Serum levels of osteoprotegerin, RANK-L & vitamin D in different stages of osteoarthritis of the knee

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Background & objectives: Osteoarthritis (OA) is the 11th leading cause of disability in the modern world, but till date, there have been no effective markers for monitoring the progression of OA. The three proteins RANK/RANK-Ligand and Osteoprotegerin (OPG) have been found to be the key regulators of bone metabolism. Interaction of RANK-Ligand with its receptor RANK triggers differentiation of osteoclasts leading to bone resorption. OPG on the other hand is protective as it is expressed by osteoblasts and bind RANKL with higher affinity preventing its interaction with RANK. The levels of these serum proteins are regulated by vitamin D and parathyroid hormones. Therefore, the present study, aimed to study the association of serum RANKL, OPG and vitamin D with disease severity in patients with knee OA.

Methods: It was a cross-sectional study where 80 (43 women and 37 men) newly diagnosed subjects with OA knee were recruited. They were classified into four grades based on K-L grading and into two groups as early (grade 1+grade 2) and advanced (grade 3 + grade 4) based on the disease progression.

Results: On comparing the biochemical parameters among the four grades decreasing vitamin D levels were seen with increasing severity of knee OA; an increasing trend of RANKL with increase in the severity of OA was seen; OPG was found to be elevated more in the early stages of OA. We also observed a strong association of RANKL/OPG ratio with disease severity.

Interpretation & conclusions: Overall the results suggest that OPG may be considered as an early marker of the diseases.

Osteoarthritis (OA) of knee is a multifactorial disease of the large joints affecting almost 16 per cent of the global population[1]. Lifetime risk estimates that nearly 50 per cent of population will develop OA by the age of 85 yr[2]. In India, OA is the 2nd most prevalent disease with an estimate of 22 to 39 per cent patients who suffer from symptomatic OA[3]. Joint pain is the most common symptom followed by stiffness, swelling and decreased range of motion. Over time, it can progress to permanent joint changes which lead to difficulty in walking and thus affects the day-to-day activities of the patients. OA can occur at various joints like hand, feet, spine, but most commonly affects the weight-bearing joints like the hip and knee. The

prevalence of knee OA in India is estimated to be 31.1 and 33.1 per cent in the rural and the urban population respectively[3,4].

Pathogenesis of OA is characterized by damage to articular cartilage, hypertrophy of bone margins, and subchondral bone sclerosis and biochemical alterations in the synovial fluid as well as the joint capsule[5-7]. Genetics, age, obesity, previous history of (h/o) any injury like anterior cruciate ligament (ACL) tear or heavy exercise are the most prevalent risk factors for OA knee attenuating the forces caused due to any kind of acute or chronic stresses within the bone[8].

Bone remodelling, a fairly constant process throughout life is a much-regulated process which requires a coordinated action between the bone-forming osteoblasts and bone-resorbing osteoclastic cells[9]. It can however, get perturbed in many pathologic conditions like rheumatoid arthritis (RA), OA and osteoporosis where there can be local or systemic disbalance in the hormone levels, pro-inflammatory cytokines or autoimmune antibodies that are known to trigger or inhibit bone resorption. The molecular mechanisms that controlled the bone homeostasis were poorly understood until the discovery of the serum proteins RANK, receptor activator of nuclear kappa – B ligand (RANKL) and Osteoprotegerin (OPG) in mid to late 1990's[10]. RANK is a type 1 transmembrane glycoprotein and belongs to a tumour necrosis factor receptor family. It is mostly located on chromosome 18q22.1. Generally expressed by the osteoclast precursors and on mature osteoclasts is made up of 616 amino acids[11,12]. It is responsible for the activation of osteoclasts once RANKL binds to its receptor RANK.

RANKL, on the other hand, is a type 2 homo-trimeric transmembrane protein mostly expressed by the osteoblasts and activated T-cells and occurs in two forms; membranous and soluble. Soluble RANKL is formed once the membranous RANKL undergoes proteolytic cleavage by metalloproteinases or by alternative splicing[13].

Osteoprotegerin generally functions as a natural decoy receptor of RANKL and was discovered by Boyle and coworkers[14]. It is known to actively inhibit bone erosion by binding with high affinity to its ligand RANKL, thereby blocking its interaction with RANK. Therefore, it is regarded as protective for the bones.

These serum proteins are further regulated by a number of osteotropic factors like vitamin D, prostaglandin E2 and parathyroid hormones. One of them is vitamin D which is a steroid hormone and a key regulator of bone metabolism which maintains the levels of both calcium and phosphates in the body. Active form of vitamin D enters circulation and interacts with vitamin D receptors, thus activating VDRE's (vitamin D response elements)[15]. These elements play a key role in blocking the transcription of nuclear factors of activated T-cells and the NFk-B pathway, thus decreasing cellular response to inflammatory factors. Therefore, due to the anti-inflammatory properties of vitamin D it is being used in the treatment of many chronic diseases like RA, systemic lupus erythematous and multiple sclerosis[16]. However, the potency of vitamin D in treating OA is quite controversial. Studies have shown conflicting results as to whether low vitamin D concentration is correlated with OA-related pain or disease progression[17]. Till date, there have been no effective markers for monitoring the progression of OA. Developing countries like India have a large burden of arthritis, leading to chronic disability among the population and poor quality of life and must therefore develop strategies for the primary prevention of this disease. Estimation of biochemical parameters can help us understand the basic aspects of pathogenesis and prognostic factors need to be well understood. But most importantly biochemical basis of these diseases must be studied. Therefore, our study, as the first in India undertook to establish an association between serum RANKL and OPG with vitamin D and disease severity in patients with knee OA.

Material & Methods

This cross-sectional study was undertaken by the departments of Biochemistry and Orthopaedics, All India Institute of Medical Sciences, Bhubaneswar, from January 2017 to March 2018 after taking the approval from the Institutional Ethics Committee. Eighty patients diagnosed with OA of the knee based on the clinical signs and symptoms such as pain, swelling, decreased ROM (range of movement) and clinical history were recruited into the study. Men and women over 50 yr of age, with newly diagnosed osteoarthritis and willing to participate in the study were included in the study.

Patients suffering from any kind of infectious disease, autoimmune, acute or chronic inflammatory condition, primary bone malignancy or metastasis were excluded from the study. Patients under treatment with any other drugs or steroids for any other indications or having any history of alcohol intake or smoking were also excluded from the study. After taking written consent and a detailed history of signs and symptoms,

co-morbidities, dietary history and drug intake history were taken and recorded on the data capture formats from each participant. The detailed examination findings including gait, joint swelling, joint effusion, range of motion, deformity and pain (by visual analogue score) were recorded.

All the patients were advised anteroposterior as well as lateral X-ray of the bilateral knee for radiological grading of disease severity, along with bilateral hip X-ray to rule out osteoporosis using Singh's index[18]. These subjects were stratified into four grades based on Kellgren and Lawrence radiological grading system[19]. They were further classified as early (grade 1 + grade 2) and advanced stages (grade 3 + grade 4). Details of the radiological grading were then obtained and documented.

As the above non-parametric observations are not observed in healthy controls, and also since the biochemical markers used in this study are affected by thyroid and bone nutrition status, therefore we did not include a healthy control group was not included in this study. Serum chemistry including renal and liver function tests, serum calcium (total), phosphorus, alkaline phosphatase and C-reactive protein level were measured using AU5800 auto analyzers, (Beckman Coulter, Brea, USA), using commercially available reagents. Routine investigations such as complete blood count and erythrocyte sedimentation rate were also estimated. The other special biochemistry tests were osteoprotegerin, vitamin D and RANKL which were estimated using ELISA.

Statistical analysis: Statistical analysis was performed using SPSS version 23 (IBM, SPSS software, USA). Data were presented as mean ± standard deviation as appropriate. The comparison between the means of the two groups early and advanced was done using unpaired student t test. One-way analysis of variance (ANOVA) was done to compare the levels within the four grades. Correlation between variables and disease severity was calculated using Pearson's correlation and $P<0.05$ was considered to be significant.

Results

Demographic features: Out of 80 patients enrolled in the study grade-wise distribution of patients was as follows: 19 of grade 1, 22 each in grade 2 and 3 respectively and 17 belonged to grade 4.

Table I shows the mean ± SD of different variables including vitamin D, RANKL and OPG in groups 1 and 2. Initially a normality test was done using Shapiro–Wilk test ($P=0.260$) and following conformation, a parametric test was applied. The corresponding P values for comparison of the serum parameters with disease severity among the two groups are listed in Table II. Vitamin D levels were found to be significantly high in group 1, *i.e.* early stage as compared to the advanced stage (group 2) ($r=-0.629$, $P<0.001$). RANKL was found to be significantly higher in the advanced stage as compared to the early stage ($r=0.512$, $P<0.001$). OPG was lower in the advanced

Table I. Biochemical parameters in the two groups		
Parameters	Early stage	Late stage
ESR (mm/h)	20.0±12.4	21.3±14.9
CRP (mg/L)	1.7±3.7	1.9±3.7
Calcium (mg/dl)	9.07±0.63	9.17±0.61
Phosphates (mg/dl)	1.8±3.05	1.1±1.7
ALP (U/L)	115.1±32	129.5±35
Vitamin D (ng/ml)	42.8±21.8	14.6±8.2***
RANKL (ng/ml)	1.08±1	3.5±2.6***
OPG (ng/ml)	750.28±261.8	616.2±299

P***<0.001. ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; ALP, alanine aminotransferase; OPG, osteprotegerin; RANKL, receptor activator of nuclear kappa-B ligand

Table II. Correlation of disease severity with serum parameters	
Serum parameters	r
ESR	0.080
CRP	0.020
Calcium	−0.015
Phosphate	−0.113
ALP	0.273
Vitamin D	−0.708***
RANKL	0.575***
OPG	−0.206
RANKL/OPG ratio	0.272*
VAS	0.512***

P *< 0.05, ***<0.001. ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; ALP, alanine aminotransferase; OPG, osteprotegerin; RANKL, receptor activator of nuclear kappa-B ligand; VAS, visual analogue score

stage (group 2) as compared to the early stage (group 1) but was not statistically significant ($r=-0.037$, $P=0.742$). RANKL/OPG ratio was also significantly higher in the advanced stage as compared to early stage ($r=0.227$, $P=0.043$).

On comparing the biomarkers among the four grades of knee OA (Table III), there was a significant difference in the vitamin D levels across the grades of OA ($P=0.004$ for α). The levels were lowest with grade 4 as compared with other groups. Although decreasing levels of vitamin D were observed across the groups, here was no significant difference between the groups. There was a significant increase in the levels of RANKL ($P<0.001$ for α) across the grades of knee OA. The RANKL/OPG ratio were significantly different in all the 4 grades. The levels were highest with grade 4 as compared with other groups. The RANKL/OPG ratio were statistically different both between and within the groups ($P=0.047$). Post hoc analysis with Bonferroni correction (Table IV) showed significant difference in vitamin D levels but not with RANKL between the four groups.

Discussion

Knee being one of the largest synovial joints in the human body is the most prone to degeneration and erosion due to its frequent use and stress leading to painful conditions like osteoarthritis[20]. Equilibrium between RANKL and OPG levels is critical for the pathophysiology of the bone. Any imbalance in their levels can cause destructive effects. Recent advances have shown that cartilage degradation can be related to subchondral bone alterations in OA[20]. Therefore, OPG and RANKL may be implicated in the pathogenesis of the disease.

In our study, vitamin D levels were found to be significantly higher in group 1 as compared to group 2. Therefore, our findings inferred that with advancing stage the patient were deficient in vitamin D levels

or decreased vitamin D levels accelerated the OA to advance stage. Jansen and Haddad[21] in 2013 also demonstrated that out of 139 patients with advanced OA awaiting total knee replacement, 24 per cent of the patients were found to be deficient in vitamin D levels. Zhang et al[22] in 2014 further showed that patients with vitamin D deficiency had an increased risk of knee OA. Similarly, Bassiouni et al[23] demonstrated that vitamin D levels were significantly decreased in patients with knee osteoarthritis.

In our study, we found the serum levels of RANKL in the early stage were significantly lower as compared to that in the advanced stage. Higher RANKL in the latter indicated increased osteoclastogenic activity. This was in accordance with Pilichou et al[24] who showed increase in serum RANKL levels in knee OA which correlated with disease severity Pantsulaia et al[25] had demonstrated higher OPG levels in patients of hand[24]. Since WNT/ beta-catenin pathway is the major signaling pathway which increases the expression of both OPG and RANKL by osteoblasts[26], increased RANKL levels may indicate increased osteoclastic activity leading to subchondral bone erosion in OA. In our study, OPG levels were found to be lower in the advanced as compared to the early stage, though not significant. From different literature searches, we could find normal levels of OPG in healthy controls to be around 0.54 ± 0.2 ng/ml. In comparison, a high concentration of OPG was found in patients with OA knee which may reflect a compensatory reaction by chondrocytes, macrophages or osteoblasts consequence of sRANKL elevation and protecting the joint[24]. Since OPG was elevated more in the early stages, this may be regarded as an early marker for OA.

In our study, the RANKL/OPG ratio was found to be significantly associated with disease severity ($r=0.272$, $P=0.015$, $n=80$), more so in the female population as compared to males. ($r=0.368$, $n=43$, $P=0.015$). These findings were in accordance with

Table III. Comparison of vitamin D, receptor activator of nuclear kappa-B ligand and receptor activator of nuclear kappa-B ligand/ osteprotegerin according to severity of osteoarthritis

Parameters	Mean±SD			
	Grade 1 (n=19)	Grade 2 (n=22)	Grade 3 (n=22)	Grade 4 (n=17)
Vitamin D**	51.48±25.5	35.3±14.3	18.7±7.59	10.21±7.11
RANKL***	0.76±0.81	1.36±1.31	2.68±2.2	4.52±2.89
RANKL/OPG*	0.01±0.038	0.002±0.005	0.14±0.6	0.61±1.43

One-way ANOVA $P^*< 0.05$, $^{**}<0.01$, $^{***}<0.001$. RANKL, receptor activator of nuclear kappa-B ligand; SD, standard deviation; OPG, osteprotegerin

Table IV. Post hoc analysis of the ANOVA

Post hoc Analysis	Grade 1			Grade 2		Grade 3
	Grade 2	Grade 3	Grade 4	Grade 3	Grade 4	Grade 4
Vitamin D	16.18 (3.1-29.2)**	32.78 (19.7-45.8)***	41.27 (27.3-55.2)***	16.60 (4.0-29.1)**	25.09 (11.6-38.5)***	8.49 (-4.9-21.9)
RANKL	-0.60 (-2.2-1.0)	-1.92 (-3.5--0.28)*	-3.76 (-5.5--2.0)***	-1.31 (-2.8-0.26)	-3.15 (-4.8--1.46)***	-1.83 (-3.5--0.14)*

Values mentioned are mean difference (95% CI). P* < 0.05, **<0.01, ***<0.001. RANKL, receptor activator of nuclear kappa-B ligand; CI, confidence interval

other studies which showed a significant association between sRANKL/OPG ratio and disease severity[24,27]. All these findings indicate that RANKL/OPG ratio may act as an accurate early indicator for increasing severity of OA in patients.

In our study, non-parametric variables such as gait, tenderness, effusion, deformity, osteophytes and range of motion were also correlated with three serum parameters,vitamin D, RANKL and OPG. Interestingly,vitamin D was found to be significantly associated with most of the variables including gait, effusion, deformity, osteophytes and ROM[28]. On the other hand, RANKL was positively correlated with deformity, osteophytes and ROM. OPG was only found to be negatively correlated with effusion. Studies like Malas *et al*[29] and Chaganti *et al*[30] in who emphasized in their study that the severe deficient group with vitamin D had thinner femoral cartilage thickness. All these findings suggest that vitamin D, RANKL and OPG have a remarkable role in the pathogenesis of OA and any derangement in levels of these serum proteins affects the severity of the disease.

These findings suggest that the molecular triad RANK/RANKL and OPG might have significant implications in the pathogenesis of OA. Furthermore, vitamin D deficiency might play a critical role in the progression and severity of the disease. Higher RANKL levels in the advanced stage indicate towards increased osteoclastogenic activity leading to subchondral bone resorption. Further higher OPG levels in early stages might indicate an increased osteoblastic activity leading to more cartilage destruction, thus imply its role in the progression of OA. Overall, it isconcluded that these serum proteins especially the RANKL/OPG ratio can be targeted as an early marker for monitoring the progression and disease severity of OA knee and can also be implicated as an important therapeutic target for the treatment of OA in the future.

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