International Journal of General Medicine

Dovepress

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ORIGINAL RESEARCH

Circulating Osteoprotegerin in Chronic Kidney Disease and All-Cause Mortality

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Background: Chronic kidney disease (CKD) is associated with cardiovascular disease (CKD), mineral and bone disorder (CKD-MBD) and high mortality. Bone-related factors such as osteopontin (OPN), osteocalcin (OC), osteoprotegerin (OPG) and fibroblast growth factor 23 (FGF23) were linked to cardiovascular complications of CKD and are expected to have predictive value in CKD patients.

Purpose: The aim of this study was to assess the relationship of OPN, OC, OPG and FGF23 to clinical characteristics and to evaluate their ability to predict mortality in patients with different CKD stages.

Methods: The following study groups were enrolled: subjects with end-stage renal disease (38 ESRD), CKD stages 3 and 4 (19 CKD3-4) and non-CKD controls (19), respectively. Blood was withdrawn once to perform the measurements and cardiac computed tomography was used to evaluate coronary calcium score (CS). Patients were followed for 5 years for the ascertainment of their all-cause mortality.

Results: Serum OPN, OC and OPG concentrations increased significantly along with the progression of renal disease. We found a significant positive correlation among these proteins. Additionally, OPN and OPG were significantly and positively correlated to CS. Serum OPG revealed the strongest correlation to the calcium turnover markers of GFR decline and was significantly associated with an increased risk of death in subjects with CKD3-4 or ESRD (HR 5.8, CI 95%).

Conclusion: Single measurement of osteoprotegerin is associated with 5-year all-cause mortality in patients with CKD3-4 or ESRD. We suggest assessing its concentration, preferably in combination with calcium score, to stratify mortality risks in CKD patients.

Keywords: calcium score, chronic kidney disease, osteocalcin, osteopontin, osteoprotegerin

Introduction

Chronic kidney disease (CKD) is associated with a high incidence of cardiovascular disease (CVD) and related mortality.[1] Another common complication of CKD that may also affect CVD is mineral and bone disorder (CKD-MBD).[2] CKD-MBD is manifested by one or a combination of: 1) abnormalities of calcium, phosphorus, parathyroid hormone, vitamin D and some bone-related cytokine metabolism, 2) abnormalities in bone turnover, and 3) vascular or other soft-tissue calcification. Decline in glomerular filtration in CKD patients causes vitamin D deficiency, derangements in calcium and phosphate homeostasis, and secondary hyperparathyroidism resulting in bone destruction and vascular calcification.[3] The latter is associated with cardiovascular morbidity and mortality.[4] It was recently reported that medial arterial calcification in CKD patients is increased and correlates with

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circulating CVD markers.[5] Furthermore, calcification score emerged as a significant predictor of long-term survival in CKD patients.[6] CKD-MBD is a complex disease that is not completely understood. However, some factors secreted by the osteocytes might play an important role in its pathophysiology. These factors are linked to cardiovascular complications of CKD and are expected to have diagnostic predictive value in patients with CKD.[7]

Bone-related factors such as: osteopontin (OPN), osteocalcin (OC), osteoprotegerin (OPG) and fibroblast growth factor 23 (FGF23) are linked to CVD development in CKD patients.[5,8–18] Plasma OPN levels are independently associated with the presence and severity of diabetic nephropathy[9] and were found to be expressed by inflammatory cells such as macrophages and highly induced during inflammatory activation.[10,11] In combination with OPG, they increase the predictability of cardiovascular outcomes.[12] Serum OC concentrations are significantly lower in non-dialysis CKD patients than in healthy individuals, and correlate with subclinical atherosclerosis in CKD patients.[13] OPG indirectly, exerts a suppressive effect on osteoclastogenesis and regulates inflammatory and immune responses.[14] It was found to be associated with increased risk of death in CKD patients and was proposed a marker of atherosclerosis and ischemic stroke.[15–18] FGF23 is physiologically involved in renal phosphorus excretion and is associated with increased mortality, left ventricular hypertrophy, endothelial dysfunction and progression of CKD.[19,20] The diversity in study designs and patient populations precludes definite conclusions as to the diagnostic value of these bone-related factors in CKD population.

The aim of this study was to assess the relationship of OPN, OC, OPG and FGF23 to clinical characteristics and to evaluate whether these biomarkers could predict mortality in patients with different CKD stages at baseline.

Materials and Methods

Patients and Study Design

The following study groups were enrolled: subjects with end-stage renal disease (ESRD, n = 38), CKD stages 3 and 4 (CKD3-4, n = 19) and non-CKD controls (n = 19). Patients were enrolled randomly from the group of patients followed-up in our single center. The inclusion criteria were ESRD or CKD 3 and 4, age >18 years, willingness to participate in the study and ability to sign the informed consent. The exclusion criteria included active infections, malignancies, acute cardiologic conditions such as myocardial infarction or atrial fibrillation and pregnancy. The study groups were unintentionally comparable with respect to sex and age. The clinical and biochemical markers of renal function differed significantly between the groups by study design. Clinical characteristics are summarized in Table 1. Fasting blood was drawn once to perform the measurements, and cardiac computed tomography (CT) was used to evaluate the coronary calcium score (CS). The CT safety was discussed with the patients and appropriate information was provided when informed consent was obtained. Patients were followed prospectively for 5 years to ascertain their all-cause mortality. None of the study patients had to be referred to further invasive diagnostics of coronary artery disease based on the CS results. Fatal events were recorded based on patients' hospital and out-patient medical records. During this 5-year period 16 patients died: 13 from the ESRD group and 3 from the CKD group. Cardiovascular causes of death were recorded in 11 ESRD and in 2 CKD3-4 patients. Mortality data were collected blind to the laboratory and CT results. The study received approval from the Ethical Committee at the Warsaw Regional Medical Chamber (Resolution No 08/10) and all individuals gave informed consent prior to enrollment. This study was conducted in accordance with the Declaration of Helsinki and was a continuation of previous investigation aimed at determination of other biomarkers.[21]

Methods

In the ESRD group, who were dialyzed 3 times per week, blood was taken before mid-week hemodialysis. Blood samples were centrifuged 10 minutes, at 1800g at +4°C and stored in small aliquots at −70°C until analysis (Sarstedt tubes, Numbrecht, Germany). The classical inflammatory markers: C-reactive protein (CRP) or high-sensitivity CRP (hsCRP) (if CRP was lower than 5 mg/l) and procalcitonin, as well as indices of calcium turnover: total and ionized calcium, phosphorus, vitamin D and parathyroid hormone (PTH) were evaluated. MDRD equation was used to estimate glomerular filtration rate (eGFR).[22,23]

Bone-Related Factors

All bone-related biomarkers were determined with the use of quantitative antibody-based immunoassays in serum samples subjected to one freeze-thaw cycle. OPN, OC and OPG were measured with Milliplex Human Bone

Table 1 Clinical Data at Baseline (Modified From[21])

Clinical Characteristics	Control Group n = 19	CKD3-4 Group n = 19	ESRD Group n = 38	*P* value
Age (years)	62 ± 9	65 ± 15	60 ± 16	0.45
Male n (%)	10 (53)	9 (47)	21 (55)	0.87
Prevalent				
Diabetes n (%)	2 (11)	7 (37)	12 (32)	0.14
Hypertension n (%)	5 (26)	16 (84)	22 (59)	< 0.001*
CVD n (%)	2 (11)	7 (37)	13 (34)	0.12
Biochemical tests [mean (SD)]				
eGFR (mL/min/1.73m^2)	91.28 (17.34)	28.59 (11.18)	6.70 (1.94)	by design
CRP (mg/l)	1.66 (1.23)	7.51 (11.68)	12.48 (31.84)	< 0.001*
hs-CRP (mg/l)	1.73 (1.19)	2.89 (3.13)	-	0.15
PCT (ng/mL)	-	0.12 (0.11)	1.13 (2.84)	< 0.001*
pH	-	7.33 (0.11)	7.37 (0.02)	0.007*
Total protein (g/dl)	-	7.45 (0.58)	6.84 (0.63)	< 0.001*
Albumin (g/dl)	-	4.09 (0.33)	3.99 (0.78)	0.61
Ionized calcium (mEq/l)	-	1.23 (0.05)	1.09 (0.10)	< 0.001*
Total calcium (mEq/l)	-	4.61 (0.30)	4.28 (0.58)	0.02*
Phosphorus (mEq/l)	-	2.17 (0.43)	3.80 (1.06)	< 0.001*
PTH (pg/mL)	-	190.53 (209.09)	539.05 (461.46)	0.003*
Vitamin D (ng/mL)	27.25 (8.05)	26.84 (7.14)	-	0.88
Alkaline phosphatase (U/l)	-	129.63 (113.24)	96.26 (33.75)	0.10

Notes: Conversion factors to SI units are as follows: for CRP and hs-CRP (µg/l) – 1000; for total protein and albumin (g/l) – 10; for 25-hydroxyvitamin D (nmol/l) – 2.496; for PTH (pmol/l) – 0.105; for phosphorus (mmol/l) – 0.0323; for total and ionized calcium (mmol/l) – 0.5. *Indicates statistical significant p < 0.05.
Abbreviations: CKD, chronic kidney disease; CRP, C-reactive protein; CVD, cardiovascular disease; ESRD, end stage renal disease; eGFR, estimated glomerular filtration rate; hs-CRP, high sensitivity C-reactive protein; PCT, procalcitonin; PTH, parathyroid hormone.

Metabolism Panel (HBN1A-51K, Millipore Sigma (formerly Millipore), Billerica, MA, USA) on the Luminex platform, following vendor's protocols. This multiplex particle-enhanced platform incorporates laser-based detection system based on flow cytometry fluidics. FGF23 was assayed with ELISA (EZHFGF23-32K, Millipore Sigma (formerly Millipore), Billerica, MA, USA). Inter-assay coefficient of variation was lower than 18% in each assay. Samples were balanced by caseness. The optical density (ELISA) or fluorescence intensity (Luminex) was matched with the use of 5-parametric logistic standard curve.[24] Forty-three percent of the FGF23 measurements were not detectable, which was more frequent in subjects with better preserved renal function. Assay sensitivity for FGF-23 was 9.9 pg/mL. Inter-assay coefficient of variations for these biomarkers was < 12%.

Biochemical Tests

Basic biochemistry measurements were performed with automatic biochemical analyzers: Cobas Integra 400 plus (Roche Diagnostics, Mannheim, Germany) and Elecsys 2010 Roche. Hs-CRP amount was determined with Roche Diagnostics test; the protein electrophoresis – with Beckman, Appraise Paragon; and blood differential test – with Sysmex SF3000 and Sysmex K4500.

Coronary Calcium Score

A 64-row CT scanner (Aquilion 64, Toshiba Medical Systems, Japan) was used to measure CS. The following protocol parameters of this non-contrast enhanced, electrocardiography-gated CT scan were applied: scan number of 40–52, slice thickness of 3 mm, tube voltage of 120 kV, and tube current of 300 mA. Time of rotation was adjusted to the heart rate. The analysis was performed quantitatively according to the Agatston algorithm using Vitrea 2 workstation V3.9 (Vital Images Inc., USA).[25] Lesions were detected based on density of at least 130 HU. Then, they were colour-marked by the software. Experienced radiologist evaluated coronary calcifications. Lesions were scored 1 to 4 depending on their density. CS was determined as a sum of products of each lesion area and its density index.[25] The mean CT radiation dose ranged from

Table 2 Circulating Bone-Related and Imaging Markers at Baseline in the Controls, CKD3-4 and ESRD Groups, Respectively. Biomarker Concentrations are Presented as Median (25th, 75th Percentile) Values. Proportion of Detectable FGF23 Values Was 5% in Controls, 26% in the CKD3-4 and 92% in the ESRD Patients

	Control Group n=19	CKD3-4 Group n=19	ESRD Group n=38	*P* value CKD3-4 vs Control	*P* value CKD3-4 vs ESRD
Bone-related markers					
Osteopontin (ng/mL)	11 (6, 15)	27 (22, 37)	38 (27, 49)	< 0.001	< 0.001
Osteocalcin (ng/mL)	8.0 (5.8, 10.5)	20 (15, 30)	102 (39, 188)	0.04	< 0.001
Osteoprotegerin (pg/mL)	451 (383, 607)	729 (477, 855)	1146 (894, 1613)	0.10	< 0.001
FGF23 (pg/mL)	-	- (-, 13)	2044 (707, 5213)	0.05	< 0.001
Imaging markers					
Coronary calcium score	NM	338 (67, 563)	375 (38, 1144)	NA	0.74

Abbreviations: CKD, chronic kidney disease; ESRD, end stage renal disease; FGF, fibroblast growth factor; NM, not measured; NA, non applicable.

0.7 to 1.4 mSv which qualifies the study as a low-dose technique. The safety of the examination was discussed with the patients.

Statistical Analysis

Descriptive characteristics were presented as a mean (standard deviation), a median (25th, 75th percentile) or proportions. Skewness and kurtosis metrics of departures from normality were checked and data were transformed to their base 10 logarithms. Cross-sectional biomarker comparisons were done with variance analysis for unbalanced design, where biomarker was considered dependent variable. Correlations were evaluated with Spearman correlation coefficients in the analyses adjusted for multiple comparisons (Bonferroni corrected alpha = 0.0029).[26] Cox proportional-hazards models tested biomarkers associations with the prospective outcome expressed as hazard ratios per one tertile change of the monotonic marker distribution (one degree of freedom). Ties in the failure time were expressed by the exact conditional probabilities. Non-detectable FGF23 values in the follow-up study group accounted for less than one-third of the biomarker values allowing us to evaluate the effect of FGF23 per tertile treated as a categorical variable. Relevant clinical covariates were considered in building the final model. Principal component analysis is an attractive data reduction approach for highly correlated data and was used here to examine the correlated circulating biomarker data in the context of the baseline CKD status.[27] The analysis was conducted with an alpha level set to $\alpha = 0.05$ with the use of softwares: SAS v. 9.4, Cary, NC and JMP Pro14.

Results

Total and ionized calcium were significantly lower, in contrast to phosphorus and PTH, which were significantly higher in ESRD than in CKD3-4 patients (Table 1). Serum concentrations of OPN, OC and OPG increased significantly along with the CKD progression (Table 2). All studied factors were significantly higher in ESRD than in CKD3-4 patients, while OPN and OC were also significantly higher in CKD3-4 group than in controls. Moreover, all bone-related factor concentrations had a significant positive correlation among each other and had an inverse correlation to eGFR (Table 3).

The principal component analysis based on the circulating biomarkers revealed decent level of discrimination between the groups and explained more than a half of the variance (Figure 1). The first principal component featured an eigenvalue of 2.758, whereas the following components had an eigenvalue below 1.0. Loadings of the first component were quite evenly distributed among 4 biomarkers (Supplemental Table 1).

Additionally, OPN and OPG showed a significant positive correlation to CS. OPN was also found to inversely correlate with total calcium. OC and FGF23 similarly significantly correlated positively to PTH and phosphorus and negatively to ionized calcium. Serum OPG revealed the strongest correlation to the clinical consequences of GFR decline. It correlated significantly with all studied calcium turnover markers, with inflammation markers and with pH, albuminemia and proteinemia (Table 3).

Among studied bone-related markers, OPG was the only to increase significantly the risk of death in subjects with CKD3-4 or ESRD. The effect of OPG per one tertile change in the crude analysis was HR (95% CI): 5.8 (2.2,

Table 3 Spearman Correlation Matrix for the Studied Biomarkers and Clinical Characteristics

	Osteopontin	Osteocalcin	Osteoprotegerin	FGF23	Calcium Score
Bone-related markers					
Osteopontin	1	0.57**	0.56**	0.49**	0.32*
Osteocalcin	0.57**	1	0.55**	0.74**	−0.16
Osteoprotegerin	0.57**	0.55**	1	0.59**	0.57**
FGF23	0.49**	0.74**	0.59**	1	0.20
Imaging marker					
Calcium score	0.32*	−0.16	0.57**	0.20	1
Clinical characteristics					
age	0.14821	−0.15	0.42**	−0.14	0.62**
eGFR	−0.61**	−0.78**	−0.68**	−0.81**	−0.02
CRP	0,21	0.17882	0.33*	0.20	0.19
Procalcitonin	0,20	0.54**	0.36*	0.65**	0.03
pH	0,07	0.34*	0.33*	0.55**	0.02
Total protein	−0.10	−0.27*	−0.38*	−0.35*	−0.05
Albumin	0.01	0.22	−0.31*	0.18	−0.21
Ionized calcium	−0.14	−0.43*	−0.50**	−0.49**	−0.19
Total calcium	−0.26*	−0.26	−0.46**	−0.16	−0.31*
Phosphorus	0.20	0.66**	0.37*	0.75**	0.04
Parathyroid hormone	0.20	0.76**	0.26*	0.69**	−0.03
Vitamin D	−0.14	−0.16	−0.11	−0.18	0.25

Notes: Correlation coefficients are presented (*Indicates coefficients significant at alpha = 0.05 and **Indicates coefficients significant at Bonferroni corrected alpha =0.0029).
Abbreviations: CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; FGF, fibroblast growth factor.

16.0) (Table 4). It remained significant after adjustment for age, sex, baseline CKD status: HR (95% CI): 5.1 (1.1, 23.3) and further, after adjustment for either CRP, total protein or phosphorus levels. An adjustment for ionized calcium resulted in borderline significance (p=0.058). The effect of OPG in the model adjusted for age, sex, baseline CKD status and coronary calcium score was HR (95% CI): 4.1 (0.8, 20.9); p=0.093. There was no interaction between OPG and the CKD status (p=0.57).

Discussion

We found that osteoprotegerin correlated with lower eGFR, calcium score and other bone-related factors, and was associated with increased 5-year all-cause mortality risk in CKD3-4 or ESRD patients. These observations are in line with the previous reports showing OPG to be elevated in non-diabetic[28,29] and diabetic[30–32] CKD patients, and to predict renal function decline, cardiovascular events and all-cause mortality.[31] OPG has been found to be elevated in association with increased 5- and 10-year kidney function deterioration risk, CKD-related hospitalization, and/or deaths in elderly women.[33] A meta-analysis of 10 studies comprising 2120 CKD patients (including 1723 with ESRD) revealed an association of elevated OPG concentrations with an increased risk of cardiovascular death.[34] Recently, it was also reported that circulating OPG was significantly associated with CKD diagnosis in hypertensive non-diabetic patients, independently from other variables.[35] Accordingly, elevated serum OPG levels were associated with higher all-cause and cardiovascular 5-year mortality risk, independent of age, CVD, diabetes, and inflammatory markers, in patients with CKD stages 3–5.[36] Most of these studies were performed in diabetic patients, and suggest OPG to be a biomarker of CKD progression.[37,38] Our findings were confirmed on mixed population of diabetic and non-diabetic subjects at different CKD stages, therefore bring novel arguments as to the use of OPG as a mortality predicting marker in CKD patients.

Of note, we found patient age to be positively correlated with OPG, but not with other bone-related markers. This is an important finding in the context of a cross-sectional study by Vik et al, who found that OPG variably correlates to eGFR depending on age and renal function. A reverse correlation was found in individuals older than the median age with reduced renal function, whereas a positive association could be observed in younger subjects with normal eGFR.[39] The mean overall age in this

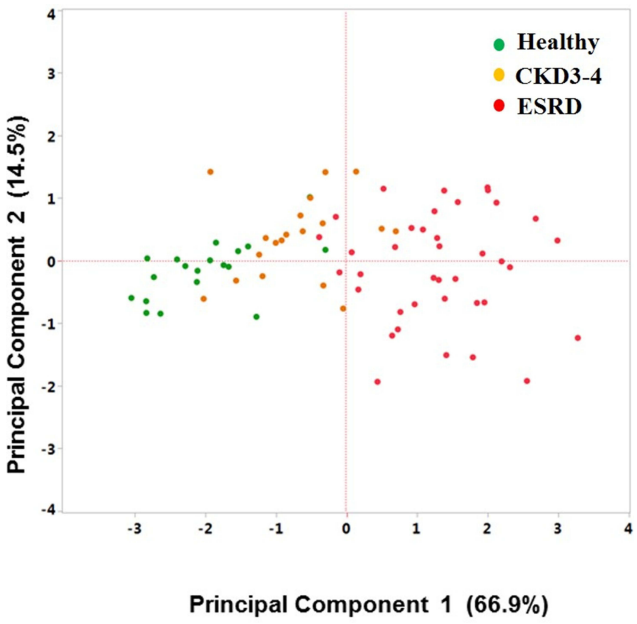

Figure 1 Score plot of principal components 1 and 2 based on the four circulating bone-related biomarkers different between study groups. Each mark represents a study subject. Subjects with ESRD are marked as red circles, those with CKD3-4 are marked as orange circles and the control group is marked with green circles. Description of the x and y axes includes the number of the principal component and the explained variance.

study was 61 years and was comparable to our study groups. This indicated that younger subjects with elevated OPG who develop CKD might have a worse prognosis, particularly because it was also positively correlated with the calcium score.

Analogically to OPG, we found OPN a significant positive correlation with declining eGFR, other bone-related factors and CS. It is known that OPN is produced by the vasculature and bone, is engaged in atherosclerotic plaque formation, and causes renal damage in animal models.[40,41] In humans, OPN levels may indicate atherosclerosis by means of plaque growth and its rupture susceptibility. Moreover, statins treatment and bypass surgery could reduce OPN concentration.[42] Therefore, it is not surprising that OPN is linked to the mortality prediction in CKD patients.[43] Of note, these associations disappeared after adjustment for markers of inflammation. For this reason, combinations of OPN with other biomarkers are sought. Our findings of positive correlations of OPN with OPG and CS are consistent with the results of the NEFRONA Study subanalysis, where the atherosclerotic plaques were assessed in 1043 patients with renal failure in relation to OPG, OPN and sTWEAK concentrations. It was found that elevated OPG or OPN along with the inferior sTWEAK levels significantly correlated to a higher risk of cardiovascular events. Moreover, it was reported that combination of the mentioned biomarkers improved cardiovascular event prognostication in patients with CKD.[12]

Interestingly, FGF23 was below the level of detection in controls and in the CKD3-4 group. Relation of serum markers to bone expression of specific proteins could partially explain this. In a recent study of patients with different CKD stages and controls, several bone remodeling markers were determined in serum and bone biopsy. This study revealed that sclerostin and PTHR1 were elevated in the earlier CKD stages, whereas FGF23 and phosphorylated b-catenin expression were higher in the advanced CKD. Moreover, significant correlations between serum and bone FGF-23 were established.[44] One of

Table 4 Cox Proportional Hazard Models for Incident All-Cause Mortality in CKD3-4 or ESRD Patients at Baseline. Crude Models are Presented. Effects of All Biomarkers are Presented per One Tertile Change of a Monotonic Biomarker Distribution, Except for FGF23, Where the Effects are Presented per One Tertile as a Categorical Variable, Respectively

Marker	Cox Analysis (CKD3-4 + ESRD)			Cox Analysis (ESRD Only)		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Osteopontin	1.74	0.95–3.18	0.075	1.42	0.74–2.7	0.29
Osteocalcin	0.64	0.34–1.22	0.17	0.61	0.32–1.19	0.15
Osteoprotegerin	**5.81**	**2.17–15.56**	**< 0.001**	**4.87**	**1.74–13.56**	**0.003**
FGF23 T1 (ref)	1.00			1.00		
T2 vs T1	1.99	0.30–13.28	0.48	1.38	0.31–6.15	0.68
T3 vs T1	1.71	0.21–13.37	0.61	1.64	0.37–7.33	0.52
Coronary calcium score	**4.65**	**2.14 −10.1**	**0.0001**	**4.09**	**1.88–8.9**	**0.0004**

Note: Bold formatting indicates statistical significance (p<0.05).
Abbreviations: CKD, chronic kidney disease; ESRD, end stage renal disease; FGF, fibroblast growth factor; HR, hazard ratio; T, tertile.

the study limitations was that our hypothesis-driven approach only focused on four biologically related biomarkers. Future untargeted studies of circulating biomarkers using emerging proteomics technologies will allow us to elucidate these biomarker relationships in greater detail.[45] Another limitation of our study is the small sample size and patient heterogeneity, which make this study prone to several types of biases. Despite the fact that we have employed a number of careful biostatistical strategies, an analysis adjusted for confounding factors is limited.

Conclusion

Serum osteoprotegerin is associated with an incident 5-year all-cause mortality in patients with CKD3-4 or ESRD. We suggest assessing its concentration, preferably in combination with calcium score, to stratify mortality risks in CKD patients.

Disclosure

Dr Joanna Kamińska report personal fees from Roche Poland, outside the submitted work; Dr Bartosz Foroncewicz report congress fee + travel from Astellas and Chiesi, outside the submitted work; Dr Krzysztof Mucha report congress fee + travel from Astellas, outside the submitted work; In addition, Dr Bartosz Foroncewicz, Dr Krzysztof Mucha and Dr Leszek Pączek have patents WO2018141975A1 and WO2017212463 pending. The authors report no other conflicts of interest in this work.

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