RESEARCH ARTICLE | JANUARY 10 2019

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AIP Conf. Proc. 2058, 020008 (2019)
https://doi.org/10.1063/1.5085521

Correlation of the MMP-9 and Inhibitor-1, TNF-A and Bone Mineral Density in Patients with Chronic Obstructive Pulmonary Disease

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Abstract. Objective: to explore the Correlation of the Matrix Metallo Preteinases-9 (MMP-9) and inhibitor-1, TNF-α and bone mineral density in the chronic obstructive pulmonary disease (COPD) patients. Methods: 90 male patients diagnosed with COPD at the stable period were selected in this study got their BMD examined by dual energy X-ray absorptiometry (DXA). According to the results of the BMD, they were dived into normal group, low bone mass group and osteoporosis group each with 30 cases. Before the selection, we should record the history, pulmonary function, severity of the illness (CAT scores), and examined the MMP-9, TIMP-1, TNF-α and serum bone alkaline phosphatase (sBAP), serum osteocalcin (sOC), serum I type Collagen cross linked C terminal peptide (sCTX). Results: During the stage period, the BMI, CAT scores of the osteoporosis group, normal group and low bone mass group were significantly different from each other (all $P<0.01$); but the difference in the age, smoking indexes, FEV1/FVC, FEV1 % Pre was not statistically significant ($P>0.05$). The BMD in the lumbar vertebrae and neck of femur in the L group and O group were respectively lower compared with the normal group ($P<0.05$; $P<0.01$). Serum level of MMP-9, ratio of MMP-9/TIMP-1, TNF-α steppedly increased in the normal group, L and O group, especial the O group ($P<0.05$; $P<0.01$). Serum level of TIMP-1 stepdly increased in the normal group, L and O group, but it did not significantly ($P>0.05$). The sBAP, sOC in the L and O group were lower than the normal group ($P<0.01$), without statistical significance ($P>0.05$). The sCTX in the O group, was significantly higher than the normal group and L group, ($P<0.01$), but the different between the L group and N group were not statistically significant ($P>0.05$). The BMI in the lumbar vertebra and neck of the furmur, FEV1% Pre and MMP-9 were significantly negative correlative (respectively $r=-0.432$, $P<0.01$; $r=-0.697$ $P<0.01$; $r=-0.226$, $P<0.05$); the BMI in the lumbar vertebra and neckle of the furmur, FEV1% Pre and sBAP were significant positive correlative (respectively $r=0.418$, $P<0.01$; $r=0.702$, $P<0.01$; $r=0.295$, $P<0.01$); sCTX presents with a positive correlation ($r=0.338$, $P<0.01$; $r=0.574$, $P<0.01$; $r=0.278$, $P<0.05$); but TIMP-1 and TNF-α were not correlative significant. FEV1% Pre and MMP-9 were negative correlative ($r=-0.226$, $P<0.05$); ratio of MMP-9/TIMP-1 were negative correlative with the sBAP, sOC ($r=-0.525$, $P<0.01$; $r=-0.460$ $P<0.01$); positive correlation with the sCTX ($r=0.635$, $P<0.01$). TIMP-1 was significantly positive correlative with the sCTX ($r=0.236$, $P<0.05$), had no correlation with the sBAP, sOC ($P>0.05$). TNF-α was negative correlative with sBAP ($r=-0.350$, $P<0.01$), positive correlation ($r=370$, $P<0.01$). TNF-α was not significant with sOC ($P>0.05$). Conclusion: During the stable stage of the male COPD patients, the enlightened MMP-9, TNF-α were relative with the markers of bone turnover, playing an important role in the osteoporosis; the raised ratio of MMP-9/TIMP-1 and TNF-α might relative with the fasten bone metabolism.

Key words: Chronic Obstructive Pulmonary Disease; Bone Mass; Osteoporosis; Matrix Metallo Preteinases-9; Biological Markers in the Bone Transformation.

International Conference on Frontiers of Biological Sciences and Engineering (FBSE 2018)
AIP Conf. Proc. 2058, 020008-1–020008-8; https://doi.org/10.1063/1.5085521
Published by AIP Publishing. 978-0-7354-1786-1/$30.00

MATERIALS AND METHODS

General Information

90 male COPD patients at the stable stage during September 2010 to May 2012 were recruited in this study, the patients were diagnosed and graded according to the Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD). The female patients were out of the choice considering the the BIM might be affected in the postmenopausal women. And all the included patients were grouped into normal group, L group and O group each with 30 cases according to the BMD degree. And the cytokines were examined.

Inclusion criteria

The patients who were over forty year old, former smokers or had smoking history could be selected in this study.

Exclusion criteria

If the patients had one/ more of the following characteristics shall excluded:

(1) With severe cardiovascular disease, hepatorenal function failure, consciousness disturbance;

(2) With asthma, pulmonary tuberculosis, bronchial dilation, interstitial lung disease, pulmonary cystic fibrosis

(3) With skeletal system disease, like fracture, osteomalacia etc;

(4) With history of rheumatic diseases, such as rheumatoid arthritis, ankylosing spondylitis etc.

(5) Endocrine and metabolic diseases like hyperthyroidism, hyperparathyroidis

(6) The patients had given glucocorticoids by oral, intake or venous injecting within 3 months;

(7) Taking drugs that affect the skeletal metabolism within 1 year;

(8) With malignant tumor history within 5 years.

The general information and medical history were collected on the enrolled day of all the patients in the group, as well as the bone mineral density (BMD) measurement. 10 mL venous blood from the patient's elbow was collected as samples on the following morning. Evaluation on life quality of patients was tested by COPD Assessment Test (CAT).

Bone Mineral Density (BMD) Measurement

The BMD of the 1-4 lumbar spines and bilateral neck of femur were measured by the DelphiTM dual energy X-ray absorptiometry (DEXA) which was produced from the Hologic Company in America (Hologic Inc., Waltham, Massachusetts, USA) in all subjects. The instruments were required to receive the detection of human body vertebral body every weekday. The detection results showed that coefficient of variation (CV) of Area was within 0.52%, and the CV of BMD was less than 0.40%. BMD measurement results include two forms of absolute value and T value (standard deviations from the reference mean). According to the diagnostic standard of osteoporosis of the World Health Organization (WHO) (Kanis et al, 1994), the following diagnosis could be made on the basis of the T values: the 1-4 lumbar spines and femoral neck of the tested parts were compared with the peak bone mass of the same sex and same racial healthy adults. ① Normal bone mass: T value $\geq$ -1.0; ② Low bone mass: for any part of the subject, -2.5 < T value < -1.0; ③ Osteoporosis: for any part of the subject, T value $\leq$ -2.5. With this standard, the enrolled patients could be classified into the following 3 groups according to the actual test results: COPD normal bone mass group (30 cases), low bone mass group (30 cases), and osteoporosis group (30 cases).

Cytokines Detection

10 mL venous blood from the all the 90 patient's elbow was collected on the following morning, and then the venous blood was centrifuged at 1000 × g for 5 min at room temperature, with its supernatant extracted and stored in -80 ºC.

ELISA method was used to determine the MMP-9, TIMP-1, and TNF-α level (Catalog No.DMP900; DTM100; DTA00C; R&D Systems, Inc., USA). Variable coefficient < 10%. The sensitivity of tested MMP-9, TIMP-1, TNF-α were 0.156 ng/ml, 0.08 ng/ml, 1.6 pg/ml respectively.

ELISA method was also used to measure the sBAP, sOC, sCTX level (Osteometer Bio Tech, Shanghai Fengxiang biological technology co., LTD). The variable coefficient of tested sBAP, sOC, sCTX were all < 10%.

The indicators detecting process must be strictly in accordance with the operating standards within the kit, the specific steps were as follows:

1. Dilution of standard substance (Table 1)

TABLE 1. Dilution of standard substance

Number	ng/ml	Amount of standard substance	Sample Diluents
1	40	0.5 ml form No. 1 tube	1 ml
2	20	0.5 ml form No. 2 tube	0.5 ml
3	10	0.5 ml form No. 3 tube	0.5 ml
4	5	0.5 ml form No. 4 tube	0.5 ml
5	2.5	0.5 ml form No. 5 tube	0.5 ml
6	1.25	0.5 ml form No. 6 tube	0.5 ml

(1) Adding 100 ul standard substance and sample into each hole in turn;
(2) Incubation for 2 h at 37 ºC;
(3) Discarding the liquid without washing the board;
2. Adding second antibody
(1) The secondary antibody was diluted with bound diluent, and the dilution ratio was 1:100;
(2) Adding 100 ul diluted second antibody in each hole;
(3) Incubation for 1 h at 37 ºC;
(4) Washing the board for three times with washing machine.
3. Adding enzyme-labelled conjugate
(1) Diluting with enzyme-labelled conjugate dilution, and the dilution ratio was 1:100
(2) Adding 100 ul diluted second antibody in each hole;
(3) Incubation for 1 h at 37 ºC;
(4) Washing the board for four times with washing machine.
4. Color reaction
(1) Adding 90 ul TMB Substrate in each hole;
(2) Incubation for 10-30 min at 37 ºC until there was obvious color change in standard substance;
(3) Adding 50 ul stop solution in each hole;
(4) Detecting with 450 nm wavelength, and then making a 4-PL curve chart. The corresponding sample concentration value was obtained

Statistical Methods

SPSS 13.0 (SPSS Inc, Chicago, USA) statistical analyzer was used for data analysis, and the measurement data was expressed with $\bar{x} \pm s$. Normality test and correlation analysis was tested by Shapiro-Wilk. One way ANOVA method was used to analyze the comparison of normal distribution of measured data between groups. Pearson analysis was used to analyze the correlation between parameters. $P<0.05$ meant that the difference was statistical significant.

RESULTS

The Comparison between General Clinical Data and Bone Mineral Density Measurement Results (Table 2)

In the stable stage of COPD, the age, BMI, smoking index, FEV1/FVC, the FEV1 percentage of predicted value, CAT score of the patients in three groups were compared between two groups. The results showed that: compared with normal bone mass group and low bone mass group, there was significant difference of BMI and CAT score in osteoporosis group ($P<0.01$); while there was no statistical significance in the age, smoking index, FEV1/FVC and FEV1 %Per ($P>0.05$). The lumbar BMD in low bone mass group and osteoporosis group were significantly decreased compared with normal bone mass group, with statistical significance ($P<0.05$, $P<0.01$). The femoral neck BMD in low bone mass group and osteoporosis group were significantly decreased compared with normal bone mass group, with statistical significance ($P<0.05$, $P<0.01$).

TABLE 2. Comparison of general clinical data of CPOD patients (90 cases) ($\bar{x} \pm s$)

	Normal bone mass group	Low bone mass group	Osteoporosis group	F-value	P-value
Age	64.10±10.13	66.07±11.45	68.33±9.68	1.233	1.296
FEV1/FVC	57.29±7.57	54.13±8.59	54.05±5.87	1.854	0.163
FEV1% Pre	55.99±7.57	54.95±7.76	52.65±7.12	1.539	0.220
Smoking index	32.23±6.62	33.77±6.64	35.70±7.67	1.851	0.163
BMI	27.27±6.44	24.04±3.11**	20.57±2.94**^^	16.942	0.000
CAT score	15.50±4.33	19.73±5.06**	24.83±5.25**^^	27.307	0.000
BMD lumbar vertebrae	1.03±0.20	0.87±0.44*	0.69±0.15**^	9.994	0.000
BMD femoral neck	0.95±0.22	0.75±0.16**	0.59±0.11**^^	31.557	0.000

Note: Compared with normal bone mass group, *P<0.05, **P<0.01;
Compared with low bone mass group, ^P<0.05, ^^P<0.01.

Comparison of Serum MMP-9/TIMP-1, TNF-A and Biochemical Markers of Bone Turnover Between the Three Groups in COPD Stable Stage

There was significant difference in serum MMP-9 level between the three groups (P<0.05). The serum MMP-9 level was increased in turn in COPD normal bone mass group, low bone mass group, and osteoporosis group. The increase in osteoporosis group was the most significant.

The serum TIMP-1 level was increased in turn in COPD normal bone mass group, low bone mass group, and osteoporosis group, the difference was not statistically significant.

The MMP-9/TIMP-1 was increased in turn in COPD normal bone mass group, low bone mass group, and osteoporosis group. There was a significant difference in osteoporosis group than in the normal bone mass group.

There was significant difference in serum TNF-α level between the three groups (P<0.05). The serum TNF-α level was increased in turn in COPD normal bone mass group, low bone mass group, and osteoporosis group. The increase in osteoporosis group was the most significant.

The serum sBAP level in the COPD group and the low bone mass group was lower than that in the normal bone mass group (P<0.01), but there was no significant difference between the low bone mass group and the osteoporosis group (P>0.05). (Figure 1)

The serum sOC level in the COPD group and the low bone mass group was lower than that in the normal bone mass group (P<0.01), but there was no significant difference between the low bone mass group and the osteoporosis group (P>0.05). (Figure 1)

Compared with normal bone mass group and low bone mass group, the serum sCTX level in osteoporosis group was increased, with significant difference. While there was no significant difference between the low bone mass group and the normal bone mass group. (Figure 2)

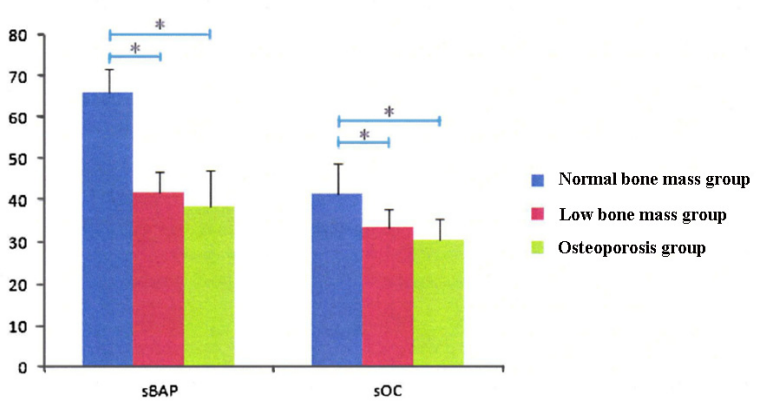

FIGURE 1. The comparison of serum sBAP, sOC level between the three groups (*: P<0.05).

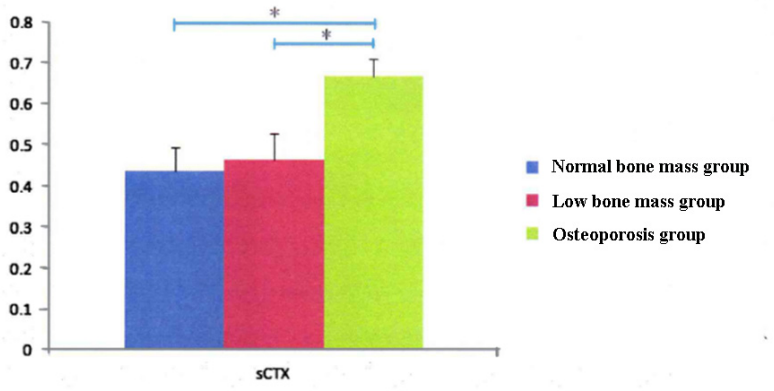

FIGURE 2. The comparison of sCTX level between the three groups (*: P<0.05)

Correlation Analysis Between BMD and Serum MMP-9/TIMP-1, TNF-A, Sbap, Soc and Sctx Level

Pearson analysis showed that: The BMD of lumbar spine, femoral neck density were significant negatively correlated with serum MMP-9 (r=0.432, P<0.01; r=0.697, P<0.01); The BMD of lumbar spine, femoral neck density were significant positively correlated with sBAP (r=0.418, P<0.01; r=0.702, P<0.01), and positively correlated with sOC (r=0.338, P<0.01; r=0.574, P<0.01), and negatively correlated with sCTX (r=0.418, P<0.01; r=0.602, P<0.01), while no significant correlation with TIMP-1 and TNF-α.

Correlation Analysis Between FEV1% Pre and Serum MMP-9/TIMP-1, TNF-A, Sbap, Soc and Sctx Level

Pearson analysis showed that: there was a negative correlation between FEV1% Pre and serum MMP-9 level (r=0.226, P<0.05); there was a positive correlation between FEV1% Pre and sBAP and sOC (r=0.295, P<0.01; r=0.278, P<0.01), and a negative correlation between FEV1% Pre and sCTX (r=0.243, P<0.05), and no significant correlation with other cytokines (TIMP-1, TNF-α).

Correlation Analysis between BMD, FEV1% Pre and Other Parameters

Pearson analysis showed that: FEV1% Pre was significant positively correlated with BMD of lumbar spine and femoral neck density (r=0.338, P<0.01; r=0.409, P<0.01); MMP-9/TIMP-1 was significant negatively correlated with sBAP and sOC (r=0.525, P<0.01; r=0.460, P<0.01), and positively correlated with sCTX (r=0.635, P<0.01); TIMP-1 was positively correlated with sCTX (r=0.236, P<0.05), and no significant correlation with sBAP, sOC (P>0.05); TNF-α was significant negatively correlated with sBAP (r=0.350, P<0.01), and positively correlated with sCTX (r=0.370, P<0.01); There was no significant correlation between TNF-α and sOC (P>0.05).

DISCUSSION

This research found that there was significant difference in serum MMP-9 level between the three groups (P<0.05). The serum MMP-9 level in COPD normal bone mass group, low bone mass group and osteoporosis group was increased in turn, and the increase in osteoporosis group was the most significant; there was a significantly negative correlation between serum MMP-9 level and FEV1% Pre (r=0.36, P<0.05); there was a significantly negative correlation between serum MMP-9 level and BMD of lumbar spine, femoral neck density (r=0.58, P<0.01; r=0.62, P<0.01). Higashimoto et al and Olafsdottir had reported that MMP-9 was negative correlated with FEV1 (r=-0.28, r=-0.11, P<0.01) (Higashimoto et al, 2009; Olafsdottir et al, 2010). In terms of the influence of osteoporosis, our results were consistent with Bolton studies (Bolton et al, 2004), which in his study, the levels of MMP-9 expression in patients with osteoporosis and healthy control group were detected and found that the level of MMP-9 in patients with

osteoporosis was significantly higher than that in healthy control group, while TIMP-1, 2 had no significant difference. Our results also conform to Qin's study (Sin et al, 2003), which found that the level of serum MMP-9 increased with the decrease of bone mineral density in postmenopausal women with osteoporosis. Through the analysis of tibial metaphyseal trabecular bone of 16 week old female wild mouse, Some researchers found that deletion of MMP-9 increased trabecular connection density, MMP-9 and MMP-2 high expression in the wild mouse vertebral trabecular bone thinning became thining, the number of trabecular bone decreased, the osteoporosis occurred, and femoral strength was also lower than that of normal wild-type mice (Leech et al, 1990).

This research also found that the expression of MMP-9 / TIMP-1 in COPD normal bone mass group, low bone mass group and osteoporosis group increased in turn, and the difference was significant between the osteoporosis group and the normal bone mass group ($P<0.05$). When the occurrences of osteoporosis, with the activation of osteoclasts, MMP-9 appeared a high expression with it, and promoted bone resorption. While there was no corresponding increase in TIMP-1 produced by osteoblast, leading to the imbalance of MMP-9/TIMP-1 ratio and increased activity of osteoclasts, while the activity of osteoblasts was relatively weakened, the bone formation slowed down, and irreversible bone loss occurred, resulting in osteoporosis. The imbalance of MMP-9 / TIMP-1 may be one of the important causes of COPD combined with osteoporosis.

In this research, the serum TNF-α level in COPD patients combined with osteoporosis were significantly higher than those in normal and osteopenia groups, with statistical significance ($P<0.05$); there was a significant positive correlation between the TNF-α and MMP-9 ($r=0.35$, $P<0.05$), Which, to some extent, confirmed the existence of COPD systemic inflammation. TNF-α was an already clear COPD systemic inflammatory factors, and the occurrence and development of multiple COPD systemic complications including osteoporosis were related with it. TNF-α was a very important inflammatory factor, which had a variety of biological functions, including regulatory response, immune response, apoptosis and anti-viral response, etc. TNF-α was also an important regulator of bone metabolism and remodeling, which might stimulate osteoclast differentiation through synergistic action. In the skeletal system, TNF-α might cause osteoporosis by a variety of ways, which could inhibit the deposition of extracellular matrix and stimulate the synthesis of matrix metalloproteinases, thereby to degrade the organic bone. And it also could promote the expression of osteolytic cytokines, such as macrophage colony stimulating factor (M-CSF), hence to stimulate the generation of osteoclast; TNF-α could inhibit the osteoblast to compound type I collagen, while estrogen could inhibit the release of TNF-α in osteoblasts.

Research of Kjensli et al found that inflammatory cytokines IL-6, TNF turned up an increased trend in the phase of damaged disease and a decreased trend in the recovery phase (Kjensli et al, 2007). Some foreign studies showed that there was a clear correlation between multiple serum inflammatory cytokines, including IL-1, IL-6, and TNF-α, and quantitatively assessed severity of emphysema under high-resolution CT. Meanwhile, inflammatory cytokines TNF-α had been proved to be important regulators of bone metabolism and remodeling. Inflammatory factors could also act directly on osteoclasts and their precursor cells, which could result in the enhancement of osteoclast activity, cause and accelerate bone destruction and bone loss.

This study showed that: the serum BAP, OC level in osteoporosis group and low bone mass group were all lower than that in the normal bone mass group ($P<0.01$), while there was no significant difference between the low bone mass group and osteoporosis group ($P>0.05$); compared with normal bone mass group and low bone mass group, the serum sCTX in osteoporosis group increased, with significant difference ($P<0.01$), while there was no significant difference between the normal bone mass group and low bone mass group ($P>0.05$); There was no significant correlation between the BMD of lumbar spine, femoral neck density and TIMP-1, TNF-α; TNF-α were negative correlated with sBAP, and positive correlated with sCTX.

The serum MMP-9 and TNF-α were consistent with the change of sCTX; BMD of lumbar spine, femoral neck density were significantly negative correlated with serum MMP-9 and sCTX, and also positive correlated with sBAP and sOC, which indicated that when COPD patients was combined with osteoporosis, the body was in the high conversion state of bone resorption and bone formation, and the function of osteoclast and osteoblast were active. It also suggested that there was a good correlation between the serums MMP-9, TNF-α and sBAP, sOC, sCTX.

FEV1% Pre was significantly negative correlated with MMP-9 ($r=0.226$, $P<0.05$); FEV1% Pre was significantly positive correlated with sBAP, sOC ($r=0.295$, $P<0.01$; $r=0.278$, $P<0.01$), and negative correlated with sCTX ($r=0.243$, $P<0.05$); FEV1% Pre was no significant correlated with other cytokines (TIMP-1, TNF-α).

Normal human bone metabolism included two complex processes, bone resorption and bone formation. In the period of bone formation, osteoblasts first developed into osteoid and then mineralized into new bone. In the period of bone resorption, osteoclasts removed the minerals from old bones. Bone metabolism made bone formation and bone resorption in a state of dynamic equilibrium under the control of many factors. When this dynamic balance was out-of-balance, it will result in the occurrence of different diseases of the skeletal system. sBAP, sOC, sCTX were the

good marker for bone resorption and osteoclast activity ever found. The results of this study showed that, compared with the control group, the bone formation index of serum sBAP and sOC level of COPD group was significantly decreased, and the bone resorption index CTX was significantly increased. BMD of lumbar spine, femoral neck BMD were significantly negatively correlated with serum MMP-9, bone resorption index sCTX, and was significantly positively correlated with bone formation index sOC, sBAP, which indicated that there was a bone metabolism disorder in elderly patients with COPD, its mechanism might be related to the enhancement of bone resorption and the weakening of the formation of new bone, which also showed the consistency of BMD and stock conversion markers.

The level of serum inflammatory factor TNF-α in osteoporosis group were significantly higher than those in the normal group and the low bone mass group, with statistical significance ($P<0.05$); TNF-α was significantly positively correlated with MMP-9. MMP-9 was the major enzyme that regulated ECM degradation and synthesis. COPD was a kind of chronic bronchitis or emphysema with airflow obstruction, while airway wall fibrosis, hyperplasia and hypertrophy of mucous glands, bronchial smooth muscle hyperplasia and progressive destruction of alveolar wall were the main pathologic features of airway remodeling in COPD. Under normal conditions, the synthesis and degradation of ECM maintained homeostasis, and the balance of MMP and tissue inhibitor of mental protein (TIMP) was the decisive factor to maintain the stability of the ECM. MMP-9 was a non-glycosylated protease, mainly expressed in the cytoplasm of fibroblasts, osteoblasts, vascular endothelial cells and some osteoclasts. However, MMP-9 and other cytokines were involved in COPD bronchial, alveolar wall damage and subsequent airway remodeling process. The imbalance of MMP-9/TIMP-1 was an important mechanism of airway remodeling. Studies had found that the positive expression rate of serum MMP-9 in postmenopausal women with osteoporosis was significantly increased, and it was also correlated with biochemical indexes of bone metabolism and BMD (Vrieze et al, 2007).

For COPD patients, due to the airway inflammation, inflammatory cells would increase the relaese of MMP-9 and then enter the blood circulation. At the same time, a large number of inflammatory cytokines, such as TNF, were released, which not only had an effect on alveolar extracellular matrix, but also on the bone matrix, leading to the imbalance of OPG/RANK/RANKL system, the increased in RANKL/OPG ratio, and the activation of osteoclasts, the high expression of MMP-9 and the increased in bone matrix absorption, and finally, the osteoporosis occurred (Cote et al, 205). The release of MMP-9 from osteoclasts entered into the circulation, aggravated the imbalance in the ratio of MMP-9 / TIMP-1 in the lungs, and increased the degradation of extracellular matrix and the destruction of the structure of the lung, resulting in increased pulmonary emphysema. MMP-9 could not only degrade the ECM of alveolar wall, but also promote inflammatory cell chemotaxis, migration and accumulation in the airway, further aggravate the inflammatory reaction, increase the airway damage, causing irreversible airflow obstruction. COPD airway inflammation and systemic inflammation, osteoporosis might form a vicious circle, leading to deterioration of the patient's condition.

BMD measurement was to evaluate the stage performance of bone mineral metabolism. Bone biochemical indexes were used to evaluate the bone metabolism indirectly, which can be more sensitive and timely to reflect bone metabolism, and the prediction, differential diagnosis, prevention, observation and treatment of osteoporosis were also essential. The clinical effect of bone turnover markers detection was an important auxiliary means for the clinical diagnosis of various metabolic bone diseases, which could prompt people to constantly explore new and more specific markers of bone turnover. Bone formation index was mainly the by-products produced by the process of bone formation and secreted enzymes. BAP was a commonly used bone formation index and a glycoprotein produced by osteoblasts, which could indirectly reflect the rate of bone formation; Osteocalcin was a specific index that reflected the coupling or uncoupling of bone formation and bone resorption. Increased osteocalcin (OC) concentration meant that the process of bone formation was stimulated, while decreased OC concentration meant that suggested that the bone absorption process was slowed. The specificity of the bone absorption index CTX could be used to monitor the patient's response to therapy.

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