based on the median MPO value). The significance of MPO in predicting mortality persisted even after adjusting for multiple factors (Table 4).

Table 2. Cox regression univariate analysis, hazard ratio, and 95% confidence intervals for survival in HD patients.

Analyses	HR (95%CI)	P-value
Age (years)	1.062 (1.047-1.077)	<0.001
Male/Female	0.981 (0.714-1.349)	0.908
Body mass index, kg/m2	0.992 (0.961-1.024)	0.615
Drinker, n (%)	1.108 (0.754-1.629)	0.601
Smoker, n (%)	0.879 (0.634-1.220)	0.441
Diabetes mellitus, n (%)	1.203 (0.887-1.632)	0.235
Hypertension, n (%)	0.723 (0.493-1.059)	0.096
Cardiovascular disease, n (%)	1.440 (1.056-1.963)	0.021
Dialysis vintage (days)	0.999869 (0.999716-1.000022)	0.093
Dialysis dose (Kt/V)	0.731 (0.377-1.417)	0.353
Hemoglobin (g/dL)	0.950 (0.868-1.038)	0.256
Ferritin (ng/mL)	1.000 (1.000-1.000)	0.846
Transferrin (μg/mL)	0.995 (0.992-0.998)	0.002
Fasting blood glucose (mg/dL)	0.999 (0.995-1.002)	0.449
Creatinine (mg/dL)	0.875 (0.819-0.935)	<0.001
Potassium (mmol/L)	0.895 (0.744-1.076)	0.238
Calcium (mmol/L)	0.832 (0.493-1.403)	0.490
Phosphorus (mmol/L)	0.771 (0.595-1.000)	0.0503
iPTH (ng/L)	0.998 (0.997-1.000)	0.012
n-ox PTH	0.986 (0.973-1.000)	0.043
Albumin (g/dL)	0.663 (0.515-0.855)	0.001
BUN (mg/dL)	1.000 (0.999-1.001)	0.479
LDL (mg/dL)	0.997 (0.993-1.002)	0.250
HDL (mg/dL)	1.005 (0.996-1.015)	0.302
hsCRP (mg/L)	1.018 (0.992-1.044)	0.179
MPO (ng/mL)	1.000035 (1.000020-1.000051)	<0.001
AOPPs (μmol/L)	1.001 (0.998-1.004)	0.445
ox-LDL (mg/dL)	1.000 (0.999-1.000)	0.451
Carbonyl proteins (ng/mL)	1.002 (1.001-1.003)	0.001

Abbreviations: iPTH: intact parathyroid hormone; n-oxPTH: non-oxidized parathyroid hormone; BUN: Blood urea nitrogen; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; hsCRP: High sensitivity C-reactive protein; MPO: Myeloperoxidase; AOPPs: Advanced oxidation protein products; ox-LDL: Oxidized low-density lipoprotein. This table was modified from our published paper: Zuo J, et al.2022 (21).

Table 3. Univariate and multivariate Cox regression analysis of carbonyl proteins, hazard ratio, and 95% confidence intervals for survival in HD patients.

Analyses	HR (95%CI)	P-value
Univariate Cox regression		
Continuous Carbonyl proteins	1.002 (1.001-1.003)	0.001
Binary Carbonyl proteins	0.564 (0.414-0.767)	<0.001
Log Carbonyl proteins	3.162 (1.684-5.937)	<0.001
Multivariable Cox regression		
Model A	1.002 (1.001-1.004)	0.001
Model B	1.002 (1.000-1.003)	0.027
Model C	1.002 (1.000-1.004)	0.015

Binary carbonyl proteins were divided according to the median of carbonyl proteins (117.85 ng/mL).

Model A was adjusted for age, hypertension, and cardiovascular disease; Model B was adjusted for serum creatinine, transferrin, phosphorus; albumin, and n-oxPTH; Model C was adjusted for the above risk factors (Model A + Model B). This table was modified from our published paper: Zuo J, et al.2022 (21).

Table 4. Cox regression univariate and multivariate analysis of MPO, hazard ratio, and 95% confidence intervals for survival in HD patients.

Analyses	HR (95%CI)	P-value
Univariate Cox regression		
Continuous MPO	1.000035 (1.000020-1.000051)	<0.001
Binary MPO	1.363 (0.998-1.862)	0.052
Log MPO	2.123 (1.394-3.234)	<0.001
Multivariable Cox regression		
Model A	1.000033 (1.000018-1.000049)	<0.001
Model B	1.000028 (1.000012-1.000044)	<0.001
Model C	1.000024 (1.000008-1.000040)	0.003

Binary MPO was divided according to the median of MPO (106.84ng/mL).

Model A was adjusted for age, hypertension, and cardiovascular disease; Model B was adjusted for serum creatinine, transferrin, phosphorus; albumin, and n-oxPTH; Model C was adjusted for the above risk factors (Model A +Model B).This table was modified from our published paper: Zuo J, et al.2022 (21).

4 Discussion

4.1 Short summary of results

This study examines four oxidative stress biomarkers to assess their ability to predict long-term mortality in HD patients (Figure 4). Significant variations were observed in the predictive capacity of various oxidative stress biomarkers in forecasting all-cause mortality in dialysis patients. Survivors exhibited lower levels of baseline carbonyl proteins compared to non-survivors (Figure 1), whereas baseline MPO, AOPPs, and oxLDL showed no significant differences between survivors and non-survivors (Figure 2). Upon conducting Cox regression analysis, considering confounding factors, it was revealed that both carbonyl proteins and MPO emerged as independent predictors of all-cause mortality in patients with HD. In contrast, AOPPs and oxLDL were not found to be independently associated with all-cause mortality.

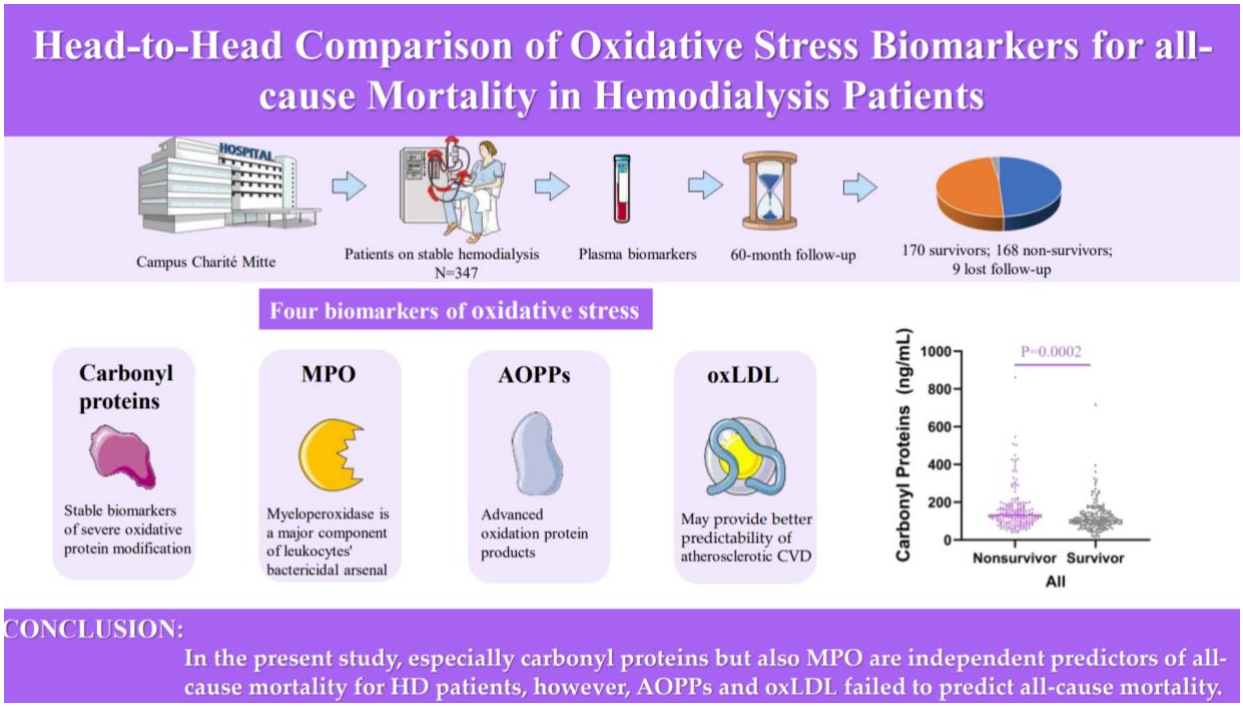


Figure 4. Graphical representation of the main findings

This graph was modified from our published paper: Zuo J, Chaykovska L, Chu C, Chen X, Hasan AA, Krämer BK, Tepel M, Hocher B. Head-to-Head Comparison of Oxidative Stress Biomarkers for All-Cause Mortality in Hemodialysis Patients. Antioxidants (Basel). 2022 Oct 2;11(10):1975 (21).

4.2 Interpretation of results

Approximately 70% of the dry mass in tissues and cells is composed of proteins, making them a major focal point susceptible to damage and posttranslational modifications (22, 23). Protein carbonylation, indicative of oxidative protein damage, arises from the direct oxidation of specific amino acid residues such as lysine, arginine, proline, and threonine. Additionally, reactive carbonyl species generated through carbohydrate and lipid oxidation can interact directly with dicarbonyl compounds, further contributing to protein carbonylation (24). Carbonylation is an irreversible process, and the modification cannot be effectively reversed by antioxidant defenses (25, 26). Carbonylation is believed to have detrimental effects on both protein function and cellular viability (27-31). Furthermore, carbonylation has the potential to induce the formation of large protease-resistant protein aggregates that are highly cytotoxic (32).

In our research, we observed elevated levels of carbonyl proteins in HD patients that did not survive. Even after adjustments for multiple risk factors, carbonyl proteins proved to be reliable indicators of overall mortality in patients undergoing dialysis. Early studies have also shown that hemodialysis patients exhibit higher levels of plasma carbonyl proteins compared to healthy individuals (33, 34). The data we obtained are consistent with a recent study that was also conducted in HD patients (35) (Table 5). The hypothesis suggests that interaction with the dialysis filter triggers the activation of neutrophils, potentially leading to heightened oxidative/carbonyl stress and inflammation after undergoing HD (36). Nonetheless, there is another study that failed to demonstrate an impact of protein carbonylation on mortality. It should be noted that this particular study only examined a small cohort of 44 patients, indicating a potential limitation in statistical power (37) (Table 5).

Myeloperoxidase (MPO), one of the heme peroxidases found in mammals, fulfills a significant function in the innate immune system by producing reactive oxidants. These oxidants play a crucial role in eliminating bacteria, yeasts, fungi, parasites, and other harmful pathogens (19, 38). Under normal physiological conditions, the production of MPO is primarily limited to the neutrophil phagosome. Upon cellular activation, MPO is

released from the lysosomal nitrogenophil granules found within neutrophils (39). This release aligns with the formation of the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase (NOX) complex on the phagosome endosomal membrane. The NOX complex efficiently generates substantial quantities of O2- from O2, utilizing intracellular NADPH as an energy source, and resulting in the occurrence of the 'oxidative burst' (40). O2- undergoes rapid spontaneous or enzyme-catalyzed dismutation, primarily catalyzed by superoxide dismutases. This dismutation process results in the formation of H2O2 and O2 (41). Subsequently, MPO employs the generated H2O2 together with chloride ions (Cl-) to produce hypochlorous acid. This hypochlorous acid plays a crucial role in the eradication of microorganisms within the phagolysosome (42, 43). But hypochlorous acid (HOCl) does not discriminate in its targeting of microbes, which results in significant collateral damage to proteins within neutrophils (44). This encompasses a range of conditions such as atherosclerosis, hypertension and other cardiovascular pathologies, neurodegenerative disease, kidney disease, respiratory disease, arthritis, colitis, and cancer (45-53). All these conditions have been associated with the involvement of MPO-induced oxidative damage.

The levels of plasma MPO seem to be elevated during HD due to oxidative stress as well (54). Activation of leukocytes at the dialysis membrane may increase MPO, and the level of MPO may be dependent on the dialysis membrane's biocompatibility (55-57). But MPO did not perform well in predicting all-cause mortality in our cohort, and unadjusted baseline values were similar in survivors and non-survivors. Multivariate Cox analysis, as well as a continuous univariate correlation analysis, showed an independent association with all-cause mortality only after adjusting for demographic and clinical factors. Furthermore, all-cause mortality hazard risk of binary transformed MPO was not statistically significant. Some clinical studies have shown that patients on maintenance dialysis have increased MPO levels, and that MPO levels were independently associated with long-term mortality (Table 5) (58, 59). But in another follow-up study of dialysis patients, MPO did not predict mortality from all causes independently (Table 5) (60).

In 1996, a groundbreaking discovery was made regarding a new biomarker for oxidative stress, known as advanced oxidation protein products (AOPPs). These products were first identified in the plasma of patients suffering from chronic uremic conditions, marking

a significant milestone in the field of oxidative stress research (61). Elevated AOPPs are seen in a variety of diseases and pathological conditions in addition to chronic uremia (62-64). The levels of AOPPs exhibit a correlation with plasma concentrations of dityrosine and advanced glycation end-products (AGE)-pentosidine. These biomarkers serve as indicators of protein damage resulting from oxygen-mediated processes. AOPPs further stimulate the generation of reactive oxygen species as a consequence of oxidative damage (65). AOPPs have been found to be more reliable than lipid peroxidation products as a measure of oxidative stress (61).

It has been shown that HD patients have elevated levels of these proteins (66). The level of AOPPs in healthy individuals and HD patients is also shown to be associated with an elevated risk of atherosclerosis-related cardiovascular events (67). However, HD patients' AOPPs were not found to be a predictor of mortality in our study. Not even a trend was evident. Our findings remained consistent with a previous study involving 112 HD patients and a follow-up period of 5.5 years (Table 5) (68). In an 8-year follow-up prospective study of 199 patients undergoing hemodialysis for ESRD, AOPPs had a significant predictive influence on both overall survival and cardiovascular survival (Table 5) (69). Furthermore, a prospective cohort study conducted across multiple centers revealed a correlation between elevated serum AOPPs levels and an increased risk of all-cause mortality among Chinese patients undergoing maintenance dialysis (Table 5) (70). Two factors could account for the varying results observed in the predictive ability of AOPPs for all-cause mortality: firstly, the initial factor is the higher likelihood and odds of mortality prediction for patients undergoing dialysis for ESRD with an 8-year follow-up; secondly, in the 8-year follow-up study, more than half of the patients were females, close to fifty percent of the individuals in our study were male. The influence of gender on the results cannot be excluded.

The accumulation of cholesterol in macrophages requires the presence of oxidized low-density lipoprotein (oxLDL), which is a modified form of LDL resulting from LDL oxidation (71). OxLDL serves as a chemoattractant for monocytes and triggers inflammatory reactions within the arterial wall (72). Increased monocyte endothelial cell adhesion, associated with high oxLDL, may contribute to CVD development in chronic renal failure patients on dialysis through another mechanism that interferes with coagulation activation

(73). Therefore, the measurement of oxLDL may provide better predictability of atherosclerotic CVD in patients with HD compared to total serum LDL cholesterol levels (74). Furthermore, oxLDL triggers cooperative signaling pathways involving scavenger receptors (SR) and toll-like receptors (TLR) in macrophages. This activation leads to the initiation of downstream pro-inflammatory signaling cascades, the production of ROS, and the maturation of interleukin-1β (IL-1β) via NLRP3 inflammasomes (75).

There is conflicting evidence regarding the levels of oxLDL in patients undergoing HD. While some studies have demonstrated increased oxLDL levels in HD patients (76-78), other studies have reported similar (79-82), or even lower oxLDL levels (83) compared to the general population. Although the correlation between oxLDL levels and stable coronary artery disease and acute coronary syndromes is recognized, the connection between oxLDL and mortality in HD patients remains a topic of debate (84). The clinical utility of oxLDL in assessing the risk of vascular complications in young HD patients is limited (79), with or without CVD (85) (Table 5). In one prospective observational study, it was found that there is no association between either oxLDL or anti-oxLDL levels and overall mortality or cardiovascular mortality in patients undergoing HD (Table 5) (71, 86). The LURIC study also showed no significant correlation between oxLDL levels and mortality in HD patients (Table 5) (87). Likewise, our study did not find any association with all-cause mortality. Once LDL undergoes significant oxidation, it transforms into a pro-apoptotic form and no longer binds effectively to the LDL receptor (LDLR) (88). Additionally, the absorption of oxLDL by macrophage scavenger receptors induces the formation of foam cells within macrophages. As a result of this mechanism, oxLDL has a limited lifespan in circulation. This could explain why there was no correlation observed between oxLDL and all-cause mortality in HD patients (89, 90).

Table 5. Clinical studies that examined the four OS markers in hemodialyzed patients.

Study	Samples	Follow-up	OS marker	Results
Zhou, Chun et al.	1394 Chinese HD patients	5.2 years	AOPPs	A higher risk of all-cause mortality was observed in Chinese maintenance HD patients with elevated levels of serum AOPP.
Song, Young Rim et al.	88 HD patients	5.2 years	Protein carbonyl	The results of multivariate analysis revealed that serum protein carbonyl levels (Hazard ratio [HR] 2.37, 95% CI 1.02–5.55, p = 0.036) independently contributed to all-cause mortality.
Suvakov, Sonja et al.	199 HD patients	8 years	AOPPs, PAB (prooxidant-antioxidant balance), MDA (malondialdehyde), GSTM1, sVCAM-1/sICAM-1	AOPP demonstrated predictive capabilities in relation to cardiovascular survival outcomes.
Wagner, Sandra, et al.	2773 patients	4 years	oxLDL	Adjusting for apolipoprotein B levels in the AURORA study rendered AOPP's prognostic potential for cardiovascular survival insignificant. Furthermore, no relationship between AOPP levels and mortality was observed in the LURIC study.
Rusu, Crina Claudia et al.	44 HD patients	9 years	bound MDA (bMDA), Protein carbonyls, ceruloplasmin, nitric oxide	Among the factors examined, only bMDA demonstrated a significant association with survival.

Hsiao, Kuang-Chih et al.	40 HD patients	5 years	Albumin, MMP-2/9 (matrix metalloproteinases), MPO, SDF-1	The predictive value of MPO for survival was not found to be statistically significant.
Lee, Young-Ki, et al.	69 HD patients	5 years	oxLDL and LPC (lysophosphatidylcholine)	No association was found between oxLDL and an elevated risk for CVD in a cohort of Korean HD patients.
Sevinc Ok, Ebru, et al.	124 HD patients	3 years	ox-LDL, anti-oxLDL	The levels of both oxidized and anti-oxidized low-density lipoprotein were not found to be associated with atherosclerosis nor mortality.
Wang, Angela Yee-Moon et al.	236 PD patients	3 years	MPO	The initial article demonstrated that the level of MPO provides substantial independent and additional predictive value for long-term mortality and cardiovascular events in patients with ESRD receiving maintenance peritoneal dialysis (PD) therapy.
Pachaly, Maria A et al.	112 HD patients	5.5 years	IL-6, AOPPs, pentosidine, homocysteine	Based on the median AOPP values, no notable differences in the survival rate were observed.
Kalantar-Zadeh, Kamyar et al.	356 MHD patients	3 years	MPO	There is a correlation between serum MPO levels and markers of inflammation, as well as an association with the prospective risk of mortality.

Abbreviations: OS: oxidative stress; CVD: cardiovascular disease; ESRD: end-stage renal disease; HD: hemodialysis; MHD: maintenance hemodialysis; MPO: myeloperoxidase; AOPPs: advanced oxidation

protein products; ox-LDL: oxidized low-density lipoprotein; PD: peritoneal dialysis. This table was modified from our published paper (21).

4.3 Embedding the results into the current state of research

This study is the pioneer in directly comparing the four ox-stress biomarkers (carbonyl proteins, MPO, AOPPs, and oxLDL) in HD patients, evaluating their association with all-cause mortality. The findings of this study unequivocally demonstrate that carbonyl proteins outperform the other biomarkers as superior predictors of all-cause mortality in HD patients. On the contrary, MPO appears to demonstrate a weaker predictive power as an all-cause mortality biomarker, whereas oxLDL and AOPPs show no significant association with all-cause mortality in HD patients. Therefore, our study can serve as a valuable resource in the selection of ox-stress biomarkers for clinical purposes, such as monitoring oxidative markers in dialysis patients or other patients, allowing for better tracking of changes in the patient's condition and for timely adjustments to treatment regimens. The measurement of oxidative markers in healthy populations can also be utilized for early prediction, prevention, and treatment of diseases. In addition, our findings can be applied in the fields of anti-aging, preventive medicine, and serve as a valuable guide for preventive and aesthetic medicine purposes.

4.4 Strengths and weaknesses of the study(s)

Our study does have certain limitations that need to be acknowledged. Firstly, we only had access to data on all-cause mortality and not specifically cardiovascular mortality. Secondly, we lacked information regarding the use of any antioxidative compounds by the patients involved in our study. In contrast to prior research, we adopted a distinct approach by directly comparing widely utilized biomarkers for oxidative stress that have not been previously evaluated side by side.

4.5 Implications for practice and/or future research

This study is the pioneer in directly comparing the four ox-stress biomarkers (carbonyl proteins, MPO, AOPPs, and oxLDL) in HD patients, evaluating their association with all-cause mortality. The findings of this study unequivocally demonstrate that carbonyl proteins outperform the other biomarkers as superior predictors of all-cause mortality in

HD patients. On the contrary, MPO appears to demonstrate a weaker predictive power as an all-cause mortality biomarker, whereas oxLDL and AOPPs show no significant association with all-cause mortality in HD patients. Therefore, our study can serve as a valuable resource in the selection of ox-stress biomarkers for clinical purposes, such as monitoring oxidative markers in dialysis patients or other patients, allowing for better tracking of changes in the patient's condition and for timely adjustments to treatment regimens. The measurement of oxidative markers in healthy populations can also be utilized for early prediction, prevention, and treatment of diseases. In addition, our findings can be applied in the fields of anti-aging, preventive medicine, and serve as a valuable guide for preventive and aesthetic medicine purposes.

5 Conclusions

To summarize, not all oxidative stress markers equally predict all-cause mortality in HD patients. In this current study, particularly carbonyl proteins and additionally MPO stand as independent predictors of all-cause mortality among HD patients. Nonetheless, AOPPs and oxLDL did not exhibit the capability to predict all-cause mortality.

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Statutory Declaration

“I, Jiao Zuo, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic [A Comparative Assessment of Oxidative Stress Biomarkers in Hemodialysis Patients for Predicting All-Cause Mortality. Eine vergleichende Bewertung von Biomarkern für oxidativen Stress bei Hämodialysepatienten zur Vorhersage der Gesamtmortalität], independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; http://www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

Declaration of your own contribution to the publications

Jiao Zuo contributed the following to the below listed publications:

Publication 1: Zuo J, Chaykovska L, Chu C, Chen X, Hasan AA, Krämer BK, Tepel M, Hocher B. Head-to-Head Comparison of Oxidative Stress Biomarkers for All-Cause Mortality in Hemodialysis Patients. Antioxidants (Basel). 2022 Oct 2;11(10):1975. https://doi.org/10.3390/antiox11101975.
Contribution (please set out in detail):
Jiao Zuo contributed to the data curation, including data collation, establishment of relevant database, sifting through the data, and analyzing data.
All the formal analysis, including spearman correlation, Mann-Whitney U test, Kaplan-Meier test. And univariate and multivariate Cox regression analysis were performed by Jiao Zuo.
All figures (Figure 1-4) and tables (Table 1-5) were all created by me.
Jiao Zuo wrote the original draft, revision of manuscript, submitted it to “Antioxidants” and writing-review and editing.

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Article

Head-to-Head Comparison of Oxidative Stress Biomarkers for All-Cause Mortality in Hemodialysis Patients

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Abstract: Oxidative stress (OS) presents even in the early chronic kidney disease (CKD) stage and is exacerbated in patients with end-stage renal disease (ESRD) undergoing maintenance hemodialysis (MHD). There is still a debate over the association between oxidative stress and mortality. Our study aims to compare head-to-head the prognostic value of different oxidative markers for all-cause mortality in hemodialysis (HD) patients. We thus enrolled 347 patients on HD in this prospective study. Four OS biomarkers were measured (carbonyl proteins, myeloperoxidase (MPO), advanced oxidation protein products (AOPPs), and oxidized low-density lipoprotein (ox-LDL)). During the 60-month follow-up period, 9 patients have been lost to follow-up and 168 (48.4%) patients died. Concerning the oxidative stress (ox-stress) byproducts, carbonyl proteins were lower in survivors (105.40 ng/mL (IQR 81.30–147.85) versus 129.65 ng/mL (IQR 93.20–180.33); $p < 0.001$), with similar results for male patients (103.70 ng/mL (IQR 76.90–153.33) versus 134.55 ng/mL (IQR 93.95–178.68); $p = 0.0014$). However, there are no significant differences in MPO, AOPP, and ox-LDL between the two groups. Kaplan–Meier survival analysis indicated that patients in the higher carbonyl proteins concentration (>117.85 ng/mL group) had a significantly lower survival rate (log-rank test, $p < 0.001$). Univariate Cox regression analysis showed a positive correlation between carbonyl proteins and all-cause mortality in the higher and lower halves. Even after adjustment for conventional risk factors, it remained a statistically significant predictor of an increased risk of death in MHD. Univariate Cox regression analysis of MPO showed that continuous MPO and Log MPO were significantly associated with all-cause mortality, except for binary MPO (divided according to the median of MPO). Multivariate Cox analysis for MPO showed that the mortality prediction remains significant after adjusting for multiple factors. In conclusion, not all ox-stress biomarkers predict all-cause mortality in HD patients to a similar extent. In the present study, carbonyl proteins and MPO are independent predictors of all-cause mortality in HD patients, whereas AOPPs and oxLDL are clearly not associated with all-cause mortality in HD patients.

Keywords: maintenance hemodialysis; oxidative stress; all-cause mortality; carbonyl proteins; myeloperoxidase; advanced oxidation protein products; oxidized low-density lipoprotein

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1. Introduction

End-stage renal disease (ESRD) patients are highly prone to acute-phase inflammation and oxidative stress, both linked with cardiovascular mortality and morbidity [1–4]. Additionally, maintenance dialysis patients have an excessively high risk of cardiovascular morbidity and mortality; even after adjustment, cardiovascular mortality has been reported

to be 10 to 20-fold higher than in the general population [5]. The development of long-term complications such as amyloidosis, atherosclerosis, and cardiovascular disease (CVD) in hemodialysis (HD) patients may be influenced by oxidative stress, which may act synergistically with inflammation [4,6,7]. There is increasing recognition that oxidative stress is an important metabolic component of ESRD [6].

The imbalance between the generation of oxidant compounds and the defense mechanisms against them causes oxidative stress, described as tissue damage [7], which leads to a greater risk of atherosclerosis and b2-microglobulin amyloidosis, as well as significant oxidative stress in ESRD patients [6]. Oxidatively modified amino acids and plasma proteins can be important in vivo oxidative stress biomarkers [8]. The half-life of oxidants is only seconds, making them highly reactive compounds. Due to this, it is generally not possible to determine them in vivo. Unlike proteins, carbohydrates, and nucleic acids, oxidant-modified lipids have lifetimes ranging from hours to weeks, making them ideal indicators of oxidant stress [9].

The available studies have shown different results regarding the predictive role of different oxidative biomarkers for all-cause mortality. In this study, we aimed to assess the prognostic value of four different oxidative stress biomarkers (carbonyl proteins, myeloperoxidase (MPO), advanced oxidation protein products (AOPPs), and oxidized LDL (oxLDL)) for all-cause mortality in HD patients.

2. Materials and Methods

2.1. Study Population

In our study, we recruited 347 patients on stable hemodialysis from two dialysis centers associated with our inpatient facility at the Campus Charité Mitte (KfH Dialysezentrum-Neukölln, Berlin, Germany, and KfH Dialysezentrum-Moabit, Berlin, Germany). Local ethics committees approved this study (approval number: S-20090061), and informed consent was obtained from all study participants.

Hemodialysis with standard bicarbonate dialysis with biocompatible membranes was administered three to four times per week to all patients. Dialysate flow rates were 500 mL/min and blood flow rates were 250–300 mL/min. All patients had a functioning permanent access. The study excluded patients with malignancies, active infections, pregnancy, or unwillingness to participate. Every patient had a functional permanent access device. A 60-month follow-up period documented all-cause deaths. Patients who received a transplant were censored at the time of transplantation.

2.2. Assays

At the study entrance, blood samples were collected before each session of hemodialysis and the blood was drawn on a fasting state at the morning. Routine blood tests (hemoglobin, ferritin, transferrin, fasting blood glucose, creatinine, potassium, calcium, phosphorus, iPTH, *n*-ox PTH, albumin, BUN, LDL, HDL, hsCRP) were assessed by standardized methods in the clinical laboratory. The plasma biomarkers were analyzed using a sandwich enzyme immunoassay: Myeloperoxidase (MPO) [K6631B, in vitro determination of Myeloperoxidase in serum and plasma (ELISA), Immundiagnostik, AG, Bensheim, Germany], advanced oxidation protein products (AOPPs) [KR7811W, in vitro determination of Advanced oxidation protein products (AOPPs) in EDTA plasma (Photometric), Immundiagnostik, AG, Bensheim, Germany], oxidized low-density lipoprotein (ox-LDL) [K7810, in vitro determination of ox-LDL (ELISA), Immundiagnostik, AG, Bensheim, Germany], and Carbonyl proteins concentrations [K7870, in vitro determination of protein-bound carbonyls in human serum and plasma (ELISA), Immundiagnostik, AG, Bensheim, Germany] according to manufacturer instructions.

2.3. Statistical Analysis

Statistical significance was defined as $p < 0.05$. All analysis was performed using SPSS version 25.0 (IBM, Armonk, NY, USA). Descriptive variables are expressed as medians

(interquartile ranges) or numbers (percentages). The Mann–Whitney U test was performed to determine the differences between the survivors and non-survivors. Cumulative survival curves were performed using the Kaplan–Meier method stratified by the median (lower and higher than values), and the log-rank test was used to compare groups' survival. After conducting one-way regression analysis, those with p values less than 0.1 were included in the final multi-factor regression equation. Among them, iPTH and noxPTH interacted with each other in regression analysis, while n-oxPTH may better reflect the hormonal function [10], so noxPTH was selected to be included in the regression equation. The analysis of the simultaneous associations between risk factors and survival time was performed using the multivariate Cox regression analysis to control for possible confounding factors. Hazard ratios (HR) and their 95% confidence intervals (CI) were calculated. According to univariate Cox Regression results, we created three models for multivariate Cox regression analysis. Model A was an adjustment for demographics (age, hypertension, and CVD); Model B was an adjustment for clinical parameters (serum creatinine, transferrin, phosphorus, n-oxPTH, albumin); Model C was an adjustment for the risk factors in both model A and model B.

3. Results

A total of 347 HD patients were included in this study; the median age was 66 years (IQR 56–75). There were 229 male patients, 117 female patients, and 1 patient with no sex indicated. In total, 130 patients had diabetes mellitus (DM) and 161 had a history of CVD. More than three-quarters of patients had hypertension (77.5%). According to the outcome, we divided the HD patients into two groups: survivors and non-survivors. Demographic and clinical data within each group are presented in Table 1. During the 60-month follow-up period, 9 patients (including 1 patient of unknown sex) were lost to follow-up, and 168 (48.4%) patients died. Among the 347 HD patients, survivors were younger, had a lower prevalence of DM and CVD, and had significantly lower hsCRP concentrations, while having higher transferrin, fasting blood glucose, intact parathyroid hormone (iPTH), non-oxidized parathyroid hormone, serum albumin, and LDL compared to non-survivors. Concerning the ox-stress byproducts, carbonyl proteins were lower in survivors (105.40 ng/mL (IQR 81.30–147.85) versus 129.65 ng/mL (IQR 93.20–180.33); $p < 0.001$) (Figure 1), and among male survivors, this trend continues (103.70 ng/mL (IQR 76.90–153.33) versus 134.55 ng/mL (IQR 93.95–178.68); $p = 0.0014$) (Figure 1). However, there are no significant differences in MPO, AOPPs, and ox-LDL between the two groups (Table 1; Supplementary Figure S1).

Table 1. Clinical and biochemical characteristics of dialysis patients.

Characteristics	All (n = 347)	Survivors (n = 170)	Non-Survivors (n = 168)	p-Value
Age (years)	66.0 (56.0–75.0)	60.50 (49.00–69.00)	71.00 (66.00–78.00)	<0.001
Sex (M/F/Unknown)	229/117/1	114/56/0	110/58/0	0.759
Body mass index, kg/m^2	24.40 (22.01–27.60)	24.20 (22.12–28.30)	24.57 (21.71–26.99)	0.541
Drinker, n (%)	62 (17.90%)	30 (17.60%)	32 (9.10%)	0.740
Smoker, n (%)	108 (31.10%)	54 (31.80%)	52 (14.80%)	0.872
Diabetes mellitus, n (%)	130 (37.50%)	55 (32.40%)	74 (21.10%)	0.027
Hypertension, n (%)	269 (77.50%)	134 (78.80%)	135 (38.50%)	0.727
Cardiovascular disease, n (%)	161 (46.40%)	82 (48.20%)	101 (28.80%)	<0.001
Dialysis vintage (days)	263.00 (31.00–1219.25)	221.00 (31.00–939.25)	351.00 (31.00–1461.00)	0.004
Dialysis dose (Kt/V)	1.04 (0.91–1.16)	1.03 (0.91–1.16)	1.04 (0.91–1.17)	0.749
Medication, n (%)				
RAAS inhibitors	88 (25.40%)	46 (27.1%)	41 (11.70%)	0.577
Beta-blockers	204 (58.8%)	116 (68.2%)	86 (24.50%)	0.001
Calcium channel blockers	104 (30.00%)	60 (35.3%)	43 (12.30%)	0.053
Erythropoietin	171 (49.30%)	82 (48.2%)	89 (25.40%)	0.414
Diuretics	194 (55.90%)	98 (57.6%)	95 (27.10%)	0.838

Table 1. *Cont.*

Characteristics	All (n = 347)	Survivors (n = 170)	Non-Survivors (n = 168)	p-Value
Hemoglobin (g/dL)	10.20 (9.10–11.63)	10.25 (9.00–11.67)	10.20 (9.20–11.70)	0.865
Ferritin (ng/mL)	532.00 (253.25–1125.88)	527.50 (225.00–1065.75)	532.00 (281.00–1235.00)	0.540
Transferrin (µg/mL)	138.00(106.00–173.00)	145.00 (121.00–173.50)	128.50 (99.00–172.25)	0.003
Fasting blood glucose (mg/dL)	108.00 (90.00–134.00)	114.50 (94.50–143.60)	104.00 (87.00–123.60)	0.006
Creatinine (mg/dL)	6.62 (4.23–8.34)	6.67 (4.15–8.53)	6.60 (4.23–7.96)	0.007
Potassium (mmol/L)	4.70 (4.10–5.28)	4.60 (4.00–5.30)	4.77 (4.21–5.26)	0.734
Calcium (mmol/L)	2.24 (2.10–2.40)	2.20 (2.09–2.40)	2.27 (2.10–2.47)	0.414
Phosphorus (mmol/L)	1.61 (1.19–2.10)	1.70 (1.22–2.12)	1.54 (1.11–2.06)	0.051
iPTH (ng/L)	49.90 (18.68–124.60)	68.19 (21.75–171.05)	39.76 (14.47–101.90)	0.003
n-ox PTH (ng/L)	5.86 (2.38–14.01)	7.18 (3.05–16.26)	4.99 (1.98–11.11)	0.003
Albumin (g/dL)	3.30 (2.90–3.70)	3.40 (3.05–3.80)	3.10 (2.80–3.60)	0.001
BUN (mg/dL)	195.12 (146.70–267.67)	201.05 (152.64–267.67)	189.63 (131.73–279.50)	0.822
LDL (mg/dL)	92.70 (72.20–121.20)	100.80 (75.05–127.40)	89.00 (70.70–112.00)	0.013
HDL (mg/dL)	39.90 (32.20–50.80)	38.60 (31.00–50.20)	42.30 (34.30–54.00)	0.435
hsCRP (mg/L)	2.60 (1.00–5.20)	2.30 (0.70–4.50)	2.80 (1.20–6.63)	0.006
MPO (ng/mL)	106.84 (67.71–188.38)	102.27 (67.37–176.37)	118.90 (69.46–199.24)	0.176
AOPPs (µmol/L)	107.79 (78.79–149.94)	109.99 (80.59–156.72)	107.57 (79.53–146.80)	0.588
ox-LDL (mg/dL)	84.90 (44.80–180.55)	87.55 (45.85–197.63)	83.10 (44.53–176.35)	0.779
Carbonyl proteins (ng/mL)	117.85 (84.73–163.18)	105.40 (81.30–147.85)	129.65 (93.20–180.33)	<0.001

Values are presented as median (IQR). Between groups (survivors versus non-survivors) comparisons were made using a nonparametric Mann–Whitney U test for continuous variables and the Chi-test for categorical variables. 1 patient who did not indicate sex showed in this table as unknown. Abbreviations: RAAS: Renin-Angiotensin-Aldosterone-System; iPTH: intact Parathyroid hormone; n-oxPTH: non-oxidized Parathyroid hormone; BUN: Blood urea nitrogen; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; hsCRP: High sensitivity C-reactive protein; MPO: Myeloperoxidase; AOPPs: Advanced oxidation protein products; ox-LDL: Oxidized low-density lipoprotein.

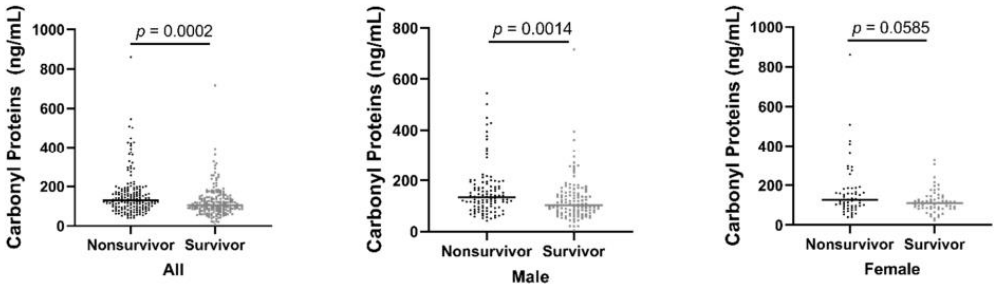

Figure 1. Plots of serum carbonyl proteins concentrations. Median serum carbonyl proteins were significantly lower in the survivors than the non-survivors using the Mann–Whitney U test.

Kaplan–Meier curves for all-cause mortality according to the median of each of four ox-stress byproduct concentrations at the baseline are presented in Figure 2. It revealed that the lower carbonyl proteins concentration group (<117.85 ng/mL) had a significantly higher survival rate (log-rank test, $p < 0.001$) in this study cohort (Figure 2). AOPPs, MPO, and oxLDL did not show statistical significance.

Then, we performed univariate and multivariate Cox regression analysis. Univariate Cox's proportional hazards regression analysis showed that age (HR = 1.062 CI 95% (1.047–1.077) $p < 0.001$), CVD (HR = 1.440 CI 95% (1.056–1.963) $p = 0.021$), transferrin (HR = 0.995 CI 95% (0.992–0.998) $p = 0.002$), creatinine (HR = 0.875 CI 95% (0.819–0.935) $p < 0.001$), phosphorus (HR = 0.771 CI 95% (0.595–1.000) $p = 0.05$), iPTH (HR = 0.998 CI 95% (0.997–1.000) $p = 0.012$), n-oxPTH (HR = 0.986 CI 95% (0.973–1.000) $p = 0.043$), albumin (HR = 0.663 CI 95% (0.515–0.855) $p = 0.001$), MPO (HR = 1.000 CI 95% (1.000–1.000) $p < 0.001$) and carbonyl proteins (HR = 1.002 CI 95% (1.001–1.003) $p = 0.001$) had a significant association with survival (Table 2). After adjustment for the conventional risk factors

of HD patients in different models (as described in the Materials and Methods section), baseline concentrations of carbonyl proteins remained a statistically significant predictor of an increased risk of death (Table 3). Continuous MPO and Log MPO were significantly associated with all-cause mortality, except binary MPO (divided according to the median of MPO) (Table 4). The mortality prediction of MPO remained significant after adjusting for multiple factors.

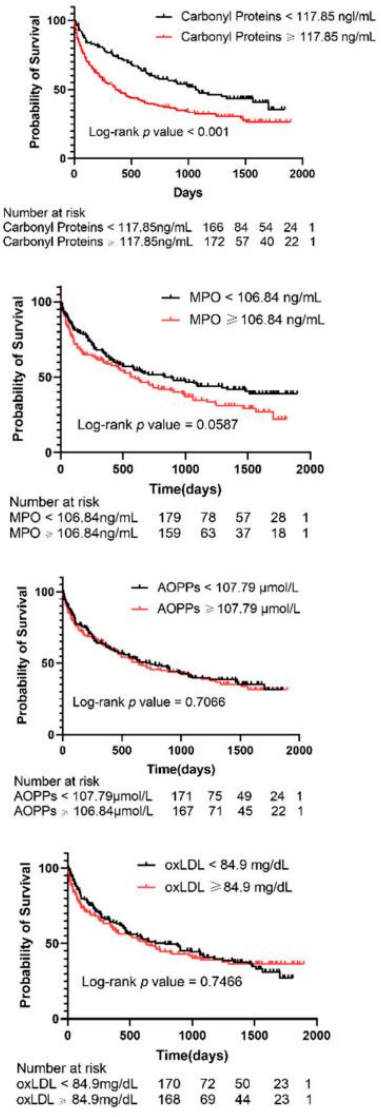

Figure 2. Kaplan–Meier survival curves for all-cause mortality. Patients were divided according to the median values of variables. Abbreviations: MPO: Myeloperoxidase; AOPPs: Advanced oxidation protein products; ox-LDL: Oxidized low-density lipoprotein.

Table 2. Cox regression univariate analysis, hazard ratio, and 95% confidence intervals for survival in HD patients.

Analyses	HR (95% CI)	*p*-Value
Age(years)	1.062 (1.047–1.077)	<0.001
Male/Female	0.981 (0.714–1.349)	0.908
Body mass index, kg/m^2	0.992 (0.961–1.024)	0.615
Drinker, *n* (%)	1.108 (0.754–1.629)	0.601
Smoker, *n* (%)	0.879 (0.634–1.220)	0.441
Diabetes mellitus, *n* (%)	1.203 (0.887–1.632)	0.235
Hypertension, *n* (%)	0.723 (0.493–1.059)	0.096
Cardiovascular disease, *n* (%)	1.440 (1.056–1.963)	0.021
Dialysis vintage (days)	0.999869 (0.999716–1.000022)	0.093
Dialysis dose (Kt/V)	0.731 (0.377–1.417)	0.353
Hemoglobin (g/dL)	0.950 (0.868–1.038)	0.256
Ferritin (ng/mL)	1.000 (1.000–1.000)	0.846
Transferrin (µg/mL)	0.995 (0.992–0.998)	0.002
Fasting blood glucose (mg/dL)	0.999 (0.995–1.002)	0.449
Creatinine (mg/dL)	0.875 (0.819–0.935)	<0.001
Potassium (mmol/L)	0.895 (0.744–1.076)	0.238
Calcium (mmol/L)	0.832 (0.493–1.403)	0.490
Phosphorus (mmol/L)	0.771 (0.595–1.000)	0.0503
iPTH (ng/L)	0.998 (0.997–1.000)	0.012
n-ox PTH	0.986 (0.973–1.000)	0.043
Albumin (g/dL)	0.663 (0.515–0.855)	0.001
BUN (mg/dL)	1.000 (0.999–1.001)	0.479
LDL (mg/dL)	0.997 (0.993–1.002)	0.250
HDL (mg/dL)	1.005 (0.996–1.015)	0.302
hsCRP (mg/L)	1.018 (0.992–1.044)	0.179
MPO (ng/mL)	1.000035 (1.000020–1.000051)	<0.001
AOPPs (µmol/L)	1.001 (0.998–1.004)	0.445
ox-LDL (mg/dL)	1.000 (0.999–1.000)	0.451
Carbonyl proteins (ng/mL)	1.002 (1.001–1.003)	0.001

Abbreviations: iPTH: intact Parathyroid hormone; *n*-oxPTH: non-oxidized Parathyroid hormone; BUN: Blood urea nitrogen; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; hsCRP: High sensitivity C-reactive protein; MPO: Myeloperoxidase; AOPPs: Advanced oxidation protein products; ox-LDL: Oxidized low-density lipoprotein.

Table 3. Cox regression univariate and multivariate analysis of carbonyl proteins, hazard ratio, and 95% confidence intervals for survival in HD patients.

Analyses	HR (95% CI)	*p*-Value
Univariate Cox regression		
Continuous Carbonyl proteins	1.002 (1.001–1.003)	0.001
Binary Carbonyl proteins	0.564 (0.414–0.767)	<0.001
Log Carbonyl proteins	3.162 (1.684–5.937)	<0.001
Multivariable Cox regression		
Model A	1.002 (1.001–1.004)	0.001
Model B	1.002 (1.000–1.003)	0.027
Model C	1.002 (1.000–1.004)	0.015

Binary carbonyl proteins were divided according to the median of carbonyl proteins (117.85 ng/mL). Model A was adjusted for age, hypertension, and CVD; Model B was adjusted for serum creatinine, transferrin, phosphorus; albumin, and *n*-oxPTH; Model C was adjusted for the above risk factors (Model A + Model B).

Table 4. Cox regression univariate and multivariate analysis of MPO, hazard ratio, and 95% confidence intervals for survival in HD patients.

Analyses	HR (95% CI)	*p*-Value
Univariate Cox regression		
Continuous MPO	1.000035 (1.000020–1.000051)	<0.001
Binary MPO	1.363 (0.998–1.862)	0.052

Table 4. *Cont.*

Analyses	HR (95% CI)	*p*-Value
Log MPO	2.123 (1.394–3.234)	<0.001
Multivariable Cox regression		
Model A	1.000033 (1.000018–1.000049)	<0.001
Model B	1.000028 (1.000012–1.000044)	<0.001
Model C	1.000024 (1.000008–1.000040)	0.003

Binary MPO was divided according to the median of MPO (106.84 ng/mL). Model A was adjusted for age, hypertension, and CVD; Model B was adjusted for serum creatinine, transferrin, phosphorus; albumin, and *n*-oxPTH; Model C was adjusted for the above risk factors (Model A + Model B).

4. Discussion

In this study, four biomarkers of oxidative stress are evaluated as predictors of long-term mortality among patients with HD. Substantial differences were seen regarding the predictive power of different oxidative stress biomarkers to predict all-cause mortality in patients on dialysis. Baseline carbonyl proteins were lower in survivors versus non-survivors (Figure 1), whereas baseline MPO, AOPPs, and oxLDL did not differ between survivors and non-survivors (Supplementary Figure S1). When performing Cox regression analysis considering confounding factors showed that both carbonyl proteins and MPO were independent predictors of all-cause mortality in HD patients. AOPPs and oxLDL, on the other hand, were not independently associated with all-cause mortality,

Proteins constitute 70% of the tissue and cell dry mass and proteins are a major target for damage/posttranslational modifications [11,12]. One of the most widely used stable biomarkers for detecting severe oxidative protein damage is carbonyl proteins, which have been found to remain elevated in the blood for up to 18 h [13]. As a sign of oxidative protein damage, protein carbonylation occurs when lysine, arginine, proline, and threonine residues are directly oxidized, and when reactive carbonyl species are produced from carbohydrate and lipid oxidation interact with dicarbonyl compounds directly [14]. The process of carbonylation is irreversible and antioxidant defenses cannot effectively reverse this modification [15,16]. It is thought that carbonylation negatively affects both protein function and cellular viability [17–21]. Additionally, carbonylation may lead to highly cytotoxic large protease-resistant protein aggregates [22]. The level of plasma carbonyl proteins is higher in hemodialysis patients than in healthy individuals [23,24]. Our study showed higher carbonyl proteins level in HD non-survivable patients. Carbonyl proteins were good predictors of all-cause mortality in dialysis patients even after adjustments for multiple risk factors. Our data are in good agreement with a recent study also performed in HD patients (Supplementary Table S1) [25]. It is hypothesized that contact with the dialysis filter activates neutrophils, likely increasing oxidative/carbonyl stress and inflammation following HD [26]. However, there is also one other study who did not show an effect of protein carbonylation on mortality. This study just analyzed 44 patients (Supplementary Table S1) [27]. The power was thus probably too low.

As a major component of leukocytes' bactericidal arsenal, myeloperoxidase (MPO), a heme enzyme synthesized and secreted by neutrophils and monocytic cells, is an important source of Reactive Oxygen Species (ROS) [28]. At inflammation sites, MPO is a major catalyst for lipid peroxidation, a process crucial to atherogenesis [29–35]. Plasma MPO levels appear to be increased during HD due to oxidative stress as well [36]. Dialysis may increase MPO through leukocyte activation at the dialysis membrane, and the degree of MPO may depend on the biocompatibility of the dialysis membrane [37–39]. A study including 356 patients on maintenance dialysis showed that increased MPO levels were independently associated with an increased risk of death and that measuring MPO may be useful for diagnosing unrecognized clinical risks (Supplementary Table S1) [40]. MPO may predict long-term mortality in HD patients was also confirmed in a comparative study (Supplementary Table S1) [41]. However, in another 5-year follow-up study of dialysis patients, MPO did not show an independent ability to predict all-cause mortality

(Supplementary Table S1) [42]. We found that MPO had limited value in predicting all-cause mortality in our cohort and, unadjusted baseline values were similar in survivors and non-survivors. Only after adjusting for demographic and clinical risk factors in multivariate Cox analysis and continuous univariate correlation analysis was independently associated with all-cause mortality. In addition, this hazard risk of binary transformed MPO for all-cause mortality lost significance.

As a result of oxidative damage, proteins can develop modifications in their spectroscopic characteristics called advanced oxidation protein products (AOPPs) [43]. The AOPPs also promotes the production of reactive oxygen species as a byproduct of oxidative damage [44]. In comparison to lipid peroxidation products, AOPPs are more accurate for the measurement of oxidative stress [43]. These proteins are highly elevated in HD patients [45]. In healthy individuals and HD patients, AOPPs has been implicated as a risk factor for atherosclerotic cardiovascular events [46]. An 8-year follow-up prospective study of 199 patients with ESRD on hemodialysis showed that AOPPs demonstrated a significant predictive impact in overall and cardiovascular survival (Supplementary Table S1) [47]. Additionally, a multi-center, prospective cohort study showed that elevated serum AOPP levels were associated with higher risk of all-cause mortality in Chinese maintenance HD patients (Supplementary Table S1) [48]. In our study, AOPPs were not found to be a predictor of mortality in HD patients. There was even no trend. Different results from AOPPs for predicting all-cause mortality could be explained by two factors: first, a higher probability and odds of death would be predicted for patients on ESRD dialysis with an 8-year follow-up; second, more than half of the patients in this 8-year follow-up study were women, whereas almost half of the patients in our study were males. However, our result was consistent with the 112 HD patients, 5.5-year follow-up study (Supplementary Table S1) [49].

Oxidized low-density lipoprotein (oxLDL), a form of LDL formed after oxidation of LDL, is necessary for macrophages to accumulate cholesterol [50]. The measurement of oxLDL may provide better predictability of atherosclerotic CVD in patients with HD than total serum LDL cholesterol [51], because the increased monocyte endothelial cell adhesion associated with high oxLDL may contribute to CVD development in chronic renal failure patients on dialysis through another mechanism that interferes with coagulation activation [52]. Some studies showed that HD patients have increased oxLDL [53–55]. In contrast, other studies have reported that the oxLDL levels of HD patients were similar to those of the general population [56–59], or even lower [60]. Although oxLDL levels were associated with stable coronary artery disease and acute coronary syndromes [61], in HD patients, the findings of the relationship between oxLDL and mortality are controversial. OxLDL has limited clinical value in identifying the risk of vascular complications in young HD patients [56], with no difference seen between CVD and non-CVD groups (Supplementary Table S1) [62], and there are also studies showing that oxLDL is not associated with coronary artery calcification in MHD patients [63]. In patients not receiving HD in the LURIC study, there was no correlation between oxLDL and mortality (Supplementary Table S1) [64]. Another prospective observational study showed that oxLDL and anti-oxLDL in HD patients are not associated with overall mortality or cardiovascular mortality [50]. Similarly, no association with all-cause mortality was found in our study. When LDL is highly oxidized, it becomes pro-apoptotic and fails to be recognized by the LDL receptor (LDLR) [65]. Alternatively, oxLDL is absorbed by macrophage scavenger receptors, causing macrophage foam cells to form. This causes oxLDL cannot last too long in circulation, perhaps that is why oxLDL was not correlated with all-cause mortality in HD patients [66,67].

This study is the first to make a head-to-head comparison of HD patients' four oxstress biomarkers (carbonyl proteins, MPO, AOPPs, and oxLDL) with all-cause mortality and clearly shows that carbonyl proteins are superior biomarkers of all-cause mortality in HD patients. MPO, on the other hand, seems to be a somewhat weaker all-cause mortality biomarker, while oxLDL and AOPPs seem to have no impact on all-cause mortality in

HD patients. Our study hence may be a useful tool to select ox-stress biomarkers for clinical use.

Our study also has clearly limitations, first we just had data on all-cause mortality but not on cardiovascular mortality. Second, we had no information of the use of any anti-oxidative substances by our patients. However, in contrast to previous studies, we used the approach of comparing key biomarkers for oxidative stress that are widely used but have never been compared head-to-head.

5. Conclusions

In conclusion, not all ox-stress markers predict all-cause mortality in HD patients with equal power. In the present study, especially carbonyl proteins but also MPO were found to be independent predictors of all-cause mortality for HD patients' however, AOPPs and oxLDL failed to predict all-cause mortality.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/antiox11101975/s1, Figure S1: Plots of serum MPO, AOPPs, oxLDL. Table S1: Clinical studies that examined the four OS markers in hemodialyzed patients. References [25,27,40–42,47–50,62,64] are cited in the Supplementary Materials.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, approved by the regional ethics committee (reference number S-20090061, approval date: 2 December 2009), the Danish Medicines Agency (EudraCT: 2008–006438–82, approval date: 15 June 2009).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available in the article and Supplementary Materials.

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Conflicts of Interest: None of the authors has any conflict of interest with regard to this study. The authors declare that there are no relationships or activities that might bias, or be perceived to bias, their work.

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Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

Publication list

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