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Estimation of some biomarkers as indicators of the incidence and prevalence of osteoporosis in Iraqi population

A Thesis

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تقدير بعض المعايير الحيويه كدلائل لحصول وانتشار هشاشة العظام في المجتمع العراقي

أطروحـــــــــــة

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بسم الله الرحمن الرحيم

﴿ قَالُوا سُبْحَانكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا
أنكَ أنتَ الْعَلِيمُ الْحَكِيمُ ﴾

صدق الله العلي العظيم

سورة البقرة / الآية 32

Dedication

Dedicated to..

dear father

My lovely mother

precious husband

My beautiful daughters and son (Zahraa, Fatema, Batool and Ahmad)

Faithful family who helped and encouraged me all the time I dedicate this work

Shaimaa

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List of Contents

List of Figures

List of Tables

List of Abbreviations

	Definition
ACP-5	Phosphatase acid type 5,Tartrate _Resistant
ACR	American College of Rheumatology
ADT	androgen-deprivation therapy
ALP	Alkaline phosphatase
BMD	Bone Mineral Density
BMI	Body mass index
BSP	Bone sialoprotein
Ca	Calcium
CatK	Cathepcin K
DXA	dual X-ray absorptiometry
FKN	fractalkine
GSN	Gelsolin
NAMS	The North American Menopause Society
NCBI	National Center For Biotechnology Information

	Definition
NOF	National Osteoporosis Foundation
OP	Osteoporosis
OR	Odds Ratio
PCR	Polymerase Chain Reaction
Po4	phosphorus
PTH	Parathyroid hormone
PTHR	PTH receptor
RANKL	nuclear factor-kappaB ligand
SNP	Single Nucleotide Polymorphism
TRAP	Tartrate-resistant acid phosphatase
U	Unit
UV-VIS spectrophotometer	Ultra Violate-Visible Spectrophotometer
VDR	vitamin D receptor
Vit.D	Vitamin D
w/v	Weight Per Volume
WHO	World Health Organization

Summary

This study was conducted at Babylon University / College of Science, period was between March 2019 to May 2021 this research was included case-control study , blood samples were collected from 100 osteoporosis patients from Marjan Medical City in Babylon Governorate, and 50 samples were collected as control group.

In present study has been studied some physiological and biochemical parameters in addition to molecular studies. The biochemical study included estimate the level of the following parameters in the serum (Fraktalkin (CX3CL1), Cathepcin K, Gelsolin and Bone sialoprotein) in addition estimate the level of (Vitamin D, Calcium, Phosphorous and Alkaline phosphatase).In present study has been studied the correlation between the levels of this vital signs with the age ,sex, BMI and T-score.

The results are shown the osteoporosis disease caused high significant ($p \leq 0.05$)in biochemical parameters in osteoporosis patients (Fraktalkin (CX3CL1),Cathepcin K, Gelsolin, Bone sialoprotein, Phosphorous and Alkaline phosphatase) when compared with healthy controls. also the results are shown marked reduce in the Vit. D and calcium level in both gender of the patients as compared with control group.

The study results showed statically increase in the level of the following parameters (CX3CL1, Cathepcin K and Gelsolin) in the female osteoporosis patients when compared with male osteoporosis patients also strong increase in the following parameters (Bone sialoprotein and Alkaline phosphatase) in both gender at ($p \leq 0.05$).

According to age, the results show significant increase in the patients of osteoporosis when divided to age categories (20-40), (41-60)

and (61-80) in the level of (CX3CL1, Bone sialoprotein and Alkaline phosphatase) compared with same age categories in the healthy group and no there is significant difference between (vit. D, Ca and P) level in the previous age categories,during divided the patients according age categories found there are significant increase ($p \leq 0.05$) in the age groups (20-40) and (41-60) in the parameters (CX3CL1, CathepcinK ,calcium and phosphorous) in osteoporosis and osteopenia patients, also there are no marked difference between (Bone sialoprotein, Gelsolin and vitamin D) in same age groups.

When comparing by BMI, the significant increase in (CX3CL1, CathepcinK, Gelsolin, Bone sialoprotein and Alkaline phosphatase) level in osteoporosis patients compared with healthy for the same (BMI) also, no there significant difference in the (vitD, Ca and P) between them. The our results showed that there is no significant difference in the most examined parameters when divided the osteoporosis according to T-score into (osteoporosis and osteopenia) except the Gelsolin. The study reveals that there great increase in the osteoporosis as compared to osteopenia, furthermore, decrease in vit D level in the osteoporosis as compared with osteopenia.

The results demonstrated that significant increase of the osteoporosis in the female as compared with male in the following parameters (Bone sialoprotein, Cathepcin K, vitamin D and Alkaline phosphatase), furthermore, there is marked increase in Gelsolin level in male patients with osteoporosis compared with female patients with osteoporosis also, no there significant difference between females and males in (CX3CL1, calcium and phosphorous) levels.

Summary

The current study signed during comparing between osteoporosis and osteopenia patients according to use BMI, non-significant difference in (Bone sialoprotein, Gelsolin ,CathepcinK, vitamin D and Alkaline phosphatase) levels when comparing between two groups, non-significant difference in (CX3CL1, calcium and phosphorous) levels between the both groups.

The current study divided the female osteoporosis patients into two groups(premenopausal and postmenopausal), the findings reveals significant increase in (CXC3CL1, Bone sialoprotein, CathepcinK, Gelsolin and Alkaline phosphatase) levels in the both groups of patients as the healthy female, during comparing between female premenopausal and postmenopausal furthermore, non-significant difference in all the parameters except (Gelsolin and Alkaline phosphatase) levels.

The results of the genetic study for ACP-5 (rs 2071484) by using the methods SSCP showed there are lack of correlation between alleles and also the genetic results showed that genotype CC have significant correlation (0.0026) with the disease so the results showed that the people who have the genotype CC have the ability for getting sick (4.5) times more than the people who have the genotypes TT and TC.

From the results showed that most of the parameters significantly associated with osteoporosis disease also the genetic study showed there is significant differences in the genotypes and how related with osteoporosis disease.

الخلاصة

أجريت هذه الدراسة في جامعة بابل / كلية العلوم للمدة من أذار2019 إلى أيار2021 وتضمن البحث جمع العينات المرضية اضافة الى عينات السيطرة ، جمعت عينات الدم من 100 مصابا بهشاشة العظام حيث تم جمع العينات من مستشفى مدينة مرجان الطبية في محافظة بابل اضافة الى 50 عينة كمجموعة سيطرة.

تم دراسة بعض المعايير الفسلجية والكيموحيوية بالأضافة للدراسة الجزيئية .تضمنت الدراسة الكيموحيوية تقدير مستويات المعايير التالية في المصل (CX3CL1,Cathepcin K, Gelsolin, Bone sialoprotein) بالاضافة قياس مستوى (فيتامين د ، الكالسيوم ،الفسفور وانزيم الفوسفاتيز القاعدي). تم دراسة العلاقة بين مستويات هذه المؤشرات الحيوية مع العمر والجنس ومؤشر كتلة الجسم وT-score.

أظهرت النتائج ان مرض هشاشة العظام تسبب في ارتفاع معنوي ($p\leq0.05$) في المعايير الكيموحيوية المدروسة للمرضى (CX3CL1, Cathepcin K, Gelsolin ,Bone sailoprotein, والفسفوروالفوسفاتيز القاعدي) عند مقارنتها مع الاشخاص الاصحاء كما أظهرت الدراسة وجود انخفاض معنوي ($p\leq0.05$) في مستوى فيتامين د والكالسيوم على التوالي في المرضى من كلا الجنسين مقارنة مع الاصحاء.

أشارت النتائج الى وجود أرتفاع معنوي($p\leq0.05$) في المعايير التالية (CX3CL1, Cathepcin K, gelsolin) في الاناث المصابات بهشاشة العظام مقارنة مع الذكور المصابين وكذلك وجود ارتفاع معنوي ($p\leq0.05$) في المعايير التالية (Bon sialoproein, والفوسفاتيز القاعدي) في كلا الجنسين.

كما اظهرت نتائج الفئات العمرية وجود ارتفاع معنوي ($p\leq0.05$) في المرضى المصابين بهشاشة العظام عند تقسيمهم الى الفئات العمرية (20-40) و(41-60) و(61-80) في المعايير التالية (Bone sialoprotein, CX3CL1 والفوسفاتيز القاعدي) مقارنة مع نفس الفئات العمرية للأشخاص الاصحاء في حين لم تسجل النتائج فروق معنوية ($p\geq0.05$) في المعايير (فيتامين د ،كالسيوم والفسفور)في الفئات العمرية السابقة.

لوحظ عند المقارنة باستخدام مؤشر كتلة الجسم (BMI) حصول أرتفاع معنوي ($p\leq0.05$) في كل من المعايير(CXC3, CathepcinK, gelsolin, bone sialoprotein

والفوسفاتيز القاعدي) في المرضى المصابين بهشاشة العظام مقارنة مع الأصحاء لنفس (BMI) وعدم وجود فروق معنوية ($p\geq0.05$) بينهم لكلا من (فيتامين د ، الكالسيوم والفسفور).

بينت نتائج الدراسة الحالية عدم وجود فروق معنوية ($p\geq0.05$) في أغلب المعايير المدروسة عند تقسيم المرضى المصابين بهشاشة العظام حسب مؤشر T-score حيث تم تقسيمهم الى مجموعتين (المرضى المصابين بهشاشة العظام osteoporosis والمرضى المصابين بقلة كتلة العظم osteopenia) ماعدا Gelsolin حيث اظهرت الدراسة وجود ارتفاع معنوي ($p\leq0.05$) في المرضى المصابين بالهشاشة مقارنة بالمرضى المصابين بقلة كتلة العظم وكذلك وجود انخفاض معنوي ($p\leq0.05$) في مستوى فيتامين د في المرضى المصابين بالهشاشة مقارنة بالمرضى المصابين بقلة كتلة العظم.

لوحظ من خلال النتائج حصول ارتفاع معنوي ($p\leq0.05$) في الاناث المصابات بهشاشة العظام osteoporosis وكذلك بقلة كتلة العظم osteopenia مقارنة بالذكور في مستوى sialoprotein (Bone) في حين وجد حصول ارتفاع معنوي ($p\leq0.05$) في مستوى (Gelsolin) في الذكور المصابين بهشاشة العظام مقارنة بالاناث المصابات ،ولم تصل الفروق بين الذكور والاناث الى المعنوية ($p\geq0.05$) في المعايير (CX3CL1 والكالسيوم والفسفور).

عند تقسيم المرضى حسب الفئات العمرية وجد حصول زيادة معنوية ($p\leq0.05$) في الفئة العمرية (20-40) والفئة العمرية (41-60) في المعايير (Bone sialoprotein وGelsolin وفيتامين د) ولم تكن هنالك فروق معنوية ($p\geq0.05$) في مستوى كلاً من (CX3CL1 وCathepcin K والكالسيوم والفسفور) لنفس الفئات العمرية.

أشارت نتائج الدراسة الحالية عند المقارنة باستخدام مؤشر كتلة الجسم (BMI) لدى المرضى المصابين بهشاشة العظام والمرضى المصابين بانخفاض كتلة العظم وجود فروق معنوية ($p\leq0.05$) في المعايير (Bone sailoprotein وGelsolin و Cathepcin K وفيتامين د والفوسفاتيز القاعدي) عند المقارنة بين المجموعتين ،وعدم وجود فروق معنوية ($p\geq0.05$) بينهم لكلا من (CX3CL1 والكالسيوم والفسفور).

تضمنت الدراسة الحالية تقسيم الاناث المصابات بهشاشة العظم الى مجموعتين (قبل سن اليأس وبعد سن اليأس) واظهرت النتائج وجود أرتفاع معنوي ($p\leq0.05$) في المعايير التالية(CX3CL1, Cathepcin K وBone sialoprotein و gelsolin والفوسفاتيز القاعدي) في كلا المجموعتين من المرضى مقارنة بالنساء الصحيحات، وعند المقارنة بين النساء

فبل وبعد سن اليأس أظهرت النتائج عدم وجود فروق معنوية ($p\geq0.05$) لكل المعايير عدا كلا من (Gelsolinوالفوسفاتيز القاعدي).

أظهرت نتائج التحليل الجيني لـ ACP-5 rs2071484 بأستخدام تقنية SSCP عدم وجود علاقة بين الاليلات وكما بينت النتائج الوراثية بأن الطراز الوراثي CC له أرتباط معنوي (0.0026) مع المرض أذ اظهرت الدراسة ان الاشخاص الذين يمتلكون الطراز الوراثي CC لهم القابلية على الاصابة بالمرض ب(4.5) مرة اكثر من الاشخاص الذين يحملون الطراز الوراثي TT, TC.

من خلال النتائج تبين أن أغلب المعايير أرتبطت معنويا بالإصابة بمرض هشاشة العظام وكذلك بينت الدراسة الجينية ان هنالك فروقا في الطرز الوراثية ومدى ارتباطها بهشاشة العظام.

Conclusion and Recommendation:

Conclusion:

1- The percentage of the distribution of disease in women higher than men in Iraq.
2- Indicators cannot be adopted (Vit.D, Ca and po4) as biological markers for osteoporosis disease because these indicators depending on the age and gender.
3- Obesity or increase in body mass index BMI have large association with osteoporosis disease.
4- Vital signs (CX3CL, bone sialoprotein, gelsolin and cathepcin K) consider positive indicators for osteoporosis disease.
5- ACP-5 rs2071484 genotype CC have potential risk for Osteoporosis more than other genotypes (TC andTT).

Recommendation:

1- Studying other genes that may have a relationship with the disease like, PTH gene, collagen alpha gene, and calcitonin gene.
2- Studying other physiological parameters that may have a relationship with the disease like osteoprotogerin, and osteocalcin.
3- Increase the sample size and included other Iraq provinces to give a complete picture about genotype distribution in Iraqi population.
4- Studying family history of genetic polymorphism related with Osteoporosis.
5- Recommended measured level of liptin hormones for people who under going from obesity and connect it with osteoporosis disease.

Chapter One
Introduction

Chapter Two
Literature Review

Chapter Three Materials and Methods

Chapter Four
The Results

Chapter Five
The Discussion

Conclusion and Recommendation

References

Appendices

1.1.Introduction

Osteoporosis is a disease affecting a skeletal system that affects bone strength (bone quality and density). Osteoporosis is predisposing and a risk factor for fractures which occur due to minor injuries,the osteoporosis shows the low density of the bone and loss of the maintenance of bone homeostasis (Ivanova *et al*.,2015). In humans, osteoporosis is the most common bone disorder and is a major global public (So¨zen *et al*., 2017). Osteoporotic fractures lead to high mortality (Cauleym , 2013).

The fractures are associated with reduced physical functions, increased disability, and poor quality of life (Johnell and Kanism, 2006). In Taiwan, Nearly (9.056.000) new cases of osteoporotic fractures occurred in 2005; between (2001- 2011) the osteoporosis prevalence increased by 7.6%(Johnell and Kanism, 2006).

Bone mineral density is a diagnostic index for osteoporosis and predicts fractures (Ioannidis *et al*.,2007). Many genetic and nongenetic factors are associated with osteoporosis (Ivanova *et al*.,2015), some of which include age (Chang *et al*.,2018), sex, menopausal status (Mo *et al*.,2017; So¨zen *et al*., 2017), educational level, coffee drinking (Cristina de Sousa *et al*.,2016; Chang *et al*.,2018), smoking, exercise, alcohol consumption , Diet and body mass index (BMI) (Ivanova *et al*.,2015; Akhlaque *et al*., 2017).

Dual Energy and peripheral dual of the X-ray Absorptiometry, Quantitative and Ultrasound Computed Tomography, and DualPhoton Absorptiometery are methods for detection of bone mineral density. The most accurate way is dual-energy x-ray, and their results are detected by T-score, which indicates osteoporosis (Leslie *et al*., 2008). The other risk factors of osteoporosis are lifestyle and Diet, Furthermore, inadequate

nutrition, intestine absorption disorder, obesity, activity, lack of movement, and smoking (Pouresmaeili *et al.*,2018).

Many studies found the important role of Ca and vit. D on the bone homeostasis as ESCEA guidelines are recommended that Ca and vit. D intake should be taken daily (Kanis *et al*., 2019). In addition to that, many nutrients have a great role in maintaining the bone's high density (Muñoz-Garach *et al*.,2020).

Furthermore, dietary decisions impact the makeup of the gut microbiota, which is a complex network of microbes capable of metabolizing meals into metabolically active substances that impact the body's homeostasis, Recently, the intestine bacterial contents of primary osteoporosis were investigated, and it was shown to differ considerably from healthy age- and sex-matched control subjects (Xu *et al*.,2020).

Acid phosphatase 5 ACP5 gene have extravagant role in the composition of a tartrate-resistant acid phosphatase TRAP5 enzyme. TRAP-5 is 35 K D glycosylated di- iron metalloenzyme responsible for the regulation of osteopontin activity, there are two isoforms from TRAP5, TRAP5a and TRAP5b. TRAP5a functions with low enzymatic action due to a loop reacting with the functional site and the more active TRAP5b is generat upon proteolytic cleavage of this loop (Mohit *et al.*,2021). Acid phosphatase 5 ACP5 is main transcribe through asingle gene that contain 5 exons in which the first three exon E51, E52, E53 have three alternate promoters (Walsh *et al*.,2003).

1.2. Aim of Study

The current study aimed to detected biomarkers in patients with osteoporosis and it association with some physiological status and molecular criteria have been a study because increase incidence of osteoporosis, for both genders which might also help in early diagnosis of cases of osteoporosis to prevent development of complication , this achieved by:

1-Estimation of some biomarkers include(Bone Sailoprotein, Human CX3C-Chemokine, Gelsolin, Cathapci K).

2-Estimation of some biochemical parameters(Vitamin D, Ca, Po4, and ALP).

3-Molecular study (DNA extraction, PCR for amplified region and SSCP for detect region sequencing to detect genotype).

2.1. Definition of Osteoporosis:

Osteoporosis is a metabolic illness of the bones in which the bones become so weak and unable to support the body due to the loss of the bone mass and the minerals over time (Hinjorgo *et al.*, 2008). It begins to set in throughout the latter years, but it begins to develop much sooner and without warning. More than 25 million individuals worldwide have been impacted by this condition, which affects mostly women. It is the most prevalent disease of ageing. Every year in the United States, people are admitted to hospitals because of osteoporosis (Whitney and Rolfes, 2002).

The prevalence of osteoporosis and osteopenia in women aged 45 to 70 years has been shown to be 16 and 34 %, respectively, (Sultan *et al.*, 2006). In the normal process of bone turnover, the rates of bone resorption and bone synthesis are comparable. Acidity causes osteoclasts to remove bone and osteoblasts to construct bone by producing the osteoid into the resorption cavity(Manolagas, 2000).

Osteoclasts have a longer life span than osteoblasts, which causes an increase the bone turnover to rise. Osteoblasts have a shorter life span than osteoclasts, which causes the rate of bone turnover to increase (Manolagas, 2000). Bone mass is the most important element in determining bone density in an older person. A woman's bone mass starts to build during the pregnancy and is typically completed by the time she reaches her forties; nevertheless, the quantity of bone acquired during adolescence is the most significant contribution to this process (Mora and Gilsanz, 2003).

When the ovarian function is no longer present after menopause, bone loss increases in the following years, and bone mass continues to fall as

we get older (Hannan *et al*., 2002). As a result, older age is a risk agent for bone loss in addition to having reached peak bone mass. Reduced body fat percentage, low body weight, or low BMI are all associated with fast bone loss and low bone density, all of which are independent risk factors for postmenopausal osteoporosis, according to the National Osteoporosis Foundation (Lane, 2006).

2.2.Criteria for Diagnosis of Osteoporosis:

An osteoporotic fracture and/or low bone mineral density (BMD) in postmenopausal females are required to diagnose osteoporosis in this population. Dual X-ray absorptiometry is the "gold standard" technique of measuring bone mineral density (DXA). Specifically, it is expressed as a number of standard deviations away from the population's mean is young adults (T-score) or away from the mean of a population of similar age (Z-score). Database of the National reference in Caucasian females aged 20–29 years is the reference range recommended by IOF, ISCD, WHO, and NOF for calculating the T-score in postmenopausal women (Dawson Hughes, 2008).

Osteoporosis is diagnosed in postmenopausal females and men over the age of 50 if the T-score of the lumbar spine, whole hip, or femoral neck is less than -2.5 on the International Skeletal Diagnostics (ISCD) scale. In certain cases, 33 % of the radius (also known as the 1/3radius) may be used to achieve the desired result (Lewiecki *et al.*,2008). The Z-score describes the number of standard deviations (SDs) by which a person's BMD differs from the average expected for his or her age and gender. It is most often used in children, females, and adolescents who are premenopausal. The presence of a Z-score less than -2 is abnormal and should be "low for age." A low Z-score in a postmenopausal woman implies that she should be evaluated for

secondary osteoporosis, which is more common after menopause (Lewiecki *et al.*,2008).

Table (2-1) T scores and WHO Diagnostic Criteria for Osteoporosis (Cosman *et al.*,2015).	
Interpretation	**T-Score**
Normal	**–1.0 and higher**
Osteopenia	**–1.0 to –2.5**
Osteoporosis	**–2.5 and lower**
Severe osteoporosis	**–2.5 and lower with one or more fragility fractures**
Reference values vary by geographical location. WHO = World Health Organization.	

2.3.Causes of primary Osteoporosis:

Primary osteoporosis is connected with a lack of sex and age hormones in the body. The progressive degeneration of the trabeculae characterizes Age-related osteoporosis in the bone over time. In addition, the drop in estrogen production in women after menopause results in a considerable rise in bone loss, according to research. In males, sexual hormone–attached globulin inactivates estrogen and testosterone, decreasing bone mineral density (Raisz, 2005).

2.4.Causes of secondary osteoporosis:

Many diseases are causes secondary osteoporosis (Management of osteoporosis in postmenopausal women: 2010). All the diseases included imbalance of sex hormones, Ca, and vit. D (Raisz,2005). The Cushing disease is one disease that increases bone loss by glucocorticoid formation at a high percentage (Kawamata *et al.*,2008). Several diseases, like rheumatoid arthritis, need the patient's administration of glucocorticoid therapy for a long time, leading to secondary osteoporosis (Buckley *et al.*,2017).

Particularly noteworthy is that glucocorticoids are regarded as the most often prescribed drugs associated with drug-induced osteoporosis (Buckley *et al*.,2017). When glucocorticoid medication is initiated, BMD is shown to fall significantly within (3-6) months after the treatment. In order to help in the selection of appropriate medication for the treatment of glucocorticoid-induced osteoporosis, the American College of Rheumatology (ACR) has developed specific guidelines (Buckley *et al.*,2017). Secondary osteoporosis may be caused by various factors that vary between men and women. A large amount of alcohol, hypogonadism, and glucocorticoid is related to osteoporosis in males than in women (Sutton *et al.*,2011).

According to Shahinian *et al.(*2005), the males who take androgens for prostate cancer have a higher risk. 19.4 % of those with high risk for fracture, while 12.6 % do not take androgens.Using data from Tannenbaum *et al.* (2002), they discovered that secondary causes of osteoporosis were present in 32.4 % of women. These secondary causes included hypercalciuria, hyperparathyroidism, Ca malabsorption, vit. D deficiency, hyperthyroidism, hypocalciuric hypercalcemia, and Cushing's disease. It is important to emphasize that abnormalities of Ca metabolism and hyperparathyroidism were responsible for 78 % of the secondary causes (Tannenbaum *et al*.,2002).

2.5. Pathophysiology:

Besides providing the body structure and organs protection, bones also serve as a reservoir for minerals like P and Ca, which are necessary for bone formation. The individuals who have developed bone will attain peak bone mass and then lose bone mass during the rest of their lives. Although genetics play a significant role in peak bone mass, a variety of modifiable variables, such as diet, activity, and certain illnesses and/or drugs, may have an impact on bone mass as well (NIH, 2015).

Bones are continually modified throughout one's life, which means they are resorbed and produce new bone. This procedure provides for the preservation of strength and its restoration. When there is an imbalance in remodelling activity, such that resorption outpaces creation, the pathophysiological alterations associated with osteoporosis might occur (Raisz,2005). It is believed that growth factors and hormones are responsible for controlling bone function. Bone remodelling is significantly influenced by oestrogen and testosterone, which are principally responsible for suppressing bone breakdown. There have also been discoveries of cytokines that impact remodelling, such as receptor activator of (RANKL), RANKL is generated and connected to RANK receptors causing the osteoclasts to become activated and mature, resulting in bone resorption (Raisz,2005) When it comes to bone production, parathyroid hormone (PTH) is critical since it indirectly stimulates the proliferation of osteoblasts by regulating calcium homeostasis (Das and Crockett, 2013).

2.6. Bone Structure:

The bones are the differentiated organ that originates from the mesenchyme tissues and are the most specialized in the body. It has a vibrant and lively feel about it. Function, the bone is primarily a structural organ that can withstand a great deal of pressure (Zang *et al.*,2017).

Three cell types in bone are found: osteocytes, osteoblasts, osteoclasts, and lining cells (Dera,2017).

2.6.1.Osteocytes:

Osteocytes are osteoblast-derived cells incorporated into the bone matrix. Between 5% and 20% of osteoblasts are converted into osteocytes (Pérez-Castrillón *et al.*,2020). The osteoblasts entrapped in the newly formed bone matrix developed into osteocytes. Osteocytes account for

approximately 95% of the bone cells; they are not divided and long-lived (Capulli *et al.*,2014).

2.6.2.Osteoblast:

It is the cells responsible for bone formation and located along the outer surface of the bone (Dera,2017). The osteoblasts are cubic cells located along the surface of the bone, 4% of the total resident bone cells known for their role in bone formation (Rizzoli,2018). These cells demonstrate the morphological properties of protein-synthesizing, including prominent Golgi apparatus abundant, rough endoplasmic reticulum and various secretory vesicles (Florencio-Silva *et al*.,2015).

2.6.3. Osteoclasts

Osteoclasts are cells that line the inside of the bone that works as bone resorption. Osteoclasts dissolve bone mineral content (BMC) by producing an acidic environment, compromising the existing bone strength. Osteoclasts secrete enzymes that break down the residual collagen bone matrix, bringing the resorption process to a conclusion (Terndrup *et al.*,2016).

2.7. Screening and diagnosis:

Osteoporosis screening recommendations published in the literature are quite variable. In general, most organizations suggest that all persons more than fifty years of age have a family history of fracture and require BMD screening (Camacho *et al*.,2016). In addition, studies recommend routine screening of the bone density for all women over the age of 65 and older men for younger women who have a greater risk of fracture than the healthy Caucasian women over the age of 65 who have no risk factors. The Endocrine Society recommended that males with more than 70 years and those between 50 and 69, who have another risk factor for secondary osteoporosis, have their bones screened (Watts *et al*.,2012).

Berry *et al*. (2013) researched 4,800 women between the ages of 45 and 54 who were randomized to either be checked for osteoporosis or not be examined for osteoporosis. The experiment proved the efficacy of screening for osteoporosis early detection. The studies found that those who have hormone therapy at large amounts, and 25.9 % have fracture risk for a long time compared to the control group.

BMD tests, particularly in the hip and lumbar spine, using (DXA) instrument, or the incidence of non-traumatic hip or vertebral fractures are the diagnosing tools for osteoporosis, according to the American Osteopathic Association (Qaseem *et al.*,2017.(NOF) advises that bone mineral density (BMD) be measured one to two years after starting therapy and every two years after that for the next five years. The study recommends that testing be done each four years (Berry *et al*.,2013). According to (NAMS), recurrent examination of females with postmenopausal who have not been treated is not suggested until two to five years have elapsed. Repeated examination in females who have osteoporosis therapy has beneficial until one to two years following commencement (Das and Crockett ,2013).

Another diagnostic tool, FRAX is a risk-assessment tool for cancer patients (Fracture Risk Assessment Tool). To early detection of the hip fracture at ten years, for preventing the fractures which occur due to osteoporosis, it takes into account risk factors such as age, race, alcohol consumption, gender, BMI, smoking, prior personal or parental history of fracture, glucocorticoids using, secondary osteoporosis, and rheumatoid arthritis (Camacho *et al.*,2016).

This technique is used in combination with other tools, like DXA scan, to evaluate which individuals are good candidates for therapy and which patients are not (the University of Sheffield. FRAX calculation tool.,2017) In spite of this, there are certain limitations to FRAX,

including the fact that it not validated for use with whole BMD, for those undergoing osteoporotic therapy, or for anyone older than the designated (40-90) years. A history of falls is also not included as a risk factor since there is no standardized metre or pharmacological data to support the reduction of fracture risk associated with a fall history. Finally, it makes no suggestions about who should be treated or who should not be treated (University of Sheffield. FRAX calculation tool,,2017).

2.8. Epidemiology of Osteoporosis:

The ageing population is increasing at an exceptional rate. This burst in population will cause a large number of individuals with OP. It has been assessed that the prevalence of OP will increase from 1/3 in people 50-60 to over 50% of people over 80 years of age (Zang *et al*.,2017). Knowing OP's global diffusion is relevant to understanding its complex aetiology within the associated gene pools of different races and ethnicities. It also highlights the serious impact of this silent killer on families and societies worldwide (Suskin *et al*.,2018).

Postmenopausal women were found more likely to diagnose osteoporosis, with 41% being found to have OP, compared to 23.7% of women premenopausal. This is expected because estrogen is a protective factor against OP. After all, it helps maintain bone density, which is lost in postmenopausal women (Ahmadieh, *et al*.,2018).

The prevalence of OP in postmenopausal Iraqi women had been reported to be 22.8 % (Gorial *et al*.,2013). The prevalence of OP among postmenopausal women (PMW) is increasing across the globe. One study showed that 28.4% of Malaysian women are osteoporotic (Hatta, *et al*.,2019).

The prevalence recorded of osteoporosis was15% in Germany and France, 9% in the UK, 38% in Japan and 16% in the USA, whereas in men, the prevalence was 3% in Canada, 4% in Japan, 1% in the UK and

8% in France. Prior studies reported that the prevalence of OP in Caucasian women older than 50 years ranged between 7.9 and 22.6 %. Meanwhile, in Taiwan Nutrition and Health Survey found that the prevalence of OP in men was 11.6%, and forearm women were 25.0% (Limin, *et al.*,2017).

OP affects the population of about 1.4 million Canadians, mostly PMW and the elderly (Al Anouti *et al*.,2019). OP fractures in India are common in both sexes and may occur younger than in the west. Data shows that vit D deficiency is widespread in India; poor sunlight exposure, skin pigmentation and a vitamin D deficient diet are apparent causes of this finding (Malhotra and Mithal,2008). About Arab countries, the prevalence of OP and osteopenia among Jordanian PMW was 37.5 and 44.6 %, respectively (Ensrud and Carolyn 2019)**.**

2.9. Risk Factor for Osteoporosis

There are two types of OP risk factors:

A- Non-modifiable risk factors:

1- Age:

Premature age is the single most important risk factor for OP. Growing older causes bone mineral density (BMD) to decline in both sexes due to the influence of hormonal alteration on the bone remodelling process. The hormones estrogen and testosterone have an effect on the intestine absorption of Ca from the blood flow. When the gut absorption of calcium is reduced, calcium storage in compact and cancellous bone is recovered in order to maintain serum calcium levels at a constant level. A loss in bone mass happens as a result of calcium absorption from the bones exceeding the quantity of calcium that can be replenished (Donna,2012).

2- Gender:

Women experience bone loss at a younger age and faster than men. Women aged 50 or more have four times the OP rate and two times the osteopenia rate compared to men (Khaled,2017).

3- Family History of Fragility Fracture:

Fragility fracture causes extreme complications and even death for all menopausal women. About 61% of fragility fractures occur in women, with the female-male ratio reaching 1.6 (Sharifadin and Deraman).

4- Race:

Conventionally, the Caucasian race has a higher risk factor for the occurrence of osteoporosis, while Asians showed the normal risk of osteoporosis (BOW *et al*.,2012).

B- Modifiable Risk Factors

1-Medication:

The use of prednisolone in a dose ≥ 5.0 mg/day for more than 3 months and other medications such as glucocorticoids, anticoagulants, anticonvulsants, aromatase inhibitors, cancer chemotherapeutic drugs, and gonadotropin-releasing hormone agonists are risk factors of OP (Foundation, 2019).

2- Short Time of Sun Exposure:

The short time of sun exposure has been supposed to afford low BMD, since enough sun exposure to ultraviolet light is essential for vitamin D synthesis, which is essential for calcium homeostasis in the human body (Chawla *et al*.,2018).

3- Smoking:

Smoking is harmful to the bone, reduce body weight, calcium absorption, reproductive hormones, and bone mineral density, induces early menopause, and elevates bone-turnover markers and fracture risk

(Bainbridge *et al*.,2004). Smokers tend to have spinal deformities more than non-smokers (Wong *et al*.,2007).

4- Consumption of Alcohol:

Consumption of alcohol increases the risk of OP by inhibiting the function of the osteoblast and reducing bone formation, therefore resulting in more hip fractures (Rajasree and Büsselberg, 2016).

5- Nutrition:

The abundant nutrients used in our daily diet can affect bone health. Bones can be affected by different mechanisms, including Adjusting bone structure, the Process of endocrine and Metabolism of bone (Cashman, 2007).

2.10.Osteoporosis in Men:

As the population ages, osteoporosis in males is becoming a more serious public health concern, and its incidence is growing (Gennari and Bilezikian,2007). Even though osteoporosis has traditionally been considered a women's health concern, the mortality and morbidity rates associated with the disease are greater in males (Gennari and Bilezikian,2007). In most males, the consequences of osteoporosis are underappreciated, and the illness is often undiagnosed and ignored when it occurs (Curtis *et al*.,2009).

Male osteoporosis is caused by different factors, like age-related sex hormone deficiency, lifestyle, genetics (inactivity, using of tobacco and alcohol at large amounts), and risk agent (taking corticosteroids for long time), all of which contribute to bone loss and microarchitectural disruption. As men get older, their ability to produce estradiol and testosterone diminishes. In contrast to women, who have a sudden reduction in oestrogen levels during menopause, which results in faster bone loss, males endure delayed bone loss with a lesser overall decrease in bone mineral density (BMD) throughout menopause. In males, bone

loss manifests itself as trabecular thinning, but bone loss manifests as a decrease in trabecular thickness (Shobha *et al*.,2010).

2.11. Estrogens and androgens:

Androgens and estrogens are generated from cholesterol and are created in the gonads and adrenal glands to regulate reproduction. Furthermore, they are locally stimulated inside target tissues, such as bone, which is beneficial (Vanderschueren *et al*.,2014). Estradiol (E2) is a hormone that is produced largely in the cells of theca and granulosa of the ovarian follicles in females. In males, only 15 % of E2 is released directly from the testicles, with the remaining 85% obtained via peripheral aromatization (Gennari *et al*.,2004).

While women go through a period of sudden fall in E2, elderly men do not go through a period of "andropause," and overall E2 concentrations stay above the threshold required to maintain bone homeostasis,It becomes the major circulating androgen when testosterone (T) is converted to the more powerful dihydrotestosterone (DHT). Testosterone (T) is produced by the Leydig cells of the testicles and operates unaltered (DHT) (Finkelstein *et al*.,2016).

Testosterone may also be transformed to E2 by the action of the aromatase (CYP19A1). Hormonal feedback between the hypothalamus and pituitary regulates the bioactivity of circulating estrogens and androgens. Gonadotropins (FSH and LH) are responsible for this regulation. Human oestrogen and androgen bioavailability is regulated by high-affinity binding to the circulating sex hormone-binding protein (SHBG), which is found in the bloodstream (Laurent and Vanderschueren, 2014).

The free fraction of circulating T, DHT, and E2 (the fraction attached to SHBG, lbumin, or other proteins) is assumed to be physiologically active. In contrast, the bound fraction is thought to be

inactive. Estrogens and androgens aid in the development of bone mass during puberty and the maintenance of bone mass after puberty is over. Increased oestrogen levels in females at menopause or increased oestrogen and androgen levels in males later in life contribute to bone loss and strength loss, which contributes to osteoporosis development, one of the most frequent metabolic illnesses of old age (Manolagas *et al.*,2013).

2.12.Cathepsin K:

Cathepsins are lysosomal cysteine proteases initially identified in the early twentieth century and are now often referred to as cathepsins. The cathepsins (cathepsin B through Z) are a family of enzymes found in humans that are defined by their structural differences, their catalytic processes, and the proteins that they cleave (cathepsin B through Z) (Turk *et al*., 2001).

(Vasiljeva *et al*.,2007) showed that many enzymes have the same papain structure and a conserved Cys-Asn-His trio of residues in the active region. CatK is one of these enzymes that is primarily released by active osteoclasts to destroy collagen and proteins during bone resorption, and it is one of the most important (Costa *et al.*, 2011).

An N-terminal signal sequence of 15 amino acids length. It is a multifunctional protein that performs many roles, including that of an enzyme (Bromme and Okamoto,1995). In addition, CatK exhibits the typical 3D structure of a CatL, which is crucial from a structural perspective, located at the top of the CatK molecule, a V-shaped gap is filled by cysteine–histidine, which serves as the catalytic diad. CatK is an enzyme that catalyzes the reaction. A proline residue may be accepted at the P2 position, which is connected with the high quantity of proline and hydroxyproline residues seen in collagen (McGrath *et al.*,1997; Novinec and Lenarcic, 2013).

As a result of the discovery that inhibiting CatK activity might reduce bone resorption without impairing bone generation (Garber, 2016), it has emerged as a promising target for anti-resorptive drug research.

2.12.1. CatK and Bone Cells:

In osteoclasts, the expression of CatK was previously discovered (Drake *et al*., 1996), despite the fact that the other cathepsins were also present. As previously reported (Troen, 2006), the catK gene expression is controlled by (RANKL)-RANK signalling, which is a key signalling pathway in osteoclastogenesis (Troen, 2006). Because of the RANK signalling stimulation in osteoclast, the transcriptional factor NFATc1 is activated, which stimuli the osteoclast gene (Balkan *et al*., 2009).

There are a variety of additional substances that potentially induce CatK expression in osteoclasts, including (TNF-a), terleukins, vit D, and parathyroid hormone (Troen, 2006). CatK is known to be capable of degrading type I collagen (Garneroetal., 1998; Kafienahetal., 1998), which accounts for 90 % of bone matrix, Bossard *et al.* (1996) discovered that the extracellular matrix glycoprotein osteonectin (which accounts for the remaining 10% of organic bone matrix) is crucial in promoting bone remodelling and preserving bone mass (Garneroetal., 1998; Kafienahetal., 1998) (Delany *et al.*, 2000).

CatK can be cleaving the triple helix and the type I collagen fibres telopeptides, which is quite an accomplishment in itself (Bromme and Okamoto, 1995; Garnero *et al.*, 1998).

2.13. Bone sialoprotein

Bone sialoprotein (BSP) is a major noncollagenous extracellular matrix protein in bone produced by osteoclasts, osteoblasts, osteocytes, and hypertrophic chondrocytes. (Paz *et al.*,2005).In vitro studies revealed that BSP might have a role in many stages of the bone modelling and remodelling process. Additionally, BSP has been shown to

stimulate cell adhesion (Flore *et al*.,1992), enhance osteoclastogenesis, and decrease bone resorption (Tye *et al*.,2003) and it's potential to nucleate hydroxyl apatite crystal production and promote mineralization. (Valverde and colleagues, 2005) More importantly, even though BSP expression is connected with de novo bone formation (Chen *et al.*, 1992) and ectopic calcification (Lekic *et al*., 1997), a high BSP level in blood under pathological circumstances has been linked to excessive bone resorption (Chen *et al*., 1997). Examples include the discovery of BSP expression in several malignant tumour cells with a propensity to metastasize to bone and the observation of elevated serum BSP levels in patients with osteotropic malignancies and tumour-associated bone loss in the literature. Bellahcene (1994) and Jung *et al*. (2004).

Several metabolic bone diseases characterized by excessive bone resorption, such as postmenopausal osteoporosis, Paget's disease, rheumatoid, and ankylosing spondylitis, have been linked to abnormally high BSP levels in serum or synovial fluid. BSP levels in serum or synovial fluid have also been linked to abnormally high BSP levels in serum or synovial fluid (Shaarawy and Hasan, 2001; Acebes *et al*.,1999). Treatments targeted at preventing bone loss in women or people with Paget's disease, on the other hand, have been shown to lower serum BSP levels (Stork *et al.*,2000; Woitge *et al*.,2000).

A phosphorylated glycoprotein with a mass of 70–80 kDa, bone sialoprotein (BSP), is generated by osteoblasts, odontoblasts, osteoclasts, and osteocytes in the bone matrix. Franzen and Heinegard (1985) discovered that it was contained in the non-collagenous part of the bovine cortical bone matrix and that it was also present in dentine, calcified cartilage, and cement (Franzen and Heinegard, 1985; Störk *et al*., 2000).

Because of the unique location in which sialoprotein is synthesized, it is extremely specific to bone tissue, opening the door to the idea of

employing it as a marker of bone metabolism. Like most bone turnover indicators, BSP exhibits concentration changes, with greater levels in the morning among days, for example. Higher quantities of oestrogen are seen in healthy persons throughout the foetal period, early childhood, and after menopause (Seibel,2005; Shaarawy and Hasan,2001).

A thorough investigation of the role of BSP and its function in bone metabolism has not yet been completed. It is currently unclear if its primary function is in the formation processes or whether it participates in both of these processes (Malaval *et al.*,2008).

Even though BSP has predominantly been isolated from osteoblasts, it has also been found in osteoclasts and osteoclast-like cell lines and in tumour cells, among other places. According to theory, osteoclasts are important in the onset of bone resorption because they make it easier for them to connect to the bone. The presence of high levels of BSP in the plasma of persons suffering from metabolic bone illnesses such as Paget's disease, multiple myeloma, or hyperthyroidism further suggests that BSP plays a role in the onset of bone resorption in these patients (Seibel *et al.*,1996).

2.14.Gelsolin (GSN):

A founding member of the gelsolin superfamily, gelsolin (GSN) is a multifunctional protein identified in various tissues and organs of both mammals and non-mammals (Silacci *et al.*, 2004). (McGough *et al.*,2003). Among other things, it is vital in breaking formed actin filaments, capping the rapidly expanding plus ends, and stimulating the development of actin filaments in humans, all of which occur under the control of calcium ions and pH, which may enhance GSN binding to actin (Zhang *et al.*,2017). Recent research revealed that GSN concentration was associated with a wide range of disorders, including cancer of the

breast, mouth, multiple sclerosis, and rheumatoid arthritis, among others (Chen *et al.*,2015; Park *et al.*,2016).

Bone resorption and bone mass and strength are both affected by GSN expression. For example, mice missing GSN expression had a decreased rate of bone resorption while having an increased rate of bone strength and mass (Chellaiah *et al*.,2000).Premenopausal Chinese women with severely variable bone mineral density (BMD) were considerably higher levels of GSN protein expression in their circulating monocytes in clinical proteomics research (Deng *et al*.,2008).

A further multi-disciplinary and integrative investigation in premenopausal Caucasian women using the CMC proteome revealed that GSN is relevant for bone mineral density in Caucasian, These findings, taken together, demonstrated the critical role played by GSN in bone mineral density (BMD) and bone metabolism. Furthermore, GSN has the ability to be secreted out of CMCs, which is a good thing. Until now, we didn't know whether plasma GSN was connected with BMD or if it might be used as a possible biomarker for OP(Deng *et al*.,2014).

2.15.Fractalkine (FKN)/ CX3CL1:

To put it another way, cytokines are a family of glycoprotein molecules that serve as cellular hormones and are produced by stimulation of the cells to exert local and/or systemic effects on a variety of different types of human cells and organs (Stenken and Poschenrieder,2015). According to research, cytokines regulate osteoclast production and function, and interactions between the immune system and bone seem to be controlled mostly by cytokine signals (Lee and Lorenzo,2006).

Chemokine, ctoyokines are tiny (8–14 kD) proteins that regulate WBC trafficking, cell adhesion, phagocytosis (cytokine secretion), cell activation, proliferation, death (apoptosis), angiogenesis, and other

processes that occur during inflammation (Yoshie *et al.*,2001). CXC and CX3C chemokines are classified into four groups based on the amount and location of conserved cysteine residues (CC, XC, CXC and CX3C, respectively) in their amino acid structure (Rossi and Zlotnik,2000). According to research, chemokines can attract osteoclast precursors to areas of resorption that are locally inflamed and contribute significantly to bone remodelling (Galliera *et al.*,2008).

2.15.1.Structure and Function of CX3CL1/CX3CR1 Signaling Axis:

CX3CL1 was discovered for the first time in 1997 (Zlotnik and Yoshie,2012).According to the most recent nomenclature, the CX3CL1 gene is the sole member of the CX3C (delta) subfamily (Nomiyama *et al*.,1998). The gene that encodes CX3CL1 in humans is located on chromosome 16q13 (Kim *et al*.,2011). It differs from other chemokines structurally. It has a motif consisting of three amino acid residues sandwiched between two cysteine residues, which forms disulfide bonds and stabilizes the tertiary structure of the molecule other chemokines do not (Pan *et al*.,1997).

The existence of two distinct CX3CL1 forms in the body dictates this cytokine's specific function in the cytokine network. CX3CL1/CX3CR1 axis biological activity manifests itself most often in highly-vascularized organs (the synovial membrane) and a variety of other tissues (Zlotnik and Yoshie,2012).

When expressed in endothelial cells, mCX3CL1 acts as an adhesion molecule, facilitating the passage of immune cells by the vascular endothelium regardless of whether or not the integrin-related mechanism is involved ,This particularly proinflammatory feature allows for a more rapid buildup of immune cells at the location of the inflammation, which is beneficial(Nomiyama *et al.*,1998).

This characteristic is particularly noteworthy since it is unique to this particular set of chemicals. The involvement of the CX3CL1/CX3CR1 axis has been demonstrated in a variety of diseases, including rheumatoid, SLE, atherosclerosis, spinal cord damage, and osteoarthritis (OA)(Muo *et al.*, 2012). When CX3CL1 interacts with CX3CR1, it is possible to detect biological CX3CL1 activity (Mizoue *et al.*,1999).

2.16.Calcium:

Bone contains roughly 1–1.3kg of calcium per healthy adult, with 99% of the calcium being found in bone 1% of the calcium in extracellular fluid and soft tissues, which are also sources of calcium. The skeleton is a primary source of calcium, and it serves as a large calcium storage reservoir. Calcium is found in plasma in three forms: free or ionized calcium, bound calcium proteins, and complexed calcium like citrate, lactate, phosphate, and bicarbonate. Albumin is a calcium-binding protein, with 1gram of albumin for each deciliter of blood binding to around 0.8 milligrammes of calcium every day(Klemm and Klein,2011).

The pH of the solution affects the binding of calcium by albumin. Albumin contains fewer calcium-binding sites when the acidic pH is, resulting in a higher free calcium concentration in the blood. When the pH is raised, the free calcium concentration decreases. It is necessary to consume and absorb enough calcium from the diet to maintain adequate calcium reserves in the body (Johnson and Kumar,1994).

It has been proven that calcium absorption in males and non-pregnant women is 25% of the calcium intake , the typical urinary loss is 22%, and the average faecal loss is 75 % of the calcium taken, with small losses through perspiration, skin, hair, and other sources. Osteomalacia and rickets are conditions caused by a lack of calcium in the diet over a lengthy period(Hunt, Johnson,2007).

2.16.1.Calcium Homeostasis:

Calcium is required for various processes such as cellular signalling, impulse transmission, and muscular contraction. Among healthy persons, the level of total calcium in the blood is carefully regulated within a limited range, with total calcium (8.8-10.4) mg/dL and free Ca (4.6-5.3) mg/dL. Ca2+ is an active form of Ca and has a level in plasma that is strictly controlled by Ca reabsorption in the renal. These actions are all controlled by parathyroid hormone and (1,25(OH)2D), which are both hormones (Blaine *et al.*,2015).

2.17.Vitamin D:

Due to its regulatory functions in Ca, vit. D and its metabolites are considered hormones and prohormones. There are two types of vit D: vit. D2 and vit. D3. Milk is fortified with vit D2 to increase its nutritional value in the USA. Vit D3 was produced from cholesterol when the skin is exposed to UV rays or obtained through specific foods (egg yolk, fish liver oils, liver, and fatty fish). Vit. D3 is essential for bone health (Winter and Kleerekoper,2012). 1-hydroxylase in the kidney converts vitamin D to 25(OH)D in the liver, the most abundant circulating form of vitamin D, and subsequently to 1,25 (OH)2D. The body synthesizes vitamin D from calcium and phosphorus (Pazirandeh and Burns,2016).

Increased bone resorption is caused by 1,25(OH)2D activated stem cells developing into osteoclasts and motivating osteoblasts to release cytokines that regulate osteoclast activity when exposed to high concentrations of the compound (Winter and Kleerekoper,2012).

Comparatively speaking, vit D has a significantly lower impact on calcium homeostasis than parathyroid hormone (PTH). A low Vit D intake causes deficient Vit D, intestine malabsorption, impaired 1-(OH) of25(OH)Dconverting,1,25 (OH)3resistance(PazirandehandBurns,2016).

The amount of 25(OH)D present in the serum offers information about that individual's Vit. D. 25(OH)D level for maintaining the skeletal system is a matter of contention (Dawson-Hughes,2016). Some experts recommend 25(OH)D levels (20-40) ng/mL (50–100nmol/L) in the serum, while others recommend 25(OH)D levels (30-50) ng/mL (75–125nmol/L) in the serum. This is based on the results of vitamin D supplementation trials (Dawson-Hughes *et al.,* 1997; Sanders *et al*., 2010) (Institute of Medicine, 2010 In general, experts believe that levels fewer than 20ng/mL are suboptimal for bone health and that levels more than 50ng/mL (125nmol/L) may raise concerns about toxic effects on the body (Sanders *et al*.,2010).

2.18. Phosphorous (po4):

Energy metabolism, bone formation, cell communication, and balance of the acid-base are just a few of the fundamental biochemical processes in which phosphate is involved (Winter and Harris,2011).

Phosphorus may be found as forms in the human body. Organic phosphate esters are typically found inside cells, which makes sense. When it comes to bone, inorganic phosphate is the most abundant component. It plays a significant function in the body's structural support and supplies phosphate to extracellular and intracellular pools. Phosphate is protein bound to a lesser extent than Mg and Ca, and the remaining portion is free phosphate (around 10%) (Endres *et al.*, 1999). Inorganic phosphate may be found in plasma in the form of both monovalent (H2PO4) and divalent (HPO42) anions. H2PO4 to HPO42 is a 1:1 ratio in acidosis, a 1:4 ratio at pH 7.4, and a 1:9 ratio in alkalosis, depending on the pH value (Endres *et al.*, 1999).

2.18.1.Homeostasis of the phosphate:

Blood phosphate acts of absorption in the gut, excretion in the renal, and the processes of deposition and resorption in the bone. A

phosphate cotransporter, sodium-dependent phosphate cotransporter 2b, primarily assists in the absorption of phosphates in the small intestine (NaPi-2b). One of the effects of 1,25(OH)2D is that it may raise the expression of NaPi-2b protein, which helps to phosphate absorption by the intestine (Razzaque, 2009).

In the epithelial cells of the kidney, PTH enhances urine phosphate production by inhibiting phosphate, which was taken by the cells. The studies showed that (FGF23) and klotho might directly inhibit sodium phosphate cotransporter activity (Hu *et al*.,2010). In addition to the gut and kidney, bone plays an important function in maintaining phosphate equilibrium in the body. During low blood phosphate levels, the bone releases extra phosphate to maintain homeostatic balance, controlled by PTH action and 1,25(OH)2D action (Lanske and Razzaque, 2014).

2.19.Alkaline Phosphatase:

Alkaline phosphatase (EC 3.1.3.1) is an enzyme that is activated in the intestine, liver, kidneys, and bone. ALP activity in the sera of healthy people is mostly derived from the liver, with the majority of the remaining activity coming from the skeleton. The osteoblast produces bALP during the osteoblastic process. This enzyme's primary can hydrolyze phosphate and inorganic pyrophosphate used in Ca-hydroxyapatite production. Paget disease is characterized by a significant rise in total ALP activity, which gives the best diagnosis of PDB(Winter and Kleerekoper,2012).

ALP levels are elevated in individuals with metabolic diseases such as osteoporosis, rickets, osteomalacia, hyperparathyroidism, renal osteodystrophy, and thyrotoxicosis. ALP levels are also elevated in people with bone metastases and acromegaly (Winter and Kleerekoper,2012).

ALP is the ideal measure for bone turnover in individuals with poor renal function because it does not rely on glomerular filtration to clear the blood. When p-nitrophenol phosphate is used to remove the phosphate from a p-nitrophenol phosphate to a p-nitrophenol at an alkaline pH of 10.4, the total ALP was detected is calculated. The rise in absorbance at 450nm is related to the amount of ALP activity present in the sample. bALP is more resistant to heat than liver ALP, which results in a larger proportion of bALP activity being lost and a greater percentage of liver ALP activity being maintained following exposure to (56–59°C) for thirty minutes(Klein and Bodenmuller,1996).

The difference between the levels of bALP activity is used to measure bALP activity. On the other hand, this approach has been criticized for its lack of repeatability and unpredictability in results. In addition, wheat germ agglutinin (WGA) may be used to precipitate bALP. However, this method failed to fully distinguish between bone and liver activity in individuals with Paget illness (Klein and Bodenmuller,1996).

Because the carbohydrate content of ALP from various cell types varies, immunological assays have been designed to assess bALP based on the carbohydrate of ALP. On an automated immunoassay analyzer, a bALP test is available (Broyles *et al.*,1998). The findings of the bALP immunoassay have higher specificity and sensitivity for bone growth than the results of the total ALP. The antibodies are not selective for bALP and may demonstrate (7–17)% cross-reactivity with hepatic ALP, depending on the concentration used (Broyles *et al*.,1998; Price *et al.*,1997). bALP is also a possibility for individuals who do not have significant liver disease, according to Gonzalez-Calvin *et al*. (2009).

2.20. Genetic and Osteoporosis:

It is a prevalent condition characterized by decreased bone mineral density (BMD) and an increased risk of fragility fractures, responsible for major morbidity and death and large healthcare expenses in industrialized nations (Cummings and Melton,2002). BMD is one of the most significant clinical markers of osteoporotic fracture risk, and it is also one of the most easily measured. According to data gathered from twin and family studies, anywhere between (50-85)% of the diversity in BMD is genetically driven (Krall and Dawson-Hughes,1993).

Numerous polymorphisms have been discovered in genes linked to low bone mineral density (BMD) or osteoporotic fractures (Liu *et al.*,2002). Just a few of these have been verified with large-scale data, though (Ioannidismz, 2003). Those that have only accounted for a tiny part of the genetic contribution to bone mineral density and susceptibility to osteoporotic fractures (Ralston *et al* .,2006) The development of peak bone mass, which occurs in early adulthood, is assumed to be primarily influenced by genetic factors, according to current thinking (Pollitzer and Anderson,1989) While some researchers have observed that bone loss may be genetically determined, others have said that it is not (Kelly *et al.*,1993).

Christian *et al*. (1989) found no indication of genetic influences on bone loss even though the bone turnover is strongly linked to genetics. (Hunte *et al*. 2001) Many genome-wide linkage searches for quantitative trait loci (QTLs) that regulate bone mineral density (BMD) have been conducted. However, only a small number of these studies have identified loci that meet the criteria for genome-wide significance. There has been only limited replication of linkage peaks between studies in the literature. (2005); (Ralston, 2005).

2.20.1. Phosphatase acid type 5,Tartrate _Resistant ACP5:

TRAP (tartrate-resistant acid phosphatase; ACP5, EC 3.1.3.2), sometimes called uteroferrin (Ek-Rylander *et al.,* 1997), was recognized for more than 50 years as a reliable marker for osteoclasts (Ek-Rylander *et al.,* 1997). A relative proenzyme (monomeric TRAP [mTRAP]), loop-TRAP, and serum trap 5a are generated. Their catalytic activity is boosted by proteolytic cleavage by members of the cathepsin family or other proteinases by a factor of at least ten, resulting in increased catalytic activity (Fagerlund *et al*.,2006; Ljusberg *et al.,* 2005).

In the ACP5 gene, instructions are provided for producing an enzyme known as tartrate-resistant acid phosphatase type 5. (TRAP). The TRAP enzyme is largely responsible for regulating the activity of a protein known as osteopontin, which is generated by bone cells known as osteoclasts and immune cells. Osteopontin is a protein that has many roles in these cells. It has been discovered that the TRAP enzyme is synthesized in two different versions (isoforms): TRAP5a is found largely in immune cells, whereas TRAP5b is found primarily in bone cells known as osteoclasts (Janckila *et al*.,2001;Fagerlund *et al*.,2006).

Ek-Rylander *et al.,* (1994); Ek-Rylander and Andersson,(2010) have shown that cleaved, active TRAP is the same in structure to osteoclastic TRAP (Janckila *et al*., 2001) ,According to the findings of research done by (Halleen *et al*.,2002), the serum activity of TRAP 5b is significantly greater in persons with osteoporosis and is negatively related to bone mineral density (BMD). It has been observed in mice that global deletion of TRAP causes disturbed endochondral ossification as well as a osteopetrotic phenotype (Hayman and colleagues, 1996; Suter and colleagues, 2001), overexpression of TRAP causes increased bone turnover as well as an osteoporotic phenotype (Hayman and colleagues, 1996; Suter and colleagues, 2001).

In addition to osteoclasts, trap is found in osteoblasts and osteocytes, and osteoblasts and osteocytes (Gradin *et al*., 2012; Qing *et al*.,2012). Bone surfaces or intracortical remodelling sites (Yamamoto and Nagai, 1998) have been shown to be associated with these lesions (Bianco *et al*.,1988; Nakano *et al*.,2004). The bone tissue of the rat was examined. A number of hypotheses have been advanced to explain the genesis and function of TRAP in these cells; one theory holds that osteoclastic TRAP from the resorption lacunae is endocytosed by the osteoblasts and/or osteocytes, while another holds that TRAP from the resorption lacunae is endocytosed by the osteoblasts and/or osteocytes. Researchers have discovered that osteoblast-like cells can engulf and inactivate osteoclastic TRAP, suggesting that this might be utilized to restrict the enzyme's activity and prevent further degradation of matrix components in the body (Perez-Amodio *et al*.,2006; Perez-Amodio *et al*.,2005).

2.20.2.Single Nucleotide Polymorphism (SNP):

Individuals with single base pair in gDNA at which different sequence alternatives (alleles) exist in normal individuals in some population(s), wherein the least frequent allele has an abundance of at least 1% or greater, are considered to have single nucleotide polymorphisms (SNPs). In contrast, single base insertion/deletion variants (indels) are not SNPs (Shahzad *et al*.,2020). According to Jehan and Lakhanpau (2006), SNP markers can be found in noncoding regions of the genome and genes (both exons and introns). Depending on the potential application, SNP markers can be selected outside exons or directly cause a genetic mutation (Jehan and Lakhanpau, 2006).

In order to genotype SNPs, a variety of methods are available, including The proper study of genetic diversity has significant consequences in several fields such as detection of the diseases, and the

mutations. Methods for analysing allelic and mutational sequence variation, such as PCR (SSCP), provide substantial benefits many other nucleic acid methods when it comes to obtaining an accurate picture of allelic and mutational variation. It describes the small-scale comparative genomics (SSCP) method involved extraction of the gDNA and DNA amplification of specific area for detection the mutations. Also covered in depth is the sequencing analysis of polymorphi bands recovered from gels that took place after they were isolated. The SSCP approach is capable of detecting limited mutations more than (450-500) bp and can be completed in as little as one day, on average. Users have proved the efficacy of this easy-to-use, inexpensive, and potentially high-throughput technology in the research of a broad variety of infections and illnesses, and it has the capability of being applied to the study of any gene from any organism. (Gasser *et al*., 2006).

3.Materials and Methods

3.1.Chemicals and Instruments

3.1.1.Chemicals:

Chemicals and biological materials used in this study are listed in Table (3-1).

Table (3-1) : Chemicals and biological materials and its Supplying company.

No.	Material	Company	country
1	100 bp ladder	Semen	China
2	The agarose powder	pronadisa	Spain
3	Boric acid	CDH	India
4	DNA loading dye	promega	USA
5	EDTA	Scr	Chain
6	Gel loading dye purple DNA	biolabs	New England
7	$Mgcl_2$	cyntol	Rusia
8	Nuclease free water	Cyntol	Russian
9	Primers	Macrogen	Korea
10	Proteinase K	Bioneer	India
11	Pcr Master mix	cyntol	Russia
18	Ethedium bromide	Intron	Korea
20	Tris Borate EDTA (TBE)	Thomas BAKer	India
21	Tris -base	Thomas baker	India
22	Humane Gelsolin ELISA Kit	BTLAB	China
23	HumanCX3C Chemokine/Fractalkine (ELISA)	BT LAB	China
24	Human Bone Sialoprotein (ELISA)	BT LAB	China
25	Human Cathepsin K (ELISA)	BT LAB	China
26	VIT D Direct (ELISA)	AccuBind ELISA Microwells	USA
27	Calcium Kit	BIOLABO	France
28	Inorganic Phosphorus Kit	BIOLABO	France
29	Alkaline Phosphatase Kit	BIOLABO	France

3.1.2.Instruments:

used apparatus and instruments are shown in Table (3-2).

Table (3-2): the used apparatus in the present study

No.	Apparatus	Company	Country
1	Autoclave	Hirayama	Japan
2	Cooling centrifuge	Hettich	Germany
3	Distillatory	Shin saeng	Korea
4	Vortex	Griffen and George Ltd	UK
5	Microwave	Argos	Germany
6	UV Transilluminator	Quantum	France
7	Thermo cycle PCR	BIOMETRA	Germany
8	Horizontal gel electrophoresis	ATTA	Japan
9	Centrifuge for Eppendorf tub	Hettich	Germany
10	Hoot plate	Medico	USA
11	Deep freezer	Almateen	China
12	Digital camera	Canon	China
13	Sensitive Electron Balance	Entric	Germany
14	Flask	Chemical-Lab	China
15	Syringe	Meheco	China
16	Different size Micropipettes µl	Dragon	German
17	Different size tips	Biobaseic	Canada
18	ELISA reader and washer	Biotek	USA
19	Eppendorf tubes	Sigma	(England)
20	Digital camera	Sony	(Japan)
21	Spectrophotometer	APEL	Japan

3.2. The methods:

3.2.1. Sample collection:

The samples are collected from the patients attended the Marjan Medical City in Babylon-Iraq (Bone Density unit). Samples in the study were collected from March 2019 to May 2021. The analysis part of the current study was carried out at the Department Biology Laboratories of the College of Science at University of Babylon.

3.2.2.Study Population :

This study used 150 patients. The samples are consist of two groups, the 1st are one hundred patients (80 and 20) of women and men respectively with OP and 2nd consist of fifty healthy individuals (31 and 19) of women and men respectively, at age (20- 80) years.

3.3. Data Collection

3.3.1. Inclusion Criteria:

Inclusion criteria in the present study includes (femals and male) their age ranged between(20-80years),according to history, physical examination, laboratory investigation and donot suffer from chronic diseases illustrated in exclusion criteria below. The participant were divided into two groups; patient group (women and man with OP) and apparently healthy control (healthy women and man), those who were admitted to Bone Density Unit in Marjan Medical City and agreed to participate in this study.

3.3.2.Exclusion Criteria:

Exclusion criteria in the present study includes any participant had:

1-Hypothyroidism,hyperthyroidism.

2-Historyofcancer.

3-Rheumatoidarthritis.

4-Thalassemia.

5- History of chronic drug users such as heparin, corticosteroids and contraceptives that can affect BMD.

3.4.Socio-Demographic and Anthropometric Measurement

A-Questionnaire:

A questionnaire was taken from the patients and case sheets including: name, age, sex, onset of disease, other diseases, and medication. Socio-demographic characteristics include height, weight, age, equivalence, miscarriage, marital status, and medical history.

B-Anthropometric Measurement:

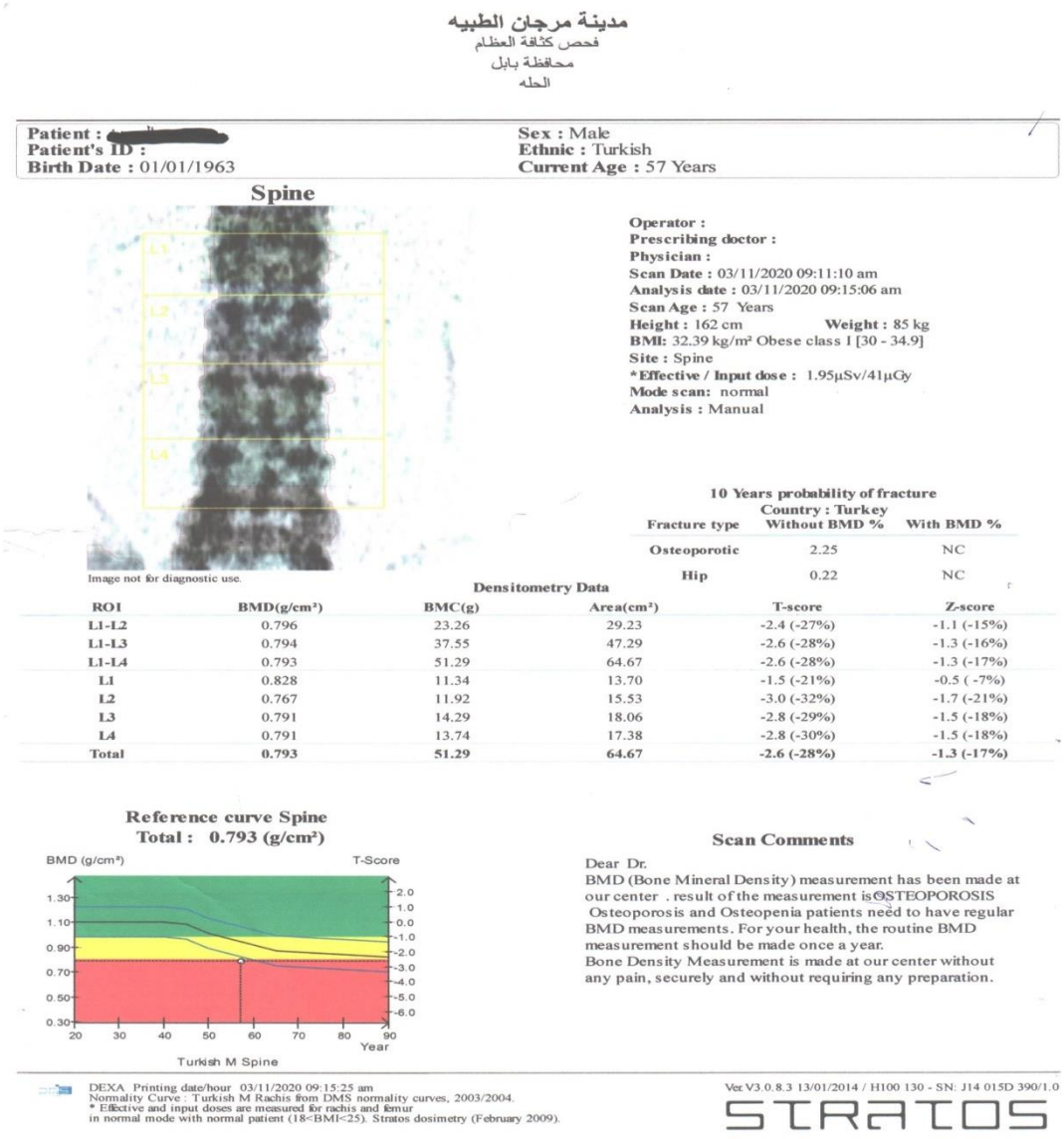
This section include body mass index, bodyweight (Kg),height (m). The body mass index (BMI) is calculated by weight (kg) divided by the square of height (m).

3.5. Diagnosis of Osteoporosis:

All the patients involved in this study had been examined by bone mineral densitometry (central DEXA type DEXXUM 3).

Procedure:

DEXA scan model DEXXUM 3 was used to take T-score at spine (L1-L4) and neck of femur.



مدينة مرجان الطبيه
فحص كثافة العظام
محافظة بابل
الحله

Patient : [redacted]
Patient's ID :
Birth Date : 01/01/1963
Sex : Male
Ethnic : Turkish
Current Age : 57 Years

Spine

Image not for diagnostic use.

Operator :
Prescribing doctor :
Physician :
Scan Date : 03/11/2020 09:11:10 am
Analysis date : 03/11/2020 09:15:06 am
Scan Age : 57 Years
Height : 162 cm **Weight :** 85 kg
BMI: 32.39 kg/m² Obese class I [30 - 34.9]
Site : Spine
***Effective / Input dose :** 1.95µSv/41µGy
Mode scan: normal
Analysis : Manual

10 Years probability of fracture
Country : Turkey

Fracture type	Without BMD %	With BMD %
Osteoporotic	2.25	NC
Hip	0.22	NC

Densitometry Data

ROI	BMD(g/cm²)	BMC(g)	Area(cm²)	T-score	Z-score
L1-L2	0.796	23.26	29.23	-2.4 (-27%)	-1.1 (-15%)
L1-L3	0.794	37.55	47.29	-2.6 (-28%)	-1.3 (-16%)
L1-L4	0.793	51.29	64.67	-2.6 (-28%)	-1.3 (-17%)
L1	0.828	11.34	13.70	-1.5 (-21%)	-0.5 (-7%)
L2	0.767	11.92	15.53	-3.0 (-32%)	-1.7 (-21%)
L3	0.791	14.29	18.06	-2.8 (-29%)	-1.5 (-18%)
L4	0.791	13.74	17.38	-2.8 (-30%)	-1.5 (-18%)
Total	**0.793**	**51.29**	**64.67**	**-2.6 (-28%)**	**-1.3 (-17%)**

Reference curve Spine
Total : 0.793 (g/cm²)

Scan Comments

Dear Dr.
BMD (Bone Mineral Density) measurement has been made at our center . result of the measurement is OSTEOPOROSIS
Osteoporosis and Osteopenia patients need to have regular BMD measurements. For your health, the routine BMD measurement should be made once a year.
Bone Density Measurement is made at our center without any pain, securely and without requiring any preparation.

DEXA Printing date/hour 03/11/2020 09:15:25 am
Normality Curve : Turkish M Rachis from DMS normality curves, 2003/2004.
* Effective and input doses are measured for rachis and femur in normal mode with normal patient (18<BMI<25). Stratos dosimetry (February 2009).

Ver.V3.0.8.3 13/01/2014 / H100 130 - SN: J14 015D 390/1.0
STRATOS

Figuer (3-1) Report Obtained from DEXA Scan

3.6. Interpretation of the Results:

For all patients (women and men) who are20years of age or older, another record called T-Score is used for diagnosis. The normal value of BMD for youth of the same age and gender (T degree is more than -1).

Osteopenia have BMD value and ranged (1- 2.5) SDs, less than the adults (T degree is less than -1).

Osteoporosis with 2.5 SDs or more, that mean BMD (T degree less than -2.5) (Iqbal,2000; World Health Organization (WHO)(2004).

3.7. Blood samples Collecting:

The blood samples were collected from control and patients by syringes (G23). Five ml of blood was obtained from each subject, blood (2) ml was kept in tube with EDTA stored in (-20˚C) for used in molecular tests and left blood (3ml) kept in disposable gel tubes which left to make clot for fifteen minutes then centrifuged at (3) thousand for ten minutes, then the obtained sera was stored at -20˚C (Barbara and Anna,2012).

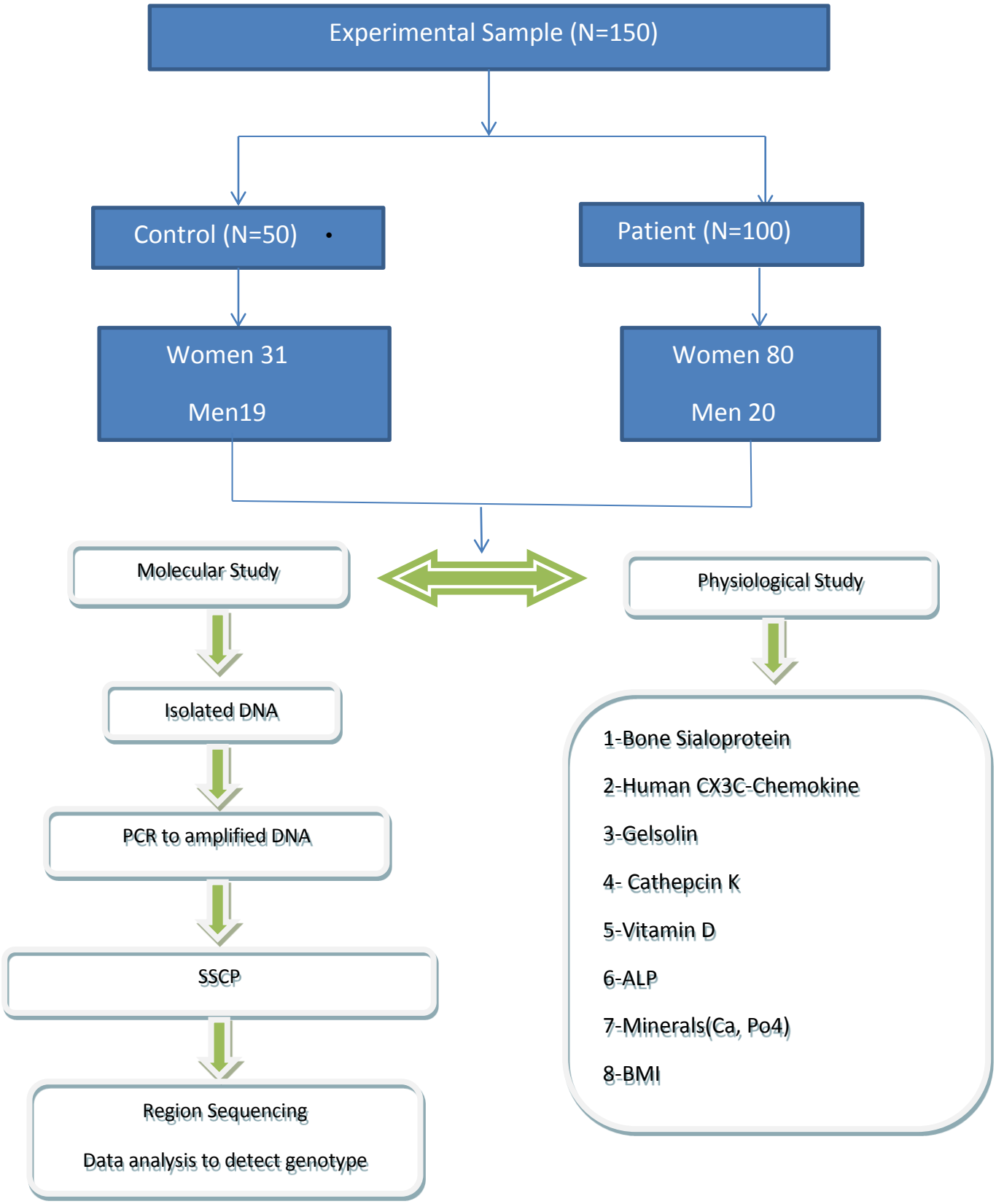


Figure (3-2):Experimental Design

3.8. Methods:

3.8.1. Determination of Human Gelsolin ELISA Kit:

Evaluating level of human Gelsolin explained in Appendix A .

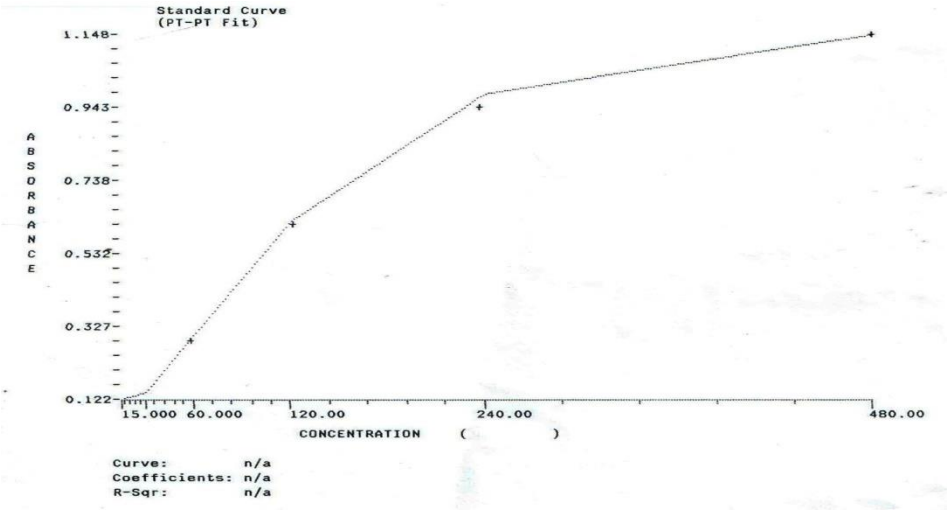


Figure (3-3) Standard Curve of gelsoline Concentration

3.8.2.Determination of Human CX3C-chemokine/ Fractalkine ELISA Kit

Assessment of human CX3C-chemokine/ Fractalkine explained in "Appendix B"

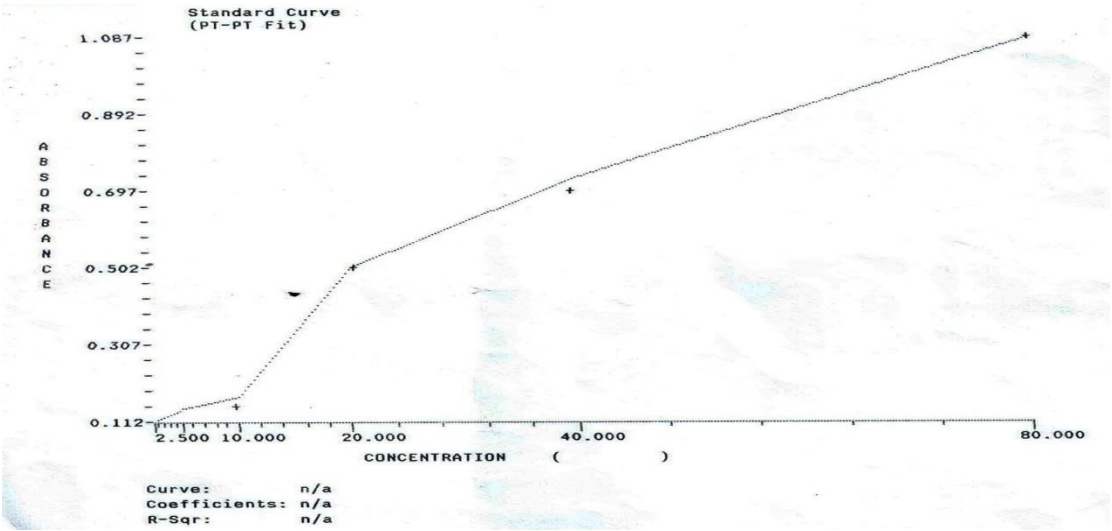
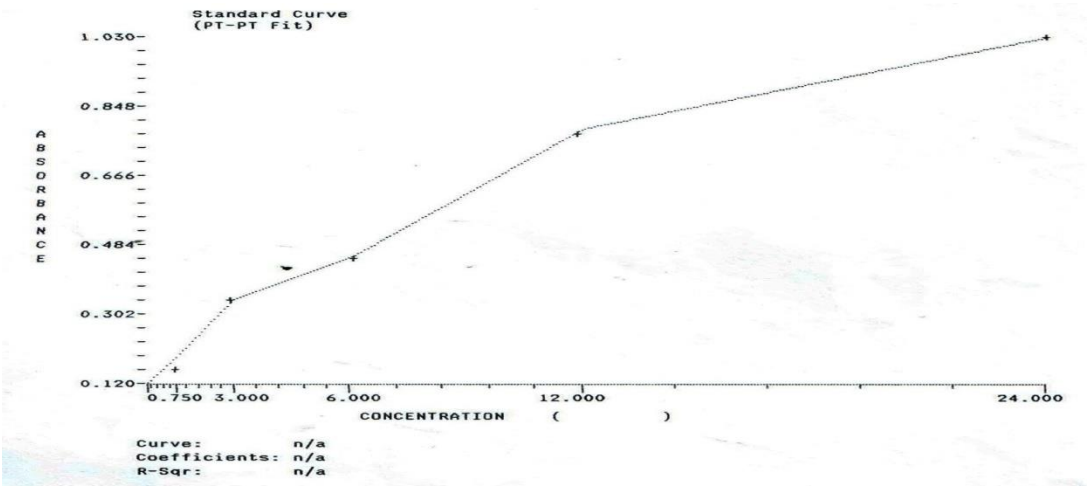


Figure (3-4): Standard Curve of CX3C Concentration -chemokine/ Fractalkine

3.8.3. Determination of Human Cathepsin K ELISA Kit

Evaluating level of human cathepcin K explained in Appendix C



Figure(3-5) Standard Curve of Cathepsin K Concentration

3.8.4. Determination of Human Bone Sialoprotein ELISA Kit:

Assessment of human Bone Sialoprotein explained in Appendix D

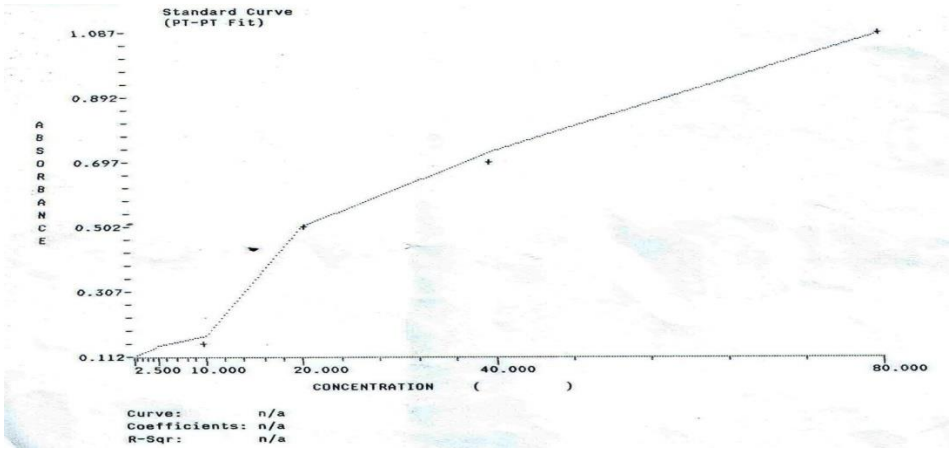


Figure (3-6) Standard Curve of Bone Sailoprotein Concentration

3.8.5. Determination of VIT D DIRECT ELISA:

Evaluating level of human cathepcin K explained in Appendix E

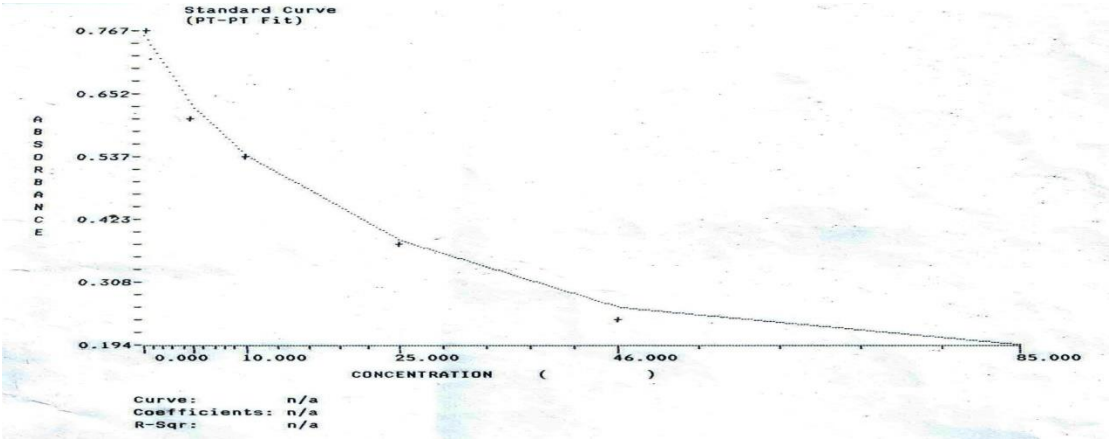


Figure (3-7) Standard Curve of VIT D Concentration

3.8.6. Determination of Calcium by CPC method

Assessment of Calcium explained in Appendix F

3.8.7. Determination of Inorganic Phosphorus:

Evaluating level of Inorganic Phosphorus explained in Appendix G

3.8.8. Determination of Alkaline Phophatase:

Assessment of Alkaline Phophatase in Appendix H

The phenol is produced due to hydrolysis of the substrate at absorbance (510) nm in the sample. Na-arsenate prevents the dilution of the colour during the methods application.

3.9. Genetic Study

3.9.1. Extraction of DNA:

gDNA are extracted from white blood cells for all the patients and healthy by using special extraction kit (Hashim and Al-Shuhaib,2020):

1- Transfer 5 mL of the blood to second tube with a 1.5 mL capacity, as indicated in the figure. 2- Repeat the process with the remaining blood. It was added and mixed well before incubating for ten minutes or for 5 minutes in an automated rotating mixer, depending on the time of day.

Using a 10000 g centrifuge at room temperature for 2 minutes, the mixture was separated. The pellet was washed with the washing buffer once again, which was inverted numerous times to remove any leftover washing buffer before centrifuging for ten seconds to remove the residual washing buffer. removing the supernatant from the solution after the experiment

2-Suspend the pellet in 200 mL of cell suspension buffer for 15 minutes, then add 400 mL of extraction buffer and mix well to ensure even dispersion in the 60 Silesian.

3-Leave the mixture at room temperature for 30 minutes before adding 100 mL of sodium acetate and spinning the mixture for 1 minute.

4- The content was centrifuged at (10000) g for 10 minutes at room temperature. Take the supernatant from a centrifuged for half minute and discard it.

5-Washed twice with 600 mL (70 percent ethanol+30 percent Tris) and centrifuged for 30 seconds each time after. The preceding process was performed twice more, followed by dehydration followed by centrifugation at 10000 g for 3 minutes to remove the washing buffer, followed by transfer of the supernatant to a fresh tube.

6- Added 100 mL of DNA elution buffer and let to sit for 5 minutes, then centrifuged at 10,000 g for 1 minute to get a clear liquid containing pure DNA.

3.9.2. Evaluated isolated DNA:

The extracted DNA was checked quality, quantity and integrity by spectrophotometry and agarose-electrophoresis the spectrophotometry of DNA was conducted by scan-drop (biometra –Germany) in which the

quantity a cheifed arranged from 30-45ng/ µl and the quality of 260/280 ratio ranged (1.7-1.85)each sample that did not produce 260-280 ratio larger than 1.7 was re extracted. On the other hand the DNA integrity was checked by resolving the extracted DNA by electrophoresis on 1% agarose.

3.9.3. Agarose gel electrophoresis:

1-Horizontal gel electrophoresis was used to create the gel (2 percent), which was made by dissolving 1.6 grammes of agarose in 80 ml and heating it in the microwave oven for 2 minutes.

2-The homogenised agarose was then chilled to 55 degrees Celsius.

3-Three to four millilitres of ethidium bromide were added to the gel and swirled together.

4-After that, the gel was placed into the gel tray and allowed to polymerize for half hour.

5-After that, the polymerized gel was moved to the electrophoresis apparatus and immersed in 0.5 TBE.

6-the pcr product (5) ml were combined with two microliters of loading dye and gently pipetted into the gel wells using a mechanical pipette.

7-In order to carry out the electrophoresis procedure, we set the apparatus to 100 volts and 50 milliamps for 60 minutes.

8- The gel was then photographed, and the picture was examined in order to estimate the molecular weight of the retrieved PCR product.

3.10. Primer design:

The designing of the primers for PCR are done depending on the following:

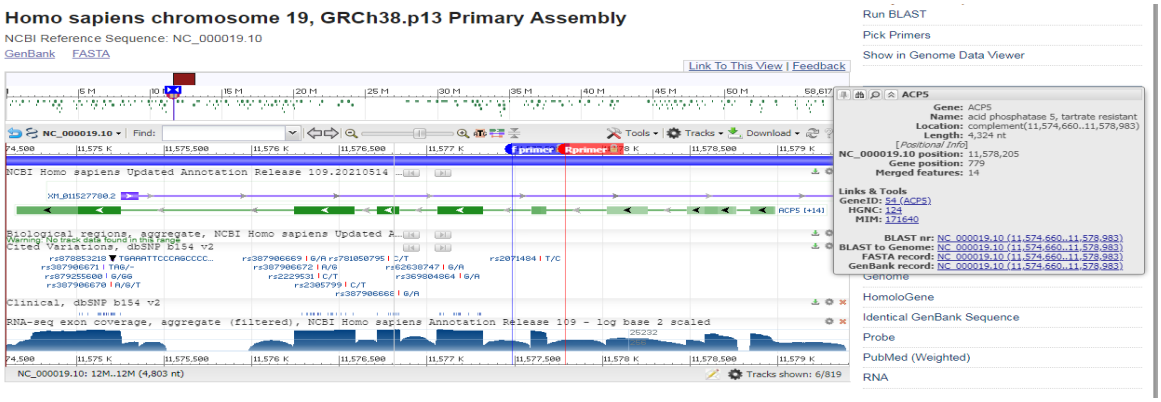
With the aid of the BLAST online tool, the primers were constructed,, the specificity of developed primers was tested by conducting a BLAST against the human genome. Next, demand criteria were used to pick the primers pair, which included length, melting temp similarity, specificity, and primers length. Following that, the mutations were buried in line with the design specifications.

By using the Oligo Calconline programmer, the capacity of the primer to create secondary structure was examined. the primer is rejected when it had five bases or more that that could form self-dimerization and/or 4 bases that could form a hairpin structure, respectively.

When using the "Multiple Primer Analyzer" online programmer from Thermo Fisher Scientific Inc., each primer pair was tested for the creation of dimers. The software was set to the number 2; if the primer pair formed dimers at this level of sensitivity, the primer pair was discarded.

Table (3-3): Primers of Tartrate resistant acid phosphatase(TRAP) or (ACP-5) gene

	Sequence (5'->3')	Length	Tm	GC%	Self-complementarity	Self-3' complementarity
Forward primer	ACAAGCTGGCTTAGGGAAGG	20	59.67	55.00	6.00	0.00
Reverse primer	TGTCCTGCTCCAGGGAAGT	19	60.15	57.89	5.00	1.00



3.10.1. Reconstituting and diluting primers:

It was formed by Macrogen–Korea and provided as the following:

1-The tube had been spined down before the cap was opened.

2-The necessary quantity of denuclease water according to the oligos manufacturer was added to the oligos to reach a concentration of 100 p moles/mL. (Master Stock).

3-After that, it was adequately mixed by Vortex in order to re-suspend the primers.

4-Ten microliters of the master solution were stock moved to a 0.2 microliter eppendroff. (Working with) a tube that carries 90 mL of denuclease water Stock.

5-Keeping of the master stock at (-20) degrees Celsius.

6-Keeping of the working stock at -20 degrees Celsius.

7- Thawing of the working stock by using ice and vortex before being used in the experiment. PCR was performed, and the results were held at -20 Co.

3.11. PCR Amplification of TRAP-5 or ACP-5 gene:

One PCR fragment was selected for amplification, which supposed to cover bp of TRAP-5gene (Table).

Table (3-4): reaction mixture for PCR

no	component	concentration	volume
1	DNA	30-45ng/ µl	2 µ
	Master mix	2.5X	8µ
	F primer	10pmol	1 µ
	R primer	10pmol	1 µ
	Mgcl	25 mM	0.5 µ
	Nucleases free water		7.5 µ
	Final reaction volume		20 µ

3.11.1. Optimization of TRAP-5 or ACP-5 Gene:

Many annealing temp are used to optimization of the primer (304pb) of TRAP-5. Annealing temp are ranged (55-67) C^0 are used to the amplification.

Table (3-5): PCR Thermocycling condition

no	stage	temperature	Incubation time	Cycle number
	Initial denaturation	95 C	5min	1
	denaturation	95 C	30sec	35 cycle
	Annealing	55-67C	30sec	
	polymerization	72 C	30sec	
	Final polymerization	72 C	5min	1

Table (3-6) optimized PCR Condition of TRAP-5 OR ACP-5 gene

no	stage	temperature	Incubation time	Cycle number
	Initial denaturation	95 C°	5min	1
	denaturation	95 C°	30sec	35 cycle
	Annealing	63C°	30sec	
	polymerization	72 C°	30sec	
	Final polymerization	72 C°	5min	1

Different annealing temp are 63 C°, It give best results of the amplification. At (304) bp amplicons, the details of its sequences were highlighted, the primers of the 304 bp amplified amplicons as Table (3-7).

Table (3-7): The 304 bp PCR amplicons used to amplify a portion of the 5′UTR sequences within the ACP5 gene located within chromosome 19 (GenBank acc. no. NG_028127.1), The gray-colored sequences referred to the position of the reverse and forward primers, respectively as placed in the negative strand. Amplicon Reference locus sequences (5′ - 3′) length DNA sequences within the ACP5 gene.

Amplicon	Reference locus sequences (5′ - 3′)	length
DNA sequences within the *ACP5* gene	*ACAAGCTGGCTTAGGGAAGGGGGGCGCGGTCTGTGAGAGGGCGAGCTGTAC CAAGATGGCCCTGCAGGCCCATTTCACCCTCCTTCCACCTAGCCTGCCCAGC ACTCACCCAGGGGGAGACACAGGCCAGTCACCGGAGGCTCTGAGAGGCTGGT GGGCTCTAGAGTAGAACTGCCGGTCCCTGAGCCTTTATTCCCTGAGGAGGAA GTGGATCATTAGTGAGGATGATGCAGTTTCTCCGAGGGCTGTCCCGGGAGCC CTCCCCTTGGGTCATGTGAGCCCTGGACTTCCCTGGAGCAGGACA**	304 bp

*** indicate to forward primer**
**** indicate to reverse primer**

3.12. polymorphism SSCP:

A. Procedure of SSCP:

1- Warm tap water was used to thoroughly clean the glass plates, which were then washed with tap water, deionized water, and ethanol before being dried in the sun. They were dried by wiping or the air.

2-The long plate was put down first on a clean surface, and then the left and right spacers were placed along the sides of the long plate.

3-Each sandwich clamp was placed at the correct side of the gel sandwich with the locating arrows pointing up and toward the glass sandwich after the single screw on each clamp was released.

4-The gel sandwich was securely clamped together. The screws were adequately tightened to ensure that the plates remained in place. It was checked to see whether the plates were properly positioned in the bottom. Otherwise, the plates and spacers were moved in order to ensure a sufficient seal was achieved. It is possible that failure to do so resulted in

gel leakage during casting, as well as buffer leakage during the whole run.

5-The grey sponge was placed into the casting hole at the front of the mould. The sandwich assembly was placed on the sponge such that the short glass plate was towards the front of the sponge. The sandwich was pressed down and kept down in order to keep it from falling apart.

6-A gel solution was made with the desired polyacrylamide percentage volume of reagents used to cast polyacrylamide gel 10% including H_2O (5 ml), TBE-5x (2 ml), 10%APS (200 µl), TEMED (10µl), 30% Acrylamide (2.4 ml).

7-The gel solution was poured into the sandwich.

8-The comb was put in the sandwich top on the wells and was left to polymerize at (25) C for half hour.

9- Removing of the comb gently.

10- Filling of the chambers with buffer and rinsed.

11- The core, along with the gel sandwiches that were linked to it, were inserted into the electrophoresis tank, enabling the core to become locked in place. The top was secured in place, and the system was linked to the chiller to cool the water. The temperature was selected and the pre-run for 20 minutes was completed in order to get the required temperature (Menounos & Patrinos, 2010).

B. Sample Preparation:

2.5µL PCR product was mixed with 2.5 µL SSCP buffer at 91C° for 10 min, and was put in the ice.

C. Electrophoresis:

1-The pre-run was terminated after the buffer had attained the correct temperature. After that, the wells were washed with running buffer once more, and 10 L of each sample was put into the wells using "long" tips.

2-The gel was operated at 5–15 degrees Celsius for 6–12 hours at a continuous power of 100 volts.

3-When the electrophoresis was finished, the power supply were switched off, the electrodes were reconnected, and the core was gently removed from the electrophoresis tank.

D. Silver Staining:

1-The gel sandwich and core were placed on a cushioned surface in order to absorb any leaks from the buffer. The gel sandwich was taken from the centre of the sandwich. The gel was gently removed off the plates, and the plates were quickly washed in deionized water thereafter.

2- The gel was dipped in a tray containing solution 1, and the tray was put on top of a shaker for at least 30 minutes to ensure thorough mixing of the solution.3- Solution 1 was poured off and the gel was briefly rinsed with deionized water.

4- Solution 2 was put in a water bath at 55 C. As soon as solution 1 was removed, solution 2 (1.5 g NaOH, 75 µL formaldehyde, 50 ml deionized water) was added for 20 min.

5- When the bands were clearly visible, the second solution was thrown and the third solution (5 ml ethanol, 45 ml deionized water and 250 µl acetic acid) was added for 5 min.

6- The gel was placed on top of the LED light, then the bands were read and the results on the gel were recorded and a photo was taken afterwards.

3.12.1. DNA Sequencing of the amplicons:

The amplicons were sequenced from right and left primers, by (Macrogen company, Korea). The chromatographs acquired from ABI sequence files were used for further analysis, verifying that variances were not caused by PCR or sequencing artefacts or other sources of error.

The virtual locations and other features of the recovered PCR fragments were determined by comparing our DNA sequences with the retrieved nearby DNA sequences of Blast.

2.12.2. Interpretation of sequencing data:

By utilising the BioEdit Sequence Alignment Editor V 7.1, the sequencing of PCR products of various samples were aligned, and examined in order to ensure that they were consistent with the relevant sequences in the reference (DNASTAR, Madison, WI, USA). A number of the observed changes in each sequenced were identified and recorded in the sequenced sample's DNA amplicons and in the appropriate place within the referencing genome.

3.13. Statistical Analysis:

The Statistical Package for Social Sciences- SPSS version 20. Numerical data were tested for normal distribution using the Shapiro–Wilk test. And statistically significant of the data were analyzed by using descriptive analysis to determine between the mean ± standard error by using T-Test and one way ANOVA by used least significant difference – LSD test to significant comparison between means. Significance was assumed for P values ≤ 0.05. the correlation analysis was done for determine the relationship among the parameters. And the figures construction by using excel program of Microsoft office2010. Multivariable logistic regression was used to determine independent indicators of SNPS and Allele By use OR and CI 95% at ($P \leq 0.05$).

4.The Results

4.1.The general characters of study groups

This study includes 100 patients with OP 20 men and 80women, and the control group consisted of 50 (19 men and 31women). This study showed that there is a significant difference at ($p \leq 0.05$) between both study groups (controland patient) in gender, as well as marked differences between the control group and the patient group in BMI, which included three groups, 18.5-24.9, 25-29.9 and >30Kg/m^2. Furthermore, the ours study revealed marked differences between the control group and the patient group at ($p \leq 0.05$). There was no significant difference($p \geq 0.05$) in age between O.P. group and the control group.

The patients with OP divided into three groups (Osteopenia, Osteoporosis and Sever osteoporosis) according to the T-score, This study showed a significant increase at($p<0.001$) in osteoporosis group compared to osteopenia and sever osteoporosis groups.The current study showed a significant difference at($p<0.001$) between patients with OP that smoking and nonsmoking and demonstrated significant different ($p<0.002$) between the OP patients and diabetes patients. The patients with OP and suffer from hypertension showed a percentage of 23%. In comparison, it was77% in patients without hypertension and showed no significant difference at ($p \geq 0.05$) are in age (41-60)(47%) percentage and at the age (20-40), (61-80)(23%),(30)% respectively. Percentage of men was (20%) while (80%) are women (23%) of patients are hypertension while the proportion (14%) of patients have diabetes . also, the results of the study revealed(64%) of the women patients are hypertension while it was(60%) in control women, as shown in table (4-1)

Table (4-1): General characteristics of the patients and healthy groups.

Characteristics	Patients No. (%)	Control No. (%)	p-value
Groups	100 (66.67%)	50(33.33%)	
Gender			
Male	20 (20%)	19 (38%)	**0.018***
Female	80 (80%)	31 (62%)	
BMI (Kg/m$^{2)}$			
healthy weight (18.5-24.9)	20 (20%)	20 (40%)	**0.028***
Overweight (25-29.9)	32 (32%)	14 (28%)	
Obesity >30	48 (48%)	16 (32%)	
Age (years)			
20-40	23 (23%)	19 (38%)	**0.080**
41-60	47 (47%)	15 (30%)	
61-80	30 (30%)	16 (32%)	
T-score			
Osteopenia	11 (11%)	-	**0.0001****
Osteoporosis	88 (88%)	-	
Sever osteoporosis	1 (1%)	-	
Smoking			
Yes	11(11%)		**0.0001****
no	89 (89%)		
Diabetes			
Yes	14 (14%)		**0.0002****
no	86 (86%)		
Hypertension			
Yes	23 (23%)		**0.001****
No	77 (77%)		
Number of birth			
1-5	29 (36.25%)	31 (100%)	**0.001****
6-10	39 (48.75%)	0	
11-15	12 (15%)	0	

*the significant level at ($p \leq 0.05$), ** the significant level at ($p \leq 0.01$)

4.2.Biochemical parameters levels in study groups

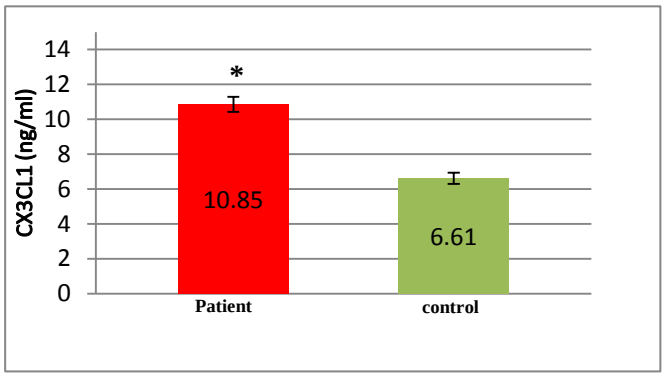
The biochemical parameters of the study groups in a table (4-2) revealed a significant increase at a level ($p \leq 0.01$) in Bone sialoprotein, FKN/CX3CL1, Gelsolin, Cathepsin K concentrations in patients compared to healthy subjects. It was also observed that the concentration of serum vitamin D generally in the patient's significant decreased at ($p \leq 0.05$)compared with the control groups, and also the concentration of serum calcium showed a significant decrease at ($p \leq 0.01$) compared with healthy groups. At the same time, the results demonstrated a significant positive differences at ($p \leq 0.01$) in phosphorous and alkaline phosphatase level in the patients compared to the control groups.

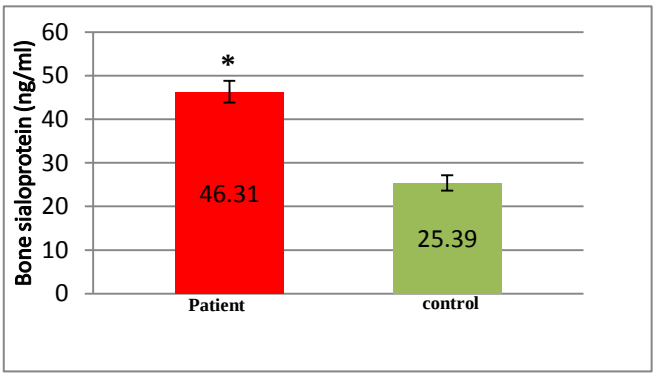
Table (4-2): levels of some biochemical parameters in osteoporosis patients and healthy control group. Mean ± S.E

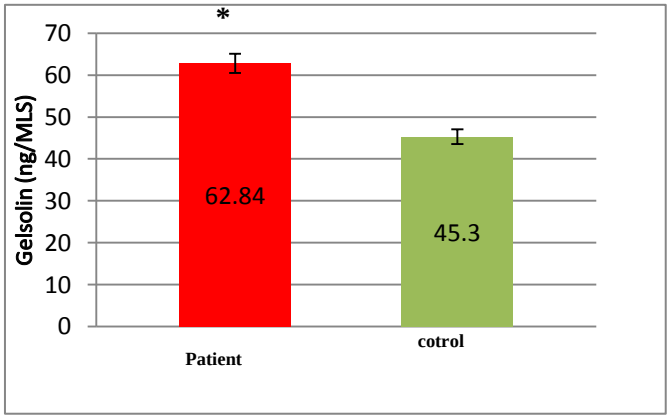
Groups / Parameters	Patient	Healthy	P-value
	Mean±S.E		
FKN/CX3CL1 (ng/ml)	10.85±0.43	6.61±0.32	**0.0001****
Bone sialoprotein (ng/ml)	46.31±2.52	25.39±1.79	**0.0001****
Gelsolin (ng/MLS)	62.84±2.31	45.30±1.76	**0.009****
Cathepsin K (ng/ml)	3.92±0.24	2.12±0.30	**0.001****
Vit. D3 (ng/ml)	16.86±1.01	20.57±1.68	**0.049***
Ca (mg/dL)	8.34±0.10	8.91±0.12	**0.001****
PO_4 (mg/dL)	4.73±0.12	3.99±0.08	**0.0001****
ALP (Iu/L)	103.73±2.11	74.43±2.98	**0.0001****

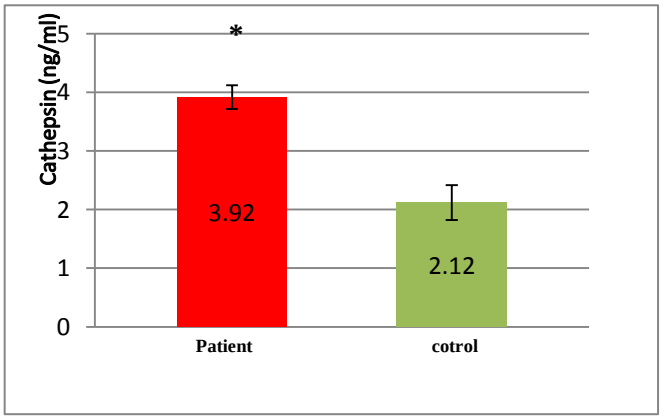
*significant difference at ($p \leq 0.05$).

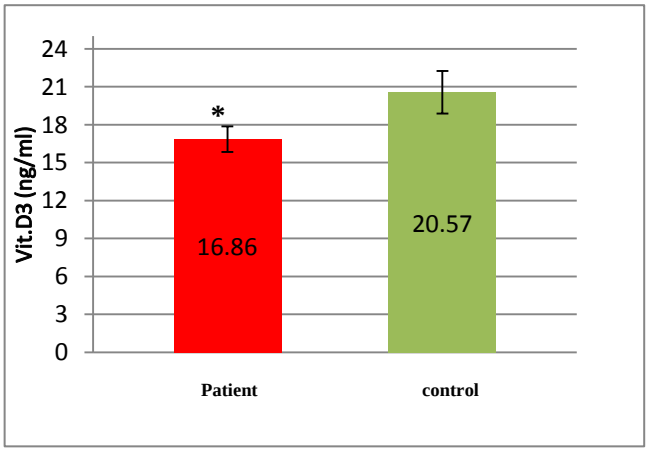
** significant difference at ($p \leq 0.01$).

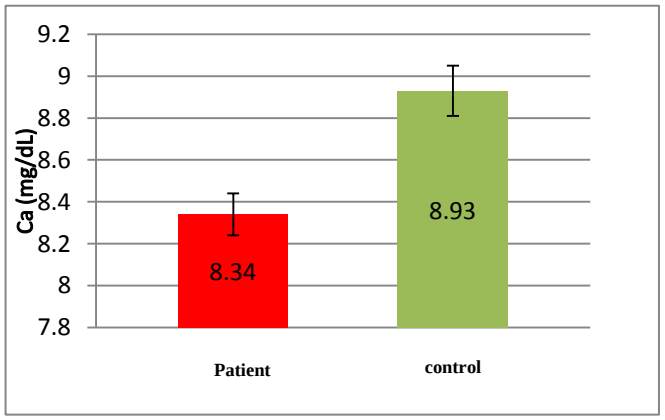


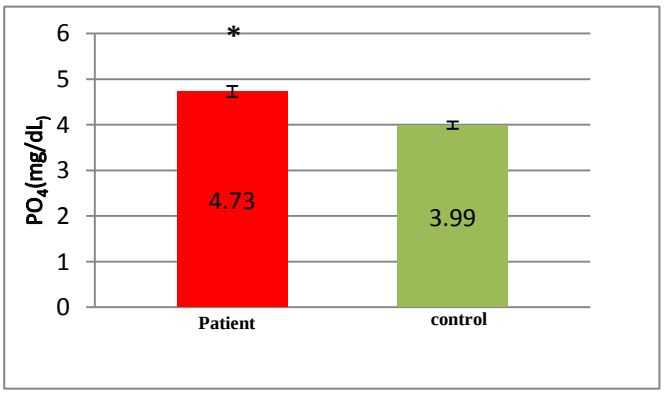


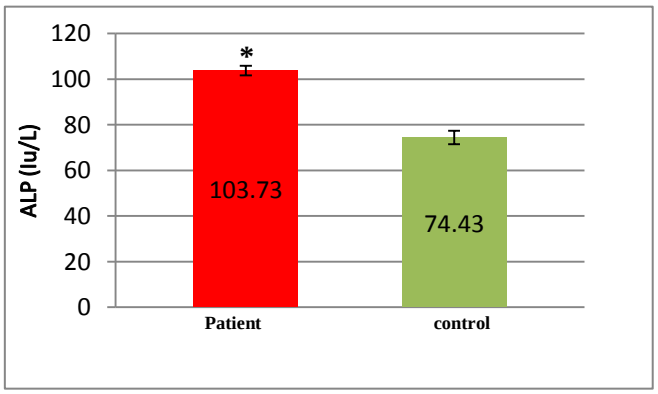












Figures (4-1) levels of some biochemical parameters in osteoporosis patients healthy control

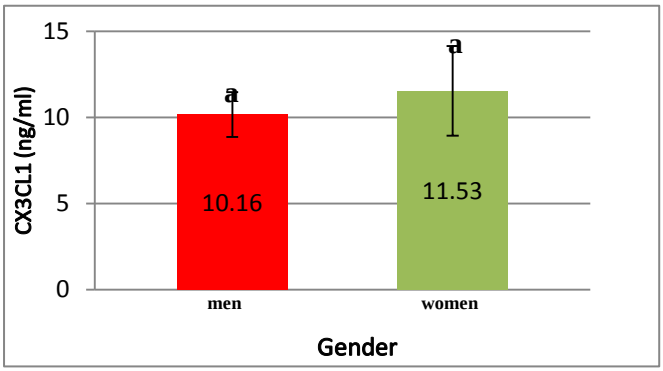
4.2.1The levels of biochemical parameters in osteoporosis patients and healthy groups according to gender (mean ± S.E.) :

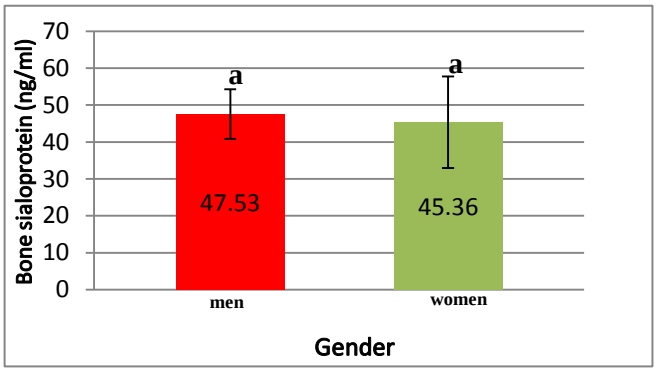
In table (4-3) describe levels of biochemical parameters showing that in osteoporosis, patients markedly significant increase in levelof biochemical parameters that measured in the study, where a significant increase($p \leq 0.05$) in FKN/CX3CL1,gelsolin and cathepcin K inwomen patients, and significant increase in bone sialoprotein,ALP concentration for both genders compared with the healthy control group.

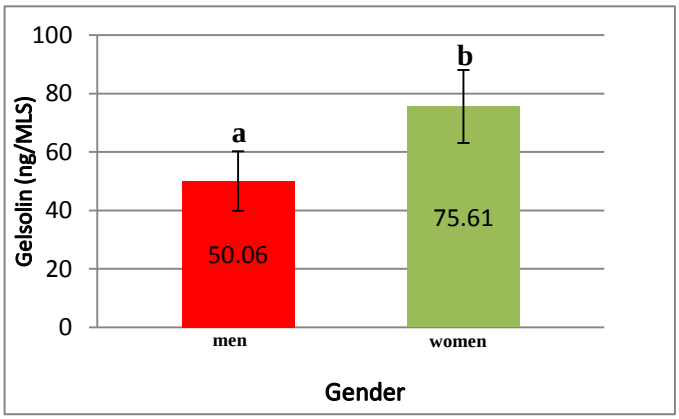
Table (4-3): levels of some the biochemical parameters inosteoporosis patients and healthy according to gender.

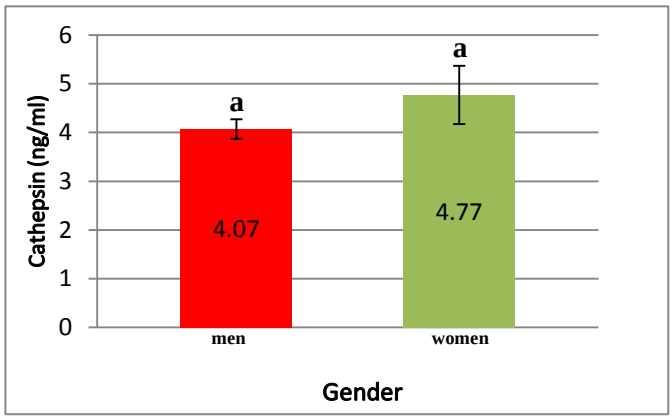
Groups / Parameters	Gender	Men	women
		Mean±S.E	
CX3CL1(ng/ml)	**Patient**	10.16±1.3	11.53±2.6
	control	9.76±1.5	3.46±0.6
p-value		**0.121**	**0.02***
Bone sialoprotein (ng/ml)	**Patient**	47.53±6.7	45.36±12.4
	control	31.45±5.1	19.32±9.8
p-value		**0.005***	**0.008***
Gelsolin (ng/MLS)	**Patient**	50.06±10.2	75.61±12.5
	control	48.13±8.1	42.46±10.5
p-value		**0.09**	**0.02***
Cathepsin K (ng/ml)	**Patient**	3.07±0.2	4.77±0.6
	control	2.99±0.1	1.25±0.8
p-value		**0.410**	**0.04***
Vit. D3(ng/ml)	**Patient**	18.44±4.3	15.26±2.2
	control	20.48±4.4	20.66±3.4
p-value		**0.215**	**0.06**
Ca (mg/dL)	**Patient**	8.46±1.3	8.22±0.8
	control	8.43±1.4	9.38±2.2
p-value		**0.687**	**0.311**
PO_4 (mg/dL)	**Patient**	4.74±0.8	4.70±0.9
	control	3.77±0.7	4.18±1.1
p-value		**0.567**	**0.244**
ALP (Iu/L)	**Patient**	93.07±15.2	114.5±17.8
	control	79.00±11.7	69.85±12.1
p-value		**0.06***	**0.002***

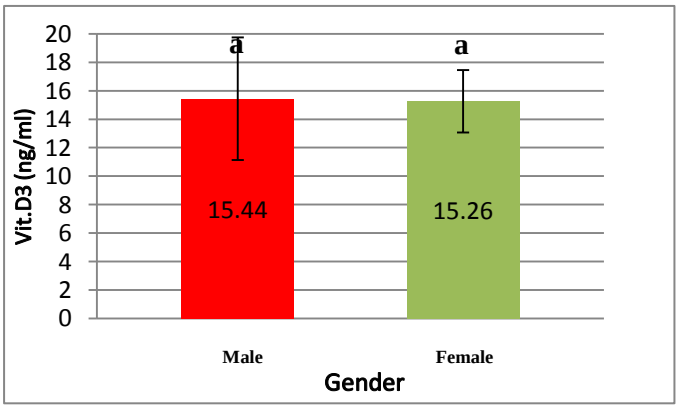
*significant level at ($p \leq 0.05$).

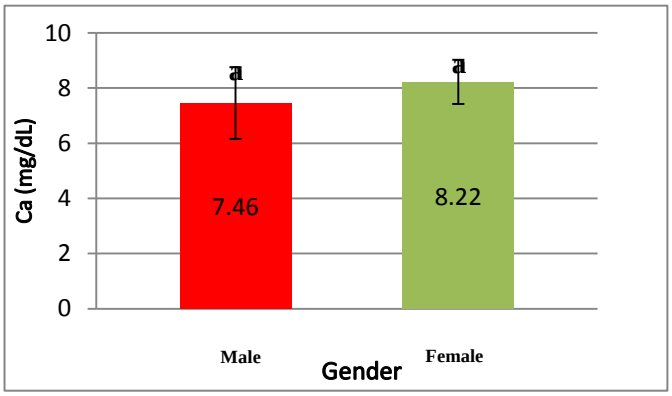


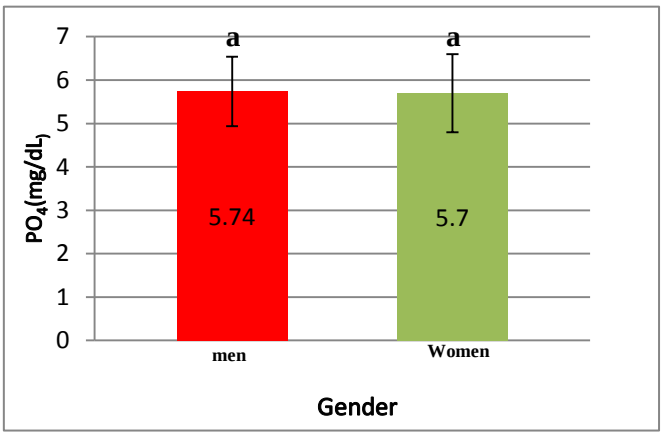


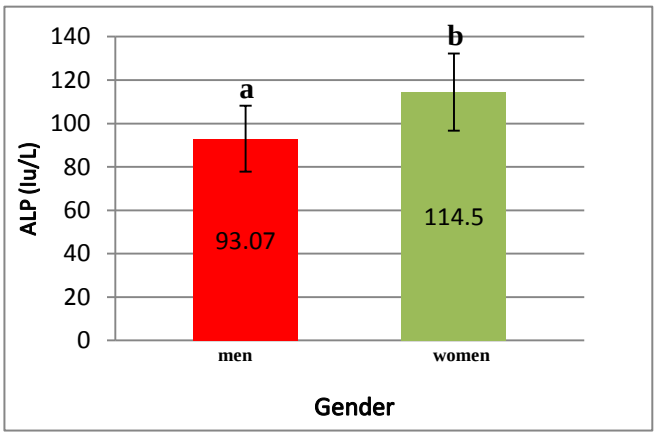












Figures (4-2): levels of some biochemical parameters in osteoporosis patients according to gender.

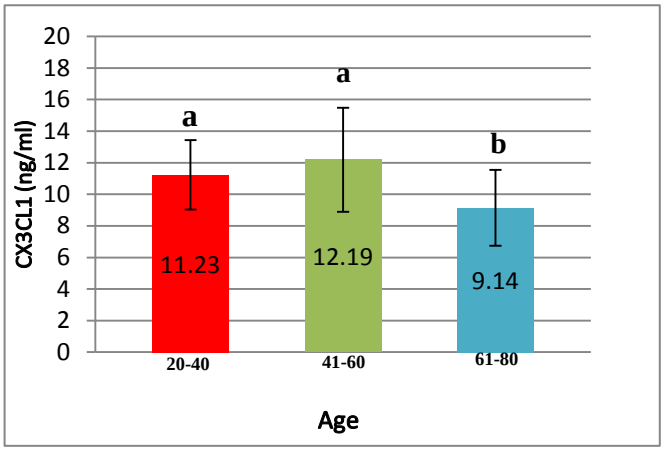
4.2.2.The levels of biochemical parameters in osteoporosis patients and healthy groups according to the age (mean ± S.E.) :

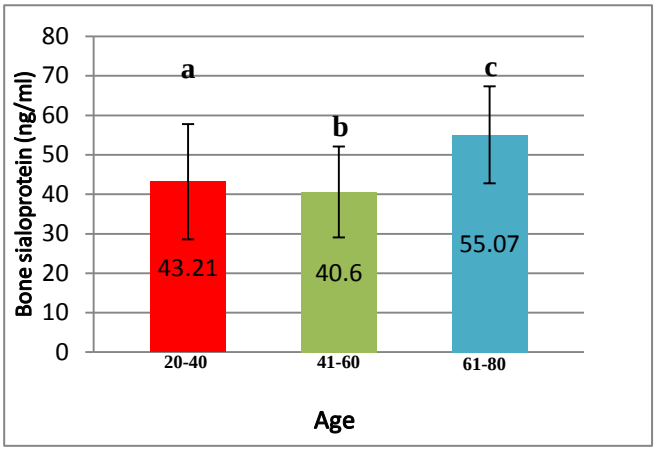
According to the age, the study groups were divided into three groups (20-40), (41-60), (61-80). This study showed there is a significant increase at ($p \leq 0.05$)in the biochemical markers (CX3CL1, bone sialoprotein) between the patients and control according to the age. This study showed a significant difference at ($p \leq 0.05$) in the (cathepsin K, gelsolin) between the patients and healthy control groups in the age groups (20-40), (61-80) while there is no significant difference in the age group (41-60).

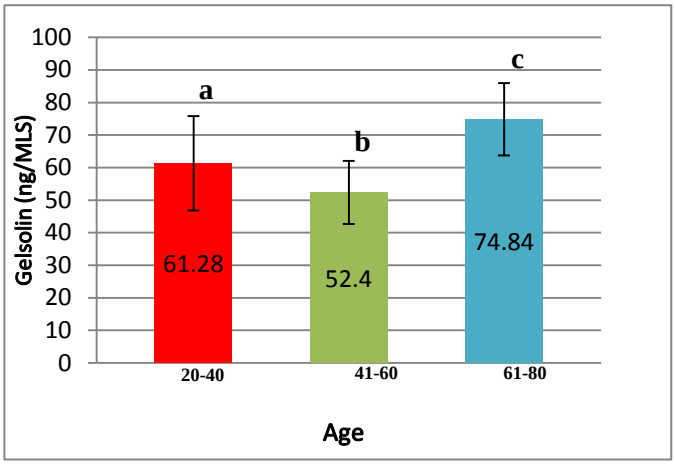
Table (4-4): levels of some the biochemical parameters in osteoporosis patients and healthy according to age.

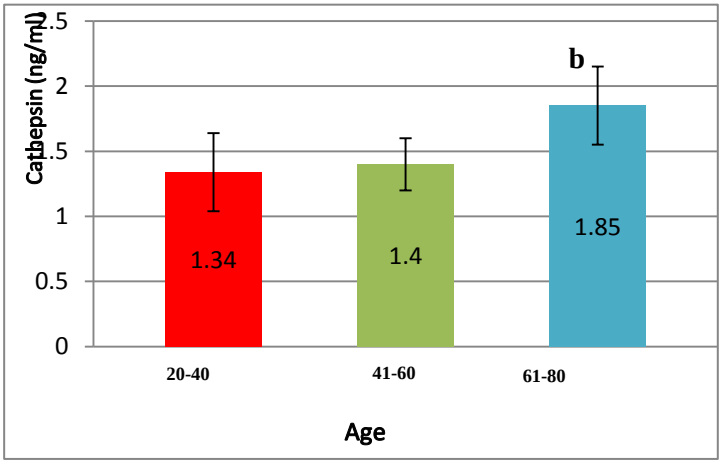
Parameters	Age (years) Groups	20-40	41-60	61-80
		Mean±S.E		
CX3CL1 (ng/ml)	Patient	11.23±2.2	12.19±3.3	9.14±2.4
	control	7.24±1.6	6.82±2.1	5.78±1.3
p-value		0.04*	0.01*	0.04*
Bone sialoprotein (ng/ml)	Patient	43.21±14.6	40.6±11.5	55.07±12.3
	control	24.04±7.2	26.54±9.8	25.60±8.2
p-value		0.002*	0.003*	0.001*
Gelsolin (ng/MLS)	Patient	61.28±14.5	52.40±9.7	74.84±11.1
	control	41.10±8.8	47.50±8.7	47.31±9.9
p-value		0.03*	0.145	0.03*
Cathepsin (ng/ml)	Patient	1.34±0.3	1.40±0.2	1.85±0.3
	control	3.40±0.7	1.80±0.3	1.15±0.1
p-value		0.04*	0.444	0.05*
Vit. D3 (ng/ml)	Patient	17.83±3.5	18.98±2.6	13.74±2.3
	control	18.61±2.9	20.72±3.4	22.37±2.6
p-value		0.614	0.291	0.06
Ca (mg/dL)	Patient	8.57±1.1	8.32±1.2	8.12±0.8
	control	8.75±1.6	9.42±1.5	8.56±0.6
p-value		0.241	0.347	0.614
PO_4 (mg/dL)	Patient	4.41±0.6	5.32±0.9	4.47±1.2
	control	4.08±0.9	4.14±0.8	3.76±0.9
p-value		0.541	0.333	0.278
ALP (Iu/L)	Patient	110.20±12.6	100.65±12.6	100.51±13.1
	control	69.06±17.5	72.24±10.9	81.99±14.5
p-value		0.001*	0.003*	0.006*

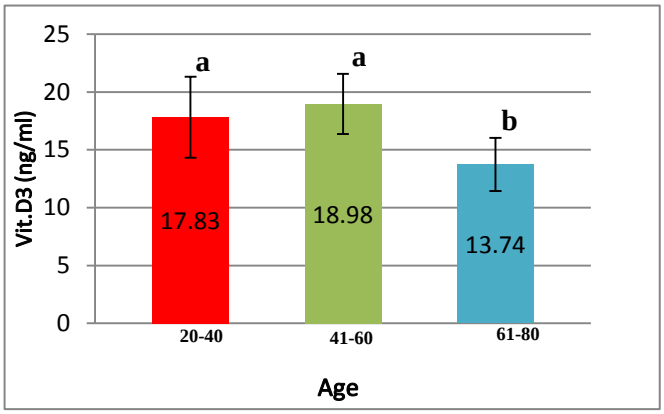
*the significant level at ($p \leq 0.05$).

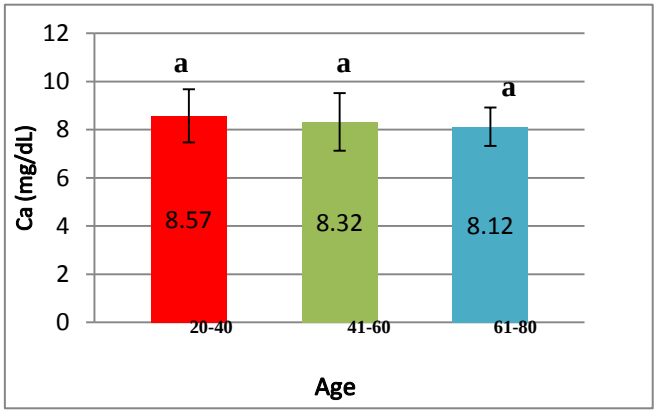


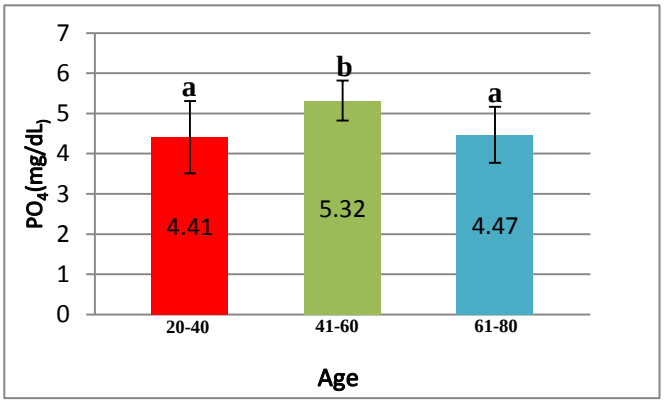


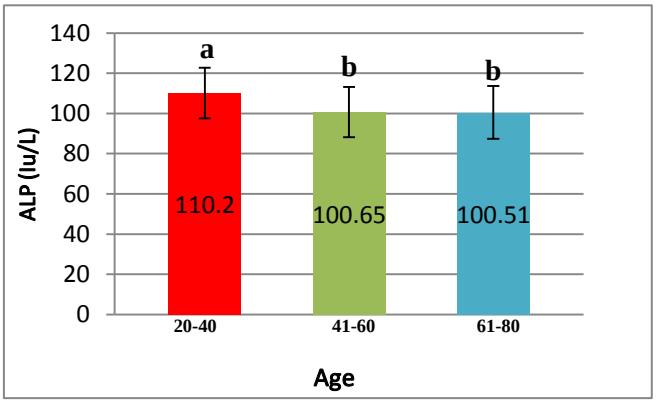












Figures (4-3): levels of some biochemical parameters in osteoporosis patient according to age.

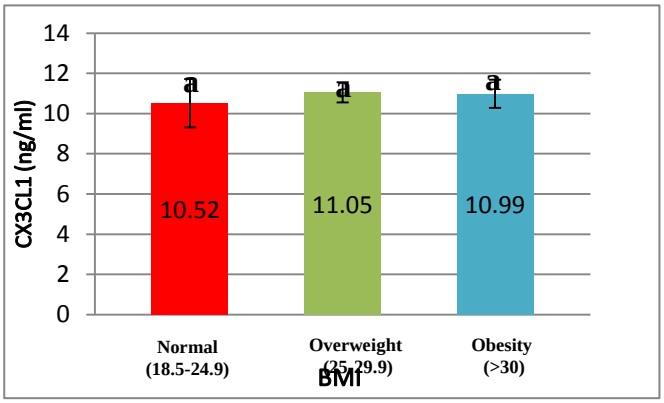
4.2.3.The levels of biochemical parameters in osteoporosis patients and healthy groups according to the BMI (mean ± S.E) :

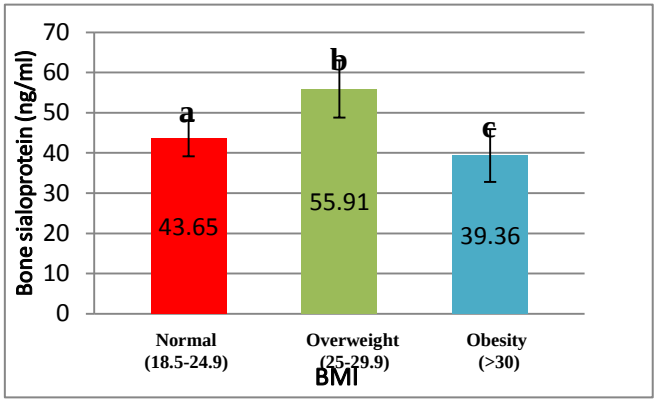
The table (4-5) demonstrated significant differences between the control and patient groups in BMI, classified for three groups, 18.5-24.9, 25-29.9 and >30. In addition, significant increase are present between the control group and the patient group at ($p \leq 0.05$) in the serum (CX3CL1, bone sialoprotein, gelsolin, cathepsin K and alkaline phosphatase) concentration.

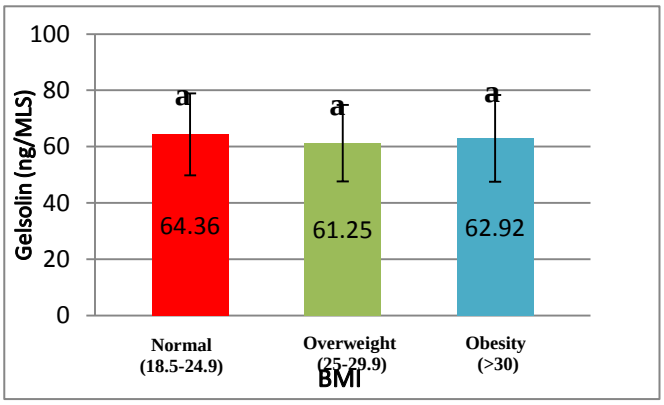
Table (4-5): levels of some the biochemical parameters in osteoporosis patients and healthy according to BMI.

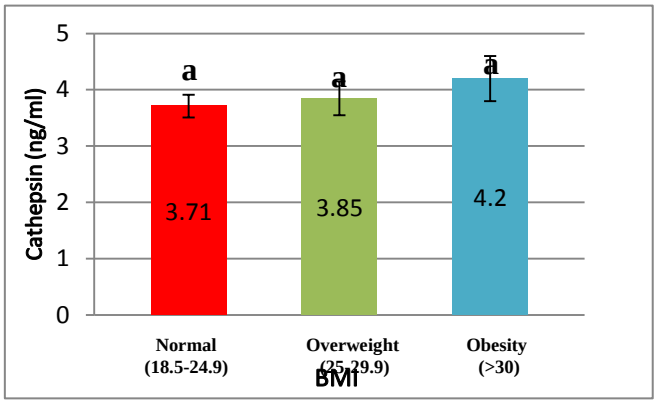
Parameters	Groups / BMI	Normal (18.5-24.9)	Overweight (25-29.9)	Obesity (>30)
		Mean±S.E		
CX3CL1 (ng/ml)	Patient	10.52±1.8	11.05±2.3	10.99±1.9
	control	6.55±0.9	6.94±1.6	6.34±1.1
p-value		**0.035***	**0.021***	**0.029***
Bone sialoprotein (ng/ml)	Patient	43.65±12.4	55.91±15.6	39.36±12.6
	control	31.65±8.9	23.41±7.7	21.11±6.3
p-value		**0.044***	**0.01***	**0.03***
Gelsolin (ng/MLS)	Patients	64.36±14.6	61.25±13.6	62.92±15.4
	control	43.50±11.9	96.55±12.7	45.85±9.7
p-value		**0.031***	**0.041***	**0.042***
CathepsinK (ng/ml)	Patients	3.71±0.2	3.85±0.3	4.20±0.4
	cotrol	2.11±0.1	2.02±0.2	2.22±0.1
p-value		**0.048***	**0.041***	**0.021***
Vit. D3 (ng/ml)	Patients	14.07±2.6	17.78±2.6	18.70±3.4
	control	20.10±2.3	19.91±5.4	21.71±4.1
p-value		**0.036**	**0.199**	**0.121**
Ca (mg/dL)	Patients	8.54±0.6	8.34±1.6	8.14±1.1
	cotrol	8.90±0.9	8.56±0.7	9.26±0.9
p-value		**0.331**	**0.271**	**0.111**
PO4 (mg/dL)	Patients	4.83±0.8	4.29±0.6	5.08±0.5
	control	4.07±0.5	3.89±0.4	4.01±0.4
p-value		**0.204**	**0.377**	**0.415**
ALP (Iu/L)	Patients	101.62±12.6	103.73±12.6	106.02±15.6
	control	71.69±13.4	71.60±10.4	80.00±12.8
p-value		**0.006***	**0.009***	**0.023***

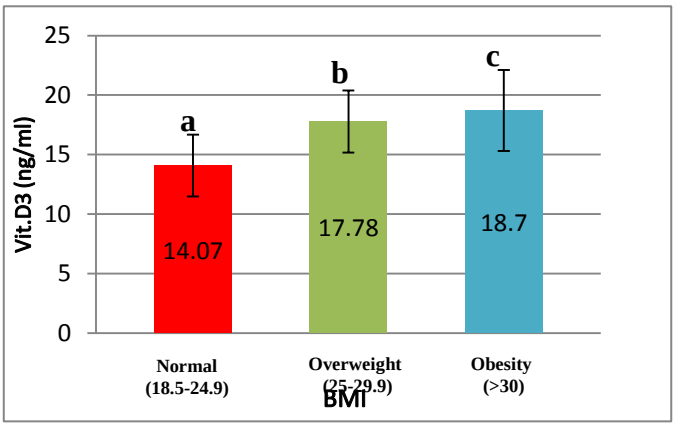
*significant level at ($p \leq 0.05$).

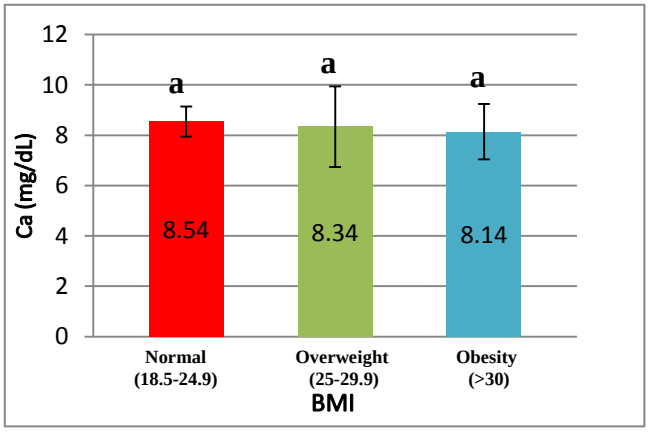


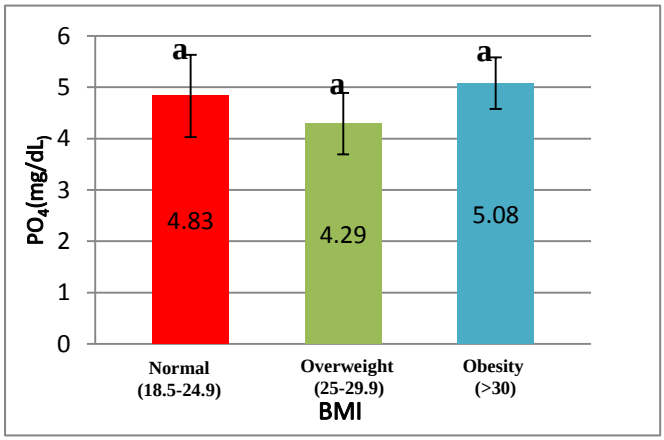


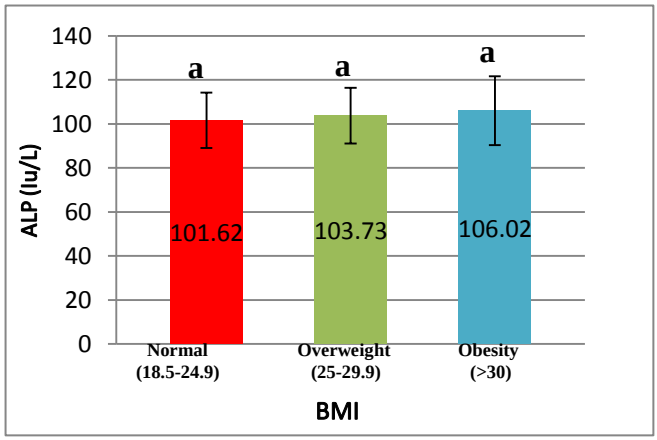












Figures (4-4): The levels of biochemical parameters in osteoporosis patient groups according to the BMI.

4.3.levels of some biochemical parameters in osteopenia and osteoporosis patients:

This study showed no significant difference between osteopenia and osteoporosis patients in serum (CX3CL1, bone sialoprotein and cathepsin K)concentration.while a significant increase ($p \leq 0.05$) in gelsolin and vit.D in osteoporosis compared to osteopenia This study showed no significant difference between osteopenia and osteoporosis patients in Ca, Po4 and ALP).

Table(4-6): levels of some biochemical parameters in osteopenia and osteoporosis patients.

Parameters	Osteopenia	Osteoporosis	p-value
	Mean±S.E		
CX3CL1(ng/ml)	10.44±1.5	11.26±2.1	**0.264**
Bone sialoprotein (ng/ml)	37.84±6.9	54.77±13.2	**0.041**
Gelsolin (ng/MLS)	52.8±12.8	72.87±7.9	**0.041***
Cathepsin K (ng/ml)	4.29±0.6	3.55±0.5	**0.161**
Vit. D3(ng/ml)	11.24±2.6	22.45±3.2	**0.009***
Ca (mg/dL)	8.48±2.1	8.19±1.41	**0.124**
PO4(mg/dL)	4.82±0.6	4.64±0.9	**0.514**
ALP (Iu/L)	99.04±11.9	108.54±15.6	**0.147**

*significant difference at ($p \leq 0.05$).

4.3.1.Levels of some biochemical parameters in osteopenia and osteoporosis patients (according to gender) (Mean ± S.E).

The results showed no significant difference in the CX3CL1 in the osteopenia and osteoporosis men and women patients. At the same time, the bone sialoprotein reveals positive significance at ($p \leq 0.05$) in the osteopenia women patients compared with osteopenia men patients. The current study demonstrated that bone sialoprotein has a significant

increase at ($p \leq 0.05$) in the osteopenia men compared with women. The study results showed no significant difference in the gelsolin concentration in osteopenia patients in both gender, while is a positive significant in the gelsolin level at ($p \leq 0.05$) in osteoporosis men patients when compared with women osteoporosis patients.

The present study revealed positive significance in the cathepsin K level in the osteopenia women patients when compared with men. In contrast, this study showed no significant difference in the cathepsin K concentration in the osteoporosis patients in both genders. This study showed a significant increase in the vitamin D concentration in the osteopenia and men osteoporosis patients compared with women patients.

The study indicates no significant difference in the (Ca and Po4) concentration in the osteopenia and osteoporosis men and women patients, and no significant difference in alkaline phosphatase concentration in the osteopenia men and women patients, while there is a significant increase in the alkaline phosphatase concentration in women osteoporosis patients when compared with men. summarized for these results in a table (4-7)

Table(4-7): Levels of some biochemical parameters in osteopenia and osteoporosis patients (according to gender) (Mean ± S.E)

Parameters	T-score / Gender	Osteopenia	Osteoporosis
		Mean±S.E	
CX3CL1 (ng/ml)	Men	10.04±1.2	11.28±1.1
	women	10.84±2.3	11.24±2.1
p-value		**0.244**	**0.178**
Bone sialoprotein (ng/ml)	Men	28.22±12.6	63.14±16.4
	women	47.45±15.6	46.40±13.5
p-value		**0.019***	**0.036***
Gelsolin (ng/MLS)	Men	55.21±13.4	84.37±16.4
	women	50.39±14.8	61.36±12.4
p-value		**0.124**	**0.033***
Cathepsin K (ng/ml)	Men	2.57±0.5	3.07±0.2
	women	6.01±0.6	4.03±0.6
p-value		**0.041***	**0.617**
Vit. D3 (ng/ml)	Men	13.98±2.4	27.3±2.3
	women	8.00±1.6	17.6±2.4
p-value		**0.012***	**0.061***
Ca(mg/dL)	Men	8.73±2.3	8.12±0.6
	women	8.22±1.7	8.26±0.9
p-value		**0.514**	**0.114**
PO_4(mg/dL)	Men	4.73±0.6	4.20±**0.6**
	women	4.90±0.5	5.07±0.1
p-value		**0.244**	**0.355**
ALP (Iu/L)	Men	94.19±0.4	98.89±12.3
	women	103.89±14.6	118.19±10.9
p-value		**0.112**	**0.009***

*significant level at ($p \leq 0.05$).

4.3.2.Levels of some biochemical parameters in osteopenia and osteoporosis patients (according to age) (Mean ± S.E).

In table (4-8) describe levels of biochemical parameters Showing that according to the age, the study groups were divided into three groups (20-40), (41-60), (61-80). The current study does not reveal the marked difference in the CX3CL1 level between osteoporosis and osteopenia according to age. The result study revealed a significant increase in the level of bone sialoprotein and cathepsin K concentration in

osteoporosis patients compared with osteopenia patients in both age groups. This study indicates a significant increase ($p \leq 0.05$) in vitamin D concentration in osteoporosis patients compared with osteopenia patients in both age groups(20-40 and 41-60). While the results no significant difference in Ca and Po4 levels between osteoporosis and osteopenia patients based on age.The study results showed significant increase in ALP concentration in osteoporosis patients compared to osteopenia patients in age group 20-40

Table(4-8): Levels of some biochemical parameters in osteoporosis and osteopenia cases based on age) (Mean ± S.E).

Parameters	T-score / Age (year)	Osteopenia	Osteoporosis	p-value
		Mean±S.E		
CX3CL1 (ng/ml)	**20-40**	10.65±1.9	11.47±2.3	**0.147**
	41-60	10.24±0.8	11.61±1.6	**0.116**
	61-80	-	10.69±2.2	-
Bone sialoprotein (ng/ml)	**20-40**	40.28±12.5	52.58±14.5	**0.042***
	41-60	35.40±6.7	74.60±12.7	**0.001***
	61-80	-	55.41±9.7	-
Gelsolin (ng/MLS)	**20-40**	59.69±12.5	77.58±12.5	**0.050***
	41-60	45.92±9.8	73.48±10.9	**0.027***
	61-80	-	67.55±9.8	-
Cathepsin K (ng/ml)	**20-40**	3.50±0.6	3.47±0.5	**0.641**
	41-60	5.09±0.7	4.07±1.1	**0.271**
	61-80	-	3.10±0.5	-
Vit. D3 (ng/ml)	**20-40**	9.13±2.3	28.47±2.3	**0.001***
	41-60	13.36±1.5	21.13±3.2	**0.026***
	61-80	-	17.76±4.1	-
Ca(mg/dL)	**20-40**	8.81±2.1	8.23±**2.1**	**0.492**
	41-60	8.16±1.6	8.16±2.0	**0.247**
	61-80	-	8.19±1.8	-
PO_4(mg/dL)	**20-40**	4.80±0.9	4.29±0.6	**0.116**
	41-60	4.84±1.1	4.84±0.8	**0.321**
	61-80	-	4.79±0.7	-
ALP (Iu/L)	**20-40**	95.21±12.6	115.84±10.6	**0.034***
	41-60	102.88±13.7	103.66±15.4	**0.248**
	61-80	-	106.13±13.7	-

*significant level at ($p \leq 0.05$).

4.3.3.Levels of biochemical parameters in osteopenia and osteoporosis patients (according to BMI) (Mean ± S.E):

According to the BMI, the patients were divided into three groups, 18.5-24.9, 25-29.9 and >30. The present study does not demonstrate a marked difference in CX3CL1 level between osteoporosis and osteopenia cases. This results indicates no significant difference in bone sialoprotein in two group patients according to BMI (18.5-24.9), but the bone sialoprotein level reveals a significant increase at ($p \leq 0.05$) between osteopenia and osteoporosis cases based on BMI (25-29.9) and there is a significant decrease in bone sialoprotein concentration in osteopenia and osteoporosis patients according to BMI (>30).

This study showed a significant rise in gelsolin concentration in osteoporosis patients compared with osteopenia patients according to BMI (18.5-24.9), while there is no significant difference in gelsolin between the two groups patients according to BMI (25-29.9) also, the gelsolin level demonstrated positive significant ($p \leq 0.05$) between osteoporosis and osteopenia cases depending to BMI (Obesity (>30). This study showed no significant difference in cathepsin K concentration between two group patients according to BMI. At the same time, there is a significant decrease in cathepsin K concentration in osteoporosis patients compared with osteopenia patients according to BMI (>30). The present study reveals positive significance at ($p \leq 0.05$) in vit. D level in osteoporosis patients compared with osteopenia patients according to BMI (Normal(18.5-24.9), while there was indicates no significant difference in vitamin D concentration between two groups patients according to BMI (Overweight(25-29.9) and Obesity (>30).

This study indicates no significant difference in (Ca, Po4)concentration between osteopenia and osteoporosis patients according BMI. The resulting study showed a significant increase at

(p≤0.05) in alkaline phosphatase concentration in osteoporosis patients and osteopenia patients according to BMI Normal(18.5-24.9) and Obesity (>30), while there is no significant difference in ALP concentration between osteoporosis patients and osteopenia patients according to BMI (25-29.9).

Table(4-9): Levels of biochemical parameters in osteopenia and osteoporosis patients (according to BMI) (Mean ± S.E).

Parameters	T-score / BMI	Osteopenia Mean±S.E	Osteoporosis Mean±S.E	p-value
CX3CL1 (ng/ml)	Normal(18.5-24.9)	9.98±2.3	10.68±2.5	0.233
	Overweight(25. -29.9)	9.72±3.4	12.16±1.6	0.171
	Obesity (>30)	11.63±1.5	10.95±1.9	0.632
Bone sialoprotein (ng/ml)	Normal(18.5-24.9)	37.50±2.6	47.62±13.5	0.061
	Overweight(25-29.9)	20.52±3.3	71.77±18.2	0.009*
	Obesity (>30)	55.49±4.1	44.93±9.8	0.047*
Gelsolin (ng/MLS)	Normal(18.5-24.9)	55.31±16.5	76.32±16.2	0.049*
	Overweight(25-29.9)	66.63±15.8	69.46±13.4	0.741
	Obesity (>30)	36.45±12.9	72.83±11.8	0.001*
Cathepsin K (ng/ml)	Normal(18.5-24.9)	2.90±0.6	3.89±0.6	0.054
	Overweight(25-29.9)	2.72±0.8	3.83±0.1	0.064
	Obesity (>30)	7.25±0.1	2.92±0.8	0.003*
Vit. D3 (ng/ml)	Normal(18.5-24.9)	7.85±2.1	29.47±2.6	0.001*
	Overweight(25-29.9)	15.58±3.4	16.20±3.1	0.711
	Obesity (>30)	10.29±1.1	21.67±2.9	0.006
Ca (mg/dL)	Normal(18.5-24.9)	8.45±0.6	8.22±1.6	0.471
	Overweight(25-29.9)	8.45±0.8	8.33±1.8	0.588
	Obesity (>30)	8.55±0.2	8.03±2.1	0.122
PO_4(mg/dL)	Normal(18.5-24.9)	4.99±0.1	4.41±1.6	0.641
	Overweight(25-29.9)	4.32±0.3	4.60±1.2	0.711
	Obesity (>30)	5.15±0.4	4.92±0.9	0.092
ALP (Iu/L)	Normal(18.5-24.9)	98.31±12.6	119.49±20.5	0.038*
	Overweight(25-29.9)	102.91±9.8	101.54±18.9	0.714
	Obesity (>30)	95.91±11.2	104.60±15.6	0.049*

*significant level at (p≤0.05).

4.4.Study of parameters study in OP women patients according to menopause:

Table (4-10) demonstrated clear marked differences between the control group and patient group based on the menopause state (p ≤ 0.05)

and ($p \leq 0.01$). The result showed there is a significant rise in the level of CX3CL1, bone sialoprotein, gelsolin, cathepsin K in both patient groups premenopausal and Postmenopausal compared with the healthy group. This study showed no significant difference in CX3CL1, bone sialoprotein and cathepsin K concentration between premenopausal and Postmenopausal patients group and healthy group, while it was a significant increase at ($p \leq 0.05$) in gelsolin concentration in Postmenopausal patients compared with premenopausal patients. Also, this study showed no significant difference in vitamin D concentration between premenopausal patients and control, while vit. D showed negative significance at ($p \leq 0.05$) in Postmenopausal cases compared with the postmenopausal control group. There is a significant increase in the level of vitamin D in premenopausal patients compared with postmenopausal patients.

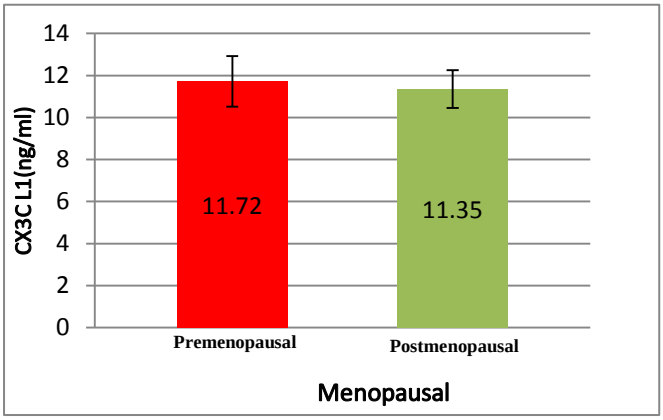
Also there was a significant decrease ($p<0.05$) in vit. In postmenopausal patients compared with postmenopausal control.The results showed no significant difference in Calcium concentration between premenopausal patients and control and postmenopausal patients and control groups. The current study showed a significant rise in the Po4 concentration in premenopausal patients compared with the healthy group. There was no significant difference between postmenopausal patients and the control group. The result study indicate there is a significant increase at ($p \leq 0.01$) in ALP concentration in premenopausal patients and postmenopausal patients compared with the control group, and ALP showed positive significance ($p \leq 0.05$) in premenopausal cases compared with postmenopausal patients.

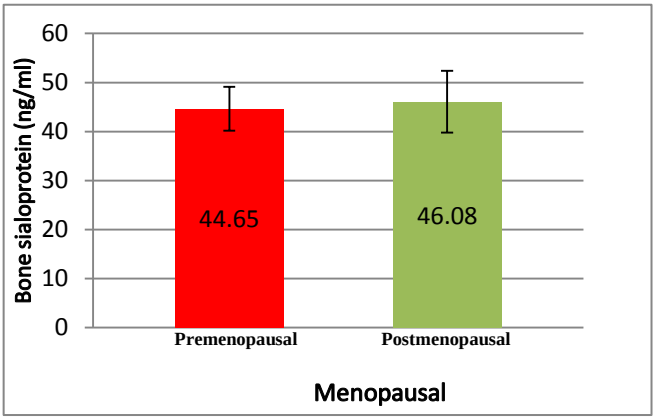
Table(4-10): Study of parameters study in O.P. women patients according to menopause.

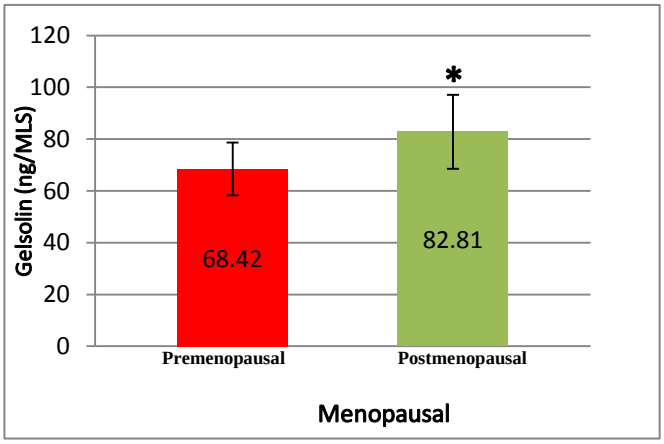
Parameters	Group / Cycle	Patient	Healthy	p-value
		Mean±S.E		
CX3CL1(ng/ml)	premenopausal	11.72±1.2	3.70±0.1	0.03*
	Postmenopausal	11.35±0.9	3.23±0.5	0.02*
		0.521	0.364	
Bone sialoprotein (ng/ml)	premenopausal	44.65±4.5	19.99±2.3	0.005**
	Postmenopausal	46.08±6.3	18.65±1.9	0.007**
p-value		0.112	0.277	
Gelsolin (ng/MLS)	premenopausal	68.42±10.2	43.24±6.8	0.04*
	Postmenopausal	82.81±14.3	41.68±6.6	0.0001**
p-value		0.03*	0.511	
Cathepsin K (ng/ml)	premenopausal	4.43±0.5	1.57±0.1	0.02*
	Postmenopausal	5.12±1.1	0.94±0.2	0.003**
p-value		0.09	0.07	
Vit. D3(ng/ml)	premenopausal	18.18±3.6	19.77±3.3	0.201
	Postmenopausal	12.30±1.7	21.55±3.7	0.04*
p-value		0.07*	0.107	
Ca(mg/dL)	premenopausal	8.37±1.2	9.40±1.3	0.417
	Postmenopausal	8.07±0.8	9.36±2.0	0.127
p-value		0.323	0.610	
PO4 (mg/dL)	premenopausal	5.82±0.2	3.15±0.6	0.05*
	Postmenopausal	5.58±0.2	3.20±0.1	0.06
p-value		0.417	0.333	
ALP (Iu/L)	premenopausal	120±11.2	72.92±12.9	0.001**
	Postmenopausal	109±10.8	66.78±11.3	0.009**
p-value		0.045*	0.142	

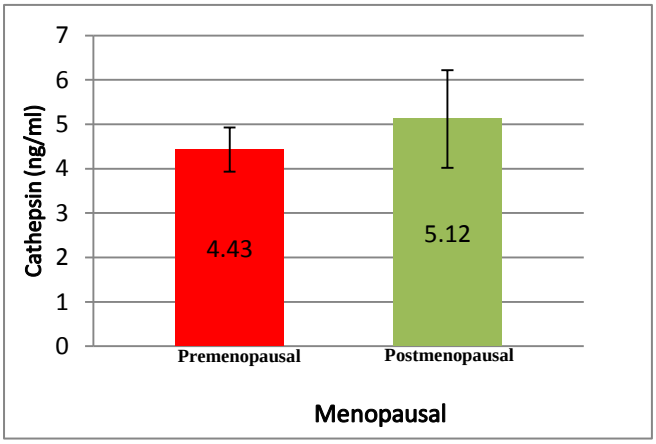
*significant level at ($p \leq 0.05$).

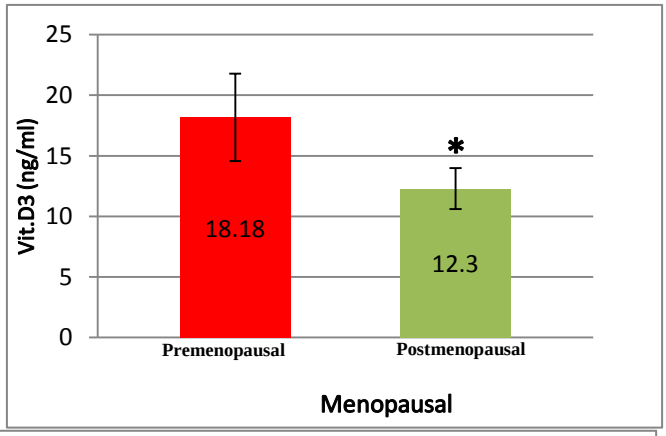
** significant level at ($p \leq 0.01$)

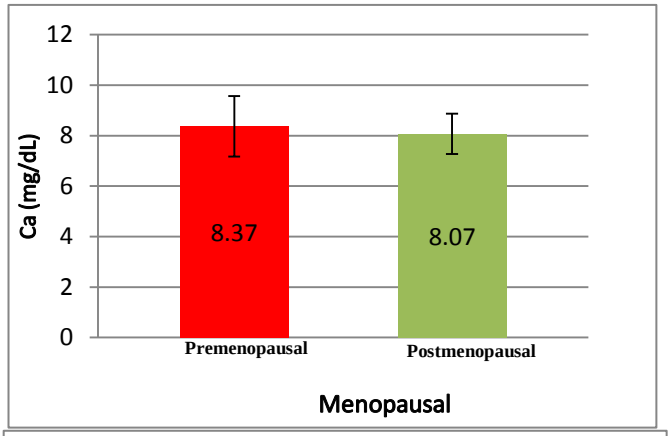


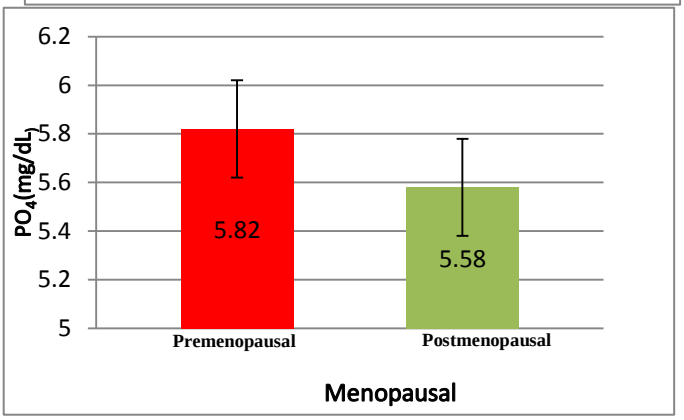


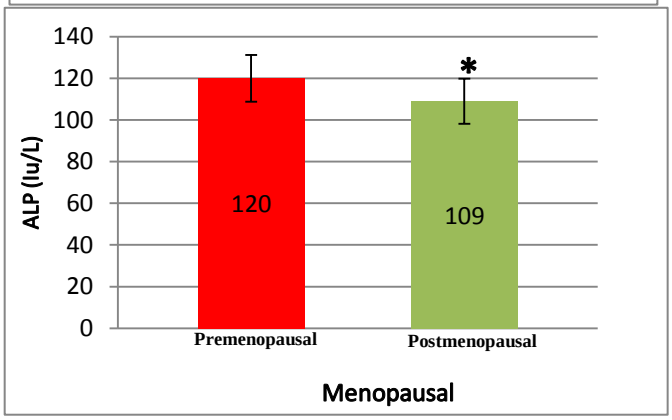












Figures (4-5): Study of parameters study in O.P. women patients according to menopause.

4.5.Correlation between study parameters:

The result of the Correlation and linear regression between study parameters show a significant negative Correlation between calcium (bone sialoprotein and phosphorous). There is a significant positive Correlation between cathepsin K and gelsolin, while there are non significant correlations between other parameters as showed in the table (4-11).

Table (4-11) : Correlation between study parameters

Parameters		Bone sialoprotein	Gelsolin	Cathepsin K	Vit. D3	Ca	PO_4	ALP
FNK	r	-0.224	-0.166	-0.112	0.031	0.061	0.229	0.138
	Sig.	.088	0.210	0.397	0.816	0.647	0.082	0.299
Bone sailopro-tein	r		0.052	0.222	0.145	-0.304*	-0.112	-0.039
	Sig.		0.694	0.091	0.272	0.019	0.398	0.771
GSN	r			0.330*	-0.093	-0.042	-0.073	-0.102
	Sig.			0.011	0.483	0.753	0.580	0.444
Cath K	r				0.012	0.224	-0.051	-0.020
	Sig.				0.929	0.088	0.703	0.880
Vit. D3	r					-0.147	0.167	-0.106
	Sig.					0.266	0.205	0.422
Ca	r						-0.331*	0.055
	Sig.						0.010	0.679
PO_4	r							-0.145
	Sig.							0.273

There was negative correlation between bone sailoprotein and calcium this result showed in the figure (4-6) and there is negative correlation between Po4 and calcium showed in figure (7-4),there was positive correlation between glsolin and cathepcin K showed in figure (4-8).

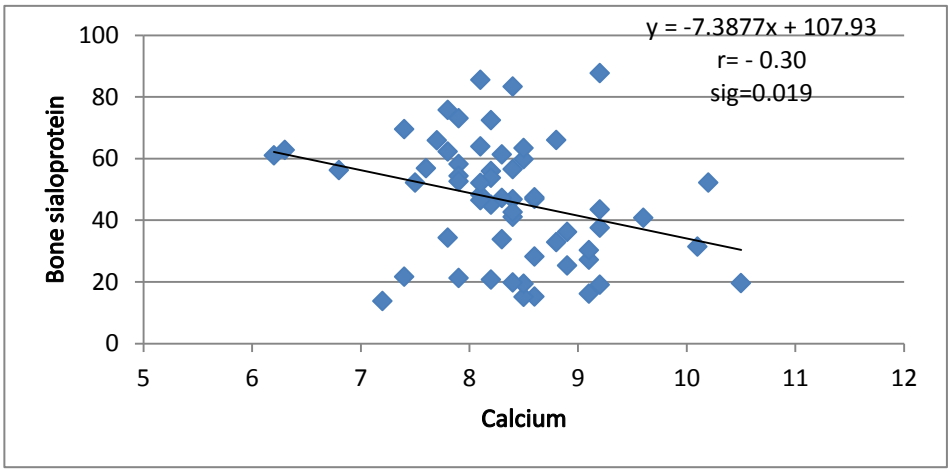


Figure (4-6): Correlation between calcium and bone sialoprotein

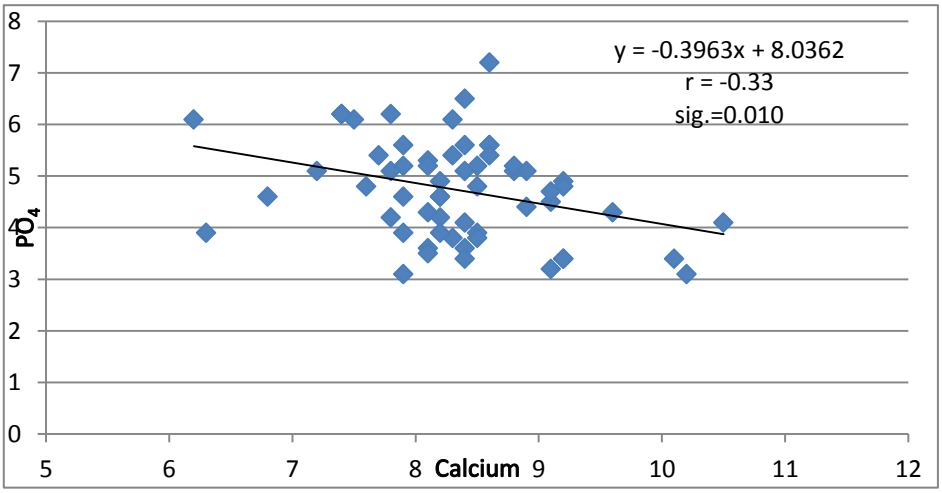


Figure (4-7): Correlation between calcium and PO_4

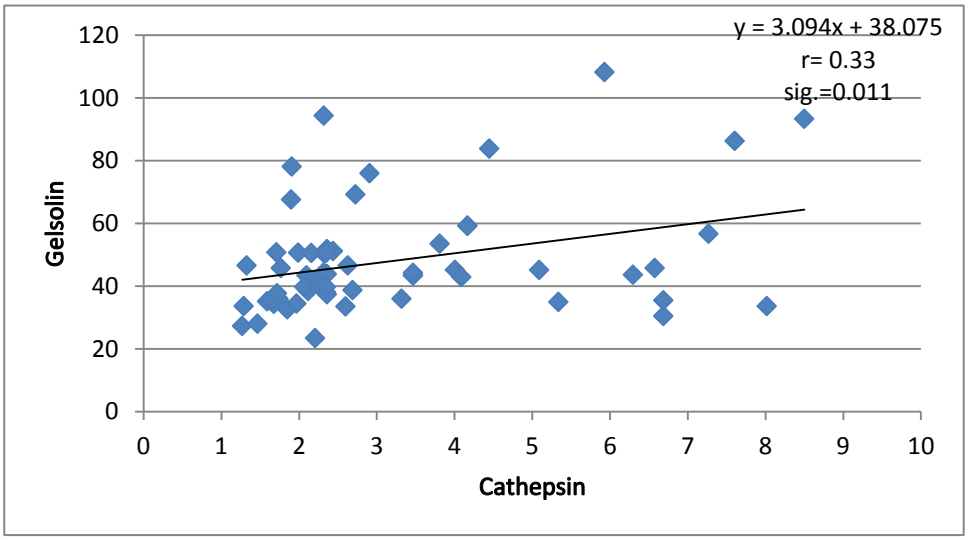


Figure (4-8): Correlation between cathepsin and gelsolin

4.6.Correlation between BMI and Age and all parameters

This study demonstrated a negative correlation between BMI and Ca and does not reveal the marked Correlation between age and BMI with other parameters.

Table (4-12): correlation between BMI and Age and all parameters.

		FNK	GSN	CathK	BSP	VITDD	Ca	PO4	ALP
BMI	r	.176	-.045	.096	.073	.084	-.338*	.121	.050
	Sig.	.195	.742	.482	.595	.541	.011	.373	.717
Age	r	-.118	-.109	-.203	.075	-.068	-.149	.099	-.037
	Sig.	.387	.422	.134	.583	.618	.273	.467	.787

***Correlation is significant at the 0.05 level .**

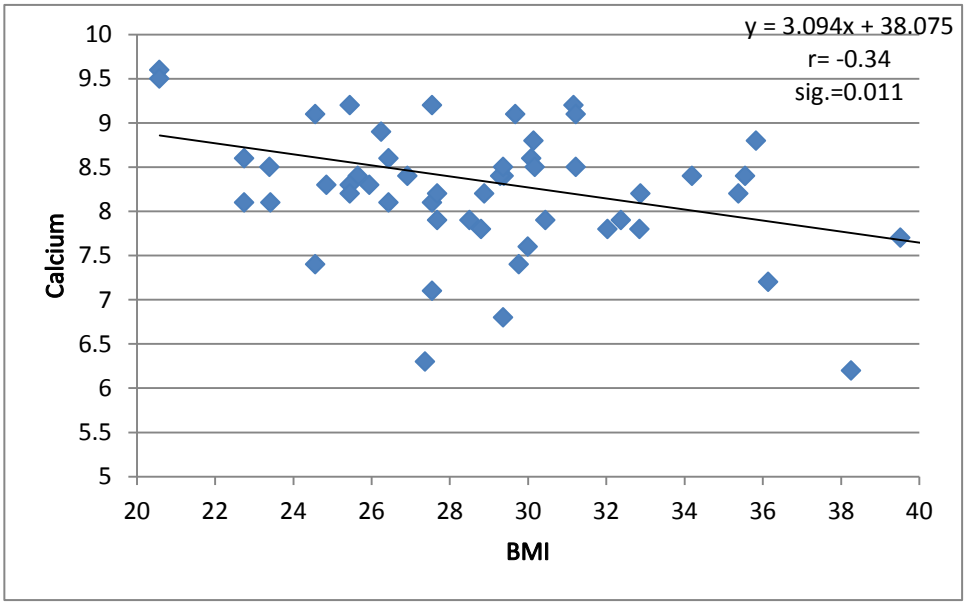


Figure (4-9): Correlation between BMI and calcium

4.7.Correlation between parameters in men osteoporosis patients

Table (4-13) showed a significant positive Correlation between FNK/CXC3L1 and Vitamin D; the present study demonstrated a positive Correlation between FNK/CXC3L1 and Cathepcin K and demonstrated a positive correlation between vitamin D and Bone sialoprotein; this study showed no significant correlation between other parameters in male osteoporosis patients.

Table (4-13): Correlation between parameters in men osteoporosis patients.

		Age	FNK	GSN	CatK	BSP	VIT-D	Ca	PO4	ALP
BMI	r	.332	.393	-.084	.113	.006	.308	-.278	-.576	-.297
	Sig.	.383	.296	.830	.772	.989	.420	.468	.105	.438
Age	r		.075	-.481	-.189	.191	.196	-.067	-.343	-.325
	Sig.		.847	.190	.627	.622	.614	.863	.366	.394
FNK	r			.303	.710*	.509	.800**	-.140	-.610	-.198
	Sig.			.428	.032	.162	.010	.720	.081	.610
GSN	r				.601	.401	.071	-.486	.124	.598
	Sig.				.087	.285	.855	.185	.750	.089
CatK	r					.462	.499	-.477	-.381	.343
	Sig.					.210	.171	.194	.311	.366
BSP	r						.714*	-.402	-.538	.199
	Sig.						.031	.283	.135	.609
VIT-D	r							.061	-.625	-.323
	Sig.							.875	.072	.397
Ca	r								.475	-.628
	Sig.								.196	.070
PO4	r									.200
	Sig.									.605

* Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

There was positive correlation between cathepcin K and CX3CL1 showed in figure (4-10), there was pstive correlation between Vit.D and CX3CL1 showed in figure (4-11) and there was positive correlation between vit. D and bone sialioprotein showed in figure (4-12).

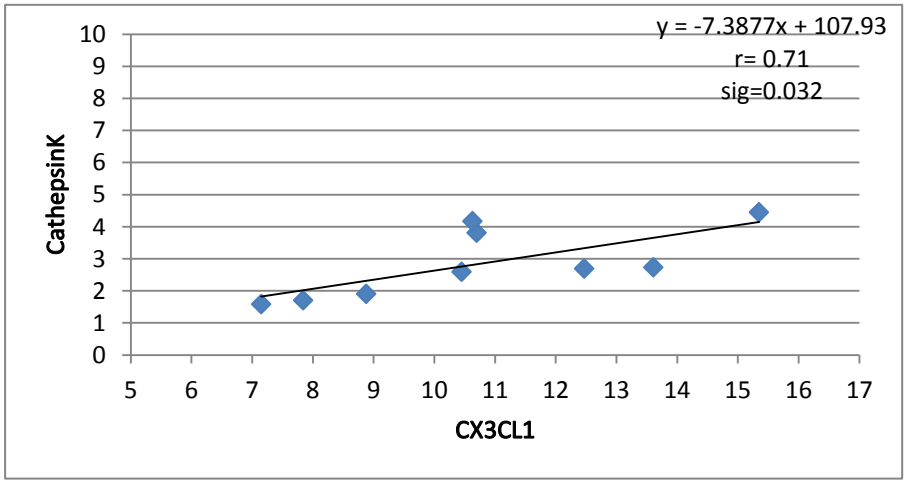


Figure (4-10): Correlation between CX3CL1 and cathepsinK

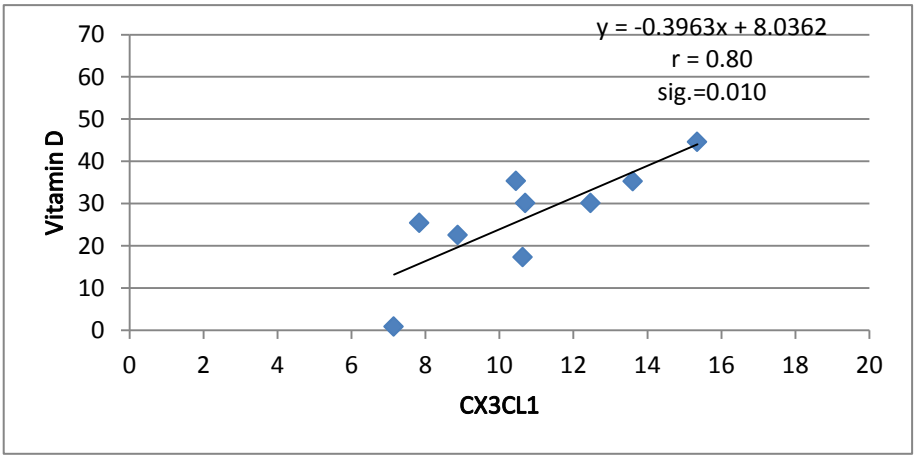


Figure (4-11): Correlation between CX3CL1 and vitamin D

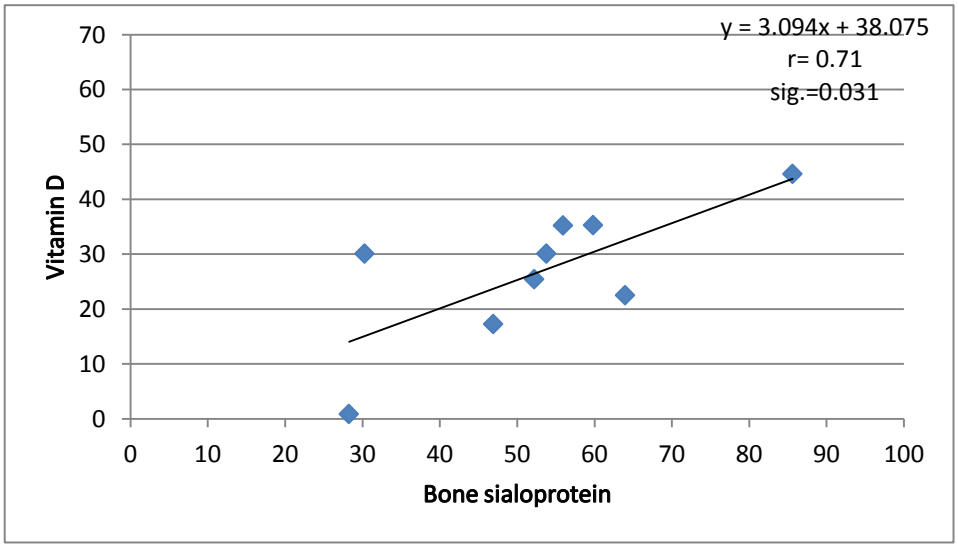


Figure (4-12): Correlation between bone sialoprotein and vitamin D

4.8.Correlation between parameters in women osteoporosis patients

The current study demonstrated a negative Correlation between Ca and BMI, showed positive Correlation between gelsolin and Bone sialoprotein, there is positive Correlation between BSP and calcium, and demonstrated negative Correlation between calcium and Phosphorous. This result is summarized in table (4-14).

Table (4-14):Correlationbetween parameters in women osteoporosis patients.

		Age	FNK	GSN	CatK	BSP	VIT-D	Ca	PO4	ALP
BMI	r	.178	.151	-.042	.065	.139	.101	-.310*	.196	-.104
	Sig.	.231	.311	.780	.666	.352	.500	.034	.186	.485
Age	r		-.159	-.069	-.251	.104	-.077	-.120	.154	-.164
	Sig.		.285	.644	.089	.487	.605	.422	.302	.272
FNK	r			-.034	-.043	.019	-.091	.099	-.268	.235
	Sig.			.822	.775	.898	.545	.510	.069	.112
GSN	r				.174	.291*	.001	.286	-.102	-.037
	Sig.				.241	.047	.995	.051	.494	.803
CatK	r					-.080	.182	-.171	.036	.182
	Sig.					.592	.221	.250	.808	.220
BSP	r						-.120	.358*	-.127	-.089
	Sig.						.421	.014	.393	.553
VIT D	r							.168	-.037	.212
	Sig.							.258	.803	.152
Ca	r								-.367*	.026
	Sig.								.011	.863
PO4	r									-.210
	Sig.									.156

* Correlation is significant at the 0.05 level (2-tailed).

There was negative correlation between bone sialoprotein and calcium and between calcium and Po4 showed in figure (4-13),(4-14). There was positive correlation between gelsolin and bone sailoprotein showed in (4-15).

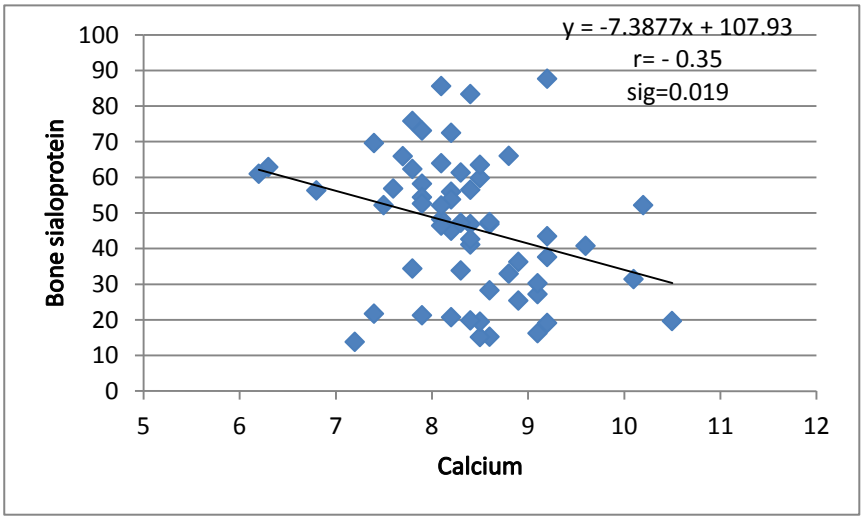


Figure (4-13): Correlation between calcium and bone

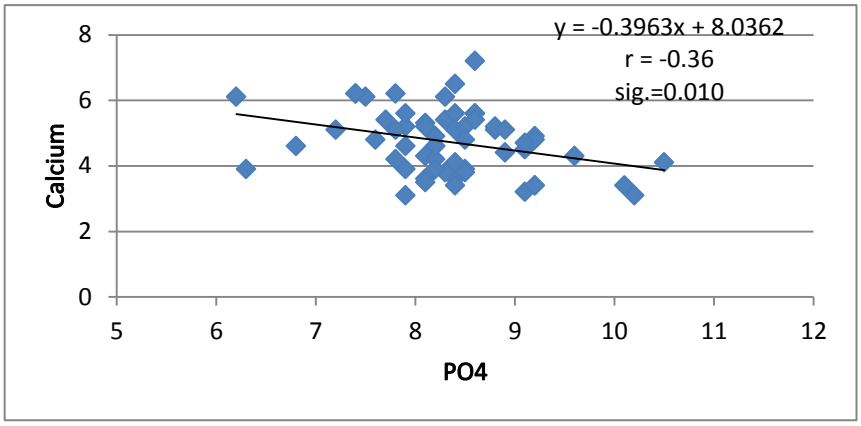


Figure (4-14): Correlation between calcium and PO_4

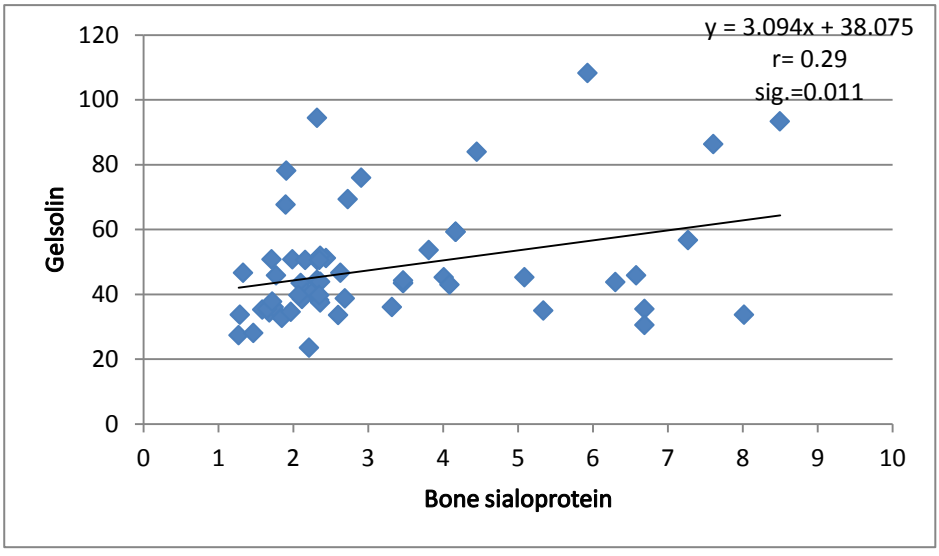


Figure (4-15): Correlation between gelsolin and bone sialoprotein

4.9.Molecular Genetic Study

4.9.1.DNA extraction

The results of the molecular genetics study showed that Deoxyribonucleic acid extracted from osteoporosis patients and the control group extracted DNA by electrophoresis on 1% agarose as shown in figure (4-16).

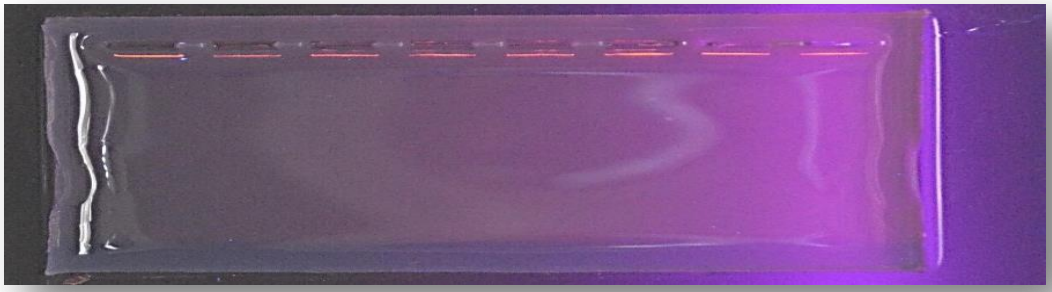


Figure (4-16)The electrophoresis pattern of gnomic DNA extracted From blood for patients with osteoporosis and control,1% agarose.100 V, 50 mA for 40 minutes.

4.9.2.Optimization of TRAP-5 or ACP-5 PCR Product.

Different annealing temperatures (gradient PCR) were used to optimize primer pairs that produced (304pb) of the TRAP-5 gene. A 55-67 C annealing temperature gradient optimised the PCR amplification.Different annealing temperatures were recorded and select the temperature 63C° because it gives the best product and is used for amplification of all the samples.For TRAP gene (rs2071484) genotyping, the genomic DNA was amplified by using specific primers and accomplished by the thermo-cycler apparatus under the optimal conditions. The results revealed that the presence asingle band (304bp)of target sequence of TRAP gene(rs2071484) showed in figure (4-17).

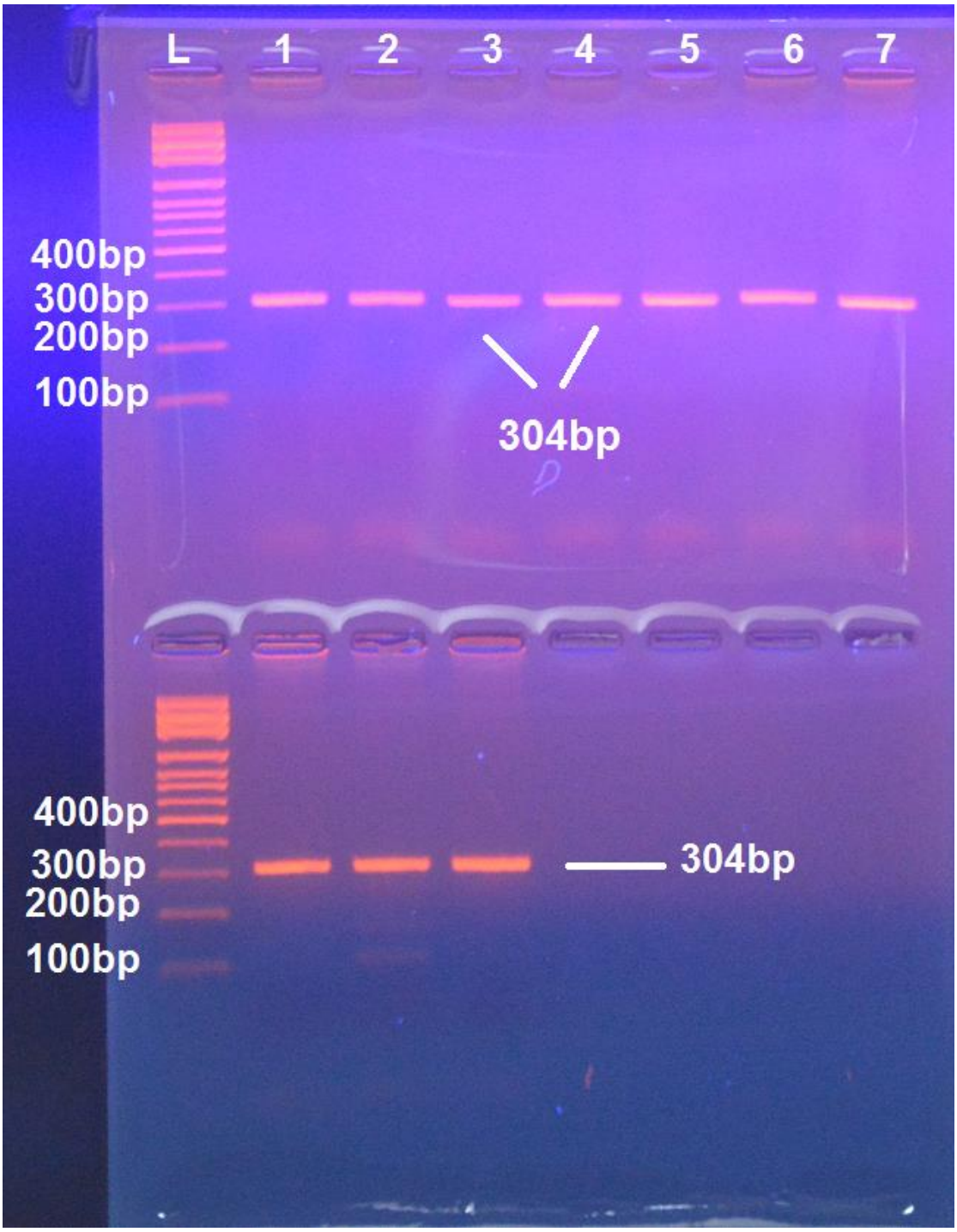


Figure (4-17)Agarose gel electrophoresis of amplified product patterens of TRAP gene (rs2071484) with specific primer DNA marker (100bp) 1-7 refer to PCR product of TRAP gene (rs2071484) of osteoporosis patients and control groups.1% agarose.100V,50mA.

Silver stained polyacrylamide gel electrophoresis which employed for TRAP-5 gene genotyping by single-strand conformation polymorphism technique (SSCP) showed in figure (4-18).

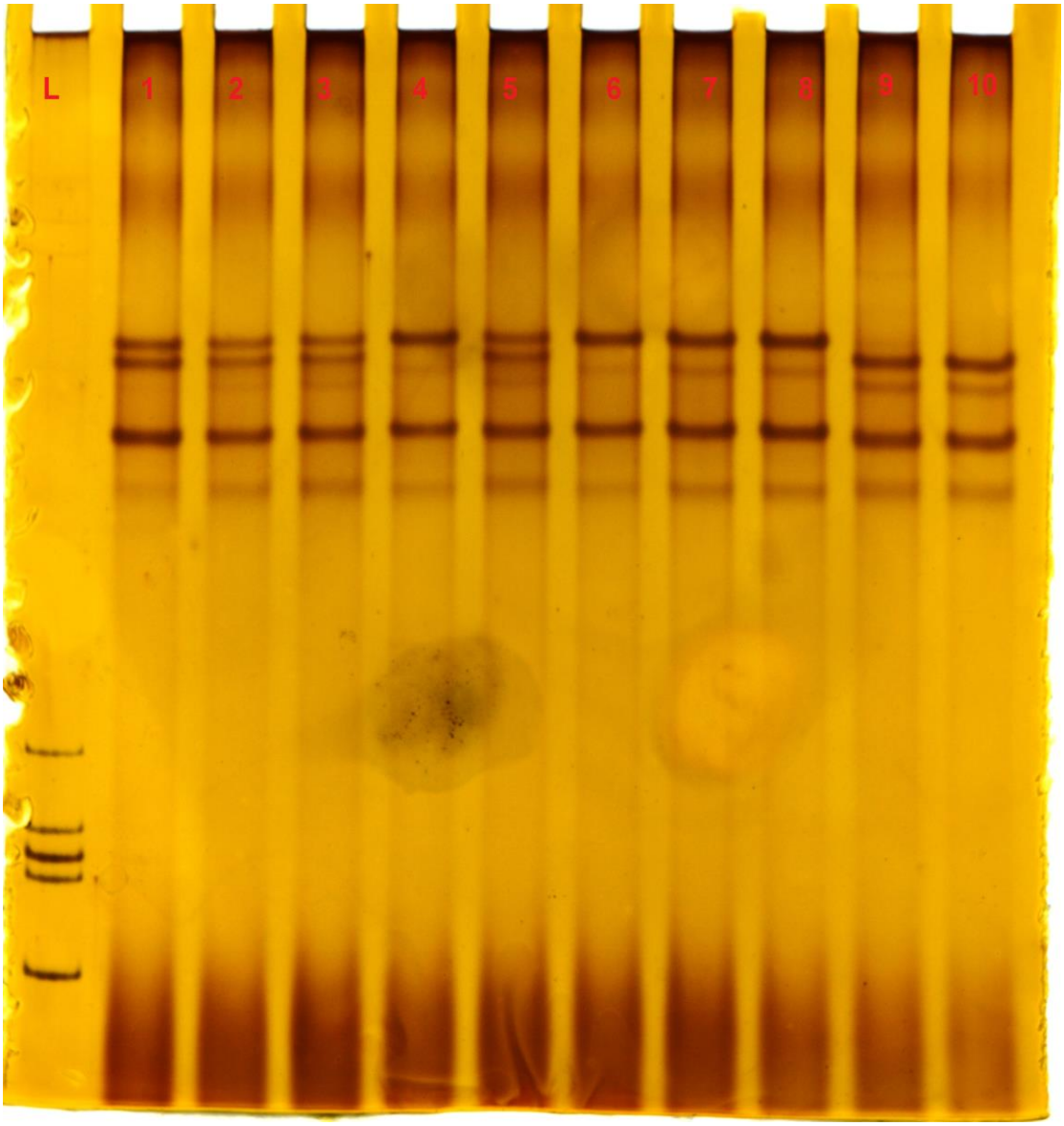


Figure (4-18) Silver stained polyacrylamide gel electrophoresis which employed for TRAP-5 gene genotyping by single-strand conformation polymorphism technique (SSCP), lane L DNA ladder; lanes 1,2,3 and 5 A pattern (T.C. genotype); lanes 4,6,7 and 8 B pattern (T.T. genotype); lanes 9 and 10 C pattern (CC genotype).

4.9.3.The genotype of TRAP-5 gene polymorphism with Allele frequency:

The allelic frequency and allelic association of (rs2071484) with osteoporosis, according to the resulting study, showed there is no allelic association as listed in the table (4-15)

Table (4-15): The allelic frequency and allelic association of (rs2071484)with osteoporosis

	All subjects		control		case		OR (95% CI)	P-value
Allele	Count	Proportion	Count	Proportion	Count	Proportion		
T	160	0.53	53	0.53	107	0.54	1.020 (0.631-1.651)	0.934
C	140	0.47	47	0.47	93	0.46	0.980 (0.606-1.586)	

On the other hand, the genotype association showed that CC genotype have a significant association with disease (p-value 0.0026), (p-value 0.0026) in which the cc genotype have an odds ratio equal to 4.47 compared to the other T.T. and T.C. genotype this indicates that C allele inherited as a recessive pathogenic allele. The table below (4-16) shows genotype frequency and association of (rs2071484) with osteoporosis under different models of inhering tins. The individual with CC genotype in which we found allelic C represent a recessive pathogenic allele has the portability to develop osteoporosis (4.5) time more than individuals with T/T and T/C genotypes.

Table(4-16)genotype frequency and association of (rs2071484) with osteoporosis under different model of inhere tins.

Model	Genotype	control	case	OR (95% CI)	P-value
Codominant	T/T	7 (14%)	35 (35%)	1.00	<0.0001
	T/C	39 (78%)	37 (37%)	**0.19 (0.08-0.48)**	
	C/C	4 (8%)	28 (28%)	1.40 (0.37-5.27)	
Dominant	T/T	7 (14%)	35 (35%)	1.00	0.0049
	T/C-C/C	43 (86%)	65 (65%)	**0.30 (0.12-0.74)**	
Recessive	T/T-T/C	46 (92%)	72 (72%)	1.00	0.0026
	C/C	4 (8%)	28 (28%)	**4.47 (1.47-13.58)**	
Overdominant	T/T-C/C	11 (22%)	63 (63%)	1.00	<0.0001
	T/C	39 (78%)	37 (37%)	**0.17 (0.08-0.36)**	

4.10.Sequencing results:

The sequencing result showed the difference in the SSCP method due to the present one SNP that appears in the human chromosome in NCBI and this SNP name ((rs2071484). This result showed that pattern A contain heterogenotype C.T., while the pattern B contain T.T. genotype and the pattern C represent CC genotype.

The alignment results of the 304 bp samples revealed the presence of only one variation in some of the analyzed samples in comparison with the referring reference DNA sequences Figure (4-19). A highly interesting nucleic acid polymorphism (SNP) was detected in this study in the investigated samples, in which Thymine was replaced with Cytosine at position 162, namely T162C.

Figure (4-19) **DNA sequences alignment of 8 genotyped samples with their corresponding reference sequences of the 304 bp amplicons of the 5′-UTR sequences of the *ACP5* gene. The symbol "ref." refers to the NCBI referring sequence, "S1-S8" refers to the genotyped samples 1 to 8, respectively.**

The sequencing chromatogram of the identified variation, as well as its detailed annotations, were documented, and the chromatogram of this sequence was shown according to its position in the PCR amplicon. However, this SNP was detected in heterozygous T/C status in both S1, S2, and S3, homozygous T/T status in S4, S5, and S6, and homozygous C/C status in both S7 and S8 Figure (4-20)

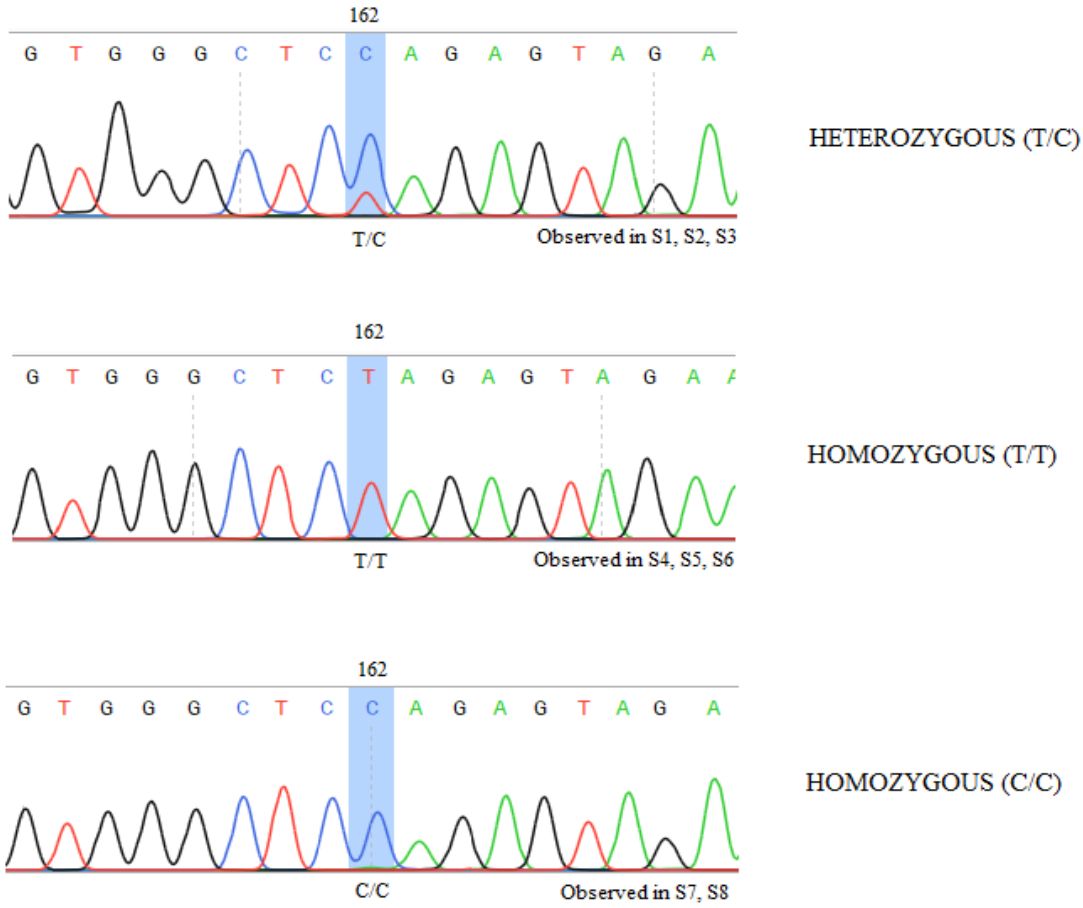


Figure (4-20) The pattern of the detected T162C SNP within the DNA chromatogram of the targeted 304 bp amplicons of the *ACP5* gene. The identified SNP was highlighted according to its position in the PCR amplicons. S1 – S3, S4 – S6, and S7 – S8 samples exhibited the T/C, T/T, and C/C states in the highlighted polymorphic locus.

To find out the nature of this SNP, a graphical representation was performed concerning the *ACP5* dbSNP database within chromosome 19. By reviewing the dbSNP engine, it was found that this detected SNP was found to be previously known as it was deposited as rs2071484 Figure (4-21).

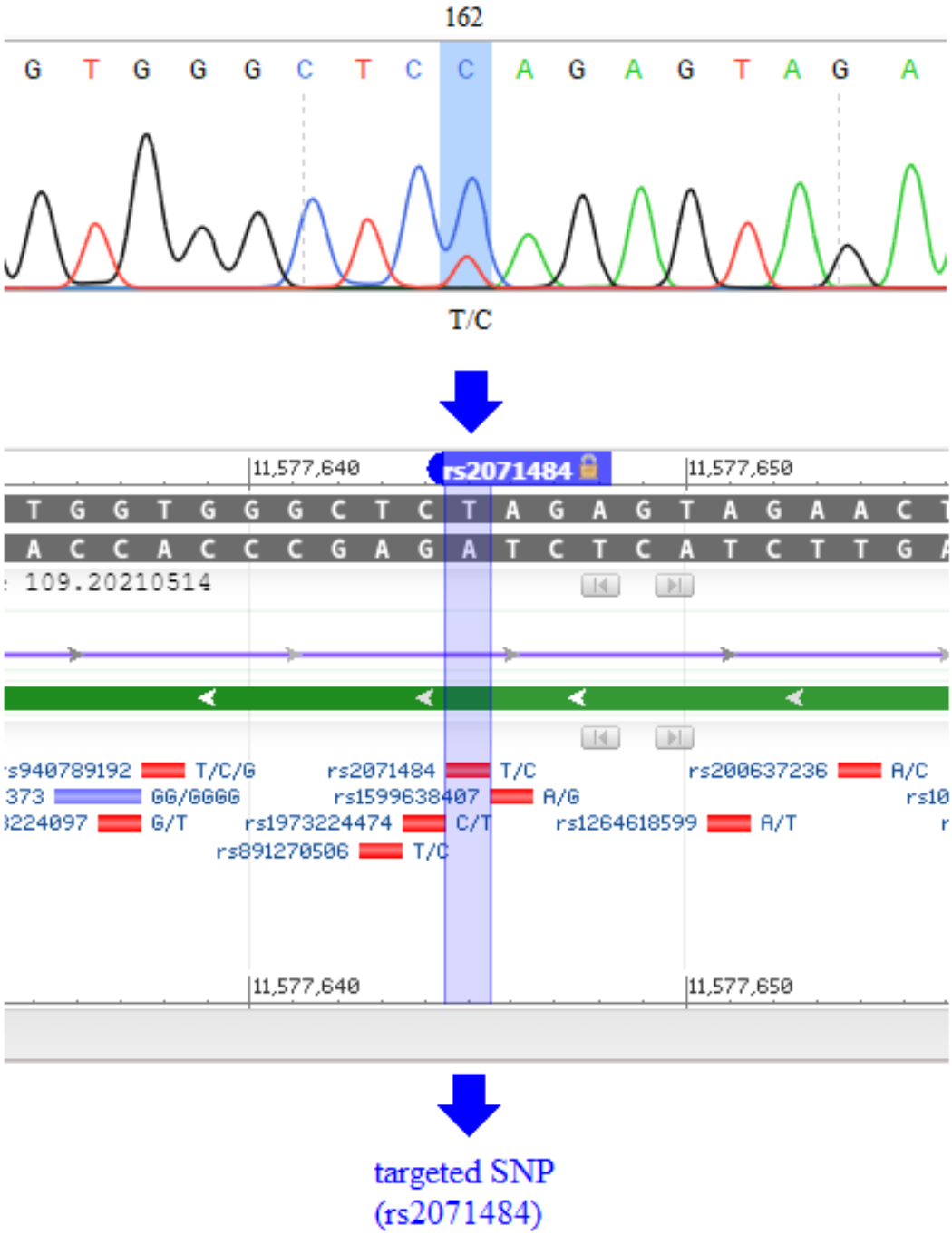


Figure (4-21) **The SNP's novelty was checking of *ACP5* genetic single nucleotides polymorphisms using the dbSNP server. The identified T162C SNP was marked with a blue colour. The GenBank acc. no. NG_028127.1 was used in the positioning of the highlighted substitution SNP. The position of the targeted sequences was found in the negative strand.**

To summarize the results obtained from the sequenced 304 bp fragments, the detailed position of the observed variation was described in the NCBI reference sequences (Table4-17).

Table(4-17). The pattern of the observed SNP in the 304 bp amplicons is designed to amplify a portion of the 5′-UTR sequences within the ACP5 gene compared to the NCBI referring sequences (GenBank acc. no. NG_028127.1). The symbol "S" refers to the sample number.

Sample No.	Zygosity status	Position in the PCR fragment	Position in the reference genome	SNP type	Variant summary
S1, S2, S3	Heterozygous (T/C)	162	6342	5 Prime UTR Variant	rs2071484
S4, S5, S6	Homozygous (T/T)	162	6342	5 Prime UTR Variant	rs2071484
S7, S8	Homozygous (C/C)	162	6342	5 Prime UTR Variant	rs2071484

5. Discussion

5.1. General characters:

5.1.1. Gender:

The results in this study recorded that women are more affected than males table (4-1). This finding, agreeing with its mention Feldstein *et al.* (2003) that Osteoporosis occurs four times in females than the males. However, some studies found that men have a high rate of Osteoporosis**.**

Gender is a predisposing factor for Osteoporosis (Vijayakumar and Bu¨sselberg, 2016). The osteoporosis risk osteoporosis is high in women (Ivanova *et al.*,2015). Data of NNHS in Taiwan (2005-2008) showed that rates of Osteoporosis were 23.9% in men and 38.3% in women (Lin and Pan,2011). One causes for sex differences in the osteoporosis rate is menopause (Vijayakumar and Bu¨sselberg, 2016). During menopause, estrogen deficiency leads to the development of Osteoporosis, bone loss, and fractures than the control group. The density of the bone was higher in men three folds than the women. The peak density commonly occurs in females, particularly in the lumbar spine. Nutrition and physical activity have great importance for preventing Osteoporosis **(**Anderson *et al.*,1996).

Males have a greater mass than females (Nieves *et al*.,2005). Thirty-six normal males and females at 18 years. The males have 8% higher bone density than the females at the hip, but the males have a high mineral density of the hip bone. This difference is not mean caused due to body weight, physical activity, and nutrition, but it is due to bone size (Jones *et al*.,1994).

The age range showed that rapid decline was (74-79) years for the males than (65-69) years for the females. The females lose bone early and positively correlate with bodyweight loss in men, women, and smokers. In the study, Osteoporosis is evaluated in lumbar bone and hip bone in (800) older males and females (74) years (Hannan *et al*.,2000). The study demonstrated that mean four-year bone loss was 0.2-3.6% in males than in females (3.4-4.8%). Osteoporosis accompanied the weight loss for males and females, but the male smokers have more to lose BMD than females at the hip bone; bone loss in the women may explain the difference between the genders.

Another study used (28) older males and females to evaluate the bone turnover; there was no difference between both genders in bone formation. However, males have high parathyroid hormone and low N-telopeptide than females (Greenspan *et al.*,1997). It is vital to note that the periosteal to the endosteal bone ratio impact bone strength. Compared to women, males have higher endosteal bone loss, but they experience larger periosteal bone growth, which causes a net loss of bone in the males (Seeman,2001). Estrogen shortage is accompanied by the development of Osteoporosis in males and females. However, it is more evident in the females and young (premenopausal) ages than males, according to research (Gennari *et al.*,2008). It is thought that around 20% of older men with Osteoporosis also have hypogonadism, which is a hormonal imbalance (Center *et al*.,1999).

Briefly stated, males and females experience bone loss, but females experience bone loss at a young and a faster rate than men, and they also have greater bone resorption indicators than males. While smoking and weight reduction are significant modifiable risk factors, they should not be the primary focus of attention when evaluating such individuals.

Compared to women, males have more periosteal growth, which helps mitigate endosteal bone loss and causes higher bone strength.

5.1.2. Body mass index:

The BMI results showed that obesity (48%) was higher than the percentage of other categories (overweight 32%). In comparison, the smallest categories percentage was (healthy weight. 20%) as presented in table (4-2), there are significant differences between them in compared to control (sig =($p \leq 0.05$). The high happening of Osteoporosis in individuals with high BMI is due to the people with a high BMI, and they have a large skeleton (Lau, *et. al*., 2000).

Other reasons that Osteoporosis and obesity are widespread diseases. Low concentration of vit D is leading to the obesity. Many studies found a relation between vit D level and BMI (Vimaleswaran *et al*., 2013). High BMI results in low level of vit D in the bloodstream, and decreased serum vitamin D levels can reduce circulating calcium and induce secondary hyperparathyroidism that can affect the incidence of disease (Cunningham, *et. al*., 2011).

The relationship between Osteoporosis and obesity is documented; the epidemiological studies demonstrated that obesity increases with increasing bone mass; after controlling for mechanical loading effects caused by total body weight, further examination in patients by body weight showed the negative connection between the mass of the fat and bone. Increasing fat mass may have no favourable impact on the mass of the bone (Zhao *et al*., 2007).

The stem cell generates differentiation of osteoblasts and adipocytes and produces adipocyte-derived hormones, leading to bone development; this cause may explain this relationship (Zhao *et al*. 2008).

Osteoporosis and obesity have related diseases (Zhao *et al.*, 2007). The adipocytes produce important factors for bone remodelling, like the aromatase, proinflammatory cytokines, estrogen synthesis enzyme (Gimble *et al*.,1996).

According to Justesen and colleagues (2001) and (Ravn *et al.,1*999), people with Osteoporosis have higher levels of fat in their bone marrow, and there is a link between obesity-related characteristics and osteoporosis-related characteristics (bone mineral density, for example). Overweight and obesity, according to WHO's definition, are defined as abnormal fat buildup that may be harmful to one's health (Bosello *et al.*,2016). Obesity has been described as an epidemic that has been steadily deteriorating over the past 50 years and has been linked to a number of medical issues. the

Primary Osteoporosis is developed as a result of increasing age or after menopause in women (senile Osteoporosis). On the other hand, secondary Osteoporosis may occur as a result of diseases that are produced by one or more medicines or by a combination of pharmaceuticals of different sorts (Mitchell *et al*.,2011). Glucocorticoids, whether Cushing or exogenous, have been shown to cause obesity and overweight in certain types of secondary osteoporosis, as observed in people who have been exposed to them for an extended period (Mitchell *et al*.,2011).

One mechanism linked with obesity that a greater BMD may explain is the increased mechanical stress and strain resulting from this state. Obese persons have increased or excess body fat mass and lean mass, and as a result, not only passive loading but also muscle-induced strain is enhanced in obese individuals. This may affect bone modelling, geometry, and density; however, the impairment in muscle strength that is associated with the fat accumulation inside the muscle (Addison *et*

al.,2014) may also reduce or lessen the beneficial effects of muscle mass and action on the bone (Addison *et al*.,2014).

Many studies revealed that the obsessed women after menopause have a high risk of fractures, such as lower limb and ankle fractures, while the risk of fracture of the wrist, pelvis and hip was decreased (Johansson *et al.*,2014). Bone density in the young-adult life is associated with risk for osteoporosis in later life. Physical activity parameters seem to be more important for bone health than nutrition factors. Therefore, high physical activity levels during childhood, adolescence, and early adult life are highly recommended to improve bone parameters (Heydenreich *et al.*,2021).

5.1.3.Age :

In this study, the percentage age groups of Osteoporosis patients doesn't show statically differences among the groups, and it was shown in the table (4-2), this was agreed with the previous study of Selma *et al*.,(2016) and disagreed with other studies show that the elderly age group has a great incidence for getting Osteoporosis according to (Zhor *et al*.,2012) and (Al-Kazzaz *et al*.,2013)**.**

Osteoporosis is a serious public health concern that affects people all over the globe. The Osteoporosis occurs one in four females and one in six males more than (65) years. In Indonesia, Osteoporosis affects 19.7% of the aged population (3.6 million individuals), accounting for 3.6 million persons (Sabrina, 2014). In Osteoporosis, there are two types of risk factors. The first is risk factors (gender and age), the second is modifiable risk factors (deficiency of Ca and P, physical activity, vit D deficiency, BMI, smoking, and alcohol), and the third is a combination of risk factors (Limpaphayom *et al*.,2001).

Widarsa *et al*. (2018) conducted another study that found that the prevalence of Osteoporosis was relatively high, accounting for 28.1% of the older people, the osteopenia was found to be as high as 54.3 %. The normal bone density rate was found to be 17.6 %. The chance of developing Osteoporosis rose with age, with the post-elderly group increasing several folds in the likelihood of developing Osteoporosis than the older. This is consistent with the ageing hypothesis, which states that the maximal bone mass is attained during the 30s when the bone remodelling process is completed. The rise in osteoclast activity, which outnumbers the increase in osteoblast activity, results in a decline in bone mass density in people over 30 years. As a result, the bone remodelling process could not attain the desired level of bone strength. This occurrence increases the chance of suffering bone density and the likelihood of experiencing bone disintegration (Osteoporosis) by a factor of two (Yamaguchi *et al*.,2008).

Another finding of this study was that bone mass growth began to decline after 45 years of age and bone density loss increases with age.

This is mainly due to age-related bone density lowering. Due to that, the bones become thin or osteoporotic during ageing, and increasing age is a contributing factor in the increased risk for incident fractures in adults (Seung-Geun *et al.,*2012). However, it is more common in females than males, especially after menopause, due to reducing estrogen hormone levels (Nandi *et al.,*2016). Reduced levels of bone minerals such as calcium will lead to the lightweight of bones, reduced density, and increased bone fragility(Anitha *et al.,*2014).

Another study was done in Pakistan at a tertiary medical care center for rehabilitation. The authors of this study reported that most of

the study population in the age group below 50 years had a normal BMD, while those in the age group above 50 years are in the osteopenic range (Akhlaque *et al*., 2017). In a recent study conducted in China on 394 participants, including 122 males and 272 females, the authors observed that there was a slow decrease of BMD with male ageing. At the same time, there was a gradual increase of BMD among females before 30-49 years and then it decreased rapidly(Zhang *et al*.,2019).

5.2.Biochemical Parameters

5.2.1.CX3CL1:

CX3CL1 is part of the CX3C family that has been identified so far (Chapman *et al* .,2000). CX3CL1/FKN is unique among chemokines, it occurs as a transmembrane protein and chemoattractant, and it is activated on monocytes, natural killer cells, and T cells in contrast to all other chemokines (Julia,2012). Only a few research have looked at the possibility of CX3CL1/FKN being involved in the bone remodelling process so far, but this is changing. Han *et al.* (2014) discovered that CX3CL1–CX3CR1 have an important role in bone resorption and osteoclast recruitment. As previously mentioned, disruption of the CX3CL1-CX3CR1 axis by using an antibody against the protein CX3CL1 was shown to suppress osteoblast-guided development of osteoclasts in the experimental (Koizumi *et al*.,2009).

Also discovered is that the CX3CR1–CX3CL1 axis is critical in maintaining osteoclastic. This is the first time this has been shown (Hoshino *et al*.,2013).

The findings of this investigation revealed a statistically significant rise in the serum CX3CL1 levels in osteoporosis patients (table) (4-2).

Other studies showed there are high effect to CX3CL1 in many disease such as Sjögren's syndrome, rheumatoid arthritis, SLE, scleroderma, as well as diseases related to vascular inflammation(Jones *et al.*,2012).

Other findings showed there is significant increase in the CX3CL1 concentration in multiple myloma MM patients. They found BM CX3CL1 concentrations in one hundred –eleven plasma cell disorders condition, wherever, active MM was (70), them monoclonal gammopathy was (16), smoldering myeloma was (25), while our study demonstrated that BM CX3CL1 level was more in MM cases when compared to MGUS and SMM, as well as, it have related with BM microvessel density. The current study dound and detected that CX3CL1 source in BM and MM cells (Marchica *et al.*,2019).

According to the findings, recent research has demonstrated that the chemokine CX3CL1 has a role in the osteoporosis etiology. CX3CL1 and CX3CR1 have distinguish between them with fifty chemokines. CX3CL1 is a chemokine that can operate as an adhesion molecule, allowing immune system cells to more easily penetrate the endothelium and reaching to the inflammation site (Wojdasiewicz *et al.*,2019). The CX3CL1/CX3CR1 is an important part of the mechanisms that contribute to BMD loss. CX3CL1/CX3CR1 is important in osteoclast maturation and binding osteoclasts with immune cells to the surface of bone tissue, among other things. It contributes to the development of inflammation and the generation of various inflammatory cytokines near the bone surface (e.g., TNF-a, IL-1a, and IL-6), which are detrimental to bone health. NTx, TRACP-5b, IL-6, and IL-1beta as well as inflammatory factors and bone turnover are raised in direct proportion to high levels of CX3CL1 in the blood serum. When it comes to the notion that inhibiting

the CX3CL1 and its receptor axis are possible target of immunological therapy, many studies are available that address the issue is inadequate. As a result, it seems appropriate to continue the study to accurately define its involvement in the bone tissue f metabolism or osteoporosis therapy (Wojdasiewicz *et al*., 2019).

Our result demonstrated a marked increase at ($p \leq 0.05$) in the serum CXC3 concentration in the female osteoporosis patients table (4-3). The resulting study showed a marked increase in CXC3 level in the premenopausal and postmenopausal osteoporosis patients compared with the healthy group agreeing with its mention Yi-Ding Chen *et al*.,(2016) that FKN level was measured after menopause in the osteoporotic cases the after menopause non-osteoporotic women and control group.

According to research , the FKN-CX3CR1 axis has a role in the pathophysiology of inflammatory illnesses and bone-resorbing. Elevated FKN concentration in osteoporosis cases was associated with decreased bone mineral density (BMD), increased bone turnover indicators, inflammatory markers, and bone fractures. According to clinical studies, osteoporosis is an endocrine disorder associated with bone mass and volume loss, and that a more comprehensive approach is needed, which should include a systemic approach in light of the significant role played by the immune response (Pietschmann *et al*.,2016). human and mice high levels of FKN expression use the soluble form of FKN to attract osteoclast generate cells, but the membrane-bound form of FKN may attach to osteoclast precursors in the lack of soluble FKN in the absence of high amounts of soluble FKN ,thus implying that FKN has important involvement in the activity of osteoclasts(Koizumi *et al*.,2009).

The amount of FKN was inversely associated with the pain's severity. Osteoporosis pain is the prevalent consequence of the disease. According to current estimates, approximately 75–85 % of people with Osteoporosis are affected by bone discomfort (Hayashi,2007). It is believed that low back pain, in particular, is the most common kind of musculoskeletal pain, especially in senior people (Woo *et al*.,2009). The improper charge of articulation and muscles that occurs in osteoporotic individuals due to increased bone loss may result in spinal deformity, which can result in long-term discomfort (Bayles *et al.*,2000). as a result, there are further functional limits, despair, and a decline in social activities (Ryan *et al*.,1994). Increased bone resorption, spinal neuropathic pain, central sensitisation, and spinal neuropathic pain are linked to chronic Osteoporosis, even though the exact causes of pain in Osteoporosis are yet unknown (Mediati *et al*.,2014). Many studies reveal that FKN/CX3CR1 has a great important role in chronic pain in various disorders, including musculoskeletal disease, inflammatory disease, and cancer (Park *et al*.,2011; Miller *et al*.,2014).

5.2.2. Bone sialoprotein (BSP):

The glycoprotein BSP is found in the bone matrix. It is one of the phosphorylated proteins that is not composed of collagen. It can only be found in mineral tissue, and it is created by the cells of the bone marrow. In addition to being critical for cell-matrix adherence, BSP also plays an activating function in osteoclast activation. According to the literature, BSP levels are elevated in individuals with rheumatoid and after menopause women (Alford and Hankenson,2006).

The study results reveal a great increase in bone sialoprotein level in osteoporosis patients compared with the control group, as shown in the table (4-2).

In the present study, BSP concentration was more level in patients with Osteoporosis than the controls, as shown in table (4-3), agreeing with (Woitge *et al*.,1999) that showed BSP concentration was high level in primary vertebral Osteoporosis more than in healthy controls. Other study showed significant increase in BSP concentration in stage I of multiple myeloma patients MM-I. In MM-I patients, the findings found that myeloma cells have affect BSP and OPN production, that take part in osteoclastic bone resorption. In MM-I patients, The concentration used as biomarker for determine the bone destruction in MM-I patients and differentiation MM-I from control (Maarouf *et al*.,2020).

Many reports demonstrated that BSP concentration is a good tool for the assessment of bone turnover and demonstrated that there is a significant correlation between bone formation such as osteocalcin and alkaline phosphatase (Fassbender *et al*., 2000) to bone resorption markers such as bone resorptive cytokines interleukin-11 (IL-11) and transforming growth factor β2 (TGFβ2) (Shaarawy and Hasan,2001). The rapid decrease of BSP concentration after bisphosphonate administration explains how the bone resorption processes occur (Seibel, 2005).

Another research conducted by Pietschmann *et al*. (2001) revealed a substantial drop in the level of BSP in males with idiopathic Osteoporosis, which is caused by an increase in bone resorption that outpaces, surpasses the rate of bone production. Low estradiol concentration and low concentration of bioavailable testosterone in men with idiopathic Osteoporosis may contribute to the increased bone

resorption seen in this condition. In men with Osteoporosis, BSP is expressed throughout the early stages of bone deposition; thus, it is tempting to infer that most parts of bone formation are normal, but there seems to be a malfunction at discrete of osteoblastic development in men with Osteoporosis. It is also necessary to examine the likelihood of a distinct metabolism of BSP in the males and females as participants in this study.

Our study showed a significant increase in serum BSP in osteoporosis patients according to age compared with the healthy group. This agrees with the mention of (Woitge *et al*.,1999) that showed a positive correlation between BSP and age. According to this study, there is a significant increase in BSP levels in premenopausal and postmenopausal osteoporosis patients compared with the healthy group. This agrees with Shaarwy and Hasan (2001), which the BSP level was increased after menopause in the osteoporosis cases more than the controls. The high level of BSP showed inactive osteoblasts cases (Fujisawa *et al*., 1993).

Another study discovered that osteoclasts express BSP-binding integrins, previously unknown (Ross *et al* .,1993). BSP seems to mediate and increase osteoclastic bone resorption (Ross *et al* .,1993). The levels of serum BSP were unaffected by the presence of alkaline phosphatase The alkaline phosphatase is used as bone developing biomarker; the BSP has not been related to new bone tissue formation (Seibel *et al*.,1996).

The present study demonstrated that the concentrations of circulating immunoassay are significantly positively correlated to bone resorption in osteoporotic after menopause. This correlation may be because the biomarkers of bone resorption included urinary-free

deoxypyridinoline (DPyr), pyridi-, and (NTX), all of which reflect osteocla (Seydin *et al.*,1993). These findings suggest that serum BSP levels may be associated with osteoclast activity in certain cases. The findings presented here suggest that the blood level of BSP in postmenopausal osteoporosis patients may be used to assess the effectiveness of antiresorptive medications and guide treatment decisions.

5.2.3. Gelsolin (GSN):

Gelsolin is a protein involved in the severing and capping of actin filaments during the organisation of the actin cytoskeleton (Zhang *et al.*,2017). It is important for podosome formation, signal transduction, active cell movement, and signal transduction, among other functions (Chellaiah *et al.*,2000). Podosomes are structures in which actin polymerization and depolymerization occur in rapid succession (Akisaka *et al.*,2001), which have a critical role in mechanical sensing, cell adhesion, mechanical sensing, and matrix remodelling in macrophages, endothelial cells, osteoclasts, and dendritic cells among other cells (Meddens *et al.*,2016). In addition to degrading the bone matrix, osteoclasts may also destroy the matrix and mineral composition, which are necessary for bone maintenance and remodelling (Qin *et al.*,2017).

The osteoclast activation for bone resorption is the mean integrity of the adhesion, motility function and actin cytoskeleton (Saltel *et al.*,2008). The result of this study showed elevated significance at ($p \leq 0.01$) in GSN concentration in osteoporosis patients compared with the healthy group in the table (4-2), and the results of the current study showed a clear increase in the GSN concentration in women osteoporosis patients compared with the healthy group in the table (4-3), this finding, agreeing with its mention Wen-Yu Wang and his colleagues (2018), that

showed in the three subgroups which included BMI and age effect on BMD.

In Subgroup 1, there was no variation in GSN concentration between the total number of individuals and between the number of men and females. In light of the fact that our earlier results on OP and GSN were derived from female participants. In Subgroup 1, tests were administered in a gender-stratified manner in accordance with the gender stratification. This study indicated that the difference in GSN level was negligible in men but was suggestively significant in females, which was surprising (Osborn *et al*.,2008).

Other reports reveals great decrease in gelsolin level in rheumatoid arthritis cases when compared with the controls. The reduced levels in combination with the presence of gelsolin-actin and actin complexes in synovial fluids, that indicated consumption of the anti-inflammatory protein in the infected joint.

The current study showed a significant increase in GSN concentration in postmenopausal osteoporosis patients compared with the healthy group agreeing with Wen-Yu Wang and his colleagues (2018), which showed that GSN is accompanied with hip BMD after menopause in Chinese women, and that means risk factor for the Osteoporosis.

The results suggest that the GSN function is likely regulated by elements that are distinct to each gender, such as sex hormones. According to previous research, GSN may act as a controller in the androgen effects on bone resorption and osteoclastogenesis in the bone matrix (Saltel *et al.*,2008). It is currently unclear if GSN interacts with oestrogen to impact bone metabolism and bone mineral density (BMD), and this will be researched more in the future. Previous research

demonstrated that GSN deficiency leads to an inability to podosomes formation, cytoskeletal architecture, and decrease osteoclast motility; reduction of the bone resorption, podosome transduction blocking; increased strength, density, and mass of the bone (Chellaiah *et al*.,2000).

5.2.4.Cathepsin K (CatK):

Cathepsin K is an enzyme produced and released by osteoclasts for bone formation. It is known to have a significant role in the breakdown of collagen, and as a result, it is a potential therapeutic target in Osteoporosis (Helali *et al*.,2013). Procathepsin K is an enzyme activated in the lysosomes by an enzyme-dependent process that involves autocatalytic at low pH before being released into the resorption ducts (Dodds *et al*.,2001). Part of the cathepsin K is likely produced inside the circulation. This cathepsin K might operate as a particular biological bio-factor of osteoclast activity in the body.

The results of the current study showed a clear increase in the cathepsin K concentration in osteoporosis patients compared with the healthy group as shown in table (4-2); this finding, agreeing with its mention Meier and his colleagues (2006), showed that serum cathepsin K level elevated in osteoporosis patients. Cathepsin K has the main role in osteoclast degradation by osteonectin and type 1 collagen. The organic matrix components correlate between high cathepsin K level and Osteoporosis, which make bone turnover become more and increase the probability of fracture occurrence.

Other studies showed increase level of cathepcin K in patients with rheumatoid arthritis, cathepsin K was localized in the giant cells, the synovial fibroblast, and the macrophages. In the healthy condition, expression of cathepsin K is restricted to the fibroblast while in

rheumatoid arthritis, the cathepsin K are appears in pannus region (Wilson *et al.*, 2009).

The previous studies demonstrated that the cathepsin K level in patients with multiple nontraumatic fractures was significantly elevated than in patients without fractures (Meier *et al*., 2006). In this study, the result showed an increase significantly in cathepsin K level in postmenopausal females with Osteoporosis compared with the healthy group that showed in the table (4-10) agreeing with (Munoz-Torres *et al.*,2009) that obtained cathepsin K concentration in women after the menopause with the Osteoporosis were higher compared to healthy postmenopausal women. These results suggested that serum cathepsin K level diagnostic tool could use as a marker for the fracture and BMD.

Our study showed no significant difference between osteopenia and osteoporosis patients in the level of cathepsin K that showed in the table (4-6) agreeing with Adolf *et al*., (2012) that Using WHO criteria of 21 postmenopausal women that have a normal BMD, 24 had Osteoporosis and 49 had osteopenia, the premenopausal women was usually have normal value of BMD. Also, the study found no significant changes in CatpK between all groups.

Holzer *et al*.,(2005) discovered statistically significant variations in CatpK levels between 101 osteoporosis patients and a healthy group, but no information was provided about the distribution of data. CatpK is expressed in high concentrations in osteoclasts, CatpK is generated in numerous tissues, and CatpK has key involvement in bone resorption. However, CatpK's functions are not exclusively restricted to bone resorption. Also discovered were CatK-positive epithelial cells of the bronchi and thyroid and synovial fibroblasts from individuals with

rheumatoid and breast cancer cells. CatK has also been discovered in cells from the ovary, chordoma, and ovarian cancers (Troen,2004).

Furthermore, macrophages and fibroblasts are shown to express the protein. Skoumal *et al*.,2005 shown that blood CatK levels are high in rheumatoid cases and that these elevated levels are associated with radiological damage in individuals with long-standing illnesses. As a result, CatK serum levels are also influenced by a variety of other disorders.

5.2.5. Vitamin D:

The Vit D level in the healthy group was (20.57±1.68) ng/mL, whereas its concentration in the osteoporosis decreased to reach (16.86±1.01) ng/mL table (4-1). This study's results agreed with a study that reported the vit D was less in the osteoporosis group than the control 53.8% vs 57.7%, without a marked difference between them. Vit D concentration in the osteoporosis group was lower (13.76 ± 7.15 ng/mL) than in the healthy (20.12 ± 7.59 ng/mL), the vit. D was nonspecific for reduction of bone mass in osteoporosis patients, and there is not a risk cause for the occurrence of osteoporosis and BMI reduction (Duan *et al*.,2020).

Another study found that 19% of people showed clinical signs of vit. D deficiency, while 38.8% of them has low concentrations of vit. D, however, 42.3% of them showed a normal level of the vit D. in the osteoporosis cases, the concentration of the vit D was (5.50 ± 5.5 ng/ml). The osteoporosis patients showed that vit D was lower than the healthy individuals (Shahnazari *et al*.,2019).

Vit D insufficiency is not evident in cases with chronic pain, but it is a regular occurrence in the general population. According to estimation, Vitamin D insufficiency is projected to affect more than half of the world's population (Wimalawansa *et al*., 2018). in China, The Vit D insufficiency prevalence in women in urban at 55.9% (Yan *et al*., 2017). Vit. D controls the balance of (P) and (Ca) in the body and the formation of skeletal bones.

Osteoporosis is a disease that is exacerbated by a lack of vitamin D. (Yuan *et al*., 2016). Vit. D insufficiency is correlated with migraine, muscle pain, osteoarthritis, aches, and cervical pain. Several chronic disorders, such as osteoporosis, high blood pressure, and cardiovascular disease, are associated with it (Eloqayli *et al.*,2018; and Song *et al*.,2018).

5.2.6. Calcium (Ca) :

The Calcium (Ca) concentration in the present study has appeared significant differences between control and case with Osteoporosis; the results showed that the concentration of Ca in control was (8.91±0.12) mg/dL. In contrast, its concentration in the case was decreased to reach (8.34±0.10) mg/dL table (4-2). The results of this study were agreed with another study that reports the Ca deficiency results in bone mass reduction, and there is a significant difference ($P<0.05$) between Ca level in the study group and control group (Duan *et al*.,2020).

In another study, patients with high calcium levels were more likely than controls to develop Osteoporosis ten years later (45 % versus 29 %). A higher proportion of patients with high calcium levels have a role in the osteoporosis occurance than patients with normal Ca levels (Dalemo *et al*.,2018). Other research (Hagstrom *et al.*, 2006) discovered a

relationship between calcium and bone mineral density, whereas others did not (Sutlovic *et al.*,2018).

In recent years, the possibilities provided by multichannel biochemical analysis have led to a rise in the use of calcium (Nordenstrom *et al.*, 2011). pHPT, among other things, produces secondary Osteoporosis, which manifests itself as low BMD in patients who showed moderate or asymptomatic pHPT (Sankaran *et al.*, 2010; and Bilezikian *et al.*,2014). Not all pHPT cases are treated surgically, but osteoporosis increases parathyroidectomy (Udelsman *et al.*, 2014).

As a result, BMD measurement includes evaluating patients suspected of pHPT (Bilezikian *et al.*,2014). The high (Ca) level is related to an increased occurrence probability of Osteoporosis. According to many studies, the screening investigation showed that blood calcium levels were adversely associated with bone mineral density (BMD). There is no connection between (PTH) and (BMD) at any of the study sites (Hagstrom *et al*., 2006). A number of other research, on the other hand, showed that there is no correlation between BMD and (Ca) (Sutlovic *et al.*, 2016).

Hypocalcemia is considered one of the most common adverse effects in denosumab treatment for Osteoporosis (Okada *et al*.,2013and Diab and Watts ,2014). The action mechanism of bone turnover due to low calcium levels occurs (with or without vit. D) is unknown. The denosumab could be resulting in hypocalcemia (Okada *et al*., 2013). Vit D and (Ca) should be taken together during treatment by the denosumab (Body *et al.*, 2015; and Sugimoto *et al*., 2015).

Our data do not reveal the marked difference in the calcium level between the osteoporosis males and the f osteoporosis with the healthy

group. Our results have come like Najlaa (2018) results, which doesn't found great difference between the osteoporotic group and control group in Ca concentration and that agrees.

5.2.7.phosphorus (PO_4):

Phosphate is a dietary precursor of acid phosphate provided by the grains and the meat. Therefore, it was added with the nutrient support factor (Oenning *et al.*,1988). The phosphate is a basic component of hydroxyapatite, which forms a basic bone structure element. The phosphate is metabolic production to the bone (Sebastian *et al.*,2002). Therefore, there is an effect relationship between phosphate concentration and bone health.

The phosphorus (PO_4) concentration in the present study has appeared significant differences between control and case with Osteoporosis; the results showed that the concentration of PO_4 in control were (3.99±0.08) mg/dL while its concentration in the case was decreased to reach (4.73±0.12) mg/dL table (4-2) The result of this study agreeing with Najlaa,(2018**)** that showed phosphorus demonstrated the marked difference between Osteoporosis and healthy.

This study showed no significant difference in PO_4 concentration in male and female osteoporosis patients compared with the healthy group agreeing with Selvapandian *et al*,(2018). Level Ca, and PO_4 in the serum were controlled by BMD phosphorus (Jayaram *et al*.,2000). Moreover, agree with Muhammad *et al*. (2018), who finds a strong difference in phosphorus level between osteoporotic males and females with a healthy group.

Dietary variables, 25(OH) vitamin D levels and kidney function were not strongly associated with serum phosphorus levels, and most of

the variance in serum phosphorus remained unexplained. The other study (Agrawal and Sharma,2013) showed that the calcium concentration phosphorus concentration was related to mean BMD. According to nutritional theory, these results are related to the nutrition and vit D concentration because the vit D deficiency contributes to Ca and P malabsorption (Gupta,1996). Physical activity and diet are important in bone health wherever exercise is critical for potential bone mass.

Consuming a large quantity of P and Ca is crucial for maintaining bone health. The proper functioning of hormones is the last critical component of bone health since they influence the supply of phosphate and calcium and the development and interruption of bone production. The parathyroid hormones hormone, oestrogen, calcitonin, growth hormone, thyroid hormone, and cortisol all have a role in the health of the bones (Anonymous, 2004). Even though phosphorus is required for bone health, a typical intake is sufficient; however, excessive supplementation has negatively impacted health. (Bonjour and colleagues, 2009)

5.2.8.Alkaline phosphatase (ALP):

Alkaline phosphates used as a biomarker for bone metabolism for several years. The alkaline phosphatase has several isoforms that generate organs such as hepatic tissues. Nearly half of ALP is produced by the hepatic tissues, while the other half is produced from the bone (Sonia *et al*.,2014).

Our result revealed a static difference in ALP concentration in osteoporosis patients compared with the control table (4-2), and the result of this study showed a significant increase in ALP concentration in male and female osteoporosis patients compared with the healthy group table (4-3) agreeing with(Ramesh *et al*.,2013; Saqib *et al*.,2018). According to

Muhammad *et al.,* (2018), ALP can be excreted from osteoblasts, which are high in ALP. It can also be found in the plasma membrane of cells in the placenta, intestine, and liver. All of which can contribute alkaline phosphatase amount. This is consistent with Muhammad *et al.* (2018) results, who discovered a statistical difference in ALP levels in osteoporotic men and women when compared.

Other studies showed a static difference in Alkaline phosphatase level between osteoporotic and the control. However, its value indicates no significant relationship between alkaline phosphatase and Osteoporosis (Najlaa ,2018). This study showed that alkaline phosphates (Ca) and (P) had not been affected by BMD. Our data showed a significant increase in ALP concentration in postmenopausal osteoporosis patients compared with the healthy group table (4-10), agreeing with Tariq *et al.*,(2019).

According to the findings, the women with osteopenic after menopause showed that alkaline phosphatase concentration was the good predictor of T-score when the alkaline phosphatase was high, T-scores was low. This indicated an imbalance between osteoclastic and osteoblastic, with increased osteoclastic in women with osteopenic increased alkaline phosphatase level. Biver *et al.* (2012) discovered that osteoporotic patients had elevated alkaline phosphatase concentrations compared to healthy controls. These elevated levels may be connected with a high occurrence of vertebral fractures.

Stepwise regression analysis was used in another investigation, and the results revealed a negative relationship between the alkaline phosphatase concentration and the bone density (Zhou *et al.*,2011). The recent research showed that ALP has an indicator for BMD in individuals

with end-stage renal illness (Bergman *et al.*,2017). As a result, measuring alkaline phosphatase and other bone turnover indicators in postmenopausal females was used for identifying the extent of bone mineral density loss (Chen *et al.*,2014).

Researchers discovered in another fascinating study that salivary alkaline phosphatase and calcium levels are similarly elevated in osteopenic and osteoporotic individuals. These levels may be utilised to diagnose the underlying bone problems in these patients (Saha *et al.*,2017). It is crucial to remember that bone density is influenced by various variables (Baig *et al.*,2015; Tariq and Lone,2017). These actions have an impact on bone via a variety of intricate routes. The presence of alkaline phosphatase in the bloodstream might be utilised to detect a reduction in bone mineral density. More longitudinal studies are advised to be conducted to determine the impact of these factors in after menopause females and in men.

Another study showed that it contributes to increased serum ALP activity; its efficacy is an indicator and not a catalyst in detecting Osteoporosis(Chinoy *et al*.,2011). Bone cells are rich in ALP as well as linked to the plasma membrane of the liver, intestines and placenta. This makes it a participant in increasing its overall blood concentration(Jayaram *et al.*,2002). ALP efficacy in serum samples of both males and females has been adopted as a tool for detecting Osteoporosis as a good indicator in cases of bone diseases such as osteoporosis and liver disease and biliary obstruction (Cavalier *et al.*,2016). A positive correlation between the effectiveness of bone-specific ALP and the speed of bone degradation was observed in a study in Hawaii, which called for a valuable marker in assessing the risk of fracture(Mahjoub and Masrour,2012).

According to all results of this study, the causing of the disease in males may be due to many reasons such as smoking ,chronic disease, environmental factors, soft drink, sun exposure, and genetic factors. Jutberger *et al.* (2014) found that smoking cigarettes are related to the poor (BMD) in males and females, as well as an increased probability of fracture (Ward and Klesges, 2001).

The processes behind the deleterious effects of smoking on bone health are not entirely understood. However, they are thought to be connected to the effect of smoking on sex hormones in both males and females, at least in part (Tanko and Christiansen,2004). It has also been proposed that smoking directly impacts skeletal remodelling and bone cells (Walker *et al.*, 2001). In an animal model, decreased bone formation was detected when exposed to nicotine. Furthermore, smoking has been shown to reduce Ca absorption and parathyroid hormone level (Krall and Dawson-Hughes, 1999, Riebel *et al.*, 1995;).

The current study showed that younger females also suffer from osteoporosis disease due to the number of pregnancies and lactation that cause take the minerals to the fetus. It has been shown that women who have had a large number of children are more likely than other women who have had fewer children to develop Osteoporosis. Because the accumulation of Ca in the neonate during pregnancy is 30 grams, with 95 % of that calcium located in the skeleton, BMD is adversely affected due to calcium loss. Furthermore, if the mother's skeleton were to serve as the sole source of calcium, it would lose approximately 3 % (30g/1000g) of its mineral per pregnancy. This impact might be particularly significant in the case of numerous pregnancies and prolonged nursing (Gur and colleagues, 2003)

Because of the high birth rate and short interval between births in our community (where the current experiment took place), a rise in the number of births harmed the BMD.

Other professionals have confirmed the validity of this theory (El Maghraoui *et al.,* 2013). It has been shown that there is a strong and statistically significant link between Osteoporosis and age, female gender, extended duration after menopause, particularly more than 10 years, and high parity number (Al mukhtar *et al*., 2014). Ca lose from maternal bone and a reduction in bone mineral density (BMD) have been seen, particularly during pregnancy and breastfeeding. However, it has been claimed that this loss will be recovered between (6–12) months. In women beyond menopause, having many births during their reproductive years and having more than one kid in adolescence has been shown to have a negative influence on after menopause bone mineral density (Kaya and colleagues, 2019).

5.3.Genetic study

5.3.1.The polymorphisms of ACP-5 gene with Osteoporosis:

According to Vijayakumar and Bu sselberg (2016), gene titraits are risk factors for Osteoporosis, with heritability accounting for around 75% of the disease (Mendoza *et al* .,2012). Furthermore, bone mineral density, an important indicator for osteoporotic fracture and osteoporosis (Chen and Xia, 2014). It is clear that hereditary factors account for around 50 % to 82 % of variability in BMD (Liu *et al*.,2012). According to current research, these genetic differences are connected with menopausal condition (Hunter *et al.*,2001). In premenopausal and postmenopausal women, the overall genetic percentage of spine BMD variation was 88% and 77%, respectively, according to the study (Hunter *et al*.,2001).

The present study discussed that presence association of ACP-5rs2071484 with or without the osteoporosis cases. There are reports showed that role of polymorphism and mutations in ACP5 or TRAP-5 gene in the diseases development. Briggs *et al.* (2011) demonstrated that compound heterozygosity for ACP5 gene mutations was found in 10 individuals with spondyloenchondrodysplasia with immune dysregulation from eight families. Testing in animals verified the absence of produced protein. According to the findings, all eight instances studied had a high interferon alpha effect, with gene expression a type I interferon.

The mice with disrupted Acp5 gene demonstrated deformities of the limb and skeleton are leading to mild osteopetrosis. Studying of the Rs2071484 polymorphism are first time in Iraq and relationship it with the osteoporosis condition, and reveals a positive relation between them (Hayman *et al.*,1996).

It was discovered that there was no allelic connection between (rs2071484) and osteoporosis in the research that resulted from this discovery. The genotype connection, revealed that CC genotype had a statistically significant link with illness (p value 0.0026). Comparing the CC genotype to TT and TC genotypes, the odds ratio for the CC genotype is 4.47. According to this, C allele is considered as a harmful allele that is recessive in nature. The CC genotype, in which we discovered allelic C to be a recessive harmful allele, is associated with a 4.5-fold increased risk of osteoporosis development compared to the TT and TC genotypes, respectively. According to this research, there is a statistically significant relationship between osteoporosis and genetic determinant. This finding is consistent with the findings of Mehrunnisa *et al.* (2017), who discovered a very significant association between the gene ACP5 and postmenopausal osteoporosis postmenopausal females in India.

The present study demonstrated strong correlation between osteoporosis and ACP5 gene. ACP5 gene is related to osteoclast-specific, the nutrition, lifestyle, and activity have roles in the osteoblasts and osteoclast differentiation. This suggests that bone resorption is controlled by a variety of variables both locally and systemically, including hormones and interleukins. A complex illness, Osteoporosis is characterised by a combination of genetic and epigenetic variables and a person's lifestyle. Osteoporosis in women is caused by their menopausal state, which is the most sensitive element. Deficiency in oestrogen and a rise in the FSH level causes bone loss, which results in Osteoporosis.

There are few studies about the ACP-5 gene, and this study considered the first study in Iraq about this gene and may be considered the first study in the world about the gene in the human and effect it in osteoporosis disease.

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Appendices

Appendix (A)

- **Determination of Human Gelsolin ELISA Kit:**

Assay Principle:

This kit is used for detection level of the gelsolin in human serum by depending on principle immunological reaction, wherever, the plate was coated with GS antibody. The gelsolin in the sample will react with gelsolin antibodies inside the wells. After that, adding of the biotinylated gelsolin Antibody for attached with sample gelsilin. After that, adding of the Streptavidin-HRP for binding with Biotinylated gelsolin antibody then incubated then removing unbound Biotinylated by washing. The substrate is added which leading to color develops when the human GS is present, as well as for stopping the reaction, adding several drops of stop solution (acid solution) then absorbed at 450 nm for measurement.

Assay Procedure:

1- the reactions are done at room temperature.

2- the strip are put in the frames.

3- the standard was added 50µl to the wells.

4- Addinf of the sample (40µl) to the wells and add anti-GS antibody (10µ), streptavidin-HRP (50µl) without adding to the standard wells.

5- Mix well, then incubated at 37C° for one hour.

6- wash the wells five times by buffer.

7- Adding of the substrate (50µl) to the wells with the substrate B 50µl to each well then incubate at 37C° for 10 minutes.

8- adding of the stop solution (50µl) to the wells, the color will changed from blue to yellow.

9- the optical density are determine for all the wells by microplate reader (450) nm during ten minutes.

Calculation of Result:

The standard curve are measured by using plotting, the OD value of the standard are represented on (Y) while the level on (X) for draw the curve depending on computer software (regression analysis).

Appendix (B)

- **Determination of Human CX3C-chemokine/ Fractalkine ELISA Kit**

Assay Principle:

This kit is used for detection level of the FKN/CX3CL1 in human serum by depending on principle immunological reaction, wherever, the plate was coated with FKN/CX3CL1 antibody. The FKN/CX3CL1 in the sample will react with gelsolin antibodies inside the wells. After that, adding of the biotinylated FKN/CX3CL1 Antibody for attached with sample gelsilin. After that, adding of the Streptavidin-HRP for binding with Biotinylated FKN/CX3CL1 antibody then incubated then removing unbound Biotinylated by washing. The substrate is added which leading to color develops when the human FKN/CX3CL1 is present, as well as for stopping the reaction, adding several drops of stop solution (acid solution) then absorbed at 450 nm for measurement.

Assay Procedure:

1- the reactions are done at room temperature.

2- the strip are put in the frames.

3- the standard was added 50µl to the wells.

4- Addinf of the sample (40µl) to the wells and add anti-GS antibody (10µ), streptavidin-HRP (50µl) without adding to the standard wells.

5- Mix well, then incubated at 37C° for one hour.

6- wash the wells five times by buffer.

7- Adding of the substrate (50µl) to the wells with the substrate B 50µl to each well then incubate at 37C° for 10 minutes.

8- adding of the stop solution (50µl) to the wells, the color will changed from blue to yellow.

9- the optical density are determine for all the wells by microplate reader (450) nm during ten minutes.

Calculation of Result:

The standard curve are measured by using plotting, the OD value of the standard are represented on (Y) while the level on (X) for draw the curve depending on computer software (regression analysis).

Appendix (C)

- **Determination of Human Cathepsin K ELISA Kit**

Assay Principle:

This kit is used for detection level of the cath-K in human serum by depending on principle immunological reaction, wherever, the plate was coated with cath-K antibody. The cath-K in the sample will react with gelsolin antibodies inside the wells. After that, adding of the biotinylated cath-K Antibody for attached with sample gelsilin. After that, adding of the Streptavidin-HRP for binding with Biotinylated cath-K antibody then incubated then removing unbound Biotinylated by washing. The substrate is added which leading to color develops when the human cath-K is present, as well as for stopping the reaction, adding several drops of stop solution (acid solution) then absorbed at 450 nm for measurement.

Assay Procedure:

1- the reactions are done at room temperature.

2- the strip are put in the frames.

3- the standard was added 50µl to the wells.

4- Adding of the sample (40µl) to the wells and add anti-GS antibody (10µ), streptavidin-HRP (50µl) without adding to the standard wells.

5- Mix well, then incubated at 37C° for one hour.

6- wash the wells five times by buffer.

7- Adding of the substrate (50µl) to the wells with the substrate B 50µl to each well then incubate at 37C° for 10 minutes.

8- adding of the stop solution (50µl) to the wells, the color will changed from blue to yellow.

9- the optical density are determine for all the wells by microplate reader (450) nm during ten minutes.

Calculation of Result:

The standard curve are measured by using plotting, the OD value of the standard are represented on (Y) while the level on (X) for draw the curve depending on computer software (regression analysis).

Appendix (D)

- **Determination of Human Bone Sialoprotein ELISA Kit**

Assay Principle:

This kit is used for detection level of the BSP in human serum by depending on principle immunological reaction, wherever, the plate was coated with BSP antibody. The BSP in the sample will react with BSP antibodies inside the wells. After that, adding of the biotinylated BSP

Antibody for attached with sample BSP. After that, adding of the Streptavidin-HRP for binding with Biotinylated BSP antibody then incubated then removing unbound Biotinylated by washing. The substrate is added which leading to color develops when the human BSP is present, as well as for stopping the reaction, adding several drops of stop solution (acid solution) then absorbed at 450 nm for measurement.

Assay Procedure:

1- the reactions are done at room temperature.
2- the strips are put in the frames.
3- the standard was added 50µl to the wells.
4- Adding of the sample (40µl) to the wells and add anti-GS antibody (10µ), streptavidin-HRP (50µl) without adding to the standard wells.
5- Mix well, then incubated at 37C° for one hour.
6- wash the wells five times by buffer.
7- Adding of the substrate (50µl) to the wells with the substrate B 50µl to each well then incubate at 37C° for 10 minutes.
8- adding of the stop solution (50µl) to the wells, the color will changed from blue to yellow.
9- the optical density are determine for all the wells by microplate reader (450) nm during ten minutes.

Calculation of Result:

The standard curve are measured by using plotting, the OD value of the standard are represented on (Y) while the level on (X) for draw the curve depending on computer software (regression analysis).

Appendix (E)

- **Determination of VIT D DIRECT ELISA:**

Principle:

The basic reagents needs solid phase of the immobilized antibody, native antigen, and Enzyme-antigen conjugate. After mixing, blood sample with native antigen and immobilized antibody, the reaction between them is demonstrated by the following.

Test Procedure:

1- preparation of all substances at (20–27^{o}C).

2- adding 25-OH Vitamin D calibrator (0.025) ml, control or specimen in the well.

3-Adding of the 25-OH Vitamin D Releasing Agent (0.100) ml to all the wells, and mixing for half minute.

4- incubated for half hour.

6- removing the microplate contents then dried.

7-Adding of the wash buffer (0.350) ml, washing three times by the manual plate washer.

8-Adding of the 25-OH Vitamin D Enzyme Reagent (0.100) ml to all the wells.

9- incubated for half hour.

10- removing the microplate contents by aspiration.

11-Adding of the wash buffer (0.350) ml for washing three times.

12-Adding of the substrate reagent (0.100) ml to all the wells. Using of the reagents for decrease the reaction time and incubate at (25) C for twenty minutes.

13-Adding of the stop solution (0.050) ml to each well and mix.

15- the absorbance of the wells are read at (450) nm at (620-630) nm.

Calculation of Result:

The standard curve draw by specific computer software, after determine concentration of 25-OH Vitamin D, the absorbance percentage for determine the level of 25-OH Vitamin D, locate the average absorbance for each unknown on the vertical axis, and read the concentration of the graph. the average absorbance as (1.033) intersects the dose response curve at 39.9 ng/ml 25-OH Vitamin D.

Appendix (F)

- **Determination of Calcium by CPC method**

Principle:

O-Cresol Phatalein Complexone is techniques used for detemination Ca level in samples. The alkaline solution reacted with Ca to produce dark complex at (absorbance at 570 nm).

Manual Procedure:

Pipette into well identified test tubes :

	Blank	Specimen blank	Standard	Assay
Reagent	1 mL		1 mL	1 mL
Saline solution		1 mL		
Demineralised water	20 µL			
Standard			20 µL	
Specimen		20 µL		20 µL
Mix well. Incubate for 2 minutes at room temperature. Use a 1 cm path length cuvette and read Standard and assays absorbance at 340 mn (334-336) against Reagent blank. Read Specimen blank against saline solution.				

Calculation: measured by:

$$\text{Result} = \frac{\text{Abs (Assay)} - \text{Abs (Specimen blank)}}{\text{Abs (Standard)}} \times \text{Standard concentration}$$

Appendix (G)

- **Determination of Inorganic Phosphorus:**

Principle:

Phosphate ions with the ammonium molybdate, at absorbance at (340) nm for determine the phosphate concentration in the sample, without removing the protein .

Manual Procedure :

Let stand reagents and specimens at room temperature.

Prepare tubes as follows :	Reagent blank	Specimen blank	Standard	Assay
Reagent R1	2mL	2 mL	2 mL	2 mL
Incubate 5 minutes at 37°C.				
Specimen				50 µL
Reagent R2 (Standard)			50 µL	
Let stand exactly 15 minutes at 37°C.				
Reagent R3	0.5 mL	0.5 mL	0.5 mL	0.5 mL
Mix well.				
Reagent R4	0.5 mL	0.5 mL	0.5 mL	0.5 mL
Specimen		50 µL		
Demineralized water	50 µL			
Mix. Incubate 10 minutes at room temperature and away from light. Read absorbances of the blank specimen, standard and assay at 510 nm against reagent blank. Coloration is stable for 45 minutes away from light.				

Calculation:

$$\text{(Kind and King units/100 mL)} = \frac{\text{Abs Assay} - \text{Abs Specimen blank}}{\text{Abs Standard}} \times 20$$

Appendix (H)

- **Determination of Alkaline Phophatase:**

Principle :

determination of the ALP is done by follows :

$$\text{Phanylphosphate} \xrightarrow{\text{Alkaline phosphatase}} \text{Phenol} + \text{Phoshate}$$

The phenol are produced due to hydrolysis of the substrate at absorbance (510) nm in the sample. Na-arsenate prevents the dilution of the colour during the methods application.

Procedure:

Temperature should be held constant as the absorbance of the dye is temperature sensitive, and done at Temperature: 37^{o}C, and wavelength: 570 nm.

	Automated analyzer	Manual procedure
Reagents	**120 µL R1 120 µL R2**	**WR : 1000 µL**
Standard, Controls, Specimen	**6 µL**	**25 µL**
Mix well. Incubate for 5 minutes at room temperature. Read absorbance at 570 nm (550-590) against reagent blank. The coloration is stable for 1 hour away from light.		

Calculation: Calculate the result as follows

$$\textbf{Result} = \frac{\textbf{Abs (Assay)}}{\textbf{Abs (Standard)}} \times \textbf{Standard concentration}$$