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# Similar to adiponectin, serum levels of osteoprotegerin are associated with obesity in healthy subjects

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ABSTRACT

An increase in serum osteoprotegerin (OPG) is associated with type 2 diabetes mellitus, the severity of vascular calcification, and coronary artery disease. Obesity is a risk factor for diabetes and cardiovascular disease, but little is known about the relationship between OPG and obesity. The purpose of this study was to determine if changes in body mass index (BMI) and insulin sensitivity influence circulating OPG in healthy subjects. A total of 100 subjects (36 lean, 41 overweight, and 23 obese) with normal glucose tolerance, blood pressure, and electrocardiogram stress test result volunteered for this study. Insulin sensitivity was estimated using a 2-hour oral glucose tolerance test with oral glucose insulin sensitivity analysis. Osteoprotegerin, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), soluble receptor activator of nuclear factor–$\kappa\beta$ ligand (sRANKL), and adiponectin were analyzed using commercially available enzyme-linked immunosorbent assays. Osteoprotegerin ($P < .01$) and adiponectin ($P < .001$) were significantly decreased in the obese compared with lean subjects. There was no significant difference between BMI categories for TRAIL or sRANKL. Controlling for age and sex, there was a significant correlation between OPG and adiponectin ($r = 0.391$, $P < .001$), BMI ($r = –0.331$, $P < .001$), waist circumference ($r = –0.268$, $P < .01$), homeostasis model assessment of insulin resistance ($r = –0.222$, $P < .05$), and oral glucose insulin sensitivity ($r = 0.221$, $P < .05$). Both OPG and adiponectin were negatively correlated with body weight, BMI, waist circumference, and fasting plasma insulin while being positively correlated with insulin sensitivity ($P < .05$). Controlling for age, sex, and BMI, TRAIL was positively related to fat mass ($r = 0.373$, $P < .001$) and waist circumference ($r = 0.257$, $P < .05$). In contrast to patients with type 2 diabetes mellitus, circulating OPG is lower in obese, but otherwise healthy subjects and is positively correlated with indices of insulin sensitivity.



Institutional approval: This study was approved by the Dublin City University Research Ethics Committee.

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## 1. Introduction

There has been a dramatic increase in the prevalence of obesity during the past 2 decades. Increased adiposity is associated with the development of insulin resistance and is a risk factor for type 2 diabetes mellitus [1] and vascular dysfunction [2,3]. Increasingly, it is recognized that adipose tissue is an active metabolic organ releasing adipokines into the circulation that influence insulin action and contribute to vascular dysfunction. Adiponectin and tumor necrosis factor (TNF)–$\alpha$ are 2 such adipokines. A reduction in adiponectin and an increase in TNF-$\alpha$ production have been shown to reduce insulin sensitivity [4-6] and increase vascular dysfunction [7].

The vascular endothelium can also produce and release glycoproteins into the circulation; yet little is know about their effect, if any, on insulin action. Osteoprotegerin (OPG) is a member of the TNF receptor superfamily [8] that is released into the circulation as a soluble glycoprotein [9] and binds to receptor activator of nuclear factor–$\kappa\beta$ ligand (RANKL) and TNF-related apoptosis-inducing ligand (TRAIL) [10,11]. Osteoprotegerin is present in a number of tissues including bone where it inhibits osteoclastogenesis, the immune system, and the vasculature. Osteoprotegerin and RANKL are highly expressed in vascular smooth muscle [12] and endothelial cells [13,14], with protein content 500 to 1000 times greater than circulating levels [15,16]. In animal studies, OPG-deficient mice exhibit severe aortic and renal calcification [17]; yet the significance of OPG and RANKL within the vasculature is not fully understood.

In humans, serum OPG correlates with the degree of underlying coronary artery calcification in patients with type 2 diabetes mellitus [18] and the severity of coronary artery disease [19-21], suggesting an important link between OPG and vascular dysfunction. TRAIL is expressed and secreted by cells of the immune system and appears to exert an effect on the vasculature. It may contribute to plague instability but others have shown the administration of TRAIL to atherogenic apolipoprotein E-/- mice induced plaque regression [22]. Despite the fact that weight gain is a risk factor for type 2 diabetes mellitus and cardiovascular disease, it is not known if obesity contributes to the changes in OPG and TRAIL observed in these studies. Therefore, the purpose of this study was to determine if differences in body mass index (BMI) and insulin sensitivity influence the concentrations of serum OPG and TRAIL in subjects who do not have cardiovascular or metabolic disease.

## 2. Materials and methods

### 2.1. Subjects

Participants were recruited by means of an open call for volunteers who were free from cardiovascular and metabolic disease and not taking any medication. In total, 136 subjects volunteered to participate in the study. Of these, 36 were excluded because of undiagnosed hypertension, impaired glucose tolerance, hyperlipidemia, and an abnormal 12-lead electrocardiogram (ECG) result at rest or in response to exercise. The final cohort that met the inclusion criteria consisted of 100 subjects, aged 22 to 74 years. This group comprised a similar number of men (n = 51) and women (n = 49); and the distribution of normal-weight (n = 36), overweight (n = 41), and obese (n = 23) subjects was similar to the Irish adult population [23]. All procedures were approved by the Dublin City University Research Ethics Committee, and subjects provided written informed consent before participation in the study.

### 2.2. Experimental design

Subjects visited the laboratory on 2 separate occasions, separated by at least 4 days. On the first occasion, they reported to the laboratory in the morning following an overnight fast. Subjects were interviewed by a physician and had anthropometric measurements and a 2-hour 75-g oral glucose tolerance test (OGTT). On the second visit, subjects reported to the laboratory approximately 3 hours after their last meal. They had a resting 12-lead ECG followed by an ECG stress test with oxygen consumption.

### 2.3. Anthropometric measures

Height and body mass were measured to the nearest 0.1 cm and 0.1 kg, respectively (SECA, Hamburg, Germany). Subjects were weighed barefoot and with minimal clothing. Body composition was estimated using 7-site skinfold measurements. Waist circumference was taken midway between the lowest rib (laterally) and the iliocristale landmark. Hip circumference was measured at the greatest protuberance of the gluteals.

### 2.4. Exercise stress test

Subjects underwent a multistage exercise treadmill test using a modified Bruce protocol. Heart rate, ECG, blood pressure, and cardiorespiratory responses were monitored throughout the exercise test. Breath-by-breath oxygen consumption was measured using indirect calorimetry (Sensormedics, Yorba Linda CA). The criteria for maximal oxygen consumption ($Vo_{2max}$) included a leveling off in the oxygen consumption, a respiratory exchange ratio greater than 1.1, and a heart rate greater than 95% age-predicted heart rate max.

### 2.5. Oral glucose tolerance test

A standard 2-hour 75-g OGTT, using the World Health Organization criteria [24], was used to confirm normal glucose tolerance. Blood samples were taken before and at 30, 60, 90, and 120 minutes after the glucose load. Blood samples were centrifuged, and serum was stored at –80$^{\circ}$C for further analysis. Total area under curve (AUC) for glucose and insulin was determined by the trapezoidal method, and homeostasis model assessment of insulin resistance (HOMA-IR) was used as an indicator of insulin resistance. Insulin sensitivity was estimated using the validated oral glucose insulin sensitivity (OGIS) predictive model [25].

### 2.6. Biochemical analysis and assays

Plasma glucose was measured using the glucose oxidase method (YSI 2300 Stat Plus, Yellow Springs, OH). Serum insulin was measured with a commercially available fluoroimmunoassay (Delphia; Perkin Elmer, Wallac, Turku, Finland). Serum levels of OPG, total soluble RANKL (sRANKL) (Biomedica, Vienna, Austria), TRAIL, and adiponectin (R&D Systems, Minneapolis, MN) were measured using commercially available enzyme-linked immunosorbent assay kits. The minimal detectable limit was 0.014 pmol·L$^{-1}$ for OPG, 0.02 pmol·L$^{-1}$ for total sRANKL, 0.246 ng·mL$^{-1}$ for adiponectin, and 2.86 ng·mL$^{-1}$ for TRAIL. The intra- and interassay coefficients of variance were less than 6% for OPG and total sRANKL and less than 5% for adiponectin and TRAIL. High-sensitivity C-reactive protein and triglycerides were measured using the Randox-Daytona automated analyzer (Randox, Antrim, Northern Ireland).

### 2.7. Statistical procedures

SPSS 15.0 for Windows (SPSS, Chicago, IL) was used for statistical analysis. Data are presented as means ± SEM. Normally distributed variables were explored using simple bivariate or partial regression. Nonnormally distributed variables including fasting glucose, insulin, 2-hour insulin, AUC glucose, AUC insulin, HOMA-IR, adiponectin, and sRANKL were log-transformed. The relationship between variables was calculated using Pearson product moment ($r$). Participants were classified as normal weight (BMI >18.5 and <25.0 kg·m$^{-2}$), overweight (BMI >25.0 and <30.0 kg·m$^{-2}$), or obese (BMI >30.0 kg·m$^{-2}$). A 1-way analysis of covariance was used to examine differences between BMI categories with age and sex as covariates. Bonferroni post hoc test was applied to determine differences among means. Statistical significance was set at $P < .05$.

## 3. Results

### 3.1. Physical characteristics

The physical and metabolic characteristics for the normal-weight, overweight, and obese subjects are presented in Table 1. Age and sex distribution was similar for the 3 groups; but there were significant differences in BMI, percentage body fat, waist circumference, and waist-to-hip ratio. In addition, the obese group had significantly higher systolic and diastolic blood pressure and lower aerobic capacity as assessed by $VO_{2max}$ (mL·kg·min$^{-1}$) compared with the other 2 groups.

### 3.2. Metabolic phenotype

All subjects had normal glucose tolerance; but the obese group had significantly higher fasting plasma glucose, insulin, and triglycerides when compared with the other 2 groups. They also had a greater glucose and insulin response to the OGTT and had lower insulin sensitivity, as determined by OGIS and HOMA-IR. Circulating adiponectin was significantly lower in men compared with women (4.99 ± 0.35 vs 10.06 ± 0.71 $\mu$g·mL$^{-1}$, $P < .001$) and in overweight and obese subjects ($P < .05$) compared with controls (Table 2).

### 3.3. Serum OPG and TRAIL concentrations

There was a significant decrease in OPG in the obese compared with normal-weight and overweight groups (Fig. 1). Circulating OPG was also lower in men than in women (5.13 ± 0.20 vs 6.07 ± 0.23 pmol·L$^{-1}$, $P = .003$). There were no significant differences between BMI categories for serum TRAIL (72 ± 5, 82 ± 4, and 82 ± 7 pg·mL$^{-1}$ for normal-weight, overweight, and obese groups) and sRANKL (3.4 ± 0.6, 3.0 ± 0.4, and 2.9 ± 0.7 pg·mL$^{-1}$ for normal-weight, overweight, and obese groups). Although there was no relationship between OPG and age, we controlled for age in addition to sex because previous studies have reported a correlation [27-29].

### 3.4. Correlation analysis

Osteoprotegerin showed a significant inverse correlation with BMI and waist circumference and a positive relationship with $Vo_{2max}$. There were also significant relationships between OPG and several other metabolic indices including a significant inverse correlation with fasting plasma glucose, fasting insulin, AUC glucose, AUC insulin, and HOMA-IR and a positive correlation with OGIS and adiponectin (Table 3). The correlation between OPG and adiponectin persisted after

**Table 1 – Subject characteristics**

| | Normal weight | Overweight | Obese |
|---|---|---|---|
| | (36) | (41) | (23) |
| Sex (male/female) | 19/17 | 19/22 | 11/12 |
| Age (y) | 44.4 ± 1.5 | 46.7 ± 2.0 | 47.2 ± 2.8 |
| BMI (kg·m$^{-2}$) | 22.8 ± 0.2 | 26.7 ± 0.2$^{*}$ | 31.4 ± 0.3$^{*,\dagger}$ |
| Waist circumference (cm) | 78.5 ± 1.2 | 89.8 ± 1.2$^{*}$ | 102.2 ± 2.0$^{*,\dagger}$ |
| Waist-to-hip ratio | 0.82 ± 0.01 | 0.87 ± 0.01$^{*}$ | 0.93 ± 0.02$^{*,\dagger}$ |
| Body fat (%) | 19.6 ± 1.15 | 27 ± 1.16$^{*}$ | 31.9 ± 1.5$^{*,\dagger}$ |
| $VO_{2max}$ (mL·kg·min$^{-1}$) | 41.5 ± 1.9 | 37.5 ± 1.5 | 29.6 ± 1.7$^{*,\dagger}$ |
| Systolic BP (mm Hg) | 117.1 ± 2.2 | 120.4 ± 1.6 | 130.3 ± 2.2$^{*,\dagger}$ |
| Diastolic BP (mm Hg) | 74.0 ± 1.5 | 76.5 ± 1.4 | 80.6 ± 2.1$^{*}$ |

Values are mean ± SEM. BP indicates blood pressure.
$^{*}$ $P < .05$ vs normal weight.
$^{\dagger}$ $P < .05$ vs overweight.

**Table 2 – Metabolic markers and indicators of insulin sensitivity**

| | Normal weight | Overweight | Obese |
|---|---|---|---|
| Fasting glucose (mmol·L$^{-1}$) | 4.8 ± 0.1 | 5.0 ± 0.1 | 5.3 ± 0.1 *,† |
| Fasting insulin (pmol·L$^{-1}$) | 26.4 ± 3.5 | 38.2 ± 4.2 * | 51.3 ± 5.6 * |
| Triglycerides (mmol·L$^{-1}$) | 0.97 ± 0.05 | 1.32 ± 0.10 * | 1.55 ± 0.15 * |
| HOMA-IR | 0.83 ± 0.12 | 1.25 ± 0.14 * | 1.8 ± 0.2 *,† |
| OGIS (mL·min·m$^{-2}$) | 533 ± 11 | 512 ± 9 | 451 ± 11 *,† |
| AUC glucose (mmol·L$^{-1}$·min) | 671 ± 18 | 738 ± 19 * | 831 ± 36 *,† |
| AUC insulin (pmol·L$^{-1}$·min) | 20487 ± 2444 | 29585 ± 4285 | 42336 ± 4979 *,† |
| hs-CRP (mg·L$^{-1}$) | 0.92 ± 0.18 | 0.92 ± 0.08 | 1.16 ± 0.15 |
| Adiponectin ($\mu$g·mL$^{-1}$) | 9.9 ± 0.9 | 6.6 ± 0.5 * | 4.8 ± 0.5 *,† |

Values are mean ± SEM. HOMA-IR was based on Matthews et al [26]; OGIS was based on Mari et al [25]. hs-CRP indicates high-sensitivity C-reactive protein.
* $P < .05$ vs normal weight.
† $P < .05$ vs overweight.

additional adjustment for BMI. A multiple, stepwise linear regression was performed to predict OPG concentrations using BMI, waist circumference, $Vo_{2max}$, fasting plasma glucose, fasting plasma insulin, AUC glucose, AUC insulin, HOMA-IR, OGIS, and adiponectin. Entry criterion for the model was set at $P < .05$. Adiponectin was the strongest predictor of serum OPG, and all other variables were eliminated from the model because they did not reach the entry criterion. Controlling for age and sex, TRAIL was significantly related to fat mass ($r = 0.255$, $P < .05$) and waist circumference ($r = 0.207$, $P < .05$). These relationships were maintained after additional adjustment for BMI ($r = 0.373$, $P < .001$ for fat mass; $r = 0.257$, $P = .011$ for waist circumference).

## 4. Discussion

The main finding from our study is that obese subjects, with normal glucose tolerance and free from cardiovascular disease, have lower circulating OPG when compared with normal-weight and overweight individuals. The purpose of this study was to examine the relationship between insulin sensitivity, obesity, and OPG in a well characterized, healthy population free from cardiovascular and metabolic disease. The positive relationship between OPG and adiponectin and the negative association with glucose-stimulated insulin secretion in this healthy cohort differ from subjects with type 2 diabetes mellitus [15,18,30,31] and nondiabetic subjects with cardiovascular disease [19,20,32-34].

Understanding the functional significance of circulating OPG has been complicated by a number of factors including the relative contribution of various tissue sources and the presence of multiple comorbidities. Following the recent link between OPG and vascular disease, we carefully screened the subjects in this study to exclude those with hypertension, hyperlipidemia, or other cardiovascular risk factors. Using this approach, we found that subtle, subclinical changes in insulin or insulin sensitivity coincide with a reduction in OPG. These results also suggest that obesity per se is not a contributory factor to increased OPG in type 2 diabetes mellitus and cardiovascular disease, despite being an independent risk factor for both conditions.

The role of obesity in the regulation of circulating OPG is not clear. Although previous studies have examined the

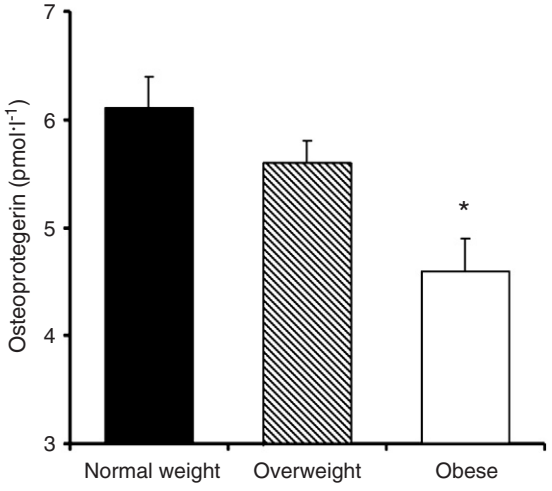


**Fig. 1 – Serum OPG for normal-weight (n = 36), overweight (n = 41), and obese subjects (n = 23). Data are presented as mean ± SEM. *Significantly different to normal-weight and overweight subjects; $P < .05$.**

**Table 3 – Age- and sex-adjusted correlations between OPG and anthropometric and metabolic indices**

| | $r$ | $P$ |
|---|---|---|
| BMI (kg·m$^{-2}$) | −0.331 | ‡ |
| Waist circumference (cm) | −0.268 | † |
| $VO_{2max}$ (mL·kg·min$^{-1}$) | 0.237 | * |
| Fasting glucose (mmol·L$^{-1}$·min) | −0.248 | * |
| Fasting insulin (pmol·L$^{-1}$·min) | −0.202 | † |
| AUC glucose (mmol·L·min) | −0.279 | † |
| AUC insulin (pmol·L·min) | −0.271 | † |
| HOMA-IR | −0.222 | * |
| OGIS (mL·min·m$^{-2}$) | 0.221 | * |
| Adiponectin ($\mu$g·mL$^{-1}$) | 0.391 | ‡ |

HOMA-IR was based on Matthews et al [26]; OGIS was based on Mari et al [25].
* $P < .05$.
† $P < .01$.
‡ $P < .001$.

relationship between adiposity and OPG in large populations, these studies have been conducted in heterogeneous populations with a variety of cardiovascular risk factors known to influence circulating OPG [35-38]. Some studies report a decrease in OPG in obese subjects compared with lean controls [39,40], but other studies have not found a relationship between OPG and BMI [41,42]. Weight gain is accompanied by a number of metabolic alterations including a decrease in insulin sensitivity. Ugur-Altan et al [39] divided a group of obese healthy subjects into tertiles based on insulin sensitivity and compared these groups with lean control subjects. When OPG was corrected for BMI, it was significantly lower in all obese tertiles compared with lean controls. They also found that OPG was significantly lower in the least insulin-sensitive obese tertile compared with the most sensitive. In the present study, insulin sensitivity was significantly lower in obese compared with overweight and normal-weight groups; and there was a positive relationship between OPG and insulin sensitivity for all subjects. If insulin sensitivity was a major regulator of serum OPG, exercise training or weight loss would be expected to induce a change in the circulating concentration. We found a positive correlation between OPG and aerobic fitness that would suggest that exercise training may increase serum OPG. However, other studies using a dietary restriction–weight loss intervention reported no change [39,41] or a further decrease in OPG [40]. Therefore, further work is required to differentiate between the impact of exercise and energy restriction on circulating OPG; and it remains unclear whether obesity or insulin sensitivity is a more important regulator of OPG.

We found an inverse relationship between fasting OPG and the AUC for glucose and insulin during the OGTT, in support of recent studies suggesting that insulin may be the primary effector of decreased OPG. Jorgensen et al [43] reported a decrease in OPG during a 4-hour hyperinsulinemic-euglycemic clamp in lean, obese, and obese type 2 diabetes mellitus subjects. The magnitude of insulin-lowering effects on OPG was reduced by approximately 50% in the obese and type 2 diabetes mellitus groups when compared with lean controls. A decrease in OPG has also been reported in lean and morbidly obese subjects following an OGTT [43,44] and in lean male subjects following a hyperglycemic clamp [45]. In this study, Knudsen et al [45] found that the decrease in OPG was related to a change in serum insulin and not glucose during the hyperglycemic clamp. In addition, the in vitro incubation of human vascular smooth muscle cells with insulin decreased OPG production compared with control samples [16]. Therefore, subtle increases in insulin secretion, as observed in this study, may be adequate to decrease OPG production in a healthy cohort.

It is possible that other factors may also contribute to the change in OPG. The positive relationship between OPG and adiponectin was robust and maintained after additional correction for BMI. Adiponectin is an adipocyte-specific endocrine protein with anti-inflammatory and insulin-sensitizing actions. Circulating adiponectin is lower in obese subjects compared with lean controls and is also decreased with cardiovascular disease and type 2 diabetes mellitus [46]. It is not yet known if adiponectin secretion and OPG appearance are directly related physiologic processes or if the positive correlation between OPG and adiponectin reported here and in other studies [41,42] is evident only in healthy cohorts. This association appears to be lost in type 2 diabetes mellitus and cardiovascular disease, as adiponectin decreases while OPG increases. It appears that certain physiologic conditions "uncouple" the positive relationship between OPG and insulin sensitivity observed in healthy groups.

This may be why an increase in circulating OPG has been consistently linked with the onset, progression, and severity of cardiovascular disease [19,20]. Osteoprotegerin is an independent risk factor for incident cardiovascular disease [33,47], heart failure [35,47], and all-cause [32] and vascular mortality [33,35,48]. When the severity of vascular disease is assessed by coronary angiography, circulating OPG increases proportionally with the number of diseased vessels [19,20]. In patients with type 2 diabetes mellitus, OPG is increased in some [32,49] but not all [43] studies. The reason for this may be related to the presence or absence of micro- and macrovascular complications. In studies that used a nondiabetic control group, circulating OPG was similar between controls and diabetic patients without vascular complications [15,31,43], whereas other studies have shown significantly higher OPG in diabetes patients with asymptomatic silent coronary artery disease [30,50] or microvascular complications including microalbuminuria [49], retinopathy [15], and neuropathy [31]. It is not known why circulating OPG is increased with type 2 diabetes mellitus and why this response is in contrast to obesity-related changes. It may be related to the increased presence of proinflammatory cytokines, as the in vitro incubation of human vascular smooth muscle cells [16] and human microvascular endothelial cells [13,51] with TNF-$\alpha$, but not glucose, increases OPG production.

Circulating TRAIL and RANKL are ligands for the soluble OPG receptor. Tumor necrosis factor–related apoptosis-inducing ligand exerts an effect on the vascular wall and is thought to influence plaque stability [22]. We found a positive relationship between TRAIL, fat mass, and waist circumference. Evidence from in vitro experiments suggest that TRAIL may promote apoptosis in vascular smooth muscle cells [22], whereas OPG appears to promote endothelial cell survival [14,52], possibly through inhibition of TRAIL-induced apoptosis [53]. Further experiments are required to determine if circulating TRAIL has a biological effect.

We did not find any significant changes or relationships with sRANKL. This may be due to the fact that our subject cohort was healthy, as other studies have reported elevated RANKL to be associated with increased [54] and decreased [55] cardiovascular disease risk. This inconsistency may also result from different assay methodologies. Several studies have measured unbound and uncomplexed sRANKL or OPG [15,49,56]; but in this study, free soluble RANKL and total OPG were measured. The OPG assay detects both monomeric and dimeric isoforms of OPG, including OPG bound to RANKL and TRAIL. It is not possible to compare the OPG concentrations from different assays because of the difficulty ascribing an exact molecular weight to the OPG isoforms. Therefore, studies that have measured uncomplexed OPG may have unintentionally excluded a large portion of the biologically active total OPG that has bound to TRAIL, RANKL, or other nonspecific ligands.

In conclusion, the results from this study suggest that an obesity-related decrease in insulin sensitivity or an increase in insulin secretion suppresses circulating OPG in healthy individuals. Observational studies, such as the present investigation, cannot prove causality but can raise hypotheses. Further study is required to determine the effect of a fall in serum OPG on vascular function in healthy subjects with increasing adiposity and also to determine why and how OPG increases in diabetic and nondiabetic patients with documented vascular disease.

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