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Osteocalcin (bone GLA protein) levels, vascular calcifications, vertebral fractures and mortality in hemodialysis patients with diabetes mellitus.

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Author Comments:	Padua, 14 October 2018 To Editor, Journal of Nephrology Dear Editor, herewith you find the manuscript Osteocalcin (bone GLA protein) levels, vascular calcifications, vertebral fractures and mortality in hemodialysis patients with diabetes mellitus, which I submit for publication as an original article in the Journal of Nephrology. Diabetes Mellitus (DM) is one of the major causes of end stage kidney disease. Patients with DM and Chronic kidney disease (CKD) have greater severity of CKD associated complications, in particular, they are inclined to micro and macro-vascular complications, and earlier and progressive bone disorders, such as osteoporosis, adynamic bone disease and fractures. Bone Gla Protein (BGP or osteocalcin, OC) is a vitamin K-dependent protein, secreted by osteoblasts and involved in the regulation of bone matrix mineralization. Levels of BGP in DM patients on hemodialysis (HD) have been poorly described. Our goal was to characterize BGP and ucBGP in DM patients on HD, compared to HD patients without DM, and their association with vascular and bone disease. To our knowledge, this is the first study in hemodialysis patients suggesting a potential protective role of BGP in the context of bone, endocrine and vascular pathway. The general message is the need to pay attention to vitamin K levels to prevent bone and vascular damage both in general population and in CKD patients, especially in DM CKD patients. Indeed we hope that our findings will raise awareness and inspire other physicians to carry out research about the importance of vitamin K and its Vitamin K Dependet Proteins in the development of vascular calcification and bone fractures, both risk factors for the main cause of morbidity and mortality. The paper is original and is not under consideration by any other journal. Thanking you for your attention. Yours sincerely Maria Fusaro, MD, PhD
Suggested Reviewers:	

Padua, 14 October 2018

To Editor, Journal of Nephrology

Dear Editor,

herewith you find the manuscript **Osteocalcin (bone GLA protein) levels, vascular calcifications, vertebral fractures and mortality in hemodialysis patients with diabetes mellitus,** which I submit for publication as an original article in the Journal of Nephrology.

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The general message is the need to pay attention to vitamin K levels to prevent bone and vascular damage both in general population and in CKD patients, especially in DM CKD patients. Indeed we hope that our findings will raise awareness and inspire other physicians to carry out research about the importance of vitamin K and its Vitamin K Dependet Proteins in the development of vascular calcification and bone fractures, both risk factors for the main cause of morbidity and mortality.

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Maria Fusaro, MD, PhD

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Keywords: Diabetes Mellitus, BGP, Vitamin K, hemodialysis.

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ABSTRACT

BACKGROUND & AIMS: Diabetes Mellitus is recognized as one of the major causes of end stage kidney disease. Bone Gla protein (BGP) is a vitamin K-dependent protein involved in bone mineralization and vascular calcifications (VC). Our goal was to characterize BGP and undercarboxylated BGP (ucBGP) in DM patients on HD, compared to HD patients without DM, and their association with vascular and bone disease.

METHODS: 387 HD patients from 18 dialysis centers in Italy. Associations of DM, levels of BGP, vitamin D and VC were evaluated. Time-to-event analysis for all-cause mortality was performed by the Kaplan-Meier.

RESULTS: Patients with DM had lower levels of total BGP (139.00 vs. 202.50 mcg/L, $p<0.001$), 25(OH)D (23.4 vs. 30.2 ng/ml, $p<0.001$), and ucBGP (9.24 vs. 11.32 mcg/L, $p=0.022$). In regression models, the geometric means of total BGP and ucBGP were 19% ($p=0.009$) and 26% ($p=0.034$) lower in diabetic patients. In univariate Cox regression analysis, DM patients had a higher risk of all-cause mortality (HR:1.83, 95% CI:1.13-2.96, $p=0.014$). Adjustment for confounders confirmed the significant DM-mortality link. We included VC and warfarin into the Cox model, the DM-mortality link was no longer significant, suggesting a role of these risk factors as causal mediators leading to increased mortality in dialysis patients.

CONCLUSIONS: HD patients have an increased mortality risk associated with DM. Furthermore, we found an association between DM and decreased BGP levels. To our knowledge this is the first study in HD patients suggesting a potential protective role of BGP in the bone, endocrine and vascular pathway.

Introduction

Diabetes Mellitus (DM) is one of the major causes of end stage kidney disease. Patients with DM and Chronic kidney disease (CKD) have greater severity of CKD associated complications compared to CKD patients with other etiologies of kidney disease. In particular, DM patients are inclined to micro and macro-vascular complications, and earlier and progressive bone disorders, such as osteoporosis, adynamic bone disease and fractures (1).

Bone Gla Protein (BGP or osteocalcin, OC) is a vitamin K-dependent protein, secreted by osteoblasts and involved in the regulation of bone matrix mineralization. Carboxylated osteocalcin (Gla-containing cBGP) is involved in bone crystal nucleation by its specific affinity to bind hydroxyapatite molecules. In contrast, ucBGP (ucBGP or ucOC) has less than 3 carboxylated residues and a lower affinity for bone tissue. An endocrine role of ucOC has been recently identified (2, 3). It seems to be able to increase insulin secretion directly by stimulating pancreatic β cells, or indirectly by promoting the release of adiponectin (4).

BGP also has a protective role in vascular calcifications in humans (5), acting under the genetic control of vitamin D. Vitamin D deficiency is highly prevalent in CKD patients, suggesting that the protective role of vitamin D on vascular calcification (6) may be mediated by a reduced expression of BGP and other vitamin K dependent proteins involved in calcification pathways, such as Matrix Gla Protein, a potent inhibitor of vascular calcifications (7).

Levels of BGP in DM patients on hemodialysis (HD) have been poorly described. Therefore, our goal was to characterize BGP and ucBGP in DM patients on HD, compared to HD patients without DM, and their association with vascular and bone disease.

Methods

We performed a secondary analysis of the VIKI (VItamin K Italian) study (8), involving 387 hemodialysis patients from 18 dialysis centers in Italy. Local ethics committees approved the study. Inclusion criteria were both genders, on hemodialysis for >1 year, and their written informed consent; exclusion criteria were patients with life expectancy < 6 months, diagnosis of cancer (with the exception of basal cell carcinoma), coagulation disorders, or conditions potentially interfering with study outcomes.

Data on DM were collected in 85 patients. We reported general features, type of dialysis, comorbidities and biochemical profiles.

Laboratory tests

Parathyroid Hormone (PTH)

Serum PTH was measured by automated LIAISON® N-Tact® PTH Assay 310910 (DiaSorin Inc., Stillwater MN, USA), a direct, 2-site, sandwich-type chemiluminescence immunoassay (CLIA) carried out on the LIAISON® (DiaSorin Inc., Stillwater MN, USA) instrument. The analytical sensitivity is 1 pg/mL and the intra-assay and inter-assay CVs were 3.7-6.3 and 3.5-5.3%, respectively.

25-OH Vitamin D

For quantitative determination of total 25-OH vitamin D (both D_2 and D_3 form) in serum, we used the automated LIAISON® 25 OH Vitamin D TOTAL Assay 310600, a direct competitive CLIA executed on the LIAISON (DiaSorin Inc., Stillwater MN, USA) instrument. The analytical sensitivity is <10 nmol/L, and the intra-assay coefficients of variation (CV) were between 2.9 and 5.5%, while the inter-assay CV is 6.3-12.9%.

Total BGP

The method for the quantitative determination of total BGP in serum was the automated LIAISON® Osteocalcin Assay 310950 (DiaSorin Inc., Stillwater MN, USA), a direct, 2-site, sandwich-type CLIA executed on the LIAISON® (DiaSorin Inc., Stillwater MN, USA) instrument. The analytical sensitivity is <0.3 ng/mL and the intra-assay CV is 3-8%, while the inter-assay CV is 4-9%.

Undercarboxylated BGP (ucBGP)

For quantitative determination of ucBGP, we used the Glu-osteocalcin Enzyme Immunoassay (EIA) Kit MK118 (Takara Bio Inc., Otsu, Shiga, Japan), a manual solid-phase EIA based on a sandwich method that utilizes 2 mouse monoclonal anti-ucBGP antibodies to detect ucBGP by a 2-step procedure. One of the mouse monoclonal anti-undercarboxylated BGPs is immobilized onto the micro-titre plate and blocked against non-specific binding. Samples are added to each well and incubated. The second step is to wash the plate and to add the second anti-BGP labelled with peroxidase (POD). The reaction between POD and substrate (H_2O_2 and 3,3', 5,5' tetramethyl-benzidine) results in color development with intensities proportional to the amount of ucBGP present. The analytical sensitivity is 0.25 ng/mL and the intra-assay and inter-assay CVs are 4.4-6.7 and 5.7-9.9%, respectively.

Total matrix GLA protein (MGP)

The quantitative determination of MGP was performed using the Human MGP—Matrix Gla Protein Kit (Biomedica Medizinprodukte GmbH & Co KG, Wien, A). It is a manual competitive ELISA method designed to detect MGP in serum. The analytical sensitivity is 0.3 nmol/L, and the intra-assay and inter-assay coefficients of variation (CVs) are 5–6 and 7–9 %, respectively.

Undercarboxylated MGP (ucMGP)

The measurement of the total undercarboxylated Matrix GLA Protein was performed by VitaK using a competitive ELISA, as described previously (9). The analytical sensitivity is 21 nmol/L, and the intra-assay and inter-assay CVs have been found to be 8.9 and 11.4 %, respectively.

Statistical analysis

Normally distributed data were summarized as mean ± standard deviation (SD), non-normally distributed data as median and interquartile range (IQR), and binary/categorical data as percentages, as appropriate. Categorical variables between two groups were compared by χ^2 test or Fisher's exact method. The comparison between medians was performed by the Mann-Whitney rank test whereas means were compared by unpaired T-Test.

To assess associations between log transformed values of total and ucBGP (outcome variables) and diabetes mellitus two multiple linear regression models were built. Variables available for the analysis were: gender, age, renal failure history, alcohol consumption, medical history (cardio and cerebrovascular disease, diabetes mellitus, malabsorption syndrome and liver disease), BMI, routine biochemical examinations, and mineral and bone disorders treatment (oral calcitriol, vitamin D analogues, calcimimetics, and phosphate-binding drugs) (table 1 supplementary). In multiple regression models we included all factors that were associated with the outcome in univariate analyses.

Time-to-event analysis for all-cause mortality related to DM was performed by the Kaplan-Meier method. To infer the involvement of DM in the pathophysiological pathway leading to death we applied an analytical approach (10): We estimated the independent relationship between DM and mortality by multiple Cox proportional-hazard models of increasing complexity. In the unadjusted analysis (Model 1) we included DM alone. In Model 2 we introduced DM patients plus potential confounders hypertension, angina, myocardial infarction, age, BMI, dialysis vintage. Finally, to unravel the potential pathogenic pathway by which diabetes mellitus could increase the mortality risk in dialysis patients we added into the multivariate Cox regression analysis two potential mediators

(that is, variables potentially involved in the pathogenesis pathway between the exposure and the outcome) of such an effect. Such mediators were peripheral vascular disease (Model 3) and warfarin (Model 4). The proportionality assumption was assessed by visual inspection and no violation was found. Hazard ratio (HR) and 95% confidence intervals (CIs) were calculated.

All statistical analyses were performed using SAS statistical package (version 9.3, SAS, Cary, NC).

Results

Main demographic and clinical characteristics of the study population are summarized in table 1. Patients with DM (85, 22%; 31 males, 54 females) had higher BMI than patients without DM (27.62 vs 23.94, $p < 0.001$). No significant differences between the two groups were observed as for age and smoking. Patients with DM had a shorter history of dialysis (37.0 vs 54.5 months; $p<0.001$), and a higher prevalence of hypertension (90.6% vs 75.2%; p=0.002), myocardial infarction (27.1% vs 16.6%; p=0.029), and aortic and peripheral vascular disease (table 2). In DM patients, mild to severe aortic calcifications were more frequently observed than in patients without DM (90.6% vs 77.8%, p=0.008) and this was also true for iliac calcifications. In particular, severe iliac calcifications were significantly worse in DM (9.4% vs 2.7%, P=0.006).

Diabetes Mellitus, total BGP and ucBGP

Patients with compared to without DM had significant lower levels of total BGP (139.00 vs. 202.50 mcg/L, $p<0.001$), ucBGP (9.24 vs. 11.32 mcg/L, p=0.022), and 25(OH) vitamin D (23.4 vs. 30.2 ng/ml, $p<0.001$) (table 1, fig 1).

Lower total BGP levels were associated with aortic calcification ($p<0.001$), iliac calcification (p=0.01) and vertebral fractures ($p<0.01$). In DM patients in treatment with warfarin (n=16, 8.8%), total BGP and ucMGP were significantly lower (56.2 vs. 152 mcg/L, $p<0.001$ and 336 vs. 616 nmol/L, p=0.038), respectively (supplementary table 2).

The regression model showed that DM patients had a statistically significant reduction of 19% of geometric mean both of total BGP (parameter estimate=-0.21092; p=0.009; R^2=0.53) and of 25.6% of geometric mean of ucBGP (parameter estimate=-0.29634; p=0.034; R^2=0.17), (table 3, table 4).

Survival Analysis

A total of 77 patients died during the follow-up (average time of observation: 2.7±0.5 years). Most patients died of cardiovascular events (n=49); other causes were infections (n=11), cancer (n=5) and

miscellaneous (n=12). Kaplan-Meier survival analysis showed that patients with DM had a lower probability of survival when compared to those with no DM (Fig. 2) and the difference was of high statistical significance (Log Rank Test, p=0.0013). Accordingly, on univariate Cox regression analysis, DM patients had a higher risk of all-cause mortality (HR: 1.83, 95% CI: 1.13-2.96, p=0.014) (fig 2). Data adjustment for confounders (hypertension, angina, myocardial infarction, age, BMI, dialysis vintage) confirmed the link between DM and mortality (HR=1.73 95% CI 1.03-2.90 p=0.038) (table 5, model 2). In models including peripheral vascular disease alone (table 5, model 3; HR=1.43 95% CI 0.82-2.48 p=0.206) or in combination with warfarin (table 5, model 4; HR=1.31 95% CI 0.75-2.28 p=0.342), the DM-mortality link was not significant. Of note, forcing total BGP or ucBGP into the model 4 in table 5, did not affect the DM-mortality link which remained not significant ($P \geq 0.28$). In these models, neither total BGP (HR: 1.00, 95% CI: 0.99-1.01, P=0.36) nor ucBGP (HR: 0.99, 95% CI: 0.98-1.01, P=0.33) significantly predicted the study outcome.

Discussion

Our study shows a significant reduction of total and ucBGP levels in patients affected by DM and CKD, in contrast to other patients affected by different nephropathies, confirmed in the regression model.

We know that BGP is not only involved in bone matrix mineralization, but it is also a mediator in endocrine pathway. The endocrine role of BGP seems to be related to its undercarboxylated form, while the process of carboxylation is necessary for the protein activation in bone tissue (11). Endocrine functions are strictly connected with glucose metabolism, thus preponderant for bone metabolism in DM patients. The endocrine role consists in regulating glucose homeostasis by promoting the secretion of insulin from pancreatic beta-cells and by increasing adiponectine expression, an anti-inflammatory protein secreted by adipocytes. These actions finally result into a greater insulin sensitivity. In fact, in mice, administration of ucOC was able to increase the insulin and adiponectin secretion and stimulated glucose and lipid catabolism (12). BGP requires vitamin K for its activity. Clinical trials have reported contrasting results about the effects of vitamin K on insulin sensitivity. A recent meta-analysis including eight trials involving 1,077 participants suggested no effect of vitamin K supplementation on insulin sensitivity (13). In contrast, other authors reported that Vitamin K2 administration could improve glycemic status in DM rats by induction of BGP gene expression (14). Moreover, Choi et al. found a positive effect of vitamin K2 supplementation in increasing insulin sensitivity in healthy young men via BGP metabolism (15).

According to the literature, in our DM population aortic and iliac calcifications were significantly more prevalent than in patients without DM. Vascular calcifications are considered strong predictors of cardiovascular disease, closely connected with morbidity and mortality (8). Evidence indicates that

vascular calcification is a process of active bone formation regulated by stimulators and inhibitors of calcification. BGP is known to be involved in preventing vascular aortic calcifications, through direct and indirect mechanisms. Indirect mechanisms are mediated by its action on insulin, in particular in the context of metabolic acidosis associated to bone reabsorption, very common in the CKD population, or by the release of adiponectin. Adiponectin may prevent the trans-differentiation of vascular smooth muscle cells into osteoblast-like cells in arterial vessels (16). Data are available not only in rats with CKD, but also in humans. In fact, higher total BGP levels were found associated with lower abdominal aortic calcification progression rate and lower mortality in a 10 year-long prospective study in elderly Caucasian subjects, showing total BGP as an independent factor of cardiovascular risk and mortality (17). Moreover, in the hemodialysis population we showed that low levels of total-BGP are associated with vertebral fractures, aortic and iliac calcifications (5). Finally, a recent review reported low BGP levels as a biomarker of abdominal aortic calcification in patients with diabetes (18).

In the univariate Cox regression analysis, DM patients were found to have a higher risk of all-cause mortality ($p=0.014$). Although we did not find that BGP levels predict mortality, we cannot exclude that their lower levels may lead to a reduced protection of bone and vascular health, increasing cardiovascular mortality. Regarding crude and adjusted HR for all-cause mortality in relation to DM (table 5), data adjustment for possible confounders (hypertension, angina, myocardial infarction, age, BMI, dialysis vintage) did not affect the significant link between DM and mortality, indicating that this association cannot be explained by confounding factors themselves. Thus, we would like to underline that when we forced into the model two other factors - vascular calcifications and warfarin - the association between DM and mortality became not significant, suggesting that vascular calcifications and warfarin use may be mediators but not confounders in the potential pathogenic pathways underlying the relationship between DM and mortality.

Finally, in the sub-analysis regarding warfarin treated DM patients, we observed reduced levels of BGP and ucMGP. The association of lower serum ucMGP, rather than total MGP, with atherosclerosis and vascular calcification has been previously reported in the CKD population (9). In a study conducted in patients affected with cardiovascular disease, Parker et al. reported that reduced kidney function was associated with lower serum ucMGP levels, suggesting MGP as a potential marker of vascular calcification and cardiovascular disease in the CKD population (19). Warfarin, acting as a vitamin K antagonist, may inhibit vitamin-K dependent proteins that are involved in bone mineralization and the prevention of vascular calcification, including BGP and MGP. We already reported that in hemodialysis patients warfarin use was associated with an increase of aortic and iliac calcifications, with significantly lower BGP levels, and ucMGP levels (20).

DM and CKD patients have a high prevalence of low vitamin D levels and our study also shows a significant reduction of vitamin D levels in DM patients. Recent evidence suggested the important role of vitamin D in the pathogenesis of DM and CKD, reporting that low vitamin D levels are associated with poor outcomes, in particular with progression of DM and cardiovascular disease (21). Moreover, in hemodialysis patients, we found a significant and independent association between low 25(OH) vitamin D levels and severe vascular calcifications, assuming a possible protective role of vitamin D on vascular calcifications by its action on vitamin K dependent proteins, such as BGP and MGP (6). In our previous study we showed that vitamin D analogues can improve vitamin K dependent protein levels. In particular, administration of vitamin D was associated with increased BGP levels in hemodialysis patients (22). These data support the potential role of vitamin D supplementation as a preventative and therapeutic agent for bone and vascular health in DM and CKD patients.

Finally, we underscore the interesting associations of total BGP with PTH and ALP, which could be explained by two factors: Vitamin D control on BGP (low vitamin D levels in DM patients could damage this function) and a higher bone turnover, resulting in higher BGP and ucBGP. All of this reveals a remarkable role of BGP as bone biomarker in the scenery of CKD-MBD.

In conclusions, in hemodialysis patients we confirmed an increased mortality associated to DM and we found an association between diabetic status and decreased BGP levels. To our knowledge, this is the first study in hemodialysis patients suggesting a potential protective role of BGP in the context of bone, endocrine and vascular pathway. Further investigations are needed to assess the clinical implications of low BGP levels in hemodialysis patients affected by DM.

Disclosures

All authors state that they have no conflicts of interest.

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264 Table 1. Patients characteristics.

	Patients With DM (n=85, 22%)	**Patients Without DM (n=302, 78%)**	**p-value**
Gender, female, n (%)	31 (36.5 %)	114 (37.7 %)	0.830
Age, years, median	68 (63, 73.50)	67 (52, 74)	0.132
Weight, kg, median	76.50 (67.75, 87.75)	67 (58.5, 75.63)	< **0.001**
Height, m, median	1.65 (1.60, 1.75)	1.68 (1.60, 1.73)	0.491
BMI, kg/cm^2, median	27.62 (24.42, 30.89)	23.94 (21.29, 26.72)	< **0.001**
Smokers, n (%) (n=370)			0.186
Yes	47 (57.3%)	187 (64.9%)	
No	25 (30.5%)	60 (20.8%)	
Ex	10 (12.2%)	41 (14.3%)	
Current or former alcohol drinkers, n (%) (n=361)	62 (21.8%)	20 (26%)	0.442
<u>Medical history</u>			
Dialysis vintage, months, median	37 (26, 58)	54.5 (29.75, 113.25)	< **0.001**
Type of dialysis, n (%)			0.138
Bicarbonate dialysis	34 (40.0%)	155 (51.3%)	
Hemofiltration (HF)	11 (12.9%)	21 (7%)	
Hemodiafiltration (HDF)	21 (24.7%)	81 (26.8%)	
Acetate free biofiltration (AFB)	16 (18.8%)	38 (12.6%)	
Other types of dialysis	3 (3.6%)	7 (2.3%)	

	Patients With DM (n=85, 22%)	**Patients Without DM (n=302, 78%)**	**p-value**
Previous kidney transplant, n (%)	2 (2.4%)	52 (17.2%)	< **0.001**
Hypertension, n (%)	77 (90.6%)	227 (75.2%)	**0.002**
Angina, n (%)	19 (22.4%)	45 (14.9%)	0.102
Myocardial infarction, n (%)	23 (27.1%)	50 (16.6%)	**0.029**
Atrial fibrillation, n (%)	14 (16.5%)	37 (12.3%)	0.310
Heart failure, n (%)	10 (11.8%)	29 (9.6%)	0.559
Peripheral vascular disease, n (%)			< **0.001**
No	33 (38.8%)	220 (72.8%)	
Asymptomatic	32 (37.6%)	66 (21.9%)	
Intermittent claudication	12 (14.1%)	16 (5.3%)	
Amputation	8 (9.5%)	0 (0%)	
Cerebrovascular accident, n (%)			0.958
No	76 (89.4%)	270 (89.4%)	
Stroke	4 (4.7%)	16 (5.3%)	
Other type	5 (5.9%)	16 (5.3%)	
Vertebral fractures, n (%)	44 (51.8%)	170 (56.3%)	0.458
Vertebral fractures among men, n (%)	30 (55.6%)	115 (61.2%)	0.458
Vertebral fractures among women, n (%)	14 (45.2%)	55 (48.2%)	0.760

	Patients With DM (n=85, 22%)	Patients Without DM (n=302, 78%)	p-value
Routine biochemical profile			
Ca, mg/dl, median	9.0 (8.7, 9.4)	9.1 (8.7, 9.6)	0.176
Ca, mg/dl, mean±SD (not normally distributed)	9.08±0.59	9.18±0.70	0.225
P, mg/dl, mean±SD (not normally distributed)	4.63±1.07	4.80±1.32	0.272
P, mg/dl, median	4.34 (3.85, 5.35)	4.65 (3.88, 5.59)	0.338
Alkaline phosphatase, U/L, median	87 (65, 111.5)	81 (64, 111)	0.432
PTH, pg/ml, median	207 (135.5, 340)	244.5 (140, 401.25)	0.169
Albumin, g/dl, median	3.9 (3.5, 4.0)	3.9 (3.5, 4.1)	0.248
CRP, mg/L, median	2.13 (0.47, 4.1)	1.65 (0.50, 5.26)	0.802
KT/V, mean±SD	1.26±0.25	1.25±0.27	0.633
Aluminium, mcg/L, median	10 (7, 16)	13.8 (8, 22)	0.057
Total cholesterol, mg/dl, median	176 (150,197.2)	165 (140, 192.3)	0.164
Triglycerides, mg/dl, median	157 (111.5, 211)	146.5 (110, 202.3)	0.349
HDL Cholesterol, mg/dl, median	39 (32, 49)	40 (33, 50)	0.652
LDL Cholesterol, mg/dl, median	96 (74, 119)	89 (69, 116)	0.513
25(OH)D, ng/mL, median	23.4 (16.5, 34.7)	30.2 (20.18, 46.78)	< **0.001**
BGP total, mcg/L, median	139 (62.40, 220.5)	202.5 (109, 362)	< **0.001**
ucBGP, mcg/L, median	9.24 (2.99,15.54)	11.32 (6.15, 18.15)	**0.022**

	Patients With DM (n=85, 22%)	Patients Without DM (n=302, 78%)	p-value
MGP total, nmol/L, median	18 (12, 31.88)	19.36 (13, 30.73)	0.582
ucMGP, nmol/L, median	541.86 (287.40, 981.5)	572.84 (285, 930)	0.634
Mg, mg/dL, median	(n=26) 2.3 (2, 2.6)	(n=113) 2.3 (2, 2.7)	0.488

Table 2. Presence and severity of vascular calcifications in patients with DM.

	Patients With DM (n=85, 22%)	**Patients No DM (n=302, 78%)**	**p-value**
Aortic calcifications, n (%)			**0.029**
None	8 (9.4%)	67 (22.2%)	
Mild + Moderate	47 (55.3%)	149 (49.3%)	
Severe	30 (35.3%)	86 (28.5%)	
Aortic calcifications: mild, moderate or severe vs none, n (%)	77 (90.6%)	235 (77.8%)	**0.008**
Iliac calcifications, n (%)			**0.012**
None	31 (36.5%)	139 (46%)	
Mild+ Moderate	46 (54.1%)	155 (51.3%)	
Severe	8 (9.4%)	8 (2.7%)	

Fig. 1. Boxplot of ucBGP, Total BGP and 25(OH)D grouped by DM.

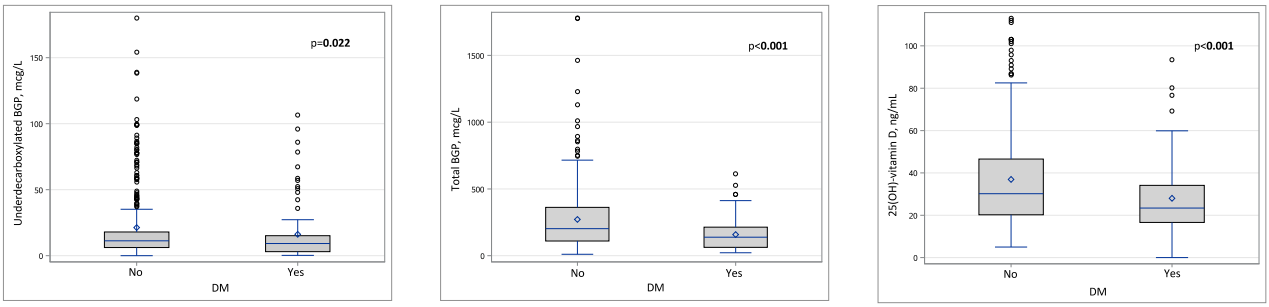

Table 3. Regression model with outcome total BGP (log-transformed).

VARIABLE	PARAMETER ESTIMATE (b)	P-VALUE
DM	-0.21	**0.009**
lgPTH	0.27	< **0.0001**
lgALP	0.29	< **0.0001**

R^2=0.53

The stepwise analysis also identified the following variables as covariates: age, BMI, smoking status, cerebrovascular accidents, hypertension, heart failure, vascular calcification, LDL cholesterol, albumin and the following therapies: aluminium, warfarin, calcimimetics, vitamin D analogues, intravenous calcitriol and antibiotics. Other variables remained out of the model.

Table 4. Regression model with outcome ucBGP (log-transformed).

VARIABLE	PARAMETER ESTIMATE (b)	P-VALUE
DM	-0.30	**0.034**
lgPTH	0.22	**0.0011**

R^2=0.17

The stepwise analysis also identified the following variables as covariates: phosphate, aortic calcifications, calcium-phosphate, KT/V, liver disease and the following therapies: intravenous calcitriol, calcimimetics, heparin, warfarin, steroids and antiepileptics. Other variables remained out of the model.

Fig. 2. Kaplan-Meier survival curves for all-cause mortality, for patients with DM (1, red line) and patients with no DM (0, blu line).

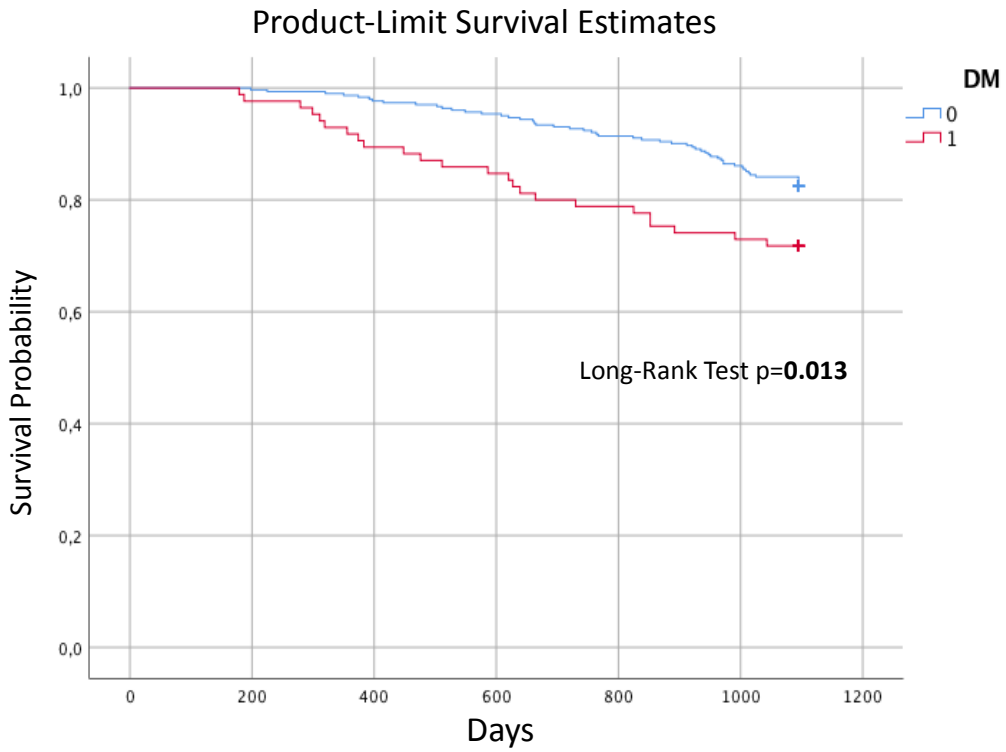

Table 5. Crude and adjusted HR for all-cause mortality in relation to DM.

	Model 1 (Unadjusted)	**Model 2 (Adjusted for confounders*)**	**Model 3 (Adjusted for confounders* and peripheral vascular disease)**	**Model 4 (Adjusted for confounders*, peripheral vascular disease and warfarin)**
Patients with DM	HR=1.83 95% CI 1.13-2.96 **p=0.014**	HR=1.73 95% CI 1.03-2.90 **p=0.038**	HR=1.43 95% CI 0.82-2.48 p=0.206	HR=1.31 95% CI 0.75-2.28 p=0.342

* Confounders: hypertension, angina, myocardial infraction, age, BMI, dialysis vintage.

CI = Confidence Interval; HR = Hazard Ratio.

Table 1 supplementary. Therapy by diabetic status.

Drugs prescribed to patients	Patients With DM (n=85, 22%)	Patients Without DM (n=302, 78%)	p-value
Warfarin (n, %)	16 (18. 8 %)	30 (9.9%)	**0.025**
Steroids (n, %)	2 (2.4%)	19 (6.3%)	0.157
Thyroid hormones (n, %)	11 (12.9%)	29 (9.6%)	0.372
Antibiotics (n, %)	8 (9.4%)	8 (2.6%)	**0.006**
Antiepileptics (n, %)	4 (4.7%)	10 (3.3%)	0.543
Statins (n, %)	40 (47.1%)	86 (28.5%)	**0.001**
Beta-Blockers (n, %)	32 (37.6%)	112 (37.1%)	0.925
Antidiabetics (n, %)	7 (8.2%)	0 (0%)	**<0.001**
Insulin (n, %)	58 (68.2%)	0 (0%)	**<0.001**
Anti-Gastric (n, %)	64 (75.3%)	233 (77.2%)	0.720
Aluminium (n, %)	16 (18.8%)	80 (26.5%)	0.148
Calcium carbonate (n, %)	35 (41.2%)	97 (32.1%)	0.120
Calcium acetate (n, %)	9 (10.6%)	12 (4.0%)	**0.017**
Sevelamer (n, %)	38 (44.7%)	125 (41.4%)	0.584
Lanthanum (n, %)	10 (11.8%)	46 (15.2%)	0.422
Oral calcitriol (n, %)	43 (50.6%)	134 (44.4%)	0.309
Intravenous calcitriol (n, %)	2 (2.4%)	134 (44.4%)	0.653

Vitamin D analogues (n, %)	12 (14.1%)	65 (21.5%)	0.131
Calcimimetics (n, %)	11 (12.9%)	64 (21.2%)	0.089

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Table 2 supplementary. Main characteristics of the Patients With DM and Warfarin VS Patients With DM and No Warfarin Use.

	Patients With DM and Warfarin Use (n=16, 18.8%)	**Patients With DM and No Warfarin Use (n=69, 81.2%)**	**p-value**
Gender, female, n (%)	8 (50 %)	23 (33.3 %)	0.212
Age, years, median	72 (64.5, 77.50)	67 (63, 72)	0.095
Weight, kg, median	75.25 (61.25, 81.0)	76.5 (69, 88)	0.220
Height, m, median	1.65 (1.60, 1.78)	1.66 (1.62, 1.75)	0.596
BMI, kg/cm^2, median	26.17 (23.04, 28.05)	28.26 (24.65, 31.45)	0.144
Smoker, n (%) (n=370)			0.429
Yes	10 (62.4%)	37 (56.1%)	
No	3 (18.8%)	22 (33.3%)	
Ex	3 (18.8%)	7 (10.6%)	
Current or former alcohol drinker, n (%) (n=77)	3 (18.8%)	17 (27.9%)	0.459
Medical history			
Dialysis vintage, months, median	42 (25, 61.5)	37 (26, 55)	0.723
Type of dialysis, n (%)			0.622
Bicarbonate dialysis	6 (37.5%)	28 (40.6%)	
Hemofiltration (HF)	2 (12.5%)	9 (13.0%)	
Hemodiafiltration (HDF)	3 (18.8%)	18 (26.1%)	
Acetate free biofiltration (AFB)	5 (31.2%)	11 (16%)	
Other types of dialysis	0 (0%)	3 (4.3%)	

	Patients With DM and Warfarin Use (n=16, 18.8%)	**Patients With DM and No Warfarin Use (n=69, 81.2%)**	**p-value**
Previous kidney transplant, n (%)	1 (6.3%)	1 (1.5%)	0.254
Hypertension, n (%)	14(87.5%)	63 (91.3%)	0.639
Angina, n (%)	5 (31.3%)	14 (20.3%)	0.343
Myocardial infarction, n (%)	2 (12.5%)	21 (30.4%)	0.146
Atrial fibrillation, n (%)	9 (56.3%)	5 (7.3%)	< **0.001**
Heart failure, n (%)	2 (12.5%)	8 11.6%)	0.919
Peripheral vascular disease, n (%)			0.420
No	7 (43.8%)	26 (37.7%)	
Asymptomatic	4 (25%)	28 (40.6%)	
Intermittent claudication	4 (25%)	8 (11.6%)	
Amputation	1 (6.2%)	7 (10.1%)	
Cerebrovascular accident, n (%)			0.301
No	14 (87.5%)	62 (89.8%)	
Stroke	0 (0%)	4 (5.8%)	
Other type	2 (12.5%)	3 (4.4%)	
Vertebral fractures, n (%)	8 (50.0%)	36 (52.2%)	0.875
Vertebral fractures among men, n (%)	6 (75%)	24 (52.2%)	0.230
Vertebral fractures among women, n (%)	2 (25%)	12 (52.2%)	0.183

	Patients With DM and Warfarin Use (n=16, 18.8%)	Patients With DM and No Warfarin Use (n=69, 81.2%)	p-value
Routine biochemical profile			
Ca, mg/dl, median	8.94 (8.55, 9.3)	9.04 (8.7, 9.4)	0.525
Ca, mg/dl, mean±SD (not normal distributed)	9.02±0.69	9.09±0.57	0.637
P, mg/dl, mean±SD (not normal distributed)	4.83±0.86	4.58±1.12	0.209
P, mg/dl, median	4.83 (4.15, 5.60)	4.34 (3.80, 5.2)	0.192
Alkaline phosphatase, U/L, median	85 (48, 124.5)	87 (65, 110)	0.601
PTH, pg/ml, median	263 (104.5, 445)	206 (142, 321.3)	0.702
Albumin, g/dl, median	3.85 (3.3, 4.0)	3.9 (3.5, 4.1)	0.357
CRP, mg/L, median	2.5 (0.36, 11.6)	1.90 (0.50, 4.0)	0.725
KT/V, median	1.21 (1.05, 1.54)	1.26 (1.10, 1.37)	0.951
Aluminium, mcg/L, median	13 (10, 30)	9 (7, 15)	0.132
Total cholesterol, mg/dl, median	181.5 (161, 205.5)	173 (150, 193)	0.331
Triglycerides, mg/dl, median	143.5 (122.5, 214.5)	161 (110, 211)	0.698
HDL Cholesterol, mg/dl, median	40.5 (32.5, 55.5)	39 (32, 47)	0.396
LDL Cholesterol, mg/dl, median	105 (76, 118)	94.5 (69, 119)	0.409
25(OH)D, median	23.9 (16.5, 47.7)	23.2 (16.6, 32.6)	0.589
BGP total, mcg/L, median	56.2 (37.40, 121.6)	152 (74.8, 230)	< **0.001**
ucBGP, mcg/L, median	12.93 (6.1,51.59)	7.94 (2.94, 13.42)	0.064

	Patients With DM and Warfarin Use (n=16, 18.8%)	Patients With DM and No Warfarin Use (n=69, 81.2%)	p-value
MGP total, nmol/L, median	16.72 (11.46, 24.12)	18.42 (12, 34.52)	0.507
ucMGP, nmol/L, median	336.00 (143, 590)	616 (310.42, 1062)	**0.038**
Mg, mg/dL, median	(n=26) 2.75 (2.7, 2.8)	(n=113) 2.3 (2, 2.6)	0.053

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