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Bone turnover markers, osteoprotegerin and RANKL cytokines in children with cystic fibrosis

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ABSTRACT

Purpose: Some scientific studies show decreased bone mineral density and increased fracture frequency in adult patients with cystic fibrosis (CF). The mechanism for early bone loss in CF patients are multifactorial: chronic pulmonary inflammation, malnutrition, reduced physical activity, delayed pubertal maturation.
The aim of this study was to assess bone metabolism markers with special attention paid to osteoprotegerin (OPG) and receptor activator of nuclear factor κB ligand (RANKL) balance in CF children.
Material and Methods: The study included 35 children with diagnosed CF and 35 healthy controls aged 5-9 years (median 7.0 years). Serum levels of fat soluble vitamins were measured by chemiluminescence (vitamin D) and HPLC (vitamins A, E) methods. Concentrations of bone metabolism markers were determined by immunoenzymatic assay.
Results: Mean levels of fat soluble vitamins (A, D, E) were lower in patients with CF compared to controls. In CF children we observed a significant ($p<0.01$) decrease in concentration of bone formation marker (osteocalcin) and similar bone resorption markers (CTX, TRACP5b) in comparison with healthy children. The serum level of OPG was significantly lower ($p<0.05$) and RANKL nearly 2-fold higher in patients with CF than in the healthy ones. The ratio of OPG to RANKL was about 2-fold lower in children with CF compared to healthy peers ($p<0.01$).
Conclusion: In CF children, an imbalance between bone formation and resorption processes occurs. An increase serum RANKL concentration coexisting with lower levels of OPG may be associated with intensification of bone resorption.

Key words: Bone formation and resorption markers, OPG/RANKL ratio, cystic fibrosis

INTRODUCTION

Cystic fibrosis (CF) is the most common lethal autosomal recessive genetic disease that causes a number of long-term health problems, including bone diseases. Some studies documented decreased bone mineral density (BMD) and increased risk of fracture in adult patients with CF [1-3]. In recent report, with the use of dual energy X-ray absorptiometry (DXA), peripheral quantitative computed tomography (pQCT) and quantitative ultrasound (QUS), normal bone mass in the majority of CF adolescents and young adults was found [4]. Data for pediatric age are limited and contradictory. Some authors observed normal bone mass in well-nourished prepubertal children with CF, but others reported reduced BMD in children with CF compared with controls [5-10]. The mechanisms for early bone loss in CF patients are multifactorial and include malnutrition, fat soluble vitamins insufficiency, reduced levels of physical activity, delayed pubertal maturation and the frequent use of glucocorticoid therapy. Additionally, chronic pulmonary inflammation associated with CF leads to increased levels of cytokines, which have stimulatory effects on bone cells

(osteoblast and osteoclast) development and activity. This condition results in uncoupling of balance between bone formation and resorption processes [11-14].

Apart from measuring BMD, biochemical bone turnover markers showing global skeletal activity have lately been developed and validated for the assessment of the dynamics of bone formation and resorption processes. Among them, products of the osteoblast activity - osteocalcin (OC), which is one of bone formation markers, and products of osteoclast activity as markers of bone resorption - type I collagen cross-linked C-telopeptide (CTX) and isoenzyme 5b of tartrate-resistant acid phosphatase (TRACP5b) are considered to be clinically useful [15,16]. Bone remodeling is mainly regulating by the cellular interactions of osteoblasts and osteoclasts. The complex of cytokines RANKL/OPG/RANK, that belongs to the tumor necrosis factor α (TNFα) receptor family, seems to be a central signalling pathway in osteoclastogenesis. Receptor activator of nuclear factor κB ligand (RANKL) is a major regulator of osteoclasts activity, which by interacting with the receptor activator of nuclear factor κB (RANK), stimulates the formation and activation of osteoclasts and resorption process. Osteoprotegerin (OPG), a glycoprotein synthesized by osteoblasts acts as a decoy receptor. OPG by binding to the RANKL, blocks the possibility of its interaction with the receptor RANK and presents anti-resorptive effect. Therefore the OPG/RANKL ratio dictates the rate of bone resorption in several pathological states, especially in inflammatory diseases [17,18].

Data regarding bone metabolism markers in children with CF are limited and obtained results are different [19-23]. To our knowledge, there are no reports describing serum OPG and RANKL values in growing patients with CF. The aim of this study was to assess bone metabolism markers with special attention paid to OPG and RANKL balance in CF children.

MATERIAL AND METHODS

The study group consisted of 35 children aged 5-9 years (median 7.0 years) with confirmed CF, attending the Cystic Fibrosis Clinic at the Institute of Mother and Child (Warsaw, Poland). The diagnosis of CF was based on clinical findings, positive sweat chloride test (>60mEq/L) and gene mutation analysis. The studied patients were in clinically stable condition without recent acute pulmonary infections. No patients in our group had diabetes, only one of them had hepatic insufficiency. None of the studied patients were receiving corticosteroids during the study and at least one month before the beginning of the study. This refers to both systemic and inhalatory steroids. All CF children, except for 2, were pancreatic insufficient and were taking pancreatic enzyme supplements (6000 U lipase/kg/day). They were on unrestricted high caloric diet in accordance with standard recommendations for CF care. Additionally, they were routinely supplemented with vitamin A (2000 IU/day), vitamin E (200 IU/day) and vitamin D_3 (400 IU/day). Calcium intake in our study group was 800 mg/day.

The control group (35 healthy children) was recruited from children (matched for age and gender) attending the outpatient clinic with minor problems other than infections and diseases that might influence bone status.

The children were not taking bone sparing drugs, did not have bone fractures and were on unrestricted diet. Demographic data (sex, age, and genotype) were collected. Pubertal stage was assessed according to Tanner criteria. To exclude the effects of puberty on bone metabolism markers, only prepubertal subjects were included in the study. Anthropometric parameters (weight, height) were performed for all children and body mass index (BMI) was calculated using the formula weight (kg)/height (m^2). The severity of CF disease was evaluated by the Shwachman-Kulczycki (S-K) scoring system. Pulmonary function was assessed by spirometric measurements, which were performed during routine check-up visits at three to six month intervals. All spirometric parameters, including Forced Expiratory Volume in one second (FEV_1), were measured using a MES JAEGER 100 spirometer in accordance with the procedures recommended by the Polish Phthysiopneumological Society. The results were recorded as percentages of the predicted values, standardized for age, height and sex. CF lung disease was assessed as mild, when FEV_1>80%, moderate FEV_1 between 40-80% and severe FEV_1<40%.

Peripheral blood was taken in the morning, at the time of routine sampling for clinical purposes and serum was obtained. Serum samples were separated and preserved at -20°C for the later examination (no longer than 2 months). Concentrations of calcium and phosphate were measured on biochemical analyzer by standard enzymatic methods. The level of 25-hydroxyvitamin D was determined by chemiluminescence immunoassay using kits from DiaSorin (USA). Concentrations of vitamins A (retinol) and E (α-tocoferol) were measured by the high-pressure liquid chromatography method (HPLC) according to Zaman et al. [24]. Serum levels of bone metabolism markers were determined by immunoenzymatic microELISA assay. Concentrations of TRACP5b and OPG were measured using available kits from Quidel (USA), OC (N-Mid Osteocalcin) and CTX (Serum CrossLaps) – kits from IDS (UK) and RANKL (total sRANKL) – kits from ImmunoDiagnostics (Germany). For quantifying total OPG we used a kit recognizing monomeric (molecular weight 60 kDa) and dimeric (molecular weight 120 kDa) forms of OPG. We measured total sRANKL using a kit which detects free sRANKL, as well as, sRANKL complexed with OPG. Intra- and inter-assay coefficients of variation of these methods were lower than 10%.

The study was performed in accordance with the ethical standards laid down in the Declaration of Helsinki and approved by the Institute of Mother and Child Ethics Committee. Written informed consent was obtained from the parents of children.

Statistical analyses were performed using the Statistica software program, version 10.0 PL. Normality of variables was performed by Shapiro-Wilk test. The symmetrically distributed data are expressed as mean ± standard deviation (SD) and the asymmetrically distributed as median with 25th and 75th percentiles. The Student`s *t* test was used to calculate differences between means and the Mann-Whitney test was used to compare asymmetric variables. Pearson`s or Spearman`s rank correlation test was used to evaluate the possible relationships between various parameters. The statistical significance was set at $p<0.05$.

Table 1. **Clinical, anthropometric and genetic characteristics of cystic fibrosis patients and healthy children.**

	Cystic fibrosis group	Control group
n	35	35
Gender (F/M)	18/17	18/17
Age (years)[b]	7.0 (5-9)	7.0 (5-9)
Body weight (kg)[a]	23.1±5.9	24.9±6.1
Body height (cm)[a]	122.2±11.9	125.7±12.4
BMI (kg/m^2)[a]	15.0±1.4	15.8±1.7
CF genotype		
ΔF508 homozygote *n (%)*	21 (60%)	
ΔF508 heterozygote *n (%)*	10 (29%)	
Non ΔF508 mutation *n (%)*	4 (11%)	
Pancreatic insufficiency *n (%)*	33 (94%)	
Shwachman-Kulczycki clinical score[a]	88.4±7.1	
FEV_1 (%)[a]	89.6±12.2	

[a] Data are presented as mean values ± SD;
[b] Data are presented as median values and ranges;
BMI – body mass index;
FEV_1 - forced expiratory volume in 1 s

RESULTS

There were no statistical differences between the CF and control groups regarding age, gender, weight, height and BMI (*Tab. 1*). Mean clinical Shwachman-Kulczycki score was 88 (ranges 70-95) indicating mild/moderate disease severity in the CF children. Pulmonary function tests assessed by FEV_1 was about 90%. CF lung disease was mild in 77% and moderate in 23% of children. The majority of patients had the ΔF508 mutation. Among them 21 (60%) were homozygous and 10 (29%) were heterozygous for the ΔF508 genotype. Four (11%) patients had non ΔF508 mutation.

Biochemical measurements regarding mineral-vitamins status and bone metabolism markers in CF and control children are shown in *Tab. 2*. Mean serum calcium and phosphate levels were within normal range in both studied groups, but the concentration of 25-hydroxyvitamin D was lower in patients with CF compared to control children ($p<0.05$). Plasma A and E vitamin levels were significantly ($p<0.01$ and $p<0.05$, respectively) lower in children with CF than in the healthy ones but stayed within the reference range. CF children, compared to controls, had significantly ($p<0.01$) lower osteocalcin level and similar CTX and TRACP5b concentrations. The serum level of OPG was significantly ($p<0.05$) lower and RANKL nearly 2-fold higher in patients with CF than in the healthy ones. Hence, the ratio of OPG to RANKL was about 2-fold lower in children with CF compared to healthy peers ($p<0.01$). We observed significant negative correlation between serum concentrations of TRACP5b and OPG ($r=-0.3603$, $p<0.05$) in CF group and in healthy children ($r=-0.3866$, $p<0.05$). Additionally, we showed significant

Table 2. **Serum fat soluble vitamins and bone metabolism parameters in CF and healthy children.**

	Children with CF (n=35)	Healthy children (n=35)	P value
Calcium (mmol/l)	2.40±0.14	2.44±0.11	0.1548
Phosphate (mmol/l)	1.53±0.15	1.49±0.17	0.3004
25OH vitamin D (ng/ml)	19.94±7.78	24.85±8.21	0.0124*
Vitamin A (μmol/l)	1.37±0.29	1.65±0.46	0.0036*
Vitamin E (μmol/l)	16.75±3.11	21.08±5.97	0.0160*
OC (ng/ml)[a]	73.53±20.62	90.15±24.16	0.0092*
CTX (ng/ml)[a]	1.754±0.585	1.700±0.480	0.6786
TRACP5b (U/L)[a]	14.02±2.16	14.74±3.78	0.3294
OPG (pmol/l)[a]	4.14±0.87	4.64±0.76	0.0132*
RANKL (nmol/l)[b]	2.477 (1.361-4.564)	1.184 (0.785-2.183)	0.0375*
Ratio OPG/RANKL	1.76±1.41	3.48±2.08	0.0045*

[a] Data are presented as mean values ± SD;
[b] Data are presented as median values and inter-quartile ranges (25-75%);
**Statistically significant ($p<0.05$)*

positive relation between OC and CTX (r=0.4924, p<0.01) in control group.

DISCUSSION

So far, data regarding biochemical bone metabolism markers in pediatric population are limited, sometimes conflicting and until recently not fully explained [19-23]. We found decreased concentration of bone formation marker (osteocalcin) in children with CF and comparable to controls levels of bone resorption markers (CTX and TRACP5b). This condition results in uncoupling of balance between bone formation and resorption processes and are unfavourable for bone development in these patients.

Baroncelli et al. [19] in prepubertal CF children showed similar levels of bone formation and elevated bone resorption markers compared to controls. Other researchers observed similar or reduced values of OC and higher levels of bone alkaline phosphatase in CF patients [8,20,21,25]. Regarding bone resorption markers, similar levels of deoxypyridinoline and hydroxyproline or elevated levels of ICTP (carboxyterminal telopeptide of type I collagen) and NTX (N-terminal cross-linking telopeptide of type I collagen) in CF children compared to the healthy controls have been reported [19,20,22]. The differences in the above results are probably due to the different nutritional status, lung disease severity of studied patients, as well as blood sample collection and problems with interpretation of bone marker results in children.

In our study, only a few significant correlations between bone metabolism markers were detected. We observed weak negative correlation between serum concentrations of TRACP5b and OPG in both groups of children and positive correlation between OC and CTX in controls. Other correlations were not statistically significant, as bone metabolism markers reflect different biological processes during skeletal growth. These markers are released during different stages of the bone formation or resorption processes.

Recently, an association between inflammation and bone disease was recognized. Little is known regarding cytokines of RANK/RANKL/OPG system in CF patients. Le Heron et al. [26] used a model of primary human osteoblast culture and demonstrated that the loss of cystic fibrosis transmembrane conductance regulator chloride channel (CFTR) activity results in decrease of OPG expression in CF patients. Shead et al. [13] showed, for the first time in adult CF patients, lower serum OPG level and similar levels of RANKL as compared to the controls. Additionally, the authors showed increased serum levels of both cytokines during infective exacerbation and their decreased levels after the completion of antibiotic therapy in patients with CF. To our knowledge, changes in serum levels of OPG and RANKL had not been previously reported in children with CF. In our study, the RANKL to OPG ratio was nearly 2-fold higher in CF children than in healthy controls, suggesting the role of RANKL as a major promoter of osteoclastogenesis.

Interpretation of serum levels of OPG and RANKL is very difficult for many reasons. Firstly, it is a methodological issue. Circulating RANKL has several potential origins and exists in different forms. RANKL is produced as a membrane-bound protein on osteoblast cells and cleaved into a soluble form by metalloproteinase. In serum, there are free RANKL and RANKL complexed with OPG, with the bound form vastly predominating. Also OPG may exist in serum in monomeric and dimeric forms. Secondly, these molecules are secreted in addition to osteoblasts by many cell types and it is unclear whether circulating concentrations of OPG and RANKL reflect their activity in the bone microenvironment [27-29].

One of the potential factors which may influence bone metabolism is nutrition, especially fat soluble vitamins' status. It is known that vitamin D plays a key role in regulation of osteoblastic cells at different stages (proliferation, maturation, mineralization) and is associated with the expression of such protein as osteocalcin, OPG and osteonectin. Recent studies suggested, that this vitamin acts also in osteoclastogenesis, but precise mechanism are not yet identified [30]. Published data have documented vitamin D deficiency, which is recognized in CF patients, both, in adulthood and in childhood [8,10,31-33]. However, the results are different and interpretation is still difficult due to a debatable optimal levels of 25-OHD required for healthy bone accretion. The CF unit protocols recommended a minimal serum value of vitamin D between 20-30 ng/ml and optimal above 30 ng/ml [34]. Only 9% of our studied patients had optimal level of this vitamin, 54% had levels between 20-30 ng/ml and 37% had levels below 20 ng/ml. Our results show, that despite supplementation, the majority of patients with CF had decreased levels of serum 25-hydroxyvitamin D. We suggest, that lower concentration of serum 25-hydroxyvitamin D in our studied children with CF may be related to their levels of OC and OPG.

Our patients were routinely supplemented with other fat-soluble vitamins (A and E) and their plasma concentrations were lower than in the healthy controls, but were within the reference range. Several studies have also reported high risk of sub-optimal vitamin K status in CF population as a result of fat malabsorption [10,35]. This vitamin is known as an essential co-factor for the post-translational gamma-carboxylation of osteocalcin, the main non-collagenous protein produced by osteoblasts. Osteocalcin in its active carboxylated form plays a significant role in bone formation, regulation of bone turnover and mineralization [21,25].

The present study has several limitations. First, the number of studied children with CF was relatively small and the results have limited statistical power. However, both studied groups were well-nourished and matched for age and gender. Secondly, we did not measure serum vitamin

K levels and undercarboxylated form of osteocalcin, but we may speculate that the decreased concentrations of total osteocalcin in CF children may be related to lower levels of this vitamin. Finally, we did not perform DXA scan in studied children. According to The European Cystic Fibrosis Bone Mineralization guidelines, the first DXA scan in CF children is recommended to be performed at the age of 8 to 10 years [36]. Our studied children were between 5 and 9 years old, the majority of them did not achieve 8 years. They were well-nourished and clinically stable, so their bone density measurements should be done at a later age.

CONCLUSIONS

In summary, in CF children an imbalance between bone formation and degradation processes can be present early, even in prepubertal period. Particularly, an increase in serum RANKL concentration coexisting with lower level of OPG may lead to the intensification of resorption processes. A careful follow up on bone status, including periodic measurement of bone turnover markers, fat soluble vitamins, as well as anthropometric measurements is required to prevent osteopenia and osteoporosis in patients with CF.

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Conflict of interest
The authors declare that they have no conflict of interest.

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