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ORIGINAL ARTICLE

Klotho and lean mass as novel cardiovascular risk factors in hemodialysis patients

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ABSTRACT

Background. Patients with chronic kidney disease (CKD) present a higher risk of cardiovascular (CV) morbidity and mortality compared with the general population. While there are several well-established traditional CV risk factors, few studies have addressed novel potential risk factors such as α-Klotho, asymmetric dimethylarginine (ADMA) and lean mass.

Methods. This was an observational, prospective, single-center, cohort study that included prevalent hemodialysis (online hemodiafiltration) adult patients. By univariate logistic regression models, univariate and multivariate Cox proportional hazards models, and Kaplan–Meier analysis, we evaluated the association between the levels of α-Klotho, ADMA and lean mass, with the risk of peripheral vascular disease (PVD), CV events and all-cause mortality in these patients.

Results. A total of 200 HD patients was included. We found that increased levels of log-α-Klotho were significantly associated with decreased odds of both PVD [odds ratio (OR) 0.521, 95% confidence interval (CI) 0.270–0.954, $P = .034$] and CV events (OR 0.415, 95% CI 0.203–0.790, $P = .01$), whereas increased levels of log-ADMA were only significantly associated with increased odds of PVD (OR 13.482, 95% CI 5.055–41.606, $P < .001$). We also found that the levels of log-α-Klotho (HR 0.357, 95% CI 0.140–0.906, $P < .05$) and lean mass (HR 0.187, 95% CI 0.042–0.829, $P < .05$), but not log-ADMA, were significantly associated with the risk of all-cause mortality, even after adjusting for possible confounding variables.

Conclusions. Novel long-term clinical associations were generated that support α-Klotho and lean mass as novel CV risk factors in hemodialysis patients.

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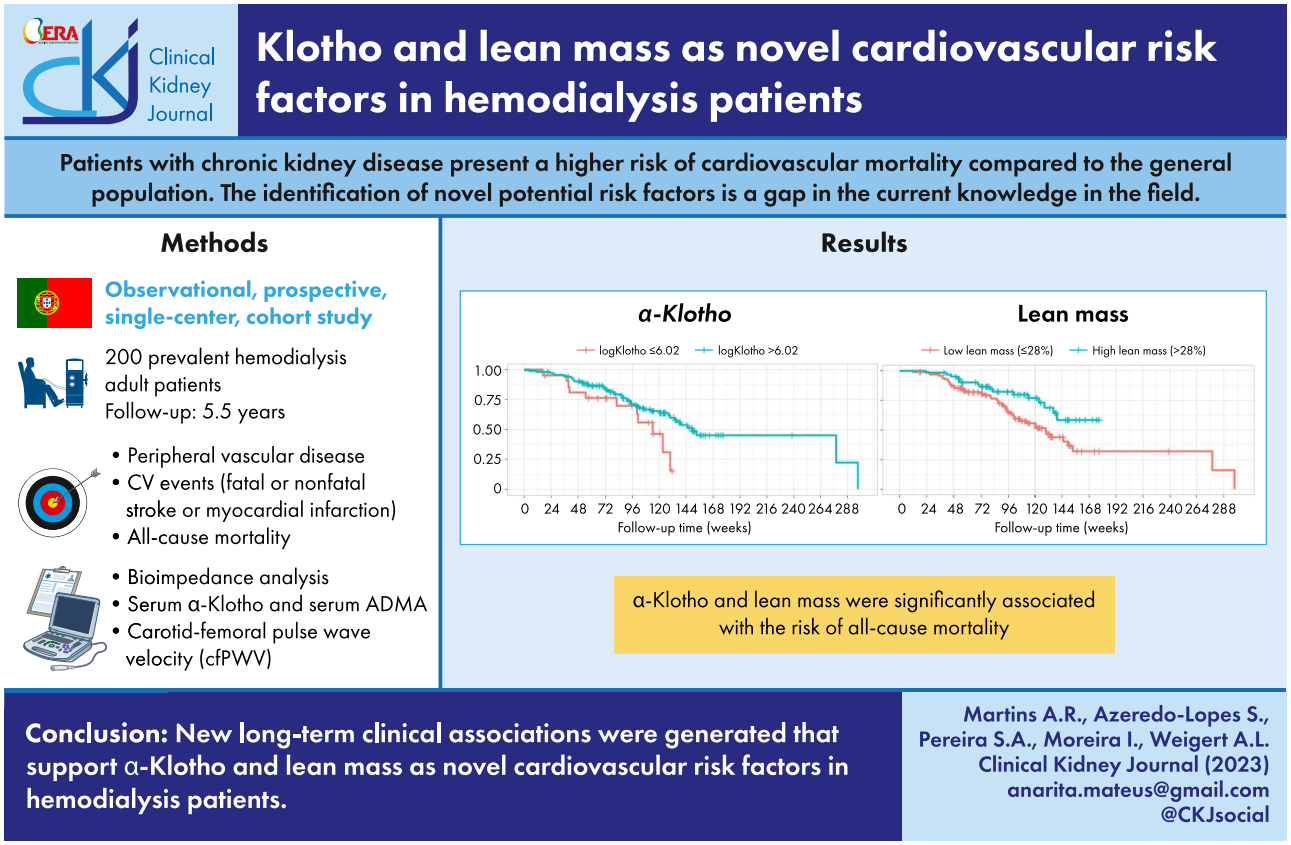
GRAPHICAL ABSTRACT



Keywords: ADMA, bioimpedance, biomarkers, mortality, peripheral vascular disease

INTRODUCTION

Chronic kidney disease (CKD) is a global public health priority associated with a high global burden and affecting 843.6 million individuals worldwide, of whom 3.9 million individuals require renal replacement therapy [1]. It is well established that patients with CKD and end-stage renal disease present a higher risk of cardiovascular (CV) morbidity and mortality, compared with the general population [2–4].

The traditional CV risk factors include age, diabetes mellitus (DM) and smoking [5], whereas specific CV risk factors including oxidative stress, chronic inflammation, CKD–mineral bone disorder, malnutrition, increased levels of fibroblast growth factor-23 (FGF23) and decreased levels of Klotho, and uremic toxins, were identified in hemodialysis (HD) patients [4].

The *α-Klotho* gene encodes a transmembrane protein that was originally implicated in the regulation of aging [6]. Since then, evidence supports that the circulating soluble-α-Klotho (α-Klotho) which results from the proteolytic cleavage of α-Klotho presents several biological functions, including modulation of endothelial nitric oxide synthesis and maintenance of endothelial integrity [7–9]. Furthermore, decreased levels of α-Klotho were reported in HD patients and have been associated with the risk of CV events [7, 10, 11], CV mortality [7, 10] and all-cause mortality [11, 12]. However, the association between the levels of α-Klotho and mortality remains unclear, as several studies failed to demonstrate a significant association between the levels of α-Klotho and all-cause mortality [7, 13, 14].

The uremic toxin asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase, inhibiting the production of nitric oxide, and playing a key role in endothelial disfunction, atherosclerosis progression and CV disease [8, 15]. Increased levels of ADMA were reported in HD patients [16, 17] and evidence supports that ADMA is implicated in the progression of CKD [18]. Furthermore, in HD patients, increased ADMA levels were associated with CV disease [19], the risk of CV events [20, 21] and all-cause mortality [20, 22].

Contrary to the general population, HD patients studies support a protective effect of a higher body mass index (BMI)—thought to reflect overweight or obesity—a phenomenon called the "obesity paradox" [23, 24]. Nevertheless, BMI is not an appropriate indicator of obesity, as it does not distinguish fat mass and muscle mass [23–27]. In fact, studies support that fat mass and lean mass are strong predictors of the outcomes of HD patients and that low fat mass [23, 27] and low lean mass [24, 27, 28] are associated with a worse survival in this population. Therefore, it remains unclear whether the protective effect of a higher BMI is due to lean mass or fat mass [24] and data are still scarce regarding the effect of lean mass in the risk of all-cause mortality in HD patients. Furthermore, there are still few studies addressing the association of the levels of α-Klotho, ADMA and lean mass

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with long-term CV outcomes and all-cause mortality in these patients.

With this study we aimed to evaluate the association between the levels of serum α-Klotho, serum ADMA and lean mass, and the risk of peripheral vascular disease (PVD), CV events and all-cause mortality in a cohort of prevalent HD patients.

MATERIALS AND METHODS

Study design and participants

This was an observational, prospective, single-center, cohort study that included prevalent HD patients. All patients signed an informed consent form prior to their voluntary inclusion in the study. Patients were recruited from November 2013 to April 2014 and followed until end of 2020 in an outpatient dialysis center in Óbidos, Portugal.

Patients >18 years old, with a duration of dialysis >6 months, and presenting a stable condition defined as the absence of an infectious condition or an active neoplasm, were included. Exclusion criteria comprised serious CV disease (cardiac function level at III or IV by New York Heart Association standards), hospitalizations or infectious diseases in the 3 months prior to study inclusion, severe malnutrition, active liver diseases or advanced cancer. None of the patients was under evaluation for kidney and pancreas transplantation, or for live donor transplantation.

All enrolled patients underwent online hemodiafiltration, three times per week and 4 h per dialysis session. All patients used standard bicarbonate dialysis fluid, dialysate flow was 500 mL/min, and blood flow ranged from 350 to 500 mL/min. Dialysis liquid ingredients were as follows: sodium 138–140 mmol/L, potassium 2.5–3.0 mmol/L, calcium 1.25 or 1.75 mmol/L, and magnesium 0.5 mmol/L.

This study was approved by the competent ethics committee from the North Lisbon Hospital Center/Faculty of Medicine from University from Lisbon (approval number 128/14) and was conducted according to the Declaration of Helsinki and Good Clinical Practice guidelines.

Clinical and demographic assessments

Baseline evaluation included medical history and physical examination. The following data were also collected: age at the beginning of the study; gender; dialysis vintage, defined as the length of time on dialysis between the start of any type of dialysis and the end of the study; death; transplantation, or transference to other dialysis clinics; follow-up time after the inclusion in the study; cause of primary kidney disease; comorbidities; and risk factors including smoking, uncontrolled hypertension, congestive heart failure, ischemic cardiomyopathy, the presence of DM, obesity (defined as BMI of ≥30 kg/m^2) and overweight (defined as BMI 25.0–29.9 kg/m^2).

In this prospective study design, each of the defined outcomes was related to the baseline value of the selected biomarkers, i.e. each patient was his/her own control. Therefore, circulating levels of α-Klotho and ADMA were quantified and body lean mass was assessed at the study baseline.

All patients performed bioimpedance analysis to estimate body composition by using the multi-frequency bioelectrical impedance analyzer, InBody S10 device (Direct Segmental Multi-Frequency Bioelectrical Impedance Analysis Method—DSM-BIA, Korea), according to the manufacturer's guidelines.

Serum α-Klotho and serum ADMA were measured by enzyme-linked immunosorbent assay (ELISA) kit (Immuno-Biological Laboratories Co., Ltd, Tokyo, Japan and Cusabio Technology LLC, Houston, USA, respectively), according to the manufacturer's protocol. Calibration curves for both serum Klotho and ADMA were calculated for a pilot sample according to the manufacturer's instructions.

Carotid-femoral pulse wave velocity (cfPWV) was measured in all patients using applanation tonometry (SphygmoCor, AtCor Medical, Sydney, Australia), in the carotid artery and in the femoral artery. All cfPWV assessments were performed at baseline before the mid-week HD session.

Outcome measurements

The primary endpoints were: (i) the presence of PVD (systemic atherosclerosis proven by clinical history, physical findings or abnormal ankle-brachial index); (ii) the occurrence of CV events defined as fatal or nonfatal stroke or myocardial infarction; and (iii) all-cause mortality.

Statistical analysis

Categorical variables are presented as absolute (*n*) and relative frequencies (%), continuous normally distributed data are expressed as mean (standard deviation), and continuous nonnormally distributed data are expressed as median and 1st quartile–3rd quartile. Normality of distributions was assessed using the Shapiro–Wilk test. Data regarding the levels of α-Klotho and ADMA were logarithmically transformed (natural logarithm) to avoid their skewed distribution and consequently facilitate the statistical analyses.

Univariate logistic regression models were adjusted to assess the association between the levels of α-Klotho and ADMA and the occurrence of PVD and CV events and were expressed as odds ratio (OR) with the corresponding 95% confidence intervals (CI).

To study the impact of the levels of α-Klotho, ADMA and lean mass on the cumulative survival probability of HD patients, survival curves were estimated with the Kaplan–Meier method, stratifying patients by log-α-Klotho ≤6.02 (low α-Klotho) and >6.02 (high α-Klotho), log-ADMA ≤0.15 (low ADMA) and >0.15 (high ADMA), and lean mass ≤28% (low lean mass) and >28% (high lean mass). These optimal cutoffs were attained using the maximally selected rank statistics that take into account the multiple testing issue [29, 30]. This test determines the optimal cutpoint for one or multiple continuous variables at once, using the maximally selected rank statistics from the *maxstat* R® package [31–33]. This is an outcome-oriented methods providing a value of a cutpoint that correspond to the most significant relation with outcome (here, survival times—time on dialysis, event-death). This interesting alternative to receiver operating characteristic curves provided by maximally selected rank statistics can be easily applied using R (maxstat package) with several advantages [31].

Comparisons between groups of patients were performed using the log-rank test. Univariate Cox proportional hazards models were additionally used to evaluate the association between α-Klotho, ADMA and lean mass with all-cause mortality. Multivariable Cox proportional hazards models with an interaction term were also applied adjusting for age and DM.

The proportion hazard assumption of Cox regression models was verified using the Schoenfeld residuals. To be in accordance with the objectives of the study, the HRs for the multivariable Cox model were estimated and interpreted at specific levels of the main effects and the interaction term. In these models,

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Table 1: Demographic and clinical characteristics of the cohort of HD patients at baseline ($n = 200$).

Characteristic	HD patients ($n = 200$)
Age, mean (SD), years	67.23 (13.53)
Sex, *n* (%)	
Female	73 (36.9)
Male	125 (63.1)
Dialysis vintage ($n = 198$), mean (SD), months	93.92 (52.90)
Follow-up time ($n = 198$), median (Q1–Q3), months	65.66 (32.90–78.60)
Renal failure etiology ($n = 146$)	
Diabetic nephropathy	33 (22.6)
Hypertensive nephrosclerosis	29 (19.9)
Chronic glomerulonephritis	17 (11.6)
Cardiorenal	15 (10.3)
Other causes	52 (35.6)
Smokers, *n* (%)	16 (8)
Hypertension ($n = 147$), *n* (%)	34 (23.1)
Congestive heart failure ($n = 189$), *n* (%)	51 (27.0)
Ischemic cardiomyopathy, ($n = 165$), *n* (%)	30 (18.2)
DM ($n = 198$), *n* (%)	76 (38.4)
Dry weight ($n = 122$), mean (SD), kg	70.19 (13.28)
BMI ($n = 144$), mean (SD), kg/m^2	26.72 (4.68)
Lean mass ($n = 160$), mean (SD), %	27.11 (5.87)
Low lean mass, *n* (%)	97 (60.6)
High lean mass, *n* (%)	63 (39.4)
Fat mass ($n = 160$), mean (SD), %	27.23 (11.71)
cfPWV ($n = 188$), mean (SD), m/s	15.38 (6.56)
α-Klotho ($n = 145$), mean (SD), pg/mL	783.25 (497.33)
Low α-Klotho, *n* (%)	22 (15.2)
High α-Klotho, *n* (%)	123 (84.8)
ADMA ($n = 145$), median (Q1–Q3), μmol/L	0.83 (0.65–1.21)
Low ADMA, *n* (%)	70 (48.3)
High ADMA, *n* (%)	75 (51.7)

n, number of patients; Q1, 1st quartile; Q3, 3rd quartile; SD, standard deviation.

the statistical significance of the estimated HRs of the interaction terms was assessed by 95% CI. The standard errors of the HR estimates, used to obtain the CIs, employed values from the variance–covariance matrix of the corresponding model fits [29].

Statistical significance was considered at $P < .05$ (two-tailed). All statistical analyses were performed using the R® statistical software [34], including the *survival* R® package [35, 36] to perform Cox proportional hazards models and the *survminer* package to obtain the survival curves [29].

RESULTS

Baseline characteristics of study population

This cohort consisted of 200 patients with a mean age of 67 (14) years, of whom 36.9% were female. The mean dialysis vintage was 94 (53) months, and the median follow-up time of patients was 66 (33–79) months.

Regarding lean mass, α-Klotho and ADMA levels, 60.6% of patients presented low levels of lean mass (≤28.0%), 15.2% of patients presented low levels of α-Klotho (logα-Klotho ≤6.02) and 51.7% of patients presented high levels of ADMA (log ADMA >0.15 μmol/L) (Table 1).

PVD occurred in 34.5% of the overall patients, in 50.0% of patients with low log-α-Klotho, in 51.4% of patients with high log-ADMA and in 32.6% of patients with low lean mass. CV events occurred in 30.3% of all the patients in the study, in 50.0% of patients with low log-α-Klotho, in 30.1% with low log-ADMA and in 31.2% of patients with low lean mass (Table 2). Ninety-two (46.5%) of the patients died during the follow-up, of which 59.8% were due to CV causes (data not shown).

Association of log-α-Klotho and log-ADMA with PVD and CV events

We observed a statistically significant association between age, the presence of DM, ischemic cardiomyopathy, cfPWV, α-Klotho, ADMA and the presence of PVD. Therefore, we found that age, α-Klotho and ADMA were significantly associated with the occurrence of PVD, with increased age being associated with an increase in the odds of PVD (OR 1.034, 95% CI 1.006–1.065, $P = .022$). Also, the increasing levels of log-α-Klotho was associated with a decrease of 48% in the odds of PVD (OR 0.521, 95% CI 0.270–0.954, $P = .034$), and the increasing levels of log-ADMA were associated with an increase of around 13.5 times in the odds of PVD (OR 13.482, 95% CI 5.055–41.606, $P < .001$).

Regarding CV events, increasing levels of log-α-Klotho were associated with a decrease of 58% in the odds of the occurrence of CV events (OR 0.415, 95% CI 0.203–0.790, $P = .01$). No association was found between the levels of log-ADMA and the odds of CV events ($P = .787$) (Table 3).

Association of log-α-Klotho, log-ADMA and lean mass with all-cause mortality

When stratifying patients by low (≤6.02) and high (>6.02) log-α-Klotho levels, a tendency for a lower survival probability ($P = .051$) (Fig. 1A) and a tendency for an increased risk of all-cause mortality were found for patients with low log-α-Klotho levels compared with patients with high log-α-Klotho levels (HR 1.933, 95% CI 0.985–3.790, $P = .055$).

When stratifying patients by low (≤0.15) and high (>0.15) log-ADMA levels, a tendency for a higher survival probability (log-rank test, $P = .065$) (Fig. 1B), and a tendency for a decreased risk of all-cause mortality were found for patients with low log-ADMA levels compared with patients with high log-α-Klotho levels (HR 0.603, 95% CI 0.350–1.040, $P = .068$).

When stratifying patients by low (≤28%) and high (>28%) lean mass values, significant differences were found in their survival probability (log-rank test, $P = .014$) (Fig. 1C). Moreover, patients with low lean mass (>28%) presented a risk of all-cause mortality 2.021 times higher when compared with patients with high lean mass (≤28%) (HR 2.021, 95% CI 1.140–3.590, $P = .016$).

Also, through the univariate analysis, the traditional CV risk factors age and presence of DM and heart disease and cfPWV were also significantly associated with the risk of all-cause mortality (Table 4). This was not the case for fat mass or arterial hypertension.

Regarding the multiple Cox proportional hazard model with the interaction term between log-α-Klotho and lean mass, after adjusting for age, DM and log-ADMA, the levels of log-α-Klotho and lean mass were significantly associated with the risk of all-cause mortality (Table 5). Therefore, when considering patients with high values of log-α-Klotho (>6.02), the risk of all-cause mortality among those with high lean mass (>28%) was estimated to decrease around 64% when compared with patients with lean mass ≤28%, assuming age, DM and log-ADMA are held constant (HR 0.357, 95% CI 0.140–0.906).

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Table 2: Absolute and relative frequencies of study endpoints in the total cohort of HD patients and stratified by low and high levels of log-α-Klotho (≤6.02 vs >6.02), log-ADMA (≤ 0.15 vs >0.15) and lean mass (≤28% vs >28%).

		Log-α-Klotho		Log-ADMA		Lean mass	
Study endpoint	Total population, *n* (%)	Low, *n* (%)	High, *n* (%)	Low, *n* (%)	High, *n* (%)	Low, *n* (%)	High, *n* (%)
PVD (*n* = 194)	67 (34.5)	11 (50.0)	39 (31.7)	14 (18.7)	36 (51.4)	31 (32.6)	19 (30.6)
CV events (*n* = 195)	59 (30.3)	11 (50.0)	31 (25.6)	2 2(30.1)	20 (28.6)	30 (31.2)	17 (27)
All-cause mortality (*n* = 198)	92 (46.5)	11 (50.0)	46 (37.4)	24 (32)	33 (47.1)	45 (46.4)	16 (25.4)

Table 3: Univariate analysis for demographic and clinical characteristics of the cohort of HD patients according to the occurrence of PVD and CV events.

	PVD				CV events			
Characteristic	No	Yes	OR (95% CI)	*P*-value[a]	No	Yes	OR (95% CI)	*P*-value[a]
Age, mean (SD), years	65.31 (14.2)	70.8 (11.4)	1.034 (1.006–1.065)	.022	66.7 (14)	68.1 (12.5)	1.008 (0.982–1.037)	.560
Sex, *n* (%)								
Female	46 (63.9)	26 (36.1)	1 [Reference]		55 (77.5)	16 (22.5)	1 [Reference]	
Male	81 (66.4)	41 (33.6)	0.896 (0.488–1.658)	.720	81 (65.3)	43 (34.7)	1.825 (0.949–3.636)	.078
Hypertension (*n* = 146), *n* (%)								
No	75 (67)	37 (33)	1 [Reference]		84 (74.3)	29 (25.7)	1 [Reference]	
Yes	19 (55.9)	15 (44.1)	1.600 (0.750–3.502)	.239	21 (61.8)	13 (38.2)	1.793 (0.785–4.012)	.160
Congestive heart failure (*n* = 186), *n* (%)								
No	95 (69.3)	42 (30.7)	1 [Reference]		105 (76.1)	33 (23.9)	1 [Reference]	
Yes	28 (57.1)	21 (42.9)	1.696 (0.861, 3.322)	.120	27 (52.9)	24 (47.1)	2.828 (1.441,5.580)	.003
DM (*n* = 194), *n* (%)								
No	88 (73.3)	32 (26.7)	1 [Reference]		88 (73.3)	32 (26.7)	1 [Reference]	
Yes	39 (52.7)	35 (47.3)	2.468 (1.345–4.569)	.003	48 (64)	27 (36)	1.547 (0.829–2.883)	.170
Ischemic cardiomyopathy (*n* = 165), *n* (%)								
No	97 (72.4)	37 (27.6)	1 [Reference]		116 (85.9)	19 (14.1)	1 [Reference]	
Yes	12 (40.0)	18 (60.0)	3.932 (1.746–9.157	.001	1 (3.3)	29 (96.7)		<.001
cfPWV (*n* = 187), m/s	12.90 (4.26)	19.50 (7.16)	1.285 (1.187–1.410)	<.001	14.6 (6.58)	16.9 (6.35)	1.051 (1.004–1.104)	.037
Log-α-Klotho (*n* = 145), pg/mL	6.580 (0.598)	6.370 (0.517)	0.521 (0.270–0.954)	.034	6.590 (0.557)	6.300 (0.589)	0.415 (0.203–0.790)	.010
ADMA (*n* = 145), μml/L	0.83 (0.34)	1.410 (1.096)	13.482 (5.055–41.606)	<.001	1.04(0.83)	1.01(0.54)	0.931 (0.485–1.495)	.787
Lean mass (*n* = 157), *n* (%)	27.40 (6.012)	26.6 (5.61)	0.978 (0.919–1.036)	.470	27.40 (6.17)	26.40 (5.11)	0.968 (0.908–1.028)	.310
Fat mass (*n* = 157), *n* (%)	27.22 (10.95)	27.00 (13.33)	0.998 (0.970–1.028)	.912	26.70 (11.20)	28.50 (12.90)	1.013 (0.984–1.044)	.385

[a]Univariate logistic regression model.
SD, standard deviation.

Similarly, when considering patients with high lean mass (>28%), the risk of all-cause mortality among those with high levels of log-α-Klotho (>6.02) was estimated to decrease 81% when compared with patients with low log-α-Klotho (≤6.02), assuming age, DM and log-ADMA are held constant (HR 0.187, 95% CI 0.042–0.829).

Again, on multivariate analyses, the traditional CV risk factors age (HR 1.055, 95% CI 1.015–1.100, *P* = .007) and DM (HR 3.476, 95% CI 1.618–7.470, *P* = .001) were significantly associated with the risk of all-cause mortality (Table 5).

DISCUSSION

In this study, we aimed to evaluate the association between the occurrence of PVD, CV events and all-cause mortality with the levels of serum α-Klotho, serum ADMA and lean mass in a cohort of prevalent HD patients. By univariate logistic regression analyses we have found that increased levels of α-Klotho were significantly associated with decreased odds of both PVD and CV events, whereas increased levels of ADMA were only significantly associated with increased odds of PVD. Furthermore, using Kaplan–Meier and Cox regression analysis, we have found that the levels of α-Klotho and lean mass but not ADMA were significantly associated with the risk of all-cause mortality, even after adjusting for the possible confounding variables age and DM. Therefore, we have found that lower levels of α-Klotho and lean mass were associated with an increased risk of all-cause mortality in HD patients.

Comparing our data with those reported for healthy volunteers also by ELISA quantification, the circulating levels of ADMA were higher than the range proposed in a systematic review and meta-analysis: 0.25 (0.18–0.31) to 0.92 (0.76–1.09) with a mean of 0.57 (0.48–0.66) (*n* = 1435) (μmol/L with 95% CI) [37].

The circulating levels of α-Klotho were lower than that reported for a cohort of 91 healthy adults aged 55–85 years, at 612 (236–919) pg/mL [38]. In our work the cutoff for low serum α-Klotho of 412 pg/mL was obtained by maximally selected rank statistics test, using death as the event. A total of 15% of patients have α-Klotho above this level, this was somehow expected as is the cut-off maximally selected to be related to mortality. A recent systematic review and meta-analysis supports that lower circulating soluble α-Klotho is associated with 88% increased risk of all-cause mortality in CKD patients [39]. Herein, six articles fulfill the eligibility criteria, of which four were on HD cohorts, with variable mean follow-up times. For instance, Otani-Takei's team (2015) in a Japanese cohort with 63 patients defined low α-Klotho as below 309 pg/mL that represented the lowest quartile [12]. In 2018 in China, the mean value from 128 patients was 455.34 pg/mL, which was used to define low α-Klotho [40]. One year later, two European studies in Spain (30 patients, 369 pg/mL [41]) and in Greece (79 patients 745 pg/mL [7]) used median α-Klotho levels as cutoff values. Therefore, the comparison of the percentage of individuals with low α-Klotho obtained

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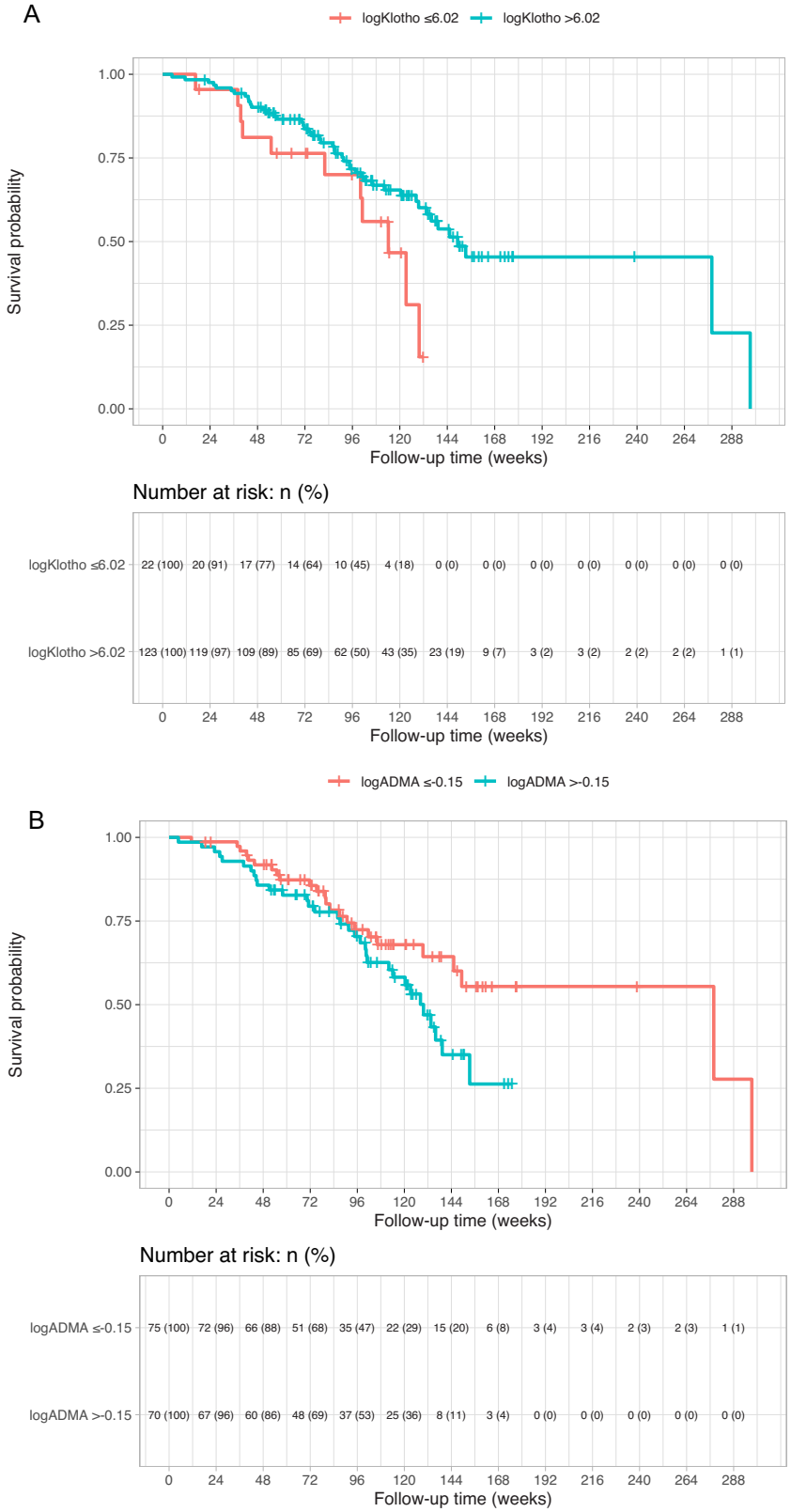

Figure 1: Kaplan–Meier estimates of the survival probabilities of HD patients with (**A**) high (blue line) and low (red line) log-α-Klotho levels (log-rank test, $P = .051$), (**B**) low (red line) and high (blue line) ADMA levels (log-rank test, $P = .065$) and (**C**) high (blue line) and low (red line) lean mass values (log-rank test, $P = .014$).

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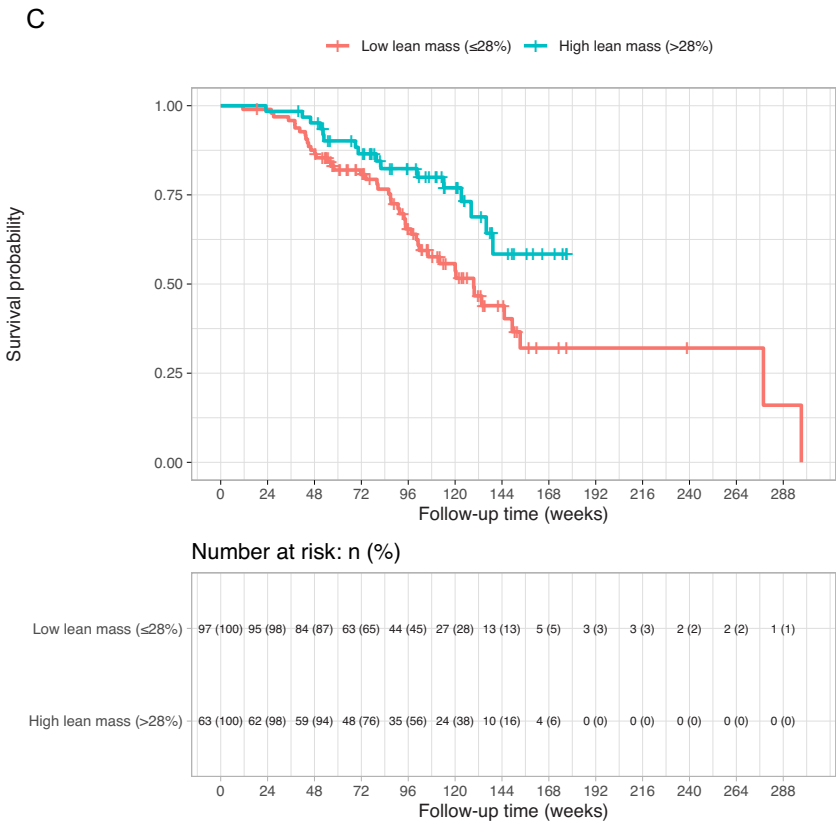

Figure 1: Continued

Table 4: Estimates of HR, corresponding 95% CI and *P*-values obtained from univariate Cox proportional hazard regression models for the association between the levels of log-α-Klotho, log-ADMA and lean mass and the risk of all-cause mortality in HD patients.

	Univariate	
Characteristic	HR (95% CI)	*P*-value[a]
Age	1.048 (1.030–1.070)	<.001
DM		
Absence	[Reference]	
Presence	2.129 (1.390–3.270)	<.001
Congestive heart failure		
No	[Reference]	
Yes	1.970 (1.270–3.050)	.002
Ischemic cardiomyopathy		
No	[Reference]	.002
Yes	1.970 (1.270–3.050)	
cfPWV	1.05 (1.02–1.08)	<.001
Log-α-Klotho		
Low	1.933 (0.985–3.790)	.055
High	[Reference]	
Log-ADMA		
Low	0.603 (0.350–1.040)	.068
High	[Reference]	
Lean mass		
Low	2.021 (1.140–3.590)	.016
High	[Reference]	

[a]Univariate Cox proportional hazards models.

Table 5: HR and respective 95% CIs for mortality, based on the multivariable Cox proportion hazard regression model with the interaction term between log-α-Klotho and lean mass.

	Univariate	
Characteristic	HR (95% CI)	*P*-value[a]
Age	1.055 (1.015–1.100)	.007
DM		
Absence	[Reference]	
Presence	3.476 (1.618–7.470)	.001
Log-ADMA		
Low	0.648 (0.313–1.340)	.243
High	[Reference]	
High log-α-Klotho*lean mass		
High lean mass	0.357 (0.140–0.906)	[a]
Low lean mass	[Reference]	
Log α-Klotho*high lean mass		
High log-α-Klotho	0.187 (0.042–0.829)	[a]
Low log-α-Klotho	[Reference]	

[a]P < .05.

in our work with other cohorts is made difficult by the difference cutoff definitions used. Unlike us, these studies did not evaluate the cutoff that best relates to the event, but rather the mean/median value or lowest quartile value.

Decreased levels of α-Klotho were previously associated with the risk of CV events [7, 10, 11], CV mortality [7, 10] and all-cause mortality in HD patients [11, 12]. However, conflicting results

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were also reported as several studies failed to show a significant association between the levels of α-Klotho and all-cause mortality in these patients [7, 13, 14]. Nevertheless, our study presented a higher follow-up time compared with the study from Nowak *et al.*, and a higher number of participants compared with the studies from Memmos *et al.* and Sági *et al.* Furthermore, in a recent systematic review with meta-analysis including eight prospective studies with 992 patients on maintenance HD, the authors confirmed the prognostic value of α-Klotho, reporting that a reduction in the levels of α-Klotho was associated with an increase in both CV events and all-cause mortality [11].

Important biomarkers of mineral and bone metabolism, and which are indissociable from α-Klotho, such as serum phosphate, calcium, vitamin D and FGF23 and the clusters/phenotypes of individuals that can arise from a comprehensive integration of this biomarkers, are relevant to pursue in the near future. However, the pertinence of investigating α-Klotho is also justified by the increased knowledge of α-Klotho mechanisms of action, such as those that are FGF23-independent and beyond bone metabolism [42, 43], as well as the controversies about α-Klotho in HD [44] which justify the clinical unmet need of generating more data.

On univariate analysis, we found that increased levels of ADMA were associated with increased odds of PVD. ADMA levels were reported to correlate with the presence of CV disease, which supports our results [19]. However, in our study we did not find a significant association between ADMA levels and CV events. As ADMA levels are biomarkers of endothelial dysfunction and atherosclerosis [8, 15], an association between ADMA and CV events was also expected. In fact, ADMA was reported as a strong independent predictor of fatal and non-fatal CV events, in which higher levels of ADMA were associated with an increased risk for these events [20]. Similar results were reported by Kumagai *et al.* [21]. Nevertheless, the lack of a significant correlation between ADMA levels and cardiac and coronary calcifications was also reported [45], which could partially explain our results. Furthermore, taking these results into account and given that higher ADMA levels are associated with a higher risk of cardiovascular outcomes in these patients [23], the conflicting results may be explained by the fact that mean ADMA levels of our sample (0.83 μmol/L) may not be sufficient to observe an association with endothelial dysfunction and CV events.

We also failed to show a significant association between ADMA levels and all-cause mortality, contrary to what has been previously reported [20, 22, 45]. Therefore, the conflicting results may be explained by the low number of events in our study, or by the different follow-up times included between studies.

We also found that higher levels of lean mass were significantly associated with an increased survival probability of HD patients and with a decreased risk of all-cause mortality both in univariate analysis and after adjusting for age and DM, similar to what was previously reported [28, 24]. However, despite showing a significant association between reduced lean body mass and an increased risk of CV mortality on multivariate analyses, Kakiya *et al.* failed to demonstrate this association regarding all-cause mortality [46]. Furthermore, the authors have reported fat mass index as a significant predictor of all-cause mortality but not of CV disease mortality [46]. In fact, other studies have reported fat mass instead of lean mass [23] or both [27] as significantly associated with the risk of all-cause mortality in HD patients. Nevertheless, in our study, we have followed HD patients for a longer period, which could partially explain the different results obtained.

Our study presented key strengths, namely the high number of HD patients included, the long follow-up time (around 5.5 years) and the simultaneous evaluation of the association of the levels of α-Klotho, ADMA and lean mass with the risk of all-cause mortality in HD patients. However, only HD patients were included, the study lacked a control group, it was a single-center study, and the recruited cohort lacks ethnic diversity, and there is evidence that lean body mass differs across race and ethnicity [24], limiting the generalizability of the study findings. In addition to the limitations listed above, the levels of α-Klotho, ADMA and lean mass were evaluated at baseline. The knowledge of "ideal" trajectory of biomarkers and lean body mass would have provided important additional information. Also, some subgroups of patients were of a reduced size, which may have limited the power for statistical analyses. Another general issue for the implementation of α-Klotho as a biomarker is the technical need to improve α-Klotho assay performance, the results of which might vary according to assay used [44].

In conclusion, we found that α-Klotho was associated with the occurrence of both PVD and CV events, while ADMA levels were only associated with the occurrence of PVD. Furthermore, α-Klotho and lean mass levels, but not ADMA levels, were independently associated with the long-term survival of HD patients. Our results may have therapeutic implications, as new clinical strategies targeting α-Klotho, lean mass and, to a lower extent, ADMA could be implemented in the management of HD patients.

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AUTHORS' CONTRIBUTIONS

A.R.M., A.L.W. and I.M. were involved in study design. A.R.M. and I.M. were involved in data collection. A.R.M. and S.A.-L. and S.A.P. were involved in data analysis and interpretation. A.R.M. and S.A.P. were involved in drafting of the manuscript. All authors were involved in the critical revision of the manuscript and provided intellectual content of critical importance to the described study. All authors approved the final version of the manuscript and take responsibility for the accuracy or integrity of any part of the work.

DATA AVAILABILITY STATEMENT

All data relevant to the study are included in the article. Further information can be obtained from the corresponding author.

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CONFLICT OF INTEREST STATEMENT

A.R.M. has received consulting fees from Viforpharma and Amgen and payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events from Viforpharma. A.R.M. also received support for attending meetings and/or travel from Viphorfarma and MSD and had a leadership or fiduciary role in the Portuguese Nephrology Society. A.L.W. has received consulting fees and payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events from Alexion, Astellas, AstraZeneca and Takeda. A.L.W. participated on a Data Safety Monitoring Board or Advisory Board of Alexion, Astellas, AstraZeneca and Takeda. All the other authors declare no conflicts of interest.

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