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Role of Osteopontin Gene Polymorphism and Certain Types of Interleukins in Pathophysiology of Osteoporosis in Iraqi postmenopausal Women

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بسم الله الرحمن الرحيم

{ٱقۡرَأۡ وَرَبُّكَ ٱلۡأَكۡرَمُ ﴿٣﴾ ٱلَّذِي عَلَّمَ بِٱلۡقَلَمِ ﴿٤﴾ عَلَّمَ ٱلۡإِنسَٰنَ مَا لَمۡ يَعۡلَمۡ ﴿٥﴾ }

صدق الله العظيم

سورة العلق, الاية 3-5

Dedication..

The laudation to the God's, his prophet Mohammed and his household prop

{Peace be upon him and his relatives and companions}

To those whom bolster, hearten and also rely in my destiny.

Whom make me surmount and moving on toward my uttermost.

To the soul of my late father, the pilgrim Salim Sultan Muzan Al-Lami

My staunched mother.

My grand instructor Prof.Dr. Jabbar Hameed Yenzeel

My sisters

To friends and the medical teams in Iraqi hospitals and laboratories, that I proposed the serving and guidance to me..

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Abstract

Osteoporosis (OP) is asymptomatic disease until the experience of falls or impacts that cause broken bones or fractures happened. Estrogen deficiency, abnormality in bone matrix components and interleukins expression can impact on bone homeostasis which gradually paved to osteoclastogenesis over activation leading to bone loss.

A current case-control study was designed to explain the alteration in certain biochemicals, bone matrix components, immunological and molecular parameters that which accompanied with OP in the postmenopausal women. The period of study's investigation was from December 2022 to July 2023. All participants provided written informed consent, and the study was approved by Department of Biology, College of Science, University of Baghdad Ethical Council (Reference code CSEC/0922/0092, on 26-9-2022). One hundred and ten postmenopausal women were randomly chosen to participate in this study based on specific criteria from Al-Wasity Hospital, Medical City and Al- Imamein Kadhimein Medical City Hospitles, Baghdad, Iraq.

The age of the participants was ranged from 45 to 70 years. Firstly, Dual Energy X-ray Absorptiometry (DEXA) scan was performed to all participants in order to assess the precence or abscense of OP disease. Out of 110 samples, 70 samples were confirmed with OP. Meanwhile, 40 samples were control. Venous blood samples were collected from all participants to estimated compelet blood count (CBC), immunological parameters as C-Reactive Protein (CRP), Rheumatoid Facter (RF), Anticyclical Citrullinated Peptide Antibody (ACPA), Interleukin-8 (IL-8), Interleukin (IL-17), and Interleukins-22 (IL-22) using enzyme link immunosorbent assay (ELISA) technique, Parathyroid Hormone (PTH), and Calicum (Ca^{+2}), bone matrix component parameters osteopontin (OPN), osteocalicn (OCN), and osteonectin (ON), in addition to,

Osteopontin gene SNP *rs11730582* polymorphism was determined by the Hight Resolution Melting (HRM) analysis.

Highly significant (P≤0.01) decrease in WBC count (6.30±0.18 k/mcL) and HGB (9.900±0.132 g/dL) were observed in OP patients as when compared with the control group, WBC was (8.26±0.27k/mcL) and HGB was (13.321±0.287 g/dL), Conversely, highly significant (P≤0.01) increase was recorded in ESR (43.73±1.76 mm/hr), PLT (437.88±7.00 $10^8$3/uL) in OP patients in compare with control, (21.10±1.25mm/hr), and (226.79±9.39 $10^8$3/uL), respectively.

However, highly significant (P≤0.01) increase was noticed in immunological parameters: CRP was (18.26±1.33 pg/ml), RF (IU/ml) results were positive, and ACPA was (23.26±2.74EU/ml) in OP patients when compared with control, CRP (2.92±0.18 pg/ml), RF (IU/ml) results were negative, and ACPA was (6.26±0.56 EU/ml). Beside to, highly significant (P≤0.01) increase was recorded in IL-8 serum level (249.08 ±19.98ng/ml), IL-17 (107.862±9.25ng/ml), and IL-22 (57.73±2.47 ng/ml) in OP patients as compared with the healthy control group were IL-8 (65.29 ± 5.62 ng/ml), IL-17 (46.135 ± 8.66 ng/ml) and IL-22 (13.57 ± 3.16 ng/ml).

The mean PTH serum level of OP patients group was (77.80±1.79pg/mL) and in control group was (35.81±2.06pg/mL), whereas a highly significant (P≤0.01) decrease in mean of Ca^{+2} serum level was recorded in OP patients (4.89±0.21 mmol/L) in compared with control group (9.63±0.13 mmol/L).

Additionally, high significant (P≤0.01) increase was detected in OPN serum level (16.88±0.77 ng/ml) and OCN (29.86±2.25ng/ml) in OP patients as compared with the healthy control group (5.95±0.41ng/ml and 10.73±0.86ng/ml), respectively. In contrast, ON serum level recorded a

highly significant (P≤0.01) decrease in the patient group (1.78±0.971ng/ml) compared with the control group (3.42±0.20ng/ml).

The frequency of homozygote TT genotype in patient's analysis was, (14.3%), (10/70) than control group (27.5%), (11/40). While, the frequency heterozygote TC and homozygote mutant CC genotypes were [TC= (71.42%) 50/70 and CC= 14.3% (10/70)] and these genotypes frequency were not significantly differet from those of the control group, (TC= 60%, (24/40), and (CC= 12.5% 5/40).

P-value was different (0.0158) significantly in genotype distribution of *rs11730582* polymorphism in OP patients whereas, the P-value of *rs11730582* was (0.1500) non-significant in control group.

In conclusion, this study found that OPN, OCN, and ON protein act as a sensitive monitoring indicator can be used in early detection of osteoporosis. And because of the capacity to activate osteoclastogenesis and promote bone resorption, IL-8, IL-17, IL-22 and *rs11730582* of *OPN* gene polymorphism may be serve as a biomarkers, pro-inflammatory and immune-stimulating factors in OP development.

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List of Abbreviations

Abbreviation	Meaning
ACPA	Anti-citrullinated protein antibodies
AhR	Arylhydrocarbon Receptor
AOP	Alcoho induce Osteoporosis
BB2	Binding buffer2
BGLAP	*Bone Gamma Carboxyglutamicacid Protien*
BGP	Bone Gla Protein
BM40	Basement membrane protein 40
BMD	Bone mineral density
BMI	Body mass index
BSP-1	Bone sialoprotein-1
BSP-2	Bone sialoprotein-2
CA II	Carbone anhydrase II
CaSR	Calcium Sense Receptor
CCL2	Cranial Cruciate Ligment2
CCL5	Cranial Cruciate Ligment5
CD4+	Cluster of differentiation 4+
CD8+	Cluster of differentiation 8+
CKD	Chronic Kidney Disease
CLCF-1	Cardiotrophin Like Cytokine Factor-1
CNTF	Ciliary Neurotrophic Factor
COPD	Chronic obstructive pulmonary disease
CT-1	Cardiotrophin-1
CXCL1	CXC chemokine Ligand1
CXCL2	CXC chemokine Ligand2
CXCL8	CXC chemokine Ligand8
dbSNP	Single nucleotide polymorphism database
dbVar	Variation database
DCN	Decorin
DEXA	Dual Energy X-ray Absorptiometry
DM	Diabetes mellutis
DMP-1	Dentin matrix protein-1

DNA	Deoxyribonucleic acid
DSPP	Dentin sialophosphoprotien
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
Eta-1	Early T-lymphocyte activation-1
FGF2	Fibroblast Growth Factor 2
FIX	Factor IX
FSH	Follical stimulating factor
FVIII	Factor VIII
GCs	Glucocorticoids
ɣδ	Gamma delta
GIO	Glucocorticoid Induced Osteoporosis
Gla	Gamma-carboxyglutamic acid
GM-CSF	Granulocyte machrophage colony stimulating factor
GnRH	Gonadotropin releasing hormone
GWAS	Genome wide association studies
HGB	Hemoglobin
HRM	High Resolution Melting
HRP	Horseradish peroxidase
HSCs	Hematopoitic stem cells
HWE	Hardy Weinberg equilibrium
IBD	Inflammatory bowel disease
IFN-α	Interferon-alfa
IFN-β	Interferon-beta
IFN-ɣ	Interferon-gamma
IGF-1	Insulin like growth factor -1
IL17R	Interleukin17 receptor
ILC3	Innate lymphoid cells type 3
kDa	Kilodalton
LIF	Leukemia Inhibitory Factor
LH	Lutenizing hormone

MCP-1	Monocyte Chemoattractant Protien-1
M-CSF	Macrophage-colony stimulating factor
MEPE1	Matrix extracellular phosphoglycoprotein-1
MEPs	Megakaryocyte-erythrocyte progenitors
MMP9	Metalloprotienase9
MPPs	Multi potent progenitors
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MSCs	Mesenchymal stem cells
MTB	Methyl Thymol Blue
NADPH	Nicotinamide adenosine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NCPs	Non collagenous proteins
NFATC1	Nuclear factor of activated Tcells-1
NF-κB	Nuclear factor- kappa B
NMC	Normalized Melting Curve
OA	Osteoarithritis
OBs	Osteoblasts
OCs	Osteoclasts
OCN	Osteocalcin
OD	Optical Density
ON	Osteonectin
OP	Osteoporosis
OPG	Osteoprotegrin
OPN	Osteopontin
OSM	Oncostatin M
OYs	Osteocytes
PBS	Polybutylene succinate
PCR	Polymerase Chain Reaction
PDGF	Platelet Dervied Growth Factor
PH	Potential of Hydrogen
PGE2	Prostaglandin2
PLTs	Platelets

PMO	Postmenopausal
PMOP	Postmenopausal osteoporosis
POP	Primary Osteoporosis
PPGF	Plasma Poor Growth Factor
PTH	Parathyroid Hormone
PTH1R	Parathyroid Hormone 1 Receptor
PWH	Patient with hemophilia
RA	Rheumatoid Arthiritis
RANK	Receptor Activator of Nuclear Factor kappa beta NFkB
RANKL	Receptor Activator of Nuclear Factor kappa beta NFkB Ligand
RGD	Arginine- glycine aspiritate domain
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
Ser/Thr	Serine-Threonine
SIBLING	Small integrin binding ligand N-linked glycoprotein
SLE	Systemic Lupus Erthymatosis
SNPs	Single nucleotide polymorphisms
SNV	Single nucleotide varation
SPARC	Secreted protein acidic and rich in cystine
SPP-1	Secreted phosphoprotein-1
SE	Standard Error
SVVYGLR	Serine-valine-valine-tyrosine-glutamate-leucine-arginine
TBS	Tris-buffered saline
T_1DM	Type 1 diabetes mellutis
T_2DM	Type 2 diabetes mellutis
TE	Tris-EDTA
TF_H	T follical helper
TGF-β	Transforming growth factor-beta
TMB	3,3',5,5'-Tetramethylbenzidine substrate
TMJ-OA	Temporomandibular Joint Ostearthritis

TNF-α	Tumor necrosis factor-alfa
TNF-β	Tumor necrosis factor-beta
TRAP	Tartate Resistant Alkaline Phosphate
UCSC	University California of Sant Croz
VBMD	Volumetric Bone Mineral Density
VEGF	Vascular endothelial growth factor
Wnt	Wingless related integration site

Chapter One Introduction

1. Introduction

1.1 Overview

Bone is an active tissue where continually replacement required. Osteoporosis-related breaking maybe hit all skeletal structure or specific regions in the body as hip, wrist or spine.

Bone microarchitecture disturbance, bone tissue degeneration, and low bone mass are the hallmarks of osteoporosis, a disorder that reduces bone strength and increases the risk of fractures. There are two forms of OP: primary osteoporosis, which develops with aging, and secondary osteoporosis, which is brought by certain systemic disorders and clinical pathologies (Clynes *et al.,* 2020).

The fractures precence was less commonly in men than in women, and the latter have a higher mortality risk after a fracture (Vilaca *et al.,* 2022). Many studies have shown that, the bone loss starts in men and women in the ages 30-40 years old and the women's bone mass and density may decrease within a year following menopause. The accelerating rate of bone loss reaches the equilibrium approximately ten years after menopause, followed by a continuous decrease in muscle mass due to aging. Though, OP can affect the people from both sexes and from all races, but the white and Asian older postmenopausal women are more likelt to have OP (Kadam *et al.,* 2018).

1.2 Research Background

Osteoporosis is a complex condition and approximately, 50-85% postmenopausal women may be at risk for illness due to etiology and genetic factors (Mitek *et al.,* 2019).

Beside to the lifestyle, the bone health can be limited by several factors. The first one is rapidly growth of skeletal system postnatal and through puberty and the second one from mid-late 20s maximal bone mass is reached. Then, the bone mass will be gradually lost due to age

advanced in both men and women and the rate of loss varies according to different conditions (Das *et al.,* 2019).

Both the development and maintenance of bone are based on twice processes; modelling and remodeling. These two processes are tightly coupled during bone turnover and collected under the term bone remodeling process (Ramesh *et al.,* 2021).

Functionally, bone remodeling has a vital role in bone quality and mass preserving, hypermineralized buildup prevention in bone, and mineral homeostasis regulation. Osteoclasts (OCs), osteoblasts (OBs) and osteocytes (OYs) are involved in bone remodeling (Bhatnagar and Kekatpure, 2022).

In women and after menopause, the balance between bone resorption and production varies when estrogen levels progressively start to decline. The hormone estrogen is one of the main factors that have a direct effect on bone cells. By promoting osteoblast maturation and osteogenic differentiation of mesenchymal stem cells (MSCs), estrogen increases bone formation during the adolescent stage. Moreover, osteoblast development is inhibited by estrogen deficiency, which decreases bone growth. These osteo-anabolic and anti-osteoclastic effects are inhibited when estrogen levels are low, which leads to ongoing bone loss (Salimi *et al.,* 2024).

The most prevalent disorders that which affect women include sarcopenia, fractures, osteoporosis, and mobility impairment. These conditions are caused by hormonal changes as well as smoking, ovariectomy, having a lean body type, not exercising, and obtaining insuficant calicum. Additionally, concuming a lot of animal protein, phosphorus, sodium, caffeine, and alcohol can raise the risk of osteoporosis (Bijelic *et al.,* 2017; Sipilä *et al.,* 2020).

For osteoporosis identification, Dual-energy X-ray absorptiometry (DEXA) or DXA is analyzed technique which assess the mineral content and density of bone and it can be carried out with computed tomography (CT) software scans for hip and spine for bone mineral density calculation (Kanis *et al.,* 2019).

In *vivo*, organic and inorganic extracellular matrix (ECM) components, immune cells and cytokines have role in bone production and resorption (Tsukasaki and Takayanagi, 2019).

The B and T lymphocytes cells are essential elements of adaptive immunity which trigger in osteoporosis. During activation, they are exposed to varied environmental stimuli (cytokines, antigens, etc.) and they undergo differentiation into avariety of subpopulation (Gong and Wu, 2021).

The B cells regulate bone metabolism by osteoprotegren (OPG) secretion which binding with receptor activator of nuclear factor kappa beta ligand (RANKL or NFkB), despite of the fact that osteoblasts are the principal source of OPG. When comparing between osteoporotic women with healthy control in Breuil *et al.* (2010) study, they discovered significantly lower quantity and size of several subpopulations of memory B cells in women with OP.

The T cells are another main driver of bone remodeling which has a role in bone homeostasis maintain. T helper cell 17 (Th17) is a form of T cell differentiation that has a certain role in osteoclast formation. Th17 cell is a core links with estrogen deficiency-resulting in bone loss inducing and promotion of osteoclast differentiation and survival. Elevated serum interleukin-17 (IL-17) in women is strongly associated with OP (Bhadricha *et al.,* 2021).

The IL-17 (especially IL-17A) can target osteocytes in presence of tumor necrosis factor-alfa (TNFα) to produce (RANKL or NFkB) ligand

and thus attract to RANK and contribute in resorption increased (Li *et al.,* 2019).

It is noteworthy that the patients with chronic obstructive pulmonary disease typically display osteoporosis and a decrease in bone mineral density are having RANKL highly expression in the neutrophils. Additionaly, it has been shown that increased in IL-8 expression promotes the development of osteoclasts (Hu *et al.,* 2017).

An alternation in CRP, interleukins IL-1β, IL-6, IL-7, IL-8, IL-17, IL-15, IL-22, IL-23 and TNF-α levels have been reported to be higher in postmenopausal women osteoporotic (PMOP) patients (Salimi *et al.,* 2024).

1.3 Problem Statements

This study was designed to explain the complication and alteration in certain biochemical, bone matrix component, immunological and molecular biomarkers that which accompanied with OP in the postmenopausal women.

1.4 Research Questions

1- What are the changes in biochemical parameters that occur with OP?

2- How does OP disease effects on biochemicals, bone matrix component and immunological parameters in the blood serum during the period of disease? And can we use them as abiomarker for early OP diagnose? What is the possibility of using the studied parameters as biomarkers for early diagnosis of OP disease?

3- How can be the polymorphism of *osteopontin* gene evaluating and identifing the area that are attached, which may be association with OP development?

4- How are the changings in biochemical and immunological parameters correlated with clinical outcome in OP patients?

1.5 Research Objectives

The research aims to evaluate and explain the role of some types of biochemicals, bone matrix components and Interleukins in the development of OP in postmenopausal women. Additionally to investigation the association of single-nucleotide polymorphism (SNP) of the *Osteopontin* gene with osteoporosis susceptibility and explain its role in OP development.

1.6 Research Significance and Justification

Osteoporosis disease caused by genetic, physiological and environmental factors and can be either primary or secondary osteoporosis. In this regard, evaluating of biochemical, bone matrix component, immunological, and molecular markers can provide valuable insights into the pathophysiology of the disease, so this study was designed to explain this problem.

1.7 Research Scope

This study includes samples of Iraqi postmenopausal women with and without osteoporosis and lasted for eight months in Baghdad city to evaluation different parameters in hospitalization patients that related to OP disease.

1.8 Thesis Outline

I. Introduction

A. The background and significance of the study

B. The aim and objectives of the study.

II. Literature Review

A. An overview of bone contents, bone remodeling and OP disease.

B. The role of bone matrix biochemical components in OP disease.

C. The impact of interleukins in OP development.

D. The association of *Osteopontin* gene polymorphism with OP presence.

III. Methodology

A. Study design, participants selection, and samples collection.

B. Methods for evaluation the biochemical parameters and bone matrix biochemical parameters by Enzyme Linked Immunosorbent Assay (ELISA).

D. Methods for immunological parameters evaluation by ELISA.

E. Method for *Osteopontin* gene polymorphism investigation by using Hight Resolution Melting (HRM) PCR Technique

IV. Results and discussions:

A. Evaluation the results and analysis of biochemical parameters, and the bone matrix components.

B. Evaluation the results and analysis of immunological parameters.

C. Investigation the results and analysis of *Osteopontin* gene polymorphisim.

D. Interpretation the results and comparison them with existing literature.

V. Conclusion and recommendations:

A. Conclusion of the present study.

B. Recommendations for future research

VI. References

Chapter Two
Literature Review

2. Literature Review

2.1 Bone Structure

The second largest organ and just behind the skin is a bone. Bone is a rigid, effecient, and connective tissue that bossted with cells, fibers, and specialized molecules. The musculoskeletal system make up from bone, muscles, and joints work that cooperate to provide the bodily movement, and this is called the musculoskeletal system. The skeleton supports the shapes of body and preserves the internal organs such as the brain, heart and lungs in the vertebrates (Rowe *et al.,* 2022).

The bone structure is involved: diaphysis and epiphysis. The diaphysis is the bone shaft that which precence between a proximal and distal ends. Additionaly, this region is a hollow known with medullary cavity that which filled with the yellow marrow. The diaphysis composes of cortical bone that which is hard, compact, much denser outer layer than trabecular bone. At the each end of the bone structure, a wider region known as epiphysis fill with spongy bone (trabecular) and red marrow. Epiphysis and diaphysis meet at a narrow region that which contains growth plate known as epiphyseal plate, a hyaline cartilage layer, figure 2.1 (Jerban *et al.,* 2020).

Bone functions include; skeletally and mobility supporttig, organs preserving, blood cells reference, mineral storage and homeostasis. Because of these vital roles, bone can repair and regenerate in the patient functionality following bone injury or damage. In maturity and just around 18 to 21 years old, the bone formation stops, the cartilage tissue replaces with the osseous tissue, and the epiphyseal plate transforms into an epiphyseal line (Wu *et al.,* 2020).

The complex structure of bone comprised from organic, inorganic, interstitial fluid and cells. The organic part of bone is about 70% contained; collagen, non- collagen structures (I, III and V), glycoproteins and proteoglycans. Whereas, 30% is inorganic minerals such as: calcium, phosphor, sodium, fluoride, bicarbonates and magnesium hydoxyapatite (HA) crystals. In addition to osteoclasts, osteoblasts, osteocytes, and bone lining cells (Tobeiha *et al.,* 2020).

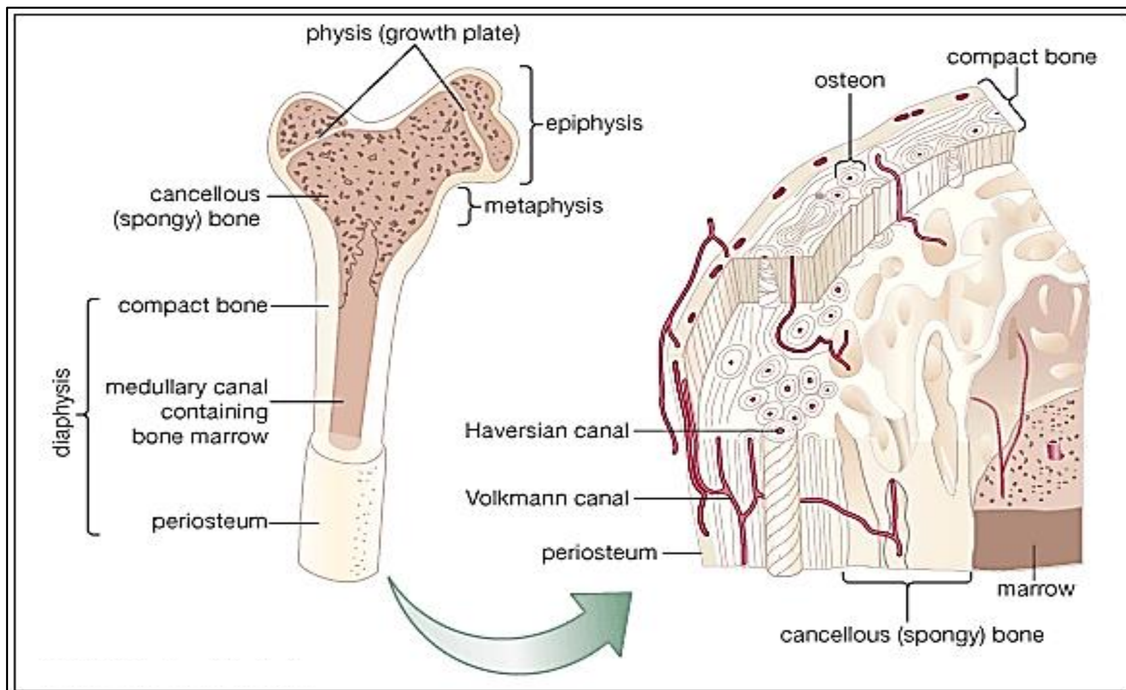


Figure (2.1): Bone tissue structure (Whedon and Heaney, 2024)

Fundamentally, bone isn't inert structure, it can be replaced over the course of the life span within process known as bone remodeling. The previous studies suggested there are different factors impact the bone strength such as hormones, physical activity, diet, weight, and lifestyle (Jerban *et al.,* 2020).

2.2 Bone Cells

2.2.1 Osteoclasts (OCs)

Osteoclasts are a giant multinucleated phagocytic bone resorbing cells, origin from fusion of mononuclear precursors belonging to the monocyte/macrophage lineage. OCs cytoplasm contains several lysosomes (vesicles and vacuoles filled with acid phosphatase and cathepsin K). Activated OCs characterized with reorganization cytoskeletal and presence of ruffled border (a specialized cell membrane) which opposes the surface of the bone tissue for acids and enzymes secretion (Yao *et al.,* 2022).

Both macrophage colony-stimulating factor (M-CSF) and RANKL are required in osteoclast's activation. Hematopoitic stem cells (HSCs) require M-CSF to differentiate into the monocyte/macrophage lineage, as well as to proliferate and

prolong the life of these progenitor cells. However, osteoclast differentiation, fusion, and maturation lifetime are all impacted by RANKL (Yao *et al.,* 2021).

2.2.2 Osteoblasts (OBs)

Mature osteoblasts, are a small mononucleated cells that are found along the bone surface. They responsible for secrtion bone matrix proteins and mineralization guidance in new bone generation. These cells comprise 4–6% of the total resident bone cells and originate from pluripotent MSCs. OBs are usually finding as cuboidal, rounded, flat and cylindrical forms, and undergo three developmental stages to become mature cells: 1) proliferation, 2) production and maturation of ECM, and 3) matrix mineralization (Oton-Gonzalez *et al.,* 2022).

2.2.3 Osteocytes (OYs)

Osteocytes are a third type of bone cells, which located within calcified bone matrix. OYs connect with the neighbor osteocytes, osteoclasts, osteoblasts, and other cells via dendritical projections that which anchor in the cell body of osteocytes to form an interconnected dendritic network. Through a process known as mechano-transduction, these dendrites projections let the osteocytes sense the changes in localized bone stresses brought on by loading or disuse and transmit this information to the osteocyte cell body (Qin *et al.,* 2020; Choi and Robling, 2021).

Osteocytes tend to express RANKL protein that which has affinity to binds with RANK receptors on surfaces of osteoclasts. RANKL is necessary for differentiation of osteoclasts from monocyte-macrophage lineage, osteoclastogenesis stimulation and bone resorption. However, osteoblast-lineage cells produce OPG to prevent RANK/ RANKL binding, OPG is an endogenous decoy receptor. Though, higher RANKL expression and/or lower OPG concentration stimulate both osteoclastogenesis and bone resorption (Yasuda, 2021).

2.3 Bone Extracellular Matrix.

Both quantity and quality are determining bone strength. Bone quantity estimated with the bone volume and bone mineral density (BMD), which is the amount of

minerals per volume unit. Whereas, bone architecture and geometry, bone turnover, cortical porosity, injury, mineralization, mineral characteristic, collagen and non-collagenous proteins are parameters determine the bone quality (Moriishi *et al.,* 2020).

According to Henry and Bordoni, (2023), found about 85–90% of collagen, proteoglycans, non-collagenous proteins (osteocalcin and osteonectin), and glycoproteins (osteopontin) are a part of organic portion of bone structure. In addition, 180–200 diverse molecules make up non-collagenous proteins (NCPs), which play a variety role in bone biology (Licini *et al.,* 2022).

Both collagenous and NCPs in bone extracellular matrix, as well as the coupling between osteoclasts and osteoblasts, work together to maintenance normal bone metabolic regulation and any anomalies in amount and structure of one or more these proteins can cause abnormalities in bone formation and resorption, then consequently leads to malformations and defects, such as osteoporosis (Licini *et al.,* 2019).

Various NCPs and type I collagen can interact and regulate by one another. For example; synthesis, assembly, and maturation of collagen type I are regulated by Decorin (DCN), ON, and Transforming Growth Factor-beta (TGF-β), which also limit degradation and maintain structure. Conversely, OCN, bone sialoprotein (BSP-2), ON, and OPN mediate bone mineralization, are regulated by type I collagen (Poundarik *et al.,* 2018).

Understanding ECM proteins expression and localization are essential to understanding bone biology in both healthy and diseased conditions. These molecules have a variety of effects on bone remodeling, including reciprocal regulation, mediation of cell-matrix interactions, and influence on cell behaviors (Licini *et al.,* 2022).

For instant, DCN, OCN, ON, BSP-2, and TGF-β are ECM proteins that which link to the structure and function of Type I collagen. DCN interacts in the formation,

maturation, and inhibition of type I collagen fibrils and the mineralization process (Bilezikian *et al.,* 2019).

2.3.1 Osteopontin (OPN)

Osteopontin is a phosphoglycoprotein that is soluble in human physiological fluids and immobilized in the bone matrix as a component of extracellular matrix. OPN is involved in several physiological processes, such as wound healing, angiogenesis, bone homeostasis, tissue refurbishing, cell adhesion, and immune response. OPN is typically known with a diverse terms, as secreted phosphoprotein-1 (*SPP1*), early T-lymphocyte activation-1 (Eta-1) and/or bone sialoprotein (BSP-1) (Martín-Márquez *et al.,* 2023).

Apolyaspartic acid sequence and serine/ threonine (Ser/Thr) phosphorylation sites that facilitate the hydroxyapatite binding and a highly conserved arginine- glycine aspiritate domain (RGD) motif that facilitate cell attachment and signaling are a characterisitic of osteopontin (Nazneen and Bai, 2020; Yim *et al.,* 2022).

Osteoblasts, osteoclasts, osteocytes, macrophage, neural, epithelial, fibroblasts, smooth muscle, T and B lymphocytes, natural killer (NKs), dendritic (DC), and endothelial cells are the cells that express OPN (Roomi *et al.,* 2022).

Particularly, in autoimmune diseases like Crohn's disease, cirrhosis, obesity, atherosclerosis, cancer, systemic lupus erythematosus (SLE), multiple sclerosis (MS), rheumatoid arthritis (RA), and osteoarthritis (OA), and inflammatory condition especially in bone, joints, lung, liver, brain, adipose tissues, blood, urine, and milk, osteopontin presents in highly concentrations (Martín-Márquez *et al.,* 2022).

Additionally, osteopontin affects many elements of carcinogenesis and metastasis; cell survival, proliferation, adhesion, migration, and invasion, in addition to, participating in a number of normal physiological processes; vascularization, immunological responses, inflammation, tissue remodeling, and cell adhesion (Jambor *et al.,* 2022).

The occurrence and progression of numerous bone-related conditions, including OP, RA, and osteosarcoma, have been shown to be closely associated with osteopetin. During bone damage, integrin receptors (αvβ3) are highly expressed on the surface of OCs. OPN mediates OCs bone resorption by binding to αvβ3, a downstream signaling molecule for RANK/RANKL activation. OPN deficiency reduces the bone resorption capacity brought on by parathyroid hormone (PTH), RANKL, and M-CSF, and it also has an impact on the proliferation of OCs (Kaleta, 2017).

Si *et al.* (2020) clinical research found that postmenopausal women's OPN serum levels serve as indicators for the early detection of osteoporosis when compared to women with normal OPN levels.

Through encouraging the release of IL-17 and interferon gamma (IFN-γ) in T cells and IL-6 in monocytes, as well as facilitating the development of T follicular helper (TFH), OPN causes harmful in autoimmune illnesses (Zhang *et al.,* 2022).

MartínMárquez *et al.* (2022) detected that OPN promotes the inflammatory process in SLE by stimulating T cells, NK, and macrophages that drive TH1 cell differentiation. It may also play a role in the development and differentiation of B cells as well as the generation of autoantibodies.

Furthermore, some evidence about the role of OPN in chronic inflammatory illnesses like RA and OA that include the deterioration of bone and cartilage has been found (Martín-Márquez *et al.,* 2023).

2.3.2 Osteocalcin (OCN)

Bone Gla Protein (BGP), osteocalcin or γ-carboxyglutamic acid (Gla), is the most prevalent non-collagenous protein in the bone matrix. It was isolated in the late 1970s, during the peak of the protein chemistry era of bone biology. Structurally, it produces by osteoblasts and has 49 amino acids in humans and 46 amino acids in rats and has molecular weight about 5 kDa (Karsenty, 2023).

Human osteocalcin *bone gamma carboxyglutamic acid protein* (*BGLAP*) gene presense on chromosome 1 specificaly at 1q25-q31, which encodes an 11kD 98 amino acid pre-pro- protein. After a series of cleavage events that eliminate an endoplasmic reticulum signal sequence and the pro-sequence, three glutamic acid residues at positions 17, 21, and 24 undergo γcarboxylation to produce the mature peptide (Saxena *et al.,* 2021).

The gamma carboxylase enzyme uses vitamin K as a cofactor to perform carboxylation, a posttranslational alteration in three glutamate acids that results in OCN with a high affinity for Ca^{+2}, inhibits the formation of hydroxyapatite, and acts as a chemoattractant of osteoclast precursors (Komori, 2020).

As a biochemical marker of bone remodeling, osteocalcin plays a role in a number of physiological processes, including the preservation of healthy bone mineralization and the slowing down of growth-cartilage mineralization (Araújo *et al.,* 2022).

In addition to promoting mineral deposition in ECM, and OCN controls, Ca^{+2} metabolism, Whereas, OPN controls the development of hydroxyapatite crystals and encourages the mineralization and creation of bones (Oton-Gonzalez *et al.,* 2022).

2.3.3 Osteonectin (ON)

Osteonectin is a 32 kDa Calcium-binding matricellular glycoprotein that is encoded by the 26.5 kb *Secreted Protein Acidic and Rich in Cysteine* (*SPARC*) gene, which is found on chromosomes 5q31–q33 and has nine introns and ten exons. Mineralized and non-mineralized tissues both express osteonectin. Basement membrane protein 40 (BM-40) is also another name for ON (Si *et al.,* 2020).

Osteonectin encoded by a gene that generates a secreted, monomeric, glycosylated polypeptide characterized with four domains: 1) an extracellular Ca+2 (EC) domain with an E-F hand motif at the C-terminus that encloses the collagen binding domain; 2) a cysteine-rich domain; 3) a hydrophilic area; and 4) an N-terminal low affinity, high capacity, calcium-binding domain that contains the mineral binding region (Rosset and Bradshaw, 2016).

While osteonectin expression is lower in adult bone homeostasis, it is a higher in immature bone tissue and linked to collagen mineralization. Interestingly, *SPARC* expresses by osteoid, periodontal ligament fibroblasts, odontoblasts (cells that create dentin), active osteoblasts, bone marrow progenitor cells, and hypertrophic chondrocytes. Moreover, *SPARC* expresses by recruited macrophages and is also present in platelet granules during damage (Depalle, *et al.,* 2021).

According to certain theories, ON can connect with collagen and HA crystals in the osteoid and release Calcium ions, which could improve the mineralization of the collagen matrix in bones. This function enhanced during homeostasis and disease (Rosset and Bradshaw, 2016).

2.4 Bone Remodeling

The older bone tissue removes and replaces with new one by matching of osteoclasts and osteoblasts in process called bone remodeling (Licini *et al.,* 2022).

Bone remodeling is a process perfectly balanced with a resorption and a formation phase in order to preserve the integrity of the skeleton. When bone resorption overwhelms bone formation, an imbalance can result which can cause a number of bone loss diseases, including; RA, bone metastatic cancer, aseptic loosening of arthroplasty, periodontitis, and OP. These diseases are characterized by a decrease in bone mass, a disruption in the microarchitecture of bone tissue, an increase in bone fragility, and a higher risk of fractures (Yao *et al.,* 2022; Cheng *et al.,* 2022).

Nonetheless, OCN, OPN, and Type I collagen work in concert to promote bone mineralization and resorption. The interplay between OPN, collagen, and OCN is regarded as essential to mature mineralization. At the resorption site, OCN facilitates the recruitment of OCs precursors and their differentiation into mature OCs (Poundarik *et al.,* 2018; Berezovska *et al.,* 2019).

On the other hand, OPN tends to connect with the cell matrix through its RGD sequences with OBs and αvβ3, which controls collagen fibrillogenesis and cell adherence to the bone surface during remodeling (Depalle *et al.,* 2021).

Bone remodeling is a multistep process which might be affects with several physiological alterations include; hormonal (i.e. estrogen deficiency), aging, drugs, and secondary diseases as diabete melitus (Zhang *et al.,* 2022).

There are four steps for bone remodeling: firstly is activation, HSCs different into mature multinucleated OCs in presence of M-CSF and RANKL (Srivastava and Sapra, 2022).

Secondly is a resorption of the old or damaged bone tissues by OCs, two sequential steps are required in Bone resorption: 1) dissolving the mineral of HA by acidification of resorbing lacuna and 2) proteolytic enzymes secretion to digest the organic part of the bone matrix (Rossi *et al.,* 2019).

Near to PH~4.5 acidic needs to dissolve hydroxyapatite crystals. The carbonic anhydride CO_2 in the matrix hydrated by Carbonic anhydrase II (CAII) into carbonic acid H_2CO_3 that which spontaneously dissociates in bicarbonates HCO_3^- and protons H^+ (Rowe *et al.,* 2022).

Hydrogen ions enter V-H^+ATPase pump in resorption lacuna and HCO_3^- replaces with chloride (Cl^-) by a HCO_3^-/Cl^- exchanger in the basolateral domain. Cl^-ions transmit into resorption lacuna across the membrane to balanced ion charge. When mineral crystals dissolve, proteolytic enzymes acidic hydrolases like cathepsin K, and matrix metalloproteinases (MMP9) can access the collagen bone matrix. Then osteoclasts released these enzymes into the resorption lacuna (Rossi *et al.,* 2019).

Thirdly, MSCs develop into OBs when transcription factors, growth factors, matrix proteins, and vascular endothelial growth factor (VEGF) are present. Fourthly, the new organic bone matrix mineralizes and the OBs settle. OBs incorporated into OYs or remain on the surface as a lining layer (Rowe *et al.,* 2022; Srivastava and Sapra, 2022).

In postmenopausal osteoporosis women, the bone resorption rate increases coupled with reducing in bone formation, resulting in accelerated in bone turnover and bone

loss, this situation occurs a specifically with the corticosteroids treated patients (Cheng *et al.,* 2022).

Bone degenerative disorders including OP and arthritis can result from aberrant bone resorption brought on by osteoclast hyperfunction, which is also essential for osseous metastases, including lung and breast cancer (Fang *et al.,* 2023).

2.5 Osteoporosis (OP)

Low bone mineral density and deteriorating bone architecture are hallmarks of osteoporosis, a systemic skeletal disease that reduces bone strength and increases fracture susceptibility (Fischer and Luntzer, 2021).

Bone fractures result as a contribution of several risk factors, i.e. age increasing, sex, smoking, alcoholic, low intake of vitamin D and calcium, weight loss, family history, hormonal factor as PTH, glucocorticoids and estrogen, early oophorectomy and immune status alteration as inflammation, autoimmune diseases and etc (Al-Abdullah, 2022).

Osteoporosis can result due to either decrease in estrogen level after menopausal period (primary, senile or age-related osteoporosis) or due to endocrine diseases (i.e., hypercortisolism, hyperthyroidism), diabetic osteoporosis, kidney diseases, hematologic diseases (i.e., multiple myeloma and malignant neoplasms infiltrating the bone), autoimmune or rheumatic diseases (i.e., inflammatory bowel disease, rheumatoid arthritis), drugs (i.e., steroids), malnutrition, malabsorption (i.e., celiac disease), or prolonged immobilization (secondary osteoporosis) (Locantore *et al.,*2020).

The pathogensity of OP is a complex and caused by increasing in bone turnover that leads to continuous and progressive bone loss. OP fracture might be occurs in a vertebral, hip, spine or forearm. In osteoporotic hip fractures, the mortality level reached up to 5% in men, while 21%–30% in women within one year. It is an important to identify and diagnose the individuals at high and very high risk of fracture to provide them with adequate therapeutic options. OP can hit whole skeletal system or certain localize (*Xu et al.,* 2021).

Li *et al.,* (2021) and Cheng *et al.,* (2022) studies reported that, the rate of osteoporosis approximately reached to double with the age 45–49 in 3.3%, and progressively increasing to 50.3% at the age of 85 years and older. At the age of ~51 years, the menopause occurred associated with variations in female sex hormones levels due to the cessation of the menstrual cycle and the deterioration of ovarian function.

Szamatowicz and Szamatowicz, (2022) found the osteoporosis is an asymptomatic. So, the diagnosis may often be made only after a fracture has occurred. In addition to above previous risk factors, osteoporosis might be related genetics, previous fracture, drugs that impair bone health as long-term of glucocorticoid therapy or long-term gonadotropin releasing hormone (GnRH) analog treatment due to cancer disease, inflammatory bowel disease, celiac disease, chronic kidney diseases, premature ovarian failure and hypogonadotropic hypogonadism, race (higher risk in white race than others), osteomalacia or malignancy and physical abuse should always be taken into account as a cause of fracture.

The fractures can be prevented by falling reduce, changing in lifestyle and nutrition, smoking and alcohol abstention, calcium and vitamin D supplementation may be get with food or as several drugs (Locantore *et al.,*2020).

Estrogen, progesterone, follicle stimulating hormone (FSH), and lutenizing hormone (LH) play fundamental roles in the menstrual cycle, oocyte maturation, ovulation, facilitating implantation and decidualization, uterine growth, mammary gland development, regulation of milk production, and sexual behavior in females (James *et al.,* 2022).

In women, the menopausal duration describes with four stages: peri-menopause, early- menopause, menopause and post- menopause. During peri-menopause, the estrogen level gradually decline. However, more amount of estrogen fall during both menopause and postmenopause stages. Both estrogen and some of FSH are

responsible for bone turnover regulation. So, for this reason, the skeletal health is directly impacted during all menopausal stages (Thapa *et al.,* 2022).

Because of ovarian atrophy, functional degeneration, and decreased estrogen secretion, postmenopausal osteoporosis develops in elder women. This leads to a decrease in bone density, changes in bone trabecular structure, bone fragility, and an increased risk of fracture in systemic bone metabolic illness (Zhou *et al.,* 2021).

2.6 Types of Osteoporosis

2.6.1 Primary Osteoporosis

The most prevalent bone disease, primary osteoporosis (POP), is characterized by reduced bone mass, microstructure destruction, increased in bone fragility, and a propensity for fracture. In postmenopausal women, primary osteoporosis arises from either estrogen insufficiency which causes bone loss, or aging (Hemmati *et al.,* 2021).

The hallmarks of postmenopausal osteoporosis are brittle fractures and bone loss due to excessive oclastogenesis, which is common in primary osteoporosis. One of the main pathogenetic factors in PMOP is estrogen shortage. Estrogen aid in the bone preserving by promote osteoclasts apoptosis and prevent their maturation. However, the balance of bone remodeling favors bone resorption in the absence of estrogen's protective action, leading to PMOP (Kim *et al.,* 2021).

Senile osteoporosis is a condition that affects persons over 70 year and has grown to be a global health concern. Senile osteoporosis is typically characterized with minimal bone turnover, meaning that bone production is greatly reduced and resorption is lowered. Yet, in recent years revealed that systemic low-grade chronic inflammation and increased inflammatory mediators like IL-6 and TNF-α typically accompany aging (De-Maeyer and Chambers, 2021).

2.6.2 Secondary Osteoporosis

The most prevalent type of secondary osteoporosis is glucocorticoid-induced osteoporosis (GIO). The cumulative adverse effects of glucocorticoids on bone depend on the underlying condition, dosage, and duration. Glucocorticoid use is

mostly indicated for inflammatory rheumatological disorders, lung conditions (such as asthma and chronic obstructive pulmonary disease), solid organ transplant immunosuppression, and inflammatory bowel illness (Aurora, 2019).

Excessive exposure to glucocorticoids causes fragility and decreased bone mineral density. When compared to postmenopausal osteoporotic, BMD declines quickly, and the risk of fracture increases within three months of supraphysiological glucocorticoid exposure (Buckley *et al.,* 2017).

In addition to causing apoptosis, glucocorticoids prevent osteoblast differentiation and development. Through elevated production of sclerostin and dickopf-1, glucocorticoids can also disrupt the wingless-related integration site signaling pathway, which in turn affects osteoblasts. Glucocorticoids can also directly lower bone morphogenic protein, which lowers osteoblast differentiation (Yao *et al.,* 2022).

According to Van-Bommel *et al.* (2018), glucocorticoid therapy can decrease the circulation of osteocalcin, which is closely linked to metabolic alterations such decreased insulin sensitivity.

A temporary decrease in OPG promotes osteoclastogenesis and unopposed RANKL activity. The dramatic drops in bone mineral density during the initial months of glucocorticoid therapy could be explained by an increase in osteoclast activity. Increased apoptosis in osteocytes results in decreased bone blood vessel volume and osteocyte-lacunar-canalicular circulation, which in turn lowers bone strength and quality. This could explain why fractures have been observed to occur at a greater BMD in GIO patients (Ebeling *et al.,* 2022).

Indirectly mechanisms, such as decreasing intestinal calcium absorption and blocking renal calcium reabsorption are another way that glucocorticoids might cause detrimental bone consequences. Excess glucocorticoids decrease insulin-like growth factor 1 (IGF-1) and suppress gonadotrophin production, which causes hypogonadism and accelerated bone loss. The effects of glucocorticoids on bone cells

are defined by an increase in osteoclast survival and activity, followed by a long-term suppression of osteoblast number and function (Compston, 2018).

2.7 Risk Factors of Osteoporosis

2.7.1 Age, Sex and Race

Tu *et al.* (2019) and Qadir *et al.* (2020) studies found, osteoporosis could be occurred in people with different ages and gender. Szamatowicz and Szamatowicz. (2022) detected the higher risk factors of fracture observed in a white individual race than others.

About 31–36% of people with age above 70 years have normal bones, while the remainders suffer from either osteopenia or osteoporosis. However, bone loss and dysfunctions increased with the age advanced individuals due to increased in osteoblasts apoptosis, proliferation declined, osteoblast differentiation impaired, osteoblast senescence increased and osteoprogenitors dysfunction (Chandra and Rajawat, 2021).

2.7.2 Genetics

Genetics play a substantial role in determining an individual's skeletal strength, bone microarchitectural properties and risk of osteoporosis. BMD is known to be a highly heritable trait. Genetic factors influence bone health in a polygenic manner and multiple gene variants, or SNPs. Recent research, especially large-scale genome-wide association studies in large cohorts, has elucidated the complexity of genetic networks that are important for bone metabolism. On the other hand, significant scientific advances have been made by studying rare monogenic forms of osteoporosis in which one mutation in a single gene with a major role in bone metabolism dominates and is alone sufficient to cause osteoporosis (Mäkitie *et al.,* 2019).

Both mutations and deoxyribonucleic acid (DNA) damaged factors throughout life consider as a two major sources of DNA disorders. DNA continuously exposes to chemical exposure, physical damage, biological agents as exogenous conditions or endogenous as error in DNA structure leading to protein encoded gene dysfunction

and its products, and these agents influence DNA integrity and functions (Chandra and Rajawat, 2021).

2.7.3 Endocrinal Disorders

All of muscle, adipose tissues and peptides contribute in energy metabolic regulation and involved in the metabolic homestasis of protiens, lipids, and glucose. In addition, the energy of metabolism and bone remodeling are influence in insulin and epinephrine. Both diabetes mellitus type 1 (T1DM) and type 2 (T2DM) are associated with the risk of fracture elevation (Araújo *et al.,* 2022).

However, PTH is a peptided hormone secreted by the parathyroid gland, which is partially embedded in the posterior surface of thyroid. PTH has an 84-amino-acid and promotes the bone formation through varity methods. PTH has a great potential in application of bone defect repair. PTH is another factor that has related role with osteoporosis. PTH also defines as parathormone or parathyrin, which is a calcium-phosphate metabolism regulated hormone. PTH/ PTH1R (parathyroid hormone receptor) binding can regulate the bone metabolism and it plays an osteogenic role by acting directly on mesenchymal stem cells, osteoblastic lineage, osteocytes, and T cells. It also participates indirectly acting on OC precursor cells and osteoclasts (Chen *et al.,* 2021).

2.7.4 Estrogen

Estrogen is a steroid hormone in female responsible for reproductive system regulation in female and secondary sex characteristics. Estrogen has three forms estrone (E1), estradoil (E2) and estriol (E3) and synthesis by granulosa cells in developing ovary's follicle. Estrogen is antagonistic to LH role in menstrual cycle (Holesh *et al.,* 2022).

Estrogen is the primary hormone that which regulate bone metabolism either directly through osteoclast estrogen receptors or indirectly through RANKL on osteoblasts, T cells, and B cells, additionaly to osteoclast develop enhanceing (Jiang *et al.,* 2021).

In postmenopausal women, estrogen has a directly role in maintenance of bone formation and bone resorption balanced after menopause. The concentration of estrogen increase with MSCs differentiation and osteoblast maturation, leading to bone formation enhanced. While, osteoclast formation inhibit and osteoclast apoptosis induce, which limits bone resorption and vas versa (Fischer and Luntzer, 2021).

The deficiency in estrogen hormone can directly affects on both cell differentiation and apoptosis, which then leading to increased bone turnover and enhanced bone resorption, resulting in osteoporosis (Xu *et al.,* 2021; Cheng *et al.,* 2022).

However, ovariaectoy after menopause, smaller and thinner bones in women than men, and falling in estrogen levels all of them are a factors that maybe cause rapidly acceleration in bone loss. Approximately 10% of bone mass reduced throughout the menopausal phase, that mean a half of women are losing bone at a higher rate, reaching 20% loss in the five to seven years following menopause. Additionally, based on bone loss rates and signs of bone resorption, about 25% of postmenopausal women can be further categorized as fast bone losers (Yong and Logan, 2021).

2.7.5 Vitamin D and Calcium

The 1α,25-dihydroxyvitamin D (1,25(OH)2D3) or calcitriol is the active form of vitamin D. VitD involved in bone development regulation and calcium homeostasis through its actions in the intestine, kidney, and bone. VitD deficiency has been associated with numerous health conditions as immune diseases, pasturing soft bones, and skeletal deformities (Mu *et al.,* 2020; Ahmad *et al.,* 2021).

Calcium plays acritical roles in skeleton mineralization, normal growth, development, bone strength and in a wide range of biological functions, such as muscle contraction, vascular contraction and vasodilatation, nerve impulse transmission and intra and extracellular signaling (Vannucci *et al.,* 2018).

Calcium and vitamin D are main supplements that which maintain bone health in postmenopausal women and recommended for people at risk of osteoporotic fracture

(Capozz *et al.,* 2020). Fruits and vegetables are rich with bioactive compounds, such as phenolic acid, flavonoids, carotenoids, vitamins and minerals, and all of them have a potential osteoprotective effects and the postmenopausal women require botanical supplements for osteoporosis management (Darling *et al.,* 2021).

2.7.6 Obesity and Adipokines

Leptin is a dipokine - like hormone, encoded by ob (*Lep*) gene of adipocytes. It is involved in energy homeostasis and bone metabolism. Leptin concentration serum level is typically elevated in obese individual, which is a leptin-resistant condition. The evidence previous studies have demonstrated that leptin regulates bone mass by both peripheral and central mechanisms (Rinonapoli *et al.,* 2021).

Obesity, hyperglycemia, lipid metabolism, and cardiovascular disease all are significantly impacted by adiponectin (another common adipokine). It's yet unclear how adiponectin signaling affects bone homeostasis. According to preclinical research, adiponectin improves bone homeostasis by increasing osteoblastic differentiation and decreasing osteoclast activity (Deepika *et al.,* 2023). Moreover, it has been demonstrated to promote osteoblastic proliferation, which is linked to an increase in alkaline phosphatase activation, type I collagen synthesis, and osteocalcin production. These markers aid in osteoblast differentiation and maturation (Damani *et al.,* 2022).

Moreover, adiponectin level in the patients suffered from chronic kidney disease (CKD), showed an inverse relationship with trabecular volumetric bone mineral density (VBMD), cortical VBMD, and cortical thickness (Deepika *et al.,* 2023).

Also, Adiponectin had been showed an inverse association with bone strength parameters in immobilized patients. A recent case-control study obtained a strong inverse relationship between adiponectin and T scores in women with osteoporosis and osteopenia (Roomi *et al.,* 2021).

In obese individual, adiponectin serum level is usually low and this condition can be induces the reduction in osteoblastogenesis and the increase in osteoclastogenesis.

In addition, adiponectin deficiency is also found in insulin-resistant diabetes people and this could be one of the links between obesity and diabetes (Yamauchi *et al.,* 2001).

2.7.7 Hemophilia

Hemophilia A and B are rare X-linked recessive hereditary bleeding diseases caused by abnormalities in the genes encoding coagulation factor VIII (FVIII) or IX (FIX). The most common type of hemophilia is type A. Spontaneous hemorrhages in joints, muscles, and soft tissues are characterize joining with hemophilia A or B disease (Castaman and Matino, 2019).

A previous study found that 27% of patients with hemophilia (PWH) had osteoporosis, while 43% had osteopenia. The risk of osteoporotic fractures in PWH was 4.37 times higher than in the general population of the same sex and age. PWH, identified with acute significant blood loss during fracture as a result of coagulation deficit, which may obviously enhance osteoporosis risk and refracture (Lin *et al.,* 2023).

Petkovic *et al.* (2022) showed strong association between hemophilia and low BMD over all ages, for instant; 27–28% of mean age 45.9 years adults have a low BMD.

2.7.8 Smoking

Cigarette smoking (CS) is an independent risk factor of osteoporosis development. In a comparison between smokers and non smokers, clinical studies clarified a significant lower BMD in smokers than non-smokers, and claimed the bone loss can increase the lifetime risk of hip fracture by 50% (Wang *et al.,* 2019[a]).

Long-term of CS using can leads to an imbalance in bone turnover, and that contribute in bone mass and bone length reduction and increased fracture risks (Al-Bashaireh *et al.,* 2018). Furthermore, cigarettes chronic consumption has been increasingly linked to impaired muscle function (Lee *et al.,* 2018).

However, several studies have observed that smoking able to inhibit the production of PTH and down-regulates vitamin D serum levels. PTH has a regulatory function in serum calcium levels regulation by bone and kidney reabsorption, while vitamin D stimulates calcium absorption from the intestine. According to gonadal hormones, in females CS reduces estrogen levels by several different methods as, a romatase enzyme activity suppression by CS components, nicotine and cotinine, CS can promotes estradiol to decompose into 2-methoxyestrone in the liver and CS enhances serum hormone-binding globulin levels, leading to a reduction in free estradiol levels in the blood (Weng *et al.,* 2022).

Also, CS aids in high ROS level, which promotes osteoclast activation and osteoblast suppression, leading to reduce in bone mass (Callaway and Jiang, 2015).

2.7.9 Alcohol

Alcohol is one of the main drink beverag that firstly metabolized into acetaldehyde and then into acetate, which is a main for energy metabolism, gut health maintenance and appetite regulation. However, excessive acetate production can leads to inhibition in gluconeogenesis and fatty acid oxidation (Zaso *et al.,* 2019).

Alcohol consumption can influence many organ systems in the body via impact nutrition or the bioactivity of ethanol and its metabolites. Heavy consumption of alcohol is related to skeletal health, fracture risk and osteoporosis (Cheraghi *et al.,* 2019).

Alcohol- induce osteoporosis (AOP) is a systemic bone metabolic disordered belonging to secondary OP. Long-term of excessive alcohol consumption considers as a one of OP risk factors. Some previous evidence showed that there were multiple direct toxic effects of alcohol on the bone cells such as changing in osteoblasts activation and proliferation based on dose-dependent manner. In addition, alcohol also has direct or indirect effect with bone mineral metabolic alteration, include: PTH, vitamin D, testosterone, and cortisol levels (Lee *et al.,* 2018).

However, AOP is a common disorder in clinical practice, with the age advanced, the rate of bone turnover increases leads to damage in osteoblast bone formation and

increased in osteoclast bone resorption, which significantly increase the incidence of osteoporosis (Cheng *et al.,* 2021[a]).

Moreover, heavy alcohol consumption may decrease estrogen receptor isoforms α and β, which prevent bone loss. Chronic alcohol drinking increases the expression of NADPH oxidase in osteoblasts, leading to increased ROS production and bone resorption (Sheng *et al.,* 2021).

2.7.10 Drugs:

Glucocorticoids are endogenous hormones and medications that have anti-inflammatory, immunosuppressive, anti-proliferative, and vasoconstrictive properties. GCs are commonly used to treat acute and chronic inflammatory conditions, lymphoproliferative diseases, chemotherapy and radiotherapy side effects, organ transplant rejection prevention, and adrenal insufficiency (Laurent *et al.,* 2022).

Chronic glucocorticoid use, in addition to treating inflammatory and autoimmune diseases, can lead to bone loss, osteoporosis, and fractures. GIO is the most common type of secondary osteoporosis (Wang *et al.,* 2019[b]; Braun *et al.,* 2020)

2.8 Immunity and Osteoporosis

Osteoporosis is a complex chronic disease caused by genetic and environmental factors. Both systemic immunological and inflammatory conditions are linked to it, either directly or indirectly (Tang *et al.,* 2022). In addition to immunological and inflammatory status, the identified novel gene candidates could serve as biomarkers or therapeutic targets (Mo *et al.,* 2022).

The bone immune response comprises interactions between bone cells and immune cells, including M0 macrophages that differentiate into pro-inflammatory M1 and anti-inflammatory M2 (figure 2.2). Phenotypes and cellular factors of MSCs, OCs, and OBs play a significant role in bone tissue pathology (Yang *et al.,* 2019[a]; Muñoz *et al.,* 2020).

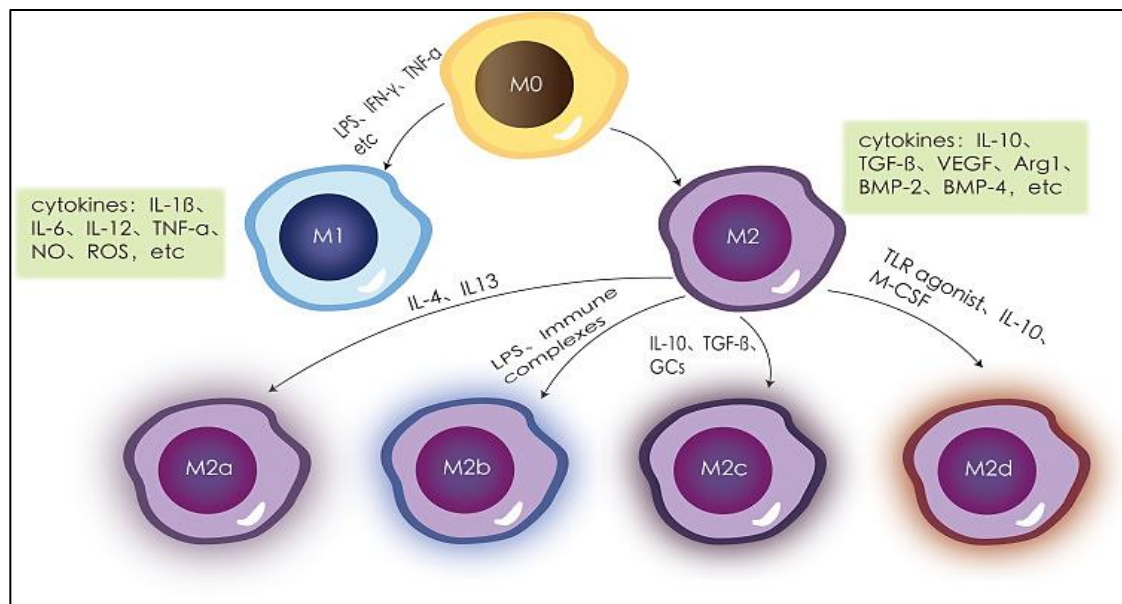


Figure (2.2): Macrophages polarized M0 into pro-inflammatory M1 and anti-inflammatory M2 (Hu *et al.*, 2023).

TNF-α, IL-6, IL-1, chemokine ligand (CXCL2, CXCL8, and CXCL1) are an examples of macrophages secresion to promote OCs differentiation and maturation. Whereas, IL-4 and IL-10 are the cytokines that secreted by M2 which inhibited OCs formation. M1 macrophages promote angiogenesis and osteogenesis in early and mid-stage MSCs, while M2 macrophages produce osteogenic substances to stimulate mineralization in osteoblast precursors and MSCs. Excessive activation of M1 macrophages can lead to persistent inflammation and compromise bone homeostasis (Hu *et al.,* 2023).

Chronic inflammation promotes bone loss and the onset of OP in addition to hormones effective on bone metabolism which causing inflammation and inducing inflammatory cell death (Jiang *et al.,* 2021).

According to Zhang *et al.* (2022), osteoporosis involves a communication between the skeletal and immune systems. Immune cells secrete soluble mediators such as cytokines, chemokines, and growth factors, which influence osteoblast and osteoclast activity. Osteoblasts and osteoclasts govern the hematopoietic stem cell niche, which is responsible for immune cell development.

However, M1 macrophages show potential to differentiate into osteoclasts and consider as the precursors of osteoclasts, so they act as osteoclast reservoir and may make bone resorption easier and severer (Yang and Yang, 2019).

While M2 macrophages tend to inhibit osteoclasts activation and differentiation, bone resorption and promote osteogenesis by IL-4 and IL-13 cytokines. M2 cytokines downregulate osteoclastic genes, involving RANK and tartrate resistant alkaline phosphatase (TRAP) thus inhibit osteoclast activation and differentiation (Wang *et al.,* 2020).

However, the dendritic cells are primary antigen-presenting cells that derived from monocyte/macrophage progenitor cells. DCs can activate adaptive immune responses, present antigens, T lymphocytes cells development regulation, differentiation, and function, contribute in Treg cells differentiation and homeostasis, which serve an essential role in promoting osteogenesis and inhibiting osteoclastogenesis *via* anti-inflammatory effect (Balan *et al.,* 2019).

In the absence of estrogen, both IL-7 and IL-15 will increased expression by DCs. IL-7 and IL-15 induce IL-17 and TNF-α production in a subset of memory T cells, independent of antigen activation (Cline-Smith *et al.,* 2020).

Whereas, neutrophils able to protective the bone formation at early stages of bone healing, secretion of many pro-angiogenic growth factors and osteogenic factors, such as fibroblast growth factor-2 (FGF-2), platelet-derived growth factor (PDGF), and TGF-β (Kovtun *et al.,* 2018).

The hyperactive neutrophils triggered by infection or injury can harm bone homeostasis, neutrophils could produce chemokines to recruit proinflammatory cells, such as T-helper 17 cells, and suppress B lymphocytes, thereby promoting inflammatory bone loss . In addition, activated neutrophils secrete RANKL and contribute in osteoclast formation and maturation (Chakravarti *et al.,* 2009: Hajishengallis *et al.,* 2016).

Mast cells (MCs) originate from hematopoietic stem cells in bone marrow and have a role in bone homeostasis regulation and disease development via cytokines and synthases. According of Kim *et al.* (2021), MCs can either directly induce osteoclast production or indirectly create tissue-destroying cytokines.

Natural killer cells stimulate osteoclast development in an M-CSF and RANKL-dependent manner to regulate bone remodeling. NK cells can either control or exacerbate osteoporosis depending on the tissue microenvironment. Cytotoxic NK cells may inhibit osteoclasts' pathogenic bone resorption (Fathollahi *et al.,* 2021). NK cells triggered by IL-15 have the potential to kill osteoclasts and prevent bone degradation (Feng *et al.,* 2015).

Estrogen deficiency increases the thymus output of T cells in peripheral blood and they suggested that T cells over activated under estrogen deficiency, particularly $CD4^+$ T cells (Abildgaard *et al.,* 2020). In addition, to the different subtypes of T cells which might play a certain role in promotion or inhibition bone resorption, activated T cells could be contribute in osteoclastogenesis by RANKL strong expression in PMOP (Tyagi *et al.,* 2014).

Th17 cells, an osteoclastogenic subset of T cells, were observed to proliferate in bone marrow and produce elevated levels of IL-17 in peripheral blood. In contrast, Treg cells play a bone-protecting role in PMOP development (Dar *et al.,* 2018).

Estrogen insufficiency can disrupt the equilibrium between Th17 and Treg cells, leading to increased Th17 and decreased Treg cells. Under estrogen shortage, Treg cells may lose their immunosuppressive function and convert to Th17 cells, resulting in an imbalance of Th17/Treg in PMOP (Lai *et al.,* 2015).

However, estrogen insufficiency can increase the number of B cells in bone marrow, leading to the formation of osteoclasts. In the absence of estrogen, B cell precursors increased, whereas in the presence of estrogen, stromal cell-dependent B cells decreased significantly. B cells from postmenopausal women's bone marrow have been found to release RANKL, which promotes osteoclastogenesis. These

findings show that estrogen deprivation stimulates B cells, leading to osteoclast development (Fischer and Luntzer, 2021).

2.8.1 Cytokines and Osteoporosis

There are specific cytokines shared a unique roles in bone activity, classified into osteoblastogenesis (bone formation) and osteoclastogenesis (bone resorption) promoters and/or inhibitors, bone-resident osteocytes, and cartilage cells (chondrocytes) such as; IL-6, IL-11, oncostatin M (OSM), leukemia inhibitory factor (LIF), cardiotrophin 1 (CT-1), ciliary neurotrophic factor (CNTF), cardiotrophin-like cytokine factor 1 (CLCF1), neuropoietin, humanin, IL-27 and some others (Sims, 2021). In addition to, IL-1α, -4, -7, -10, -12, -13, -18, -23, TNF-α, -β, IFN-α, -β, and IFN-γ (Saxena *et al.,* 2021; Srivastava and Sapra, 2022).

After menopausal period there was an increase occurred in inflammatory cytokines levels, as osteoclastogenesis –induced interleukins: IL-1β, IL-6, IL-7, IL-9, IL-15, IL-17, IL-21, IL-22, IL-23, IL-31, TNF-α, ROS and RANKL. While, decreased via elevation in IFN-γ and IL-4, IL-5, IL-10, IL-12, IL-13, IL-33, IL-35, TGF-β, FGF2, Plasma Poor Growth Factor (PPGF), Cranial Cruciate Ligment (CCL2, and CCL5) and IGF-1 (Fischer and Luntzer, 2021; Zhang *et al.,* 2022).

Both IL-31 and IL-33 are Th2 cytokines that might be have a role in osteoporosis. IL-31 influence in the myeloid progenitor differentiation into osteoclasts and increase proinflammatory cytokines, such as TNF-*α* and IL-6. In Contrast to IL-31, IL-33 inhibits bone resorption by inhibiting RANKL osteoclastic gene expression, and that may become one of the key points in osteoporosis treatment (De-Martinis *et al.,* 2020).

IL-10 is anti-inflammatory cytokine that suppresses osteoclast formation and bone resorption produced by M2, IL-10 may lead to bone resorption after spinal cord injury (Kitaura *et al.,* 2020; Azevedo *et al.,* 2021).

The hypoestrogenism is a potent stimulation for increased in inflammatory mediator production. In particular, estrogen deficiency can lead to activation certain

immune cells as macrophages, monocytes and T cells, which stimulate osteoclast and inhibit osteoblast by inflammatory cytokines secretion (Sen *et al.,* 2020).

However, hypoestrogenism, caused by ovarian senescence during menopause, leads to accelerated bone loss, followed by gradual loss of strength, density, and bone quality, increasing the risk of osteoporosis and fractures. Aging increases proinflammatory cytokines including IL-1β, IL-6, and TNF-α, contributing to the "inflamm-aging" phenomena in elders (Damani *et al.,* 2022).

Santo *et al.* (2012) reported that was relationship between hormones, cytokines and exercises or physical training and could be used with osteoporosis elderly patients as a non-drug preventive strategy. Six-month of exercise were able to regulate hormones, reduce bone loss in the elderly, and increased in serum level of IL-2, IL-10, IL-12, IL-13, IL-18, and IFN (protective cytokines against bone resorption). While significantly decrease in serum levels of IL-1, IL-6, and TNF-*α*.

2.8.1.1 Interleukin-8 (IL-8)

Its CXC subfamily of chemokine familt characterized by the spacing of the first-two cysteine residues in their structures. It plays essential role in regulation of inflammatory response. It was described as a chemo-attractant of neutrophils. Macrophages, epithelial cells, airway smooth muscle cells, and endothelial cells are the sources of IL-8. Over-expression of IL-8 not only noticed with cancer cells, promoting tumor growth, angiogenesis and metastasis, but also serves a critical role in osteoclast formation and bone metastasis specially, in patients with breast cancer (Liu *et al.,* 2019).

Also, recent clinical data found IL-8 level in serum and synovial fluid maybe elevated in RA patients.TNF-α and IL-8 noticed their ability in induction of osteoclast formation. IL-8 up-regulates RANKL expression in osteoblast and binds directly with CXCR1 on cells for osteoclastogenesis activation (Kitaura *et al.,* 2020; Haider *et al.,* 2021).

Kany *et al.* (2019) reported that postmenopausal osteoporosis is characterized by rapid bone loss and IL-8 has been implicated among other pro-inflammatory cytokines to play a role in bone remodeling. There was a significant IL-8 increase in post-menopausal women with osteoporosis and bone loss.

RANKL-expressing neutrophils are increased in male patients with chronic obstructive pulmonary disease (COPD), and furthermore, associated with bone mineral density and lung function, suggesting that these cells play a role in osteoclastogenesis in COPD. Plasma levels of IL-8 were increased in COPD patients and correlated with RANKL expression by neutrophils (Hu *et al.,* 2017).

2.8.1.2 Interleukin-17 (IL-17)

It's a pro-inflammatory cytokine. Secreted from T helper- 17 (Th-17). Encoded gene located at the short arm of chromosome 6 in position 6p12. T, γ and δ cells, neutrophils, innate lymphoid cells, macrophages, mast cells, natural killer cells, and B cells are other sources of IL-17. IL-17 family includes six members: IL-17A to IL-17F (Kitaura *et al.,* 2020; Jameel *et al.,* 2022).

All of these interleukins are isoforms and sharing approximately 50% of amino acid sequence and have a special receptor called (IL-17R) begin with IL-17RA to IL-17RE. IL-17RA is the most available member, related to owing proinflammatory properties. The recent studies suggested that, IL-17 has essential role in osteoclastogenesis stimulation (Srivastava and Sapra, 2022).

In the last years, new evidences reported relationship between immune system and bone cellular metabolism in humans affected with bone disease, such as osteoporosis, psoriasis, periodontal disease, RA and inflammatory bowel disease (IBD). Now, IL-17 might be known as a bone loss inducer in postmenopausal osteoporosis, and it represents across link between estrogen deprivation and increased immune reactivity (AL-Tai, 2015; Amarasekara *et al.,* 2021).

IL-17A, C, and F are usually excite host defense response and promote autoimmune inflammatory, whereas IL-17E (IL-25) induces Th2 polarization with

allergic response and has been shown to present anti-inflammatory response (Min *et al.,* 2020).

Th17 mainly activated by TGF-β, IL-6, IL-1β and IL-23, Th17primary secret IL-17, IL-21 and IL-22. Th17 has direct or indirect osteoclastogenic effects through secreting higher levels of interleukins; IL-1, IL-6, RANKL, TNF and low levels of IFN-γ and IL-17-mediated stimulation of osteoclast-related molecules on a wide variety of target cells. Osteoclastogenesis stimulated by osteocytes/osteoblasts RANK upregulation which promote production of higher levels of RANKL. IL-17 acts as across link between T lymphocytes and osteocytes by modulating production of RANKL. Many studies found the osteoporotic patients Th17 cell population has been increased (Srivastava *et al.,* 2018).

IL-17, along with IL-6, IFN-γ and TNF-α, M-CSF, and monocyte chemoattractant protein-1 (MCP-1), promote bone resorption and osteoblast inhibition, while IL-4, IL-10, TGF-β, and IL-12 suppress osteoclasts and osteogenesis promotion (Amjadi and Akhavan, 2019; De-Martinis *et al.,* 2020).

New research indicates that IL-17/IL-17RA in osteocytes requires parathyroid hormone to regulate bone. According to Li *et al.*, (2019), IL-17 increases osteocyte sensitivity to parathyroid hormone and stimulates osteocytic RANKL production.

2.8.1.3 Interleukin- 22 (IL-22)

Interleukin-22, α-helical cytokine is linked to various bone loss illnesses, including rheumatoid arthritis, peri-implantitis, periodontitis, osteoporosis, and diabetes. Th17 and Th22 cells generate IL-22, which is encoded on the 12q15 chromosome alongside IFN-γ and IL-26 genes. IL-22 synthesis is facilitated by IL-17, IL-23, IL-1β, aryl-hydrocarbon receptors, and Notch signaling. IL22 levels may be elevated in people with bone mass loss (Woś and Tabarkiewicz, 2021).

Another study has been shown, IL-22-produce cluster of (CD) 4^{+} T cell (Th22) population and has been found to be elevating in patients with RA as a comparing to

healthy controls, and it is also correlated with the disease activity score (Min *et al.,* 2020).

Whereas, IL-22 level increased in periodontitis patients as a compare with both gingivitis patients and healthy individuals. This finding joined with increasing in osteoclastogenesis and bone resorption observed in periodontitis patients (Díaz-Zúñiga *et al.,* 2017).

TNF-α, IL-17, and IL-22 are influence osteoclastogenesis and osteoblastogenesis via affecting other systems such as RANK, RANKL, OPG, and others (Resende *et al.,* 2020).

Recent findings indicate that the IL-22 cytokine increases RANKL expression, leading to OC differentiation and bone resorption in sub-chondral bone in TMJ-OA and periodontitis. IL-22 cytokine could be used to treat bone-related illnesses, such as osteoporosis (Woś and Tabarkiewicz, 2021; Srivastava and Sapra, 2022).

2.9 *Secreted Phosphoprotein-1*(*SPP1*) Gene

*S*ecreted phosphoprotein-1 is a member of the SIBLING (Small Integrin Binding Ligand N-linked Glycoprotein) family, which also includes DMP-1, BSP-2, DSPP, and MEPE-1 and encoded human OPN (Nazneen and Bai, 2020; Roomi *et al.,* 2022). *SIBLING* is located on human chromosome 4q21-q25, has comparable exon structures, and has an RGD sequence. *SPP1*, found on chromosome 4q21-q25, has an open reading frame of 942 nucleotides and produces three mRNA variants: *OPN-a, OPN-b, and OPN-c.* According to Castello *et al.* (2017) and Eremo *et al.* (2020), *OPN-a* has all seven exons, but *OPN-b* lacks exon 5 and *OPN-c* has a loss of exon 4, figure (2.3).

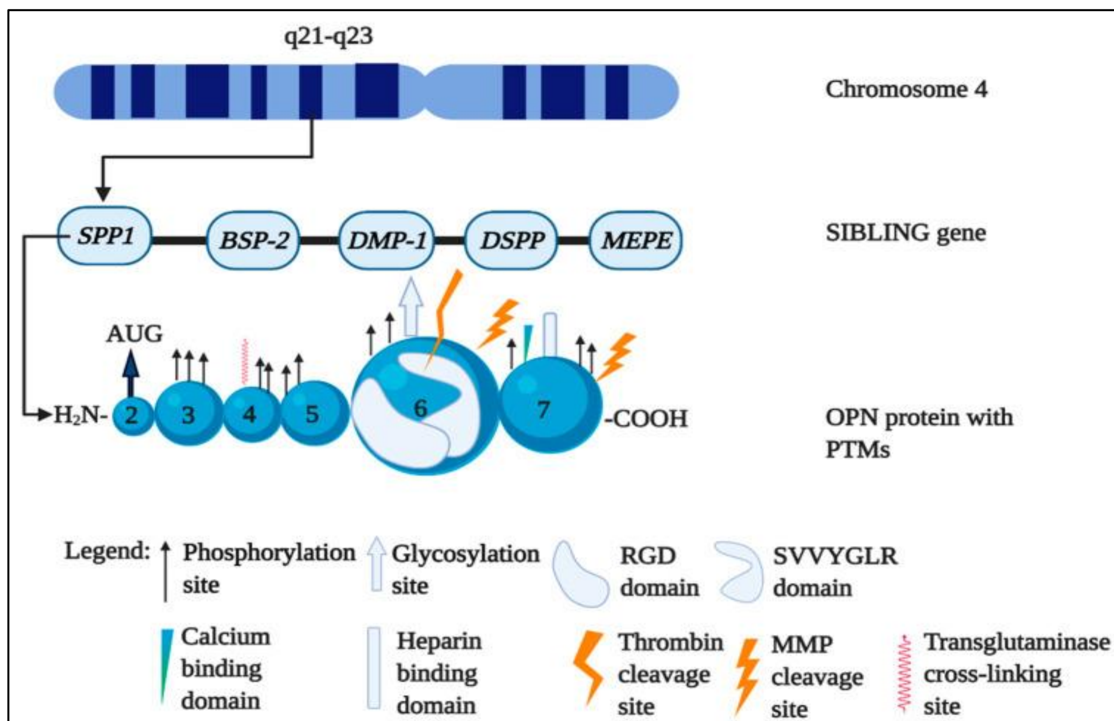


Figure (2.3): Human *secreted phosphoprotien-1* (*SPP-1*) gene location and the post-translational modification (PTM) sites in osteopontin (OPN) protein (Nazneen and Bai, 2020)

Nevertheless, OPN transcript undergoes alternative splicing, resulting in two variants: *OPN-4,* which deletes both exons 4 and 5, and *OPN-5*, which includes an extra region derived from exon 3 (Gimba *et al.,* 2019).

The translated OPNa has 314 amino acids, OPNb (300 amino acids), and OPNc (284 amino acids), whereas OPN4 (273 amino acids) and OPN5 (327 amino acids) were recently found (Lamort *et al.,* 2019).

Osteopontin undergoes numerous posttranslational changes including phosphorylation, glycosylation, and sulfation. In humans, OPN is primarily phosphorylated in the Golgi apparatus by Fam20C kinase, which also phosphorylates OPN in the extracellular matrix. The phosphorylation process affects the interaction of OPN with hydroxyapatite, which is related to bone remodeling and has been linked to macrophage movement and host cell contacts (Si *et al.,* 2020; Mateos *et al.,* 2021).

Osteopontin receptors such as αvβ1, αvβ3, αvβ5, αvβ6, α4β1, α4β7, α5β1, and α9β1 integrins, as well as CD44, bind to specific RGD domains and interact with αv

integrins (particularly αvβ1, αvβ3, and αvβ5) and SVVYGLR (serine-valine-valine-tyrosine-glutamate-leucine) (Jámbor *et al.,* 2022).

Osteopontin expression and function are affected by various factors, including posttranslational modifications (phosphorylation, O-linked glycosylation, sialylation, tyrosine sulfation), hormones (calcitriol, retinoid acid, steroids), pro-inflammatory cytokines, growth and differentiation factors (epidermal growth factor, platelet-derived growth factor, transforming growth factor beta), and promoter genetic polymorphisms (Kaleta, 2017).

The variations in *SPP1* gene may play a significant role in osteoporosis and the SNPs may affect the production of *SPP1* protein structure, *SPP1* serum level, change or reduce *SPP1* protein and bone formation (Chen *et al.,* 2014).

SPP1 plays a role in various biological functions, including cell adhesion, migration, immunological response, and tissue regeneration. It also contributes to the development of disorders such as bone catabolism, chronic inflammatory diseases, and autoimmune diseases. Previous research suggests that *SPP1* triggers TGFβ1 to initiate the transition of MSCs into fibroblasts (Faqeer *et al.,* 2023).

SPP1 polymorphisms may contribute to osteoporosis by affecting osteogenesis and bone remodeling. Chen *et al.* (2014) found that functional SNPs can impact *SPP1* protein synthesis and bone formation.

Chapter Three

Methodology

3. Methodology

3.1 Participants Selection

The period of this investigation study was from December 2022 to July 2023. All participants provided written informed consents, and the study was approved by the University of Baghdad's, Department of Biology's ethical council (reference code CSEC/0922/0092, on 26-9-2022). 110 postmenopausal women were randomly chosen to participate in this study based on specific criteria from Al-Imamein Kadhimein Medical City, AlWasity and Medical City hospitals, Baghdad, Iraq.

3.1.1 Inclusion Criteria

This study was included patients:

1- Postmenopausal women with varying body weights and heights.

2- Their ages were between 45-70 years old.

3.1.2 Exclusion Criteria

This study was excluded patients:

1- Women with menopausal or premenopausal period.

2- Women with autoimmune diseases.

3- Women with acute and infectious disease, for instant: covid-19, and ovary as well as uterine resection.

4- Women with hereditary diseases.

5- Women with hormonal imbalance.

6- Women refused to join the study.

3.1.3 Demographical and Clinical Cases

According to the conditions (as in inclusion criteria paragraph in above), 110 women participated in this study. The demographic data of patients including their cities of living, age, race/ethnicity (white or other), weight, height, marital state, healthy state if they had (chronic diseases, autoimmune diseases, diabetes, infectious disease), smoking, Alcoholic, family history, fractures history, glucocorticoids or certain type of medicine uptake. hyperparathyroid, estrogen deficiency…etc were

collected from face to face interviews, baseline questionnaire and their medical files, (appendix 2).

DEXA scan was used to determine the bone density and investigate the presence or absence of OP. However, for each sample, a DEXA scan was performed on the lumbar spine and femoral neck to evaluate bone health and osteoporosis resembled with T and Z scores. 40 samples were controls while, 70 samples were proven with OP disease. Additionaly, the BMI kg/m^2 was calculated by dividing the weight (kg) by height (m^2), (appendix 3).

3.1.4 Diagnosis of Osteoporosis

All samples examined with a DEXA san apparatus (Japan) to ensure the osteoporosis presence or absence. It is a radiological test used for assessing bone health and osteoporosis by examined the lumbar spine and femoral neck with T and Z scores. T-scores compare the bone density of individual to that of a healthy young adult, while Z-scores compare bone density to the average for age, sex, and ethnicity, (appendix, 4, 5 and 6). According to T- score values, the results explaining as:

- Over -1 as normal.
- Between -1.0 and -2.5 as osteopenia.
- Below -2.5 as osteoporosis.

When BMD declines by more than -2.5 standard deviations, osteoporosis is diagnosed. However, BMI is a body mass index used to obesity identification, calculated by specific equation that which applied by divided the weight by height squared (kg/m^2).

$$\textbf{BMI (kg/m}^2\textbf{)} = \frac{\textbf{Weight (kg)}}{\textbf{Height (m}^2\textbf{)}}$$

3.1.5 Blood Samples Collection

Ten ml of venous blood were collected from each participant by vein puncher using ten ml disposable srynge, each blood sample were divided into the following:

1- Six ml of blood were placed in a gel tube and left at room temperature for 15 min then centrifugated at 3000 rpm for 10 min to serum separation to measurement the following parameters:

   A. Two ml of serum for biochemicals test to assessment the levels of; CRP, RF, ACPA, Ca^{+2} and PTH.

   B. Two ml of serum for biochemical tests, investigation human OPN, OCN, and ON.

   C. Two ml of serum for immunological tests, investigation the levels of human interleukin-8,-17, and-22 based on Sandwich-ELISA technique

2- Two ml of blood kept in ethylene-diamine-tetra-acetic-acid (EDTA) tubes for haematological test, CBC test.

3- Two ml reminder of blood kept in ethylene-diamine-tetra-acetic-acid (EDTA) tubes and stored in -20C° until use, for *OPN* gene *rs11730582* polymorphism. (Figure 3.1).

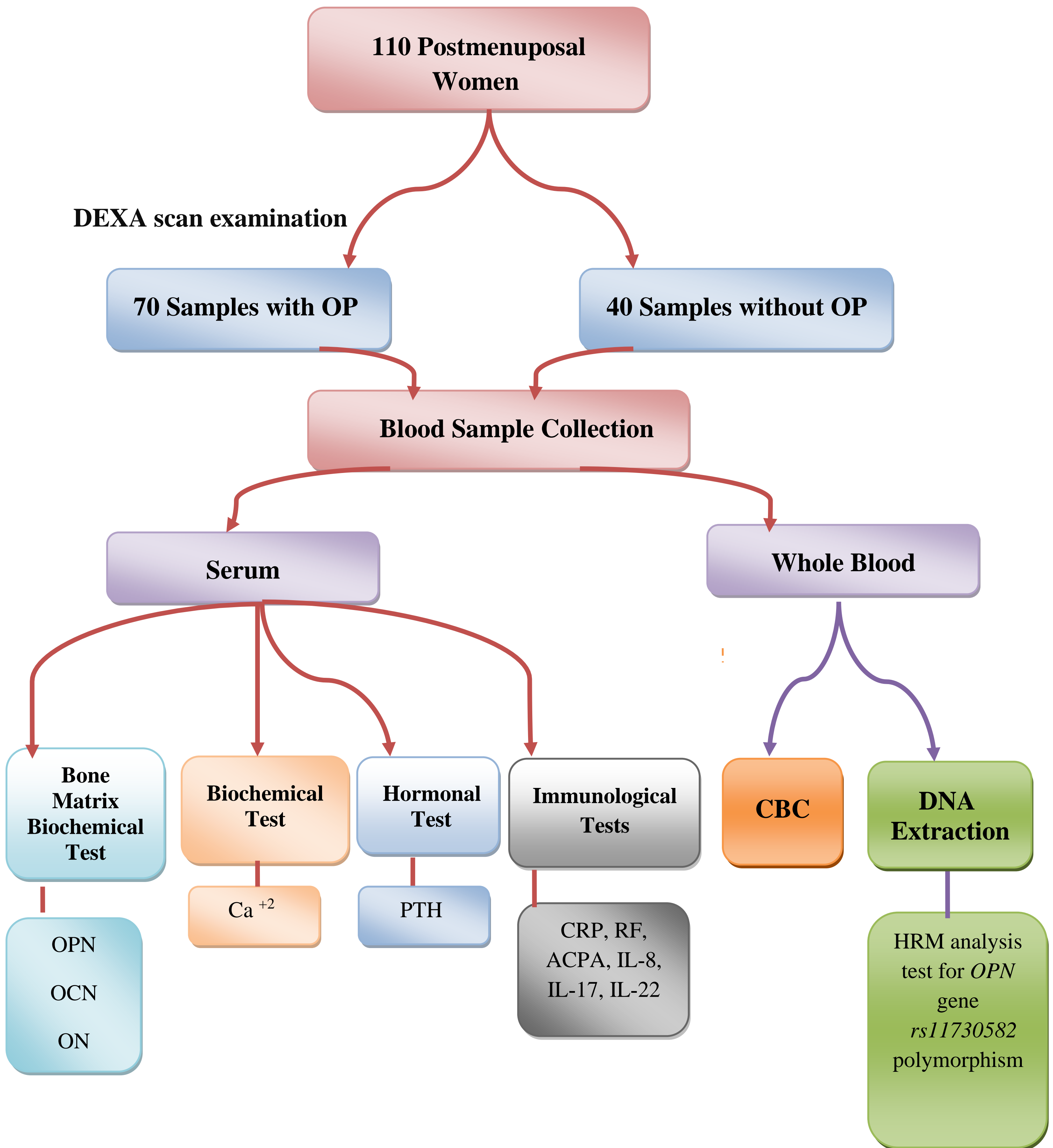


Figure (3.1): The study design

3.2 Materials

3.2.1 Apparatus and Equipments:

All the apparatus and equipments were used in this study are summarized in table (3-1).

Table (3-1): The apparatus and equipment used to perform the study.

Equipment's Name	Company	Country of Origin
Centrifuge	Hermelh	Germany
Centrifuge Tubes (Sterile) - 1.5ml	Bio basic	Canada
Cover Plate		
Deep Freeze	Liebherr	Germany
DEXA Scan	French	French
Disposable Syringe 5ml	AZMDF	China
EDTA Tubes (2ml)	ARTH AL.RAFIDAIN	Iraq
Elecrical Shaker		Germeny
ELISA Washer	tecan	USA
ELISA Reader	tecan	USA
Eppendorf Tubes (2ml)	Biobasic	Canada
Gel and Clot Activator Tubes(6ml)	ARTH AL.RAFIDAIN	Iraq
Gilson Blue Pipette Tips (200 μl)	-	China
Gilson White Pipette Tips (200 μl)	-	China
Gilson Yellow Pipette Tips (200 μl)	-	China
Incubation	Faithful	-
Incubation	JRAD	-
Micropipettes Multi Channels (5-50μl)	Dragon	Germany
Microplate Reader	Biotek	USA
Microplate Reader	Human	Germany
Micropipettes Single Channel (10μl-100μl)	Dragon	Finland
Micropipette 1.0-10ul	NEXTY-10	Japan
Micropipette 100-1000ul	Slamed	Japan
Micropipette 50 – 200ul	Slamed	Japan
Nanodrop Spectrophotometer	Thermo Fisher Scientific	USA
Plan Tubes	-	China
Refrigerator	Hitachi	Japan
Spectrophotometer	Labomed	USA
Sysmex XP-300	-	Japan
Vortex Mixer	Stuart scientific	UK
Water Bath	GFL	Germany

3.2.2. Laboratory kits.

The kits used in the study are shown in table 3-2:

Table (3-2): The laboratoery kits that used in the study and their manufactures.

Kit's Name	Company	Country of Origin
C- Reactive Proten ELISA kit (BPE193)	BT LAB	China
Rheumatoid Factor (RF) Latex kit (098100)	BIOLABO	France
Human Anti-Cyclical Citrullinated Peptide Anibody (ACPA) ELISA kits (ED0108Hu)	BT LAB	China
Human Parathyroid Hormonal (PTH) ELISA kit (MBS263675)	MYBioSource	USA
Human Calcium (Ca^{+2}) ELISA kit (MBS2540479)		
Human Osteopontin (OPN) ELISA kit (YLA0342HU)	ShanghaiYL Biont	China
Human Osteocalcin/ bone gla protein (OT/BGP) ELISA kit (YLA1183HU)		
Human Osteonectin (ON) ELISA kit (YLA1345HU)		
Human Interleukin-8 (IL-8) ELISA kit (E0098Hu)	BT LAB	China
Human Interleukin-17 (IL-17) ELISA kit (E0142Hu)		
Human Interleukin-22 (IL-22) ELISA kit (In-Hu2165)		
EasyPure® Genomic DNA Kit (TransGen, EE101-01)	Biotech	China
TransStart® Tip Green qPCR Super Mix for HRM	QIAGEN	Germany

3.3. Methods

3.3.1 Hematological Test

Two ml of blood containing within EDTA anticoagulant tube shaking for 15-20 minutes with electrical shaker to prevent agglutination. EDTA tubes inserted into the digital counting apparatus manufactured by Sysmex XP-300 Corporation, Japan for CBC measurements, and the findings were shown within a minutes.

3.3.2 Immunological Tests

3.3.2.1 Determination of C-Reactive Protein (CRP) Concentration

*** Principle**

This kit uses an Enzyme-Linked Immunosorbent Assay (ELISA) to measurement CRP in human. An anti-Human CRP monoclonal antibody from mice has been pre-

coated onto the assay plate then CRP-containing sample attaches itself to the antibodies coated on the plate wells when it is introduced. After that, the wells are filled with a mouse anti-CRP antibody conjugated with horseradish peroxidase to attachment with the CRP in the samples. After wells washing, substrate solutions are added and the color intensity is directly correlated with the concentration of human CRP. An acidic stop solution is added to finishing the process, and at 450 nm the absorbance is measured.

* **Procedure:**

1- All reagents, standard solutions, samples and Detection Antibody Solutions were prepared as instructed.
2-All reagents were brought to room temperature before uses.
3- One hundred µl of standard or sample were added per well.
4- The plate was covered with a sealer and incubated for 2 hours at room temperature.
5- Three hundred µl of 1× Wash Buffer were added to each well and soaked for 1 minute. Then aspirated or decanted the liquid and patted the wells dry with clean paper towels.
6- Repeating the process twice for a total of three washes.
7- One hundred µl of the Detection Antibody Solution were added to each well, sealed the plate and incubated for 1 hour at room temperature.
8- The aspiration/wash as in Step 3 was repeated.
9- Two hundred µl of Substrate Solution (Pre-mixed in reagent preparation step) were added to each well, incubated for 20 minutes at room temperature and protected from light.
10- Fifty µl of Stop Solution were added to each well to stop the reaction. The solution color in the wells should change from blue to yellow.
11- Determine the optical density (O.D value) of each well immediately with a microplate reader set to 450 nm within 10 minutes after adding the stop solution, (appendix 7).

3.3.2.2 Determination of Rheumatoid Factor (RF)

*** Principle**

For evaluating the presence of RF in serum sample, a suspension of latex particles was used and coated with human gamma globulin. The presence or absence of RF is indicated by the presence or absence of a visible agglutination. Human pure IgG globins are coated on specific polystyrene latex particles to create RF, a sensitive, standardized preparation. When the RF protein is directed toward IgG globin determinants, it acts as an IgM. The serum that containing RF was combined with the latex reagent through latex agglutination, an antigen-antibodies reaction can be easily observed.

***Procedure:**

1- Test reagents and samples were putted at room temperature.
2- Reagent vial mixed gently.
3- One drop (50 µl) from all serum samples was added onto each card's circles.
4- One drop of RF-Latex Reagent was added to each circle next to the sample to be tested.
5- The contents of each circle was mixed with a disposable stirrer
6- A slide was rotated with the circles directions for 2 minutes.
7- Observe agglutination: -ve= no visible agglutination and +ve = visible agglutination.

3.3.2.3 Determination of Human Anti-Cyclical Citrullinated Peptide Antibody (ACPA) Level.

*** Principle**

Human anti-Cyclical Citrullinated Peptide Anibody ELISA kit utilized with double antigen sandwich ELISA depending on ACPA antibody-ACPA antigen complexes (immunosorbency).

The microtiter plate has been coated with a target antigen. Positive/Negative Controls or samples are added to the wells and incubate. Antibodies of the samples

bind with the antigen on the plate. During a washing step, unbound antibodies are washed away. A Horseradish Peroxidase (HRP) conjugated to detection antibody added and incubated. Unbound HRP is washed away during a washing step. Then TMB substrate is added and color develops. By addition of acidic stop solution and color changes the reaction is stopped and the yellow color can be measured at 450 nm.

***Procedure:**

1- Test reagents and samples were putted at room temperature.
2- One hundred µl of each standards or samples were added to their corresponding wells.
3- The wells of plate sealed with adhesive tape strip, and incubated at 37℃ for 90min.
4- ELISA plate washed for 2 times
5- One hundred µl of prepared antigen were added to each well, sealed with adhesive tape strip, and incubated at 37℃ for 60min.
6- ELISA plate washed for 3 times.
7- One hundred µl from prepared enzyme conjugate were added to each well other than the blank wells, Sealed with adhesive tape strip, and incubated at 37℃ for 30min.
8- ELISA plate washed for 5 times.
9- One hundred µl of the prepared colour reagent A were added to individual wells (also into blank well), then incubated and protected the plate from light at 37 C°.
10- One hundred µl of chromogenic reaction (colour reagent B) should be controlled to within 30 min.
11- One hundred µl from colour reagent C were added to each individual well (also into blank well).
12- Mixed well and read O.D at 450nm.

3.3.2.4 Determination of Human Interlukin-8 (IL-8) Concentration

*** Principle**

Human IL-8 ELISA kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with human IL-8 antibody. IL-8 present in the sample is added and binds to antibodies coated on the wells. And then biotinylated human IL-8 Antibody is added and binds to IL-8 in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated IL-8 antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and colour develops in proportion to the amount of human IL-8. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

***Procedure:**

1- All of reagents, standard solution and samples prepared and brought at room temperature before using.
2- Fifty µl from standard were added to standard well.
3- Fourty µl from samples were added to sample wells, then 10µl of anti IL-8 antibody, and 50µl of streptavidin-HRP was added to sample and standard wells (not blank control well). Mixed, covered with a sealer and incubated at 37°C for 60 minutes.
4- The plate was washed 5 times with wash buffer. Soaked wells with 300ul wash buffer for 30 seconds to 1 minute for each wash.
5- Fifty µl of substrate solution A and 50µl substrate solution B were added to to each well. Covered with sealer and incubated for 10 minutes at 37°C in dark.
6- Fifty µl of stop Solution were added to each well. (The blue colour would change into yellow).
7- O.D read with a microplate reader at 450 nm within 10 minutes, (appendix 8).

3.3.2.5 Determination of Human Interleukin-17 (IL-17) Concentration

*** Principle**

Human IL-17 ELISA kit is an Enzyme-Linked Immunosorbent Assay. The plate has been pre-coated with human IL-17A antibody. IL-17A present in the sample is added and binds to antibodies coated on the wells. And then biotinylated human IL-17A Antibody is added and binds to IL-17A in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated IL-17A antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of human IL-17A. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

***Procedure:**

1- All of reagents, standard solution and samples prepared and brought at room temperature before using.
2- Fifty µl from standard were added to standard well.
3- Fourty µl from samples were added to sample wells, then 10µl of anti IL-17 antibody, and 50µl of streptavidin-HRP was added to sample and standard wells (not blank control well). Mixed, covered with a sealer and incubated at 37°C for 60 minutes.
4- The plate was washed 5 times with wash buffer. Soaked wells with 300ul wash buffer for 30 seconds to 1 minute for each wash.
5- Fifty µl of substrate solution A and 50µl substrate solution B were added to to each well. Covered with sealer and incubated for 10 minutes at 37°C in dark.
6- Fifty µl of stop Solution were added to each well. (The blue colour would change into yellow).
7- O.D read with a microplate reader at 450 nm within 10 minutes, (appendix 9).

3.3.2.6 Determination of Human Interleukin-22 (IL-22) Concentration

*** Principle**

The human IL-22 ELISA kit is an Enzyme-Linked Immunosorbent Assay. The plate has been coated with human IL-22 antibody. The sample contains IL-22 binds to antibodies coated on the wells. The sample subcequantly treated with the biotinylated human IL-22 Antibody. Then Streptavidin-HRP is added and binds to the Biotinylated IL-22 antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. After adding the substrate solution, the color develops in proportionly to the amount of human IL-22. To termination the reaction acidic stop solution was added and absorbance was measured at 450 nm.

***Procedure:**

1- In the microelisa stripplate, left a well as blank. In sample wells, 40μl sample dilution buffer and 10μl sample were added (dilution factor is 5).
2- Concentrated washing buffer diluted with distilled water (30 times for 96T and 20 times for 48T).
3- Peeled off closure plate, aspirated and refilled with the washing solution for 30 seconds. This step repeated for 5 times.
4- Fifty µl HRP- conjugate reagent was added to each wells except blank control well).
5- Incubating at 37°C as in step3.
6- Washing as in step5.
7- Fifty µl of chromogen solutions A and B were added to each well, mixed, shaked, and incubated in 37°C for 15 minutes. (Avoid light during colouring).
8- Fifty μl of Stop Solution were added to each well to terminate the reaction, the colour should change from blue to yellow immediately.
9- O.D read at 450nm using microtiter plate reader, (appendix, 10).

3.3.3 Hormonal Test

3.3.3.1 Determination of Parathyroid hormone (PTH) Concentration

*** Principle**

Human parathyroid hormonal kit utilized with double-sandwich ELISA technique to detection human PTH serum level. Samples and biotinylated antibodies are added into ELISA plate wells and washed with Polybutylene succinate (PBS) or Tris-buffered saline (TBS). The Avidin-peroxidase conjugates are added to the wells. TMB substrate used for coloration after the enzyme conjugate has already been thoroughly washed out of the wells by PBS or TBS. TMB reacts to form a blue product from the peroxidase activity, and finally turns to yellow after addition of the stop solution (Colour Reagent C). The colour intensity and quantity of target analyte in the sample are positively correlated.

***Procedure:**

1- Test reagents and samples were putted at room temperature.
2- One hundred µl of standards or samples were added for each corresponding wells.
3- ELISA plate washed for 2 times.
4- One hundred µl of prepared biotinylated antibody were added to each well, Sealed with adhesive tape strip, and incubated at 37°C for 60min.
5- ELISA plate was washed for 3 times.
6- Prepared Enzyme Conjugate was added to each well other than the blank wells (100µl for each), sealed with the adhesive tape strip, and incubated at 37°C for 30min.
7- ELISA plate washed for 5 times.
8- One hundred µl of the prepared Color Reagent A were added to individual wells (also into blank well), incubate protected from light at 37°C. The chromogenic reaction (colour Reagent B) should be controlled to within 30 min.
9- One hundred µl of Color Reagent C were added to each individual well (also into blank well).
10- Mixed well and read the OD at 450nm, (appendix 11).

3.3.4 Biochemical Test

3.3.4.1 Determination of Calcium (Ca^{+2}) Concentration.

*** Principle**

The concentration of Calicum was detected by Human Calicum ELISA kit. Calcium ion in the sample binds to Methyl Thymol Blue (MTB) in alkaline solution and form blue complex. Calcium content can be calculated by measuring the O.D value at 610 nm.

***Procedure:**

1- Ten µl of Standard solution with different concentrations were added into the wells.
2- Ten µl of sample were added into the wells.
3- From working solution 1 (250 µl) were added into the wells.
4- Mixed fully and standard for 5 min at room temperature.
5- Measured O.D value at 610 nm with Microplate Reader, (appendix 12).

3.3.5 Bone Matrix Biochemical Tests

3.3.5.1 Determination of Human Osteopontin Concentration

*** Principle**

Human osteopontin (OPN) ELISA kit used enzyme-linked immune sorbent assay (ELISA) based on the biotin double antibody sandwich technology to assay OPN. Osteopontin was added to the wells, which are coated with OPN monoclonal antibody and incubated. Anti-OPN antibodies labeled was added with biotin to unite with streptavidin-HRP, which forms immune complex. Unbound enzymes removed after incubation and washing. Substrate A and B were added. Then the solution will turn blue and change into yellow with the effect of acid. The shades of solution and the concentration of human OPN are positively correlated.

***Procedure:**

1- All reagents, samples and standards prepared
2- Samples, standards and ELISA solutions were added and let to reaction for 60 minutes at37 °C.

3- The plate washed 5 times. Chromogen solution A and B were added and incubated for 10 minutes at37 ℃; for colour development.

4- Stop solution was added and read O.D within 10 minutes, (appendix 13).

3.3.5.2 Determination of Human Osteocalcin Concentration.

*** Principle**

Human osteocalcin uses enzyme-linked immune sorbent assay (ELISA) based on the Biotin double antibody sandwich technology to assay the Human Osteocalcin/Bone gla protein (OT/BGP). Add OT/BGP protein to the wells, which are pre-coated with OT/BGP monoclonal antibody and then incubate. After that, add anti OT/BGP antibodies labelled with biotin to unite with streptavidin-HRP, which forms immune complex. Remove unbound enzymes after incubation and washing. Add substrate A and B. Then the solution will turn blue and change into yellow with the effect of acid. The shades of solution and the concentration of OT/BGP are positively correlated.

*** Procedure:**

1- All of reagents, samples and standards prepared

2- Samples, standards and ELISA solutions were added and let to reaction for 60 minutes at 37 °C.

3- The plate washed 5 times, Chromogen solution A and B were added and incubated for 10 minutes at 37 °C; for color development.

4- Stop solution was added and read O.D within 10 minutes, (appendix, 14).

3.3.5.3 Determination of Human Osteonectin Concentration

*** Principle**

Human osteonectin ELISA kit uses enzyme-linked immune sorbent assay (ELISA) based on the Biotin double antibody sandwich technology to assay the Human Osteonectin (ON). Add ON to the wells, which are pre-coated with ON monoclonal antibody and then incubate. After that, add anti ON antibodies labelled with biotin to unite with streptavidin-HRP, which forms immune complex. Remove unbound enzymes after incubation and washing. Add substrate A and B. Then the solution will

turn blue and change into yellow with the effect of acid. The shades of solution and the concentration of ON are positively correlated.

***Procedure:**

1- All of reagents, samples and standards prepared
2- Samples, standards and ELISA solutions were added and let to reaction for 60 minutes at 37 °C.
3- The plate washed 5 times, Chromogen solution A and B were added and incubated for 10 minutes at 37 °C; for color development.
4- Stop solution was added and read O.D within 10 minutes, (appendix 15).

3.3.6 Molecular Study

3.3.6.1 SNP Selection

Osteopontin gene *rs11730582* polymorphism chose to investigate the relationship with development of osteoporosis pathogenecity in postmenopausal women. Based on gene card, the genetic information of *OPN* gene collected from National Center for Biotechnology Information (NCBI), https://www.ncbi.nlm.nih.gov/snp/rs11730582. Table (3-3).

Table (3-3): *OPN gene* and *rs11730582* polymorphism information.

OPN Gene	
Organism	*Homo Sapiens*
Gene	*Spp-1*
Gene Full Name	Secreted Phosphoprotein-1
Gene Type	Protein coding
Product	Osteopontin (OPN)
Function	-Attachment of osteoclasts to the mineralized bone matrix. The encoded protein secrets and binds hydroxyapatite with high affinity. -This protein is a cytokine that up-regulates the expression of interferon-gamma and interleukin-12.
Location	Chromosome 4, LOC124900730 transcript, 2KB_upstream_variant region
Alleles	T/A/C
Sources	3505 single nucleotide polymorphism database (dbSNP) 160 genetic variation database (dbVar) 36 Clinical variation 3629 without clinical variation

rs11730582 polymorphism	
Variation ID	*rs11730582*
Variation Type	Single nucleotides variation (SNV), length 1
Mutation at Allele	T/C
Location	chromosome 4 GCF_000001405.40: NC_000004.12 @ 87975269 GCF_000001405.25: NC_000004.11 @ 88896421
Variant Position	2KB upstream variant
Sequence	GAGTAGTAAAGGACAGAGGCAAGTT[T/C]TCT GAACTCCTTGCAGGCTTGAAC.
Clinical Significant in Pathogenesis	Not provided
1000 G MAF	C = 0.32995, specific ≥ 0.05
GO_ESP MAF	Not specific
ExAC MAF	Not specific
SNPs Beside It	At right rs1439234044 At left rs1178732042 At below rs1725330579

3.3.6.2 Primer Design

The primer had been checked with the University California of Santa Croz (UCSC) and reference sequences in the NCBI database. They were synthesized and lyophilized by Al pha ADN (S.E.N.C., Canada).Table (3-4).

Table (3-4): Information of primer kit

Primer	Sequence (5′→3′ direction)	primer size bp	Product size bp	Ta°C
OPN gene				
SNP rs11730582				
Forward	GCTAAGCTTGAGTAGTAAAGGACAGA	20	339	74
Reverse	TTATTGAAGAGCCAGAAGGCTA	20	339	62

The required primers, as given in Table (3-5), were synthesized in the following manner: After dissolving the lyophilized material in nuclease-free water per manufacturer's instructions, a stock solution of 100µl was made and stored at -20°C. After diluting 10µl of each primer stock solution in 10µl of nuclease-free water, a working solution with a concentration of 10µl was created and stored at -20°C until used.

Table (3-5): Preparation of primer work solution

Components	Concentrations
Distal water	180 µl
Spp1 rs11730582 HRM reverse	10 µl
Spp1 rs11730582 HRM forward	10 µl
Nuclease free	10 µl

3.3.6.3 DNA Extraction

About 2 ml venous blood sample from each participant was collected in ethylene-diamine-tetraacetic acid (EDTA) tubes. Genomic DNA was obtained from peripheral blood leukocyte samples using the DNA blood extraction kit (*EasyPure*® Genomic DNA Kit. Table (3-6).

Table (3-6): The easypure® genomic DNA kit's components.

Components	(50 rxns)
Lysis Buffer 2 (LB2)	6 ml
Wash Buffer 2 (WB2)	12 ml
Binding Buffer 2 (BB2)	28 ml
Clean Buffer 2 (CB2)	55 ml
Elution Buffer (EB)	25 ml
RNase A (20 mg/ml)	1 ml
Proteinase K (20 mg/ml)	1 ml
Genomic Spin Columns with Collection Tubes	50 each

*** Procedure:**

1- In 110 empty eppendrof tubes, 20 µl of RNase A to the lysate (to obtain RNA-free genomic DNA) was added and incubated at room temperature for 2 min.

2- Followed with 20 µl of Proteinase K to the lysate then mixed well by vortexing and incubated at room temperature for 2 minutes.

3- Five- hundred µl of binding buffer (BB2) was added and immediately mixed by vortexing for 5 seconds and incubated at room temperature for 10 minutes.

4- Two- hundred µl of blood from each blood sample were added then incubated in thermostatic water bath for 15 minutes at 56°C.

5- The contents of each tubes were transferred into genomic spin columns and centrifugated for 1 min at (20000 rpm).

6- Then washing with 500 µl cleaning buffer and centrifugated at 20000 rpm for 1 min this step repeated two times for impurities removing.
7- Dehydration the tubes for 2 min at 12000 rpm.

3.3.6.4 DNA Concentration and Purity Assessment

To assess the concentration of DNA samples with acceptable integrity, a Nanodrop spectrophotometer 2000c (Thermo Fisher Scientific) was used with basic computerized software control and data recording. A blank solution of TE buffer was used beforehand. The Nanodrop was loaded with two microliters of isolated DNA to evaluate its concentration in ng/µl. The concentration was between 75-110 ng/µl. The Nanodrop spectrophotometer 2000c was also used to determine DNA purity, with the absorbance of the sample measured at two wavelengths (260 and 280nm). The A260/A280 ratio was 1.7-1.9, indicating a pure DNA sample.

3.3.6.5 Genotyping by High-Resolution Melting (HRM) Analysis

OPN gene's genetic variation was chosen to investigate the relationships with osteoporosis in Iraqi postmenopausal women. The SNP detection *rs11730582* was achieved by using HRM real-time PCR.

1. Green: Often used to represent the wild-type homozygote (the normal or reference sequence).
2. Red: Often used to represent one type of homozygote (e.g., a mutant homozygote).
3. Blue/Yellow: Often used to represent the heterozygote, showing the combined melting behavior of the two different alleles. Figure (3.2).

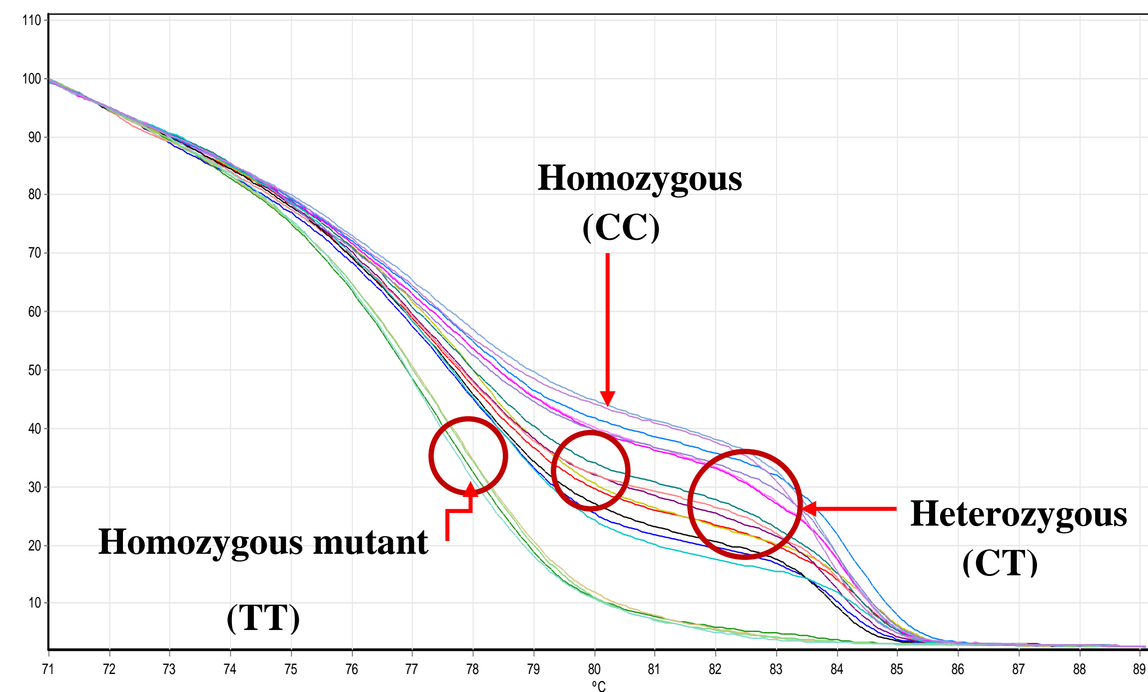


Figure (3.2): The result output of HRM for the three genotypes in *rs11730582* SNP

3.3.6.6. High Resolution Melting (HRM) Analysis

The rotor gene Q Real-time PCR System (QIAGEN, Germany) was utilized for qPCR-HRM, followed by HRM analysis at 0.2°C scaling from 55 to 95 °C.

Duplicates of the 2xTransStart® Tip Green qPCR Super Mix Synthetic SNP sequences were used. To discover allelic differences, qPCR-HRM was performed on triple synthetic controls. Normalized melting curves (NMC) and differential curves (DC) were created using the HRM Tool in the integrated software.

The data were analyzed by software version (Rotor-Gene Q software 2.3.5.1). The amplification was done by 20 µl of reaction volume containing Master Mix, SNP genotyping assay (containing Sequence-specific forward and reverse primers to amplify the polymorphic sequence of interest, distal water and genomic DNA. As in table (3-7).

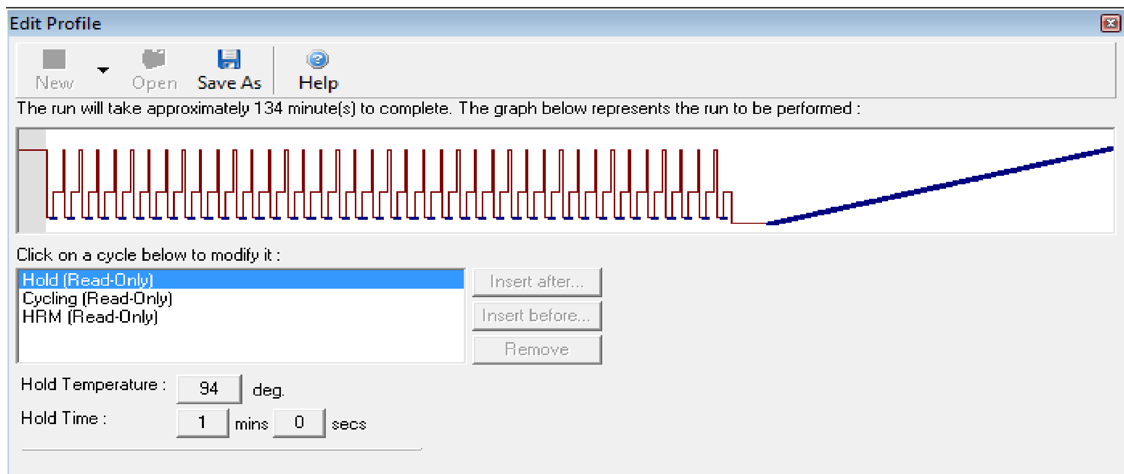
Table (3-7): The HRM SNP experiment uses quantitative real-time PCR components.

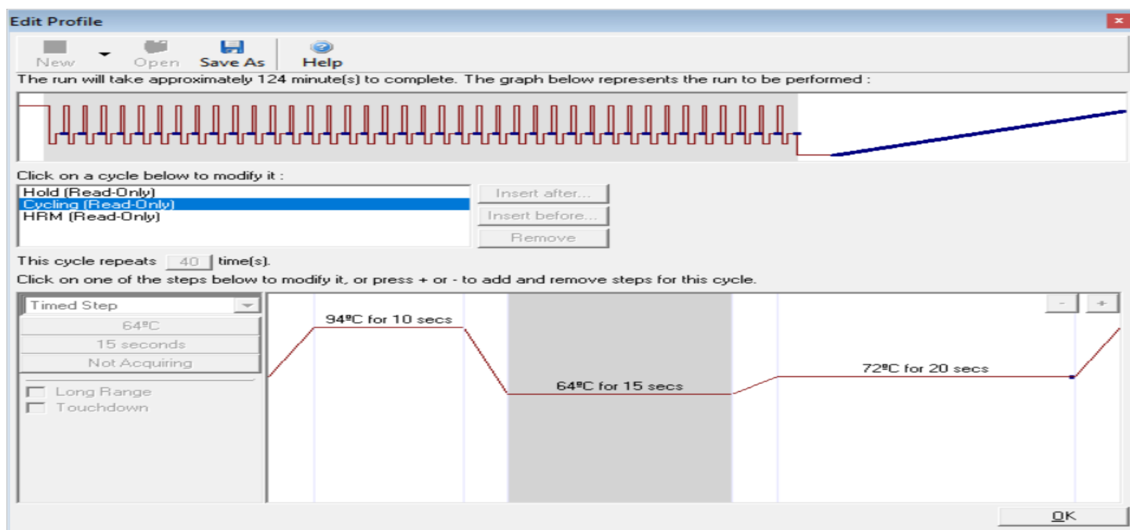
Components	20 µl rxn
Master mix	10
Nuclease free water	4
Forward Primer (10 µM)	1
Reverse Primer (10 µM)	1
DNA	4

According to the thermal profile, the cycling protocol was programmed for the following optimized cycles, as given in Table (3-8):

Table (3-8): The thermal profile of HRM genotyping.

Step	Temperature (ºC)	Time (sec.)	Cycles
Enzyme activation	94	30	1
Denaturation	94	5	40
Anneling	64	15	
Extension	72	20	
HRM	55-95	0.2 sec for 1 degree	





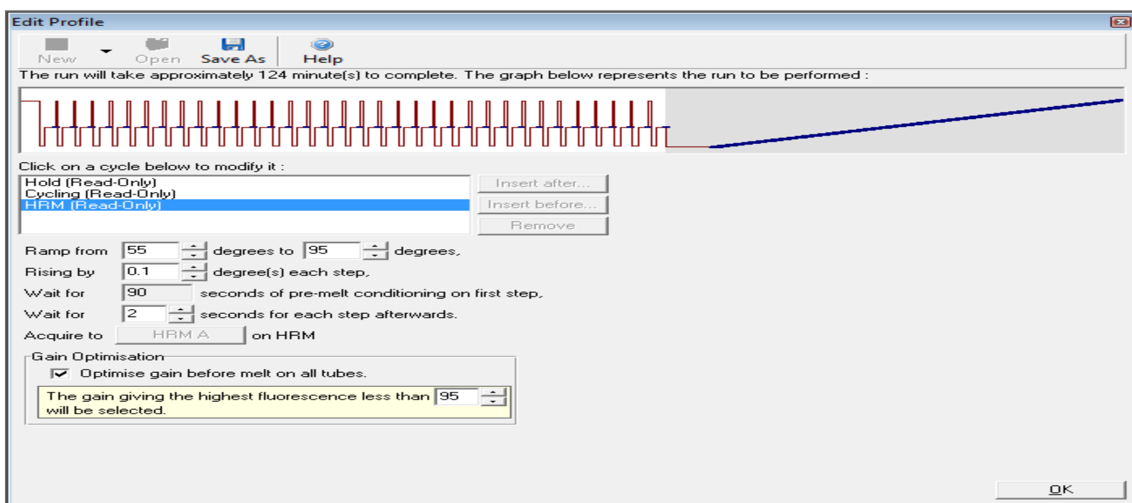


Figure (3.3): Thermal profile used for HRM genotyping. The images were directly taken from the qPCR machine.

High resolution melting analysis is a sensitive molecular approach for genotyping and mutation detection. HRM detects SNPs and point mutued in amplified DNA fragments by measuring fluorescence as temperature changes. Alleles have different melting curves, which can be compared to reference samples. Using a saturating DNA dye helps detect heteroduplexes based on changes in melting curve shape (Smith *et al.,* 2009).

High resolution melting analysis produces specific and sensitive DNA melt curve profiles for both exploratory mutation scanning (discovery of unknown genetic variation) and routine detection of known variants (targeted genotyping with probes or reference curve-based genotyping of defined multiple SNPs for human genome analysis and mutation search) (Stomika *et al.,* 2017).

3.4 Statistic Analysis

Duncan test, IBM SPSS 28.00 version software (2021), chi-square test and an independent-sample *t*-test were utilized between different variables to examine data's mean and standard error (SE). Probability in this study is (0.05 and 0.01). The receiver operating characteristic (ROC) curve analysis was used to evaluate the correlation between interleukins-8, -17 and -22 serum levels indicators for osteoporosis. However, WinPepi 11.65 program was used for Hardy-Weinberg equilibrium probability, Fisher’s exact probability, Odd ratio, 95% confidence intervals, genotyping and alleles frequencies calculation.

Chapter Four

Results and Discussion

4. Results and Discussion

4.1 DEXA Scan

According to the patient's questionnaires and interviews, the OP disease is asymptomatic condition until the first fracture occurs joined with substantial pain, suffering, disability, and even death. DEXA scan's results showed 70 samples were +ve provided with OP disease with duration ranged from 1-15 years managed into four groups. While, forty samples were -ve results (healthy control).

4.2 Demographics and Baseline Clinical Characteristics

All participants were female and 100% identified as white race. The majority of patients (86.25%) claimed residing on the outskirts of Baghdad, while rest came from the city center. There were no recorded occurrences of smoking or alcoholism among the 110 participants, including OP patients and controls.

4.2.1 Distribution of Osteoporotic Patients According to Age Groups

The present study reported non- significant differences in the mean ± SE of the age in OP patients (63.01±1.09 years) as compared with control group (60.43±1.14 years), however, out of 70 OP patients, the greater percentages of participants were recorded in 27 women with the age group ranged between 60-69 years old (38.57%), 19 women with 50-59 years old (27.14%),18 women with ≥70 years old(25.71%) and the lowest percentage was 6 women with 45-49 years old (8.57%), (table 4-1) and (appendix 16).

Table (4-1): Sorting of OP patients according to their ages.

Age groups (years)	Patients, N=70	
	NO. of Patients	% Percentage
45-49	6	8.6%
50-59	19	27.1%
60-69	27	38.6%
≥70	18	25.7%
**P≤0.01= High significant		

In this study, all participant patients were elders postmenopausal women and no significant differences was observed when compared with healthy. The results of

other studies were in line and agreed with the present results such as Cui *et al.* (2019) found the overall prevalence of osteoporosis in the elderly women of the world is 35.3%.

Zhang *et al.* (2020) study detected that female gender is an independent risk factor for osteoporosis in the elders. This increase may be related to a decrease in postmenopausal estrogen in women

Salari *et al.* (2021) detected the prevalence OP disease increases and become a serious problem among the elders.

Ma *et al.* (2018) suggested the women over age of 50 are 5 times more likely to develop osteoporosis than the normal population.

Al-Swat, (2017) discovered that males are typically less likely to get osteoporosis and to seek treatment for fractures, and the women frequently get fractures 5–10 years earlier than men may be attributed to the differences in bone size between the sexes, diet and weight, and the degree of physical activity.

4.2.2 Distribution of Osteoporosis According to Duration of Disease

The higher percentage of osteoporosis duration was recorded in women with 1-3 years of disease (61.42%), from 4-7 years of disease (22.85%), from 8-11 years of disease (17.14%) and from 12-15 years of disease (4.28%), (table 4-2) and (appendix 17).

Table (4-2): Duration of OP disease among the patients

Duration of Disease	Patients, N=70	
	No. of Patients	% percentage
From 1-3 years	43	61.4
From 4-7 years	16	22.9
From 8-11 years	8	11.4
From 12-15 years	3	4.3
**P≤0.01= High significant		

The present study revealed that most of patients who joined in this study were suffered from sever osteoporosis and the duration of disease ranged between 1-3 years and 4-7 years. The results suggested that the early years of disease and insufficient period of treatment may be the reasons leaded to the percentages increased.

However, in this study, the bone fractures ranged between mild to severe and the bone was required splinting or a casting. Fractures healing time may be depending on a few factors including; causes of broken, region of the bone that was broken, type of fracture, which treatment required and if there are some injured experienced occur.

However, Age increasing in elder women possibly rises the risk of osteoporosis and this attributed may not only to hormonal deficiency in postmenopausal women but also to a decreasing in bone wall thickness and an increase in bone resorption. In addition, a healthy lifestyle is essential for keeping the bones strong, low levels of physical activity and prolonged periods of inactivity can contribute to an increased rate of bone loss.

4.2.3. Distribution of Osteoporotic Patients According to BMI and Classes of Obesity

The results showed highly significant ($P \leq 0.01$) increased in the mean ± SE of BMI in OP patients (34.22 ± 0.86 kg/m^2) as a comparison with control (24.83 ± 0.43 kg/m^2). That elevation was noticed in the OP patients who had overweight, obese class I, II and III. Approximately 18.57% was recorded in patients with overweight, 37.14% in patients with obesity class I, 25.71% in patients with obesity class II and 14.28% in patients with obesity class III. While the lowest one was 4.28% in patients with normal weight, (table 4-3) and (appendix 18).

Table (4-3): Distribution of OP patients according to BMI

BMI groups	Patients , N=70		
	BMI values kg/m^2	No.of Patients	% Percentage
Normal	18.5-24.9	3	4.3
Overweight	25-29.9	13	18.6
Obese classI	30-34.9	26	37.1
Obese classII	35-39.9	18	25.7
Obese classIII	≥40	10	14.3
**P≤0.01= High significant			

World Health Organisation (WHO) considered the person with a weight 30 kg/m^2 BMI or higher had obese, while a person with a BMI between 25- 29 kg/m^2 had overweight (Gkastaris *et al.,* 2020). The study's findings were matched with those of Gkastaris *et al.* (2020) and Wang *et al.* (2022), who found that obesity increases adipocytes and adipose tissue, which may be linked to OP. Obesity is also linked to low-grade chronic inflammation, with high levels of CRP, TNF-α, and IL-6, which may contribute to bone loss.

A study by López-Gómez *et al.* (2016) found that there may be a bidirectional relationship between obesity and bone mass. Obesity can either benefit or impair bone metabolism. Obesity is related with pro-inflammatory cytokines (e.g. IL-6, TNF-α) and adipocytokines (e.g. adiponectin, leptin), which have been proven to negatively impact bone health.

Meyer *et al.* (2016) detected that abdominal fat increases the risk of hip fractures. However, El-Miedany *et al.* (2024) observed that overweight and obese patients had comparable levels of OP. Post-menopausal women showed a statistically significant difference, whereas men did not.

In contrast, there are other studies was having a nother opinion and disagreed with the study findings as a study by Liu *et al.* (2023) suggested the overweight and obesity were associated with lower odds of developing OP, whereas underweight was

associated with higher odds and the central obesity did not show a significant association with osteoporosis.

Ha and Baek's (2020) study detected that a BMI between 23.0 and 24.9 kg/m2 is the ideal range for reducing the risk of type 2 diabetes and osteoporosis. Higher BMI reduces the risk of OP but increases the risk of developing type 2 diabetes. If the BMI falls below this level, the condition reverses. According to Kurniawan *et al.* (2018) study noticed that the greater BMI is associated with type 2 diabetes but not OP due to a strong correlation between fat mass and insulin resistance.

Obese postmenopausal women in this study had a higher risk of fractures that contribute to OP, including humoral, ankle, lower limb, hip, pelvic, and wrist fractures, than thinner women. The study's findings hypothesized that numerous factors may overlap and operate together as risk factors, progressively leading to OP illness. Obese individuals may have adequate vitamin D and calcium stores, but a decrease in 25-OH vitamin D circulation levels due to vitamin sequestration in adipose tissue may impair bone formation and quality (architecture). Usually vitamin D deficiency is typically accompanied by greater dietary phosphorus levels, which may be connected with increased PTH levels and may independently alter bone metabolism.

The second factor could be adipocyte, adipose tissue, and adipokine accumulation. Obese individuals may experience low-grade chronic inflammation, including immune cell activation, elevated proteins, and interleukins, due to their large fat stores in specific tissues. This inflammatory reaction may cause rapid bone loss and is linked to OP disease. Other investigations provide scientific support for this proposal. Leptin can reduce serotonin levels in hypothalamic neurons, leading to decreased bone production. The hypothalamus neuropeptide regulates food consumption and energy, as well as bone remodeling (Gkastaris *et al.,* 2020; Rinonapoli *et al.,* 2021).

According to Ali *et al.* (2020) study, obesity increases the risk of various diseases, including cardiovascular disease (CVD), osteoarthritis, musculoskeletal disorders,

gall stones, psychological disorders, psychosocial problems, sleep apnea, diabetes, hypertension, liver disease, and certain cancers. These diseases often shorten people's lives.

Yang *et al.* (2019[b]) suggested that the experimental studies which achieved in *vivo* on the mice with adiponectin-deficiency showed a reduction in bone mass and increasing in adiposity. While in vitro research, Chen *et al.* (2018) found that adiponectin inhibits osteoclastogenesis by modulating the RANKL/OPG ratio and decreasing the NF-kB and p38 signaling pathways, which are necessary for osteoclast formation.

Lyu *et al.* (2023) suggested that approximately 24.7% of obese postmenopausal women with OP may have diabetes or prediabetes in the freedom trial. Whereas, in a study by Chen *et al.* (2021), detected that obese individuals over 60 with prediabetes were found to have OP or osteopenia, with roughly 60% of women and 40% of men affected.

According to Kalra *et al.* (2022), revealed that the individuals with type 1 diabetes have reduced BMD, increasing their risk of fractures and osteoporosis. Conversely, BMD in type 2 diabetics can be deceiving.

Clinical investigations on humans have found a negative correlation between adiponectin, bone mineral density, and fracture risk. Stojanovic *et al.* (2018) found an inverse connection between adiponectin and BMD in postmenopausal women, with a reduction in adiponectin levels among those with osteoporosis.

In a large population-based study in Pomerania, Kadric *et al.* (2018) found that chemerin levels were inversely connected to bone quality in obese men and women, but not in overweight and lean individuals. The study found a favorable link between chemerin levels and OP risk.

The evidence suggested that excess fat storage and release of active adipokines (e.g., leptin, adiponectin, resistin, visfatin, and schelatin) might cause chronic problems and affect WBC, ESR, CRP, and TNF-α blood serum levels. Obese persons

may develop chronic diseases such as hypertension and diabetes. Inflammation can disrupt the balance of osteoclasts and osteoblasts, leading to OP disease. The inflammation has possibly detrimental effect directly or indirect on bone which can causes an increasing in the risk of fractures and complications lead to osteoporotic-enhanced inflammation and reflex on several alterations in titers of specific serum's molecule, specific proteins and immune cells activation (Jafaripour *et al.,* 2020).

4.3. CBC in OP Patients and Control

In CBC results, highly significant (P≤0.01) decrease noticed in a WBCs count (6.30±0.18 k/mcL) and HGB (9.900±0.132 g/dL) in OP patients as a compare with the control group, WBCs was (8.26±0.27k/mcL) and HGB was (13.321±0.287 g/dL), whereas, highly significant (P≤0.01) increase was recorded in ESR (43.73±1.76 mm/hr), PLTs (437.88±7.00 $10^8$3/uL) in OP patients in compare with control, ESR (21.10±1.25mm/hr), and PLTs (226.79±9.39 $10^8$3/uL), (table 4-4) and (appendix 19, 20, 21 and 22).

Table (4-4): CBC value in OP patients and control

	Mean ±SE			
Group	**WBCs (k/mcL)**	**HGB (g/dL)**	**ESR (mm/hr)**	**PLT ($10^8$3/uL)**
Patients	**6.30±0.18**	**9.900±0.132**	**43.73±1.76**	**437.88±7.00**
Control	**8.26±0.27**	**13.321±0.287**	**21.10±1.25**	**226.79±9.39**
T-test	**1.09****	**2.77****	**8.95****	**18.056****
P-value	**0.0001****	**0.0001****	**0.0001****	**0.0001****
****P≤0.01= High significant**				

Kim *et al.* (2011) discovered a significant reduction in platelet count in postmenopausal women from Seoul, Korea. Although Li *et al.* (2022) found that osteoporotic patients and mice had higher WBC counts in their peripheral blood, there was no change in other blood cell indices between the two groups and these findings are consistent with the current investigation.

The current study supports Ganidağlı *et al.* (2023) finding which noticed that greater ESR levels are related with OP.

A study by Chen *et al.* (2023) found that platelets are cytoplasmic fragments formed from megakaryocytes that play significant roles in bone homeostasis, production, and resorption. They propose that in cases of bone loss, the body may require more platelets than lymphocytes.

Recent investigations suggest a complex link between hematopoiesis and bone homeostasis in typical stable states. Studies by Pinho and Frenette (2019) and Pissarra *et al.* (2021) detected that bone marrow creates a particular responsible for maintaining bone homeostasis and forming blood cells.

Aprile *et al.* (2020) found that hematopoiesis regulates a specific microenvironment that affects bone and blood physiology.

The present study examined the blood parameters in postmenopausal women with OP diseases and identified two major findings. The first one is ESR and PLTs percentages remained dependently associated with the presence of OP. Second, WBCs and HGB were negatively associated with BMD of the spine and hip areas.

4.4. Levels of CRP, RF, and ACPA in Osteoporotic Patients and Control.

Highly significant (P≤0.01) increase was recorded in CRP (18.26±1.33 pg/ml), RF (IU/ml) was positive and ACPA (23.26±2.74EU/ml) in OP patients, respectively, when compared with control, CRP (2.92±0.18 pg/ml), RF (IU/ml) was negative, and ACPA (6.26±0.56 EU/ml), respectively. (Table 4-5) and (appendix 23, 24, and 25).

Table (4-5): Serum levels of CRP, RF, and ACPA in osteoporotic patients and control.

Groups	Mean±SE		
	CRP (pg/ml)	RF (IU/ml)	ACPA (EU/ml)
Patients	18.26±1.33	Positive	23.26±2.74
Control	2.92±0.18	Negative	6.26±0.56
T-test	8.617**		4.652**
P-value	0.0001**		0.0001**
**P≤0.01= High significant			

Several studies agreed with the current findings such as Ye *et al.* (2022) indicate a link between elevated CRP levels and reduced BMD levels.

Wang and He, (2020) and Tao *et al.* (2021) found that elevated CRP levels can increase the expression of inflammatory markers such as IL-6 and TNFα. These markers can enhance osteoclast activity and suppress osteoblast function, leading to decreased bone mass and structural impairment.

Huang *et al.* (2023) observed that higher CRP levels were associated with decreased BMD and bone strength in the femoral neck.

Khinda *et al.* (2022) cross-sectional investigation discovered that the postmenopausal women with osteopenia and/or OP have considerably higher CRP levels than women with normal BMD.

Little-Letsinger (2023) found that serum high-sensitivity C-reactive protein (hsCRP) is associated with low BMD, accelerated bone resorption, bone loss, and increased fracture risk.

Mun *et al.* (2021) found risk ratios of 1.54-1.57 for those with the highest tertile of hsCRP in a meta-analysis of research on the connection between hsCRP and fracture risk.

Yan *et al.* (2024) discovered a significant negative connection in CRP-TBS Townes-Brocks syndrome. CRP is a commonly used blood measure in clinical settings. Combining it with BMD can increase fracture risk assessment precision.

The current study contradicted Ilesanmi-Oyelere *et al.* (2019) finding that postmenopausal women with OP have lower CRP levels than those with osteopenia or normal BMD.

Damani *et al.* (2022) found that postmenopausal women with OP, osteopenia, or normal BMD had similar CRP levels, despite having decreased body mass.

Greendale *et al.* (2021) detected that within-woman increases in CRP during premenopause, early perimenopause, and late postmenopause indicate quicker BMD reduction in the next ~2 years. However, the magnitude of the influence on BMD is minor. Changes in CRP in the BMD pathway are unlikely to significantly impact fracture risk in women with similar characteristics to those investigated. CRP may affect bone turnover, size, and microarchitecture, potentially increasing the risk of fractures.

Moreover, this study investigated an elevation in RF and ACPA levels in OP patients as compare with healthy control, this evidence was agreed with Kocijan *et al.* (2017) and Llorente *et al.* (2017) suggested that OP may be linked to auto-antibodies such ACPA and RF in RA, in addition to inflammation. Untreated patients with early RA with ACPA showed significantly higher levels of receptor activator of nuclear factor kappa-B ligand in serum and synovial fluid compared to those with negative results. ACPA may cause localized bone loss and erosion, leading to an increase in osteoclasts.

Bugatti *et al.* (2016) observed that RF was linked to decreased BMD in the lumbar and hip areas, but only at high titers. Even at modest RF titrations, ACPA had a detrimental effect on the lumbar Zscore. Sargın *et al.* (2019) discovered that having ACPA and RF is linked to decreased systemic BMD, particularly in the femoral neck.

Monitoring OP in RA patients with seropositive status may be a more appropriate method.

Hwang *et al.* (2019) observed that individuals with RF had reduced BMD in the lumbar spine, even after controlling for potential confounding variables. Epidemiological research suggests that RF positive can negatively impact bone metabolism.

Furthermore, Kareem *et al.* (2021) found that RA is a significant risk factor for OP. Factors such as long-term glucocorticoid treatment, chronic joint inflammation, calcium malabsorption, age (post-menopausal women and older men over 50 years), genetics, and the estrogen hormone all increase the risk of OP and fractures in RA patients.

4.5 Concentration of IL-8, IL-17 and IL-22 in OP Patients and Control

Interleukin-8, IL-17 and IL-22 displayed a remarkable highly significant ($p \leq 0.01$) increased in osteoporotic patients as a compared to the control group. Highly significant ($P \leq 0.01$) increase was recorded in IL-8 serum level (249.08 ±19.98ng/ml), IL-17 (107.862 ± 9.25ng/ml), and IL-22 (57.73 ± 2.47 ng/ml), respectively in OP patients as compared with the healthy control group were IL-8 (65.29 ± 5.62 ng/ml), IL-17 (46.135 ± 8.66 ng/ml) and IL-22 (13.57 ± 3.16 ng/ml), respectively, the findings pertaining to the measurements of interleukins are succinctly summarized in, (table 4-6) and (appendix 26).

Table (4-6): The concentrations of IL-8, IL- 17 and IL- 22 in OP patients and control

Group	Mean±SE		
	IL-8 (ng/ml)	IL-17 (ng/ml)	IL-22 (ng/ml)
Patients	249.08 ±19.98	107.862 ± 9.25	57.73 ± 2.47
Controls	65.29 ± 5.62	46.135 ± 8.66	13.57 ± 3.16
T-test	6.81**	4.42**	10.88**
P-value	0.0001	0.0001	0.0001
** P≤0.01= High significant			

In this study and as a comparison between patients and control groups noticed an elevation in IL-8, IL-17 and IL-22 in OP patient serum levels were recorded and this notification agreed with several previous and recent studies and suggested that IL-8 might stimulate both osteoclastogenesis and bone resorption.

Hwang *et al.* (2019) found that IL-8 enhances osteoclast development, driven by RANKL, in an autocrine way. In laboratory settings, this effect can be mitigated by employing IL-8-specific antibodies or IL-8 receptor inhibitors. Human osteoclasts produce large levels of IL-8.

Xu *et al.* (2023) discovered that IL-8 generated by osteoblasts stimulates RANKL-induced osteoclastogenesis in an autocrine manner and can be blocked in vitro by anti-IL-8 antibodies or IL-8 receptor inhibitors. Although, human osteoclasts produce high quantity of IL-8.

Lam *et al.* (2021) proposed that pro-inflammatory biomarkers such as IL-1β, IL-6, IL8, and TNF-α have an inverse connection with BMD measures, indicating increased osteoclast activity. They suggested that these indicators may be connected with bone loss over time.

Kitaura *et al.* (2020) found that RA patients with ACPA have higher levels of IL-8 in their circulation. ACPA may attach to the progenitor cells of bone-resorbing osteoclasts are stimulated to differentiate. The application of an IL-8 neutralizing

antibody reduced ACPA-induced osteoclast development, highlighting the critical involvement of IL-8 in this process. The study found that IL-8 increases osteoclast differentiation and osteoblast-mediated osteoclastogenesis by increasing the expression of RANKL, a major regulator of the process.

According to Amarasekara *et al.* (2021) detected that IL-8 activates RANK-mediated NFATc1 by acting as an autocrine regulator of osteoclastogenesis.

According to Sousa *et al.* (2016) considered that the rising in IL-8 has been linked to postmenopausal women's increased bone resorption and resulting lower bone density. This implies that by encouraging osteoclast activation, IL-8 actively contributes to bone metabolism.

The systemic inflammatory environment may be a mechanism driving RANKL expression by neutrophils in COPD, according to recent findings by Xu *et al.* (2023), which also showed that IL-8 raises plasma levels and is linked to an increase in RANKL neutrophils in COPD patients. Since neutrophils produce RANKL, it is hypothesized that they contribute to osteoclastogenesis, which increases bone loss associated with COPD. These results demonstrate the role of IL-8 in bone metabolism and point to IL-8 targeting as a possible treatment or prophylactic approach for bone disorders. However, more investigation and clinical trials are required for IL8-targeted therapeutic approaches due to the intricate and reciprocal effects of IL-8.

Bhadricha *et al.* (2021) demonstrated that IL-17 levels were higher in active people with OP illness, the results of this study show a noteworthy and highly statistically significant increase in IL-17 in those suffering from osteoporosis. Higher Th17 cell counts result in higher IL-17 production. Consequently, this promotes the production of osteoclasts, the cells that break down bone. Furthermore, macrophages release other inflammatory cytokines such IL-6, IL-1, and TNF-α in response to elevated IL-17. These inflammatory chemicals have a well-established ability to interfere with the natural remodeling process of bones, which eventually leads to reduced bone density.

According to research by Shamsuldin *et al.* (2020) and El-Mallah *et al.* (2021), found that people with OP had significantly higher levels of IL-17 and significantly lower levels of estradiol than people without the condition at ($P<0.01$).

According to Barbulescu *et al.* (2019), IL-17 and IL-23 may upset the equilibrium of bone formation by influencing osteoclasts directly or indirectly, resulting in extensive bone loss and accelerating bone erosion.

Cheng *et al.* (2022) found that the extremely inflammatory cytokine IL-17 causes cartilage matrix to be destroyed and activates OCs. Today, IL-17 is thought to have a significant role in Th1 cell secretion in the joint's synovium. Between Th1/Th2 cell subsets, there is mutual inhibition and dynamic equilibrium. When this dynamic balance is upset, the body will become diseased because it will be in a Th1/Th2 drift state where either Th1 or Th2 is dominating.

According to the current investigation, OP patients had noticeably higher serum levels of IL-22. When compared to people in good health, the condition is more common among patients. According to a study by Jethwa and Bowness (2016), IL-17A and IL-17F may not be very potent inflammatory cytokines by themselves; rather, their capacity to draw immune cells is what gives them their notable inflammatory effects. Furthermore, the synergistic association they have with other pro-inflammatory cytokines like TNF, IL-1β, IFN-γ, GMCSF, and IL-22 is strongly linked to their strong effects.

Remarkably, Kim *et al.* (2012) discovered that fibroblast-like synoviocytes can express significant quantities of RANKL, which can then trigger the activation and differentiation of osteoclasts. This is comparable to TNF-α, IL-22, IL-1, IL-6, and prostaglandin E2 (PGE2).

In inflammatory settings, El-Zayadi *et al.* (2017) demonstrated that IL-22 influenced human MSC migration and proliferation, with MSC osteogenesis only taking place when IFN-γ/TNF was absent.

The study by Min *et al.* (2020) demonstrated that IL-22 and IL-25 were correlated in the plasma and synovial fluid samples of RA patients. Taking into account IL-25's regulatory role and the previously mentioned correlation with IL-22, IL-25 may be upregulated in response to pathogenic cytokines like TNF-α, IL-17A, and IL-22 and counteract the effects of the pro-inflammatory cytokines.

4.5.1 Receiver Operating Curve Characteristic (ROC) Analyses

The serum levels of IL-8, IL-17, and IL-22 may be able to reliably identify patients with OP, according to ROC analysis. Area under curve (AUC) of IL-8 = 0.925, 95% CI = 0.882-0.969, p-value = 0.001, cut-off values of IL-8 = 150.05, sensitivity = 80% and specificity = 100%), and AUC of IL-17 = 0.997, 95% CI = 0.991-1.0, p-value = 0.001, cut-off values of IL-17 = 49.11, sensitivity = 85% and specificity = 100%), respectively, the Area under curve (AUC) of IL-22 = 0.997, 95% CI= 0.990- 1.0, p- value= 0.001, cut-off values of IL-22= 38.39, sensitivity= 80% and specificity= 100%). Table (4-7) and Figures (4.1, 4.2, 4.3).

Table (4-7): Receiver operating curve characteristic (ROC) analyses of interleukins-8, -17 and -22 in serum to identify clinical abatement

Test variables	AUC	Probability	Sensitivity	Specificity	Cut-off value
IL-8	0.925	0.001	0.80	1.0	150.05
IL-17	0.997	0.001	0.85	1.0	49.11
IL-22	0.997	0.001	0.80	1.0	38.39

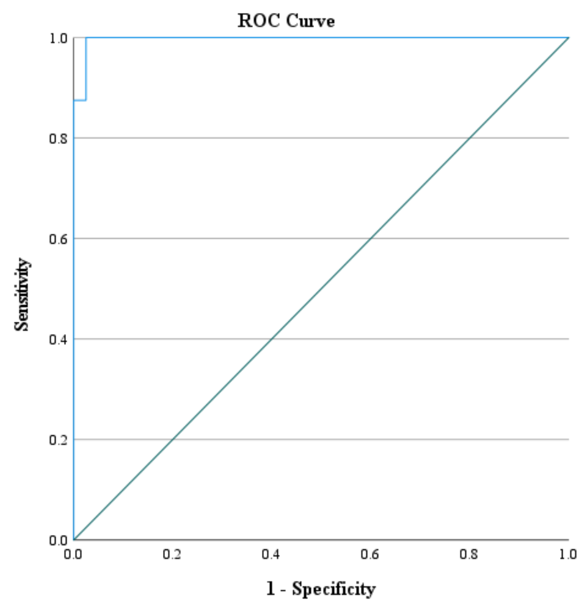


Figure (4.1): ROC curve of IL-8 level in individuals with OP (area under the curve = 0.925; 95% confidence interval = 0.882 – 0.969; *p* ≤ 0.001; sensitivity = 80%; specificity = 100%).

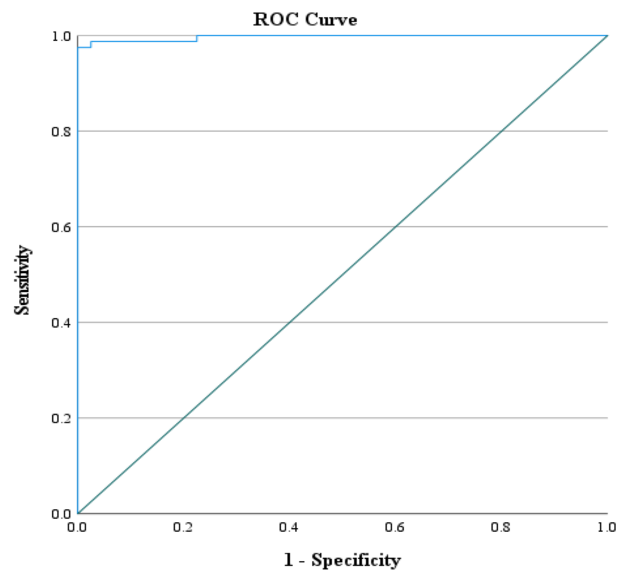


Figure (4.2): ROC curve of IL-17 level in individuals with OP (area under the curve = 0.997; 95% confidence interval = 0.991 – 1.0; *p* ≤ 0.001; sensitivity = 85%; specificity = 100%).

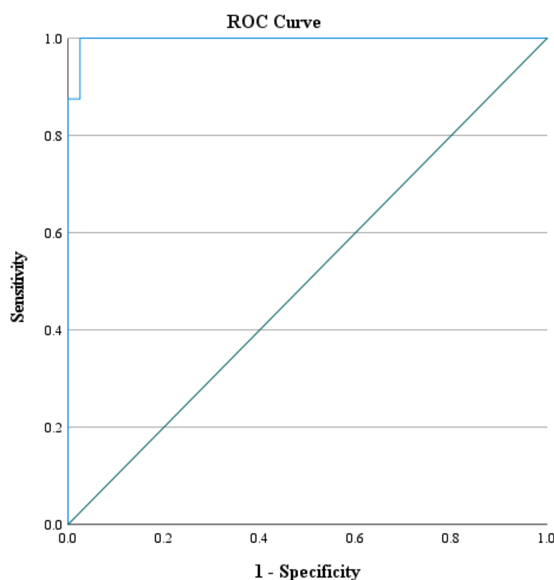


Figure (4.3): ROC curve of IL-22 level in individuals with OP (area under the curve = 0.997; 95% confidence interval = 0.990 – 1.0; *p* ≤ 0.001; sensitivity = 80%; specificity = 100%).

Data of ROC analyses for interleukins-8, -17, and -22 appeared that it is a potentially useful diagnostic biomarker for OP disease in postmenopausal women. The sensitivity and specificity of IL-8 in serum were found to be 80% and 100%, respectively, for differentiating between IL-17, 85% and 100%, and the sensitivity and specificity of IL-22 in serum were found to be 80% and 100%, respectively.

Additionally, IL-8,-17 and -22 have demonstrated potential in predicting flare outcomes in OP patients. The results of this investigation demonstrated a statistically significant positive link between the levels of IL-8,-17, -22 and OP.

4.6 Level of PTH and Ca^{+2} in OP Patients and Control

However, the current investigation showed a highly significant (P≤0.01) increase in PTH serum level between mean ± SE of OP patients group (77.80±1.79pg/mL) and control group (35.81±2.06pg/mL), whereas a highly significant (P≤0.01) decrease in Ca^{+2} serum level was recorded in the mean ± SE of OP patients (4.89±0.21 mmol/L) and control group (9.63±0.13 mmol/L). (Table 4-8) and (appendix 27 and 28).

Table (4-8): Serum levels of PTH and Ca^{+2} in osteoporotic patients and control.

Groups	Mean±SE	
	PTH (pg/ml)	Ca^{+2}(mmol/L)
Patients	77.80±1.79	4.89±0.21
Control	35.81±2.06	9.63 ± 0.13
T-test	14.75**	2.53**
P-value	0.0001**	0.0001**
**P≤0.01= High Significant		

However, this present study agreed with Qu *et al.* (2024) found that parathyroid glands typically secrete PTH to maintain calcium homeostasis when the concentration of extracellular calcium drops. PTH is produced by accelerating bone resorption to release calcium and phosphorus into the circulation, increasing calcium reabsorption in the renal tubules, and encouraging the kidneys' production of active vitamin D.

Jilka *et al.* (2010) and Silva and Bilezikian, (2015) studies revealed that PTH can promote bone formation at low dosages, while it can activate bone resorption and lead to net bone loss at high dosages.

Zahmatkesh, (2023) suggested that a high level of PTH can cause long-term bone resorption, which stimulates osteoclasts, bone-removing cells and decreases bone density. Studies by Akber and Yenzeel, (2023) and Marques and Moreira, (2023) suggested that PTH hypersecretion causes hyperparathyroidism, a disorder that affects calcium metabolism. Abnormal PTH elevation can increase blood calcium levels.

According to Lou *et al.* (2018) study detected that PTH is the first bone anabolic medication approved to treat OP and prevent fracture healing delays caused by age.

Zhao-Nan *et al.* (2019) discovered that high-risk populations, including the elderly, malnourished, and postmenopausal women, have a better chance of fracture therapy with PTH.

Shabnam *et al.* (2021) found that all ill patients, regardless of gender or age group, had lower Ca^{+2} levels in their blood compared to controls. During OP and osteopenia, blood Ca^{+2} levels were found to be associated with OP. Determining this metal may aid in clinical examination and diagnosis of osteoporosis.

Overactive thyroid glands or thyroid abnormalities can impact bone turnover, while pituitary gland disorders can lead to OP. Chronic elevated PTH levels can be caused by benign tumors in the parathyroid glands or CKD (English *et al.,* 2024). PTH promotes bone resorption by boosting RANKL expression and osteoblast activity (Magagnoli *et al.* 2024).

As a looking back to present data found that PTH level was greater and Ca^{+2} level was lower in OP patient, in contrast with the group that is not afflicted. According to a previous studies were done by Kuzma *et al.* (2021) found that bone remodeling involves a closely knit community of osteoblasts and osteoclasts that alternately produce new bone and resorb existing bone. $Ca_{10}(PO_4)_6(OH)_2$ is one of the main building block in the bone matrix that which provide the structural integrity and calcium- phosphate reservoir. Parathyroid hormone secreted as a response to hypocalcemia; when extracellular calcium level is low and phosphate level is high, PTH will release into blood circulation and stimulate bone resorption via osteoblast release of RANKL, which considered as osteoclastogenesis drivers. When Ca^{2+} binds with Ca^{2+} sense receptor (CaSR) in parathyroid glands, PTH release and stimulates the kidney to reabsorb Ca^{2+} (together with magnesium) and inhibit the re-absorption of phosphate and bicarbonate. As response to PTH activation, both calcium and phosphate are released to the blood during bone resorption, while Ca^{+2} and PO_4 deposit into bone during bone formation.

Decreasing number and expression of CaSR in hypertrophied parathyroid glands may be related to the proliferation of parathyroid tissue. Calcium balance is

wholebody calcium retention or deficit total calcium inputs subtract total body loses. A positive balance may increase vascular calcification and cardiovascular events, while a negative balance may increase the risk of OPand fracture (Hsu *et al.,* 2020).

However, continuous elevation in PTH serum level secretion and Ca^{+2} releases from the bone might lead to an increase in net bone resorption, along with other factors such as RANKL increase and OPG (decoy receptor) decrease in osteoblasts and osteocytes. Both calcium and PTH possibly have a complementary role in bone homeostasis management and an inverse correlation with OP development and any imbalanced occurs in bone remodeling can leads to abnormality in bone formation. The individual with OP will have increased in the rates of bone resorption and impaired in osteogenesis.

A study by Rejnmark *et al.* (2020) found that PTH increases osteoblast activity via the Wnt/beta-catenin signaling pathway. PTH's effects on bone are complex because it has both catabolic and anabolic properties.

This study showed that several biomarkers may be indicative OP development. An elevation in bone matrix biochemicals (OPN and OCN), and interleukins secretion for a long-term specific inflammation may lead to increased fragility beside to age advance, sex, Ca^{+2} content deficiency, PTH level elevation and medication statement.

4.7 Concentration of OPN, OCN and ON in osteoporotic Patients and Control

Highly significant ($P \leq 0.01$) increase was recorded in OPN serum level (16.88±0.77 ng/ml) and OCN (29.86±2.25ng/ml) in OP patients as compared with the healthy control group, whereas OPN level (5.95±0.41ng/ml) and OCN level (10.73±0.86ng/ml) in the control group. In contrast, ON serum level recorded a highly significant ($P \leq 0.01$) decrease in the patient group (1.78±0.971ng/ml) compared with the control group (3.42±0.20ng/ml). (Table 4-9) and (appendix 29).

Table (4-9): The concentrations of OPN, OCN and ON in osteoporotic patients and control

Group	Mean ± SE		
	Osteopontin (ng/ml)	Osteocalcin (ng/ml)	Osteonectin(ng/ml)
Patients	16.88 ± 0.77	29.86 ± 2.25	1.78 ± 0.971
Control	5.95 ± 0.41	10.73 ± 0.86	3.42 ± 0.20
T-test	5.81**	6.215**	1.22**
P-value	0.0001	0.0001	0.0001
** P≤0.01= High significant			

This work supported by Si *et al.* (2020) findings which found that high levels of OPN are a risk factor for OP, positively regulating OCs and inhibiting bone mineral deposition. A lack of OPN can increase fracture sensitivity in OP patients.

Shamsulddin *et al.* (2020) found a substantial rise in OP levels in OP patients compared to controls, with a weak inverse connection.

Jian-Chun *et al.* (2020) found that serum levels of OPN and β-CTX are independent indicators of hip fracture risk. Elevated serum levels of OPN and β-CTX have been linked to a higher risk of hip fracture among the elderly.

Kim *et al.* (2018) discovered that OPN can impact the adherence and dissemination of OCs. OPN activates PLCγ, which releases Ca^{+2} and increases free Ca^{+2} levels in the cytoplasm. The Ca^{+2} signal causes dephosphorylation of nuclear factor of activated T cells 1 (NFATc1), revealing nuclear localization signals and promoting OC survival through transcriptional modifications.

Si *et al.,* (2020) found that postmenopausal women have greater levels of OPN, which may enhance the risk of OP fractures. Without OPN, fracture toughness might decrease by 30%, highlighting the need of OPN and it plays a crucial role in reducing fracture crack formation and maintaining bone durability.

In contrary, Bai *et al.,* (2022) suggested the low activity of OPN could increase the fractures with osteoporosis especially for those postmenopausal women.

Ali and Al-Attabi, (2022) showed that OPN and OCN play a role in their effect on the bones leading to fragility, and therefore can be used to detect and evaluate the disease.

Also, the results showed an elevation in OCN level beside to OPN in OP patients as compare with control group. This revealed was agreed with study by Hamdi, (2013) which concluded an increase in OCN serum level in postmenopausal women with OP plays an important role in development of primary OP.

Mayur *et al.* (2018) suggested that OCN is a sensitive bone formation marker that can be used to diagnose or screen for OP in post-menopausal women. Lower serum levels of OCN after menopause may contribute to OP risk.

As a result fracture risk can be predicted and reduced, Rai *et al.* (2018) found that osteocalcin is a promising diagnostic for detecting OP. Postmenopausal women with fractures have significantly lower serum OCN levels compared to postmenopausal and premenopausal women.

Alam *et al.* (2019) revealed that the serum OCN levels correlate with bone production and may serve as a better predictor of OBs function than BMD.

According to Liu *et al*. (2019) showed there is no significant difference in the pooled OCN level among postmenopausal women (PMO) and controls. In addition, OCN is not a reliable predictor of PMO's high bone turnover state.

Fasihi *et al.* (2021) found that the high levels of alkaline phosphatase and serum OCN in postmenopausal women contribute to early bone density loss among middle-aged women. These factors can predict bone density and identify women aged 35-65 who are at risk for OP.

In CKD patients, OP severity was determined independently by age, female sex, intact PTH, and serum OCN levels. A multivariate linear regression model shows a

favorable correlation between serum OCN levels and intact PTH. OCN may be a bone turnover marker in people with CKD (Chi *et al.,* 2022).

Furthermore, the current study recorded decline in ON level in OP patients as comparsion with control. This result was agreed with one study only, which was achieved by Baloğlu and Özkorkmaz, (2019), depending on their data found ON may enhance bone formation by regulating collagen fiber synthesis, extracellular matrix development, osteoblast differentiation, and osteoclast activity.

According to Rosset and Bradshaw (2016), ON regulates calcium release by binding to collagen and HA crystals and influencing collagen mineralization during bone formation.

Delany *et al.* (2000) found that ON-null animals had lower collagen I content, BMD, and osteoblast/osteoclast numbers in bone, as well as impaired biomechanical qualities. ON regulates bone remodeling and helps preserve bone mass.

A correlation was noticed between high serum level of OPN, OCN and OP and osteoporotic fractures in postmenopausal women. This analysis supports the idea that serum OPN and OCN levels might be used for the early and improved detection of fracture risk, especially in postmenopausal women, with or without osteoporotic vertebral fractures.

The study found that OPN and OCN may serve as a reliable signal for better prognosis of OP in postmenopausal women. Elevated serum levels of OPN and OCN may contribute to the development of primary OP in post-menopausal women, acting as a surrogate marker. OPN and OCN fragments may stimulate osteoclast production in the bone matrix when combined with RANKL and MCSF. These fragments may play a role in osteoclast maturation, particularly during the last phases of differentiation, which can lead to bone fragility and fracture.

4.8 The Correlation Coefficient between Biomarkers

Highly positive significant correlation at ($P \leq 0.01$) was recorded between OPN, and CBC, CRP, PTH and Ca^{+2} parameters. While, a positive significant was recorded at ($P \leq 0.05$) with RF, in contrary, no significant noticed with ACPA.

Highly positive significant correlation at (P≤0.01) was recorded between OCN, and PLTs, WBCs, HGB, RF, ACPA, PTH and Ca^{+2} parameters. While, a positive significant was recorded at (P≤0.05) with CRP, and, no significant noticed with ESR.

However, non-significant correlation was observed between ON and all parameters that used in this study, (table 4-10).

Table (4-10): Correlation between bone matrix biochemicals parameters, CBC, and biochemical parameters.

Parameters		Correlation coefficient-r	P-value
OPN	CBC		
	ESR	.526**	.000**
	PLTs	.597**	.000**
	WBCs	.314**	.001**
	HGB	.528**	.000**
	Biochemicals		
	CRP	.486**	.047**
	RF	.192*	.046*
	ACPA	.157	.106 NS
	PTH	.571**	.001**
	Ca^{+2}	.529**	.001**
OCN	CBC		
	ESR	.151	.118 NS
	PLTs	.470**	.000**
	WBCs	.331**	.000**
	HGB	.395**	.000**
	Biochemicals		
	CRP	.191*	.047*
	RF	.322**	.001**
	ACPA	.308**	.001**
	PTH	.533**	.000**
	Ca^{+2}	.533**	.000**
ON	CBC		
	ESR	.014	.888 NS
	PLTs	.041	.672 NS
	WBCs	.061	.528 NS
	HGB	.044	.651 NS
	Biochemicals		
	CRP	.007	.941 NS
	RF	.055	.575 NS
	ACPA	.044	.651 NS
	PTH	.027	.722 NS
	Ca^{+2}	.013	.895 NS

**(P≤0.01)= High significant
*(P≤0.05)= Significant
N.S= non significant

The study results suggested a close connection that might be precent between hematopoietic function and bone homeostasis, but indirectly links.

Cummings and Melton (2002) found that the creation of functional blood cells depends on both hematopoietic and nonhematopoietic cells in the bone marrow, and that bone homeostasis depends on a dynamic balance between osteogenesis by osteoblasts and osteoclastogenesis by osteoclasts.

Nonhematopoietic stromal cells, such as osteoblasts and osteoprogenitor cells support the preservation of HSCs, according to research by (Morrison and Scadden, 2014) and (Bowers *et al.*, 2015).

According to De Sanctis *et al.* (2018), hematological abnormalities can result in bone deformities, improving the patients' chances of long-term survival and causing more consequences like OP.

Yuan *et al.* (2020) discovered that in an aging human population, hematological decline is accompanied with a fall in BMD. Additionally, osteoporotic patients had a markedly decreased ability for macroautophagy in the hematopoietic system, which resulted in OP.

Osteopontin was shown to play a part in bone remodeling, migration, adhesion, and survival according to Smane and Pilmane (2016) study, OPN deficiency has been shown in animal models to modify the function of several cell types, leading to late bone remodeling, changed matrix architecture, and delayed early vascularization.

Moreover, a remarkable correlations at ($P \leq 0.01$) were noticed between IL-8, and ESR, PLTs, HGB, CRP, PTH and Ca^{+2} parameters, and a significant correlation at ($P \leq 0.05$) was observed bwtween IL-8 and WBCs. Whereas no significant correlation was detected between IL-8, RF, and ACPA.

In addition, a positive correlation at ($P \leq 0.01$) between IL-17, and ESR, PLTs, CRP, PTH and Ca^{+2} parameters, while, non- significant correlation was observed between IL-17, WBCs, HGB, RF and ACPA.

Furthermore, a positive correlation at (P≤0.01) was detected between IL-22, and all parameters that performed in this study. (Table 4-11).

Table (4-11): Correlation between immunological, CBC, and biochemical parameters

Parameters		Correlation coefficient-r	P-value
IL-8	CBC		
	ESR	.491**	.000**
	PLTs	.453**	.000**
	WBCs	.190*	.049*
	HGB	.444**	.000**
	Biochemicals		
	CRP	.299**	.002**
	RF	.124	.200 NS
	ACPA	.149	.123 NS
	PTH	.415**	.000**
	Ca^{+2}	.385**	.000**
IL-17	CBC		
	ESR	.312**	.001**
	PLTs	.306**	.001**
	WBCs	.147	.128 NS
	HGB	.247**	.010 NS
	Biochemicals		
	CRP	.886**	.000**
	RF	.047	.630 NS
	ACPA	.082	.397 NS
	PTH	.288**	.003**
	Ca^{+2}	.278**	.004**
IL-22	CBC		
	ESR	.458**	.000**
	PLTs	.649**	.000**
	WBCs	.272**	.004**
	HGB	.508**	.000**
	Biochemical		
	CRP	.420**	.000**
	RF	.283**	.003**
	ACPA	.288**	.003**
	PTH	.620**	.000**
	Ca^{+2}	.643**	.000**

**(P≤0.01)= High significant
*(P≤0.05)= Significant
N.S= non-significant

The interleukins-8,-17 and -22 were significantly correlated with most of CBC and biochemicals parameters that which examined in this study. Subsequently, IL-8

serum level was greater among PMOP patients that studied. These results hypothised the correlation between these parameters related to the presence of inflammation that was created inside the bone of OP patients. Several studies pointing out that IL-8, IL-17 and IL-22 are abled to activate the blood components that which having a roles in immunity system in a relations of respiratory burst, enzyme discharge, superior activity, and phagocytosis, which are all crucial in proinflammatory purposes, such as Zlotnik *et al.* (1998) discovered that interleukin-8 is exclusively released during inflammatory responses and is produced by a variety of cell types, including monocytes, lymphocytes, granulocytes, fibroblasts, endothelial cells, epithelial cells, hepatocytes, mesangial cells, and chondrocytes. IL-8 is stored in the granules of platelets and is released quickly in an inflammatory environment, according to research by Balkwill and Burke, (1989).

A study by Matsushima *et al.* (2022) found that IL-8 in the first identified as a chemoattractant of neutrophils involved in acute inflammation. It was later revealed to be chemotactic for endothelial cells that play a significant role in angiogenesis. IL-8 stimulatory effect on tumor growth is facilitated by these two functions. Li *et al.* (2015) revealed that IL-17 facilitates the interaction between T cells and osteocytes because it controls the development of osteocytic RANKL, a crucial effect of PTH on osteocytes.

Nevertheless, PTH promotes the release of cytokines and growth factors by immune and bone cells. These mediators include TGFβ, IL-6, and TNF (Koh *et al.,* 2011), which guide the development of naïve CD4+ cells into Th17 cells (Sugita *et al.,* 2012; Basu *et al.,* 2013). PTH may cause Th17 cells differentiation and the cytokine IL-17A generation and may function as an upstream mediator that is essential to the PTH-induced bone loss (Li *et al.,* 2015).

Ruiz de Morales *et al.* (2021) suggested that Th17 is a source of IL-17, IL-22, IFN-γ and granulocyte-macrophage colony-stimulating factor secretion (GM-CSF) and T helper17 have been implicated in the pathogenesis of postmenopausal OP.

Interestingly, Perez *et al.* (2020) was reported several hematopoietic cell types are a sources of IL-22 production, as gamma delta T cells, NKs and innate lymphoid cells type 3 (ILC3) within innate compartment, and within the adaptive immune system, Th17 cells describe to be an important source of IL-22.

In addition, a liner correlation at (P≤0.01) was found between immunological (IL-8, IL-17, and IL-22) and bone matrix biochemical parameters (OPN and OCN), whereas, non-significant correlation between these interleukins and ON parameter. (Table 4-12).

Table (4-12): Correlation between interleukins and bone matrix biochemical parameters

Parameters		Correlation coefficient-r	P-value
Immunologicals	Bone Matrix Biochemical Components		
IL-8	OPN	.516**	.000**
	OCN	.275**	0.004**
	ON	.038	.697 NS
IL-17	OPN	.256**	.005**
	OCN	.243**	.011**
	ON	.052	.591 NS
IL-22	OPN	.473**	.000**
	OCN	.454**	.000**
	ON	.002	.987 NS

**(P≤0.01)= High significant
*(P≤0.05)= Significant
N.S= non-significant

This investigation study has reported significantly higher levels in IL-8, IL-17, IL-22 and OPN and OCN in patients with OP in compared with healthy control, and have shown a positive correlation between these parameters, but an exception with ON.

This study suggested that because of abnormality in interleukins-8,-17 and -22 that probably effect on bone remodeling imbalance, OCs activation maybe an OPN and OCN dependent manners joining with high-level expression of αvβ3 receptors on OCs surface. As depending on to the facts that were previously discovered that OPN tends to bind with αvβ3 in order to mediate OCs bone resorption.

Moorman *et al.* (2020) was reported that osteopontin is a multifunctional protein that is expressed immune system cells, fibroblasts, nerve cells, osteoblasts, osteoclasts, and endothelial cells.

According to Zhao *et al.* (2018) found that OPN has a role in a variety of physiological functions, including angiogenesis, wound healing, innate and adaptive immunological responses, and bone remodeling.

Furthermore, Tsai *et al.* (2017) observed that OCs can be activated by IL-17, leading to cartilage matrix degradation. At the moment, IL-17 is thought to have a significant role in Th1 cell secretion in the joint synovium. On the other hand, the primary ECM elements in bone construction are OPN, OCN, and ON. OPN can improve cellular immunity, suppress Th2 cells and humoral immune function, and promote Th1 cell development by binding with a similar receptor on the surface of T cells. A chain of events results in chronic inflammation and the degeneration of bone and cartilage are set off by an imbalance in Th1/Th2 cells and the amounts of released cytokines.

Raineri *et al.* (2021) detected that OPN can interact with inducible T-cell costimulatory ligand, CD44, and integrin family receptors.

Si *et al.* (2020) suggested that the osteopontin may have an impact on the ratio of Th1/Th2 cells and may also trigger Th17 cell differentiation via T cell surface receptors. These two situations may then have an impact on IL-17 levels, which in turn may promote OC activity and bone resorption, ultimately resulting in bone fracture.

According to Mizokami *et al.* (2017) study found that the process of bone resorption can increase the release of OCN into circulation by decreasing its affinity for hydroxyapatite. In addition to being a hormone that controls energy and glucose metabolism, circulating OCN's serum concentration can be employed as a biochemical marker of bone growth.

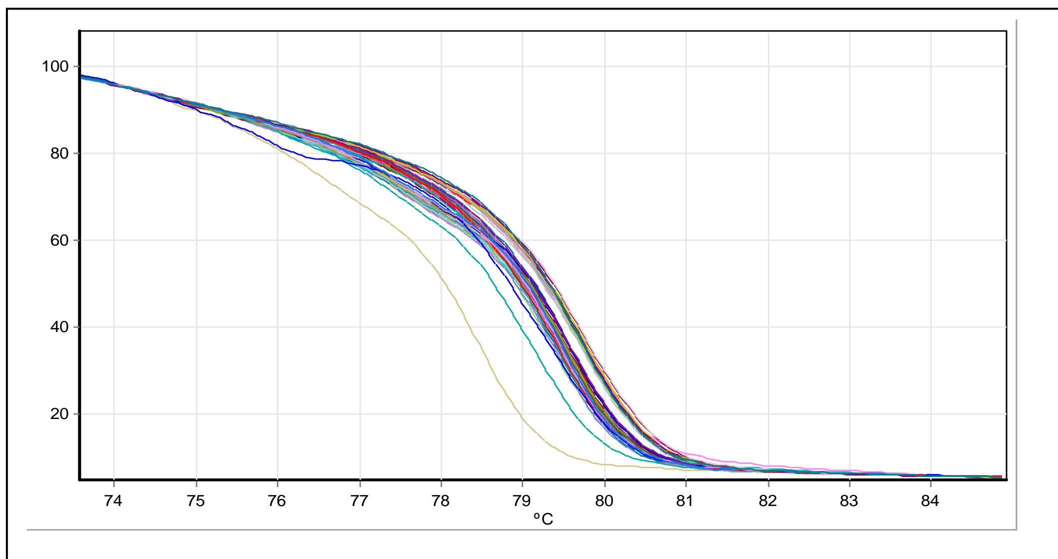
4.9 Molecular Results

4.9.1 Genomic DNA Extraction

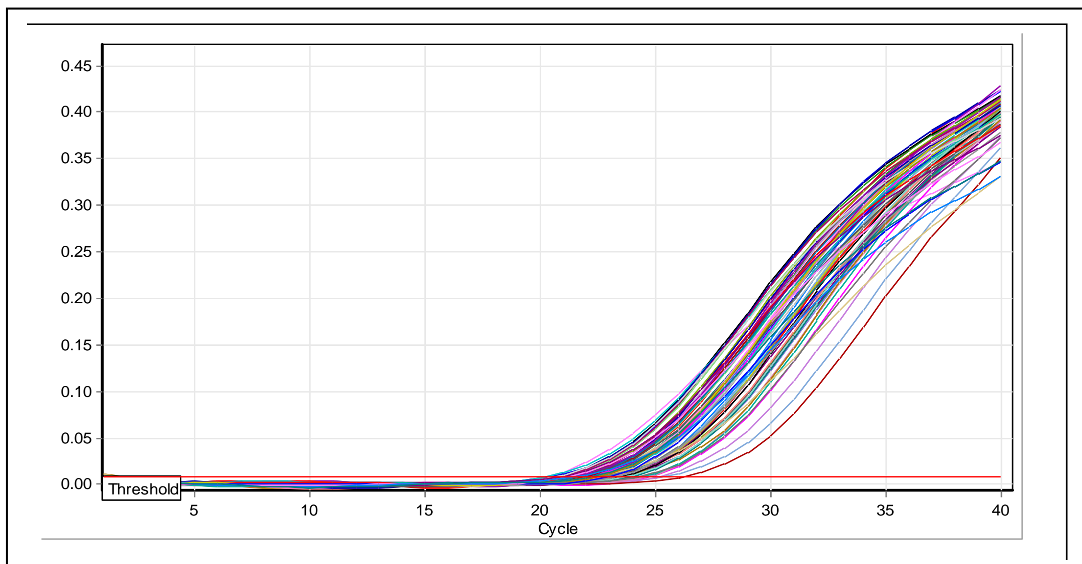
Genomic DNA was extracted from the blood samples of OP patients and control subjects by using Easy Pure Genomic DNA kit. Both DNA concentration and purity were determined by using Nanodrop spectrophotometer 2000c, samples absorbance read at 260 and 280nm wavelengths within the range1.6-1.8, suggestive that DNA sample was pure (Lucena-Aguilar *et al.,* 2016).

4.9.2 *SPP1* Genotyping by Using HRM Real-Time PCR

DNA samples of all study groups were genotyped for *rs11730582* SNP, detection was achieved by using HRM real-time PCR. Figures (4-4) and (4-5).

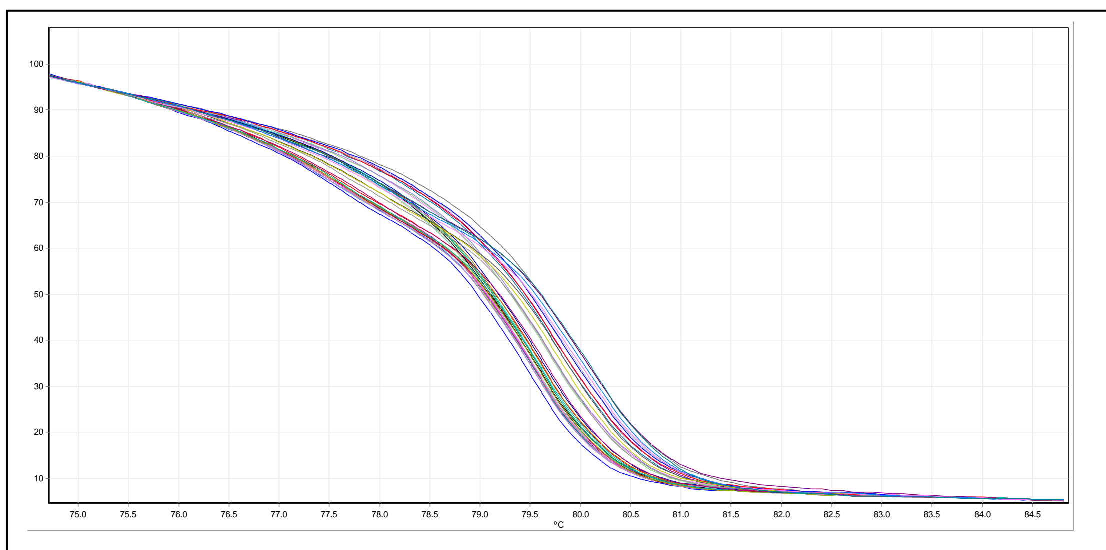


(A)

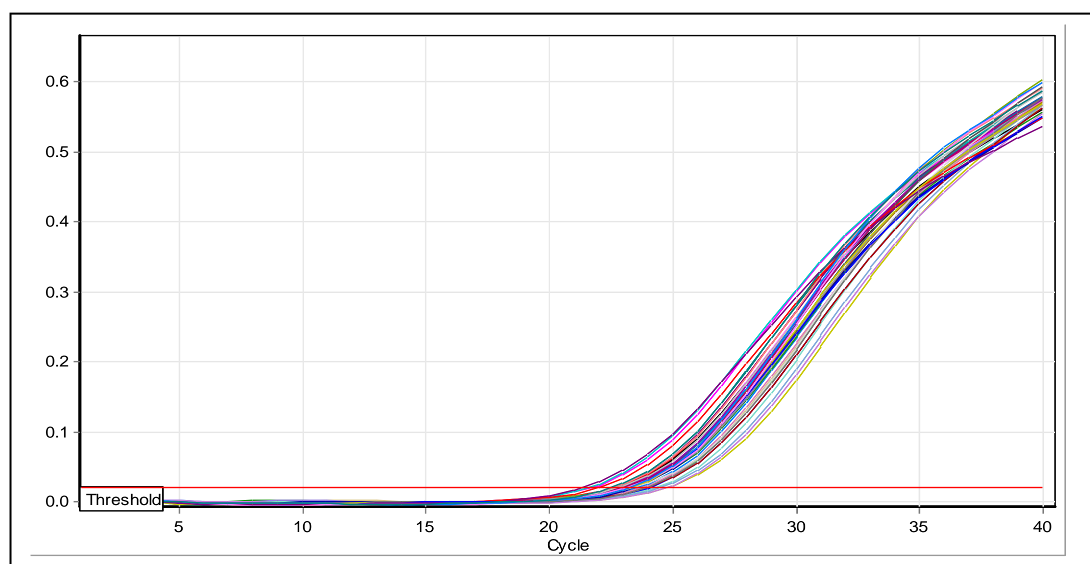


(B)

Figure (4.4): The results output of HRM for TT, TC, CC genotypes in *SPP1* SNP *rs11730582* in patients



(A)



(B)

Figure (4.5): The result output of HRM for the TT, TC, CC genotypes in *SPP1* SNP *rs11730582* in control

The resulting output of thermocycler of the HRM analysis process for *rs11730582* SNP three genotypes represented with (TT, TC and CC), and there were corresponding two alleles (T and C). Out of 110 samples, the frequency of homozygote TT genotype in patient's analysis and as reported in tables (4-13) was, (14.3%) 10/70 than control group (11/40, 27.5%).

While, the frequency heterozygote TC and homozygote mutant CC genotypes were [TC= (71.42%) 50/70 and CC= 14.3% (10/70)]. The frequencies of these genotypes

did not differ significantly from the control group, (TC= 60%, (24/40), and (CC= 12.5% 5/40) respectively. The SNP *rs11730582* showed high odds ratio (1.14) in TT and CC (1.0) genotypes which represent positive association with OP, and odds ratio for the TC was (0.9) which represent negative association with the risk of OP according to odds ratio could be considered as a protective genotype. T allele considered an etiological allele according to the odds ratio and may be related with risk of OP, while the C allele was a protective allele.

Table (4-13): Genotypes and alleles frequency for *OPN rs11730582*

rs11730582 genotyping	Control group	Patients group	OR (95% CI)	EF or PF	Probability
T	46 (58.0%)	90 (56.0%)	1.05 (0.61-1.80)	2.9%	0.891
C	34 (43.0%)	70 (44.0%)	0.95 (0.55-1.63)	2.2%	0.891
TT	11(27.50%)	10 (14.3%)	1.14 (0.49-2.66)	3.3%	0.826
TC	24 (60.0%)	50 (71.4%)	0.90 (0.42-1.94)	6.2%	0.843
CC	5 (12.50%)	10 (14.3%)	1.0 (0.32-3.11)	0.0%	1.0
Total	40 (100.0%)	70 (100.0%)			

Genotype distributions for *rs11730582* polymorphism in OP patients and controls conformed to the HWE *P*-value=0.0158 in patients whereas p=0.1500 N.S in controls. Logistic regression analyses revealed that significantly difference between *rs11730582* polymorphism in patients and controls. *rs11730582* polymorphism increase the risk of OP under the allelic model, The current study showed that TT and CC genotypes might be related with osteoporosis development and allele T may be related with the risk of osteoporosis. (Table 4-14).

Table (4-14): Expected frequency of genotypes and alleles of *rs11730582* using Hardy Weinberg Equilibrium (HWE)

SPP1 genotyping *rs11730582* frequency	Control group		Patients group	
	Observed	Expected	Observed	Expected
TT	11(27.50%)	13.23 (33.06%)	10 (14.3%)	15.31 (21.87%)
TC	24 (60.0%)	19.55 (48.88%)	50 (71.4%)	39.38 (56.25%)
CC	5 (12.50%)	7.23 (18.06%)	10 (14.3%)	15.31 (21.87%)
Total	40 (100.0%)	40 (100.0%)	70 (100.0%)	70 (100.0%)
P-HWE	0.1500		0.0158	

However, no significant differences between *OPN* gene *rs11730582* genotypes, age, race, and gender. Whereas, no significant differences noticed between the three genotypes and Mean± SE (Kg/m^2) of BMI among the individual of the same groups and between TT and TC and BMI between patients and controls, but in contrary a significant difference detected between CC and BMI, p= 0.03. (Table 4-15).

Table (4-15): The Correlation of *rs11730582* genotypes and alleles frequency and Mean± SE of BMI between OP patients and control.

SPP1 genotyping *rs11730582* and alleles frequency	BMI mean ± SE (Kg/m^2)		Probability
	Control group	Patients group	
TT	23.44 ± 1.01 ^{A}	34.59 ± 2.23 ^{A}	0.000007
TC	25.23 ± 0.56 ^{A}	34.62 ± 0.87 ^{A}	2.38 x 10^{-8}
CC	24.72 ± 0.67 ^{A}	35.28 ± 2.31 ^{A}	0.003
(Duncan test): Similar letters indicate there were no significant differences between the genetic patterns among the same group			

The current study tried to testing and understanding the correlation between *rs11730582* polymorphism and *OPN* concentrations. Among individuals of the same group, no significant differences noticed in the Mean ± SE of OPN concentrations and three *rs11730582* genotypes. Whereas high significant differences

detected between the genotypes homozygote TT and homozygote mutant CC and the Mean ± SE between patients and controls whereas p-value= 0.00372 for TT and p= 0.002 for CC, while no significant with TC. (Table 4-16).

Table (4-16): The correlation between genotyping and alleles frequency of *rs11730582* and Mean±SE of OPN concentrations between OP patients and control

SPP1 genotyping *rs11730582* and alleles frequency	Osteopontin (OPN) Mean ± SE (ng/ml)		Probability
	Control group	Patients group	
TT	6.98 ± 0.67 ^{A}	15.26 ± 1.29 ^{AB}	0.00372
TC	5.81 ± 0.54 ^{A}	18.05 ± 1.13 ^{A}	4.01
CC	4.38 ± 0.94 ^{A}	15.02 ± 1.11 ^{AB}	0.002

(Duncan test): Similar letters indicate there were no significant differences between the genetic patterns among the same group

The current findings were in conflict with other studies as Jing *et al.* (2013) reported that an elevated risk of ischemic stroke was linked to the *rs11730582* polymorphism.

Pérez-Hernández *et al.* (2021) suggested that the *rs11730582* and *rs2728127 OPN* gene polymorphisms in Mexican people are linked to a number of aberrant metabolic characteristics in both controls and patients with premature coronary artery disease (PCAD).

Cheema *et al.* (2015) found in patients with type 2 diabetes, the polymorphism was linked to a lower risk of diabetic nephropathy.

As demonstrated by Honsawek *et al.* (2009), OPN in plasma and synovial fluid was linked to progressive joint degradation in knee OA and could be a biochemical marker for assessing the severity of OA disease.

Jing *et al.* (2013) found that the *OPN* gene variations were linked to both the radiographic severity and the risk of OA.

A study by Chen *et al.* (2017) among a Chinese population was attempted to determine whether SPP1 *rs17524488* and *rs11730582* were associated with

susceptibility of hip OA. This study was undertaken as a comparison with other studies and approaches. 389 patients with hip OA and 315 controls were enrolled in the trial. The results of the investigation showed that *rs17524488* delG/insG was more susceptible to hip OA. On the other hand, there is no correlation between *rs11730582* and the likelihood of developing hip OA.

There was no significant correlation between the *rs11730582* polymorphism and the existence of CAD in Lin *et al.* (2019) analysis of 536 patients with CAD, 86 patients with peripheral artery disease (PAD), and 617 controls.

In a study of 1092 individuals with essential hypertension, Hou *et al.* (2014) examined the relationship between four *OPN* gene polymorphisms, including *rs11730582*, and left ventricular hypertrophy (LVH); they found no discernible changes between the groups they examined.

Liu *et al.* (2015) detected that the *OPN* promoter *rs11730582* (-443C/T) polymorphism did not significantly correlate with cancer risk.

Eventhough OPN is a significant clinical predictor of many diseases, only a small number of genetic association studies have been conducted to far to ascertain its role as an osteoporosis disease risk marker. To properly understand this intricate and multifaceted disease, a genetic approach is required. In order to determine their relationship to the osteoporosis risk factors in the population of postmenopausal women in Iraq, this study examined one polymorphism, *rs11730582* of the *OPN* gene in 40 healthy individuals and 70 postmenopausal women with osteoporosis.

The human genome is full with genetic polymorphisms, and mutation in a single amino acid could be cause the alteration in the structural and functional characteristics of the OPN protein. Because SNPs are widely distributed throughout a genome, they are the most often employed molecular markers in genetic disease research. SNPs can change the encoded amino acids, be silent, or be in non-coding areas. By altering messenger RNA structure (stability), proteins, and promoter activity (gene expression), they can result indiseases. Therefore, finding and

examining several gene variations could help to better understand how they affect gene function and a person's health (Robert and Pelletier, 2018). This genetic variant in *rs11730582* SNP may influence how OPN protein is synthesized using different amino acids, which alters the protein domain's efficacy.

The current findings found that both immunological cells and other cell types have high levels of OPN expression. Adaptive and innate immune responses are both regulated by this pleiotropic protein. Numerous studies indicate that OPN plays a role in the etiology of certain illnesses. Furthermore, several data indicate that *OPN* gene variation may play a part in the etiology and/or clinical presentation of chronic and immune-mediated illnesses. Nevertheless, other studies were unable to find any connections between *OPN* SNPs and these disorders.

However, Ethnical, environmental, and as-yet-unidentified factors are the largest contributors to these disparities. Furthermore, some research does not satisfy the strict requirements for large-cohort, non-biased trials that are currently in place. Future studies should concentrate on choosing the most appropriate study groups to examine how *OPN* variations contribute to the etiology and development of illnesses. Ethnic, gene-environmental, and gene-gene interactions must all be considered in studies of *OPN* polymorphisms.

In addition, it's necessary to clarify and investigate the function of *OPN* polymorphism variations in diseases as a target for monitoring and treatment. Understanding of certain SNPs in *OPN* may assist develop genetic profiles for disease susceptibility so that preventative measures can be taken from childhood to adulthood.

Numerous diseases, such as Crohn's disease, urolithiasis, osteoarthritis in the knee, breast cancer, and diabetic nephropathy, have been linked to variations in the *Osteopontin* gene. OPN overexpression has been found in patient blood and can serve as a biomarker for both diagnosis and prognosis of OP disease as well as a number of

other illnesses, including diabetic nephropathy (*Xiao et al.,* 2016), intestinal metaplasia (Chang, *et al.,* 2017), and breast cancer (Zakhary *et al.,* 2018).

Last but not least, and according to Hardy-Weinberg state, in the absence of evolutionary forces, such as mutations, immigration/emigration, natural selection, sexual selection, and a large population, the allele and genotype frequencies of a population will remain constant. The findings of the study were not subjected to the Hardy-Weinberg Equilibrium (HWE) idea; for example, a small number of participants may change the HWE, which could impact the population's unsuitable genotype distribution.

Chapter Five
Conclusions and Recommendations

5. Conclusions and Recommendations

5.1. Conclusions

1- A significant elevation was observed in biochemicals (ESR, PLTs, CRP, RF, ACPA and PTH), bone matrix components (OPN and OCN) and in immunological parameters as (IL-8, IL-17 and IL-22) in women with OPdisease. can be used a prognostic for OP disease. Increase of these parameters in OP patients, could be participate in development of OP. An elevation in interleukins-8,-17 and -22 levels in postmenopausal women with osteoporosis explain the importance of these interleukins in affect the immune response activation and in progression of OP disease,

2- IL-8, IL-17, and IL-22 may act as one of the main bio-markers for osteoporosis due to their ability to stimulate osteoclastogenesis and bone resorption.

3- TT genotype *of Osteopontin* gene (SNP *rs11730582*) polymorphism has a significant effect in both OPN expression and functions, and with osteoporosis risk in postmenopausal women.

4- Current diagnostic parameters that used in this study can aid in enhancing early OP detection and improve treatment outcomes.

5- Understanding biochemicals and cytokines influences on bone health is essential for developing targeted therapies that address the underlying mechanisms of bone loss.

5.2. Recommendation

The folloing suggestions are recommended:

1- Future study performed with a larger sample size and for longer period of time.
2- Studying another bone biochemical matrix as, Keratocan, Periostin and etc.
3- Study another interlukines, e.g. IL-4, IL-10, IL-18, IL-23, and etc.
4- Farther genetic study for another gene related with OP, e.g. vitamin D encoding gene, and etc.
5- Study more genetic regions for *OPN* gene as; *rs1439234044*, *rs1725330579*, etc.

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Appendices

University of Baghdad
College of Science
Ethics Committee
csec@sc.uobaghdad.edu.iq

September 26, 2022
Ref.: CSEC/0922/0092

To: Ms. Reem Salim Sultan Al-Lami

Proposal title: Role of Osteopontin Gene Polymorphism and Certain Types of Interleukins in Pathophysiology of Osteoporosis in Iraqi postmenopausal Women

Approval letter

Dear **Ms. Reem Salim Sultan Al-Lami,**

The College of Science Research Ethics Committee has recently reviewed and discussed your application to conduct the research proposal and documents outlined below in the Department of Biology.

1. The proposal description.
2. Consent form.

The College of Science Ethics committee approves the research proposal to be conducted in the presented form. None of the investigator and co-investigator participating in this study took part in the decision-making and voting procedure for this study.

The College of Science Ethics committee expects to be informed about the progress of the study, any serious adverse events occurring in the course of the study, any revision in the protocol and patient information/informed consent and ask to be provided with a copy of the final report.

This Ethics committee is working in accordance with College of Science guidelines on biomedical research.

Truly yours,

Prof. Harith Jabbar Fahad Al-Mathkhury, Ph.D
The Chair of The College of Science Research Ethics Committee

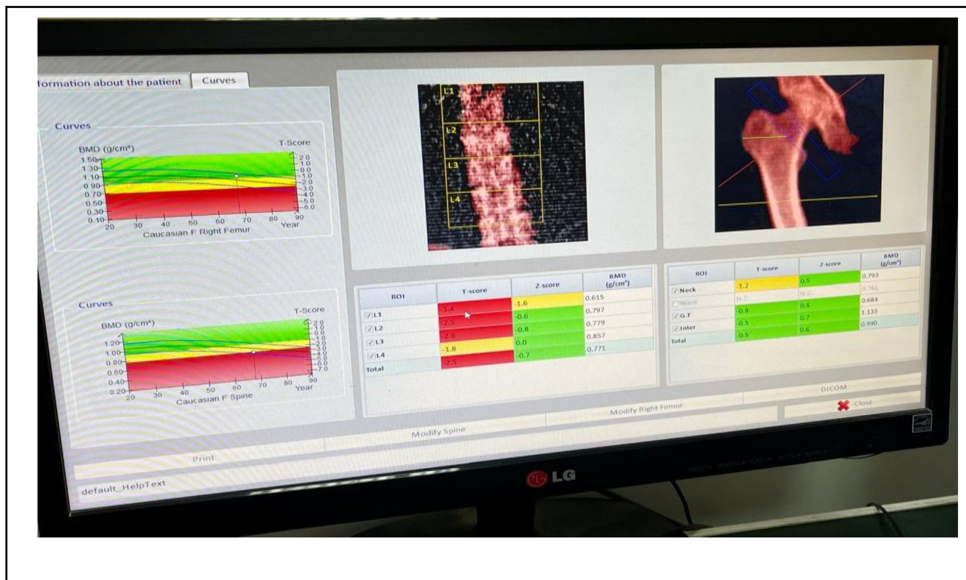
Appendix (1): Ethical Aproval Letter

A questionnaire Form			
Sure name:		Given name:	
Address:			
Phone number:			
Hospital:			
Sample Number:			
Age:	Gender:	Marital status	
		Married ☐	single ☐
Weight :		Length:	

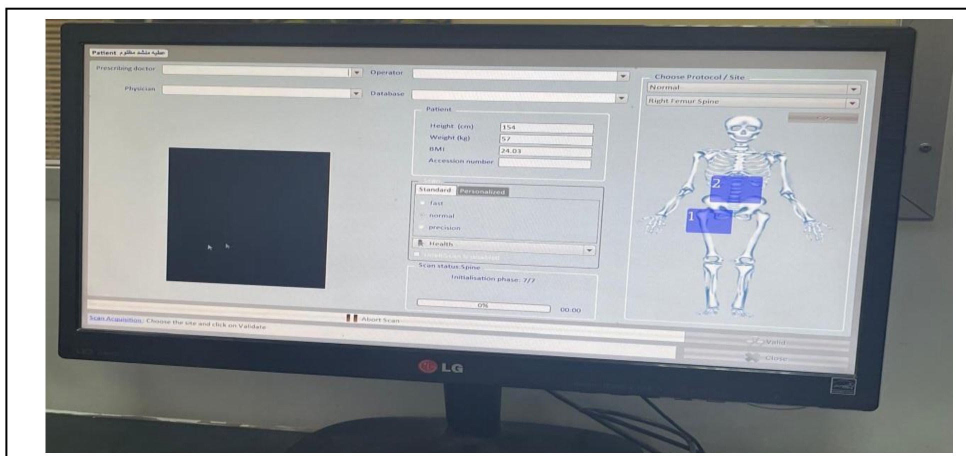
Healthy status		
	Yes	NO
Chronic Diseases		
Diabetes		
Autoimmune Disease		
Smoking		
Al-coholic		
Duration of Disease		
Type of medicine		

Appendix (2): Questionniar model that used in this study

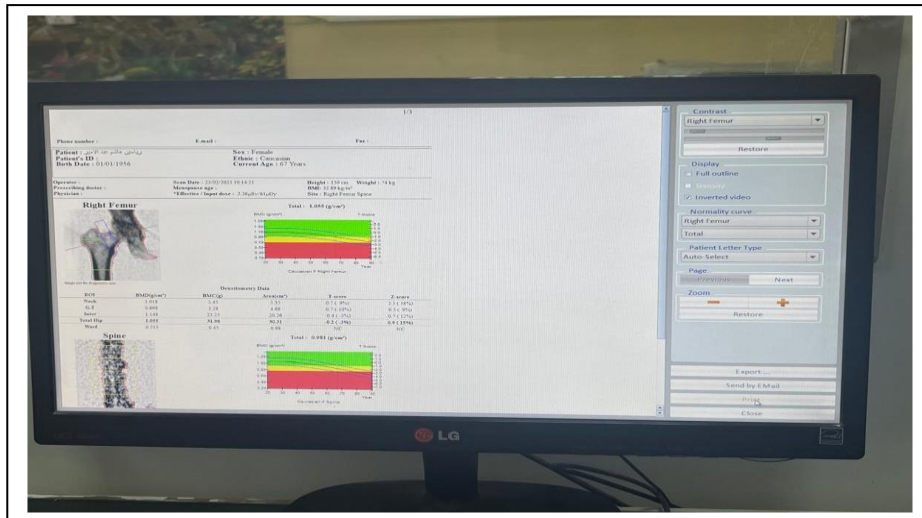
Appendix (3): Dual-energy X-ray absorptiometry (DEXA or DXA) scan.



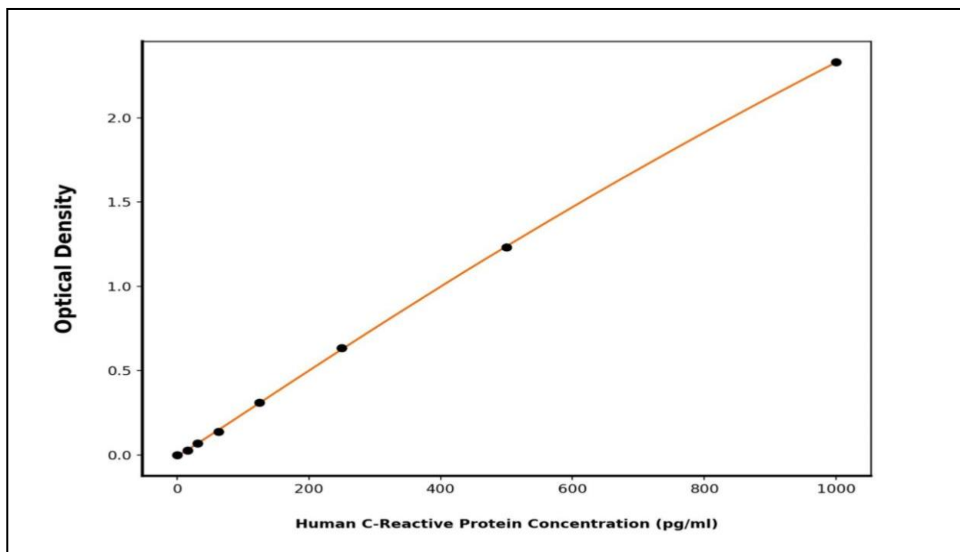
Appendix (4): Software analyze result of computed tomography



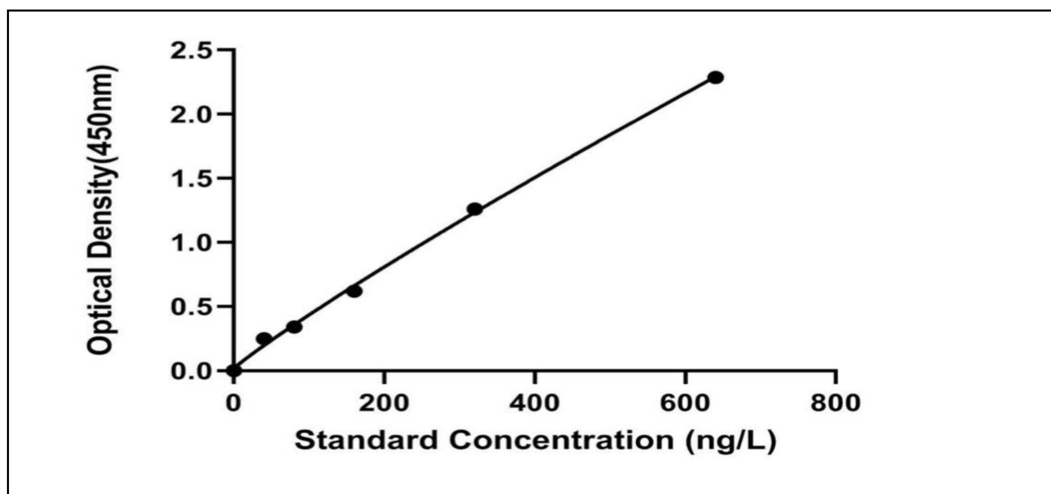
Appendix (5): Patient's information filling page in scan program



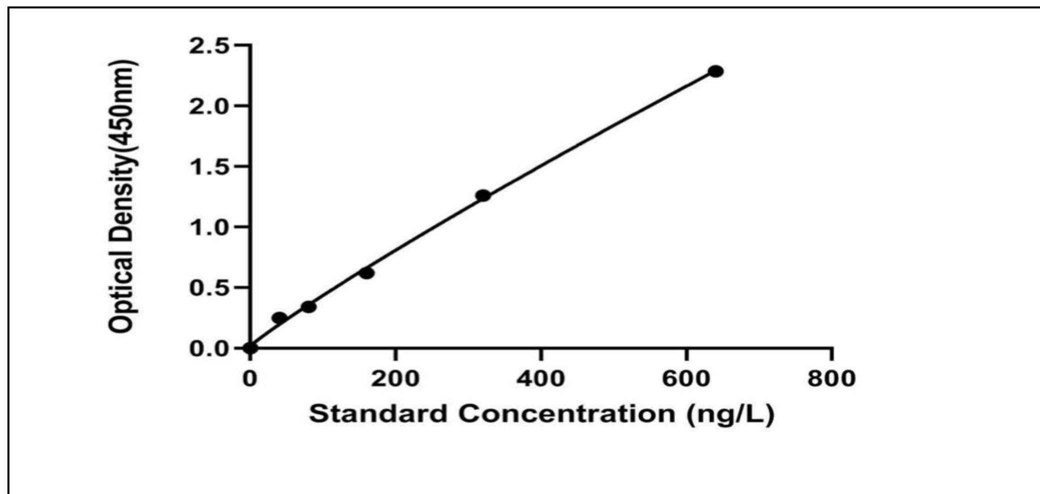
Appendix (6): Final clinical report of DEXA scan before printing



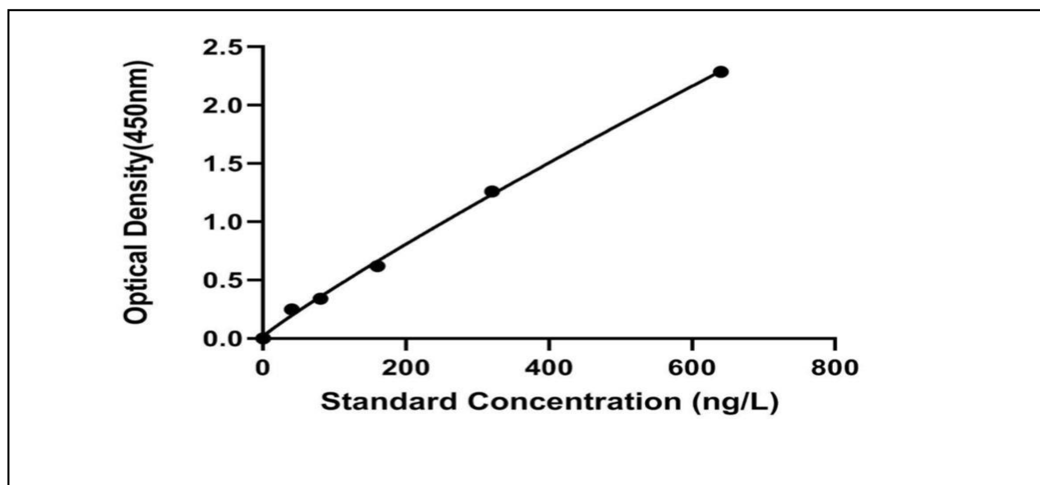
Appendix (7): Standard curve of C- reactive protein (CRP)



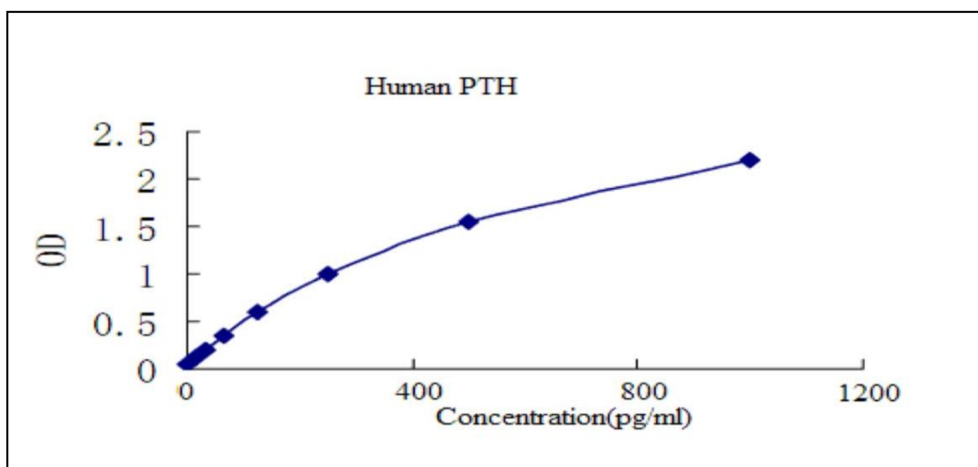
Appendix (8): Standard carve of IL-8



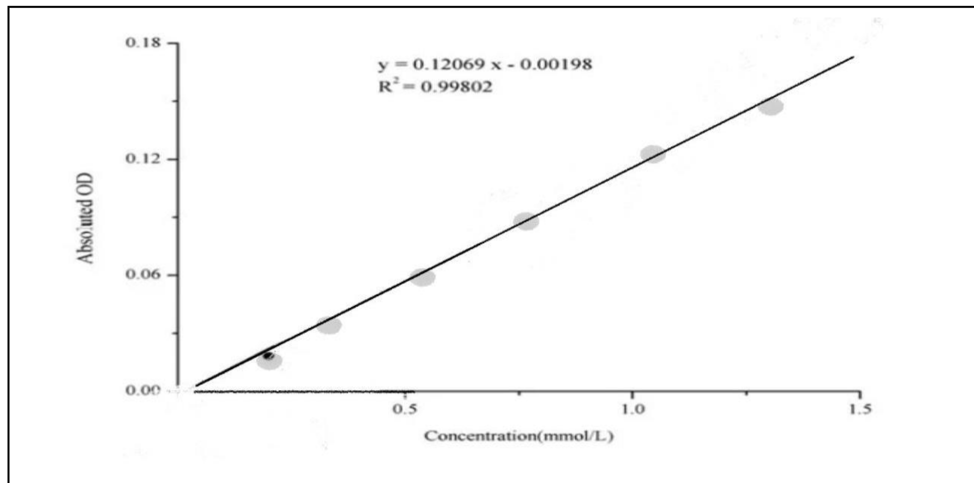
Appendix (9): Standard carve of IL-17



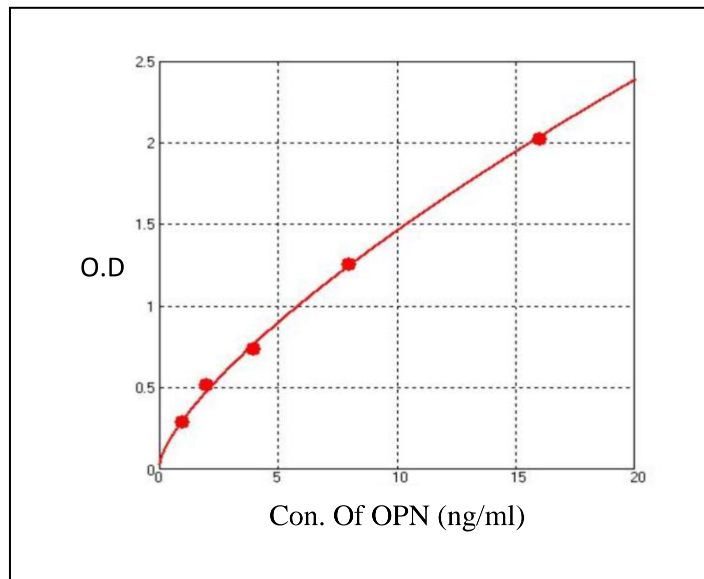
Appendix (10): Standard carve of IL-22



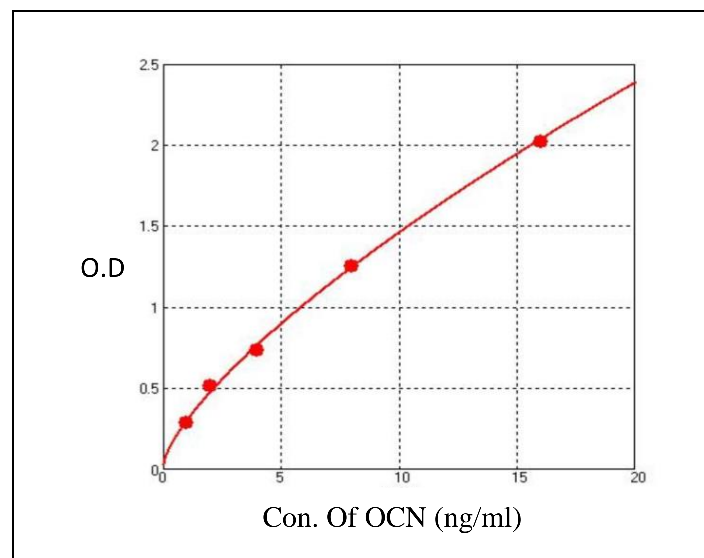
Appendix (11): Standard curve of parathyroid hormone (PTH)



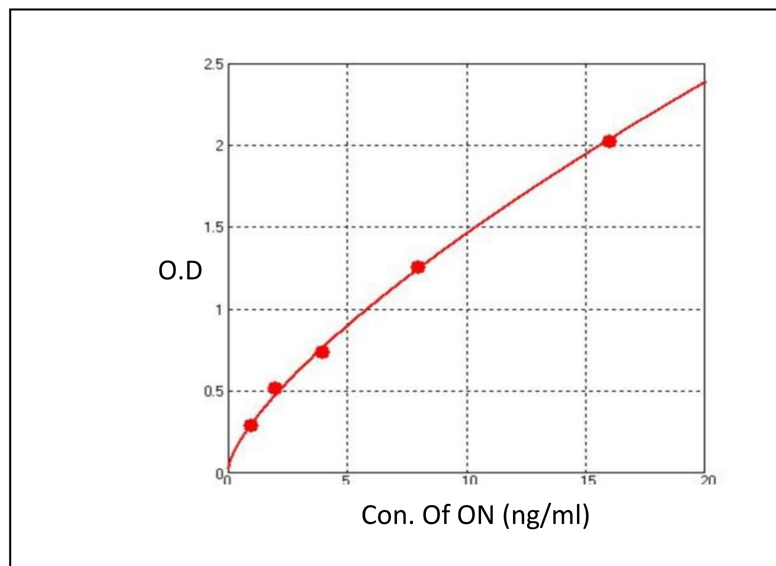
Appendix (12): Standard curve of Calicum (Ca^{+2})



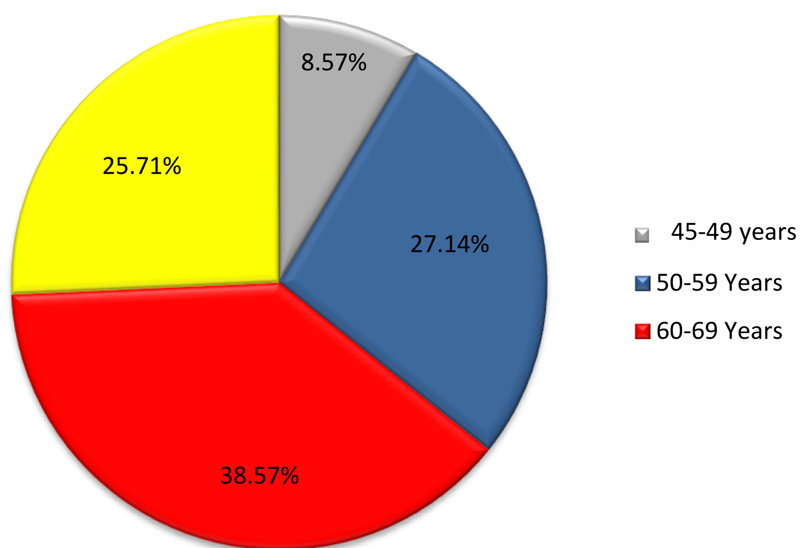
Appendix (13): Standard curve of osteopontin (OPN)



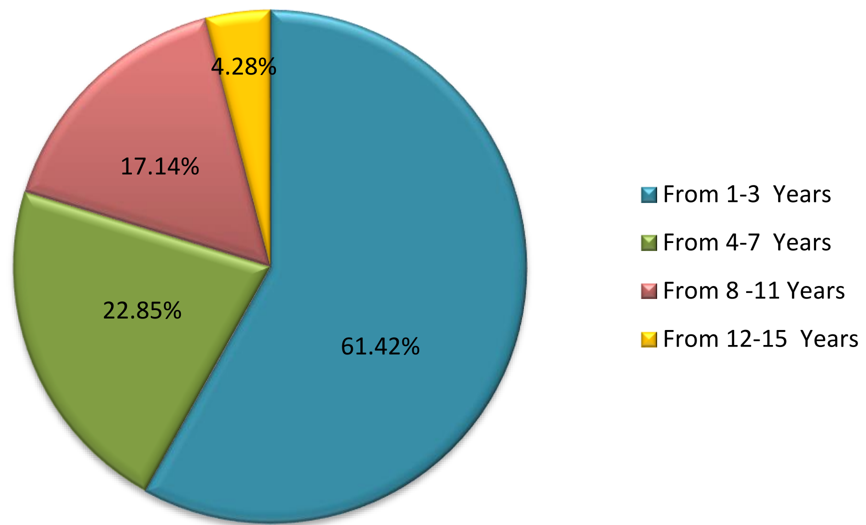
Appendix (14): Standard curve of osteocalicin (OCN)



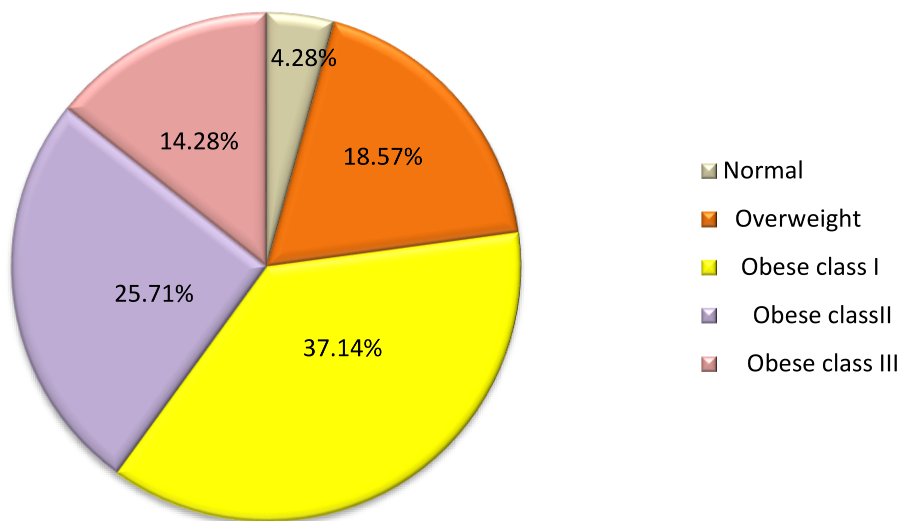
Appendix (15): Standard curve of osteonectin (ON)



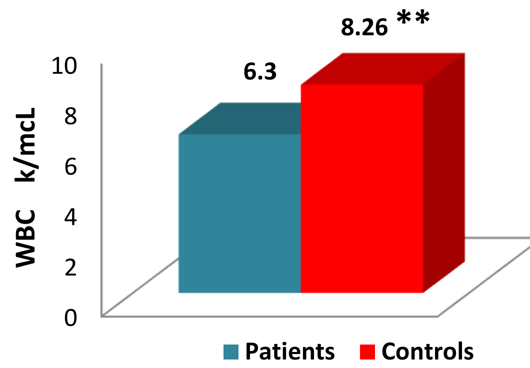
Appendix (16): Distribution of OP patients according to age groups.



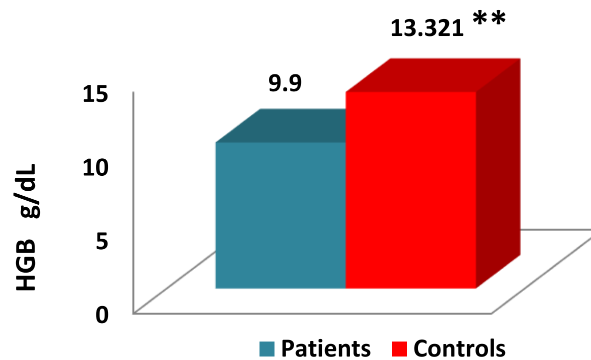
Appendix (17): Distribution of OP patients according to duration of disease



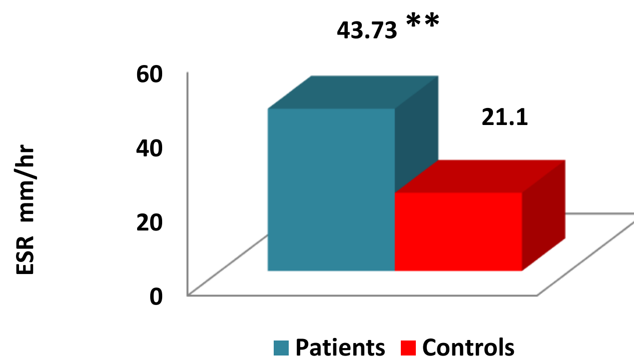
Appendix (18): Distribution of OP patients according to BMI and classes of obesity



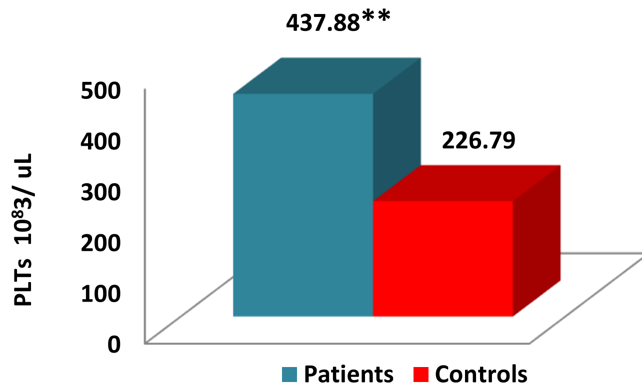
Appendix (19): WBCs count in OP patients and control



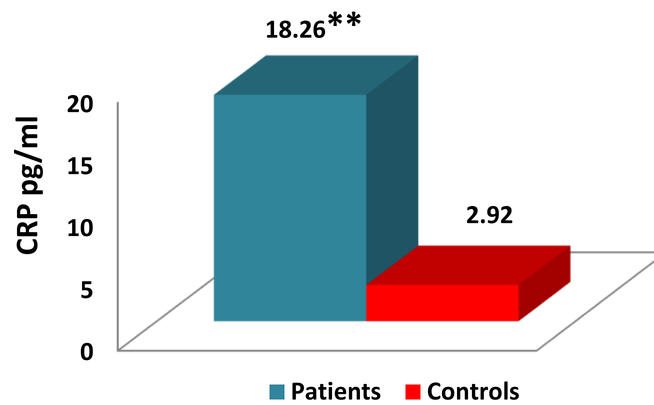
Appendix (20): Concentration of HGB in osteoporotic patients and control



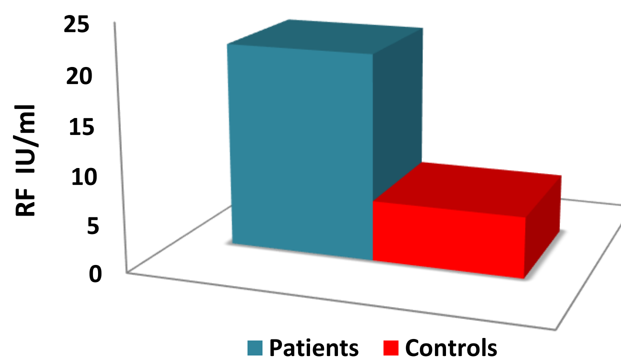
Appendix (21): Level of ESR in osteoporotic patients and control



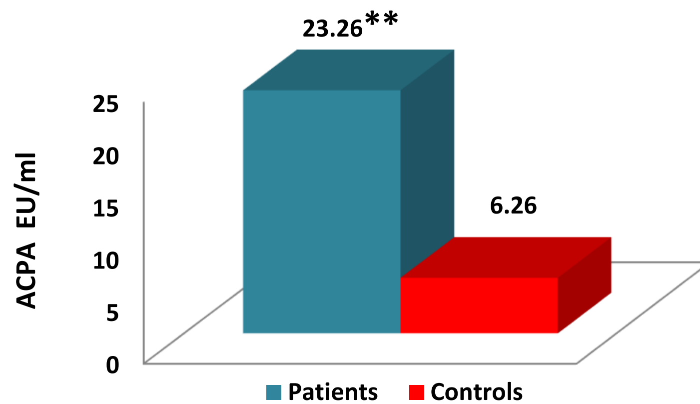
Appendix (22): PLTs count in OP patients and control



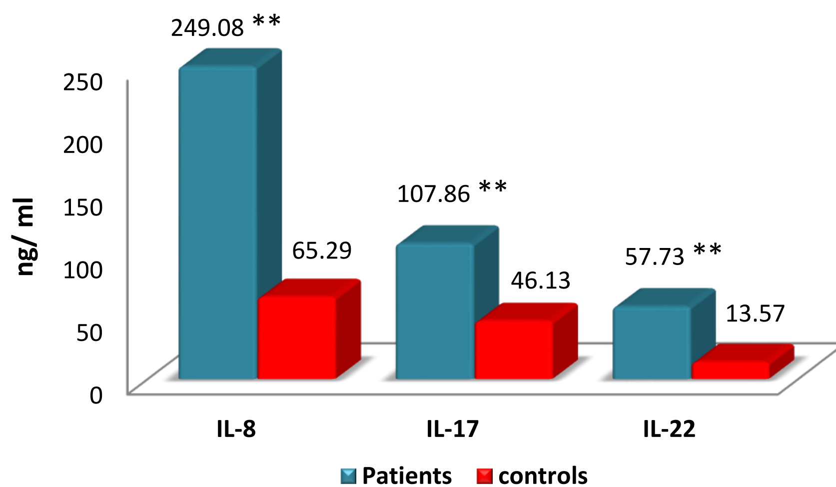
Appendix (23): Concentration of CRP in osteoporotic patients and control



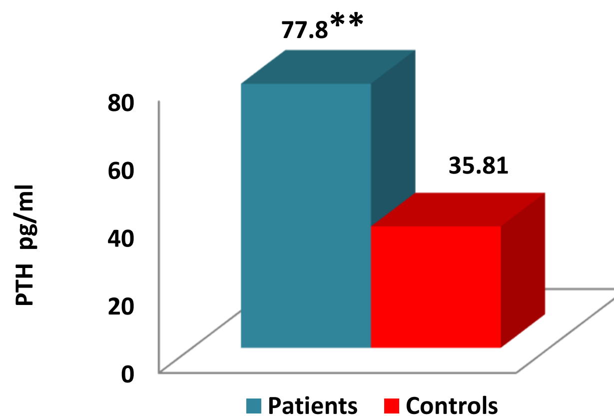
Appendix (24): Serum level of RF in osteoporotic patients and control



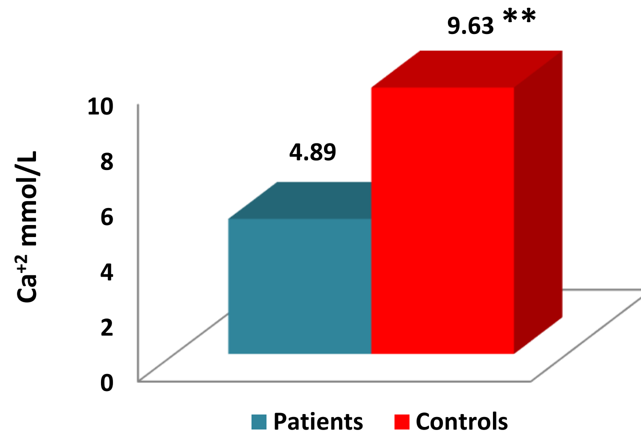
Appendix (25): Serum level of ACPA in osteoporotic patients and control



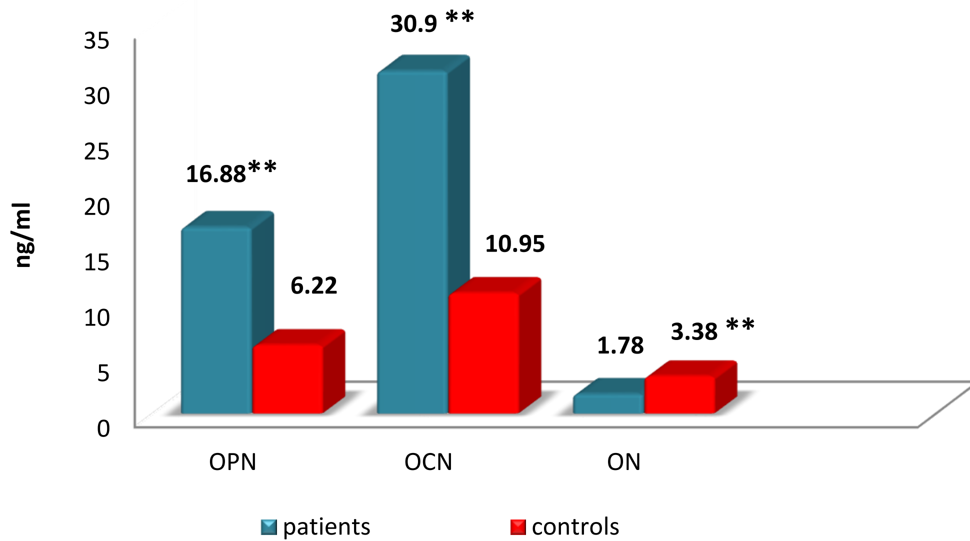
Appendix (26): Concentration of IL-8, -17 and -22 in OP patients and control



Appendix (27): Concentration of PTH in osteoporotic patients and control



Appendix (28): Concentration of Ca^{+2} in osteoporotic patients and control



Appendix (29): Concentration of OPN, OCN and ON in OP patients and control

المستخلص

يعد هشاشة العظام مرض غير مصاحب بأعراض لحين حدوث سقوط او التعرض لعوامل تؤدي الى التسبب بكسرفي العظام . يمكن ان يؤثر هرمون الاستروجين والتغييرات في مكونات مصفوفة العظم و التعبير عن الانترلوكين على عملية توازن العظم والذي يمهد تدريجياً الى تنشيط الخلايا الاكلة للعظم مما يؤدي الى فقدانه.

صممت هذه الدراسة الحديثة (مجموعتي الضوابط والمرضى) لتفسير التغييرات في بعض المواد الكيميائية الحيوية ومكونات مصفوفة العظام و المعايير المناعية و الجزيئية التي رافقت مرض هشاشة العظام في النساء بعد انقطاع الطمث. اجريت هذه الدراسة بين كانون الاول 2022 الى تموز 2023 . قدمت جميع المشاركات موافقة خطية وتمت الموافقة على هذه الدراسة من قبل اللجنة الاخلاقية لقسم علوم الحياة, كلية العلوم, جامعة بغداد (الرمز المرجعي/CSEC0922/0092 , في 26/ 9/ 2022). تم الأختيار عشوائياً 110 نساء في فترة ما بعد انقطاع الطمث من مستشفى مدينة الامامين الكاظمين (ع) الطبية, ومستشفى الواسطي ومستشفى مدينة الطب في بغداد, العراق للمشاركة في هذه الدراسة بناءَ على معاييرمحددة.

تراوحت اعمار جميع المشاركات بين 45-70 عاماً. في البداية, تم اجراء فحص قياس امتصاص الاشعة السينية ثنائي الطاقة DEXA لجميع المشاركات للتأكد من وجود وعدم وجود مرض هشاشة العظام. من بين 110 عينة, تم تأكيد 70 عينة مصابة بهشاشة العظام. في حين, هناك 40 عينة لم تكن مصابة بالمرض.

تم جمع عينات الدم الوريدي من جميع المشاركات لتقييم: عدد الدم الكامل CBC و المعاييرالمناعية الحيوية مثل بروتين C التفاعلي CRP, والعامل الروماتويدي RF , والاجسام المضادة لببتيد السيترولين الدورانيACPA و انترلوكين 8 (IL-8), و انترلوكين 17 (IL-17) وانترلوكين 22 (IL-22) بأستخدام تقنية ال ELISA , وهرمون غدة جارالدرقية PTH, والكاليسيوم Ca^{+2} ومكونات مصفوفة العظام الأوستيوبونتين OPN, و الأوستيوكالسين OCN, والأوستيوناكتين ON, . بالاضافة الى دراسة دور التعدد الشكلي لجين الاوستيوبونتين *rs11730582* بأستخدام تقنية تحليل الذوبان عالي الدقة **HRM.**

تم تسجيل انخفاض عالي المعنوية (P≤0.01) في معدل خلايا الدم البيضاء (6.30±0.18 k/mcL) و الهيموغلوبين (9.900±0.132 g/dL) في المرضى المصابين بهشاشة العظام بالمقارنة مع مجموعة الضوابط حيث كانت معدل خلايا الدم البيضاء (8.26±0.27 k/mcL) و الهيموغلوبين (13.321±0.287 g/dL) .بينما, تم تسجيل ارتفاع عالي المعنوية (P≤0.01) في معدل ترسب كريات الدم الحمراء (43.73±1.76 , mm/hr) ومعدل الصفيحات الدموية (10^83/uL) 437.88±7.00) في المرضى المصابين بهشاشة العظام بالمقارنة مع مجموعة الضوابط حيث كان معدل ترسب كريات الدم الحمراء (21.10±1.25mm/hr) ومعدل الصفيحات الدموية (10^83/uL) 226.79±9.39).

تم تسجيل زيادة عالية المعنوية ($P\leq0.01$) في قياس المعايير الكيميائية الحيوية كمعدل بروتين C التفاعلي (18.26±1.33 pg/ml) و العامل الروماتويدي (موجباً) , و الاجسام المضادة لببتيد السترولين الدوراني (23.26±2.74EU/ml) في المرضى المصابين بهشاشة العظام مقارنةً بمجموعة الضوابط حيث كان معدل بروتين C التفاعلي (2.92±0.18 pg/ml) وسجلت نتيجة سالبة للعامل الروماتويدي , في حين كان معدل الاجسام المضاة لببتيد السترولين الدوراني (6.26±0.56 EU/ml).

كما اظهر نتائج انترلوكين 8و 17و 22 زيادة عالية المعنوية وملحوضة ($p\leq0.01$) في المرضى المصابين بالمقارنة مع مجموعة الضوابط. حيث سجلت زيادة عالية المعنوية في المستوى المصلي لانترلوكين 8 (249.08±19.98ng/ml), وانترلوكين 17(107.862±9.25ng/ml), وانترلوكين 22 (57.73±2.47 ng/ml), في المرضى المصابين بهشاشة العظام بالمقارنة مع مجموعة الضوابط حيث كان مستوى انترلوكين 8 (65.29 ± 5.62 ng/ml) وانترلوكين 17 (46.135 ± 8.66 ng/ml) وانترلوكين 22 (13.57 ± 3.16) ng/ml.

كما لوحظت زياد عالية المعنوية في معدل هرمون غدة الجار الدرقية في المرضى المصابين بهشاشة العظام (77.80±1.79pg/mL) بينما كان في مجموعة الضوابط (35.81±2.06pg/mL), كما سجل انخفاض عالي المعنوية ($P\leq0.01$) في مستوى الكالسيوم المصلي في المرضى المصابين (4.89±0.21 mmol/L) مقارنة مع مجموعة الضوابط (9.63±0.13 mmol/L).

الى جانب ذلك, تم الكشف عن زيادة عالية المعنوية في المستوى المصلي لكل من الاوستيوبونتين (16.88±0.77 ng/ml) والاوستيوكالسين (29.86±2.25ng/ml) في المرضى المصابين بالمقارنة مع مجموعة الضوابط حيث كان معدل الاوستيوبونتين (5.95±0.41ng/ml) و الاوستيوكالسين (10.73±0.86ng/ml). على العكس من ذلك, سجل انخفاض عالي المعنوية ($P\leq0.01$) في المستوى المصلي لبروتين الاوستيوناكتين في مجموعة المرضى المصابين (1.78±0.971ng/ml) بالمقارنة مع مجموعة الضوابط حيث كان معدل بروتين الاوستيوناكتين يساوي (3.42±0.20ng/ml).

كما ان تكرار النمط الوراثي TT المتماثل في تحليل المرضى المصابين كان 10/70 (14.3%) من مجموعة الضوابط (11/40, 27.5%) . في حين ان التكرار الغير متجانس TC والنمط الجيني المتجانس الهجين CC [TC= (71.42%) 50/70 and CC= 14.3% (10/70)]. لم يكن تكرار الانماط الجينية مختلفاً اختلاف كبير عن تلك الموجودة في مجموعة الضوابط حيث ان (TC= 60% 24/40) and (CC= 12.5% 5/40) على التوالي.

بالاضافة الى ذلك,لوحظ اختلاف عالي المعنوية (*P*-value=0.0158) في توزيع الانماط الوراثية لتعدد الاشكال *rs11730582* في مرضى هشاشة العظام بالمقارنة مع مجموعة الضوابط (P-value=0.1500).

في الختام, توصلت هذه الدراسة ان بروتين الاوستيوبونتين والاوستيوكالسين والاوستيوناكتين مؤشرات مراقبة حساسة يمكن استخدامها للكشف المبكر عن هشاشة العظام . وبسبب القدرة على تنشيط الخلايا الاكلة للعظام وتعزيز عملية امتصاص العظام تعد الانترلوكينات 8و 17و22 بالاضافة الى التعدد الشكلي في جين الاوستيوبونتين ***rs1173058 2*** مؤشرات حيوية وعوامل محفزة للالتهاب تساهم في تحفيز المناعة للمناعة وتطوير امراضية هشاشة العظام.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة بغداد

كلية العلوم

قسم علوم الحياة

دور تعدد الاشكال في جين الاوستيوبونتين وبعض انواع البين ابيضاض في الفسيولوجيا المرضية لتنخر العظام في النساء العراقيات بعد سن اليأس

أطروحة

مقدمة الى مجلس كلية العلوم/ جامعة بغداد وهي جزء من متطلبات نيل درجة الدكتوراه في علوم الحياة/ علم الحيوان

تقدم بها

ريم سالم سلطان اللامي

بكالوريوس علوم الحياة / جامعة بغداد/ كلية العلوم (٢٠١٦-٢٠١٧)

ماجستير علوم الحياة / جامعة بغداد/ كلية العلوم (٢٠٢٠-٢٠٢١)

بأشراف

أ.د. جبار حميد ينزيل

١٤٤٦هـ **٢٠٢٥م**