

Osteoprotegerin Serum Levels in Kawasaki Disease: An Additional Potential Marker in Predicting Children with Coronary Artery Involvement

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ABSTRACT. *Objective.* Emerging evidence from *in vitro* studies and mouse genetics attributes to osteoprotegerin (OPG), a member of the tumor necrosis factor receptor family, an important role in vascular biology. We evaluated serum levels of OPG in a group of children with Kawasaki disease (KD), before immunoglobulin (IVIG) infusion and at 3-month followup.

Methods. Fifty patients (38 boys, 20 girls, median age 3.6 yrs, range 4 mo–7.4 yrs) fulfilling criteria for the diagnosis of KD, 30 febrile controls with infectious diseases, 18 patients with juvenile systemic lupus erythematosus (JSLE), and 40 healthy controls were enrolled. All KD patients received IVIG treatment within the first 10 days of illness, and aspirin. Coronary artery abnormalities (CAA) were reported in 6 out of 58 patients; all were male and younger than 5 years. Serum OPG was measured by ELISA in patients with KD before IVIG and at 3-month followup (median time 3.2 mo, range 3–3.5).

Results. At baseline and at the 3-month followup, KD patients had significantly higher OPG serum levels than febrile controls ($p < 0.001$ and $p < 0.004$, respectively), JSLE patients ($p < 0.0001$), and healthy controls ($p < 0.0001$). At baseline, KD patients who developed CAA had higher OPG serum levels than those without CAA ($p = 0.0001$); this difference was not present at 3-month followup. The optimal OPG cutoff value of 123.2 pg/ml was a significant predictor for CAA, with a sensitivity of 100% (6/6), a specificity of 96% (50/52), and a positive predictive value of 75% (6/8).

Conclusion. High OPG levels might be the result of compensatory production during acute and sub-acute phases of KD. OPG assay might be an additional clinically useful marker to monitor and differentiate patients who develop, from those who do not develop, such coronary artery abnormalities. (J Rheumatol 2005;32:2233–8)

Key Indexing Terms:

OSTEOPROTEGERIN KAWASAKI DISEASE CORONARY ARTERY ABNORMALITIES

Kawasaki disease (KD) is an acute febrile systemic vasculitis, affecting young children and infants, and involving small and medium size vessels, with fibrinoid necrosis of the medium-size muscular arteries. It is a self-limiting disease, but it can lead to life-threatening complications due to the risk of coronary artery aneurysms and myocardial infarction¹. Emerging evidence from *in vitro* studies and

mouse genetics attributes an important role in vascular biology to osteoprotegerin (OPG), a soluble decoy receptor of the tumor necrosis factor (TNF) receptor superfamily. A glycoprotein present only in soluble form, OPG is able to prevent binding of RANK ligand (RANK-L) to its specific cellular receptor RANK (expressed on osteoclasts) and its precursors. The term OPG derives from its ability to protect bone; as a receptor antagonist, it inhibits osteoclast differentiation and activation, thus reducing bone resorption². The overproduction of OPG in transgenic mice results in severe osteopetrosis, while in contrast, OPG-deficient mice exhibit an overall decrease in total bone density together with the early development of calcified lesions in the media of the aorta and the renal arteries³. This peculiar phenotype can be completely prevented by restoration of the gene⁴; and in another animal model the systemic administration of OPG was able to prevent vascular lesions⁵. OPG expression can be demonstrated in the media of large arterial vessels⁶, and different vascular cell types such as coronary smooth muscle cells and endothelial cells have been implicated as cellular sources and targets of vascular OPG production⁷. Thus,

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in endothelial cells, OPG has been found to act as autocrine, anti-apoptotic, and survival factor⁸. It has recently been hypothesized that the OPG/RANK-L system may be involved in endothelial homeostasis and in the development of vascular diseases⁹. We determined serum concentrations of OPG in children with KD, before and after intravenous immunoglobulin (IVIG) treatment.

MATERIALS AND METHODS

Clinical characteristics of study population. Our study population included 58 consecutive Caucasian patients (38 boys, 20 girls, median age of 3.6 years, range 4 mo–7.4 yrs), all fulfilled the diagnosis of KD¹⁰, and were attending our Rheumatology Unit from January 1999 to March 2004.

All children promptly received the currently recommended treatment (IVIG 2 g/kg in a single infusion), within the first 10 days from the onset of fever; aspirin (50–80 mg/kg) during the acute phase of the disease, and 3–5 mg/kg up to normalization of erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), platelet (PLT) count, and coronary alterations.

Forty sex- and age-matched children, attending our outpatient clinic for musculoskeletal pain, without inflammatory conditions, acted as healthy controls after the exclusion of rheumatic, endocrine, or metabolic diseases.

Every year, we update a healthy control database with children that attend our unit for arthralgias and/or musculoskeletal pains and review routine clinical, laboratory, and instrumental investigations for possible rheumatic diseases. During routine laboratory tests and after parental informed consent, we collect serum samples for each patient, which are subsequently stored at –20°C.

From this database, we selected Caucasian subjects who were younger than 8 years at the time they came to our attention; then we selected for matched cases with KD children only healthy controls who met the following criteria: same sex, same age (\pm 3 mo), and admitted in the same calendar year.

Thirty sex- and age-matched febrile control patients with non-vasculitic diseases were selected: 16 children with bacterial infections (8 with pneumonia, 3 with urinary tract infections, 5 with pharyngitis), and 14 children with viral infections (8 with influenza, 4 with rotavirus, 2 with Epstein-Barr virus). Bacterial infections were diagnosed based on urine, stool, or blood culture, together with the clinical signs and a chest radiograph when indicated. Viral infections were diagnosed based on changes in virus-specific antibodies in paired samples presenting a profile suggestive of acute infection, in addition to clinical signs.

Sixteen patients with juvenile systemic lupus erythematosus (JSLE) were enrolled as controls with non-febrile inflammatory illness and childhood systemic vasculitis. The diagnosis in all 16 children had been established before enrollment in the study. Approval was obtained from the Ethical Committee of Meyer Hospital, and parents gave informed consent.

Cardiac evaluation in KD patients. Patients with KD underwent electrocardiogram and 2D-echo Doppler on suspicion of KD, before hospital discharge, at the second, fourth and eighth week, and according to the degree of coronary involvement; children with coronary artery abnormalities (CAA) were closely followed until normalization of their condition.

CAA were defined as the internal lumen diameter greater than 2 standard deviations above the expected mean calculated for body surface area on the basis of the study by De Zorzi and colleagues¹¹.

Osteoprotegerin serum assays. Serum samples from patients with KD, febrile controls, JSLE patients, and healthy controls were collected during routine blood tests and subsequently stored at –20°C until OPG was measured.

Blood samples for routine laboratory tests, including ESR, CRP, white blood cell count, hemoglobin, PLT, total protein with albumin, and OPG concentrations were obtained in all study subjects.

Serum samples from patients with KD were drawn during acute phase of illness [before IVIG administration, at least 5 days after fever onset

(median 6, range 5–7 days)], and at followup 3 months after the first day of fever onset (median 3.2 mo, range 3–3.5 mo), when patients no longer exhibited clinical features of KD and laboratory tests (ESR, CRP, and PTL count) were normalized.

In patients with bacterial and viral infections, blood samples were obtained during acute stage of disease: at onset of the febrile state, soon after admission, and before any administered treatment; OPG was subsequently measured only after a definitive diagnosis was achieved.

In patients with JSLE, blood samples were drawn when patients exhibited clinical and/or laboratory features of active disease, at the time of first outpatient visit, or on hospital admission, before receiving any steroid treatment.

OPG concentrations were determined in serum using a highly sensitive, commercial sandwich ELISA provided by Immundiagnostik (Bensheim, Germany). Measurements were performed in samples according to manufacturer's instructions and as described in detail^{12,13}. The lower limit of detection of this assay is 2.8 pg/ml; the intraassay and interassay ($n = 16$) coefficient of variation is $< 10\%$. All assays were measured blinded to any clinical information and performed in duplicate.

Statistical analysis. An *a priori* power analysis was completed using GPower program¹⁴. Two-tailed p values were employed. A large expected difference was estimated for the sample (the effect size $f = 0.40$, as per Cohen¹⁵). In addition, power was set at 0.90, meaning there would be a 90% probability of reaching statistical significance if the obtained differences were truly present in the population. Results from the power analysis showed that 96 participants in all groups combined would be required. Data are reported as the mean \pm SD. Mann-Whitney U test, Wilcoxon signed-rank test for paired samples, and analysis of covariance (ANCOVA) with LSD correction were used to evaluate the mean differences (\pm SD) of OPG between groups, considering the following covariates for ANCOVA: sex, age, body mass index, and timing of IVIG infusion from the disease onset for children with KD. Spearman rank correlation test was used to determine correlation coefficients between OPG levels, ESR, CRP, hemoglobin, and PTL count values. A receiver operating characteristic curve (ROC) was constructed for determination of optimal cutoff values of OPG and CRP for predicting the development of CAA. Nonparametric tests were used, where necessary, due to the small size of our groups and to the skewness of our data. Levels of $p < 0.05$ were considered statistically significant. Analyses were performed on SPSS for Windows, version 11.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

Clinical data of KD patients. All 58 KD patients fulfilled criteria for diagnosis. The following clinical major manifestations are reported, in decreasing order. Typical fever was present in all patients, ranging from 38.5°C to 40°C, with median duration 6 days; a polymorphous rash was observed in 55 patients (94.8%); conjunctival and oral mucosal alterations were reported in 54 (93.1%) and 53 (91.3%) patients, respectively. Extremity changes with digital periungual peeling were present in 51 (87.9%) out of 58 patients, while acute cervical lymphadenopathy was observed in 36 (62%) patients.

Twenty-two children presented other clinically associated manifestations at the onset and/or during the clinical course of the disease: diarrhea, vomiting, abdominal pain in 11 (18.9%), arthralgia/arthritis in 10 (17.2%), and gall bladder hydrosis in 9 (15.5%) subjects.

Six boys younger than 5 years developed coronary artery abnormalities during the second to fourth week of the disease. Four of these patients, who had normal echocardi-

grams at 4-month followup, did not present alterations at the subsequent evaluations, while 2 with persistent alterations, albeit with reduced diameter, are still being followed.

The disease course was typical in all enrolled children, and the outcome was favorable.

The clinical and laboratory data of all subjects (study group and controls) are summarized in Table 1.

Osteoprotegerin assay. The mean concentrations of serum OPG in KD patients, before and after IVIG infusion, febrile controls, patients with JSLE, and healthy subjects are summarized in Table 1. At baseline and at the 3-month followup, KD patients had significantly higher OPG serum levels than febrile controls ($p < 0.001$ and < 0.004 , respectively), patients with JSLE ($p < 0.0001$), and healthy controls ($p < 0.0001$) (Figure 1). In KD patients no significant difference of OPG serum levels between acute phase and convalescence was detected. No significant correlation was found among OPG serum values and mean white blood cell count, ESR, CRP, hemoglobin, PLT count, or albumin at baseline and at 3-month followup.

Before IVIG infusion, KD patients with CAA did not differ from those who never developed CAA regarding number of diagnostic criteria, mean fever duration, timing of IVIG infusion, mean white blood cell count, hemoglobin, ESR, and PLT; while patients with CAA had higher concentrations of CRP (15.8 ± 9.9 mg/dl vs 5.7 ± 6.6 mg/dl; $p < 0.01$) and lower concentrations of albumin (3.0 ± 0.3 g/dl vs 3.8 ± 0.4 g/dl; $p < 0.001$). At baseline, KD patients who developed CAA had higher OPG serum levels than those without CAA (141.7 ± 10.2 vs 96.1 ± 13 ; $p < 0.0001$); this difference was not present at 3-month followup. In patients with CAA, a significant reduction of OPG serum levels was observed at 3-month followup when compared to baseline (141.7 ± 10.2 vs 96.6 ± 13.9 ; $p < 0.02$); in contrast, such a reduction was not found in those patients who never developed CAA (Figure 2).

ROC analysis was used to examine diagnostic accuracy of OPG, CRP, and albumin levels of patients with acute KD

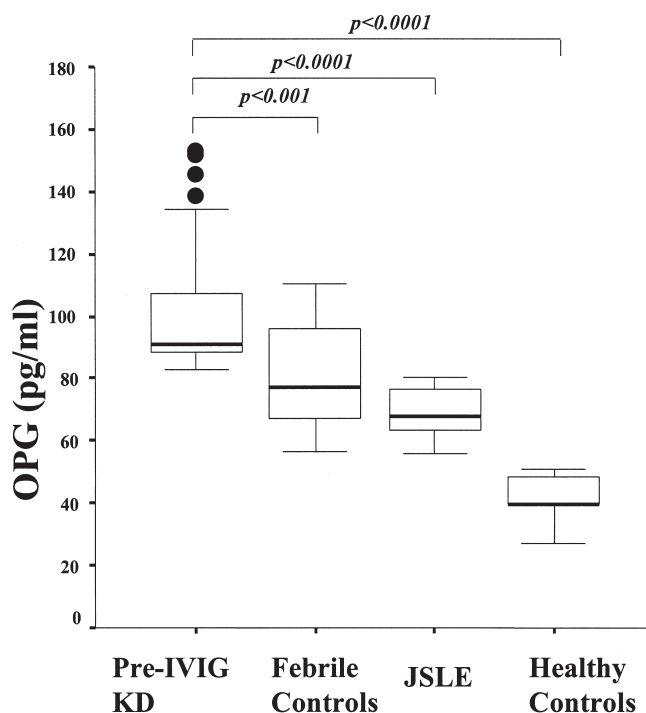


Figure 1. Serum osteoprotegerin (OPG) levels in healthy controls, febrile controls, and Kawasaki disease (KD) subjects, at baseline. The central line represents the distribution median, boxes span 25th to 75th percentiles, and error bars extend from 10th to 90th percentiles. Dots are values higher than the 90th percentile.

to predict subsequent development of CAA. The area under the ROC curve (AUC) was 0.98 for OPG, 0.93 for albumin, and 0.84 for CRP; thus all variables were statistically significant predictors for CAA (OPG AUC, $p < 0.0001$; albumin AUC, $p < 0.005$; and CRP AUC, $p < 0.01$). Thus, all these parameters resulted statistically significant predictors for CAA. The optimal cutoff value for predicting CAA was 123.2 pg/ml for OPG, 3.6 g/dl for albumin, and 6.61 mg/dl for CRP. A serum OPG concentration > 123.2 pg/ml had a sensitivity of 100% (6/6), specificity of 96% (50/52), and a

Table 1. Clinical and laboratory data of all subjects, study group and controls.

Group	No. of Patients	F/M	Age, yrs (range)	WBC, $\times 10^3/\text{mm}^3$	Hb, g/dl	PLT, $\times 10^3/\text{mm}^3$	CRP, mg/dl (NV ≤ 0.35 mg/dl)	Albumin, g/dl (NV > 3.7 g/dl)	OPG, pg/ml
Kawasaki disease	58	20/38	3.6 (4 mo–7.4 yrs)						
Baseline				14.5 ± 6.3	9.4 ± 2.6	397 ± 120	8.31 ± 8.6	3.7 ± 0.51	101.1 ± 20.3
Followup				10.2 ± 1.4	12.4 ± 1.1	686 ± 90	0.37 ± 0.4	4.4 ± 0.6	96.6 ± 16.5
Febrile controls	30	11/19	3.8 (2 mo–7.2 yrs)	13.4 ± 7.1	12.1 ± 1.2	622 ± 110	2.4 ± 3.5	4.3 ± 0.6	$80.7 \pm 17.3^{\dagger,*}$
Juvenile SLE	16	9/7	8.6 (6.4–9.4 yrs)	6.2 ± 3.2	11.4 ± 2.3	326 ± 150	0.45 ± 0.3	4.5 ± 0.7	$68.4 \pm 7.9^{**}$
Healthy controls	40	11/29	3.4 (5 mo–7.6 yrs)	7.4 ± 2.4	12.3 ± 1.2	356 ± 124	0.35 ± 0.1	4.6 ± 0.4	$40 \pm 7.8^{**}$

CRP: C-reactive protein; WBC: white blood cell count; Hb: hemoglobin; PLT: platelet count; NV: normal value. * $p < 0.004$, $^{\dagger} p < 0.001$, ** $p < 0.0001$.

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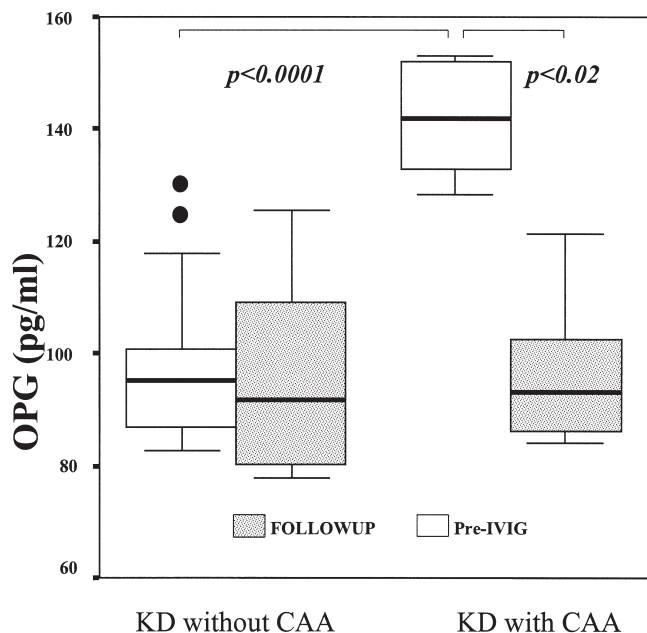


Figure 2. Serum osteoprotegerin (OPG) levels in Kawasaki disease (KD) subjects with and without coronary artery abnormalities (CAA), at baseline and at 3-month followup. The central line represents the distribution median, boxes span 25th to 75th percentiles, and error bars extend from 10th to 90th percentiles. Dots are values higher than the 90th percentile.

positive predictive value of 75% (6/8); serum albumin value < 3.6 g/dl had a sensitivity of 100% (6/6), specificity of 65% (34/52), and positive predictive value of 25% (6/24); serum CRP value > 6.61 pg/ml provided a sensitivity of 100% (6/6), specificity of 71% (37/52), and positive predictive value of 29% (6/21). Thus, the optimal cutoff value of OPG was superior to those of albumin and CRP with respect to specificity and positive predictive value. In our analysis, the OPG assay showed higher diagnostic accuracy than albumin ($p < 0.005$) and CRP ($p < 0.001$) in predicting CAA in patients with KD.

Indeed, some patients who had only one variable greater than or equal to the optimal cutoff point for OPG and CRP, or less than or equal to the cutoff point for albumin, were false-positive predictors of CAA, while KD patients with all 3 variables positive for each specific optimal cutoff point were definitively true-positive (children with KD who then developed CAA).

Combining this set of cutoff points identified for OPG, albumin, and CRP by ROC analysis, the positive predictive value and the specificity in predicting CAA development among this cohort of KD children reached 100%: i.e., no KD patient with all 3 findings of OPG values < 123.2 pg/ml, albumin values > 3.6 g/dl, and CRP values < 6.61 pg/ml developed CAA.

Two children who did not develop CAA showed OPG values > 123.2 pg/ml: the first also had CRP values > 6.61

pg/ml, but albumin values were > 3.6 g/dl; the second had albumin values < 3.6 g/dl, but CRP values were < 6.61 pg/ml.

On the other hand, when considering only OPG and CRP cutoff levels, the positive predictive value was only 85% (6/7), with a sensitivity of 100% (6/6) and a specificity of 98% (51/52). We achieved the same results combining only the OPG and albumin optimal cutoff values. Therefore, in predicting CAA, the combination of OPG and CRP optimal cutoff values was not superior to that of OPG and albumin.

DISCUSSION

In our study, OPG serum levels were measured in children with KD: OPG serum levels significantly higher than in febrile controls, JSLE patients, and healthy controls were found in the entire group of KD patients, and, before IVIG therapy, in particular in those who later developed CAA.

It has to be considered that an important feature of OPG regulation *in vivo* is a significant increase of OPG serum levels with aging in healthy people¹⁶, therefore a more definitive interpretation of OPG serum values should also take into account the age, and not just the disease, of studied subjects¹². Indeed, it has been shown that in children mean OPG levels are significantly lower than in adults: we previously confirmed this pattern in a group of 40 healthy children, age-matched with juvenile idiopathic arthritis patients, in whom the mean concentration of serum OPG was 39.6 ± 11 pg/ml¹³.

As discussed in an article on osteoporosis and cardiovascular diseases¹⁷, increased OPG serum levels may reflect an incomplete compensatory self-defence mechanism, in our setting to control endothelial injury in KD patients, especially in those with coronary alterations.

In the vascular system, increased OPG production may in fact indicate endothelial damage, and/or insufficient protective mechanism to prevent further vascular damage, especially in patients with CAA.

Further support for OPG as a vascular regulator comes from an epidemiologic study of 490 elderly women: Browner and colleagues¹⁸ reported the relative risk of cardiovascular mortality to be increased more than 4-fold in women within the highest quintile of serum OPG concentration. This finding may be due to increased OPG concentrations, representing a compensatory self-defence mechanism that incompletely controls the immune mechanisms that contribute to arterial calcification and atherosclerosis.

A similar interpretation was made by Yano and co-workers, when they found that women with osteoporosis and increased biochemical markers of bone turnover had serum concentrations of OPG higher than age-matched controls without osteoporosis¹⁹. Although the precise mechanism remains unclear, vasculitis during KD might enhance production or release of this protein from the endothelium within the vascular system.

It has been reported that OPG administration exerts pro-

tective vascular effects in various animal models³⁻⁵; thus, considering these potential protective properties for vascular biology, its elevated serum levels may represent a biological mechanism attempting to reduce endothelial damage. Due to possible development of CAA, this mechanism seems in some way insufficient or incomplete.

Alternatively, or in addition, inflammatory mechanisms and mediators, e.g., TNF- α , may both promote vascular disease and increase OPG serum levels.

The possibility that high OPG levels could be secondary to decreased clearance also cannot be completely ruled out.

Since KD patients showed higher OPG levels than JSLE and febrile controls not associated with vasculitis, elevated OPG levels may not be related only to inflammation per se, nor can they be considered as a mere measure of acute inflammation. This idea has also been supported by our finding of lack of correlations between OPG and inflammatory markers, such as mean white blood cell count, ESR, and CRP.

Considering OPG as an indirect, potential marker of endothelial damage, the lack of normalization of OPG levels in KD children as a group at 3-month followup may lend support to observations that KD vasculitis is an ongoing process, still present even when the systemic inflammation is fading and the risk of coronary involvement is reduced. From this point of view, the putative protective mechanism of OPG on the vascular environment still appears incomplete.

Children with KD show significantly higher OPG values than those with JSLE: therefore it is unlikely that elevated OPG serum levels are related to the vasculitis phenomenon in general. This finding might add a possible clue to the pathogenesis of KD injury and suggest a closer relationship between the OPG/RANK-L system and KD.

Interestingly, we observed that in patients developing CAA, mean OPG serum values were significantly elevated in the acute phase of the disease, when compared to convalescence, while in children without CAA, no significant difference in OPG levels was observed between the acute phase and followup. Although a small number of subjects were studied, ROC analysis revealed serum OPG concentration as a significant predictor of development of CAA in KD, with a sensitivity and specificity of 100% and 96%, respectively, when the optimal cutoff value is set at 123.2 pg/ml.

Taken together, these data also seem to suggest the potential usefulness of serum OPG assay in differentiating KD patients who develop CAA from those who do not develop such coronary alterations. Variables such as the blood cell count, hemoglobin, albumin, and CRP, TNF- α and interleukin 6 concentrations have been reported to predict the later development of coronary aneurysms²⁰⁻²³, thus several scoring systems have been developed to identify children at high risk for CAA^{21,24,25}.

A combination of OPG levels with some of these variables might enhance the accuracy of predicting coronary involvement in KD. In our analysis, due to the lack of reciprocal correlation between the 3 identified variables, the combined use of OPG, albumin, and CRP predicts the development of CAA better than one variable alone, clearly distinguishing false-positive from true-positive patients.

Although the mechanism for the effects of OPG on the vascular system is unknown, it is likely that the compensatory self-defence response is overexpressed in patients with CAA. Consistent with these findings, increased OPG serum levels have recently been associated with the presence and severity of coronary artery disease, suggesting the potential contribution to the pathogenesis and progression of vascular diseases^{26,27}.

In regard to OPG levels in healthy controls, a direct comparison between these studies and our findings is hampered by age-related differences since, as stated, children display much lower OPG circulating levels than adults.

Nonetheless, the finding of increased levels of OPG in patients with coronary artery diseases seems consistent with the highest OPG levels reported in our children with CAA and KD, prompting similar conclusions: higher OPG serum levels in patients with coronary involvement might reflect an incomplete compensatory mechanism in response to endothelial damage.

A major limitation of our study is related to the measurement of total serum OPG. As far as we know, there is no commercial kit available to specifically detect the free fraction of OPG, allowing identification of free OPG from that complexed to its ligand, RANK-L. Therefore increased serum OPG levels in KD children, in particular in those with coronary alterations, may be due to increased free OPG, increased OPG/RANK-L complexes, or, more likely, to both. Considering this, in order to confirm the potential modulating role of the OPG/RANK-L system in KD vascular injury, a RANK-L assay seems mandatory. The report that RANK-L serum levels are lower in patients with coronary artery disease suggests additional evidence of insufficient counter-regulatory mechanisms for OPG/RANK-L in vascular diseases²⁸.

Finally, the observation of increased OPG levels in KD compared to other febrile illnesses might be clinically helpful in the differential diagnosis with bacterial and/or viral infections. Our results, however, do not allow a clear distinction because of some overlapping results, due to the standard deviations of OPG values in these 2 groups of diseases (see Figure 1). Even though appropriate statistical analysis for multiple comparisons (post hoc multiple comparisons) detected a p value < 0.001, it has to be considered that 3 patients with febrile infectious diseases showed OPG values higher than the mean of OPG values for KD (101.7 pg/ml, 102.1 pg/ml, 110.5 pg/ml), and the lowest value in KD patients (82.6 pg/ml) almost corresponds to the mean of

febrile controls. This caveat seems not to apply to JSLE and healthy controls: these groups clearly present different distributions regarding OPG levels.

To better understand the clinical significance of our results, OPG needs to be evaluated in a larger cohort of KD patients, especially in those with coronary damage, with longer followup, and concomitant RANK-L assays should be performed. Nonetheless, our data suggest that OPG serum assay may help differentiate KD from other childhood febrile illnesses. As a predictor of the later development of coronary aneurysms, OPG might be an additional biochemical marker to identify KD children at risk of coronary artery abnormalities. Further studies will also be necessary to investigate a possible role of osteoprotegerin in the pathogenesis of coronary and endothelial injury in Kawasaki disease.

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