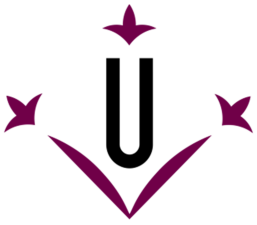

Combination of biomarkers of vascular calcification and sTWEAK to predict cardiovascular events in chronic kidney disease

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Abstract

Background and objectives: Vascular calcification and atherosclerosis have been related with an excess of cardiovascular mortality associated with chronic kidney disease (CKD). Different proteins such as osteoprotegerin (OPG), osteopontin (OPN) are involved in both conditions. In addition, soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK) is a proinflammatory cytokine that has been related to cardiovascular disease. We hypothesized that circulating levels of OPG, OPN and sTWEAK may relate to a higher prevalence of cardiovascular outcomes in patients with CKD.

Design, setting, participants, & measurements: Baseline circulating levels of OPG, OPN and sTWEAK were measured in 565 patients with CKD stages 3-5D [age, 61(48-68) years old, median (IQR); 359 men] without any previous CV event from The National Observatory of Atherosclerosis in Nephrology (NEFRONA) Study. Patients were followed for cardiovascular outcomes (follow-up of 3.11±1.26 years).

Results: After the follow-up, 30 fatal and 33 nonfatal cardiovascular events occurred. At baseline, OPG and OPN levels were increased and sTWEAK concentrations were decreased in CKD patients suffering a fatal or nonfatal cardiovascular event. In a Cox model, after controlling for potential confounding factors, patients with OPG or OPN above and sTWEAK below optimal cut-off points obtained from ROC analysis had a higher risk of fatal and nonfatal cardiovascular events [HR: 2.62 (1.59-5.04); p<0.01; 2.88 (1.59-5.24); p<0.005; 2.00 (1.04-3.84), p<0.05; respectively]. In addition, when CKD patients were grouped according to the number of biomarkers above (OPG and OPN) or below (sTWEAK) of their cut-off points, the combination of the three biomarkers had the highest risk for fatal and nonfatal cardiovascular events [HR: 10.25 (3.51-29.91); p<0.001].

Conclusions: Combination of OPG, OPN and sTWEAK impacted the predictability of cardiovascular outcomes.

Introduction

Chronic kidney disease (CKD) is associated with a high incidence of cardiovascular events (CVE) and mortality (1). In fact, CVE and mortality increase progressively as glomerular filtration rate decreases (2-3). Traditional risk factors such as hyperlipidemia, hypertension, diabetes and smoking fail to fully explain the increased CV risk in CKD patients (4). Tools able to improve CV risk assessment are needed in CKD patients. In this context, the addition of vascular calcification (VC) scores to traditional risk factors improves CV risk assessment in CKD patients (5).

Vascular calcification is the result of an accumulation of calcium and phosphate salts within the arterial wall as well as in cardiac valves. Data from animal models have identified different factors such as osteopontin (OPN) and osteoprotegerin (OPG) as factors that may regulate calcification in the arterial wall (6-7). OPG is a key cytokine that belongs to the tumor necrosis factor (TNF) receptor superfamily, which has a range of pleiotropic effects on bone metabolism, endocrine function and the immune system (8). OPG inhibits osteoclastic bone resorption by binding to the receptor activator of nuclear factor-κB ligand (RANKL), acting as a decoy receptor to competitively inhibit RANKL interaction with its receptor, RANK (9). Circulating OPG levels have been associated with the presence of VC and all-cause mortality in CKD patients (10). In addition, elevated serum OPG levels increased the risk of CVD and all-cause mortality in elderly women, and the association was more evident in women with poorer renal function (11). On the other hand, OPN belongs to the small integrin-binding ligand N-linked glycoprotein family (12). This protein is produced and secreted by different cell types such as macrophages, T cells, renal and vascular smooth muscle cells (VSMCs) as well as osteoblast and osteoclast (13). In CKD, elevated levels of OPN predicted overall and cardiovascular mortality, although this effect was lost after adjustment for inflammatory biomarkers (14).

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is a proinflammatory cytokine of the TNF-superfamily that circulates in serum as a soluble form (sTWEAK). Different studies have demonstrated a key role of this cytokine in atherosclerotic plaque development, progression and rupture (15-17). Recently, it has been demonstrated that TWEAK also participates in

VSMC calcification (18). In addition, loss-of function experiments have shown that TWEAK increases atherosclerotic plaque calcification (15). Finally, circulating soluble TWEAK (sTWEAK) levels have been associated with cardiovascular outcomes in CKD patients (19-20).

The National Observatory of Atherosclerosis in Nephrology (NEFRONA) Study is a multicenter, observational, prospective study designed to analyze the prevalence of atherosclerosis and its associated risk factors in patients with CKD (21). In this study, we evaluated the association between selected calcification biomarkers, sTWEAK and their combination with cardiovascular outcomes in the NEFRONA population.

Material and Methods

Study Population

Participants included 565 CKD 3-5D patients from NEFRONA Study (21). Briefly, the study included male and female without history of CVD (acute myocardial infarction, angina pectoris, hemorrhagic or ischemic stroke, atherosclerosis, and abdominal aortic aneurysm). CKD patients in this sub-study were enrolled within 57 Spanish primary care centers distributed in 32 different regions from Spain. The exclusion criteria included previous CV events, pregnancy, having received any organ transplantation, active infections and, having a life expectancy of <1 year.

Events

Primary outcomes were CVD events according to the International Classification of Diseases of the World Health Organization, which included myocardial infarction, unstable angina, transient ischemic attack, cerebrovascular accident, arrhythmia, congestive heart failure, peripheral artery disease or amputation for vascular disease, and aorta aneurism. CV mortality was defined as cerebrovascular accident (ischemic or hemorrhagic), myocardial ischemia and infarction, hyperkalemia or arrhytmia, sudden death, hemorrhage due to aneurysm rupture and mesenteric infarct. Non-CV mortality causes included neoplasia, accident, infection, uremic, non-determined or unknown.

The local Ethics Committee of the Hospital Arnau de Vilanova approved the protocol. The authors adhere to the declaration of Helsinki and patients were included after providing informed consent.

Clinical and biochemical data

Patients were asked to complete a questionnaire at recruitment including clinical history of hypertension, dyslipidemia and diabetes, CV risk factors and medication use. Biochemical parameters were obtained from a routine fasting blood test. Serum sTWEAK levels was determined in duplicate with a commercially available ELISA kit (Bender MedSystems), and OPG and OPN levels by multiplex kits (Merck Millipore).

Statistical Analysis

Statistical analyses were performed using SPSS 11.0 (SPSS Inc, Chicago, IL)

statistical package. Normally distributed variables were expressed as mean±SD, and non-normally distributed variables were expressed as medians (IQR, expressed as the 25^{th} and 75^{th} percentile). Between-group comparisons were assessed for nominal variables with the chi-squared test and Mann-Whitney U test. Spearman rank correlation was used to determine correlations between variables. A receiver operator characteristic curve analysis was done to determine the OPG, OPN and sTWEAK cut-off points and maximum sensitivity and highest specificity for prediction of cardiovascular event. A categorical variable was generated containing the information of the number of biomarkers that were over (or under, in the case of sTWEAK) the cut-off point. Time to event analysis of CV outcomes was done using the Cox proportional hazards model, including adjustment for potential confounding factors. Data are presented in the form of Hazard ratios (HRs) and 95% confidence intervals (95% CIs). Statistical differences in c statistics were compared using the method by DeLong et al. (25); 95% CIs were calculated for each comparison. Kaplan-Meier curves were used to compare time to outcome according to a multimarker score. *P* value <0.05 was considered statistically significant.

Results

Patient characteristics

Characteristics of patient population according to cardiovascular outcomes are summarized in Table 1. Patients free for CV event included CKD stage III (N=157), stage 4-5 (N=152) or being in dialysis (N=193). Patients suffering a CV event included CKD stage III (N=10), stage 4-5 (N=16) or being in dialysis (N=37). The baseline levels of OPG, OPN and sTWEAK in the overall population were median (IQR): 119 (86-174) pg/mL, 109 (66-190) ng/mL and 343 (214-514) pg/mL, respectively.

There were significant differences among CKD patients suffering a CV event regarding age, SBP, glucose concentrations, eGFR, diabetes, and circulating OPG, OPN and sTWEAK levels (Table 1).

Univariate association of OPG, OPN and sTWEAK are given in Table 2. The three biomarkers correlated with cholesterol and LDL-c concentrations, eGFR and ABI. In addition, OPG was positively correlated with OPN. OPG and OPN were negatively associated with sTWEAK.

OPG, OPN, sTWEAK and CV outcomes

Cardiovascular outcomes were determined from the day of examination onward, with a mean follow-up of 3.11±1.26 years**.** Sixty-two patients died; 30 died from cardiovascular mortality (cancer [N=12], infection [N=16], and others [N=12]). Causes of cardiovascular disease were myocardial infarction (N=11), mesenteric infarction (N=7), sudden death (N=4), stroke (N=5), or other CV-related causes (N=3). OPG and OPN levels were higher in patients suffering a fatal CV event compared with those free for fatal CV event [OPG median (IQR): 212 (120-293) versus 118 (85-168) pg/mL; p<0.001; OPN median (IQR): 160 (112-231) versus 106 (112-231) ng/mL; p=0.002]. ~~However~~ In contrast, sTWEAK concentrations were lower in patients suffering a fatal CV event compared with those free for fatal CV event [median (IQR): 211(139-367) versus 350 (221-517); p=0.01].

Because of the limited number of fatal CV events registered, we analyzed the effect of OPG, OPN and sTWEAK levels on the prediction of CV events for a composite of fatal and nonfatal CV events (N=63). During the follow up, 33 additional nonfatal CV events were registered; unstable angina (N=8),

myocardial infarction (N=6), cerebrovascular accident (N=5); intermittent claudication (N=8), and other CV event (N=6). Characteristics of patient population according to cardiovascular outcomes are summarized in Table 1. There were significant differences among CKD patients suffering a CV event regarding age, SBP, glucose concentrations, eGFR, diabetes, and circulating OPG, OPN and sTWEAK levels.

ROC curves were generated by logistic regression model to assess the prediction values of baseline OPG, OPN and sTWEAK levels (Fig. 1A). The ROC curve of patients with fatal and nonfatal CV events showed an area under the curve (AUC) of 0.69 (95%CI: 0.61-0.77; p<0.001) for OPG. A OPG threshold level >179 pg/mL had the highest combined sensitivity (56%) and specificity (81%) for the identification of individuals suffering a CV event. Similar AUC were found for OPN [AUC=0.63 (95% CI: 0.57-0.70); p<0.001] and sTWEAK [AUC=0.63 (95%CI: 0.56-0.69); p<0.001]. A OPN threshold level of >11.4 ng/mL and sTWEAK threshold concentration <307 pg/mL had the highest sensitivity (73% and 67%, respectively) and specificity (57% and 58%, respectively) for the identification of patients suffering a CV event. Kaplan-Meier curves showed a significant association of OPG or OPN above and sTWEAK below cut-off points with probability to suffer a CV event (p<0.001 for all) (Fig. 1B).

The predictors for time-to-cardiovascular event (N=63, including a composite of fatal and nonfatal) were studied by univariate and multivariate Cox analysis. In univariate Cox, age, diabetes, eGFR, being on dialysis, insulin treatment, OPG or OPN above and sTWEAK below of their cut-off points obtained from ROC analysis were significant predictors of outcome (Table 3). Multivariate Cox was used to study the effect of variables that were statistically significant in the univariate analysis. After that, only OPG or OPN above and sTWEAK below of their cut-off points persisted as independent predictors of CV events (Table 3).

Since the three biomarkers correlate with each other and are independent predictors of CV events, a multimarker combination was developed according to the number of biomarkers whose values were above (OPG and OPN) or below (sTWEAK) of their cut-off points. Thus, a patient could have a multimarker score of 0 to 3. The score value was 0 in 31.2% patients, 1 in

34.5%, 2 in 23.0% and 3 in 11.3%. Kaplan-Meier curve showed a significant association of multimarker score with probability to suffer a CV event (Fig. 2A). Using this score in the Cox proportional hazards model, score 2 or 3 were significant predictors of outcome (Table 3). After adjustment by variables statistically significant in the univariate analysis, both score 2 or 3 persisted as independent predictors of CV events (Table 3).

Finally, to assess the clinical usefulness of multimarker score in predicting CV outcomes in CKD patients, we created multivariable regression models with or without multimarker score. The model with conventional CV risk factors included age, sex, hyperlipidemia, hypertension, diabetes, and smoking status as well as eGFR. According to c statistics, the model including the multimarker score has shown a significant improvement from HR (95%CI): 0.71 (0.65-0.78) to 0.79 (95%CI, 0.72-0.85) in accuracy of CV events prediction (p=0.013) (Fig. 2B).

Discussion

In this work**,** we investigated OPG, OPN and sTWEAK serum levels as predictors of CV outcomes in CKD patients with or without CV risk factors but without any history of CVD. Specifically, we have observed that higher levels of calcification biomarkers OPG or OPN while lower levels of sTWEAK were associated with significantly greater risk of fatal and non-fatal CV events. In addition, we demonstrated that a combination of these biomarkers improves CV events prediction in CKD patients, being the first report showing the predictive value of this panel of biomarkers.

It has been demonstrated that OPG is directly secreted from the vascular wall (22). OPG can modulate inflammation, apoptosis and calcium deposition in atherosclerotic plaques and cardiac valves (22). The beneficial role of OPG has been demonstrated in genetically modified mice. Thus, genetic deletion of osteoprotegerin in ApoE knockout mice accelerates advanced atherosclerotic lesion progression and calcification (23). In addition, osteoprotegerin injection in ApoE deficient mice promotes fibrous cap formation, contributing to plaque stabilization (24). In contrast, high levels of OPG have been associated with increased aortic and coronary calcification in patients (25), with vascular calcification in experimental models (CITA Panizo,..Valdivielso. Circ Res. 2009 104:1041-8) and with CV events in CKD patients (14, 26). Accordingly, we have demonstrated that OPG levels are negatively associated with eGFR and that high concentrations of OPG can predict CV events in the NEFRONA population. The discrepancy between the potential beneficial effects of OPG and their high levels observed in CKD patients could be explained by a compensatory mechanism to counteract vascular calcium deposition in the arterial wall of CKD patients.

OPN is also expressed and secreted from the vascular wall and bone tissue, plays a role in atherosclerotic plaque development (27) and contributes to kidney damage in mice (28). Furthermore, OPN has been used as a marker of phenotypic transformation of VSMC in to osteoblast-like cells, (Lau Adv Chronic Kidney Dis. 2011 March ; 18(2): 105–112) and its expression is increased in human aortic valvular lesions (O'Brien KD, Osteopontin is expressed in human aortic valvular lesions. Circulation. 1995;92:2163–2168). However, its effects on vascular calcification are not clear. Thus, whereas

OPN levels increase in calcified aortas human (Chen et al Mol Cell Biochem. 392(:65-76) and in experimental models of vitamin D toxicity and uremia (Moe, Kidney Int. 2003 63:1003-11), OPN is able to inhibit in vitro calcification, (Wada, Circ Res. 1999 84(:166-78) and OPN deficient cells show increased VC levels (Speer, Cardiovasc Res. 2005 66:324-33).

In humans, OPN levels have been shown to be a good marker of the atherosclerotic process, reflecting not only atherosclerotic extension but also plaque susceptibility to rupture. In addition, treatments aimed to reduce atherosclerosis (statins, by pass, etc) also reduced OPN levels.(Wolak, Atherosclerosis. 2014 Oct;236(2):327-37) OPN concentrations have been associated with the prediction of total and cardiovascular mortality in CKD patients (14). However, this association was lost after adjustment for inflammatory markers. In the present paper, we observed that OPN negatively correlate with both eGFR and OPG and we demonstrated that OPN levels predict CV events in CKD patients, independently of inflammatory markers such as CRP or sTWEAK.

sTWEAK is a proinflammatory cytokine that participates in the development, progression and rupture of atherosclerotic plaques (15-17). In addition, it has been recently reported and active role of TWEAK on vascular calcification. Thus, loss-of-function experiments have demonstrated that TWEAK diminished atherosclerotic plaque calcification (15). In addition, TWEAK favors phosphate-induced calcification in cultured VSMC (18). However, effects of TWEAK and OPG in vascular calcification should be independent since it has been demonstrated that TWEAK induces macrophage differentiation into osteoclasts in the presence of OPG, indicating a different mechanism of action (29). Confirming data of previous works (19-20), we observed that sTWEAK levels are independently associated with CV events in CKD patients.

The most important finding of our study is the association of a combination of biomarkers of vascular calcification (OPG and OPN) and inflammation (sTWEAK) with the risk of CV events. This combination showed a good discriminative power, suggesting that marker combinations integrating different biological mechanisms might better stratify CKD patients. Despite the independent association that we observed between multimarker score and

risk of fatal and non-fatal CV events, the inclusion of this multimarker score improves clinical risk prediction in our population. Our findings support the potential inclusion of these biomarkers in risk prediction algorithms.

Finally, we want to highlight some limitations of our study for a correct interpretation of the results. Only CKD patients without history of CV events were included in the study. This was a necessary intentional bias because the study was aimed to primary prevention of CV events. A relative low number of fatal and non-fatal CV events was reported during the follow-up, which should limit the statistical power of our analysis.

In conclusion, OPG, OPN and sTWEAK impacted the predictability of cardiovascular outcomes. The information provided by these biomarkers was additive because the risk of developing a CV event increased along with the number of them altered. Thus, a multimarker score including OPG, OPN and sTWEAK concentrations was independently and statistically associated with CV outcomes in CKD patients.

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The NEFRONA Study investigators are listed in the Supplemental Material.

Figure Legends

Figure 1.- Effect of OPN, OPG and sTWEAK on cardiovascular outcomes.

A) Receiver-operating characteristic curves for cardiovascular prediction concerning OPG (red), OPN (green) or sTWEAK (blue) concentrations.

B) Kaplan-Meier plot of event-free patients when patients were grouped according to OPG or OPN above and sTWEAK below of their optimal cut-off points.

Figure 2.- Effect of multimarker score on cardiovascular outcomes.

A) Kaplan-Meier plot of event-free patient for cardiovascular prediction according to multimarker score.

B) Receiver-operating curves for cardiovascular events prediction. Curves are on the basis of logistic regression models incorporating conventional cardiovascular risk factors (age, sex, hyperlipidemia, hypertension, diabetes mellitus, smoking status, and eGFR) with or without multimarker score.

Table 1.- Characteristics of CKD patients according to cardiovascular outcomes.

	No (N=948)	Yes (N=95)	p
Age, years	61 (50-68)	65 (58-70)	<0.001
Male, N (%)	588 (62)	69 (73)	0.02
BMI, Kg/m^2	28 (25-31)	28 (26-32)	0.39
SBP, mmHg	140 (128-155)	149 (136-162)	0.001
DBP, mmHg	80 (74-89)	81 (72-89)	0.96
Cholesterol, mg/dL	177±39	180±50	0.61
LDL-cholesterol, mg/dL	100±33	103±44	0.50
HDL-cholesterol, mg/dL	47 (38-58)	43 (34-52)	0.02
Triglycerides, mg/dL	126 (91-177)	138 (104-181)	0.17
Glucose, mg/dL	98 (87-115)	117 (99-156)	<0.001
Hs-CRP, mg/L	1.83 (0.90-4.22)	3.25 (1.19-7.84)	0.006
eGFR, mL/min/1.73 m^2	26 (12-40)	21 (9.3-37)	0.08
ABI	1.04 (0.96-1.15)	1.00 (0.88-1.18)	0.10
c-IMT, mm	0.72 (0.61-0.87)	0.84 (0.68-1.15)	<0.001
Smoker, N (%)	532 (56)	61 (64)	0.08
Dyslipemia, N (%)	607 (64)	69 (73)	0.06
Medical Treatment, N (%)	534 (56)	62 (65)	
Diabetes Mellitus, N (%)	254 (27)	51 (54)	<0.001
Medical Treatment, N (%)	247 (26)	46 (48)	
Hypertension, N (%)	869 (92)	90 (95)	0.20
Medical Treatment, N (%)	843 (89)	86 (91)	
Familial CVD, N (%)	78 (8)	11 (12)	0.18
OPG, pg/mL	463 (254-672)	514 (278-992)	0.009
OPN, ng/mL	20.4 (11.0-39.5)	24.1 (12.9-53.4)	0.043
sTWEAK, pg/mL	399 (261-585)	293 (211-448)	<0.001

BMI: Body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; LDL: low-density lipoprotein; HDL: high-density lipoprotein; eGFR: estimated glomerular filtration rate; hs-CRP: high-sensitivity C-reactive protein; ABI: Ankle-brachial index; c-IMT: carotid intima/media thickness.

Table 2.- Spearman correlations coefficients between OPG, OPN, sTWEAK levels, and selected parameters in CKD patients.

	OPG		OPN		sTWEAK	
	r	p	r	p	r	p
Age, years	0.15	<0.001	-0.05	0.12	-0.02	0.42
BMI, kg/m^2	-0.13	<0.001	-0.13	<0.001	0.03	0.27
SBP, mm Hg	0.07	0.11	-0.02	0.95	0.03	0.25
DBP, mm Hg	-0.08	0.01	-0.01	0.85	-0.04	0.18
Cholesterol, mg/dL	-0.17	<0.001	-0.08	0.01	0.10	0.001
HDL-c, mg/dL	0.01	0.72	0.01	0.69	0.11	0.001
LDL-c, mg/dL	-0.19	<0.001	-0.10	0.003	0.15	<0.001
Triglicerydes, mg/dL	-0.05	0.14	-0.02	0.53	-0.08	0.02
Glucose, mg/dL	-0.04	0.17	-0.13	<0.001	0.01	0.86
Hs-CRP, mg/L	0.11	0.001	0.06	0.07	-0.04	0.22
eGFR, mL/min/1.73m^2	-0.48	<0.001	-0.42	<0.001	0.36	<0.001
c-IMT, mm	0.16	<0.001	-0.04	0.25	-0.13	<0.001
ABI	0.11	<0.001	0.08	0.01	-0.05	0.10
OPG, pg/mL	--------	--------	0.48	<0.001	-0.22	<0.001
OPN, ng/mL	0.48	<0.001	--------	--------	-0.11	0.001
sTWEAK, pg/mL	-0.22	<0.001	-0.11	0.001	--------	--------

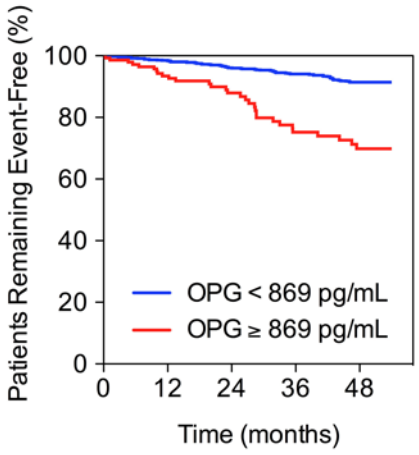
BMI: Body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; LDL: low-density lipoprotein; HDL: high-density lipoprotein; eGFR: estimated glomerular filtration rate; hs-CRP: high-sensitivity C-reactive protein; ABI: Ankle-brachial index; c-IMT: carotid intima/media thickness.

Table 3.- Univariate and multivariate Cox regression analyses predicting cardiovascular outcomes.

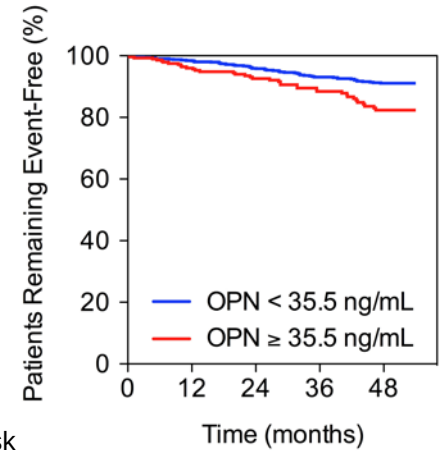
	Crude		Adjusted Model 1		Adjusted Model 2	
	Hazard Ratio CI (95%)	p	Hazard Ratio CI (95%)	p	Hazard Ratio CI (95%)	p
Age, per 10 years	1.34 (1.10-1.63)	0.004	1.24 (1.01-1.52)	0.04	1.25 (1.02-1.54)	0.03
Gender, male vs female	1.61 (1.02-2.53)	0.04	1.77 (1.11-2.84)	0.02	1.95 (1.22-3.14)	0.005
Current Smoker, yes vs no	1.39 (0.92-2.12)	0.12	----------	---------	----------	---------
Hypertension, yes vs no	1.45 (0.59-3.57)	0.42	----------	---------	----------	---------
Diabetes, yes vs no	2.83 (1.89-4.23)	<0.001	2.05 (1.11-3.76)	0.02	2.02 (1.10-3.71)	0.02
Hyperlipidemia, yes vs no	1.25 (0.79-1.96)	0.34	----------	---------	----------	---------
Familial CVD, yes vs no	1.43 (0.76-2.68)	0.27	----------	---------	----------	---------
eGFR, per 10 mL/min/1.73m^2	0.79 (0.69-0.90)	<0.001	0.97 (0.80-1.18)	0.79	0.92 (0.75-1.12)	0.39
Dialysis, yes vs no	2.64 (1.70-4.08)	<0.001	1.19 (0.58-2.46)	0.64	0.93 (0.42-2.09)	0.87
Hs-CRP, >3 mg/L	1.84 (1.22-2.77)	0.003	1.45 (0.95-2.21)	0.08	1.39 (0.91-2.14)	0.13
Antihypertensive drugs, yes vs no	0.98 (0.50-1.96)	0.97	----------	---------	----------	---------
Hypolipemic drugs, yes vs no	1.29 (0.85-1.97)	0.24	----------	---------	----------	---------
Oral antidiabetic drugs, yes vs no	1.01 (0.54-1.90)	0.96	----------	---------	----------	---------
Insulin treatment, yes vs no	2.82 (1.86-4.27)	<0.001	1.33 (0.71-2.51)	0.37	1.40 (0.74-2.63)	0.30
OPG > 869 pg/mL	3.98 (2.58-6.15)	<0.001	1.96 (1.07-3.62)	0.03	----------	---------
OPN > 35.5 ng/mL	2.02 (1.34-3.06)	0.001	1.69 (1.06-2.71)	0.03	----------	---------
sTWEAK < 370 pg/mL	2.62 (1.71-4.01)	<0.001	2.09 (1.30-3.34)	0.002	----------	---------
0 Biomarkers	1.00	--------			1.00	--------
1 Biomarker	1.86 (1.10-3.15)	0.02	----------	---------	1.44 (0.84-2.49)	0.19
2 Biomarkers	1.95 (0.96-3.78)	0.07	----------	---------	1.46 (0.65-3.30)	0.36
3 Biomarkers	13.02 (7.16-23.69)	<0.001	----------	---------	9.95 (3.99-24.79)	<0.001

Multivariable Cox analysis included variables that were statistically significant in the univariate analysis. HR, Hazard ratio; 95% CI, 95% confidence interval;
Model 1 adjusted also by OPG, OPN or sTWEAK based in their optimal cut-off points.
Model 2 adjusted also by number of biomarkers above (OPG and OPN) or below (sTWEAK) of their optimal cut-off points.
BMI: Body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; LDL: low-density lipoprotein; HDL: high-density lipoprotein; eGFR: estimated glomerular filtration rate; hs-CRP: high-sensitivity C-reactive protein.

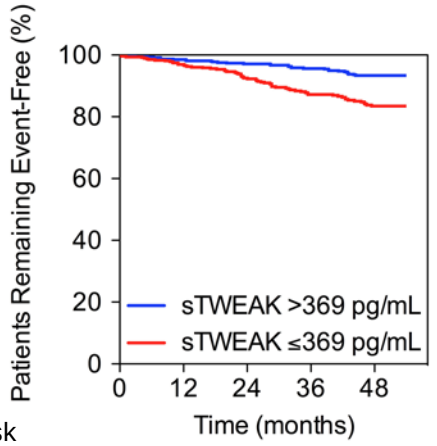
A



Number at risk					
Above cut-off point	893	832	742	675	569
Below cut-off point	150	120	83	64	49

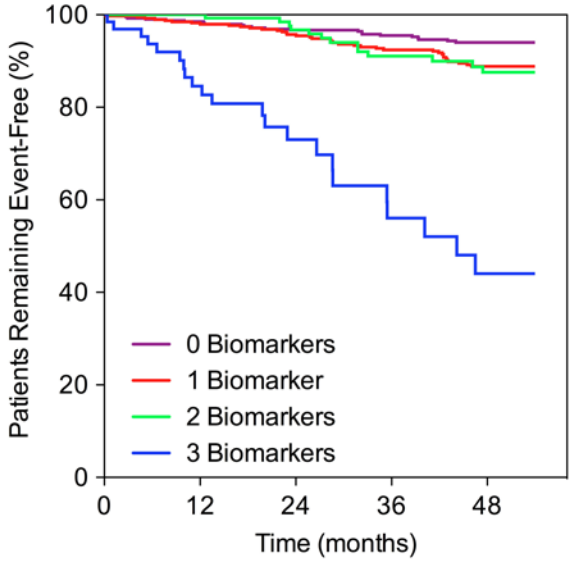


Number at risk					
Above cut-off point	742	698	630	575	495
Below cut-off point	301	254	195	165	121



Number at risk					
Above cut-off point	558	523	476	441	376
Below cut-off point	485	429	349	298	240

B



Number at risk					
0 Biomarkers	405	387	360	335	291
1 Biomarker	406	329	329	300	252
2 Biomarkers	166	147	112	89	67
3 Biomarkers	66	46	25	17	9