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The influence of Klotho protein and prooxidant–antioxidant balance combination on the mortality of HD patients

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Abstract

Purpose End-stage renal disease patients on chronic hemodialysis (HD) have a shortened life expectancy compared to the general population. The aim of this study was to evaluate a possible link between three new and emerging factors in renal pathophysiology: Klotho protein, telomere length in peripheral blood mononuclear cells (TL) and redox status parameters before HD (bHD) and after HD (aHD), and to test mortality prediction capability of these emerging parameters in a population of HD patients.

Methods The study included 130 adult patients with average age 66 (54–72), on HD (3 times per week; 4–5 h per session). Klotho level, TL, routine laboratory parameters, dialysis adequacy and redox status parameters: advanced oxidation protein products (AOPP), prooxidant–antioxidant balance (PAB), superoxide anion ($O_2^{\cdot -}$), malondialdehyde (MDA), ischemia-modified albumin (IMA), total sulfhydryl group content (SHG), and superoxide dismutase (SOD) were determined.

Results Klotho concentration was significantly higher aHD; 68.2 (22.6–152.9) vs. bHD 64.2 (25.5–119.8) ($p=0.027$). The observed increase in TL was not statistically significant. AOPP, PAB, SHG, and SOD activity were significantly increased aHD ($p>0.001$). The patients with the highest mortality risk score (MRS) had significantly higher PAB bHD ($p=0.002$). Significantly lower $O_2^{\cdot -}$ ($p<0.001$), SHG content ($p=0.072$), and IMA ($p=0.002$) aHD were found in patients with the lowest MRS values. Principal component analysis revealed redox balance-Klotho factor as a significant predictor of high mortality risk ($p=0.014$).

Conclusion Decreased Klotho and TL attrition as well as redox status disturbance could be connected with higher mortality rate in HD patients.

Keywords Hemodialysis · Klotho protein · Oxidative stress · Telomeres

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Introduction

End-stage renal disease (ESRD) is the last stage of chronic kidney disease with estimated glomerular filtration rate (eGFR) less than 15 mL/min/1.73 m^2. As a consequence, renal replacement treatments such as dialysis or kidney transplantation are necessary to prolong patient life [1]. This stage is associated with a number of systemic complications already initiated in the earlier stages of chronic kidney disease (CKD) and have worsened during progression to ESRD. These include chronic low-grade inflammation, malnutrition, anemia, mineral and bone disorder, acidosis, hypervolemia, and hyperkalemia [2].

Klotho protein participates in a number of protective processes and plays a role in the pathophysiology of a number of aging-related diseases [3]. Klotho is a transmembrane protein expressed on the cell surface. It forms a binary complex with fibroblast growth factor 23 (FGF23) receptor (FGFR) and enhances FGF23 binding affinity. Klotho is also responsible for phosphate, calcium, and vitamin D homeostasis; it participates in inflammatory signaling, energy metabolism, and other processes such as cardiovascular homeostasis and hematopoiesis [2–4]. In addition to a membrane-bound form, soluble Klotho acts independent of FGF23 as an endocrine and paracrine hormone. Soluble Klotho arises from metalloproteinase ADAM 10 and ADAM 17 proteolytic cleavage of the extracellular domain of membrane-bound Klotho [5]. Soluble Klotho exhibits antioxidative activity mediated by inhibition of insulin/insulin-like growth factor-1 (IGF-1) and transforming growth factor-β1 (TGF-β1) signaling pathways [3, 4] and also by activating forkhead box (FoxO) transcription factors and by inhibition of Toll-like receptor 4 (TLR4) [6].

Hallmarks of ESRD may be associated with the decline in Klotho and impaired function of the FGF23/Klotho axis [2, 7].

Hemodialysis (HD) patients have a shortened life expectancy compared to the general population. Common chronic co-morbidities such as cardiovascular disease (CVD) and acute conditions such as infections are linked to higher death rates. Besides the well-known risk factors associated with cardiovascular mortality in HD patients, oxidative stress has emerged as a novel risk factor [8]. HD patients themselves are more susceptible to oxidative stress due to advanced age and co-morbidities than the general population. In addition, a contribution of the HD process itself via bio-incompatibility of material for extracorporeal circulation or dialysate endotoxins favorizing oxidative stress generation cannot be ruled out [9]. Data from previous studies in HD patients showed that reduced antioxidative defense potential, malnutrition, and inflammation are associated with increased mortality [10, 11].

Another emerging biomarker associated with senescence and age-related diseases is telomere length. As the hallmarks of ESRD (chronic inflammation, aging, unregulated renin–angiotensin system, and oxidative stress) may lead to lymphocyte telomere attrition, resulting in immune cell senescence and decreased T-cell response, increased susceptibility to kidney infection, consequential injury, and impaired renal regeneration are evident [12].

In our study, we aimed to determine the changes in Klotho abundance, telomere length in peripheral blood mononuclear cells (TL in PBMC), and redox status parameters before and after HD, and to evaluate a possible link between these three new and emerging factors in renal disease pathology. The second aim of this study was to test mortality prediction capability of all three measured parameters in a population of HD patients.

Materials and methods

A total of 130 ESRD patients undergoing chronic HD treatment at the Clinical Department of Nephrology, Clinical Hospital Center "Zvezdara" were enrolled in this study. Patients were on a regular HD program (3 times per week, 4–5 h per session) on bicarbonate dialysis (91 patients), hemodiafiltration or expanded hemodialysis with biocompatible polysulfone hemodialysis membrane Fx-Cor Diax (Fresenius Medical Care) (28 patients) and Theranova (Baxter) for expanded hemodialysis (11 patients). This class of membrane was introduced to effectively target the removal of large and middle molecules such as β2-microglobulin when connected to a Polyflux dialyzer (Gambro).

The major cause of ESRD was hypertension in 56 (43%), diabetes mellitus in 30 (23.1%), glomerulonephritis in 14 (10.8%), polycystic kidney disease in 14 (10.8%), and other causes in 16 (12.3%) patients.

EDTA blood samples were taken from all patients before HD (bHD) and after HD (aHD) session and transported to the laboratory of the Faculty of Pharmacy (Department of Medical Biochemistry) in an ice block-containing coolbox within 1–2 h after being drawn. Blood samples in vacutainers with serum separator gel were obtained from all subjects before a HD session and aliquoted immediately after centrifugation at 3000 × g for 15 min and stored at − 70 °C until analysis.

Complete blood count was determined on a XN 1000 hematological analyzer (Sysmex Corporation, Kobe, Japan) and routine biochemical parameters were determined on a Roche/Hitachi Cobas c501 automated analyzer (Roche Diagnostic, Mannheim, Germany).

From aliquoted EDTA plasma, Klotho was measured using a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (R&D Systems, DuoSet ELISA, Minneapolis, USA).

Peripheral blood mononuclear cells (PBMCs) from whole blood were isolated using Ficoll-Paque® gradient (Cytiva, Global Life Sciences Solutions, Marlborough, USA) and immediately stored at – 80 °C until further processing. The genomic DNA from PBMCs was extracted using a commercial DNA kit (Flexi GENE DNA kit, Qiagen). The TL was determined using the Cawthon method with modifications and albumin as a single copy gene [13]. As a final result TL was calculated as the T/S ratio. TL PCR measurements used a real-time 7500 PCR system (Applied Biosystems, Foster City, CA, USA).

Redox status was determined on an ILAB 300 + autoanalyzer (Instrumentation Laboratory, Milan, Italy) by measuring prooxidants and products of its activity: advanced oxidation protein products (AOPP), prooxidant–antioxidant balance (PAB), superoxide anion ($O_2^{\cdot -}$), malondialdehyde (MDA), ischemia-modified albumin (IMA), total sulfhydryl groups (SHG) and superoxide dismutase (SOD). The intra-assay and inter-assay coefficients of variance for all assays were below 3.0% and 5.6%, respectively. The level of AOPP was obtained according to the Witko-Sarsat method, using a reaction with glacial acetic acid and potassium iodide [14]. PAB was determined by a modified PAB test using 3, 3′, 5, 5′-tetramethylbenzidine as a chromogen [15]. Levels of $O_2^{\cdot -}$ were measured as a rate of reduction of nitroblue tetrazolium according to the method by Auclair and Voisin [16]. The concentration of MDA was determined as thiobarbituric acid reactive substance (TBARS) in plasma samples [17]. The IMA level was measured using the rapid and colorimetric method developed by Bar-Or et al. [18]. Levels of SHG in plasma were measured by the Ellman method, using DTNB (dinitrodithiobenzoic acid) as a reagent [19]. The method of Misra and Fridovich, based on the ability of the enzyme to inhibit autooxidation of epinephrine in alkaline medium, was used to measure plasma SOD activity [20].

To calculate the 2-year mortality score, we used Floege's risk score originating from the Framingham Heart Study. It is based on the laboratory parameters such as hemoglobin, ferritin, C-reactive protein, serum albumin, creatinine and calcium levels, vascular access and actual blood flow as HD parameters and clinic-epidemiological data such as age, smoking status, body mass index, history of cardiovascular and cancer disease, and etiology of primary illness. These data were converted into risk score points using a risk point calculator for estimation of all-cause mortality and the patients were subsequently categorized into: low-(< 5 points), intermediate- (5–9 points) and high-(> 9 points) risk groups [21].

This study was conducted according to the ethical standards following the Declaration of Helsinki. Participants provided written informed consent. All participants completed a questionnaire with information on age, sex, weight, height, tobacco and alcohol consumption, physical activity, nutrition, and therapy. The Ethic Committee of University Clinical Hospital Center "Zvezdara" approved the study protocol.

Statistical analysis

Complete statistical analyses were performed using SPSS software, version 18.0 (IBM, Armonk, New York, USA). We have performed post hoc study power calculation, regarding Klotho protein values obtained in this current study in the two related groups (before and after dialysis session). This analysis revealed the study power of 0.8, which is the minimal condition for the study validity. We have used the program G*Power version 3.1.9.4 University Kiel, Germany for the study power calculation. For a distribution normality check, we used the Kolmogorov–Smirnov test. Continuous variables with normal distributions were expressed as mean ± standard deviation; these variables were compared with the ANOVA test. Categorical variables were reported as numbers (percentages). Data with non-normal distribution were expressed as median and interquartile range (IQR, 25–75th percentile values). These variables were compared using the Mann–Whitney *U* test or Kruskal–Wallis test, according to the number of groups. To compare paired means of variables we used Wilcoxon's paired test. Factorial principal component analysis (PCA) was performed to decrease the number of variables into an adequate number of factors compressed according to its variance level similarity. Binary logistic regression analysis followed the PCA and using the PCA-derived scores, it was possible to find the significant predictors of mortality score. All probabilities were two tailed and the level of statistical significance was defined as $p < 0.05$.

Results

The baseline clinical, socio-demographic, and biochemical characteristics of HD patients are summarized in Table 1.

The median age of the patients was 66 years and the majority of them were male (75.4%). Average Kt/V was above 1.4, which is the cutoff value for efficient HD treatment [1].

The redox status parameters bHD and aHD are listed in Table 2.

Table 1 Demographic, clinical characteristics, and laboratory data of the examined patients

Parameter	All patients ($N=130$)
Age (years)	66 (54–72)
Gender (male/female) *n* (%)	98/32 (75.4/24.6)
BMI (kg/m^2)	25.7 ± 4.7
Smokers, *n* (%)	37 (28)
Primary renal diagnosis	
Hypertension *n* (%)	56 (43.1)
Glomerulonephritis *n* (%)	14 (10.8)
Diabetes mellitus *n* (%)	30 (23.1)
Polycystic kidney disease *n* (%)	14 (10.8)
Other n (%)	16 (12.3)
Dialysis duration (months)	40.0 (20.0–91.0)
Kt/V	1.48 (1.37–1.67)
PTH (pg /mL)	263 (135–488)
Creatinine (µmol/L)	882 (749–1032)
Urea (µmol/L)	23.4 (20.9–27.5)
Albumin (g/L)	39 (37–41)
Calcium (mmol/L)	2.3 (2.2–2.4)
Phosphate (mmol/L)	1.7 (1.3–1.9)
Leukocyte count (10^9/L)	6.5 (5.3–8.1)
Hemoglobin (g/L)	107 (98–113)

Values are represented as mean ± SD when data follow a normal distribution or as median and interquartile range (IQR) when data follow a non-Gaussian distribution

BMI body mass index, *PTH* parathyroid hormone, *Kt/V* number used to quantify hemodialysis treatment adequacy

Table 2 Redox status parameters, Klotho protein, and TL before and after a hemodialysis session

Parameter	Before HD	After HD	*p*
AOPP, µmol/L	48.2 (30.4–69.6)	76.7 (60.2–90.3)	< 0.001
PAB, U/L	46.4 (38.9–57.0)	69.4 (55.7–80.5)	< 0.001
MDA, µmol/L	1.53 (1.19–2.23)	1.28 (1.04–1.50)	0.921
IMA, ABSU	0.10 (0.07–1.4)	0.12 (0.09–1.85)	< 0.001
$O_2^{\cdot-}$, µmol NBT/min/L	27 (18–38)	26.5 (19–35.5)	0.994
SOD,U/L	136 (129–142)	151 (141–158)	< 0.001
SHG, mmol/L	0.26 (0.22–0.33)	0.41 (0.31–0.56)	< 0.001
Klotho protein pg/mL	64.2 (25.5–119.8)	68.2 (22.6–152.9)	0.027
TL, T/S	0.92 (0.70–1.14)	0.95 (0.69–1.23)	0.209

Data presented as median and IQR; *p* from Wilcoxon's paired test

HD hemodialysis, *AOPP* advanced oxidation protein products, *PAB* prooxidant–antioxidant balance, *MDA* malondialdehyde, *IMA* ischemia-modified albumin, *ABSU* absorbance units, O_2^- superoxide anion, *SOD* superoxide dismutase, *SHG* sulfhydryl groups, *TL* telomere length in peripheral blood mononuclear cells

We found a significant increase in the levels of several redox status parameters aHD. Prooxidative species AOPP and IMA, as well as PAB were significantly increased aHD ($p<0.001$). We did not find a difference in the MDA concentration bHD and aHD ($p=0.921$). Total SHG content and SOD activity were significantly increased after HD ($p<0.001$). Klotho concentration was also significantly higher after HD ($p=0.027$). The observed increase in TL did not reach statistical significance ($p=0.209$).

Using Floege's risk point calculator to estimate all-cause mortality risk over the next 2 years, we divided the HD patients into low- (1), intermediate- (2) and high- (3) risk groups (Table 3).

Patients with the highest MRS scores bHD had significantly higher PAB compared with low and intermediate MRS patients ($p<0.01$). We found significantly lower superoxide anion aHD in patients with the highest MRS scores in comparison to patients with the lowest MRS values ($p<0.01$). Also, there have been significantly lower values of SHG content aHD and albumin level in HD patients with the highest MRS scores compared to HD patients with the lowest MRS.

PCA was applied to clinical/demographic and redox status parameters bHD (AOPP, IMA, $O_2^{\cdot-}$, MDA, SHG, and SOD; Klotho protein and TL) as disease determinants and possible emerging disease-risk parameters, respectively, to obtain a smaller number of factors which could be important predictors of high mortality risk score (Table 4). Kaiser–Meyer–Olkin parameter (> 0.500) and Bartlett's tests ($p<0.001$) confirmed this model's adequacy. Five significant factors emerged in this analysis, accounting for 65.4% of the total variance. The first factor referred to as "prooxidative factor" explained 16.3% of total variance. The distribution of other factor effects was as follows: "Protein-related redox factor" (15.6%), "HD-related factor" (13.9%), "antioxidative factor" (10.2%), and "redox balance-Klotho-related factor" (9.4%).

PCA analysis provided the scores which presented numerical values of every extracted factor. We used these scores as new variables for multivariate binary logistic regression analysis. Our results showed that significant predictors of high mortality score (low *vs.* high tertile of MS score) was the fifth factor consisting of PAB and Klotho ($p=0.014$). The detailed results of this analysis are shown in Table 5.

Discussion

Our study examined the link between the level of secreted Klotho, TL, redox status parameters, and cardiovascular outcome prediction in HD patients. By simultaneously analyzing routine laboratory parameters and emerging

Table 3 Comparison of different MRS levels according to demographic, clinical and laboratory parameters

Parameter	Low risk	Intermediate risk	High risk	*p*
MRS	1.0 (− 0.5 to 2.0)	5.0 (4.0–6.0)	9.0 (7.5–11.0)	
N	56	34	40	
Age (years)	54.5 (50.0–61.5)	67.5 (58–72.6)[aaa]	72.5 (70.0–79.0) [aaa]	< 0.001
Gender (m/f) *n* (%)	42/14 (42.9/43.8)	23/11 (23.5/34.4)	33/7 (33.7/21.9)	0.334
Smoking status	21/35 (56.8/37.69)	9/25 (24.3/26.9)	7/33 (18.9/35.5)	0.097
BMI (kg/m^2)	25.47 ± 4.12	26.06 ± 5.05	25.66 ± 5.11	0.846
Dialysis duration (months)	34.5 (12.0–82.0)	43.5 (27.0–115.0)	43.0 (19.0–85.0)	0.495
Klotho bHD, pg/mL	64.6 (30.7–114.6)	77.1 (22.6–121.2)	54.6 (21.1–158.8)	0.898
Klotho aHD, pg/mL	71.2 (26.3–176.4)	47.6 (19.6–126.4)	78.5 (31.4–147.0)	0.528
TL bHD, T/S	0.92 (0.71–1.15)	0.94 (0.77–1.10)	0.87 (0.66–1.14)	0.585
TL aHD, T/S	0.99 (0.78–1.25)	0.83 (0.66–1.17)	1.09 (0.73–1.29)	0.132
PAB bHD, U/L	43.3 (37.7–48.3)	50.9 (44.6–58.8)[a]	53.4 (39.9–66.5)[aa]	< 0.01
PAB aHD, U/L	63.5 (51.9–77.4)	71.1 (64.6–78.6)	74.8 (5.4–87.9)	0.138
AOPP bHD, µmol/L	47.6 (29.4–76.2)	48.9 (33.5–68.5)	48.3 (30.2–64.4)	0.875
AOPP aHD, µmol/L	77.1 (60.6–90.9)	75.6 (60.6–83.0)	78.6 (60.4–91.6)	0.746
MDA bHD, µmol/L	1.44 (1.19–2.17)	1.53 (1.15–2.04)	1.68 (1.44–2.43)	0.338
MDA aHD, µmol/L	1.63 (1.00–18.40)	1.37 (0.88–22.27)	1.56 (0.98–26.41)	0.471
IMA bHD, ABSU	0.107 (0.073–0.150)	0.109 (0.072–0.139)	0.083 (0.069–0.117)[a]	0.062
IMA aHD, ABSU	0.133 (0.093–0.274)	0.109 (0.093–0.134)	0.116 (0.095–0.146)	0.072
$O_2^{\cdot-}$ bHD	29.0 (18.0–38.5)	25.0 (19.0–32.0)	26.0 (17.0–38.0)	0.673
$O_2^{\cdot-}$ aHD	32.0 (25.5–42.0)	22.0 (16.5–28.0)[aaa]	22.0 (16.0–29.0)[aaa]	< 0.001
SHG bHD, mmol/L	0.268 (0.232–0.378)	0.255 (0.219–0.320)	0.243 (0.195–0.300)[a]	0.067
SHG aHD, mmol/L	0.465 (0.360–0.643)	0.393 (0.291–0.494)	0.360 (0.277–0.517)[a]	< 0.01
Total protein, mmol/L	69 (66–72)	67 (66–73)	68 (63–72)	0.611
Albumin, mmol/L	40 (38–42)	39 (36–41)	38 (34–40)[aaa]	< 0.01

p from the Kruskal–Wallis test, followed by Mann–Whitney *U* test

MRS mortality risk score, *bHD* before hemodialysis, *aHD* after hemodialysis, *TL* telomere length in peripheral blood mononuclear cells, *PAB* prooxidant–antioxidant balance, *AOPP* advanced oxidation protein products, MDA malondialdehyde, *IMA* ischemia-modified albumin, *ABSU* absorbance units, O_2^- superoxide anion, *SHG* sulfhydryl groups

[a,aa,aaa] $p < 0.05$, 0.01, 0.001 vs. low-risk group, respectively

Table 4 The factors extracted by PCA

Factors	Included variables with loadings	Factor variability (%)
Prooxidative factor	MDA (0.844) $O_2^{\cdot-}$(0.753) AOPP (− 0.699)	16.3
Protein-related redox factor	IMA (0.851) SHG (0.835)	15.6
Hemodialysis related factor	Kt/V (0.816) Gender (0.655) Hemodialysis duration (0.644)	13.9
Antioxidative factor	TL (0.792) SOD (0.653)	10.2
Redox balance-Klotho-related factor	PAB (0.853) Klotho (− 0.528)	9.4

Values of redox status parameters are before dialysis treatment

MDA malondialdehyde, $O_2^{\cdot-}$ superoxide anion, *AOPP* advanced oxidation protein products, *SHG* sulfhydryl groups, *IMA* ischemia-modified albumin, *Kt/V* number used to quantify hemodialysis treatment adequacy, *TL* telomere length in peripheral blood mononuclear cells, *SOD* superoxide dismutase, *PAB* prooxidant–antioxidant balance

Table 5 Univariate logistic regression analysis of mortality score predictors

Factors	B (SE)	Wald coefficient	OR (95% CI)	*p*
Prooxidative factor	− 0.193 (0.271)	0.507	0.824 (0.485–1.403)	0.477
Protein-related redox factor	− 0.479 (0.327)	2.149	0.619 (0.326–1.175)	0.143
Hemodialysis related factor	− 0.046 (0.248)	0.035	0.955 (0.588–1.551)	0.851
Antioxidative-telomere factor	0.057 (0.249)	0.052	1.059 (0.649–1.726)	0.820
Redox balance-Klotho-related factor	0.923 (0.377)	6.007	2.52 (1.20–5.27)	0.014

OR odds ratio, *CI* confidence interval

markers of oxidative stress and biological aging we found a combination of parameters for earlier categorization of HD patients who are at higher mortality risk.

Disturbed prooxidant and antioxidant balance develops in early stages of CKD and is exacerbated as the disease progresses to ESRD [22]. This sequence of adverse events is evident in the kidneys as they are an important source of antioxidants. However, uremic toxin accumulation, which promotes reactive oxygen species (ROS) production resulting in a vicious circle of inflammation and oxidative stress, abrogates the normal protective effect of kidney function. Further redox disbalance is caused by inadequate clearance of prooxidative substances, lower production of antioxidants and the elimination of the latter during HD [23]. Analysis of redox status parameters bHD and aHD showed a significant negative impact of HD on oxidative stress burden with increased levels of oxidative stress markers and decreased antioxidant defense [24, 25]. In our study, AOPP and PAB, as prooxidative species, were significantly increased aHD which is in accordance with results from Alamdary and coworkers. It was probably due to the HD process itself [24]. These are in contrast to findings that oxidation markers diminished aHD, which was considered as a favorable effect of HD on plasma aminothiols [25]. Thus, HD effects on oxidative stress remain controversial. In addition, previous studies have reported that the type of dialysis membrane used during hemodialysis procedure may play a significant role in OS production. Our results regarding dialysis membrane type showed inconsistent change of several redox status parameters, but did not influence Klotho neither TL values. Certainly, this important phenomenon should be a subject of separate study with higher number of subjects per each membrane type group [26]. Along with the prooxidants increase aHD, we noticed increases in both SOD activity and SHG, probably caused by compensatory mechanism activation, governed by nuclear factor erythroid 2-related factor 2 (NRF2) activity towards the antioxidant response element (ARE), whose activation in turn increases expression of several antioxidant enzymes/proteins genes [27]. The N-terminal site of albumin is very susceptible to biochemical modification, induced by oxidative stress, which leads to formation of modified albumin termed ischemia-modified albumin. Similar to the study of Kiyici and coworkers, we found significantly higher values of IMA aHD. This observation may be ascribed to the low concentration of albumin, thus lower protective potency of this plasma protein moiety together with oxidative stress exacerbation induced by the HD process leading to IMA (aHD) elevation [28].

Bearing in mind that kidneys participate in the production, uptake, and elimination of circulating Klotho, it is reasonable to assume that such processes are damaged and inadequate in patients with any kidney disease, but especially in ESRD [29]. Our findings of higher soluble Klotho aHD disagree with the results of Hibab and coworkers who found an elevated level of soluble Klotho bHD. This may be explained by different study designs, lower number, and different characteristics of HD patients [30].

It is well known that telomeres undergo shortening with age and in conditions associated with oxidative stress and inflammation. ESRD patients suffer from chronic inflammation, oxidative stress and uremic toxicity as a consequence of kidney failure. These conditions along with the effect of HD could explain patients telomere attrition, as seen by others [31, 32] and also in our study. When comparing the TL between ESRD patients and healthy controls from a previous study we noticed significantly shorter TL in patients 0.92 (0.70–1.14) *vs.* healthy controls 1.329 (1.096–1.624), data not shown [33]. Stefanidis and coworkers found no difference in TL in HD patients compared to healthy controls, but they confirmed TL shortening after years on HD [34]. In addition, some authors found reduced TL in HD patients in comparison to healthy controls [35]. Both of the above studies included a relatively small number of HD patients. Since previous studies by Vukasinovic and coworkers showed a significant reduction in TL after primary percutaneous coronary intervention immediately after a heart attack, we expected HD will also affect TL. A slight increase aHD was found but was not statistically significant in comparison with bHD value [33].

Although advances in both HD technology and patient care during the last two decades have been achieved, there is only a minor increase in the survival of ESRD patients, while mortality still remains high. Data from the United States Renal Data System (USRDS) annual report show the markedly foreshortened projected lifespan for patients

with ESRD relative to those without. In individuals aged 40–44 years, for example, there is a > 25-year projected lifespan difference between males receiving HD (For males receiving HD, life expectancy is 11.1 years, and for males in the general population, life expectancy is 36.5 years). The difference for females is even higher [36]. Several models for predicting mortality rate have been developed for HD patients, but they are not in routine clinical use due to a lack of robustness, generalizability, and applicability. In our study, we used Floege's risk point calculator to stratify patients into low-, intermediate-, and high-mortality risk groups.

PCA and subsequent logistic regression analysis enabled the selection of significant predictors of mortality. Initial analysis group-tested variables in 5 factors: prooxidative, protein related, HD related, antioxidative and redox balance-Klotho related. The prooxidative factor included MDA, $O_2^{\cdot -}$, and AOPP which unequivocally confirmed connection between ROS ($O_2^{\cdot -}$) and oxidatively modified lipid such as protein biomolecules whose derivatives are end products of lipid peroxidation and protein products of advanced oxidation. Similar analysis was conducted by Antolini and coworkers and their results showed that ROS were involved in protein molecules modification [37]. PCA extracted AGEs, carbonyls, and AOPP as one factor, connected by the same level of variation. Protein-related redox factor contained SHG content and IMA, which are both protein products and their lower value could be explained by the significantly lower albumin value in the highest MRS tertile subgroup [38]. Free SHG /free thiols are considered as the main plasma antioxidants in humans, playing very important physiological roles as part of the antioxidant defense system. SHG represents a reflection of a favorable redox status [39, 40]. Bearing in mind the long-term nature of renal disease, it was expected that PCA analysis could confirm not only the same variability in Kt/V and duration of HD, but also gender distribution with males dominating in our group of patients. The antioxidant–telomere factor extracted by PCA also confirmed the connection between the antioxidative enzyme SOD and TL.

PCA analysis revealed the connection between redox balance (measured through the prooxidative factor PAB) and soluble Klotho. Importantly, it demonstrated significant prediction of a high mortality score in a group of HD patients. The direct influence of oxidative stress on diminishing Klotho concentration in CKD was seen by Podkowinska and coworkers [41]. A key link between diminishing Klotho concentration and increased oxidative stress markers (8-hydroxy-2-deoxyguanosine, MDA and 8-isoprostanoid) was noted [41]. Oh and coworkers explained an inverse relationship between Klotho concentration and oxidative stress markers in peritoneal dialysis patients as having a role to alleviate inflammation burden as one of the main characteristics of progressive renal disease [42]. Additionally, in our study, logistic regression revealed a fifth factor (redox balance-Klotho-related factor) as a significant predictor of high mortality risk. This confirmed Klotho's significance in basic ESRD mechanisms. Some study limitations should be mentioned. First, we only had a relatively small sample size, so that future studies with greater number of patients would be needed to further expand our observations. Also, various underlying diseases (e.g., diabetes mellitus or glomerulonephritis) that caused kidney failure could have influenced our results. Finally, all patients received some treatment (e.g., ACE inhibitors, diuretics, corticosteroids hypoglycemic drugs, and erythropoietin) which could also influence the results.

Our current study focused on the link between routine laboratory parameters and the new emerging markers of oxidative stress and biological aging. The search for a combination of parameters for earlier recognition of HD patients who are at higher mortality risk was our key goal. We conclude that lowered Klotho along with TL attrition and oxidative stress increases with decreased antioxidative defense potential which together represent the clinical characteristic of HD patients that could be connected with higher mortality rate.

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Data availability The data supporting this study's findings are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study has complied with all the relevant national regulations, institutional policies and that listed in the Helsinki Declaration of Ethical Principles for Medical Research. The study was approved by the Ethic Committee of "Zvezdara" University Clinical Hospital Center on 22.01.2021.

Informed consent Informed consent was obtained from all individuals included in this study.

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