Osteoprotegerin Is an Independent Predictor of Vascular Events in Finnish Adults With Type 1 Diabetes

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OBJECTIVE—Osteoprotegerin (OPG) is involved in the process of vascular calcification. We investigated whether OPG is associated with the development and progression of diabetes complications in adults with type 1 diabetes (T1D).

RESEARCH DESIGN AND METHODS—Serum OPG was measured in 1,939 adults with T1D participating in the Finnish Diabetic Nephropathy (FinnDiane) Study. Patients with end-stage renal disease (dialysis or transplantation) at baseline were excluded from analysis. Data on cardiovascular (CV) events and mortality during follow-up were verified from hospital discharge registries (ICD codes) and the Finnish National Death Registry, respectively. The follow-up time was 10.4 ± 2.0 (mean ± SD) years.

RESULTS—Only patients with macroalbuminuria and/or renal impairment had elevated OPG concentrations, when compared with participants without overt kidney disease. Patients with retinopathy or CV disease also had higher OPG concentrations, but this was attributable to their higher frequency of chronic kidney disease. OPG predicted an incident CV event (hazard ratio 1.21 [95% CI 1.01–1.45]; $P = 0.035$) and peripheral vascular disease/amputation events (1.46 [1.13–1.88]; $P = 0.004$) during follow-up.

CONCLUSIONS—We showed that serum OPG is an independent predictor of CV complications. OPG may be directly involved in extraosseous calcification, resulting in stiffening of the arteries and subsequent vascular insufficiency in patients with T1D.

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Arterial calcification is strongly associated with the development and progression of vascular stiffening and arteriosclerosis leading to cardiovascular disease (CVD). This process is accelerated in patients with diabetes or chronic kidney disease (CKD) and especially in those with both (1). Many of the key regulators of bone mineralization also appear to be key mediators of osteogenic transformation of vascular smooth muscle cells and arterial calcification in diabetes (2,3). One of the most well known is osteoprotegerin (OPG) (4,5). OPG concentrations are positively correlated with coronary calcification (6), vascular stiffness (7), and the presence of unstable plaque (8) in nondiabetic individuals and an increased risk of cardiovascular (CV) mortality in patients with diabetes (9,10). In this study, we further explore the association between circulating concentrations of OPG and CV outcomes in a large well-characterized cohort of patients with type 1 diabetes (T1D) exploring mortality, coronary, stroke, and amputation events.

RESEARCH DESIGN AND METHODS

Study participants

This study is part of the ongoing Finnish Diabetic Nephropathy (FinnDiane) Study, with the aim to identify genetic, clinical, and environmental risk factors for diabetic nephropathy in patients with T1D. The study was initiated in 1997, and follow-up data have been collected since 2004 either by re-examination of the patients or review of the medical files. Detailed description of the follow-up protocol has been described previously (11). T1D was defined as an onset of diabetes before the age of 40 years and permanent insulin treatment initiated within 1 year of diagnosis. For this study, outcomes were ascertained in patients in the FinnDiane prospective cohort recruited between 1997 and 2004, in whom serum OPG was estimated on baseline samples ($N = 1,939$). Furthermore, patients with end-stage renal disease (ESRD; dialysis or transplantation) at baseline were excluded from analysis, as risk factors for adverse outcomes are clearly different in these patients. The ethical committees of all participating centers approved the study protocol. Written informed consent was obtained from each patient, and the study was performed in accordance with the Declaration of Helsinki as revised in the year 2000.

Cohort characteristics

At baseline, all patients also underwent a thorough clinical investigation in connection with a regular patient visit to their attending physician. Data on medication and diabetes complications were registered with the use of a standardized questionnaire, which was completed by the physician based upon medical files. Blood pressure was measured twice in the sitting position after a 10-min rest, and the average of these two measurements

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Table 1—*Demographic and biochemical characteristics of the study subjects*

	Normoalbuminuria	Microalbuminuria	Macroalbuminuria
N	1,296	322	228
Male sex (%)	46	60*	57†
Age (years)	35.8 ± 0.3	37.9 ± 0.7†	41.9 ± 0.7*
Diabetes duration (years)	17.0 ± 0.3	24.4 ± 0.6*	27.5 ± 0.5*
HbA_{1c} (%)	8.2 ± 0.04	8.7 ± 0.08*	9.1 ± 0.1*
Insulin dose (IU/kg)	0.7 ± 0.01	0.8 ± 0.08†	0.7 ± 0.01
BMI (kg/m^2)	24.7 ± 0.1	25.8 ± 0.2*	26.0 ± 0.3*
Waist-to-hip ratio	0.8 ± 0.01	0.9 ± 0.01*	0.9 ± 0.01*
SBP (mmHg)	129 ± 1	135 ± 1*	146 ± 1*
DBP (mmHg)	78 ± 1	81 ± 1*	83 ± 1*
AER (mg/24 h)	8 (6–12)	53 (22–104)*	452 (170–1,242)*
Serum creatinine (mmol/L)	82 (73–91)	87 (78–97)*	109 (93–142)*
eGFR (mL/min/1.73 m^2)	86 ± 1	81 ± 1	50 ± 2*
Total cholesterol (mmol/L)	4.8 ± 0.1	5.0 ± 0.1*	5.6 ± 0.1*
Triglycerides (mmol/L)	1.0 (0.7–1.3)	1.1 (0.8–1.6)	1.5 (1.1–2.2)*
HDL cholesterol (mmol/L)	1.3 ± 0.1	1.2 ± 0.1	1.1 ± 0.1*
Antihypertensive treatment (%)	12	63*	95*
Laser-treated diabetic retinopathy (%)	13	48*	83*
Smoking (%)	20	28†	30*
CVD (%)	4	7†	24*
C-reactive protein (mg/L)	3.8 ± 0.2	6.2 ± 0.7*	5.2 ± 0.6†
OPG (mg/L)	1.6 ± 0.1	1.7 ± 0.1	2.1 ± 0.1*

Data are presented as mean ± SEM and percentages except for AER, triglycerides, and serum creatinine, for which median and interquartile range is presented. DBP, diastolic blood pressure; SBP, systolic blood pressure. *$P < 0.001$ vs. normoalbuminuria group. †$P < 0.05$ vs. normoalbuminuria group.

was used in the analysis. Height, weight, and waist-to-hip ratio were recorded, and blood was drawn for the measurements of HbA_{1c}, lipids, and creatinine. HbA_{1c} and creatinine were determined by standardized assays at each center and glomerular filtration rate (GFR) estimated using the Chronic Kidney Disease Epidemiology Collaboration formula (12,13). Serum lipid and lipoprotein concentrations were analyzed centrally by automated enzymatic methods (Hoffmann-La Roche, Basel, Switzerland). Urinary albumin was determined in one sample using an immunoturbidimetric method (Hitachi 911 analyzer; Roche Diagnostics, Basel, Switzerland). In addition, serum OPG was measured by a sandwich time-resolved immunofluorometric assay using commercially available antibodies (R&D Systems, Minneapolis, MN), as previously described (14).

Ascertainment of outcomes

Renal status was defined based on the urinary albumin excretion rate (AER) in three overnight or 24-h urine collections. Normal AER was defined as <20 μg/min or <30 mg/24 h, microalbuminuria as 20 μg/min ≤ AER <200 μg/min or 30 mg/24 h ≤ AER <300 mg/24 h, and macroalbuminuria as AER ≥200 μg/min or ≥300 mg/24 h. ESRD was defined as patients undergoing dialysis or having received a kidney transplant. Identification of the CVD until the end of 2010 was obtained by linking the FinnDiane data with the Hospital Discharge Register (HDR) and the Finnish Cause of Death Registry. The HDR lists all discharged hospital patients, each patient's unique personal identifier (assigned to every resident of Finland), dates of admission and discharge, and up to four diagnoses with the ICD and procedure codes that are based on the Nordic Classification of Surgical Procedures. Completeness and accuracy of the HDR concerning vascular disease has been shown to be very high (15). CVD was defined as a history of myocardial infarction, a coronary artery procedure (bypass surgery or angioplasty), stroke, or a peripheral artery procedure (bypass surgery or angioplasty), which was verified on the basis of ICD discharge codes specifying CV events. Limb amputations were further ascertained on the basis of ICD discharge codes specifying amputation, regardless of the presence or absence of documented peripheral vascular disease (PVD). Deaths from any cause through to 17 March 2010 were identified via a search of the Finnish National Death Registry and center databases. All deaths were confirmed with death certificate

Table 2—*Cox regression analysis for the predictive value of serum OPG for all-cause mortality, after adjusting for factors associated with serum OPG concentrations, as well as other factors independently associated with the studied event*

	All-cause mortality	*P* value
Age (years)	1.06 (1.04–1.08)	<0.001
Duration of diabetes (years)	1.03 (1.01–1.04)	0.005
Albumin excretion rate (mg/24 h)	1.00 (1.00–1.01)	<0.001
Waist-to-hip ratio	47.7 (5.07–448.9)	0.001
Triglycerides (log)	3.65 (1.72–7.78)	0.001
Current smoking	1.48 (1.22–1.65)	0.001
OPG	1.13 (0.93–1.38)	0.21

OPG is analyzed in SD from the mean. Data are hazard ratios (95% CI).

Table 3—*Cox regression analysis for the predictive value of serum OPG for incident CVD, after adjusting for factors associated with serum OPG concentrations, as well as other factors independently associated with the studied event*

	Incident CVD	*P* value
Age (years)	1.07 (1.05–1.10)	<0.001
Duration of diabetes (years)	1.04 (1.02–1.06)	<0.001
Waist-to-hip ratio	9.36 (1.05–88.0)	0.045
Triglycerides (log)	3.81 (1.72–8.44)	0.001
Microalbuminuria	1.72 (1.09–2.70)	0.001
Macroalbuminuria	3.35 (2.17–5.16)	<0.001
OPG	1.21 (1.01–1.45)	0.035

OPG is analyzed in SD from the mean. Data are hazard ratios (95% CI).

data. In each case, vitality status was verified from the Finnish National Death Registry and cause of death classified on the basis of death certificates. The causes of death in this cohort were CV death (49%), cancer-related death (8%), infection-related death (9%), and other causes (34%).

Statistical analysis

Continuous data are expressed as mean ± SEM. Differences in the mean among groups were compared using two-way ANOVA. Pairwise multiple comparisons were made with the Student-Newman-Keuls post hoc analysis to detect significant differences between groups. A *P* value <0.05 was considered statistically significant. To evaluate the independent predictors of all-cause mortality, CV, coronary, stroke, and amputation events, we used multivariate Cox proportional hazards models. Model selection from candidate variables was accomplished by minimization of the Akaike and Bayesian information criteria (16). Overall, Cox model fit was assessed by: *1)* approximation of cumulative Cox-Snell residuals to (-log) Kaplan-Meier estimates, residual plots, and specific testing of the proportional hazards assumption (17) and 2) Harrell's C statistic (18) and added-variable goodness-of-fit tests (19). Cox model performance was adjudged by the explained variation using 5,000 bootstrap repetitions of the whole data set, adjusting for covariates (20). The potential for multiple colinearity was tested using the variance inflation factor and condition number, in which a variance inflation factor <10 and condition number <30 are desirable (21).

RESULTS

Clinical characteristics of the study subjects

The FinnDiane cohort in whom serum OPG was estimated comprised 1,939 adult patients with T1D without ESRD. The cohort characteristics at baseline have been previously described in detail (11) and are summarized in Table 1. Briefly, approximately half of the participants were males (51%). The mean age of the participants was 39 years, with a median duration of diabetes of 20 years. Forty-seven percent of patients had hypertension (defined by the use of antihypertensive agents and/or a blood pressure >140/90 mmHg). At baseline, 17% had a urinary AER in the microalbuminuric range, 13% had a urinary AER in the macroalbuminuric range, and 65% had a urinary AER in the normoalbuminuric range. A further 5% of study participants were unclassified because of an inadequate number of urine collections, and 12% of patients had an estimated GFR (eGFR) <60 mL/min/1.73 m^2, most of whom also had macroalbuminuria.

Serum OPG concentrations and their determinants

Serum OPG concentrations did not differ between patients with T1D and normoalbuminuria or microalbuminuria (Table 1). However, patients with macroalbuminuria had higher OPG concentrations than those with microalbuminuria or normal AER. Patients with moderate to severe renal impairment (eGFR <60 mL/min/1.73 m^2) also had higher OPG concentrations that those without renal impairment (1.9 ± 0.1 vs. 1.4 ± 0.1 μg/mL; $P < 0.05$). In addition, after adjusting for renal function, OPG remained independently correlated with high-sensitivity C-reactive protein, a marker of systemic inflammation ($P < 0.01$), and HbA_{1c}, a marker of chronic glycemic control ($P < 0.05$; data not shown). Notably age, sex, body mass, blood pressure, and lipid concentrations were not associated with OPG after adjusting for renal function. Patients with established CVD had higher OPG concentrations than those without macrovascular disease (2.0 ± 0.1 vs. 1.7 ± 0.1 mg/L; $P < 0.001$). However, the observed difference in serum OPG in patients with T1D with or without CVD was attributable to an excess of patients with CKD and eliminated after adjusting for renal function. Furthermore, patients with retinopathy requiring laser treatment had higher OPG concentrations than those without severe retinal disease (1.9 ± 0.1 vs. 1.6 ± 0.1 mg/L; $P < 0.001$).

OPG and all-cause mortality in patients with T1D

A total of 166 patients (9%, 0.8 per hundred person-years) died during follow-up (mean of 10.2 years). Serum OPG concentrations at baseline were higher in patients who died due to an all-cause mortality (2.0 ± 0.1 vs. 1.6 ± 0.1 mg/L; $P = 0.006$). However, after adjusting for factors associated with serum OPG concentrations, as well as other factors independently associated with all-cause mortality, OPG was no longer associated with all-cause mortality on multivariate Cox regression analysis (*P* not significant; Table 2).

OPG and incident CVD in patients with T1D

During the follow-up period (mean of 10.5 years), 190 patients experienced their first CV event ever (of 1,844 patients without prior CVD; 1.0 per hundred patient-years). Again, serum OPG concentrations at baseline were higher in patients who had an incident CVD event (2.2 ± 0.1 vs. 1.6 ± 0.1 mg/L; $P < 0.001$). After adjusting for factors associated with serum OPG concentrations, as well as other factors independently associated with CVD, OPG remained significantly associated with incident CVD on multivariate Cox regression analysis ($P = 0.03$; Table 3, Fig. 1*A*).

OPG and incident coronary heart disease in patients with T1D

During the follow-up period (mean of 10.5 years), 152 patients experienced their first coronary event (of 1,868 patients without prior coronary heart disease [CHD]; 0.8 per hundred patient-years). Furthermore, serum OPG concentrations at baseline were increased in patients who had an incident CVD event (2.2 ± 0.1 vs. 1.3 ± 0.1 mg/L; $P = 0.002$). After adjusting for factors associated with serum OPG concentrations, as well as other factors independently associated with CHD,

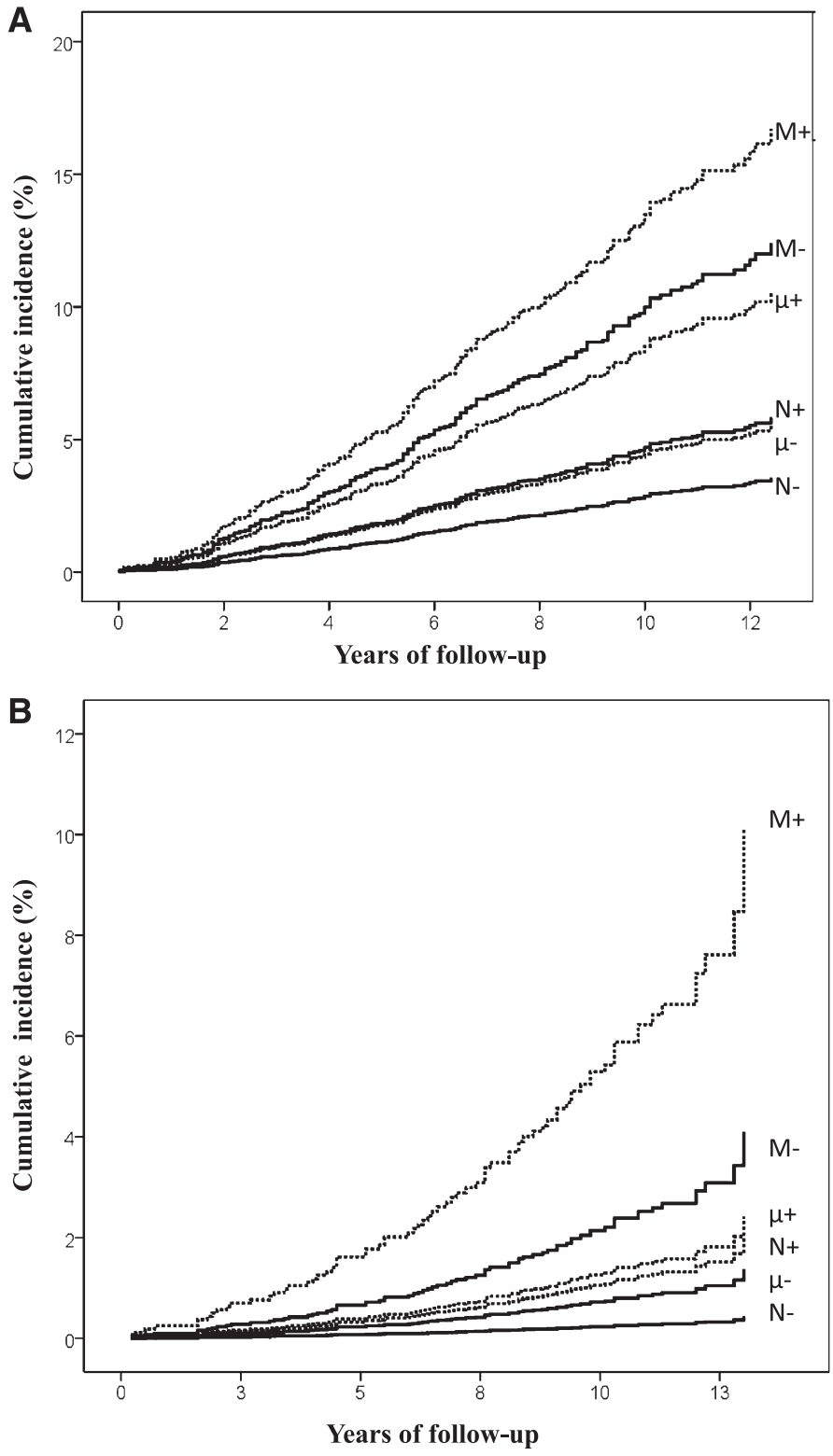

Figure 1—*The relationship of serum OPG concentrations (above and below the mean) with new-onset CVD* (A) *and amputation/leg revascularization* (B)*, stratified according to the stage of albuminuria, and adjusted for other risk factors. μ−, patients with microalbuminuria and OPG concentrations below median; μ+, patients with microalbuminuria and OPG concentrations above median; M−, patients with macroalbuminuria and OPG concentrations below median; M+, patients with macroalbuminuria and OPG concentrations above median; N−, patients with normal AER and OPG concentrations below median; N+, patients with normal AER and OPG concentrations above median.*

the association of OPG with incident CHD was of borderline significance on multivariate Cox regression analysis (P = 0.07; Table 4).

OPG and incident stroke in patients with T1D

During a mean of 10.3 years of follow-up, 71 patients experienced their first stroke event (of 1,903 patients without a prior stroke; 0.3 per hundred patient-years). Again, serum OPG concentrations at baseline were higher in patients who had an incident stroke (2.2 ± 0.1 vs. 1.6 ± 0.1 mg/L; P = 0.02). After adjusting for factors associated with serum OPG concentrations, as well as other factors independently associated with stroke, OPG was not associated with stroke on multivariate Cox regression analysis (P = 0.7; Table 5).

OPG and incident PVD in patients with T1D

During a mean of 10.2 years of follow-up, 80 patients had a leg revascularization procedure or an amputation (any cause) as their first CV event (of 1,922 patients without prior CVD; 0.4% per hundred patient-years). Serum OPG concentrations at baseline were higher in patients who had an incident leg revascularization procedure or amputation (2.0 ± 0.1 vs. 1.6 ± 0.1 mg/L; $P < 0.001$). After adjusting for factors associated with serum OPG concentrations, as well as other factors independently associated with PVD events, OPG was independently associated with leg revascularization procedure or an amputation on multivariate Cox regression analysis (P = 0.004; Table 6, Fig. 1*B*).

CONCLUSIONS—OPG has been widely implicated in the process of vascular calcification and progressive CVD. OPG concentrations are positively correlated with coronary calcification (6), vascular stiffness (7), and the presence of unstable plaque (8) as well as all-cause and CV mortality in elderly women (22), as well as men with coronary artery disease (23). Previous studies have suggested that OPG concentrations are also associated with CV mortality in patients with diabetes (9,10,24). The current study extends these findings to show that OPG predicted not only incident CVD and PVD events but was also associated with the risk of all-cause mortality in patients with T1D.

Table 4—*Cox regression analysis for the predictive value of serum OPG for incident CHD, after adjusting for factors associated with serum OPG concentrations, as well as other factors independently associated with the studied event*

	Incident CHD	*P* value
Age (years)	1.09 (1.06–1.12)	<0.001
Duration of diabetes (years)	1.05 (1.03–1.07)	<0.001
Triglycerides (log)	6.17 (2.76–13.8)	<0.001
Macroalbuminuria	2.70 (1.67–4.37)	<0.001
OPG	1.22 (0.98–1.50)	0.066

OPG is analyzed in SD from the mean. Data are hazard ratios (95% CI).

Although it has been suggested in small studies that children with T1D have increased OPG concentrations compared with nondiabetic individuals (25), in our large adult cohort, OPG concentrations were not directly elevated by T1D. Elevation of the OPG concentration was only observed in those with overt nephropathy or established macrovascular disease. The mechanisms by which OPG concentrations are increased in these settings are unclear (26). The retention of peptides like OPG and cystatin C associated with renal impairment provides part of the answer. In our study, OPG levels were certainly higher in patients with moderate to severe renal impairment (eGFR <60 mL/min/1.73 m^2). However, after adjusting for renal function and albuminuria, OPG levels remained a significant predictor of adverse outcomes. Indeed, some of the risk attributable to renal disease may be medicated through accelerated vascular calcification. This may be one reason why eGFR was eliminated as an independent predictor of adverse outcomes in this cohort. The independent association of OPG with high-sensitivity C-reactive protein, a marker of systemic inflammation ($P < 0.01$), may also have contributed to elevated OPG levels in patients with overt complications, as inflammation is also elevated in patients with complications and inflammation may also drive the expression of OPG.

OPG is a soluble member of the inflammatory tumor necrosis factor receptor super family. It acts as a receptor for the receptor activator of nuclear factor-κB ligand (RANKL) and interferes in its binding to cell-surface RANK (27). OPG has been shown to block the differentiation of osteoclasts, the bone-resorbing cell type. OPG also has an important regulatory role in endocrine function and the immune system (4). In addition, the OPG–RANKL–RANK axis has recently been linked to atherosclerosis (5). OPG is present in blood vessels (4,28) and especially in the smooth muscle cells of the vascular media (29,30), which are thought to be the major source of OPG in the arterial wall. OPG-deficient mice develop early arterial calcification (31,32). Consequently, the elevation of circulating OPG concentrations in proatherogenic settings has been thought to represent a compensatory phenomenon to limit further vascular damage. However, by inhibiting the regulatory pathways signaled through the RANK receptor, and particularly those of the atheroprotective ligand tumor necrosis factor-α–related apoptosis-inducing ligand (TRAIL) (33), elevated OPG concentrations may also have direct actions on vascular function, as well as the development and progression of vascular disease. Indeed, it has been previously shown that human full-length OPG induces the proliferation of rodent vascular smooth muscle cells and augments atherogenesis in diabetic apolipoprotein E knockout mice (34). In addition, it has been demonstrated that OPG is able to initiate transforming growth factor-β–dependent changes in vascular smooth muscle cells, stimulating proliferation, inflammation, and fibrogenesis. Such data suggest that the increases in adverse vascular outcomes associated with elevated OPG concentration in our patients with T1D may be causally linked.

Vascular calcification is strongly associated with PVD in patients with diabetes, including the risk for amputation. Vascular calcification score on plain radiographs of the feet is a predictor of peripheral arterial disease in patients with CKD. However, our study is the first to show an independent link between OPG and peripheral vascular events (amputation or revascularization). Although amputation may have multiple etiologies, we chose to use this more pragmatic outcome as it is often difficult to determine the relative contribution of infection, neuropathy, or vascular insufficiency as the cause of a foot ulcer or subsequent need for amputation.

While OPG was higher in those participants with severe retinal disease in our much larger patient cohort, after adjusting for the confounding effects of renal impairment, this difference was eliminated. Similarly, no association between OPG and diabetic retinopathy was reported in a cohort of 200 patients with T1D (35). By contrast, OPG has previously been associated with maculopathy in patients with type 2 diabetes (T2D) (24). The reasons for this difference between T1D and T2D is unclear, although it may be due to confounding effects of renal impairment in older patients with T2D.

In conclusion, we demonstrate that serum OPG is independently associated with CV complications and mortality in adults with T1D. There are also sufficient experimental data to support a causal link. Blocking the actions of OPG consequently may therefore offer one potential intervention to slow the development and progression of vascular disease in diabetes. Indeed, complete blockade of OPG in mice causes early onset osteoporosis and arterial calcification (32). This suggests

Table 5—*Cox regression analysis for the predictive value of serum OPG for incident stroke, after adjusting for factors associated with serum OPG concentrations, as well as other factors independently associated with the studied event*

	Incident stroke	*P* value
Age (years)	1.06 (1.02–1.10)	0.002
Sex (male/female)	1.57 (1.16–1.78)	0.014
Microalbuminuria	3.26 (1.37–7.75)	0.008
Macroalbuminuria	11.2 (5.11–24.3)	<0.001
Pre-existing CVD	3.79 (1.71–8.40)	<0.001
OPG	1.06 (0.77–1.46)	0.73

OPG is analyzed in SD from the mean. Data are hazard ratios (95% CI).

Table 6—*Cox regression analysis for the predictive value of serum OPG for incident amputation or peripheral revascularization, after adjusting for factors associated with serum OPG concentrations, as well as other factors independently associated with the studied event*

	Incident PVD	*P* value
Age (years)	1.04 (1.01–1.06)	0.001
Waist-to-hip ratio	205.1 (41.3–1,010)	<0.001
Microalbuminuria	3.75 (1.85–7.61)	<0.001
Macroalbuminuria	7.88 (4.07–15.3)	<0.001
OPG	1.46 (1.13–1.88)	0.004

OPG is analyzed in SD from the mean. Data are hazard ratios (95% CI).

that a better target for the prevention of vascular disease may be to augment its ligands, TRAIL and RANKL. For example, studies using TRAIL in apolipoprotein E knockout mice with T1D have shown promising reductions in atherogenesis (33), possibly by overcoming the inhibitory effects of its decoy-receptor OPG.

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