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ORIGINAL ARTICLE

The role of serum osteoprotegerin and receptor–activator of nuclear factor-κB ligand in metabolic bone disease of women after obesity surgery

José A. Balsa[1,6] · Christian Lafuente[1] · Jesús M. Gómez-Martín[1] · Julio Galindo[2] · Roberto Peromingo[2] · Francisca García-Moreno[2] · Gloria Rodriguez-Velasco[2] · Javier Martínez-Botas[3,4] · Diego Gómez-Coronado[3,4] · Héctor F. Escobar-Morreale[1,5] · José I. Botella-Carretero[1,4]

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Abstract Metabolic bone disease may appear as a complication of obesity surgery. Because an imbalance in the osteoprotegerin and receptor–activator of nuclear factor-κB ligand system may underlie osteoporosis, we aimed to study this system in humans in the metabolic bone disease occurring after obesity surgery. In this study we included sixty women with a mean age of 47 ± 10 years studied 7 ± 2 years after bariatric surgery. The variables studied were bone mineral density, β-isomer of C-terminal telopeptide of type I collagen cross-links (a bone resorption marker), the bone formation markers osteocalcin and N-terminal propeptide of procollagen 1, serum osteoprotegerin and receptor–activator of nuclear factor-κB ligand. Serum osteoprotegerin inversely correlated with the bone remodeling markers osteocalcin, β-isomer of C-terminal telopeptide of type I collagen cross-links and N-terminal propeptide of procollagen 1. The osteoprotegerin and receptor–activator of nuclear factor-κB ligand ratio also correlated inversely with serum parathormone and osteocalcin. Bone mineral density at the lumbar spine was associated with age ($\beta = -0.235$, $P = 0.046$), percentage of weight loss ($\beta = 0.421$, $P = 0.001$) and osteoprotegerin and receptor–activator of nuclear factor-κB ligand ratio ($\beta = 0.259$, $P = 0.029$) in stepwise multivariate analysis ($R^2 = 0.29$, $F = 7.49$, $P < 0.001$). Bone mineral density at the hip site was associated only with percentage of weight loss ($\beta = 0.464$, $P < 0.001$) in stepwise multivariate regression ($R^2 = 0.21$, $F = 15.1$, $P < 0.001$). These data show that the osteoprotegerin and receptor–activator of nuclear factor-κB ligand system is associated with bone markers and bone mineral density at the lumbar spine after obesity surgery.

Keywords Metabolic bone disease · Osteoprotegerin · Receptor–activator of nuclear factor-κB ligand · Osteopenia · Obesity surgery

✉ José I. Botella-Carretero
joseignacio.botella@salud.madrid.org

[1] Department of Endocrinology and Nutrition, Hospital Universitario Ramón y Cajal, Universidad de Alcalá, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Carretera de Colmenar Km. 9.1, 28034 Madrid, Spain

[2] Department of Digestive and General Surgery, Hospital Universitario Ramón y Cajal, Universidad de Alcalá, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain

[3] Department of Biochemistry-Research, Hospital Universitario Ramón y Cajal, Universidad de Alcalá, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain

[4] Centro de Investigación Biomédica en Red-Fisiopatología de Obesidad y Nutrición (CIBERobn), Hospital Universitario Ramón y Cajal, Universidad de Alcalá, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain

[5] Centro de Investigación Biomédica en Red Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Hospital Universitario Ramón y Cajal, Universidad de Alcalá, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain

[6] Present Address: Department of Endocrinology and Nutrition, Hospital Infanta Sofía, Madrid, Spain

Introduction

Obesity is a major health problem showing increasing prevalence worldwide [1] with a subsequent increase in all-cause mortality [2]. In recent years, the use of obesity surgery for the treatment of severe obesity has increased, as it results in more successful weight loss and long-term weight maintenance when compared with weight loss

strategies based on diet and lifestyle changes [3]. Long-term outcomes after obesity surgery have shown resolution of obesity-associated complications such as diabetes mellitus, hypertension, dyslipidemia, sleep apnea, polycystic ovarian syndrome in women and hypogonadism in men in a high percentage of patients [4–7].

However, even though modern bariatric surgical techniques show relatively few complications, they are not free of long-term nutritional and metabolic issues [8–10]. Metabolic bone disease may present early after obesity surgery as an increase in markers of bone turnover that is not related to 25-hydroxyvitamin D and parathyroid hormone (PTH) concentrations [11–13]. Although the mechanisms involved are largely unknown, the cause may be related to the rapid and intense weight loss taking place during the first months after surgery [12]. In addition, late metabolic bone disease may also develop after patients reach a steady body weight. Chronic malabsorption of calcium and vitamin D results in secondary hyperparathyroidism that is more severe after Scopinaro's biliopancreatic diversion (BPD) than after laparoscopic Roux-en-Y gastric bypass (LRYGB) or restrictive procedures [9, 11]. This in turn exposes these patients to a higher risk of elevated bone turnover markers and osteoporosis.

Osteoprotegerin (OPG) is a glycoprotein [14, 15] generally considered to be produced in many different tissues and cell types, and, through its binding to the receptor–activator of nuclear factor-κB ligand (RANKL), exerts an inhibitory effect on osteoclastic bone resorption [16]. Hence, osteoclast activity depends, at least in part, on the relative balance of RANKL and OPG. Studies in animal models of bone disease show that RANKL inhibition leads to marked suppression of bone resorption and to increases in cortical and cancellous bone volume, density and strength [17, 18]. RANKL inhibitors also prevent the focal bone loss that occurs in animal models of rheumatoid arthritis and bone metastasis [17].

Osteoporosis in humans has also been related to an imbalance in the OPG/RANKL system [19] leading to the development of drugs such as denosumab that selectively target the inhibition of RANKL [20]. A recent study explored the OPG/RANKL system in an animal model of obesity surgery [21] but, to the best of our knowledge, no human studies have been reported so far. Therefore, we aimed to study in humans the role of the OPG/RANKL system in metabolic bone disease occurring in the long term after obesity surgery.

Materials and methods

Patients

We included 60 women with a mean age of 47 ± 10 years who had previously undergone a bariatric surgical procedure, including 40 women receiving BPD (some of these patients were also included in previous published studies of our group [8, 9]) and 20 women receiving LRYGB. The follow-up time after surgery was 7 ± 2 years. The reason for selecting a 2:1 ratio was the fact that there are fewer patients with long-term follow-up after LRYGB than with BPD in our center. Patients presenting with kidney or liver failure or receiving any treatment for osteoporosis were excluded. All patients were receiving calcium and calcifediol supplementation at average doses of 2.1 ± 0.7 g/day and 6160 ± 2388 IU/day, respectively, for patients with BPD, and 1.2 ± 0.6 g/day and 2742 ± 1128 IU/day for patients with LRYGB, with the aim of avoiding secondary hyperparathyroidism. The study was approved by the Ethics Committee of the Hospital Universitario Ramón y Cajal, and informed consent was obtained from every participant.

Analytical procedures

Patients were evaluated after a 12-h overnight fast. Serum and plasma were obtained by venipuncture and immediately frozen and stored below −25 °C until assayed. Serum total calcium, phosphorus, magnesium and creatinine levels were measured using the Architect ci8200 analyzer (Abbot Diagnostics, Berkshire, UK). A commercial enzyme-linked immunosorbent assay (ELISA) was employed for the measurement of 25-hydroxyvitamin D concentrations (IDS Ltd., Boldon, UK). Serum intact PTH, the β-isomer of C-terminal telopeptide of type I collagen cross-links (β-CTX, a bone resorption marker), and the bone formation markers osteocalcin and N-terminal propeptide of procollagen 1 (P1NP) were measured by electrochemiluminescence (Elecsys 2010, Roche Diagnostics, Basel, Switzerland). Their intra- and inter-assay coefficients of variation were below 10 %. We defined vitamin D deficiency as serum concentrations below 20 ng/mL, and vitamin D insufficiency as concentrations between 20 and 30 ng/mL. The normal range for PTH was 15–65 pg/mL, for β-CTX was <0.45 ng/ml, for serum osteocalcin was 15–45 ng/mL and for P1NP was 12–62 ng/mL, as established by the Central Laboratory of the Hospital.

Serum concentrations of RANKL and OPG were determined using commercially available ELISA kits—AmplisRANKL (BI-20452) and Osteoprotegerin (BI-20403) from Biomedica Gruppe—according to the manufacturer's instructions. The sensitivity of the assays was 0.4–1.4 pg/mL for RANKL and OPG, respectively, and intra-assay coefficients of variation were below 10 %.

Bone mineral density (BMD) was assessed at the L1–L4 lumbar spine and total hip by dual-energy X-ray absorptiometry (DXA) using a Hologic QDR 4000 instrument (Hologic Inc, Waltham, MA, USA). A standardized

procedure for patient positioning was used. The longitudinal precision of BMD scans was assessed using phantoms. The phantom percent coefficient of variation for BMD at the lumbar spine was less than 1 %, indicating an adequate adjustment of the densitometer. Osteoporosis was suggested by *T* scores below −2.5 and osteopenia was defined by *T* scores between −1.0 and −2.5.

Statistics

Data are presented as mean ± SD, unless otherwise stated. For continuous variables, logarithm or square root transformations were applied to ensure normality whenever possible. Unpaired *t* tests or Mann–Whitney *U* tests were then used to compare the central tendencies of the different groups, as appropriate. When needed, we corrected the comparisons introducing covariates in a general linear model. To evaluate the association between discontinuous variables we used χ^2 or Fisher's exact tests. Bivariate correlation was employed to study lineal association between two quantitative variables using Pearson's or Spearman's tests as appropriate. Backwards stepwise multiple linear regression models were employed as described below in order to correct for multiple comparisons and variables after univariate tests. Analyses were performed by using SPSS for Windows, version 15 (SPSS, Inc., Chicago, IL, USA). *P* values less than 0.05 were considered statistically significant.

Results

Table 1 summarizes the clinical and biochemical characteristics of the patients. Secondary hyperparathyroidism was present in 38 women (63.3 %), 39 women (65 %) had vitamin D insufficiency and 27 women (45 %) had vitamin D deficiency. BMD scans revealed osteopenia at the lumbar spine in 35 women (58.3 %), osteopenia at the hip level in 13 (21.7 %) and osteoporosis at lumbar spine and hip site in 4 (6.7 %) and 1 (1.7 %) women, respectively. Secondary hyperparathyroidism was more frequent in women presenting with osteopenia at the lumbar spine compared with women with normal BMD scans at this level (68.3 vs 31.7 %, $P = 0.046$). When considering the surgical technique performed previously, 29 women (72.5 %) receiving BPD presented secondary hyperparathyroidism compared with only 9 women (23.7 %) treated with LRYGB ($P = 0.049$).

Even though there were no differences in the proportion of patients with or without osteopenia or osteoporosis at either lumbar or hip sites between different surgical techniques ($p < 0.05$ for all comparisons), *T* scores at both the lumbar spine and the hip site, and *Z* scores and BMD at the hip site, were lower in patients receiving BPD compared with those treated by LRYGB (Table 2). However, after correcting these comparisons for differences in the postsurgical follow-up time and serum PTH levels among these groups (Table 2), the aforementioned differences lost statistical significance ($P = 0.467$, $P = 0.282$, $P = 0.236$ and $P = 0.256$, respectively).

We then conducted bivariate correlation analyses between OPG, RANKL, OPG/RANKL ratio and other continuous variables considering all women as a whole, irrespective of the technique used for obesity surgery (Table 3). Serum OPG inversely correlated with the bone remodeling markers osteocalcin, β-CTX and P1NP. The OPG/RANKL ratio also inversely correlated with serum PTH and osteocalcin. BMD scan parameters did not correlate significantly with either OPG, RANKL or the OPG/RANKL ratio, showing only a trend for an inverse correlation of serum RANKL with BMD at the lumbar spine and a trend for a

Table 1 Clinical and analytical characteristics of the included women ($n = 60$)

Type of surgical technique	
Laparoscopic gastric bypass (%)	33.3
Scopinaro's biliopancreatic diversion (%)	67.7
Menopause (%)	45.0
Smoking habit (%)	41.2
Age (years)	47 ± 10
Percentage of BMI lost after surgery	34 ± 10
Postsurgical follow-up (years)	7.0 ± 2.3
Serum creatinine (mg/dL)	0.7 ± 0.1
Serum calcium (mg/dL)	9.1 ± 0.5
Serum phosphate (mg/dL)	3.5 ± 0.5
Serum PTH (pg/mL)	84 ± 55
25-Hydroxyvitamin D (ng/mL)	26 ± 16
1,25-Dihydroxyvitamin D (ng/mL)	74 ± 5
Serum osteocalcin (ng/mL)	33 ± 13
Serum β-CTX (ng/mL)	0.5 ± 0.2
Serum P1NP (ng/mL)	57 ± 28
Lumbar spine *T* score	−1.1 ± 1.2
Lumbar spine *Z* score	−0.5 ± 1.2
Lumbar spine BMD (g/cm^2)	0.9 ± 0.2
Hip *T* score	−0.2 ± 1.1
Hip *Z* score	0.2 ± 1.0
Hip BMD (g/cm^2)	0.9 ± 0.1
Serum RANKL (pg/mL)	3.4 ± 9.8
Serum OPG (pg/mL)	105 ± 38
Ratio OPG/RANKL	160 ± 123

Data are mean ± SD unless otherwise stated

β-CTX C-terminal telopeptide of type I collagen cross-links, *P1NP* N-terminal propeptide of procollagen 1, *BMD* bone mineral density, *RANKL* receptor–activator of nuclear factor-κB ligand, *OPG* osteoprotegerin

Table 2 Comparison of clinical and analytical data between types of surgical techniques

	LRYGB ($n = 20$)	BPD ($n = 40$)	P value
Menopause (%)	45	45	1.000
Smokers (%)	43	20	0.085
Age (years)	47 ± 13	47 ± 9	0.837
Percentage of BMI lost after surgery	−30.9 ± 12.3	−35.4 ± 8.8	0.156
Postsurgical follow-up (years)	5.7 ± 1.3	7.7 ± 2.4	0.001
Serum creatinine (mg/dL)	0.7 ± 0.1	0.7 ± 0.1	0.936
Serum calcium (mg/dL)	9.1 ± 0.4	9.1 ± 0.5	0.713
Serum phosphate (mg/dL)	3.3 ± 0.5	3.6 ± 0.5	0.051
Serum PTH (pg/mL)	66 ± 23	94 ± 62	0.027
25-Hydroxyvitamin D (ng/mL)	25 ± 15	26 ± 17	0.826
1,25 Dihydroxyvitamin D (ng/mL)	70 ± 46	76 ± 57	0.895
Serum osteocalcin (ng/mL)	31 ± 10	34 ± 15	0.487
Serum β-CTX (ng/mL)	0.4 ± 0.2	0.5 ± 0.3	0.285
Serum P1NP (ng/mL)	47 ± 19	62 ± 31	0.022
Lumbar spine T score	−0.61 ± 1.20	−1.29 ± 1.15	0.043*
Lumbar spine Z score	−0.05 ± 1.18	−0.68 ± 1.16	0.058
Lumbar spine BMD (g/cm^2)	0.94 ± 0.26	0.91 ± 0.13	0.831
Hip T score	0.27 ± 0.93	−0.39 ± 1.04	0.026*
Hip Z score	0.62 ± 0.81	0.01 ± 1.06	0.034*
Hip BMD (g/cm^2)	0.98 ± 0.11	0.90 ± 0.13	0.030*
Serum RANKL (pmol/L)	3.8 ± 13.0	3.2 ± 7.8	0.551
Serum OPG (pg/mL)	103 ± 37	105 ± 39	0.828
Ratio OPG/RANKL	176 ± 137	152 ± 116	0.506

Data are mean ± SD unless otherwise specified

LRYGB laparoscopic Roux-en-Y gastric bypass, *BPD* Scopinaro's biliopancreatic diversion, *BMI* body mass index, *PTH* parathyroid hormone, *β-CTX* C-terminal telopeptide of type I collagen cross-links, *P1NP* N-terminal propeptide of procollagen 1, *BMD* bone mineral density, *RANKL* receptor–activator of nuclear factor-κB ligand, *OPG* osteoprotegerin

* Statistical significance was not retained when correcting for postsurgical follow-up time and serum PTH levels

direct correlation of the OPG/RANKL ratio with BMD at the lumbar spine, which were close to reaching statistical significance.

In contrast, BMD at the lumbar spine and the hip site correlated with the percentage of BMI lost after surgery ($r = 0.428$, $P = 0.001$ and $r = 0.463$, $P < 0.001$, respectively). Furthermore, T scores at the lumbar spine correlated negatively with age ($r = -0.297$, P = 0.022) and serum PTH levels ($r = -0.346$, $P = 0.036$) and positively with the percentage of BMI lost ($r = 0.317$, $P = 0.015$), whereas T scores at the hip site correlated only with the percentage of BMI lost ($r = 0.452$, $P < 0.001$).

We then performed stepwise multivariate analyses in order to correct for possible confounding factors in the results of bivariate correlations. When osteocalcin was introduced as the dependent variable, and age, percentage of BMI lost, time of postsurgical follow-up, OPG, RANKL, OPG/RANKL ratio and serum PTH as independent variables, the model retained OPG ($\beta = -0.325$, $P = 0.005$) and percentage of BMI lost ($\beta = -0.303$, $P = 0.008$) as associated variables ($R^2 = 0.360$, $F = 10.112$, $P < 0.001$). When β-CTX was introduced as the dependent variable, and using the same independent variables, the model retained only OPG ($\beta = -0.337$, $P = 0.007$) as the associated variable ($R^2 = 0.207$, $F = 7.158$, $P = 0.002$). When P1NP was introduced as the dependent variable, and using the same independent variables, the model retained only the percentage of BMI lost ($\beta = -0.302$, $P = 0.012$) as the associated variable ($R^2 = 0.285$, $F = 7.177$, $P < 0.001$).

Finally, when BMD at the lumbar spine was introduced as the dependent variable, and age, percentage of BMI lost, time of postsurgical follow-up, OPG, RANKL, OPG/RANKL ratio, serum PTH, osteocalcin, β-CTX and P1NP as independent variables, the model retained age, percentage of BMI lost and the OPG/RANKL ratio as variables explaining the variability observed in the BMD at the lumbar spine (Table 4). A similar model introducing BMD at the hip site as dependent variable, and using the same independent variables, retained only percentage of BMI lost as the determinant of hip BMD (Table 4).

Discussion

The relative expression of RANKL and OPG plays a key role in the regulation of osteoclast activity and in the bone remodeling cycle [22]. Osteoblasts express RANKL, which binds to a receptor that is expressed on the surface of osteoclast precursor cells, leading to formation of the mature osteoclast [23]. OPG acts as a decoy receptor by binding and neutralizing RANKL, inhibiting osteoclastogenesis and osteoclast activity and inducing osteoclast apoptosis [22, 23]. Therefore, it seems of interest to study the possible role of the OPG/RANKL system in the metabolic bone disease that occurs after obesity surgery in many patients.

The present results suggest that the OPG/RANKL system might play a role in metabolic bone disease after bariatric surgery in humans, and are in conceptual agreement with previous findings showing that metabolic bone disease after menopause is related to an imbalance in the OPG/RANKL system [19].

Table 3 Bivariate correlations for OPG, RANKL and other continuous variables

	OPG		RANKL		OPG/RANKL ratio	
	r	*P*	*r*	*P*	*r*	*P*
Age	0.209	0.109	−0.062	0.638	0.117	0.374
Supplemented calcium intake*	0.127	0.296	−0.076	0.530	0.075	0.537
Percentage of BMI lost after surgery	0.066	0.618	−0.047	0.722	0.049	0.712
Serum creatinine	−0.093	0.482	−0.247	0.057	0.220	0.091
Serum calcium	0.066	0.618	0.021	0.876	0.005	0.970
Serum PTH	−0.231	0.076	0.209	0.110	−0.264	0.042
25-Hydroxyvitamin D	0.126	0.341	0.037	0.381	0.017	0.896
1,25 Dihydroxyvitamin D	0.149	0.265	0.137	0.304	−0.094	0.481
Serum osteocalcin	−0.406	0.001	0.181	0.170	−0.288	0.027
Serum β-CTX	−0.355	0.006	0.082	0.538	−0.177	0.180
Serum P1NP	−0.324	0.012	0.124	0.348	−0.214	0.103
Lumbar spine *T* score	0.093	0.689	0.106	0.424	−0.062	0.639
Lumbar spine *Z* score	0.207	0.116	0.105	0.427	−0.044	0.741
Lumbar spine BMD	0.132	0.318	−0.226	0.086	0.246	0.060
Hip *T* score	0.036	0.787	0.034	0.799	−0.030	0.821
Hip *Z* score	0.063	0.636	0.040	0.764	−0.031	0.818
Hip BMD	0.053	0.692	0.025	0.853	−0.016	0.903

OPG osteoprotegerin, *RANKL* receptor–activator of nuclear factor-κB ligand, *BMI* body mass index, *PTH* parathyroid hormone, *β-CTX* C-terminal telopeptide of type I collagen cross-links, *P1NP* N-terminal propeptide of procollagen 1, *BMD* bone mineral density

* Only mean dose of the supplemented calcium intake was considered, with no measurement from dietary calcium sources

Table 4 Multivariate analysis for bone mineral density

Dependent variable	Independent variables retained by the model	R^2	*F*	*β*	*P*
Lumbar spine BMD		0.29	7.49		<0.001
	OPG/RANKL ratio			0.259	0.029
	Percentage of BMI lost after surgery			0.421	0.001
	Age			−0.235	0.046
Hip BMD		0.21	15.1		<0.001
	Percentage of BMI lost after surgery			0.464	<0.001

BMD bone mineral density, *BMI* body mass index, *OPG* osteoprotegerin, *RANKL* receptor–activator of nuclear factor-κB ligand

The lack of differences between surgical techniques in the OPG/RANKL system is also in accordance with the results of an animal model addressing metabolic bone disease after obesity surgery [21]. No postsurgical differences were found in the RNA expression of OPG or RANKL from the femoral neck of Goto–Kakizaki rats which underwent gastrojejunal bypass or sleeve gastroplasty, between surgical techniques or with non-operated animals [21]. However, in this animal study only osteocalcin expression was measured as a bone marker, and bone scans were not performed [21].

We have shown previously that secondary hyperparathyroidism and the loss of weight after obesity surgery determine a high rate of bone turnover that is associated with decreasing BMD in patients after BPD [9], and that calcium malabsorption may produce secondary hyperparathyroidism after BPD even in patients with high levels of vitamin D [8]. The findings of the present study are consistent with our previous results [9], as serum levels of vitamin D did not correlate with BMD or the OPG/RANKL system, but PTH showed an inverse correlation with the OPG/RANKL ratio.

We have also previously found that several bone markers inversely correlated with BMD at the lumbar spine after BPD [9], and the present study has confirmed those results

also after LRYGB. Furthermore, we found significant correlations of several bone markers with OPG and/or the OPG/RANKL ratio. This is in conceptual agreement with other studies that have demonstrated an association of the OPG/RANKL system with several bone markers [22, 24, 25].

The variables associated with BMD at the lumbar spine in the present study were the OPG/RANKL ratio and also the patients' age and the percentage of BMI lost after surgery. Interestingly, the percentage of BMI lost after obesity surgery may persist as an independent variable that determines a higher rate of bone turnover. We speculate that, since patients who lose more weight are more likely to have poorer absorption, the possibility exists that deficiencies in some micronutrients such as vitamin K, vitamin C or different metals may contribute to maintaining a higher rate of bone turnover. In fact, deficiency of these micronutrients has been associated with bone loss [26]. An alternative hypothesis may be that massive weight loss, apart from lowering body fat mass, also diminishes free-fat mass, including both muscle and bone mass, as has also been demonstrated after dieting, especially when concomitant exercise has not been performed [27].

An important limitation of our study is that, because we excluded patients on drug treatment for osteoporosis, only very few patients with osteoporosis were included. The exclusion of these patients was necessary since many of the drugs used for the treatment of osteoporosis interfere with the OPG/RANKL system [28, 29]. Hence, our present results are mainly applicable to patients with osteopenia, and future studies should confirm our results in patients with osteoporosis after bariatric surgery. Nevertheless, the involvement of the OPG/RANKL system may be even more important in patients with osteoporosis, as already shown for idiopathic or postmenopausal osteoporosis [30–32]. Another potential limitation is that we could not include a control group of obese non-operated patients with the same characteristics of those undergoing surgery, as the latter were usually younger, with higher degrees of obesity and with more metabolic complications. Furthermore, although patients were followed up longitudinally, the cross-sectional measurement of variables at one time point after bariatric surgery precludes any causal assumption between the OPG/RANKL system and metabolic bone disease.

In addition, calcium supplementation was different between patients, especially for those treated by LRYGB or BPD, as we adjusted the calcium dose trying to overcome secondary hyperparathyroidism. Although we included each patient's mean calcium dose in the statistical analysis, we did not record dietary sources, and it was not possible to assess the exact amount of calcium absorbed by the intestine after malabsortive surgery [8] in our study. This is another limitation of the study, as calcium supplementation has been shown to improve the serum OPG/RANKL ratio [33]. Most of the factors known to stimulate osteoclast formation and activity induce RANKL expression by osteoblastic stromal cells [34] and PTH receptor signaling in osteoblasts and osteocytes can increase the RANKL/OPG ratio, increasing both osteoclast recruitment and osteoclast activity [35]. As shown by our results, serum PTH correlated with the OPG/RANKL system, but, as calcium malabsorption is a key factor in the development of secondary hyperparathyroidism after obesity surgery [8], we cannot exclude the possibility that calcium malabsorption per se might have been the driving factor for the alterations in the OPG/RANKL system shown in our study.

In conclusion, the OPG/RANKL system may be associated with bone markers and BMD at the lumbar spine in patients after obesity surgery. Future studies should confirm our results and also investigate the clinical utility of drugs which selectively target the inhibition of RANKL for the treatment of these patients.

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Compliance with ethical standards

Conflict of interest José A. Balsa, Christian Lafuente, Jesús M. Gómez-Martín, Julio Galindo, Roberto Peromingo, Francisca García-Moreno, Gloria Rodriguez-Velasco, Javier Martínez-Botas, Diego Gómez-Coronado, Héctor F. Escobar-Morreale declare no conflict of interest. José I. Botella-Carretero, the corresponding Author and Principal Investigator of this study, also declares no conflict of interest.

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