Cigarette smoking is associated with decreased Bone Gla-protein (BGP) levels in hemodialysis patients

Maria Fusaro[1,2], Maurizio Gallieni[3], Andrea Aghi[4], Giorgio Iervasi[1], Maria Antonietta Rizzo[5], Andrea Stucchi[6], Marianna Noale[7], Giovanni Tripepi[8], Thomas Nickolas[9], Nicola Veronese[10], Fabrizio Fabris[4], Sandro Giannini[4], Lorenzo Calò[11], Antonio Piccoli[11], Maria Cristina Mereu[12], Laura Cosmai[13], Alberto Ferraro[14], Fiorenza Magonara[15], Michela Spinello[16], Stefania Sella[4], Mario Plebani[17].

[1] National Research Council (CNR) – Institute of Clinical Physiology (IFC), Pisa Via G. Moruzzi 1, 56124, Pisa, PI, Italy

[2] Department of Medicine, University of Padova Italy; Via Giustiniani 2, 35128, Padova, PD, Italy

[3] Nephrology and Dialysis Unit, Ospedale San Carlo Borromeo, Department of Clinical and Biomedical Sciences "Luigi Sacco", University of Milan, Milan, Italy

[4] Department of Medicine, Clinica Medica 1, University of Padova, Padova, Italy

[5] Nephrology and Dialysis Unit, Ospedale di Circolo di Busto Arsizio, ASST Valle Olona, Italy

[6] Nephrology and Dialysis Unit, IRCCS Multimedica, Sesto San Giovanni (Milano), Milan, Italy

[7] National Research Council (CNR), Neuroscience Institute, Padua, Italy

[8] Clinical Epidemiology and Physiopathology of Renal Diseases and Hypertension, CNR, Institute of Biomedicine, Reggio Calabria, Calabria, Italy

[9] Department of Medicine, Division of Nephrology, Columbia University Medical Center, New York, New York

[10] Department of Medicine (DI-MED), Geriatric Section, University of Padua, Italy

[11] Nephrology Unit, University of Padua, Padua, Italy

[12] Ospedale N.S. di Bonaria, S. Gavino Monreale, Italy

[13] Istituti Ospitalieri di Cremona, Cremona, Italy

[14] Unità di Nefrologia di Castelfranco Veneto, Treviso, Italy

[15] Unità di Nefrologia e Dialisi, Ospedale Alto Vicentino Santorso, Vicenza, Italy

[16] Unità di Nefrologia-Emodialisi O.C. Piove di Sacco Ulss 6, Padova, Italy

[17] Laboratory Medicine Unit, Department of Medicine, University of Padova, Padova, Italy.

Corresponding Author:

Maria Fusaro, National Research Council (CNR) – Institute of Clinical Physiology (IFC), Pisa Via G. Moruzzi 1, 56124, Pisa, PI, Italy; and Department of Medicine, University of Padova Italy; Via Giustiniani 2, 35128, Padova, PD, Italy, E-mail: dante.lucia@libero.it.

ABSTRACT

Background: Bone gamma-carboxyglutamic acid (Gla)-protein (BGP or osteocalcin) is a vitamin K-dependent protein involved in the regulation of bone mineralization. Smoking is a risk factor for osteoporosis.

Methods: We carried out a secondary analysis of the vitamin K Italian (VIKI) study to investigate the effects of cigarette smoking on BGP levels in patients with end stage renal disease. Data were collected in 370 haemodialysis patients, 37% (136) smokers (or ex-smokers) and 63% (234) non-smokers. Vascular calcifications and vertebral fractures (quantitative morphometry) were identified on spine radiographs.

Results: Smokers had significantly lower BGP levels (152 *vs* 204 μg/L, p= 0.003). Smokers had lower phosphate levels (4.25 (3.7, 5.3)) *vs* (4.86 (4.0, 5.6)) mg/dl, respectively, p= 0.008), albumin (3.8 (3.5, 4.0)) *vs* (3.9 (3.6, 4.2)) g/dL, respectively, p= 0.001). Lower BGP levels were associated with aortic calcification (p< 0.001), iliac calcification (p=0.042) and vertebral fractures (p=0.023). The regression model showed that smoking is associated with a significant reduction of total BGP levels by about 18% (p=0.01).

Conclusion: This is the first clinical study in a haemodialysis population which identifies cigarette smoking as a potential inhibitor of BGP activity, a protective agent in bone and vascular health.

Keywords: BGP, smoke, haemodialysis, vascular calcification, vertebral fractures.

INTRODUCTION

Bone gamma-carboxyglutamic acid (Gla)-protein (BGP), also called osteocalcin, is a small protein produced by osteoblasts under the control of vitamin D, which modulates the expression of BGP gene. Its specific affinity to bind hydroxyapatite molecules in bone is permitted by γ-carboxylated Gla residues [1]. As a result, carboxylated (Gla-containing) cBGP normalizes bone crystal nucleation [2]. BGP knockout mice exhibit a bone phenotype, characterized by small increases in cortical thickness (hyperostosis), and reduced bone strength, without differences in bone mineral content [3]. However, their bone contains immature hydroxyapatite crystals [4], indicating a role of BGP in promoting normal bone mineralization. Indeed, an *in vitro* study reported that osteoblasts markedly increase their expression of BGP under mineralizing conditions, compared with non-mineralizing cultures [5], indicating that BGP regulates matrix mineralization.

Smoking is a risk factor for osteoporosis [6] and vascular calcification [7]. Nicotine and non-nicotine tobacco smoke components have been shown to depress osteoblast activity. Gao et al. showed that smoke exposure inhibited bone formation and increased bone resorption. Lumbar spine and femur Bone Mass Density (BMD) was lower in 4-month smoke-exposed female rats than controls. However, there was no significant difference in serum BGP levels between smoke-exposed rats and controls **[**8**].**

The objective of this secondary analysis of the vitamin K Italian (VIKI) study, an observational study designed to assess the prevalence of vitamin K deficiency in haemodialysis patients, was to assess the association between cigarette smoking and BGP levels in an end stage renal disease (ESRD) population. We hypothesized that BGP levels would be lower in smokers than never smokers, and that lower BGP levels would be related to fractures and severity of prevalent vascular calcification.

PATIENTS AND METHODS

This study is a secondary analysis of the VIKI study, involving 18 dialysis centres in Italy [9]. All the local ethics committees approved the study, which was conducted according to the regulations in force related to observational studies. Approval dates ranged from July 14, 2008 to October 26, 2009. Patient enrolment took place between November 2008 and November 2009, and follow-up to assess vital status was performed in December 2011. We included adult patients of both genders who had been on haemodialysis for >1 year, provided that they gave their informed consent, in writing, for the use of their medical records for the study. We excluded patients who had a life expectancy <6 months, cancer (with the exception of basal cell carcinoma), coagulation disorders, or conditions that according to the investigator could interfere with the study outcome. Study subjects were administered a questionnaire to ascertain smoking status (i.e. current, past or never smoker).

Laboratory determination

Parathyroid hormone (PTH)

The method for quantitative determination of PTH in serum was the 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 (Dia-Sorin 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® (Dia- Sorin 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 the undercarboxylated form, we used Glu-osteocalcin Enzyme Immuno Assay (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 colour 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.

Vertebral fractures and vascular calcification assessment

A radiograph of the thoracic and lumbar regions of the spinal column (D5 to L4) in the latero-lateral view with the patient in the lateral recumbent position was obtained. A vertebral fracture (VF) was considered to be present when the height of the vertebral body was reduced by at least 20% (4 mm) [10].

Vascular calcifications (VC) were quantified by measuring the length of calcific deposits along the abdominal aortic wall (mild 0.1-5 cm, moderate 5.1-10 cm and severe >10 cm). The presence of calcifications of the iliac arteries was evaluated through the same radiograph (mild 0.1-3 cm,

moderate 3.1-5 cm, and severe >5 cm) [11].

Statistical analysis

The data are shown as mean ± standard deviation (SD) for quantitative variables or median and interquartile range (IQ) for not normal or strongly asymmetric variables, and frequency percentages for all discrete variables. Normal distribution of continuous variables was tested using the Shapiro-Wilk test. For discrete variables, the differential distribution between present or past smokers and non-smokers was analysed by χ^2 test or Fisher's exact method. For quantitative variables, the comparisons were evaluated using Generalized Linear Models (Levene's test was performed to test the homoschedasticity; when its assumption was violated, the Welch's ANOVA was used) or the non-parametric Wilcoxon sum rank test.

Multiple regression models were defined, to assess the strength of the association between total and undercarboxylated BGP (outcomes) and smoking status; each outcome underwent logarithmic transformation. Among available variables (gender, age, renal failure history, alcohol consumption, medical history (cardio- and cerebrovascular disease, diabetes mellitus, malabsorption syndrome and liver disease), BMI, results from routine biochemical examinations, and mineral and bone disorders treatment (oral calcitriol, vitamin D analogues, calcimimetics, and phosphate-binding drugs)), factors included as independent variables were those associated with the outcome at the univariate level ($p \leq 0.10$) or associated with smoking status ($p \leq 0.10$).

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

RESULTS

*Cohort characteristics by smoking status (**Table 1**).*

Data on smoking status were collected in 370 patients: 136 (36.8%) were current or former smokers and 234 (63.2 %) never smoked. Current and former smokers were more commonly men, had greater use of alcohol, and had greater prevalence of angina (23.5 *vs* 13.2%, p=0.011) and myocardial infarction (27.2 *vs* 15.0%, p=0.006). Smokers also had lower levels of phosphate [4.25

(3.72, 5.30)] *vs* [4.86 (4, 5.58)] mg/dl respectively, p= 0.008), albumin [3.8 (3.5, 4.0)] *vs* [3.9 (3.6, 4.2)] g/dL respectively, p= 0.001).

Variables associated with BGP levels

Total but not undercarboxylated levels of BGP were lower in smokers than non-smokers (152 *vs* 204 μg/L, p= 0.003). Stratification by gender demonstrated that median [IQR] levels of BGP were lower in male smokers [147 μg/L (83.8, 247)] compared with non-smokers [206 μg/L (97.2, 355.3)], p = 0.005. In contrast, differences between female smokers [187 μg/L (107, 361.9)], and non-smokers [204 μg/L (115, 403.3)] were not significant, p = 0.79. In linear regression modelling, adjusted for gender, age, BMI, alcohol consumption, angina, myocardial infarction, atrial fibrillation, heart failure, cerebrovascular accidents, diabetes mellitus, vertebral fractures, calcifications, biochemical profile (P, Ca-P product, albumin, high-density lipoprotein (HDL) cholesterol, alkaline phosphatase (ALP), parathyroid hormone (PTH), C-reactive protein (PCR), drugs (vitamin D analogues, calcimimetics, warfarin, steroids, antibiotics, insulin, statins), a history of smoking was associated with an 18% lower level of median BGP, and smoking accounted for 53% of the variability in BGP levels (parameter estimate -0.199; p = 0.0105; R^2 = 0.53). Other variables significantly associated with BGP were found to be atrial fibrillation (parameter estimate -0.374, p=0.0010), diabetes mellitus (-0.328, p=0.0108), age (-0.010, p=0.0006), BMI (-0.0375, p<0.0001), alkaline phosphatase (0.349, p<0.0001), PTH (0.234, p<0.0001), calcimimetics (0.308, p=0.0005) and warfarin use (-0.429, p=0.0002); gender was not significantly associated with BGP (p=0.5761).

Relationships between BGP, vascular calcifications and fractures

We also explored relationships between vascular calcifications, vertebral fracture, smoking status and BGP. Calcifications of the abdominal aorta and iliac arteries were present in 79.7% and 54.9% of our cohort, respectively. Prevalence of calcifications at both sites were similar between groups regardless of smoking history. Lower total BGP levels were associated with aortic (p< 0.001) and iliac (p=0.042) calcification, and severe aortic vascular calcifications were associated with lower

total BGP levels (218 *vs* 165 µg/L, p=0.002). Vertebral fractures were prevalent in 54% of patients and did not differ by smoking status. Lower total levels of BGP were associated with vertebral fractures (p=0.023) **(Figure 1**).

DISCUSSION

This study shows a significant association of BGP and cigarette smoking, especially in males, as well as a possible protective role of BGP in bone metabolism and vascular health. To our knowledge, this is the first clinical study conducted in a haemodialysis population suggesting that smoking interferes with BGP activity and therefore bone and vascular health.

The carboxylation of BGP is responsible for protein activation in bone tissue, while the undecarboxylated form (ucBGP) seems to be less important for bone metabolism and more active in extra-skeletal sites [12]. In fact, BGP may be involved in the aortic calcification process indirectly by its action on insulin, in particular in the presence of metabolic acidosis with bone resorption, where an increase of ucBGP is observed. ucBGP is able to promote the secretion of insulin from pancreatic beta-cells [13]. Moreover, BGP may also induce the release of adiponectin, an anti-inflammatory adipokine secreted by adipocytes, as described in mice [14] and chronic kidney disease (CKD) patients [15]. Adiponectin is able to prevent the transdifferentiation of vascular smooth muscle cells (VSMCs) into osteoblast-like cells in arterial vessels [16]. On the other hand, an in vitro study suggested the BGP overexpression may be associated with increased cartilage and vascular calcification with a hypoxia-inducible factor 1 mediated mechanism [17].

In humans, a 10 years prospective study in elderly Caucasian men showed that BGP levels might be an independent indicator of cardiovascular risk and mortality, with higher total BGP concentrations being associated with lower abdominal aortic calcification (AAC) progression rate and lower mortality [18]. Moreover, we showed that low levels of BGP are associated to vertebral fracture (VF), aortic and iliac calcifications [19].

We showed that vitamin D analogues and calcimimetics are able to improve vitamin K dependent

protein levels. In particular, calcimimetics and vitamin D analogues use was associated with increased BGP levels in haemodialysis patients, suggesting a role of these drugs in preserving vitamin K-dependent protein activity, thus contributing to bone and vascular health in CKD patients [20].

Nowadays, smoking is widely prevalent and is known to be strictly connected with cardiovascular disease by promoting vascular damage and progression of arterial ageing: for example, smoking stimulates the formation of reactive oxygen radical species and oxidative stress, inducing early vascular ageing [21].

Some studies have identified smoking as a risk factor for osteoporosis, showing inhibition of osteoblast activity both in vitro and in animal studies [8]. In humans, meta-analyses of the effects of smoking on bone status demonstrated decreased bone mass in current smoker compared with non-smoker populations [22, 23]. Nevertheless, the effect of nicotine on bone remains controversial; the negative impact of smoking on bone status might be associated to cigarette smoke constituents (such as toxic heavy metals, polychlorinated biphenyls, dioxin, polycyclic aromatic hydrocarbons) other than nicotine alone [24].

By the regression model, we found that smokers had levels of BGP that were about 20% lower than those of patients who never smoked. In our model, gender was not significantly associated with total BGP. To our knowledge, data about the relationship between smoking and BGP are limited in human populations. Available studies suggested that smoking could be associated with depressed osteoblast activity and with reduced BGP gene expression in humans. In particular, consistent with our findings, Laroche et al. observed that BGP levels were significantly lower in smokers than in non-smokers; the difference was significant in males but not in females [25,26].

Chassanidis et al. identified in smokers a remarkable decrease of the gene expression of BMP-2, -4, and -6, which play an important role in the maintenance of bone mass, resulting in reduced BGP synthesis [27**]**.

Another study highlighted that cigarette smoke extract can lead to inhibition of osteoblastic

differentiation of cultured human periosteum-derived cell, as well as inhibition of alkaline phosphatase activity, mineralization and Runx2 transactivation of the periosteum-derived cells. The role of BGP was not investigated [28].

In conclusion, we report for the first time a significant association between smoking and lower BGP levels in males affected by advanced CKD. In the same population, low BGP levels are associated with fractures and vascular calcifications. Larger longitudinal studies should evaluate the effects of smoking on BGP activity and their consequences on bone and vascular health.

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Conflict of interest:

Maria Fusaro, Maurizio Gallieni, Andrea Aghi, Giorgio Iervasi, Maria Antonietta Rizzo, Andrea Stucchi, Marianna Noale, Giovanni Tripepi, Thomas Nickolas, Nicola Veronese, Fabrizio Fabris, Sandro Giannini, Antonio Piccoli, Maria Cristina Mereu, Laura Cosmai, Alberto Ferraro, Fiorenza Magonara, Michela Spinello, Stefania Sella and Mario Plebani declare that they have no conflict of interest.

References

1. Fusaro M, Crepaldi G, Maggi S, et al. Vitamin K, bone fractures, and vascular calcifications in chronic kidney disease: an important but poorly studied relationship. J Endocrinol Invest 2011; 34:317-23.

2. Amizuka N1, Li M, Kobayashi M, et al. Vitamin K2, a gamma-carboxylating factor of gla-proteins, normalizes the bone crystal nucleation impaired by Mg-insufficiency. Histol Histopathol 2008;23:1353-66.
3. Ducy P, Desbois C, Boyce B, et al. Increased bone formation in osteocalcin-deficient mice. Nature 1996; 382: 448-52.
4. Brennan-Speranza TC, Conigrave AD. Osteocalcin: an osteoblast-derived polypeptide hormone that modulates whole body energy metabolism. Calcif Tissue Int 2015;96:1-10.
5. Lian J, Stewart C, Puchacz E, et al. Structure of the rat osteocalcin gene and regulation of vitamin D-dependent expression. Proc Natl Acad Sci USA 1989; 86:1143-7.
6. Law MR, Hackshaw AK. A meta-analysis of cigarette smoking, bone mineral density and risk of hip fracture: recognition of a major effect. BMJ 1997; 315:841-6.
7. Hirooka N, Kadowaki T, Sekikawa A, et al. Influence of cigarette smoking on coronary artery and aortic calcium among random samples from populations of middle-age Japanese and Korean men. J Epidemiol Community Health 2013; 67: 119-24.
8. Gao SG, Li KH, Xu M, et al. Bone turnover in passive smoking female rat: relationships to change in bone mineral density. BMC Musculoskelet Disord 2011; 12:131.
9. Fusaro M, Noale M, Viola V et al. Vitamin K, vertebral fractures, vascular calcifications, and mortality: vitamin K Italian (VIKI) dialysis study. J Bone Miner Res 2012; 27: 2271-28.
10. Genant HK, Jergas M, Palermo L, et al. Comparison of semiquantitative visual and quantitative morphometric assessment of prevalent and incident vertebral fractures in osteoporosis The Study of Osteoporotic Fractures Research Group. J Bone Miner Res 1996; 11: 984-96.
11. Witteman JC, Grobbee DE, Valkenburg HA, et al. J-shaped relation between change in diastolic blood pressure and progression of aortic atherosclerosis. Lancet 1994; 343: 504-7.
12. Neve A, Corrado A, Cantatore FP. Osteocalcin: skeletal and extraskeletal effects. J Cell Physiol 2013; 228:1149-53.

13. Ducy P, Schinke T, Karsenty G. The osteoblast: A sophisticated fibroblast under central surveillance. Science 2000; 289:1501-4.
14. Lee NK, Sowa H, Hinoi E, et al. Endocrine regulation of energy metabolism by the skeleton. Cell 2007; 130: 456-69.
15. Bacchetta J, Boutroy S, Guebre-Egziabher F, et al. The relationship between adipokines, osteocalcin and bone quality in chronic kidney disease. Nephrol Dial Transplant 2009; 24:3120-5.
16. Luo XH, Zhao LL, Yuan LQ, Wang M, Xie H, Liao EY. Development of arterial calcification in adiponectin-deficient mice: adiponectin regulates arterial calcification. J Bone Miner Res 2009; 24:1461-8.
17. Idelevich A, Rais Y, Monsonego-Ornan E. Bone Gla protein increases HIF-1-dependent glucose metabolism and induces cartilage and vascular calcification. Arterioscler Thromb Vasc Biol 2011; 31:e55-71.
18. Confavreux CB, Szulc P, Casey R, et al., Higher serum osteocalcin is associated with lower abdominal aortic calcification progression and longer 10-year survival in elderly men of the MINOS cohort. J Clin Endocrinol Metab 2013; 98: 1084-92.
19. Fusaro M, Gallieni M, Noale M, et al. The relationship between the Spine Deformity Index, biochemical parameters of bone metabolism and vascular calcifications: results from the Epidemiological VERtebral FRACtures iTalian Study (EVERFRACT) in dialysis patients. Clin Chem Lab Med 2014; 52: 1595-603.
20. Fusaro M, Giannini S, Gallieni M, et al. Calcimimetic and vitamin D analog use in hemodialyzed patients is associated with increased levels of vitamin K dependent proteins. Endocrine 2016; 51:333-41.
21. Anderson C, Majeste A, Hanus J, Wang S. E-cigarette aerosol exposure induces reactive oxygen species, DNA damage, and cell death in vascular endothelial cells. Toxicol Sci 2016; 154:332-40.

22. Kanis J A, Johnell O, Oden A, et al. Smoking and fracture risk: a meta-analysis. Osteoporosis Int 2005;16:155-62.
23. Ward KD, Klesges RC. A meta-analysis of the effects of cigarette smoking on bone mineral density. Calcif Tissue Int 2001;68: 259-70.
24. César-Neto JB, Duarte PM, Sallum EA, Barbieri D, Moreno H Jr, Nociti FH Jr. A comparative study on the effect of nicotine administration and cigarette smoke inhalation on bone healing around titanium implants. J Periodontol 2003; 74: 1454-9.
25. Laroche M, Lasne Y, Felez A, et al. Osteocalcin and smoking. Rev Rhum Ed Fr 1994; 61: 433-6.
26. Fujiyoshi A, Polgreen LE, Gross MD, Reis JP, Sidney S, Jacobs DR Jr. Smoking habits and parathyroid hormone concentrations in young adults: The CARDIA study. Bone Rep 2016;5:104-109.
27. Chassanidis CG1, Malizos KN, Varitimidis S, et al. Smoking affects mRNA expression of bone morphogenetic proteins in human periosteum. J Bone Joint Surg Br 2012; 94:1427-32.
28. Sung IY, Park BC, Hah YS, et al. FOXO1 is involved in the Effects of Cigarette Smoke Extract on Osteoblastic Differentiation of Cultured Human Periosteum-derived Cells. Int J Med Sci 2015;12:881-90.

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329 Table 1. Main patient characteristics.

	Patients Smoker or ex-smoker (n=136)	**Patients Non-Smoker (n=234)**	**p**
Gender, female, n (%)	31 (22.8 %)	108 (46.2 %)	< **0.001**
Age, years, mean ± standard deviation	65.4± 3.5	63.6 ±14.4	0.241
Weight, kg, mean ± standard deviation	72.7±14.9	68.8±14.0	**0.012**
Height, m, mean ± standard deviation	1.69±0.09	1.66±0.09	**0.001**
Height, m, median	1.70 (1.62, 1.75)	1.65 (1.60, 1.72)	< **0.001**
Body mass index (BMI), kg/m^2, mean ± standard deviation	25.3±4.3	24.9±4.4	0.411
Current or former alcohol drinker, n (%)	56 (41.2%)	24 (10.3%)	< **0.001**
<u>Medical history</u>			
Dialysis vintage, months, median	45 (26.5, 77.5)	52 (29, 99)	0.105
Type of dialysis, n (%)			
Bicarbonate dialysis	59 (43.4%)	118 (50.4%)	
Haemofiltration (HF)	10 (7.4%)	22 (9.5%)	0.448
Haemodiafiltration (HDF)	43 (31.6%)	56 (23.9%)	
Acetate free biofiltration (AFB)	21 (15.4%)	31 (13.2%)	

	Patients Smoker or ex-smoker (n=136)	**Patients Non-Smoker (n=234)**	**p**
Other types of dialysis	3 (2.2%)	7 (3.0%)	
Previous kidney transplant, n (%)	13 (9.6%)	36 (15.4%)	0.111
Hypertension, n (%) IPA	111 (81.6%)	177 (75.6%)	0.182
Angina, n (%)	32 (23.5%)	31 (13.2%)	**0.011**
Myocardial infarction, n (%)	37 (27.2%)	35 (15.0%)	**0.006**
Atrial fibrillation, n (%)	14 (10.3%)	35 (15.0%)	0.202
Heart failure, n (%)	20 (14.7%)	17 (7.3%)	0.222
Diabetes Mellitus, n (%)	35 (25.7%)	47 (20.1%)	0.207
Peripheral arterial disease, n (%)			
None	84 (61.8%)	160 (68.4%)	
Asymptomatic	35 (25.7%)	56 (23.9%)	0.343
Intermittent claudication	14 (10.3%)	13 (5.6%)	
Amputation	3 (2.2%)	5 (2.1%)	

	Patients Smoker or ex-smoker (n=136)	**Patients Non-Smoker (n=234)**	**p**
Cerebrovascular accident, n (%)			
None	118 (50.0%)	216 (92.3%)	0.050
Stroke	12 (8.8%)	7 (3.0%)	
Other type	6 (4.4%)	11 (4.7%)	
Vertebral fractures, n (%)	74 (54.4%)	127 (54.3%)	0.979
Vertebral fractures among men, n (%)	59 (43.4%)	78 (33.3%)	0.379
Vertebral fractures among women, n (%)	15 (48.4%)	49 (45.4%)	0.766
Aortic calcifications, n (%)	115 (84.6%)	180 (76.9%)	0.078
Iliac calcifications, n (%)	71 (52.2%)	132 (56.4%)	0.433
<u>Routine biochemical profile</u>			
Calcium, mg/dl, mean ± standard deviation	9.098±0.526	9.181±0.709	0.199
Phosphate, mg/dl, median	4.25 (3.72, 5.30)	4.70 (4, 5.58)	**0.008**
Alkaline phosphatase, U/L, median	85.50 (64.50, 111.50)	83.50 (65, 112)	0.582
Parathyroid hormone (PTH), pg/ml, median	248.5 (140, 411)	243.5 (140, 381)	0.777
Albumin, g/dl, median	3.8 (3.5, 4.0)	3.9 (3.6, 4.2)	**0.001**

	Patients Smoker or ex-smoker (n=136)	**Patients Non-Smoker (n=234)**	**p**
C-reactive protein (PCR), mg/L, median	1.50 (0.42, 4.76)	1.90 (0.52, 5.50)	0.476
Kt/V mean ± standard deviation	1.22 ± 0.28	1.28 ± 0.27	0.056
Aluminium, mcg/L, median	12.5 (7.1, 20) (for 60 patients)	12 (8, 21) for (104 patients)	0.806
Total cholesterol, mg/dl, median	164.50(139.5, 190.5)	169.5 (144, 195)	0.587
Triglycerides, mg/dl, median	159 (109, 206)	142.5 (113, 208)	0.588
High-density lipoprotein (HDL) Cholesterol, mg/dl, median	39 (32, 49)	41 (33, 51.5)	0.071
Low-density lipoprotein (LDL) Cholesterol, mg/dl, median	90 (70, 116)	91 (70, 117)	0.920
25(OH)-vitamin D, median	27.9 (17.3, 42.6)	29.9 (20.1, 46)	0.131
Bone Gla-protein (BGP) total, μg /L, median	152.00 (91.70, 251)	204.50 (111, 362)	**0.003**
Bone Gla-protein (BGP) undercarboxylated, μg /L, median	10.37 (4.01,16.10)	11.09 (5, 21.23)	0.212

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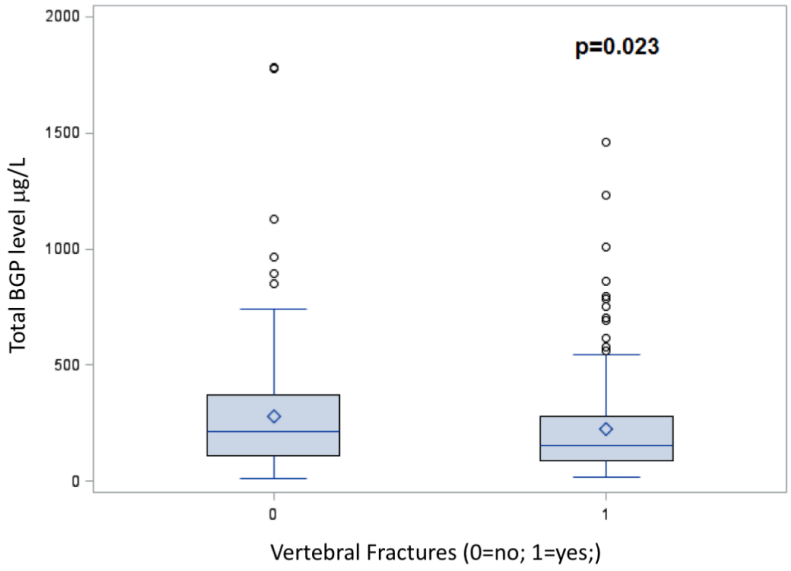
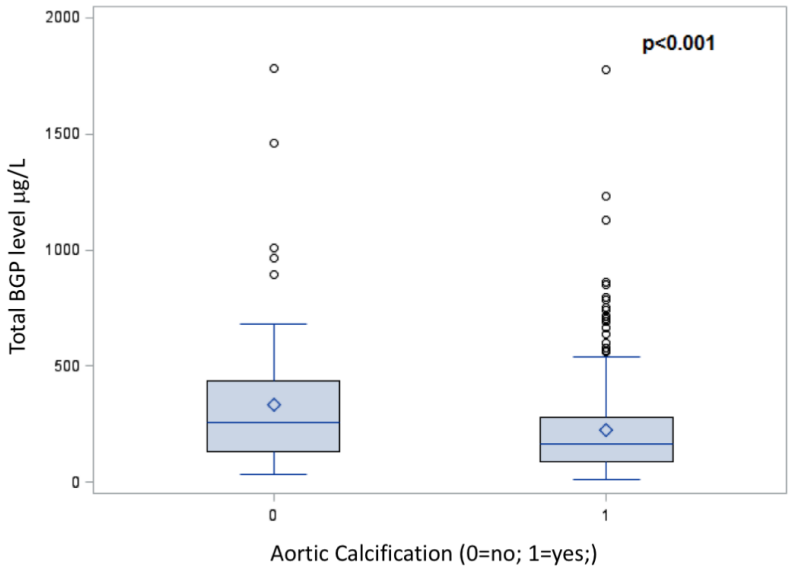
Figure. 1. Median total BGP levels and association with (a) vertebral fractures, (b) aortic calcification, and, (c) iliac calcification.

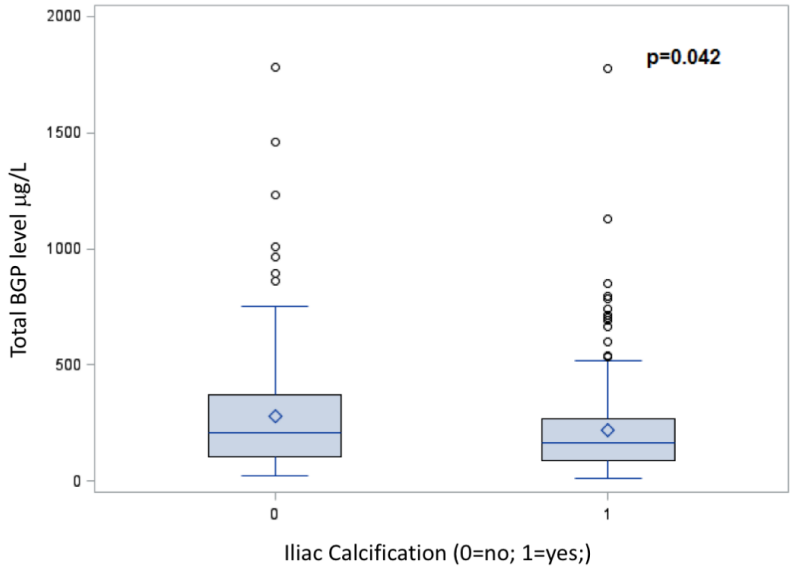
(a)



(b)



(c)



SUPPLEMENT TABLE 1. Therapy.

Drugs prescribed to patients	Patients Smoker or ex-smoker (n=136)	Patients Non-Smoker (n=234)	p
Warfarin (n, %)	16 (11.8%)	28 (12.0%)	0.954
Steroid (n, %)	3 (2.2%)	17 (7.3%)	**0.038**
Thyroid Hormones (n, %)	16 (11.8%)	20 (8.5%)	0.314
Antibiotics (n, %)	7 (5.1%)	9 (3.8%)	0.553
Antiepileptic (n, %)	7 (5.1%)	6 (2.6%)	0.193
Statin Therapy (n, %)	58 (42.6%)	67 (28.6%)	**0.006**
Beta-Blockers (n, %)	43 (31.6%)	92 (39.3%)	0.138

Antidiabetics (n, %)	2 (1.5%)	4 (1.7%)	0.861
Insulin (n, %)	25 (18.4%)	31 (13.2%)	0.184
Anti-Gastric (n, %)	108 (79.4%)	180 (76.9%)	0.822
Aluminium (n, %)	21 (15.4%)	74 (31.6%)	**0.001**
Calcium Carbonate (n, %)	52 (38.2%)	76 (32.5%)	0.262
Calcium Acetate (n, %)	9 (6.6%)	10 (4.3%)	0.325
Sevelamer (n, %)	55 (40.4%)	100 (42.7%)	0.666
Lanthanum (n, %)	22 (16.2%)	33 (14.1%)	0.589
Oral Calcitriol (n, %)	65 (47.8%)	103 (44.0%)	0.482
Intravenous Calcitriol (n, %)	6 (4.4%)	5 (2.1%)	0.214
Vitamin D Analogues (n, %)	20 (14.7%)	54 (23.1%)	0.052
Calcimimetics (n, %)	28 (20.6%)	45 (19.2%)	0.752

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