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Listă 10 publicaţii relevante

Candidat pentru obţinerea atestatului de abilitare în Domeniul Medicină:

RUSU CRINA CLAUDIA

1. Rusu CC, Kacso I, Moldovan D, Potra A, Tirinescu D, Ticala M, Maslyennikov Y, Urs A, Bondor CI. Exploring the Associations Between Inflammatory Biomarkers, Survival, and Cardiovascular Events in Hemodialysis Patients and the Interrelationship with Nutritional Parameters—The Experience of a Single Transylvanian Dialysis Center. Journal of Clinical Medicine. 2025; 14(4):1139. https://doi.org/10.3390/jcm14041139 (ISI Q1 FI= 3) Pag.3

2. Rusu CC, Kacso I, Moldovan D, Potra A, Tirinescu D, Ticala M, Orasan R, Budurea C, Anton F, Valea A, Bondor CI, Carsote M. Leptin Is Associated with Testosterone, Nutritional Markers, and Vascular Muscular Dysfunction in Chronic Kidney Disease. Int J Mol Sci. 2024; 25(14):7646. doi: 10.3390/ijms25147646. (Q1 ISI FI=4,9) Pag. 20

3. Rusu CC, Anton F, Valea A, Bondor CI. N-Terminal Pro-Brain Natriuretic Peptide Correlates with Ghrelin and Acyl-Ghrelin in Pre-Dialysis Chronic Kidney Disease. International Journal of Molecular Sciences. 2024 23;25(11):5696.https://doi.org/10.3390/ijms25115696 (ISI Q1 FI=4,9) Pag. 37

4. Rusu CC, Kacso I, Moldovan D, Potra A, Tirinescu D, Ticala M, Rotar AM, Orasan R, Budurea C, Barar A, Anton F. Triiodothyronine and Protein Malnutrition Could Influence Pulse Wave Velocity in Pre-Dialysis Chronic Kidney Disease Patients. Diagnostics. 2023 Jul 24;13(14):2462. https://doi.org/10.3390/diagnostics13142462; (ISI Q1 IF=3) Pag. 50

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Exploring the Associations Between Inflammatory Biomarkers, Survival, and Cardiovascular Events in Hemodialysis Patients and the Interrelationship with Nutritional Parameters—The Experience of a Single Transylvanian Dialysis Center

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Abstract: Background/Objectives: In hemodialysis (HD), inflammatory biomarkers are discussed as prognostic markers for survival and cardiovascular events (CVEs). The results of the studies are not uniform and there are particularities related to population groups and comorbidities. In addition, it is known that inflammation determines protein malnutrition and less about the effect of adipose tissue on inflammation in HD. This study investigates the relationship between inflammatory molecules and nutritional biomarkers, and CVE and survival in HD patients. **Methods:** We included, in an observational, longitudinal study, 65 patients with chronic HD (53 without diabetes and 22 smokers), with a mean age of 60.1 ± 12.4 years. High-sensitivity C-reactive protein (hs-CRP), interleukin 1 beta, tumor necrosis factor alpha (TNF-alpha), interleukin 6, soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK), soluble CD163 (sCD163), and fibroblast growth factor 21 were determined. We recorded survival and cardiovascular events for 60 months. Univariate and multivariate analyses were performed. **Results:** Hs-CRP was significantly associated with survival ($p = 0.014$) in the total group. In smokers and former smokers, TNF-α lower than 368.34 pg/mL was associated with better survival. In multivariate analysis, hs-CRP was correlated with adipose tissue biomarkers ($p = 0.006$), and sCD163 was correlated with total and LDL cholesterol ($p = 0.002$). In addition, in univariate analysis, sTWEAK was correlated with serum albumin ($p = 0.026$, $r = -0.30$). **In conclusion**, in HD patients, hs-CRP was significantly associated with survival, and low TNF-alpha values in smokers and former smokers were linked to better survival. Hs-CRP was also correlated with adipose tissue biomarkers, CD163 was correlated with total and LDL cholesterol, and albumin was inversely associated with sTWEAK. The relation between inflammatory molecules and adipose tissue biomarkers was less identified in HD patients until now.

Keywords: kidney disease; diagnosis; biomarkers; nutrition; survival; cardiovascular events; hemodialysis

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1. Introduction

In patients on chronic hemodialysis (HD), the elevated risk of morbidity and mortality from cardiovascular disease (CVD) can be attributed to both traditional and non-traditional

CKD-specific risk factors. Among the non-traditional factors, malnutrition, inflammation, and atherosclerosis (ATS) are components of the malnutrition–inflammation–atherosclerosis (MIA) syndrome [1–3]. In HD patients, protein malnutrition is frequently observed and is associated with increased morbidity and mortality [4,5]. Inflammation in CKD is a protective physiological response [6], but it can also become maladaptive, uncontrolled, and persistent over time [7]. As CKD progresses, patients have an increasingly inflammatory state [8]. In chronic HD patients, inflammation can occur secondarily to dialysis membranes, central venous catheters, oxidative stress, cellular aging, hypoxia, fluid overload, sodium overload, immune dysfunction, intestinal dysbiosis, and retention of uremic toxins [9,10]. Inflammatory molecules are produced by immunocompetent cells and cardiomyocytes, endothelial cells, adipocytes, and vascular smooth muscle cells [11,12]. Numerous inflammatory markers have been evaluated for their impact on CV pathology and survival in patients with chronic HD, including molecules such as high-sensitivity C-reactive protein (hs-CRP), tumor necrosis factor alpha (TNF-α), and interleukin 6 (IL-6). In contrast, interleukin 1 beta (IL-1β), the weak inducer of apoptosis similar to soluble tumor necrosis factor (sTWEAK), soluble CD163 (sCD163), and fibroblast growth factor 21 (FGF 21) have been less studied. IL-1β, IL-6, TNF-α, and hs-CRP are hallmarks of low-grade inflammation in CKD [13]. TNF-α regulates adipose tissue metabolism [12,14], and IL-6 stimulates the hepatic synthesis of acute-phase proteins, including CRP [15]. sTWEAK, a member of the TNF family, circulates in plasma as a soluble form [16], can induce smooth muscle cell proliferation in the arterial wall [17], and is involved in all pathogenetic phases of ATS [18]. Like sTWEAK, CD163 is a transmembrane protein with a soluble variant that can be measured in serum [19]. sTWEAK can bind and block sCD163 [20]. sCD163 levels are increased in obese and hypertensive patients [21,22] and are directly correlated to the severity of ATS in CKD [21,22]. The liver and adipose tissue secrete fibroblast growth factor 21 (FGF21), an endocrine-like cytokine [23]. Elevated FGF21 levels have been associated with the development of major acute cardiovascular events [24,25]. The cytokines in HD patients can predict survival rates and cardiovascular events. However, the predictive ability of different competing molecules has been less frequently evaluated [9,26–30] and may vary depending on population characteristics and comorbidities. In addition, a few studies focusing on hemodialysis patients have examined the relationship between inflammatory mediators and nutritional status in a dual manner: which mediators are related to adipose tissue and which are associated with muscle mass. Furthermore, exploring the correlations between different inflammatory molecules may provide insights into the pathogenetic cascades involved in CVD in HD patients.

This study aims to evaluate the relationship between inflammatory molecules (hs-CRP, IL-6, TNF-α, IL-1β, sCD163, sTWEAK, and FGF21) and cardiovascular events and survival in chronic hemodialysis patients within a specific sample from a single center. In addition, we will examine the relationship between these inflammatory mediators, including novel ones, and nutritional status, assessed by anthropometric and laboratory parameters. We will also explore the connections between different inflammatory molecules.

2. Materials and Methods

We performed a comprehensive observational study on a cohort of HD patients randomly selected from a chronic dialysis center in Cluj-Napoca. We included patients with prevalent HD and a minimum age of 18 years, with at least 6 months of HD maintenance treatment (HD duration), and without residual renal function (thus, residual renal function cannot be discussed as a factor influencing nutrition, respectively, in the inflammation markers of the studied patients). Exclusion criteria were acute inflammation, terminal neoplasia, previous renal transplantation, immunosuppressive treatment, and active hepatitis.

We registered demographic data, HD duration, and comorbidities (diabetes, hypertension, CVD, hepatitis B or C infection, smoking status, and medication) from their medical records. Additionally, we recorded clinical data: age, weight, height, systolic blood pressure (SBP) and diastolic blood pressure (DBP) (pre-dialysis values), history of CVD, waist circumference (WC) (cm), and triceps skinfold thickness (TST) (mm). Pulse pressure was calculated with the following formula: (PP): PP = SBP − DBP (mmHg).

To evaluate body mass index (BMI), we used the formula BMI = weight (kg)/height^2 (m^2). The other anthropometric parameters were measured by bioimpedance using the Body Composition Monitor, a certified device (manufactured by Fresenius Medical Care, Bad Homburg, Germany) that provided body composition as follows: lean tissue mass (LTM) (kg) and adipose tissue mass (ATM) (kg) [31].

We followed patients prospectively for 60 months or until death or transplantation. Throughout this time, we documented general mortality, fatal cardiovascular events (myocardial infarction, congestive heart failure, stroke, and sudden death), and nonfatal cardiovascular events every six months. According to other authors, the term stroke can be replaced by cerebrovascular insult [32]. We calculated the survival time (ST) as the interval between the study entry and death and the time to a cardiovascular event (TCVE) as the interval between the study entry and the first recorded cardiovascular event.

2.1. Laboratory Parameters

All biochemical analyses were performed after an overnight fast between 7.00 and 9.00 a.m., always during a midweek non-dialysis day. Current measurements at the initiation of this study include serum electrolytes, albumin, creatinine, uric acid, iron profile (iron, transferrin, and ferritin), lipid profile (total cholesterol, triglycerides (TGs), and HDL cholesterol), hs-CRP, alkaline phosphatase, intact parathormone (iPTH), and transaminases. Pre-dialysis and post-dialysis urea levels were used to calculate Kt/V. Serum calcium was corrected (cCa) for albumin according to the following formula: cCa (mg/dL) = serum calcium (mg/dL) + 0.8 × [4.0-serum albumin (g/dL)]. Hepatitis virus B and C detection was performed by electrochemiluminescence for HBs antigen (HBs Ag) and hepatitis C virus antibodies (HCV Ab). We already determined Serum IL-6, IL-1β, TNF-α, sTWEAK, sCD163, and FGF21 with an enzyme-linked immunosorbent assay (ELISA) using commercially available kits (R&D System, Minneapolis, MN, USA). The minimum detection limit for TNF-α was 15.6 pg/mL, for IL-6—3.2 pg/mL, for IL-1β—10.2 pg/mL, and for sTWEAK—10 pg/mL, and the intra- and inter-assay coefficients of variation were 7.9% and 9.1%, respectively; for sCD163, the minimum detectable level was 0.613 ng/mL, and the intra- and inter-assay coefficients of variation were 5.1 and 3.5%, respectively, while for FGF21, the detection limit was 7 pg/mL and the intra- and inter-assay coefficients of variation were 8 and 8.7%.

2.2. Dialysis Prescription

All included patients received conventional HD treatment three times a week, 4–5 h per session (only 3 patients had 5 h/session, the rest, 4 h/session), and were dialyzed with disposable synthetic dialyzers (polysulfone) and heparin as a standard anticoagulant. A nephrologist guided the dialysis prescription, achieving a Kt/V value $\geq$ 1.4. Erythropoietin was administered according to a standardized algorithm. Antihypertensive treatment was indicated for persistent post-dialysis or inter-dialysis blood pressure > 150/90 mmHg, and ultrafiltration during HD was performed based on dry weight.

2.3. Statistical Analysis

Numerical characteristics were summarized as mean ± standard deviation or median (25th–75th percentile) according to the normal and non-normal distribution, and qualitative

characteristics were expressed as numbers and percentages. Pearson's or Spearman correlation coefficient was used for analysis if correlations between two continuous variables were present. The inflammatory markers were analyzed in the multivariate regression models adjusted for age and dialysis duration for the total group. Collinearity was evaluated using the Variance Inflation Factor (VIF). For those variables with high VIFs, a dimension reduction was made with factor analysis. The resulting composite variables were introduced in the multivariate linear model as an independent variable.

The univariate Cox proportional hazards regression analysis examined the relationships between different independent factors and survival time without an event. For the inflammatory markers that significantly impacted survival time in Cox proportional hazards regression analysis, we also evaluated their effect on survival using Kaplan–Meier analysis. The quantitative variables were transformed into dichotomous variables using a rounded cut-off value. The cut-off values were found with the receiving operating characteristics curve, using the maximum sensitivity and specificity. The calculation of cumulative survival probabilities was plotted using the Kaplan–Meier method, and the curves were compared using the log-rank test. Significant variables significant in the univariate analysis were included in a multivariate Cox proportional hazards regression model (Enter). The models were adjusted for age and duration of dialysis. The hazard ratio (HR) and their 95% confidence intervals (CIs) were calculated. Separate multivariate Cox proportional hazards regression models were analyzed with all the inflammatory markers as independent variables.

For the analysis in this study, SPSS version 25.0 was used [33]

2.4. Ethical Issues

All patients provided written informed consent. The study was conducted according to the Declaration of Helsinki and approved by the Ethics Committee of "Iuliu Hatieganu" University of Medicine and Pharmacy Cluj-Napoca 348/26 September 2017.

3. Results

3.1. Total Group Description

We enrolled 65 randomly selected patients who met the inclusion and exclusion criteria. Two (3.1%) patients were excluded due to missing data regarding their transfer to other hospitals; 63 remained in the study, 53 did not have diabetes, and 22 were current and former smokers. The mean follow-up period was 51.81 ± 15.77 months. The number of patients who died in the follow-up period was 17 [26.2%; 95% CI (16; 37.9)], and 24 [36.9%; 95% CI (26.1; 50)] patients had CVE.

The clinical and laboratory patients' characteristics at the time of inclusion in the study are shown in Table 1. In the study, we had a small number of patients with diabetes mellitus (15.4%), with an average age of 60.78 ± 11.68 years, 63% male.

Table 1. Baseline characteristics of hemodialysis patients.

Parameter	Total Group (n = 63)
Age (years)	60.78 ± 11.68
Male, n (%)	36 (63)
Current and former smokers, n (%)	22 (34.9)
Diabetes mellitus, n (%)	10 (15.4)
Hypertension, n (%)	40 (63.5)
SBP (mmHg)	142.27 ± 19.99
DBP (mmHg)	76 (70; 80)

Table 1. *Cont.*

Parameter	Total Group (n = 63)
PP (mmHg)	67.19 ± 17.37
Body mass index (kg/m^2)	27.98 (23.94; 30.93)
Waist circumference (cm)	97.81 ± 14.56
Triceps skinfold thickness (mm)	3 (2.5; 4)
LTM (kg)	30.66 (25.52; 36.26)
ATM (kg)	41.64 ± 16.3
Dialysis duration (months)	68 (29.5; 90.5)
Kt/V	1.56 (1.39; 1.74)
LDL-cholesterol (mg/dL)	100.57 ± 38.21
Total cholesterol (mg/dL)	169 (149.5; 196.5)
HDL-cholesterol (mg/dL)	41 (31.6; 48.34)
Fasting glucose (mg/dL)	94 (88.1; 113)
Corrected Calcium (mg/dL)	8.77 (8.29; 9.18)
Phosphorus (mg/dL)	4.87 (4.09; 5.93)
Alkaline phosphatase (UI/L)	74.5 (54.78; 94.44)
iPTH (pg/mL)	300 (166.85; 789.75)
Serum albumin (g/L)	3.93 ± 0.24
Hemoglobin (g/dL)	11.39 ± 1.12
White blood cells (n/mm^3)	6330 (5415; 7315)
hs-C reactive protein (mg/dL)	0.61 (0.25; 1.23)
TNF-α (pg/mL)	288.53 (241.91; 359.83)
IL-6 (pg/mL)	275.04 (234.58; 348.34)
IL-1β (pg/mL)	48.29 (12.72; 252.31)
FGF 21 (pg/mL)	23.34 (19.75; 37.62)
sTWEAK (pg/mL)	3738.59 (3346.57; 4721.51)
sCD163 (ng/mL)	1070 (620; 1500)

Mean ± standard deviation; median (25th–75th percentile); SBP—systolic blood pressure, DBP—diastolic blood pressure, PP—pulse pressure; LTM—lean tissue mass; ATM—adipose tissue mass; iPTH—intact parathormone; hs-C reactive protein—high-sensitivity C reactive protein; TNF-α—tumor necrosis factor alfa; IL-6—interleukin 6; IL-1β—interleukin 1 beta; FGF 21—fibroblast growth factor 21; sTWEAK—soluble tumor necrosis factor-like weak inducer of apoptosis; sCD163—soluble CD163.

3.2. Correlations Between Inflammatory Markers and Nutritional and Lipid Parameters

The correlations between inflammatory markers and nutritional and lipid parameters are presented in Table 2.

We found significant correlations between the following inflammatory mediators: hs-CRP with sCD163, sTWEAK with FGF 21, sTWEAK with TNF-α, and TNF-α with IL-6 (Table 2). In addition, we found significant correlations between the following inflammatory mediators and markers of lipid metabolism: WBC with triglycerides, sCD163 with triglycerides, FGF21 with triglycerides, sCD163 with Total cholesterol, and sCD163 with LDL cholesterol (Table 2).

Of the total group, in multivariate linear regression, hs-CRP remained significantly statistically correlated with WBC total ($p = 0.034$) and with the composite variable from BMI and ATM ($p = 0.006$) after adjusting with age and diabetes duration. WBC remained significantly statistically correlated with hs-CRP ($p < 0.001$) and TG ($p = 0.035$). When sCD163 was analyzed in multivariate linear regression, LDL cholesterol and total cholesterol were highly associated and were reduced to a composite variable, which remained statistically significantly associated with sCD163 in multivariate regression ($p = 0.002$). TNF-α remained significantly statistically correlated with IL-6 ($p = 0.005$).

Table 2. Correlations between inflammatory biomarkers and nutritional and lipid parameters in the study group and subgroups divided by gender, smoking status, and diabetes.

Inflammatory Marker	Nutritional/ Inflammatory/ Lipid Markers	Spearman/Pearson Coefficient of Correlation r	*p*
hs-CRP (total group)	ATM	0.34	0.007
hs-CRP (nondiabetic group)	ATM	0.31	0.026
hs-CRP (nonsmoking subgroup)	ATM	0.4	0.004
hs-CRP (nonsmoking subgroup)	WC	0.33	0.016
hs-CRP (total group)	BMI	0.26	0.046
hs-CRP (diabetic subgroup)	BMI	0.64	0.048
WBC (total group)	ATM	0.29	0.021
WBC (nondiabetic group)	ATM	0.29	0.041
WBC (total group)	WC	0.26	0.043
WBC (smoking subgroup)	Pre-dialysis creatinine	0.84	0.002
sCD163 (total group)	TST	0.48	0.048
sCD163 (nonsmoking subgroup)	TST	0.34	0.023
sTWEAK (total group)	Serum albumin	−0.30	0.026
sTWEAK (nondiabetic group)	Serum albumin	−0.39	0.007
sTWEAK (smoking subgroup)	WC	−0.68	0.043
IL-1β (diabetic subgroup)	LTM	−0.75	0.03
IL-1β (nondiabetic group)	Serum albumin	0.032	0.02
FGF21 (nonsmoking subgroup)	LTM	0.34	0.023
hs-CRP (total group)	sCD163	0.45	0.001
hs-CRP (total group)	WBC	0.55	<0.001
sTWEAK (total group)	FGF 21	0.37	0.006
sTWEAK (total group)	TNF-α	0.35	0.01
TNF-α (total group)	IL-6	0.35	0.004
WBC (total group)	Triglycerides	0.26	0.037
sCD163 (total group)	Triglycerides	0.28	0.038
sCD163 (total group)	Total Cholesterol	−0.31	0.022
sCD163 (total group)	LDL-cholesterol	−0.43	0.001
FGF 21 (total group)	Triglycerides	0.29	0.035

LTM—lean tissue mass; ATM—adipose tissue mass; TST—Triceps skinfold thickness; BMI—body mass index; WC—waist circumference; WBC—white blood cells; hs-CRP—high-sensitivity C reactive protein; TNF-α—tumor necrosis factor alfa; IL-6—interleukin 6; IL-1β—interleukin 1 beta; FGF 21—fibroblast growth factor 21; sTWEAK—soluble tumor necrosis factor-like weak inducer of apoptosis; sCD163—soluble CD163.

3.3. Survival Time and Time to the First CVE on Total Group

Table 3 presents the effect of inflammatory molecules on survival time and on time to a cardiovascular event (TCVE) resulting from the univariate Cox proportional hazards regression analysis. Diabetes mellitus influences the time to CVE (TCVE); hs-CRP, iPTH, and calcium were other factors affecting survival time. Diastolic blood pressure was a protective factor that influenced time without CVE.

We analyzed the independent factors' relationship with *survival time* in a multivariate Cox proportional hazards regression analysis. All the variables significant in the univariate Cox proportional hazards regression analysis, calcium, IPTH, and hs-CRP, were taken in the model and adjusted with age, kt/v, and dialysis duration. The hs-CRP [HR = 1.48, 95% CI (1.0, 1.99), p = 0.010] and calcium [HR = 2.83, 95% CI (1.30, 6.16), p = 0.009] remained significant in predicting survival time. For the *time to CVE*, the variables that remained significant in the model were diabetes mellitus [HR = 4.47, 95% CI (1.19, 16.72), p = 0.026], smoking (current and former) [HR = 4.88, 95% CI (1.80, 13.23), p = 0.002], and DBP [HR = 0.94, 95% CI (0.89, 0.98), p = 0.010].

Table 3. Factors influencing survival time and time to the first CVE (TCVE).

Parameter	Total Survival Time (n = 63)		TCVE (n = 63)	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Dialysis duration (months)	1.002 (0.99; 1.01)	0.646	1 (0.99; 1.01)	0.733
Age (years)	1.02 (0.98; 1.07)	0.307	1.03 (0.99; 1.07)	0.136
Male	1.27 (0.46; 3.48)	0.649	0.85 (0.38; 1.91)	0.699
Current and former smokers	0.87 (0.30; 2.49)	0.789	2.40 (1.07; 5.36)	**0.033**
Diabetes mellitus	1.81 (0.58; 5.62)	0.303	3.13 (1.23; 7.96)	**0.016**
Hypertension	1.36 (0.47; 3.92)	0.569	2.38 (0.94; 6)	0.067
SBP (mmHg)	0.99 (0.97; 1.01)	0.355	1 (0.98; 1.03)	0.703
DBP (mmHg)	1.01 (0.96; 1.06)	0.791	0.97 (0.92; 1)	**0.039**
PP (mmHg)	0.98 (0.95; 1.01)	0.230	1.02 (1; 1.05)	0.091
Body mass index (kg/m^2)	0.94 (0.85; 1.05)	0.269	1.03 (0.96; 1.105)	0.395
LTM (kg)	0.96 (0.89; 1.03)	0.244	1 (0.95; 1.05)	0.962
Waist circumference (cm)	0.98 (0.95; 1.01)	0.222	1.01 (0.98; 1.03)	0.732
Triceps skinfold thickness (mm)	0.77 (0.49; 1.19)	0.240	1.01 (0.75; 1.36)	0.961
ATM (kg)	0.99 (0.97; 1.03)	0.888	1.01 (0.99; 1.04)	0.346
Kt/V	0.9 (0.19; 4.19)	0.895	0.91 (0.26; 3.19)	0.888
Total cholesterol (mg/dL)	0.99 (0.98; 1.01)	0.210	0.99 (0.98; 1)	0.193
LDL cholesterol (mg/dL)	0.99 (0.98; 1.00)	0.149	0.99 (0.98; 1)	0.113
HDL cholesterol (mg/dL)	1.01 (0.99; 1.04)	0.435	0.98 (0.94; 1.01)	0.217
Triglycerides (mg/dL)	1 (0.99; 1.00)	0.379	1 (1;1.01)	0.191
Fasting glucose (mg/dL)	1.003 (0.99;1.02)	0.599	1.01 (1; 1.02)	**0.043**
Calcium (mg/dL)	2.69 (1.36; 5.33)	**0.004**	0.94 (0.50; 1.80)	0.86
Phosphorus (mg/dL)	1.18 (0.90; 1.57)	0.236	1.13 (0.9; 1.43)	0.292
iPTH (pg/mL)	1.001 (1.0001;1.001)	**0.027**	1 (1; 1)	0.142
Alkaline phosphatase (UI/L)	1 (0.99; 1.01)	0.788	1 (1; 1)	0.791
Hemoglobin (g/dL)	1.14 (0.72; 1.78)	0.580	1.01 (0.68; 1.49)	0.969
Serum albumin (g/L)	0.58 (0.07; 4.34)	0.586	0.25 (0.04; 1.02)	0.12
hs-C reactive protein (mg/dL)	1.36 (1.06; 1.73)	**0.014**	1.18 (0.9; 1.56)	0.239
White blood cells (n/mm^3)	1.0002 (1; 1.001)	0.141	1 (1; 1)	0.174
TNF-α (pg/mL)	1 (1; 1.003)	0.669	1 (1; 1)	0.884
IL-6 (pg/mL)	1 (0.99; 1.003)	0.872	1 (1; 1)	0.947
IL-1β (pg/mL)	1 (1; 1)	0.195	1 (1; 1)	0.684
FGF 21 (pg/mL)	0.99 (0.956; 1.03)	0.496	1 (1; 1)	0.482
sTWEAK (pg/mL)	1 (1; 1)	0.830	1.21 (0.28; 0.53)	0.176
sCD163 (ng/mL)	1 (1; 1.001)	0.660	1 (1; 1)	0.414

TCVE—time to cardiovascular event; HR—hazard ratio; CI—confidence interval; SBP—systolic blood pressure, DBP—diastolic blood pressure, PP—pulse pressure; LTM—lean tissue mass; ATM—adipose tissue mass; iPTH—intact parathormone; hs-C reactive protein—high-sensitivity C reactive protein; TNF-α—tumor necrosis factor alfa; IL-6—interleukin 6; IL-1β—interleukin 1 beta; FGF 21—fibroblast growth factor 21; sTWEAK—soluble tumor necrosis factor-like weak inducer of apoptosis; sCD163—soluble CD163; *p*-value—bold for factors significantly associated with survival or cardiovascular events in HD patients.

The survival time was significantly different in patients with hs-CRP < 0.3 mg/dL compared to those with hs-CRP $\geq$ 0.3 mg/dL (Figure 1) (p = 0.024). In 18 patients with hs-CRP < 0.3 mg/dL, only one (5.56%) had the event (death) compared to 15 (33.3%) events in 45 patients with hs-CRP $\geq$ 0.3 mg/dL.

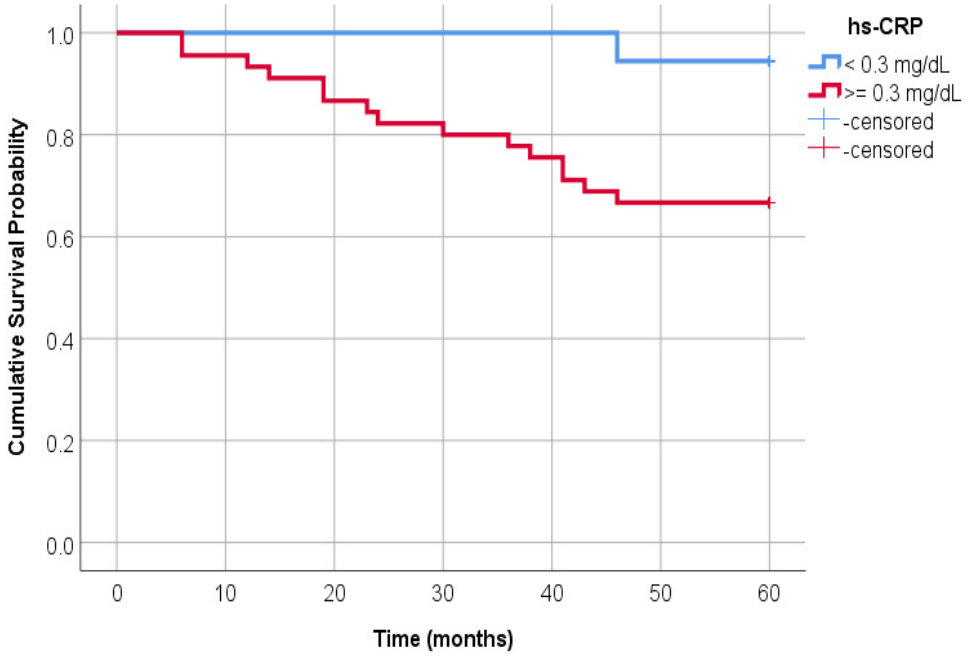

Figure 1. Comparative survival curves between subjects with hs-CRP less than 0.3 mg/dL and those with hs-CRP greater than or equal to 0.3 mg/dL.

3.4. Survival Time and Time to the First CVE on Subgroups with and Without Diabetes

3.4.1. Survival Time on Subgroups with and Without Diabetes

Univariate survival analysis in the *subgroup of diabetes* patients (n = 10) was not performed due to a small sample size.

Univariate survival analysis in the *subgroup of those without diabetes* (n = 53) revealed statistically significant factors in predicting the total survival time as follows: LTM HR = 0.91, 95% CI (0.82, 0.998), $p = 0.045$; iPTH HR = 1.001, 95% CI (1.00, 1.001), $p = 0.026$; calcium HR = 2.98, 95% CI (1.38, 6.42), $p = 0.005$; near statistically significant hs-CRP HR = 1.48, 95% CI (0.95, 2.31), $p = 0.081$; SBP HR = 0.98, 95% CI (0.95, 1.004), $p = 0.088$; and PP HR = 0.97, 95% CI (0.93, 4.00), $p = 0.077$. In multivariate analysis, after adjusting for age and dialysis duration, LTM HR = 0.87, 95% CI (0.75, 0.996), $p = 0.026$; and iPTH (HR = 1.001, 95% CI (1.00, 1.002), $p = 0.012$ remained statistically significant.

3.4.2. CVE-Free Survival Time on Subgroups with and Without Diabetes

Univariate survival analysis in the subgroup of those *without diabetes* (n = 53) revealed statistically significant factors in predicting CVE-free survival time as follows: sTWEAK HR = 1.0002, 95% CI (1.00002, 1.0005), $p = 0.029$; smoking (current and former) HR = 3.21, 95% CI (1.26, 8.20), $p = 0.015$; near statistically significant serum albumin HR = 0.14, 95% CI (0.02, 1.24), $p = 0.077$; and DBP HR = 0.96, 95% CI (0.91, 1.00), $p = 0.060$. In multivariate analysis, both of the following remained statistically significant: sTWEAK HR = 1.0002, 95% CI (1.00004, 1.0005), $p = 0.021$; and smoking (current and former) HR = 2.99, 95% CI (1.17, 7.63), $p = 0.022$, but after adjusting with the age and dialysis duration only the smoking remained significant.

We did not find a significant statistical cut-off for sTWEAK in this group.

sTWEAK was significantly different between patients with and without diabetes ($p = 0.014$) (Figure 2). STWEAK was lower in patients with diabetes.

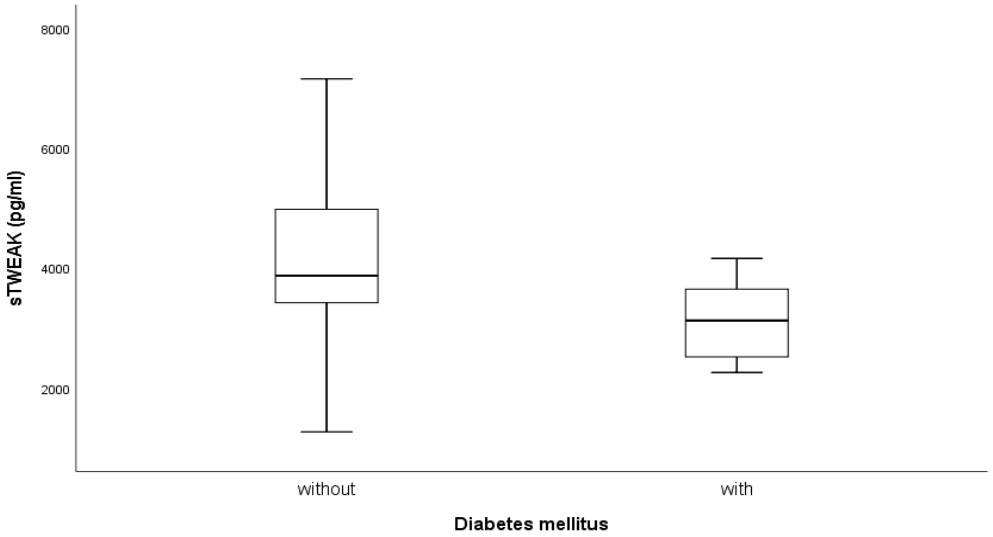

Figure 2. Comparative values of sTWEAK between subjects with diabetes mellitus and without.

3.5. Survival Time and Time to the First CVE on Subgroups of Smokers, Former Smokers, and Non-Smokers

3.5.1. Survival Time on Subgroups of Smokers, Former Smokers, and Non-Smokers

Univariate survival analysis in the subgroup of smokers and former smokers (n = 22) revealed statistically significant factors in predicting total survival time as follows: TNF-α HR = 1.02, 95% CI (1.003, 1.04), $p = 0.026$; sCD163 HR = 1.001, 95% CI (1.00, 1.003), $p = 0.036$; and hs-CRP HR = 1.40, 95% CI (1.05, 1.87), $p = 0.022$. These results had a very small power ≤ 0.1. Multivariate analysis was not performed due to a small sample size.

The survival time was significantly different between the smokers and former smokers with TNF-α less than 368.34 pg/mL and those with TNF-α higher or equal to 368.34 pg/mL (Figure 3) ($p < 0.001$). In nineteen patients with TNF-α < 368.34 pg/mL, only two (10.52%) had the event (death) compared to three (100%) events in three patients with TNF-α ≥ 368.34 pg/mL.

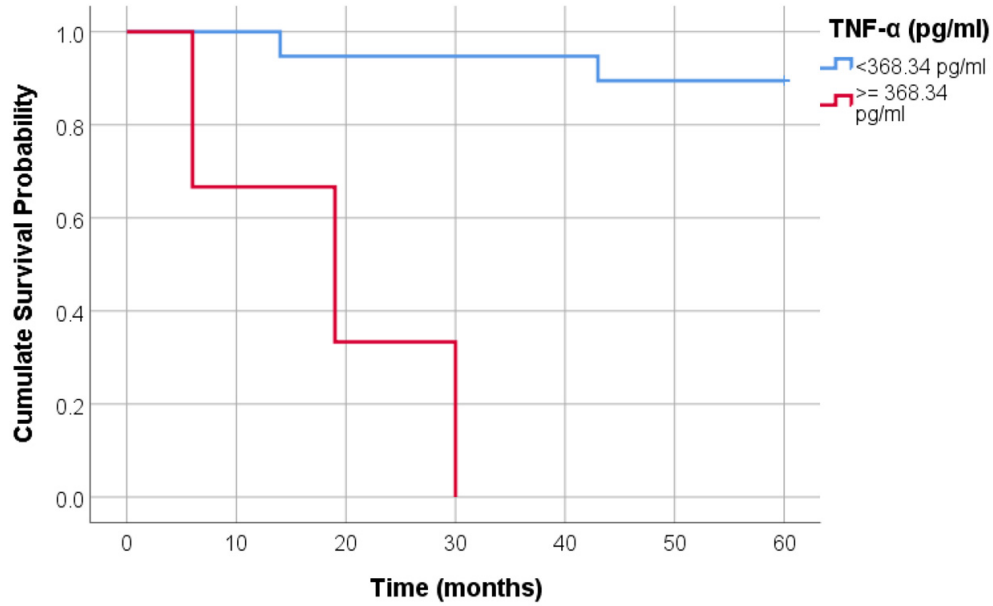

Figure 3. Comparative survival curves between subjects with TNF-α less than 368.34 pg/mL and those with TNF-α higher or equal to 368.34 pg/mL.

We did not find a significant statistical cut-off for sCD163 in this group.

Univariate survival analysis in the non-smoking subgroup (n = 41) shows that no inflammatory mediators influenced survival time and cardiovascular events.

3.5.2. CVE-Free Survival Time on Subgroups of Smokers, Former Smokers, and Non-Smokers

Univariate survival analysis in the subgroup of smokers and former smokers (n = 22) did not highlight statistically significant factors in predicting CVE-free survival time.

Univariate survival analysis in the non-smoking subgroup (n = 41) did not highlight statistically significant inflammatory mediators predicting CVE-free survival time.

4. Discussion

This study showed that CRP was associated with survival in our HD patients. In addition, patients with hs-CRP levels of less than 0.3 mg/dL in the entire hemodialysis group and TNF-alpha levels of less than 368.34 pg/mL in smokers had a survival advantage in our study. Hs-CRP was correlated with adipose tissue biomarkers, a relationship that has been less defined in HD patients until now.

Hs-CRP is an inflammatory marker that is easily monitored in clinical practice. Similarly to our study, it negatively influences survival in the general population [34] and CKD, including ESRD patients [27,35,36]. In addition, hs-CRP can predict cardiovascular events in HD patients and is superior to pentraxin 3 in this prediction [35]. However, not all studies have the same results on this issue; there is still debate about which inflammatory marker better predicts cardiovascular mortality or cardiovascular events in HD patients. Thus, in one review, pentraxin 3 was associated with CVE and mortality in people with CKD [37]. IL-6 alone, or with hs-CRP, has also been associated with all-cause and cardiovascular mortality in other studies, and it was a superior predictor of malnutrition compared with other cytokines in ESRD [11,28,38,39]. In another analysis of ESRD patients, hs-CRP predicted malnutrition better than IL-6 [40]. Elevated levels of TNF-α and IL-1β have also been associated with reduced survival in chronic HD patients [41], and TNF-α has been associated with left ventricular hypertrophy in these patients [42]. Our study found that TNF alpha levels less than 368.34 pg./mL in HD smoker patients were associated with better survival. SCD163 values, too, in the same group of patients, influenced survival. It is known that smoking significantly increases all-cause mortality in dialysis patients [43], and our results indicate that one possible mechanism is the increased inflammatory process. The differences between the results of studies on inflammatory markers predictive of survival and CVE are probably due to the regional characteristics of each hemodialysis group related to lifestyle and dietary habits that influence the intestinal microbiota and, consequently, the inflammatory process. There were also differences in mean age between studies (in one review, the age range was between 45 and 87.4 years [44], in the percentage of DM, which was 15% in our study and other research [39] and around 50% in some studies [35,38], and even in the number of smokers among patients. Finally, in our opinion, based on our results and the views of other authors [35,36,39,44–46], CRP still proves to be an optimal and cheaper option for monitoring the inflammatory state in ESRD patients with good prognostic capacity. CRP is an acute-phase protein synthesized in hepatocytes and released in response to IL-6, IL1-β, and IL-17, secondary to signals associated with tissue damage [47,48]. The first phase is a slow release of CRP (basal levels) as the CRP pre-form, pCRP, is stored in intracellular vesicles and is converted in acute insults to the active form, mCRP [49]. In our study, we did not find correlations between hs-CRP and IL-6 or IL-1β. Other authors have also observed that in CKD (pre-dialysis and dialysis stage), there is not always a correlation between hs-CRP and IL-6 or IL-1β [50–52].

This may be because, besides hepatic synthesis of CRP in response to IL-6, other factors, such as genetic factors and dialysis-related factors (immune activation), can affect CRP levels [51,53]. However, we have noted that elevated hs-CRP is associated with elevated sCD163, a monocyte/macrophage-derived biomarker that reflects macrophage activation during inflammation [54]. The concentration of sCD163 in blood [40] is associated with acute and chronic inflammatory processes, fat metabolism, and CVD [55]. Regarding the role of CRP, there have been conflicting reports, and both pro- and anti-inflammatory and pro- and anti-thrombotic activities have been described [56]. CRP has only remained a diagnostic inflammatory marker because the mechanism of action is uncertain. Among patients receiving statins in the general population, high-sensitivity CRP was a stronger predictor of the risk of future cardiovascular events and death than LDL cholesterol [57].

In our study, diabetes, although present in a small percentage of patients with all associated metabolic disorders, significantly influenced CVE, as in other studies [35,58]; no inflammatory marker significantly influenced CVE in the whole group. In the subgroup analysis, we noted that although sTWEAK and smoking in nondiabetic patients were associated with CVE-free survival time in univariate and multivariate analysis, only smoking remained significantly related to CVE-free survival time after adjusting for age and duration of dialysis. More extensive studies in nondiabetic HD patients are needed to re-examine the relationship between sTWEAK and CVE. In patients undergoing chronic hemodialysis, lower levels of sTWEAK have been associated with higher carotid intima-media thickness [59] and vascular muscle dysfunction [60]. sTWEAK values decrease as CKD progresses and are particularly low in patients on hemodialysis [61]. Serum levels of sTWEAK depend on two factors: Fn 14 (a highly inducible cell surface receptor) [30] and sCD163 (a scavenger for sTWEAK) [62].

Inflammatory mediators had a dual relationship with nutritional markers in our study. We observed that high adipose mass (expressed by BMI, adipose tissue mass, waist circumference, and triceps skinfold thickness) was associated with high hs-CRP, WBC, and sCD163. In addition, in multivariate analysis, even after adjustment, hs-CRP remained related to markers of adipose tissue. This relation has been less remarked upon in other studies in HD [63]. Adipocytes are now being analyzed as the epicenter of a global pandemic of metabolic diseases [64]. White adipose tissue, the most abundant type of fat in humans, is located subcutaneously in the viscera and bone marrow. It contains dysfunctional adipocytes that secrete inflammatory cytokines and other cell types, including macrophage-like immune cells [63]. These cells produce substances that act in a paracrine and endocrine manner, affecting local and systemic metabolic responses [65,66]. Macrophage infiltration into adipose tissue in CKD has been reported to be independent of BMI [67–69].

Adipocytes involved in the onset of the inflammatory syndrome are also regulators of lipolysis and lipogenesis, which explains the correlations between inflammatory mediators and markers of lipid metabolism [70]. Accordingly, our study found high triglycerides associated with high sCD163 and high FGF21, resulting in an atherogenic metabolic profile. Total cholesterol and LDL cholesterol among dialysis patients also relate to nutritional status, not only to atherogenic risk. High total cholesterol levels have been associated with increased survival in chronic HD patients as an expression of the reverse epidemiology phenomenon [71]. In addition, survival in chronic HD patients is related to low inflammation [27]. In our research, we identified associations between low levels of sCD163 (low inflammation expression) and high levels of total cholesterol and LDL cholesterol in the total group (values related to good nutritional status); the association was interpreted in the same phenomenon of reverse epidemiology of cardiovascular risk in HD patients.

In addition to direct correlations between inflammatory and adipose tissue markers, many studies have shown that inflammatory markers are inversely associated with protein nutritional markers in HD patients [72,73]. This association suggests that inflammation favors protein malnutrition or, conversely, malnutrition favors the inflammatory process, both detrimental pathways for HD patients. Among HD patients, high hs-CRP has been observed to be associated with lower albumin levels [9,26], but not in our study. The absence of correlations between serum albumin and hs-CRP in our research most likely reflects a specific characteristic of the population sample studied, in which hs-CRP correlates predominantly with adipose tissue markers. However, we observed high values of other inflammatory markers, such as sTWEAK, to be associated with low albumin. We also found other markers of protein malnutrition related to inflammatory markers in subgroup analysis. In nondiabetic patients, high IL-1β was associated with low albumin, and in diabetic patients, with low lean tissue mass. In the smoking subgroup, high WBC was related to low pre-dialysis creatinine. Creatinine levels in HD patients predominantly reflect muscle mass, not renal function. Low pre-dialysis creatinine levels, as markers of protein malnutrition in chronic HD, predicted mortality [35–40]. Similarly to our results, other studies have described the relationship between inflammation and protein nutritional markers. Kaysen et al., in the HEMO study, found that CRP levels were associated with serum albumin and creatinine concentrations [74]. In addition, Johansen et al. [75] showed that during a year of longitudinal observation of 54 HD patients, CRP influenced albumin change and IL-1β modulated the change in phase angle over time. All these relationships support the malnutrition–inflammation–atherosclerosis syndrome, and this favors CVD. The pathogenesis of ATS involves an inflammatory process; in HD patients, there is a microinflammatory state. The systemic inflammatory response can increase the expression of soluble intracellular adhesion molecules and vascular endothelial growth factors [76,77] and alter blood lipids, vascular endothelium, and plasma protein composition. Lipoprotein composition and adhesion molecule changes trigger vascular injury [78–81].

The correlations between inflammatory molecules may suggest some pathogenic links and possible therapeutic targets. Above, we presented the association between CRP and sCD163. Among the other correlations, we remarked that high levels of FGF21 are associated with high levels of sTWEAK; these, in turn, are associated with high levels of TNF-α. FGF21 is associated with sTWEAK among chronic dialysis patients, which was also observed in another study by us [82]. One explanation is that, in CKD patients, elevated FGF21 levels may be secondary to a state of FGF21 resistance and have been observed to be related to the dysregulation of FGF21 receptor signaling [83]. It has been postulated that inflammation may be one of the culprits [84,85]. On the other hand, FGF21 needs βKlotho as a cofactor for its activity. sTWEAK and TNF-α reduce Klotho expression in various tissues and cells, including adipocytes, by activating NFkB and, secondarily, favor reduced FGF21 activity [86]. In turn, reduced FGF21 activity may increase compensatory synthesis. Klotho is related to FGF 23, which can exacerbate the inflammatory state [87] and promote CV disease. Also, in our study, increased levels of TNF-α were associated with increased levels of IL-6, an association explained by the pathogenic cascade of the hepatic synthesis of inflammatory cytokines [12,14]. Therefore, inflammatory molecules interact and are progressively activated in CKD, which explains their impact on survival and CVD and may raise questions about which inflammatory markers can be potential therapeutic targets.

Our research has certain limitations.

First, the small sample size may have produced bias or instability in the results. It should be noted that examining specific subgroups still leads to an even smaller sample size, increasing the likelihood that statistically significant results are due to chance. Studies with small sample sizes, like this one, face significant challenges related to statistical robustness

and the reliability of results. Secondly, all participants were from a single hemodialysis center; thus, the generalizability of findings may be restricted. Lastly, the simple analysis of correlations between parameters precludes causality. Given its exploratory nature and methodological limitations, the study's findings should be interpreted cautiously. Based on the findings of this study, future studies should be designed to explore the causal relationships between inflammation and nutrition in HD patients to facilitate better-targeted treatment.

Although it has some limitations, this study is valuable because it analyzes seven diagnostic inflammatory biomarkers and highlights the association of hs-CRP with survival in our hemodialysis (HD) patients. In addition, identifying inflammatory biomarkers associated with survival and cardiovascular events in different population groups and according to comorbidities is essential for personalizing treatments for hemodialysis patients. Our study also suggests a dual relationship between inflammation and nutrition: adipose tissue markers are associated with inflammation. At the same time, protein nutritional markers are linked to inflammation in HD patients. The relationship between inflammatory molecules and adipose tissue biomarkers has been less defined in chronic HD patients; most research has highlighted protein malnutrition associated with inflammation. Finally, although our sample size is small, the 60-month follow-up period enhances the significance and robustness of our findings.

5. Conclusions

CRP, a classic inflammatory biomarker, was significantly associated with survival among our HD patients. In addition, low TNF- α was associated with better survival in smokers and former smokers among HD patients. In contrast, newer biomarkers, such as IL-6, IL-1β, sCD163, sTWEAK, and FGF 21, did not show a similar influence. CRP was correlated with adipose tissue biomarkers, CD163 was correlated with total and LDL cholesterol, and albumin was inversely associated with sTWEAK. The correlation of adipose tissue and protein nutritional biomarkers with inflammatory biomarkers suggests a complex, dual relationship between inflammation and nutrition in hemodialysis patients. The relation between inflammatory molecules and adipose tissue biomarkers has been less defined in HD patients. Our findings are based on HD patients from a specific single-center sample, and extensive, multicentric studies are necessary for confirmation.

Author Contributions: Conceptualization, C.C.R.; Data curation, C.C.R., D.T., A.U. and C.I.B.; Formal analysis, C.C.R., D.T., M.T. and C.I.B.; Investigation C.C.R., I.K., A.P. and Y.M.; Methodology, C.C.R., A.P., M.T. and C.I.B.; Project administration, C.C.R.; Resources, C.C.R., D.M., A.U. and D.T.; Software, Y.M., M.T. and C.I.B.; Supervision, C.C.R. and I.K.; Validation, C.C.R., I.K., D.M. and D.T.; Visualization, D.M., A.P., Y.M. and A.U.; Writing—original draft, C.C.R., M.T. and C.I.B.; Writing—review and editing, C.C.R., D.M. and I.K. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The research data supporting this study's findings are not publicly available. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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Leptin Is Associated with Testosterone, Nutritional Markers, and Vascular Muscular Dysfunction in Chronic Kidney Disease

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Abstract: Chronic kidney disease (CKD) causes specific hormonal disturbances, such as variations in leptin and testosterone levels and function. These disturbances can promote errors in signaling interaction and cellular information processing and can be implicated in the pathogenesis of atherosclerosis. This study investigates the factors that affect leptin in CKD patients and examines how leptin is related to markers of vascular disease. We conducted a cross-sectional study of 162 patients with CKD in pre-dialysis and dialysis stages. We recorded clinical and laboratory data, including leptin, testosterone, and subclinical atherosclerosis markers like brachial–ankle pulse wave velocity (ba PWV) in pre-dialysis CKD patients and flow-mediated vasodilation (FMD) and nitroglycerin-mediated vasodilation (NMD) in hemodialysis (HD) patients. Leptin was significantly correlated with testosterone in CKD pre-dialysis stages ($p < 0.001$) and also in HD ($p = 0.026$), with adipose tissue mass in pre-dialysis stages ($p < 0.001$), and also in HD ($p < 0.001$). In women HD patients, leptin correlated with NMD ($p = 0.039$; r = −0.379); in all HD patients, leptin correlated with C reactive protein ($p = 0.007$; r = 0.28) and parathormone ($p = 0.039$; r = −0.220). Our research emphasizes the connection between leptin, adipose tissue, and testosterone in all stages of CKD. Leptin was associated with NMD in HD women and correlated with inflammatory syndrome and parathyroid hormone in all HD patients.

Keywords: hormone; testosterone; leptin; chronic kidney disease; atherosclerosis

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1. Introduction

Chronic kidney disease (CKD) leads to specific metabolic disorders, including hormonal disturbances and persistent inflammatory syndrome. These disorders increase the risk of atherosclerosis and cardiovascular disease to an extremely high level, which exceeds the risk in the general population, worsens the progression of CKD, and leads to high mortality rates. Hormonal disturbances, including modifications in leptin, ghrelin, testosterone, and prolactin levels, are commonly seen in CKD patients. In humans, circulating leptin comes primarily from visceral and subcutaneous adipose tissue [1] but can also be secreted

by normal human osteoblasts, subchondral osteoblasts, placental syncytiotrophoblasts, and gastric epithelium [2,3]. In blood, leptin circulates in free and protein-bound forms; biologically active leptin exists in the free form. At the renal level, it is freely filtered glomerular; then, it is captured tubularly and degraded [4,5]. The basic mechanism of tubular reabsorption of leptin is receptor-mediated endocytosis [6]. Thus, it reaches the urinary level in a small amount.

The leptin receptor LepR is found in various tissues, such as adipose tissue, heart, muscle, lung, small intestine, liver, and the central nervous system, especially the hypothalamus [7]. Leptin is a hormone that plays a crucial role in regulating food intake [8]. Through neurohormonal mechanisms, increasing leptin can reduce food intake and improve insulin sensitivity. Leptin also helps in the maturation of the gonads, the regulation of the immune system, and mineral bone metabolism [8]. This hormone intervenes in metabolic regulation in several ways: (1) by significantly reducing appetite, it reduces energy intake [9,10], (2) it increases energy consumption by stimulating the activity of the sympathetic nervous system in the cardiovascular system or by increasing thermogenesis [11–15], (3) it increases lipolysis and the use of lipids as energy fuel [11,13], and (4) it increases glucose turnover and glucose absorption in brown adipose tissue, brain, and heart [16,17].

In addition to all the favorable effects of leptin mentioned above, some studies have indicated that its functions are altered in certain pathological conditions, possibly secondary to errors in intercellular signaling. Leptin can increase oxidative stress, promote endothelial dysfunction, and increase arterial stiffness [18]. As a result of these phenomena, the vulnerability of the atherosclerotic plaque and the risk of thrombosis increase [19]. Statins and antidiabetic drugs (including sitagliptin, liraglutide, and empagliflozin) can modify leptin levels [18]. In addition, leptin can stimulate the activity of the sympathetic nervous system, leading to hypertension [20]. The correlation of leptin with blood pressure values and adipose tissue mass brought into discussion a new possible therapeutic intervention for treating hypertension associated with obesity [21].

Leptin levels are high in CKD patients as an active and free form. However, not all CKD patients have high serum leptin levels, but after adjusting for adipose mass and age, hyperleptinemia was identified in all patients [5]. The high values of leptin in CKD are not solely due to kidney failure; other factors, such as inflammation, reduced erythropoietin levels, hyperinsulinemia, the type of dialysis, dialyzer membrane, and diet, may also be involved [2,22,23].

Hyperleptinemia in CKD has been shown to contribute to insulin resistance, protein-energy wasting, and atherogenic lipid profiles [24–26]. In peritoneal dialysis, it was associated with left ventricular hypertrophy [27]. In chronic hemodialysis (HD), low leptin levels have been associated with increased mortality [28] and a higher risk of cardiovascular events [29], and high leptin values were associated with vascular access dysfunction [30]. In patients with end-stage kidney disease, higher leptin values were recorded in those with a history of stroke. Still, among patients with stroke, serum leptin values were lower in those who also had congestive heart failure [18].

Thus, the role of leptin in CKD is not fully understood due to limited research and conflicting data. Therefore, further research is needed to clarify its role in CKD and its impact on cardiovascular disease. The purpose of this study is to evaluate the factors that influence the level of leptin in CKD patients in different stages and to determine the predictor of leptin levels. Additionally, the study aims to investigate the relationship between leptin levels and subclinical markers of atherosclerosis, such as nitroglycerin-mediated vasodilation (NMD), flow-mediated vasodilation (FMD), and pulse wave velocity (PWV) in CKD.

2. Results

The average age of patients in the pre-dialysis stage was 68 years, while in the HD group, it was 61.5 years. Among the patients receiving chronic HD, 56.8% were men, while in the pre-dialysis CKD group, 51.3% were men. Additionally, 19.5% of patients undergoing

chronic HD and 37.8% of those with pre-dialytic CKD had diabetes. Table 1 shows the characteristics of the patients and the comparison between groups.

When comparing nutritional markers, leptin, and testosterone levels in men and women in both chronic HD patients and in pre-dialysis patients, we found that higher testosterone levels in men were associated with increased lean tissue mass (LTM), decreased adipose tissue mass (ATM) (only in HD group), and lower leptin levels (see Table 2).

Table 1. Comparisons between groups according to estimated glomerular filtration rate (eGFR) and dialysis treatment.

Variable	Group A Hemodialysis (n = 88)	Group B Std. V KDIGO (n = 23)	Group C Std. IV KDIGO (n = 26)	Group D Std. III KDIGO (n = 25)	*p*
Male sex n (%)	50 (56.8)	12 (52.2)	12 (46.2)	14 (56)	0.803
Age (years)	61.5 (54, 71) [b,c]	65 (60, 70)	69 (62, 78)	70 (65, 75)	**0.003**
WC (cm)	98 (84, 108)	104 (93.5, 111)	96 (90, 106)	105 (104, 108)	0.094
BMI (kg/m^2)	27.54 (23.2, 31)	27.3 (25.6, 29.9)	28.25 (24.85, 29.6)	29.1 (27.7, 33.5)	0.133
LTM (kg)	29.71 (25.2, 37.64)	33.9 (29.25, 52.65)	39.5 (26.25, 45)	35.35 (26.6, 42.45)	0.065
ATM (kg)	41.75 (26.51, 51.38)	35.3 (23.75, 38.75) [e]	35.5 (32.15, 43.7) [f]	46.8 (40.45, 60.65)	**0.009**
SBP (mmHg)	142.32 ± 21.29	155.76 ± 24.48	145.81 ± 23.88	144.67 ± 18.21	0.140
DBP (mmHg)	75 (67, 80) [a,b,c]	94 (86, 98)	80 (74, 92)	82 (80, 98)	**<0.001**
PP (mmHg)	68.28 ± 18.77	62.18 ± 18.06	62.33 ± 20.16	58.67 ± 15.41	0.115
Diabetes n (%)	17 (19.5) [a,b,c]	10 (43.5)	10 (38.5)	8 (32)	0.059
Hypertension n (%)	62 (70.5) [b]	18 (85.7)	24 (100)	19 (90.5)	**0.005**
Fasting glucose (mg/dL)	93.89 (87.43, 113)	99 (91, 129.5)	108 (90, 139.5)	101 (94, 122)	0.180
Triglycerides (mg/dL)	134 (95.33, 181.16)	131 (99.5, 170.5)	148 (116, 188)	126 (93, 159.5)	0.675
LDL-cholesterol (mg/dL)	100.21 ± 36.88	120.53 ± 51.74	123.35 ± 46.66	127.84 ± 38.3	**0.015**
Total cholesterol (mg/dL)	173.15 ± 40.6	173.89 ± 39.16	173.96 ± 32.66	188.33 ± 35.58	0.386
HDL-cholesterol (mg/dL)	39.61 (30.75, 47.64)	41 (32.5, 47.5)	35.5 (28, 41)	45 (39, 49.5)	0.111
Hemoglobin (g/dL)	11.5 (10.65, 12.3) [c]	11 (10.3, 11.8) [e]	12.05 (10.3, 13.2)	13.4 (12, 14)	**0.001**
Ferritin (ng/mL)	567 (333.03, 782.65) [a,b,c]	178.5 (63.5, 351)	98 (34, 186)	88.5 (61.5, 157)	**<0.001**
Hs-CRP (mg/dL)	0.59 (0.23, 1.33)	0.5 (0.2, 1.27)	0.53 (0.29, 1.19)	0.38 (0.23, 0.89)	0.660
WBC (no./mmc)	6440 (5530, 7815) [c]	7770 (6120, 9800)	7315 (6200, 8370)	8630 (6500, 8930)	**0.005**
Bicarbonate level (mEq/L)	21.4 (18.45, 24.25)	19.15 (16.3, 20.85)	19.9 (17.3, 23.6)	20 (17.7, 22.3)	0.068
Calcium (mg/dL)	8.9 (8.32, 9.2)	8.66 (8.04, 9.1) [c]	9.23 (8.64, 9.68)	9.32 (8.94, 9.62)	**0.003**
Phosphorus (mg/dL)	4.71 (3.98, 5.89) [b,c]	4.72 (4.18, 5.81) [d,e]	3.7 (3.16, 4.48)	3.15 (2.91, 3.53)	**<0.001**
AP (UI/L)	73 (55.92, 95.43)	86 (70, 98)	83 (77.5, 107.5)	75 (70, 110.5)	0.175
iPTH (pg/mL)	286.75 (164.65, 729.65) [b,c]	283.95 (151.85, 398.85) [d,e]	104.1 (52.15, 153.1)	104.2 (78.3, 152.45)	**<0.001**
Creatinine (mg/dL)	8.59 (7.3, 10.4) [a,b,c]	4.7 (4.04, 6.33) [d,e]	2.4 (2.09, 2.51) [f]	1.56 (1.37, 1.68)	**<0.001**
Albumin (g/dL)	3.91 (3.7, 4.08) [b,c]	3.76 (3.56, 4.13) [d]	3.57 (3.47, 3.82) [f]	4.38 (3.9, 4.46)	**0.002**
eGFR (mL/min/m^2)		10.17 ± 3.11 [d,e]	23.63 ± 4.3 [f]	41.68 ± 8.13	**<0.001**
Testosterone (ng/mL)	2.1 (0.7, 4)	2.7 (0.6, 3.55)	0.65 (0.4, 3.6)	0.9 (0.4, 3.2)	0.076
Leptin (ng/mL)	5.99 (1.57, 31.14)	3.41 (1.9, 9.67) [e]	14.48 (3.86, 37.9)	19.98 (6.33, 39.44)	**0.020**
Betablockers n (%)	50 (56.8)	12 (54.5)	13 (52)	14 (66.7)	0.774
ACEI + ARB	37 (42)	7 (31.8)	14 (56)	11 (52.4)	0.319
Statin n (%)	16 (18.2)	8 (42.1)	6 (24)	7 (33.3)	0.111
Antiagregants n (%)	32 (36.4)	4 (19)	10 (40)	5 (23.8)	0.301
Ba PWV (cm/s)	-	9.98 ± 2.5	11.12 ± 2.25	10.65 ± 2.11	0.324

Table 1. *Cont.*

Variable	Group A Hemodialysis (n = 88)	Group B Std. V KDIGO (n = 23)	Group C Std. IV KDIGO (n = 26)	Group D Std. III KDIGO (n = 25)	*p*
NMD (%)	7.25 (2.27, 12.5)	-	-	-	-
FMD (%)	8.33 (4.31, 14.29)	-	-	-	-

[a] $p < 0.05$ when comparing group a with group b; [b] $p < 0.05$ when comparing group a with group c; [c] $p < 0.05$ when comparing group a with group d; [d] $p < 0.05$ when comparing group b with group c; [e] $p < 0.05$ when comparing group b with group d; [f] $p < 0.05$ when comparing group c with group d; significant *p* value with bold; std.—stadium; n—number of people; no.—number of cells; BMI—body mass index; SBP—systolic blood pressure; DBP—diastolic blood pressure; PP—pulse pressure; eGFR—estimated glomerular filtration rate; LTM—lean tissue mass; ATM—adipose tissue mass; LDL—low-density lipoprotein; HDL—high-density lipoprotein; iPTH—Intact parathyroid hormone; Ba PWV—brachial–ankle pulse wave velocity; ACEI—angiotensin-converting enzyme inhibitors; ARB—angiotensin II receptor blockers; WC—waist circumference; NMD—nitroglycerin-mediated vasodilatation; FMD—flow-mediated vasodilation; hs-CRP—high-sensitive C-reactive protein; AP—alkaline phosphatase; WBC—white blood cell; arithmetic mean ± standard deviation; median (25th–75th percentile).

Table 2. Comparisons between men and women in group A with dialysis treatment and in pre-dialysis group.

Variable	Group A Hemodialysis (n = 88)			Group Pre-Dialysis (n = 74)		
	Women (n = 38)	Men (n = 50)	*p*	Women (n = 36)	Men (n = 38)	*p*
Age (years)	66 (56, 75)	59 (52, 68)	**0.032**	70 (65, 77)	67 (61, 71)	0.122
WC (cm)	99 (80, 115)	97.5 (85, 104)	0.622	105 (91, 107)	104 (97, 109)	0.254
BMI (kg/m^2)	29.12 (24.38, 36.48)	26.54 (22.83, 29.53)	**0.010**	29.85 (26.4, 33.3)	28.2 (26.75, 29.1)	0.168
LTM (kg)	25.98 (22.47, 27.96)	35.56 (30.12, 43.41)	**<0.001**	27.1 (24.7, 34.7)	42.4 (36, 52.7)	**<0.001**
ATM (kg)	43.44 (30.15, 62.45)	39.65 (24.95, 46.62)	**0.013**	40.9 (35.1, 55.2)	36.6 (30.2, 44.1)	0.167
SBP (mmHg)	139.26 ± 18.81	144.64 ± 22.92	0.243	143 (127, 162)	151 (130, 170)	0.254
DBP (mmHg)	70 (67, 78)	80 (70, 82)	**0.020**	80.5 (74, 96)	91 (81, 99)	**0.022**
PP (mmHg)	68.66 ± 19.23	68 ± 18.6	0.872	66 (44, 74)	57 (50, 72)	0.856
Diabetes n (%)	6 (15.8)	11 (22.4)	0.437	13 (36.1)	15 (39.5)	0.766
Hypertension n (%)	26 (68.4)	36 (72)	0.715	26 (86.7)	35 (97.2)	0.169
Fasting glucose (mg/dL)	93.5 (87.2, 110)	94 (87.65, 115)	0.150	108.5 (99, 139)	97 (89, 124.5)	0.896
Triglycerides (mg/dL)	145.97 (102, 225)	125.69 (94.05, 163.51)	0.080	148 (114.5, 171.5)	126 (93, 155.5)	0.541
LDL-cholesterol (mg/dL)	106 ± 36.3	95.81 ± 37.06	0.201	106.8 (76.2, 140)	136 (105, 163)	0.055
Total cholesterol (mg/dL)	184.89 ± 40.36	164.22 ± 38.83	**0.017**	171.5 (153.5, 201)	176 (155, 195.5)	0.754
HDL-cholesterol (mg/dL)	41.25 (30.14, 49)	36.5 (31.69, 45.8)	0.374	42 (32.5, 48.5)	39 (33, 46)	0.778
Hemoglobin (g/dL)	11.05 (10.6, 11.6)	12 (10.8, 12.6)	**0.015**	11.55 (10.75, 12.5)	13 (10.3, 14.8)	0.306
Ferritin (ng/mL)	665.7 (402.34, 846.6)	472 (317.5, 723)	**0.033**	91 (55, 186)	106 (63, 279.5)	0.679
Hs-CRP (mg/dL)	0.61 (0.34, 1.43)	0.53 (0.21, 1.32)	0.440	0.43 (0.2, 0.91)	0.52 (0.34, 1.42)	0.236
WBC (no./mmc)	6210 (5530, 7440)	6495 (5260, 7880)	0.947	7065 (6160, 8875)	7800 (6410, 9060)	0.146
Bicarbonate level (mEq/L)	22.8 (18.2, 24.1)	20.5 (18.6, 24.5)	0.330	19 (17.3, 21.1)	19.6 (16.5, 22.3)	0.673
Calcium (mg/dL)	8.97 (8.4, 9.2)	8.79 (8.24, 9.2)	0.303	9.3 (8.93, 9.68)	8.79 (8.44, 9.3)	0.314
Phosphorus (mg/dL)	4.5 (3.8, 5.19)	5.12 (4.08, 6.84)	0.127	4.08 (3.48, 4.88)	3.56 (2.93, 4.5)	0.249
AP (UI/L)	79.61 (61, 100)	66.2 (51.83, 94.44)	0.130	89.5 (78, 106)	77 (68, 105.5)	0.095
iPTH (pg/mL)	266.5 (158.7, 481.85)	336 (185.7, 798)	0.179	140 (94.35, 260.95)	118.4 (87.5, 202.6)	0.176
Creatinine (mg/dL)	8.1 (6.8, 8.73)	9.42 (7.7, 11.3)	**<0.001**	2.18 (1.6, 3.44)	2.47 (1.8, 4.5)	0.225
Albumin (g/dL)	3.87 (3.62, 4)	3.94 (3.74, 4.13)	0.053	3.79 (3.61, 4.07)	3.76 (3.49, 4.26)	0.875
eGFR (mL/min/m^2)				23 (13.5, 32)	26 (12, 38)	0.354
Testosterone (ng/mL)	0.7 (0.5, 1.3)	3.65 (2.5, 4.8)	**<0.001**	0.5 (0.3, 0.6)	3.3 (2.3, 4.3)	**<0.001**

Table 2. *Cont.*

Variable	Group A Hemodialysis (n = 88)			Group Pre-Dialysis (n = 74)		
	Women (n = 38)	**Men (n = 50)**	***p***	**Women (n = 36)**	**Men (n = 38)**	***p***
Leptin (ng/mL)	24.3 (5.43, 49.47)	3.49 (0.57, 9.58)	**<0.001**	35.5 (13.93, 54.97)	3.82 (1.54, 7.68)	**<0.001**
Betablockers n (%)	21 (55.3)	29 (58)	0.797	19 (63.3)	20 (52.6)	0.376
ACEI + ARB	14 (36.8)	23 (46)	0.804	15 (50)	17 (44.7)	0.666
Statin n (%)	7 (18.4)	9 (18)	**0.002**	10 (35.7)	11 (29.7)	0.609
Antiagregants n (%)	17 (44.7)	15 (30)	0.155	7 (24.1)	12 (31.6)	0.503
Ba PWV (cm/s)				10.6 (9.7, 12.2)	10.65 (9.05, 11.95)	0.516
NMD (%)	9.68 (4.65, 14.09)	4.34 (1.83, 11.69)	**0.014**			
FMD (%)	11.11 (4.55, 15.38)	8.16 (4.08, 14.29)	0.454			
HD duration	69 (34, 88)	54 (22, 83)	0.034			

Significant *p* value with bold; n—number of people; no.—number of cells; BMI—body mass index; SBP—systolic blood pressure; DBP—diastolic blood pressure; PP—pulse pressure; eGFR—estimated glomerular filtration rate; LTM—lean tissue mass; ATM—adipose tissue mass; LDL—low-density lipoprotein; HDL—high-density lipoprotein; iPTH—intact parathyroid hormone; Ba PWV—brachial–ankle pulse wave velocity; ACEI—angiotensin-converting enzyme inhibitors; ARB—angiotensin II receptor blockers; WC—waist circumference; NMD—nitroglycerin-mediated vasodilatation; FMD—flow-mediated vasodilation; hs-CRP—high-sensitive C-reactive protein; AP—alkaline phosphatase; WBC—white blood cell; HD—hemodialysis; arithmetic mean ± standard deviation; median (25th–75th percentile).

2.1. Hemodialysis Group

The level of leptin in the group treated with HD was directly related to body mass index (BMI), ATM, the inflammatory syndrome indicated by white blood cells (WBCs) and high-sensitive C-reactive protein (hs-CRP), triglycerides, and low-density lipoprotein (LDL) cholesterol. It was inversely related to testosterone levels, intact parathyroid hormone (iPTH), and LTM. The multivariate analysis showed that leptin levels remained significantly associated with ATM, hs-CRP, and testosterone levels (see Table 3).

Table 3. Correlation and multivariate linear regression between the parameters and leptin in hemodialysis (HD) group A.

Variable	Women in Group A (n = 38)		Men in Group A (n = 50)		Group A		Multivariate Analysis in Group A	
Leptin (ng/mL)	**Correlation Coefficient**	***p***	**Correlation Coefficient**	***p***	**Correlation Coefficient**	***p***	**B Coefficient 95%CI (Lower, Upper)**	***p***
Age (years)	0.166	0.320	0.159	0.269	0.210	**0.049**		
WC (cm)	0.624	**<0.001**	0.620	**<0.001**	0.558	**<0.001**		
DBP (mmHg)	−0.048	0.773	−0.168	0.243	−0.184	0.086		
Triglycerides (mg/dL)	0.152	0.361	0.201	0.162	0.235	**0.028**		
LDL-cholesterol (mg/dL)	0.004	0.983	0.271	0.057	0.221	**0.038**		
Total Cholesterol (mg/dL)	−0.056	0.740	0.367	**0.009**	0.270	**0.011**		
iPTH (pg/mL)	−0.122	0.464	−0.236	0.098	−0.220	**0.039**		
hs-CRP (mg/dL)	0.193	0.246	0.337	**0.017**	0.284	**0.007**	0.35 (0.07, 0.63)	**0.016**
WBC (no/mmc)	0.104	0.534	0.444	**0.001**	0.253	**0.017**		
Testosterone (ng/mL)	−0.153	0.359	0.230	0.108	−0.377	**<0.001**	−1.83 (−3.44, −0.23)	**0.026**
BMI (kg/m^2)	0.713	**<0.001**	0.713	**<0.001**	0.737	**<0.001**		
LTM (kg)	−0.262	0.112	−0.271	0.065	−0.439	**<0.001**		

Table 3. *Cont.*

Variable	Women in Group A (n = 38)		Men in Group A (n = 50)		Group A		Multivariate Analysis in Group A	
Leptin (ng/mL)	**Correlation Coefficient**	***p***	**Correlation Coefficient**	***p***	**Correlation Coefficient**	***p***	**B Coefficient 95%CI (Lower, Upper)**	***p***
ATM (kg)	0.716	**<0.001**	0.787	**<0.001**	0.751	**<0.001**	0.86 (0.61, 1.12)	**<0.001**
NMD (%)	−0.379	**0.039**	−0.137	0.392	−0.177	0.139		

Significant *p* value with bold; n—number of people; BMI—body mass index; DBP—diastolic blood pressure; LTM—lean tissue mass; ATM—adipose tissue mass; LDL—low-density lipoprotein; iPTH—intact parathormone; WC—waist circumference; NMD—nitroglycerin-mediated vasodilatation; FMD—flow-mediated vasodilation; hs-CRP—high-sensitive C-reactive protein, WBC—white blood cell.

Figure 1 illustrates the inverse correlation between leptin values and testosterone in the HD group A.

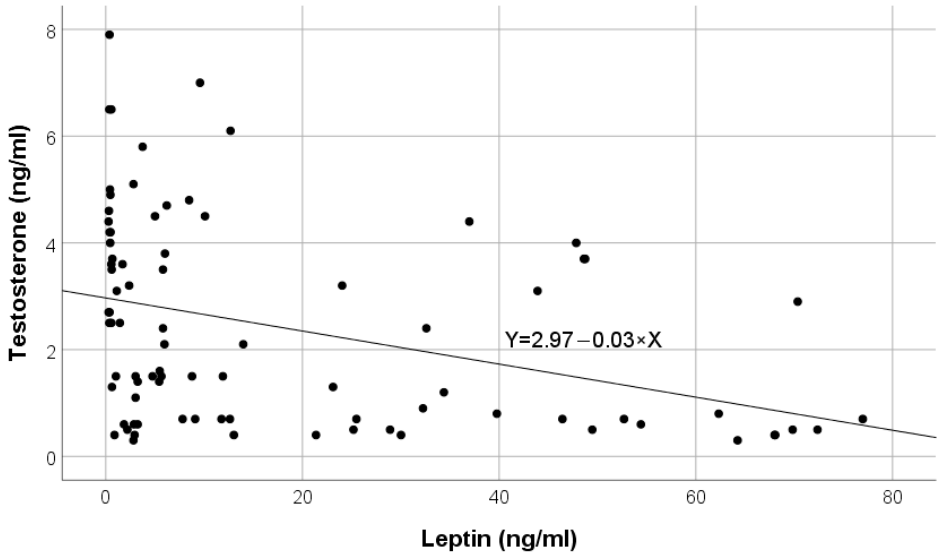

Figure 1. Variation in testosterone according to leptin in hemodialysis (HD) group A.

The direct correlation between leptin values and ATM in the HD group A is shown in Figure 2.

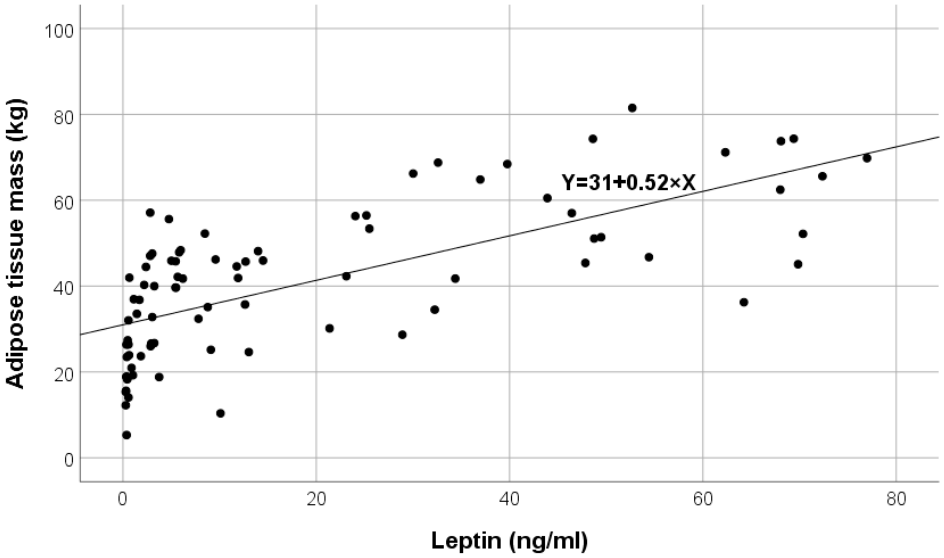

Figure 2. Variation in adipose tissue mass (ATM) according to leptin in hemodialysis (HD) group A.

Testosterone in HD group A was directly correlated with markers of protein metabolism such as pre-dialysis serum creatinine, serum phosphorus, and LTM but also with hemoglobin and diastolic blood pressure, and inversely with age, LDL cholesterol and total cholesterol, ferritin, and leptin. Multivariate analysis showed that testosterone remained significantly associated with hemoglobin and leptin. Interestingly, in the group of chronic HD men, testosterone correlated only with hemoglobin and NMD (see Table 4).

Table 4. Correlation between the parameters and testosterone in hemodialysis (HD) group A.

Variable	Women in Group A (n = 38)		Men in Group A (n = 50)		Group A		Multivariate Analysis of Group A	
Testosterone (ng/mL)	**Correlation Coefficient**	***p***	**Correlation Coefficient**	***p***	**Correlation Coefficient**	***p***	**Correlation Coefficient**	***p***
Age (years)	−0.119	0.475	−0.080	0.580	−0.221	**0.039**		
SBP (mmHg)	−0.150	0.369	0.185	0.199	0.179	0.095		
DBP (mmHg)	0.076	0.651	0.212	0.138	0.298	**0.005**		
Creatinine (mg/dL)	0.022	0.894	−0.079	0.585	0.269	**0.011**		
LDL cholesterol (mg/dL)	0.103	0.539	−0.142	0.324	−0.182	0.090		
Total cholesterol (mg/dL)	0.154	0.357	−0.170	0.238	−0.235	**0.027**		
Phosphorus (mg/dL)	0.279	0.090	0.140	0.331	0.223	**0.037**		
Ferritin (ng/mL)	0.149	0.371	−0.225	0.116	−0.236	**0.027**		
Hemoglobin (g/dL)	0.174	0.295	0.330	**0.019**	0.256	**0.016**	0.84 (0.38, 1.30)	**<0.001**
Leptin (ng/mL)	−0.153	0.359	0.230	0.108	−0.377	**<0.001**		
BMI (kg/m^2)	−0.032	0.850	0.231	0.114	−0.200	0.064		
LTM (kg)	−0.147	0.378	0.145	0.332	0.510	**<0.001**	0.09 (0.03, 0.15)	**0.006**
NMD (%)	0.199	0.291	−0.316	**0.044**	−0.145	0.226		

Significant *p* value with bold; n—number of people; SBP—systolic blood pressure; DBP—diastolic blood pressure; LDL—low-density lipoprotein; BMI—body mass index; LTM—lean tissue mass; NMD—nitroglycerin-mediated vasodilatation.

2.2. Pre-Dialysis Group

The leptin values in the pre-dialysis group correlated directly with BMI, ATM, serum calcium, serum bicarbonate, and eGFR, and inversely with testosterone levels, diastolic blood pressure (DBP), ferritin, and LTM. In the multivariate analysis, the leptin level remained significantly associated with ATM, BMI, LTM, and testosterone levels (see Table 5).

Table 5. Correlation between the parameters and leptin in the pre-dialysis chronic kidney disease (CKD) patients (n = 73).

Variable	Women in Group Pre-Dialysis (n = 36)		Men in Group Pre-Dialysis (n = 38)		Group Pre-Dialysis		Multivariate Analysis on Group Pre-Dialysis	
Leptin (ng/mL)	**Correlation Coefficient**	***p***	**Correlation Coefficient**	***p***	**Correlation Coefficient**	***p***	**B Coefficient 95%CI (Lower, Upper)**	***p***
Testosterone (ng/mL)	−0.238	0.162	−0.113	0.499	−0.570	**<0.001**	−6.09 (−8.87, −3.30)	**<0.001**
DBP (mmHg)	−0.191	0.349	−0.205	0.253	−0.299	**0.021**	-	
Triglycerides (mg/dL)	0.426	0.061	0.065	0.748	0.254	0.084	-	
Calcium (mg/dL)	0.277	0.119	0.166	0.327	0.364	**0.002**	-	
Phosphorus (mg/dL)	−0.181	0.329	−0.317	0.064	−0.078	0.533		
iPTH (pg/mL)	−0.425	**0.027**	−0.109	0.545	−0.173	0.185		
Fasting glucose (mg/dL)	0.522	**0.009**	0.293	0.083	0.302	**0.019**	0.16 (0.04, 0.03)	**0.013**
Ferritin (ng/mL)	−0.169	0.453	−0.323	0.076	−0.294	**0.033**	-	
Bicarbonate level (mEq/L)	0.383	0.308	0.538	**0.021**	0.408	**0.034**	-	

Table 5. *Cont.*

Variable	Women in Group Pre-Dialysis (n = 36)		Men in Group Pre-Dialysis (n = 38)		Group Pre-Dialysis		Multivariate Analysis on Group Pre-Dialysis	
Leptin (ng/mL)	**Correlation Coefficient**	***p***	**Correlation Coefficient**	***p***	**Correlation Coefficient**	***p***	**B Coefficient 95%CI (Lower, Upper)**	***p***
BMI (kg/m^2)	0.709	**<0.001**	0.505	**0.003**	0.595	**<0.001**	4.56 (3.02, 6.10)	**<0.001**
LTM (kg)	0.048	0.818	−0.094	0.628	−0.384	**0.004**	−0.97 (−1.4, −0.54)	**<0.001**
ATM (kg)	0.685	**<0.001**	0.446	**0.015**	0.591	**<0.001**	0.81 (0.48, 1.15)	**<0.001**
eGFR (mL/min/1.73 m^2)	0.416	**0.012**	0.340	**0.037**	0.264	**0.023**	-	

Significant *p* value with bold; n—number of people; DBP—diastolic blood pressure; LDL—low-density lipoprotein; eGFR—estimated glomerular filtration rate; iPTH—intact parathyroid hormone; BMI—body mass index; LTM—lean tissue mass; ATM—adipose tissue mass.

The inverse correlation of leptin values with testosterone in the pre-dialysis group is shown in Figure 3.

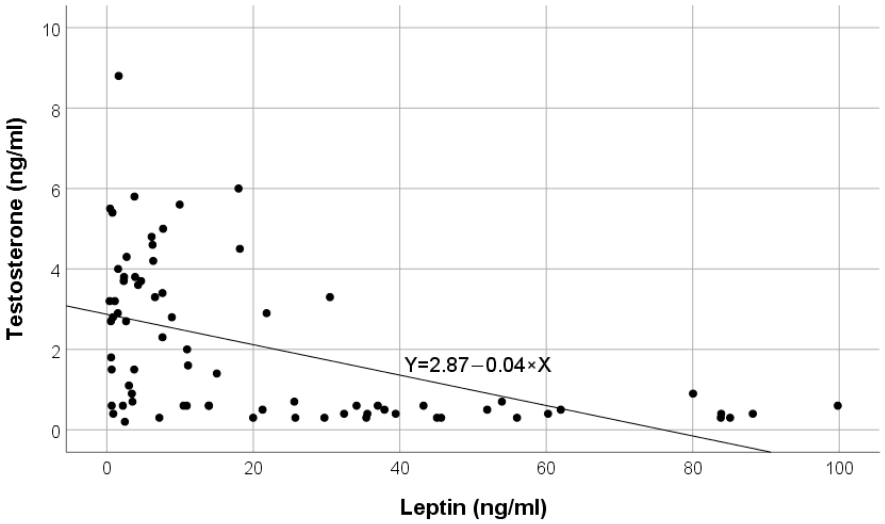

Figure 3. Variation in testosterone according to leptin in pre-dialysis chronic kidney disease (CKD) patients.

Figure 4 shows the direct correlation of leptin values with ATM in the pre-dialysis group.

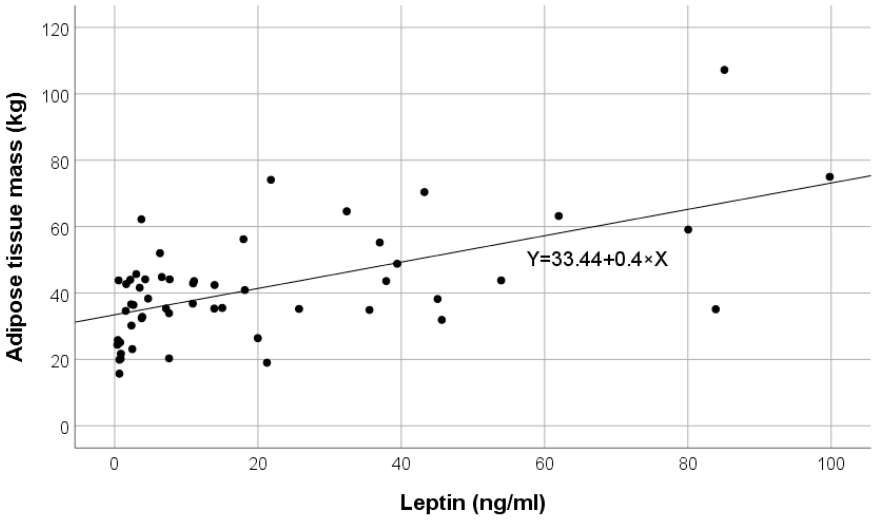

Figure 4. Variation in adipose tissue mass (ATM) according to leptin in pre-dialysis chronic kidney disease (CKD) patients.

Testosterone in the pre-dialysis group was directly correlated with markers of protein metabolism, such as LTM, and inversely with leptin and calcium. Multivariate analysis showed that testosterone remained significantly associated with LTM and leptin. Interestingly, in the group of men in the pre-dialysis stages of CKD, testosterone was not associated with any of the monitored parameters (see Table 6).

Table 6. Correlation between the parameters and testosterone in pre-dialysis chronic kidney disease (CKD) patients.

Variable	Women in Group Pre-Dialysis (n = 36)		Men in Group Pre-Dialysis (n = 38)		Group Pre-Dialysis		Multivariate Analysis on Group Pre-Dialysis	
Testosterone (ng/mL)	**Correlation Coefficient**	***p***	**Correlation Coefficient**	***p***	**Correlation Coefficient**	***p***	**B Coefficient 95%CI (Lower, Upper)**	***p***
Leptin (ng/mL)	−0.238	0.162	−0.113	0.499	−0.570	**<0.001**	−0.03 (−0.05, −0.01)	**0.009**
WC (cm)	0.573	**0.040**	0.027	0.913	0.233	0.200		
SBP (mmHg)	−0.228	0.262	0.124	0.491	0.255	0.051		
LDL-cholesterol (mg/dL)	0.154	0.529	0.086	0.670	0.264	0.076		
Calcium (mg/dL)	−0.173	0.335	−0.056	0.743	−0.296	**0.013**		
LTM (kg)	0.227	0.275	0.022	0.911	0.450	**0.001**	0.05 (0.01, 0.09)	**0.029**
eGFR (mL/min/1.73 m^2)	−0.336	**0.045**	0.054	0.746	−0.065	0.585		

Significant *p* value with bold; n—number of people; WC— waist circumference; SBP—sistolic blood pressure; LDL—low-density lipoprotein; eGFR—estimated glomerular filtration rate; LTM—lean tissue mass.

3. Discussion

According to our study, leptin levels in all patients were significantly influenced by their adipose tissue mass and serum testosterone regardless of the CKD stage. This suggests that the main factor influencing leptin levels in the CKD patients studied is their body fat mass [31–33] not renal function. A higher value of adipose tissue mass was linked to higher levels of leptin in the blood, which is consistent with findings from other studies. The association of elevated leptin, an anorexigenic hormone, with high ATM values suggests the possibility of leptin resistance [34]. Adipocytes secrete leptin in CKD due to several mechanisms.

First, adipose tissue can lead to hyperinsulinism, which may increase serum leptin [35]. Second, the fatty acid profile specifically altered in CKD can increase leptin gene expression in subcutaneous adipose tissue and cause hyperleptinemia. The link between fatty acid disorders in CKD and the leptin gene also explains the relationship between leptin and lipids in our dialysis patients. Such correlations have been identified in CKD and the general population [26,36–38]. Thirdly, leptin mRNA is high in adipose tissue, which can cause increased serum leptin [39]. It is noted that visceral adipose tissue is particularly associated with hyperleptinemia in CKD [33], but in the general population, it was shown that leptin is mainly produced by subcutaneous white adipose tissue [40]. We mention a positive correlation between waist circumference, a marker of visceral adipose tissue, and leptin in our chronic HD patients.

High leptin levels associated with high adipose tissue mass may favor hypogonadism in the general population and chronic renal failure. This mechanism can explain our study's inverse correlation between leptin and testosterone [39,41–44]. In fact, high leptin levels can increase estrogen levels, leading to increased aromatase activity. High aromatase activity can inhibit the hypothalamic–pituitary–gonadal axis and reduce testosterone levels [42]. In addition, in our study, HD women patients with higher ATM levels had higher leptin levels and lower muscle mass than males. The difference in circulating leptin levels between genders is known and may be explained by the higher proportion of adipose tissue and an increased production rate of leptin per unit mass of adipose tissue in women compared to men in the general population [45]. In the case of CKD patients, the findings are conflicting. Some studies show higher levels of leptin in women [46], while in diabetes

patients with nephropathy, no gender-based variations in leptin levels were observed [47]. In addition, it was noted that the impact of leptin on weight regulation differs between genders [48]. Testosterone stimulates muscle mass formation by increasing muscle protein production and promoting myoblast differentiation [41]. In our study, muscle tissue mass was a common predictor of testosterone values in the pre-dialysis and dialysis stages. Interestingly, in men with chronic HD, the only marker associated with testosterone was hemoglobin, while in pre-dialysis, none of the analyzed parameters was correlated with serum testosterone. In normal physiology, leptin and testosterone levels may not have an inverse relationship. In situations where there is a mild to moderate leptin deficiency or in states of energy deficit such as fasting, the administration of leptin in a physiological dose can restore the level of androgenic and estrogenic hormones through its effect on the hypothalamic–pituitary–gonadal axis [1,49]. This means that low testosterone levels are associated with low leptin levels. After leptin administration, an increase in testosterone levels was also observed, indicating that the two hormones are directly correlated. These effects highlight the important role of leptin in neuroendocrine regulation in situations where there is a lack of energy and where the gonadal system is suppressed to conserve energy [50] and a lack of normal hormonal signaling in CKD patients.

It is also interesting to note that in our study, the mean testosterone level was found to be higher in the dialytic stage compared to the pre-dialysis stages, with no significant differences in the number of men, especially compared to the patients in stage 3 KDIGO (Kidney Disease Improving Global Outcomes) classification. In other studies, it is emphasized that the number of patients with hypogonadism and low testosterone increases with the progression of CKD [51]. However, several aspects were observed during the analysis of our study patients. First, in stage 3, KDIGO CKD patients were significantly older, and testosterone levels declined with age [52]. Second, stage 3 patients had more abundant ATM and higher serum leptin values, and we discussed the interrelationship between these parameters above.

Testosterone positively affects male cardiovascular health in a concentration-dependent manner in the general population [53], but studies on CKD are limited [54,55]. We found that elevated testosterone levels in male HD patients are associated with low NMD values, a marker of vascular smooth muscle dysfunction, and thus not with cardiovascular protection as in the general population. This association in men, which is somewhat inconsistent with data in the general population, could be related to differences in the cellular response to testosterone in CKD. Vascular endothelial cells contain receptors for androgen hormones, and their activation may vary by sex or other conditions [56].

In addition to the relationship of leptin with adipose tissue and serum testosterone, many other factors and multiple metabolic disorders may condition leptin levels in CKD patients. We observed that serum leptin was related to markers of the inflammatory syndrome and mineral and bone metabolism in the whole group. We recorded associations between leptin, eGFR, and blood glucose in pre-dialysis CKD patients.

In our chronic HD patients, we observed a significant association between leptin levels and hs-CRP as a marker of inflammatory syndrome. We found that patients with high hs-CRP and WBC values also had high leptin values, which is consistent with other studies' findings [32]. It should be noted that the relationship between inflammatory syndrome and leptin is quite complex and warrants further analysis. On the one hand, studies have shown that in CKD, tumor necrosis factor (TNF) alpha can trigger the production and release of leptin from adipose tissue [57]. Simultaneously, other inflammatory molecules such as hs-CRP, interleukin (IL)-6, and IL-10 can also rise [31]. On the other hand, hyperleptinemia can induce the synthesis of inflammatory mediators and reactive oxygen species [58,59]. As the CKD severity rises, leptin, IL-6, and TNF alpha levels can also rise [60]. Some researchers believe leptin, TNF alpha, and IL-6 are pro-inflammatory cytokines activating the nuclear transcription factor kappa B. This activation can reduce protein synthesis and stimulate the ubiquitin-mediated proteolytic system, causing protein degradation [61]. In

addition to the one mediated by testosterone, this mechanism would explain the association of high leptin values with reduced muscle mass in CKD patients.

The leptin levels in our study were directly correlated with factors such as ATM, triglycerides, increased glucose level, and markers of the inflammatory syndrome. These correlations suggest that hyperleptinemia in CKD is associated with an increase in the risk of atherosclerosis. It was mentioned that atherosclerosis is an indirect effect of high leptin levels [62]. Surrogate markers such as NMD, FMD, and PWV are used to detect subclinical atherosclerosis. In our study, we found that chronic HD women with high leptin values had low NMD values, indicating a relationship between hyperleptinemia and vascular smooth muscle dysfunction, which has not been noted in other studies. In the general population, the reduction in ATM was found to lead to a reduction in leptin levels associated with an improvement in endothelial function (increase in FMD) but without any effect on NMD [63]. Experimental data and clinical studies in CKD indicate that leptin in high concentrations can produce endothelial dysfunction by modifying the f-actin cytoskeleton through a mechanism involving the protein kinase B/glycogen synthase kinase β (AKT/GSK3β), nitric oxide, and β-catenin pathway [64,65]. We did not record associations of serum leptin with endothelial dysfunction, but we followed only FMD and not molecules such as vascular cell adhesion protein 1 (V-CAM 1) and intercellular adhesion molecule 1 (I-CAM 1), as in other studies [64].

Apart from these links to atherosclerosis, leptin, a hormone produced by adipose tissue and osteoblasts, plays a crucial role in mineral and bone metabolism. Studies have shown that leptin can stimulate osteoblasts, which are responsible for bone restoration [13], and inhibit osteoclast differentiation [66]. This positive effect on bones is supported by research showing that leptin is associated with reduced bone turnover and improved bone mineral density in patients with end-stage renal disease [67]. In chronic HD patients, leptin levels, serum albumin, and body weight correlate positively with bone mineral density [63,68]. In the same way, our findings in chronic HD patients showed an inverse correlation between leptin and iPTH, which is a hormone that can increase bone turnover and reduce bone mineral density. However, high leptin levels in CKD are not always associated with positive effects on bone. Bone resistance to leptin in CKD has been discussed due to leptin's two modes of action: a direct stimulatory effect on bone and an opposite indirect effect via the central nervous system [66].

In the dialytic stage, inflammatory syndrome markers and mineral and bone metabolism markers, such as iPTH, significantly correlated with the leptin level. The factors associated with serum leptin in CKD in pre-dialysis stages are similar to those identified in the general population.

Leptin, a glomerular filtered and tubularly metabolized hormone, may increase in CKD patients as eGFR decreases, as shown in other studies [69,70]. In contrast, our research found that leptin levels increased with increasing eGFR and in parallel with increasing ATM. The difference between our study and other results is that we did not adjust leptin levels for age or ATM [70]. Renal function has been observed to only partially influence leptin levels in CKD patients, the main determinant being leptin overproduction in adipose tissue due to hyperinsulinemia and chronic inflammation [35,71]. In turn, leptin can influence kidney function. It can stimulate the synthesis of TGF beta, a molecule with a fibrogenesis role, causing the progression of CKD [72]. Hyperleptinemia may predict a decline in renal function in men over time regardless of the presence of diabetes or hypertension [73].

This study is significant because it highlights the connections of leptin in all phases of CKD and reveals relationships that have not been emphasized in other research. Specifically, it highlights the strong correlation of leptin with ATM and testosterone in pre-dialysis and dialysis patients, the gender variation in leptin in CKD, and the association of leptin with vascular smooth muscle dysfunction as an atherosclerosis marker in women undergoing chronic HD. The multiple connections of leptin in CKD indicate that it intersects with multiple metabolic pathways and pathogenic cascades. Future research will determine whether leptin can be a potential therapeutic target in CKD patients.

Our study has several limitations. First, it included a relatively small number of patients in each stage of CKD, so further studies are needed to confirm the interactions of leptin in this population group. Second, the study was observational by design, so the findings need to be confirmed in a prospective study. Third, there was no control group. Additionally, due to the nature of our cross-sectional data, this study was limited in interpreting causality.

4. Materials and Methods

4.1. Participants

We conducted a cross-sectional observational study involving 162 patients with CKD: 88 patients were in chronic HD treatment in Nefromed Dialysis Center Cluj-Napoca, and 74 patients were in the pre-dialysis stages of CKD in the monitorization of Cluj County Emergency Clinical Hospital Department of Nephrology. All included patients met the inclusion and exclusion criteria and signed informed consent. All procedures in the study followed institutional and national research committee ethical standards and the 1964 Declaration of Helsinki and its subsequent amendments. The Ethics Committee of "Iuliu Hatieganu" University of Medicine and Pharmacy Cluj-Napoca approved the study with IRB number 348/26 September 2017.

The inclusion criteria for pre-dialysis patients were age $\geq$ 18 years, diagnosis of CKD stage 3–5 pre-dialysis, defined according to the KDIGO [74], and no kidney transplant for at least six months, having stable renal function in the last three months (<5 mL/min/1.73 m^2 change in eGFR). The inclusion criteria for HD patients were prevalent HD patients, age > 18 years, and duration of HD at least 6 months (HD vintage). All patients were on a thrice-weekly HD (4–5 h) regimen.

Exclusion criteria for all patients were the following: acute inflammation, severe neoplasia with a life expectancy of <6 months, hepatitis virus infection, and any other serious chronic or acute diseases requiring treatment or absence of data.

4.2. Methods

Demographic data, comorbidities (diabetes, hypertension), and medication at enrollment were obtained from medical records. We also registered clinical data: age, weight, height, systolic blood pressure (SBP), and DBP. Hypertension was diagnosed according to SBP/DBP $\geq$ 140/90 mmHg, as well as the use of relevant medications. We registered laboratory data (such as urea, creatinine, sodium, potassium, calcium, phosphorus, total cholesterol, LDL-cholesterol, high-density lipoprotein (HDL) cholesterol, blood glucose, iPTH, hs-CRP, hemoglobin, WBC count, calcium, phosphorus, ferritin, albumin, alkaline phosphatase, serum bicarbonate, and serum creatinine in the pre-dialysis stage). Additionally, we have determined the levels of leptin, free testosterone, and prolactin using ELISA, with a minimum detection limit of 0.15 ng/mL for leptin and 0.06 pg./mL for testosterone.

We calculated pulse pressure (PP), PP = SBP − DBP, and BMI based on the formula BMI = weight (kg)/[height (m^2)]. Nutrition was also assessed by bioimpedance using the Body Composition Monitor, which is a certified device (manufactured by Fresenius Medical Care, Bad Homburg, Germany) that showed LTM (kg) and ATM (kg) and also calculated lean and fat tissue indexes (LTI and FTI, respectively) [75].

In addition, we measured vascular function in HD patients using markers such as NMD for vascular smooth muscle function and FMD for endothelial function. NMD and FMD were evaluated using high-resolution ultrasound: GE Logiq3 (General Electric Company, Fairfield, CT, USA). In pre-dialysis CKD patients, we evaluated the arterial stiffness expressed by PWV with the Mobil-O-Graph NG device (Medexpert Ltd., Budapest, Hungary).

Our published articles [76,77] described the procedure for measuring NMD, FMD, and PWV.

4.3. Statistical Analysis

The data were presented using the following parameters: qualitative data with absolute and relative frequencies; normally distributed quantitative data with averages and standard deviations; non-normally distributed quantitative data with medians, the 25th, and the 75th percentiles.

Data analysis procedures included conducting an ANOVA test (if normal distribution and variance equality were present) or a Kruskal–Wallis test for the comparison of four means, followed by post-doc analysis, with the use of Scheffe post-doc analysis for ANOVA and the Bonferroni correction for Kruskal–Wallis. A Chi-square test was conducted to compare four frequencies, which was followed by Bonferroni correction. For correlation analysis, the Pearson correlation coefficient was calculated for normally distributed data, and the Spearman correlation coefficient was calculated for non-normally distributed data.

Only significant or almost significant correlations were presented in tables. All the variables significantly or almost significantly correlated with leptin or testosterone were considered as independent variables for multivariate analysis. In multivariate linear analysis, the dependent variables were leptin and testosterone. Only the significant variables in the multivariate models were reported in the tables.

IBM SPSS Statistics for Windows, Version 25.0. IBM Corp., Armonk, NY, USA was used for the analysis. The significance threshold of 0.05 was taken.

5. Conclusions

Our study shows the connection between leptin, adipose tissue, and testosterone in all stages of CKD. We also note that hyperleptinemia in our CKD patients was linked to other proatherogenic factors, such as dyslipidemia and hyperglycemia in all CKD stages and with vascular smooth muscle dysfunction in chronic hemodialyzed women. Additionally, we observe the gender variation in leptin in CKD: in advanced stages of CKD, the inflammatory syndrome impacts leptin values, and leptin is associated with parathormone.

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Data Availability Statement: The research data supporting this study's findings are not publicly available. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: Author Remus Orasan and Cristian Budurea were employed by the company Nefromed Dialysis Center. The remaining authors declare that the research was conducted in the absence of any commercial or financial relation-ships that could be construed as a potential conflict of interest.

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N-Terminal Pro-Brain Natriuretic Peptide Correlates with Ghrelin and Acyl-Ghrelin in Pre-Dialysis Chronic Kidney Disease

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Abstract: Pro-B amino-terminal natriuretic peptide (NT-proBNP) is a diagnostic marker for heart failure (HF), a severe complication of chronic kidney disease (CKD). However, its significance in CKD is not clear, as other factors, such as renal function, may also have an impact. Recent studies have shown that ghrelin treatment is effective in HF in the general population, but the impact of ghrelin on cardiac function in CKD patients is still unknown. Our study aimed to investigate the factors associated with NT-proBNP in pre-dialysis CKD patients and to evaluate the correlation between NT-proBNP and ghrelin and acyl-ghrelin, molecules determined using ELISA methods. In a cross-sectional observational study, we included 80 patients with pre-dialysis CKD, with a mean age of 68 years and 50% men. The median values for NT-proBNP were 351.8 pg/mL, for acyl ghrelin 16.39 pg/mL, and for ghrelin 543.32 pg/mL. NT-proBNP was correlated with ghrelin (p = 0.034, r = 0.24), acyl-ghrelin (p = 0.033, r = −0.24), estimated glomerular filtration rate (p = 0.027, r = −0.25), serum urea (p = 0.006, r = 0.31), and ferritin (p = 0.041, r = 0.28). In multivariate analysis, ghrelin (p = 0.040) and blood urea (p = 0.040) remained significant predictors for NT-proBNP levels. NT-proBNP was a significant predictor for acyl-ghrelin (p = 0.036). In conclusion, in pre-dialysis CKD patients, a high value of NT-proBNP was associated with a high value of total ghrelin and a low value of acyl-ghrelin.

Keywords: heart failure; biomarkers; pro-brain natriuretic peptide; ghrelin; acyl-ghrelin; chronic kidney diseases

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1. Introduction

Chronic heart failure (HF) affects 25% of pre-dialysis chronic kidney disease (CKD) patients, and its prevalence increases as the disease progresses, reaching up to 65–70% in end-stage renal disease [1], and it has a major impact on patient survival [2]. It is essential to assess the diagnostic and prognostic biomarkers for HF in CKD patients. Additionally, it is crucial to propose novel treatment options for this disease. Natriuretic peptides are molecules linked to myocardial dysfunction and are known as biomarkers. Among them, B-type natriuretic peptide (BNP) and pro-B amino-terminal natriuretic peptide (NT-proBNP) are commonly tested [3] and are important indicators for diagnosis, prognosis,

and treatment monitoring in chronic HF. NT-proBNP is superior to BNP as a prognostic value [4–7]. After being secreted from the coronary sinus, BNP and NT-proBNP are either enzymatically degraded by neutral endopeptidase and dipeptidyl peptidase-4 or excreted by the kidneys [8]. NT-proBNP is mainly cleared through glomerular filtration by the kidney, which explains the strong correlation between renal function and NT-proBNP levels [9]. In patients with CKD, besides heart and renal failure, other factors, such as hyperhydration, left ventricular hypertrophy, anemia [3,10], age, inflammation, and malnutrition, can increase the levels of NT-proBNP [11–15]. In these patients, the ratio between the cardiac secretion of NT-proBNP linked to different stimuli and the retention of NT-proBNP due to renal dysfunction is unknown [16]. Thus, the level of NT-proBNP has a lower specificity and sensitivity for the diagnosis of acute HF in CKD 3–5 stages [17]. Fu et al. suggest that NT-proBNP depends on cardiac function in CKD patients with an estimated glomerular filtration rate (eGFR) of 45–60 mL/min/1.73 m^2, while for an eGFR < 45 mL/min/1.73 m^2, it depends on renal function [18]. There are no established NT-proBNP values for diagnosing HF in patients with CKD [19]. However, NT-proBNP is an independent predictor of mortality in CKD patients, and it can also be useful for cardiovascular risk stratification in these patients [20], being significantly associated with the risk of incident HF [21].

The high prevalence and severity of HF in pre-dialysis CKD patients may be explained by hormonal imbalances (such as ghrelin, prolactin, etc.) and the specific inflammatory syndrome associated with CKD. In this case, certain molecules that act as markers of HF in CKD may be linked to hormone levels, such as ghrelin or prolactin levels. Identifying such associations could have therapeutic implications, since ghrelin treatment is effective for HF in the general population, and perhaps we can use this treatment in CKD patients as well.

Ghrelin is an appetite-stimulating hormone secreted in the small intestine and stomach in response to fasting and weight loss, and its activation involves the acylation of amino acid 3 [22–24]. There are two major forms of circulating ghrelin: acyl-ghrelin, which is orexigenic, and des-acyl ghrelin, which has possible anorexigenic effects [25–29]. Ghrelin is primarily metabolized and excreted by the kidneys. Several studies have found that patients with CKD have higher levels of circulating ghrelin than the general population. This is because the kidneys are less able to break down ghrelin in people with CKD. Other factors, such as nutritional status, inflammation, age, and sex, can also influence ghrelin levels in CKD patients [30–32]. Ghrelin has several functions, including carbohydrate and energy metabolism and gastrointestinal, cardiovascular, pulmonary, and immune functions. It can also stimulate osteoblast proliferation and bone formation [33]. Reduced levels of total ghrelin and acyl-ghrelin in end-stage kidney disease patients are associated with higher rates of mortality and cardiovascular morbidity, particularly when combined with inflammatory and nutritional markers [34,35]. Studies on chronic hemodialysis patients indicate that higher levels of acyl-ghrelin are associated with higher body mass index (BMI) and better survival, regardless of appetite, nutritional status, and inflammation [36]. High levels of NT-proBNP were correlated with elevated levels of ghrelin in obese dialysis patients [37] and with low levels of acyl-ghrelin in male hemodialysis patients [38]. To the best of our knowledge, no studies have examined the relationship between ghrelin/acyl-ghrelin and NT-proBNP in pre-dialysis CKD stages.

In CKD patients, the level of prolactin is higher than in the general population. This is due to reduced renal elimination and increased production caused by decreased sensitivity to dopaminergic inhibition [39]. Hyperprolactinemia has been associated with general and cardiovascular morbidity and mortality in CKD patients [40,41].

Among the markers of inflammation associated with cardiovascular diseases in pre-dialysis CKD patients, the role of cytokines such as interleukin 1 beta (IL-1 beta) [42] is known, but their relationship with NT-proBNP or hormonal status has not been studied.

This study aimed to evaluate the factors that can influence the level of NT-proBNP in pre-dialysis CKD patients, especially the relationship between NT-proBNP and endogenous ghrelin, and acyl-ghrelin levels.

2. Results

2.1. Patients' Characteristics

We recorded clinical and laboratory data for patients with CKD, and we observed an equal number of women: men, 1/3 patients with diabetes, a mean age over 65 years, and almost all patients with hypertension. Parameters that may influence cardiovascular disease in CKD patients were also recorded. These included blood pressure values; nutritional markers such as adipose tissue mass, lean tissue mass, body mass index (BMI), serum albumin, lipid fractions, and fasting glucose; markers of mineral and bone metabolism, including serum calcium, phosphorus, alkaline phosphatase, and intact parathormone; markers of the inflammatory syndrome; the level of hemoglobin; and renal function expressed by the estimated glomerular filtration rate. Hormonal markers, including NT-proBNP, were also studied.

Demographical, clinical, and laboratory patient characteristics are presented in Table 1.

Table 1. Characteristics of participants (n = 80), arithmetic mean ± standard deviation/median (25th; 75th percentile).

Parameter	Group (n = 80)
Age (years)	68 (62; 75)
Male, n (%)	40 (50.0)
Diabetes mellitus, n (%)	32 (40.0)
Hypertension, n (%)	65 (88.8)
SBP (mmHg)	144 (126; 162)
DBP (mmHg)	86.89 ± 12.65
PP (mmHg)	59 (45; 73)
eGFR (mL/min/1.73 m^2)	27 (15; 39.5)
Body mass index (kg/m^2)	28.65 (26.55; 30.75)
Lean tissue mass (kg)	35 (27.05; 43.6)
Adipose tissue mass (kg)	40.9 (34.25; 47.25)
Total cholesterol (mg/dL)	173 (153.5; 196.5)
LDL cholesterol (mg/dL)	100.16 ± 30.64
HDL cholesterol (mg/dL)	40 (33; 49.5)
Triglycerides (mg/dL)	133 (93; 169)
Fasting glucose (mg/dL)	103 (92; 133.5)
Serum bicarbonate (mmol/L)	19.6 (17.7; 22.3)
Calcium (mg/dL)	9.17 (8.61; 9.61)
Phosphorus (mg/dL)	3.62 (3.1; 4.52)
iPTH (pg/mL)	118.3 (87.5; 207)
Alkaline phosphatase (UI/L)	84 (72; 104)
Hemoglobin (g/dL)	12.27 ± 2.39
Serum albumin (g/L)	3.82 (3.51; 4.19)
Urea (mg/dL)	85 (56.5; 114.5)
Ferritin (ng/mL)	93 (53; 200)
hs-CRP (mg/dl)	0.45 (0.22; 1.19)
White blood cells (no./mm^3)	7642.83 ± 2322.96
IL-1 beta (pg/mL)	6.92 (6.38; 12.49)
NT-proBNP (pg/mL)	351.8 (232.77; 610.59)
Ghrelin (pg/mL)	543.32 (270.69; 857.88)
Acyl-ghrelin (pg/mL)	16.39 (14.04; 24.85)
Prolactin (ng/mL)	5.64 (3.66; 9.06)
Angiotensin-converting enzyme inhibitor/angiotensin receptor blocker, n (%)	36 (45)

SBP: systolic blood pressure; DBP: diastolic blood pressure; PP: pulse pressure; eGFR: estimated glomerular filtration rate; iPTH: intact parathyroid hormone; hs-CRP: high-sensitivity C-reactive protein; IL-1 beta: interleukin-1 beta; NT-proBNP: amino-terminal pro-B-type natriuretic peptide; no.: number.

All patients were Caucasian. No patient included was under angiotensin receptor/neprilysin inhibitor (ARNI) medication.

2.2. Determinants of NT-proBNP

High values of NT-proBNP were statistically significantly associated with high values of ghrelin (shown in Figure 1), high levels of blood urea, high levels of ferritin, and high values of LDL cholesterol, and near statistically significantly correlated with high prolactin values. Low levels of NT-proBNP were statistically significantly associated with high levels of acyl-ghrelin (shown in Figure 2) and with high levels of eGFR (Table 2). In the multivariate analysis, it was noted that only ghrelin levels and blood urea remained significantly associated with NT-proBNP (Table 2).

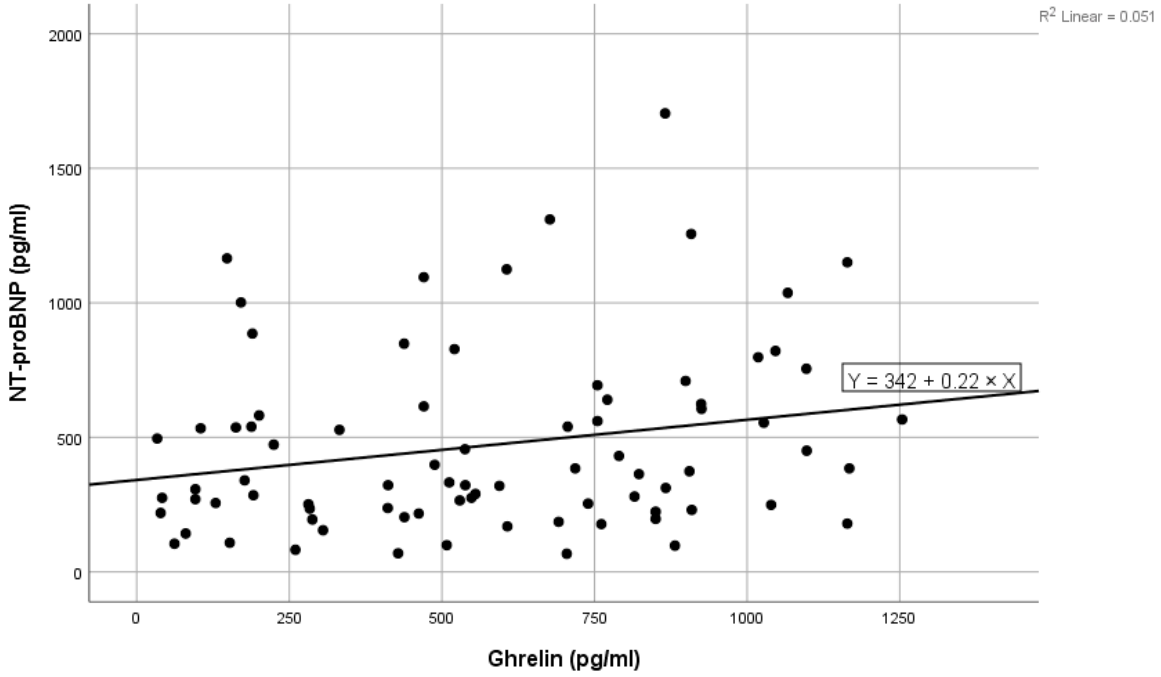

Figure 1. Positive linear correlation between NT-proBNP and ghrelin in the total group.

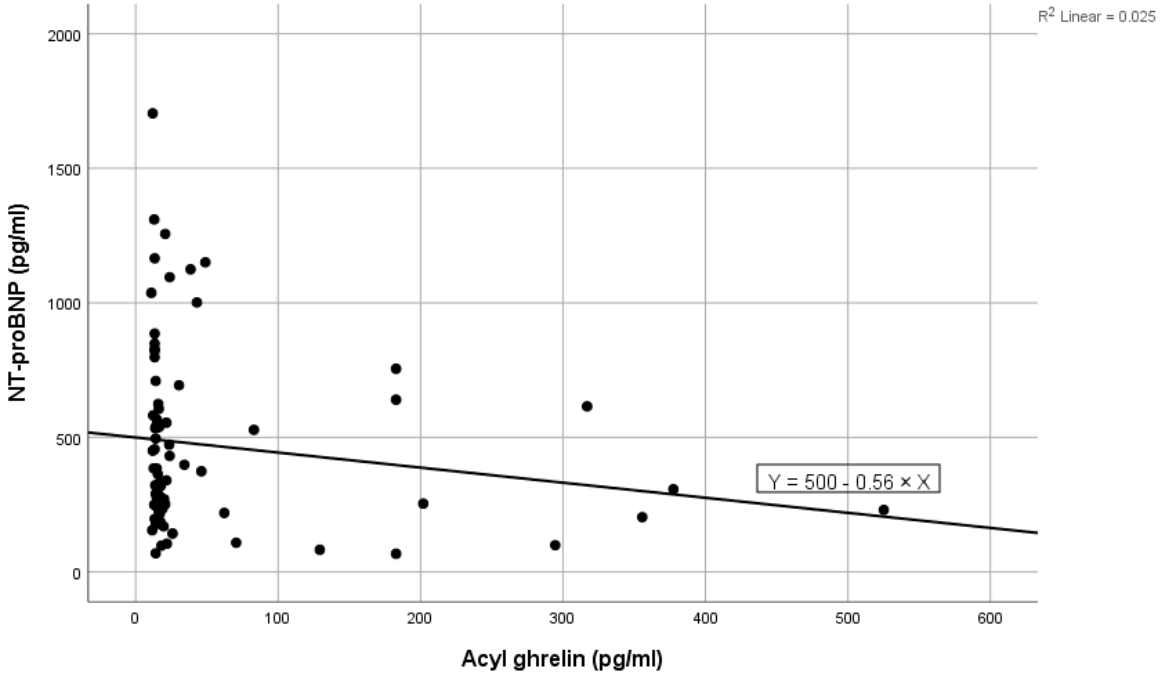

Figure 2. Negative linear correlation between NT-proBNP and acyl-ghrelin in the total group.

Table 2. The NT-proBNP correlation with other parameters.

Parameter	Univariate Analysis		Multivariate Analysis	
	Coefficient of Correlation	*p*	B Coefficient 95% CI	*p*
Ghrelin (pg/mL)	0.24	0.034	0.30 (0.02; 0.59)	0.040
Acyl-ghrelin (pg/mL)	−0.24	0.033	-	-
Prolactin (ng/mL)	0.21	0.068	-	-
eGFR (mL/min/1.73 m^2)	−0.25	0.027	-	-
Blood Urea (mg/dL)	0.31	0.006	2.35 (0.11; 4.59)	0.040
Ferritin (ng/mL)	0.28	0.041	-	-
LDL cholesterol (mg/dL)	0.36	0.012	-	-

eGFR: estimated glomerular filtration rate; CI: confidence interval.

Using receiver operator curve analysis for NT-proBNP, the concentration of 287.35 pg/mL (the area under the curve = 0.65, 95% confidence interval (CI) 0.52–0.78, $p = 0.029$, sensitivity = 0.75, specificity = 0.55) was identified as the optimal cut-off value in relation with eGFR (=30 mL/min/1.73 m^2). In conclusion, when comparing NT-proBNP for the group with eGFR < 30 mL/min/1.73 m^2, statistically significantly more subjects had NT-proBNP > 287.35 pg/mL than those in the group with eGFR $\geq$ 30 mL/min/1.73 m^2 with NT-proBNP > 287.35 pg/mL [33 (75%) vs. 13 (44.8%), $p = 0.009$].

2.3. Determinants of Ghrelin

Regarding ghrelin in the analysis of correlations, it was observed that high values of ghrelin were statistically significantly associated with high values of BMI, adipose tissue mass, triglycerides, fasting glucose, and prolactin. Low levels of ghrelin were statistically significantly associated with lean tissue mass, HDL cholesterol, eGFR, and DBP (Table 3). In the multivariate analysis, it was noted that triglycerides and BMI levels remained significantly associated with ghrelin (Table 3).

Table 3. The ghrelin correlation with other parameters.

Parameter	Univariate Analysis		Multivariate Analysis	
	Coefficient of Correlation	*p*	B Coefficient 95% CI	*p*
Body mass index (kg/m^2)	0.44	<0.001	5.67 (0.18; 11.17)	0.043
Adipose tissue mass (kg)	0.46	<0.001	-	-
Lean tissue mass (kg)	−0.35	0.006	-	-
Triglycerides (mg/dL)	0.32	0.022	0.47 (0.51; 0.88)	0.029
HDL cholesterol (mg/dL)	−0.31	0.039	-	-
Fasting glucose (mg/dL)	0.29	0.021	-	-
eGFR (mL/min/1.73 m^2)	−0.23	0.043	-	-
Prolactin (ng/mL)	0.32	0.004	-	-
NT-proBNP (pg/mL)	0.24	0.034	-	-
DBP (mmHg)	−0.35	0.004	-	-

eGFR: estimated glomerular filtration rate; NT-proBNP: amino-terminal pro-B-type natriuretic peptide; DBP: diastolic blood pressure; CI: confidence interval.

2.4. Determinants of Acyl-Ghrelin

For high acyl-ghrelin, we noted correlations with low levels of NT-proBNP and with high levels of IL-1 beta, triglycerides, triglycerides, and serum bicarbonate (Table 4). The multivariate analysis showed that only NT-proBNP remained significantly associated with acyl-ghrelin (Table 4).

Table 4. The acyl-ghrelin correlation with other parameters.

Parameter	Univariate Analysis		Multivariate Analysis	
	Coefficient of Correlation	*p*	B Coefficient 95% CI	*p*
NT-proBNP (pg/mL)	−0.24	0.033	0.24 (0.02, 0.46)	0.036
IL-1 beta (pg/mL)	0.94	<0.001	-	-
Triglycerides (mg/dL)	0.29	0.044	-	-
Serum bicarbonate (mmol/L)	0.47	0.015	-	-

NT-proBNP: amino-terminal pro-B-type natriuretic peptide; IL-1 beta: interleukin-1 beta; CI: confidence interval.

3. Discussion

Our research found that ghrelin, acyl-ghrelin, prolactin, eGFR, blood urea, ferritin, and LDL cholesterol were correlated with NT-proBNP levels in pre-dialysis CKD patients. After performing multivariate analysis, two molecules, ghrelin and blood urea, remained significant predictors for NT-proBNP. As far as we know, associations between NT-proBNP and ghrelin have not been described previously in this group of patients. The relationship between ghrelin and NT-proBNP has been observed in the general population, but the data are contradictory. In one study, it was noted that high NT-proBNP values were associated with hyperghrelinemia in the elderly and that hyperghrelinemia was associated with severe HF assessed by ultrasound [43]. In another study, researchers found a correlation between high ghrelin levels and low NT-proBNP levels, indicating no association with HF [44]. None of the studies specified which form of ghrelin was being studied: acyl-ghrelin, des-acyl ghrelin, or total ghrelin. This lack of specificity may be a cause of conflicting data. It appears that the acetylated form is required for ghrelin activity [45]. According to a recent review by Hosoda, there are multiple molecules derived from ghrelin, each with a different number of fatty acids. These molecules have been found to inhibit sympathetic activity, stimulate parasympathetic activity, and improve cardiac function in patients with HF by working through growth hormone and insulin growth factor-1 [46]. Not only does ghrelin act through growth hormones, but receptors for ghrelin have also been discovered in the cardiovascular system [47,48]. Experimental studies have shown that ghrelin has a vasodilator effect by acting on calcium-sensitive potassium channels [49] and that ghrelin produces vasodilation on isolated blood vessels precontracted with endothelin [50]. In other research where total ghrelin and acyl-ghrelin were distinguished, the authors noted that total ghrelin can have a maladaptive effect, promoting adipose tissue growth and glucose intolerance [51,52]. In pre-dialysis CKD patients, such an effect may be relevant. In our study, we found that high levels of total ghrelin were associated with high BMI, high adipose tissue mass, high levels of glucose, high levels of triglycerides, high levels of prolactin, reduced levels of HDL cholesterol, and reduced muscle mass, and thus with cardiovascular risk factors. BMI and triglyceride levels were the most significant factors in determining total ghrelin levels in our study. All these correlations of total ghrelin suggest that high levels of total ghrelin are linked to an increased risk of cardiovascular disease. In addition, we observed that total ghrelin levels tend to increase with decreasing kidney function, whereas eGFR does not affect acyl-ghrelin levels.

The prolactin mentioned above was directly correlated with ghrelin and other atherosclerosis risk factors in our study. It can regulate vessel formation and cardiac remodeling [38], leading to disrupting cardiac angiogenesis, HF, and increasing mortality [53].

As mentioned earlier, the level of NT-proBNP in our study was associated with the blood urea level. In fact, in CKD, the blood urea level is not only a marker of renal function, such as eGFR, but can also be influenced by factors such as appetite, the presence of a hypercatabolic state due to metabolic acidosis, and inflammatory syndrome [54]. Concerning ferritin, it is considered a marker of inflammatory syndrome besides its role in iron metabolism, and probably as an inflammatory marker, it is directly correlated with NT-proBNP in our study, similar to other data in the literature [55].

Regarding acyl-ghrelin, it is known that it has a cardiovascular protective effect due to its antioxidant and anti-inflammatory properties [45]. In our study, we observed that it was linked to inflammatory mediators such as IL-1 beta, but not to classic ones such as high-sensitivity C-reactive protein (hs-CRP). Similarly, previous research conducted on chronic HD patients did not identify any connections between acyl-ghrelin and hs-CRP, TNF alpha, or IL-6 [56]. It was observed in our study that acyl-ghrelin was directly correlated with IL-1 beta; we consider it possible through a compensatory mechanism. It is known that chronic inflammation can affect ghrelin levels in humans and rats. In the rat model of adjuvant-induced arthritis, a compensatory variation in ghrelin level was observed. Similar findings were recorded in patients with rheumatoid arthritis [57]. On the other hand, ghrelin administration can inhibit the expression of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, which are induced by leptin in human T lymphocytes. It appears that ghrelin and leptin are part of a regulatory network that controls immune cell activation and inflammation. Additionally, ghrelin has potent anti-inflammatory effects and acts as a key signal linking the metabolic axis with the immune system [58].

Low acyl-ghrelin levels were also associated with low levels of serum bicarbonate and therefore with metabolic acidosis, another cardiovascular risk factor in CKD. Acidosis increases endothelin-1 and aldosterone production, furthering CKD progression and cardiovascular pathology [59]. Additionally, our study found that higher levels of acyl-ghrelin are linked to lower levels of NT-proBNP, which suggests that high acyl-ghrelin is linked to good cardiac function. NT-proBNP was found to be the primary determinant of acyl-ghrelin levels in our study, highlighting the importance of the relationship between these two molecules in pre-dialysis CKD patients. Recent studies have shown that administering synthetic acyl-ghrelin can increase cardiac output in individuals with HF and reduce ejection fraction, without significant side effects in the general population [60,61]. Knowing these molecular mechanisms, the relationships that we identified could be the premises of a new treatment for HF in CKD. We have shown that reduced acyl-ghrelin values are associated with increased NT-proBNP values. NT-proBNP is a biomarker of HF with a major predictive role for cardiovascular disease, even in CKD patients. Therefore, the administration of synthetic acyl-ghrelin as a medication can also be discussed to improve cardiac function in these patients. Acyl-ghrelin receptors are widely distributed in cardiac and skeletal muscle and endothelium [47]. In rat HF models, ghrelin increased cardiac output and fractional contractility [62] in a load-independent fashion and without Ca^{2+} mobilization [60].

Despite the potential benefits in managing cardiovascular disease that we discussed above in patients with CKD, determining the cut-off value of NT-proBNP for HF diagnosis remains a challenge. Although the current data show that elevated NT-proBNP levels in pre-dialysis and dialysis patients mainly indicate cardiovascular disease and are linked to the risk of future cardiovascular events in CKD [63–65], the diagnostic value of elevated NT-proBNP provides moderate or no prediction of heart failure in CKD patients, especially in advanced stages [66,67]. There are no recommendations in the guidelines for cut-off values of NT-proBNP for HF diagnosis in different stages of CKD. Studies have shown that cut-off values for NT-proBNP are greater in CKD [68] and increase as CKD progresses to stage 5, reaching thousands in dialysis patients [69–71]. In fact, elevated levels of NT proBNP in CKD may also indicate a high risk of CKD progression in advanced stages [72,73]. In our study, operator curve analysis yielded a 287.35 pg/mL cutoff for NT-proBNP for eGFR less than or greater than 30 mL/min/1.73 m^2, double the normal laboratory limit of 125 pg/mL. However, we did not look at the advanced stages of CKD.

The results of this study are medically significant, and we can highlight several aspects. Firstly, our study has identified certain molecules that can affect NT-proBNP levels in pre-dialysis CKD patients. Secondly, the study sheds light on the relationship between acyl-ghrelin and NT-proBNP and suggests a new molecular mechanism for HF in CKD. Furthermore, recent studies have shown that acyl-ghrelin administration as medication could improve cardiac function in HF in the general population. In this context, the

relationship identified in our study between acyl-ghrelin and NT proBNP could be a basis for acyl-ghrelin treatment in HF in pre-dialysis CKD patients. Additionally, we have remarked on associations that have not been published before in this group of patients. These findings could contribute to better management of HF in CKD patients.

The study has several limitations. First, it included a relatively small number of patients, so further studies are needed to confirm the correlations and associations between NT-proBNP and ghrelin/acyl-ghrelin levels. Second, there was no control group. Third, the study was observational by design, so the findings need to be confirmed in a prospective interventional study. Fourth, due to the nature of our cross-sectional data, this study was limited in interpreting causality.

4. Materials and Methods

4.1. Participants

We performed a cross-sectional observational study including patients with pre-dialysis CKD. Of the 82 patients randomized in the Cluj County Emergency Clinical Hospital Department of Nephrology, 80 met the inclusion and exclusion criteria after giving written informed consent. All procedures in the study followed institutional and national research committee ethical standards and the 1964 Declaration of Helsinki and its subsequent amendments.

The inclusion criteria were age $\geq$ 18 years, diagnosis of CKD stage 3–5 pre-dialysis, and no kidney transplant for at least six months defined according to the Kidney Disease Improving Global Outcomes guidelines [74], having stable renal function in the last three months (<5 mL/min/1.73 m^2 change in eGFR), without changes in cardiac medication in the same period.

The exclusion criteria were the following: acute inflammatory processes, severe neoplasia with a life expectancy of <6 months, chronic or acute diseases that require medication changes, or absence of data. Demographic data, comorbidities (diabetes, hypertension), and medication at enrollment were obtained from medical records. We also registered clinical data: age, weight, height, systolic blood pressure (SBP), and diastolic blood pressure (DBP). Hypertension was diagnosed according to SBP/DBP $\geq$ 140/90 mmHg as well as the use of relevant medications. We calculated the pulse pressure (PP) using the formula

$$PP = SBP - DBP \text{ (mmHg)}. \quad (1)$$

4.2. Anthropometric Nutritional Parameters Assessment

Body mass index was calculated using the formula

$$BMI = \text{weight (kg)}/\text{height}^2 \text{ (m}^2\text{)}. \quad (2)$$

In addition, nutritional status was assessed by bioimpedance using a certified device (manufactured by Fresenius Medical Care, Bad Homburg, Germany), Body Composition Monitor, which recorded lean tissue mass (kg) and adipose tissue mass (kg) [75].

4.3. Laboratory Parameters

All laboratory data were collected between 7:00 and 9:00 a.m. after an overnight fast. Current measurements at baseline included serum electrolytes, albumin, urea, creatinine, lipid profile (total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol), blood glucose, inflammatory markers (high-sensitivity C-reactive protein (hs-CRP), ferritin), intact parathyroid hormone (iPTH), hemoglobin, and white blood cells).

The current laboratory data were recorded, and the methods used for determination were the following: serum electrolytes using potentiometry, albumin, urea, creatinine, lipid profile (total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol), blood glucose, ferritin using spectrophotometry, high-sensitivity C-reactive protein (hs-CRP) using latex immunoturbidimetry, intact parathyroid hormone (iPTH) using chemiluminescence, and hemoglobin and white blood cells using impedance spectroscopy, spectrophotometry, and

flow cytometry. Interleukin-1 beta, NT-proBNP, ghrelin, and acyl-ghrelin were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available kits similar to those used in other studies (R&D System, Minneapolis, MN, USA). IL-1 beta, NT proBNP, and ghrelin were measured in serum and acyl ghrelin in plasma. For total ghrelin, the catalog number was DY8149-05, and the intra-assay coefficient of variation was 3.6%; for acyl-ghrelin, the catalog number was RA194062400R, and the intra-assay coefficient of variation was 6.9%; for IL1-beta, the catalog number was RAF048R, and the intra-assay coefficient of variation was 5.1%; for prolactin, the catalog number was DKO011 and the intra-assay coefficient of variation was $\leq$4.0%; for NT proBNP the catalog number was MBS355233. The minimum detectable levels were as follows: less than 6.1 pg/mL for IL-1 beta, 7.8 pg/mL for NT-proBNP, 1.5 ng/mL for prolactin, 100 pg/mL for ghrelin, and 10 pg/mL for acyl-ghrelin. We used the online equation for eGFR based on creatinine, age, sex, and a coefficient for race [76].

4.4. Statistical Analysis

Two patients with NT-proBNP > 4000 were excluded; the respective values were considered input errors. The data of 82 patients were analyzed.

The variables measured on quantitative scales were described using the mean or median if they did not follow the normal distribution. The correlation was reported after calculating the Pearson and Spearman correlation coefficients. The Pearson correlation coefficient was reported for linear relationships, and the Spearman correlation coefficient was reported for non-linear ones.

For the variables correlated with NT-proBNP, a cut-off was found to be related to the median NT-proBNP with the help of receiver operator curves. The cut-off was considered to be the value for which the receiver operator curve had the sum of maximum sensitivity and specificity. For each cut-off, the area under the curve, lower and upper limits of the 95% confidence interval (CI) of the area under the curve, sensitivity, and specificity were reported. The same procedure was followed for the cut-off NT-proBNP in relation to the median of the other variables correlated with it.

Multivariate analysis was performed using linear regression. All significantly correlated or almost significantly correlated quantitative variables in the multivariate linear analysis were analyzed.

Two-sided *p*-values were considered. The level of statistical significance was considered to be $\alpha = 0.05$. Data analysis was performed using SPSS 25.00 version.

5. Conclusions

In conclusion, in pre-dialysis CKD patients, total ghrelin and blood urea levels were found to be significant predictors of NT-proBNP level. High NT-proBNP values were associated with low acyl-ghrelin values. Increased ghrelin levels were linked with proatherogenic markers, while decreased acyl-ghrelin values were associated with metabolic acidosis and low IL-1 beta. NT-proBNP was a significant predictor for acyl-ghrelin level in our patients.

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Triiodothyronine and Protein Malnutrition Could Influence Pulse Wave Velocity in Pre-Dialysis Chronic Kidney Disease Patients

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Abstract: Cardiovascular diseases (CVD) are the first cause of chronic kidney disease (CKD) mortality. For personalized improved medicine, detecting correctable markers of CVD can be considered a priority. The aim of this study was the evaluation of the impact of nutritional, hormonal and inflammatory markers on brachial-ankle Pulse Wave Velocity (PWV) in pre-dialysis CKD patients. A cross-sectional observational study was conducted on 68 pre-dialysis CKD patients (median age of 69 years, 41.2% with diabetes mellitus, 52.9% male). Laboratory data were collected, including levels of prolactin, triiodothyronine, TGF α, IL-6, and IL-1β. The high values of brachial-ankle PWV were associated with reduced muscle mass ($p = 0.001$, $r = -0.44$), low levels of total cholesterol ($p = 0.04$, $r = -0.26$), triglycerides ($p = 0.03$, $r = -0.31$), triiodothyronine ($p = 0.04$, $r = -0.24$), and prolactin ($p = 0.02$, $r = -0.27$). High PWV was associated with advanced age ($p < 0.001$, $r = 0.19$). In the multivariate analysis, reduced muscle mass ($p = 0.018$), low levels of triiodothyronine ($p = 0.002$), and triglycerides ($p = 0.049$) were significant predictors of PWV, but age ($p < 0.001$) remained an important factor. In conclusion, reduced triiodothyronine together with markers of malnutrition and age were associated with PWV in pre-dialysis CKD patients.

Keywords: pulse wave velocity; chronic kidney disease; malnutrition; inflammation triiodothyronine; prolactin

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1. Introduction

High cardiovascular risk in chronic kidney disease (CKD) patients is associated with accelerated atherosclerosis, endothelial dysfunction, and arterial stiffness (AS) and it has major consequences on survival and quality of life. Arterial stiffness is a negative prognostic factor for CKD progression [1] and for associated cardiovascular diseases, contributing to the increase in medical services costs [2–4]. Previous studies have suggested that therapeutic modification of AS can improve cardiovascular mortality in CKD [5,6]. AS was influenced by angiotensin-converting enzyme (ACE) inhibitors/angiotensin II receptor blockers (ARB), vitamin D in the pre-dialysis CKD patients [6,7], and by hypotensive medication combined with the reduction of calcium in the dialysis solution in hemodialysis

patients [8]. A relationship between increased AS and declining kidney function was shown [8].

According to experts, the PWV remains a standard parameter for the assessment of AS [9,10]. There are different devices for measuring PWV, based on tonometry, oscillometry, and magnetic resonance [11].

AS is characterized by chronic structural modifications in the arterial wall expressed by elastin fragmentation and media calcification, but molecular changes in the intimal layer may also occur through the atherosclerotic inflammatory process [12,13].

Hyperphosphatemia, the fluctuations of calcium such as hyper and hypocalcemia, hyperparathyroidism, the reduction of alpha Klotho, and the increase in FGF-23 are the main determinants of AS in CKD. Classic cardiovascular risk factors also intervene: hypertension, obesity, dyslipidemia, advanced age (through decreased endothelium nitric oxide availability and increased production of vasoconstrictors), diabetes mellitus (DM), and hyperuricemia [10–12]. In addition, advanced glycation end products (AGEs) that accumulate in CKD activate Nuclear Factor Kappa B, favoring the activation of the vascular inflammatory cascade and promoting the vessel's stiffening by stimulating fibrosis and proliferation of the vascular smooth muscle cells [14,15]. There was also an association of serum glucose concentrations with PWV, independent of the diabetic status [16].

In fact, AS in CKD is based on an enormously increased cardiovascular risk due to, on the one hand, the additional cardiovascular risk factors (oxidative stress, protein malnutrition, alteration of the phospho-calcium balance, etc.) and, on the other hand, due to certain particularities of the classic cardiovascular risk factors such as the appearance of the reverse epidemiology phenomenon [17]. It is well known that malnutrition and inflammation are associated with atherosclerosis (malnutrition inflammation atherosclerosis syndrome) in these patients [18], and it can be a major determinant of vascular stiffness in CKD. There are few data in the literature about the correlation between nutritional markers and PWV in pre-dialysis and dialysis CKD patients. Thus, in a study that evaluated pre-dialysis CKD patients from Korea, it was shown that reduced muscle mass was associated with high brachial-ankle PWV [19]. In addition, another study revealed that hydration status and blood pressure might be major determinants of PWV in hemodialysis patients [20], while in peritoneal dialysis patients a significant association between nutritional markers and PWV was described, suggesting that malnutrition could be the major contributor to vascular dysfunction [21]. It was noted that body mass index (BMI), body fat mass, waist-hip ratio, abdominal circumference, neck circumference, and visceral fat are positively correlated with PWV in the general population [22].

There are also studies that have shown that the hormonal changes occurring in CKD could also influence cardiovascular morbidity and AS. Prolactin is a hormone which is considered as a uremic toxin by some authors. It accumulates with loss of renal function, and it is associated with cardiovascular diseases in the general population and CKD population as well [23]. Hyperprolactinemia is implicated in biological processes such as insulin resistance, metabolic syndrome, inflammation modulation, endothelial dysfunction, and lastly, accelerated atherosclerosis [24,25]. A 27% increased risk of cardiovascular events was observed for each 10 ng/mL prolactin elevation in non-dialysis CKD patients [26].

The presence of subclinical hypothyroidism was also recorded in CKD. It was associated with general mortality in advanced CKD [27]. Low triiodothyronine levels are the most common laboratory finding followed by subclinical hypothyroidism in CKD patients. Hypothyroidism can cause vascular calcification and endothelial damage [28,29].

Currently, it is still not clear how prolactin and triiodothyronine influence cardiovascular diseases in CKD, and if they affect PWV, in fact, what are the most important factors that influence PWV in pre-dialysis CKD patients.

That is why the aim of this study was to evaluate the impact of some inflammatory, nutritional, and hormonal markers on PWV in pre-dialysis CKD patients, and as a second aim in the subgroup of the patients with diabetes.

2. Materials and Methods

2.1. The Participants

We conducted a cross-sectional observational study on a cohort of pre-dialysis CKD patients. The patients were selected from those admitted to the Department of Nephrology, County Clinical Emergency Hospital Cluj, and taken into this study based on the inclusion and exclusion criteria. All patients provided written informed consent. The study methodology was in accordance with institutional and national research ethical standards and with the 1964 Helsinki Declaration and its subsequent amendments.

Inclusion criteria were the following: patients aged $\geq$ 18 years, diagnosed with CKD for at least 6 months, defined according to Kidney Disease: Improving Global. Outcomes (KDIGO) guidelines, with estimated glomerular filtration rate (eGFR) less than 60 mL/min, (predialytic stage), having a stable renal function during 3 months prior to study (change in eGFR < 5 mL/min/1.73 m^2), and no change in medication during the same 3 months.

The exclusion criteria were the following: cancer patients with a life expectancy <6 months, acute inflammatory diseases, terminal neoplasia, hepatitis viral infection, and any other chronic or acute diseases that required changes in treatment during 3 months prior to study.

The patient's clinical data: age, weight, height, systolic blood pressure (SBP), and diastolic blood pressure (DBP), comorbidities (diabetes, hypertension) and the medication data were registered. The diagnosis of hypertension was established on the basis of BP values, namely SBP/DBP $\geq$ 140/90 mmHg as well as on the basis of the use of hypotensive drugs. We calculated pulse pressure (PP) as the difference between the SBP and DBP.

2.2. Evaluation of Anthropometric Parameters

In addition to body mass index (BMI), nutritional status was assessed by bioimpedance using the Body Composition Monitor, a certified device (manufacturer by Fresenius Medical Care, Bad Homburg, Germany) which provided body composition as follows: lean tissue mass (LTM) (kg), and adipose tissue mass (ATM) (kg) [30].

2.3. Laboratory Parameters

Blood samples were collected in the morning after 8 h fasting. Serum electrolytes, albumin, creatinine, lipid profile, inflammatory markers, intact parathormone (iPTH) and the medular response (hemoglobin and white blood cells) were determined. Serum IL-6, IL-1β, TNF-α, prolactin and triiodothyronine were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (R & D System, Minneapolis, MN, USA). The minimum detection limit for TNF-α was 15.6 pg/mL, for IL-6–3.2 pg/mL, for IL-1β–10.2 pg/mL, for prolactin 1.5 ng/mL, and for triiodothyronine < 0.1 ng/mL). Low-density lipoprotein-cholesterol (LDL-cholesterol) was calculated according to the Friedewald formula: LDL cholesterol = total cholesterol-(HDL-cholesterol + triglycerides/5).

2.4. Assessment of Arterial Stiffness

Brachial-ankle PWV was evaluated to assess arterial stiffness with the Mobil-O-Graph NG device (Medexpert Ltd., Budapest, Hungary), based on an oscillometric method. The device gave the augmentation pressure, augmentation index, central SBP, central DBP, and PWV. Brachial BP [31] was initially recorded, then the cuff was automatically re-inflated above DBP for approximately 10 s and brachial pulse waves were recorded with a high-fidelity pressure sensor (MPX5050, Freescale Halbleiter Deutschland GmbH, Muenchen, Germany). Brachial BP was used to calibrate the pulse waveform. Finally, the aortic pulse wave form was reconstructed by the software (HMS version 5.1) using an ARCSolver algorithm [32,33]. The aortic pulse wave was decomposed into forward traveling (incident) and backward traveling (reflected) pulse waves for wave separation analysis. PWV was estimated by mathematical models based on the characteristic impedance and age and assuming a three-element Windkessel model [32,33].

2.5. Statistical Analysis

Data were presented using different statistical measures depending on the nature of the variables. For normally distributed variables the mean ± standard deviation (SD) was reported. For non-normally distributed variables, median (25th–75th percentile) was used. Nominal variables were expressed as absolute and relative frequencies.

To examine the relationships between quantitative variables, either the Spearman or Pearson coefficient of correlation was employed. Spearman coefficient of correlation was used when the relationship was non-linear or when the outliers were present.

In the multivariate linear regression analysis, PWV was considered the dependent variable. Independent variables included those that showed significant correlation in the univariate analysis and those previously identified in relevant literature as influencing PWV levels were considered. However, SBP was excluded from the model due to multicollinearity.

To compare two groups, different statistical tests were employed based on the nature of the variables. The *t*-test or Mann–Whitney U test for quantitative variables depending on their distribution (normal and non-normal, respectively), while the Chi-square test or Fisher exact test was used for qualitative variables. A *p*-value less than 0.05 was considered statistically significant.

3. Results

In this study 80 patients were selected, from which six patients were excluded: four with acute infection, one with acute myocardial infarction, and one with malignancy. Another six patients were excluded due to missing data. Finally, 68 patients remained in the study.

3.1. Patients' Characteristics

The demographical, clinical and laboratory patients' characteristics are presented in Table 1. In our group, the median (25th, 75th percentile) age was 69 (62.5, 76) years; 41.2% had diabetes and 52.9% were men.

Table 1. Characteristics of participants.

Parameter	Group (*n* = 68)
Age (years)	69 (62.5, 76)
Male, *n* (%)	36 (52.9)
Diabetes mellitus [1], *n* (%)	28 (41.2)
Hypertension, *n* (%)	60 (89.6)
SBP (mmHg)	144 (126.5, 162)
DBP (mmHg)	87.07 ± 12.42
PP (mmHg)	58 (45.5, 72.5)
eGFR (mL/min/1.73 m^2)	27 (15, 42)
Body mass index (kg/m^2)	28.6 (26.4, 30.35)
LTM (kg)	38.25 ± 12.16
ATM (kg)	41.74 ± 13.77
Total cholesterol (mg/dL)	177.49 ± 37.45
LDL-cholesterol (mg/dL)	98.73 ± 28.61
HDL-cholesterol (mg/dL)	43.86 ± 12.94
Triglycerides (mg/dL)	125 (91.5, 166)
Fasting glucose (mg/dL)	103 (92, 131)
Calcium (mg/dL)	9.2 (8.64, 9.69)
Phosphorus (mg/dL)	3.66 (3.14, 4.56)
iPTH (pg/mL)	108.85 (84.85, 227.35)
Alkaline phosphatase (UI/L)	80 (72, 96.5)
Hemoglobin (g/dL)	12.46 ± 2.22
Serum albumin (g/L)	3.89 ± 0.49
hs-C reactive protein (mg/dL)	0.47 (0.23, 1.19)
White blood cells (no./mm^3)	7625 (6340, 9050)

Table 1. *Cont.*

Parameter	Group (*n* = 68)
TNF-α (pg/mL)	4.4 (2.94, 6.8)
IL-6 (pg/mL)	2.44 (1.7, 3.55)
IL-1β (pg/mL)	7.06 (6.45, 12.99)
Prolactin (ng/mL)	4.83 (3.1, 7.76)
Triiodothyronine (ng/mL)	1.2 (0.9, 1.3)
Brachial-ankle PWV (m/s)	10.55 ± 2.17
ACEI/ARB, n (%)	29 (42.6)

[1] data about 66 patients. Arithmetic mean ± standard deviation; *n*—number of people; no.—number of cell; SBP—systolic blood pressure; DBP—diastolic blood pressure; PP—pulse pressure; eGFR—estimated glomerular filtration rate; LTM—lean tissue mass; ATM—adipose tissue mass; LDL—low-density lipoprotein; HDL—high-density lipoprotein; iPTH—intact parathormone; PWV—pulse wave velocity; ACEI—angiotensin-converting enzyme inhibitors; ARB—angiotensin II receptor blockers.

3.2. Determinants of Brachial-Ankle PWV

In the analysis of correlations, it was observed that high values of brachial-ankle PWV were associated with reduced values of muscle mass ($p = 0.001$, r = −0.45), low levels of total cholesterol ($p = 0.042$, r = −0.26), triglycerides ($p = 0.023$, r = −0.34) and, respectively, low levels of the hormonal engage: triiodothyronine ($p = 0.04$, r = −0.25) (Figure 1) and prolactin ($p = 0.026$, r = −0.27) (Figure 2). Additionally, increasing brachial-ankle PWV was directly associated with high values of SBP ($p < 0.001$, r = 0.56), PP ($p < 0.001$, r = 0.57) and advanced age ($p < 0.001$, r = 0.92), all of these findings are described in Table 2 listed below.

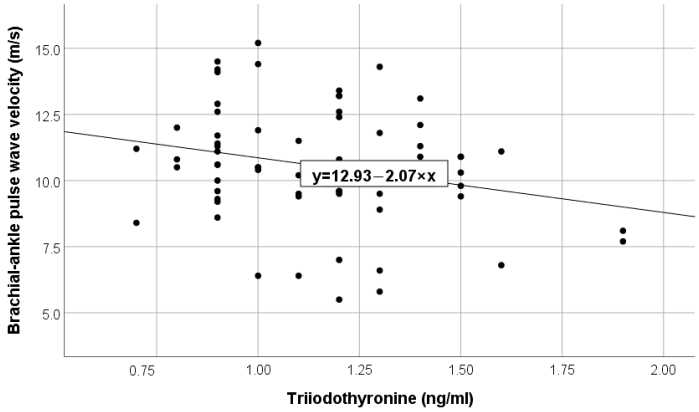

Figure 1. Negative linear correlation between PWV and triiodothyronine in the total group.

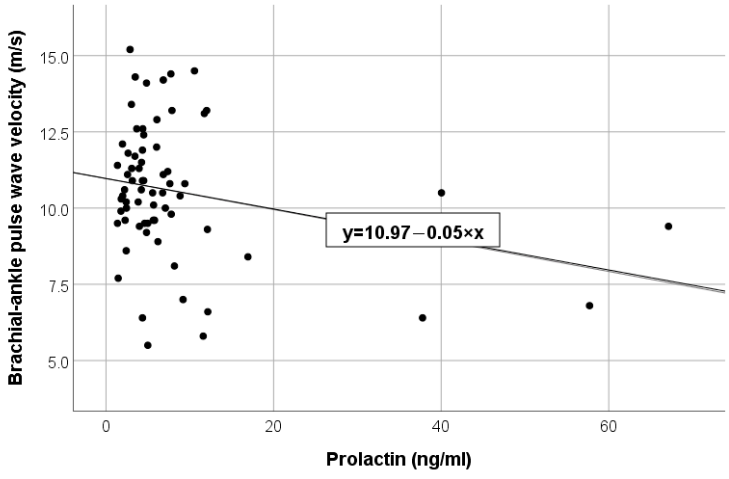

Figure 2. Negative linear correlation between PWV and prolactin in the total group.

Table 2. The brachial-ankle PWV correlation in the group.

Parameters	r—Coefficient of Correlation	*p*
Age (years)	0.92	<0.001
SBP (mmHg)	0.56	<0.001
PP (mmHg)	0.57	<0.001
Lean tissue mass (kg)	−0.45	0.001
Prolactin (ng/mL)	−0.27	0.026
Triiodothyronine (ng/mL)	−0.25	0.040
Total Cholesterol(mg/dL)	−0.26	0.042
Triglycerides (mg/dL)	−0.34	0.023

SBP—systolic blood pressure, PP—pulse pressure.

In the multivariate analysis it was noted that muscle mass ($p = 0.019$) and triiodothyronine ($p = 0.014$), PP ($p = 0.013$), and triglycerides ($p = 0.024$), respectively, remained with a significant impact on brachial-ankle PWV, but its strongest determinant was age ($p < 0.001$).

3.3. Analysis of the Subgroups

Diabetic vs. non-diabetic patients were analyzed (Table 3). In the DM subgroup there were significantly higher values of SBP ($p = 0.010$) and PP ($p = 0.009$) identified, significantly higher ATM ($p = 0.032$), and significantly higher IL-1β levels ($p = 0.015$).

Table 3. Comparisons between diabetes vs. non-diabetes groups ($n = 66$).

Parameter	Non-Diabetes Subgroup ($n = 38$)	Diabetes Subgroup ($n = 28$)	*p*
Age (years)	68.5 (61, 76)	68 (62.5, 76)	0.689
Male, *n* (%)	17 (45.9)	13 (46.4)	0.969
Hypertension, *n* (%)	34 (89.5)	24 (88.9)	0.940
SBP (mmHg)	138 (121, 156)	150.5 (138.5, 172)	0.010
DBP (mmHg)	82 (76, 98)	89 (80, 97.5)	0.508
PP (mmHg)	49.5 (41, 68)	63 (52, 74)	0.009
eGFR (mL/min/1.73 m^2)	26 (19, 38)	27.85 (12.5, 47)	0.910
BMI (kg/m^2)	28.45 (25.5, 29.9)	29.5 (27.1, 31.55)	0.109
LTM (kg)	38.35 ± 12	38.13 ± 12.63	0.948
ATM (kg)	38.27 ± 12.19	46.22 ± 14.62	0.032
Total cholesterol (mg/dL)	185.97 ± 36.06	168.62 ± 36.58	0.072
Triglycerides (mg/dL)	130 (94, 159.5)	124 (85.5, 175.5)	0.961
Calcium (mg/dL)	9.34 (8.67, 9.76)	9.17 (8.78, 9.47)	0.410
Phosphorus (mg/dL)	3.63 (3.13, 4.41)	3.7 (3.3, 5.22)	0.425
TNF-α (pg/mL)	4.6 (3.11, 6.03)	4.02 (2.69, 8.3)	0.678
IL-6 (pg/mL)	2.44 (1.7, 3.27)	2.2 (1.6, 4.36)	0.830
IL-1β (pg/mL)	6.86 (6.38, 9.96)	9.54 (6.79, 19.35)	0.015
Prolactin (ng/mL)	4.98 (3.07, 8.16)	4.66 (3.91, 7.24)	0.912
Triiodothyronine (ng/mL)	1.2 (0.9, 1.3)	1.1 (0.9, 1.35)	0.568
Brachial-ankle PWV (m/s)	10.14 ± 2.42	10.94 ± 1.58	0.135

Arithmetic mean ± standard deviation; SBP—systolic blood pressure; DBP—diastolic blood pressure; PP—pulse pressure; eGFR—estimated glomerular filtration rate; LTM—lean tissue mass; ATM—adipose tissue mass; PWV—pulse wave velocity.

In the DM subgroup, brachial-ankle PWV was directly correlated with inflammatory markers (TNF alpha $p = 0.012$, r = 0.46; IL-6 $p = 0.034$, r = 0.40), age ($p < 0.001$, r = 0.39) and serum phosphorus ($p = 0.012$, r = 0.39), but not with the eGFR (Table 4).

In the multivariate analysis the parameters which were correlated significantly with brachial-ankle PWV were included in our study, and it was noted that only age ($p < 0.001$) remained statistically significantly associated with PWV.

Table 4. The brachial-ankle PWV correlation in the subgroup of patients with DM.

Parameters	r—Coefficient of Correlation	*p*
Age (years)	0.89	<0.001
Phosphorus (mg/dL)	−0.51	0.014
eGFR (mL/min/1.73 m^2)	0.11	0.593
TNF-α (pg/mL)	0.47	0.012
IL-6 (pg/mL)	0.40	0.034

eGFR—estimated glomerular filtration rate.

4. Discussion

Age was the strongest determinant of arterial stiffness in the studied group, even though triiodothyronine and prolactin values were also correlated with brachial-ankle PWV. Similar data were published from the CRIC study, in which the worsening of AS with age, the reduction of eGFR, and the increase in PP in CKD were noted [34]. Additionally, in our study, strong correlations of PWV with SBP and PP values were obtained. It is known that hypertension, diabetes mellitus, and CKD are the major determinants of the loss of elasticity and reduced compliance of the vascular wall and, consecutively, increased arterial stiffness. Impaired collagen-elastin ratio, calcification of blood vessels, endothelial dysfunction, increased intima media-thickness, and genetic determinants can produce arterial wall remodeling [35]. All these factors have a prevalence that increases with age.

In addition, it is known that malnutrition is among the risk factors for atherosclerosis and, implicitly, for the increase in AS in CKD. In our study, we remarked that reduced values of muscle mass, therefore protein malnutrition, were associated with the increase of AS. In the longitudinal analysis, in the CRIC study, serum albumin concentration, which is another marker reflecting the protein nutritional status, was predictive of changes in PWV over time [34]. In addition, Harada et al. [36] observed that malnutrition in CKD was a factor associated with vascular calcifications and, consecutively, arterial stiffness, while Cordeiro, in a study, emphasized that another parameter reflecting the nutritional status, the abdominal fat, was associated with coronary artery calcification in non-dialysis dependent CKD patients [37] and then stiffening. In addition, secondary to the reverse epidemiology phenomenon of cardiovascular risk factors in CKD, we noticed that reduced values of lipid markers were associated with increased PWA, not with cardiovascular protection. Therefore, low values of total cholesterol and triglycerides may show a poor nutritional status in this population group.

Regarding the low values of T3 in CKD, they can express a deficit of thyroid function, known in this group of patients, most of the time subclinical (without having a thyroid disease as a substrate) and this could be associated with increased cardiovascular risk. Low values of T3 were associated with AS in our study, consistent with other studies in which FT3, was inversely associated with arterial stiffness in CKD patients [38]. In fact, Klotho synthesis seems to be influenced by the thyroid hormone level [39], and Klotho has a vascular protective effect by reducing vascular calcification. Therefore, the alteration of thyroid hormones in CKD may increase vascular calcifications by reducing the protective effects of Klotho [38]. In addition, overt hypothyroidism has been associated with altered vascular function and altered endothelial-dependent vasodilation [40], partly because of the lack of vasodilatory effect of triiodothyronine (subsequent vasodilation was reported when triiodothyronine increases the NO production by endothelial and smooth muscle cells) [41–43]. An increase in central arterial stiffness may be due the overt hypothyroidism as previous studies have shown.

Prolactine was reported to be correlated with PWV. Carrero et al. observed increased prolactin levels in subjects with endothelial dysfunction/stiffness and which further increased the risk of cardiovascular events and mortality [26]. The increase in prolactinoma in CKD is determined by the reduction of its metabolism, by the increased secretion of PRL in the uremic state and by the reduced availability of dopamine in the brain. Secondary to the decrease in dopaminergic activity, there can be an increase in the release of norepinephrine

with a negative result on the endothelial function and other organs, favoring myocardial hypertrophy, hypertension, and other cardiovascular diseases [44]. On the other hand, prolactin retention can inhibit the production of gonadotropic hormone, and consequently induce a testosterone deficiency in male patients with CKD, and through this mechanism, atherosclerosis. Prolactin retention was indeed linked to increased intima–media thickness, atherosclerotic plaque occurrence, systemic inflammation, and cardiovascular risk [45,46].

As evidence in our study, not the high values, but the low values of prolactin, a polypeptide hormone, were associated with the increase in PWA and we consider this type of association as an effect of the protein malnutrition present in the patients enrolled in the study. Moreover, in the study by Haring et al., they noted the association of low prolactin values with increased left ventricular mass, these changes only affecting males [47], without finding a clear explanation. Prolactin has 23 kDa and can induce angiogenesis. After proteolytic cleavage, a 5.6–18 kDa, isoform of prolactin, called vasoinhibins, appears with antiangiogenic properties [48]. Thus, the balance between prolactin and vasoinhibins regulates vascular functions [49].

In diabetic patients, PWV is higher than in the general population and promotes an increase in general and cardiovascular mortality [50]. If a diabetic patient has CKD, s/he has also all specific CKD cardiovascular risk factors and PWV increases additionally, with the impact being more significant. In the present study, the analysis of the subgroup of diabetic patients highlighted several aspects. First, age was also the strongest determinant of PWA values. Second, we did not identify significant differences between PWV in diabetics vs. non-diabetics, although we identified several cardiovascular risk markers that were significantly modified in the DM group. Thus, IL-1β (an inflammatory marker) was significantly higher as well as SBP, PP, and adipose tissue mass (expressed by ATM). Third, other inflammatory markers, TNF alpha, and IL-6, as well as phosphorus, a marker of mineral and bone metabolism, were found among the factors significantly associated with the PWA value in the subgroup with DM.

Other studies also reported that inflammatory markers such as fibrinogen and IL-10 were independently associated with PWV [34,51]. Moreover, it is known that micro-inflammation is present in CKD from the early stages and that there is a link between inflammation and atherosclerosis regarding malnutrition (malnutrition inflammation atherosclerosis syndrome). Several possible pathophysiological pathways can explain the association between chronic inflammation and arterial wall disease [14]. Initially, the circulation of inflammatory mediators favors leukocyte migration into the arterial wall [52]. Then, macrophages' activation by different factors amplifies the inflammatory reaction. This inflammatory cascade then alters the endothelium's function that interacts and conditions the remodeling of the tunica media, further along with changing the artery's mechanical properties [53]. Moreover, endothelial cells decrease the usual production of nitric oxide (NO) and increase endothelin (E1), favoring arterial stiffness.

Regarding the connection between phosphorus and AS as noted in our study, it is probably via vascular calcification. In fact, CKD alters hormonal processes that regulate phosphate levels (intestinal absorption, renal excretion by remaining nephrons, bone metabolism modulated by vitamin D, fetuin-A, Klotho, and fibroblast growth factor 23 (FGF-23); all these processes mentioned favoring hyperphosphatemia [54]. Excessive levels of phosphorous and calcium are endogenous minerals capable of stimulating the phenotypic transformation of vascular smooth muscle cells into osteoblast-like cells [55]. Experimental studies indicate that arterial medial calcification-related vascular alterations develop in the early stages of CKD [56].

All these processes initiated in pre-dialysis stages of CKD explain the significant cardiovascular changes detected in dialysis CKD patients [57].

In conclusion, in pre-dialysis CKD patients, age is the strongest determinant of PWV even among diabetic patients. Reduced triiodothyronine and prolactin values are associated with arterial stiffness, while also being markers of malnutrition. Inflammatory markers and hyperphosphatemia influenced PWV in diabetic patients. No variations of PWV

were recorded with eGFR or determined by the DM presence. Therefore, we speculate that if we detect and treat the inflammatory syndrome, respectively the malnutrition, the triiodothyronine, and prolactin levels, probably the value of PWV can be influenced. We believe that knowing the factors that influence PWV as a marker of AS, can help to administer a personalized treatment.

The study has some limitations, the first being the relatively small number of included patients, which makes additional studies necessary in order to validate the correlations and associations between PWV and the level of triiodothyronine, prolactin, and inflammatory markers with nutritional status. Secondly, due to the nature of our cross-sectional data, this study was limited in what we can infer about the causality of the results. Thirdly, by design it was an observational study and the conclusions need to be confirmed in the future, possibly by larger prospective studies.

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NEPHROLOGY - ORIGINAL PAPER

Ghrelin and acyl ghrelin levels are associated with inflammatory and nutritional markers and with cardiac and vascular dysfunction parameters in hemodialysis patients

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Abstract

Purpose Exogenous ghrelin is associated with cardiovascular protection in experimental and human studies. Nevertheless ESRD patients have increased ghrelin levels and severe cardiovascular comorbidities. This study aims to elucidate the metabolic factors influencing endogenous ghrelin/acyl ghrelin levels and to analyze the relation between endogenous ghrelin/acyl ghrelin levels and cardiac and vascular function markers in hemodialysis patients.

Methods The cross-sectional study was conducted in hemodialysis patients ($n=88$); 50 of them were men, mean age 61.1 ± 13.5 years, 17% had diabetes. We assessed nutritional and inflammatory status and analyzed the determinants of ghrelin/acyl ghrelin and their relation with cardiac and vascular function.

Results Ghrelin is correlated with IL-1β ($r=0.88$, $p<0.0001$), triglycerides, total cholesterol (TC), and Kt/V. IL-1β is the strongest predictor of ghrelin levels ($p<0.0001$). Acyl ghrelin is correlated with TC ($r=0.36$, $p=0.001$), LDL-cholesterol, serum bicarbonate, body mass index. TC is the strongest predictor for acyl ghrelin levels ($p=0.038$). Patients with high ghrelin levels had significantly decreased nitroglycerin-mediated dilation ($p=0.05$) and higher IL-1β levels ($p<0.001$); increased NT-proBNP is associated with lower levels of acyl ghrelin ($r=-0.33$, $p=0.02$) in male patients.

Conclusion The inflammatory marker IL-1β is in our study the strongest predictor of ghrelin levels while the nutritional marker-total cholesterol is the strongest predictor for acyl ghrelin levels in HD patients. High endogenous ghrelin level is associated with high IL-1β and with vascular smooth muscle cell dysfunction. Low acyl ghrelin level is associated with high NT-proBNP (a cardiac dysfunction marker) in male HD patients. There is a direct correlation between endogenous ghrelin level and inflammatory markers, which is not related with cardiovascular protection.

Keywords Ghrelin · IL-1β · Nutritional status · Inflammation · Cardiovascular disease · Chronic kidney failure

Introduction

Ghrelin is an orexigenic intestinal peptide, secreted mainly by the stomach and small bowel. There are two major forms of circulating ghrelin: acyl ghrelin, the active form which is orexigenic, and des-acyl ghrelin with anorexigenic effect [1]. Ghrelin is mainly metabolized and excreted by the kidneys.

Most of the studies (the majority of them analyzing total circulating ghrelin) found higher values in chronic kidney disease (CKD) patients than in the general population [2–4]. The increased levels are partly due to reduced degradation by the kidneys but also seem to be determined by the nutritional status, inflammation, age, and gender [2–4]. Studies showed that in CKD patients only des-acyl

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ghrelin is increased [5] (possible involved in anorexia that these patients are experiencing), while acyl ghrelin levels are decreased [6].

Many studies evaluated the role of ghrelin and its subtypes. Experimental studies demonstrated that ghrelin might have anti-inflammatory and immunomodulatory effects. It inhibits angiotensin II-induced expression of IL-6, IL-1β, TNF-α, IL-8, and monocyte chemoattractant protein-1 (MCP-1) in the endothelial cells of the human umbilical vein [7]. In clinical studies, the correlation between ghrelin/acyl ghrelin and inflammatory cytokines is less clear. Some studies found that there is no correlation between them [6, 8], while others found that endogenous ghrelin was positively correlated with inflammatory markers in both non-CKD [9, 10] and CKD patients [11].

Other clinical and experimental studies revealed associations between ghrelin and its subtypes with cardiovascular disease markers. In this way in the general population, clinical studies showed that low ghrelin levels are associated with carotid atherosclerosis in male patients [12]. HD patients which also presented protein-energy wasting (PEW) and low ghrelin levels had higher inflammatory markers and higher cardiovascular mortality risk [3, 8]. In addition, high N-terminal pro B-type Natriuretic Peptide (NT-proBNP) level, an index of heart failure, was found to be correlated with high ghrelin levels in obese dialysis patients [13].

Exogenous administration of ghrelin in experimental and human studies enhances exercise capacity, improves left ventricular function, ameliorates endothelial function, increases myocardial contractility, and limits atherosclerosis progression [14, 15]. Ghrelin might inhibit the migration of endothelial cells driven by angiotensin II [16] as well as the endothelial dysfunction induced by homocysteine in human endothelial cells [17].

Although most studies reported an increase in endogenous ghrelin levels in dialysis patients [2–4], in some experimental and clinical studies, high ghrelin levels are associated with cardiovascular protection [8, 12, 14, 15]. In dialysis patients, cardiovascular disease remains the main cause of morbidity and mortality. In our opinion, this might be due complex vascular lesions in advanced CKD that overcome the protective effect associated with increased levels of ghrelin or due to increased des-acyl ghrelin levels without cardiovascular effects.

On the other hand, it seems that experimentally exogenous ghrelin administration in animal studies and in humans resulted in favorable nutritional and CV effects [14–17].

These findings suggest the possibility of ghrelin treatment in the clinical setting, while some unanswered questions are still present in HD patients: which CV disease might be targeted, in which situations exogenous ghrelin is benefic depending on the endogenous levels, and which are the metabolic factors that influence endogenous ghrelin.

The present study aims to elucidate the metabolic factors influencing endogenous ghrelin/acyl ghrelin levels and to analyze the relation between endogenous ghrelin/acyl ghrelin levels and cardiac and vascular function markers in hemodialysis patients.

Method

Patients

We conducted a cross-sectional observational study on a cohort of HD patients. Of the 192 patients on conventional HD treatment in Nefromed Dialysis Center Cluj, 88 patients met the inclusion criteria after they provided written informed consent. All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Inclusion criteria were as follows: prevalent HD patients, age > 18 years, duration of hemodialysis maintenance at least 6 months (HD vintage). Exclusion criteria were as follows: acute inflammatory processes, terminal neoplasia, hepatitis viral infection, and any other serious chronic or acute diseases requiring treatment. All patients were on three times weekly HD (4–5 h) regimen. Patients' demographic data, etiology of end stage renal disease (ESRD), HD vintage, comorbidities (diabetes, hypertension, smoking status), and medication upon enrolment were obtained from medical documents. We registered also clinical data: age, weight, height, pre dialysis systolic blood pressure (SBP), and diastolic blood pressure (DBP) as well as previous cardiovascular disease (angina or infarction, coronary revascularization, stroke or documented peripheral arterial disease).

Nutritional assessment

We registered triceps skinfold thickness (TST) (mm) and waist circumference (WC) (cm) and we calculated body mass index (BMI) as BMI = weight (kg)/height^2 (m^2) and pulse pressure (PP) with formula: PP = SBP − DBP (mmHg). Beside the anthropometric measurements, nutritional status was evaluated by bioimpedance, using the Body composition monitor that furnishes data on body composition as follows: lean tissue mass (LTM) (kg), adipose tissue mass (ATM) (kg), lean tissue index (LTI) (kg/m^2) and fat tissue index (FTI) (kg/m^2) as described in the literature [18].

Laboratory parameters

All biochemical analyses were performed between 7.00 and 9.00 a.m. during a midweek dialysis day, after an overnight

fast. Current measurements at the initiation of the study included serum electrolytes, albumin, pre-dialysis creatinine, lipid profile [total cholesterol, triglycerides (TGL) and HDL-cholesterol (HDL-chol), high-sensitivity C-reactive protein (hs-CRP), intact parathormone (iPTH), hemoglobin, and white blood cell (WBC)]. Pre- and post-dialysis urea levels were used to calculate Kt/V. LDL-cholesterol (LDL-chol) was calculated with Friedewald formula: LDL-cholesterol = total cholesterol-(HDL-chol + TG/5).

For the measurement of ghrelin, acyl ghrelin, IL-1β, IL-6, TNF-α, NT-proBNP, a sample of the venous blood was centrifuged at 5000 rotations/min for 3 min, frozen within 1 h of collection and stored at − 80 °C in triplicate Eppendorf tubes.

Serum ghrelin, acyl ghrelin, IL-6, IL-1β, TNF-α, and NT-proBNP were determined by enzyme-linked immunosorbent assay (ELISA), using commercially available kits similar to those used in other studies (R&D System, Minneapolis, MN, USA). The minimum detectable level of ghrelin was 123.5 pg/ml, for acyl ghrelin − 31.2 pg/ml, TNF-α − 15.6 pg/ml, IL-6 − 3.2 pg/ml, IL-1β − 10.2 pg/ml, and NT-proBNP − 7.8 pg/ml.

Endothelial function

Endothelial function was evaluated using high-resolution ultrasound: GE Logiq 3 (General Electric Company, Fairfield, CT, USA), with a 5–10 MHz linear transducer. The brachial artery in the arm without arteriovenous fistula was examined. The ultrasound examiner was blinded to other patients' data. Measurements were performed according to protocols described in other studies [19]; we determined flux-mediated dilation (FMD) and nitroglycerin-mediated dilation (NMD).

Dialysis prescription

All patients were managed by nephrologists and were dialyzed with bicarbonate-based dialysate, volumetric ultrafiltration control, single use synthetic (polysulphone) dialyzers, and heparin as standard anticoagulant. Dialysis prescription was guided to achieve a Kt/V goal value ≥ 1.4.

Statistical analysis

Data were presented as a mean ± standard deviation (SD) for normally distributed variables, median (25^{th}–75th percentile) for non-normally distributed variables, or absolute or relative frequencies for nominal variables.

The association between quantitative variables was measured with Spearman or Pearson coefficient of correlation. Spearman coefficient of correlation was used if the association was not linear or in case of the presence of outliers.

For multivariate linear regression with ghrelin/acyl ghrelin as dependent variables, all variables that were significantly correlated with them in univariate analysis and those previously found in the literature to influence ghrelin levels were considered as independent variables.

To compare the two groups, we used t test or Mann–Whitney test for quantitative variables according to their normal/non-normal distribution and Chi-square test/Fisher exact test for qualitative variables. We compared the group of patients with ghrelin levels lower than 25th percentile (inferior Quartile) with the group of patients with ghrelin levels higher than 75th percentile (superior Quartile). $p < 0.05$ was considered significant.

Results

Patients' characteristics

Demographical, clinical, and biological patients' characteristics are presented in Table 1. 76 patients (82.8%) had an arteriovenous fistula and 12 patients (17.2%) a semi-permanent transcutaneous access. The etiology of ESRD was chronic glomerulonephritis in 8% of patients, diabetes in 20%, vascular nephropathy in 11%, tubulo interstitial diseases in 11%, and unknown cause in 50% of cases.

Current medication comprised the following: erythropoietin-stimulating agents (ESA)—80% of patients, intravenous iron—46% of patients, statins—18% of patients, beta blockers—57% of patients, calcium channel blockers—25% of patients, angiotensin-converting enzyme inhibitors or angiotensin receptor blockers—42% of patients, antiplatelet therapy—36% of patients, calcium-based phosphorus binders in 74% and non-calcium-based phosphorus binders in 31% of patients.

Determinants of ghrelin and acyl ghrelin in HD patients

Ghrelin was positively correlated with triglycerides ($r = 0.31$, $p = 0.004$), total cholesterol ($r = 0.23$, $p = 0.03$), IL-1β ($r = 0.88$, $p < 0.0001$), and Kt/V ($r = 0.25$, $p = 0.02$).

In multivariate linear regression with ghrelin as dependent variable, we took into account the independent variables that were significantly correlated with ghrelin in univariate analysis and those previously found in the literature to influence ghrelin levels. IL-1β was the most significant predictor for ghrelin in multivariate linear analysis (Table 2; Fig. 1).

Acyl ghrelin was positively correlated with total cholesterol ($r = 0.36$, $p = 0.001$), LDL-cholesterol ($r = 0.39$, $p < 0.001$), serum bicarbonate ($r = 0.31$, $p = 0.004$), BMI ($r = 0.24$, $p = 0.03$), fat tissue index ($r = 0.28$, $p = 0.009$), and negatively with lean tissue mass ($r = -0.29$, $p = 0.008$).

Table 1 Demographic, clinical, and biochemical characteristics of the patients ($n=88$)

Parameter	Value
Age (years)	61.1 ± 13.5
HD vintage (months)	63.4 (6–200)
Male [*n* (%)]	50 (57)
Diabetes mellitus [*n* (%)]	17 (19.3)
Hypertension [*n* (%)]	62 (70)
SBP (mmHg)	142.0 ± 21.3
DBP (mmHg)	74.0 ± 12.2
Cardiovascular disease [*n* (%)]	22 (25)
Smoking [*n* (%)]	11 (12.5)
FMD (%)	7.2 (−7.2 to 31.2)
NMD (%)	8.3 (−13.7 to 38.4)
Body mass index (kg/m^2)	27.5 (18.6–43.7)
Waist circumference in males (cm)	95.6 ± 15.4
Waist circumference in females (cm)	97.2 ± 18.1
Triceps skinfold thickness (mm)	3.0 (1.00–8.00)
LTI (kg/m^2)	11.3 (4.7–18.1)
FTI (kg/m^2)	14.8 (1.6–31.8)
Kt/V	1.5 ± 0.32
Total cholesterol (mg/dl)	177.106 ± 43.38
LDL-cholesterol (mg/dl)	103.15 ± 38.81
HDL-cholesterol (mg/dl)	36.00 (30.54–47.64)
Triglycerides (mg/dl)	139.00 (97.65–195.75)
Hemoglobin (g/l)	11.5 ± 1.2
Serum albumin (g/l)	3.9 ± 0.3
CRP (mg/dl)	0.59 (0.1–1.2)
Fasting glucose (mg/dl)	94.0 (68.4–115.3)
White blood cells (n/mm^3)	6440 (3210–10,500)
Ghrelin (pg/ml)	571.2 (142.5–14157.4)
Acyl ghrelin (pg/ml)	69.3 (6.3–1195.3)
TNF-α (pg/ml)	283.2 (166.7–11287.2)
IL-6 (pg/ml)	272.4 (143.9–2096.6)
IL-1β (pg/ml)	47.1 (3.6–1052.6)
NT-proBNP (pg/ml)	9557.2 (2374.8–10903.7)

Data are presented as arithmetic mean ± standard deviation; median (25th–75th percentile)

SBP systolic blood pressure, *DBP* diastolic blood pressure, *PP* pulse pressure, *FMD* flow-mediated dilation, *NMD* nitroglycerin-mediated dilation, *LTI* lean tissue index, *FTI* fat tissue index, *CRP* C-reactive protein, *TNF-α* tumor necrosis factor-α, *IL-6* interleukin-6, *IL-1β* interleukin-1β, *NT-proBNP* N-terminal pro B-type natriuretic peptide

Although acyl ghrelin is a fraction of total ghrelin, these two molecules have different determinants and showed no correlation ($p=0.38$, $r=-0.09$).

In multivariate linear regression with acyl ghrelin as dependent variable, we took into account the independent variables that were significantly correlated with ghrelin in univariate analysis and those previously found in the literature to influence ghrelin levels. Only total cholesterol was a significant predictor for acyl ghrelin in multivariate linear analysis (Table 2).

We observed the correlations between the studied hormones and measured inflammation and nutritional markers in males and females. In males, acyl ghrelin was correlated with NT-proBNP ($r=-0.33$, $p=0.02$), while in females significant correlations were found with nutritional markers (Table 3).

Statistically no significant variations of ghrelin and acyl ghrelin levels were found in relation with cardiovascular disease, diabetes mellitus, gender, or smoking.

FMD and NMD in the brachial artery are presented in Table 4. Patients with high ghrelin levels had significantly decreased nitroglycerin-mediated dilation ($p=0.05$) and higher IL-1β ($p<0.001$). In the group of patients with high ghrelin levels, we found significantly better dialysis efficiency ($p=0.01$) and less smokers ($p=0.02$).

Discussion

In our study, inflammatory cytokine IL-1β is the strongest predictor of ghrelin levels in HD patients. To our knowledge, this association has never been described in dialysis patients.

Ghrelin and acyl ghrelin seem to have an important role in the pathogenesis of inflammation-malnutrition-atherosclerosis, mainly the PEW syndrome, these molecules being correlated with both inflammatory and nutritional markers and possible with other factors that contribute in the development of atherosclerosis [20]. Ghrelin and acyl ghrelin currently are available as exogenous experimental therapies; this makes them potential candidates as interventions in the pathogenesis of atherosclerosis. The positive effect of exogenous ghrelin might be mediated through protection of endothelial cells by inhibiting proinflammatory cytokines and preventing atherosclerosis development [16, 17].

The relation between inflammatory markers and ghrelin has been described both in the general population and in CKD patients [21]. It was shown that administration of ghrelin in patients without CKD inhibits the inflammatory response in heart failure by bringing a decrease in TNF-α and IL-1β levels and in the expression of matrix metalloproteinase [22]. So, exogenous ghrelin administration induces a decrease in cytokine levels [23]. But, other studies showed that increased values of endogenous ghrelin can be associated with increased cytokine levels in non-CKD [9, 10, 24] as well as in CKD patients [25].

The interplay between ghrelin and inflammatory markers is complex and a double-way interaction between ghrelin and IL-1β has been identified. On the one hand, experimental data suggested that in acute inflammation IL-1β acts on gastric mucosal cells which produce prostacyclins as a second messenger to reduce ghrelin production [26].

Table 2 Multivariate linear regression analysis for determinants of ghrelin and acyl ghrelin

	Unstandardized coefficients	Standardized coefficients		*p*	95% confidence interval for B	
	B	Std. error	Beta		Lower bound	Upper bound
Ghrelin						
IL-1β	0.003	0.000	0.815	**< 0.0001***	0.003	0.004
TGL	0.001	0.001	0.093	0.154	0.000	0.003
Total chol	−0.001	0.002	−0.047	0.453	−0.005	0.002
Kt/V	0.516	0.238	0.132	**0.033**	0.043	0.990
Gender	0.150	0.166	0.058	0.369	−0.180	0.480
Age	0.010	0.006	0.102	0.093	−0.002	0.021
Acyl ghrelin						
Gender	−0.038	0.357	−0.014	0.915	−0.749	0.672
Age	−0.018	0.011	−0.178	0.124	−0.040	0.005
Total chol	0.008	0.004	0.229	**0.038**[#]	0.000	0.015
Serum bic	0.003	0.002	0.198	0.063	0.000	0.006
LTM (kg)	−0.028	0.020	−0.194	0.169	−0.069	0.012

Statistically significantly different values between quartile inferior and superior are in bold

IL-1β interleukin-1β, *TGL* triglycerides, *LTM* lean tissue mass

*IL-1β is the most significant predictor for ghrelin levels

[#]Total cholesterol is the most significant predictor for acyl ghrelin levels

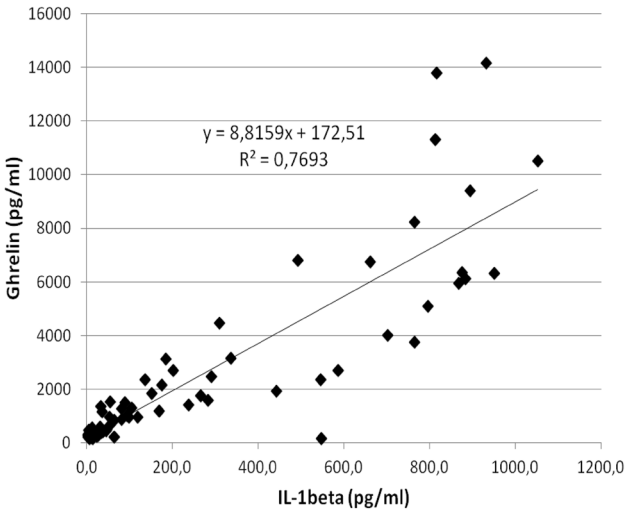

Fig. 1 Correlation between ghrelin and IL-1β in hemodialysis patients ($r = 0.88$, $p < 0.0001$)

On the other hand, some experimental studies showed that ghrelin release from T cells and/or monocytes regulates levels of IL-1β, IL-6, TNF-α [27]. In addition to this, ghrelin can inhibit nuclear factor kappa B [NF-kB] activation and as a consequence TNF-α secretion [28]. Thus, an acute inflammation can influence total endogenous ghrelin, while reactively increased ghrelin can modify cytokine levels. Moreover, inflammatory status might change bioactivity and ghrelin metabolism in HD patients [25]. It seems that in the inflammatory response in CKD, as well as in other chronic diseases, the main effects are induced by IL-1β and IL-6 [29].

In accordance with other authors' findings [6], we did not find a correlation between acyl ghrelin and inflammatory markers.

Besides the correlations with the inflammatory process, ghrelin is able to influence nutritional status by several mechanisms. First, it regulates fat distribution and energy metabolism in muscle and liver. Muscle mass can be influenced by ghrelin levels in part due to suppressed muscle proteolysis and possibly related to anti-inflammatory effects [30]. Secondly, by influencing inflammatory cytokines [31], ghrelin can control protein wasting present in many conditions, including advanced CKD.

Thirdly, ghrelin is a hormone that regulates appetite. It has been shown that exogenous daily ghrelin positively influences energy balance in dialysis patients [32]. Numerous clinical studies confirmed the ghrelin effect on nutritional status, showed to be related to BMI, muscle mass in healthy adults [33] and fat mass in CKD patients [34]. Acyl ghrelin is also capable to influence nutritional status. In our study, similar to other findings [23, 35], acyl ghrelin is directly correlated with BMI. But concerning the direct correlation found in our study between acyl ghrelin and body fat (expressed by FTI in the whole group and TST in males) and the inverse correlations between acyl ghrelin and LTM in all patients and in the females group, the results in the literature are contradictory [6, 23]. In fact, adipose tissue might have a dual role: it might be suggestive of an adequate nutritional status with favorable effects

Table 3 Correlations of ghrelin and acyl ghrelin in HD males and females (only statistically significant parameters were included in the table)

	Females ($n=38$)	Males ($n=50$)
Ghrelin	– TGL ($r=0.38$, $p=0.02$) – LDL-chol ($r=0.36$, $p=0.03$) – Total chol ($r=0.51$, $p<0.001$) – Hb ($r=-0.36$, $p=0.03$) – Kt/V ($r=0.37$, $p=0.02$) – Serum bic ($r=-0.38$, $p=0.019$) – IL-1β ($r=0.91$, $p<0.001$) – TNF-α ($r=-0.36$, $p=0.03$)	– TGL ($p=0.36$, $p=0.01$) – IL-1β ($r=0.86$, $p<0.001$)
Acyl ghrelin	– LDL-chol ($r=0.41$, $p=0.001$) – Total chol ($r=0.41$, $p=0.01$) – Serum bic ($r=0.34$, $p=0.04$) – LTM ($r=-0.36$, $p=0.029$)	– LDL-chol ($r=0.36$, $p=0.01$) – NT-proBNP ($r=-0.33$, $p=0.02$) – TST ($r=0.32$, $p=0.02$)

TGL triglycerides, *Hb* hemoglobin, *Serum bic* serum bicarbonate, *IL-1β* interleukin-1β, *TNF-α* tumor necrosis factor-α, *LTM* lean tissue mass, *NT-proBNP* N-terminal pro B-type natriuretic peptide, *TST* triceps skin fold thickness

Table 4 Comparative analysis of clinical and biochemical characteristics of patients in inferior quartile versus superior quartile

Parameter	Ghrelin < 304 (pg/ml) Inferior quartile ($n=22$)	Ghrelin ≥ 2350 (pg/ml) Superior quartile ($n=23$)	*p*
Age (years)	60.1 ± 12.5	61.3 ± 12.9	0.73
Male (%)	40	60	0.18
Cardiovascular disease (%)	62	38	0.17
Diabetes mellitus (%)	56	44	1
Smoking (%)	**73**	**27**	**0.02***
Hypertension (%)	47	53	0.66
SBP (mmHg)	145 (110–175)	137 (90–270)	0.13
DBP (mmHg)	70.7 (50–93)	74.1 (49–90)	0.28
TST (mm)	4.1 (2–8)	3.5 (2–6)	0.26
Body mass index (kg/m²)	30.7 (19.1–40.1)	27.8 (20.1–42.8)	0.08
Waist circumference (cm)	100.8 ± 16.0	95.5 ± 17.61	0.29
FTI (kg/m^2)	18.3 (1.6–29.8)	15.3 (3.6–31.7)	0.20
LTI (kg/m^2)	11.6 (4.7–17.7)	11.7 (6.9–18.1)	0.91
FMD %	9.8	6.2	0.09
NMD %	**13.5**	**8.33**	**0.05***
Total cholesterol (mg/dl)	170.0 ± 33.4	178.6 ± 48.4	0.49
LDL-cholesterol (mg/dl)	96.4 (42.9–144.2)	98.2 (20.3–184.4)	0.88
HDL-cholesterol (mg/dl)	42.8 (25–126)	38.1 (19.8–60)	0.66
Triglycerides (mg/dl)	148.4 (48.6–345.0)	201.0 (42–725.9)	0.17
Fasting glucose (mg/dl)	102.0 (72.9–239)	100.2 (73–145)	0.56
Kt/V	**1.4 ± 0.4**	**1.6 ± 0.23**	**0.01***
CRP	1.5 (0.2–2.8)	2.6 (0.1–3.8)	0.13
IL-1β (pg/ml)	**38.3 (3.6–547.9)**	**649.8 (136.6–1052.6)**	**< 0.001***
TNF-α (pg/ml)	308.2 (188.2–546.7)	330.4 (160.6–1267.5)	0.35
IL-6 (pg/ml)	286.8 (152.3–509.2)	394.3 (143.9–2096.6)	0.65

Statistically significantly different values between quartile inferior and superior are in bold

Data are presented as arithmetic mean ± standard deviation; median (25th–75th percentile)

SBP systolic blood pressure, *DBP* diastolic blood pressure, *TST* tricipital skinfold thickness, *FTI* fat tissue index, *LTI* lean tissue index, *FMD* flow-mediated dilation, *NMD* nitroglycerin-mediated dilation, *CRP* C-reactive protein, *IL-1β* interleukin-1β, *TNF-α* tumor necrosis factor-α, *IL-6* interleukin-6

*Parameters presenting statistically significant differences in patients with high versus low values of ghrelin

in the long term, but it can also be a source of inflammatory factors (adipokines). In our study, acyl ghrelin is mainly correlated with markers of nutritional status, not with inflammatory mediators, so this is why we consider the relation between high AG values and high fat mass is an expression of good nutritional status.

The effects of ghrelin and acyl ghrelin on atherogenesis might involve lipid metabolism. The correlations between ghrelin, acyl ghrelin, and lipid metabolism (triglycerides, total cholesterol, LDL-cholesterol) have been less studied. In a study performed in CAPD patients, the authors found no correlations between ghrelin/acyl ghrelin and triglycerides, total cholesterol or LDL-cholesterol [36], which differed from our study data. We found that ghrelin and acyl ghrelin levels are associated with lipid metabolism markers, total cholesterol being the most important predictor of acyl ghrelin levels. In fact, in dialysis patients, lipid metabolism markers, especially cholesterol levels, express mainly the inverse epidemiology phenomenon present in these patients, fact that might explain our findings: the association between low levels of acyl ghrelin and total and LDL-cholesterol, which in this setting are nutritional markers [37].

In addition, it was interesting to analyze in our research the correlation between acyl ghrelin and NT-proBNP (Table 3) in the male patients subgroup: the increase in NT-proBNP was associated with lower levels of acyl ghrelin. This is to our knowledge the first study concerning acyl ghrelin and NT-proBNP in HD patients. It is known that heart failure is associated with increased release of NT-proBNP from ventricular myocytes (NT-proBNP is an indicator of ventricular wall stress) [38]. In HD patients, NT-proBNP has been identified as a mortality predictor [39] and correlates with left ventricular systolic dysfunction [40]. These findings have suggested NT-proBNP as a marker of heart dysfunction in HD patients. On the other hand, clinical studies on heart failure in patients with normal renal function [41] and in obese HD patients [13] found correlations between ghrelin and NT-proBNP. It is known that ghrelin pretreatment in mice with inherited dilated cardiomyopathy reduces the expression of ventricular proBNP mRNA and leads to significantly prolonged life span [42]. In consequence, ghrelin can improve ventricular wall stress and protect the heart from ischemia [43]. Moreover, high acyl ghrelin levels in HD patients have been associated with lower cardiovascular and all-cause mortality [23]. Taken together these data may suggest that in our study, in male patients, the decreased acyl ghrelin levels, correlated with the increased NT-proBNP showed that heart dysfunction was associated with low acyl ghrelin levels. But the significance of this correlation should be cautiously accepted since in HD patients NT-proBNP displays wide variations [44], and is influenced by volemic status, [39], nutritional markers (muscular mass) [45], as well as residual renal function [46].

The vascular structural alteration secondary to atherosclerosis can be evaluated in early stages by measuring endothelial function. In experimental studies, ghrelin appeared to have a protective effect on endothelial cells [16, 17] while in clinical studies correlations have been found between ghrelin and endothelial dysfunction in coronary microcirculation in dialysis patients [47]. In more advanced stages of vascular pathology, nitroglycerin-mediated dilation (NMD) in the brachial artery can be assessed, as an indicator of vascular muscular function. Impaired NMD is thought to be related with vascular structural changes and smooth muscle cells dysfunction [48], and can reflect arterial compliance [49]. Studies showed that in the general population and in dialysis patients, NMD is a good predictor for cardiovascular morbidity and mortality [49, 50] and is associated in HD patients with inflammatory markers [51, 52]. In our study, high levels of ghrelin and IL-1β are associated with reduced NMD (superior quartile) (Table 4), but not with endothelial dysfunction as expressed by FMD. It might be that the increase in ghrelin levels is a compensatory mechanism to increased values of IL-1β and is associated with smooth muscle cell dysfunction as suggested by NMD alteration.

These findings suggest the possibility that ghrelin and acyl ghrelin might be used as biomarkers of subclinical cardiovascular disease in HD patients. Their levels are influenced by nutritional and inflammatory status.

Some studies showed that acute smoking increases ghrelin levels [53] and that quitting smoking induces increased levels of acyl ghrelin [54]. We observed an increased number of smokers in the group with low levels of ghrelin and not in those with increased levels. In our study, smokers as compared to non-smokers did not have significantly different values of ghrelin and acyl ghrelin. Blood was drawn after 12 h of smoking withdrawal, in order to circumvent the possible influence of acute smoking on ghrelin and acyl ghrelin values.

The increase in dialysis efficiency as expressed by Kt/V might be associated with increased appetite and nutritional status. In our study, although we found significantly better dialysis efficiency in patients with higher levels of ghrelin which consequently might increase appetite and improve nutritional markers, high ghrelin is not correlated with nutritional markers but is correlated with IL-1β. This finding further emphasizes the impact of inflammation on ghrelin in HD patients.

Limitations

First, because of the cross-sectional design, the present analysis is limited in its ability to establish causal correlations. Secondly, as our cohort was relatively small and consisted of prevalent patients, our findings need confirmation in larger patient cohorts.

Thirdly, we did not directly determine the level of des-acyl ghrelin, which could have brought more clarifications into the interaction between ghrelin, inflammation, and cardiovascular disease.

Conclusion

The inflammation marker IL-1β is in our study the strongest predictor of ghrelin levels while the nutritional marker-total cholesterol is the strongest predictor for acyl ghrelin levels in HD patients. The high level of total endogenous ghrelin has been associated with high level of IL1 beta and with vascular smooth muscle cell dysfunction. In HD patients, low acyl ghrelin levels are associated with high NT-proBNP, a cardiac dysfunction marker.

Our results suggest that endogenous ghrelin does not have the same significance or the same effects as exogenous ghrelin. Although many nutritional and cardiovascular benefits of ghrelin are well known, our study on HD patients shows that increased total endogenous ghrelin is associated with an inflammatory status and does not offer cardiovascular protection. On the other hand determining the levels of endogenous acyl ghrelin, which is more specifically linked with nutritional and cardiac markers, might also be useful. More studies are required to follow the effects on cardiovascular disease of exogenous administration of ghrelin in chronic HD patients, related to endogenous ghrelin/acyl ghrelin levels and inflammatory and nutritional status.

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Compliance with ethical standards

Conflict of interest The authors report no conflict of interest.

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NEPHROLOGY - ORIGINAL PAPER

The metabolic hormone FGF21 is associated with endothelial dysfunction in hemodialysis patients

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Abstract
Purpose Finding new, reliable biomarkers of cardiovascular risk in hemodialysis (HD) patients is of utmost importance. Fibroblast growth factor 21 (FGF21) has been recently associated with atherosclerosis in the general population. The relationship between markedly elevated FGF21 levels in HD patients and endothelial dysfunction is unknown. The aim of the study was to assess the determinants of FGF21, the correlation between FGF21 and tumor necrosis factor TNF-like weak inducer of apoptosis (sTWEAK) and the correlation between FGF21 and endothelial dysfunction in HD patients.
Methods A cross-sectional observational study was conducted in 70 HD patients (mean age 59.9 ± 12.5 years, 14.3% diabetes mellitus, 57.1% male) from Nefromed Dialysis Center Cluj. We registered clinical and biological data, and serum FGF21 levels were measured by ELISA. Endothelial function was evaluated by brachial flow-mediated dilation (FMD). An analysis based on stratification of FGF21 values into quartiles was performed.
Results FGF21 levels were directly correlated with sTWEAK, tricipital skinfold thickness (TST), systolic blood pressure (SBP), total cholesterol and triglycerides. In multivariate linear analysis, only sTWEAK and SBP remained significantly associated with FGF21. FGF21 values in the inferior quartile were directly correlated with HDL-cholesterol, while FGF21 values in the superior quartile were directly correlated with SBP, pulse pressure and sTWEAK. FMD was significantly higher in the inferior quartile as compared to the superior quartile.
Conclusions High FGF21 values in our patients are correlated with atherosclerosis risk factors: hypercholesterolemia, hypertriglyceridemia, hypertension, increased TST and increased levels of sTWEAK. Endothelial dysfunction is associated with high FGF21 in HD patients.

Keywords Endothelial dysfunction · Hemodialysis · Atherosclerosis risk · FGF21 · sTWEAK

Introduction

Fibroblast growth factor 21 (FGF21) belongs to the FGF family and is mainly produced by the liver but also by adipose tissue, skeletal muscle and pancreatic β-cells [1]. FGF21 influences lipid and glucose metabolism, is regulated by nutritional status [2] and is considered to be a metabolic hormone.

In animal models, the effects of FGF21 on glucose and lipid metabolism are impressive. In diabetic rhesus monkeys, FGF21 therapy significantly improved lipid profile with decrease in triglycerides and low-density lipoprotein cholesterol and increase in high-density lipoprotein cholesterol [3]. FGF21 stimulates glucose uptake in adipocytes in an insulin-independent manner [4]. Experimental studies have shown an antiobesity effect of FGF21 secondary to a decrease in leptin levels in FGF21 transgenic mice [5, 6].

In contrast to experimental studies, in humans elevated circulating FGF21 is associated with metabolic syndrome

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[7], dyslipidemia [8], progression of diabetes type 2 associated nephropathy [9], increased inflammation markers [10] and different stages of atherosclerosis [11, 12]. In women with diabetes mellitus type 2, high FGF21 levels correlate with femoral intima-media thickness and lower extremity atherosclerosis [11]. Elevated serum FGF21 levels are associated with carotid atherosclerosis [12], coronary arterial disease [13, 14], acute myocardial infarction [15] and hypertension [16]. The association of FGF21 with atherosclerosis lesions suggests its potential value as a cardiovascular risk biomarker.

In chronic kidney disease (CKD), FGF21 levels progressively increase as kidney function declines [17]; in long-term dialysis, patients as compared to general population 8–15 times higher levels of FGF21 have been described [8, 18]. It is yet not known whether in CKD patients FGF21 levels are associated with atherosclerosis similar to the general population. In continuous ambulatory peritoneal dialysis, patients FGF21 levels were found to be inversely correlated with brachial flow-mediated dilation, known marker of endothelial dysfunction [19]. Further studies are needed to evaluate the association between FGF21 and atherosclerosis markers in hemodialysis patients.

Tumor necrosis factor TNF-like weak inducer of apoptosis (sTWEAK) is a less commonly studied cytokine that has been found to be associated with proatherosclerosis factors and endothelial dysfunction in CKD patients [20].

The aim of the study was to assess the determinants of FGF21, the correlation between FGF21 and sTWEAK and the correlation between FGF21 and endothelial function in HD patients.

Materials and methods

Patients

We conducted a cross-sectional observational study on a cohort of HD patients. Of the 180 patients on conventional HD treatment in Nefromed Dialysis Center Cluj, 70 patients met the inclusion criteria and agreed to participate in this study. Inclusion criteria were: prevalent HD patients, age >18 years, duration of maintenance hemodialysis at least 6 months (HD vintage). Exclusion criteria were: acute inflammation, neoplasia, hepatitis virus infection and any other serious chronic or acute diseases requiring treatment. All patients were on thrice weekly HD (4–5 h) regimen. Patients' demographic data, etiology of end-stage renal disease (ESRD), HD vintage, comorbidity conditions (diabetes, hypertension, smoking status), antihypertensive treatment, statins, antiplatelet therapy, erythropoietin treatment and intravenous iron upon enrollment were obtained from medical documents. We registered also clinical data: age, weight, height, predialysis systolic blood pressure (SBP), diastolic blood pressure (DBP), triceps skinfold thickness (TST) (mm) and waist circumference (WC) (cm). We registered previous cardiovascular disease (angina or infarction, coronary revascularization, stroke or documented peripheral arterial disease). We calculated body mass index (BMI) as BMI = weight (kg)/height^2 (m^2) and pulse pressure (PP) with formula: PP = SBP − DBP (mmHg).

Laboratory parameters

All biochemical analyses were performed after an overnight fast between 7.00 and 9.00 a.m. during a midweek dialysis day. Current measurements at the initiation of the study included serum electrolytes, albumin, predialysis creatinine, lipid profile (total cholesterol, triglycerides (TG) and HDL-cholesterol), C-reactive protein (CRP), intact parathormone (iPTH), hemoglobin and white blood cell (WBC). Pre- and post-dialysis urea levels were used to calculate Kt/V. LDL-cholesterol was calculated with Friedewald formula: LDL-cholesterol = total cholesterol − (HDL-cholesterol + TG/5). For the measurement of FGF21 and sTWEAK, a sample of the venous blood was centrifuged at 5000 rotations/min for 3 min; the obtained serum was refrigerated at −80° Celsius in triplicate Eppendorf tubes.

Serum FGF21 and sTWEAK were determined by enzyme-linked immunosorbent assay (ELISA), using commercially available kits similar to those used in other studies [10, 20]: RD191108200R Biovender R&D for FGF21 and Bender MedSystem kit, Vienna, Austria, for sTWEAK. The minimum detectable level of FGF21 was 7 pg/ml and of sTWEAK 10 pg/ml. For FGF21, intra- and interassay coefficients of variation were 8 and 8.7%, respectively, and for sTWEAK, intra- and interassay coefficients of variation were 7.9 and 9.1%, respectively.

Endothelial function

Endothelial function was evaluated using high-resolution ultrasound: GE Logiq 3 (General Electric Company, Fairfield, CT, USA), with a 5- to 10-MHz linear transducer. The brachial artery in the arm without arteriovenous fistula was examined. The ultrasound examiner was blinded to other patient's data. All vasoactive medications were withheld 24 h before the procedure, and examination was performed after 14 h of overnight fasting. After 15 min of rest, the brachial artery diameter was assessed 5 cm above the antecubital fossa; the baseline diameter of the artery was registered as the mean of three consecutive measurements. A pneumatic tourniquet was inflated 50 mmHg above the systolic pressure. After 5 min, the cuff was deflated and flow measurements were taken at 1 and 10 min post-deflation.

The diameters at 1 and 10 min were also obtained as the mean of three consecutive measurements. After further 15 min, measurements were repeated and again 3 and 4 min after administration of sublingual 0.5 mg nitroglycerin. FMD was calculated as the percent change in brachial artery diameter post-deflation compared with baseline resting diameters. NMD was calculated as the percent change in brachial artery diameter post-nitroglycerin administration compared with baseline resting diameter [21, 22].

Dialysis prescription

All patients were managed by nephrologists and were dialyzed with bicarbonate-based dialysate, volumetric ultrafiltration control, single-use synthetic (polysulfone) dialyzers and heparin as standard anticoagulant. Dialysis prescription was guided by a goal of achieving a value of Kt/V $\geq$ 1.4.

Statistical analysis

Data are presented as mean $\pm$ SD for normally distributed variables or median (25th–75th percentile) for non-normally distributed variables, or absolute or relative frequencies for nominal variables. We compared the group of patients with FGF21 levels lower than 25th percentile (inferior quartile) with the group with FGF21 higher than 75th percentile (superior quartile). The statistical comparison was made using *t* test for variables with normal distribution or the Mann–Whitney rank sum test for the others. For identifying correlations between two continuous variables, Pearson's correlation coefficient or Spearman's correlation coefficient was used.

The associations between FGF21 and other clinical and biological parameters were analyzed with multivariate linear regression (ENTER method—the model contains all the variables that in univariate analysis had $p \leq 0.05$). Standardized beta and unstandardized B coefficient of regression equation, standard error and 95% confidence interval for B were reported. $p \leq 0.05$ was considered statistically significant. Statistical analyses were performed using Statistica 7.0.

Table 1 Demographic, clinical and biochemical characteristics of patients ($n = 70$)

Parameter	Value
Age (years)	59.93 ± 12.49
HD vintage (months)	67.50 (30.75–88.25)
Male *n* (%)	40 (57.1)
Diabetes mellitus *n* (%)	10 (14.3)
Hypertension *n* (%)	49(70)
SBP (mmHg)	140.90 ± 20.44
DBP (mmHg)	72.89 ± 11.11
PP (mmHg)	68.01 ± 19.57
Cardiovascular disease *n* (%)	17 (24.3)
Smoking *n* (%)	22 (31.4)
FMD (%)	8.93 (2.26–13.33)
NMD (%)	8.71 (3.93–15.17)
Body mass index (kg/m^2)	26.58 (23.71–31.09)
Waist circumference (cm)	98.88 ± 15.97
Triceps skinfold thickness (mm)	3.75 (3.00–4.00)
Kt/V	1.60 ± 0.37
Total cholesterol (mg/dl)	177.106 ± 43.38
LDL-cholesterol (mg/dl)	103.15 ± 38.81
HDL-cholesterol (mg/dl)	36.00 (30.54–47.64)
Triglycerides (mg/dl)	139.00 (97.65–195.75)
Hemoglobin (g/l)	11.41 ± 1.06
Serum albumin (g/l)	3.92 ± 0.25
CRP (mg/dl)	0.54 (0.23–1.00)
Ca (mg/dl)	8.86 ± 0.66
P (mg/dl)	4.70 (4.07–6.10)
iPTH (pg/ml)	318.00 (155.78–785.63)
Fasting glucose (mg/dl)	94.00 (87.20–115.29)
White blood cells (n/mm3)	6225 (5472–7345)
FGF21 (pg/ml)	23.34 (19.75–34.55)
sTWEAK (pg/ml)	3686.16 (3100.08–4984.87)

Data are presented as arithmetic mean ± SD; median (25th–75th percentile)

SBP systolic blood pressure, *DBP* diastolic blood pressure, *PP* pulse pressure, *FMD* flow-mediated dilation, *NMD* nitroglycerin-mediated dilation, *CRP* C-reactive protein, *Ca* calcium, *P* phosphate, *iPTH* intact parathormone, *FGF21* fibroblast growth factor 21, *sTWEAK* soluble tumor necrosis factor TNF-like weak inducer of apoptosis

Results

Patients characteristics

Demographical, clinical and biological characteristics of the patients are presented in Table 1. Fifty-eight patients (82.84%) had an arteriovenous fistula, and 12 patients (17.14%) had a tunneled transcutaneous access. The etiology of ESRD was chronic glomerulonephritis in 22% of patients, diabetes in 14%, vascular nephropathy in 20%, tubulo-interstitial diseases in 17%, polycystic kidney disease in 13% and unknown in 14%.

Current medication comprised: erythropoietin-stimulating agents (ESA) 81% of patients, intravenous iron 46% of patients, statins 20% of patients, beta blockers 57% of patients, calcium channel blockers 16% of patients, angiotensin-converting enzyme inhibitors or angiotensin receptor blockers 41% of patients and antiplatelet therapy 38% of patients.

Table 2 Multivariate linear regression analysis for determinants of FGF21

	Unstandardized coefficients		Standardized coefficients		95% Confidence interval for *B*	
	B	SE	*β*	*p*	Lower bound	Upper bound
TST	6.30	1.26	0.04	0.77	−36.24	48.85
SBP	3.40	1.46	0.30	**0.02**	0.48	6.31
DBP	1.71	2.47	0.09	0.49	−3.22	6.65
TGL	−0.03	0.26	−0.01	0.92	−0.55	0.49
Total cholesterol	0.78	0.68	0.15	0.26	−0.58	2.14
sTWEAK	0.03	0.01	0.29	**0.02**	0.01	0.06

Bold values highlight significant statistical variation of parameters

TST tricipital skinfold thickness, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *TGL* triglycerides, *sTWEAK* soluble tumor necrosis factor TNF-like weak inducer of apoptosis

Determinants of FGF21 in HD patients

FGF21 was correlated with sTWEAK ($r = 0.32$ $p = 0.007$), TST ($r = 0.27$, $p = 0.03$), SBP ($r = 0.28$, $p = 0.02$), DBP ($r = 0.26$, $p = 0.03$), total cholesterol ($r = 0.28$, $p = 0.02$) and TG ($r = 0.28$, $p = 0.02$). No statistically significant variations in FGF21 in relation to cardiovascular disease, diabetes mellitus, gender, smoking or the above-mentioned medications were found.

In multivariate linear regression with FGF21 as dependent variable, we took into account only the independent variables that were significantly correlated with FGF21 in univariate analysis. sTWEAK and SBP were significant predictors for FGF21 in multivariate linear analysis (Table 2).

The analysis of correlations in the inferior quartile has shown that FGF21 correlated with HDL-cholesterol ($r = 0.53$, $p = 0.03$). FGF21 in the superior quartile correlated with SBP ($r = 0.57$, $p = 0.01$), PP ($r = 0.61$, $p = 0.008$) and sTWEAK ($r = 0.52$, $p = 0.03$).

FMD was significantly higher in patients with lower FGF21 levels (inferior quartile) as compared to patients with higher FGF21 (superior quartile) (11.36 (6.94–13.94) vs. 6.86 (2.33–11.11) %, $p = 0.04$), while NMD was nearly statistically significant higher in the same group (14.09 (10.00–20.51) vs. 8.16 (4.76–12.28) %, $p = 0.07$) (Table 3). We found no differences between inferior and superior quartile in relation to gender, diabetes mellitus, cardiovascular disease or medication.

Discussion

The association between FGF21 and atherosclerosis risk factors [16, 23] or atherosclerosis lesions in different stages [11] has been described mainly in patients with normal renal function. In our study, in HD patients we found strong correlations between high FGF21 and proatherosclerosis markers (high levels of total cholesterol, triglycerides, SBP and DBP and sTWEAK as inflammation marker), as well as associations between high FGF21 and endothelial dysfunction (an early stage of atherosclerosis).

To our knowledge, these associations have never been studied in HD patients.

Understanding the relation between sTWEAK, a molecule belonging to tumor necrosis factor family and FGF21 might contribute to understanding cellular mechanisms involved in FGF21 effects.

FGF21 acts through interaction with specific FGF receptors that are selectively complexed with a cofactor, βKlotho [24], whose expression is predominantly detected in metabolically active tissues: liver, white adipose tissue, pancreas [25]. This interaction determines the tissue specificity of FGF21 actions [26]. There are data suggesting the existence of Klotho-independent FGF21 signaling pathway(s) where undefined cofactors are involved [27].

TWEAK is a cytokine involved in tissue injury and repair, whose actions are mediated through interaction with its receptor, fibroblast growth factor-inducible 14 (Fn14) [28], and is cleared from the bloodstream by binding to its specific receptor and by the macrophages through a specific ligand, CD163 [29]. The soluble form sTWEAK has been found to be associated with endothelial dysfunction in CKD patients [20], while in a previous study we found that sTWEAK/sCD163 ratio is associated with proatherosclerosis factors and previous cardiovascular disease in HD patients [30]. sTWEAK and TNFα downregulate Klotho expression in vivo and in vitro through activation of NFkB pathway [31, 32] and induce inflammatory cytokines expression [33]. There are also data that shows that in adipocytes TNFα reduces βKlotho expression and hence results in impaired FGF21 action [34]. Consequently, the association between sTWEAK and FGF21 found in our study might be explained by a possible interaction via βKlotho modulation:

Table 3 Comparative analysis of clinical and biochemical characteristics of patients in inferior quartile versus superior quartile

Parameter	FGF21 < 19.75 (pg/ml) Inferior quartile ($n = 17$)	FGF21 ≥ 34.55 (pg/ml) Superior quartile ($n = 18$)	p
Age (years)	60.3 ± 10.62	59.17 ± 12.54	0.77
Male (%)	52.9	61.1	0.65
Cardiovascular disease (%)	23.5	11.1	0.40
Diabetes mellitus (%)	5.9	11.1	1.00
Smoking (%)	11.8	0.0	0.22
Hypertension (%)	82.4	61.1	0.20
SBP (mmHg)	142.00 ± 22.98	143.22 ± 20.35	0.87
DBP (mmHg)	69.53 ± 10.42	77.67 ± 9.19	**0.02**
PP (mmHg)	72.47 ± 25.70	65.56 ± 16.75	0.36
TST (mm)	3.00 (2.00–4.00)	4.00 (3.00–4.00)	0.17
Body mass index (kg/m^2)	25.39 (22.51–29.55)	27.35 (25.30–31.25)	0.39
Waist circumference (cm)	100.63 ± 17.66	100.56 ± 13.51	0.99
FMD (%)	11.36 (6.94–13.94)	6.86 (2.33–11.11)	**0.04**
NMD (%)	14.09 (10.00–20.51)	8.16 (4.76–12.28)	0.07
Total cholesterol (mg/dl)	159.53 ± 34.06	193.06 ± 45.55	**0.02**
LDL-cholesterol (mg/dl)	93.01 ± 30.63	115.32 ± 43.34	0.09
HDL-cholesterol (mg/dl)	36.00 (33.76–48.00)	38.00 (33.90–43.00)	0.69
Triglycerides (mg/dl)	102.00 (92.23–146.00)	151.84 (124.00–239.00)	**0.02**
Fasting glucose (mg/dl)	102.76 ± 24.95	103.94 ± 31.04	0.53
CRP (mg/dl)	0.54 (0.27–0.98)	0.51 (0.28–0.94)	0.80
White blood cells (n/mm^3)	5640 (4710–6330)	6350 (5720–7780)	0.09
Kt/V	1.59 ± 0.44	1.57 ± 0.38	0.89
sTWEAK (pg/ml)	3383.46 (2840.21–4161.90	449.62 (3295.29–5985.67)	0.07

Bold values highlight significant statistical variation of parameters

Data are presented as arithmetic mean ± SD; median (25th–75th percentile)

SBP systolic blood pressure, *DBP* diastolic blood pressure, *PP* pulse pressure, *TST* tricipital skinfold thickness, *FMD* flow-mediated dilation, *NMD* nitroglycerin-mediated dilation, *CRP* C-reactive protein, *sTWEAK* soluble tumor necrosis factor TNF-like weak inducer of apoptosis

sTWEAK-induced βKlotho downregulation impairs the action of FGF21 which needs βKlotho as a cofactor. In our study, sTWEAK is a strong determinant of FGF21 levels; their interaction might be important for vascular homeostasis.

In addition, we found that FGF21 levels correlated with other atherosclerosis risk factors. High serum levels of FGF21 are associated with high levels of total cholesterol, triglycerides, SBP and DBP. Although FGF21 administered in experimental studies improved glucose and lipid metabolism [3] in the clinical setting in dialysis patients as well as in the general population high levels of FGF21 were found to be associated with atherosclerosis risk factors [2, 8, 26] and metabolic syndrome [35], similar to our findings. In our patients, low levels of FGF21 (inferior quartile) were associated with HDL-cholesterol which has protective effects against atherosclerosis and only high FGF21 levels (superior quartile) were positively correlated with SBP, PP and inflammation markers such as sTWEAK. It has been speculated that increased FGF21 level in cardiovascular risk populations is a compensatory mechanism in the presence of atherogenic milieu [8]. In CKD patients, the increase in FGF21 levels is due to impaired renal excretion and might be a reactive mechanism which counterbalances metabolic stress and insulin resistance present in these patients [36, 37].

Since FGF21 is produced by adipose tissue, an increase in adipose mass could lead to higher levels, as suggested by the direct correlation between FGF21 and TST in our study.

Moreover, as mentioned above, we found in our patients an association between high levels of FGF21 and endothelial dysfunction, association previously found in continuous ambulatory peritoneal dialysis patients [19]. FGF21 could be produced by endothelial cells in response to stress, and its elevated levels may be a signal of endothelial cell injury [12].

The observed correlations between FGF21 and inflammation markers that influence vascular structure and

function and atherosclerosis risk factors, as well as between FGF21 and endothelial dysfunction, suggests that in HD patients FGF21 is associated with cardiovascular risk and early atherosclerosis, most probably as part of the metabolic response mechanisms induced by the atherogenic milieu.

Conclusions

High FGF21 values in HD patients are associated with increased sTWEAK and cardiovascular risk factors: hypercholesterolemia, hypertriglyceridemia, hypertension and increased TST. Endothelial dysfunction is associated with high FGF21. These findings can propose FGF21 as a biomarker for early atherosclerosis in HD patients.

Our study has certain limitations. Firstly, since it is cross-sectional and observational we could evaluate only the associations between various parameters and not the pathogenetic mechanisms. Secondly, the study comprises a relatively small number of patients, while the statistical approach is valid. Our findings might lead to further studies with larger sample size.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. The results of this paper have not been published previously in whole or part.

Ethical approval All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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RESEARCH ARTICLE

Soluble CD40 ligand in haemodialysis patients: survival impact and cardiovascular prognostic role

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ABSTRACT

Context: Soluble CD40 ligand (sCD40l) can predict cardiovascular events (CVE) and mortality in haemodialysis (HD) patients (short-, medium-term follow-up studies).
Objective: To evaluate the relationship between sCD40l and survival, CVE and mortality in HD patients on long-term follow-up.
Methods: We registered 46 HD patients' baseline characteristics, mortality and CVE for 108 months.
Results: SCD40l correlated positively with C-reactive protein, was higher in survivors, but had no impact on survival and was not predictive for CVE or CV mortality.
Conclusion: The levels of sCD40l have no influence on survival or CVE and mortality in HD patients in a long-term follow-up.

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Introduction

Chronic kidney disease is associated with substantially increased risk for cardiovascular (CV) morbidity and mortality, only partially explained by classical cardiovascular risk factors. It has been proposed that enhanced vascular inflammation might explain the mechanisms underlying the increased susceptibility of HD patients to develop atherosclerosis and CV events (Appel, 2004; Panichi et al., 2008; Ritz & McClellan, 2004). In this regard various mediators were studied, amongst them soluble CD40 ligand and CD40 receptor, involved in many processes: inflammation, immune system (of crucial interest being cell signalling in innate and adaptive immunity) (Aloui et al., 2014), atherosclerosis and atherothrombosis (Antoniades et al., 2009; Packard & Libby, 2008; Panichi et al., 2012). CD40l is a 33 kDa type II transmembrane protein, structurally related to tumour necrosis factor (TNF) superfamily. CD40l is constitutively highly expressed by multiple haematopoietic and non-hematopoetic cells: platelets, macrophages, lymphocytes and several cells of the vasculature, including endothelial cells and smooth muscle cells (Elgueta et al., 2009; Graf et al., 1995; Zhang et al., 2013). CD40l may be cleaved into a soluble form (sCD40l) that has a cytokine-like activity. Active CD40l at the cell surface or in its soluble form is composed of homotrimers, like other members of the TNF family (Locksley et al., 2001). This multimeric conformation of CD40l is of crucial importance for effective interaction with CD40 and the subsequent intracellular signalling (Anand et al., 2003). Activated sCD40l in macrophages induces the production of reactive oxygen and nitrogen species that contribute to the destruction of intracellular pathogens (Bhadra et al., 2011). sCD40l induces signals that activate T cells (CD8) with a role in chronic viral infections (Aloui et al., 2014; Bhadra et al., 2011). Both forms of CD40l activate classical as well as alternative NF-kB pathways (Chen et al., 2008b). Besides its role in immunity processes (Aloui et al., 2014) this molecule has a dual prothrombotic and proinflammatory role, induces endothelial dysfunction with decreased NO synthesis and augmented oxidative stress (Chen et al., 2008a; Cipollone et al., 2005; Desideri & Ferri, 2005). In hypertension (Ferroni & Guadagni, 2008; Patel et al., 2006; Penno et al., 2009; Sonmez et al., 2005; Yuan et al., 2010), diabetes (Cipollone et al., 2005; Lajer et al., 2010) and hypercholesterolaemia (Cipollone et al., 2002) elevated or abnormal values are described. Many studies reported the impact of sCD40l in CV disease. In this regard, the prognostic value in acute coronary syndrome was shown (Aukrust et al., 1999; Heeschen et al., 2003), as well as the interrelations of sCD40l with Framingham score for coronary disease (Verma et al., 2005), cardiac hypertrophy (Derks et al., 2013), and the relation of sCD40l with cardiovascular risk (Schönbeck, 2001; Vishnevetsky et al., 2004). The correlation between sCD40l and all-cause mortality and between sCD40l and cardiovascular morbidity and mortality was also studied in HD patients (Sirolli et al., 2001; Tripepi et al., 2005). SCD40l was found in atherosclerotic plaques in HD patients (Campean et al., 2007), and some studies demonstrated that sCD40l has predictive value for acute and chronic CV events in these patients (short and medium follow-up) (Desideri et al., 2011; Hocher et al., 2007; Lim et al., 2008). Hocher et al. (2007) found that serum

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values of sCD40l in HD patients are predictive for CV events but not for general mortality in a 52-month follow-up study. Desideri et al. (2011) showed that sCD40l is predictive for combined cardiovascular morbidity and mortality in HD in a relatively short-term follow-up study (24 months). Hocher and Desideri found no impact of sCD40l on general mortality.

Interestingly, high values of sCD40l were generally associated with CV events and CV mortality, but in a recent study (Haller et al., 2013), in a group of patients with renal arterial stenosis and reduced glomerular filtration rate (mean 40 ± 19 ml/min), higher values with a tendency towards statistical significance of sCD40l were found to be associated with better survival. These studies show that the impact and predictive value on long-term survival in HD patients of sCD40l is not clear. All these findings point towards the necessity of supplemental studies.

The aim of this study was the analysis of the impact of sCD40l on survival and CV events and mortality in HD patients in a long-term follow-up and the analysis of sCD40l values associated with better survival.

Methods

Patients

We conducted a prospective cohort study, carried in HD patients, with the aim to investigate the possible predictive role of circulating sCD40l levels on survival and cardiovascular morbidity and mortality rates during a long-term follow-up (108 months). All measurements were performed during a midweek non-dialysis day.

Of the 90 patients on conventional HD treatment in Nefromed Dialysis Centre, Cluj-Napoca, 46 patients met the inclusion criteria and also agreed to participate in this study in 2005. Inclusion criteria were: prevalent HD patients, age >18 years, duration of maintenance haemodialysis at least 6 months (HD vintage), without residual renal function. We excluded patients with acute inflammation processes, terminal neoplasia, previous renal transplant or immunosuppressive or antiviral treatment. All patients were on thrice weekly HD (4–5 h) regimen. Dry weight was targeted to achieve a normotensive oedema-free state. Patients' demographics data, cause of end stage renal disease (ESRD), duration of maintenance haemodialysis, and comorbidity conditions (diabetes, hypertension, virus hepatitis B or C infection, smoking status, antihypertensive medication, especially angiotensin system inhibitors and statins) upon enrolment were obtained from charts and medical documents. We also registered clinical data: age, weight, height, systolic blood pressure, diastolic blood pressure (predialysis values). Blood pressure (BP) was measured with an upper arm mercury sphygmomanometer according to the recommendations of the American Heart Association. We measured arm systolic blood pressure (SBP) and diastolic blood pressure (DBP) in reclined position after 10 min of rest. We registered previous cardiovascular disease (cardiac disease evaluated by: electrocardiogram with Q-wave infarction, or myocardial enzyme elevation, coronary revascularisation, typical history of angina with abnormal coronarography, neurological disease with: new onset focal neurological deficit, with or without computed tomography or magnetic resonance imaging evidence of cerebral infarction, or carotid stenosis with or without endarterectomy, or lower extremity arterial disease with revascularisation or amputation, new onset of intermittent claudication confirmed by Doppler or arteriography findings. We calculated body mass index (BMI) as BMI = (weight (kg)/height^2 (m^2) and pulse pressure (PP) with the formula: PP = SBP − DBP (mmHg).

All patients enrolled in the study were followed prospectively for 108 months. During the follow-up period, we registered every 6 months the general and cardiovascular mortality (myocardial infarction, congestive heart failure, stroke and sudden death) and cardiac events (electrocardiogram with Q-wave infarction, or myocardial enzyme elevation, coronary revascularisation, typical history of angina with abnormal coronarography) vascular events (neurological disease and lower extremity arterial disease with same method as the registration of previous cardiovascular disease). Patients were surveyed till the time of transfer to other dialysis units, transplantation or the end of the observation period.

All clinical examinations performed took place in the Dialysis Centre where the patients had three sessions in a week. Five authors have recorded all the current events (including cardiovascular events or deaths) taken from the patients' medical documents from the medical centres, where they had investigations and treatment.

Laboratory parameters

All biochemical analyses were performed after an overnight fast between 7.00 and 9.00 AM always during a midweek non-dialysis day. The samples were collected into chilled EDTA (ethylendiaminotetraacetic acid) vacutainers placed immediately on ice and centrifuged within 30 min at −4 °C and the plasma was stored at −80 °C. Measurements at the initiation of this study included serum electrolytes, albumin, creatinine, uric acid, iron profile (iron, transferrin and ferritin), lipid profile (total cholesterol, triglycerides (TG) and HDL-cholesterol), C-reactiveprotein (CRP), alkaline phosphatase, intact parathormone (iPTH). Pre-dialysis and post-dialysis urea levels were used to calculate *Kt/V*. Serum calcium was corrected (cCa) for serum albumin according to the formula: cCa (mg/dl) = serum calcium (mg/dl) + 0.8 * (4.0-serum albumin (g/dl)), LDL-cholesterol was calculated with Friedewald's formula: LDL-cholesterol = total cholesterol − (HDL-chol + TG/5). Soluble CD40 ligand was determined by commercial ELISA kit (enzyme linked immunosorbent assay), detection limit 0.06 ng/ml, Bender Medical System, Vienna, Austria. Hepatitis virus B and C detection was performed by electrochemiluminiscence for HBs antigen (HBs Ag) and hepatitic C virus antibodies (HCV Ab).

Dialysis prescription

All patients were managed by nephrologists and were dialysed with bicarbonate based dialysate, volumetric ultrafiltration control, single use synthetic (polysulphone) dialysers and heparin as standard anticoagulant. Dialysis prescription was guided by a goal of achieving a value of $Kt/V \geq 1.4$.

Erythropoietin was prescribed via a standardised algorithm. Antihypertensive drugs were prescribed for patients having post-dialysis or inter-dialysis blood pressure persistently above 150/95 mmHg, at dry weight.

Statistical analysis

Continuous variables are presented as mean ± SD or median (25th–75th percentile) and categorical variables are expressed as percentages unless otherwise stated. Normal distribution was tested with the Kolmogorov–Smirnov test for large samples or with the Shapiro–Wilk test for small samples. For comparison of continuous variables between two groups, the Student *t*-test or the Mann–Whitney test were used. For identifying correlations between two continuous variables, Pearson's correlation coefficient or Spearman's correlation coefficient were used. To estimate the relation between one dependent variable and several independent quantitative variables, multivariate linear regression (enter method) was used. Cox's proportional hazards regression analysis was used to examine the associations between variables and survival time. Variables that were significant in the univariate analysis were included in a multivariate Cox proportional hazards regression model. Hazard ratios (HR) and their 95% confidence intervals (CI) were calculated. We compared survival curves with log-rank test and we presented the curves with the Kaplan–Meier plot. The cut-off was obtained with receiver operating characteristic (ROC) curve analysis. We found cut-off value with best sensitivity and specificity. $p < 0.05$ was considered statistically significant. Statistical analyses were performed using Statistica 7.0 (StatSoft Inc., Tulsa, OK).

Ethical issues

All patients signed an informed consent before entering the study. Their privacy was respected. The study protocol conformed to the ethical guidelines and was approved by the University Ethics Committee.

Results

Demographical and clinical characteristics of the study population (46 patients) are reported in Table 1. 44 patients (95.6%) had an arterio-venous fistula, and 2 (4.1%) patients had a semipermanent transcutaneous access. Comorbidity conditions included 44.4% hypertension, hepatitis virus infection 43.5% (20 patients, 3 HBs Ag positive, 17 HCV Ab positive) and no diabetes mellitus. sCD40l levels were not different between subgroups with or without hepatitis virus infection (18.0 (13.70–22.85) ng/ml versus 17.8 (13.55–24.00) ng/ml, $p = 0.97$). We registered only 4 patients with statin treatment (8.7%).

Only CRP correlated with sCD40l levels ($r = 0.39$, $p = 0.01$), after evaluation of all clinical and laboratory parameters registered. We did not find correlations of sCD40l with total cholesterol ($r = -0.09$, $p = 0.55$), LDL-cholesterol ($r = -0.14$, $p = 0.37$) or statistically significant differences of sCD40l between patients with and without statin treatment 16.8 (14.50−22.78) ng/ml versus 18.0 (13.45−23.10) ng/ml, $p = 0.97$.

Total mortality rate was 41.3% (19 patients), 11 cardiovascular deaths (cardiovascular mortality rate 23.9%), 8 (17.3%) deaths of other causes (infections, malignances, hyperkaliemia and gastro-intestinal bleeding).

The analysis of clinical and laboratory parameters of survivors and deceased patients showed significantly higher levels of sCD40l in survivors (Figure 1) and higher levels of PP and cCa in non-survivors (Table 1).

Univariate Cox proportional hazard analysis of factors predicting mortality is presented in Table 2. ROC Curves [Area Under the Curve (AUC) = 0.67, $p = 0.05$] detected that cut-off value for sCD40l was 22.2 pg/ml to predict survival (sensitivity

Table 1. Baseline characteristics of patients and comparison of clinical and biochemical profiles of survivors versus non-survivors.

Parameters	Total ($n = 46$)	Non-survivors ($n = 19$)	Survivors ($n = 27$)	p
Age (years)	59.04 ± 12.12	64.00 ± 11.60	56.56 ± 11.41	**0.02**
Body mass index (kg/m^2)	23.23 (20.75–26.85)	23.05 (20.39–26.75)	23.56 (22.10–27.24)	0.27
Gender (male) (%)	25/46 (54.3%)	12/19 (63.2%)	13/27 (48.1%)	0.31
Smoking (%)	23/46 (50.0%)	8/19 (42.1%)	15/27 (55.6%)	0.37
SBP (mmHg)	129.00 ± 20.60	132.89 ± 20.83	126.15 ± 20.36	0.28
DBP (mmHg)	80.00 (70.00–87.50)	75.00 (70.00–85.00)	80.00 (70.00–90.00)	0.37
PP (mmHg)	50.00 (40.00–60.00)	55.00 (47.50–62.50)	50.00 (40.00–51.25)	**0.049**
Previous CV events	23/46 (50.0%)	12/19 (63.2%)	11/27 (40.7%)	0.13
Hypertension	20/46 (44.4%)	10/19 (52.6%)	10/27 (37.0%)	0.35
Total chol (mg/dl)	166.93 ± 41.76	176.10 ± 41.99	160.48 ± 41.14	0.22
HDL-chol (mg/dl)	42.30 (31.35–52.45)	48.4 (30.2–61.2)	39.85 (32.68–49.33)	0.29
LDL-chol (mg/dl)	86.41 ± 29.24	94.43 ± 23.41	80.55 ± 32.03	0.12
Tryglicerides (mg/dl)	163.50 (109.50–234.75)	143.00 (76.00–228.00)	179.00 (120.00–256.00)	0.16
HD vintage (months)	85.00 (56.00–139.75)	125.00 (85.00–166.00)	60.00 (26.00–104.00)	**0.002**
Kt/V	1.30 (1.20–1.60)	1.20 (1.20–1.50)	1.30 (1.20–1.80)	0.29
Hemoglobin (g/l)	11.24 ± 1.57	11.36 ± 1.45	11.15 ± 1.67	0.65
Serum albumin (g/l)	4.21 ± 0.30	4.14 ± 0.35	4.26 ± 0.24	0.19
CRP (mg/dl)	0.36 (0.15–1.20)	0.34 (0.24–1.51)	0.47 (0.13–0.86)	0.72
cCa (mg/dl)	8.62 ± 1.32	9.01 ± 1.32	8.34 ± 1.29	0.09
P (mg/dl)	6.76 ± 2.11	6.76 ± 2.22	6.76 ± 2.07	1.00
Serum iPTH (pg/ml)	532.00 (207.00–929.00)	532.00 (231.50–1215.50)	539.50 (187.28–823.25)	0.69
sCD40l (ng/ml)	18.00 (13.90–23.10)	16.00 (12.20–19.20)	20.00 (14.20–24.10)	**0.046**
HV positive	20/46 (43.5%)	11/19 (40.7%)	9/27 (47.4%)	0.19

Data are presented as mean ± SD or median; SBP: systolic blood pressure; DBP: diastolic blood pressure; PP: pulse pressure; cCa: calcium corrected by serum albumin; P: phosphate; iPTH: intact parathyroid hormone; CRP: C-reactive protein; HV: hepatitis virus.
Bold numbers indicate the values of $p < 0.05$ (statistically significant comparisons).

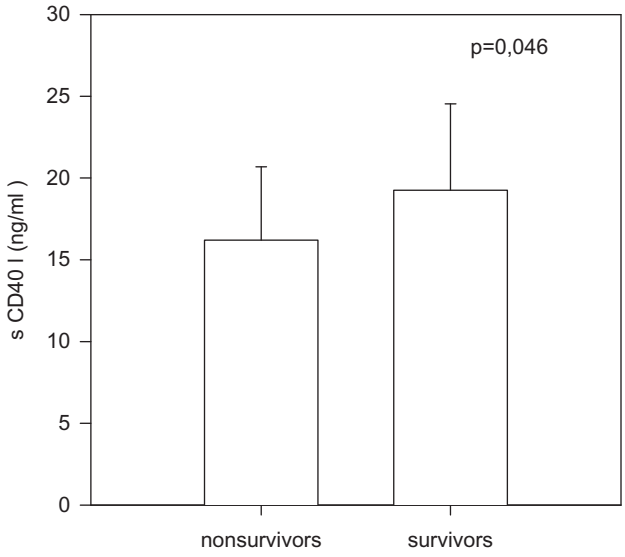

Figure 1. Baseline sCD40 l (log scale) and survival in HD patients.

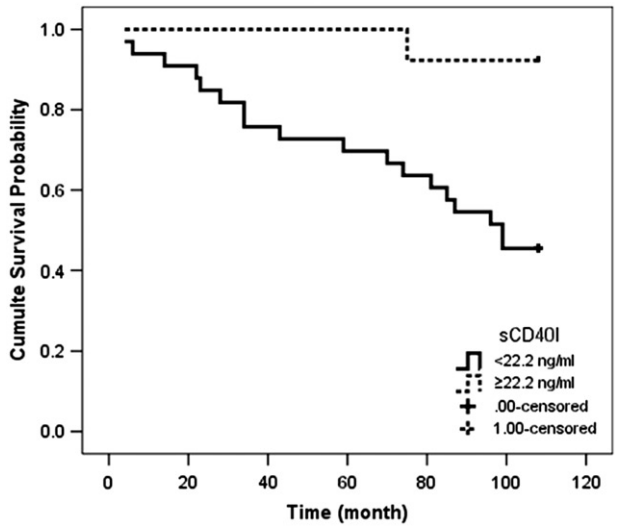

Figure 2. Kaplan–Meier survival curves stratified by sCD40l (cut-off obtained with ROC curve): sCD40l $<$ 22.2 ng/ml versus sCD40l $\geq$ 22.2 ng/ml, $p = 0.007$.

Table 2. Univariate Cox proportional hazard analysis of factors predicting mortality in HD patients.

Parameters	Hazard ratio (95% CI)	p Value
Gender (male)	1.70 (0.67–4.33)	0.27
Smoking	0.67 (0.27–1.67)	0.39
Age (years)	1.04 (1.00–1.08)	**0.049**
HD vintage (months)	1.00 (1.00–1.01)	**0.035**
SBP (mmHg)	1.02 (0.99–1.04)	0.20
DBP (mmHg)	0.99 (0.96–1.03)	0.67
PP (mmHg)	1.06 (1.01–1.10)	**0.015**
BMI (kg/m^2)	0.93 (0.82–1.05)	0.22
Previous CV disease	0.91 (0.78–1.01)	0.4
Total chol (mg/dl)	1.01 (1.00–1.02)	0.26
sCD40l (ng/ml)	0.91 (0.83–0.99)	**0.03**
LDL-cholesterol (mg/dl)	1.01 (1.00–1.03)	0.17
CRP (mg/dl)	1.44 (0.96–2.16)	0.08
Serum albumin (g/dl)	0.19 (0.03–1.14)	0.07
Serum iPTH (pg/ml)	1.00 (1.00–1.01)	0.65
cCa (mg/dl)	1.64 (1.07–2.51)	**0.02**
P (mg/dl)	1.01 (0.82–1.25)	0.92

SBP: systolic blood pressure; DBP: diastolic blood pressure; PP: pulse pressure; cCa: calcium corrected by serum albumin; P: phosphate; iPTH: intact parathyroid hormone; CRP: C-reactive protein.
Bold numbers indicate the values of $p < 0.05$ (statistically significant comparisons).

$= 0.44$, specificity $= 0.96$). Kaplan–Meier analysis curve show that survival was significantly different between the group with sCD40l $<$ 22.2 ng/ml as compared to the group with sCD40l $>$ 22.2 ng/ml (test log-rank $p = 0.007$) (Figure 2). In the non-survivors group 1 (0.05%), patient had sCD40l levels over the cut-off value versus 12 (44.4%) from the survivors group ($p = 0.004$). Median survival time was 99 months for the group with sCD40l over 22.2 pg/ml ($p = 0.007$). In the multivariate Cox proportional hazards regression model, including all possible demographic, inflammatory, HD-related confounders and comorbidities found to be significant in univariate analysis, we found that survival was significantly influenced by cCa [HR $= 1.99$, 95% CI (1.19−3.34), $p = 0.008$], age [HR $= 1.06$, 95% CI (1.01−1.11), $p = 0.03$], but not HD vintage [HR $= 1.004$, 95% CI (1.004−1.008), $p = 0.06$], PP [HR $= 1.03$, 95% CI (0.98−1.08), $p = 0.27$], and sCD40 l [HR $= 0.94$, 95% CI (0.84−1.04), $p = 0.21$]. Multivariate Cox proportional hazard analysis of factors predicting survival using the same parameters, but replacing sCD40l with cut-off sCD40l $>$22.2 ng/ml validated the statistically significant impact of cCa [HR $= 1.94$, 95% CI (1.17−3.24), $p = 0.01$], and not of sCD40l $>$ 22.2 pg/ml [HR $= 0.15$, 95% CI (0.02–1.22), $p = 0.08$], PP [HR $= 1.03$, 95% CI (0.98–1.08), $p = 0.21$], age [HR $= 1.04$, 95% CI (0.99–1.09), $p = 0.09$] and HD vintage [HR $= 1.004$, 95% CI (1.000–1.008), $p = 0.08$].

During the follow-up period, 18 (39.1%) patients experienced non-fatal cardiovascular events: 10 (55.6%) cardiac events and 8 (44.4%) vascular events.

We analysed the impact of sCD40l on global CV events [HR $= 0.97$, 95% CI (0.89−1.06), $p = 0.54$], cardiac events [HR $= 0.98$; 95% CI (0.89−1.09), $p = 0.73$] and vascular events [HR $= 0.96$, 95% CI (0.81−1.14), $p = 0.64$] using univariate Cox regression. sCD40l proved not to be predictive for CV events in our population. CV deaths represented 57.9% (11 deaths of 19) of overall mortality. sCD40l was not predictive for CV mortality in our study [HR $= 0.88$, 95% CI (0.76–1.007), $p = 0.06$].

Discussion

The high incidence of CV complications in patients with chronic renal failure (CRF) is partly explained by more aggressive atherosclerosis, i.e. increased incidence and severity of lesions with higher tendency to calcification. The pathogenesis of this accelerated atherosclerosis, however, is not completely understood. Among other risk factors, chronic micro-inflammation may be involved (Kacso et al., 2010; Panichi et al., 2012). The inflammatory status might induce thrombocyte activation and release of CD40l. The interaction CD40–CD154 (CD40 ligand) might favour an inflammatory process with activation of cells and adhesion molecules and atherosclerotic plaque instability via the induction of various cytokines, chemokines, growth factors and coagulation factors.

Significantly higher sCD40l levels in HD patients as compared to healthy controls was found in most of the studies in the literature and the positive correlation between sCD40l

levels and inflammatory markers might be secondary to chronic microinflammation. The positive correlation between sCD40l and CRP, observed in our study, might be the reflection of this phenomenon. Lim et al. (2008) noticed positive correlations between sCD40l and inflammatory and oxidative stress markers in HD patients: soluble vascular adhesion molecule, malondialdehyde, protein carbonyl, but not with CRP. Considering there is no correlation between CRP and sCD40l as showed in the previous studies, it was hypothesised that thrombocyte activation with sCD40l release might be induced by other inflammation mediators than CRP. In contrast, in our study, the positive correlation between sCD40l and CRP might suggest a CRP induced thrombocyte activation or an increase in both mediators (CRP and sCD40l) secondary to inflammation. The mentioned correlations between sCD40l and inflammatory and oxidative stress mediators identified in other studies as well as in our study might be explained by the malnutrition-inflammation syndrome (MIA) present in HD patients.

Some authors observed that sCD40l levels seem to be influenced by body weight, cholesterol levels, statin treatment and smoking (Cipollone et al., 2002; Desideri & Ferri, 2003; Malyszko et al., 2004; Strippoli et al., 2008). In our study, as in other previous studies, we identified no significant correlations between sCD40l and total cholesterol or LDL-cholesterol (Desideri et al., 2011; Lim et al., 2008). In uraemic populations, quite distinct from non-uraemic populations, the association between cholesterol and atherosclerosis is weaker.

sCD40l has been recently proposed as a CV prognostic factor in HD patients, due to its ability to predict non-fatal and fatal atherothrombotic events, but not all-cause mortality in a 4-year follow-up study (Hocher et al., 2007). Desideri et al. (2011) found in a shorter (2 years) follow-up study a significant increase in CV events and mortality but not in general mortality associated with higher levels of sCD40l.

Our long-term follow-up study yielded interesting results. In our study, sCD40l levels did not predict CV events and CV mortality or all-cause mortality. This finding might be explained by different characteristics of our study group: first, our follow-up period is much longer than in previous studies and it might be that the predictive value of sCD40l on CV events and mortality is valid only on short and medium term. Second, the HD patients included in our study have a different CV profile as compared to the most important previous studies that observed the impact of sCD40l on CV events and CV and all-cause mortality (Desideri et al., 2011; Hocher et al., 2007). The age in our group is lower by 6 to 10 years than that in other groups, none of the patients had diabetes mellitus, previous CV disease was present in around 50% of our patients and was slightly lower in previous studies, HD vintage and CRP were higher in our patients. Moreover, in our patients, mineral metabolism changes were more severe: higher serum phosphorus and iPTH. This CV profile might have completely different consequences on survival, CV events and mortality than that found in previous studies (Hocher et al., 2007), might dominate the prognosis and reduce the role of sCD40l to an innocent bystander, secondary increased or with a yet undetermined role. Third, the statistical analysis we applied was different in our study as compared to the study of Desideri et al. (2011), who analysed composite end points: non-fatal and fatal cardiovascular events. We analysed CV events and mortality separately. Studies in general population showed that sCD40l is associated with other inflammatory markers but is not in itself a strong independent risk marker for either stroke or myocardic infarction (Jefferis et al., 2011). Fourth, it is possible that the high percentage of patients with hepatitis virus B and C included (50% in non-survivors and 33% in survivors) might have influenced sCD40l levels, although we did not find a correlation between these two parameters. Even the higher levels of sCD40l found in our study as compared to previous ones might be explained by different mechanisms of activation in our study, possibly related to hepatitis virus infection.

Moreover, Haller et al. (2013) in a study on renal artery stenosis patients with decreased GFR shows that in non-survivors sCD40l levels are lower than in survivors in a short-term follow-up (12 months). We also found significantly lower levels of sCD40l in non-survivors as compared to survivors, without an obvious explanation, possibly an epiphenomenon without a direct connection with survival. Other parameters (higher serum calcium, older age, higher HD vintage) seem to have a higher influence on survival in our patients. The cut-off value obtained by the ROC curve and the results of Kaplan–Meier analysis, also underlines the association between higher sCD40l values and better survival.

Regarding serum calcium, several studies found its impact on survival and CV events and mortality in HD patients. The effect of mineral metabolism disturbances (hyperphosphataemia, hypercalcaemia, secondary hyperparathyroidism) on survival and CV disease has been repeatedly demonstrated (Tagawa et al., 2014; Tentori et al., 2008), making our findings less surprising. A cardiovascular profile comprising profound mineral metabolism changes and the well-known effect of mineral metabolism on survival in HD patients explains the major impact of serum calcium on survival.

These findings demonstrate the importance of proper analysis of cardiovascular risk in each population before undiscriminately applying new prognosis markers in different patient groups since their peculiarities have certain consequences on follow-up and treatment.

Conclusions

In HD patients, sCD40l levels correlate with CRP and are higher in survivors. This marker has no influence on survival or cardiovascular events and mortality in HD patients in long-term follow-up. The limit of this study is relatively low number of patients. We underline the necessity of larger prospective studies that might confirm or not our findings and might shed more light on the prognostic role of sCD40l in HD patients.

Disclosure statement

The authors report no declarations of interest.

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Original papers

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Nitroglycerin mediated dilation evaluated by ultrasound is associated with sTWEAK in hemodialysis patients.

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Abstract

Aims: The main cause of death in hemodialysis (HD) patients is cardiovascular disease. Ultrasound assessment of the brachial artery dysfunction is easily achievable and can non-invasively detect atherosclerosis in various stages. In HD patients the cardiovascular risk profile is different and the determinants of brachial arterial function can be distinct comparing with general population. The aim of the study is to assess the determinants of arterial brachial function (flow-mediated and nitroglycerin-mediated dilation) evaluated by ultrasound in HD patients and their relation with tumor necrosis factor-like weak inducer of apoptosis (sTWEAK) described as atherosclerotic marker in chronic kidney disease patients. **Material and methods:** We conducted a cross-sectional observational study on 54 hemodialysis patients. We recorded clinical and biological data and we measured sTWEAK serum levels by ELISA. We evaluated the arterial brachial function by measurement of flow-mediated and nitroglycerin-mediated dilation, using B mode ultrasound. **Results:** The determinants of flow-mediated dilation were: Kt/V ($r=0.47$, $p<0.001$), LDL-cholesterol ($r=0.29$, $p=0.04$), and total cholesterol ($r=0.31$, $p=0.02$). Flow-mediated dilation correlated with nitroglycerin-mediated dilation ($r=0.70$, $p<0.001$). In multivariate analysis kt/V was the only significant predictor for flow-mediated dilation ($p=0.04$). Nitroglycerin-mediated dilation correlates with sTWEAK ($r=-0.30$, $p=0.03$), systolic blood pressure ($r=-0.28$, $p=0.04$) and pulse pressure ($r=-0.31$, $p=0.02$). In multivariate analysis sTWEAK was the only significant predictor for nitroglycerin-mediated dilation ($p=0.04$). **Conclusions:** The main determinant of nitroglycerin-mediated dilation was sTWEAK. In addition, decreased nitroglycerin-mediated dilation was associated with higher systolic blood pressure and pulse pressure. The main determinant of FMD was Kt/V. Increased flow-mediated dilation was associated with better dialysis efficiency and high total cholesterol and LDL-cholesterol.

Keywords: hemodialysis, vascular ultrasound, biomarkers

Introduction

In hemodialysis (HD) patients the main cause of death is cardiovascular disease. For this reason the detection of atherosclerosis (ATS) in preclinical phases using various techniques and biomarkers is a necessity. Among these techniques, the endothelial function and vascular smooth muscle function evaluated with ultrasound flow-mediated dilation (FMD) and nitroglycerin-mediated dilation (NMD) are currently used. In addition, the panel of cardiovascular risk factors is different in HD patients, with more severe inflammation and ATS. Considering this, the parameters of FMD and NMD may be different compared to the general population.

Endothelial dysfunction is one of the initial changes in the development of ATS and an important predictor of cardiovascular disease in chronic kidney disease patients [1]. FMD has become the most widely used technique to measure endothelial function [2]. FMD is a non-invasive

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method, easily accessible, and with good correlation with invasive epicardial vascular function evaluation [3] but still requires better standardization [4].

Vascular smooth muscle dysfunction assessed by brachial NMD, was used initially as a control test for FMD measurements in order to differentiate endothelium-dependent from endothelium-independent vasodilation because both endogenous NO and administered nitroglycerin act on vascular smooth muscle cells. However, several studies have proved that both FMD and NMD are modified in subjects with cardiovascular risk factors and coronary heart disease [5,6]. In addition NMD proved to be a good predictor of cardiovascular mortality and morbidity both in the general population and in dialysis patients [7,8]. Impaired NMD is thought to be related with structural vascular alterations and smooth muscle cells dysfunction as a result of ATS [9] and can reflect arterial compliance [8].

Measurement of brachial FMD and NMD with ultrasound is generally achieved by grey scale, but in some studies Doppler exam is used to assess peak flow velocity (basal and after reactive hyperemia) [1].

Even if in principle FMD and NMD measurements seem simple, the procedures are technically challenging and require training and standardization. Most crucial are the study preparation, image acquisition, site selection, sphygmomanometer probe position, cuff occlusion time and correct description of the FMD response [4].

Mechanisms responsible for vascular disorders in HD patients are unclear, controversial, and presumed to be multifactorial. Endothelial dysfunction involves dysregulation of multiple pathways [10], one of them possibly mediated by tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK). This, a member of TNF family, is synthesized as a type II transmembrane glycoprotein and circulates in plasma as a soluble form (sTWEAK) [11]. This molecule can induces smooth muscle cells proliferation in the arterial wall [12] and seems to be involved in all pathogenetic phases of ATS [13].

A few studies found that in chronic kidney disease (CKD), sTWEAK correlates with brachial FMD and NMD. In HD patients the effect of sTWEAK on FMD and NMD was studied only in patients with no diabetes mellitus and no previous cardiovascular disease [10], with the emphasis mostly on endothelial dysfunction and less on muscular vascular dysfunction. Further studies are needed in order to assess the influence of this molecule on vascular dysfunction in HD patients.

The **aim** of the study is to assess the determinants of FMD and NMD in hemodialysis patients and to evaluate the relation between FMD, NMD, and sTWEAK described as atherosclerotic marker in CKD patients.

Materials and methods

Patients:

We conducted an analytical cross-sectional observational study, on a cohort of HD patients. All measurements were performed during a midweek non-dialysis day. Of the 110 patients on conventional HD treatment in Nefromed Dialysis Center Cluj, 54 patients met the inclusion criteria and also agreed to participate in this study in 2014. Inclusion criteria were: prevalent HD patients, age>18 years, duration of maintenance hemodialysis at least 6 months (HD vintage) and no residual renal function. We excluded patients with acute inflammation processes, terminal neoplasia, and viral hepatitis. All patients were on thrice weekly HD (4-5 h) regimen. Patients' demographics data, etiology of end stage renal disease (ESRD), HD vintage, and comorbidity conditions (diabetes, hypertension, smoking status, antihypertensive treatment, statins, antiplatelet therapy, erythropoietin treatment, intravenous iron) upon enrolment were obtained from medical documents. We registered clinical data: age, weight, height, systolic blood pressure (SBP), diastolic blood pressure (DBP) (predialysis values), triceps skinfold thickness (TST) (mm), waist circumference (WC) (cm). We also registered previous cardiovascular disease (angina or infarcts, coronary revascularization, stroke or documented peripheral arterial disease). We calculated body mass index (BMI) as BMI = (weight (kg)/height² (m^2) and pulse pressure (PP) with formula: PP=SBP-DBP (mmHg).

All patients signed an informed consent before entering the study. Their privacy was respected. The study protocol conformed to the ethical guidelines and was approved by the University Ethics Committee, and was in accordance with the ethical standards of the World Medical Association, Declaration of Helsinki, revised in 2000, Edinburgh.

Laboratory parameters

All biochemical analyses were performed after an overnight fast between 7.00-9.00 a.m. always during a midweek dialysis day. Current measurements at the initiation of the study include serum electrolytes, albumin, predialysis creatinine, lipid profile (total cholesterol, triglycerides (TG) and HDL-cholesterol), C-reactive protein (CRP), intact parathormone (iPTH), hemoglobin, white blood cell (WBC). Pre- and post-dialysis urea levels were used to calculate Kt/V. LDL-cholesterol was calculated with Friedewald formula: LDL-cholesterol=total cholesterol-(HDL-chol+TG/5). sTWEAK was determined with commercially available enzyme-linked immunosorbent assay kits (Bender MedSystem, Vienna, Austria kit for sTWEAK). The minimum detectable level

of sTWEAK was 10 pg/ml. Intra- and interassay coefficients of variation were 7.9% and 9.1% respectively.

Ultrasonography

Vascular measurements were performed using high-resolution ultrasound: GE Logiq 3 (General Electric Company, Fairfield, CT, USA), with a 5–10 MHz linear transducer. The brachial artery in the arm without arteriovenous fistula was examined. The ultrasound examiner was blinded to other patient's data. All vasoactive medications were withheld 24 hours before the procedure and examination was performed after 14 hours of overnight fasting. After 15 minutes of rest before the examination started, the brachial artery diameter was assessed 5 cm above the antecubital fossa. Longitudinal images were obtained and the depth and gain setting were optimized to identify the anterior and posterior intimal interfaces between the lumen and vessel wall. The optimal grey scale image was obtained when the transducer was perpendicular on the vessel. Three adjacent measurements were recorded. In longitudinal view, both walls showed up distinctly over a certain range on a straight and non-branching arterial segment. The transition from the intima to the lumen showed a weak signal, while the adventitia had a high amplitude signal. The media (between the 2 layers) had relatively low reflectivity and appeared as a hypo-echoic band on the images recorded with ultrasound systems with sufficient resolution [14] (fig 1). A pneumatic tourniquet was inflated 50 mmHg above the systolic pressure for 5 minutes before deflating it. After 5 minutes the cuff was deflated and flow measurements were made at 1 minute and 10 minutes post deflation. After further 15 minute measurements were repeated and again 3 and 4 minutes after administration of sublingual 0.5 mg nitroglycerin [2,4]. FMD was calculated as the percent change in brachial artery diameter post deflation compared with baseline resting diameters. NMD was calculated as the percent change in the brachial artery diameter post nitroglycerin administration compared with baseline resting diameters [2,4,15].

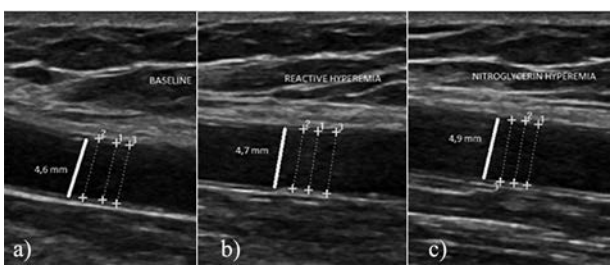

Fig 1. High resolution linear ultrasound measurement of brachial artery diameter a) before stimulus (baseline), b) 1 min after hyperemic stimulus (reactive hyperemia), and c) after nitroglycerin administration (nitroglycerin hyperemia).

Dialysis prescription

All patients were managed by nephrologists and were dialyzed with bicarbonate based dialysate, volumetric ultrafiltration control, single use synthetic (polysulphone) dialyzers and heparin as standard anticoagulant. Dialysis prescription was guided by a goal of achieving a value of Kt/V≥1.4

Statistical analysis

Data are presented as a mean±standard deviation (SD) for normally distributed variables or median (25th-75th percentile) for non-normally distributed variables, or absolute or relative frequencies for nominal variables. The statistical comparison was performed using t-test for variables with normal distribution or the Mann-Whitney Rank Sum Test for the others. For identifying correlations between two continuous variables, Pearson's correlation coefficient or the Spearman correlation coefficient were used. The associations between NMD and FMD and oth-

Table I. Demographic, clinical and biochemical characteristics of patients (n=54)

Parameter	Value
Age (years)	60.4±1.7
HD vintage (months)	59.4±5.8
Male n (%)	31(57.4)
Diabetes mellitus n (%)	9 (16.7)
Hypertension n (%)	39 (72.2)
SBP (mmHg)	144.3±2.6
DBP (mmHg)	72.9±1.6
PP (mmHg)	71.4±2.6
Cardiovascular disease n (%)	12 (22.2)
Smoking n (%)	8 (14.8)
FMD (%)	8.9±1.2
NMD (%)	10.3±1.4
Body mass index (kg/m²)	27.4 (23.3-32.4)
Waist circumference (cm)	99.1±2.3
Triceps skinfold thickness (mm)	4 (3-4)
Kt/V	1.5±0.5
Total chol (mg/dl)	172.6±5.9
LDL-chol (mg/dl)	101.4±5.5
HDL-chol (mg/dl)	38.5±1.3
Triglycerides (mg/dl)	130.7 (97.6-180.9)
Hemoglobin (g/l)	11.4±0.1
Serum albumin (g/l)	3.9±0.1
CRP (mg/dl)	0.5 (0.2-0.9)
Ca (mg/dl)	8.9±0.1
P (mg/dl)	4.7 (4.1-6.1)
iPTH (pg/ml)	270.5 (146.9-658.1)
sTWEAK (pg/ml)	3686.2 (3086.1-4984.9)

Data are presented as mean ± standard deviation or median (25th-75th percentile); SBP: systolic blood pressure, DBP: diastolic blood pressure, PP: pulse pressure, FMD: flow-mediated dilation, NMD: nitroglycerin-mediated dilation, CRP: C-reactive protein, Ca: calcium, P: phosphate, iPTH: intact parathormone

er clinical and biological parameters were analyzed with multivariate linear regression analysis (ENTER method - the model contains all the variables that in univariate analysis had p≤0.1). Standardized Beta and unstandardized B coefficient of regression equation, standard error and 95% confidence interval for B were reported. p≤0.05 was considered statistically significant. Statistical analyses were performed using Statistica 7.0.

Results

Patient characteristics

The demographical, clinical and biological characteristics of the patients are presented in Table I. Forty five patients (83.3%) had an arterio-venous fistula and the rest a semi-permanent transcutaneous access. The etiology of ESRD was chronic glomerulonephritis (21.5%), diabetes (16.7%), vascular nephropathy (11.9%), tubulo-interstitial diseases (11.4%), polycystic kidney disease (7.2%), and unknown (31.4%). We noted the treatment: 22% patients with statin, 17% patients under calcium-channel blockers, 37% patients with renin angiotensin system inhibitors, 57% patients with beta blockers, 85% patients with erythropoietin, 46% patients with intravenous iron, and 35% patients with antiplatelet therapy.

Determinants of FMD in HD patients

FMD significantly correlated with Kt/V (r=0.47, p<0.001), LDL-cholesterol (r=0.29, p=0.04), total cholesterol (r=0.31, p=0.02), with NMD (r=0.70, p<0.001) and approached statistical significance in its correlation with SBP (r= -0.25, p=0.07). Multivariate linear regression analysis (ENTER method) for FMD showed that Kt/V was the only predictor for FMD (p=0.003) (Table II).

Determinants of NMD in HD patients

NMD significantly correlated with sTWEAK (r= -0.30, p=0.03), SBP (r= -0.28, p=0.04) and PP (r= -0.31 p=0.02). Multivariate linear regression analysis (ENTER method) for NMD showed that sTWEAK was the only predictor for NMD (p=0.04) (Table II). We found no correlation of FMD with sTWEAK and no statistically significant variations of FMD or NMD depending on presence of diabetes mellitus, smoking, antihypertensive medication or statins.

Discussions

Vascular disorders are common in HD patients and have a major impact on survival. Vascular ultrasound evaluation may reveal endothelial dysfunction, vascular calcification, intima-media thickening, with important prognostic value in these patients [15]. Previous studies in HD patients assessed especially markers of endothelial dysfunction, considered as an early stage of ATS and were less focused on vascular smooth muscle dysfunction as marker of the severity of atherosclerosis. Accordingly, the main aim of our study was to assess the relationship between impaired NMD (a parameter that evaluates vascular smooth muscle dysfunction) and high levels of sTWEAK (a molecule shown to be involved in all ATS stages), a relationship that was confirmed by multivariate analysis. This association suggests that sTWEAK might be a marker of vascular smooth muscle dysfunction in HD patients. It is known that sTWEAK induces smooth muscle cells proliferation in the arterial wall [12]. Consecutively a relative decrease in NO derived from nitroglycerin can occur [9] leading to impaired vascular smooth muscle function.

Whether low or high levels of sTWEAK are related to the NMD decrease in HD patients is not clear. In our study high levels of sTWEAK were associated with diminished NMD while others authors found that low sTWEAK levels were related to smaller NMD in HD patients [10]. In experimental studies increased sTWEAK in mice through genetic approaches resulted in the development of dilated cardiomiopathy with cardiac dysfunction and death [16], which indicates that high levels of sTWEAK are associated with cardiovascular pathology.

Table II. Multivariate linear regression analysis (ENTER method) for nitroglycerin-mediated dilation and flow-mediated dilation.

		Unstandardized Coefficients		**Standardized Coefficients**		**95% Confidence Interval for B**	
		B	**Std. Error**	**Beta**	**p**	**Lower Bound**	**Upper Bound**
NMD	SBP	-0.042	0.12	-0.08	0.72	-0.286	0.201
	PP	-0.03	0.119	-0.075	0.74	-0.28	0.201
	sTWEAK	-0.001	0.0006	-0.281	**0.04**	-0.002	0
FMD	SBP	-0.001	0.066	-0.002	0.988	-0.133	0.132
	Total chol	-0.032	0.062	-0.157	0.607	-0.158	0.093
	LDL-chol	-0.075	0.064	0.337	0.245	-0.053	0.203
	Kt/V	12.094	3.779	0.485	**0.003**	4.478	19.71

FMD: flow-mediated dilation, NMD: nitroglycerin-mediated dilation, SBP: systolic blood pressure, PP: pulse pressure

In addition, the differences between our results (high sTWEAK related with impaired NMD) and the results of other studies in HD patients (low sTWEAK related with impaired NMD) can have several explanations; first, by the characteristics of patients studied. Our group, comprising 17% patients with diabetes mellitus and 22% with previous CVD is different from those in previous studies that included only patients without diabetes mellitus and no previous CVD. It is known that in diabetes patients vascular smooth muscle function is significantly impaired by the diabetes itself [17]. Second, our patients might have genetic particularities which are responsible for circulating levels of sTWEAK. These particularities may cause defective shedding of sTWEAK in the bloodstream [10]. Third, in our study the association between high sTWEAK and impaired NMD might be due to the presence of a microenvironment which is either associated with low levels of sCD163 (sTWEAK scavenger) or with the overcoming of the capacity of sCD163 to bind and internalise sTWEAK. Moreover, all data available to date on sTWEAK come from observational studies and are somewhat contradictory. Patients with chronic diseases (including CKD) have been found to have lower values of sTWEAK than the general population [18-20], but in HD patients, higher levels of sTWEAK are associated with increased mortality [21].

Besides the relationship with sTWEAK, impaired NMD (increased vascular smooth muscle dysfunction) was associated in our study with high SBP and PP in agreement with previous studies in patients with normal renal function [9] and CKD patients. SBP and PP are markers of vascular stiffness, frequently present in HD patients, that can induce vascular smooth muscle dysfunction. In fact high blood pressure in HD patients was noted as an independent risk factor for vascular smooth muscle dysfunction [17].

Although we found a relation between NMD and sTWEAK we did not find a correlation between FMD and sTWEAK in our group of patients. Others have noticed in HD and predialysis CKD patients a decrease in FMD accompanying the decrease in sTWEAK [10,22]. A potential explanation for the difference may be related to the characteristics of the patients. As already mentioned in previous studies HD patients were included without diabetes mellitus and previous CVD or predialysis CKD patients which can have less severe vascular disorders. In addition, NMD was significantly correlated with the intima-media thickness of the brachial artery in other studies [9].

On the other hand, in our study the determinants of FMD were Kt/V (as measure of dialysis efficiency), total cholesterol and LDL-cholesterol. Endothelial function improves as dialysis efficacy is improving, as has been previously shown [23], but increased HD vintage may predispose to ED [24]. Hemodialysis sessions diminish some of the risk factors associated with impaired endothelial function (excessive fluid and micro molecular toxins) and correct metabolic disorders in these patients, explaining the correlation between FMD and kt/V. However, in hemodialysis, the increased oxidative stress, inflammation and vascular calcifications may cause in time a greater arterial injury [24].

The correlation of LDL-cholesterol and total cholesterol with endothelial function was found by other authors, but in HD patients the type of correlation is different than that in the patients with normal renal function. Low levels of LDL-cholesterol and total cholesterol are associated with better endothelial function [25] in the general population. Surprisingly, in HD patients high levels of LDL-cholesterol are associated with better endothelial function (high FMD) [26], in agreement with our results. In fact in HD patients, as opposed to the patients with normal renal function, LDL and total cholesterol are mainly nutrition markers. It is known that dialysis patients with the lowest levels of LDL and total cholesterol are at very high risk for all-cause and cardiovascular mortality, likely because of the confounding by inflammation and malnutrition [27]. Moreover, it has been demonstrated that in CKD patients LDL-oxidation is not a major factor of endothelial dysfunction, more important are the inflammatory status [28], serum phosphate [29], or vitamin D levels [30].

The limits of our study are: first this is a cross-sectional observational study and we could evaluate only the associations between various parameters and not the pathogenetic mechanisms. The second limitation of our study is the relatively small cohort, while more extensive studies might assess the role of sTWEAK in evaluating the severity of vascular dysfunction and cardiovascular prognosis.

In **conclusion** the main determinant of NMD was sTWEAK. The correlation between sTWEAK and NMD in our study suggests novel links between sTWEAK and vascular smooth muscle dysfunction in HD patients. Consequently, measuring sTWEAK (as a predictor of vascular smooth muscle dysfunction) might contribute to better understanding the cardiovascular and survival prognosis in HD patients. Moreover, ultrasound assessment of brachial artery reactivity proves once again to be a readily available method that can improve the knowledge of the CV prognosis in HD patients. We consider that these methods can be used in clinical practice. Practitioners may use brachial artery FMD and NMD to as-

sess response to therapy and to individualize patient risk modification programs.

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NEPHROLOGY - ORIGINAL ARTICLE

The association of high sCD163/sTWEAK ratio with cardiovascular disease in hemodialysis patients

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Abstract
Purpose Cardiovascular disease (CVD) is the most common cause of death in hemodialysis (HD) patients. Transmembrane proteins that circulate as soluble form such as tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) and CD163 have been proposed in previous studies as CVD biomarkers in chronic kidney disease patients. In HD patients, since studies are scarce, the role of these proteins is not completely understood. We tested the hypothesis that sTWEAK, sCD163 or sCD163/sTWEAK ratio could be associated with cardiovascular disease in HD patients.
Methods We recorded current clinical and biological data, and we measured sTWEAK and sCD163 serum levels by ELISA in 70 hemodialysis patients. Univariate analysis and multivariate (logistic regression) analysis were used to identify the relation between sTWEAK, sCD163 and sCD163/sTWEAK ratio and CVD.
Results In univariate analysis, CVD in HD patients is associated with higher sCD163/sTWEAK ratio ($p = 0.04$), sCD163 ($p = 0.07$), CRP ($p = 0.04$), age ($p = 0.07$), smoking ($p = 0.09$) and vascular calcifications ($p = 0.10$). In multivariate analysis, only logarithm of sCD163/sTWEAK ratio ($p = 0.04$) and smoking ($p = 0.03$) was significantly associated with CVD. The levels of these molecules and their ratio were correlated with atherosclerosis risk factors: diabetes mellitus, high fasting glucose, tricipital skinfold thickness and CRP as well as (for sCD163/sTWEAK) intravenous iron therapy.
Conclusions Cardiovascular disease is associated with increased sCD163/sTWEAK ratio. To our knowledge, this is the first report about this relationship in HD patients.

Keywords sCD163/sTWEAK ratio · Hemodialysis · Cardiovascular disease · Atherosclerosis

Introduction

Chronic kidney disease patients (CKD) have a markedly increased mortality rate, mainly due to cardiovascular disease (CVD). New biomarkers emerge in the search for early diagnosis tools and targeted prophylactic treatments. Among the molecules proposed as CV risk biomarkers in patients with and without CKD are tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK), a member of the TNF superfamily that is synthesized as a type II transmembrane glycoprotein and circulates in plasma as a soluble form (sTWEAK) [1], and its scavenger CD163 [2].

TWEAK gene is expressed in many tissues, including healthy vasculature, and the molecule exerts specific biological effects after binding to its receptor, fibroblast growth factor-inducible 14 (Fn14) [3, 4]. Fn14 expression is low or undetectable in healthy tissues and is upregulated in context of disease such as the arterial wall in atherosclerosis [5]. sTWEAK induces various and sometimes contradictory effects depending on cell type, cell cycle and the

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presence of an inflammatory microenvironment [6], resulting in cell proliferation and migration, angiogenesis [7, 8] or osteoclastogenesis [9], while in an inflammatory milieu apoptosis [10]. The sTWEAK–Fn14 pathway is involved in atherogenesis [11].

sTWEAK is removed from circulation through uptake by the specific receptor and by CD163, a monocyte–macrophage surface receptor which acts as a scavenger for sTWEAK [12]. Like TWEAK, CD163 is a transmembrane protein that has a soluble variant, sCD163, which can be measured in serum and reflects total membrane-bound CD163 pool [2]. CD163 is expressed only by monocytes and macrophages and has the capacity to bind and internalize hemoglobin–haptoglobin complexes [13], uptaken from hemoragic atherosclerosis (ATS) plaques. sTWEAK mimics the structure of the hemoglobin–haptoglobin complex [14] and is able to bind and block sCD163. The biological functions of sCD163 are not fully understood, but it is known that in inflammation with macrophage activation, serum levels of sCD163 rapidly increase due to metalloproteinase-mediated cleavage near the macrophage membrane [15].

sTWEAK and sCD163 are associated with the increased CV disease burden present in CKD. Some studies have found that sTWEAK is associated with endothelial dysfunction [16, 17], coronary and carotid atherosclerosis [16–19] and with cardiovascular events [16] in CKD patients.

sCD163 levels are increased and associated with ATS severity in CKD [20], but also in obese and hypertensive patients [21]. In addition, sCD163/sTWEAK ratio was described as a potential ATS biomarker in asymptomatic peripheral artery disease patients as it is associated with incipient atherothrombosis [22].

The few available studies that evaluate sTWEAK and sCD163 in HD patients do not offer complete understanding of all the factors that regulate these molecules. In diabetes mellitus [23, 24] and predialysis and dialysis CKD [25], sTWEAK levels are lower than in general population, a finding without a clear explanation. Moreover, in HD patients, although serum levels are lower than in general population, higher sTWEAK predicts mortality [26].

The aim of this study is to evaluate the relationship between sTWEAK, sCD163 and sCD163/sTWEAK ratio and cardiovascular disease in HD patients and to assess the determinants of the levels of these molecules in this group of patients.

Patients and methods

Patients

We conducted an analytical cross-sectional observational study, carried on a cohort of HD patients on conventional HD treatment in Nefromed Dialysis Center Cluj. From the 140 patients, 70 were selected in accordance with the inclusion and exclusion criteria and agreed to participate in this study. All patients signed an informed consent before entering the study. Their privacy was respected. The study protocol conformed to the ethical guidelines and was approved by the University Ethics Committee.

Inclusion criteria were: prevalent HD patients, age >18 years and hemodialysis vintage of at least 6 months. We excluded patients with acute inflammation processes (CRP > 3 mg/dl), terminal neoplasia, previous renal transplantation, immunosuppressive treatment, hepatitis B or C virus infection.

From the 140 prevalent HD patients, four patients were younger than 18 years and 19 patients refused to participate. In accordance with the criteria mentioned above, we excluded 47 patients, 12 due to acute inflammation processes, 3 patients with terminal neoplasia, 2 patients with previous renal transplantation, 1 patient with immunosuppressive treatment for polyangiitis and 29 patients with hepatitis B or C virus infection.

Patients' demographics data, etiology of end-stage renal disease, hemodialysis vintage, comorbidity conditions (diabetes, hypertension, CVD), smoking status and medication were obtained from medical documents. We also registered clinical data: age, weight, height, blood pressure (predialysis values), triceps skinfold thickness (TST) (mm) [27] and waist circumference (cm).

Cardiovascular disease was registered as follows: Coronary artery disease was evaluated by: electrocardiogram with Q-wave infarction, or myocardial enzyme elevation, or coronary revascularization, or typical history of angina with abnormal coronarography. Cerebrovascular disease was evaluated by the presence of focal neurological deficit, or carotid stenosis and lower extremity arterial disease were evaluated by the presence of revascularization or amputation, or intermittent claudication confirmed by Doppler or arteriography findings.

We calculated body mass index as BMI = (weight (kg)/height^2 (m^2) and pulse pressure as PP = SBP-DBP (mmHg).

Dialysis prescription

All patients were on thrice weekly HD (4–5 h) regimen with bicarbonate-based dialysate, volumetric ultrafiltration control, single-use synthetic (polysulfone) dialyzers and heparin as standard anticoagulant. Dialysis prescription was guided by the goal of achieving a value of Kt/V $\geq$ 1.4.

Laboratory parameters

All biochemical analyses were performed after an overnight fast between 7.00 and 9.00 a.m. always during a midweek

non-dialysis day. The serum was separated by centrifugation with 10,000 rotations/minute for 3 min. Samples for fasting glucose, total and high-density lipoprotein (HDL) cholesterol, triglycerides, calcium, phosphate (automated colorimetric enzymatic method), parathormone (electrochemiluminescence immunoassay ECLIA method), standard hematology panel (automated flow cytometry method) and C-reactive protein (CRP) and albumin (immunoturbidimetric method) were shipped and assessed right away by the same authorized laboratory. Pre- and post-dialysis urea levels were used to calculate Kt/V. Serum calcium was corrected (cCa) for albumin according to the formula: cCa (mg/dl) = serum calcium (mg/dl) + 0.8 × (4.0-serum albumin (g/dl), LDL cholesterol was calculated with Friedewald formula: LDL cholesterol = total cholesterol-(HDL cholesterol + TG/5).

For the measurement of sTWEAK and sCD163, a sample of the venous blood was centrifuged and the serum was refrigerated at −80° Celsius in triplicate Eppendorf tubes. sTWEAK and sCD163 were determined with commercially available enzyme-linked immunosorbent assay kits (Bender MedSystem, Vienna, Austria kit for sTWEAK, Human CD163 Quantikine ELISA Kit, DC 1630, R&D for sCD163). The minimum detectable level of sTWEAK was 10 pg/ml. Intra- and interassay coefficients of variation were 7.9 and 9.1 %, respectively. For sCD163, the minimum detectable level was 0.613 ng/ml and intra- and interassay coefficients of variation were 5.1 and 3.5 %, respectively. Vascular ultrasound was performed to detect arterial calcification as described in the literature [28].

Statistical analysis

Data are presented as mean ± SD for normally distributed variables or median (25th–75th percentile) for non-normally distributed variables, or absolute or relative frequencies for nominal variables. The statistical comparison was made using *t* test for variables with normal distribution or the Mann–Whitney rank sum test for the others. Chi-square or Fisher's exact test was used to test the relationship between qualitative variables. For identifying correlations between two continuous variables, Pearson's correlation coefficient or Spearman's correlation coefficient was used. We used multivariate linear regression analysis (stepwise method—the final model contains only statistically significant variables) to determine the dependence of the molecules studied on other continuous quantitative variables. All quantitative variables were analyzed as independent variables in multivariate linear regression. The associations between CVD and other clinical and biological parameters were analyzed with multivariate logistic regression (ENTER method—the model contains all the variables that in univariate analysis had $p \leq 0.10$. Standardized beta and unstandardized B coefficient of regression equation, standard error and 95 % confidence interval for B were reported). A receiver operating characteristic (ROC) curve was designed to identify a cutoff value of sCD163/sTWEAK that best predicted CVD, according to the maximum of the Youden Index. Because sCD163/sTWEAK ratio was not normally distributed, logarithm to base 10 was computed from it. $p \leq 0.05$ was considered statistically significant. Statistical analyses were performed using Statistica 7.0.

Results

Patient characteristics

The demographical, clinical and biological characteristics of the patients are presented in Tables 1 and 2. In total, 58 patients (82.8 %) had an arteriovenous fistula and 12 patients (17.2 %) had a semipermanent transcutaneous access. The etiology of ESRD was chronic glomerulonephritis in 21.5 % of patients, diabetes in 14.2 %, vascular nephropathy in 14.3 %, tubulo-interstitial diseases in 11.4 %, polycystic kidney disease in 7.2 % and unknown in 31.4 % of cases. Comorbidity conditions and current medication are presented in Table 1.

Table 1 Demographic and clinical characteristics of patients ($n = 70$)

Parameter	Value
Age (years)	59.9 ± 12.5
HD vintage (months)	70.1 ± 46.5
Male [*n* (%)]	40 (57.1)
Diabetes mellitus [*n* (%)]	10 (14.3 %)
Hypertension [*n* (%)]	49 (70 %)
Cardiovascular disease [*n* (%)]	17 (24.3 %)
Smoking [*n* (%)]	8 (11.4)
ESAs [*n* (%)]	57 (81.4)
Statins [*n* (%)]	14 (20.0)
Beta blockers [*n* (%)]	40 (57.1)
Calcium channel blockers [*n* (%)]	11 (15.7)
ARBs or ACE inhibitors [*n* (%)]	29 (41.4)
Antiplatelet therapy [*n* (%)]	27 (38.5)
Intravenous iron [*n* (%)]	32 (45.7)

Data are presented as arithmetic mean ± standard deviation

ESA erythropoiesis-stimulating agents, *ACE inhibitors* angiotensin-converting enzyme inhibitors, *ARB* angiotensin receptor blockers, *n* number

Determinants of sTWEAK, sCD163 and sCD163/sTWEAK

sTWEAK was inversely correlated with fasting glucose ($r = -0.32$, $p = 0.01$), and a tendency to an inverse correlation with sCD163 ($r = -0.22$, $p = 0.07$) was observed.

Table 2 Clinical and biochemical characteristics of patients ($n = 70$)

Parameter	Value
SBP (mmHg)	140 (126.5–155.8)
DBP (mmHg)	73.5 (67–80)
PP (mmHg)	68.5 (52–80)
Body mass index (kg/m^2)	26.6 (23.7–31.1)
Waist circumference (cm)	100.5 (89–110)
Triceps skinfold thickness (mm)	3.8 (3–4)
Arterial calcifications [*n* (%)]	14 (20 %)
Kt/V	1.6 ± 0.4
Total cholesterol (mg/dl)	177.1 ± 43.4
LDL cholesterol (mg/dl)	103.15 ± 38.8
HDL cholesterol (mg/dl)	39.7 ± 14.3
Triglycerides (mg/dl)	139.0 (97.7–195.8)
Hemoglobin (g/l)	11.5 (10.8–12.2)
WBC (n/mm^3)	6225 (5472–7345)
Serum albumin (g/l)	3.9 ± 0.3
CRP (mg/dl)	0.54 (0.20–1.00)
cCa (mg/dl)	8.9 ± 0.6
P (mg/dl)	4.7 (4.1–6.1)
iPTH (pg/ml)	318.0 (155.8–785.6)
sTWEAK (pg/ml)	3686 (3100–4985)
sCD163 (ng/ml)	1090 (690–1765)
sCD163/sTWEAK	0.27 (0.16–0.51)

Data are presented as arithmetic mean ± standard deviation; median (25th–75th percentile)

SBP systolic blood pressure, *DBP* diastolic blood pressure, *PP* pulse pressure, *cCa* calcium corrected by serum albumin, *CRP* C-reactive protein, *P* phosphate, *iPTH* intact parathormone, *WBC* white blood cells, *n* number

Serum value of sCD163 directly correlates with fasting glucose ($r = 0.32$, $p = 0.01$), CRP ($r = 0.36$, $p < 0.001$), TST ($r = 0.28$, $p = 0.02$), predialysis serum urea ($r = 0.30$, $p = 0.01$) and inversely correlates with LDL cholesterol ($r = -0.24$, $p = 0.05$).

In multivariate linear regression analysis, sTWEAK was significantly associated with serum glucose, age and serum albumin (Table 3), while sCD163 was significantly associated with predialysis urea and LDL cholesterol (Table 3). sCD163/sTWEAK ratio correlated with parameters previously found as being significant for at least one of the molecules.

sCD163/sTWEAK ratio significantly correlated with CRP ($r = 0.25$, $p = 0.03$), predialysis serum urea ($r = 0.27$, $p = 0.02$), triglycerides ($r = 0.34$, $p < 0.001$), fasting glucose ($r = 0.38$, $p < 0.001$) and inversely correlated with total cholesterol ($r = -0.24$, $p = 0.05$) and LDL cholesterol ($r = -0.25$, $p = 0.04$).

sTWEAK levels were significantly lower in diabetes mellitus patients as compared to non-diabetics (3012.5 ± 690.3 vs. 4436.5 ± 2003.1 pg/ml, $p = 0.005$), while sCD163 and sCD163/sTWEAK ratio were significantly higher in diabetes patients (1676.0 ± 596.9 vs. 1230.7 ± 789.1 ng/ml, $p = 0.02$ and 0.61 ± 033 vs. 0.33 ± 0.29, $p = 0.003$), respectively.

No significant variations of sTWEAK, sCD163 and sCD163/sTWEAK were found depending on the presence of arterial calcifications, gender or smoking status in all patients.

No influence on sTWEAK, sCD163 and sCD163/sTWEAK was found in the presence or absence of treatment with calcium channel blockers, beta blockers, ARB, ACEI, statins, antiplatelet or ESA therapy.

Subgroup analysis according to the presence or absence of CVD

sCD163/sTWEAK ratio was significantly higher in the subgroup with CVD (0.47 ± 0.30 vs. 0.34 ± 0.29 $p = 0.04$),

Table 3 Multivariate linear regression analysis (stepwise method) for sTWEAK and sCD163

	Unstandardized coefficients		Standardized coefficients	*p*	95 % confidence interval for B	
	B	SE	Beta		Lower bound	Upper bound
sTWEAK						
Fasting glucose	−0.01	0.001	−0.57	<0.001	−0.011	−0.005
Age	−0.42	0.177	−0.26	0.02	−0.774	−0.064
Serum albumin	0.01	0.004	0.25	0.03	0.001	0.016
sCD163						
Predialysis urea	11.72	3.17	0.41	0.0005	5.39	18.06
LDL cholesterol	−6.51	2.22	−0.32	0.005	−10.94	−2.07

which are older (63.59 ± 7.03 vs 58.85 ± 1.76 years $p = 0.07$) and have higher CRP (0.87 ± 0.56 vs. 0.67 ± 0.57 mg/dl, $p = 0.04$). Patients with CVD vascular calcifications are more frequent (35.3 vs. 15 %, $p = 0.09$) as well as smoking (23.5 vs. 7.5 %, $p = 0.10$) (Table 4).

According to multivariate logistic regression analysis with CVD as a dependent variable, introducing variables that were significant or near significance in simple regression analysis (CRP, age, presence of vascular calcifications, log sCD163/sTWEAK, smoking) as independent variables, the identified risk factors for CVD were log sCD163/sTWEAK (OR 9.76, 95 % CI (1.05–90.83), $p = 0.04$) and smoking (OR 8.49, 95 % CI (1.30–55.56), $p = 0.03$).

The cutoff value for sCD163/sTWEAK to best predict CVD, detected by ROC curves, was 0.19 (sensitivity = 88.2 % specificity = 55.7 %). Plasma sCD163/sTWEAK ratio had a good predictive power for CVD, with an area under ROC curve of 0.66. In the group without CVD, 31 patients of 53 (58 %) had sCD163/sTWEAK over 0.19, and in the group with CVD, 15 patients of 17 (88 %) had sCD163/sTWEAK over the cutoff value ($p = 0.02$).

The comparison between subgroups above and below the cutoff value of sCD163/sTWEAK ratio showed that all diabetic patients had sCD163/sTWEAK ratio values above the cutoff ($p = 0.01$). The patients with sCD163/sTWEAK ratio above the cutoff had significantly lower values of total cholesterol and LDL cholesterol ($p = 0.04$, respectively, $p = 0.002$), higher CRP and fasting glucose ($p = 0.03$, respectively, $p = 0.001$) and more frequent intravenous iron treatment ($p = 0.04$) (Table 5).

Table 4 Comparison of clinical and biochemical profiles of hemodialysis patients with and without cardiovascular disease (CVD)

Parameter	With CVD ($n = 17$)	Without CVD ($n = 53$)	p
Age (years)	63.6 ± 7.1	58.8 ± 13.8	**0.07**
HD vintage (months)	67 (26.5–101.0)	68 (31.7–87.0)	0.89
Diabetes (%)	4 (23.5 %)	6 (11.3 %)	0.97
SBP (mmHg)	145 (134–158)	140 (126–157)	0.84
DBP (mmHg)	70 (61–80)	75 (68–80)	0.24
PP (mmHg)	70 (58–88)	66 (51–80)	0.32
WC (cm)	95 (89–107)	102 (89–113)	0.82
BMI (kg/m^2)	25.8 (23.7–31.4)	27.0 (23.5–31.2)	0.90
TST (mm)	4 (3–4)	3 (3–4)	0.25
Smoking [*n* (%)]	4 (23.5)	4 (7.5)	0.10
Arterial calcifications [*n* (%)]	6 (35.3)	8 (15.0)	0.09
cCa (mg/dl)	9.0 ± 0.8	8.8 ± 0.6	0.30
P (mg/dl)	5.1 (4.2–6.7)	4.5 (3.9–5.6)	0.10
iPTH (pg/ml)	272.0 (188.0–686.7)	325.6 (146.6–768.7)	0.80
Kt/V	1.5 (1.2–1.7)	1.6 (1.4–1.9)	0.20
Total cholesterol (mg/dl)	179.9 ± 46.2	167.4 ± 33.2	0.32
LDL cholesterol (mg/dl)	100.1 ± 27.1	104.1 ± 42.5	0.65
HDL cholesterol (mg/dl)	37.8 ± 9.7	40.6 ± 15.5	0.53
Triglycerides (mg/)	140 (99.7–215.2)	120.4 (95.3–172.0)	0.41
CRP (mg/dl)	0.6 (0.5–1.1)	0.4 (0.2–1.0)	**0.04**
Serum albumin (g/dl)	3.8 ± 0.3	3.9 ± 0.2	0.30
Hemoglobin (g/l)	11.2 ± 1.3	11.4 ± 0.9	0.50
sTWEAK (pg/ml)	3569 (2896–4279)	3713 (3188–5714)	0.36
sCD163 (ng/ml)	1440 (960–1940)	1070 (625–1500)	**0.07**
sCD163/sTWEAK ratio	0.33 (0.23–0.74)	0.24 (0.14–0.43)	**0.04**

Data are presented as arithmetic mean ± standard deviation; median (25th–75th percentile)

Bold *p* values are statistically significant

SBP systolic blood pressure, *DBP* diastolic blood pressure, *PP* pulse pressure, *WC* waist circumference, *BMI* body mass index, *TST* triceps skinfold thickness, *cCa* calcium corrected for serum albumin, *P* phosphate, *iPTH* intact parathormone, *CRP* C-reactive protein, *n* number

Discussion

The main finding of our study is the important relationship between sCD163/sTWEAK ratio and CVD in HD patients, confirmed in multivariate analysis, and reflected by the comparison of subgroups of patients with and without CVD. Previous studies in CKD found that increased levels of sCD163 and decreased sTWEAK levels are associated with more severe atherosclerosis [20]. Although in our patients with CVD, we found lower levels of sTWEAK and higher sCD163, we did not find a statistically significant association between sTWEAK or sCD163 and CVD. Apparently in our patients not the actual levels of sTWEAK and sCD163 seem to be significant for the development of CVD but the interplay between the two molecules, which results in more or less sTWEAK scavenging.

To our knowledge, the relation between sCD163/sTWEAK ratio and CV disease has never been studied in HD patients, although it has been shown that in patients with peripheral arterial disease decreased sTWEAK and increased sCD163/sTWEAK ratio reflects the progression of atherothrombosis [22] and is associated with long-term cardiovascular mortality [29]. Considering the results of our study and previous data, we appreciate that in HD patients, sCD163/sTWEAK ratio might be a better CVD marker than the two molecules separately.

Regarding the relationship between sTWEAK and atherosclerosis, different studies in CKD patient results are

Table 5 Comparison of patients according to cutoff value for sCD163/sTWEAK

Parameter (UM)	SCD163/sTWEAK ≤0.19 ($n = 24$)	SCD163/sTWEAK ≥0.19 ($n = 46$)	p
Age (years)	59.4 ± 14.7	60.2 ± 11.3	0.81
HD vintage (months)	64.5 (25.5–79.5)	74.5 (43–96.3)	0.18
Diabetes [*n* (%)]	0 (0)	10 (100)	**0.01**
Hypertension [*n* (%)]	14 (58.3)	35 (76.1)	0.12
SBP (mmHg)	140 (128–159)	141 (122–156)	0.83
DBP (mmHg)	75 (68–80)	72 (66–80)	0.61
PP (mmHg)	60 (51–82)	70 (54–80)	0.99
WC (cm)	98 (89–108)	102 (89–114)	0.69
BMI (kg/m^2)	25.8 (23.5–30.6)	26.9 (23.8–32.2)	0.88
TST (mm)	3 (2–4)	4 (3–4)	0.20
Smoking [*n* (%)]	2 (8.3)	6 (13.3)	0.70
Arterial calcifications [*n* (%)]	4 (18.2)	10 (22.7)	0.47
cCa (mg/dl)	8.8 ± 0.5	8.9 ± 0.7	0.85
P (mg/dl)	4.6 (3.7–6.2)	4.7 (4.1–6.1)	0.70
iPTH (pg/ml)	290.3 (149.4–681.7)	334.8 (155.8–821.4)	0.65
Kt/V	1.5 (1.4–1.9)	1.6 (1.4–1.9)	0.62
Total cholesterol (mg/dl)	191.3 ± 44.8	169.3 ± 41.1	**0.04**
LDL cholesterol (mg/dl)	122.2 ± 39.5	93.2 ± 34.8	**0.002**
HDL cholesterol (mg/dl)	41.4 ± 9.4	38.8 ± 16.2	0.12
Triglycerides (mg/dl)	125.7 (88.8–177.8)	145.9 (100.6–244.8)	0.18
CRP (mg/dl)	0.3 (0.2–0.9)	0.6 (0.3–1.1)	**0.03**
Serum albumin (g/dl)	3.9 ± 0.2	3.9 ± 0.2	0.93
Hemoglobin (g/l)	11.5 ± 0.9	11.4 ± 1.1	0.6
Glucose (mg/dl)	90.3 ± 12.4	116.1 ± 42.9	**0.001**
Calcium channel blockers [*n* (%)]	1 (4.1)	10 (21.7)	0.08
Intravenous iron [*n* (%)]	7 (29.2)	25 (54.3)	**0.04**

Data are presented as arithmetic mean ± standard deviation; median (25th–75th percentile)

Bold p values are statistically significant

SBP systolic blood pressure, *DBP* diastolic blood pressure, *PP* pulse pressure, *WC* waist circumference, *BMI* body mass index, *TST* triceps skinfold thickness, *cCa* calcium corrected for serum albumin, *P* phosphate, *iPTH* intact parathormone, *CRP* C-reactive protein, *n* number

contradictory: Some describe an association between sTWEAK and atherosclerosis [16], while others do not [26]. In experimental studies in mice, high sTWEAK levels are involved in the development of dilated cardiomyopathy with cardiac failure and early death [30] and the sTWEAK–Fn14 interaction has proatherogenic effects in cell cultures, observations that might shed some light on the pathogenesis of atherosclerosis [31]. In our study, although we did not find a direct relationship between sTWEAK and CVD, we observed an association of low levels of sTWEAK with atherogenic factors such as high fasting glucose, diabetes mellitus, age and lower serum albumin. Consequently, we can suppose that concurrently with these factors, sTWEAK might be involved in the development of atherosclerosis. Low sTWEAK levels associated with increased sCD163 suggest that the cause of the decrease in sTWEAK levels in our patients might be macrophage binding and internalization via sCD163, as mentioned by other authors [12, 29]. The reduction in sTWEAK levels in inflammatory diseases is a compensatory mechanism that protects against Fn14 activation by sTWEAK. Low sTWEAK associated with high sCD163 has been noticed in all atherosclerosis stages [12, 20].

Besides the associations of low levels of sTWEAK with atherogenic factors, we found that high sCD163 levels are associated with atherosclerosis risk factors such as increased TST and fasting glucose, inflammatory syndrome, diabetes mellitus. TST, easy to measure, is an indicator of adipose tissue. In CKD patients, adipocytes can release chemokines that attract monocytes to infiltrate adipose tissue as resident macrophages and become the source for sCD163 [32, 33]. In consequence, sCD163 in CKD correlates with fat mass and inflammatory cytokines [32]. Increased levels of sCD163 associated with higher CRP described in our study might favor atherosclerosis. In addition, the association of high sCD163/sTWEAK ratio with intravenous iron treatment is

probably secondary to increased inflammatory syndrome induced by this treatment as stated by other authors [34].

Interestingly, high levels of sCD163 and sCD163/sTWEAK ratio were associated with low LDL cholesterol and total cholesterol in our study. This association might be the effect of reverse epidemiology of CV risk characteristic to CKD patients and of the presence of malnutrition. It is known that dialysis patients with the lowest levels of LDL and total cholesterol are at very high risk for all-cause and cardiovascular mortality, likely because of confounding by inflammation and malnutrition [35].

In conclusion, CVD in HD patients is associated with increased sCD163/sTWEAK ratio. These molecules and their ratio were correlated in HD patients with atherosclerosis risk factors: diabetes mellitus, increased fasting glucose and higher tricipital skinfold thickness, increased CRP and intravenous iron therapy (for sCD163/sTWEAK).

sCD163/sTWEAK ratio can be proposed as a biomarker for CVD in HD patients. Larger studies are needed to confirm the findings of our study.

Our study has certain limitations: first, since our study is cross-sectional and observational, we could evaluate only the associations between various parameters and not the pathogenetic mechanisms. Secondly, we do not have data concerning the prognostic value of sTWEAK, sCD163 and sCD163/sTWEAK for CVD events occurrence. We consider that the results of our study are valid due to the homogeneity of the group and the conclusive multivariate analysis.

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Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest.

Ethical standard All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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THE CALCIUM PHOSPHORUS PRODUCT IS A BETTER INDICATOR FOR SURVIVAL THAN IMMUNOREACTIVE PARATHORMONE IN CHRONIC HEMODIALYSIS PATIENTS WITH RENAL FAILURE. POSSIBLE ROLE OF SERUM ALBUMIN LEVEL

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Abstract

Introduction. Recent studies suggest that nutritional status can modify the association between high iPTH and mortality, especially in diabetics and older hemodialysis patients (HDP).

Aim. To assess the impact of mineral metabolism parameters in the survival of HDP in our area and to evidence the factors that influence iPTH levels in our HDP, which are younger and have less frequently diabetic nephropathy as the cause of chronic renal failure than in most published studies.

Patients and Methods. A prospective cohort study of 126 HDP was recorded for demographic, clinical and laboratory data, and after 24 months, the general mortality. Patients were divided in two groups, survivors and non-survivors, and each of groups classified according to the time on hemodialysis (THD). The groups of non-survivors and survivors with THD more than 10 year-period were compared to the groups with less than 10 year vintage, regarding the albumin levels, iPTH levels, phosphate-calcium metabolism markers, age and sex.

Results. We observed the better survival only for calcium phosphate product less than 55 mg^2/dL^2 (p=0,02). The iPTH level seems to be conditioned by albumin levels. For THD<10 years, iPTH levels are greater in survivors (p=0.01); in this subgroup we observed higher levels of serum albumin (p<0.001), the patients were younger (p<0.001), and had 5-fold lower frequency of diabetes. For THD>10 years, iPTH levels are greater in non-survivor patients (p=0.02), as well as calcium, phosphorus and calcium phosphorus product.

Conclusions. Calcium-Phosphorus product is a better indicator for survival in HDP in our area than immunoreactive PTH levels. Immunoreactive PTH as prognostic factor might be better evaluated in association with calcium phosphorus metabolism parameters and albumin levels too, even in younger and lower percent-diabetic HDP groups.

Key words: iPTH, hemodialysis, albumin levels, phosphate-calcium metabolism markers.

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INTRODUCTION

High serum immunoreactive parathormone (iPTH) levels are present in chronic kidney disease patients even from the stage 3 and reach a maximum in stage 5 (renal replacement therapy). The hyperparathyroidism is induced (1) by low active vitamin D levels, hypocalcemia, and persistent hyperphosphatemia and it is classically considered an important risk factor for cardiovascular morbidity and general mortality (2). The optimum iPTH levels, associated with the lowest morbidity and mortality in chronic hemodialysis patients (CHDP), are still a controversy and a subject of continuous debate.

Previous studies (3, 4) have shown that the iPTH levels between 100 and 300 pg/mL are associated with better survival and a better nutrition status (expressed by higher serum creatinine and albumin levels) than serum iPTH levels below 65 pg/mL. Considering those results, the actual guidelines recommend that chronic hemodialysis patient should have iPTH levels between 150 and 300 pg/mL (5).

The increase of iPTH over the recommended levels, associated with hypercalcemia and hyperphosphataemia, can induce extraosseous calcifications (6) and cardiovascular events (7). Recent data suggest that not even the actual recommended levels are fully satisfactory. A meta-analysis published in 2009, on 35 studies, showed that a iPTH level over 476.1 pg/mL is associated with higher risk of cardiovascular events in dialysis patients (2). The highest mortality rate was observed in HDP with iPTH over 600 pg/mL (4). Other investigators have shown a high risk of negative outcome in patients with iPTH below 200 pg/mL (8). At this moment a new modification of the optimum levels of iPTH is being discussed in this category of patients. Previous studies have shown that nutrition deficit can reverse the classical relation iPTH-survival in older and diabetic HDP (9, 10). There are not enough studies that analyze the influence of the nutritional indices on iPTH levels on survival in younger HDP and in groups that have a smaller number of diabetics.

The hemodialysis patients in our area have lower frequency of diabetes mellitus associated with chronic kidney disease (8% in our area *vs.* 40% in USA and 38% in Japan) (3, 11). They are also 10 years younger than in other studies (11) and have particular nourishment habits. These characteristics can be validated in differences related to nutrition and the levels of calcium, phosphate and iPTH. This study wants to assess, in our area, the impact of mineral metabolism parameters in survival for HDP, and to show the factors which influence iPTH levels in our HDP, which are younger and have less frequently diabetic nephropathy as the cause of chronic renal failure than in most published studies.

PATIENTS AND METHODS

A prospective cohort study of 126 chronic hemodialysis patients (CHDP) was conducted in Nefromed Dialysis Center Cluj-Napoca (mean age 54.9±12.7 years;

63.8 % males; 8% diabetics). All patients treated by chronic hemodialysis in Nefromed Dialysis Center Cluj were included. The exclusion criteria were: parathyroidectomised patients, acute infectious diseases, neoplasms; 10 patients were transplanted or transferred during the study, and therefore they were excluded. The institutional ethics committee approved the study protocol.

The clinical and laboratory data, which we recorded at enrollment, are: demographic data, weight, complete diagnosis, and time on hemodialysis (THD), calcium-phosphate metabolism markers: calcium (Ca), phosphate (P), iPTH, nutritional markers: serum albumin, predialytic serum creatinine, inflammation markers: C-reactive protein (CRP). For all laboratory markers we used the fasting blood samples. Laboratory data were performed using a modular Cobas 6000 analyzer. Calcium, phosphate, albumin were assessed by a photometric method, alkaline phosphatase (AP) by using a colorimetric method, CRP by immunoturbidimetry. For iPTH an analyzer Roche/Hitachi Cobas systems/Elecsys was used employing the electrochemiluminescence assay "ECLIA". All reagent kits were provided by Roche. We reviewed records of the characteristics of the hemodialysis (HD) sessions: all patients were treated with standard hemodialysis, using synthetic polysulphone membrane, mean arterial blood flow 287.1±13.2 mL/min, mean session length 4.5±0.6 hours; mean time on HD was 59.3±56.6 months, mean HD dose 10.6±1.9 hours/week. The HD efficacy was estimated by Kt/V calculated with the formula: Kt/V=0.024*(100-100* postdialytic urea (mg/dL)/predialytic urea)-0.27. If Kt/V ≥ 1.2 hemodialysis was considered efficient. Body mass index (BMI) was calculated with the formula: BMI= weight (kg)/height²(m²).We followed these patients for 24 months and we registered the deaths of all causes. At the end of the study, the initial group was divided into two groups: A-survivors (n=89) and B-non-survivors (n=27): and then it

Table 1. Characteristics of the study population at enrollment

Parameters	
Age (years)	54.9±12.7
Male gender (%)	63.8
Time on hemodialysis (months)	59.3±56.6
Diabetes mellitus (%)	8
BMI (kg/m²)	24.0±4.3
Hemodialysis doses (hours/week)	10.6±1.9
Kt/V	1.3±0.2
Serum albumin (g/dl)	4.1±0.4
Predialitic serum creatinine (mg/dl)	10.3±2.8
Ca (mg/dl)	8.6±1.3
P (mg/dl)	6.8±2.2
CaxP (mg²/dl²)	58.5±21.9
iPTH (pg/ml)	730.8±695.6
AP (U/l)	326.9±250.4
CRP (mg/dl)	1.0±2.8

BMI-body mass index, iPTH-immunoreactive parathormone, CRP-C-reactive protein, AP-alkaline phosphatase

was divided into four groups after the time on HD: group A1 (n=71) survivors with time on HD<10 years; group B1 (n=18) survivors with time on HD>10 years; group C1 (n=21) non-survivors with time on HD<10 years; group D1 (n=6) non-survivors with time on HD>10 years. The treatment with active vitamin D and calcium or non-calcium containing phosphate binders was given based on the actual guidelines.

Statistical analysis was performed using Sigma Stat, Sigma Plot version 11, and Epi Info, software. Data are presented as mean ± SD. The cutoff values for serum Ca, P, CaxP product and iPTH were set according to the target ranges recommended by the K/DOQI guidelines.Continuous variables were compared using t test or Man-Whitney Rank Sum Test, discrete variables were compared using Fisher exact and Chi square test. Parameters susceptible to correlate with iPTH levels were introduced in a linear regression analysis. Total survival according to calcium phosphate metabolism markers levels were estimated with the Kaplan Meier method, followed by the log-rank test. In order to analyze the impact of calcium phosphorus metabolism in survival we used Log Rank Test. Statistical significance was $p<0.05$.

RESULTS

The baseline clinical characteristics of the 116 patients are presented in Table 1. Their mean age was 54.9±12.7 years, mean time on hemodialysis was 59.3±56.6 months, and 8% of them had diabetes mellitus. Mean serum Ca, P, and iPTH were 8.6±1.3 mg/dl, 6.8±2.2 mg/dl, 730.8±695.6 pg/ml, respectively. 61.2% of the patients were treated with calcium-containing phosphate binders, 30.2% received sevelamer hydrochloride and combination therapy with calcium-containing phosphate binders and sevelamer hydrochloride was given in 8.6% of the patients. 25.8% received vitamin D analogs.

Table 2. Characteristics at enrollment of survivors and non-survivors patients

Parameter	Group B (NSP) n=27	Group A (SP) n=89	p+
age (years)	62.4±12.1	52.9±1.2	NS
DM %	22.2	5.05	NS
Male sex %	74.0	6.6	NS
P(mg/dL)	7.7±2.2	6.5±2.1	p=0.01
Ca (mg/dL)	9.0± 1.2	8.4±1.2	p=0.04
CaxP (mg^2/dL^2)	69.4±12.3	55.3±10.2	p=0.06
iPTH pg/mL	599.9±688.0	768.3±486.1	NS
AP (U/L)	314.3±239.2	342.4±255.4	NS
Albumin (g/dl)	3.9±0.5	4.2±0.3	P=<0.001

SP survivor patients, NSP non-survivors patients.

DM-diabetes mellitus, CaxP-calcium phosphorus product.

AP-alkaline phosphatase.

p+ t test or Man-Whitney Rank Sum test for continuous variable.

Table 3. Risk Ratio and calcium phosphate metabolism marker

Parameter	Risk ratio (RR)	p++
P>5.5mg/dL	1.2	0.08
CaxP>55mg²/dL²	1.22	0.04

p++ Chi square or Fisher exact test for discrete variable.
RR>1: shows that the levels of parameters is a risk factor for death, and RR<1 shows that the levels of parameters is a protective factor.

All of our patients were Caucasians. The analysis between group A and B (Table 2) showed statistically significant differences between survivors vs. non-survivors patients concerning calcemia (8.4±1.2 mg/dL *vs* 9.0±1.2 mg/dL, p=0.04), phosphatemia, (6.5±2.1 mg/dL *vs* 7.7±2.2 mg/dL, p=0.01) calcium phosphorus product (55.3±10.2 mg²/dL² *vs* 69.4±12.3 mg²/dL² p=0.06). We did not find statistically significant differences regarding age, diabetes mellitus frequency, sex, iPTH or alkaline phosphatase between the two groups although alkaline phosphatase and iPTH values were greater in survivors.

Analysis of Risk Ratio (RR) (Table 3), regarding calcium and phosphorus in our patients, showed increased risk of death for calcium phosphate product >55 mg²/dL² (RR=1.22, p=0.04), and for phosphatemia >5.5 mg/dL (RR=1.2, p=0.08), but not for calcemia >10.2 mg/dL, or for iPTH > 300, 400 or 600 pg/mL. Consecutively, we observed the better survival (statistically significant) only for calcium phosphate product less than 55 mg²/dL².(p=0.02) (Fig. 1) and not for calcemia less than 10.2mg/dL, phosphatemia less than 5.5mg/dL, iPTH less than 300 pg/mL.

When we compared the four groups (incriminated in the modification of the relation iPTH — survival and morbidity, (Table 4) we observed that there were no

Table 4. Characteristics at enrollment of patients in the THD depending groups

Parameter	Group A1-SPTHD<10y	Group B1-SPTHD>10y	Group C1-NSP THD<10y	Group D1-NSP THD>10y
iPTH (pg/mL)	800.7±717.6	631.1±541.9	426.3±486.1	1700.0±775.0
Albumin(mg/dL)	4.2±0.3	4.3±0.4	3.8±0.6	4.0±0.2
Creatinine (mg/dL)	10.4±2.8	11.4±2.1	9.5±2.8	10.2±2.6
CRP (mg/dL)	1.1±1.0	1.3±2.3	1,1±0.9	0.4±0.1
Age (years)	53.8±12.4	48.7±10.4	64.7±10.4	42.2±14.1
DM (%)	4.8	0	26.1	0
Male sex (%)	57.9	61.5	81	83.4
Ca (mg/dL)	8.4±1.2	8.1±1.6	8.9±1.4	9.4±0.5
P(mg/dL)	6.5±2.1	7.2±1.2	7.6±2.4	8.6±0.7
CaxP(mg²/dL²)	54.1±22.1	57.2±17.6	61.2±29.2	81.9±10.2
Kt/V	1.3±0.7	1.3±0.1	1.3±0.2	1.3±0.2
AP (U/L)	332.2±261.1	245.1±87.7	287.5±241.6	617.0±99.1

THD time on hemodialysis, SP survivors patients, NSP non-survivors patients, DM-diabetes mellitus, CaxP-calcium phosphorus product, CRP-C-reactive protein, AP-alkaline phosphatase.

Table 5. Differences between values markers at enrollement for survivors and non-survivors patients with THD<10 years

Parameter	Group A1 survivors	Group C1 Non-survivors	p+
iPTH(pg/mL)	800.7±717.6	426.3±486.1	p=0.01
AP (U/L)	332.2±261.1	287.5±241.6	NS
Albumin (mg/dL)	4.2±0.3	3.8±0.6	p<0.001
Age (ani)	53.8±12.4	64.7±10.4	p<0.001
DM (%)	4.8	26.1	0,03

p+ - t test or Man-Whitney Rank Sum test for continuous variable.

diabetic patients in the groups with time on hemodialysis more than 10 years. Mortality rate was 21.6% if time on hemodialysis was less than 10 years and 31.6% if time on hemodialysis was more than 10 years, but without statistical significance. Male proportion was greater in the non-survivors group, but without a statistical significance. The Kt/V was similar in the four groups.

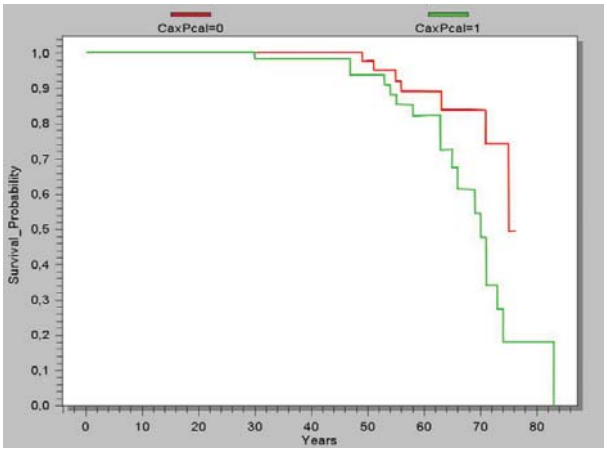

Fig.1.Cumulative survival probability according to CaxP product (cut-off value 55 mg²/dl²) p=0.02

When we analyzed only the groups with time on hemodialysis less than 10 years (Table 5 and Fig. 2), we found statistically significant differences between group A1 and C1, regarding the levels of iPTH (800.7±717.6 pg/mL *vs* 426.3±486.1 pg/mL, p=0.01), serum albumin (4.2±0.3 g/L vs. 3.8±0.6 g/L, p<0.001) and age (53.8±12.4 years *vs* 64.7±10.4 years, p<0.001).The alkaline phosphatase presented the same kind of variation as iPTH (higher in group A1), but without statistical significance. Diabetes mellitus was present 5-fold more in group C1 (26.1% vs. 4.8%, p=0.03).

There are no significant differences regarding BMI, predialytic serum creatinine, CRP, serum calcium, serum phosphate, calcium-phosphate product, sex. We did not find the correlation in group A1 between iPTH value and the other parameters: serum albumin, serum creatinine, BMI, phosphate-calcium metabolism markers, CRP, age, time on hemodialysis when using linear regression.

For the groups with time on hemodialysis more than 10 years, there are statistically significant higher levels of iPTH (Fig.3) and AP in group D1 *versus*

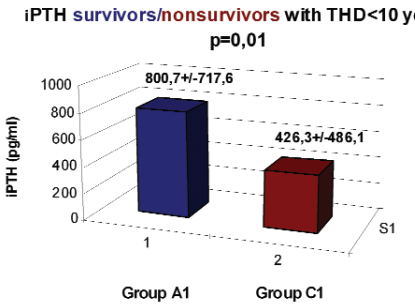

Figure 2. iPTH levels in survivors and non-survivors patients with THD<10 years.

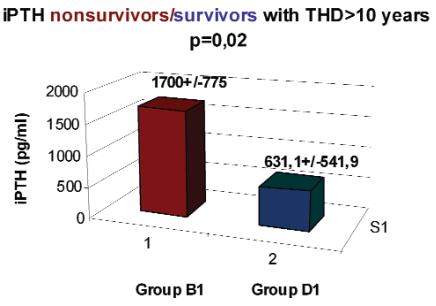

Figure 3. iPTH levels in survivors and non-survivors patients with THD>10 years.

group B1 (1700.0 ± 775.0 pg/mL vs. 631.1 ± 541.9 pg/mL, p=0.02 for iPTH and 617.0 ± 99.1 U/L *vs* 245.1 ± 87.7 U/L, p<0.001 for AP) incriminated in the modification of the relation iPTH — survival and morbidity, but there are no significant differences regarding age, sex, serum albumin, creatinine and BMI.

Serum calcium, phosphate and Ca x P product are higher in non-survivors patients (groups C1 and D1 *vs* groups A1 and B1) with a statistical significance for phosphatemia (7.6±2.4 mg/dL *vs* 6.5±2.1 mg/dL, p=0.03) if time on HD is less than 10 years and for the CaxP product (81.9±10.2 mg^2/dL^2 *vs* 57.2±17.6 mg^2/dL^2, p=0.01) if time on HD is more than 10 years.

We didn't find any correlation between phosphatemia or calcium phosphorus product and hemodialysis dose or Kt/V, in any of the groups.

DISCUSSION

This study showed that the levels above the KOQI target of CPMM such as phosphatemia > 5.5mg/dL and calcium-phosphorus product >55 mg^2/dL^2, in chronic hemodialysis patients, were correlated with mortality and survival, the same kind of variation observed in other studies (2,10,12-15). This correlation was not evident for iPTH values. The levels of iPTH in the studied patients seemed to be related not only to the markers of the calcium-phosphate metabolism. Albumin levels, age, the time on hemodialysis (HD) can also condition the high levels of iPTH. When we compared the groups of survivors and non-survivors, we found for time on hemodialysis less than 10 years higher values of iPTH at enrollment in the survivors group and for time on hemodialysis more than 10 years, higher values of iPTH at enrollment in the non-survivors group. In the survivors group with THD less than 10 years we had the same kind of variation for iPTH levels with albumin levels. In the non-survivors group, with THD more than 10 years iPTH levels at enrollment had the same kind of variation with calcium-phosphate metabolism markers. We found no direct correlation between iPTH and time on HD, as other studies did (16).

In survivors group with time on HD less than 10 years, the higher serum albumin

level can be determined by the younger age and the lower frequency of the diabetes mellitus. In this category of patients, the albumin level probably was associated with survival advantage and a higher iPTH level. We did not find a direct correlation between iPTH and nutritional markers and we could not identify any association between serum iPTH, calcium and phosphorus. This kind of variation does not seem to confirm the classic relation of high iPTH values with mortality, well known in chronic hemodialysis patients (12-14). Some authors obtained a different type of relation iPTH-mortality, too (lower iPTH values associated with higher mortality rate in HDP). This phenomenon is known as "reverse epidemiology" or altered risk factors patterns (18).

It was observed that the presence of diabetes mellitus can be associated with lower iPTH levels at patients with normal renal function (17) and at hemodialysis patients too (9,18). It can also be associated with an inflammatory syndrome, carbonil and oxidril stress and it determines a higher mortality (18,19). In our study, the proportion of diabetics was lower than in other studies, and especially in the survivors group with time on hemodialysis less than 10 years. This proportion increased in the non-survivors group, where we found the lower albumin values at enrollment and an older age. These can be associated with lower level of iPTH. Other nutrition markers, besides albumin, such as BMI and serum creatinine, were not significantly modified in the groups with time on hemodialysis less than 10 years. There were no correlations with CRP as an inflammatory marker. *In vitro*, inflammation has been shown to suppress PTH (20, 21). The inflammation can produce malnutrition and influence iPTH level (22).

Regarding age, this can have an inverse variation than iPTH in HDP (16,23), by the nutrition modification. In older patients, a lower protein intake can determine a lower phosphatemia and lower iPTH levels in relation with a precarious nutrition (24-26). In our patients we did not find this correlation. Contrary to previous studies, we identified higher phosphatemia at enrollment in the non-survivors group, where the level of albumin was significantly lower and where we found the association older age-lower iPTH values. An inverse relation of iPTH with age was previously described, even independent from the nutrition or inflammatory status (23).

These results suggest that more satisfactory albumin levels seem to be an important factor that conditioned the high iPTH level in younger and less diabetic survivor patients, with time on HD shorter. Some authors sustain that only the 7-84 PTH fraction can be modified by the nutrition status, but not the 1-84 fraction (27).

If the time on HD is longer, the relation iPTH-albumin level is changed. For a time on HD longer than 10 years, the values of iPTH, calcium-phosphate product and alkaline phosphatase were higher at enrollment in the non-survivors group. Only in this group the total alkaline phosphatase values had a statistically significant variation between survivors and non-survivors patients. Bone AP is known to be an important biochemical marker of bone formation (28). In our study we did not measure bone alkaline phosphatase, and probably for this reason the level of alkaline phosphatase did not always have the same variation as iPTH. We did not identify any significant difference regarding age, male sex, serum albumin levels and others nutrition markers and no patient had diabetes. In these groups with time on hemodialysis more than 10 years, the classical association between high iPTH values, high alkaline phosphatase values, calcium-phosphate metabolism markers and mortality, observed in the other studies (2, 12-14) seems to be confirmed.

There were no statistically significant differences between the groups with time on hemodialysis more or less than 10 years, regarding mortality rate. The optimum levels of iPTH in HDP (probably higher than those still recommended) are under evaluation. The iPTH levels do not have to be interpreted singularly. Even in younger and lower percent-diabetic HDP groups, an evaluation of the albumin levels seems useful, besides the calcium-phosphate metabolism markers. The relations observed between iPTH, albumin levels and mineral metabolism need further investigation and confirmation in larger studies.

In conclusion, calcium-phosphorus product is a better indicator for survival in HDP in our area than immunoreactive PTH. Immunoreactive PTH as prognostic factor is better evaluate in association with calcium phosphorus metabolism parameters and albumin levels too, even in younger and lower percent-diabetic HDP groups.

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