Baghdad Science Journal

Volume 22 | Issue 9 Article 1

9-16-2025

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How to Cite this Article
Hassan, Zahraa Salim and Farhan, Layla Othman (2025) "Investigating the Effects of Cystatin D on Osteoporosis in Iraqi Patients," *Baghdad Science Journal*: Vol. 22: Iss. 9, Article 1.
DOI: https://doi.org/10.21123/2411-7986.5042

BAGHDAD SCIENCE JOURNAL 2025;22(9):2838–2845

RESEARCH ARTICLE

Investigating the Effects of Cystatin D on Osteoporosis in Iraqi Patients

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ABSTRACT

The common systemic skeletal disorder known as osteoporosis (OP) is characterized by low bone mass and microarchitectural deterioration of bone tissue, which makes bones brittle and prone to breaking. Because of Cystatin D (CST5) peptide potential to regulate a significant class of proteolytic enzymes that play a crucial part in pathophysiological processes, cystatins have drawn a lot of attention in the past ten years, as well as their real efficacy in preventing the growth of bacteria and viruses in vivo. This study evaluated the serum cystatin D (CST5) diagnostic accuracy in osteoporosis and osteopenia patients. One hundred twenty participants were involved, (40) patients with osteoporosis, (40) with osteopenia, and (40) as healthy individuals. The age range for all patients was (40–60) years for both females and males whom did not suffer from any significant diseases and underwent an examination of osteoporosis activity by using dual energy x-ray absorptiometry (DXA scan), and the determination of level of serum CST5 and vitamin D3 (Vit D3) by ELISA technique. Calcium (Ca) was measured using a spectrophotometer. The results of this study showed significantly higher CST5 concentration in patients with osteoporosis and osteopenia compared with healthy individuals with P-value < 0.001. Serum CST5 may be useful for diagnosing osteoporosis because ROC curve analysis indicated that it can reliably distinguish osteoporosis patients from healthy individuals with great area under the curve (AUC $= 1.000$, $p < 0.001$). Finally, serum CST5 is an accurate marker that is able to differentiate osteoporosis and osteopenia patients from control.

Keywords: Cystatin D, Osteoporosis, Osteopenia, T-score, Vitamin D3

Introduction

The metabolic bone disease known as osteoporosis is characterized by a reduction in bone mass,[1,2] and its potency by inducing bone micro-architectural deterioration. One major consequence of weak bones is decreased bone mineral density (BMD).[3,4] Brittle and broken bones a higher risk of fracture,[5] and a change in the architecture of the bone. Osteoblasts are responsible for the production of new bone, whereas osteoclasts are in charge of bone resorption. It is thought that osteoporosis results from an imbalance between these cells. Old bones will be swapped out[6] to maintain better with the skeleton condition as complex remodeling occurs often in bone. Osteoporosis primarily affects women, with a significantly higher incidence rate among the elderly.[1,7]

An estimated 200 million women worldwide suffer from osteoporosis, and one in three women over 50 and one in five men over 50 will suffer fractures as a result of the condition. The most frequent fractures caused by osteoporotic stress injuries are those of the distal forearm (wrist), proximal femur (hip), and vertebrae (spine). There are numerous causes of osteoporosis, but bone loss associated with estrogen shortage, such as that which happens after menopause, is the most prevalent one.[8–10]

In postmenopausal women, it was discovered that the prevalence of osteopenia and osteoporosis was 44.20% and 30.50%, respectively.[3,11] During the

Received 25 December 2023; revised 16 April 2024; accepted 18 April 2024.
Available online 16 September 2025

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https://doi.org/10.21123/2411-7986.5042

stage of menopause, the rate of bone remodeling increases two- to four-fold due to the drop in estrogen levels. A phase of faster bone depletion and Ca exhaustion from the skeleton into the extracellular fluid are caused by increased bone resorption. These modifications result in an imbalance of Ca throughout the body, aggravating bone losses.[8,12] Osteoporosis is caused by abnormal bone tissue function.[13]

Ca -phosphate homeostasis and optimal growth for bones are significantly influenced by Vit D3. In humans, photosynthetic pigmentation is the primary source of Vit D3.[14] Vit D3 deficiency increases the risk of osteoporotic fracture. Actually, by controlling Ca transport proteins in the small intestine, physiologically active Vit D3 improves Ca intestinal absorption while promoting osteoclastic maturation and bone growth,[15] those sufficient serum Ca concentrations must be maintained for the proper mineralization of bone. For the duration of the anti-osteoporotic therapy, patients with osteoporosis should have adequate Vit D3 levels maintained.[16] It is involved in bone growth and remodeling by osteoblasts and osteoclasts.[17]

Osteoporosis and osteopenia are measured using the dual energy X-ray absorptiometry (DXA),[18] at the lumbar and femoral neck spine regions vertebrae (L1–L4) depending on T-scores obtained according to WHO guidelines.[19] Patients were classified as follows: normal (no bone loss) Osteoporosis was identified if the T-score fell between –2.5 and –2.5. standard deviation (SD); osteopenia was identified if the T-score was observed between –1.5 and –2.5 SD. If the T-score was less than 1 standard deviation below young adults' healthy peak bone density for the same sex. The data sets of these three groups were contrasted considering the influence of aging.[3]

The CST5 is a group of proteins, belonging to family II of cystatins,[20] this family consists of approximately 120 amino acid residues (14 KDa) and two intrachain disulfide bonds, it is considered one of the salivary proteins secreted from the parotid glands,[21] found in many human tissues and body fluids.[22,23] The inhibitor CST5 inhibits the cathepsin family of cysteine proteases. Some cathepsins sometimes leaks from damaged lysosomes of apoptotic cells,and also defense against microbial pathogens utilizing cysteine proteases, cell homeostasis depends on the balance between cathepsin cysteine and its inhibitors from the cystatin family in many diseases including neoplasia, rheumatoid arthritis, osteoporpsis, Alzheimer's and Parkinson's disease, and atherosclerosis contribute to the cause of cancer and numerous alternative illnesses.[24,25] CST5 is stimulated by vitamin D receptors (VDR). Interestingly, because Vit D3 suppresses tumors in a range of tumor types, CST5 is a crucial mediator of tumor suppression in colorectal cancer.[26–28] It also has a suppressive effect on decrease human leukemic cell proliferation and enhance apoptosis.[29] Cystatins also have multiple functions in neurodegenerative diseases as well as the pathogenesis and physiology of tumor formation and progression.[30] Research has shown that people with human immunodeficiency virus type 1(HIV-1) and bladder cancer (HIV-1) exhibit low levels of CST5 expression.[31] Recently, a previous study referred to the important role of CST5 in regulating the activity of mastocytosis. It highlighted the potential role of CST5 in modifying the function of these cells and considering it a biomarker for the disease.[32] Another study conducted to examine the effect of CST5 on the reproduction of the human coronavirus, a decrease in virus productivity was observed, indicating that CST5 is a strong inhibitor of the reproduction of the coronavirus.[33] As for the saliva of healthy people who were suffering from oral dryness, low concentrations of protease inhibitor enzyme (CST5) were observed.[34] A study revealed a correlation between lower serum levels of cystatin D and relapses of periodontitis following surgery, reduced levels of cystatin-D may cause osteoclasts to resorb bone, and/or increased activity of cathepsin may result in tissue destruction and trigger auto-immune processes.[35–37]

It is interesting to note that CST5 over-expression prevents bone osteoclasts degradation by preventing the NF-κB pathway being activated. Studies have indicated that osteoporpsis may be relieved by blocking the NF-κB pathway.[1,38,39] It is unclear, nevertheless, how serum CST5 relates to osteoporosis, osteoopenia, and healthy individual's groups. Therefore, the purpose of this research was to examine the connections between serum CST5 levels and additional osteoporosis-related variables in Iraqi patients. Understanding these relationships could help with osteoporosis early detection and treatment.

Materials and methods

Osteoporosis and osteopenia patients

This study assessed the possibility of using CST5 as a osteoporosis marker by looking into the protein level of CST5 in peripheral whole blood from 120 participants, 40 with osteoporosis, 40 with osteopenia as study groups and 40 healthy as control group. Written consent was obtained from each participant before they began the study, all patients were aged (40–60) years for both females and males whom did not suffer from any significant diseases and underwent an examination osteoporosis activity by using dual energy x-ray absorptiometry (DXA scan) with T-score $\leq$ –2.5

for osteoporosis, T-score between (–1 and –2.4) for osteopenia,and less than (1) for healthy individuals, and determination of the level of serum CST5 and Vit D3 by ELISA technique. Ca was measured using a spectrophotometer. People suffering from diabetes, heart disease, rheumatoid arthritis, kidney diseases, cancer, hysterectomised women. Addison's disease and many other diseases were excluded from this research. All participants were selected from the National Joint Center at Yarmouk Teaching Hospital in Baghdad for the period between (September and October) 2023.

This study was approved by the National Health Ministry of Iraq's Center in the interest of education and human development, the Yarmouk Iraqi Teaching Hospital Committee of Ethics, and the University of Baghdad Ethical Committee. The ethical standards were adhered to the procedures depending on (Helsinki Declaration).

Sample collection, and clinical laboratory analysis

The entire samples were collected from the participants without using a tape to apply pressure to the vein when drawing the blood sample, leaving them to coagulate in a clot activator tube for 15 minutes at room temperature, and then separated for 5 minutes using a centrifuge to obtain serum which was stored in 2 ml Eppend or f containers as well as keeping them at –4 ˚C. Concentrations of serum CST5 (Cloud-Clone Corp., USA, SEJ325Hu), Vit D3(Cloud-Clone Corp., USA, CEA920Ge) were measured by using an ELISA plate reader from Germany's Human. Serum Ca (LINEAR CHEMICALS, SPAIN) concentration evaluated using A spectrophotometer.

Statistical analysis

Variations in values such as CST5 concentration and other parameters in osteoporosis, osteopenia as study groups and healthy as control group were statistically analyzed using version 26 of SPSS. The median (25th and 75th percentiles) of the data was used; non-normally distributed numerical variables were found using the Mann-Whitney and Kruskal Wallis tests. The Spearman correlation coefficient was used to look at the relationships between the variables. A statistically significant p-value was defined as one that was less than 0.05. ROC curve method was utilized to assess the serum CST5 level cut-off value. Additionally, calculations were made for the specificity, sensitivity, negative predictive value, and positive predictive value. P-values less than 0.05 were regarded as significant.

Results and discussion

Characteristics of osteoporosis, osteopenia patients and healthy individuals groups

The distribution of average ages for the osteoporosis patients 57.00(52.00–59.00) and the osteopenia patients 56.00(51.25–59.00) was compared with the healthy individuals 55.00(45.00–58.75), respectively, with p-value (.648). The average BMI of individuals with osteoporosis 28.19 (24.52–33.24) kg/m_2, and the osteopenia patients 30.59(28.002–32.98) was also compared with the healthy individuals groups with 30.48(26.66–34.73) kg/m_2,the statistical significance was the same with p-value (0.273). Distinctive sociodemographic and aspects of the osteoporosis, osteopenia and the healthy individuals' group are mentioned in Table 1.

The researcher noted there was a clear significant differences (inverse relationship) between the distributions of average Vit D3, Ca for the osteoporosis patients 6.44(4.05–9.44) (ng/dl), 7.99(7.81–8.148)(mg/dl) and osteopenia patients 12.21(9.82-13.04)(ng/dl),8.17(7.946–8.72)(mg/dl) respectively with 66.99(38.32–79.09) (ng/dl), 9.32(8.72–9.75) (mg/dl) healthy individuals groups, p-value (0.001). Also there was a significant difference in Vit D3 serum concentration between the osteoporosis and osteopenia patients' groups as displayed in Table 2. It was observed that the concentration of Vit D3 decreased as osteoporosis increased.

Serum CST5 levels were noticeably higher in osteoporosis patients with (8.70(5.85–9.14) ng/ml) and osteopenia patients (3.75[3.37–4.35] ng/ml) more than in healthy individuals group (2.71(2.35–2.96) ng/ml) with p-value (0.001). Also the statistical analysis showed that the osteoporosis Group and the osteopenia Group have important differences as

Table 1. Sociodemographic and distinguishing characteristics osteoporosis, osteopenia patients and healthy individuals' group.

Variables	Osteoporosis patients (n = 40)	Osteopenia patients (n = 40)	Healthy individuals (n = 40)	P-value
Age (year)	57.00(52.00–59.00)	56.00(51.25–59.00)	55.00(45.00–58.75)	.648
BMI (kg/m^2)	28.19 (24.52–33.24)	30.59(28.002–32.98)	30.48 (26.66–34.73)	0.273
WHR	0.925 (0.908–0.945)	0.918 (0.896–0.938)	0.920 (0.899–0.934)	0.407

The median (25th and 75th percentiles) of the obtained data were evaluated using the Mann-Whitney test at the 0.05 threshold; The two independent means differed significantly from one another.

Table 2. Studied parameters characteristics in osteoporosis, osteopenia patients and healthy individuals' group.

Variables	Osteoporosis patients(n = 40)	Osteopenia patients (n = 40)	Healthy individuals (n = 40)	P-value
Vit D3 (ng/dl)	6.44 (4.05–9.44) a.c	12.21 (9.82–13.04) b.c	66.99 (38.32–79.09	0.0001
Ca (mg/dl)	7.99 (7.81–8.148) a.c	8.17 (7.946–8.72) b.c	9.32 (8.72–9.75)	0.001
T/score%	–2.95 (-3.27 – –2.60) a.c	–1.60 (-1.87 – –1.40)b.c	0.050 (-0.800–0.375)	0.001
Cystatin D (ng/ml)	8.70 (5.85–9.14) a,c	3.75(3.37–4.35) b,c	2.71(2.35–2.96)	0.001

The median (25th and 75th percentiles) of the obtained data were evaluated using the Mann-Whitney test at the 0.05 threshold; The two independent means differed significantly from one another.
(a) Shows how Osteoporosis Group and control differ significantly from each other.
(b) Shows that the Osteopenia Group and Control have a significant difference.
(c) Shows that the Osteoporosis Group and the Osteopenia Group have important differences.

displayed in Table 2. It was observed that the concentration of CST5 in osteoporosis Group higher more than in osteopenia patients.

The correlation analysis of CST5 with additional parameters

The CST5 along with additional indicators (Vit D3, Ca) in osteoporosis, osteopenia a correlation study was conducted with patients and a healthy individual's group. There was negative correlation between CST5 with Vit D3[(r = –0.356)(p = 0.024)], andq Ca[(r = –0.347) (p = 0.028)] in Osteopenia group while no correlation other groups (osteoporosis, and healthy individuals) as shown in Table 3.

Vit D3 and Ca concentration showed a significant difference between the osteoporosis and osteopenia patients comparable with the healthy individuals group, the decline in vitamin D levels in patients with osteoporosis was more severe than in patients with osteopenia comparable with the healthy individuals' group, this decrease in concentrations is consistent with other studies conducted by Abeer, et al. [40] While it does not agree with a study conducted by Layla, et al, which showed high Vit D3 levels in severe osteoporosis patients with no significance in calcium levels. [4] Vit D3 which is provided through food or the cutaneous synthesis, undergoes a hydroxylation process in the liver to convert it to the biologically active form 25-hydroxyvitamin D [25(OH)D], which is later converted in the kidneys to 1,25-dihydroxyvitamin D [1,25(OH)2D] named calcitriol which is linked to the calcium homeostasis and phosphate absorption in the intestine maintaining adequate levels of calcium and phosphate in the bloodstream. [16] Ca is a major component of hydroxyapatite, the mineral compound that makes up bone tissue. This process is necessary for bone mineralization and strength. [15] Vit D3 also binds to its receptor, known as the vitamin D receptor (VDR), found on the surface of bone cells. This binding stimulates the production of proteins involved in bone formation, such as osteocalcin and collagen. Ultimately, this leads to increased bone matrix formation and mineralization, which promotes bone growth and density and reduces osteoporosis [14]. The results of this study indicated the role of vitamin D in preventing osteoporosis, as all people with osteoporosis suffer from low levels of vitamin D, and it may be one of the main causes of the disease.

Results in this study showed a highly significant difference in the increasing in of CST5 levels in osteoporosis and osteopenia Iraqi patients groups compared with the healthy individuals' group. T-score was significantly increased in osteoporosis patients. A previous studies pointed to the role of CST5 in inhibits the cathepsin family of cysteine proteases, they are enzymes involved in the degradation of bone matrix proteins, by inhibiting cathepsin activity, cystatin D may help maintain bone tissue integrity and prevent excessive bone resorption. [22,24] The role of CST5 for osteoporosis by inhibiting the NF-κB pathway,which showed lower levels of CST5 in cases of osteoporosis, lower level of CST5 in those studies an indication of its consumption in inhibiting osteoclasts degradation in cases of osteoporosis as a defensive role for the body. [1] In this study, we found high concentrations of anti-inflammatory protein CST5 in osteoporosis and osteopenia patients compared to healthy people, indicating its defensive activity of the body against the causes of osteoporosis, and this confirms its diagnostic role for the disease. We also suggest

Table 3. Correlation analysis of CST5 with Vit D3,and Ca in the osteoporosis, osteopenia and healthy individuals' group.

Parameters	Osteoporosis R value(P value)	Osteopenia R value(P value)	Healthy individuals R value(P value)
Vit D3 (ng/ml)	–0.261(0.103)	–0.356(0.024)*	0.274(0.087)
Ca (mg/dl)	–0.251(0.119)	–0.347(0.028)*	–0.360(0.023)

The correlation is significant = 0.05.

Table 4. CST5 AUC and validity in distinguishing.

Variable	AUC	P-Value	cut off value	Sensitivity	Specificity	Accuracy	PPV	NPV
CST5(A)	1.000	0.001	>3.343	100.0	100.0	1.000	100.0	100.0
CST5(B)	0.967	0.001	> 3.315	87.5	97.5	0.850	97.2	88.6

A: AUC and the ability to discriminate between osteoporosis sufferers and healthy individuals.
B: AUC and the ability to discriminate between osteopenia sufferers and healthy individuals.

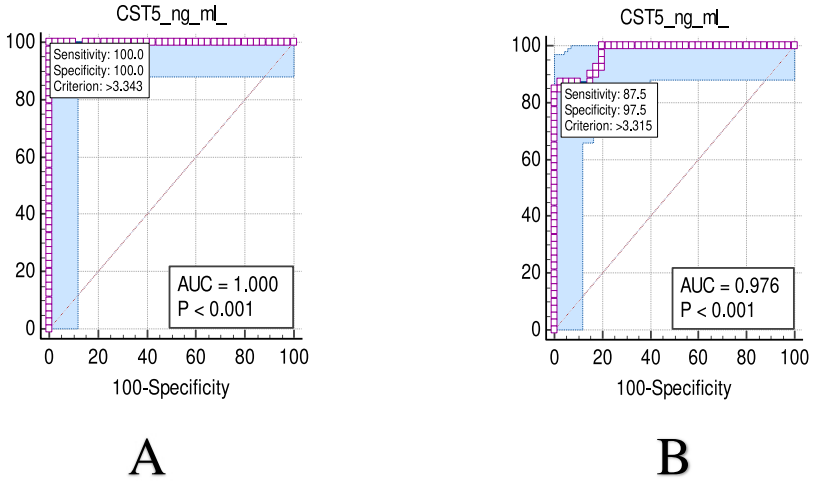

Fig. 1. A: The ROC curve evaluation regarding the serum concentration's predictive value of CST5 in osteoporosis (n = 40) versus control (n = 40) (AUC is 1.000; 95%), p < 0.001. B: The ROC curve analysis for the serum concentration's predictive value of CST5 in osteopenia (n = 40) in contrast to control (n = 40) (AUC is 0.976; 95%), p < 0.001.

further research in the future to discuss the potential therapeutic possibilities of CST5 for osteoporosis patients.

ROC curve evaluation

Utilizing ROC curve analysis, assess how well the serum CST5concentration could differentiate osteoporosis patients from healthy participants Table 4, Fig. 1A. With higher validity (great specificity and sensitivity), the ROC curve pertaining to osteoporosis was significantly above the diagnostic test. The optimal level of accurate prediction of osteoporosis was obtained by the using ROC curve with an AUC of 1.000 ($p < 0.001$) for the presence of an osteoporosis diagnosis. The ROC curve demonstrated increased validity (high specificity and sensitivity) and was noticeably greater than the diagnostic examination. for osteopenia. Utilizing ROC curve analysis, the evaluation of the efficacy of the serum CST5 concentration in differentiating osteopenia patients from healthy individuals Table 4, Fig. 1B. The ROC curve's AUC for the diagnosis of osteoporosis was 0.976 ($p < 0.001$), indicating a a strong likelihood of accurate diagnosis at a fair level.

Conclusion

Patients with osteoporosis and osteopenia have significantly higher levels of CST5 and significantly lower levels of (Vit D, Ca). It was also observed that levels of cystatin concentration were higher in patients with osteoporosis than in individuals suffering from osteopenia in contrast to the healthy subject group and that the disease's activity is reflected in this marker's levels. In order to diagnose osteoporosis, serum CST5 levels may be used as a useful biological marker. However, after more research, we discovered that large-scale prospective studies and ongoing clinical studies are necessary to validate these results. Furthermore, more research is required to fully understand the therapeutic role and clinical function of CST5 in osteoporosis.

Acknowledgment

We are extremely thankful to the AL-Yarmouk Teaching Hospital, National Center for Joints, Iraq, for providing us with the approval for this project, without which the present study would not have been possible.

Authors' declaration

- Conflicts of Interest: None.
- We hereby confirm that all the figures and tables in the manuscript are ours. Besides, the figures and images, which are not ours, have been given the permission for re-publication attached with the manuscript.
- No animal studies are present in the manuscript.
- No human studies are present in the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at University of the Baghdad.

Authors' contribution statement

Z. S. H. performed the conceptualization, analysis, visualization, data curation, research, first draft writing, L. O. F. made the conceptualization, testing, data analysis, project management, editing, review, and visualization.

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دراسة تأثير السيستاتين د على هشاشة العظام لدى المرضى العراقيين

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المستخلص

يتميز اضطراب الهيكل العظمي الجهازي الشائع المعروف باسم هشاشة العظام (OP) بانخفاض كتلة العظام وتدهور البنية الدقيقة لأنسجة العظام، مما يجعل العظام هشة وعرضة للكسر. بسبب قدرة البيتيد Cystatin D (CST5) على تنظيم فئة كبيرة من الإنزيمات المحللة للبروتين التي تلعب دورًا حاسمًا في العمليات الفيزيولوجية المرضية، فقد لفت CST5 الكثير من الاهتمام في السنوات العشر الماضية، بالإضافة إلى فعاليته الحقيقية في منع النمو البكتيريا والفيروسات في الجسم الحي. قيمت هذه الدراسة دقة تشخيص مصل السيستاتين D CST5 لدى مرضى ليونة العظام وهشاشة العظام. مائة وعشرون مشاركا (40) مريضا بهشاشة العظام، (40) بليونة العظام، و (40) من الأفراد الأصحاء، جميع المرضى تتراوح أعمارهم بين (40-60) سنة للإناث والذكور الذين لم يعانون من أي أمراض موثرة وخضعوا لفحص هشاشة العظام باستخدام قياس امتصاص الأشعة السينية ثنائي الطاقة (DXA scan)، وتحديد مستوى CST5 وفيتامين D3 في المصل بتقنية ELISA. تم قياس الكالسيوم Ca باستخدام مقياس الطيف الضوئي. أظهرت نتائج هذه الدراسة تركيز CST5 أعلى بكثير في المرضى الذين يعانون من هشاشة العظام وليونة العظام مقارنة بالأفراد الأصحاء الذين لديهم قيمة. P <0.00 قد يكون CST5 مفيدًا لتشخيص هشاشة العظام لأن تحليل منحنى ROC أشار إلى أنه يمكنه التمييز بشكل موثوق بين مرضى هشاشة العظام والأفراد الأصحاء الذين لديهم مساحة كبيرة تحت المنحنى(p <0.00، AUC = 1.000) وأخيرًا، يعتبر مصل CST5 علامة دقيقة قادرة على التمييز بين مرضى هشاشة العظام وليونة العظام عن الافراد الاصحاء.

الكلمات المفتاحية: السيستاتين د، هشاشة العظام، ليونة العظام، T-score، فيتامين د.