

# Screening and validation of serum protein biomarkers for early postmenopausal osteoporosis diagnosis

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Received December 13, 2016; Accepted July 27, 2017

DOI: 10.3892/mmr.2017.7620

**Abstract.** Postmenopausal osteoporosis is one of the most prominent worldwide public health problems and the morbidity is increasing with the aging population. It has been demonstrated that early diagnosis and intervention delay the disease progression and improve the outcome. Therefore, searching for biomarkers that are able to identify postmenopausal women at high risk for developing osteoporosis is an effective way to improve the quality of life of patients, and alleviate social and economic burdens. In the present study, a protein array was used to identify potential biomarkers. The bone mineral densities of 10 rats were dynamically measured in an ovariectomized model by micro-computed tomography assessment, and the early stage of osteoporosis was defined. Through the protein array-based screening, the expression levels of six serum protein biomarkers in ovariectomized rats were observed to alter at the initiation stage of the postmenopausal osteoporosis. Fractalkine, tissue inhibitor of metalloproteinases-1 and monocyte chemotactic protein-1 were finally demonstrated to be increased in the serum of eight enrolled postmenopausal osteoporosis patients using ELISA assay and were correlated with the severity of progressive bone loss. These biomarkers may be explored as potential early biomarkers to readily evaluate and diagnose postmenopausal osteoporosis in the clinic.

## Introduction

Osteoporosis is a common bone metabolic disease characterized by systemic bone loss, impaired bone microstructure and

reduced bone strength (1,2). Clinically, it typically manifests as chronic pain and increased susceptibility to low-traumatic fractures, resulting in compromised life quality and increased patient mortality (3-5). Postmenopausal osteoporosis is the primary form of osteoporosis which results from estrogen deficiency, leading to excessive bone absorption and inadequate bone formation (6). Bone mineral density (BMD) measurement using dual energy X-ray absorptiometry is currently the gold standard for confirmative diagnosis of osteoporosis. However, the early stage of osteoporosis is usually asymptomatic and >50% of low-traumatic patients do not meet the diagnostic criteria of osteoporosis, according to BMD value (7), therefore, the search for more sensitive markers for osteoporosis is essential and will be beneficial for evaluation and treatment for osteoporosis patients, particularly at the early stages.

Previously, investigations using osteoporotic serum have suggested carboxy-terminal crosslinking telopeptide of type I collagen and procollagen type I N propeptide as biomarkers of bone resorption and bone formation (8). The serum cathepsin K has been explored as a biomarker of osteoclast activity, whereas fibroblast growth factor-23 and sclerostin were considered osteocyte factors (9-11). In addition, with the rapid development of omics technologies, various osteoporosis associated biomarkers have been discovered. Micro (mi)RNA-133a and miR-422a in circulating monocytes have been demonstrated to act as potential nucleic biomarkers for postmenopausal osteoporosis (12,13). However, all these candidates are not fully specific or sensitive as early-stage markers of osteoporosis. Recently developed protein array technology is sensitive and specific in detecting trace amounts of proteins in body fluid and has been successfully applied in the discovery of various disease associated biomarkers (14-16). Therefore, the present study hypothesized that protein array screening in combination with osteoporosis models may provide useful information in identifying biomarkers for early osteoporosis diagnosis.

The present study successfully established the ovariectomized rat as a postmenopausal osteoporosis model, which demonstrated progressive bone loss starting from four weeks following surgery. In the protein array screening, B7-2,  $\beta$ -nerve growth factor ( $\beta$ -NGF), Fractalkine, interferon- $\gamma$  (IFN- $\gamma$ ), tissue inhibitor of metalloproteinases-1 (TIMP-1) and monocyte chemotactic protein-1 (MCP-1) were observed to be increased with the development of osteoporosis in the rat serum. Validation in human samples demonstrated that

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**Key words:** osteoporosis, serum biomarker, Fractalkine, tissue inhibitor of metalloproteinases-1, monocyte chemotactic protein-1

Fractalkine, TIMP-1 and MCP-1 were increased in serum of patients suffering from osteoporosis or decreased bone density compared with that of healthy people with normal bone mineral density. Overall, the present study identified a novel panel of serum protein markers that may successfully predict the development and severity of postmenopausal osteoporosis.

### Materials and methods

**Animals and experimental procedures.** A total of 10 female Sprague-Dawley rats, (age, 3 months; weight, 239±17.5 g), were obtained from the Experimental Animal Center at the Fourth Military Medical University (Xi'an, China), and were housed under specific pathogen-free conditions (20°C, 12-h light/dark cycle and 50-55% humidity) with free access to food and water. They were randomly divided into two groups, an ovariectomy group (OVX, n=5) and a sham group (Sham, n=5). A total of 5 rats of OVX group underwent an ovariectomy to establish the postmenopausal osteoporosis model. The rats were anesthetized intraperitoneally with pentobarbital at a dose of 40 mg/kg. The ovariectomy surgery was performed using the dorsal approach (17). All 5 rats in the sham group underwent the same operation with the exception of the ovary ablation. Blood samples were obtained from the angular veins on each animal at 2-week intervals for 2 months following the operations (2, 4, 6 and 8 weeks). Serum samples were collected by centrifugation and stored at -80°C for further analysis. There was no significant difference in total body weight between the 2 groups at the differing time points. All experimental procedures conducted using animals were approved by the Ethics in Animal Research Committee of the Fourth Military Medical University (permission code 20110405-5).

**Patients.** A total of 24 women aged 57-68 from Xijing Hospital (Xi'an, China) were recruited as human subjects and signed informed-consent documents, prior to being enrolled in the present study. Subjects were excluded if they had a history of cancer, cardiovascular disease or diabetes mellitus. None of the subjects had been diagnosed with metabolic bone diseases such as osteoarthritis and rheumatoid arthritis or had been treated with medication known to impact upon bone metabolism, such as hormone therapy, bisphosphonates or calcitonin. Patients had normal hepatorenal function and were not suffering from any endocrine disturbances, hypercalcemia or urolithiasis. The human investigations were approved by the ethics committee of the Fourth Military Medical University. A total of 8 subjects had normal BMD ( $T_m \geq -1.0$ ) and 8 had low BMD ( $-2.5 < T_m < -1.0$ ). The other 8 subjects were postmenopausal osteoporosis patients ( $T_m \leq -2.5$ ). BMD of all subjects for the lumbar spine (L1-4) and total hip (femoral neck, trochanter and intertrochanteric region) were measured using dual energy X-ray absorptiometry (DXA) scanners. Blood samples were collected from subjects and centrifuged at 1,500 x g for 10 min at room temperature. Supernatant sera were obtained and stored at -80°C for further analysis.

**Micro-computed tomography (micro-CT) assessment.** Micro-CT scanning was performed *in vivo* on each animal at

Table I. Correlation between potential early postmenopausal osteoporosis biomarker and bone mineral density in ovariectomized rats.

Biomarker	r	P-value
B7-2	-0.914	0.086
Fractalkine	-0.971	0.029 <sup>a</sup>
IFN $\gamma$	-0.976	0.024 <sup>a</sup>
$\beta$ -NGF	-0.888	0.112
MCP1	-0.979	0.021 <sup>a</sup>
TIMP1	-0.971	0.029 <sup>a</sup>

<sup>a</sup>P<0.05. IFN- $\gamma$ , interferon- $\gamma$ ;  $\beta$ -NGF,  $\beta$ -nerve growth factor; MCP-1, monocyte chemoattractant protein-1; TIMP-1, tissue inhibitor of metalloproteinases-1.

2 week intervals for 2 months following the ovariectomy or sham operations (2, 4, 6, and 8 weeks). Rats were anesthetized with pentobarbital at a dose of 40 mg/kg during each measurement. The distal femurs were scanned using Pre-Clinical Inveon Micro-CT (Siemens Healthineers, Erlangen, Germany) with an 8 mm resolution, a 50 kV tube voltage and a 0.1 mA tube current. Reconstruction and three-dimensional quantitative analyses were determined using the software provided by a desktop micro-CT system (Inveon Research Workplace 2.2; Siemens Healthineers). The scanning regions were confined to the distal metaphysis and extended 2.0 mm proximally from the proximal tip of the primary spongiosa. The following three-dimensional indices in the defined region of interest were analyzed: BMD, trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and relative bone volume over the total volume (BV/TV, %). The operator who conducted the scan analyses was blinded to the procedure associated with the subjects.

**Cytokine antibody array analysis.** Rat serum samples were assessed for the presence of 27 cytokines using Quantibody Rat Cytokine Array 3 kit (RayBiotech Inc., Norcross, GA, USA). Antibody array membranes were first blocked with Tris-buffered saline containing 0.05% Tween-20, supplemented with 5% skimmed milk at room temperature for 1 h, to which rat sera were subsequently added for a final 10-fold dilution, following the manufacturer's protocol. The cytokine expression levels were detected and quantified on a fluorescent scanner (Axon GenePix; Molecular Devices, LLC, Sunnyvale, CA, USA).

**ELISA assay.** Human serum Fractalkine, TIMP-1 and MCP-1 expression levels were measured using sandwich ELISA assay kits (P78423, P01033 and P13500; RayBiotech Inc.) according to the manufacturer's protocol. Total protein concentration was calculated using the Bradford protein assay method.

**Statistical analysis.** Statistical analyses were performed using SPSS software, version 15.0 (SPSS Inc., Chicago, IL, USA). Quantitative data are presented as the mean  $\pm$  standard deviation. Statistical tests were two-sided; the differences between

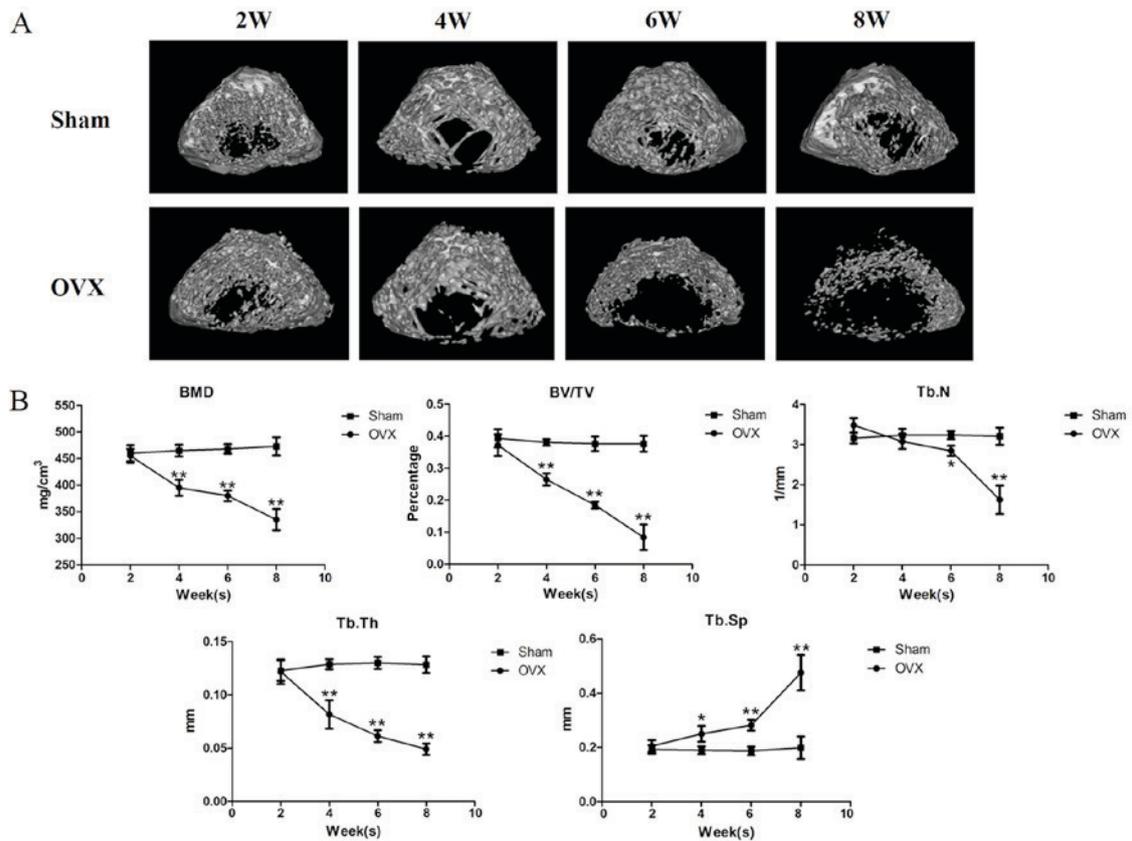


Figure 1. Establishment and confirmation of early osteoporosis in ovariectomized rat models. (A) Micro-CT analysis within the distal metaphyseal femur region at 2, 4, 6 and 8 weeks following sham or ovariectomy operations. Magnification x40 of the original section. (B) Dynamic alterations of BMD, BV/TV, Tb.N, Tb.Th and Tb.Sp in rats that received either sham or ovariectomy operations. \* $P < 0.05$  and \*\* $P < 0.01$  vs. sham group ( $n = 5$ ). OVX, ovariectomy; BMD, bone mineral density; BV/TV, relative bone volume over the total volume; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; OVX, ovariectomy group.

two groups were assessed using Student's t-tests, and analysis of variance followed by the Bonferroni post-hoc test was conducted to analyze multiple comparisons among groups. Pearson correlation was employed to determine the linear relationship between two variables.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Establishment and confirmation of early osteoporosis in ovariectomized rat model.** To determine when early postmenopausal osteoporosis occurs, the present study measured BMD and other osteoporosis parameters starting from two weeks following surgery. Distal femurs of the rats were scanned using micro-CT and trabecular bone structures were reconstructed based on the images (Fig. 1A). The results revealed that there was no difference in BMD between the OVX and the sham group 2 weeks following the ovariectomy. A gradual reduction of BMD in the ovariectomized rats was observed 4 weeks post-surgery and reached  $>25\%$  reduction 8 weeks following surgery (Fig. 1B). No observable BMD alteration was detected in the sham group during this time course. Consistent with the decreased BMD values, quantitative analysis of further osteoporosis parameters revealed a significant decrease in BV/TV and Tb.Th with a similar pattern to BMD from 4 weeks post-surgery ( $P < 0.01$ ). Tb.N, as a marker of trabecule number, decreased later and revealed a significant reduction from

6 weeks following surgery ( $P < 0.05$  and  $P < 0.01$ ). A prominent increase in trabecular space marker, Tb.Sp, was also observed from 4 weeks following surgery ( $P < 0.05$  and  $P < 0.01$ ). These data suggested that initiation of rat postmenopausal osteoporosis occurred 4 weeks post-surgery.

**Screening of postmenopausal osteoporosis-associated biomarkers using protein array.** A total of 27 proteins, B7-2,  $\beta$ -NGF, cytokine-induced neutrophil chemoattractant (CINC)-1, CINC-2, CINC-3, ciliary neurotrophic factor, Fractalkine, granulocyte-macrophage colony-stimulating factor, intercellular adhesion molecule-1, IFN- $\gamma$ , interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-10, IL-13, chemokine (C-X-C motif) ligand 5, L-selectin, MCP-1, platelet derived growth factor subunit A, Prolactin R, receptor for advanced glycation end products, calmodulin-binding kinesin-like protein-1, TIMP-1, tumor necrosis factor- $\alpha$  and vascular endothelial growth factor, were included in the protein array screening assay, based on previous literature. By analyzing the fluorescence intensities, the results demonstrated that the serum levels of B7-2, Fractalkine, IFN- $\gamma$ ,  $\beta$ -NGF, MCP-1 and TIMP-1 increased following ovariectomy (Fig. 2). Further statistical study revealed that TIMP-1 significantly increased at 4 weeks following the ovariectomy, almost in parallel with the aforementioned significant alterations in bone mineral density at 4 weeks. Other biomarkers increased significantly at 6 weeks post-surgery, two weeks later than when the alterations

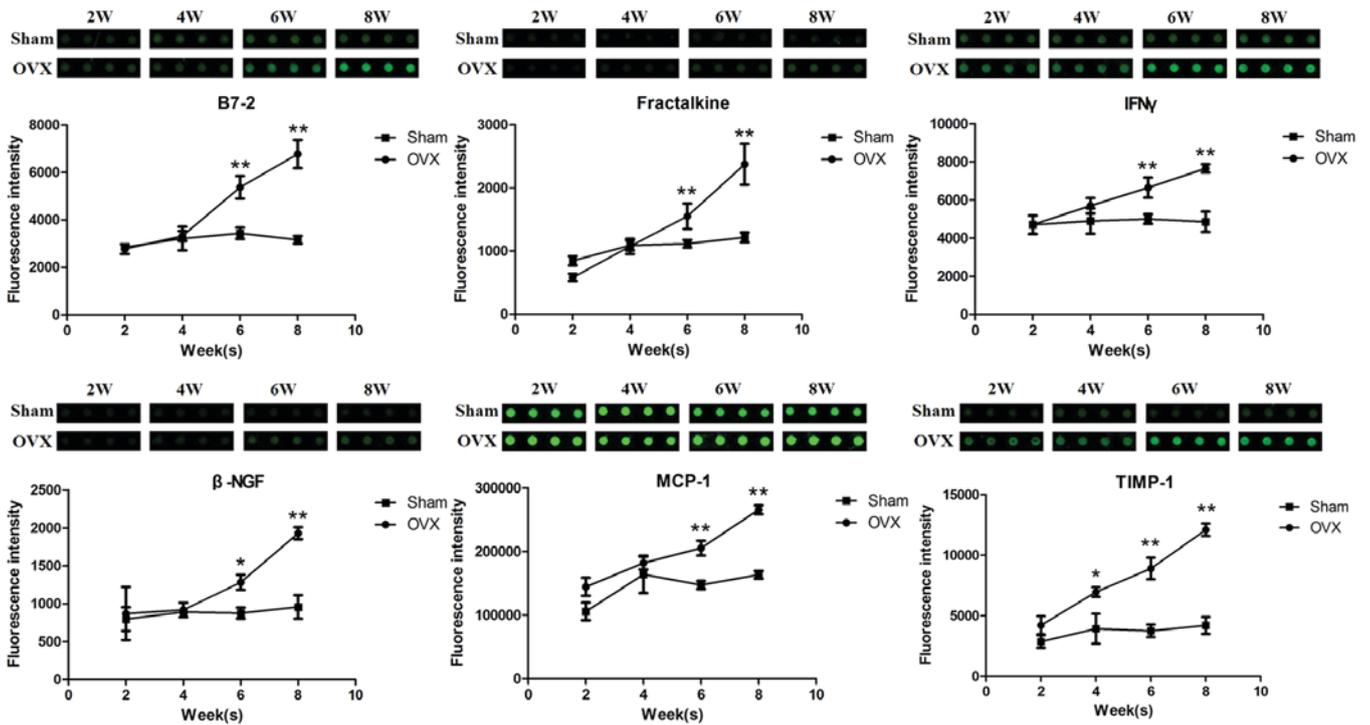


Figure 2. Screening results of postmenopausal osteoporosis-associated biomarkers using protein array. Upper panel: fluorescence intensity diagram of B7-2, Fractalkine, IFN- $\gamma$ ,  $\beta$ -NGF, MCP-1 and TIMP-1 in sera obtained from sham or ovariectomized rats at 2, 4, 6 and 8 weeks following surgery in the protein array screening. Each sample repeated in four slots. Lower panel: dynamic alterations of serum biomarkers in the sham or ovariectomized rats post-surgery. \*P<0.05, \*\*P<0.01 vs. sham group. n=5. IFN- $\gamma$ , interferon- $\gamma$ ;  $\beta$ -NGF,  $\beta$ -nerve growth factor; MCP-1, monocyte chemotactic protein-1; TIMP-1, tissue inhibitor of metalloproteinases-1; OVX, ovariectomy group.

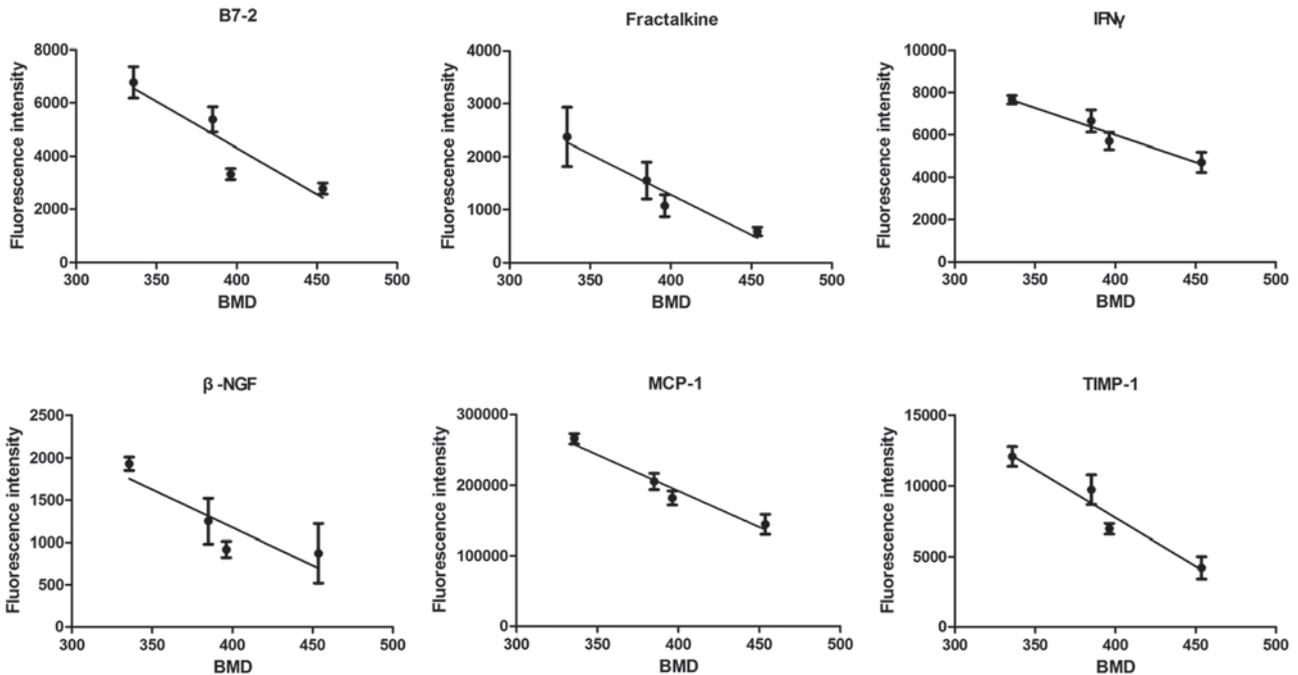


Figure 3. Correlation between serum B7-2, Fractalkine, IFN- $\gamma$ ,  $\beta$ -NGF, MCP-1 and TIMP-1 expression levels and bone loss. The fluorescence intensity of B7-2, Fractalkine, IFN- $\gamma$ ,  $\beta$ -NGF, MCP-1 and TIMP-1 in the protein array screening was plotted against the BMD of the rats following surgery. BMD, bone mineral density; IFN- $\gamma$ , interferon- $\gamma$ ;  $\beta$ -NGF,  $\beta$ -nerve growth factor; MCP-1, monocyte chemotactic protein-1; TIMP-1, tissue inhibitor of metalloproteinases-1.

in bone mineral density were observed (P<0.05 and P<0.01). The levels of other candidate proteins did not significantly alter in the screening during this time course.

*Fractalkine, IFN- $\gamma$ , MCP-1 and TIMP-1 correlate with the severity of bone loss in the ovariectomized rat model. In the postmenopausal osteoporosis rat model, eight weeks following*

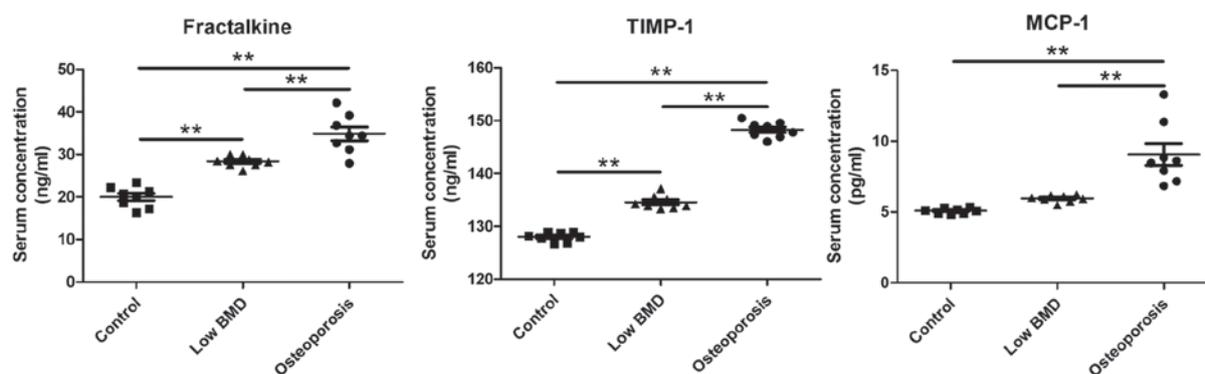


Figure 4. Validation of early postmenopausal osteoporosis biomarkers in human serum samples. The serum levels of Fractalkine, TIMP-1 and MCP-1 in healthy control, patients suffering from osteopenia and patients suffering from postmenopausal osteoporosis. \*\* $P < 0.01$  as indicated.  $n = 8$ . TIMP-1, tissue inhibitor of metalloproteinases-1; MCP-1, monocyte chemoattractant protein-1; BMD, bone mineral density.

ovariectomy is usually defined as the standard period for the presentation of obvious bone loss. Six biomarkers were observed to be elevated in the serum during the early stage of osteoporosis in our model. However, whether they were also correlated with osteoporosis progression remained unclear. To verify the association of these six proteins with the progression of postmenopausal osteoporosis, the present study analyzed the correlation between the protein levels and the severity of bone loss of the ovariectomized rats. As presented in Fig. 3 and Table I, Fractalkine, IFN- $\gamma$ , MCP-1 and TIMP-1 were negatively correlated with the BMD of the ovariectomized rats ( $P < 0.05$ ), whereas the results for B7-2 and  $\beta$ -NGF were not significant.

*Validation of early postmenopausal osteoporosis-associated biomarkers in human samples.* To further verify the clinical significance of the potential early postmenopausal osteoporosis serum biomarkers, the present study detected the serum levels of Fractalkine, TIMP-1 and MCP-1 using commercially available ELISA kits in patients. The different serum protein levels among people with normal BMD ( $T_m \geq -1.0$ ,  $n = 8$ ), patients with reduced BMD that did not reach the osteoporosis diagnosis criteria ( $-2.5 < T_m < -1.0$ ,  $n = 8$ ) and patients suffering from postmenopausal osteoporosis ( $T_m \leq -2.5$ ,  $n = 8$ ) were compared and analyzed. The results demonstrated that these three candidates, Fractalkine, TIMP-1 and MCP-1, increased as the BMD decreased from normal people to patients with reduced BMD and postmenopausal osteoporosis with significant differences (Fig. 4).

## Discussion

Postmenopausal osteoporosis is one of the most common skeletal diseases lacking practical methods for early diagnosis and intervention to reduce the possibility of low-traumatic fracture. In the present study, through a protein array screening in the ovariectomy osteoporosis rat model, a total of 6 serum protein markers were identified with expression levels that altered in parallel with the development of early postmenopausal osteoporosis. Of the six potential markers, Fractalkine, TIMP-1 and MCP-1 were further validated to be increased in serum at the early stage of osteoporosis in human samples.

Postmenopausal osteoporosis is gradually acquired following the menopause and there is currently no consent on parameters that define the early stage of this disease. The World Health Organization classification of osteoporosis using the BMD T score (normal if  $BMD \geq -1.0$ ; low bone mass or alternatively osteopenia if  $-2.5 < BMD < -1.0$ ; osteoporosis if  $BMD \leq -2.5$ ; severe or established osteoporosis if  $BMD \leq -2.5$  with history of fragility fracture) is useful for clinically diagnosing and evaluating the response to treatment of osteoporosis patients (7). However, the BMD T score is not sensitive in identifying members of the population that are at a high risk of developing osteoporosis, prior to the prominent occurrence of bone loss. To establish the early stage of postmenopausal osteoporosis, the BMD value of the rat femur was measured post ovariectomy. The majority of parameters were still comparable 2 weeks following the surgery, slight alterations appeared 4 weeks following surgery and the trends extended to the end of the observation period, at 8 weeks following the ovariectomy. Therefore, the time window of 2-4 weeks following ovariectomy in the rat may be considered as the early stage of postmenopausal osteoporosis development, and serum biochemical alterations may be explored for discovery of biomarkers predicting the upcoming osteoporosis. This time window for early postmenopausal osteoporosis may not be strict, however it does provide helpful information for screening early postmenopausal osteoporosis associated markers.

Following the establishment of the 2-4-week period following ovariectomy as the early stage of postmenopausal osteoporosis genesis, the present study focused on investigation of the biomarkers that demonstrated increased trends during this period. A total of 6 protein markers were observed to exhibit this trend, and the elevation of Fractalkine, TIMP-1 and MCP-1 were further verified with investigations using serum from osteopenia and osteoporosis patients.

Fractalkine, additionally termed C-X3-C motif ligand 1, is the only member of the CX3C chemokine subfamily that functions through binding with the CX3C receptor 1 (18). Fractalkine is a membrane-bound protein that may be released into serum by metalloproteinase, thus it acts as an adhesion molecule and potential chemoattractant (19,20). Certain studies have previously linked Fractalkine with bone metabolism. In mouse, ionizing irradiation augments Fractalkine production in skeletal vascular endothelium,

which promotes recruitment of osteoclast precursor cells resulting in enhanced osteoclastogenesis and bone absorption (21). The membrane-bound form of Fractalkine has been demonstrated to be critical for the osteoblast-osteoclast interaction and the osteoblast-induced osteoclast differentiation (22,23). Furthermore, it has been reported that circulating Fractalkine levels are associated with the severity of postmenopausal osteoporosis (24). The present study demonstrated that serum Fractalkine concentration was elevated not only in postmenopausal osteoporosis patients, however additionally in osteopenia patients, compared with the healthy control. The study further provided evidence that demonstrated Fractalkine progressively increased from osteopenia to osteoporosis, acting as a potential warning marker for early postmenopausal osteoporosis.

TIMP-1 belongs to the inhibitor of metalloproteinases (TIMPs) family and works together with the metalloproteinases (MMPs) to remodel the extracellular matrices (25). Coordination degradation of bone matrices by MMPs and TIMPs is critical in bone formation and absorption. It has been reported that TIMP-1 levels are elevated in chronic obstructive pulmonary disease (COPD) and early rheumatoid arthritis (RA) patients with bone loss (26,27). COPD and RA are associated with systemic chronic inflammation and it has previously been demonstrated that dysregulation of immunity contributes to postmenopausal osteoporosis (28). The present study revealed that serum TIMP-1 was increased in osteopenia and osteoporosis patients. In a previous study (29), the researchers excluded TIMP-1 as a potential biomarker for osteoporosis diagnosis. However, the authors may have misinterpreted their data, as a negative correlation exists between TIMP-1 level and BMD, in addition to the other two serum biomarkers of osteoporosis in their study.

MCP-1 is one of the most important chemokines that recruits different kinds of immune cells during inflammation and infection. Gene polymorphism analysis demonstrated that the MCP-1 A2518G polymorphism is correlated with osteopenia and osteoporosis risk in postmenopausal women (30). To the best of the author's knowledge, the present study, for the first time, observed that MCP-1 was elevated in the serum of postmenopausal osteoporosis patients and prominently increased during the progression of osteopenia to osteoporosis, suggesting that MCP-1 may serve as a potential biomarker for early postmenopausal osteoporosis diagnosis.

In conclusion, through protein array screening in ovariectomy postmenopausal osteoporosis models and validation in human samples, the present study identified Fractalkine, TIMP-1 and MCP-1 as potential candidate biomarkers for early postmenopausal osteoporosis diagnosis. Further verification of these biomarkers in clinical large-data trials will help to enable establishment of novel therapeutic strategies for readily evaluating and diagnosing postmenopausal osteoporosis at an early stage.

### Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant no. 81572192) and the Natural Science Foundation of Shannxi Province (grant no. 606308942,029).

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