Elevated plasma osteoprotegerin levels are associated with venous thrombosis and bleeding in patients with polycythemia vera

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Summary

Patients with polycythemia vera (PV) have an increased risk for the development of thrombohemorrhagic complications. The pathogenesis of these complications is still unclear. An important role in vascular disease has recently been attributed to osteoprotegerin (OPG). It has been shown that various tissues of the cardiovascular system produce OPG, and there is growing evidence of an association between elevated serum OPG levels and cardiovascular morbidity. We evaluated if OPG was associated with an increased risk of venous thrombosis or bleeding complications in a cohort of 114 PV patients. The analysis consisted of a retrospective and a prospective part. In the retrospective univariate analysis, a one unit change in OPG caused the odds of venous thrombosis to increase by 40% (p=0.005) and the odds of bleeding to increase by 52% (p=0.001). Multivariate analysis only slightly attenuated the association to 33% (p=0.03) and 37% (p=0.013) for venous thrombosis and bleeding, respectively. OPG was also related to the development of the combined outcome of venous thrombosis and bleeding in the prospective analysis (log-rank-test: p=0.017). This is the first report that links the occurrence of venous thrombosis or bleeding to elevated OPG levels.

Keywords

Osteoprotegerin, polycythemia vera, thrombosis, bleeding, vascular complications

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Introduction

Arterial and venous thromboembolic complications, microvasculatory disturbances and bleeding are common complications in polycythemia vera (PV). The incidence of thrombotic events has been estimated to range from 4 to 11 events per 100 patient years once the diagnosis is established (1). Often, thromboembolic events lead to the diagnosis of PV. The pathophysiologic mechanism of thromboembolism in this myeloproliferative disorder, however, remains elusive (2). Apart from an elevated hematocrit (3) and of rare genetic mutations (e.g. factor V Leiden or prothrombin gene mutation), no laboratory parameter is predictive of thromboembolism in patients with PV (4, 5). Patients with secondary polycythemia, in contrast, despite having high hematocrit levels, do not have an elevated risk of thrombosis (6). Even though patients with PV have fewer conventional risk factors for atherosclerosis, it has been shown that endothelial function is impaired in patients with PV (7). Von Willebrand factor and thrombomodulin, plasma markers of endothelial activation/damage, were significantly elevated in PV patients (8). Atherogenesis with the loss of the endothelial barrier function are possible downstream effects of endothelium perturbation (9). Consequently, endothelial dysfunction together with a procoagulate state may be responsible for vascular events observed in this patient collective. An emerging candidate as biochemical marker of vascular disease is osteoprotegerin (OPG) since OPG levels are augmented in cardiovascular disease (10, 11). This connection of OPG with vascular biology has recently been found (12). OPG is a soluble receptor of the tumor necrosis factor (TNF) receptor superfamily (13) and has primarily been discovered as a member of the central cytokine system regulating bone density. OPG acts as a decoy receptor for the receptor activator of NF-κB ligand (RANKL) and prevents RANKL/RANK interaction and the associated bone resorption (14). The regulation of bone density, however, does not seem to be the only physiologic function of this cytokine system. Besides an involvement in mammary gland development (15) it plays a yet undefined role in the vascular system. A possible significance of OPG for vascular physiology was first suspected when $OPG^{-/-}$ mice exhibited a marked calcification of the aorta and renal arteries together with a distinct de-

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crease in bone density (16). Tissues of the cardiovascular system [endothelial cells (17, 18), heart (13), arterial vascular wall (13), vascular smooth muscle cells (19)] produce OPG besides various other tissues (13, 20). Recent clinical studies have reported a significant correlation between elevated serum OPG levels and arterial vessel disease in patients with coronary artery disease (10, 21), stroke (11) and diabetes (11, 22). In the present study, we assessed the association between OPG-values and the occurrence of vascular complications in 114 patients with PV.

Patients, materials and methods

Patients

The present study involved 114 patients (57 male, 57 female; median age 67 years, range 28–92) with the diagnosis of PV based on the polycythemia vera study group criteria (23). One hundred and two patients were simultaneously enrolled in a prospective clinical trial. In this trial, the patients were randomized to receive either low dose aspirin (100mg/day) or placebo, or were merely prospectively observed if they presented with clear indications or contraindications for aspirin (ECLAP study) (24). Of the 102 patients, 37 were randomized and 65 patients were only observed prospectively. Clinical and laboratory data were collected prospectively following a defined protocol and entered into a registry. Twelve subsequent PV patients who were observed in the same fashion were also included for the present study. All 114 patients were treated according to the following therapeutic guidelines: after a venous thromboembolic event, patients were treated with anticoagulant (warfarin) therapy for 3–6 months; patients with an arterial thromboembolic event received aspirin, those patients with recurrent arterial thromboembolic events, atrial fibrillation or congestive heart failure were on long term warfarin; patients with venous or arterial thromboembolic events were also treated with myelosuppressive agents (busulfan n=10, hydroxyurea n=46, pipobroman n=2) or with interferon alpha (n=30) to reduce platelet count to a value lower than $600x10^9$/L. Patients without history of thrombosis were treated with myelosuppressive agents only if classified as high-risk patients (platelet count higher than $1.500x10^9$/L or platelet count higher than $600x10^9$/L and age above 60 years). Patients were phlebotomized to maintain a hematocrit level between 40% and 45%. They were invited for a follow up visit at least every 6 months where the physician in charge assessed if a thromboembolic or a bleeding event had occurred. The mean observation time of the patients was 102 months (± 68 months SD). Vascular events before the diagnosis of PV were determined retrospectively using validated questionnaires and reviewing clinical records. The prevalence of a cardiovascular complication in a patient was noted when the following events were determined either retrospectively or prospectively: A) arterial thrombosis: acute myocardial infarction, cerebral insult, transient ischemic attack, peripheral arterial vessel disease or stenocardiac pain due to coronary artery disease; B) venous thrombosis: deep vein thrombosis, pulmonary embolism; C) bleeding complication: severe hemorrhage. Microvascular disturbances and superficial phlebitis were not considered. Written informed consent was obtained from all patients.

Plasma OPG measurement

Blood samples were collected from all patients and heparinized plasma was frozen at –70°C until use. All assays were measured blinded to any clinical information. OPG was detected by a sandwich enzyme immunoassay using a mouse monoclonal antibody and a rabbit polyclonal antibody for detection, and recombinant human OPG as standard material (Biomedica, Vienna, Austria). All samples were measured in duplicate and the results were averaged. The assay detects both monomeric and dimeric forms of OPG, including OPG bound to its ligand. The sensitivity of the assay was 0.14pmol/l (defined as »0« + 3SD). To show that OPG levels are a relatively constant plasma entity, repeated OPG measurements were performed. In 25 patients repetitive samples in 1 year intervals, up to 4 years, were available. The coefficient of variation of OPG was 14.5% (4 patients were followed for 1 year, 9 patients for 2 years, 10 patients for 3 years and 2 for 4 years).

Statistical analysis

Because OPG was measured at a point in time when some patients had already developed the outcomes of interest while others would develop the events later on during the observational period, we used two approaches for the statistical analysis of our data: a retrospective part (including all patients), as well as a prospective part (including only those participants who had remained free of the outcomes of interest until OPG measurement). Because the numbers of myocardial infarctions, strokes, transient ischemic attacks, and peripheral arterial events were low, we decided to use a composite outcome comprising of all of these events taken together. We think that from a pathophysiolocical standpoint this approach is warranted.

In the retrospective analysis which included PV patients who had their OPG values taken either before or after the event, OPG levels of patients with arterial thrombotic events or venous thrombotic events or bleeding complications were compared to OPG levels of PV patients without the respective complication (Fig. 1). For venous thrombotic events and bleeding complications we then used multiple logistic regression analysis since

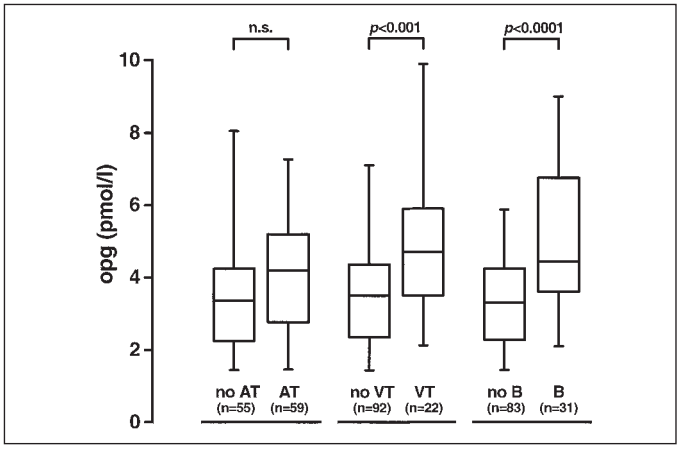

Figure 1: Retrospective analysis. OPG levels of PV patients with arterial thrombotic events (AT) or venous thrombotic events (VT) or bleeding complications (B) are compared to OPG levels of PV patients without the respective complication.

patients with these 2 event entities showed significantly higher OPG values than the patients without the respective events.

Assessment of confounding

We used a well established method (25) for the assessment of confounding of the association between OPG and the respective outcomes. Using this method, we first fit a univariate model, examining the univariate association of OPG with the outcomes of interest. Then, we carried out multiple bivariate analyses with OPG and one of the following covariates together in the model: age (years), sex, creatinin level, platelet count, systolic blood pressure (mmHg), diastolic blood pressure (mmHg), cholesterol

Table 1: Patients' characteristics and prevalence of risk factors for thrombosis or bleeding.

Characteristic	*Patients without venous thrombosis* n=92	*Patients with venous thrombosis* n=22	*Total* n=114	*p* for difference of groups*
Female	45/92 (48.9%)	12/22 (54.6%)	57/114 (50%)	0.635
Age, years	63.7 (±13.9)	69.9 (±9.4)	64.8 (±13.4)	**0.044**
Disease duration, months	102.0 (±71.8)	100.1 (±50.1)	101.6 (±68.0)	0.905
BMI, kg/m2	25.6 (±3.7)	24.6 (±3.3)	25.5 (±3.7)	0.261
Smoker	10/91 (11.0%)	2/22 (9.1%)	12/113 (10.6%)	0.857
Ex smoker	7/91 (7.7%)	1/22 (4.6%)	8/113 (7.1%)	0.775
Diabetes	4/92 (4.4%)	1/22 (4.6%)	5/114 (4.4%)	0.968
Creatinine, mg/dl	1.06 (±0.23)	1.09 (±0.25)	1.06 (±0.24)	0.552
Platelet count at diagnosis, $\times 10^9$/l	498 (±322)	551 (±347)	508 (±326)	0.504
Platelet count, $\times 10^9$/l	432 (±200)	452 (±236)	436 (±206)	0.679
Hematocrit at diagnosis, %	53.8 (±7.1)	51.2 (±6.5)	53.3 (±7.0)	0.126
Protein C, %	82.7 (±24.4)	60.6 (±28.1)	78.1 (±26.6)	**0.009**
Protein S, %	74.6 (±28.9)	60.8 (±25.5)	71.8 (±28.6)	0.138
Homocystein, µmol/l	13.6 (±6.2)	16.2 (±7.8)	14.1 (±6.6)	0.226
Antithrombin III, %	91.5 (±13.5)	93.9 (±12.8)	92.0 (±13.3)	0.561
Factor V Leiden mutation	3/70 (4.0%)	5/20 (25%)	8/90 (8.9%)	**0.004**
Prothrombin mutation	1/67 (1.49%)	4/19 (21.1%)	5/86 (5.8%)	**0.001**
OPG, pmol/l	3.63 (±1.66)	5.06 (±2.32)	3.9 (±1.9)	**0.001**

Characteristic	*Patients without bleeding event* n=83	*Patients with bleeding event* n=31	*Total* n=114	*p* for difference of groups*
Female	40/83 (48.2%)	17/31 (54.8%)	57/114 (50%)	0.528
Age, years	62.5 (±13.8)	71.2 (±9.6)	64.8 (±13.4)	**0.002**
Disease duration, months	85.7 (±63.6)	144.2 (±61.4)	101.6 (±68.0)	**0.0001**
BMI, kg/m2	25.9 (±3.7)	24.2 (±3.2)	25.5 (±3.7)	**0.03**
Smoker	8/82 (9.8%)	4/31 (12.9%)	12/113 (10.6%)	0.736
Ex smoker	6/82 (7.32%)	2/31 (6.5%)	8/113 (7.1%)	0.818
Diabetes	2/83 (2.4%)	3/31 (9.7%)	5/114 (4.4%)	0.092
Creatinine, mg/dl	1.07 (±0.25)	1.04 (±0.20)	1.06 (±0.24)	0.524
Platelet count at diagnosis, $\times 10^9$/l	502 (±348)	459 (±292)	508 (±326)	0.545
Platelet count, $\times 10^9$/l	457 (±214)	378 (±173)	436 (±206)	0.070
Hematocrit at diagnosis, %	53.5 (±6.9)	52.7 (±7.4)	53.3 (±7.0)	0.813
Protein C, %	76.0 (±25.3)	81.9 (±28.9)	78.1 (±26.6)	0.421
Protein S, %	69.4 (±30.1)	76.0 (±25.9)	71.8 (±28.6)	0.404
Homocystein, µmol/l	14.2 (±6.6)	14.0 (±6.5)	14.1 (±6.6)	0.922
Antithrombin III, %	91.7 (±13.8)	92.6 (±12.5)	92.0 (±13.3)	0.799
Factor V Leiden mutation	7/64 (10.9%)	1/26 (3.8%)	8/90 (8.9%)	0.284
Prothrombin mutation	3/60 (5%)	2/26 (7.7%)	5/86 (5.8%)	0.624
OPG, pmol/l	3.45 (±1.53)	5.13 (±2.20)	3.9 (±1.9)	**0.0001**

* p was calculated by student's t-test or chi square test. Bold p-values signify statistical significance ($p<0.05$). Values are given as mean ± 1 standard deviation or number of patients per total number of patients.

level (mg/dl), diabetes (yes/no), homocysteine (µmol/l), body mass index (BMI, kg/m^2), the factor V-Leiden mutation (yes/no), the prothrombin mutation (yes/no), protein C activity (percent), protein S activity (percent), antithrombin III (percent), the intake of warfarin/marcoumar, duration of disease, and the presence or absence of arterial thromboembolic events. Confounding was judged to be present if the crude point estimate was changed by more than 10% by the second covariate. None of the above variables influenced the association between OPG and the outcomes at this 10% cut-off and, consequently, no confounding was judged to be present. We, therefore, decided to present adjusted results for age and gender only. The appropriateness of model-fit was assessed using the Hosmer-Lemeshow goodness-of-fit-test.

In the prospective part of our analysis we only evaluated those patients who had their OPG values taken before the first thrombotic or bleeding event and compared them to the remaining subjects who never experienced a thrombotic or bleeding event either before OPG-measurement or thereafter. Follow-up time started at the point in time when OPG was measured. We used a composite outcome consisting of bleeding and thrombotic complications. This was done since each outcome individually would have provided too few events.

We used the Kaplan-Meier method to compare patients above and below the mean OPG level. The log-rank test was used to calculate the associated statistical significance. The STATA software package was used for all analyses (*Stata Statistical Software: Release 7.0*. College Station, TX: StataCorp LP).

Results

Baseline characteristics

The patients' characteristics and the prevalence of other risk factors for thrombosis or bleeding are shown in table 1 (patients are divided into groups with or without venous thrombotic or bleeding event). Patients with venous thrombosis had significantly lower protein C levels, a significantly higher prevalence of the factor V Leiden mutation, and a significantly higher prevalence of the prothrombin mutation than control subjects without thrombosis. Patients with bleeding event(s) had a significantly longer disease duration and a significantly lower BMI than control subjects without bleeding event. Patients of both symptomatic groups were significantly older than patients without the respective events.

Retrospective analysis for the assessment of the risk for thrombosis and bleeding

It is known that age and renal insufficiency can influence systemic OPG levels, however, neither the above mentioned nor any of the other tested variables (see statistical methods section) proved to be confounders in the association of plasma OPG levels with venous thrombotic events or bleeding complications.

When the effect of OPG on the development of venous thrombosis was assessed in our retrospective cohort of 114 PV patients, we found that a change of 1pmol/l in OPG plasma levels caused the odds of developing thrombosis to increase by 40% (OR=1.4, p=0.005) in the univariate analysis (table 2). In the multivariate analysis, adjusting for age and gender, this effect was only slightly attenuated to 33% (OR=1.33, p=0.03). In other

Table 2: Effect of osteoprotegerin (OPG) on the development of venous thrombosis, bleeding, and their combined outcomes using logistic regression analysis. Odds ratios for OPG and age given for a one unit change of the respective, continuous variable. The odds ratio for the gender variable compares females to males. The multivariate models contains all three variables at a time. Upon comparison of the univariate- and multivariate models, it can be seen that age and gender do not confound the associations between OPG and the respective outcomes.

Venous Thrombosis

	univariate analysis		multivariate analysis	
	OR	95% CI	OR	95% CI
OPG (unit = 1pmol/l)	1.4	1.11 - 1.78	1.33	1.03 - 1.73
age (unit= 1year)	1.04	1.0002 - 1.09	1.02	0.97 - 1.07
gender (male vs. female)	1.42	0.55 - 3.7	1.17	0.42 - 3.27

Bleeding

	univariate analysis		multivariate analysis	
	OR	95% CI	OR	95% CI
OPG (unit = 1pmol/l)	1.52	1.2 - 1.92	1.37	1.07 - 1.76
age (unit= 1year)	1.06	1.02 - 1.11	1.04	0.99 - 1.09
gender (male vs. female)	1.19	0.53 - 2.7	0.83	0.33 - 2.09

Thrombosis & Bleeding

	univariate analysis		multivariate analysis	
	OR	95% CI	OR	95% CI
OPG (unit = 1pmol/l)	1.51	1.2 - 1.91	1.32	1.03 - 1.68
age (unit= 1year)	1.07	1.03 - 1.12	1.05	1.01 - 1.1
gender (male vs. female)	1.34	0.63 - 2.84	0.88	0.38 - 2.06

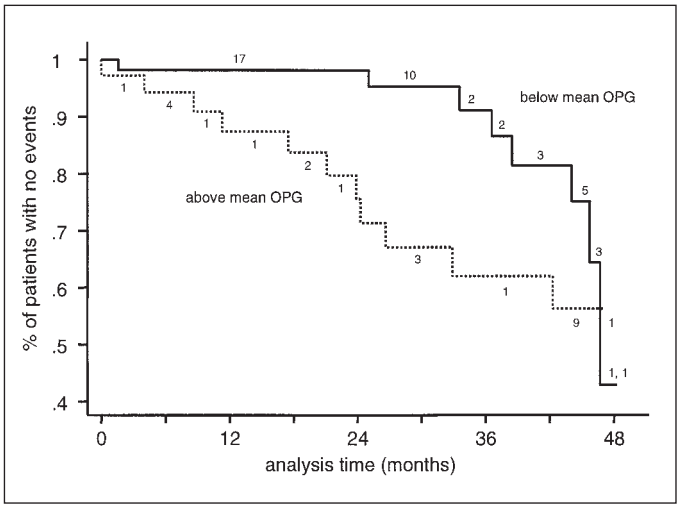

Figure 2: Kaplan-Meier event free survival estimates. Kaplan-Meier plot assessing the effect of OPG on the combined outcome of venous thrombosis and bleeding complications. Event free survival curves are shown for patients above mean OPG values (3.9pmol/l) and below mean OPG values. Log-rank-test: p=0.017. The numbers above or below the respective survival curves represent censored individuals at the respective points in time. Analysis time is given in months.

words, using the univariate associations, a 1pmol/l change in OPG plasma levels has the same effect on the occurrence of a thrombotic complication as does an increase in age of 8.6 years. And a change in OPG of one standard deviation increases the risk of developing venous thrombosis by as much as 92%. Similar findings were obtained for bleeding complications (univariate OR=1.52, p=0.001; multivariate OR=1.37, p=0.013). With respect to the risk of developing a bleeding complication, a 1pmol/l change in OPG was equivalent to ageing 7.2 years and a change of one standard deviation of OPG causes the risk of bleeding to increase by 125%. The results of an analysis of a combined outcome (thrombosis and bleeding) are also shown in table 2. In a retrospective analysis of arterial thrombotic complications, there was no significant association between plasma OPG levels and the occurrence of arterial thrombotic events. Plasma OPG levels were slightly elevated in patients with arterial thrombotic event(s) compared to patients without arterial thrombotic event (4.16 ±1.82 vs. 3.63 ±1.93, p=0.13).

Analysis of event free survival

In order to increase the number of events in the prospective analysis we used a combined outcome of venous thrombosis and bleeding complications. We only included those individuals in the analysis who had no history of venous thrombosis or bleeding. When we compared the 52 individuals with below mean OPG plasma levels (3.9pmol/l) to the 35 participants with OPG plasma levels above the mean (Fig. 2), we found that higher OPG plasma levels were associated with decreased event free survival in a statistically significant manner using the Kaplan-Meier method (log-rank-test: p=0.017).

Discussion

In this cohort of 114 PV patients we found that elevated OPG plasma levels were associated with adverse vascular complications. This is the first report that links elevated OPG levels to thrombosis in the venous vascular system or to bleeding complications. No data exists on a possible association of OPG levels and venous thrombosis. So far, the attention of clinical studies evaluating systemic OPG levels in vascular disease has been focussed on arterial vessel disease. PV patients presenting with arterial vascular complications showed increased OPG levels, although this association was not statistically significant. Our study cohort is representative of all PV patients seen at our hospital. Cases (symptomatic patients), as well as controls (asymptomatic patients) come from the same population of patients. We consider the presence of any biases which could jeopardize study validity as highly unlikely. The possibility of a type-I error always has to be taken into account when presented with positive research results. Nevertheless, we think that a false positive result is not very likely in light of the strength of the association and the consistency across strata of bleeding and venous thrombosis. Interpreting the results of our prospective analysis shown in figure 2, one has to take into account, that, firstly, the longer the time of observation after OPG measurement, the bigger is the variation from the initial OPG value. It is known that OPG levels increase over time. A second reason for decreasing accuracy towards the right side of the graph is that an increasing number of patients are censored. At the time of the overlap of the 2 graphs after approximately 4 years of follow up, only 4 patients of the initial 87 patients were still under observation. This fact results in a relatively high degree of uncertainty with respect to precision of the two survival functions at the last weeks of follow-up. Therefore, the predictive value of OPG after about three years of OPG-measurement will have to be evaluated in larger cohorts of PV patients with longer follow-up times.

Osteosclerosis seen in myeloproliferative disorders might be a consequence of an upregulation of OPG in the bone marrow environment. In a thrombopoietin stimulated myeloproliferative mouse model the observed osteosclerosis was the consequence of an inhibition of osteoclastogenesis via an increased production of OPG (27). In patients with PV, the up-regulation of OPG might therefore be induced by growth factors and cytokines which are involved in the pathogenesis of chronic myeloproliferative disorders. Because it has been suggested that megakaryocytes express OPG (28), we evaluated a possible role of platelets for the increase of OPG plasma levels in our cohort of PV patients. We could, however, not observe a correlation of the peripheral blood platelet count with plasma OPG levels (Pearson coefficient of correlation: r=-0.149). Alternative sites of OPG production besides the bone marrow environment could be tissues of the vascular system. Endothelial cells (17,18), arterial vascular wall (13) and vascular smooth muscle cells (19) are known to produce OPG. Although it is known that OPG levels are elevated in vascular disease, no explicit trigger for OPG production within vascular tissue is evident. Several signalling pathways, however, seem to be involved. Platelet-derived growth factor (PDGF) as well as basic fibroblast growth factor (bFGF), TNF-α and interleukin (IL)-1β upregulate OPG in vascular smooth muscle cells (19). Proinflammatory cytokines such as TNF-α or IL-1β also upregulate OPG expression in human microvascular endothelial cells (18).

The following pathogenetic mechanisms of increased OPG levels in vascular disease are proposed: It was shown that RANKL suppresses apoptosis of primary cultured endothelial cells (17). With OPG being a soluble decoy receptor for RANKL, elevated serum OPG levels could antagonize this patronizing effect during the evolution of vascular disease and hereby play an active role in the development of vascular complications. Alternatively, OPG might have a protective role in endothelial hemostasis. Malyankar et al. (29) could show that OPG prevents endothelial cells from apoptosis induced by growth factor deprivation. OPG production might, therefore, constitute a compensatory, protective reaction in response to vascular damage. Another hypothesis would be that OPG, if not totally absent as seen in $OPG^{-/-}$ mice, does not by itself effect the vasculature in a clinically significant way, but that certain inflammatory mediators trigger systemic OPG production and deteriorate vascular function likewise (i.e. promote atherosclerosis, advance a procoagulatory endothelial state or a bleeding tendency).

We could show that OPG levels are a relatively constant parameter over time. Irrespective of the underlying pathophysiologic mechanism of increased OPG levels in vascular disease, it can be postulated with support of our data, that OPG is a useful measure for event prediction. Further studies are needed to detect possible OPG protein polymorphisms to enable a differenti-

ation of skeletal and vascular wall-derived OPG. In future, OPG measurement could, thus, prove to be a useful test as marker for disease activity and prognosis in vascular diseases. Considering the high expectations towards the therapeutic use of OPG or other RANKL binding molecules for bone absorbing diseases, the role of the OPG/RANKL/RANK system in vascular physiology should be clarified.

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