Persistent increase of osteoprotegerin levels after cortisol normalization in patients with Cushing’s syndrome

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Abstract

Objective: Osteoprotegerin (OPG) has been identified as a decoy receptor that inhibits osteoclast differentiation and, more recently, as a paracrine regulator of vascular calcification. OPG is suppressed by glucocorticoids (GC); however, results from experimental and clinical studies are not univocal. The aim of this study was to evaluate OPG and bone metabolism in patients with Cushing’s syndrome (CS) before and after cure.

Design and methods: Twenty-six patients with CS (all women, mean age: 39.1 ± 11.9 years) and 24 age- and gonadal status-matched healthy women were studied for bone mineral density, bone metabolism, OPG, and receptor activator of nuclear factor-kB ligand at baseline. Twelve patients were also studied 6–18 months after surgery, with persistent normalization of cortisol levels.

Results: OPG was significantly higher and osteocalcin (OC) was significantly lower in CS patients than in controls (OPG: 4.17 ± 1.23 vs 2.95 ± 0.79 pmol/l, P = 0.00001; OC: 15.0 ± 6.1 vs 18.8 ± 6.8 ng/ml, P = 0.04 in CS and controls respectively). After cure, we found no difference in OPG levels, despite a significant increase in OC levels (from 16.4 ± 11 to 37.2 ± 15 ng/ml, P = 0.03).

Conclusion: Patients with CS showed increased OPG serum levels that remained unchanged after recovery, despite a restoration of bone formation. We speculate that high levels of OPG could reflect the persistent damage of the GCs on cardiovascular system.

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Introduction

Osteoporosis is one of the major features of either exogenous or endogenous glucocorticoids (GCs) excess (1). GCs increase bone resorption by stimulating osteoclastogenesis and inhibit bone formation. The latter is considered the prevalent effect, and is due both to a decrease in osteoblastic cells replication and differentiation, and to the apoptosis of mature osteoblasts (2–4). The reduction in bone formation during GCs excess is reflected by low levels of serum osteocalcin (OC) and alkaline phosphatase (ALP), both markers of bone formation (5). The increased bone resorption has been ascribed to an increased expression of receptor activator of nuclear factor-kB ligand (RANKL) and to a decreased expression of its decoy receptor osteoprotegerin (OPG) (6–9). Differences in the circulating concentration of OPG and soluble RANKL have been observed in several diseases. An enhanced RANKL/OPG ratio may be a crucial paracrine mechanism for increased bone resorption during GC excess (10). Hofbauer et al. demonstrated a decrease in OPG and an increase in RANKL production by osteoblast-like cells in response to dexamethasone in vitro (11, 12). In vivo studies have shown that GCs suppress serum OPG in patients with renal disease (13). On the contrary, the chronic endogenous GCs excess observed in Cushing’s syndrome (CS) seems to be associated with an increase in OPG levels (14).

Osteoporosis and vascular diseases are commonly associated (15). Experimental studies suggest that OPG and RANKL may be common mediators that affect both bone metabolism and vascular integrity (16). The role of OPG and RANKL as paracrine regulators of vascular calcification is reinforced by the fact that these factors are produced also by vascular endothelial cells (17). Human studies have assessed that, in patients with coronary artery disease, serum OPG was higher in subjects with significant arterial stenosis than in those without (18). Chronic cortisol hypersecretion causes a series of systemic alterations such as hypertension, insulin resistance, dyslipidemia, prothrombotic state, and central obesity. These complications increase the cardiovascular risk and cause a severe atherosclerotic damage that develops with the acquired metabolic syndrome. Both osteoporosis and cardiovascular risk...
are common features in CS (19–22). The remission from hypercortisolism is not followed by the improvement of the metabolic and vascular damage that persists as in the active phase of the disease (23). The recovery of bone mass after remission of the disease occurs a long time after the normalization of cortisol levels (24–26); commonly, the biochemical markers of bone turnover are more precocious than the changes of bone mineral density (BMD) in reflecting bone metabolism (27).

In order to evaluate the effects of GCs on bone in CS and the modifications that occur in the biochemical parameters of bone turnover before and after cure, we evaluated a group of patients with CS at baseline and for a follow-up period ranging from 6 to 18 months. Our attention was focused on OPG and RANKL serum levels with the aim to evaluate a possible role of this system on the damages produced by GCs excess, since few data are available after the normalization of cortisol levels.

**Subjects and methods**

**Subjects**

We retrospectively studied 26 patients (all women, mean age 39.1 ± 11.96 years, range 15–67) affected by overt CS, referred to our center between November 2004 and August 2006 (mean duration of disease: 29 ± 16 months). Twenty-four healthy age- and gonadal status-matched women were selected as controls. None of them were receiving medications or had medical conditions affecting bone metabolism. The diagnosis of cortisol excess was suspected on clinical grounds and confirmed by laboratory evaluation (28): high levels of serum cortisol in the morning with lack of physiological circadian rhythm (mean values: 24.3 ± 6.3 µg/dl at 0800 h (normal range: 9.4–26.7 µg/dl) and 18.9 ± 7.2 µg/dl at 2300 h (normal range: 1.8–12.7 µg/dl)), high 24-h excretion of urinary free cortisol (UFC) (mean values: 476 ± 306 µg/day, normal values: 13.7–70.4 µg/day), lack of serum cortisol suppression after low dose (1 mg) of dexamethasone (mean values after dexamethasone: 12.8 ± 8.4 µg/dl, normal response: <1.8 µg/dl).

Twenty-three patients had pituitary-dependent CS, confirmed by detectable plasma ACTH levels (mean values 53.1 ± 19.8 pg/ml, normal value < 50 pg/ml) and pituitary adenoma at magnetic resonance imaging; three patients had ACTH-independent CS with suppressed plasma ACTH levels (mean ACTH values 24.8 ± 8.9 pg/ml) and computed tomography evidence of adrenal lesion.

Eighteen patients were followed up longitudinally for 6–18 months. Twelve of them recovered after surgery, with persistent normalization of serum and urinary cortisol levels (mean values 14 ± 5 µg/dl at 0800 h and 36.8 ± 24.6 µg/day respectively) as well as restoration of plasma cortisol suppression after 1 mg dexamethasone (mean values 1.2 ± 0.8 µg/dl); three of them needed a short-term GC replacement therapy for about 1–2 months (mean daily dose of cortisone acetate: 37.5 mg). Six patients underwent medical treatment (ketoconazole) without reaching the normalization of UFC. For this reason, here we present data obtained only in the 12 cured patients.

All patients gave informed consent to use their clinical and laboratory data for scientific purpose.

**Methods**

Patients and controls underwent medical history and physical examination, serum and urinary sample collection for endocrine evaluations, and dual-energy X-ray absorptiometry (DEXA) at lumbar spine and proximal femur. Body mass index (BMI) and blood pressure were measured using standard methods. Fasting glucose, plasma cholesterol, and triglycerides were analyzed with common laboratory methods. Pathological blood pressure was considered above 130/85 mmHg and impaired fasting glucose above 6.1 mmol/l, according to the National Cholesterol Education Program criteria (29).

In CS, serum and UFC were determined by a RIA kit (Cortisol Bridge, Adaltis, Italy), detection limit = 0.36 µg/dl, inter- and intra-assay coefficient of variation (CV): 5.7 and 3.5% respectively; plasma ACTH was measured by IRMA (ACTH IRMA, Scantibodies Laboratory, San Diego, CA, USA), detection limit = 3 pg/ml, inter- and intra-assay CV: 2.5 and 3.8% respectively.

Bone metabolism parameters were studied at baseline in all CS patients and in controls, and 6–18 months after surgical intervention in the subgroup of 18 CS patients who underwent surgical or medical treatment; however, only the 12 cured patients were the objects of the follow-up study.

Serum calcium, phosphorus, creatinine, urinary calcium, and ALP were determined with common laboratory methods. Parathyroid hormone (PTH) was measured by IRMA (Intact PTH Bridge, Adaltis, Italy), detection limit = 10 pg/ml, inter- and intra-assay CV: 4 and 3.5%; 25-hydroxyvitamin D was determined by RIA method (25-hydroxyvitamin D_23_1 RIA KIT, DiaSorin, Saluggia, Italy): detection limit = 1.5 ng/ml, inter- and intra-assay CV: 9%; serum OC was determined by IRMA (Human Osteocalcin Bridge, Adaltis, Casalecchio di Reno, BO, Italy): detection limit = 0.3 ng/ml, inter- and intra-assay CV: 5 and 3% respectively; serum OPG and serum RANKL levels were measured by immunoenzymatic assay (Osteoprotegerin and sRANKL Biomedica Gruppe, Vienna, Austria): detection limit = 0.14 and 0.4 pmol/l, inter- and intra-assay CV: 10 and 8% respectively; and serum crosslaps were determined with immunoenzymatic assay (Serum CrossLaps ELISA, Nordic Bioscience Diagnostics, Herlev, Denmark): detection limit = 0.010 ng/ml, inter- and intra-assay CV: 5.4 and 5% respectively. Fasting blood samples were collected from 0800 to 1000 h.
Table 1 Clinical and laboratory data of the subjects.

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=26)</th>
<th>Controls (n=24)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>39.1±11.9</td>
<td>39.9±3.0</td>
<td>NS</td>
</tr>
<tr>
<td>PM</td>
<td>8</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.2±4.8</td>
<td>23.2±3.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>20 (77)</td>
<td>7 (29)</td>
<td>0.01</td>
</tr>
<tr>
<td>IFG, n (%)</td>
<td>15 (58)</td>
<td>0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>5.83±0.63</td>
<td>4.75±0.62</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.69±0.85</td>
<td>0.82±0.32</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>1.64±0.24</td>
<td>1.91±0.49</td>
<td>0.016</td>
</tr>
</tbody>
</table>

Values are expressed as mean±S.D. M, male; F, female; PM, postmenopausal women; BMI, body mass index (normal >25, overweight 25–30, and obese >30); IFG, impaired fasting glucose (plasma fasting glucose >6.1 mmol/l); total cholesterol: normal <5.18 mmol/l; HDL: normal >1 mmol/l; triglycerides: normal <1.59 mmol/l; NS, not significant.

Dual-energy X-ray absorptiometry

Lumbar spine (L2–L4) and left femoral BMD were measured by DEXA (Hologic QDR 4500 C densitometer, Waltham, MA, USA). In vitro CV, calculated by performing 20 scans of the Hologic anthropometric spine phantom, was 0.6%, and in vivo CV, calculated by performing two scans in ten healthy volunteers at spine and femoral site, was 1.2 and 2.0% respectively.

Table 2 Parameters of bone metabolism and bone mineral density in patients and controls.

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=26)</th>
<th>Controls (n=24)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca (mmol/l)</td>
<td>2.30±0.08</td>
<td>2.27±0.09</td>
<td>NS</td>
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<tr>
<td>P (mmol/l)</td>
<td>0.98±0.12</td>
<td>1.12±0.17</td>
<td>0.001</td>
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<tr>
<td>Creatinine (µmol/l)</td>
<td>78.4±10.4</td>
<td>71.7±8.98</td>
<td>0.02</td>
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<tr>
<td>ALP (UI/l)</td>
<td>80.3±20.5</td>
<td>77.2±3</td>
<td>NS</td>
</tr>
<tr>
<td>OC (ng/ml)</td>
<td>15.0±6.1</td>
<td>18.8±6.8</td>
<td>0.04</td>
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<tr>
<td>PTH (pg/ml)</td>
<td>50.7±15.6</td>
<td>44.6±14.6</td>
<td>NS</td>
</tr>
<tr>
<td>OPG (pmol/ml)</td>
<td>4.17±1.23</td>
<td>2.95±0.79</td>
<td>0.0001</td>
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<tr>
<td>OPG/RANKL</td>
<td>13.81±10.93</td>
<td>4.24±2.82</td>
<td>0.0001</td>
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<tr>
<td>RANKL (pmol/ml)</td>
<td>0.58±0.32</td>
<td>1.35±1.2</td>
<td>0.028</td>
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<tr>
<td>sCL (ng/ml)</td>
<td>0.70±0.36</td>
<td>0.74±0.38</td>
<td>NS</td>
</tr>
<tr>
<td>25OHD (ng/ml)</td>
<td>17.7±6.9</td>
<td>30.1±11.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lumbar BMD (g/cm²)</td>
<td>0.870±0.090</td>
<td>1.010±0.100</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lumbar T-score</td>
<td>−0.86±0.058</td>
<td>−0.15±0.83</td>
<td>0.009</td>
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<tr>
<td>Lumbar Z-score</td>
<td>−0.34±0.73</td>
<td>0.26±0.68</td>
<td>0.0043</td>
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<tr>
<td>Femoral neck BMD</td>
<td>0.720±0.02</td>
<td>0.810±0.110</td>
<td>0.0058</td>
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<tr>
<td>Femoral neck T-score</td>
<td>−1.66±0.95</td>
<td>−0.91±0.88</td>
<td>0.0058</td>
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<tr>
<td>Femoral neck Z-score</td>
<td>−0.92±0.94</td>
<td>−0.28±0.73</td>
<td>0.01</td>
</tr>
<tr>
<td>Total femur BMD</td>
<td>0.82±0.110</td>
<td>0.910±0.110</td>
<td>0.0058</td>
</tr>
<tr>
<td>Total femur T-score</td>
<td>−1.44±0.93</td>
<td>−0.65±0.94</td>
<td>0.0044</td>
</tr>
<tr>
<td>Total femur Z-score</td>
<td>−0.71±0.92</td>
<td>−0.05±0.78</td>
<td>0.0009</td>
</tr>
</tbody>
</table>

Values are expressed as mean±S.D. Ca, serum calcium (normal range 2.2–2.6 mmol/l); P, serum phosphorus (normal range 0.87–1.45 mmol/l); Creatinine, serum creatinine (62–115); ALP, alkaline phosphatase (normal 55–128 UI/l); OC, osteocalcin (normal 4–26 ng/ml); PTH, parathyroid hormone (normal 10–65 pg/ml); OPG, osteoprotegerin (normal 0–30 pmol/l); RANKL, receptor activator for NF-kB ligand (normal 0–8 pmol/l); sCL, serum crosslaps (normal 0.2–1.5 mg/ml); 25OHD, 25-hydroxyvitamin D (sufficient >30 ng/ml); BMD, bone mineral density; NS, not significant.

Statistical analysis

For descriptive statistics, PRISM 4.0 Graph Pad (San Diego, CA, USA) program was used. Statistical analysis was carried out using the Mann–Whitney test for unpaired data and the Wilcoxon test for paired groups. The effectiveness of the pairing was tested by calculating the Spearman nonparametric coefficient. To assess the differences in discrete variables, χ² test was used. Linear regressions analysis was also carried out. A general linear model (GLM) multivariate analysis was performed to adjust data for BMI using SAS program. Data are given as mean±S.D. A value of P<0.05 was considered significant.

Results

Clinical and laboratory characteristics of the study population are shown in Table 1. BMI was significantly higher in CS than in controls (P=0.0003). OPG levels and OPG/RANKL ratio were significantly higher (P=0.0001 in both cases), and RANKL was significantly lower (P=0.028) in CS patients than in controls. 25OHD levels were significantly lower in CS patients than in controls (P<0.0001). BMD was significantly lower in CS than in controls both at spine and femoral levels (Table 2).

Figure 1 OPG and OPG/RANKL ratio in patients with active Cushing’s syndrome and after remission. OPG, osteoprotegerin; RANKL, receptor activator for NF-kB ligand.
A significantly positive correlation between sCL and OC levels was found ($r^2 = 0.43$, $P < 0.01$).

In patients with remission of CS, both OPG and OPG/RANKL ratio remained high, without statistical differences before and after recovering (Fig. 1). A significant increase in OC levels was observed after the normalization of cortisol levels (mean values from 16.4 ± 11 to 37.2 ± 15 ng/ml; $P = 0.03$; Fig. 2), while no differences in RANKL and sCL serum levels were found before and after recovering. In our patients, metabolic parameters did not significantly improve after remission as shown in Table 3.

The results were confirmed for every parameter after adjusting for BMI.

### Discussion

At baseline, OC, but not sCL, was lower in CS patients and the two markers were positively correlated, maintaining a coupled state. The low OC levels, without any significant change of sCL, could be due to the period that occurred from onset of the disease and the time of the first observation since the resorption increases in the first phase of GIO, which is difficult to establish in CS. After cure, OC was markedly increased compared with baseline levels in CS patients, without substantial modifications of sCL levels. The restart of bone formation is the sign of the recovery of bone health.

In CS, we found increased levels of OPG and OPG/RANKL ratio compared with controls, persistent after the normalization of cortisol levels.

In *in vitro* studies, GC were able to suppress OPG mRNA expression and protein secretion and contemporarily to upregulate RANKL mRNA expression in various osteoblastic cell models (11–12).

Clinical studies have shown that systemic GC therapy decreases OPG serum levels during a short observational phase. In patients with renal disease, a decrease in both OPG and OC serum circulating levels was shown, with a parallel increase in bone resorption markers during the first two weeks of GCs treatment (13). In addition, during a 3-month period of high-dose GC treatment (60 mg prednisolone per day), it was observed that OPG decreased after 2 weeks and returned to baseline after 3 months, while soluble RANKL serum levels increased (30). In contrast, considering CS, in three studies, OPG serum levels were found to be higher in patients compared to healthy controls (14, 31, 32). The increased OPG levels in CS might be a compensation to protect bone from the damage that occurs in long and uncontrolled endogenous cortisol excess, while the decrease in OPG in GC-treated patients could reflect the direct effect of GC on osteoblastic cells.

Since in treated patients we observe a dramatic increase of OC without any significant change in OPG serum levels, we hypothesize that the high levels of OPG in CS patients could be related to the persistent cardiovascular damage rather than to bone status. In fact, RANKL–OPG system also acts as a paracrine regulator of vascular calcification (33). Observational studies revealed that coronary artery disease in men is associated with high serum OPG and low serum RANKL (17). These findings are in accordance with an epidemiologic study by Browner *et al* who demonstrated that increased serum OPG was associated with an increased cardiovascular mortality (34). More recently, Shargorodsky *et al* in a multiple linear regression analysis demonstrated that OPG was an independent predictor of pulse wave velocity and aortic augmentation index, resulting as a marker of subclinical atherosclerosis in a population of osteoporotic postmenopausal women (35). High serum OPG was also observed in microvascular complication associated with

<table>
<thead>
<tr>
<th>Active phase</th>
<th>Recovered</th>
<th>$P$</th>
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<tr>
<td>(n=12)</td>
<td>(n=12)</td>
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</tr>
<tr>
<td>Hypertension (%)</td>
<td>9 (75)</td>
<td>8 (66)</td>
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<tr>
<td>Diabetes (%)</td>
<td>8 (67)</td>
<td>4 (33)</td>
</tr>
<tr>
<td>Overweight (%)</td>
<td>7 (58)</td>
<td>5 (41)</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>6 (50)</td>
<td>5 (41)</td>
</tr>
</tbody>
</table>

Values are numbers (percentage). Hypertension: blood pressure > 130/85 mmHg; overweight: BMI > 25 g/cm²; dyslipidemia: high triglycerides (> 1.7 mmol/l) ± low HDL (< 1.04; according to ATPIII classification).
diabetes and in the increased intimamedia-thickness of the carotid arteries (36, 37).

While many studies confirmed the recovery of bone health after cure (24–26), several studies gave evidence that both metabolic syndrome and cardiovascular risk persist despite the normalization of cortisol secretion during longer follow-up periods (1 year and more) (19, 22, 23). According to our results, Kristo et al. in a longer follow-up period (range 5–69 months) observed the persistence of high OPG values after CS remission. The authors correlated the absence of OPG normalization with an increased inflammation pattern persistence, which might represent a pro-atherogenic profile (32).

In our study, metabolic complications in CS patients were still substantially unchanged after cortisol normalization (67% of patients had hypertension, 33% had diabetes, and 40% had dyslipidemia), so we can suppose the persistence of a high cardiovascular risk despite a lack of direct evidence of vascular damage.

A more consistent number of patients and a more complete evaluation of the cardiovascular risk, including the measurement of the intima-media thickness, are needed to confirm our hypothesis, in order to evaluate the possibility that OPG might be considered a useful biochemical marker of cardiovascular risk in CS patients.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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