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A Case control study to determine Macrophage migration inhibitor, and N-telopeptides of type I bone collagen Levels in the sera of osteoporosis patients

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Abstract:

This study focused on determining the markers of Macrophage migration inhibitor (MIF), as well as the N-telopeptides of type I bone collagen (NTX), and some other parameters (alkaline phosphatase (ALP), vitamin D (Vit D), calcium (Ca), phosphorus (P), and magnesium (Mg), and their correlation with other parameters in osteoporosis. One hundred ten subjects were involved in the current study. There were two groups of patients: group I (30) women with severe osteoporosis and group II (30) women with mild osteoporosis. For comparison, 50 apparently healthy individuals were included as a control. Serum levels of MIF, and NTX were significantly higher in groups I and II as compared to the control group, which indicate that these two parameters were related to disease. Moreover MIF, and NTX were organized in one cluster when applying cluster analysis test to all the studied groups. This indicates that in most of the studied samples the two parameters were related to each other as well as to osteoporosis. Magnesium showed a significant decrease in its level in both groups as compared to the control. On the other hand, alkaline phosphatase (ALP) showed a significant increase in its activity in both studied groups as compared to the control. Vitamin D level manifested significant difference between group I and group II, with a significant decrease in its level when comparing group II with the control group. The MIF, NTX was highly associated with osteoporosis patients, in addition to Mg and Vit-D. On the other hand, Ca and P levels did not alter in a significant way with osteoporosis which may be considered as a risk factor as long as they are organized in one cluster with MIF, NTX, Mg, and Vit D in all the studied patients. Both markers showed a clear cut-off value using the ROC curve in which the best cutoff value of NTX was 166.8 pg/ml, and the best cutoff value of MIF was 6.6 ng/ml according to ROC analysis.

Keywords: Alkaline phosphatase, Bone, Macrophage migration inhibitor, N-telopeptides of type I bone collagen, Osteoporosis, Vitamin D.

Introduction:

Osteoporosis is characterized by the World Health Organization (WHO) as a progressive systemic skeletal disorder identified by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and fracture susceptibility. The perfect standard for osteoporosis diagnosis is dual-energy X-ray absorptiometry (DXA), checked by bone mineral density (BMD) [1–3]. Several parameters play a critical role in bone metabolism such as the macrophage migration inhibitor (MIF). Macrophage migration inhibitor is a pro-inflammatory cytokine that prevents macrophages from the random motion. T-lymphocytes release was the first explanation for this factor. Since then, however, several studies have shown several other forms of cells, including epithelial cells, macrophages, and endothelial cells, synthesize MIF. MIF has been known for a long time to be involved in inflammatory disorders, such as systemic lupus erythematosus, psoriasis, atherosclerosis, and diabetes. Multiple studies have shown that over-expression of MIF has occurred in different tumors, such as lung, colorectal, breast, and prostate. Overall, by regulating both cell proliferation and invasiveness, over-expression appears to play a large function in tumor growth [4,5]. Macrophage migration inhibitor is a cytokine that is expressed by immune as well as non-immune

cells, this is well known for its prophylactic special effects and is regarded as a negative regulator of immunosuppressive glucocorticoid actions [6]. The progression of many acute inflammatory and autoimmune disorders has also been impaired by MIF [7], and a few chronic inflammatory metabolic conditions, too. Through the action of their enzymatic tautomerase and oxidoreductase, MIF can exert inflammatory effects [8]. In addition to controlling inflammation, several lines of evidence suggest that MIF may also be related to energy metabolism. It is reflected in adipose and liver metabolism [9,10]. Other markers are involved in osteoporosis like N-telopeptides of type I bone collagen NTX.

This marker is a marker for bone resorption derived from collagen I [11]. Collagen type I is synthesized as a procollagen type I, which makes up 90% of bone protein. The amino-terminal [Terminal N-propeptide of type I collagen (P1NP)] and carboxy-terminal propeptide (P1CP) cleavage occur during extracellular treatment of type I procollagen [12]. The benefit of using NTX in everyday usage is that it is less sensitive than other bone turnover manufacturers to circadian shifts and food intake. High NTX shows a bone resorption increase. Studies have shown that NTX, apart from a bone resorption marker, is an effective predictor of postmenopausal female fracture risk [13]. The aim of the study to investigate the link of serum MIF, and NTX with osteoporosis, and the involvement of other routine parameters.

Materials and Methods:

Patients

Levels of MIF and NTX levels in patients with osteoporosis in Iraq were determined in a case-control study. One hundred ten individuals with an age range between 40-55 years were registered in this study. 60 Iraqi patients with osteoporosis were recruited between November 2019-March 2020 from Baghdad Teaching Hospital, DXA Unit/ Baghdad /Iraq. There were two groups of patients in this study: group I included 30 individuals who were diagnosed with severe osteoporosis, and group II included 30 individuals with mild osteoporosis. These patients were without heart, liver, kidney, and hypertension diseases. Individuals who were apparently healthy and free of acute illness were chosen as controls(n=50). Smoker and drinker individuals were excluded from this study. Body mass index (BMI) was calculated for the three studied groups.

Samples:

A venipuncture with a 10 ml disposable syringe was used to obtain 10 ml of blood., then the blood was collected into a gel tube to collect serum (after clotting, at room temperature, blood was centrifuged to 3000 rpm and then separated, divided into five aliquots and stored at -20-degree C until tested).

Determination of osteoporosis-related parameters:

Serum of MIF and NTX were assessed by sandwich enzyme immunoassay technique, using the kit supplied by Shanghai biological, China. Colorimetric methods were used for measuring serum levels of Magnesium, calcium, Inorganic phosphorus. as well as the activity of alkaline phosphatase. Vitamin D was measured using an electric chemiluminescent protein test.

Statistical analysis

A statistical analysis SPSS 26 was carried out. Log transfer data were used. The main findings were described using a general descriptive statistic, where the groups were compared using a one-way analysis of variance test. An independent T test was also used. Dendrogram and Multiple Regression were used to identify the similarity between variables. Also, the receiver operating characteristic curve (ROC) analysis was used to find the cutoff value for the markers.

Results and Discussion:

The sample population's anthropometric and biochemical characteristics are listed in Tab 1. The results showed a significant difference between patient's groups [severe osteoporosis, mild osteoporosis] and the control group. T-score % was significantly increased in women with the severe osteoporosis group when compared to the mild osteoporosis group and control group, and they were significantly increased with mild osteoporosis group when matched with healthy women. Activity of ALP was significantly increased in group I and II when compared to control. According to One way ANOVA test Vit D showed a significant difference between group I and group II, as well as to significant decrease in its level in group II as compared to control. At the same time when one-way ANOVA was run on Mg data, the result showed a significant decrease in women with the severe osteoporosis group and mild osteoporosis when compared to the control group. In contrast, BMI, P, and Ca showed no significant difference between patients groups [severe osteoporosis, mild osteoporosis] and control group Tab1.

Table 1. Clinical data in patients having osteoporosis (GI severe osteoporosis, GII mild osteoporosis) and control group.

Parameter	GI Severe osteoporosis [Mean±SE] (n=30)	GII Mild osteoporosis [Mean±SE] (n=30)	Control [Mean±SE] (n=50)	p-value
Age(year)	47.56 ± 2.26 a	46.83 ± 2.34b	42 ± 0.98	<0.005*
BMI(Kg/m^2)	31.22±1.17	29.58±1.65	31.99± 1.03	0.393
ALP (U/L)	110.06±10.55 a	98.61±10.24 b	64.58±3.68	< 0.001*
P (mg/dl)	3.36± 0.16	3.43±0.14	3.64±0.091	0.25
Ca (mg/dl)	8.61±0.15	8.73±0.13	8.71±0.11	0.81
Vit D(ng/ml)	19.74±0.040 c	12.61±0.040 b	22.97±0.05	<0.01*
Mg (mg/dl)	1.309±0.10a	1.505±0.08 b	2.11±0.04	<0.001*
T-score %	32.10±1.36 a,b,c	21.26±0.60 a,b,c	7.02±0.70 a,b,c	<0.001*

*** The significant difference between the three separate ways of using the 0.05 level ANOVA measure.**
a) Point to a significant difference between Group I and control
b) Point to a significant difference between Group II and control
c) Point to a significant difference between GroupI and GroupII

The results of the two studied parameters (MIF and NTX) are presented in Tab 2. Significant decrements in MIF, and NTX levels were found when compared to svere osteoporosis, and mild osteoporosis with control (p< 0.001), although no significant differences were found between the patient groups themselves Tab 2.

Table 2. MIF, and NTX levels severe osteoporosis, mild osteoporosis patients and control.

Parameter	Group I Severe osteoporosis [Mean±SE] (n=30)	Group II Mild osteoporosis [Mean±SE] (n=30)	Control [Mean±SE] (n=50)	p-value
MIF (ng/ml)	4.56±0.07 a	4.90±0.06 b	8.71±0.04	<0.001*
NTX (pg/ml)	120±0.07a	106.5±0.06 b	194.98±0.05	<0.001*

*** The significant difference between the three separate ways of using the 0.05 level ANOVA measure.**
a) Point to a significant difference between Group I and control
b) Point to a significant difference between Group II and control
c) Point to a significant difference between Group I and Group II

Macrophage migration inhibitor is known as an osteoclastogenesis inhibitor which reduces both the number of precursing osteoclast cells and the fusion into the mature multinucleated osteoclasts of mononuclear precursors. So, the reduction in MIF level indicated the severity of osteoporosis. In osteoarthritis, MIF may have a protective function [14]. Our findings conflict with the study conducted by Kim et al that showed that plasma MIF levels in the lumbar spine and proximal femur are inversely correlated with BMD and showed that MIF may be a potential biomarker of human bone metabolism [15].

The physiological function of MIF is to counteract the inhibiting effects of steroids on inflammatory and immune reactions. MIF is an inflammation-inducing cytokine that, when produced, induces the release of other cytokines, for example, IL-1 β, TNF-α, IFNγ, and IL-6 from macrophages that cause a rapid immune response [16,17].

The decrease of NTX level in our study agrees with some reviews and disagrees with others. Serum NTX has been determined, although most studies evaluate NTX in urine because NTX is stable in urine at room temperature for up to 24 h and is not impaired by food intake, also prevents intrusive blood-associated venipuncture and may be preferred by patients [18].

We determined NTX in serum because of the difficulty of keeping the urine sample for a long period until the time of its measurement. This difference in outcome may be due to pre-analytical variables such as bone resorption with significant circadian variation and NTX serum concentration peak early in the morning between midnight and 8 a.m. with a nadir in the afternoon. Seasonal variations in bone turnover are often noted, with a peak in bone remodeling occurring during the winter months, although the degree of pairing varies with premenopausal women with the highest seasonal variation [18,19]. The urinary markers were

more easily affected by bone fractures, Kenji Takahara et al noticed [20]. And bone resorption markers, such as urinary NTX and serum NTX, showed a peak increase within 3 to 5 weeks of vertebral fracture [21].

The cluster analysis of Multivariate

The purpose of the Cluster Analysis is to group variables. The tool aims to achieve group variables by searching for variables that are related or dependent on others, placing them together in a cluster or section, and separating them from other variables that are different from each other. One of the most traditional cluster analysis is wards method. This method is typically displayed using a dendrogram. In this test, there is no prior assumption of the clustering making the cluster analysis be used to discover the similarities via studied variables. According to coefficients, the variables in all studded groups are distributed in 2 clusters with mild shifting in some groups. Fig 1: A describes the multivariable cluster analysis using osteoporosis patients' data. The results identified the variables as classified into 2 clusters. The first cluster included the most well-known diagnostic tests Vit D, and Mg, as well as MIF, NTX, T-score. Cluster two included ALP. A fusion of the first cluster with the second one taking place to form a single group. Fig 1 :B describes the multivariable cluster analysis using mild osteoporosis data. The results identified the variables as classified into 2 clusters. The first cluster included T-score, NTX, Mg, Vit D, MIF, P, and Ca. Cluster two included ALP. Fig 1:C describes the multivariable cluster analysis using control data. The results identified the variables as classified into 2 clusters. The first cluster included Mg, NTX, P, T-score, MIF, Vit D, and Ca. Cluster two included ALP.

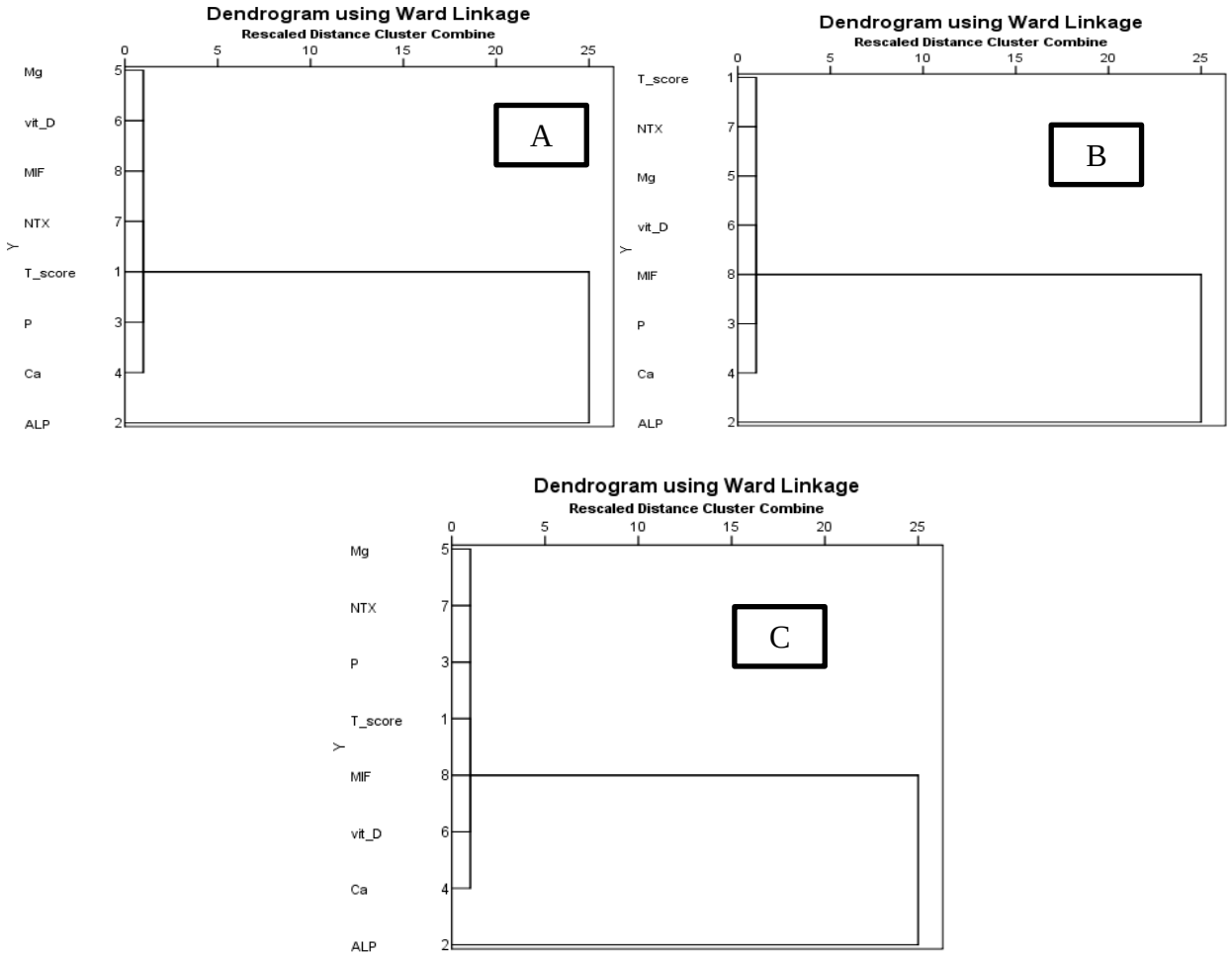

Figure 1. The cluster analysis of Multivariate for all parameters for : [A] Severe Osteoporosis patients, [B] Mild osteoporosis patients,and [C] control.

Multiple regression of studies groups

The multivariate regression method was used to identify the degree of association of the independent variables to the dependent variable. Multivariate analysis revealed that the significant predictor of T-score in severe osteoporosis and mild osteoporosis were p, and Ca respectively as shown in Tab 3 .

Table 3. Multiple regression of ALP, Phosphorus, calcium, vitamin D, magnesium, N-telopeptides of type I bone collagen, and Macrophage migration inhibitor with dependent variable T- score % for patient's groups and control.

Parameter	Group1 Severe osteoporosis	Group2 Mild osteoporosis	control
ALP	Not dependent	Not dependent	Not dependent
P	↑	Not dependent	Not dependent
Ca	Not dependent	Not dependent	↑
Vit D	Not dependent	Not dependent	Not dependent
Mg	Not dependent	Not dependent	Not dependent
NTX	Not dependent	Not dependent	Not dependent
MIF	Not dependent	Not dependent	Not dependent
↑↑↑, $3 \leqq t < 3.5$ and $p < 0.05$			
↑↑, $2.5 \leqq t < 3.0$ and $p < 0.05$			
↑, $2 \leqq t < 2.5$ and $p < 0.05$			
↓, $-2.5 \leqq t < -2$ and $p < 0.05$			
↓↓, $-3 \leqq t < -2.5$ and $p < 0.05$			
↓↓↓, $-3.5 \leqq t < -3$ and $p < 0.05$			

The study shows dependence between Phosphorus and T-score% ($t < 2.5$ and $p < 0.05$) in severe osteoporosis group and no dependence between Phosphorus and T-score in mild osteoporosis group, in contrast, depended in Ca ($t < 2.5$ and $p < 0.05$) with T-score% in the control group, As shown in Tab 3.

Receiver Operating Characteristic (ROC)

Receiver Operating Characteristic curve is a statistical model that is designed to find the sensitivity and specificity which are appropriate with a diagnostic test via a plot concern with the relation between sensitivity versus 1-specificity. The cut-off points estimation to know whether the test results are positive, which are corresponding to numerous points on the plotted curve [22,23].

Table 4. The ROC curve for MIF, and NTX between patients and control.

Marker	AUC	95%CI AUC	Cut-off value	P-value
NTX	0.697	0.595-0.799	166.8 pg/ml	0.001
MIF	0.713	0.616-0.809	6.6 ng/ml	0.001

The area under the ROC curve (AUC) for the MIF was 71% the Receiver Operating Characteristic (ROC) curve, and 95 percent CI AUC is 0.616-0.809 while it is found to be the best cut-off point at 6.6 ng / ml . This means that the test value higher than 6.6 ng/ml considers healthy conditions whereas the value is less than 6.6 ng/ml represents the abnormal case as shown in Tab 4, Fig 2. As mentioned ROC curve the AUC for the NTX was 0.69%, and 95%CI AUC is 0.595-0.799 while the best cut–off point was found to be 166.8 pg/ml. This means that the test value higher than (166.8 pg/ml) considers healthy conditions whereas the value is less than 166.8 pg/ml represents the abnormal case as shown in Tab 4, Fig 3 .

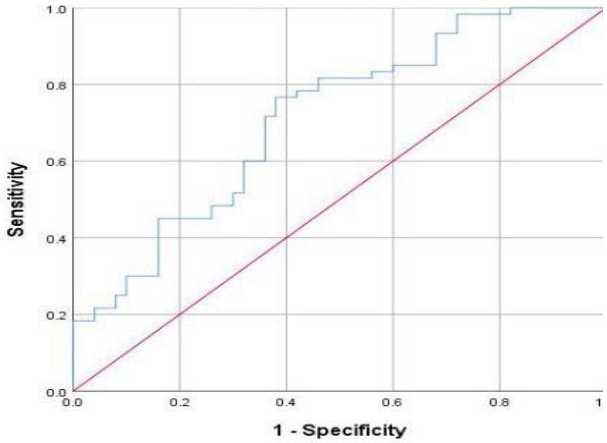

Figure 2. The Roc curve for Macrophage migration inhibitor.

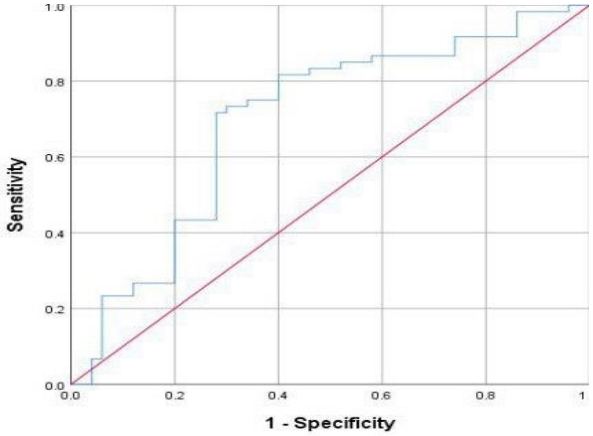

Figure 3. The Roc curve for N-telopeptides of type I bone collagen.

Conclusion:

The two main parameters in the current study (MIF, NTX) are highly associated with osteoporosis patients, in addition to Mg, and Vit-D. On the other hand, Ca, and P levels are alterated significantly with osteoporosis which may be considered as a risk factor as long as they are organized in one cluster with MIF, NTX, Mg, and Vit D in all the studied patients. The best cutoff value of NTX is 166.8 pg/ml, and the best cutoff value of MIF is 6.6 ng/ml according to ROC analysis.

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- The author has signed an animal welfare statement.
- Authors sign on ethical consideration's approval
- Ethical Clearance: The project was approved by the local ethical committee in University of Baghdad.

Authors' contributions:

Layla Othman Farhan: conceptualisation, analysis, Visualization, curation of data, study, writing – original draft.

Ekhlass M. Taha: conceptualization, testing, project management, data analysis, writing-review & editing, Resources, visualization.

Ahlam M. Farhan: Conceptualization, Analysis, Resources, Visualization, Editing Writing.

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دراسة مراقبة الحالة لتحديد مثبط هجرة البلاعم ومستويات التلوببتايد النوع الاول – كولاجين في مصل مرضى هشاشة العظام

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الخلاصة :

ركزت هذه الدراسة على تحديد علامات مثبط هجرة البلاعم (MIF) ، بالإضافة إلى N-telopeptides من النوع الأول من كولاجين العظام (NTX) ، وبعض المعلمات الأخرى (الفوسفاتيز القلوي (ALP) ، فيتامين د (فيتامين د) ، الكالسيوم (Ca) والفوسفور (P) والمغنيسيوم (Mg) وارتباطهم بالمعايير الأخرى في مرض هشاشة العظام. شارك في الدراسة الحالية (110) شخص. كانت هناك مجموعتان من المرضى في هذه الدراسة: المجموعة الأولى (30) امرأة يعانون من هشاشة العظام الشديدة والمجموعة الثانية (30) من النساء المصابات بهشاشة العظام الخفيفة. تم تضمين 50 فردًا سليمًا كمجموعة تحكم . تم تقدير MIF و NTXباستخدام مقايسة الممتز المناعي المرتبط بالإنزيم (ELISA). مستويات المصل من MIF و NTX كانت أعلى بشكل ملحوظ في المجموعتين الأولى والثانية مقارنة بالمجموعة الضابطة، مما يشير إلى أن هذين المعيارين مرتبطان بالمرض، علاوة على ذلك تم تنظيم MIF و NTX في مجموعة واحدة عند تطبيق اختبار التحليل العنقودي في جميع المجموعات المدروسة، وهذا يشير إلى أنه في معظم العينات المدروسة ارتبطت المعلمتان ببعضهما البعض وكذلك مرتبطة بنقص العظم. أظهرت كلتا العلامات قيمة قطع واضحة باستخدام منحنى ROC. أظهر المغنيسيوم انخفاضًا معنويًا في مستواه في كلا المجموعتين مقارنةً بمجموعة التحكم. بينما أظهر الفوسفاتيز القلوي (ALP) زيادة معنوية فيه فهو نشاط في كلا المجموعتين المدروستين مقارنة بمجموعة التحكم. ظهر اختلاف معنوي في مستوى فيتامين (د) بين المجموعة الأولى والمجموعة الثانية، مع انخفاض معنوي في مستواه عند المقارنة بالمجموعة الثانية مع مجموعة السيطرة. ارتبط MIF و NTX ارتباطًا وثيقًا بمرضى هشاشة العظام، وكذلك Mg و Vit-D. من ناحية أخرى، لم تتغير مستويات الكالسيوم والفوسفور بشكل كبير مع هشاشة العظام التي يمكن اعتبارها عامل خطر طالما أنها منظمة في مجموعة واحدة مع MIF و NTXو Mgو Vit D في جميع المرضى الذين خضعوا للدراسة. كان أفضل cut off لـ NTX هو 166.8 بيكوغرام / مل، وكان أفضل cut offلـ MIF هو 8.41 نانوغرام / مل وفقًا لتحليل ROC.

الكلمات المفتاحية: العظام، انزيم الالكلاين فوسفتيز، مثبط هجرة البلاعم، تلوببتايد -N من النوع الأول كولاجين العظام، هشاشة العظام ،فيتامين د.