# Osteoprotegerin and soluble receptor activator of nuclear factor κB ligand in children with inflammatory bowel disease

## Stężenie osteoprotegeryny i liganda receptora aktywującego czynnik jądrowy κB w przebiegu nieswoistych zapaleń jelit u dzieci

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**Key words:** osteoprotegerin, receptor activator of nuclear factor κB ligand, children, inflammatory bowel disease, bone mineral density.

**Słowa kluczowe:** osteoprotegeryna, receptor aktywujący czynnik jądrowy κB, dzieci, nieswoiste zapalenie jelit, gęstość mineralna kości.

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## Abstract

**Introduction:** Osteoprotegerin (OPG) and receptor activator of nuclear factor κB ligand (RANKL) are cytokines that play a key role in bone metabolism but also in the immune system. Ulcerative colitis (UC) and Crohn's disease (CD) are autoimmune inflammatory bowel diseases (IBD), affecting digestive system but also bone mineralization.

**Aim:** To evaluate serum concentrations of osteoprotegerin and the sRANKL and their relationship with certain parameters in children with IBD.

**Material and methods:** The study included 93 children treated at the Department of Pediatrics, Gastroenterology, Hepatology and Nutrition, Medical University of Gdansk: 18 with UC, 24 with CD, 51 healthy children. The OPG and sRANKL were detected with a commercial ELISA kit. Bone mineral density (BMD) was assessed by means of dual energy X-ray absorptiometry (DXA) in IBD patients.

**Results:** The higher concentrations of osteoprotegerin were in the IBD group (range 0.20-0.73 pmol/l, SD 1.35, median 4.10 pmol/l) than in the control group (0.03-0.2 pmol/l, SD 1.72, median 3.61 pmol/l). The concentration of sRANKL could be determined only in a fraction of patients; imore often measurable ($p$ = 0.001) and of higher values in IBD children compared to the control group ($p$ < 0.02). Children with more active disease had lower osteoprotegerin concentrations. Nearly 29% of patients had decreased BMD, more often boys than girls ($p$ < 0.036). Children with lo-

## Streszczenie

**Wstęp:** Osteoprotegeryna (OPG) i ligand receptora aktywującego czynnik jądrowy κB (*receptor activator of nuclear factor κB ligand* – RANKL) to cytokiny biorące udział w metabolizmie tkanki kostnej oraz procesach immunologicznych. We wrzodziejącym zapaleniu jelita grubego (ZJG, *ulcerative colitis* – UC) i chorobie Leśniowskiego-Crohna (ChLC), należących do grupy nieswoistych zapaleń jelit (NZJ), toczący się proces chorobowy może dotyczyć, poza układem pokarmowym, także tkanki kostnej.

**Cel:** Ocena stężenia OPG i sRANKL w surowicy dzieci z NZJ oraz stwierdzenie zależności między tymi cytokinami a wybranymi parametrami klinicznymi u chorych na NZJ.

**Materiał i metody:** W badaniu wzięło udział 93 dzieci leczonych w Klinice Pediatrii, Gastroenterologii, Hepatologii i Żywienia Dzieci Gdańskiego Uniwersytetu Medycznego, w tym 18 z ZJG, 24 z ChLC i 51 zdrowych dzieci z grupy kontrolnej. Stężenia OPG i sRANKL oznaczano metodą ELISA. Gęstość mineralną kości (*bone mineral density* – BMD) u dzieci z NZJ mierzono metodą podwójnej absorpcjometrii promieniami X (*dual energy X-ray absorptiometry* – DXA).

**Wyniki:** Stężenie OPG było większe u dzieci chorych (0,20–0,73 pmol/l, SD 1,35, mediana 4,10 pmol/l) niż u dzieci z grupy kontrolnej (0,03–0,2 pmol/l, SD 1,72, mediana 3,61 pmol/l). U części pacjentów stężenia sRANKL były niemierzalne; jednak więcej oznaczeń ($p$ = 0,001) i większe wartości stwierdzono u dzieci chorych ($p$ < 0,02). U blisko 29% dzieci z NZJ odnotowano zmniejszenie wartości BMD, przy czym częściej u chłopców

wer BMD had significantly lower sRANKL concentration ($p < 0.03$).
**Conclusions:** Evaluation of OPG and sRANKL does not seem to be useful in diagnosis of IBD in children with disorders of bone mineralization. The complete meaning of these cytokines remains unclear.

niż dziewcząt ($p < 0,036$). Dzieci ze zmniejszonymi wartościami BMD miały ponadto mniejsze wartości sRANKL ($p < 0,03$).
**Wnioski:** Wydaje się, że oznaczanie stężeń OPG i sRANKL nie jest użyteczne w diagnostyce NZJ u dzieci z zaburzeniami mineralizacji kości. Ustalenie pełnego znaczenia tych cytokin wymaga dalszych badań.

## Introduction

There is strong evidence for the existence of complex relationships between the skeletal and immune systems. Disturbed bone mineralization can be either primary bone pathology or secondary to other diseases. Osteoporosis is the most prevalent metabolic bone disorder in adults; there are not reliable data on osteoporosis in children [1]. Since the bone tissue develops mostly in childhood, the adequate conditions for its formation in this period of life are of great concern.

A milestone in understanding the complicated processes that regulate bone metabolism was achieved in 1997, when osteoprotegerin (OPG) was discovered [2]. Osteoprotegerin belongs to the tumour necrosis factor receptors (TNFR) superfamily and is the only known soluble molecule in this group [2, 3].

Receptor activator of nuclear factor κB ligand (RANKL) is another TNF cytokine, present in three biological isoforms: soluble, cytoplasmic and the most active – primarily membrane-bound [4, 5]. The receptor activator of nuclear factor κB (RANK) is functionally related to RANKL [6]. Expression of these three molecules is ubiquitous and can be modified by many different factors [7].

It has been proven that RANKL-RANK interaction is crucial for initiation and maintenance of osteoclastogenesis and can be completely inhibited by OPG [8]. Moreover, it must be strongly emphasized that anti-RANKL therapy has already been applied in osteoporosis treatment [9]. The majority of information about the OPG-RANKL-RANK triad originates from studies in adults. Considering the paediatric population, there are no reliable data about concentrations of RANKL with only a few reports on OPG [10-17].

The aetiopathogenesis of inflammatory bowel diseases (IBD), especially ulcerative colitis (UC) and Crohn's disease (CD), is complex [18]. These conditions are diagnosed even in young children and infants; the average age of children suffering from UC and CD in Poland is 8.2 and 10 years, respectively [19].

Inflammatory bowel diseases are associated with many complications; lower bone mineral density (BMD) is one of them. Malabsorption, chronic inflammation, reduced physical activity, delayed puberty and iatrogenic factors – especially of glucocorticoids – are all considered in the pathomechanism of low BMD [20].

## Aim

The aim of this study was to evaluate the OPG-RANK-RANKL system and to estimate the relationship between concentrations of OPG and soluble RANKL (sRANKL) and particular clinical and biochemical parameters in children with inflammatory bowel diseases.

## Material and methods

The study included 93 children: 18 patients with UC, 24 with CD and 51 healthy children of comparable sex and age (control group) (Table I).

The patients were hospitalized in the Department of Paediatrics, Paediatric Gastroenterology, Hepatology and Nutrition, Medical University of Gdansk, between 2006 and 2008. The study was approved by the Commission of Bioethics of the Medical University of Gdansk, number 406/2006. Written informed consent was given by patients' parents and children over 16 years.

The exclusion criteria were as follows: positive medical history of any autoimmune disease or chronic bone

**Table I.** Characteristics of the studied population
***Tabela I.*** *Charakterystyka grupy badanej*

| Group | *N* | Girls | Boys | Min. age [years] | Max. age [years] | Mean age [years] | SD | Median [years] |
|---|---|---|---|---|---|---|---|---|
| UC | 18 | 2 | 16 | 4.08 | 18 | 14.8 | 3.87 | 17.0 |
| CD | 24 | 11 | 13 | 5 | 18 | 14.6 | 3.26 | 16.0 |
| Control | 51 | 23 | 28 | 4.4 | 17.75 | 12.8 | 3.84 | 13.7 |
| Total | 93 | 36 | 57 | 4.08 | 18 | 13.7 | 3.78 | 14.7 |

*N – number, SD – standard deviation, UC – ulcerative colitis, CD – Crohn's disease, min. – minimum, max. – maximum*

disease (especially osteoporosis), cigarette smoking, chronic glucocorticoid therapy (including inhalations), immunomodulating or anti-inflammatory medications – drugs other than those required for IBD therapy.

Analysis was based on the medical records, anamnesis and physical examination. Inflammatory bowel disease was recognized on the basis of clinical symptoms, endoscopic and histopathological examination. The disease activity of UC and CD was estimated according to the Truelove-Witts index and Paediatric Crohn's Disease Activity Index (PCDAI), respectively [21, 22]. The nutritional state was evaluated by means of Cole's index (CI) estimated as (BMI/50$^{th}$ centile BMI) × 100%. Laboratory tests included full blood count, erythrocyte sedimentation rate (ESR), faecal occult blood, serum concentration of iron, albumin, C-reactive protein (CRP), calcium, phosphorus, osteoprotegerin and soluble RANK-ligand. Concentrations of OPG and sRANKL were determined with a commercial ELISA kit (Biomedica, BI-20402 and BI-20422H); the other parameters were measured by conventional laboratory methods.

Bone mineral density of lumbar spine was measured in children suffering from IBD by means of dual energy X-ray absorptiometry (DXA) (Lunar). The reference values for BMD were based on the Polish reference dataset for children [23]. Diminished BMD was recognized when the BMD *Z*-score was below –2.

### Statistical analysis

Statistical analyses were carried out using Mann-Whitney *U* test, precise Fisher's test and $\chi^2$ (Pearson chi-square); Statistica software was used.

## Results

The IBD duration ranged from 1 to 109 months (mean 38.9, SD 26.22, median 40 months). Crohn's disease was diagnosed in 85% of girls, which was significant ($p < 0.02$). The UC activity was mild in 66.67% and moderate in 33.3% of patients; CD remission was observed in 50% of children, mild activity in 45.83% and moderate only in 1 child. Malnutrition was observed more often in children with IBD ($p < 0.04$), and in more children with CD than those with UC ($p < 0.01$) (Table II).

**Table II.** State of nutrition in studied population, as Cole's Index
***Tabela II.*** *Stan odżywienia w badanej grupie wyrażony jako współczynnik Cole'a*

| Cole's Index (%) | IBD Group | Control group | Total |
|---|---|---|---|
| Severe undernutrition (below 75) | 3 | 1 | 4 |
| Undernutrition (75-85) | 3 | 3 | 6 |
| Norm (85-115) | 31 | 37 | 68 |
| Overweight (115-120) | 2 | 3 | 5 |
| Obesity (above 120) | 3 | 7 | 10 |
| Total | 42 | 51 | 93 |

*IBD – inflammatory bowel disease*

All the IBD patients were treated with some aminosalicylate medication at the time of investigation. Altogether 40 patients (95%) had glucocorticoid therapy recorded. Seven children were receiving glucocorticoids at the time on analyses. Azathioprine was administered to 26 patients. Biological treatment with infliximab was applied to 8 patients (2 UC and 6 CD); 2 of them have already been given 6 doses. The period between analysis and the last drug infusion ranged from 1 to 297 days.

The serum concentrations of OPG in the studied population are presented in Table III. In the control group higher OPG levels (above 3.61 pmol/l) were more prevalent in boys than in girls ($p = 0.036$); there was no such sex-related difference in the IBD group. There were no other significant differences between the groups.

Concentrations of sRANKL in the serum were very low in all groups, in many cases below the detection limit (0.08 pmol/l) of the ELISA kit we used. We observed that in the IBD group, compared with the control group, sRANKL concentrations were more often detected and were of higher values (levels > 0.01 pmol and above 0.08 pmol/l respectively) ($p < 0.02$). In addition, sex-related differences were observed only within the IBD group; sRANKL was detected more frequently in boys (21%) than in girls (15%) ($p < 0.024$). In the UC group higher sRANKL concentrations (> 0.08 pmol) were more preva-

**Table III.** Serum concentration of osteoprotegerin (pmol/l) in studied population
***Tabela III.*** *Stężenie osteoprotegeryny (pmol/l) w grupie badanej*

| Group | Mean | SD | Min. | Max. | Median | *N* |
|---|---|---|---|---|---|---|
| IBD | 4.09 | 1.35 | 0.20 | 7.3 | 4.10 | 42 |
| Control | 3.91 | 1.72 | 0.03 | 10.2 | 3.61 | 51 |

*SD – standard deviation, min. – minimum, max. – maximum*

lent in children with moderate compared to mild disease activity (33.3% vs. 8.3%; $p < 0.015$) and in children with CD remission (25%) than with active disease (both mild and moderate; 16.7%) ($p < 0.039$). In children suffering from CD mean serum concentration of OPG was significantly lower in those children who had an sRANKL level above 0.08 pmol/l ($p < 0.048$). Higher levels of sRANKL were observed more often in malnourished children compared with well-nourished patients ($p < 0.04$). In the IBD group children who had an elevated fibrinogen level in serum had lower concentrations of sRANKL ($p < 0.04$), whereas children with normal CRP levels had higher sRANKL concentrations in serum ($p < 0.026$). No other laboratory parameters were significantly correlated with sRANKL levels. Lower OPG concentrations in serum were observed in children with increased ESR ($p < 0.053$). There was no correlation between concentration of OPG and any type of therapy recorded. However, the concentration of sRANKL was higher than 0.08 pmol/l in all patients who were biologically treated ($p < 0.026$) and who received azathioprine ($p < 0.039$).

Decreased bone mass (BMD *Z*-score below –2) was diagnosed in 28.5% of children with IBD (5 UC, 7 CD; 11 boys and 1 girl). The majority of children with low sRANKL serum concentration had decreased BMD ($p < 0.032$). There was no correlation between serum concentrations of OPG and BMD.

## Discussion

In recent years scientific studies have revealed new cytokines that play a key role in bone metabolism: osteoprotegerin and receptor activator for nuclear factor κB ligand. Although these cytokines have been under investigation for over a decade, there are only a few studies concerning their role in the paediatric population. Furthermore, the reference values of serum concentrations of these cytokines in children still remain to be determined.

Taking into consideration that the prevalence of IBD in children is increasing and that bone tissue is often affected in these diseases, we aimed to evaluate OPG and sRANKL concentrations and their correlation with some biochemical and clinical parameters in paediatric patients suffering from IBD.

The results that we obtained for OPG in sera of healthy children correspond with previous reports [17]. Contrary to Kudlacek *et al.*, we did not find any correlation between the concentration of OPG and the age of the children [24]. The differences may result from the diversity of the studied populations and different methodologies (ethnicity, assay kits, blood storage conditions, etc.).

We found very low sRANKL concentrations in all children; the majority of the concentrations fell below the detection limit. Similar results have been reported previously [25]. This phenomenon remains to be elucidated. It is known that soluble RANKL is only a small fraction of the total amount of this cytokine. Although there are more sensitive techniques enabling detection of all RANKL forms, they are not commonly available in clinical practice. Furthermore, there is also no consensus about the stability of these cytokines in serum and plasma [26]. It is therefore important to determine reference values for specific populations and laboratories.

Considering the small number of determined values (in 21% of patients), it was impossible to indicate a reference range for sRANKL concentration in serum of healthy children participating in our study. This could explain the lack of statistical significance between estimated sRANKL levels. In such circumstances an analysis of the positive incidence of sRANKL was applied in this study.

Concentrations of OPG and sRANKL in sera of IBD children were higher than in the control group, with no differences between boys and girls. We found that CD children had higher concentrations of OPG than UC children. Sylvester *et al.* provided somewhat corresponding data; higher serum OPG levels were in children with CD than in the control group [16].

Elevated OPG levels were observed previously in various diseases that are not directly related to bone metabolism [14, 16, 20-22]. Therefore the question arises whether OPG is a cytokine exclusively involved in bone metabolism or, rather, it is an epitome of the inflammatory or autoimmune process.

Taking it into consideration, BDM, markers of inflammation, disease duration as well as the medical treatment and their relationship with OPG and sRANKL in children with IBD were investigated. We observed decreased BMD of lumbar spine in 28.5% of children with IBD. This is in agreement with previous studies [20]. Furthermore, we also noted a trend towards lower bone mass in children with CD compared to UC. The differences in bone mass between CD and UC patients have been described previously [27, 28]. Malnutrition was significant in the IBD group, especially in CD patients. We did not find any significant correlation between the nutritional state and BMD, even though in well-nourished children BMD was often normal. It should also be noted that malnourished children had higher sRANKL concentrations. Another puzzling result that we obtained was the increased incidence of higher sRANKL concentrations in boys with lower BMD compared to girls. This observation is consistent with the assumption that sRANKL levels increase during bone destruction. Unlike other authors, we did not find any correlation

between either OPG or sRANKL concentration and glucocorticoid therapy [29]. On the other hand, we found higher sRANKL concentrations in children who underwent infliximab therapy. The biological treatment was required due to more severe disease – such, one could speculate, that sRANKL might, indirectly, reflect inflammatory process intensity.

It appears that in IBD there are at least three possible sources of OPG (osteoblasts, T cells and intestinal cells). It is not unanimous whether elevated levels of this cytokine result from bone loss compensation or rather reflect the immune system response. In the latter case, perhaps, OPG could become another marker of inflammation. Although in our study we found some significant relations between concentrations of OPG and fibrinogen, sRANKL and C-reactive protein and fibrinogen levels, these data are inconclusive.

There are also not enough reliable data about the stability and metabolism of OPG and sRANKL and the diagnostic costs are still very high.

Our investigation has some disadvantages. It must be noted that the number of patients participating in this study was relatively small. In addition, the studied groups were heterogeneous due to the disease duration, activity and different stages of treatment at the time of the evaluation. Therefore, despite the fact that we noted differences in serum levels of OPG and more often measurable concentrations of sRANKL in the children with more active disease, prospective studies with larger groups of patients are necessary.

Nevertheless, these cytokines represent potential and promising opportunities for the development of treatment strategies in the future and therefore certainly warrant further studies.

## Conclusions

Diminished bone mineralization is prevalent in children with IBD. Osteoprotegerin and soluble RANKL may reflect bone mineralization in children with IBD. There was no correlation between serum concentration of osteoprotegerin and the age of children in the studied population, although higher values were observed in girls. Evaluation of serum concentrations of osteoprotegerin and soluble receptor activator of nuclear factor κB ligand in children with inflammatory bowel disease does not seem to directly correspond with the disease activity. However, further investigations are necessary.

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