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Article

Lymphocyte Phenotypes and Protein-Bound Uremic Toxins as Determinants of Clinical Outcomes in Hemodialysis Patients

Theodoros Tourountzis [1,2,†], Georgios Lioulios [2,†], Stamatia Stai [2], Steven Van Laecke [3], Eleni Moysidou [2], Michalis Christodoulou [2], Ariadni Fouza [2] Asimina Fylaktou [4], Konstantia Kantartzi [5], Griet Glorieux [3] and Maria Stangou [2,*]

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Abstract

Background/Objectives: The impact of protein bound uremic toxins (PBUT) and lymphocyte alterations in morbidity and mortality in patients on hemodialysis (HD) is of great concern. Aim of this study was the assessment of association between PBUT, immunosenescencent lymphocytes' phenotype and clinical events [cardiovascular, severe infections (hospitalization due to infection, respiratory infection), all-cause mortality] during 2-year follow-up. **Methods**: In this prospective observational study, lymphocytes' phenotype of 54 patients on HD and 31 age-matched controls, was analyzed by flow cytometry, and simultaneously, PBUT serum levels [hippuric acid (HA), indoxyl sulfate (IxS), p-cresyl sulfate (pCS), p-cresyl glycuronide (pCG), in-dole-3-acetic acid (IAA), 3-carboxy-4-methyl-5-propyl-2-furanpropionate (CMPF)] were quantified by ultra-performance liquid chromatography. **Results**: Patients with increased levels of free IxS, total and free HA had higher mortality within 2-year follow-up period ($p = 0.049$, $p = 0.01$, $p = 0.01$, respectively). In patients who experienced cardio-vascular events, higher concentrations of CMPF ($p=0.015$) were observed. Higher total and free HA levels associate with increased all-cause mortality in patients on HD, independently of age, dialysis vintage, decreased count of CD4+CD45RA+CD31+ and naïve B cells (CD19+IgD+CD27-). **Conclusions**: In patients on HD, increased levels of total and free HA associate with an increased risk of death.

Keywords: protein bound uremic toxins; immunosenescence; morbidity; mortality; hemodialysis

1. Introduction

Morbidity and mortality are increased in patients on hemodialysis (HD). Factors associated with increased all-cause mortality are, among others, increased age, diabetes mellitus, cardiovascular disease and increased C-reactive protein [1]. The increased risk for cardiovascular disease in patients on HD are due to hypervolemia, uremic cardiomyopathy, secondary hyperparathyroidism, lipid disorders, anemia and accumulation of protein bound uremic toxins (PBUT) [2]. Central venous

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catheter at the commencement of HD is a crucial factor associated with hospitalizations due to all-cause infections, bacteremia and HD access infections [3].

Uremic toxins were classified, based on their physicochemical characteristics and their protein binding, into three categories by the European Uremic Toxin Work Group: (1) water-soluble low molecular weight molecules (< 500 Dalton), (2) middle molecules (≥ 500 Dalton) and (3) protein bound solutes. The latter, also called PBUT, include among others, indoxyl sulfate (IxS), p-cresyl sulfate (pCS), p-cresyl glycuronide (pCG), hippuric acid (HA), indole-3-acetic acid (IAA), 3-carboxy-4-methyl-5-propyl-2-furanpropionate (CMPF), and they have low molecular weight [4,5]. Following the improvements in HD membranes and other removal techniques, the classification was recently finetuned [6]. PBUT are not sufficiently removed with current methods of HD, due to their high protein binding. As a result, they accumulate in these patients and they correlate with comorbidity and mortality [7]. Uremic toxins are associated with kidney and cardiovascular damage, endothelial dysfunction, susceptibility to infections, intestinal dysbiosis and cognitive impairment, which may lead to higher mortality and lower quality of life in patients with chronic kidney disease (CKD) [6,8–10].

Immunosenescence includes dysregulations of innate and adaptive immune system which is related with aging. It can cause sensitivity to infections, increased risk of malignancy and poor response after vaccination [11]. Senescence of T cells is associated to low-grade inflammation (inflammaging) and both are involved in the pathogenesis of age-related conditions, such as cardiovascular disease, cancer and neurodegenerative disorders [12]. End stage kidney disease (ESKD) patients experience a premature senescence of immune system which is related to other comorbidities. Possible immunosenescencent alterations, in patients on HD are decreased number of T, naïve T and $CD4^+$ naïve T cells, decreased percentage of T cells and increased percentage of $CD8^+$ central-memory T cells. Decreased count of naïve T cells, may influence all-cause mortality in patients on HD [13].

The main aim of this study was the evaluation of association between PBUT serum levels, lymphocytes' phenotype and clinical outcome including cardiovascular events, severe infections (hospitalization due to infection, respiratory infection) and all-cause mortality, in patients on HD, during a 2-year follow-up period.

2. Materials and Methods

2.1. Study Design

This is a prospective observational study, conducted in the First Department of Nephrology of Aristotle University of Thessaloniki, in collaboration with the Department of Immunology, General Hospital "Hippokratio", Thessaloniki, Greece, from 4/2022 to 3/2024. Adult patients on thrice weekly chronic maintenance HD for at least 6 months were eligible for the study. Patients with history of diabetes mellitus, recent infection or vaccination (last 3 months), autoimmune disease or recent medication with immunosuppressive drugs (last 12 months) and active malignancy or history of malignancy during the last 5 years were excluded from the study, as these conditions may have been associated to potential alterations in immunological status. All patients provided informed consent prior to enrollment and demographic and clinical data were recorded. For the analysis of immune phenotype, total blood samples were collected before the initiation of a mid-week dialysis session, and analyzed with flow cytometry as described below. At the same time point, plasma was collected, after centrifugation of whole blood in Ethylene diamine tetraacetic acid tubes for 10 minutes at 2095×g, and preserved in -60°C for evaluation of PBUT levels with ultra-performance liquid chromatography. Subsequently, the patients were observed for a 24 months follow-up period and future clinical events were recorded.

Informed consent was obtained from all subjects involved in the study. The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of the Medical School of the Aristotle University of Thessaloniki

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(protocol code 134/2023, 10 May 2023). All research activities were performed with coded, pseudonymized tissue samples and data.

2.2. Clinical Events

During the 24-month follow-up period clinical events were recorded, including cardiovascular ones, hospitalization due to infection, respiratory infection and all-cause mortality. Cardiovascular events were defined as a composite of the first occurrence of congestive heart failure, acute myocardial infarction, coronary heart disease, atrial fibrillation and peripheral arterial disease, during the follow-up period. Hospitalization due to infection was defined as a composite of the occurrence of different kinds of infections (bacteremia, pneumonia and other pulmonary infection, urinary tract infection, soft tissue infection, vascular access infection, osteomyelitis, viral or fungal infection) that required hospitalization, during the follow-up period. Respiratory infection was defined as bacterial, viral or unknown origin or etiology infection of the respiratory tract that required treatment (such as antibiotics), during the follow-up period. All-cause mortality was defined as a composite of recorded causes of death (cardiac arrest, cardiovascular disease, infection, unknown, others) during the follow-up period.

2.3. Laboratory Methods

2.3.1. Analysis of PBUT

The blood preparation for total PBUT concentration was performed by ultra performance liquid chromatography and has been described in details previously [14]. The detailed explanation of the uremic toxins analysis process can be found in the Supplementary Materials.

2.3.2. Analysis of Lymphocytes

Whole blood samples were immediately transferred to the laboratory, where kept at room temperature until processing, for no longer than 12 hours. Blood samples were stained with the following conjugated antibodies and analyzed with flow cytometry, according to manufacturer's recommendations, as described previously [14]. Percentages and counts of T cells subpopulations were determined according to the expression of surface markers as following: early differentiated cells (recent thymic emigrants, naïve), memory cells (central memory, effector memory), advanced differentiated senescent cells [effector memory re-expressing CD45RA (EMRA)], terminally differentiated senescent cells (EMRACD28-). B cells were classified as naïve, IgM memory, switched memory and double negative. The detailed explanation of flow cytometry analysis process can be found in the Supplementary Materials.

In the same of immune profile and PBUT evaluation, additional laboratory parameters: complete blood count, serum urea (enzymatic method, in mg/dL), serum creatinine (enzymatic kinetic method, in mg/dL), parathyroid hormone (chemiluminescent microparticle immunoassay method, in pg/mL), serum phosphorus (colorimetric method, in mg/dL,), serum calcium (colorimetric method, in mg/dL), serum lactate dehydrogenase (enzymatic kinetic method, in IU/L), serum albumin (colorimetric method, in g/dL), ferritin (chemiluminescent microparticle immunoassay method, in ng/mL), C-reactive protein (immunostaining method, in mg/L) and lipids [serum cholesterol (enzymatic method, in mg/dL), serum triglycerides (enzymatic method, in mg/dL), serum high-density lipoprotein cholesterol (enzymatic method, in mg/dL), serum low-density lipoprotein cholesterol (enzymatic method, in mg/dL)].

2.4. Statistical Analysis

The statistical processing and analysis of the data was performed with the statistical package for social sciences (SPSS) 27 IBM Corp, Armonk, NY, USA, for Windows. The level of statistical significance (p) was set below 0.05. The qualitative variables were described using absolute (n) and

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relative frequency (%). Kolmogorov-Smirnov and Shapiro tests were used to estimate the normality distribution of continuous variables. Normally distributed parameters were expressed as mean ± standard deviation (SD), while non-normally distributed parameters were expressed as the median and range. A Mann-Whitney U test was performed to estimate the differences in parameters between two groups (patients and control group, patient who experienced or not a clinical event), and a Spearman's correlation test was used to assess the correlations between non-parametric variables. The Spearman's coefficient and multiple regression analysis were performed to estimate the correlation between PBUT serum levels and clinical events. Kaplan-Meier and Cox regression model analysis were performed for future clinical events of patients on HD.

3. Results

3.1. Patients' Characteristics

Fifty-four patients on HD and 31 healthy age-matched controls, were included in the study. The mean age of the patient group (23 females, 31 males) was 51.3 ± 16.9 years and of the control group (15 females, 16 males) 51.3 ± 17.2 years (p=0.993). The median HD vintage was 67 (20.7-95.2) months, 24/54 (44.4%) patients were on online hemodiafiltration and 30/54 (55.6%) on HD. Mean body mass index was at upper normal limits, 24.5 ± 3.6 kg/m^2. Dialysis related details, comorbidities and causes of ESKD are depicted in Table 1.

Table 1. Dialysis related details, comorbidity and causes of end stage kidney disease (ESKD) in patients.

Parameters	Patients
Dialysis related details	
Online hemodiafiltration/Hemodialysis	44.4%/55.6%
Duration of dialysis' session (min)	240 (240-240)
Kt/V	1.45 (1.31-1.56)
Fistula/Central venous catheter	79.2%/20.8%
Comorbidity	
Arterial hypertension	66.7%
Dyslipidemia	31.5%
Hypothyroidism	11.1%
Peripheral arterial disease	9.3%
Coronary heart disease	7.4%
Atrial fibrillation	7.4%
Stroke	7.4%
Primary cause of ESKD	
Primary glomerulonephritis	29.6%
Not known	27.8%
Obstructive uropathy	16.7%
Autosomal dominant polycystic kidney disease	13%
Hypertension	5.5%
Alport syndrome	3.7%
Other	3.7%

Laboratory parameters of the patients and control group are shown on Table 2.

Table 2. Laboratory parameters of patients and control group. P values are referred to differences between patients and control group.

Parameters	Patients	Control group	*p*
Whole blood count			
Hemoglobin (g/dL)	11.8 ± 0.9	13.7 ± 1.1	< 0.001

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Hematocrit (%)	36.1 ± 3.1	40.7 ± 3.1	< 0.001
White blood cells (cells/μL)	7100 (5500-8325)	6200 (5300-7100)	0.046
Neutrophils (cells/μL)	4550 (3475-5500)	3400 (2700-4200)	0.001
Lymphocytes (cells/μL)	1400 (1175-1800)	2100 (1600-2500)	< 0.001
Platelets (10^3/μL)	227 (184.2-265)	216 (199-245)	0.729
Urea (mg/dL)	127 (111.5-151.5)		
Creatinine (mg/dL)	9.4 (7.3-10.9)		
Parathyroid hormone (pg/mL)	206.5 (106.2-370.7)		
Phosphorus (mg/dL)	4.3 (3.7-5)		
Calcium (mg/dL)	9.1 (8.8-9.3)		
Albumin (g/dL)	4.1 (3.9-4.3)		
C-reactive protein (mg/L)	2.3 (1.4-4.2)		

3.2. PBUT and Peripheral Lymphocytic Phenotype of Patients on HD

Increased levels of serum PBUT were observed in patients compared with control group (p < 0.001), (Table 3).

Table 3. Patients' and control group serum concentration levels of protein bound uremic toxins. P values are referred to differences between patients and control group.

Parameters	Patients	Control group	*p*
Total IxS* (mg/dL)	2.21 (1.27-3.34)	0.06 (0.04-0.09)	< 0.001
Free IxS* (mg/dL)	0.15 (0.09-0.27)	0.0004 (0.0004-0.0004)	< 0.001
Total pCS* (mg/dL)	1.25 (0.84-1.66)	0.07 (0.04-0.13)	< 0.001
Free pCS* (mg/dL)	0.09 (0.06-0.13)	0.004 (0.004-0.005)	< 0.001
Total pCG* (mg/dL)	0.22 (0.09-0.39)	0.001 (0.0013-0.0017)	< 0.001
Free pCG* (mg/dL)	0.2 (0.08-0.35)	0.002 (0.0011-0.0017)	< 0.001
Total HA* (mg/dL)	3.1 (1.66-5.37)	0.1 (0.04-0.2)	< 0.001
Free HA* (mg/dL)	1.48 (0.7-2.8)	0.03 (0.03-0.04)	< 0.001
Total IAA* (mg/dL)	0.12 (0.09-0.167)	0.03 (0.02-0.03)	< 0.001
Free IAA* (mg/dL)	0.04 (0.03-0.05)	0.006 (0.0056-0.0069)	< 0.001
CMPF* (mg/dL)	0.17 (0.1-0.38)	0.07 (0.035-0.144)	< 0.001

* indoxyl sulfate (IxS); p-cresyl sulfate (pCS); p-cresyl glucuronide (pCG); hippuric acid (HA); in-dole-3-acetic acid (IAA); 3-carboxy-4-methyl-propyl-2-furanpropanoic acid (CMPF).

Patients on HD had increased white blood cells compared to control group; 7100 (5500-8325) vs. 6200 (5300-7100) cells/μL (p=0.046). Moreover, reduction of CD4+ [679.5 (483-862.2) vs. 999 (786-1237) cells/μL (p < 0.001)], and CD8+ cells [377 (261.7-531.5) vs. 451 (296-746) cells/μL (p=0.13)] was found in patients on HD. Furthermore, reduced numbers of B cells and their subpopulations were found in patients on HD in comparison to healthy individuals, namely: CD19+ 91 (52.2-131.2) vs. 248 (163-388) cells/μL (p < 0.001); naïve (IgD+CD27-) 56.5 (27.7-96.2) vs. 144 (91-258) cells/μL (p < 0.001); IgM memory (IgD+CD27+), 5 (3-10.2) vs. 23 (11-32) cells/μL (p < 0.001); switched memory (IgD-CD27+) 13 (7.7-18.5) vs. 35 (22-61) cells/μL (p < 0.001); double negative (IgD-CD27-) 7 (4.7-12) vs. 26 (14-43) cells/μL (p < 0.001), (Table 4).

Table 4. Differences in T and B lymphocyte subpopulations between patients and control group. P values are referred to differences between patients and control group.

Parameters	Patients	Control group	*p*
White blood cells (cells/μL)	7100 (5500-8325)	6200 (5300-7100)	0.046
CD4+ (cells/μL)	679.5 (483-862.2)	999 (786-1237)	< 0.001
CD8+ (cells/μL)	377 (261.7-531.5)	451 (296-746)	0.13
CD19+ (cells/μL)	91 (52.2-131.2)	248 (163-388)	< 0.001

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Naïve (IgD+CD27-) (cells/μL)	56.5 (27.7-96.2)	144 (91-258)	< 0.001
IgM memory (IgD+CD27+) (cells/μL)	5 (3-10.2)	23 (11-32)	< 0.001
Switched memory (IgD-CD27+) (cells/μL)	13 (7.7-18.5)	35 (22-61)	< 0.001
Double negative (IgD-CD27-) (cells/ μL)	7 (4.7-12)	26 (14-43)	< 0.001

3.3. Association with Clinical Events

3.3.1. Association of Immune Phenotype with Mortality and Morbidity

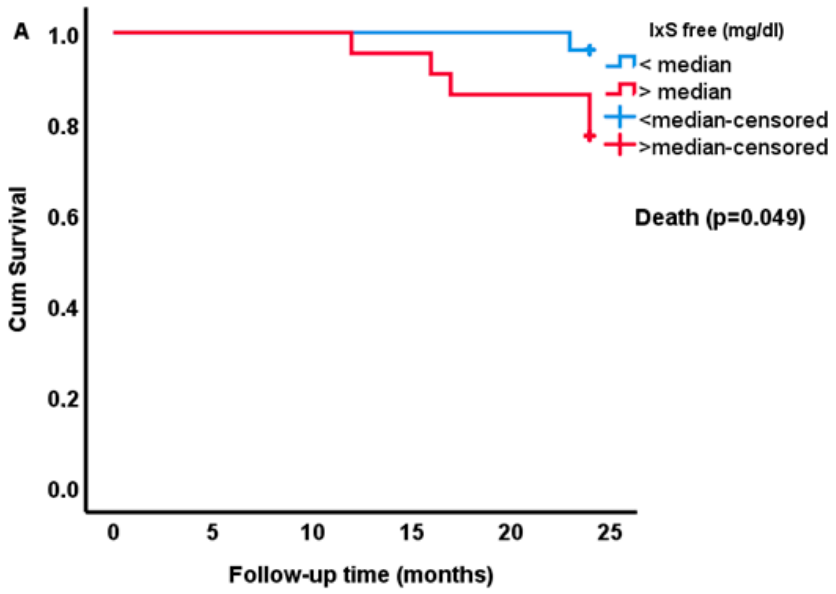
Patients on HD who died in the 2 years follow up (n = 6), compared to those remained alive (n = 48), had significantly reduced count of naïve B cells (CD19+IgD+CD27-) [23.5 (11.25-54.25) vs. 58.5 (30-98.75), p = 0.032 respectively], but no significant changes in the rest subpopulations of T lymphocytes, and also no differences in B lymphocytes. Besides, patients' morbidity, including cardiovascular events or severe infections were not associated with differences in baseline lymphocyte subtype.

3.3.2. Multiple Regression Analysis

Dialysis vintage was positively correlated with increased levels of total (r = 0.379, p = 0.005) and free HA (r = 0.392, p = 0.003), but not with IxS (r = 0.252, p = 0.066). Additionally, significant effect of increased levels of total and free HA in mortality remained in cox regression analysis, either examined alone as continuous variables [p = 0.023, 95% confidence interval (CI) 1.024-1.366, p = 0.008, 95% CI 1.077-1.628, respectively] or in combination with age, dialysis vintage, total number of CD4+CD45RA+CD31+ and CD19+IgD+CD27- lymphocytes (p = 0.041, 95% CI 1.007-1.444, p = 0.01, 95% CI 1.093-1.948, respectively). In contrary, the significance of the association between increased levels of free IxS and mortality, disappeared after analyzed with cox regression with the above-mentioned parameters.

3.3.3. Association of PBUT with Mortality and Morbidity

Log rank tests were run to determine if there were differences in the clinical events distribution (cardiovascular events, hospitalization due to infection and all-cause mortality) for the PBUT that were examined. Kaplan-Meier survival analysis tests found that patients' survival was significantly different when they were clustered based on median levels of free IxS, total HA and free HA [$\chi^2(2) = 3.849$, p = 0.049, $\chi^2(2) = 6.721$, p = 0.01, $\chi^2(2) = 6.721$, p = 0.01 respectively] (Figure 1).



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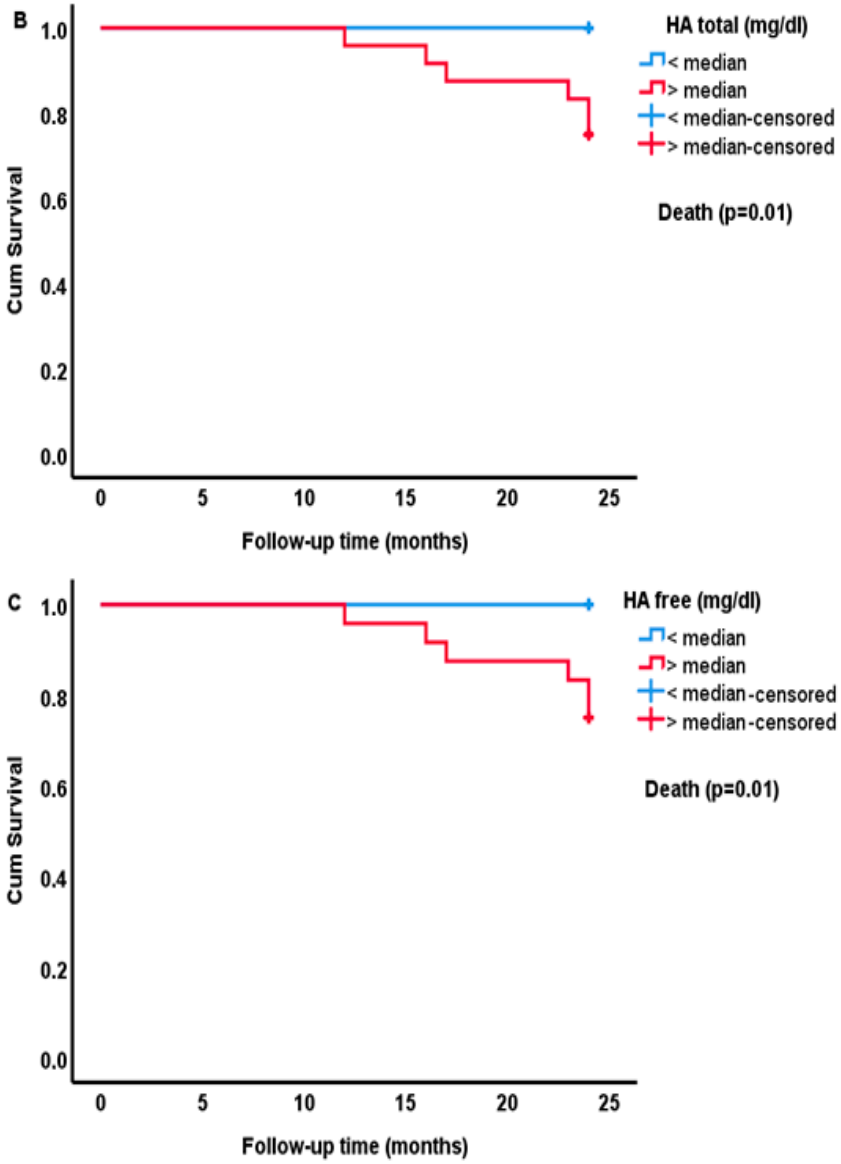

Figure 1. Kaplan-Meier curves of estimated survival probability for 2 different groups of patients on hemodialysis, according to protein bound uremic toxins serum levels [indoxyl sulfate (IxS) (A), total (B) and free (C) hippuric acid (HA)].

Moreover, no significant correlations were found between pCS and IAA levels with mortality in this study. A logistic regression model, describing the effect of each PBUT on death probability could explain 44% (Nagelkerke R^2) of the variance in death and correctly classified 90.7% of cases. Patients with increased levels of free IxS, total and free HA were more likely to die within a 2-year follow-up period ($p = 0.047$, $p = 0.013$, $p = 0.003$, respectively). In patients who died during follow-up period (n = 6, 11.11%, all of them in the second year), increased levels, compared to those remained alive, of total HA 6.42 (4.54-9.08) vs. 2.75 (1.46-4.49) mg/dl ($p = 0.007$); free HA 4.79 (2.47-6.4) vs. 1.36 (0.58-2.43) mg/dl ($p = 0.004$); free IxS 0.29 (0.14-0.42) vs. 0.13 (0.08-0.24) mg/dl ($p = 0.045$); total pCG 0.35 (0.25-0.74) vs. 0.19 (0.08-0.37) mg/dl ($p = 0.045$); free pCG 0.33 (0.23-0.65) vs. 0.18 (0.08-0.33) mg/dl ($p = 0.042$), were found, respectively (Figure 2). However, in these patients, increased levels of total pCS 1.34 (1.13-1.62) vs. 1.21 (0.8-1.68) mg/dl and free pCS 0.11 (0.08-0.25) vs. 0.08 (0.05-0.12) were also found, but were not statistically significant ($p = 0.457$, $p = 0.16$, respectively).

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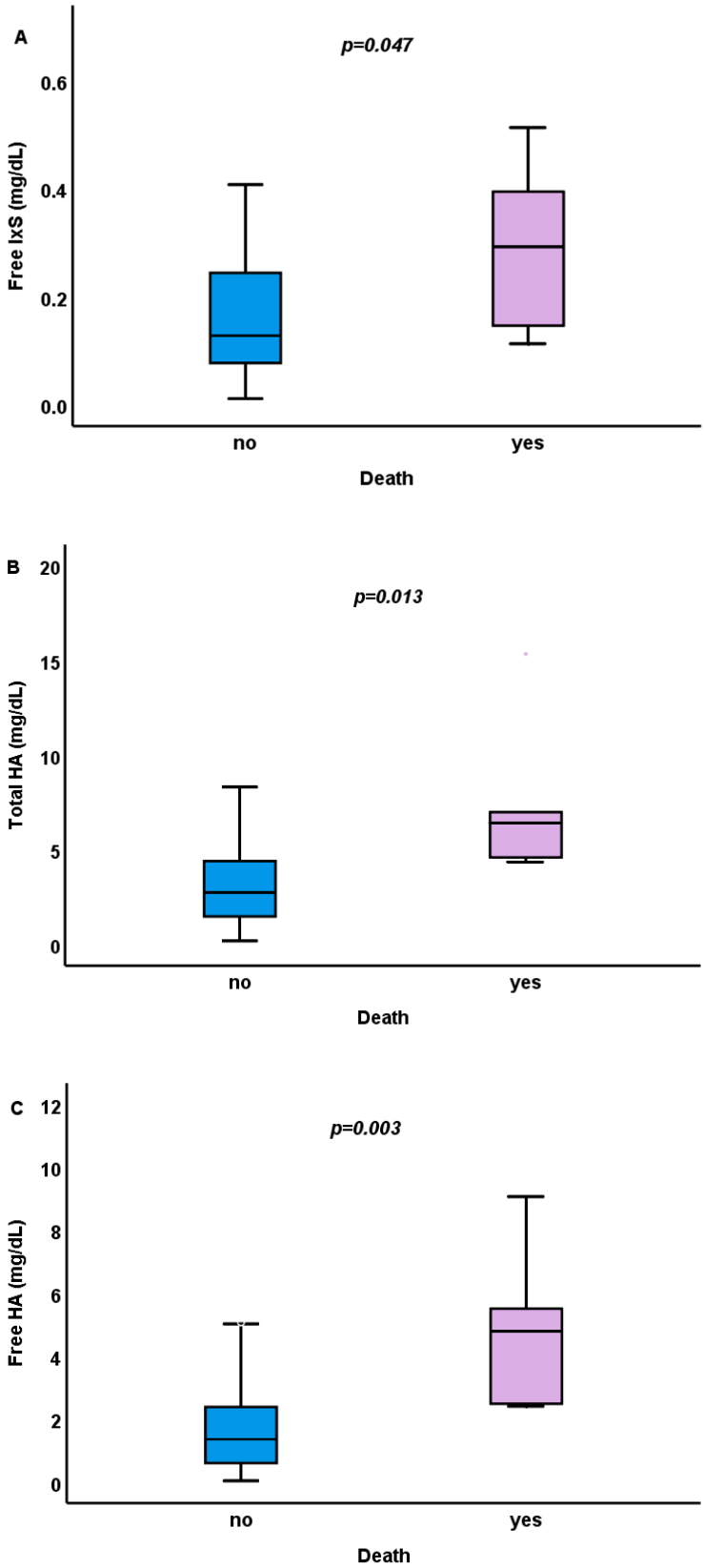

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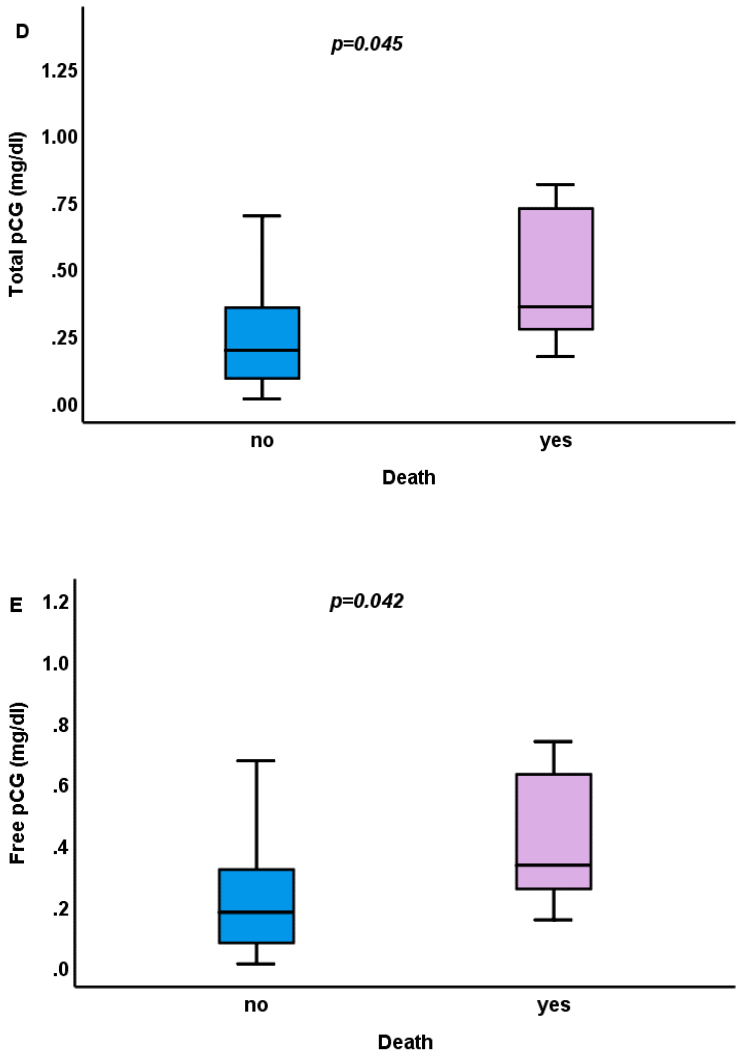

Figure 2. Differences in serum levels of free indoxyl sulfate (IxS) (A), total (B) and free (C) hippuric acid (HA), total (D) and free (E) p-cresyl glycuronide (pCG) in patients on hemodialysis who died during 2-year follow up-period.

Regarding cardiovascular events, our study pointed out the potential importance of CMPF. Patients who experienced one or more cardiovascular events during follow up (n = 11) had a higher concentration of CMPF [0.39 (0.18-0.85) vs. 0.15 (0.1-0.29) mg/dl (p = 0.015)] (Figure 3).

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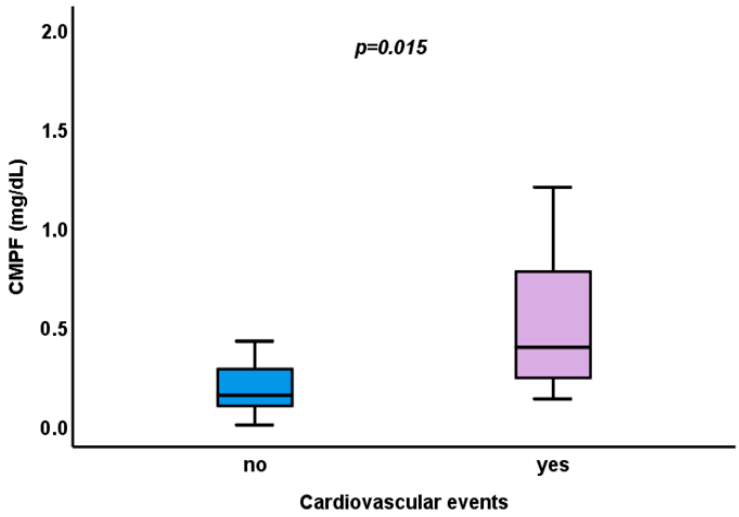

Figure 3. Differences in serum levels of 3-carboxy-4-methyl-5-propyl-2-furanpropionate (CMPF), in patients on hemodialysis who experienced a cardiovascular event during 2-year follow up-period.

PBUT levels were not different among patients on HD with and without severe infections during the 2 years follow up.

4. Discussion

In this study, we tried to investigate the possible association between immune alterations in HD, including increased serum PBUT levels and phenotypic changes of lymphocytes, and morbidity and mortality of these patients. During a two year follow up, we found mortality in patients on HD was associated with increased levels of total and free HA, free IxS, total and free pCG. Also, patients on HD with reduced naïve CD4 subpopulations were more likely to die within the next 2 years. However, multiple regression analysis showed that increased levels of free and total HA, and free IxS were the only independent factors associated with increased risk of death, independently of age, dialysis vintage, and immune-phenotypic changes.

Results from previous studies have revealed rather controversial results. Some investigators showed that free serum levels of pCS and total IxS level were associated with increased all-cause mortality and also cardiovascular mortality in patients on HD [15,16]. Increased total pCS levels, also, associated with cardiovascular events, such as death from cardiac cause, myocardial ischemia, non-fatal myocardial infarction, ischemic stroke, peripheral vascular disease and all-cause mortality in patients on HD, who were older than 65 years [17]. However, in the same population, kynurenic acid, IxS, IAA, pCG and HA plasma concentrations were not associated with either all-cause mortality or cardiovascular events [18].

HA is derived from the catabolism of dietary polyphenols of plant based food by the gut microbiota [19]. Excretion of HA is compromised in age related conditions, such as cognitive impairments, sarcopenia and hypomobility [19], and it has been proposed as a hallmark of aging and may have harmful effects on the kidney and the brain [20]. Despite its low molecular weight (179,2 Da), the kinetics of HA are similar to those of larger molecules, due to its high protein binding, mainly to albumin, and has low clearance through dialysis [21,22]. IxS is originated from the breakdown of tryptophan by microbes in the colon. IxS is also a small molecule, however, as bound to more than 90% with plasma proteins, it has a limited clearance by HD, resulting in peripheral blood accumulation [23]. Elevated levels of IxS are associated with cardiovascular damage in patients with uremia, with underlying mechanisms being the contribution to endothelium dysfunction, vascular oxidative stress, inordinate inflammatory responses, calcification and thrombosis [24]. PCS is derived from the bacterial metabolism in the gut, and is associated with a plethora of biological toxic

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outcomes, including contribution to mechanisms that are comprised in renal (such as toxic effects on renal tubular cells) and cardiovascular damage [25]. Both IxS and pCS trigger arterial calcification in CKD and associate with endothelial dysfunction and oxidative stress [26]. CMPF is a furan fatty acid metabolite and a biomarker of fish intake. It can interact with free oxygen radical, which provoke cell damage [27].

CMPF concentration was correlated with cardiovascular events, in this study. Several PBUTs have been previously described to have positive correlation with increased cardiovascular risk in HD, although the results are still controversial. Indoxyl sulfate was related with symptomatic peripheral arterial disease, after adjusting for its known risk factors, but not with other major adverse cardiovascular events in HD [28]. HA is found to be related with left ventricular hypertrophy, which is associated with high risk of death in this population [29]. Moreover, HA contributes to endothelial dysfunction in vitro and in vivo, resulting in progression of cardiovascular disease [21]. Serum total IxS levels had a positive association with all-cause mortality and severe infections [30], and plasma pCS may forecast the first incidence of ischemic stroke in population on HD [31]. In addition, increased PBUT levels are also found to correlate with other manifestations, such as cognitive impairment, attributed to increased IAA levels [32]. However, results from other studies, indicate that pCS and IxS are not associated with cardiovascular outcomes, such as cardiac death and first cardiovascular event [33].

Regarding changes in lymphocytes and their subpopulations, the only subtype which showed close association with death, was that of naïve B cells (CD19+IgD+CD27-), found significantly reduced in HD patients who died during follow up. Previous studies have demonstrated and highlighted the close association between lymphocytic alterations and death in patients on HD. Therefore, low absolute number and percentage of T lymphocytes, low CD4+ naïve and increased CD8+ central memory lymphocytes were associated with all-cause mortality, with low count of naïve T lymphocytes being the only independent factor [13]. Additionally, CD19+ B cell lymphocytopenia (< 100 cells/μL) may be an independent predictor of all-cause and cardiovascular mortality in HD. Some patients in the previous study had received immunosuppression due to previous renal transplantation, glomerulonephritis, autoimmune diseases and other causes [34]. Another study, found that decreased CD4+ naïve T lymphocyte count was independently related with cardiovascular events (coronary artery disease, congestive heart failure, stroke, peripheral arterial disease), while low CD8+ naïve count was independently related with severe infection episodes (infectious diseases that required treatment with intravenous antibiotics in hospital or emergency ward) in patients on HD [35]. The neutrophil to lymphocyte ratio may predict mortality and cardiovascular events (myocardial infarction, ischemic heart disease, peripheral vascular disease, acute limb ischemia, mesenteric ischemia, stroke, deep venous thrombosis, pulmonary embolism), but not severe infections (infections with sepsis and / or requiring hospitalization and respiratory infection treated with antibiotics) in patients on HD, and had positive association with the IxS [36]. Decreased memory B cells are irrespectively correlated with higher risk of infections, mainly respiratory ones, in patients on HD [37].

A major limitation of this study is the small number of participants, both patients and control group. Secondly, due the low number of the clinical events we cannot surely reckon for other confounders in the analysis. Interestingly though, patients with increased morbidity were excluded, namely those with diabetes mellitus and / or active malignancy, as parameters that potentially affect lymphocytes' phenotype and function. Accordingly, our results are not representative of the whole cohort of HD population, but rather of those non-diabetic patients being in a stable condition on HD. Furthermore, since it is an observational study, we could not prove causality of the relation between serum PBUT levels and clinical events, but can simply describe correlation.

5. Conclusions

In patients on HD, increased levels of PBUT, mainly free and total HA, were associated with increased risk of death within two years, independently of age, dialysis vintage, and immune-

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phenotypic changes. Patients on HD who died in the follow up period, had significantly reduced count of naïve B cells, compared to those remained alive. Further studies are needed to investigate methods of efficient removal of HA within HD, strategies of lower production of HA by gut microbiota and examination of dietary changes for reduction of HA intake, without losing the benefits of plant-based foods, such as dietary fibers.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Analysis of lymphocytes; analysis of protein bound uremic toxins; Figure S1: Gating strategy for CD4 and CD8 cells and their subsets, based on the presence of CD45RA, CCR7, CD31, CD57, and CD28; Figure S2: Gating strategy for CD19+ cells and their subsets based on the presence of IgD and CD27.

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

Data Availability Statement: The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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Abbreviations

The following abbreviations are used in this manuscript:

PBUT	protein bound uremic toxins
HD	hemodialysis
HA	hippuric acid
IxS	indoxyl sulfate
pCS	p-cresyl sulfate
pCG	p-cresyl glycuronide
IAA	indole-3-acetic acid
CMPF	3-carboxy-4-methyl-5-propyl-2-furanpropionate
CKD	chronic kidney disease
ESKD	end stage kidney disease
EMRA	effector memory re-expressing CD45RA
CI	confidence interval

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