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Quantifying RANKL and OPG levels in healthy children: A large cross-sectional analysis

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ABSTRACT

Background: There have been new advances in understanding bone remodeling on a molecular level including the RANKL-OPG pathway, leading to advancements in targeted therapeutic intervention. There is however limited data in pediatrics with little known on normative values in healthy children.

This is the largest cohort to quantify RANKL, OPG, and RANKL: OPG levels in healthy children as well as study the influence of age, gender, Tanner stage, and BMI in this population.

Methods: Healthy subjects, 1–21 years of age, were recruited from general pediatric clinics affiliated with CHLA and in collaboration with samples stored from a previously completed study. Healthy children were defined as those with no chronic disease, daily medication, or fractures in the past six months. Free soluble RANKL and OPG levels were quantified using a sandwich ELISA.

Results: Three hundred samples were collected with overall serum concentrations of RANKL, OPG and RANKL: OPG of 0.28 pmol/L, 3.56 pmol/L and 0.08 pmol/L, respectively. Serum RANKL and RANKL: OPG concentrations were significantly different by age ($p = 0.0001$ and 0.0027, respectively). There was an overall downward trend by age except in the 11–15-year age group where a slight increase was noted. RANKL concentrations were also significantly different between Tanner stages, with highest concentrations seen at Tanner 3 ($p = 0.0481$), and zBMI ($p = 0.001$). OPG was inversely correlated with zBMI, but not influenced by gender, age, or Tanner stage.

Conclusion: We showed significant difference in RANKL levels by age, Tanner stage, and zBMI. OPG was inversely correlated with zBMI. Insight into circulating levels of RANKL, OPG and RANKL: OPG in healthy children may be a potential tool to better understand disease states in pediatrics. Future studies are needed to evaluate the clinical significance of RANKL and OPG levels for diagnostic and therapeutic purposes in this population.

1. Background

There have been new advances in understanding bone remodeling on a molecular level. One such pathway of interest is the RANKL (transmembrane protein receptor activator of NF-kB ligand) and OPG (osteoprotegerin) pathway [1]. RANKL, secreted by osteoblasts and expressed by cells of the mesenchymal lineage and activated T cells, functions by binding to its cognate receptor of RANK, leading to osteoclast differentiation and activity. In addition, it prevents osteoclast apoptosis, further enhancing bone resorption [2,3]. The binding effects of RANK-RANKL can be neutralized by OPG, a non-signaling glycoprotein also secreted by osteoblasts. OPG acts as a decoy receptor, binding RANKL and preventing attachment to the RANK receptor, thereby blocking its catabolic effects [4]. The balance between RANKL and OPG is thus important in bone homeostasis and disruption of these coordinated cycles may lead to increased bone resorption, microarchitecture changes, and increased fracture risk [4]. In adults, elevated RANKL levels have been implicated in conditions such as post-menopausal osteoporosis with current treatment now including Denosumab, a monoclonal antibody against RANKL, which has shown gains in bone mineral density as well as reductions in bone turnover [4–7].

Pediatric trials with this targeted, bone remodeling therapy are now

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Table 1
RANKL, OPG, and RANKL: OPG concentration values are listed with their respective p-values. The number of patients is expressed as (n). The values for each variable are the median with the range expressed in parentheses. Body mass index z-score is expressed as zBMI.

Variables	Categories	RANKL median (IQR)	p-Value	OPG median (IQR)	p-Value	RANKL/OPG median (IQR)	p-Value
Overall		0.28 (0.19–0.38)		3.56 (2.92–4.25)		0.08 (0.05–0.12)	
Gender			0.0930		0.4963		0.1404
	Male (n = 173)	0.29 (0.20–0.39)		3.53 (3.03–4.13)		0.08 (0.05–0.13)	
	Female (n = 127)	0.27 (0.18–0.37)		3.59 (2.82–4.46)		0.08 (0.05–0.11)	
Age group			0.0001		0.0646		0.0027
	< 6 years (n = 34)	0.36 (0.23–0.48)		3.44 (3.09–4.58)		0.09 (0.05–0.17)	
	6–10 years (n = 86)	0.28 (0.20–0.37)		3.78 (3.23–4.23)		0.08 (0.05–0.11)	
	11–15 years (n = 114)	0.30 (0.21–0.41)		3.39 (2.88–4.04)		0.09 (0.06–0.14)	
	16–21 years (n = 66)	0.21 (0.14–0.31)		3.53 (2.61–4.08)		0.07 (0.04–0.10)	
zBMI group[a]			0.0001		0.0123		0.0001
	< −2.0 (n = 3)	0.17 (0.09–0.22)		5.27 (3.16–5.40)		0.04 (0.02–0.05)	
	−2.0–1.0 (n = 230)	0.27 (0.18–0.36)		3.62 (3.07–4.37)		0.07 (0.05–0.11)	
	> 1.0–2.0 (n = 43)	0.41 (0.26–0.48)		3.39 (2.79–3.84)		0.11 (0.08–0.14)	
	> 2.0 (n = 15)	0.30 (0.22–0.40)		3.01 (2.24–3.64)		0.10 (0.08–0.18)	
Tanner Stage			0.0481		0.2914		0.5123
	1 (n = 110)	0.28 (0.20–0.41)		3.70 (3.15–4.40)		0.08 (0.05–0.12)	
	2 (n = 31)	0.28 (0.21–0.36)		3.44 (3.07–4.13)		0.08 (0.06–0.11)	
	3 (n = 20)	0.36 (0.29–0.52)		3.63 (3.10–4.52)		0.10 (0.08–0.15)	
	4 (n = 41)	0.27 (0.18–0.32)		3.48 (2.67–3.96)		0.07 (0.05–0.12)	
	5 (n = 89)	0.26 (0.16–0.36)		3.50 (2.79–4.14)		0.08 (0.05–0.12)	

[a] BMI z-score was categorized based on WHO classification: Underweight: < −2.0; Normal Weight: −2.0 to 1.0; Overweight: > 1.0–2.0; Obese: > 2.0.

underway despite limited data on RANKL and OPG levels in pediatrics. There are limited studies focusing on disease burden which showed elevated RANKL levels in children with chronic illness, immobility, poor nutrition, and glucocorticoid exposure [2,4]. However, there are very few studies that have defined normative values in healthy children. Most normative data have been briefly described in the setting of small sample controls for comparison to a disease of interest. For example, Wasilewska et al. evaluated RANKL and OPG levels in healthy subjects among 70 healthy children 6 months to 19 years of age, finding that serum levels of RANKL and RANKL:OPG ratios were influenced by physical factors such as gender and body weight while OPG levels were not [8]. While they were able to use this data for controls in another study, [2] their cohort was too small to derive any reference values. Other studies show similarly limited cohorts of healthy children, generally with narrower age ranges for enrolled subjects [9,10].

Thus, it is imperative to establish normative data of RANKL and OPG in children in a large cohort. This is the largest analysis of serum RANKL, OPG and the ratio of RANKL to OPG concentration in healthy children. Our aim was to quantify overall RANKL, OPG, and RANKL: OPG levels in healthy children as well as study the influence of age, gender, and physical determinants of health such as pubertal status and body mass index (BMI).

2. Materials and methods

We conducted a cross-sectional study with sample attainment in collaboration with a previous study conducted by Olney et al. [11]. Two hundred and fifty-eight healthy children between 12 months and 20 years of age were enrolled. Some patients had two samples drawn at different time points, both of which were used in the final analysis. Subjects were recruited either from sibling involvement or through fliers, newspaper and internet advertisements. Healthy subjects were defined as those with no chronic disease or daily medication use. Anthropometric data and Tanner staging were collected for each subject as previously described [11].

Additional subjects were recruited from general pediatric clinics affiliated with the Children's Hospital Los Angeles (CHLA). Healthy subjects, 1–21 years of age with no known medical conditions or daily medications were enrolled. Patients were excluded if they had sustained a fracture in the past six months.

The study was approved by the Institutional Review Board at the Children's Hospital Los Angeles.

2.1. Biochemical determinants

Blood samples from CHLA and outside collaboration were collected upon consent and processed within 4–6 h. Samples were centrifuged, serum aliquoted, and frozen at −80 °C until assayed [11].

Free soluble RANKL levels were quantified using a sandwich enzyme-linked immunosorbent assay (ELISA) (Biomedica Medizinprodukte GmbH & Co KG, Vienna, Austria). The limit of detection for this assay was 0.01 pmol/L. Intra- and inter-assay precision was ≤5% and ≤3%, respectively. OPG was also measured using a sandwich ELISA (Biomedica Medizinprodukte GmbH & Co KG, Vienna, Austria). The limit of detection for this assay was 0.07 pmol/L, with intra- and inter-assay precision of ≤3% and ≤5%, respectively. The RANK: OPG ratio was calculated for each patient by dividing the values of RANKL by OPG.

2.2. Statistical analysis

The distribution of study variables was summarized using descriptive statistics. Median and inter-quartile range (IQR) were used due to the non-normality of the distribution. The Wilcoxon ranksum test was used to examine gender differences in RANKL, OPG, and the RANKL: OPG concentration. The Kruskal-Wallis test was used to examine differences among subjects by age, BMI and Tanner stage. Statistical significance was set a two-sided 0.05 level. All computations were done in Stata/SE 15.1 (College Station, Texas).

3. Results

A total of 300 samples were collected with descriptive statistics shown in Table 1. Nine subjects did not have data for Tanner Stage or BMI and were excluded from those analyses. Fifty-eight percent of the cohort were male. The average age of subjects was 11.64 years with a standard deviation of 4.84 years. The overall median RANKL concentration was 0.28 pmol/L (IQR 0.19–0.38). The overall median OPG concentration was 3.56 pmol/L (IQR 2.92–4.25) and the median RANKL:OPG ratio was 0.08 pmol/L (IQR 0.05–0.12).

Age was categorized into four groups: < 6 years, 6–10 years, 11–15 years and 16–21 years of age. RANKL and RANKL: OPG were

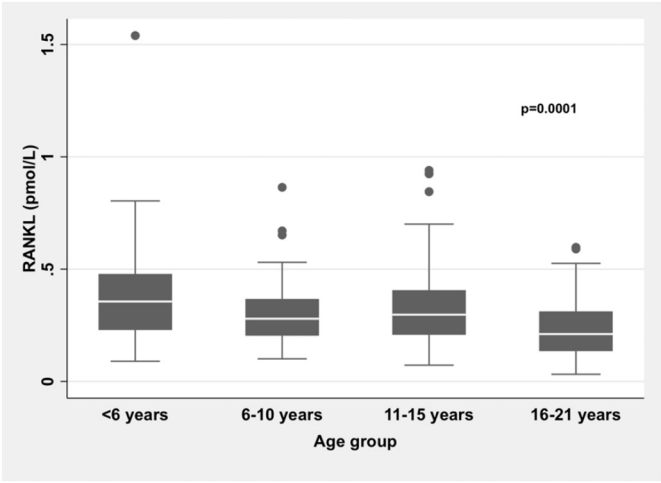

Fig. 1. Box plots of median and interquartile range (IQR) of RANKL by age. The white bar is representative of the median with the error bars being the 25th and 75th percentiles of the IQR. The dots represent outliers. For subjects < 6 years of age, median RANKL concentration level was 0.36 pmol/L, for 6–10 years was 0.28 pmol/L, 11–15 years was 0.30 pmol/L and 16–21 years was 0.21 pmol/L.

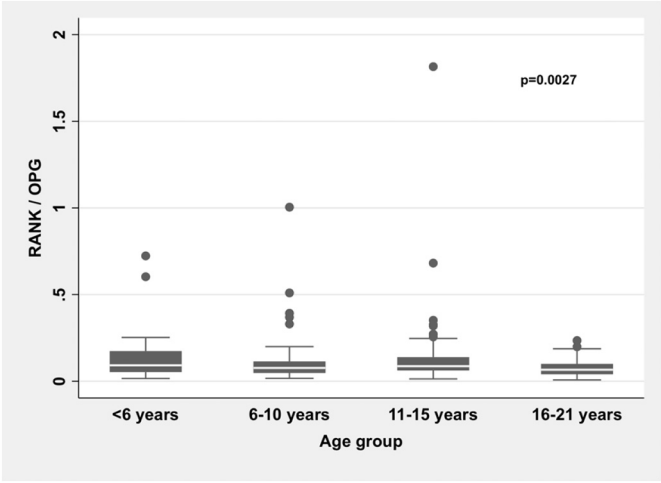

Fig. 2. Box plots of median and IQR of RANKL: OPG by age group. For subjects < 6 years of age, median RANKL: OPG concentration level was 0.09 pmol/L, for 6–10 years was 0.08 pmol/L, 11–15 years was 0.09 pmol/L and 16–21 years was 0.07 pmol/L.

significantly different by age (Table 1). Fig. 1 shows a downward trend of RANKL with age, with a median concentration of 0.36 pmol/L for ages < 6 years to 0.21 pmol/L for ages 16–21 years old. There is however, an increase in the 11–15 age group (median = 0.30 pmol/L) compared to the 6–10-year group (median = 0.28 pmol/L). Fig. 2 highlights RANKL: OPG values, which showed a similar trend to RANKL. RANKL: OPG levels were highest in the < 6 years age group and lowest in the 16–21 year group. There is again a slight increase in the RANKL: OPG concentration in the 11–15-year age group compared to the 6–10-year age group. OPG stayed relatively constant over time with no significant difference among age groups. There were no significant differences in RANKL, OPG and RANKL/OPG concentration based on gender (Table 1).

RANKL concentrations were also significantly different between Tanner stages (Fig. 3). Tanner stage 1 and 2 both had median concentrations of 0.28 pmol/L. The highest RANKL concentration overall was seen at Tanner stage 3, with a median concentration of 0.36 pmol/L. RANKL levels then decline at Tanner stages 4 and 5, with Tanner stage 5 having the lowest median value of 0.26 pmol/L.

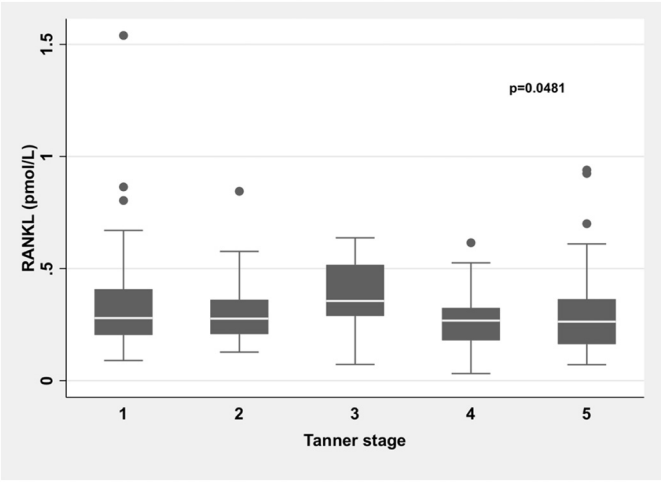

Fig. 3. Box plots of median and IQR of RANKL by Tanner Stage. For subjects in Tanner Stage 1, the median RANKL concentration was 0.28 pmol/L, Stage 2 was 0.28 pmol/L, Stage 3 was 0.36 pmol/L, Stage 4 was 0.27 pmol/L and subjects in Stage 5 had a median value of 0.26 pmol/L/.

Body mass index was categorized into four groups based on the World Health Organization z-score classification (Table 1) [12]. The vast majority of subjects were of normal weight with a BMI z-score (zBMI) of −2.0 to 1.0 (n = 230). There were significant differences in RANKL, OPG, and RANKL: OPG levels based on BMI z-score (Table 1). RANKL and RANKL: OPG generally trended up as BMI increased with the overweight and obese subjects having higher levels than normal and underweight subjects. OPG levels were inversely correlated with zBMI, with the highest levels seen in the underweight group and lowest levels in the obese group.

4. Discussion

This is the largest pediatric study evaluating RANKL, OPG, and RANKL: OPG in healthy subjects as well as categorizing by gender, age, pubertal and anthropometric data. We found significant differences in RANKL and RANKL: OPG concentration by age, with a general decrease in levels over time with the exception of the 11–15 age group. This is similar to a trend observed in adults, with RANKL and RANKL: OPG ratios decreasing with age in adults, especially in postmenopausal women [13]. This decline of serum RANKL has been suggested to be protective in reducing bone resorption and turnover in older age groups [8,13]. Interestingly, we found a rise in RANKL and RANKL: OPG levels in the 11–15 age group. This small increase may be due to the large rate of bone growth and remodeling at this time, with peak rates of bone growth occurring between ages 11–13 in females and 13–15 in males [14]. OPG was fairly constant over time, similar to other smaller studies showing a lack of correlation of OPG with age in children [8,15].

We did not find significant difference in RANKL, OPG, and RANKL:OPG between genders. This is in contrast to Wasilewska et al. who found RANKL to be three times higher in males than females in their cohort [8]. More recent studies, however, also showed absence of statistical significance in RANKL between males and females [16]. Our OPG findings were also supported by a smaller study from Buzi et al. who similarly found no differences in OPG by gender in children [9].

RANKL concentrations were also significantly different among Tanner stages, with peak RANKL concentration at Tanner stage 3. There may be an overlap here with the increase seen in the 11–15 age group, suggesting that sex hormones may have influenced the rise in RANKL. There is evidence that hormones such as estrogen influence RANK and RANKL expression, leading to possible increase in RANK and RANKL concentrations during puberty [17]. In addition, peak height velocity occurs around Tanner 3–4 in boys (mean 13.5 years) and in Tanner 2–3

in girls (mean 11.5 years), which may be when bone turnover markers are expected to peak [18,19]. Wasilewska et al. similarly found an increase in RANKL levels in Tanner stage 3 patients compared to Tanner stage 1 [8]. However, they also found an increase in the Tanner stage 5 group in contrast to our findings, as this group had the lowest RANKL levels in our cohort. This may be due to the downward trend of RANKL levels we observed in the older age group in our study. There was no significant difference in OPG by Tanner stage. This lack of significance for OPG by Tanner stage is interesting as estrogen is a known regulator of OPG expression, with decreased levels of estrogen leading to decreased OPG levels and increased RANKL:OPG ratios [20]. Further studies are needed to address this issue.

In addition, we found significant differences in RANKL, OPG and RANKL: OPG by zBMI. There has been conflicting data in the literature regarding BMI and its influence on RANKL/OPG levels. Similar to our study, Wasilewska et al. found a positive correlation between body weight percentiles and RANKL [8]. Serrano-Pina et al. in contrast, showed no difference in serum RANKL concentrations in low-weight, normal-weight and obese patients, though their cohort was much smaller [16]. In terms of OPG, previous studies have also cited an inverse relationship between BMI and OPG [21,22] consistent with our results, including a recent large cohort by Stanik et al., who analyzed this relationship in 1325 pediatric subjects [23]. Other smaller studies have reported no association between OPG and BMI [8,9,24]. It has been proposed that obesity can be confounded by decreased physical activity, which may negatively influence bone turnover and therefore OPG production [24]. In addition, excess weight itself may also correlate to lower bone mass, causing weaker osteoclast activity [24] and subsequently lower levels of RANKL and OPG overall. Thus, RANKL and OPG may not be effective tools in evaluating the role of weight on bone turnover in pediatrics.

There are limitations to our study. The stability of RANKL and whether there is diurnal variation has not been well-established. Hence, it is unclear whether there is optimal timing for sample collection as random sampling was done. RANKL is also predominantly found in bound form, either complexed with OPG or other circulating factors [3]. We quantified free soluble RANKL levels, the clinical utility of which compared to total RANKL levels has yet to be established [3]. Previous authors have similarly quantified free soluble RANKL [9,13,16,22] whereas others report measuring total serum RANKL levels [8], perhaps accounting for the conflicting results seen in the literature. Furthermore, clinical applications of free soluble RANKL and OPG assays are limited due to the uncertainty of the tissue source of the RANKL being measured and whether circulating levels reflect levels in general or those in specific areas of interest, particularly in bone since RANKL and OPG act at the paracrine level [3,25]. Lastly, other markers of bone remodeling were not concurrently evaluated for comparison.

However, our study has addressed limitations of other studies. The large size of our cohort allowed for more statistical power with a smaller margin of error. We also increased the upper limit of our age range to include adolescents up to 21 years of age where other studies typically included children and teen groups up to 19 years of age [8,9]. While peak bone mass occurs during puberty, bone mass accumulation can continue for up to seven years after peak height velocity, suggesting a need to continue to analyze bone turnover markers at later time points [19].

This sizeable study of RANKL, OPG, and RANKL: OPG with parameters of physical development in healthy children is important to establish standards in these biochemical markers and to potentially create pediatric reference data. This may be a valuable clinical tool in evaluating children with specific disease subsets, in particular bone disorders where standard pediatric data is often unavailable.

5. Conclusion

This is the largest data on RANKL, OPG and RANKL: OPG in healthy subjects 1–21 years of age to date. Because no clear reference range exists, our study sought to provide insight into circulating levels of RANKL, OPG and RANKL: OPG in healthy children, as a potential tool to better understand disease states. We showed significant difference in RANKL and RANKL: OPG by age, Tanner stage, and zBMI. There was no difference in these levels with gender. OPG was only influenced by zBMI, with no difference in any other parameter of physical development. Future studies are needed to evaluate the clinical significance of RANKL and OPG levels for diagnostic and therapeutic purposes in the pediatric population.

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee. Informed consent was obtained for all subjects.

Declaration of Competing Interest

Sara Akhtar Ali, Harsimar Kang, Robert Olney, Leigh Ramos-Platt, Anna Ryabets-Lienhard, Senta Georgia, and Pisit Pitukcheewanont have no conflicts of interest.

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