BRITISH JOURNAL OF BIOMEDICAL SCIENCE, 2016
VOL. 73, NO. 3, 121–128
http://dx.doi.org/10.1080/09674845.2016.1209897

Serum CX3CL1/fractalkine concentrations are positively associated with disease severity in postmenopausal osteoporotic patients

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ABSTRACT

Background: The chemokine (C-X3-C motif) ligand 1 (CX3CL1), also called fractalkine (FKN), has recently been reported to be involved in osteoclastogenic process and pathological bone destruction.

Objective: This study aimed to investigate the link between serum CX3CL1/FKN levels with disease progression of postmenopausal osteoporotic patients.

Methods: A total of 53 women with postmenopausal osteoporosis (PMOP group), 51 postmenopausal non-osteoporotic female patients (PMNOP group) and 50 premenopausal non-osteoporotic healthy women of childbearing age (control group) were enrolled in the study. The bone mineral density (BMD) for all subjects was determined via dual-energy X-ray absorptiometry of the lumbar spine, femoral neck, internal trochanter, total hip, greater trochanter and Ward's triangle. The levels of FKN in the serum were examined using the enzyme-linked immunosorbent assay method. The serum bone resorption markers TRACP-5b, NTX levels, inflammation markers IL-1β and IL-6 as well as oestrogen-2(E2) were also detected in all participants. The visual analogue scores (VAS) and Oswestry Disability Index (ODI) for low back pain were recorded in PMOP females for evaluation of osteoporotic pain and function.

Results: FKN levels were significantly higher in postmenopausal osteoporotic patients compared with postmenopausal non-osteoporotic females (139.8 ± 44.3 pg/mL VS 116.5 ± 23.1 pg/mL, $p < 0.05$) and healthy controls (139.8 ± 44.3 pg/mL VS 109.7 ± 19.4 pg/mL, $p < 0.05$). Serum FKN concentrations were negatively associated with BMD at femoral neck ($r = -0.394$, $p = 0.004$), total hip($r = -0.374$, $p = 0.006$), internal trochanter($r = -0.340$, $p = 0.013$), greater trochanter($r = -0.376$, $p = 0.006$), Ward's triangle($r = -0.343$, $p = 0.012$), L1–L4 lumbar spine($r = -0.339$, $p = 0.013$) and positively associated with VAS ($r = 0.321$, $p = 0.019$) and ODI ($r = 0.377$, $p = 0.005$) scores, bone turnover makers (TRACP-5b:$r = 0.341$, $p = 0.012$; NTX:$r = 0.364$, $p = 0.007$)as well as inflammation markers (IL-1β: $r = 0.396$, $p = 0.003$; IL-6:$r = 0.355$, $p = 0.009$) in postmenopausal osteoporotic patients.

Conclusions: Serum FKN may serve as a novel biomarker for assessing disease progression and a new potential therapeutic target for anti-resorptive treatment in osteoporosis patients.

ARTICLE HISTORY
Received 21 April 2016
Accepted 12 May 2016

KEYWORDS
Osteoporosis; disease severity; fractalkine

Introduction

Osteoporosis is a silent systemic progressive disease characterised by a decrease in bone mass per unit volume, leading to a high risk of fracture and various subsequent complications.[1] The consequences of osteoporosis are always related to loss of life quality, heavy burden of social and financial cost, and even excess mortality, which can occur at any age all over the world.[2] Although osteoporosis can be partially prevented and treated today, its pathophysiology remains largely unknown.

From the point of view of cellular pathophysiology, osteoporosis results from a preponderance of activity of osteoclasts over that of osteoblasts.[3] Recently, osteoimmunology represents an emerging area focused on the mutual interactions between bone and the immune system,[4] and osteoclasts have been regarded as the centre of osteoimmunological process due to their hematopoietic origin as well as strong activation through cytokines.[5] In an inflammatory surrounding, activated T cells are able to stimulate osteoclastic secretion of receptor activator of nuclear factor-κB (RANKL) via up-regulation of various cytokines leading to activation and aggravation of bone resorption processes.[6,7]

Cytokines are a class of glycoprotein compounds acting as cellular hormones which are released by activated cells to exert local and/or systemic effects on various kinds of human cells and tissues.[8] Cytokines have been demonstrated to regulate osteoclast formation and function, and interactions of the immune system with bone appear to be mediated mainly by cytokine signals.[9] As one important subfamily of cytokines,

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chemokines are small (8–14 kD) proteins regulating leucocyte trafficking, cell adhesion, phagocytosis, cytokine secretion, cell activation, proliferation, apoptosis, angiogenesis, etc. during inflammation.[10] Chemokines are generally divided into four groups (CC, XC, CXC and CX3C) according to the number and position of conserved cysteine residues in their amino acid structure.[11] Chemokines have been shown to recruit osteoclast precursors to locally inflamed sites of resorption and play crucial parts in bone remodelling.[12]

CX3CL1 (also called fractalkine, FKN) is the only known member of the CX3C subfamily.[13] Unlike all other chemokines, CX3CL1/FKN exists as both a transmembrane protein and a soluble chemoattractant and is widely expressed on natural killer (NK) cells, monocytes and T cells.[14] So far the potential involvement of CX3CL1/FKN in bone remodelling processes has been explored in only a few studies. Han et al. found that the CX3CL1–CX3CR1 axis plays a pivotal role in osteoclast recruitment and subsequent bone resorption.[15] Also, inhibition of the CX3CL1-CX3CR1 axis through using anti-CX3CL1 mAb could inhibit osteoblast-guided differentiation of osteoclasts *in vitro*.[16]. In addition, it has been identified that the CX3CR1–CX3CL1 axis plays an important role in the maintenance of osteoclastic precursors.[17]

All these previous works implicate that FKN may play a crucial role in the pathogenesis and progression of osteoporosis. However, there are no studies available illustrating the association between FKN levels and progression of osteoporosis. The goal of our study was to examine whether FKN levels are altered in osteoporotic patients and the relationship between the level of circulating FKN and the degree of osteoporosis as well as clinical indices in postmenopausal females.

Material and methods

Subjects

This study was approved by the Ethics Committee of our hospital and performed according to the Declaration of Helsinki. Written informed consent was obtained from all participants. From August 2015 to March 2016, a total of 53 female patients diagnosed with PMOP from outpatients of Department of Endocrinology, Nanjing Medical University Affiliated Wuxi Second Hospital were enrolled in the study. Osteoporosis was identified in all patients according to the diagnostic criteria of World Health Organization for osteoporosis.[18] Additionally, 51 age-matched postmenopausal non-osteoporotic female patients and 50 premenopausal non-osteoporotic healthy women of childbearing age were recruited. The PMNOP and PMOP group had experienced natural menopause; menopause was defined as 1 year with no menstrual bleeding. Participants were excluded if they had a history of cardiovascular disease, cancer and metabolic bone disease such as osteoarthritis and rheumatoid arthritis. None of the patients had been treated with medication known to affect bone metabolism, hormone therapy, bisphosphonates or calcitonin before entry into the study. They had normal hepatorenal function and were free from endocrine disturbances including diabetes mellitus and thyroid disorders. There were no significant abnormalities in urinary calcium excretion, no history of hypercalcuria or urolithiasis in all groups.

Bone mineral density measurements

Bone mineral density was examined by dual-energy X-ray absorptiometry (DXA, Prodigy Advance, General Electric Company, Fairfield, CT, USA). Sites of the L1–L4 lumbar spine, femoral neck, internal trochanter, total hip, greater trochanter and Ward's triangle were measured in proper position. BMD (g/cm^2) was calculated from the bone mineral content (BMC) and bone area (BA). All measurements and analyses were performed by the same experienced physician.

Definition of clinical severity

The visual analogue scale (VAS) ranged from 0 to 10 was used for evaluating degree of pain in osteoporotic patients, where 0 represents no pain and 10 indicating extremely pain. The VAS scores are broadly used for severity of pain in various diseases including osteoporotic pain.[19] The Oswestry Disability Index (ODI) for low back pain was also applied to assess the clinical severity in patients.[20] The ODI is scored from 0 to 50 points, where 0 indicates no pain and normal physical function, and higher scores represent more severe pain and worse function.[21] The Oswestry Disability Index is very widely used and the feasibility and validity have been well established among patients.[22]

Laboratory methods

Venous blood samples were taken in the morning between 8:00 am and 9:00 am after an overnight fast. The samples were centrifuged for 10 min at approximately 3000 r/min within 30 min, and serum was separated and stored at −80 °C prior to analysis. Serum FKN, tartrate resistant acid phosphatase (TRACP-5b), IL-1 and IL-6 levels were measured by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA). Intra-assay coefficients of variation in our laboratory were 4.3, 3.9, 4.6 and 3.5%, respectively, whereas inter-assay variability was all <6%. Serum cross-linked N-telopeptides of type I (NTX) were examined by automated Roche electrochemiluminescence system with 4.2% intra-assay coefficient of variation and 5.6% inter-assay variability. The TRACP-5b and NTX both determine bone resorption.[23,24] IL-1 and IL-6 are regarded as the public 'classic' bone-resorbing proinflammatory cytokines and may determine osteoclastic activities of osteoporosis.[25,26]

Statistical analysis

Statistical analysis was conducted using the Statistical Package for Social Sciences software, version 18.0 for Windows (SPSS, Inc., Chicago, IL, USA). All data were conveyed as means ± standard deviation of the mean (SD) or median (interquartile range). The Kolmogorov–Smirnov test was used to for data normality analysis, while the one-way analysis of variance (ANOVA) was applied to compare the differences of serum FKN levels as well as other biochemical indexes among PMOP, PMNOP and healthy controls. Bartlett's test was used to test the homogeneity of group variances, followed with Tukey's or Tamhane's post hoc tests, where appropriate. The correlations between FKN levels in the serum with BMD and biochemical indexes were determined using Pearson's or Spearman's correlation coefficient (*r*). *P* value less than 0.05 was considered to be statistically significant.

Results

Demographic and clinical characteristics

The main characteristics and biochemical data of the study population are listed in Table 1. There were no statistically significant differences between patients and controls for age between and age at menopause between PMOP and PMNOP group. And also differences of body mass index among PMOP, PMNOP and control do not reach significance (Table 1).

As demonstrated in Table 1, PMOP patients had higher serum FKN levels compared with PMNOP patients and controls (Table 1, Figure 1). In addition, the PMOP also had much higher serum TRACP-5b, NTX, IL-1 and IL-6 levels compared with the PMNOP and control (Figure 1, Table 2).

Correlation of serum FKN levels with BMD, VAS scores, ODI scores and biochemical indexes

We further investigated the relationship between serum levels of FKN and severity of osteoporosis in PMOP. The FKN levels were negatively correlated with BMD at the left femoral neck, greater trochanter, internal trochanter, total hip, Ward's triangle and L1–L4 lumbar spine (Figure 2). The serum FKN levels also positively correlated with VAS scores and ODI scores (Figure 3). Meanwhile, we also examined the association of FKN levels with biochemical indexes. FKN levels in serum were positively associated with bone resorption markers TRACP-5b and NTX as well as inflammation markers IL-1β and IL-6 levels (Figure 4). These correlations remain significant after adjusting by age and BMI (Table 3).

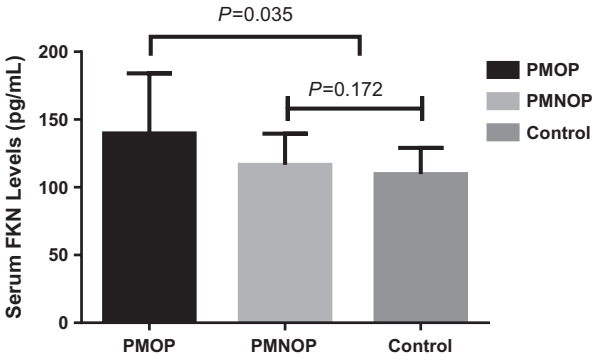

Figure 1. Comparison of serum FKN levels (pg/mL) in PMOP, PMNOP and premenopausal non-osteoporotic healthy women. One-way ANOVA was used in the comparisons. *$p < 0.05$. Data were expressed as mean ± SD.

Discussion

In this study, we found that FKN concentration in serum was elevated in postmenopausal osteoporotic patients compared with postmenopausal non-osteoporotic females and healthy controls. Elevated serum FKN levels in osteoporosis patients were correlated with attenuated BMD, increased bone turnover markers, inflammation markers and more pain and functional disability evaluated by VAS scores and Oswestry disability index. To the best of our knowledge, this study is the first to demonstrate the association of FKN concentration in serum with the disease severity of postmenopausal osteoporosis.

In recent years, authors looking to embrace a consensus of opinion related to the pathogenesis of osteoporosis in their works have been devoting increasingly more attention to the importance of an inflammatory component.[27,28] Advances in the basic and clinical sciences over these years have substantiated the belief

Table 1. Baseline clinical characteristics.

	PMOP	PMNOP	Control
Age	64.5 ± 5.9**	65.9 ± 6.3**	45.2 ± 4.4
Age of menopause	52.1 ± 3.1	52.5 ± 2.9	/
BMI	23.9 ± 3.2	23.8 ± 3.7	23.6 ± 4.0
TRACP-5b (U/L)	6.0 ± 1.1**#	4.8 ± 1.2*	3.5 ± 0.7
NTX (μmol BCE/L)	24.6 ± 8.3**#	18.7 ± 6.4*	15.2 ± 4.7
IL-1β (pg/mL)	28.2 ± 4.7*	24.1 ± 4.0*	22.5 ± 3.4
IL-6 (pg/mL)	114.3 ± 22.6**#	102.7 ± 15.8*	76.3 ± 17.4
FKN (pg/mL)	139.8 ± 44.3*#	116.5 ± 23.1	109.7 ± 19.4

Notes: BMI, body mass index; PMOP, postmenopausal osteoporotic female patients; PMNOP, postmenopausal non-osteoporotic female patients; Control, premenopausal non-osteoporotic healthy women of childbearing age. Values are expressed as the mean value ± SD.
*$p < 0.05$ VS control; **$p < 0.01$ VS control;
#$p < 0.05$, compared with the PMNOP group.

Table 2. Comparisons of the bone mineral density at the left femoral neck, greater trochanter, internal trochanter, total hip, Ward's triangle and L1–L4 lumbar spine among the healthy control, PMNOP and PMOP groups (Unit: g/cm^2).

Group	BMD(Neck)	BMD(Troch)	BMD(Inter)	BMD(Htot)	BMD(Ward)	BMD(L1–4)
Control	1.03 ± 0.22	0.78 ± 0.17	1.14 ± 0.24	1.11 ± 0.17	0.78 ± 0.18	1.25 ± 0.28
PMNOP	0.95 ± 0.21*	0.70 ± 0.15*	1.03 ± 0.22*	0.99 ± 0.19*	0.66 ± 0.24*	0.87 ± 0.34*
PMOP	0.85 ± 0.19**#	0.58 ± 0.19**#	0.95 ± 0.20**#	0.87 ± 0.16**#	0.65 ± 0.20**	0.76 ± 0.22**#

Notes: All data were presented as mean ± standard deviation. BMD, bone mineral density; Htot, total hip; Inter, internal trochanter; L1–4, L1–L4 lumbar spine; Neck, left femoral neck; Troch, greater trochanter; Ward, Ward's triangle.
*$p < 0.05$, compared with the healthy control (NC) group; **$p < 0.01$, compared with the control group;
#$p < 0.05$, compared with the PMNOP group.

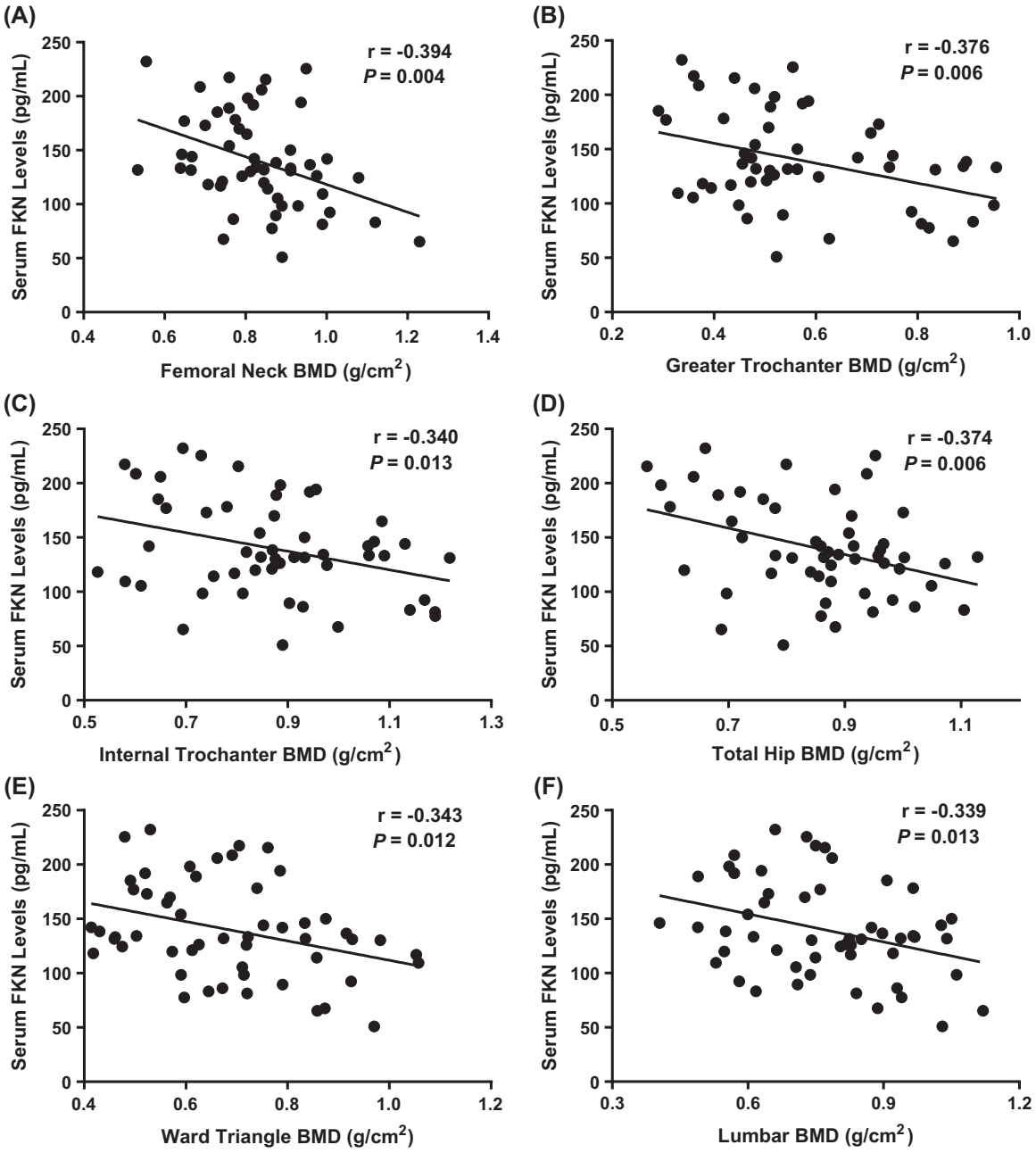

Figure 2. Correlation of serum FKN levels with bone mineral density at the left femoral neck (A), greater trochanter(B), internal trochanter(C), total hip(D), Ward's Triangle(E) and L1–L4 lumbar spine(F) in PMOP patients.

that osteoporosis is not merely a endocrine problem associated with loss of bone mass and volume, but that a wider approach is needed – including a systemic angle – in view of a significant contribution of the immune response.[29,30]

Chemokines, as one class of the most important cytokines, have been reported to play an important role in osteoclastogenesis.[12] Chemokines could be induced by RANKL during osteoclast differentiation and strongly promote the formation of TRAP$^+$ multinuclear cells.[31]

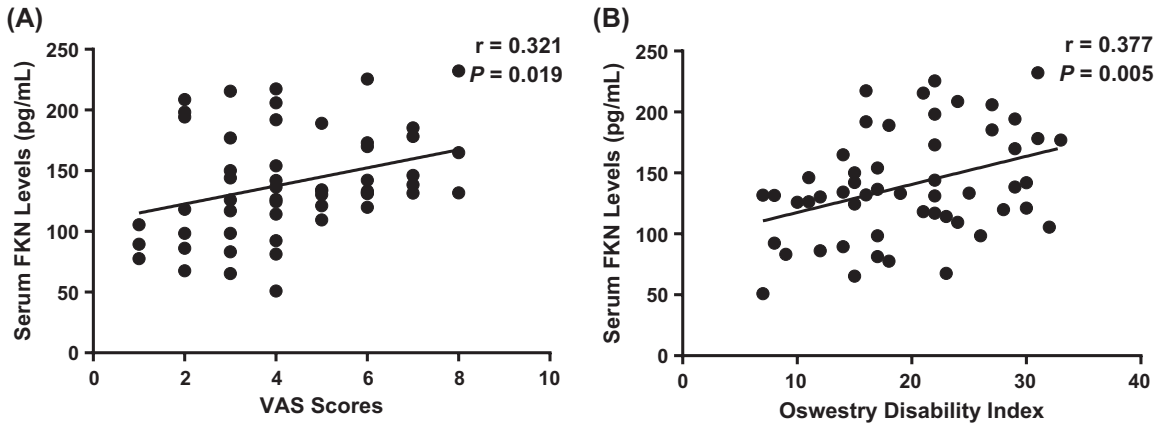

Figure 3. Correlation of serum FKN levels with VAS scores and Oswestry Disability Index in PMOP patients.

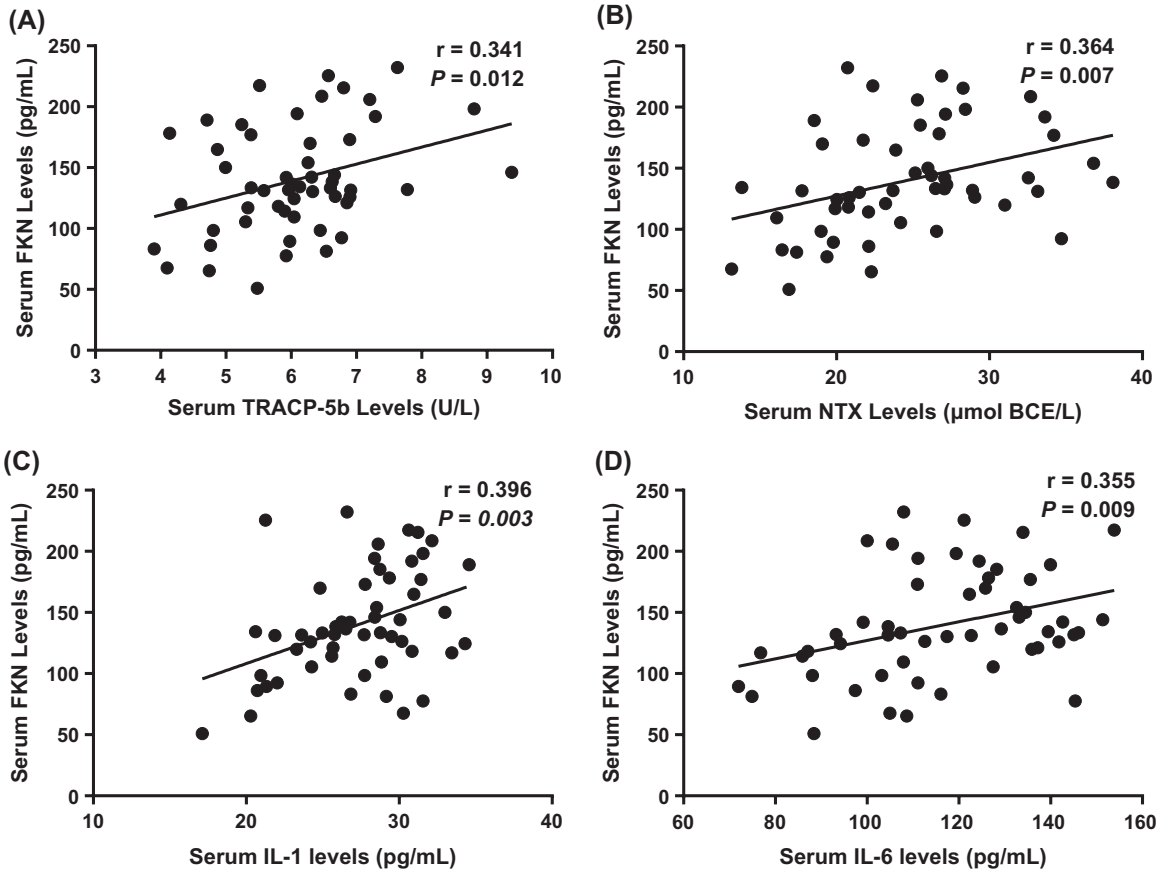

Figure 4. Correlation of serum FKN levels with bone turnover markers TRACP-5b, NTX and inflammation markers IL-1 and IL-6 in PMOP patients.

Also, chemokines could recruit osteoclast precursors.[32] CX3CL1/ fractalkine(FKN) is a membrane-bound chemokine and the only known member of the CX3C subfamily. A previous study showed that treatment with anti-CX3CL1 Ab can reduce the bone resorption.[16] Osteoblasts of both mice and humans expressing FKN at high levels may also use the soluble form of FKN to attract osteoclast precursors and the membrane-bound FKN to adhere with osteoclast precursors,[16] implicating the important role of FKN in osteoclasts activity.

In our study, we found that FKN levels are positively linked with BMD at sites of the L1–L4 lumbar spine, femoral neck, internal trochanter, total hip, greater trochanter and Ward's triangle. Dual-energy X-ray absorptiometry is used for the measurement of BMD of the spine and hip. It has gradually become the golden standard for the diagnosis of osteoporosis, assessment of fracture risk and monitoring of response to treatment.[33] This method is widely available with readily interpretable results.[34]

We also found that FKN levels were positively correlated with degree of pain evaluated by VAS scores and functional ability assessed by Oswestry Disability Index. Pain seems to be one of the most common complications of osteoporosis. It is estimated that 75–85% of the patients suffering from osteoporosis is disturbed by bone pain,[35,36] in particular low back pain is thought

Table 3. Correlations of serum FKN levels with anthropometric parameters, BMD, VAS scores, ODI scores and biochemical indexes in PMOP patients adjusted by age and BMI.

	Serum FKN levels (pg/mL)		Serum FKN levels (pg/mL) *	
Variables	*r*	*P*	*r*	*P*
BMI	0.049	>0.05	/	/
Age	0.351	0.01	/	/
Femoral neck BMD	−0.394	0.004	−0.348	0.01
Greater trochanter BMD	−0.376	0.006	−0.322	0.02
Inter trochanter BMD	−0.340	0.013	−0.301	0.031
Total hip BMD	−0.374	0.006	−0.333	0.015
Ward's triangle BMD	−0.343	0.012	−0.307	0.028
Lumbar spine BMD	−0.339	0.013	−0.294	0.035
VAS scores	0.321	0.019	0.281	0.041
ODI scores	0.377	0.005	0.328	0.018
Serum IL-1β levels	0.396	0.003	0.353	0.009
Serum IL-6 levels	0.355	0.009	0.311	0.016
Serum TRACP-5b levels	0.341	0.012	0.305	0.027
Serum NTX levels	0.364	0.007	0.327	0.018

*Adjusted by age and BMI.

to be the prevalent musculoskeletal pain, particularly in elderly populations.[37] In osteoporotic patients, abnormal charging of articulation and muscles caused by progressive bone loss can result in vertebral deformity, thus leading to chronic pain,[38] which again leads to further functional limitations, depression and reduction in social activities.[39] Although mechanisms of pain in osteoporosis are poorly known, increased osteoclastic bone resorption, maintenance of central sensitisation and spinal neuropathic pain have been implicated in chronic pain in osteoporosis.[40] A growing number of works suggest that FKN may be key in mediating neuron–microglia interactions in the dorsal horn of the spinal cord during nociceptive transmission via its sole receptor CX3CR.[41,42] FKN is able to induce nociceptive behaviours via the CX3CR1 receptor on microglia, which induces activation of p38 mitogen-activated protein kinase (MAPK)-mediated pathways.[43] Several studies have also shown that FKN/CX3CR1 play a crucial role in chronic pain in various diseases including musculoskeletal disease,[44] inflammation disease [45] and cancer,[46] implicating its pivotal role in the maintenance of pain in any conditions.[47]

Tartrate resistant acid phosphatase-5b (TRACP-5b) and cross-linked N-telopeptides of type I collagen (NTX) both determine bone resorption. Serum tartrate resistant acid phosphatase 5b (TRACP 5b) is an isozyme of osteoclast origin. Measurement of TRACP 5b activity is used as an index of osteoclast activity.[23] NTX can also be measured both in serum and urine, as a biochemical marker of bone resorption.[24] In this study, we examined serum NTX in serum, since blood can be drawn simultaneously for all other routine biochemical and haematological tests. Accordingly, both were positively correlated with serum FKN levels in the osteoporotic patients in our study. Interleukin (IL)-1β and IL-6 are both known as the most powerful proinflammatory cytokines and function as strong stimulators of *in vitro* and *in vivo* bone resorption via upregulation of RANKL that stimulates the osteoclastogenesis.[25,26] Notably, we also found significant correlations between serum FKN levels with IL-1β and IL-6 levels.

There were some limitations to our study. First, this was a cross-sectional study including a limited number of subjects; further, longitudinal studies with a larger population sample should be conducted. Second, we only examined the level of FKN in serum. Investigation of other chemokines may provide more valuable information on the role that chemokines play in osteoporosis.

In conclusion, we found that serum FKN levels were significantly elevated in postmenopausal osteoporosis patients and we identified a positive correlation between serum and FKN levels with the disease severity in female patients with postmenopausal osteoporosis. Measurement of FKN levels in the serum can be served as an alternative approach to assess the risk and severity of osteoporosis in addition to the use of traditional methods.

This work represents an advance in biomedical science because it shows that serum FKN levels are positively related to disease severity in osteoporosis, implicating immune responses are important in pathophysiology of osteoporosis (Table 4).

Table 4. Summary.

What is known about this subject:
- FKN was identified as a potential osteoclast activity and bone resorption marker.

What this paper adds:
- Serum FKN levels were raised in postmenopausal osteoporotic females
- Elevated serum FKN levels were negatively correlated with BMD and positively related to bone-resorption activity and clinical severity in postmenopausal osteoporotic females
- Serum FKN may serve as a novel biomarker for assessing disease progression and a new potential therapeutic target for anti-resorptive treatment in osteoporosis patients.

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