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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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Full Title:	Osteocalcin (bone GLA protein) levels, vascular calcifications, vertebral fractures and mortality in hemodialysis patients with diabetes mellitus.
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Abstract:	<p>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.</p> <p>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.</p> <p>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.</p> <p>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.</p>
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Author Comments:	<p>Padua, 14 October 2018</p> <p>To Editor, Journal of Nephrology</p> <p>Dear Editor,</p> <p>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.</p> <p>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.</p> <p>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.</p> <p>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.</p> <p>The paper is original and is not under consideration by any other journal.</p> <p>Thanking you for your attention.</p> <p>Yours sincerely Maria Fusaro, MD, PhD</p>
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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Yours sincerely

Maria Fusaro, MD, PhD

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

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

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

3 **ABSTRACT**

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

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10 vitamin D and VC were evaluated. Time-to-event analysis for all-cause mortality was performed by
11 the Kaplan-Meier.

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

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

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33 **Introduction**

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

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

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

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

54

55 **Methods**

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

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

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65

66 **Laboratory tests**

67 *Parathyroid Hormone (PTH)*

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

73 *25-OH Vitamin D*

74 For quantitative determination of total 25-OH vitamin D (both D₂ and D₃ form) in serum, we used
75 the automated LIAISON® 25 OH Vitamin D TOTAL Assay 310600, a direct competitive CLIA
76 executed on the LIAISON (DiaSorin Inc., Stillwater MN, USA) instrument. The analytical sensitivity
77 is <10 nmol/L, and the intra-assay coefficients of variation (CV) were between 2.9 and 5.5%, while
78 the inter-assay CV is 6.3-12.9%.

79 *Total BGP*

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

84 *Undercarboxylated BGP (ucBGP)*

85 For quantitative determination of ucBGP, we used the Glu-osteocalcin Enzyme Immunoassay (EIA)
86 Kit MK118 (Takara Bio Inc., Otsu, Shiga, Japan), a manual solid-phase EIA based on a sandwich
87 method that utilizes 2 mouse monoclonal anti-ucBGP antibodies to detect ucBGP by a 2-step
88 procedure. One of the mouse monoclonal anti-undercarboxylated BGPs is immobilized onto the
89 micro-titre plate and blocked against non-specific binding. Samples are added to each well and
90 incubated. The second step is to wash the plate and to add the second anti-BGP labelled with
91 peroxidase (POD). The reaction between POD and substrate (H₂O₂ and 3,3', 5,5' tetramethyl-
92 benzidine) results in color development with intensities proportional to the amount of ucBGP present.
93 The analytical sensitivity is 0.25 ng/mL and the intra-assay and inter-assay CVs are 4.4-6.7 and 5.7-
94 9.9%, respectively.

95

97 *Total matrix GLA protein (MGP)*

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

102 *Undercarboxylated MGP (ucMGP)*

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

106 **Statistical analysis**

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

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

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

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

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

134

135 **Results**

136 Main demographic and clinical characteristics of the study population are summarized in table
137 1. Patients with DM (85, 22%; 31 males, 54 females) had higher BMI than patients without DM
138 (27.62 vs 23.94, $p < 0.001$). No significant differences between the two groups were observed as for
139 age and smoking. Patients with DM had a shorter history of dialysis (37.0 vs 54.5 months; $p < 0.001$),
140 and a higher prevalence of hypertension (90.6% vs 75.2%; $p = 0.002$), myocardial infarction (27.1%
141 vs 16.6%; $p = 0.029$), and aortic and peripheral vascular disease (table 2). In DM patients, mild to
142 severe aortic calcifications were more frequently observed than in patients without DM (90.6% vs
143 77.8%, $p = 0.008$) and this was also true for iliac calcifications. In particular, severe iliac calcifications
144 were significantly worse in DM (9.4% vs 2.7%, $P = 0.006$).

145

146 *Diabetes Mellitus, total BGP and ucBGP*

147 Patients with compared to without DM had significant lower levels of total BGP (139.00 vs.
148 202.50 mcg/L, $p < 0.001$), ucBGP (9.24 vs. 11.32 mcg/L, $p = 0.022$), and 25(OH) vitamin D (23.4 vs.
149 30.2 ng/ml, $p < 0.001$) (table 1, fig 1).

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

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

157

158 **Survival Analysis**

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

161 miscellaneous (n=12). Kaplan-Meier survival analysis showed that patients with DM had a lower
162 probability of survival when compared to those with no DM (Fig. 2) and the difference was of high
163 statistical significance (Log Rank Test, p=0.0013). Accordingly, on univariate Cox regression
164 analysis, DM patients had a higher risk of all-cause mortality (HR: 1.83, 95% CI: 1.13-2.96, p=0.014)
165 (fig 2). Data adjustment for confounders (hypertension, angina, myocardial infarction, age, BMI,
166 dialysis vintage) confirmed the link between DM and mortality (HR=1.73 95% CI 1.03-2.90 p=0.038)
167 (table 5, model 2). In models including peripheral vascular disease alone (table 5, model 3; HR=1.43
168 95% CI 0.82-2.48 p=0.206) or in combination with warfarin (table 5, model 4; HR=1.31 95% CI
169 0.75-2.28 p=0.342), the DM-mortality link was not significant. Of note, forcing total BGP or ucBGP
170 into the model 4 in table 5, did not affect the DM-mortality link which remained not significant
171 ($P \geq 0.28$). In these models, neither total BGP (HR: 1.00, 95% CI: 0.99-1.01, P=0.36) nor ucBGP (HR:
172 0.99, 95% CI: 0.98-1.01, P=0.33) significantly predicted the study outcome.
173

174 **Discussion**

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

178 We know that BGP is not only involved in bone matrix mineralization, but it is also a mediator
179 in endocrine pathway. The endocrine role of BGP seems to be related to its undercarboxylated form,
180 while the process of carboxylation is necessary for the protein activation in bone tissue (11).
181 Endocrine functions are strictly connected with glucose metabolism, thus preponderant for bone
182 metabolism in DM patients. The endocrine role consists in regulating glucose homeostasis by
183 promoting the secretion of insulin from pancreatic beta-cells and by increasing adiponectine
184 expression, an anti-inflammatory protein secreted by adipocytes. These actions finally result into a
185 greater insulin sensitivity. In fact, in mice, administration of ucOC was able to increase the insulin
186 and adiponectin secretion and stimulated glucose and lipid catabolism (12). BGP requires vitamin K
187 for its activity. Clinical trials have reported contrasting results about the effects of vitamin K on
188 insulin sensitivity. A recent meta-analysis including eight trials involving 1,077 participants
189 suggested no effect of vitamin K supplementation on insulin sensitivity (13). In contrast, other
190 authors reported that Vitamin K2 administration could improve glycemic status in DM rats by
191 induction of BGP gene expression (14). Moreover, Choi et al. found a positive effect of vitamin K2
192 supplementation in increasing insulin sensitivity in healthy young men via BGP metabolism (15).
193 According to the literature, in our DM population aortic and iliac calcifications were significantly
194 more prevalent than in patients without DM. Vascular calcifications are considered strong predictors
195 of cardiovascular disease, closely connected with morbidity and mortality (8). Evidence indicates that

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

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

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

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

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

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

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254 **Disclosures**

255 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
<u>Routine biochemical profile</u>			
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

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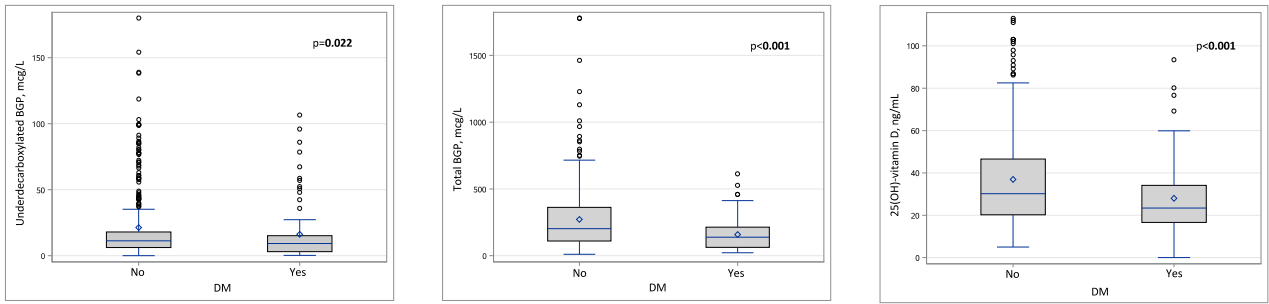
268 Table 2. Presence and severity of vascular calcifications in patients with DM.

	Patients With DM (n=85, 22%)	Patients No DM (n=302, 78%)	p-value
<hr/>			
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%)	

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



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

VARIABLE	PARAMETER ESTIMATE (b)	P-VALUE
DM	-0.21	0.009
IgPTH	0.27	< 0.0001
IgALP	0.29	< 0.0001

294 $R^2=0.53$

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

299

300 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

301 $R^2=0.17$

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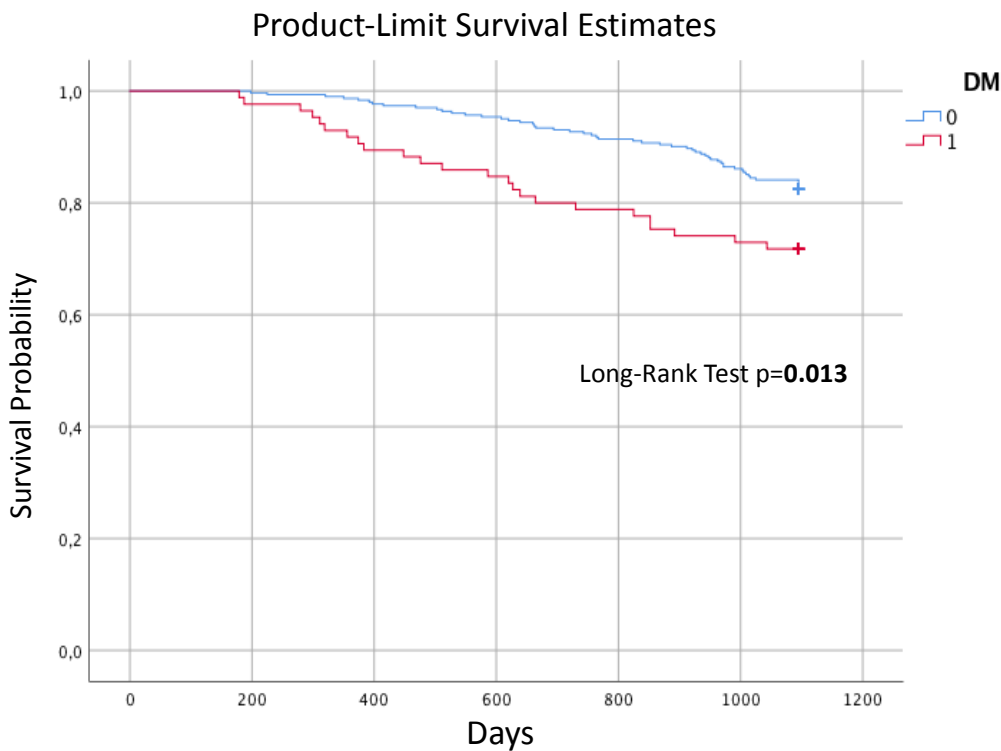
303 The stepwise analysis also identified the following variables as covariates: phosphate, aortic calcifications, calcium-
304 phosphate, KT/V, liver disease and the following therapies: intravenous calcitriol, calcimimetics, heparin, warfarin,
305 steroids and antiepileptics. Other variables remained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

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

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319 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

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

322 CI = Confidence Interval; HR = Hazard Ratio.

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Table 1 supplementary. Therapy by diabetic status.

Drugs prescribed to patients	Patients	Patients	p-value
	With DM (n=85, 22%)	Without DM (n=302, 78%)	
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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329 **Table 2 supplementary.** Main characteristics of the Patients With DM and Warfarin VS Patients With DM
 330 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
<u>Medical history</u>			
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
<u>Routine biochemical profile</u>			
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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