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Associations of Serum Osteoprotegerin Levels with Diabetes, Stroke, Bone Density, Fractures, and Mortality in Elderly Women*

WARREN S. BROWNER, LI-YUNG LUI, AND STEVEN R. CUMMINGS

General Internal Medicine Section, San Francisco Veterans Affairs Medical Center (W.S.B., L.-Y.L.); and Departments of Epidemiology and Biostatistics (W.S.B., L.-Y.L., S.R.C.) and Medicine (W.S.B., S.R.C.), University of California, San Francisco, California 94143

ABSTRACT

Osteoprotegerin (OPG) and its ligand are cytokines that regulate osteoclastogenesis and that may be involved in the regulation of vascular calcification. We examined whether serum OPG levels were associated with stroke, mortality, and cardiovascular risk factors, including diabetes, as well as with bone mineral density and fractures in a sample of 490 participants in a prospective cohort of white women, at least 65 yr of age. We found that OPG levels, assayed blinded from serum obtained at baseline, were about 30% greater in women with diabetes (mean ± SD, 0.30 ± 0.17 ng/mL) than in those without diabetes (0.23 ± 0.10 ng/mL; $P = 0.0001$). OPG levels were associated with all-cause mortality [age-adjusted odds ratio, 1.4/SD (0.11 ng/mL) increase in serum OPG level; 95% confidence interval, 1.2–1.8] and cardiovascular mortality (odds ratio, 1.4; 95% confidence interval, 1.1–1.8); these effects were not confounded by diabetes. OPG levels were not associated with baseline bone mineral density or with subsequent strokes or fractures. The association of serum OPG levels with diabetes and with cardiovascular mortality raises the possibility that OPG may be a cause of or a marker for vascular calcification. (*J Clin Endocrinol Metab* **86:** 631–637, 2001)

OSTEOPROTEGERIN (OPG) and its ligand are recently identified cytokines that regulate osteoclastogenesis (1–7). OPG ligand binds to receptors on the surface of preosteoclasts and stimulates their differentiation into active osteoclasts, leading to bone resorption. OPG is a soluble faux receptor for the ligand, thereby inhibiting osteoclastogenesis. *opg*-deficient mice develop osteoporosis, presumably because the unopposed action of OPG ligand stimulates osteoclasts and leads to excessive resorption of bone. In addition, these mice develop premature arterial calcification, suggesting that the OPG system also has effects in the regulation of vascular calcification (3).

OPG ligand and OPG are members of the tumor necrosis factor (TNF) and TNF receptor superfamilies (8, 9). They each have several other names, in part because they have other functions, including regulation of lymphocytes and apoptosis (10) and in part because they were independently identified by several groups of investigators. Thus, OPG is also known as osteoclastogenesis inhibitory factor (OCIF), follicular dendritic cell-derived receptor-1, and TNF receptor-like molecule, whereas OPG ligand is also known as osteoclast differentiation factor, receptor activator of NF-κB ligand (RANK ligand), and TNF-related activation-induced cytokine (TRANCE).

The vascular effects of OPG in humans, such as whether there is an association between OPG levels and vascular disease or cardiovascular risk factors, are not known. One previous study from Japan (11) reported that serum OPG levels were associated with bone mineral density, but did not examine the association between OPG and fractures. We tested these hypotheses using a case-control design that was nested within a larger prospective study. We used serum that had been obtained from participants at a baseline examination and compared OPG levels in those who died or suffered a stroke or fracture during follow-up with levels in randomly selected controls. We ascertained whether serum OPG levels were associated with selected medical conditions that are associated with atherosclerosis, such as diabetes, hypertension, cigarette smoking, hyperlipidemia, and the use of hormone replacement therapy, and studied the association of OPG levels with bone mineral density.

Subjects and Methods

Subjects

Ambulatory women, 65 yr of age or older, who had not previously had bilateral hip replacements were recruited from September 1986 to October 1988 at four clinical centers: The Kaiser-Permanente Center for Health Research (Portland, OR), University of Minnesota in Minneapolis, University of Maryland in Baltimore, and University of Pittsburgh (12). Men and black women were excluded because of their relatively low incidence of osteoporotic fractures. Written informed consent was obtained from all participants after the appropriate institutional review boards had approved the study protocol.

Measurements

Participants completed a questionnaire that was reviewed by an interviewer during the 3-h baseline examination. Unless otherwise noted, variables were dichotomized (yes/no). The questionnaire asked about use of cigarettes (in pack-years), college education, current use of estrogen replacement therapy, and physician-diagnosed diabetes mellitus. At a baseline examination, we measured knee height (to avoid the effects of vertebral osteoporosis on total height), weight, and blood

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Address all correspondence and requests for reprints to: Dr. W. S. Browner, California Pacific Medical Research Institute, 2340 Clay Street, Room 114, San Francisco, California 94115. E-mail: warren@cooper.cpmc.org.

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pressure; we calculated a modified body mass index. Hypertension was defined as taking a diuretic medication or having a measured blood pressure greater than 160/90 mm Hg.

During the baseline examination, blood was collected between 0900–1400 h after participants, who had been instructed to eat a nonfat breakfast to prevent lipemia, had been seated for 10 min. Serum was stored for approximately 1 week at −20 C, then was shipped on dry ice for subsequent storage at −190 C (13). All assays were measured blinded to any clinical information. We measured serum OPG levels with an enzyme-linked immunosorbent assay using a mouse monoclonal antibody as capture antibody and a rabbit polyclonal antibody for detection (Amgen, Inc., Thousand Oaks, CA). The assay detects both monomer and dimeric forms of OPG, including OPG bound to its ligand. The predominant circulating form of OPG that this assay detects in human serum has not been determined. The detection limit of this assay is 0.05 ng/mL, and more than 97% of adults have detectable levels of OPG. Intra- and interassay variabilities are less than 15%. All samples were measured in duplicate and averaged; results differing by more than 20% were reassayed. OPG levels were missing, due to sample unavailability, in four women. All other serum assays were performed by Endocrine Sciences, Inc. (Calabasas Hills, CA). We measured fructosamine levels using a standard colorimetric assay based on the ability of ketoamines to reduce nitro blue tetrazolium to formazan; the upper limit of normal was 285 μmol/L. A participant was considered to have diabetes if she reported a history of physician-diagnosed diabetes or if the serum fructosamine level was more than 285 μmol/L. C-reactive protein levels were measured with rate nephelometry. Osteocalcin (bone Gla protein) levels were measured using a RIA that uses a highly specific polyclonal antibody developed at Endocrine Sciences, Inc. Intact PTH levels were measured using a standard immunoradiometric assay for the biologically active [PTH-(1–84)] peptide. Calcium levels were measured by atomic absorption. Total cholesterol, high density lipoprotein (HDL) cholesterol, and triglyceride levels were measured using an automated chemistry analyzer; low density lipoprotein (LDL) cholesterol levels were estimated.

Measurements of bone mineral density

Bone mineral density was measured at baseline in the os calcis (heel) and at the proximal and distal radius using single photon absorptiometry (OsteoAnalyzer, Siemens-Osteon, Wahiawa, HI); approximately 2 yr later, bone mineral density was measured using dual energy x-ray absorptiometry (QDR-1000, Hologic, Inc., Waltham, MA) at the hip and spine (14).

Follow-up

Each participant or her designated proxy returned a postcard to the clinical center every 4 months. These were supplemented by phone calls for late postcards as well as an annual questionnaire that asked about incident strokes and fractures. We reviewed death certificates and hospital discharge summaries for those who died. Causes of death were coded by ICD9-CM (International Classification of Diseases-Clinical Modification) codes by a blinded investigator at the coordinating center; cardiovascular disease included codes 394–440. We obtained medical records for participants who reported strokes or transient ischemic attacks. Using a case-control design that was designed primarily to ascertain predictors of stroke, cases of thrombotic stroke [n = 243, including 81 who died during follow-up (41 from stroke)] that occurred from baseline until February 18, 1998, were validated. Two investigators independently reviewed each potential case; disagreements were resolved by consensus. Controls (n = 247) were randomly selected from the entire cohort, of whom 36 died during follow-up, for a total of 117 deaths. All decisions about clinical events were made blinded to any knowledge of assay results.

Analysis

We estimated the associations between serum OPG levels (and other potential risk factors) with dichotomous outcome (*e.g.* stroke, cardiovascular mortality, and fractures) using logistic regression models and using linear regression models to look for associations with continuous variables (*e.g.* modified body mass index and serum fructosamine levels). Multivariate logistic models were adjusted for age as well as for potential confounders of the associations between serum OPG levels and the outcomes. Confounders were defined as potential risk factors for mortality, cardiovascular disease, or stroke (*i.e.* age, history of hypertension, diabetes, pack-years of smoking, use of estrogen replacement therapy, modified body mass index, and serum levels of HDL and LDL cholesterol and C-reactive protein) or for fractures (*i.e.* age, pack-years of smoking, use of estrogen replacement therapy, and modified body mass index) that were associated (at $P < 0.05$) with serum OPG levels. We also examined multivariate models that included all of these predictor variables. Odds ratios with 95% confidence intervals are reported. We also used models with quadratic terms as well as dividing participants into quintiles of OPG levels to look for J- and U-shaped associations. Mean levels of continuous variables were compared with Student's *t* test or ANOVA, as appropriate. Categorical variables were compared using the χ^2 test. Statistical significance was set at $P < 0.05$.

Because of the unusual design of this study, there was an excess number of participants who suffered strokes during follow-up. Thus, we performed analyses of the associations between OPG levels and clinical outcomes separately in the originally defined cases and controls. Power was reduced in these stratified analyses, so although the results were similar to those presented, some results that had been significant in the overall analyses were no longer significant in the stratified analyses. Measurements of bone mineral density using single photon absorptiometry at baseline were available in 483 (distal radius) to 488 (os calcis) of the 490 women; follow-up measurements of bone mineral density using dual energy x-ray absorptiometry were available in 439 (spine) to 445 (hip) women. Serum measurements were missing in at most 9 of the 490 women.

Results

Not surprisingly, subjects who suffered strokes or died during follow-up were older and more likely to have a history of hypertension or diabetes (Table 1). Of the 117 participants who died during follow-up, 81 were included in the 243 cases of stroke. There were 154 women who suffered fractures during follow-up, including 34 with wrist fractures and 28 with hip fractures.

Serum OPG levels were roughly normally distributed among these elderly women (Fig. 1). The mean (± SD) OPG level was 0.24 ± 0.12 ng/mL; the median value was 0.22 ng/mL, with an interquartile range (25^{th} to 75^{th} percentile) of 0.16 to 0.29 ng/mL. Only one woman had an OPG level that was not measurable. OPG levels increased with age ($r = 0.18$, $P < 0.0001$), from a mean of 0.23 ± 0.12 ng/mL in women 65 to 74 yr old, to 0.26 ± 0.10 ng/mL in women 75 to 84 yr old, to 0.28 ± 0.12 ng/mL in women 85 yr of age or older ($P = 0.01$).

OPG levels and cardiovascular risk factors

We found no difference in serum OPG levels by current smoking (Table 2). There were no correlations between serum OPG levels and body mass index ($r = 0.04$; $P = 0.39$), serum LDL ($r = -0.07$; $P = 0.11$) or HDL cholesterol levels ($r = 0.02$; $P = 0.61$), or serum C-reactive protein levels ($r = 0.05$; $P = 0.25$). OPG levels were slightly greater in women with hypertension (Table 2; $P = 0.03$).

Serum OPG levels were correlated with serum fructosamine levels ($r = 0.24$; $P < 0.0001$). This correlation was apparent only in women with diabetes (Fig. 2). OPG levels were about 30% greater in women with diabetes, either based on self-report of a physician diagnosis (0.29 ± 0.15 ng/mL; n = 56) or a serum fructosamine level greater than 285 μmol/L (0.32 ± 0.25 ng/mL; n = 13) than in the 413 women without diabetes (0.23 ± 0.10 ng/mL; $P < 0.0001$). OPG levels

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TABLE 1. Characteristics of subjects at enrollment, stratified by case (stroke)-control status and by vital status at end of follow-up

Characteristic	Case-control status		Vital status	
	Control (n = 247)	Stroke (n = 243)	Alive (n = 373)	Dead (n = 117)
Age (yr; mean ± SD)	71 ± 5	73 ± 5[a]	71 ± 5	75 ± 6[a]
Hypertension (%)	35	54[a]	39	60[a]
Current smoking (%)	9	10	9	11
Smoking (pack-yr)	27 ± 26	27 ± 22	25 ± 22	32 ± 29[b]
Diabetes (%)	7	22[a]	10	30[a]
College education (%)	38	39	42	26[b]
Current use of hormone replacement (%)	11	18[c]	14	15
Systolic blood pressure (mm Hg; mean ± SD)	142 ± 18	150 ± 21[a]	144 ± 18	155 ± 22[a]
Wt (kg; mean ± SD)	68 ± 12	67 ± 12	68 ± 12	66 ± 13
Modified body mass index (kg/m^2; mean ± SD)[d]	278 ± 47	279 ± 48	279 ± 47	274 ± 50
Fructosamine (μmol/L; mean ± SD)	249 ± 35	262 ± 56[b]	249 ± 32	278 ± 74[a]
LDL cholesterol (mmol/L; mean ± SD)	3.85 ± 0.80	3.90 ± 0.93	3.93 ± 0.85	3.72 ± 0.88[c]
HDL cholesterol (mmol/L; mean ± SD)	1.40 ± 0.36	1.34 ± 0.36	1.40 ± 0.36	1.34 ± 0.39
C-Reactive protein (μg/L; mean ± SD)	3,900 ± 5,800	5,400 ± 9,100[c]	4,300 ± 6,400	5,800 ± 10,500

Significance is indicated for comparisons between stroke cases and controls, and between those who lived or died during follow-up.
[a] $P < 0.0001$.
[b] $P < 0.01$.
[c] $P < 0.05$.
[d] Based on knee height, not total height (see *Subjects and Methods*).

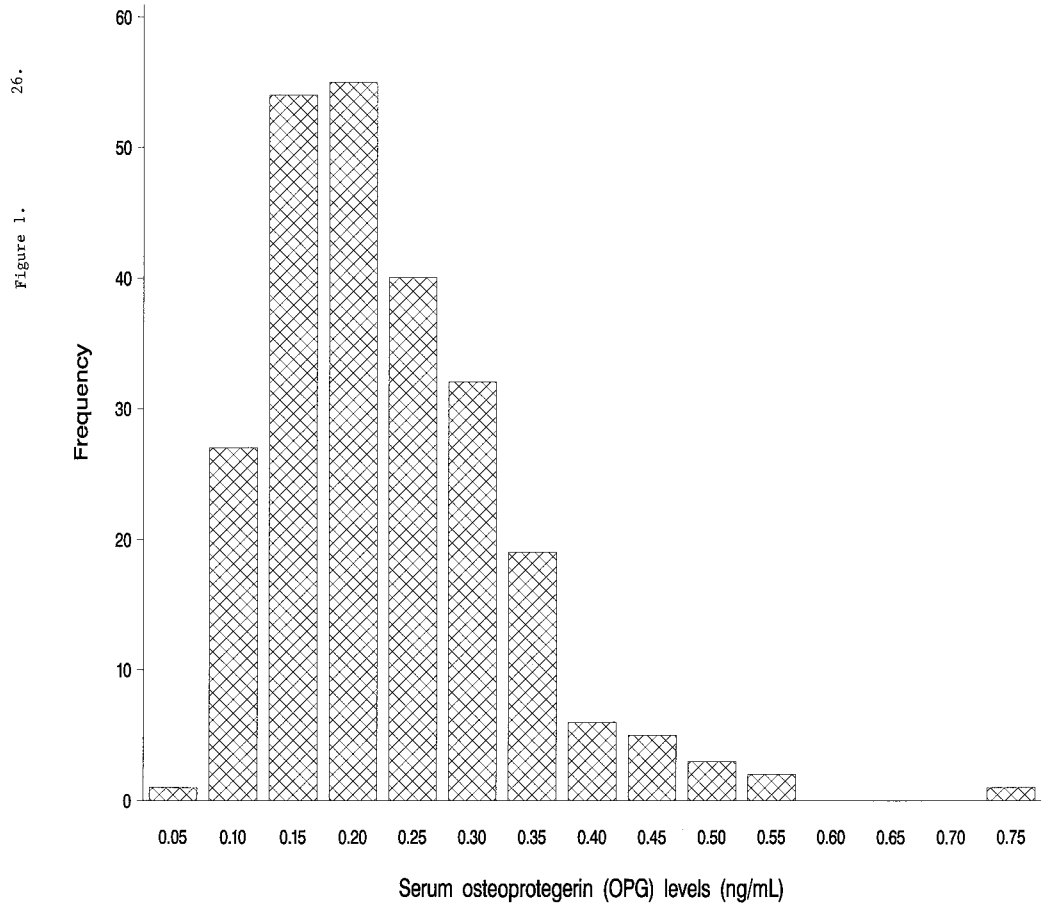

FIG. 1. Distribution of serum OPG levels in the 247 controls (women aged 65 yr and older, randomly selected from the cohort).

were also greater in the 70 women who were current users of hormone replacement therapy than in the 408 nonusers (Table 2; $P = 0.01$). Adjustment for age and body mass index had little effect on the associations between OPG levels and diabetes or hormone replacement therapy, but did account for the apparent associations with stroke and education.

Associations between OPG levels and subsequent mortality or stroke

Greater serum OPG levels were associated with increased all-cause and cardiovascular mortality (Table 3). The association between OPG and mortality was slightly diminished

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TABLE 2. OPG levels by selected characteristics of participants

Characteristic	Osteoprotegerin level (ng/mL)		*P*
	Among participants with characteristic	Among participants without characteristic	
At baseline			
Hypertension	0.25 ± 0.11	0.23 ± 0.12	0.03
Diabetes	0.30 ± 0.17	0.23 ± 0.10	<0.001
Cigarette smoking	0.27 ± 0.14	0.24 ± 0.11	0.08
Hormone replacement	0.27 ± 0.18	0.23 ± 0.10	0.01
College education	0.23 ± 0.09	0.25 ± 0.13	0.04
Body mass index (>305 kg/m^2)[a]	0.25 ± 0.11	0.24 ± 0.12	0.46
During follow-up			
Stroke	0.25 ± 0.13	0.23 ± 0.10	0.03
Death	0.28 ± 0.13	0.23 ± 0.11	<0.001

Values are the mean ± SD.
[a] Represents the upper quartile of modified body mass index.

in a multivariate model that adjusted for hypertension, diabetes, education, and the use of hormone replacement therapy, which were potential confounders of the association. There was no association between serum OPG levels and the risk of incident thrombotic strokes (Table 3), although OPG levels were associated with the risk of fatal strokes [age-adjusted odds ratio, 1.4/SD (0.11 ng/mL) increase; 95% confidence interval, 1.0–1.8; $P = 0.03$].

OPG levels were greater in women who died during follow-up regardless of whether they had diabetes (Fig. 3). The association between serum OPG levels and mortality was largely confined to women with OPG levels of 0.32 ng/mL or greater (the highest quintile). These women had a 3.0-fold (95% confidence interval, 1.5–6.2; $P = 0.02$) greater odds of mortality, including a 4.4-fold (95% confidence interval, 1.5–13; $P = 0.007$) greater odds of cardiovascular mortality, than those with levels of 0.15 ng/mL or less (the lowest quintile).

Associations between OPG levels and fractures, bone mineral density, and measures of calcium metabolism

OPG levels were not associated with the risk of subsequent fractures of all types (Table 3). In *post-hoc* analyses, there was a significant association between OPG levels and subsequent hip fractures (age-adjusted odds ratio, 1.3; 95% confidence interval, 1.0–1.7; $P = 0.03$), but not wrist fractures (age-adjusted odds ratio, 1.0; 95% confidence interval, 0.7–1.4; $P = 0.98$).

We found no significant correlations between serum OPG levels and bone mineral density at any of the five measurement sites: os calcis ($r = 0.00$; $P = 0.97$), distal radius ($r = 0.03$; $P = 0.53$), proximal radius ($r = -0.01$; $P = 0.83$), total hip ($r = -0.03$; $P = 0.58$), or spine ($r = 0.01$; $P = 0.77$). OPG levels were inversely correlated with serum osteocalcin levels ($r = -0.20$; $P = 0.0001$) and were weakly correlated with serum calcium ($r = 0.10$; $P = 0.03$) and PTH levels ($r = 0.09$; $P = 0.05$). In multivariate age-adjusted analyses, both fructosamine ($P = 0.0001$) and osteocalcin levels ($P = 0.0004$) were independently associated with OPG levels.

Discussion

We found that serum levels of OPG were greater in women with diabetes and in those who subsequently died of cardiovascular disease during follow-up than in control women. These associations were not affected by adjustment for age, body mass index, or other cardiovascular risk factors, including hypertension, smoking, and serum lipid levels. OPG levels were not associated with levels of C-reactive protein, suggesting that OPG is not only a nonspecific marker of inflammation.

Why should OPG levels be greater in women with diabetes than in control subjects? One possibility is that levels of serum glucose or glycosylated proteins affect the assay for OPG, but we found no correlation between levels of OPG and fructosamine in women without diabetes, suggesting that this is an unlikely explanation. Another hypothesis is that serum OPG levels reflect ongoing vascular disease, which is more common in patients with diabetes and in those who subsequently die. OPG levels, however, were not associated with the risk of nonfatal stroke. It is also possible that OPG levels are affected by an underlying condition that is common to both diabetes and vascular disease (15–17).

If *opg*-deficient mice, which have no measurable OPG in their blood, develop premature arterial calcification (mainly in the media of large vessels) (3) that is preventable by restoration of the gene (18), why are greater OPG levels in humans associated with diabetes and with an increased, rather than a decreased, risk of cardiovascular disease? One hypothesis is that increased serum OPG levels in humans are a response to rather than a cause of atherosclerosis or vascular calcification, perhaps in an attempt to regulate those processes. Another explanation is that the greater OPG levels are a result of decreased clearance of OPG, perhaps because of increased binding of OPG ligand. The results of this epidemiological study cannot be used to distinguish between these or other potential explanations.

OPG levels were also greater in women who were using hormone replacement therapy. This was not a randomized trial, however, and it is possible that OPG levels are a marker for health conditions that affected the likelihood that a woman used hormone replacement therapy rather than a consequence of the biological effects of estrogen.

Previous studies have suggested that patients with diabetes and peripheral vascular disease are more likely to have medial artery (macrovascular) calcification, which may be associated with an increased risk of vascular events (19–21).

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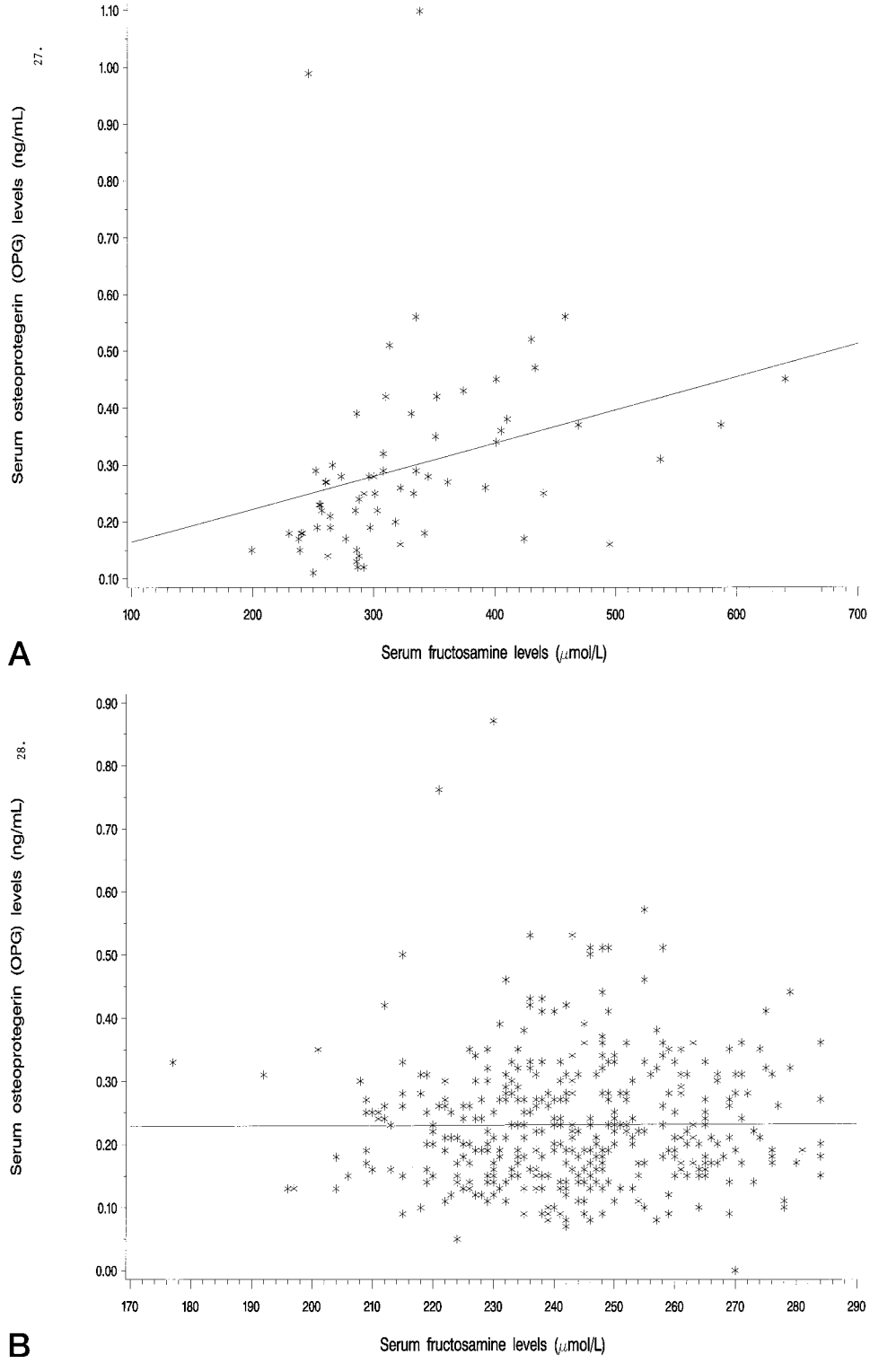

FIG. 2. Scatter plot of serum OPG levels (y-axis) and serum fructosamine levels (x-axis) in 482 women aged 65 yr and older. Women with diabetes (n = 69; defined by self-report or a serum fructosamine level >285 μmol/L) are shown in A. Those without diabetes (n = 413) are shown in B; there is a change in the scale of the axes. There is a significant correlation between serum levels of OPG (nanograms per mL) and fructosamine (micromoles per L) among women with diabetes (OPG = 0.107 + 0.00058× fructosamine; r = 0.29; P = 0.02), but not in women without diabetes (OPG = 0.223 + 0.000028× fructosamine; r = 0.005; P = 0.92).

It is important to emphasize, however, that we did not measure vascular calcification directly, at either the macrovascular or intimal level, and that the apparent similarity between the effects of diabetes in humans and those of *opg* deficiency in mice may well be coincidental.

We were unable to confirm the results of a recent report from Japan that found an association between OPG levels and bone mineral density (11). Those investigators indicated that OPG circulates as both a monomer and a homodimer; it remains to be determined whether the assay that we used measures the same form(s) of OPG as in that study (11). We did not find that OPG levels were associated with the risk of subsequent fractures, except perhaps that greater OPG levels were associated with an increased risk of hip fractures in a

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TABLE 3. Associations between serum OPG levels, mortality, and incident stroke and fracture during follow-up

Outcome	No. of events[a]	Odds ratio[b] (95% confidence interval)	*P*
Adjusted for age			
All-cause mortality	116	1.4 (1.2–1.8)	0.001
Cardiovascular mortality	55	1.4 (1.1–1.8)	0.009
Incident stroke	241	1.1 (0.9–1.4)	0.2
Fracture	154	1.1 (0.9–1.4)	0.3
Adjusted for age and current use of hormone replacement therapy			
All-cause mortality	112	1.4 (1.2–1.8)	0.001
Cardiovascular mortality	54	1.4 (1.1–1.8)	0.01
Adjusted for age, diabetes, history of hypertension, college education, and current use of hormone replacement therapy[c]			
All-cause mortality	110	1.3 (1.0–1.6)	0.04
Cardiovascular mortality	53	1.3 (1.0–1.7)	0.06
Multivariate-adjusted[d]			
All-cause mortality	106	1.3 (1.0–1.6)	0.06
Cardiovascular mortality	51	1.4 (1.0–1.8)	0.05

[a] Numbers of events differ slightly among models because of missing data.
[b] Per SD (0.11 ng/mL) increase in serum osteoprotegerin level.
[c] These variables were associated with OPG levels at $P < 0.05$.
[d] Adjusted for age, diabetes, history of hypertension, college education, current use of hormone replacement therapy, pack-years of smoking, modified body mass index, and serum levels of HDL and LDL cholesterol and C-reactive protein.

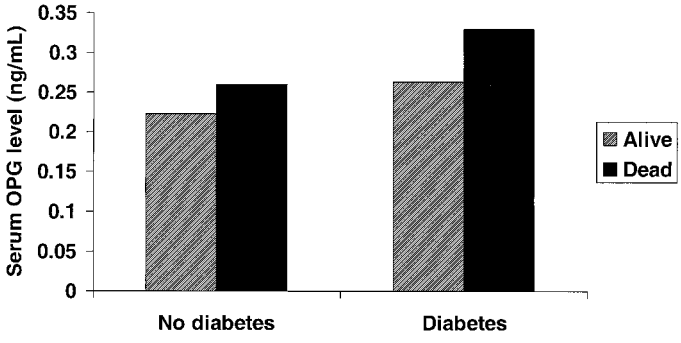

FIG. 3. Mean OPG levels in elderly women, stratified by diabetes at baseline and mortality during follow-up. The differences in OPG levels between those who survived and those who died were significant among women without diabetes ($P = 0.04$) and those with diabetes ($P = 0.01$).

post-hoc analysis that involved only 28 women with hip fractures; this finding should be examined in other studies.

We found an inverse correlation between serum levels of osteocalcin and OPG. Osteocalcin is a small protein (molecular weight, 5800) that is synthesized by osteoblasts, and serum osteocalcin levels are a marker of bone formation (22). Osteocalcin and its messenger ribonucleic acid have also been identified in platelets (23). We cannot determine, however, whether OPG and osteocalcin have a true biological (*e.g.* counterregulatory) relation or are both affected by an unmeasured third factor. The inverse correlation that we observed between serum osteocalcin and fructosamine levels ($r = -0.23$; $P = 0.0001$) is consistent with a previous finding that osteocalcin levels increased with better glucose control in 16 middle-aged men with diabetes (24). Adjustment for serum fructosamine levels, however, did not affect the association between osteocalcin and OPG levels.

Our study has several other important limitations. We enrolled only elderly white women who were ambulatory at the time of the baseline examination. This study was primarily designed to look at risk factors for stroke, as reflecting in our sampling scheme. It is plausible, albeit unlikely, that oversampling stroke cases, compared with other women in the cohort, may have affected the estimated magnitude of the association between OPG level and mortality, as stroke deaths were overrepresented. There was no association, however, between OPG level and the risk of stroke, and our analyses had similar results, albeit with less power due to smaller sample sizes, when they were restricted to only control subjects. In addition, our results should be interpreted with caution; some of the statistically significant findings may have been due to chance.

Serum samples had been stored for several years before the assays were performed, and we cannot verify the long-term stability of OPG levels in frozen sera. However, we were able to assay OPG levels in all but one specimen. Moreover, degradation of OPG in serum would have made it more difficult to find an association among OPG levels, mortality, and diabetes. Because an assay was not available, we did not measure levels of OPG ligand in our samples. It seems reasonable to assume that these levels are important, and that their measurement would enhance our understanding of the effects of OPG.

Our results raise the possibility that the OPG system may be involved in vascular calcification in humans, as has been seen in genetically altered laboratory animals (3) and with other regulators of bone formation and resorption (25–32). Additional research is needed to confirm these findings in another sample, to clarify the importance of OPG ligand, and to determine whether serum OPG levels are a cause or an effect of vascular disease. OPG levels, at least as we measured them, were not associated with bone mineral density or overall fracture risk.

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