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Determination of NF-κB and RANKL levels in peripheral blood osteoclast precursor cells in chronic kidney disease patients

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Abstract

Purpose Chronic kidney disease (CKD) is a progressive condition characterized by irreversible loss of functional nephron mass due to variety of causes; an inevitable complication of CKD is metabolic bone disease, and this pathology is called as renal osteodystrophy (ROD). In this study, we aimed to determine the levels of serum sRANKL and intracellular NF-κB levels in peripheral blood osteoclast precursor cells in patients with stage 3 CKD.

Materials and methods Forty-one male patients aged 35–60 with CKD identified as stage 3 according to GFR calculated on the basis of creatinine values and 27 healthy male subjects with age ranging from 40 to 60 as control group were included in this study. Levels of biochemical parameters, vitamin D3, parathyroid hormone, bone mineral density, sRANKL and NF-κB were determined by using photometric, electrochemiluminescence, HPLC, ELISA and flow cytometric methods in control and patient groups, respectively.

Results When stage 3 CKD patients were compared with controls, patients with stage 3 CKD had statistically significantly higher iPTH levels, but they had statistically significantly lower vitamin D3 levels. However, the other biochemical parameters, bone mineral density, sRANKL and NF-κB levels did not reveal any significance.

Conclusion In conclusion, vitamin D3 and iPTH levels seem to be important parameters for evaluating the early stages of ROD. The lack of statistically significant differences in the levels of sRANKL and NF-κB suggests that these parameters are not sufficient in the evaluation of bone metabolism in the early stages of renal failure.

Keywords Chronic kidney disease · NF-κB · Renal osteodystrophy · sRANKL

Introduction

Chronic kidney disease (CKD) is one of the major public health problems all over the world not only for the patients but also for the physicians. It has high costs of treatment and monitoring; furthermore, follow-up and the treatment of the cases are very complex [1, 2].

CKD is a pathophysiological process caused by many different etiologies which leads to progressive and irreversible decline both in nephron numbers and functions which often results in end-stage renal disease (ESRD). As CKD progresses, undesirable results not only occur in the kidney, but kidney-specific risk factors may also lead to cardiovascular events and diseases. Impaired kidney function and raised concentrations of albumin in urine, seen in CKD patients, increases the risk of cardiovascular disease two to four times [3, 4].

CKD-mineral bone disorder and/or traditional osteoporosis causes bone histological abnormalities called renal osteodystrophy (ROD). ROD is defined as the complex bone lesions formed in advanced stage CKD patients and in the majority of patients with ESRD. The pathological changes in the bone are associated with the alterations in

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levels of calcium (Ca^{+2}), phosphorus (P), vitamin D (Vit D_3) and parathyroid hormone (PTH). The hypocalcemia, hyperphosphatemia and the elevated PTH levels in CKD patients lead to secondary hyperparathyroidism (sHPT). The high turnover of the bone lesions begins to form due to the evolving sHPT, characterized by an increased number and activity of osteoclasts as well as the increased osteoblastic activity [5, 6].

Osteoclasts are multinucleated cells involved in bone resorption, originating from hematopoietic precursor cells in monocytes/macrophages series. Although majorly located in bone marrow and spleen, it has been shown with studies that osteoclast precursor cells are also present in the peripheral circulation. Furthermore, advanced studies on evaluating osteoclastogenesis report that these cells in the peripheral circulation are developed from monocytes-bearing $CD14^{+}$/$CD16^{-}$ surface markers [7–9].

The molecular mechanism of osteoclastogenesis has become more clear with the discovery of the signal system of nuclear factor kappa B receptor activator (RANK)/nuclear factor kappa B receptor activator ligand (RANKL)/osteoprotegerin (OPG). RANKL binds to its receptor, RANK, on the osteoclast precursor cells to stimulate them to differentiate into mature osteoclasts [6, 9]. OPG inhibits bone resorption by acting as a decoy receptor for RANKL. OPG treats as an effective inhibitor of osteoclast differentiation, activation and survival by binding to RANKL with high affinity to prevent the interaction with RANK. Many intracellular signaling pathways are activated when RANKL binds to RANK. One of the most important pathways involved in the osteoblast differentiation is nuclear factor kappa B (NF-kB)-mediated signaling pathway. It has been demonstrated that, RANKL, which is a regulatory factor in bone metabolism, plays an important role in the pathogenesis of many diseases resulting in bone resorption [6, 10, 11].

It is known that in patients with CKD, secondary hyperparathyroidism and consequent bone resorption becomes evident at values below the glomerular filtration rate (GFR) of 30 ml/min [3]. For this reason, studies on the evaluation of ROD have generally been performed in ESRF patients. It has been shown that histopathologic changes in the bone begin to develop in early stages. Although the gold standard method for the diagnosis of pathological changes of the ROD in the bone is, bone biopsy clinicians tend to avoid using this procedure since it is invasive and painful for patients. For this reason, researchers are trying to determine new biochemical parameters and radiological methods that may have high diagnostic value for the evaluation of ROD in early stages [12–14].

RANKL has an important role in pathological bone destruction and is also thought to be effective in the pathogenesis of ROD. There are limited numbers of studies in literature evaluating the role of RANKL in bone pathologic changes of CKD patients; furthermore, the results obtained from these studies are contradictory [15–18]. In addition, there is a lack of study evaluating the NF-κB signaling pathway which is one of the important metabolic pathways involved in osteoclastogenesis in these patients. Taking all these into account, co-evaluation of intracellular NF-κB levels in osteoclast precursor cells and serum RANKL levels may be important in determining the efficacy of signaling pathway in the pathogenesis of ROD. Flow cytometric analysis of peripheral blood may be convenient in clinical use since it is a noninvasive method for measuring NF-κB levels.

In this study, we aimed to detect the intracellular NF-κB in peripheral blood osteoclast precursor cells and serum sRANKL levels in clinically intermediate-grade (stage 3) CKD patients, to determine the relationship between accurate clinical parameters of ROD (Ca^{+2}, P, PTH, Vit D_3 levels) which have not previously been used for this purpose.

Materials and methods

Forty-one male patients who applied to the Mersin University Medical Faculty Hospital (MEUMFH), Department of Nephrology, aged between 35 and 60, assessed as grade 3 CKD according to the GFR values calculated via creatinine levels and 27 healthy male subjects who applied to the MEUMFH, Department of Physical Therapy and Rehabilitation, aged between 40 and 60 were included in this study. The study was performed only in males to avoid the possible effects of post-menopausal osteoporotic changes in women.

Patients using Vit D, TNFR antagonists, systemic steroids and phosphorus-binding drugs before and during the study, individuals with autoimmune disease, acute infections and malignancy were excluded from the study.

Sample collection

Peripheric venous blood of the patients and controls were collected both into ethylendiaminetetraacetic acid (EDTA) containing tubes and into gelled tubes in the morning, after 12 h fasting. Following the centrifugation of the gelled tube samples, creatinine, Ca^{+2}, P (Cobas c501, Roche Diagnostics Mannheim, GmbH, Germany) and PTH levels (Moduler E170, Roche Diagnostics Mannheim, GmbH, Germany) were measured by the autoanalyzer immediately, in serum. The GFR values of the patient and control groups were calculated with a package program, MDRD calculator, by entering the serum creatinine values, age, sex and ethnicity information [19]. 1 ml of the separated serum was placed into capped Ependorf tubes, and stored at − 20 °C, until the serum sRANKL (Cat. No. RD193004200R, Biovendor Research and Diagnostics, Czech Republic) levels were quantitatively assayed by ELISA method. After

centrifugation of the EDTA-containing blood samples, plasma was protected from the light and stored at − 20 °C until Vit D_3 levels were analyzed on a HPLC system (Cat. No: 38038, Chromosystems Diagnostics, GmbH, Germany). NF-κB levels were measured from the blood samples in EDTA-containing tubes by the BD FACSCalibur flow cytometry device on the same day.

Determination of NF-κB levels

Following assessment of NF-κB levels in the FSC/SSC histogram according to the monitored size and granule content of the cells, monocytes were separated from other peripheral blood cells based on their characteristic appearance and location, and monocyte gating was determined. γ_1/γ_{2a} (FITC/PE) surface staining was used as a negative control. 10,000 cells were counted in each gate. Studies were performed with the gated monocyte population on the FSC/SSC histogram (Fig. 1). Rate of the CD14 (BD Pharmingen™ CD14 Per-CP-Cy5.5 I (Cat. No: 550787), BD Biosciences, Belgium) and CD16 (BD Pharmingen™ CD16 FITC (Cat. No. 555406), BD Biosciences Belgium) fractions were evaluated at the monocyte gate. NF-κB (BD Pharmingen™ NF-KPA-B (PS529) PE I (Cat. No: 558423, BD Biosciences, Belgium) levels were also determined in osteoclast precursor cells by assessing the $CD14^+CD16^-NF\text{-}\kappa B^+$ ratio by taking the CD14 gate (Fig. 2). Following the evaluations, the calculation of the results in absolute numbers/μL by applying the blood count results for each parameter was performed with the formula given below.

$$\text{Absolute number}/\mu\text{L} = \frac{\text{Number of leukocytes} \times \%\text{monocytes} \times \%\text{antibody positivity}}{10.000}.$$

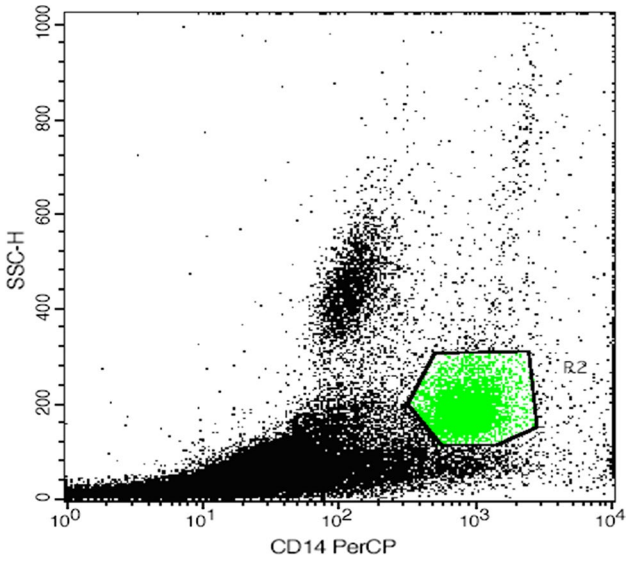

Fig. 2 Marked area indicates $CD14^+$ monocytes in total PBMC

Statistical analysis

Normal distribution of biochemical parameters in patients and control groups was evaluated by Shapiro–Wilk test. Mean and standard deviation values were given as descriptive statistics. Numerical values and percentages have been calculated as descriptive statistics for categorical data. Correlation coefficient was calculated for the test of binary linear relationships between parameters. Student *t* test was used to determine the differences of the parameters mean values between the patient and control groups. Statistical significance was $p < 0.05$. SPSS 11.5 (Statistical Package for Social Sciences version) package program was used to evaluate the data.

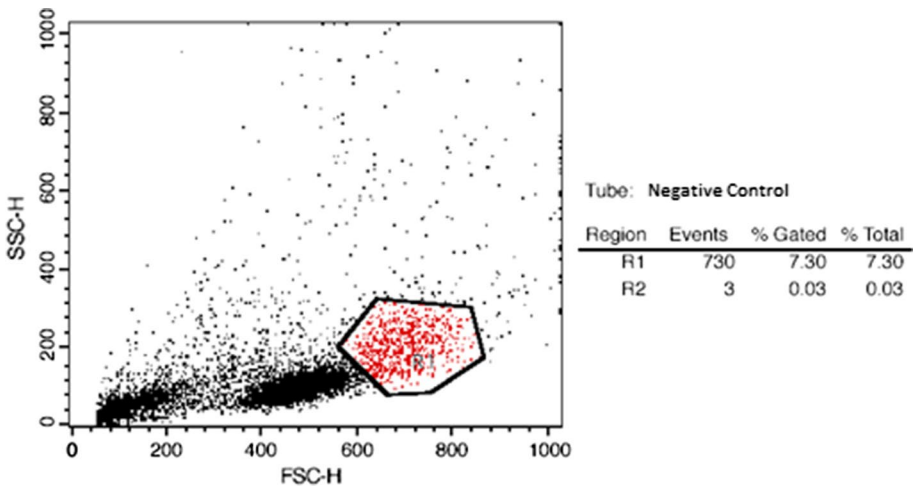

Fig. 1 Marked area indicates the percentage of monocytes in total peripheral blood mononuclear cell (PBMC)

Table 1 Mean values of biochemical parameters, GFR and sRANKL between patients and control groups

Parameters	Patient ($n=41$)	Control ($n=27$)	p
iPTH (pg/mL)	92.98 ± 57.14	33.18 ± 9.95	**<0.001**
Ca (mg/dL)	9.43 ± 0.41	9.60 ± 0.33	0.076
P (mg/dL)	3.32 ± 0.42	3.42 ± 0.49	0.396
Creatinine (mg/dL)	1.77 ± 0.35	0.86 ± 0.15	**<0.001**
GFR (ml/min/1.73 m^2)	44.58 ± 9.00	110.55 ± 22.43	**<0.001**
Vit D_3 (µg/L)	17.41 ± 7.48	34.22 ± 9.446	**<0.001**
sRANKL (pmol/L)	2.66 ± 2.72	2.45 ± 1.83	0.723

p Significance between groups

$p < 0.05$ indicates statistical significance for bold

Results

The study was conducted with 41 male patients with moderate renal insufficiency and 27 healthy male subjects. Mean ages of patients and healthy controls were (54.43 ± 7.89) and (50.40 ± 5.56), respectively. Mean values of biochemical parameters GFR and sRANKL between patients and control groups are given in Table 1.

PTH and Vit D_3 levels in CKD patients were found to be statistically lower than control group, but no statistically significant difference was found between Ca^{+2}, P, sRANKL and *T* score parameters. The comparison of flow cytometric results of patient and control groups is shown in Table 2.

When percent values and absolute number/µL levels were compared, there was no statistically significant difference between the flow cytometric analysis results of the patient and control groups. The percentage of monocytes in total peripheral blood mononuclear cell (PBMC) is given in Fig. 1.

Correlation between parameters in patient group is given in Table 3. There were statistically significant negative correlation between iPTH-GFR, and Vit D_3-Ca^{+2} values and a weak positive correlation between sRANKL and $CD14^+CD16^-NF\text{-}kB^+$ percentage and absolute number/µL in the patient group.

Discussion

Early diagnosis and prevention of ROD is extremely important in terms of reducing health care costs and decreasing mortality, especially since ROD is an inevitable complication in CKD patients [20].

Biochemical parameters and radiological methods used in ROD diagnosis are not sufficient enough to reveal the existing bone disease [21]. Therefore, a more healthy interpretation of bone destruction would be possible through new parameters. For this purpose, this study uses clinically proven ROD parameters and BMD values for early-stage CKD patients which were evaluated together with sRANKL

Table 2 Comparison of flow cytometric results of patient and control groups

Parameters	Patient ($n=41$)	Control ($n=27$)	p
$CD14^+$ %	74.59 ± 8.66	72.53 ± 10.66	0.383
$CD14^+$ (absolute number/µL)	462.59 ± 136.69	421.10 ± 160.60	0.258
$CD14^+CD16^-$ %	65.82 ± 10.28	67.41 ± 10.51	0.538
$CD14^+CD16^-$ (absolute number/µL)	407.09 ± 123.44	390.90 ± 149.11	0.628
$CD14^+CD16^-NF\text{-}kB^+$ %	3.21 ± 1.22	3.12 ± 1.06	0.735
$CD14^+CD16^-NF\text{-}kB^+$ (absolute number/µL)	19.78 ± 8.91	17.83 ± 7.71	0.354

p Significance between groups

Table 3 Correlation between parameters in patient group

Parameters	GFR		iPTH (pg/mL)		Vit D_3 (µg/L)		sRANKL (pmol/L)	
	r	p	r	p	r	p	r	p
Ca^{+2} (mg/dL)	0.286	0.069	− 0.428	0.006	0.169	0.290	0.015	0.927
P (mg/dL)	− 0.259	0.103	0.213	0.187	0.179	0.262	− 0.271	0.087
iPTH	− 0.574	0.000	1		− 0.398	0.011	− 0.082	0.616
Vit D_3	− 0.002	0.991	− 0.398	0.011	1		− 0.086	0.591
sRANKL	0.108	0.501	− 0.082	0.616	− 0.086	0.591	1	
$CD14^+CD16^-NF\text{-}kB^+$ %	− 0.071	0.661	0.075	0.644	0.032	0.842	0.324	0.039
$CD14^+CD16^-NF\text{-}kB^+$ (absolute number/µL)	− 0.053	0.743	0.228	0.157	− 0.170	0.288	0.388	0.012

r correlation coefficient

and NF-κB levels in peripheral blood osteoclast precursor cells, and all the results were compared to healthy controls.

It is considered that sHPT becomes effective from the early stages of renal failure and continues to increase in a directly proportional manner with severity of failure [2]. Additionally, it is justified that measurement of serum PTH levels should participate in routine screening for early-stage renal disease [22].

In a study performed with bone biopsy to evaluate ROD types in predialysis patients, histomorphometric experiments were conducted and it was revealed that changes mainly due to sHPT. Also the correlation between PTH elevation and pathological changes in bone was greater than those of other biochemical parameters in these patients [23].

The study by Pitt et al. showed a twofold increase in iPTH values with GFR > 40, a fourfold increase in GFR 20–40 patients, and an eightfold increase in GFR < 20 in CKD patients [24]. Furthermore, another study being conducted under higher amounts of CKD patients showed that while stage 3 CKD patients had threefold increase in iPTH levels, the increase in stage 4 CKD patients was fourfold [25].

In our study, we observed a significant increase in the mean iPTH values of patients compared to healthy controls which were consistent with the results obtained from the literature. In this sense, our results also support that PTH elevation is effective on the pathogenesis of early-stage ROD.

It is known that changes in mineral metabolism leads to the elevation of serum PTH levels, proportional to the grade of renal failure in early stages. Hyperphosphatemia is responsible for the development of sHPT and is caused by impaired phosphate excretion along with kidney damage. Even though PTH levels are elevated, normal limits of serum Ca^{+2} and P are maintained by renal compensatory effects until the last stages of renal failure [2].

In a study conducted by Jiang et al. with 60 CKD patients, there was no statistically significant difference in Ca^{+2} levels in stage 1–3 patients, while stage 4–5 patients had significantly lower Ca^{+2} levels than stage 1. In the same study, it was shown that P levels of stage 1–4 patients did not differ significantly whereas stage 5 patients had significantly higher P levels than to stage 1 [26].

Ramos and colleagues reported a significant decrease in Ca^{+2} levels, a significant increase in P levels and a positive correlation between iPTH and *p* values in predialysis patients with GFR < 60 mL/min who were followed for 1 year [27].

In our study, there was no statistically significant difference in Ca^{+2} and P values between patient and control groups, and we observed a statistically significant negative correlation between iPTH and Ca^{+2} values in the patient group, similar to the literature. In this sense, our results also support that the PTH elevation in the early stages plays an important role in early compensation in mineral metabolism. Furthermore, the negative correlation between iPTH and Ca^{+2} values indicates that the increase in PTH secretion is associated with a decrease in Ca^{+2} levels.

In a study performed in the predialysis CKD patients with serum creatinine values > 1.5 mg/dl, it was shown that there was a positive correlation between the decrease in GFR and the levels of Vit D_3, suggesting that the reduction in Vit D_3 levels in CKD patients may be effective in the development of secondary hyperparathyroidism [28]. In another study performed by Ramos et al. [27] in stage 3–5 CKD patients, it was shown that there was a negative correlation between the levels of Vit D_3 and the levels of P and iPTH, similar with the study as already mentioned above.

In our study, we found that Vit D_3 levels were significantly lower in the patient group than of the control group, consistent with the literature. We also observed a significant negative correlation between Vit D_3 and PTH levels. It is thought that early increase in PTH levels can be regarded as a consequence for deficiency in Vit D_3, and the decrease in Vit D_3 levels are effective from early stages [2]. So it is visible that the results we obtained in our study are in accordance with this hypothesis.

Following the identification of RANK/RANKL/OPG signaling system in osteoclastogenesis and its importance, serum sRANKL levels have been shown to be associated with diseases characterized by pathological bone destruction such as RA and osteoporosis. The demonstration of RANKL's role in pathological changes of bone has led to being a new therapeutic target. It is stated that RANKL inhibition can be used as a new treatment option in diseases characterized by bone destruction [29].

RANKL activity and its effects in ROD development are not fully understood. There are limited studies on RANKL levels in CKD patients, and the results obtained from these studies are contradictory. It has been reported that sRANKL concentrations can be higher, lower or within normal range in CKD patients when compared to healthy individuals [30]. It is also stated that serum levels can be measured at high levels due to the decrease in sRANKL excretion in renal failure. Therefore, it is thought that serum sRANKL values may not accurately reflect bone pathologic changes in CKD [16].

Avbersek-Luznik et al. have shown increased levels of sRANKL in HD patients compared to healthy controls. They found that patients with high PTH levels had higher levels of sRANKL than those with low PTH levels in patients grouped by PTH levels. They indicated that the results they obtained from their study support the stimulating effect of PTH in the synthesis of sRANKL, as demonstrated by in vitro studies. According to the PTH levels in patients with the same diuresis, the impairment of excretion in the molecule cannot be involved in the elevation of serum sRANKL levels due to the different sRANKL values they obtained [15].

Unlike this study, Doumouchtsis et al. showed a positive correlation between low iPTH and sRANKL levels and a negative correlation between low iPTH and OPG levels while there was a positive correlation between high iPTH and OPG levels, and a negative correlation between high iPTH and sRANKL levels, in their study which consists 104 HD patients separated into two groups according to iPTH levels. They pointed out that the positive correlation between iPTH and OPG in the group with high iPTH levels might have been under the influence of compensatory mechanisms [31].

Albalate et al. [18] did not note any difference in serum sRANKL levels between the HD patients and healthy controls, but a significant increase in OPG levels in the HD patients group. In another study performed with HD patients, it was shown that serum sRANKL levels were slightly lower than of the healthy control group, while OPG levels significantly increased [32].

Compensation mechanisms in the bone resorption process were thought to be effective in altered serum sRANKL levels in these patients, in relation to the marked increase in OPG levels, in both studies.

Our study differs from other studies performed with HD patients since ours include patients in early stages. The other difference is the inclusion of predialysis patients, with the comparison of results not only done in HD patients but also in healthy controls. In this sense, this will be the first study which compares the sRANKL levels of the patients in early stages of renal failure and healthy controls in English literature. According to the data in the literature, the relationship between sRANKL levels and other parameters involved in bone pathologic changes suggests that this molecule may play a role in the development of ROD in CKD patients. Conflicting results in ESRD patients show that sRANKL excretion disorders or compensatory mechanisms are effective in advanced renal failure, as noted in the literature. Although the mechanism and the role sRANKL in CRF remain unclear, they might partly represent a compensatory mechanism in bone remodeling in ROD.

Therefore, we think that it is more meaningful to evaluate sRANKL levels in early-staged patients in which renal function is not completely impaired to clarify its roles in ROD development and its availability for early diagnosis.

In our study, there was no significant difference in sRANKL levels between patient and control groups. Our results suggest that serum sRANKL levels do not play an important role in the pathogenesis of ROD in early stage and cannot be used as a new parameter in early diagnosis. However, the small number of patients in our study is insufficient for evaluating the results. Further studies including larger patient groups are needed to obtain more accurate results.

It is known that many RANKL/RANK interactions activate intracellular signaling pathways in the osteoclast precursor cells. Among them, NF-κB-mediated signaling pathway is thought to be extremely important for osteoclastogenesis. Regarding over-production and activation of osteoclasts, it is believed that NF-κB is associated with bone diseases and may become an impressive therapeutic target in the treatment of osteolytic diseases regulated by signaling pathways [33].

More detailed understanding of the cellular events involved in changes of bone turnover due to renal insufficiency will be possible by highlighting a number of biochemical markers used in bone formation and degradation. In this respect, we think that it may be important to evaluate the levels of NF-κB protein, which is a cellular molecule, also with the sRANKL levels, in pathological bone changes in CKD patients.

It is thought that the changes in NF-κB mRNA levels do not completely reflect the changes in protein levels due to the fact that NF-κB dimers formed by cytokine-mediated signaling are transported back to the cytoplasm from the nucleus and involves in signal transduction. It is stated that detailed studies are needed on the levels of these proteins both in the nucleus and cytoplasmic extracts, for more accurate identification of the regulation of osteoclast formation and activity by transcription factors [34]. We aimed to determine intracellular NF-κB protein levels by flow cytometric method in order to evaluate the pathway of NF-κB signaling in the pathogenesis of ROD.

Significant progress in immunophenotyping has made it easier to identify circulating leukocyte subgroups and disease pathology with CD cellular markers [35]. The study performed by Komano et al. [7] which demonstrates the development of the peripheral blood osteoclast precursor cells from $CD14^{+}CD16^{-}$ monocytes leads us to determine NF-κB protein levels by flow cytometric method.

There is no study concerning NF-κB protein levels in peripheral blood osteoclast precursor cells in English literature. In addition, there is also no study which evaluates the NF-κB activation in bone metabolic changes in CKD patients.

In our study, we determined NF-κB protein levels by examining the percentages and absolute numbers/μL of $CD14^{+}CD16^{-}NF\text{-}\kappa B^{+}$ cells. We observed that there was no statistically significant difference between NF-κB protein levels in patient and control groups, and defined a positive correlation between sRANKL and NF-κB levels in the patient group. These results suggest that the determination of NF-κB signaling pathway activation in early-stage ROD development is not an effective parameter for the assessment of pathophysiology.

It can be concluded that intracellular NF-κB levels and serum sRANKL levels are related in osteoclast precursor cells according to the positive correlation between sRANKL and NF-κB levels in the patient group. Further studies

involving more patients in various stages of the disease may provide benefits in RANKL and NF-κB pathway evaluation.

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Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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