Elevated levels of plasma osteoprotegerin are associated with all-cause mortality risk and atherosclerosis in patients with stages 3 to 5 chronic kidney disease

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Abstract

Osteoprotegerin (OPG) regulates bone mass by inhibiting osteoclast differentiation and activation, and plays a role in vascular calcification. We evaluated the relationship between osteoprotegerin levels and inflammatory markers, atherosclerosis, and mortality in patients with stages 3-5 chronic kidney disease. A total of 145 subjects (median age 61 years, 61% men; 36 patients on hemodialysis, 55 patients on peritoneal dialysis, and 54 patients with stages 3-5 chronic kidney disease) were studied. Clinical characteristics, markers of mineral metabolism (including fibroblast growth factor-23 [FGF-23]) and inflammation (high-sensitivity C-reactive protein [hsCRP] and interleukin-6 [IL-6]), and the intima-media thickness (IMT) in the common carotid arteries were measured at baseline. Cardiac function was assessed by color tissue Doppler echocardiography. After 36 months follow-up, the survival rate by Kaplan-Meier analysis was significantly different according to OPG levels ($\chi^2 = 14.33; P = 0.002$). Increased OPG levels were positively associated with IL-6 ($r = 0.38, P < 0.001$), FGF-23 ($r = 0.26, P < 0.001$) and hsCRP ($r = 0.24, P = 0.003$). In addition, OPG was positively associated with troponin I ($r = 0.54, P < 0.001$) and IMT ($r = 0.39, P < 0.0001$). Finally, in Cox analysis, only OPG (HR = 1.07, 95%CI = 1.02-1.13) and hsCRP (HR = 1.02, 95%CI = 1.01-1.04) were independently associated with increased risk of death. These results suggested that elevated levels of serum OPG might be associated with atherosclerosis and all-cause mortality in patients with chronic kidney disease.

Key words: Atherosclerosis; Chronic kidney disease; Inflammation; Mortality; Osteoprotegerin

Introduction

The mortality of patients with chronic kidney disease is high, and cardiovascular disease is a major cause of premature deaths. Traditional risk factors that are highly prevalent in patients with chronic kidney disease cannot alone explain the poor outcome of the disease and non-traditional risk factors, such as inflammation, oxidative stress, and factors linked to vascular calcification or ossification, have therefore come into focus. Recent studies have indicated that osteoprotegerin (OPG), a soluble decoy receptor of the osteoclast activator (RANKL), has a pivotal role as an important regulatory molecule in vascular disease, such as arterial calcification and atherosclerosis (1,2).

OPG is a member of the tumor necrosis factor receptor family and a decoy receptor that blocks the interaction between the receptor activator of nuclear factor-κB (RANK) with its ligand (RANKL), thus inhibiting osteoclast differentiation and activity (3,4). Despite this apparent protective effect on the vascular system and bone, the increased levels of circulating OPG in patients with chronic kidney disease are reported to be associated with both aortic calcification and increased mortality (5). Moreover, it has also been demonstrated that OPG levels might play a pathophysiological role in the development of left ventricular hypertrophy and systolic dysfunction in patients without chronic kidney disease (5).
Interestingly, OPG can also be detected in atherosclerotic lesions and in humans elevated OPG concentrations have been associated with aortic plaque as well as an increased prevalence and severity of coronary artery disease. Omland et al. (6) reported that OPG levels were strongly predictive of long-term mortality and hospitalizations caused by heart failure in patients without chronic kidney disease with acute coronary syndrome, independent of conventional risk factors. We hypothesized that there is an association between increased serum OPG levels and the presence of atherosclerosis and that serum OPG levels could predict mortality in patients with stages 3 to 5 chronic kidney disease.

Material and Methods

Patients
All clinically stable dialysis patients and all stages 3 to 5 chronic kidney disease patients at the Pró-Renal Foundation in Curitiba (Paraná State, Brazil) were considered for enrollment. The exclusion criteria were: dialysis treatment less than 1 month, age younger than 16 years, and the presence of HIV or hepatitis B/C infection, or chronic inflammatory diseases. A total of 145 of 208 patients (hemodialysis=36; peritoneal dialysis=55; stages 3 to 5 chronic kidney disease=54) fulfilled the inclusion and exclusion study criteria, and agreed to participate in the study. The causes of kidney disease were chronic glomerular nephritis (31%), diabetic nephropathy (14%), hypertensive nephrosclerosis (33%), and other causes (22%). All hemodialysis patients had an arteriovenous fistula and were treated by conventional hemodialysis three times a week (3.5 to 4 h per session), with modified celluloseic membranes (cellulose acetate or derivatized cellulose membranes). Peritoneal dialysis patients were treated by standard continuous ambulatory peritoneal dialysis (4 exchanges, using 2 or 2.5 L of solutions with 1.5, 2.5, and 4.25% glucose). The medication of 84% of patients included antihypertensive medication (β-blocking agents, calcium channel blockers, angiotensin-converting enzyme inhibitors, or angiotensin receptor blockers), while 91% of patients received erythropoietin. Most patients also used other drugs commonly prescribed for terminal chronic kidney disease, such as phosphate binders, vitamin D supplementation, and iron saccharate, while only a few patients were taking lipid-lowering medication. All patients gave written informed consent and the ethics committee of the Hospital Evangelico de Curitiba approved the study protocol.

Study design
All patients underwent a baseline investigation comprising blood sampling and cardiovascular assessment, and were then followed for 36 months for analysis of survival.

Cardiovascular assessment

Intima-media thickness (IMT) and standard echocardiography. The IMT was estimated using semiautomatic edge detection software (GE Vingmed Ultrasound AS, Norway) according to the recommendations of the American Society of Echocardiography (7). All ultrasound examinations were performed using an M3S multifrequency transducer and a Vivid 1 cardiac ultrasound unit (GE Vingmed Ultrasound AS) linked to a PC workstation with the preinstalled EchopacPc version 0.8 software (GE Vingmed Ultrasound AS). All two-dimensional and Doppler variables were acquired and analyzed according to the guidelines of the American Society of Echocardiography (8,9). The left ventricular (LV) mass was calculated according to the Penn convention and the left ventricular mass index (LVMI) was calculated by normalizing ventricular mass to height to the power 2.7. LV hypertrophy was defined as LVMI ≥49 g/m²² for men and ≥45 g/m²² for women (8,9).

Laboratory analyses and clinical assessments

Blood samples were collected from patients after they had fasted overnight. They were collected from hemodialysis patients in the midweek and from patients with stages 3 to 5 chronic kidney disease undergoing peritoneal dialysis at regular clinic visits. Plasma and serum were stored at −70°C pending biochemical analysis. Serum concentrations of parathyroid hormone, interleukin-6, and high-sensitivity C-reactive protein (hsCRP) were quantified using immunometric assays on an Immulite automatic analyzer (Siemens Medical Solutions Diagnostics, USA). Other circulating risk markers were measured by using a commercial ELISA kit (R&D Systems, Inc. USA): serum OPG (R&D Systems Inc.) and plasma fibroblast growth factor-23 (FGF-23) (Millipore Corporate Headquarters, USA). The serum level of cardiac troponin I level was analyzed by an immunometric assay, using an Immulite 1000 Analyzer (Siemens Medical Solutions Diagnostics) according to the manufacturer’s instructions. Serum albumin, creatinine, uric acid, urea, potassium, calcium, phosphate, and total and high-density lipoprotein cholesterol concentrations were determined using a Konelab 20XT centrifuge analyzer (Thermo Electron Corporation, Finland). All analyses were performed at the Renal Medicine
Laboratory, Clinical Research Center, Karolinska Institute, Stockholm, Sweden.

Statistical analysis

Data are reported as median (interquartile range) or mean±SD, as appropriate. Statistical significance was set at P<0.05. Correlations (r) were calculated using the non-parametric Spearman rank test. Survival analysis used the Kaplan-Meier survival curve or the Cox proportional hazard model. The results of univariate and multivariate Cox regression analyses are reported as hazard ratios with 95%CI. The model was adjusted for age, sex, inflammatory markers, and the presence of diabetes mellitus. The proportional hazard assumption was checked using −log-log plots. Multivariate regression linear analyses were used to assess independent predictors of IMT. For this analysis, age, presence of diabetes, and levels of interleukin-6 and OPG were dichotomized according to their median values. All statistical analyses were performed using the SAS statistical software (Version 9.2, SAS Institute Inc., USA).

Results

Clinical and biochemical data

Clinical characteristics of the 145 patients are summarized in Table 1. The levels of OPG presented a median value of 8.9 pM (1.8-33.2 pM). Patients were divided into two groups according to the median level of OPG (Table 2). Patients with a higher OPG were older and had significantly increased plasma levels of interleukin-6, hsCRP, FGF-23, troponin I, and parathyroid hormone. Moreover, patients with increased OPG had significantly higher IMT and LV mass than patients with lower OPG. Finally, serum albumin levels were significantly reduced in patients with higher OPG plasma levels.
hormone, LVMI, and IMT but were negatively associated with peak systolic velocity and serum albumin concentration. A multivariate regression analysis was performed predicting for increased IMT to test whether the associations between IMT and OPG and interleukin-6 were a reflection of aging or whether they were confounded by sex or the presence of comorbid conditions, such as diabetes mellitus. Results showed that the association of IMT with OPG was independent of these factors with the exception of interleukin-6 (Table 4).

OPG levels and clinical outcome
During 3 years of follow-up, 40 patients died due to cardiovascular causes (n=25), malignancies (n=3), infectious diseases (n=7), and other causes (n=5). There was a significant difference in mortality between pre-dialysis (n=4), peritoneal dialysis patients (n=21), and hemodialysis patients (n=15) (χ² = 45.86; P<0.0001). Twenty-three patients who underwent kidney transplantation were censored. Patients were divided into two groups according to the median plasma OPG level to compare survival rates. According to the Kaplan-Meier curve, patients with elevated OPG levels presented a significantly increased all-cause mortality risk than patients with lower OPG levels (log rank = 19.75; P<0.0001). Cox regression analysis showed that patients

Table 2. Clinical, laboratory and echocardiographic findings, according to the median of osteoprotegerin (OPG) levels (8.1 pM).

|                  | Higher OPG | Lower OPG | P   |
|------------------|------------|-----------|-----|
| Age (years)      | 63.5 (25-92) | 55 (16-83) | 0.001 |
| Gender (% males) | 57         | 64        | NS  |
| DM (%)           | 30         | 29        | NS  |
| Death (%)        | 38         | 8         | <0.0001 |
| eGFR (mL/min)    | 6.3 (2.7-41.6) | 20.4 (2.6-47.7) | <0.0001 |
| Hb (g/dL)        | 11.2 ± 0.2 | 12.3 ± 0.2 | <0.01 |
| hsCRP (mg/dL)    | 5.9 (0.3-74.9) | 2.9 (0.3-54.4) | <0.01 |
| IL-6 (pg/mL)     | 6.9 (0.1.95.1) | 2.9 (0.10-98.7) | <0.0001 |
| TNF-α (pg/mL)    | 18.6 (7.3-56.2) | 15.5 (7.1-43.5) | <0.05 |
| PTX3 (pg/mL)     | 4.4 (1.3-18.7) | 3.1 (1.4-15.1) | <0.0001 |
| PTH (ng/mL)      | 221 (31-2086) | 150 (19.4-1040) | <0.001 |
| SRAGE (pg/mL)    | 2360 (742-5778) | 1651 (471-5180) | <0.001 |
| S100A12 (ng/mL)  | 62.5 (5.0-464.2) | 36.7 (12.2-399.4) | <0.05 |
| Phosphate (pg/mL)| 1.6 (0.5-3.6) | 1.3 (0.8-3.0) | <0.05 |
| FGF 23 (pg/mL)   | 618 (61.79.560) | 181 (44-63,700) | <0.01 |
| Fetuin (pg/mL)   | 0.40 (0.20-0.73) | 0.42 (0.13-0.54) | NS  |
| Troponin I (ng/mL)| 0.10 (0.001-0.48) | 0.04 (0.001-0.48) | <0.0001 |
| 8-OH-dG (ng/mL)  | 0.270 (0.004-0.78) | 0.11 (0.002-1.454) | <0.0001 |
| E’ (cm/s)        | 4.4 ± 1.9 | 5.3 ± 2.3 | <0.05 |
| PSV (cm/s)       | 4.5 (0.9-9.8) | 4.7 (2.1-8.3) | NS  |
| LVM (g)          | 62 (30-160) | 57 (30-99) | 0.05 |
| IMT (cm)         | 1.04 ± 0.43 | 0.83 ± 0.29 | <0.001 |

Data are reported as means ± SD and median (interquartile range). See Table 1 for abbreviations. Statistical analyses were carried out using the t-test and the Wilcoxon signed rank test (NS: not significant).

Table 3. Spearman rank correlation between plasma inflammatory and vascular calcification markers.

|          | IL-6     | S-Alb   | TNF-α   | hsCRP   | Age     | Phosphate |
|----------|----------|---------|---------|---------|---------|-----------|
| Age      | -0.005 (0.95) | -0.002 (0.79) | -0.22 (0.006)* | 0.001 (0.98) | -0.31 (0.0001)* |
| hsCRP    | 0.63 (<0.0001) | -0.21 (0.001)* | 0.20 (0.01)* | 0.0019 (0.98) | 0.07 (0.37) |
| IL-6     | -0.19 (0.01)* | 0.32 (<0.0001)* | 0.63 (<0.0001)* | -0.005 (0.95) | 0.17 (0.03)* |
| Fetuin   | -0.19 (0.01)* | 0.32 (<0.0001)* | -0.17 (0.02)* | -0.09 (0.27) | -0.007 (0.35) | 0.08 (0.31) |
| OPG      | 0.38 (<0.0001)* | -0.40 (<0.0001)* | 0.14 (0.07) | 0.24 (0.003)* | 0.36 (<0.0001)* | 0.14 (0.07) |
| FGF-23   | 0.27 (0.0005)* | -0.22 (0.006)* | 0.34 (<0.0001)* | 0.19 (0.01)* | -0.39 (<0.0001)* | 0.51 (<0.0001)* |

IL-6: interleukin-6; S-Alb: serum albumin; TNF-α: tumor necrosis factor alpha; hsCRP: high-sensitivity C-reactive protein; OPG: osteoprotegerin; FGF-23: fibroblast growth factor-23.
with higher OPG levels had an increased all-cause mortality risk, which persisted after adjustment for age, sex, diabetes mellitus, and levels of hsCRP and serum albumin (Table 5).

**Discussion**

In this study, serum OPG levels (a soluble member of the tumor necrosis factor receptor superfamily) were found to be associated with age, IMT, LV mass, elevated levels of serum troponin I, and a tendency of increased LV hypertrophy, as verified by increased LV mass evaluated by echocardiography. Moreover, the chief finding of this study was that elevated OPG levels were independently associated with all-cause mortality as well as with atherosclerosis assessed by increased IMT, as demonstrated by multivariate linear regression analysis.

The Dallas Heart Study (10) showed that plasma OPG levels were independently associated with coronary artery calcification and aortic plaque in a sample of patients aged 30 to 65 years who did not have chronic kidney disease, and demonstrated that OPG associates with development of atherosclerosis in the general population. However, it seems that OPG might also play a role in the development of atherosclerosis in the presence of renal failure. Sigrist et al. (11) showed that increased OPG levels, independently of elevated hsCRP, were associated with mortality in patients with stages 4 and 5 chronic kidney disease. Conversely, Matsubara et al. (12) reported that elevated OPG levels were associated with clinical outcome in patients with chronic kidney disease, and speculated that inflammation might have a possible additive effect on survival in patients with increased OPG levels. Similarly, this study also shows that OPG is associated with inflammatory markers, such as interleukin-6 and hsCRP. Taking into account that OPG has an active role in the vascular cytokine system, these associations seem biologically plausible. In fact, OPG expression is induced in vascular smooth muscle cells by pro-inflammatory cytokines (IL-1 and TNF-α).

It is still debated whether increments in OPG levels reflect a protective and counter-regulatory effect or whether such a relation might simply reflect the level of vascular inflammatory processes that underlie the development and evolution of the atherosclerotic process (13,14). Interestingly, an independent association between OPG and the presence of atherosclerosis, assessed by increased IMT, was verified in this study, using a multiple regression model; serum OPG concentration was the only variable that was independently associated with IMT. This finding, which is in accordance with other studies in dialysis patients, supports a possible

| Parameter                        | Parameter estimate | Standard error | P     |
|----------------------------------|--------------------|----------------|-------|
| Intercept                        | 0.751              | 0.027          | <0.0001 |
| Age (>65 years)                  | 0.061              | 0.026          | 0.021* |
| Sex (male)                       | 0.049              | 0.025          | 0.056  |
| Diabetes mellitus (presence)     | -0.035             | 0.026          | 0.187  |
| OPG (>8.9 pM)                    | 0.053              | 0.025          | 0.041* |
| IL-6 (>4.1 pg/mL)                | 0.047              | 0.025          | 0.068  |

The adjusted $r^2$ of the model was 0.14. Categories for age, osteoprotegerin (OPG), and interleukin-6 (IL-6) were calculated according to the median value of the group.

**Table 5.** Cox regression analysis for osteoprotegerin (OPG) levels regarding all-cause of mortality in 145 patients with chronic kidney disease stages 3 to 5.

| Parameter                        | Parameter estimate | Standard error | P     | HR (95%CI) | P     | HR (95%CI) | P     |
|----------------------------------|--------------------|----------------|-------|------------|-------|------------|-------|
| OPG (per pM)                     | 1.07 (1.01-1.13)   | 0.01           | 1.10 (1.04-1.14) | <0.0001 | 0.01   | 1.10 (1.04-1.14) | <0.0001 |
| Age (per year)                   | 1.00 (0.9-1.03)    | 0.60           | 1.90 (0.97-3.9)  | 0.05    | 0.60   | 1.90 (0.97-3.9)  | 0.05    |
| Gender (F vs M)                  | 0.48 (0.22-1.01)   | 0.05           | 2.20 (1.09-4.5)  | 0.02    | 0.05   | 2.20 (1.09-4.5)  | 0.02    |
| hsCRP (per mg/dL)                | 1.02 (1.00-1.04)   | 0.02           | 1.05 (1.01-1.04) | 0.03    | 0.02   | 1.05 (1.01-1.04) | 0.03    |
| Albumin (per g/L)                | 0.94 (0.88-1.01)   | 0.12           | 0.91 (0.88-0.97) | 0.006   | 0.12   | 0.91 (0.88-0.97) | 0.006   |
| DM (presence vs absence)         | 1.34 (0.61-2.95)   | 0.46           | 0.80 (0.39-1.7)  | 0.56    | 0.46   | 0.80 (0.39-1.7)  | 0.56    |

Output of a Cox regression analysis with the absence of clinical history of cardiovascular disease taken as the reference. OPG: osteoprotegerin; hsCRP: high-sensitivity C-reactive protein; DM: diabetes mellitus.
association of OPG level with atherosclerosis, and might indicate a deleterious effect of OPG on endothelial integrity (15-17). It has been hypothesized that, in the vascular system, up-regulation of OPG causes a TNF-α sensitization of the endothelial cells, in which monocytes migrate into the vascular intima. In addition, OPG might play a role in the pathogenesis of plaque formation and thrombogenesis through a synergistic effect of OPG and von Willebrand factor in endothelial cells of patients with coronary artery disease (6).

It has also been postulated that OPG is secreted in the cardiovascular system (18). In fact, OPG has been found to be expressed in the heart and the vascular walls in rodents (19). The hypothesis that OPG might reflect advanced cardiovascular disease has been described in several studies in populations without chronic kidney disease (20-23). The Tromsø study (20), a cohort study with 12 years of follow-up, performed in the general population, revealed that serum OPG concentration was significantly associated with incident myocardial infarction, ischemic stroke, and all-cause mortality, independently of traditional cardiovascular risk factors. Moreover, OPG has also been considered an indicator of myocardial failure and an independent predictor of mortality in patients with acute coronary syndrome. The demonstration of an independent association between OPG levels at baseline and the incidence of hospitalizations owing to heart failure suggests that OPG concentration could be a predictor of the development of heart failure. Ueland et al. (21) reported that increased expression of components of the OPG/RANKL/RANK axis is associated with the development of heart failure through the mediation of matrix degradation, and that inflammation and ventricular remodeling might play a role in this process.

We could also verify an intriguing relationship between increased serum OPG and troponin I levels, and between increased OPG concentration and increased LV mass, as assessed by standard echocardiography, possibly reflecting an association between OPG level and myocardial damage in patients with chronic kidney disease. A population-based study (6) showed that elevated circulating OPG levels were predictive of future cardiovascular events, while in another study in a population without chronic kidney disease (18), circulating OPG levels were found to be increased both in patients with unstable angina and among those with myocardial infarction with ST-segment elevation. It has also been demonstrated that OPG levels are elevated in subjects without chronic kidney disease with only mildly impaired LV systolic function, suggesting that activation of the OPG/RANKL/RANK system is an early phenomenon in the process of ventricular dysfunction and heart failure development, in particular post-infarction heart failure.

An experimental study performed by Shaker et al. (23) demonstrated strong immunostaining of OPG/RANKL/RANK within thrombus material obtained at the site of plaque rupture during acute myocardial infarction. That study showed increased RANKL expression in T-cells from unstable angina patients, accompanied by enhanced expression of its corresponding receptor in monocytes, and the authors suggested that increased RANKL levels could contribute to inflammation, leukocyte recruitment, and matrix degradation within atherosclerotic lesions. Based on these experimental and clinical findings, it seems plausible that these mediators might not only be markers of inflammation, but also contributors to atherogenesis and plaque destabilization.

Other studies have focused on the association between increased OPG levels and LV hypertrophy. Omland et al. (6) demonstrated that OPG level was independently associated with LV function in both sexes, and with LV hypertrophy and concentric remodeling in men, in the general population. A possible link between inflammation, pressure overload, and LV hypertrophy was proposed by these authors, based on the possibility that the observed link between OPG level and LV mass might also reflect the ability of OPG to mirror pressure-independent inflammatory mechanisms in the development of LV dysfunction. The results of a close association between levels of OPG and inflammatory markers and LV mass in the current study support this putative link.

Some limitations of this study should be noted. First, we studied a cohort of prevalent dialysis patients who, by definition, are survivors; this could be a bias for the survival analysis. Second, a single sample at a certain time point may fail to reflect the natural course of the process being studied. Third, we did not measure circulating RANKL levels. Finally, in view of the relatively low numbers of patients and clinical events, as well as the cross-sectional design of the study, which limits the ability to establish causal relationships (especially in the evaluation of atherosclerosis and cardiovascular assessment), our results need verification and should be interpreted with caution.

In summary, we report that in this study with Brazilian patients with chronic kidney disease, circulating OPG levels were independent prognostic indicators of all-cause mortality risk, being closely related to the degree of renal insufficiency and atherosclerosis. As previously proposed by Morena et al. (3), we also suggest that follow-up of this marker could be potentially useful in the clinical evaluation of patients with chronic kidney disease and, perhaps, represent a possible target for future therapeutic intervention. Further and larger studies, in patients with chronic kidney disease, are necessary to confirm this hypothesis.

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Elevated osteoprotegerin levels in chronic kidney disease

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