

# Elevated Serum Levels of the Decoy Receptor Osteoprotegerin in Children with Langerhans Cell Histiocytosis

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**ABSTRACT:** Langerhans cell histiocytosis (LCH) is characterized by an accumulation of dendritic Langerhans cells in granulomatous lesions in the bone, skin, and other sites in the body. The pathogenesis of the disease remains unknown. We measured serum levels of the decoy receptor osteoprotegerin (OPG), an important regulator of bone metabolism as well as immune responses, in 18 children with single system (SS) or multi-system (MS) forms of LCH and 20 pediatric controls. OPG levels were significantly increased in LCH patients at diagnosis when compared with controls, and pretreatment levels of OPG were elevated in MS compared with SS patients. Moreover, OPG levels in LCH patients were elevated in patients with involvement of risk organs (liver, lungs, hematopoietic system, or spleen) when compared with patients without risk organ involvement, indicative of an association between OPG values and disease severity. We also observed a positive correlation between serum levels of OPG and tumor necrosis factor (TNF)- $\alpha$ , a pro-inflammatory cytokine, at the time of onset of disease. These findings show, for the first time, that serum OPG levels are elevated in LCH patients, and suggest that OPG may play a role in the pathogenesis of this enigmatic disease. (*Pediatr Res* 59: 281–286, 2006)

Langerhans cell histiocytosis (LCH) affects mainly young children and features an accumulation of CD1a<sup>+</sup> dendritic Langerhans cells (LCs) in the skin, bone, and occasionally other organs, including lymphoid tissues, lungs, and digestive tract (1). Clinically, LCH ranges from self-healing lesions to a multi-system involvement with organ dysfunction resistant to current therapies (2). Previous studies have shown that LCs from bone lesions and/or chronic forms of the disease are functionally immature, whereas LCs in patients with self-healing or isolated cutaneous disease possess a more mature phenotype (3). Nevertheless, the etiology of LCH remains unknown, and it is even debated whether LCH is a reactive

condition, linked to an abnormal response of LCs and other immune cells to offending pathogens, or a neoplastic disease (4,5).

Abundant levels of tumor necrosis factor (TNF)- $\alpha$  and other pro-inflammatory cytokines, and the co-stimulatory molecules CD40 and CD40 ligand (CD40L), are seen in LCH lesions (6–8) thus creating a veritable “cytokine storm” that may account for many of the systemic symptoms in these patients, as well as some of the long-term sequelae (pulmonary abnormalities, CNS dysfunction, etc) (9,10). Moreover, our recent studies have revealed elevated levels of TNF- $\alpha$  in the serum of LCH patients (11), and we have shown the addition of anti-TNF- $\alpha$  treatment to conventional chemotherapy to be beneficial in a child with severe LCH(12).

Osteoprotegerin (OPG) is a member of the TNF receptor superfamily, and functions as an inhibitor of bone resorption by binding to RANKL (receptor activator of NF $\kappa$ B ligand), thereby blocking the interaction between RANKL and RANK (13). OPG is also a decoy receptor for TRAIL (TNF-related apoptosis-inducing ligand) (14), a pro-apoptotic factor involved in killing of cells in the immune system, including dendritic cells (DCs) (15). OPG is produced by numerous cell types, such as osteoblasts, DCs, and B cells, and its expression is up-regulated upon CD40 ligation (16). OPG is present as a secreted factor in serum, and has also recently been detected in human milk (17). Importantly, OPG has been suggested to function as a crucial regulator not only of bone metabolism, but also of B cell and DC function (18).

Previous studies have shown that serum OPG levels are reduced in multiple myeloma (MM) patients with lytic bone lesions (19); conversely, treatment of mice with established myeloma with recombinant OPG was found to prevent the development of osteolytic bone disease (20). Furthermore, recent studies have disclosed elevated serum OPG levels in rheumatoid arthritis (RA) patients (21). Interestingly, anti-TNF- $\alpha$  treatment resulted in the normalization of OPG values in the latter group, thus supporting the notion that pro-inflammatory cytokines control the production of OPG. De-

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**Abbreviations:** CD, Crohn's disease; DC, dendritic cell; LCH, Langerhans cell histiocytosis; MGC, multinucleated giant cell; MS, multi-system; OPG, osteoprotegerin; RA, rheumatoid arthritis; SS, single system

regulated OPG levels could contribute to osteoclast activation and/or impairment of DC function in LCH patients. The purpose of the current study was to determine the serum levels of OPG in a well-defined cohort of LCH patients.

## METHODS

**Patients and controls.** Serum samples were obtained at onset of disease from 18 LCH patients who fulfilled the Histiocyte Society criteria for diagnosis, stratification, and treatment (2). The following clinical stages were considered: single system (SS) or multi-system (MS) disease, with or without involvement of risk organs (liver, lungs, hematopoietic system, or spleen); in addition, a comparison was made between patients who presented with or without bone involvement at the time of diagnosis (Table 1). For some cases, consecutive serum samples were analyzed, at diagnosis and/or during follow-up of treatment; a total of 50 samples from 30 LCH patients were thus included in the study. Control samples were obtained from 20 children undergoing minor elective surgery (median age 3 y; range 1–13). The study protocol was approved by the ethical committee of the Hospital Garrahan in Buenos Aires and Karolinska Institutet in Stockholm. Informed parental consent was obtained in all cases.

**Cytokine measurements.** Concentrations of OPG were measured by a sandwich ELISA using a set of specific antibodies and recombinant human OPG standard from R&D Systems (Minneapolis, MN), according to the manufacturer's instructions. The performance range was 62.5–4000 pg/mL and the detection limit was 30 pg/mL. Serum levels of sIL-2R and IL-1Ra were estimated using commercial ELISA kits obtained from R&D Systems, as previously described (11,22). TNF- $\alpha$  concentrations were determined by chemiluminescent immunometric assay using an IMMULITE automated analyzer (Diagnostic Products, Los Angeles, CA), according to the manufacturer's instructions.

**Statistical analysis.** The 2-tailed Mann-Whitney *U*-test was used to compare the levels of OPG in different groups of patients and controls. Correlations between cytokine concentrations (sIL-2R, IL-1Ra, TNF- $\alpha$ ) and OPG were assessed by the Spearman rank order test. Data are reported as mean values  $\pm$  SEM or as median values and range. Results were considered statistically significant when *P* values were  $< 0.05$ . All calculations were performed using SPSS 11.5 software (Chicago, IL).

## RESULTS

**Serum levels of OPG at diagnosis.** The OPG levels in our cohort of LCH patients at the time of diagnosis (*i.e.*, before

treatment) were  $1901 \pm 250$  pg/mL ( $n = 18$ ) and in controls  $427 \pm 41$  pg/mL ( $n = 20$ ). OPG levels in all except two of the LCH patients were above 843 pg/mL, the highest value detected in the control group, and the difference between the two groups was significant ( $p < 0.001$ ) (Fig. 1A). Statistically significant differences were also observed between patients with SS ( $n = 8$ ) and MS ( $n = 10$ ) forms of the disease, when monitored at the time of diagnosis ( $1266 \pm 157$  pg/mL vs.  $2409 \pm 363$  pg/mL;  $p = 0.021$ ) (Fig. 1B). LCH patients with bone involvement at the time of diagnosis ( $n = 13$ ) were found to have lower levels of OPG when compared with those without bone lesions ( $n = 5$ ) ( $1727 \pm 296$  pg/mL vs.  $2353 \pm 444$  pg/mL), but this difference did not show statistical significance (Fig. 1C). Patients with involvement of risk organs were found to have significantly higher OPG levels compared with individuals without risk organ involvement [risk organs ( $n = 10$ ),  $2534 \pm 308$  pg/mL vs. no risk organs ( $n = 8$ ),  $1109 \pm 166$  pg/mL vs.;  $p = 0.001$ ] (Fig. 1D). However, it should be noted that only 2 of the patients with MS disease did not have risk organ involvement at the time of diagnosis, whereas four of the eight MS patients with risk organ involvement had bone lesions; hence, the most valid comparison may be between patients with MS and SS disease, and patients with or without bone lesions, respectively.

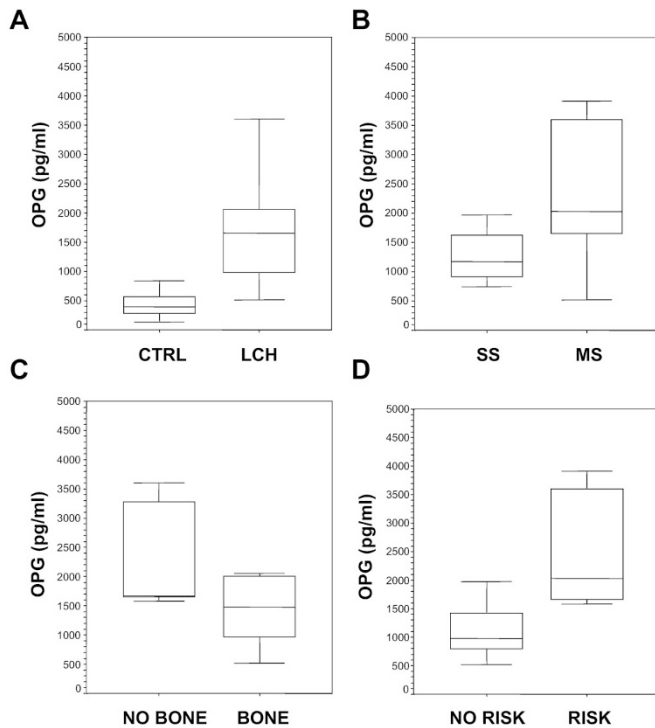
**OPG levels upon treatment.** Paired samples obtained at the time of diagnosis and after initiation of treatment were available from a subset of LCH patients (Table 1). A decrease in serum OPG levels was seen in two of three patients who were classified as active disease better (ADB) at the time of follow-up, whereas in two patients considered as active disease intermediate (ADI) at follow-up (*i.e.*, nonresponders), OPG levels were moderately increased (Fig. 2). In one child (patient nine) who displayed severe lung involvement after 6 wk of treatment and was classified as active disease worse (ADW),

**Table 1.** LCH patient characteristics and serum concentrations of cytokines

Patient	Age (m)/		Organ involvement	OPG	TNF- $\alpha$	sIL-2R	IL-1Ra	Response at follow-up
	Sex	Stage		(pg/ml) at diagnosis	(pg/ml) at diagnosis	(pg/ml) at diagnosis	(pg/ml) at diagnosis	
1	171/M	SS	Bone	1366	2	850	600	-
2	47/M	MS	Bone/skin	515	-	-	190	ADB (6 weeks)
3	47/F	SS	Bone	971	11	5500	92	-
4	177/M	MS	Bone/skin	1480	2	1500	1000	-
5	16/F	MS-RO	Skin/liver/spleen/hematopoietic	3598	42	27 500	5200	-
6	74/M	SS-RO	Lungs	1665	-	14 000	1000	ADW (6 weeks)
7	137/M	SS-MF	Bone	981	-	7000	-	-
8	30/F	SS	Bone	1970	18	1250	90	ADB (12 weeks)
9	27/M	MS-RO	Bone/skin/liver/spleen/hematopoietic/DI	1838	-	40 000	1180	ADI (6 weeks)
10	9/M	MS-RO	Skin/liver/spleen/hematopoietic	3270	41	-	26 000	-
11	132/M	SS	Bone	853	-	-	-	-
12	25/M	MS-RO	Bone/skin/liver/spleen/lungs/hematopoietic/DI	3764	-	-	-	ADB (1 year)
13	21/M	MS-RO	Skin/liver/spleen/hematopoietic	2057	35	35 000	1800	-
14	22/F	SS-RO	Lungs	1583	24	5500	1600	-
15	7/F	MS-RO	Bone/skin/liver/lungs/hematopoietic	3911	-	-	-	-
16	10/M	MS-RO	Bone/skin/liver/hematopoietic	2003	173	23 750	10 000	-
17	121/M	SS	Bone	739	-	-	-	-
18	10/F	MS-RO	Bone/skin/liver/spleen/lymph nodes/hematopoietic	1651	-	-	-	ADI (6 weeks)

Clinical stage: SS, single system (single site); SS-MF, single system (multifocal disease); MS, multi-system without risk organ involvement; MS-RO, multi-system with risk organ involvement; DI, diabetes insipidus.

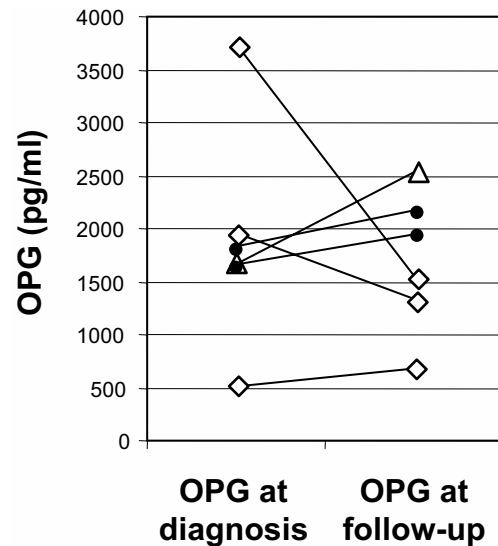
Clinical response: ADB, active disease better; ADI, active disease intermediate; ADW, active disease worse.



**Figure 1.** Elevated serum levels of OPG in LCH patients. (A) Serum OPG levels in pediatric controls (CTRL) ( $n = 20$ ) and in LCH patients (LCH) ( $n = 18$ ), as determined by ELISA. The median value in patients was 1658 pg/mL (range 515–3911) and 396 pg/mL (range 133–843) in controls ( $p < 0.001$ ). (B) OPG values in LCH patients with single system (SS) ( $n = 8$ ) and multi-system (MS) ( $n = 10$ ) forms of the disease. Median values were 1173 and 2030 pg/mL, respectively ( $p = 0.021$ ). (C) OPG values in LCH patients grouped according to whether bone involvement was present ( $n = 13$ ) or absent ( $n = 5$ ) at the time of diagnosis (BONE vs. NO BONE). Median values were 1480 and 1655, respectively ( $p = 0.301$ ). (D) OPG values in LCH patients displaying no risk organ involvement (NO RISK) ( $n = 8$ ) vs involvement of risk organs (RISK) ( $n = 10$ ) at the time of diagnosis (median values 976 and 2030 pg/mL, respectively) ( $p = 0.001$ ). Boxes (A–D) = 25<sup>th</sup> and 75<sup>th</sup> percentiles; horizontal lines within boxes = median values; vertical bars below and above boxes = range.

the serum level of OPG was significantly increased (Fig. 2). Additional, consecutive serum samples were available from 2 of these children. For patient 12, who improved upon treatment, OPG levels were initially reduced from 3764 to 1566 pg/mL. This patient was also assessed 2 y after diagnosis, following continued clinical improvement, at which time OPG levels had decreased further to 600 pg/mL. OPG levels in patient 18, who presented with MS disease, were assessed on six occasions and decreased gradually from 1651 to 679 pg/mL during the course of treatment; this child exhibited signs of progressive disease yet responded well to a combination of chemotherapeutic drugs and anti-TNF- $\alpha$  treatment (etanercept) and was classified as having nonactive disease (NAD) 2 y after diagnosis. Of note, the evolution of bone lesions in patient 12 (middle ear bone involvement, and osteolytic lesions of the skull and left femur) and patient 18 (middle ear bone involvement only) during the course of treatment was favorable in both cases.

**Other markers of inflammation.** We have previously shown that pretreatment serum levels of soluble IL-2 receptor (sIL-2R), IL-1 receptor antagonist (IL-1Ra), and TNF- $\alpha$  are



**Figure 2.** Serum levels of OPG upon treatment of LCH patients. The graph represents OPG values at the time of diagnosis and at follow-up in six LCH patients (consult Table 1 for details). Patients classified as ADB (active disease better,  $\diamond$ ) at the time of follow-up were considered to be responders, whereas patients classified as ADI (active disease intermediate,  $\bullet$ ) or ADW (active disease worse,  $\triangle$ ) were considered to be nonresponders. For comparison, the median value of OPG in pediatric controls was 396 pg/mL (range 133–843) ( $n = 20$ ) (Fig. 1).

elevated in children with LCH (11,22). In the present study, we analyzed serum levels of these cytokines in LCH patients at the time of diagnosis [TNF- $\alpha$  ( $n = 9$ ), sIL-2R ( $n = 12$ ), IL-1Ra ( $n = 11$ )] as well as in pediatric controls ( $n = 14$  for TNF- $\alpha$  and sIL-2R measurements, and  $n = 7$  for IL-1Ra measurements). All three cytokines were significantly elevated in LCH patients when compared with controls, in line with our previous observations (Table 2). Furthermore, in a subset of LCH patients ( $n = 8$ ) for whom serum concentrations of the three cytokines as well as OPG were available at the onset of disease (Table 1), a significant positive correlation was observed between OPG and TNF- $\alpha$  ( $r = 0.826$ ;  $p = 0.011$ ). In addition, a similar trend was observed when sIL-2R

**Table 2.** Serum concentrations of cytokines in LCH patients and controls

	LCH patients*	Controls	P values‡
TNF- $\alpha$	24 (2–173)	7.5 (2–10)	0.016
sIL-2R	10 500 (850–40000)	610 (343–800)	0.0001
IL-1Ra	1000 (90–10000)	190 (47–420)	0.0268
OPG†	1658 (515–3911)	396 (133–843)	0.001

\* Data are reported as median (range); all concentrations are shown as pg/ml.

For the purpose of analysis, for cytokine concentrations below the detection limit of the assay, a value halfway between zero and the lower limit of detection was used.

† OPG values are identical to those reported in Figure 1, and are included here for comparison ( $n = 18$  and 20, for patients and controls, respectively).

‡ The non-parametric Mann-Whitney test was used to determine differences between patients and controls [TNF- $\alpha$ :  $n = 9$  (patients) and  $n = 14$  (controls); sIL-2R:  $n = 12$  (patients) and  $n = 14$  (controls); IL-1Ra:  $n = 11$  (patients) and  $n = 7$  (controls)].



and IL-1Ra values were compared with TNF- $\alpha$  values in this cohort of LCH patients ( $r = 0.795$ ;  $p = 0.018$ , and  $r = 0.778$ ;  $p = 0.023$ , respectively).

## DISCUSSION

OPG is a secreted protein that regulates bone metabolism as well as immune responses. In the present study, we show that serum concentrations of OPG are significantly elevated in LCH patients at the time of diagnosis, when compared with pediatric controls. Moreover, OPG levels in LCH patients were found to correlate with serum levels of TNF- $\alpha$ , an important pro-inflammatory cytokine. In a subset of patients, OPG levels were shown to decrease upon treatment; however, the latter data should be interpreted with caution due to the small number of patients included in the follow-up analysis. Nevertheless, these results indicate, for the first time, that serum levels of the decoy receptor OPG are elevated in LCH, and provide evidence of a correlation between OPG levels and other markers of inflammation.

LCH lesions feature both osteoclast-like multinucleated giant cells (MGCs) and antigen-presenting LCs (4). A very recent immunohistochemical study suggested that the cytokine environment in these lesions may allow local formation rather than attraction of osteoclast-like MGCs; such cells could, in turn, contribute to the tissue destruction seen in LCH patients (23). Hence, these investigators were able to demonstrate the expression of two OPG-interacting molecules, RANK and RANKL, in bone and nonbone lesions in LCH patients. OPG expression was not determined. Nevertheless, based on these findings, MGCs and osteoclast-inducing cytokines were suggested to be suitable as a potential target for the treatment of LCH. Indeed, we and others have recently shown that various osteoclastogenic factors are highly expressed in the serum and/or in lesions in LCH patients (8,24). The aforementioned observations provide a rationale for the use of bisphosphonates (potent inhibitors of bone resorption) in LCH patients with osteolytic lesions and bone pain (25,26). Moreover, the recent demonstration that pamidronate stimulates OPG production by human osteoblasts (27) further supports the notion that the RANKL-OPG axis may play an important role in this context. Further studies of RANK and RANKL expression, and of serum levels of OPG, in LCH patients before and after treatment with bisphosphonates are thus warranted.

Serum levels of OPG were previously shown to be decreased in MM patients with osteolytic lesions (19), and subsequent investigations showed that OPG was internalized and degraded by MM cells, thereby providing a possible explanation for the low levels of OPG in myeloma patients (28). Our current results indicate that LCH patients with bone lesions have lower serum levels of OPG when compared with patients without bone lesions; however, overall, LCH patients were found to have elevated serum concentrations of OPG in comparison to pediatric controls. At first glance, increased levels of the decoy receptor OPG in patients with bone lesions argues against a role for the RANKL-OPG system in the development of bone loss in LCH. However, increased OPG

levels could also represent a continuing homeostatic response, attempting to reverse established osteopenia and RANKL-driven osteoclastogenesis, thus maintaining normal bone mass. In support of this contention, recent studies of other conditions of chronic inflammation have provided further evidence of a paradoxical relation between OPG and bone lesions. For instance, in patients with Crohn's disease (CD) and ulcerative colitis, increased OPG concentrations were detected (29,30). These results may appear counterintuitive since OPG interferes with osteoclast differentiation, conferring skeletal benefit, whereas inflammatory bowel disease is frequently associated with bone loss. However, up-regulation of OPG could serve as a protective host response that partially offsets the adverse skeletal effects created by the inflammatory state. Alternatively, elevated OPG in CD patients may reflect T cell activation (30). Compelling evidence for an important role of OPG in maintaining bone homeostasis during chronic inflammation comes from an animal model of spontaneous osteopenia and colitis (31). In this model, inhibition of RANKL-RANK interactions with exogenous recombinant OPG was shown to reverse skeletal abnormalities and reduce colitis. These findings suggest that the RANKL-OPG system may play a dual role in inflammatory bowel disease, influencing not only the bone but also gut pathology.

Numerous studies have shown that OPG has the potential to modulate osteoclast activation as well as the activation and survival of DCs (13,32). Moreover, transdifferentiation of immature DCs into functional osteoclasts in the presence of RANKL was recently reported (33). Indeed, pro-inflammatory cytokines such as TNF- $\alpha$  were shown to promote cell fusion during DC transdifferentiation and lead to the formation of larger MGCs than RANKL alone. The dual role of OPG in bone homeostasis and immune modulation is thus suggestive of a dual role for OPG in the pathogenesis of LCH, insofar as OPG may affect osteoclast-like MGCs or LCs, or both. However, our results could also imply a compensatory response to enhanced osteoclast activation (34), or may be viewed as a sign of chronic immune activation, in line with previous findings in RA patients and in patients with CD (21,29,35). Our current observation that OPG levels are higher in MS than in SS patients supports the latter view. Furthermore, previous results from our laboratory have suggested that elevated erythrocyte sedimentation rate (ESR) and thrombocytosis, two signs of chronic immune activation, is common in LCH patients with active disease (36). Interestingly, in a recent study of patients with RA, a direct correlation was seen between serum OPG and ESR and the Larsen score (a disease activity score), supporting the notion that serum OPG may be linked to, or driven by, systemic inflammation (37). We also show herein that OPG levels are elevated in patients with risk organ involvement at the time of diagnosis, suggesting that OPG levels are reflective of overall disease activity in these children. However, more patients need to be evaluated before general conclusions can be drawn concerning differences between patients with or without risk organ involvement, since there were only two patients with MS disease who had no risk organ involvement whereas four of the eight MS patients with risk organ involvement had bone lesions.

LCH is associated with high expression of several cytokines, both in serum and in granulomatous lesions in these patients (6,8,11,22,38). We have previously reported that pre-treatment serum levels of TNF- $\alpha$ , sIL-2R, and IL-1Ra are elevated in LCH (11,22). Similarly, in the present study, these immunomodulating cytokines were highly expressed in LCH patients at the time of onset of disease; moreover, a significant positive correlation was observed between TNF- $\alpha$  and OPG serum concentrations, at the time of diagnosis. These results thus demonstrate, for the first time, a correlation between OPG values and the expression of an important pro-inflammatory cytokine (TNF- $\alpha$ ) in children with LCH. We have also recently reported that TNF- $\alpha$  is abundantly expressed in CD1a<sup>+</sup> LCs in lesions from LCH patients (8). Interestingly, in the latter study, the children with the highest proportion of cells expressing TNF- $\alpha$  were shown to have risk organ involvement. Other investigators have shown that OPG expression is up-regulated by TNF- $\alpha$  in a time- and dose-dependent manner in rheumatoid synovial fibroblasts (39). Thus, although TNF- $\alpha$  is known to stimulate bone destruction, TNF- $\alpha$ -induced up-regulation of OPG could perhaps contribute to self-protection from bone destruction in RA patients. Moreover, a recent study has shown that anti-TNF- $\alpha$  treatment of RA patients resulted in a normalization of serum OPG levels (21). Successful treatment of LCH with etanercept, a soluble receptor fusion protein that functions as a TNF- $\alpha$  antagonist, has been reported in a limited number of cases to date (12; the present study), and the usefulness of this novel treatment has been called into question by other investigators (4); nevertheless, it would be of considerable interest to determine serum levels of OPG in LCH patients before and after treatment with anti-TNF- $\alpha$  agents, to assess whether OPG levels are regulated by TNF- $\alpha$  in these children.

In conclusion, our investigations are the first to show that serum levels of OPG are elevated in LCH. We also show that other serum markers of inflammation, including TNF- $\alpha$ , are elevated in these patients, and a correlation between serum OPG and TNF- $\alpha$  was observed. Larger series of patient sera should be evaluated for OPG expression to extend these results. Furthermore, although an involvement of the RANKL-OPG system in the development of bone lesions in LCH patients cannot be excluded, we favor the view that elevated OPG is a sign of inflammation and perhaps reflective of disease activity in these children. Further studies of OPG and OPG-interacting molecules (40) may yield novel targets for the treatment of LCH, and may also provide additional insights into DC biology.

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