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"Study of Macrophage Erythroblast Attacher Gene Polymorphism in Association with its Protein as an Index of Postmenopausal Osteoporosis"

A Thesis

Submitted to the Council of College of Medicine – University of Kerbala in Partial Fulfilment of the Requirements for the Degree of Master in
(Clinical Chemistry)

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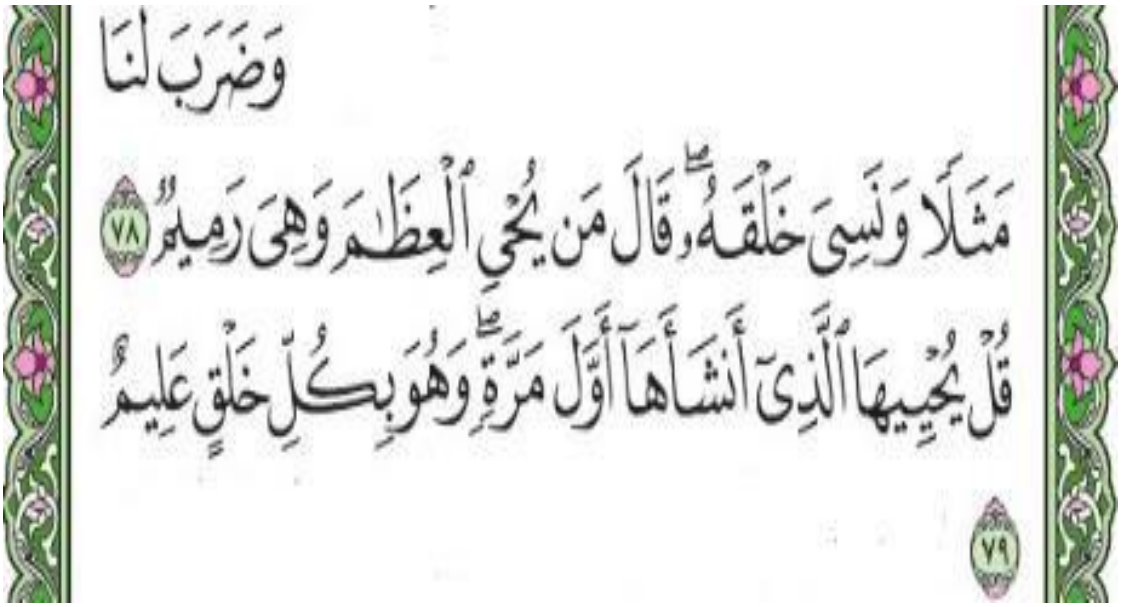

صدق الله العلي العظيم

سورة يس ٨٧- ٩٧

Dedication

To my parents, my family and all peoplewho
might get benefit from this study
I dedicate this work.

Hussein

Acknowledgments

First and foremost, I thank God Almighty for His grace upon him by granting me the ability to complete this stage.

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Supervisors' Certification

We certify that this M.Sc. thesis entitled: -

" Study of Macrophage Erythroblast Attacher Gene Polymorphism in Association with its Protein as an Index of Postmenopausal Osteoporosis "

was prepared under our supervision in laboratories at the Department of Biochemistry – College of Medicine / University of Kerbala as a partial fulfillment of the requirements of Master Degree in **Clinical Chemistry**.

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We the examining committee, certify that we have read this M.Sc. thesis entitled:

(Study of Macrophage Erythroblast Attacher Gene Polymorphism in Association with its Protein as an Index of Postmenopausal Osteoporosis)

We have examined the postgraduate student **(Hussein Ali Hussein Alwan)** in its M.Sc. thesis content and, in our opinion; it meets the standard for the degree of master in **(Clinical chemistry)** and it is adequate with **(Excellent)**.

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Summary

Osteoporosis represents an increasing global health problem, with the highest incidence rates in postmenopausal women. Osteoporosis is a typical skeletaldisorder described by low bone mass, which leads to reduced bone strength and an enhanced risk of fractures. The several genes involved in postmenopausal osteoporosis, Single Nucleotide Polymorphism rs10025665 in Macrophage Erythroblast Attacher gene (MAEA) has been suggested to be associated with osteoporosis.

This study aims to investigate the association of MAEA gene polymorphism with postmenopausal osteoporosis women in Kerbala province and to determination the alkaline phosphatase isoenzyme in sera of osteoporosis and compare it with osteopenia group.

The study design was a cross sectional study that included 160 women who were diagnosed by a physician and were divided into four groups: each group consisted of forty women as in the following: Postmenopausal Osteoporosis with type 2 diabetes mellitus(T2DM), postmenopausal Osteoporosis with hypertension (HTN), postmenopausal Osteoporosis without (diabetic Mellitus/hypertension) and postmenopausal osteopenia group. All groups of women were collected from the Al-Hassan center for Endocrinology and Osteoporosis Center at Al-Hussein Medical City in Kerbala City, for the period from November 2021 to March 2022.

They were diagnosed with osteoporosis by measured T-score through DEXA scan at lumbar spine regions confirmed osteoporosis. Osteoporosis consisted of four categories that were collected at admission and the disease severity wasassessed using WHO criteria. The patients were diagnosed previously with hypertension and patients with T2DM are based on physicians' diagnosis before their current visit to the outpatient. The patients were diagnosed with osteoporosis when they first visited the Osteoporosis Center.

The results of Vitamin D3 showed a significant difference that observed decreased Serum Vitamin D3 in all osteoporosis groups when compared with the osteopenia group($P<0.05$). The results of Magnesium showed a significant decrease in all Osteoporosis groups compared to the osteopenia group ($P<0.05$).

The results of the MAEA protein showed a significant decrease in osteoporosis without (T2DM, and HTN) when compared with osteopenia group ($P.value<0.01$), also showed a significant increase in osteoporosis with T2DM when compared with osteopenia group ($P.value<0.01$), while there is no significant difference between osteoporosis with HTN and osteopenia group. The results of bone alkaline phosphatase (BALP) showed a significant increase in osteoporosis and osteoporosis with hypertension when compared with the osteopenia group $p.value<0.05$ and nonsignificant in osteoporosis with T2DM.

The genotypes of MAEA SNP rs10025665 were significantly associated with MAEA protein in (AA) and (AG) genotypes($p.value<0.05$). There was a nonsignificant association between genotypes of SNP rs10025665 and bone alkaline phosphatase in (AA), (AG), and (GG) genotypes ($p.value>0.05$). There was no significant association between the investigation MAEA SNP rs10025665 and the risk of osteoporosis in the study population.

Lower levels of serum MAEA protein in osteoporosis without (T2DM, and HTN) and High levels of enzyme bone alkaline phosphatase in postmenopausal women areassociated with the development of osteoporosis in Iraqi women.

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Abbreviations

Abbreviation	Meaning
1,25(OH)2D	1,25-dihydroxycholecalciferol (calcitriol)
25(OH)D3	25-Hydroxyvitamin D3
AGEs	Advanced glycation end products
ACEIs	Angiotensin converting enzyme inhibitors
bp	Base pair
BBA	Binding buffer
BP	Blood pressure
BMI	Body mass index
BALP	Bone alkaline phosphatase
BMD	Bone Mineral Density
Ca	Calcium
CaSR	Calcium sensing receptor
CVD	Cardiovascular disease
CLD	Cell lysis buffer detergent
CLIA	Chemiluminescence immunoassay analyzer
CI	Confidence interval
DHEA	Dehydroepiandrosterone
dNTPs	Deoxynucleotide triphosphates
DNA	Deoxyribonucleic acid
DBP	diastolic blood pressure
DEXA	Dual-energy x-ray Absorptiometry
DBP	Vitamin D Binding protein
ELISA	Enzyme linked immunosorbent assay
E2	Estradiol
E3	Estriol
ERs	Estrogen receptors
E1	Estrone
EtBr	Ethidium bromide
EDTA	Ethylene diamine tetra acetic acid
FGF23	Fibroblast growth factor 23
FFS	Fragility fractures
GWAS	Genome wide association study
GLUT	Glucose transporter
HWE	Hardy Weinberg equilibrium
HF	Heart Failure

HRP	Horseradish peroxidase
HTN	Hypertension
Ig	Immunoglobulin
Pi	Inorganic phosphate
IGF1	Insulin like growth factor 1
IL	Interleukin
IU	International Units
LH	Luteinizing hormone
MAEA	Macrophage Erythroblast Attacher
Mg	Magnesium
MBDs	Metabolic bone diseases
mcg	Micrograms
MRAs	Mineralocorticoid receptor antagonists
NOF	National Osteoporosis Foundation
OCN	Osteocalcin
OP	Osteoporosis
PTH	Parathyroid hormone
P	Phosphorus
PCR	Polymerase Chain Reaction
PMOP	Postmenopausal osteoporosis
RANKL	Receptor Activator for nuclear factor Kb ligand
RANK	Receptor activator of nuclear factor kappa-B
rs	Reference SNP
RLUS	Relative light unit
RAAS	Renin Angiotensin Aldosterone system
SNP	Single Nucleotide Polymorphism
SD	Standard deviation
SBP	Systolic blood pressure
TDs	Thiazide diuretics
TSH	Thyroid stimulating hormone
TALP	Total Alkaline phosphatase
TGF	Transforming growth factor
TNF	Tumor necrosis factor
T2DM	Type 2 Diabetes Mellitus
UVB	Ultraviolet B
VDR	Vitamin D receptor
CWD	Column Wash Solution detergent
WHO	World Health Organization
Zn	Zinc

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Chapter One

Introduction and Literature Review

1. Introduction.

One of the newer global health problems is osteoporosis **(Li *et al*., 2021)**. It is a silent illness that results in bone fractures **(Anthamatten *et al.*, 2019)**. Osteoporotic fractures are turning into a significant source of morbidity and death in Iraq due to a rise in life expectancy, which is comparable to the western world **(Al-Rukabi *et al*., 2020)**. However, it may now be possible for early detection and rapid treatment, which may lead to a better outcome for the patient and may prove less harmful to the environment.

With the change in eating habits, people are not getting adequate nutrition for the well-being of the bones, and the lack of activity in Iraq increases the prevalence of the disease **(Mohammed *et al*., 2021)**. In the United States, thereare already 34 million osteoporosis sufferers, with 80% of them predicted to be women **(Ruiz-Esteves *et al*., 2022)**. To compare the prevalence of osteoporosis indifferent groups, older adults' fracture rates are the most informative data. However, quantifiable data from underdeveloped nations are limited since osteoporosis typically does not pose a life-threatening concern.

Despite this, the current consensus is that there are roughly 1.66 million hip fractures per year worldwide, that the incidence will more than double by 2050 due to the growing elderly population **(Veronese *et al*., 2021)**, and that the age- adjusted incidence rates are significantly higher in wealthy developed nations than in developing nations in Africa and Asia **(Kartikey *et al*., 2019)**. Therefore, our study aimed to investigate some genetics and biomarkers in one of the most important problems which is osteoporosis.

1.1. General Metabolic Bone Disease.

Metabolic bone diseases (MBDs) represent an umbrella term enveloping a wide spectrum of clinically different diseases, with a common condition of an aberrant bone chemical milieu that causes defects in bone and skeleton. It is usually characterized by a dramatic clinical manifestation commonly reversible once the underlying cause is treated. Disruptions in bone mineralization lead to a group of diseases such as Osteomalacia or rickets, whereas imbalances in the bone remodeling process lead to the occurrence of other types of diseases, such as osteoporosis and Paget's disease of bone (**Iolascon *et al.*, 2020 and Kahla *et al.*, 2021**).

1.2. Osteoporosis.

Osteoporosis (OP) represents an increasing global health problem, with the highest incidence rates in postmenopausal women and elderly men (**Salamanna *et al.*, 2021**). Osteoporosis is a systemic bone disease characterized by osteopenia and compromised bone microstructure, resulting in increased bone fragility and susceptibility to fracture (**Coll *et al.*, 2021 and Xu *et al.*, 2021**).

1.2.1. Classification of Osteoporosis.

Taking into account the causes that produce it, osteoporosis can be classified as primary and secondary.

1.2.1.1. Primary Osteoporosis.

It is the most common type of osteoporosis. This diagnosis is established after evaluating the patient, when the cause/s that can be related, are not found. In turn, primary osteoporosis can be subdivided into:

A. Juvenile OP

B. Postmenopausal OP (PMOP)

C. Age-related OP

D. Idiopathic forms OP

1.2.1.2. Secondary Osteoporosis.

When the loss of bone mass is caused by another disease, or by the use of particular drugs. Fractures occur most frequently at the level of the hip bones, vertebrae of the spine, and wrist. Vertebral fractures can cause loss of height of the spine as a whole and deformity of the rib cage. Depending on the results obtained in bone mineral density (BMD), the results can be classified according to the T scale, which refers to the mean bone density of the healthy population of the same sex and 20 years of age in the following categories (**Colangelo *et al.*, 2019 and Litwic *et al.*, 2021**):

1.2.1.3. Osteopenia.

When the BMD presents a standard deviation between -2.5 to -1, on the T scale. This variety generally does not require drug treatment.

1.2.1.4. Osteoporosis.

If the bone mineral density is equal to -2.5 or less than -2.5 standard deviations on the T scale this condition is osteoporosis as shown in (Fig.1.1).

1.2.1.5. Established Osteoporosis.

When there is osteoporosis and it has caused a fracture (**Mäkitie *et al.*, 2019; Ding *et al.*, 2020**). Osteoporosis can be classified into two main groups by considering the factors affecting bone metabolism: Primary osteoporosis and secondary osteoporosis.

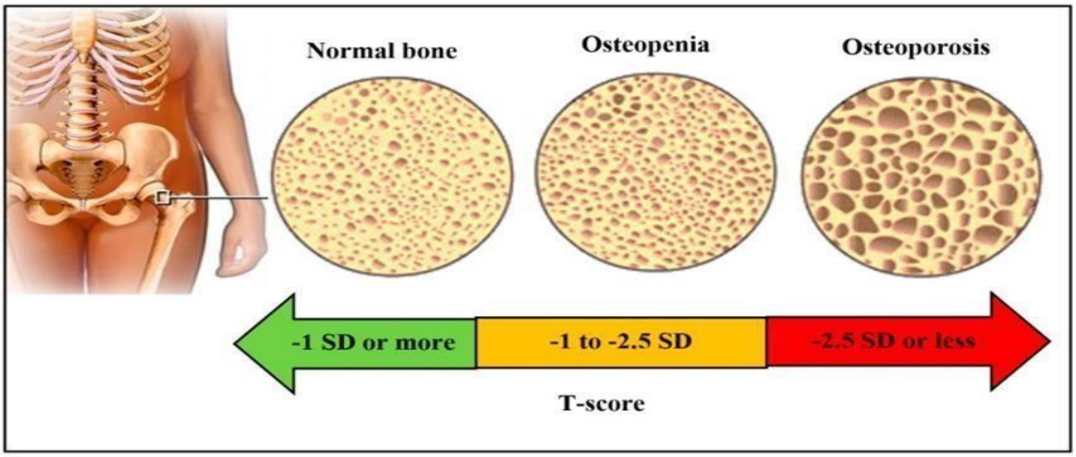

Fig. (1.1): Comparative view of normal bone, osteopenia, and osteoporosis (Rani *et al.*, 2020).

1.2.2. Risk Factors for Osteoporosis

Factors that increase the risk of developing osteoporosis are calcium and vitamin D3 deficiencies due to malnutrition, sedentary life or lack of physical activity, and tobacco and/or alcohol consumption (**Kim *et al.*, 2020**). In 1994, the WHO established diagnostic criteria for determining osteoporosis through a bone mineral density (BMD) test. Through this method, they ascertained that if a BMD T-score result was –2.5 or less and if the patient's low bone mass had a BMD T-score between −1 and −2.5, then osteoporosis could be diagnosed (**Matzkin *et al.*, 2019**).

However, following more recent research on the disease, it has been noted that BMD tests are a somewhat limited clinical indicator for diagnosis, as determining osteoporosis requires an overall assessment of a patient's fracture risk. Several fracture risk assessment tools currently exist, including the Fracture Risk Assessment Tool (FRAX), Garvan Fracture risk calculator, and Qfracture Scores-2016. When used with a BMD test, these tools can help calculate a more accurate overall fracture risk (**Compston *et al.*, 2019**).

1.2.3. Prevention of Osteoporosis

The best strategy for treating osteoporosis is prevention. Bone, or bone tissue, is a very dynamic living tissue throughout life, which is constantly formed (ossification), grows, and remodels (bone turnover) (**Allen *et al.*, 2019**). For these processes (formation, growth, and remodeling), important for the integrity or strength of the bone, hormonal activities, certain nutrients (calcium, phosphorus, magnesium, vitamin D3) and physical activity take part (**Groslan*det al.*, 2019**). Therefore, they are recognized as factors that play an important role in the prevention and treatment of osteoporosis.

It is necessary to achieve optimal bone formation in the youngest and then avoid loss of bone mass in adulthood and old age. To achieve these goals, it is necessary to carry out a diet that provides the essential nutrients for the formation, growth, and maintenance of bones (**Price *et al.*, 2012**). It is important to guarantee the consumption of the daily needs or minimum requirements of calcium (1300 mg/day), phosphorus (1250 mg/day), magnesium (420 mg/day), and vitamin D3 (20 mcg/day equivalent to 800 IU/day), through normal nutrition (daily consumption of foods that contain these nutrients) or supplementation. Perform the appropriate physical exercise for each age through frequent outdoor activity, which ensures prudent sun exposure, for the synthesis of vitamin D3 in the skin, but avoiding overexposure, due to the risks it has on skin health (**Kanis *et al.*, 2020 and Burden *et al.*, 2021**).

Osteoporosis usually does not cause any symptoms. For this reason, it has been called the “silent epidemic”. However, the error of considering that the loss of bone mass causes musculoskeletal pain is widespread, and patients are frequently referred to a specialist for this reason with suspected osteoporosis, especially pre-menopausal women or young people. The main clinical manifestations of osteoporosis are due to its complications, such as fractures,

which mainly occur in the spine, wrists, and hips. They are caused by minor trauma, such as a simple fall. This is why they are called brittle fractures. They produce the same symptoms as other fractures in the same location and are characterized by the presence of pain, deformity, and functional impotence (**Brenneman**, **2016** and **Kennel *et al.*,2020).**

1.2.4. Causes of Osteoporosis.

Primary osteoporosis is the most common form of osteoporosis and includes both postmenopausal and age-related osteoporosis. By contrast, secondary osteoporosis is a consequence of systemic disease or pharmacological intervention and its etiology includes:

A. Endocrine disorders (adrenal insufficiency, Cushing's syndrome, diabetes, hyperprolactinemia, hypogonadism) (**Kinoshita**,**2015**).

B. Connective tissue disease, e.g. rheumatoid arthritis (**Adami *et al.*, 2019**).

C. Genetic diseases, including Osteogenesis imperfect (**El-Gazzar *et al.*, 2021**).

D. Drugs, including glucocorticoids, antiepileptics, anticoagulants, chemotherapy, and gonadotrophic-releasing hormone agonists/antagonists (**Kenkre *et al.*, 2018**).

E. Metabolic disorders, including renal and liver disease (**Polyzos *et al.*, 2021**).

F. Gastrointestinal and nutritional disorders, e.g. parenteral nutrition, gastrectomy or post-gastric bypass, malabsorption, and pancreatic insufficiency (**Voderholzer *et al.*, 2020**).

G. Disorders of the bone marrow, e.g. myeloma (**Esposito *et al.*, 2022**).

H. Idiopathic hypercalciuria, calcium deficiency (**Sakhaee *et al.*, 2021**).

The most common causes of secondary osteoporosis are glucocorticoid treatment and immobilization (**Litwic *et al.*, 2021**), While osteoporosis has many and diverse causes, uncoupling of the bone remodeling cycle and increased bone resorption relative to formation is a common underlying pathophysiological mechanism. The excess skeletal resorption results in structural deterioration and increased fragility (**Ru *et al.*, 2020**).

Microscopically sites of osteoclastic bone resorption are incompletely repaired by newly formed bone, resulting in progressive bone loss and increasing cortical porosity (**Kenkre *et al.*, 2018**). Osteoporosis is commonly encountered in older women with no underlying risk factors. However, this does not necessarily mean that it is attributed only to old age.

Many diseases have strong evidence to be considered as risk factors attributing to the development of osteoporosis, even in the young female population. These include conditions like hyperthyroidism, hyperparathyroidism, and chronic kidney disease (**Keen *et al.*, 2022).** Vitamin D3 is essential for managing bone strength and calcium absorption.

It is a significant factor considered as an additional risk for osteoporosis in countries lacking tropical weather, which is vital for vitamin D synthesis. Other contributing factors to the development of osteoporosis are the judicious use of certain medications, known as secondary risk factors for osteoporosis. Important to mention are corticosteroids, thyroxine, antacids, and chemotherapies,especially aromatase inhibitors (**Mitek *et al.*, 2019**).

In general, the causes of osteoporosis can be simply classified into primary and secondary. Primary is generally age-related and influenced by hormonal and dietary elements, while secondary osteoporosis is a result of various medical conditions and medications (**Keen *et al.*, 2022**).

A. Primary causes of osteoporosis: can be further subclasses into:

Primary type 1 osteoporosis is a result of hormonal changes occurring in postmenopausal women, influenced by the change of balance in estrogen and progesterone levels, which are responsible for the symbiosis of osteoblastic and osteoclastic activity. Estrogen regulates osteoclastic activity and progesterone in osteoblastic (**Noirrit-Esclassan *et al.*, 2021**). This is common after 10-15 years after menopause, usually in the age group of 50-70 years. Because of decreased estrogen levels in postmenopausal women, there is more bone loss than significant bone formation (**Keen *et al.*, 2022**).

Primary type 2 osteoporosis is also termed senile osteoporosis (**Simpson *et al.*, 2016**) and is generally a result of chronic low dietary calcium or sometimes factors that influence calcium metabolism like hyperparathyroidism (**Keen *et al.*, 2022**).

B. Secondary: Certain Medical conditions increase bone remodeling leading to interference or disruption of bone reformation. There is a resultant bone loss as a consequence of imbalance from new bone production and loss. Some of the common conditions promoting osteoporosis are diabetes, intestinal malabsorption, Liver disease, and metastatic bone disease (**De Martinis *et al.*, 2021**). In addition to medical conditions, medications that can cause osteoporosis is steroid use(**Taqui *et al.*, 2021**).

1.2.5. Pathophysiology of Osteoporosis.

Bone metabolism is a complex mechanism that revolves around the needful bone turnover involving bone formation (osteoblastic activity) and resorption (osteoclastic activity). This cycle continues throughout life and has phases of rapid bone formation to phases of increased resorption. This complex process takes weeks to months. It can be simplified for a better understanding of bone mineralization and formation (**Keen *et al.*, 2022**). As a

result of mechanical loading, low calcium from primary or secondary causes leads to microfractures beginning the activation phase. In this phase, pre- osteoclasts are attracted to remodeling sites that fuse to form multinucleated osteoclasts (**Bolamperti *et al.*, 2022**).

This starts the resorption phase with newly generated osteoclasts breaking down the bone, releasing growth factors locked inside the matrix, and then triggering apoptosis, or programmed cell death. All of this led to the construction of a resorption pit, followed by the removal of waste from activated macrophages and the changeover to the formation phase. To cover the hole left by the cavity or pit, an osteoid matrix made of collagen is deposited. About 3-6 months are required for the full mineralization of this matrix (**Bala *et al.*, 2010**), which is aided by osteoblasts secreting vesicles in an environment with higher calcium and phosphorous ion concentrations. Before new bone is formed, the old bone matrix needs to be fully eliminated for optimal remodeling. Factors that contribute to osteoporosis are as follows:

Osteoporosis has a significant hormonal influence, especially in postmenopausal women, where decreased estrogen levels harm bone formation by increasing the sensitivity to the bone resorption effect of parathyroid hormone. This can lead to rapid bone loss as compared to bone formation leading to osteoporosis. There are other risks and environmental factors that can potentiate this bone loss (**Stoffers *et al.*, 2019**). Bone remodeling is the process by which old bone is replaced by new bone. The normal bone remodeling process consists of the resting phase, activation, resorption, reversal, and formation. In the activation phase of remodeling, osteoclasts are recruited to the surface of the bone. In the resorption phase, osteoclasts generate an acidic microenvironment between the cell and the surface of the bone dissolving or resorbing the mineral content of the bone. In the reversal phase, osteoclasts undergo apoptosis and

osteoblasts are recruited to the bone surface. In the formation phase, osteoblasts then deposit collagen; this is mineralized to form new bone (**Kenkre *et al.*, 2018 and Peretti *et al.*, 2022**).

1.2.6. Diagnosis of Osteoporosis.

If osteoporosis is detected early, it can be treated and prevented from getting significantly worse. However, many people are not diagnosed until they experience complications from fractures. Because osteoporosis is generally an age-related condition, screening is recommended for all women over age 65 and men over age 70 to determine the need for diagnostic testing (**Force, 2018**). Osteoporosis is diagnosed by the evaluation of the findings of the bone mineral densitometry test, which measures the amount of bone mass in the skeleton (**Morse *et al.*, 2019**). Its measurement is usually carried out at the level of the central skeleton (lumbar spine and/or neck of the femur) using specific radiology equipment (dual-DXA radiological densitometry). To evaluate the possible secondary causes of osteoporosis, basic and complementary tests are carried out; the latter, depending on the clinical suspicion:

1.2.6.1. Basic tests.

Various investigations can be employed for OP diagnosis including **(Ciuffi *etal.*, 2020):**

A. Complete blood count with the count, leukocyte, and sedimentation rate.

B. Coagulation study, to see if it is normal or is accompanied by some alterations.

C. Complete biochemical investigations, including serum levels of calcium, phosphorus, creatinine, alkaline phosphatase, sodium, and potassium.

D. Serum levels of TSH and vitamin D3.

E. Total protein and albumin levels, with associated proteinogram to detect the possible presence of gammopathies.

Bone mineral density is most often described as a T-score or Z-score, both of which are units of standard deviation (SD). The T-score describes the number of SDs by which the BMD in an individual differs from the mean value expected in young healthy individuals. The operational definition of osteoporosis is based on the T-score for BMD assessed at the femoral neck and is defined as a value for BMD 2.5 SD or more below the young female adult mean (T-score less than or equal to − 2.5 SD). The Z-score describes the number of SDs by which the BMD in an individual differs from the mean value expected for age and sex. It is mostly used in children and adolescents (**Ferrari *et al.*, 2012 and Kanis *et al.*, 2019**).

1.2.6.2. Additional Investigations.

additional investigation plays an important role such as (**Zhang *et al.*, 2021**):

A. Determination of serum levels of parathormone, bone-specific alkaline phosphatase, prolactin, and immunoglobulins.

B. Biochemical markers of bone remodeling, such as the C-terminal propeptide of type I procollagen.

C. Fasting serum T3, T4, and plasma cortisol levels.

D. 24-hour urine study, to quantify total calcium and phosphorus elimination in one total day.

E. Determination of insulin growth factor type 1.

1.2.6.3. X-ray.

A plain X-ray report of low bone density is a strong predictor of osteopenia or osteoporosis but is not diagnostic. Changes in plain radiographs showing osteopenia, and wedging in vertebrae can be a clue of underlying fracture risk.

1.2.6.4. Dual-Energy X-Ray Absorptiometry Bone Density Scan.

The most common test used for the measurement of bone health and osteoporosis is the Dual-energy x-ray Absorptiometry (DEXA) bone density scan. It is a radiological diagnostic test and is universally classified by T and Z scores. T.score is the measurement of bone density compared to younger people and estimates the risk of fracture, whereas Z. score measures the bone density of the comparable age group.

Most guidelines use the T. score as a standard approach for estimating fracture risk assessment and treatment. T. score above -1 is Normal, a score of -1.0 to -2.5 is classified as osteopenia, and anything less than -2.5 is osteoporosis. These numerical values are the standard deviations of comparison (**Löffler *et al.***, **2019 and Alawi *et al.***, **2021**). Dual-energy X-ray Absorptiometry (DEXA) and quantitative computed tomography (QCT) of the lumbar spine were considered as preferred methods for the evaluation of BMD.

DEXA was recommended by the World Health Organization (WHO) as a gold standard for diagnosing osteoporosis. Because of its availability, relatively minimal radiation exposure, and simplicity of use, DEXA is the most commonly employed quantitative radiologic method to assess bone mass (**Adams**, **2009**). The standard test for diagnosing osteoporosis is the estimation of bone mineral density (BMD) in the proximal femur and lumbar spine with DXEA.

The US Preventive Services Task Force has recommended screening for osteoporosis with BMD to prevent osteoporotic fractures in women 65 years and older. The early diagnosis of osteoporosis is important for the prevention of

osteoporotic fractures because therapeutic drug treatments fractures because treatments are more effective in the early stages of the condition before fractures have appeared (**Yamamoto *et al.*, 2020**).

1.2.7. Complications of Osteoporosis.

Osteoporotic fractures are associated with high morbidity, increased mortality risk, and significant economic impact. Multiple fractures can severely impair the quality of life in females and can be a severe complication of osteoporosis. Fractures of the hip and spine can limit and disable the patients. Some might have to undergo surgical interventions, including neurosurgical if fractures cause any spinal canal stenosis or cord compression. Patients with hip fractures have an increased risk of mortality in first-year post fractures (**Saeki *et al.*, 2021** and **McClung *et al.*, 2018**).

1.2.8. Post-Menopausal Osteoporosis.

It is one of the major health problems associated with menopause-related estrogen deprivation (**Roeca *et al.*, 2021**). WHO defines natural menopause as at least 12 consecutive months of amenorrhea not due to physiologic and pathologic causes (**Ambikairajah *et al.*, 2022**). Statistics show that the mean age of natural menopause is 51 years in industrialized nations, compared to 48 years in poor and non-industrialized nations (**Sapre *et al.*, 2014**). In a normal menstrual cycle, the ovaries produce estrogens (i.e. oestradiol), androgens (i.e. testosterone), and progesterone in a cyclical pattern under the control of follicle-stimulating hormone (FSH) and luteinizing hormone (LH),which are secreted by the pituitary gland **(Briden *et al.*, 2020)**. The depletion of the ovarian reserve with age, a consequence of the finite number of oocytes and the consequently reduced levels of estrogen, leads to an increase in circulating follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels (**Rozenberg *et al.*, 2020**).

Adult women have less bone mass than adult men, and after menopause, they initially lose it more rapidly than men of comparable age. Consequently, theyare more prone to the development of serious osteoporosis. The cause of boneloss after menopause is primarily estrogen deficiency, and estrogen treatment arrests the progress of the disease (**Barrett**, **2019**).

1.2.9. Pathogenesis of Postmenopausal Osteoporosis.

Healthy bone requires continuous remodeling which is pivotal for bone density maintenance. It is estimated that nearly 10% of the bone is updated by this process every year (**Lerner**, **2006**). Osteoclasts and osteoblasts are two types of cells that essentially form the bone multicellular unit, coordinating well to regular the balance of bone resorption and bone formation (**Ji *et al.***, **2015**). This process is regulated by several hormones, including parathyroid hormone, calcitonin, $1,25(OH)_2D3$, and estrogen.

At menopause, the normal bone turnover cycle is impaired by estrogen deficiency. This may be due to the presence of estrogen receptors in osteoclast progenitor cells and multi-nucleated osteoclasts. The osteoclastic resorption activity increases while the osteoblastic activity decreases. As a result, the amount of bone resorbed exceeds the amount deposited, which leads to a net loss of bone. The increase in overall bone resorption is due to a weakened inhibition effect due to the reduction of available estrogen in both osteoclastogenesis and osteoclast activity (**Lerner**, **2006**).

There are two phases of bone loss in women: The first occurs predominantly in the trabecular bone and starts at menopause. It results from estrogen deficiency and leads to a disproportionate increase in bone resorption as compared with formation. This phase could be defined as menopause-related bone loss (**Rogers *et al.***, **2002**). This is age-related bone loss, which is the only phase that also happens in men. During the menopausal transition period, the average reduction

in BMD is about 10%. Approximately half of women are losing bone even more rapidly, perhaps as much as 10%–20% in those 5–6 years around menopause. About 25% of postmenopausal women can be classified as fast bone losers, and they could be discovered by the measurement of bone loss and bone resorption markers (**Noirrit-Esclassan *et al.*, 2021**).

1.3. Chronic Disease as a Risk Factor for Osteoporosis.

1.3.1. Diabetes Mellitus.

Diabetes mellitus is a metabolic condition characterized by elevated blood glucose levels due to insulin resistance, deficiency, or both (**AL-Badry *et al.*, 2020**). There are several types of diabetes but the most prevalent type is type 2 diabetes (T2DM) which is account for (90-95%) of diagnosed DM and occurs more commonly in adults and elderly people (**Zheng *et al.*, 2018**).

The most important physiological event in T2DM is insulin resistancewhich is a condition in which cells do not respond to insulin properly, as the disease progress, a lack of insulin may develop as a result of β-cell dysfunction (**Oguntibeju**, **2019**). The most common risk factors for T2DM are a combination of obesity and physical inactivity (**Glovaci *et al.*, 2019**).

Type 2 diabetes mellitus and osteoporosis are associated with severe morbidity, increased mortality, and important social costs, mainly due to their chronic consequences **(Valderrábano *et al*., 2018)**. Epidemiological data indicate that T2DM is associated with an increased risk of fractures, suggesting that skeletal fragility should be considered among the chronic complications of T2DM **(Russo *et al*., 2016)** and, in turn, T2DM should be considered among the causes of endocrine osteoporosis **(Eller-Vainicher *et al*., 2019)**.

Osteoporosis and T2DM are affected by aging and often coexist in the elderly **(Paschou *et al*., 2017)**. T2DM affects bone metabolism and strength by influencing osteoblast and osteoclast. The imbalance between osteoblast and osteoclast might cause osteoporosis **(Sassi *et al*., 2018)**. It has been confirmed that there are insulin receptors on the surface of both cells, **(Fulzele *et al*., 2010)** and insulin signaling can regulate the bone formation of osteoblasts and bone resorption of osteoclasts **(Conte *et al*., 2018)**. Insulin appears to have an anabolic effect on bone.

This effect is exerted when insulin binds to insulin receptors on osteoblasts. Through the action of intercellular signaling molecules known as insulin receptor substrates, osteoblast proliferation and differentiation are enhanced, and bone formation increases **(Adil *et al*., 2017)**. As well, T2DM might affect bone quality and quantity, leading to a change in the structural properties of bone mass.

T2DM affects bone homeostasis, so related fractures are considered a result of T2DM **(Ferrari *et al*., 2018)**. In vitro, it has been shown that the physiological concentration of insulin can increase the proliferation rate of osteoblasts, collagen synthesis, alkaline phosphatase production, and glucose uptake and inhibit osteoclast activity **(Rahman *et al*., 2021** and **Wang *et al*., 2021)**.

1.3.2. Hypertension.

Hypertension (HTN) has been identified by WHO as one of the most significant risk factors for morbidity and mortality worldwide and is responsible for the deaths of approximately nine million people annually. High blood pressure is largely asymptomatic, especially in the early stages, leading to its description asa ‟silent killer‟ (**WHO**, **2013**).

Hypertension is the leading preventable risk factor for cardiovascular disease (CVD) and all-cause mortality worldwide (**Mills *et al.*, 2020**). Cardiovascular disease (CVD) is mainly caused by arteriosclerosis, including coronary heart disease, hypertension, and other vascular diseases. Osteoporosis and CVD are common clinical diseases. With changing lifestyles and an aging population, the incidence has increased significantly and seriously threatens the physical and mental health of elderly individuals. It was previously thought that the two were independent of each other, but epidemiological studies have proven a correlation between them (**Rodríguez-Carrio *et al.*, 2019**).

Recently, with further study of the relationship between osteoporosis and CVD, it has been confirmed that they share common risk factors among elderly individuals, especially in postmenopausal women. Age, smoking, lack of physical exercise, vitamin D deficiency, and diabetes mellitus are considered common risk factors for osteoporosis and CVD (**Covic *et al.*, 2018** and **Chai *et al.*, 2021**). The common pathogenesis of vascular calcification and bone mineralization involves inflammation, and lipid metabolism (**Veronese *et al.*, 2017** and **García-Gómez *et al.*, 2020**).

1.3.2.1. Classification of Hypertension.

A. **Primary (essential) hypertension:** makes up 90–95% of cases and it is defined as high blood pressure with no obvious underlying cause.
B. **Secondary hypertension:** the remaining 5–10% of cases and it is defined as high blood pressure due to an identifiable cause like chronic kidney disease, narrowing of the aorta or kidney arteries, or an endocrine disorder such as excess aldosterone, cortisol, and or catecholamine (**Dhruv, 2020).**

1.3.2.2. Causes of Primary and Secondary Hypertension.

Blood pressure rises with aging and the risk of becoming hypertensive in later life is relatively high. Several environmental factors affect blood pressure. lack of exercise, obesity, stress, and depression all have a role in individual cases. Research proved that kidney diseases are the most common secondary cause of hypertension. Hypertension can also be due to endocrine conditions, such as Cushing's syndrome (**Al-Tu'ma *et al.*, 2016** and **Williams *et al.*,2018**).

1.3.2.3. The Renin–Angiotensin–Aldosterone System and Osteoporosis.

The main role of the RAAS system is to maintain blood pressure stability by regulating body fluid and electrolyte balance. Studies have found that RAAS activity is associated with osteoporosis. Some research results suggest that RAAS

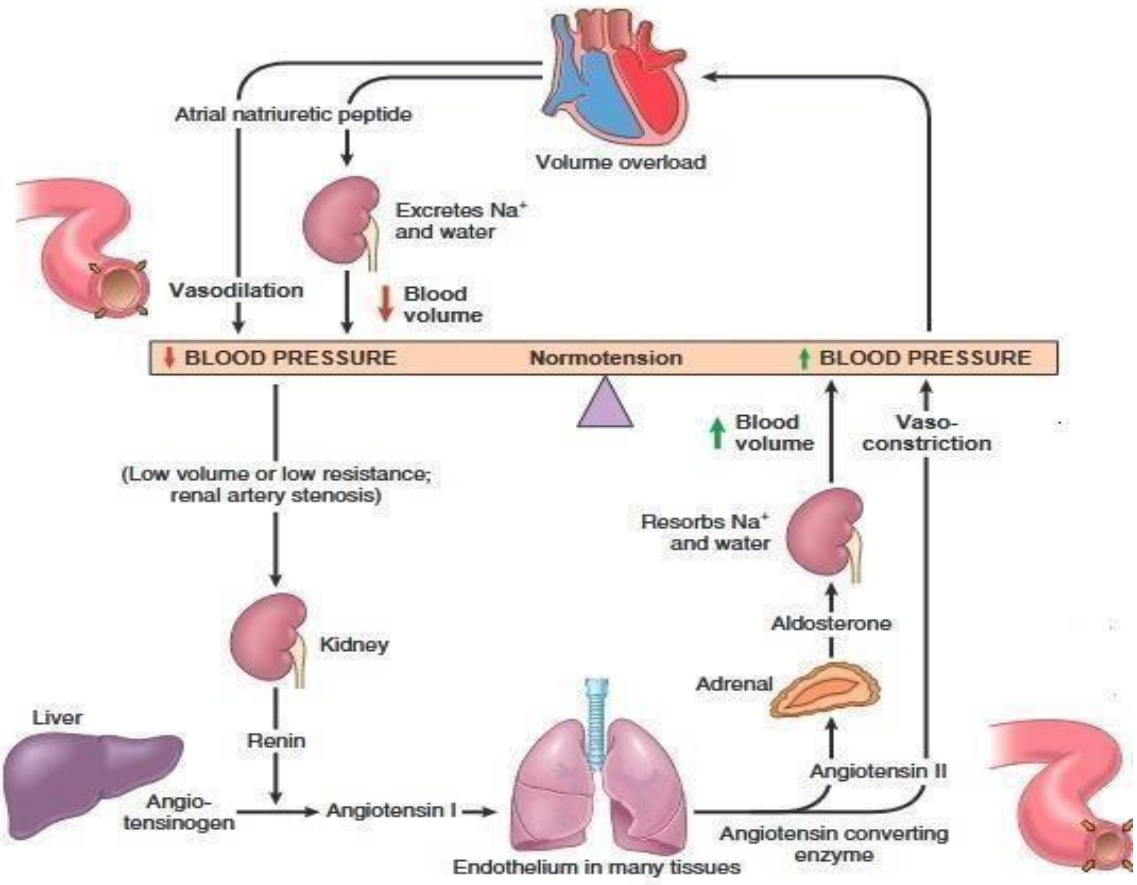

Fig.(1.2): Interplay of renin-angiotensin-aldosterone and atrial natriuretic peptide in maintaining blood pressure homeostasis (Klatt et al., 2021).

blockers do not reduce osteoporotic fractures and even increase fracture occurrence **(Zhang *et al*., 2017).** See figure (1.2).

Hypertension is thought to be linked to bone health through chronic elevation in the levels of parathyroid hormone, angiotensin II, and catecholaminesincluding adrenaline. In addition, hypertensive patients have decreased intestinal absorption, increased urinary calcium excretion, and decreased plasma vitamin D concentrations, which promote PTH continuous secretion Also, sustainedelevation of PTH contributes to bone resorption by increasing osteoclast differentiation **(Hu *et al*., 2020 and Hou *et al.*, 2022)**.

1.4. Vitamin D3.

Vitamin D3 is a steroid hormone, which makes it unique among vitamins. It's fat-soluble and comes in two types vitamin D2 and vitamin D3.

1.4.1. Chemistry of Vitamin D3.

Vitamin D2 and vitamin D3 are generated by the action of UVB, splitting a single (9,10) carbon-carbon bond in their respective precursor's ergosterol and cholesterol thus generating a secosteroid or "cut" steroid. The chemical structure of vitamin D3, therefore, has close similarities with that of the other cholesterol-derived hormones such as cortisol, aldosterone, testosterone, and estrogen (**Obaid *et al.*, 2020 and Rhodes *etal.*, 2021**).

The structural difference between vitamin D2 and vitamin D3 is the side chain of D2 contains a double bond between carbons 22, and 23, and a methyl group on carbon 24 (**Slominski *et al.*, 2014**). After the generation of cholecalciferol (or ergocalciferol), further hydroxylation is required in the liver and the kidney to generate the active 1,25-dihydroxy-cholecalciferol. Vitamins D2 and D3 are hydroxylated at position 25 in the liver to form 25-hydroxyvitamin D,
the major circulating form of vitamin D, and ultimately to the fully active moiety

by hydroxylation at position 1 in the kidney to form 1,25-dihydroxy vitamin D3 **(Pludowski *et al*., 2018)**.

In the blood approximately 85% of vitamin D3 is bound to vitamin D binding protein (DBP), 15% to albumin and just 0.03% of 25(OH)D3 and 0.4% of total 1,25(OH)2D3 are free vitamin D3 **(Daniel *et al*., 2019)**. It is thought that in most cells only free vitamin D3 can enter the cell. Cellular entry by protein-bound vitamin D3 is dependent on the expression of the cell surface receptor proteins megalin and cubulin **(Chun *et al*., 2019)** and is largely restricted to the kidney, parathyroid, and placenta. Free vitamin D3 diffuses through the plasma membrane and binds to the vitamin D receptor (VDR) in the cell nucleus where the vitamin D/VDR complex **(Carlberg *et al*., 2020)**. See figure (1.3),(1.4).

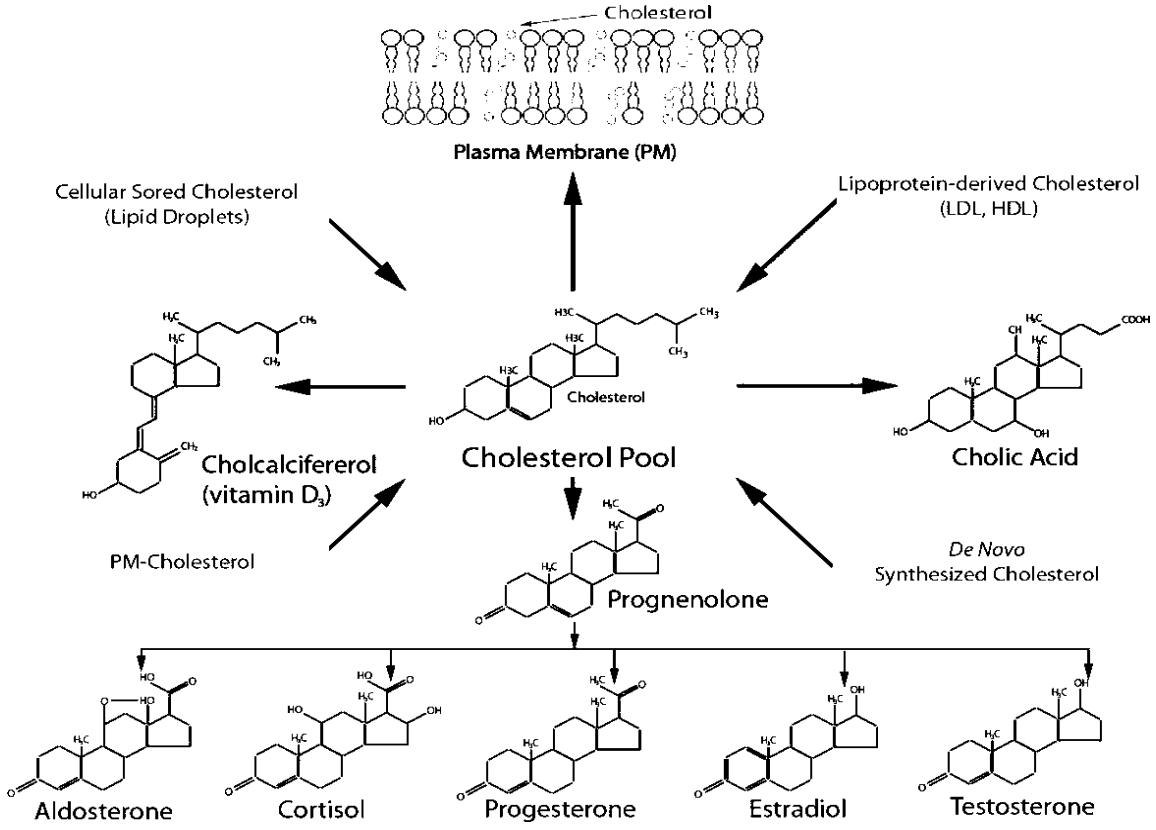

Fig. (1.3): Steroids, vitamin D3, and bile acids are all potential sources of cholesterol for product formation and membrane biogenesis (Hu *et al.*, 2010).

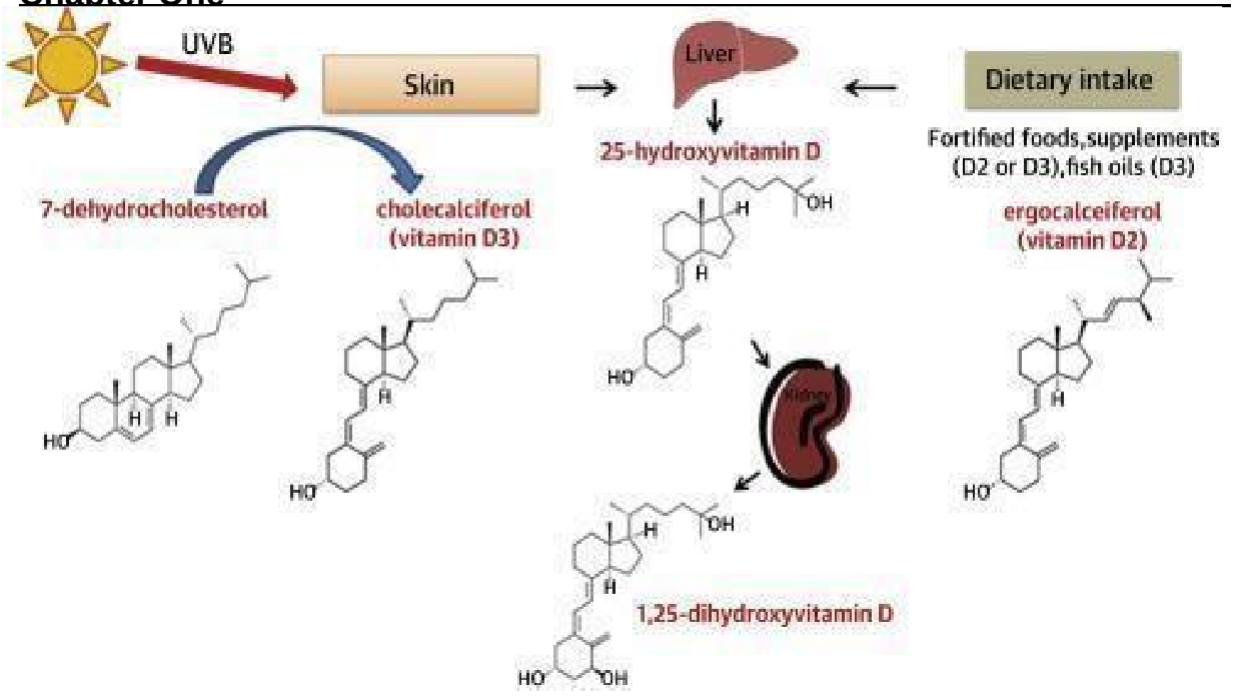

Fig. (1.4): Vitamin D3 forms, photobiosynthesis, and activation (Al Mheid *et al.*, 2017).

1.4.2. Functions of Vitamin D3.

The major function of $1,25(OH)_2D3$ is to regulate calcium homeostasis,other biological activities include the regulation of proliferation and differentiation of several cell lines including keratinocytes, endothelial cells, and osteoblasts. Most of these biological functions are mediated via the vitamin D3 nuclear receptor (VDR) which acts as a transcription factor regulating the transcription of target genes (**Khammissa *et al.*, 2018**). Calcium requires $1,25(OH)_2D3$ for adequate absorption and utilization (**Adhikari *et al.*, 2020**).

Calcium is brought into the enterocyte through a calcium transporter, it binds to a calcium-binding protein (calbindin D 28k) (**Zhao *et al.*, 2022**) this allows calcium to be transported through the enterocyte to the extracellular fluid and bumped out of the enterocyte into the blood stream via an ATP dependent calcium pump (**Wongdee *et al.*, 2019**), when $1,25(OH)_2D3$ enters the enterocytes and binds to vitamin D receptor which is a transcriptional factor and enters the nucleus where it'll activate a genetic program resulting in increasing the gene

expression of calcium transporter so that allows the enterocytes to uptake more calcium, and also increase the expression of calbindin D. All these processes is stimulated by parathyroid hormone (PTH) which stimulate the activation of vitamin D in the kidney (**Daniel D Bikle**, **2021**).

1.4.3. Vitamin D3 Activation.

For most people, the main source of vitamin D3 is the synthesis of the skin after exposure to UV-B radiation (290-315 nm). UVB radiation acts on the upper epidermis of the skin to convert 7-dehydrocholesterol to cholecalciferol through photolysis of the B-ring structure and subsequent isomerization. Maximum production is reached after 10-15 minutes of sun exposure in summer, depending on skin pigmentation (**Hanel *et al.***, **2020**).

Then, vitamin D3 is converted into 25-hydroxycholecalciferol (25(OH)D3) by 25-hydroxylase. This process mainly occurs in the liver but is not limited to this. The final stage of the formation of active 1,25-dihydroxycholecalciferol ($1,25(OH)_2$ D3) is mediated by the enzyme 25-hydroxyvitamin D3 1α-hydroxylase, which is found in proximal tubular cells (**Bikle *et al.***, **2020**).

1.4.4. Vitamin D Receptors.

Vitamin D receptor (VDR) is required for the majority of vitamin D3 actions, with $1,25(OH)_2$D3 as the primary ligand. Vitamin D receptor is considered a transcription factor. Intestinal epithelial cells, osteoblasts, parathyroid cells, and distal renal tubules all express VDR, which is important in calcium and phosphate balance(**Daniel D Bikle**, **2020**).

1.4.5. Vitamin D3 and Bone Metabolism.

Vitamin D3 promotes bone health and helps to avoid osteoporosis and fractures (**LeBoff *et al.*, 2020**). Vitamin D3 deficiency and inadequate dietary vitamin D3 are frequent in older people and are linked to an increasingly higher risk of fractures (**Kupisz-Urbańska *et al.*, 2021**). Vitamin D receptors (VDR) are found in osteocytes, osteoclasts, and osteoblasts, and they help to maintain bone health by regulating bone remodeling (**Hou *et al.*, 2018**).

Through autocrine and paracrine mechanisms, the metabolites of vitamin D3 [1,25$(OH)_2$D3], control the osteoblastogenesis of bone marrow stromal cells (**Jing Li *et al.*, 2020**). Osteoclasts are differentiating from macrophages. Osteoclast differentiation is controlled by vitamin D3, either directly or indirectly (**Shymanskyi *et al.*, 2018**). When compared to active vitamin D3, 25(OH)D3 can help with bone growth and maturation while also inhibiting the generation of osteoclasts (**Hou *et al.*, 2018**).

As a result, vitamin D3 influences the activity of osteoblasts, osteoclasts, and osteocytes, as well as bone formation, resorption, and quality. Vitamin D3 can be converted to its active form in osteoblasts, osteoclasts, and chondrocytes, based on the complexity of its active role in bones. Overexpression of 1α- hydroxylase in mature osteoblasts causes increased bone mass in both men and women, since bone formation rises while bone resorption remains constant, resulting in trabecular bone thickening (**Bouillon *et al.*, 2022**).

1.5. Estrogen Hormone.

Estrogens (E2) are hormones that regulate adipose tissue metabolism by controlling food intake, energy expenditure, and body distribution (**Bracht *et al.*, 2020**). Estrogens have widespread effects on several organs around the body and therefore play a role in a variety of physiological functions and disorders. Estrogens can act on receptors in both the cytoplasm and the plasma membrane to

mediate protein expression involving cell proliferation and metabolism (**Taheri *et al.*, 2020**). Estrogens are present in three forms: estrone (E1, has one OH group), estradiol (E2, has two OH group), and estriol (E3, has three OH group). Estradiol is the most extensively studied, as it plays key roles in reproductive phase functioning.

There are three receptors that have distinct presences and functions around the body (**Eaton *et al.*, 2019 and Al-Ghadban *et al.*, 2021**). Oestrogens and androgens exert an influence on skeletal homeostasis during growth and adulthood (**Mills *et al.*, 2021**). The postmenopausal decline in estrogenconcentration is a well-known factor causing a decrease in bone mass (**Ilovayskaya *et al.*, 2020**). Nonetheless, significant bone loss starts immediately after achieving peak bone mass regardless of changes in sex steroid concentrations (**David *et al.*, 2022**).

After menopause, due to a decrease in oestradiol concentration, loss of bone density accelerates. At the beginning of menopause loss of estrogen action on osteoclasts leads to trabecular perforation and loss of connectivity (**Jäckle *et al.*, 2020**). Estrogen affects bone through the following mechanisms:

A. Lowering the sensitivity of bone mass to PTH **(Cosman *et al*., 1993)** (parathyroid hormone), thus reducing bone resorption.

B. Increasing the production of calcitonin, thus inhibiting bone resorption **(Yamazaki *et al*., 1986)**.

C. Accelerating calcium resorption by the intestine **(Civitelli *et al*., 1988)**.

D. Reducing the calcium excretion from the kidney, and estrogen can also have direct effects on the bone since there are estrogen receptors **(Bartl *et al*., 2009)**.

1.5.1. Estrogen Deficiency-Related to Postmenopausal Osteoporosis.

1.5.2. Molecular Mechanisms of Actions.

It is known that bone remodeling is accomplished by osteoblasts, osteoclasts, and osteocytes. The negative imbalance of bone remodeling, in which bone resorption exceeds bone formation, results in osteoporosis. At a cellular level, several mechanisms contribute to bone loss related to estrogen deficiency. See fig (1-5).

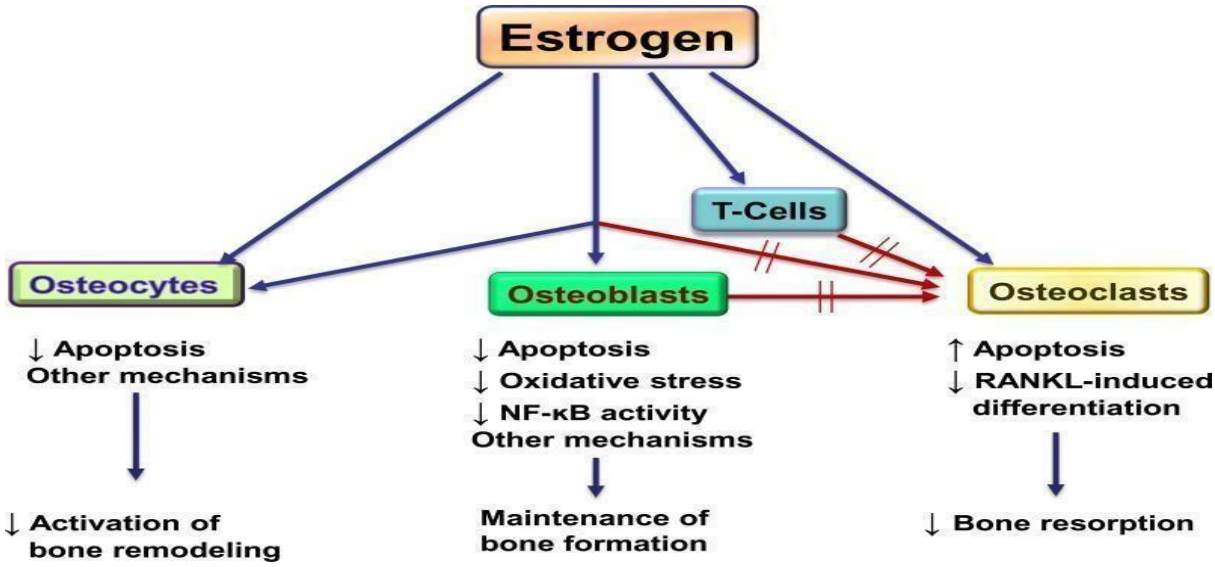

Fig. (1.5): Working model for estrogen regulation of bone turnover via effects on osteocytes, osteoblasts, osteoclasts, and T-cells (Khosla *et al.*, 2012)

1.5.3. Estrogen Signaling.

Estrogen receptors (ERs) are highly expressed in osteoblasts, osteoclasts, and osteocytes, offering protective effects in bone. Estrogen binds with ERs, which regulate the expression of estrogen targets genes-encoding proteins such as IL-1, insulin-like growth factor 1 (IGF1), and TGFβ (**Engin *et al.***, **2019**). Furthermore, ERs can also suppress the action of nuclear factor-κβ ligand (RANKL), thus inhibiting osteoclast formation and bone resorptive activity (**Abu-Amer**, **2013**). Study has also shown that estrogen deficiency directly affects cell differentiation and apoptosis (**Riggs**, **2000**). The net effects of estrogen deficiency are increased

bone turnover and enhanced bone resorption, which results in osteoporosis (**Jiao Li *et al.*, 2020**). The main effect of estrogen is the inhibition of bone remodeling, likely via the osteocyte. Estrogen also inhibits bone resorption, by direct effects on osteoclasts, although effects of estrogen on osteoblast/osteocyte and T cell regulation of osteoclasts also play a role (**Cheng *et al.*, 2022**).

1.5.4. Sources of Estrogen.

In pre-menopausal women, the ovaries are the principal source of estradiol. However, in postmenopausal women, the main source of estrogen is estrone which is converted from the adipose tissue, and the dehydroepiandrosterone (DHEA), which itself is produced by the adrenal cortex glands. Exogenous compounds with estrogen-like activities include contraceptive medication, hormone replacement therapy, phytoestrogens, and industrial chemicals (**Wehbe *et al.*, 2020; Hetemäki *et al.*, 2021**).

1.6. Minerals.

Bone growth and metabolism are also modulated by zinc, calcium, phosphorus, and magnesium. It is assumed that both deficiency and excess of trace elements may be risk factors for the development of bone diseases such as osteoporosis (**Wang *et al.*, 2020**).

1.6.1. Calcium.

Calcium(Ca) is a nutrient necessary for the proper functioning of the human body. This macro element, which influences many extracellular and intracellular processes, is essential for the development, growth, and maintenance of bone, and the stability of the cellular cytoskeleton (**Beto, 2015**). Ca regulates the activity of intracellular enzymes and participates in neuronal conduction through the ion channels. The total content of Ca in an adult human body is about 1200 g, which is about 2% of body weight. About 99% of the body's Ca is found in the bones and the teeth, stored as hydroxyapatite, which is responsible for the

mineralization of tissues (**Fischer *et al.*, 2018**). The main factors that keep blood Ca on constant levels are (1,25(OH)2 D3) and parathyroid hormone (PTH)(**Bergwitz *et al.*, 2010**).

The concentration of Ca in bones is determined by a variety of issues, among them sex, age, diet, physical activity, smoking, ethnicity, as well as genetic and endocrine factors (**Theobald, 2005**). The elderly, and especially postmenopausal women show a reduced capacity to absorb Ca (**Bhattarai *et al.*, 2020**). The older a woman is, the lower level of hormones she has and the weaker her bones (**Alswat, 2017**).

1.6.2. Magnesium.

Magnesium is essential for the function of many key organs and has an important role in the physiology of humans and other mammals. The presence of Mg is vital in bone and teeth structures and has a role in more than 300 enzymes as a cofactor, including binding to ATP for kinase reactions, the permeability of excitable membranes, and neuromuscular transmission (**Long *et al.*, 2014 and Zoroddu *et al.*, 2019**). More than half of the body's Mg requirement (60%) is stored in the bones, and the remaining 30-40% is stored in skeletal muscles and soft tissues, while only 1% is stored in bodily liquids (**Romani, 2013**).

Mg deficiency can result in endothelial dysfunction that damages bone health. In addition, Mg deficiency leads to bone remodeling and osteopenia (**Castiglioni *et al.*, 2013**). Mg deficiency indirectly affects bone structure through the regulation of PTH levels and serum $1,25(OH)_2D3$ levels, which finally results in hypocalcemia. Because Mg acts as a cofactor in the creation of PTH, a low Mg level decreases the secretion of PTH, which results in $1,25(OH)_2D3$ deficiency (**Kerstetter *et al.*, 2011**). Magnesium can intervene with calciotropic hormone function and is known as a neutralizer of calcium (**Song *et al.*, 2007**).

1.6.3. Phosphorus.

Phosphorus is one of the essential elements of the human body and is required for a diverse range of processes, such as ATP synthesis, signal transduction, and bone mineralization. The vast majority (85%) of phosphorus in the body exists as a component of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] in the extracellular matrix of bone and teeth **(Fukumoto**, **2014)**.

In contrast, intracellular phosphorus accounts for 14% of total body phosphorus, and only 1% is present, mostly as inorganic phosphate (Pi), in extracellular fluids (**Serna *et al.***, **2020**). Biochemical mineralization happens in hard tissues such as bone and cartilage, where many tissue-specific cells are involved in this mineralization process. Within bone, osteoblasts that are wrapping the osteoid are accountable for hydroxyapatite production (**Karpen**, **2018**).

1.6.4. Zinc.

Zinc is an essential component of human body. Over 85 percent of body Zn total is found in skeletal muscles and bones (**Konduru *et al.***, **2014**), whilezinc contained in plasma represents only 0.1 percent of the total, and its concentration, strictly regulated, varies from about 10 to 15 μmol/l. Zinc is a constituent of various enzymes that play a role in maintaining the structural integrity of proteins and in regulating gene expression; there are almost 100 specific enzymes that depend on Zn for catalytic activity (**Hongfang *et al.***, **2020**).

Its biological functions can be divided into three categories: catalytic, structural, and regulatory. Among the many functions performed by Zn (for example includes the formation of bones and muscles. It also has an important role in stimulating the synthesis of alkaline phosphatase(ALP) in osteoblasts (**Rondanelli *et al.***, **2021**). Bone is a reservoir of minerals and trace elements such as Zn (**Harkness *etal.***, **2019**). Zn occurs in the mineral component of bone,

probably in hydroxyapatite (**Chaudhry *et al.*, 2022**). It plays an integral role in numerous osteogenic enzymes, including ALP. Zn acts as a local regulator of an osteoblast to form the bony framework for organic matrix formation. Zn increases the activity of bone ALP and activates osteoblast tyrosine kinase. Deficiency in the Zn during postmenopausal conditions leads to bone growth retardation and osteopenia (**Bhardwaj *et al.*, 2018**).

1.7. Enzymes and Osteoporosis.

1.7.1. Alkaline Phosphatase.

ALP is a group of enzymes, located on the outer layer of the cell membrane; they catalyze the hydrolysis of organic phosphate esters present in the extracellular space. Zinc and magnesium are important cofactors of this enzyme. Although ALP is present in different body tissues and has different physiochemical properties, they are true isoenzymes because they catalyze the same reaction. The majority of ALP in serum (more than 80%) is released from the liver and bone, and in small amounts from the intestine. Even though ALP is present in many tissues throughout the body, their precise physiological function remains largely unknown (**van der Doelen *et al.*, 2019** and **Pinart *et al.*, 2020**).

The isoenzymes of ALP are classified as tissue-specific and tissue-nonspecific types. ALP found in the intestine, placenta, and germinal tissue are tissue-specific. This means they are found only in the tissues where they are expressed in physiological conditions (**Sato *et al.*, 2021**). They may also contribute to the circulating pool of serum ALP under specific situations when there is increased stimulation of their production. The tissue-nonspecific ALP form most of the fraction circulating in serum and, therefore, is of clinicalinterest. A single gene encodes it and is expressed in the liver, bone, and kidneys. Intestinal ALP is coded by a separate gene, which is different from the gene that codes for placental ALP and the Regan isoenzyme (**Cristoferi *et al.*, 2018**).

The two isoforms of tissue non-specific ALP, liver-specific ALP, andbone-specific ALP (BALP) exist in almost equal proportion in serum. Physiologically, BALP adheres to the osteoblastic cell membrane with only a small amount released in serum. Its concentration in serum rises only in cases of increased remodeling of bone. A glycoprotein that is found on the surface of osteoblasts, BALP reflects the biosynthetic activity of these bone-forming cells. BALP is a sensitive and reliable indicator of bone metabolism (**Kress**, **1998**).

1.8. Genetic study.

1.8.1. Macrophage Erythroblast Attacher Gene Polymorphism.

The genome-wide association study (GWAS) design has accelerated the discovery of gene-phenotype associations for complex disorders. GWAS focusing on osteoporosis and BMD have been intensively conducted in the last decade. Many publications on BMD and osteoporosis related phenotypes have been published, and more than 200 association signals derived from approximately 90 loci that achieved genome-wide significance have been reported (**Buniello *et al.***, **2019**).

A large proportion of these loci are located within five major bone metabolism-related pathways, including the mesenchymal cell differentiation, Despite hundreds of association signals discovered by large-scale GWAS, all of these identified variants together could only explain 10–20% of the variance in bone phenotypes (**Trajanoska *et al.***, **2019**).

1.8.2. Molecular Diagnostic of Macrophage Erythroblast Attacher Gene Polymorphism.

Gene macrophage erythroblast attacher (MAEA) is located on chromosome 4p16.3 and is expressed in a wide range of human cells, including osteoblasts and osteoclasts, which are important for bone metabolism. A recent cross-sectional study suggested an association between single nucleotide polymorphism (SNP) rs6815464, genetic polymorphism of MAEA, and low BMD of the total hip but not of the lumbar spine or femoral neck in Japanese women (**Che *et al.*, 2019**). However, large- scale and well-designed replication studies in other populations are still needed to confirm this association signal (**Cai *et al.*, 2021**).

1.8.3. Macrophage Erythroblast Attacher Protein.

Macrophage Erythroblast Attacher was discovered in 1994 as an integral membrane protein that mediates the attachment of the erythroid cells to macrophages and is essential for bone marrow hematopoiesis and with small extracellular and large cytoplasmic domains. MAEA protein is localized in the cell surface, nucleus, and plasma membrane of macrophages. MAEA is essential for the maturation of erythroid cells and macrophages. MAEA contributes to nuclear structure rearrangements and cell division both in macrophages and erythroblasts (**Hanspal *et al.*, 1998** and **Che *et al.*, 2019**).

1.8.4. Role of Polymerase Chain Reaction in Clinical Diagnosis.

Polymerase chain reaction (PCR) was invented by Mullis in 1983 andpatented in 1985. Its principle is based on the use of DNA polymerase which is an in vitro replication of specific DNA sequences (**Bhanothu *et al.*, 2019**). This method can generate tens of billions of copies of a particular DNA fragment (thesequence of interest, DNA of interest, or target DNA) from a DNA extract (DNA template). The PCR is widely used for diagnostic purposes to detect the

presence of a specific DNA sequence of this or that organism in a biological fluid (**Mukhtar *et al.*, 2021**).

PCR is a fabulous diagnostic tool (**Bhat *et al.*, 2022**). It is already widely used in the detection of genetic diseases. The amplification of all or part of a gene responsible for a genetic disease makes it possible to reveal the deleterious mutations, their positions, their sizes, and their natures. It is thus possible to detect deletions, inversions, insertions, and even point mutations, either by direct analysis of PCR products by electrophoresis or by combining PCR with other techniques. Although other diagnostic tools are effective at detecting these diseases, PCR has the enormous advantage of producing very reliable and rapid results from minute biological samples in which the presence of the pathogen is not always detectable with other techniques (**Kadri**, **2019**).

The general principle of PCR includes annealing, reverse transcription, and enzymatic action, used these days is similar to the protocol followed for the first time. It's widely used for diagnostic purposes, to detect a particular gene in a biological fluid, to make a fingerprint, to check food quality, for the identification of different varieties of plants and animals, to diagnose heredity and infectious disease. The PCR has become one of the most important techniques in modern biological and medical science (**Wang *et al.*, 2017**).

Aims of the Study:

This study aims:

1. To investigate the association of MAEA gene polymorphism with postmenopausal osteoporosis (PMOP) in Kerbala province women.
2. To investigate the association between genetic polymorphisms of MAEA and postmenopausal osteoporosis and compared with osteopenia group.
3. To determine if serum encode MAEA protein levels associated with risk SNP for osteoporosis.
4. Determination of alkaline phosphatase isoenzyme in sera of osteoporosis and compared with osteopenia group.
5. To study the association between MAEA gene and bone ALP isoenzyme and association between MAEA gene and vitamin D3 in osteoporosis and compared with osteopenia group.

Chapter Two

Subjects, Materials and Methods

2. Materials and Methods

2.1. Materials

2.1.1. Patients

This study was conducted on 160 women who were diagnosed by a physician and were divided into four groups: 40 of them were postmenopausal Osteoporosis women with diabetes mellitus, 40 of them were postmenopausal Osteoporosis women with hypertension and 40 of them were postmenopausal Osteoporosis women without (diabetic Mellitus/hypertension), and 40 of them were the postmenopausal Osteopenia group. Osteoporosis and osteopenia groups were collected from the Al-Hassan center for Endocrinology and diabetes mellitus and Osteoporosis Center at Al-Hussein Medical City, Kerbala Health Directorates, Kerbala/ Iraq from Nov. 2021 to March. 2022 with age ranged between (50-86) years and they are classified as shown in Fig (2-1).

Table (2-1): WHO definition of osteoporosis and osteopenia (Kanis, 1994).

Normal	T-score 0 to – 1
Osteopenia	T-score between –1 to –2.5
Osteoporosis	T-score of ≤ –2.5
Severe osteoporosis	T-score of ≤ –2.5 along with fracture

They were diagnosed with osteoporosis by using a measured T-score by Dual-energy x-ray absorptiometry scan (DEXA) at lumbar spine regions (L1–L4 vertebrae) confirmed osteoporosis and osteopenia. Osteoporosis consisted of four categories that were collected at admission and the disease severity was assessed using WHO criteria (Table 2-1). The National Osteoporosis Foundation provides guidelines on the most up-to-date recommendations regarding diagnostic criteria (**Dawson-Hughes *et al.*, 2008**).

The current investigation was approved by local medical ethics and all participants, informed consent before the onset of the study. The women registered their data such as name, age, weight, and height.

2.1.2. Osteopenia Group

Forty women in the group do not have (type 2 diabetes, hypertension, and osteoporosis).

2.1.3. Study design

The study design was a cross sectional study that included 160 postmenopausal women, 120 of them are Osteoporosis patients and 40 have the osteopenia group.

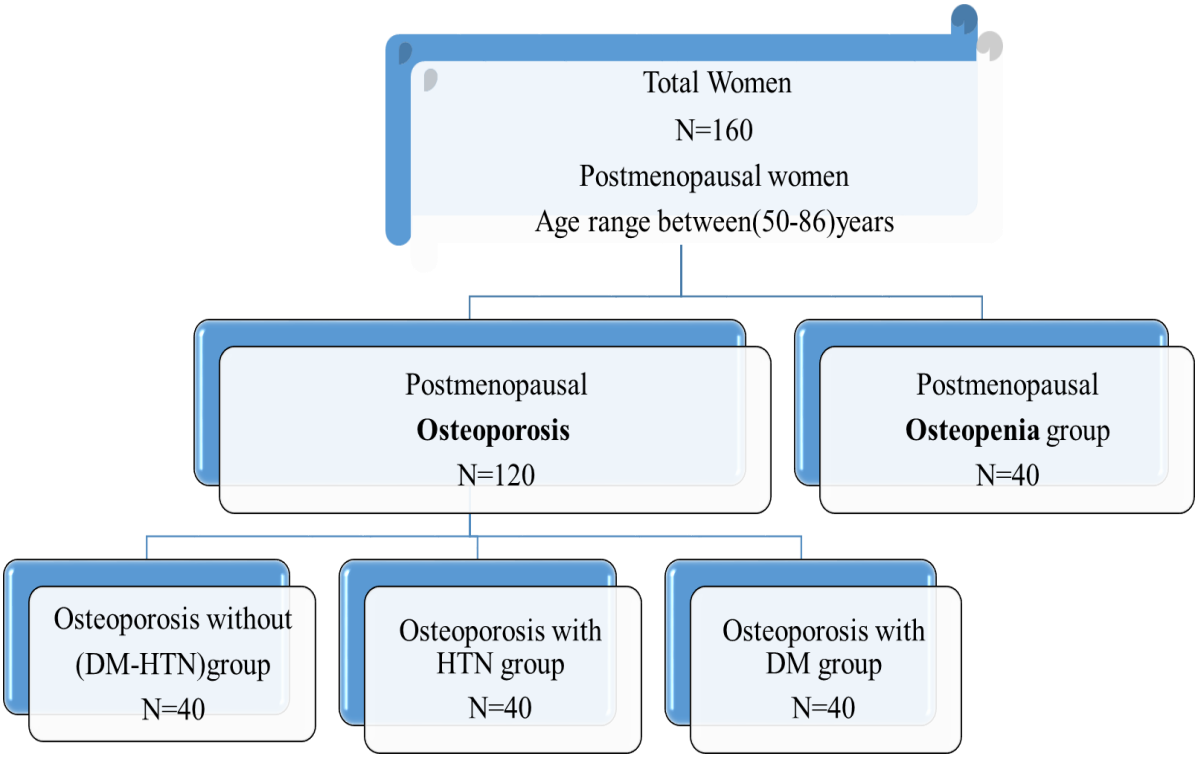

Fig. (2.1): Scheme of subjects groups

Exclusion criteria:

Exclusion criteria were as follows: individuals with a history of medication for the treatment of PMOP or medication known to affect bone metabolism within 6 months. patients with diseases known to affect bone metabolisms, such as severe malabsorption syndrome, chronic liver disease, inflammatory bowel disease, hypercalcemia, Paget's bone disease, active kidney stones, Osteogenesis imperfecta, and pituitary disease.

Women who were identified with surgical menopause, hormone replacement therapy, and type 1 diabetic Mellitus women were excluded from the study. Patients with osteoporosis, such as rheumatoid arthritis, Osteomalacia, multiple myeloma, and gout. Patients who have been continuously treated with bisphosphonates or PTH for more than 1 month within 1 year and patients who have continuously used estrogen receptor modulators within 6 months. Patients who have continuously received calcitonin, estrogen, corticosteroids, calcitriol, and other drugs that can change bone metabolism within 3 months, Patients with severe kidney diseases, peptic ulcer, immune diseases, malignant tumors, any type thalassemia diseases, and other serious underlying diseases, smoker. patients with factors that affect the measurement results of BMD, such as a history of lumbar spine fixation surgery, ankylosing spondylitis, amputation surgery, and bone fracture were excluded.

Inclusion criteria:

Inclusion criteria were as follows: only patients with postmenopausal women and age of females $\geq$ 50 years and newly diagnosed. Osteoporosis patientswith T2DM that have DM duration $\geq$5, Osteoporosis patients with hypertension, Osteoporosis patients without (T2DM / Hypertension) and osteopenia group without (T2DM, Hypertension, and osteoporosis) were enrolled in the study.

2.1.4. Blood Specimens Collection

Five milliliters of venous blood were drawn from each patient's group by medical syringes, during the morning (8-11 a.m.). The first part (3 ml) was put into gel tubes and left at room temperature for nearly thirty minutes forclotting, then centrifuged at 3000 rpm for 10 minutes to separate serum which was divided into two tubes. Serum was pipetted out into sterile Eppendorf tubes.

The first one was used to determine 25(OH)D3 and Estrogen (E2) by CLIA and use a smart 120 analyzer to determine Calcium (Ca), Zinc (Zn), Magnesium (Mg), Phosphorus (P) concentration, and activity levels of total alkaline phosphatase (TALP). The second tube was stored at -20 ^{o}C until it was used for the determination of bone-specific alkaline phosphatase (BALP) activity level and Macrophage Erythroblast Attacher protein (MAEA) levels by Enzyme-linked immunosorbent assay (ELISA) Technique.

The second part (2 ml) of blood was collected in an EDTA tube and was stored by freezing at -20 ^{o}C until used for DNA extraction and then performing various molecular analyses concerning MAEA gene polymorphism by polymerase chain reaction and related techniques.

2.1.5. Instruments and Tools

Various instruments and laboratory tools are used in this study as shown in Table (2-2).

Table (2-2): Instruments and tools used in this study

No.	Instruments	Company	Country
1	Analyzer smart120	Genotek	USA
2	Balance	Sartorius	Germany
3	Chemiluminescence immunoassay CL900i	Mindray	China
4	Cobas e 411 Analyzer System	Roche	Germany
5	Deep freeze	Nikai	Japan
6	Different glassware	Different sources	China
7	Dual-energy X-ray absorptiometry	DMS Stratos	France
8	Electrophoresis System	Drawell	China
9	ELISA microplate reader	Biotek	USA
10	Incubator	Biobase	China
11	Microwave Oven	Samsung	Korea
12	Mini Centrifuge	Biobase	China
13	Nanodrop spectrophotometer	Quawell Q5000	China
14	Refrigerated Centrifuge	Biobase	China
15	Refrigerator	Nikai	Japan
16	Thermal Cycler (Veriti)	Thermo Fisher Scientific	USA
17	UV Transilluminator	Biobase	China
18	Vortex mixer	Quality Lab System,	England

2.1.6. Chemicals and Kits

Various biochemical kits were used in this study. Table (2-3) shows the kits used with their sources.

Table (2-3): Chemicals and kits used in this study with their suppliers

No.	Chemicals and kits	Company	Country
1	ReliaPrep Blood gDNA miniprep Sys.	Promega,	USA
2	100bp DNA Ladder 250µl	Promega,	USA
3	Tag Green Master Mix	Promega,	USA
4	Loading dye	Promega,	USA
5	Ethidium Bromide Solution	Promega,	USA
6	Agarose,100g	Promega,	USA
7	TBE (Tries Borate EDTA) Buffer,10X,1000ml	Promega,	USA
8	Water, nuclease –Free 25ml	Promega,	USA
9	Primers	Macrogen	Korea
10	BALP ELISA Kit	Pars Biochem	China
11	MAEA protein ELISA Kit	Sunlong	China
12	Magnesium Kit	Giesse diagnostics	Italia
13	Zinc Kit	Giesse diagnostics	Italia
14	Calcium Kit	Giesse diagnostics	Italia
15	ALP Kit	Giesse diagnostics	Italia

16	Phosphorus	Giesse diagnostics	Italia
17	Estrogen E2 Kit	Mindary biomedical	China
18	25(OH) Vitamin D Kit	Shenzhen biomedical	China

2.1.7. Primers

Table (2-4) indicates the sequence of primers used in the molecular analysis of this study.

Table (2-4): Primers used in molecular study

Primer Name	Sequence	Annealing Temp .°C	Product Size (bp)
MAEA- rs10025665-F	5- GGTGAGACGGGCTACACTCAA-3	61	258
MAEA- rs10025665-F	5- GGTGAGACGGGCTACACTCAG-3	61	258
MAEA- rs10025665-R	5- CATTTCCACAACCTGGCAGCTC-3	61	258

2.2. Methods

2.2.1. Measurement of Body Mass Index

Obesity was defined using the body mass index (BMI). The WHO BMI range (18.5- 24.99 kg/m^2) did not adequately describe the degree of fatness. The WHO categorization for adults was utilized in this investigation, as illustrated in Table (2-5) **(Seo *et al.*, 2019)**. The BMI was measured by dividing weight in kilograms by the length of an individual in square meters: **BMI, (kg/m^2) = (weight in kg) / (height in meters^2).**

Table (2-5): Estimation of body mass index

Weight status	BMI (kg/m²)
Underweight	< 18.5 kg/m^2
Normal weight	18.5 to 24.9 kg/m^2
Overweight	25.0 to 29.9 kg/m^2
Obesity class I	30.0-34.99 kg/m^2
Obesity class II	35.0-39.99 kg/m2

2.2.2. Bone Mineral Density Measurements

In the lumbar spine (first to fourth lumbar vertebrae) the BMD was measured by DEXA Scan at the Osteoporosis Center of Al-Hussein Medical City, Kerbala/Iraq. All of the women in the study have their DEXA measurements for the first time and have no prior history of low bone density, osteoporosis, or treatment for osteoporosis. All of the BMD values were expressed in g/cm2. According to the criteria of diagnostic categories published by a WHO study group in 1994, the normal category has a BMD within one standard deviation of the reference mean (young adults). The BMD is more than one SD below the reference mean in osteopenia. However, with osteoporosis, the BMD is 2.5 SD or lower than the reference mean.

Represented by the following T-score (WHO, 1994):

I. If T-score ≤ -2.5 this means osteoporosis.
II. Osteopenia if -2.5 < T-score< -1
III. Normal if T-score > -1 (**Pavone *et al.*, 2017**).

2.2.3. Measurement of Serum 25-Hydroxy Vitamin D3 Level

The quantitative determination of 25-OH Vitamin D3 in human serum. The method can be used for samples over the range of 3.0-150ng/ml. The test was performed on the Fully-auto Chemiluminescence immunoassay (CLIA) analyzer Cobas e411 System.

Principle

The 25(OH)D3 assay was a competitive chemiluminescence immunoassay. The 25(OH)D3 assay was a two incubation Chemiluminescence immunoassay for the quantitative determination of a total of 25(OH)D3 in human serum. In the first incubation, the 25(OH)D3 was dissociated from its binding protein by the displacing reagent and binds to the 25(OH)D3 antibody. On magnetic microbeads forming an antibody-antigen complex. Following a second incubation, the 25(OH)D3 labeled ABEI was added. The rest unbound material is removed during a wash cycle. Subsequently, the Starter 1+2 was added to initiate a flash chemiluminescence reaction. The resulting chemiluminescent reaction was measured as a relative light unit (RLUs), which is inverse proportional to the concentration of 25(OH)D3 present in the sample (or calibrator/control, if applicable).

Procedure

Resuspension of the magnetic microbeads takes place automatically when the kit is loaded successfully, ensuring the magnetic microbeads are resuspended homogenous before use. To ensure proper test performance, strictly adhere to the operating instructions of the Maglumi Fully-auto CLIA analyzer. Each test parameter was identified via an RFID CHIP on the Reagent Kit.

Calculations:

The results automatically calculated the 25-OH Vitamin D concentration in each sample using a calibration curve that was generated by a 2-point calibration master curve procedure. The results are expressed in ng/ml.

Interpretation of test result.

Factors such as dietary intake, race, UV exposure, season, and age are allknown to affect the normal levels of 25-OH Vitamin D. A review of the literature suggests the following ranges for the classification of 25-OH Vitamin D status see table (2-6):

Table (2-6): Classification of Vitamin D status (Turpeinen *et al.*, 2003).

Vitamin D Status	25(OH)D3
Deficiency	< 10 ng/ml
Insufficiency	10-29 ng/ml
Sufficiency	30-100 ng/ml
Toxicity	>100 ng/ml

The result may differ between laboratories due to variations in population and test method. It recommended that each laboratory establish its expected ranges (**Turpeinen *et al.*, 2003**).

2.2.4. Measurement of serum Estradiol Level

The E2 assay is a CLIA for the quantitative determination of E2 in human serum. The test has to be performed on the Fully-auto CLIA Mindray analyzer (CL900i).

Principle:

The E2 assay is a competitive binding immuno-enzymatic assay to determine the level of estradiol. In the first step, a sample, paramagnetic microparticle coated with goat anti-rabbit IgG, sample treatment solution, and polyclonal

anti-estradiol antibody (rabbit) was added to a reaction vessel. After incubation, E2 in the sample binds to the anti-estradiol antibody. In the second step, E2- alkaline phosphatase conjugate was added to the reaction vessel. E2 in the sample competes with the E2-alkaline phosphatase conjugatefor binding sites on the anti-estradiol antibody. The resulting antigen: antibody complexes were bound to goat anti-rabbit IgG on the microparticle, which was magnetically captured while other unbound substances were removed by washing. In the third step, the substrate solution is added to the reaction vessel. It was catalyzed by E2-alkaline phosphatase conjugate in the immunocomplex retained on the microparticle.

The resulting Chemiluminescent reaction was measured as RLUs by a photomultiplier built into the system. The amount of E2 present in the sample was inversely proportional to the RLUs generated during the reaction. The E2 concentration may be determined via a calibration curve.

Procedure:

Preparatory all of the materials were needed for the assay. Before loading the E2 (CLIA)reagent kit for the first time on the machine, the unopened reagent container should be gently inverted at least 30 times to resuspend the microparticles that were settled during shipment or storage. Inspect the bottle visually to check that the microparticles have been resuspended. It was not suggested to use this bottle of reagent if the microparticles cannot be resuspended. For a single test, 35 µL of sample is required. To ensure proper test performance, strictly adhere to the operating instructions of Mindray Fully-auto CLIA analyzer. The cup containing serum was entered into the equipment and analyzed automatically.

Calculations

The results automatically calculated the analyte concentration of each sample on the master calibration curve read from the barcode, and a 4-Parameter Logistic Curve Fitting (4PLC) with the relative light units generated from three-level calibrators of defined concentration values. The results are shown in the unit of Pg/ml.

Interpretation of test result

They determine the reference range of the E2 assay. the variation in geography, race, sex, and age, it is highly recommended that each laboratory should establish its reference range.

Table (2-7): the reference range of E2 assay.

Category		N	Central 95% Range
Males		130	＜25-84 pg/ml
females	Follicular phase	125	20-138 pg/ml
	Ovulation phase	42	100-440 pg/ml
	Luteal phase	128	31-317 pg/ml
	Post menopause	134	<25-84 pg/ml

Normal value ranges may vary slightly among different laboratories. Some labs use different measurements or test different samples (**Yen**, **1991**).

2.2.5. Determination of Serum Calcium Level

Principle

In pH neutral medium, calcium forms with Arsenazo III (1, 8-Dihydroxy-3, 6- disulpho-2,7-naphthalene-bis(azo)-dibenzenearsonic acid) a stable blue-violet complex. The intensity of the color is directly proportional to the amount of calcium present in the sample. The quantitative determination of calcium in serum by Chemistry Analyzer Smart 120 by Colorimetric method.

Calcium + Arzenazo III---------- ► Blue colored complex

Reagents- working solutions:

Table (2-8): Kit Components

Reagent (A) Ca Volume = 50 ml	Buffer Arsenazo III	100mmol/l 0.13 mmol/l
Standard Volume = 10 ml	Calcium Sodium Azide	10 mg/dl (2.5mmol/l) 14mmol/l

Procedure:

The Polystyrene cup containing serum was entered into the equipment and analyzed automatically.

Calculations

The concentration of the serum Ca Level was measured by using a Smart 120 analyzer at Wavelength (600-630 nm).

Reference Range

Serum/plasma: 8.6 – 10.3 mg/dl (2.15 – 2.57 mmol/l)

Interpretation of test result

An instrument for smart 120 tests calculated the test result automatically and displayed the Calcium result of the test sample in terms of mg/dl (**Donald *et al*., 1975 and Ramakrishnan *et al*., 2012).**

2.2.6. Determination of Serum Magnesium Level

The Quantitative determination of Magnesium in serum was done by smart 120 analyzers by Colorimetric Method.

Principle

With Arsenazo, magnesium formed a red-orange complex, whose color intensity was proportional to the magnesium concentration in the sample.

Reagents- working solutions:

Table (2-9): Kit Components

Reagent (A) Mg Volume = 50 ml	Good buffer Arsenazo EGTA Sodium azide	100 mM 0.17 mM 0.1 mM 10 mM
Standard Mg Volume = 10 ml	Magnesium solution	2 mEq/l (2.5 mg/dl)

Procedure:

The Polystyrene cup containing serum was entered into the equipment and analyzed automatically.

Calculations

The concentration of the serum Mg level was measured by using a Smart 120 analyzer.

Reference Range

Adults: 1.7 – 2.5 mg/dl

Interpretation of test result

An instrument for smart 120 tests calculated the test result automatically and displayed the Magnesium result of the test sample in terms of mg/dl (**Young *etal.*, 1975**).

2.2.7. Determination of Serum Phosphorus Level

The Quantitative determination of phosphorus in serum by smart 120 analyzers by ammonium molybdate method.

Principle

The phosphate ions reacted with ammonium molybdate and formed a phosphomolybdate complex whose absorbance at 340 nm is proportional to the phosphorus quantity in the sample. An acid pH was necessary for the formation of complexes.

Phosphorus + Ammonium molybdate ► Phosphomolybdate complex

Reagents- working solutions:

Table (2-10): Kit Components

Reagent (A) Volume =100/250 ml	Ammonium molybdate Sulfuric acid 96 %	0.5 mmol/l 150 mmol/l
Standard Volume = 10 ml	Phosphorus Sodium azide	5 mg/dl (1.615 mmol/l) 14 mmol/l

Procedure:

The Polystyrene cup containing serum was enter into the equipment and analyzed automatically.

Calculations

The concentration of the serum Phosphorus level was measured by using a Smart 120 analyzer at Wavelength (340 nm).

Reference Range

Adults: 2.5 – 4.5 mg/dl (0.81 – 1.45 mmol/l)

Interpretation of test result

An instrument for smart 120 tests calculated the test result automatically and displayed the Phosphorus result of the test sample in terms of mg/dl (**Tietz *et al.*, 1994 and Ramakrishnan *et al.*, 2012**).

2.2.8. Determination of Serum Zinc Level

The Quantitative determination of the serum Zinc auto analyzer was done by direct Colorimetric Method.

Principle

Zinc reacts with the 5-Br-PAPS (2-(5-Bromo-2-pyridiylazo)-5-[N-n-propyl-N-(3-sulfopropyl)amino]phenol, disodium salt, dehydrate in a buffered solution and forms a colored compound. The intensity of this colored complex was proportional to the zinc concentration in the sample and is measured at 546-565 nm to obtain zinc concentration. Any interference due to copper, iron, cobalt, and other trace elements present in the sample was eliminated using specific masking agents. The name of the device used smart 120 analyzers.

Reagents- working solutions:

Table (2-11): Kit Components

Reagent (A)Zn Volume=40ml	Buffer Surfactant complexing	200mmol/l
Reagent (B)Zn Volume=10ml	Buffer 5-Br-PAPS	200mmol/l 0.2mmol/l
Standard Zn Volume=10ml	Zn derivative	200 µg/dl (30.6µmol/l)

Procedure:

The Polystyrene cup containing serum was entered into the equipment and analyzed automatically.

Calculations

The concentration of the serum Zn level was measured by using a Smart 120 analyzer.

Reference Range

Adults: 70 – 150 µg/dl (10.7 – 22.9 µmol/l)

Interpretation of test result

An instrument for smart 120 tests calculated the test result automatically and displays the Zinc result of the test sample in terms of µg/dl (**Tietz *et al.*, 1994**).

2.2.9. Determination of Serum Total Alkaline Phosphatase Level.

Principle

ALP catalyzed the hydrolysis of p-nitrophenyl phosphate in an alkaline medium to give p-nitrophenol and phosphate. By measuring the variation of absorbance of p-nitrophenol over a defined interval of time it was possible to calculate ALP activity in the sample by measuring spectrophotometrically 405 nm to quantify the activity of the enzyme present in the sample**.** Assay standardized by DGKC (Deutsche Gesellschaft Fur Klinische Chemie). This was an enzymatic colorimetric method of determination by the Chemistry analyzer Smart 120.

p-Nitrophenyl phosphate + H_2O ---------- ► Phosphate + p-Nitrophenol

The p-nitrophenol released was directly proportional to the catalytic ALP activity. It is determined by measuring the increase in absorbance.

Reagents- working solutions:

R1 – Alkaline Phosphatase Buffer, D.E.A. (Diethanolamine) buffer pH 10 (25°C) 1 mol/L , Magnesium Chloride 0.5 mmol/L

R2 – Alkaline Phosphatase Substrate p-nitrophenyl phosphate 10 mmol/L

Procedure:

The Polystyrene cup containing serum was entered into the equipment and analyzed automatically**.**

Calculations

The concentration of the serum ALP was measured by using a Smart 120 analyzer.

Reference Range

Adults: 98 – 279 U/l

Interpretation of test result

An instrument for smart 120 tests calculated the test result automatically and displayed the ALP result of the test sample in terms of (U/l) (**Bergmeyer**, **1970**).

2.3. Determination of Serum Bone ALP Activity Level:

Principle

The ELISA Kit used Sandwich-ELISA as the method. The kit assay Human BALP level in the sample used Purified Human BALP antibody to coat microtiterplate wells, make solid-phase antibody, then add BALP to wells, Combined BALP antibody which with HRP labeled became antibody-antigen-enzyme- antibody complex, after washing Completely, Add TMB substrate solution, TMB substrate became blue color at HRP enzyme-catalyzed, the reaction was terminated by the addition of a sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm. The concentration ofBALP in the samples was then determined by comparing the optical density (O.D.) of the samples to the standard curve. The name of the device used was the ELISA microplate reader. See fig. (2-4).

Other supplies required

1. Micro-plate reader with 450nm wavelength filter High-precision transfer pipette, EP tubes, and disposable pipette tips Incubator capable of maintaining 37°C.
2. De-ionized or distilled water.
3. Absorbent paper loading slot.

The method of work

1. The sample used was serum.
2. Reagent preparation.
3. All reagents were kept at room temperature (15-30 °C) before use, take out the necessary strips and reagents were for the present experiment.
4. 20 ml of concentrated wash buffer was diluted with 580 ml of de-ionized or distilled water to prepare 600 ml of wash buffer, if crystals had formed in the concentrate, it was warmed in a 37°C water bath, and then it was mixed gently until the crystals had completely dissolved.

Procedure

1. The sample was diluted and added to the Standard: set 10 Standard wells on the ELISA plates coated, and added Standard 100µl to the first and the second well, then added Standard dilution 50µl to the first and the second well, mixed; taken out 100µl from the first and the second well then added it to the third and the forth well separately. then added standard dilution 50µl to the third and the fourth well, and mixed; then taken out 50µl from the third and the fourth wells discarded, added 50µl to the fifth and the sixth well, then added standard dilution 50µl to the fifth and the sixth well, mixed;taken out 50µl from the fifth and the sixth well and added to the seventh and the eighth well, then added standard dilution 50µl to the seventh and the eighth well, mixed; taken out 50µl from the seventh and the eighth well and

added to the ninth and the tenth well, added standard dilution 50μl to the ninth and the tenth well, mixed, taken out 50μl from the ninth and the tenth well discarded (added Sample 50μl to each well after Diluting, (density: 135 ng/L, 90 ng/L，60 ng/L，30 ng/，15 ng/L，7.5 ng/L).

2. Added sample Set blank wells separately (blank comparison wells don't add sample and HRP-Conjugate reagent; other each step operation is same). The testing sample well. added Sample dilution 40μl to the testing sample well,then added testing sample 10μl (sample final dilution is 5-fold), added sample to wells, don't touch the well wall as far as possible, and Gently mixed.

3. Incubated: After closing the plate with the Closure plate membrane, incubated for 30 min at 37 ℃.

4. Configurated liquid: 30-fold wash solution diluted 30-fold with distilledwater and reserve.

5. Washing：Uncover the Closure plate membrane, discard the Liquid, dry by swing, added washing buffer to every well, still for the 30s then drain, repeated 5 times, and dry by pat.

6. Added enzyme：Added HRP-Conjugate reagent 50μl to each well, except the blank well.

7. Incubated: Operation with 3.

8. Washing：Operation with 5.

9. Color： Added Chromogen Solution A 50ul and Chromogen Solution B to each well, evade the light preservation for 15 min at 37℃

10. Stop the reaction：Added Stop Solution 50μl to each well, Stop the reaction(the blue color changed to yellow color).

11. Assay： taken blank well as zero, read absorbance at 450nm after

Added Stop Solution and within 15min.

Interpretation of test result

Determined the optical density of each well at once with a microplate reader set to 450 nm.

Calculation

The standard density was taken as horizontal, and the OD value for vertical, draw the standard curve on graph paper, find out the corresponding density according to the sample OD value by the Sample curve, multiplied by the dilution multiple, or calculate the straight line regression equation of the standard curve with the standard density and the OD value, with the sample OD value in the equation, calculate the sample density, multiplied by the dilution factor see fig (2-2).

Reference range

Women: 4 ng/l -100 ng/l (**Suzuki *et al.*, 1999**).

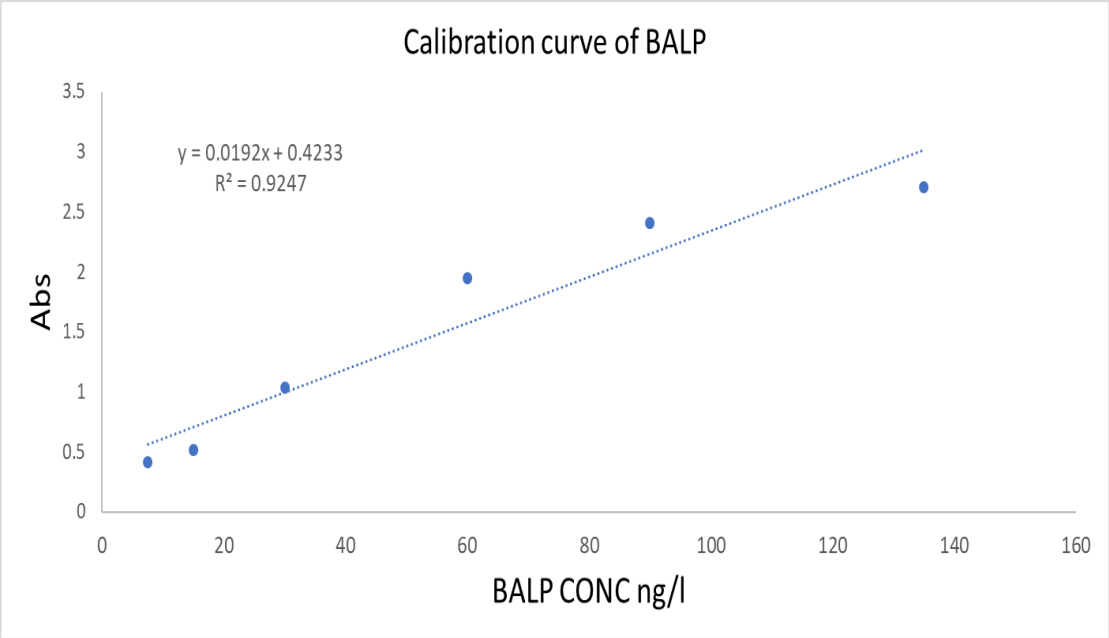

Fig. (2.2): Calibration curve of Bone specific alkaline phosphatase.

2.3.1. Determination of Serum Human Macrophage Erythroblast Attacher protein Level (MAEA).

Principle

This ELISA kit used Sandwich-ELISA as the method. The Microelisa strip plate provided in this kit had been pre-coated with an antibody specific to MAEA. Standards or samples were added to the appropriate Microelisa strip platewells and combined with the specific antibody. Then a Horseradish Peroxidase (HRP)-conjugated antibody specific for MAEA was added to each Microelisa strip plate well and incubated. Free components are washed away. The TMB substrate solution was added to each well. Only those wells that contain MAEA and HRP-conjugated MAEA antibodies would appear blue and then turn yellow after the addition of the stop solution. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm. The OD value was proportional to the concentration of MAEA. The results can be calculated for the concentration of MAEA in the samples by comparing the OD ofthe samples to the standard curve. The name of the device used was the ELISA microplate reader. See fig (2-3) and (2-4).

Procedure

1. Dilution of Standards: Diluted the standard in small tubes first, then pipette the volume of 50µl from each tube to the microplate well, each tube.
2. Used two wells, a total of ten wells.

Table (2-12): Dilution of Standards

MAEA CONC pg/ml	standard No.	details
300	1	300µl original standard+150µl standard diluents
200	2	300µl standard No.1 + 150µl standard diluents
100	3	150µl standard No.2 + 150µl standard diluents
50	4	150µl standard No.3+ 150µl standard diluents
25	5	150µl standard No.4+ 150µl standard diluents

3. In the Microelisa strip plate, left a well empty as blank control. In sample wells, 40µl Sample dilution buffer and 10µl sample are added (dilution factor is 5). Samples were loaded onto the bottom without touching the well wall. Mixed well with gentle shaking.
4. Incubation: incubated for 30 min at 37℃ after being sealed with a Closure plate membrane.
5. Dilution: diluted the concentrated washing buffer with distilled water (30 times for 96T.
6. Washing: carefully removed the Closure plate membrane, aspirated, and refilled with the wash solution. Discarded the wash solution after resting for 30 seconds. Repeated the washing procedure for 5times.
7. Added 50 µl HRP-Conjugate reagent to each well except the blank well.
8. Incubation is described in Step 3.
9. Washing as described in Step 5.

10. Coloring: Added 50 µl Chromogen Solution A and 50 µl Chromogen Solution B to each well, mixed with gentle shaking, and incubated at 37℃ for 15 minutes. avoided light during coloring.

11. Termination: added 50 µl stop solution to each well to terminate the reaction. The color of the well should change from blue to yellow.

12. Read absorbance O.D. at 450nm using a Microtiter Plate Reader. The OD value of the blank control well is set as zero. The assay was carried out within15 minutes after adding the stop solution.

Assay range: 10 Pg/ml-400pg/ml.

Calculation

Known concentrations of Human MAEA Standard and its corresponding reading OD were plotted on the log scale (x-axis) and the log scale (y-axis) respectively. The concentration of Human MAEA in the sample was

determined by plotting the sample's O.D. on the Y-axis. The original concentration was calculated by multiplying the dilution factor.

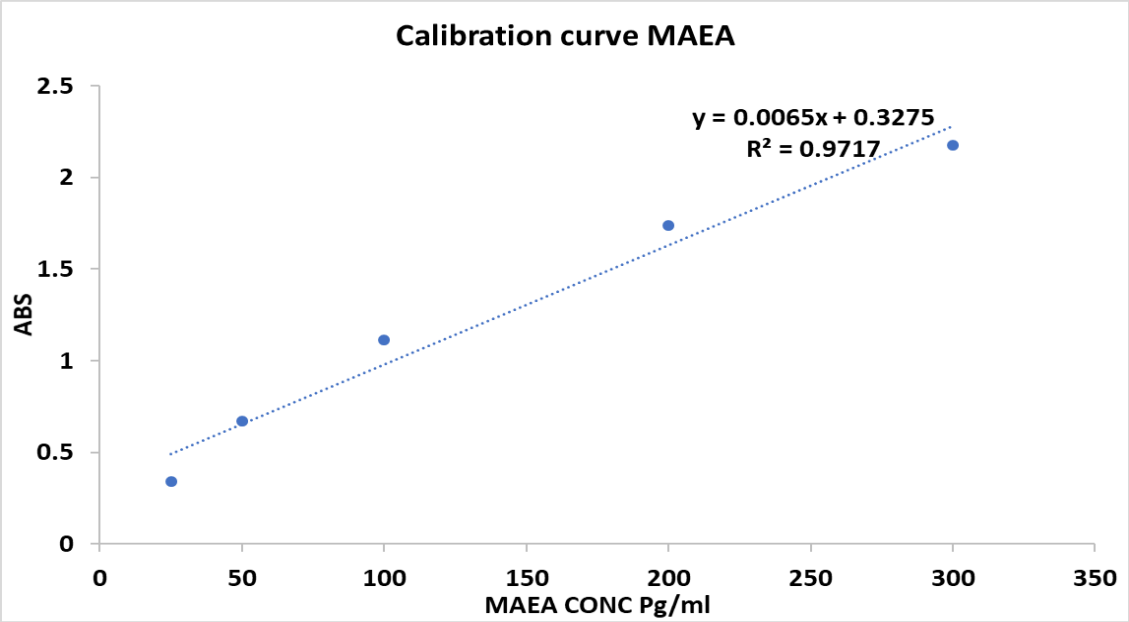

Fig. (2.3): Calibration curve of Macrophage Erythroblast Attacher protein

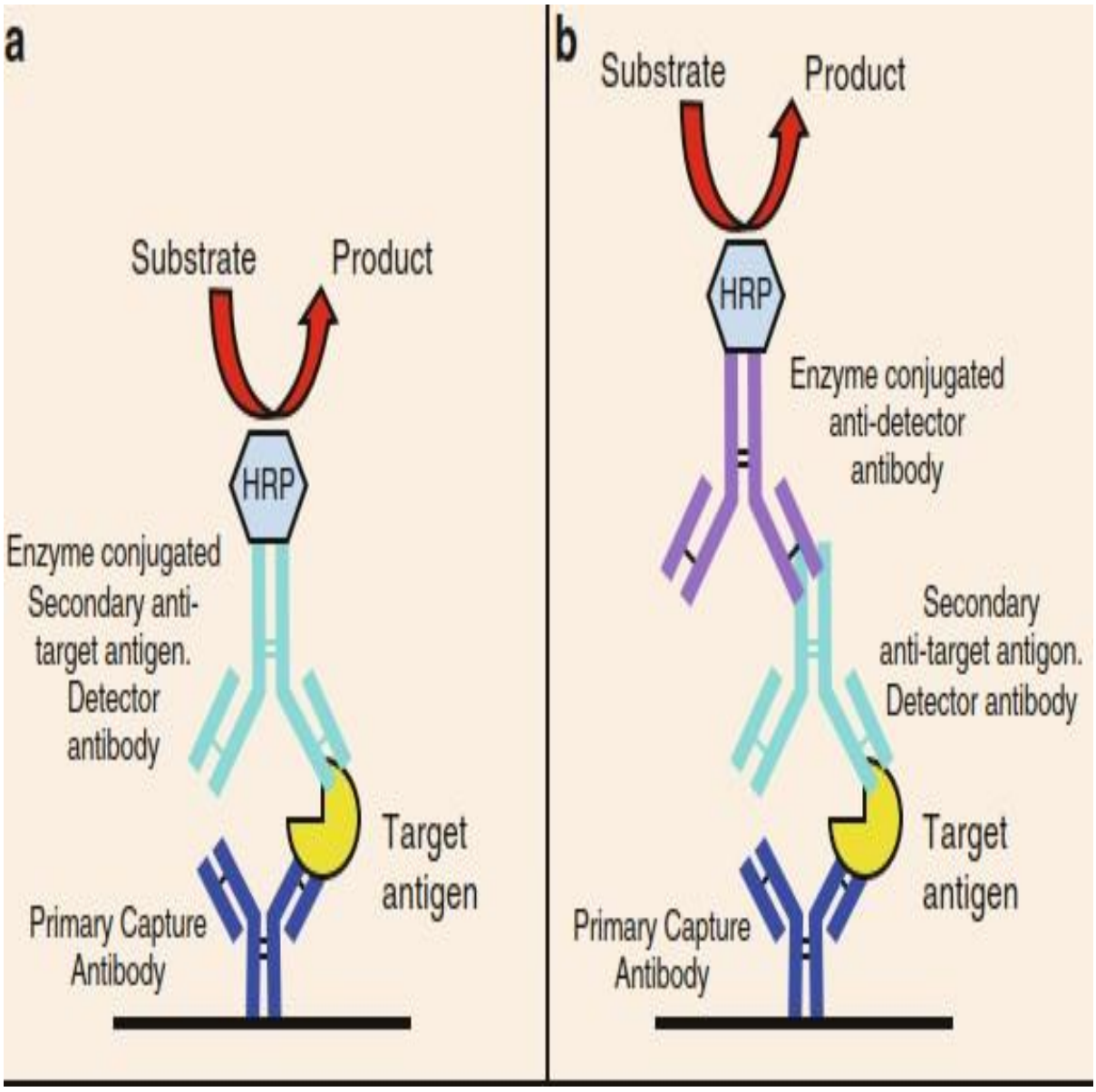

Fig. (2.4): Colorimetric Sandwich ELISA Kit Assay Principle (Hnasko, 2015).
(a) Direct sandwich ELISA
(b) indirect sandwich ELISA

2.4. Molecular Analysis

2.4.1. DNA Extraction

DNA was extracted from whole blood that was collected from women groups by using the ReliaPrep™ Blood gDNA Miniprep Kit (Promega).

Description

The ReliaPrep™ Blood gDNA Miniprep System provides a fast, simple technique for the preparation of purified and intact DNA from mammalian blood. Samples were processed using a binding column in a microcentrifuge tube. Up to 200µl of blood can be processed per purification.

Principle

The principle DNA extraction kit used proteinase K to lyse cells and degrade protein, allowing DNA to bind to the matrix of the spin column. Contaminants are removed using the awash buffer and purified genomic DNA was eluted by elution buffer. The purified DNA was suitable for usein a polymerase chain reaction (PCR) and agarose gel analysis.

Genomic DNA Extraction Protocol

1. Thoroughly mixed the blood sample for at least 10 minutes in a rotisserie shaker at room temperature. If the blood has been frozen, The sample was left to dissolve completely before mixing for 10 minutes.

2. Dispensed 20 µl of Proteinase K (PK) Solution into a 1.5ml microcentrifuge tube.

3. Added 200 µl of blood to the tube containing the PK Solution, and briefly mixed.

4. Added 200 µl of Cell Lysis Buffer (CLD) to the tube. Cap and mixed by vortexing for at least 10 seconds. this vortexing step is essential for obtaining good yields.

5. Incubated at 56 °C for 10 minutes.

6. While the blood sample is incubated, put a ReliaPrep™ Binding Column into an empty Collection tube.

7. Removed the tube from the heating block. Added 250µl of Binding Buffer (BBA), cap the tube, and mixed by vortexing for 10 seconds with a vortex mixer.

8. Added the contents of the tube to the ReliaPrep™ Binding Column, cap it,and put it in a microcentrifuge.

9. Separated by Centrifuge for 1 minute at maximum speed (14,000 rpm). Checked the binding column to make sure the lysate has completely passed through the membrane.

10. Removed the collection tube containing flowthrough, and discarded the liquid as hazardous waste.

11. Put the binding column into a fresh collection tube. Added 500µl of Column Wash Solution to the column, and separated by centrifuge for 3 minutes at maximum speed (14,000 rpm). Discarded the flowthrough.

12. Repeated Step 11 twice for a total of three washes.

13. Put the column in a new 1.5ml microcentrifuge tube.

14. Added 150µl of Nuclease-Free Water to the column. Separated by Centrifuge for 1 minute at maximum speed (14,000 rpm).

15. Discarded the ReliaPrep™ Binding Column, and saved eluate. (**Chacon Cortes *et al.*, 2014**).

2.4.2. Polymerase Chain Reaction

PCR can amplify a small amount of template DNA or RNA into large quantities in a few hours. This was performed by mixing the DNA with primers onside of the DNA (forward and reverse), free nucleotides (dNTPs for DNA, NTPs for RNA) Taq polymerase (of the species Thermus aquatics, a thermophile whose polymerase can withstand extremely high temperatures) and buffer. The

temperature was then alternated between hot and cold to denature and re-anneal the DNA, with the polymerase adding new complementary strands each time (**Butt *et al*., 2018).** There were three steps to any PCR that are cycled about 25- 35 times as shown in figure (2-5) which include:

1. **Denaturation**: It is the uncoiling of double-stranded DNA into two single strands at 94-98 °C.
2. **Annealing**: This step occurs at 55-65°C. A pair of short (17-26bp) oligonucleotide sequences (primers) anneal to the ends of the template strands of the DNA and begin the reaction.
3. **Extension**: This step occurs at 72-74°C and entails the extension of the primers to form a new strand that is complementary to the template strand.

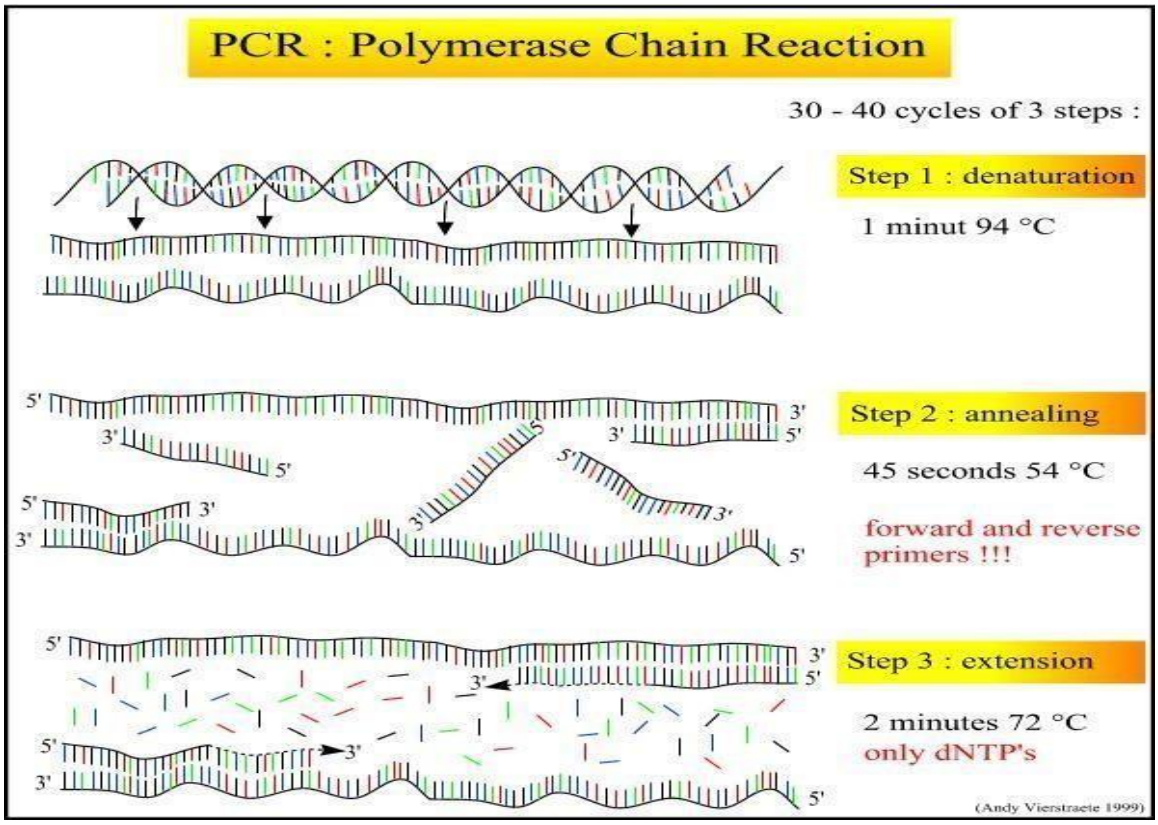

Fig. (2.5): PCR mechanism and experimental steps (Theis *et al.*, 2007).

2.4.3. Estimation of DNA concentration and purity

The concentration and purity of extracted DNA were estimated by Quawell Q5000 Nanodrop spectrophotometer to detect the quality of samples for downstream application. Quawell Q5000 is a micro-volume UV-Vis spectrophotometer. Its full-spectrum (200-900nm) allowed Q5000 to measure nucleic acids and purified proteins, and perform general laboratory UV-Vis absorbance measurements.

A unique technology used in the Q5000 holds 0.5-2 µl samples between upper and lower measurement surfaces without the use of a cuvette and measuresthe sample in about 8 seconds with a high degree of accuracy and reproducibility, DNA concentration values were detected.

Principle

Nucleic acids and proteins had absorbance maximums at 260 and 280 nm respectively. The ratio of absorbance at these wavelengths has been used as a measure of purity in both nucleic acid and protein extractions.

A ratio of 1.8-2.0 was generally accepted as pure for DNA. Similarly, absorbance at 230 nm Was accepted as being the result of another contamination; therefore, the ratio of (A260/A230) was also frequently calculated.

The (260/230) values for pure nucleic acids were often higher than the respective (260/280) values expected (260/ 230) values were commonly in the range of 2.0-2.2. see fig (2-6).

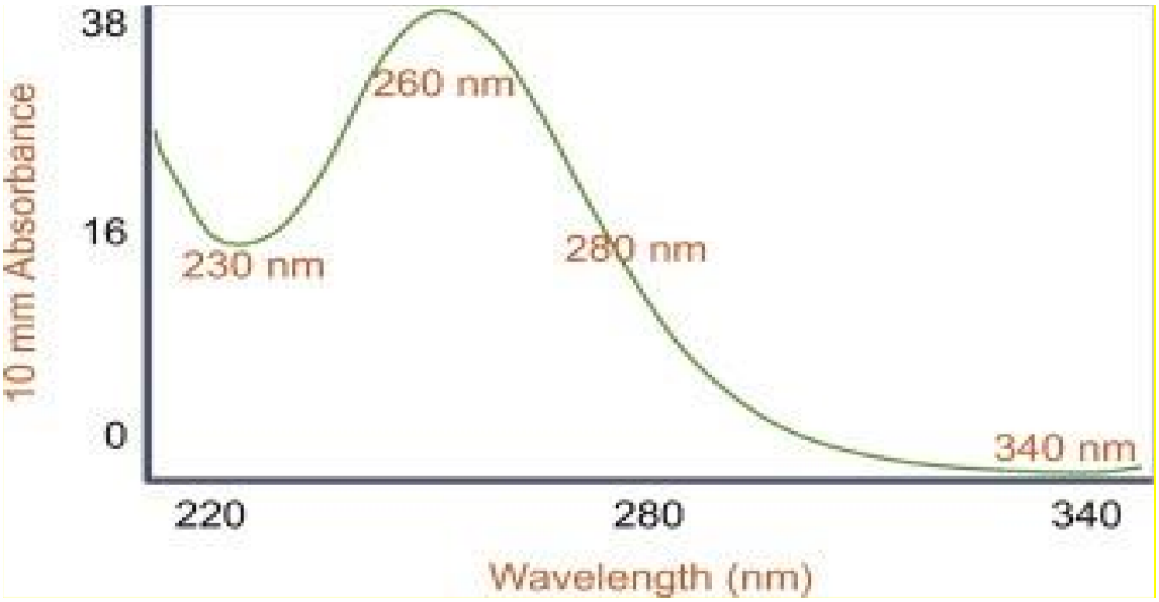

Fig. (2.6): The maximum absorbance of nucleic acids occurs at a wavelength of 260 nm (Lin, 1992).

2.4.4. Primer preparation

Macrogen Company supplied these primers in a lyophilized form. Lyophilized primers were dissolved in nuclease-free water to give a final concentration of 100pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10µl of primer stock solution (stored at freezer -20 °C) to 90 µl of nuclease-free water to obtain a working primer solution of 10 Pmol/µl. See table (2-13),(2-14) and (2-15).

Table (2-13): Primer preparation

Primer Name	Vol. of nuclease-free water (µl)	Concentration (Pmol/µl)
MAEA- rs10025665-F	**250**	**100**
MAEA- rs10025665-F	**250**	**100**
MAEA- rs10025665-R	**250**	**100**

The PCR component is shown in table (2-14):

Table (2-14): Components of PCR of the presented work.

Master mix components	Stock	Unit	Final	Unit	Volume µl 1sample)	Total Volume (160 samples)
Master Mix	2	X	1	X	13	2080
Forward primer	10	µM	1	µM	1.5	240
Reverse primer	10	µM	1	µM	1.5	240
Nuclease Free Water	-	-	-	-	6	960
DNA	-	ng/µl	-	ng/µl	3	-
Total volume	-	-	-	-	25	-
Aliquot per single r x n	-	-	-	-	22 µl of Master mix per tube and add 3 µl of Template	-

2.4.5. Program of Polymerase Chain Reaction

The following table indicated the program PCR performed.

Table (2-15): Allele-specific–PCR program for detection of MAEA gene forrs10025665.

Steps	°C	M: S	Cycle
Initial Denaturation	95	03:00	1
Denaturation	95	00:30	30
Annealing	60	00:35	
Extension	72	00:55	
Final extension	72	05:00	1
Hold	12	10:00	1

°C : Temperature ; M : Minute ; S : Second

2.4.6. Optimization of PCR Conditions

Different volumes of primer (0.5 µl,1 µl, 1.5 µl,) with different volumes of template DNA (1 µl,2 µl,3 µl,4 µl,5 µl) and different temperatures of primer (54°**C**,56°**C**,58°**C**,60°**C**,61°**C**,64°**C**,66°**C**) and different experiments of the reaction conditions were trailed to optimize the conditions of the reaction. PCR tube was centrifuged for 30 seconds at 2000 xg in a micro-centrifuge to mix solutions well at room temperature then tubes were placed in the thermocycler to start the reaction. Programs of the PCR protocol reaction for MAEA gene polymorphism for rs10025665 were illustrated in a table (2-15). In rs10025665 (258) bp band indicated the presence of the allele; if no amplification product, this means the absence of an allele in the gel. see table (2-16).

Table (2-16). Bands obtained from the amplification product of MAEA, rs10025665 SNP

Genotype		**No. of bands**	**Size of bands(bp)**
Heterozygous	**rs10025665**		**rs10025665**
	AG	2	258 A allele 258 G allele
Homozygous mutation	GG	1	258 G allele
Wild type	AA	1	258 A allele

2.4.7. Electrophoresis

This technique was used to check whether the methods like PCR had worked properly. Electrophoresis involved running a current through a gel loaded with the molecules of interest. The movement of the samples was directed based on the charge that the molecule carries. Based on the size and charge, the molecules would travel through the gel at different speeds, allowing them to be separated from one another as shown in figure **2-7**. Since all the DNA molecules possessed the same amount of charge per mass, gel electrophoresis separates them based on size only (**Liang *et al.***, **2018**). Electrophoresis through agarose gels was the standard method used to separate, identify, and purify DNA fragments(**Liang *et al.***, **2018**). The agarose gel electrophoresis was done according to Harisha method(**Shafi Sofi *et al.***, **2013**). The percentage of agarose used depended on the size of fragments to be resolved, where an agarose gel percentage were normally in the range of 0.5 % to 2 %. The ethidium bromide staining was done according to Robinson and Lafleche method (**Farouk *et al.***, **2015**).

Materials

1. Agarose powder.
2. 10 X of TBE (Tris-Borate EDTA buffer).
3. Ethidium bromide (10 mg/ml) [EtBr].
4. Electrophoresis equipment and power supply.

Agarose Gel Electrophoresis

After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification. PCR was completely dependable on the extracted DNA criteria.

Preparation of agarose gel:

1. 1.5 gram of agarose was weight and placed into a conical flask, and then 100 ml of 1X (TBE) buffer was prepared and added. this gel was used to detect the band of the PCR product.
2. The solution was heated to boiling (using a microwave) until all the gel particles were dissolved and the solution was clear then the solution was allowed to cool to about 55 ºC before pouring.
3. Two µl of ethidium bromide was added to the dissolved agarose and mixed. Ethidium bromide stains DNA by intercalating between the bases of DNA.
4. Gel chambers were sealed with tape.
5. The comb was placed in the gel chamber about 1 inch from one end of the tray.
6. The gel solution was cast into the chamber and allowed to solidify for about 20 minutes at room temperature.
7. Carefully the dams and the comb were removed, and then the chamber is placed in a horizontal electrophoresis system in about 350 ml of 1X TBE (electrophoresis buffer). when small (9 wells) or large (18 wells) electrophoresis trays are used respectively.
8. The gel was covered completely with the buffer to prevent overheating of the gel.
9. At this step, the samples had been loaded (5 µl) on each well with extreme caution to avoid damage to the wells .

10. The cathode (black electrode) was connected to the well side of the unit as the migration is toward the anode (red electrode) on the other side.

11. Electrophoresis was done at 60 volts, for 35 minutes or until dye markers have migrated an appropriate distance, depending on the size of the DNA to be visualized.

12. Ladder 100 bp bands were used as the standard for comparison with bands that resulted in allelic gene migration through gel electrophoresis.

13. For DNA detection, (3 µl) of the loading dye was mixed with a Ladder (10 µl) before putting it into the wells while the PCR product doesn't need loading dye addition. The micropipette was used to place the samples into adjacent wells carefully with a steady hand **(Lee *et al*., 2012)**.

2.4.8.Photo Documentation

Agarose gel was placed above the UV transilluminator device and exposed to UV light and the photos were captured using a digital camera and visualized by a PC connected to the transilluminator. The UV transilluminator device was covered with a protective shield to avoid the risk of exposure to UV light when the light is on.

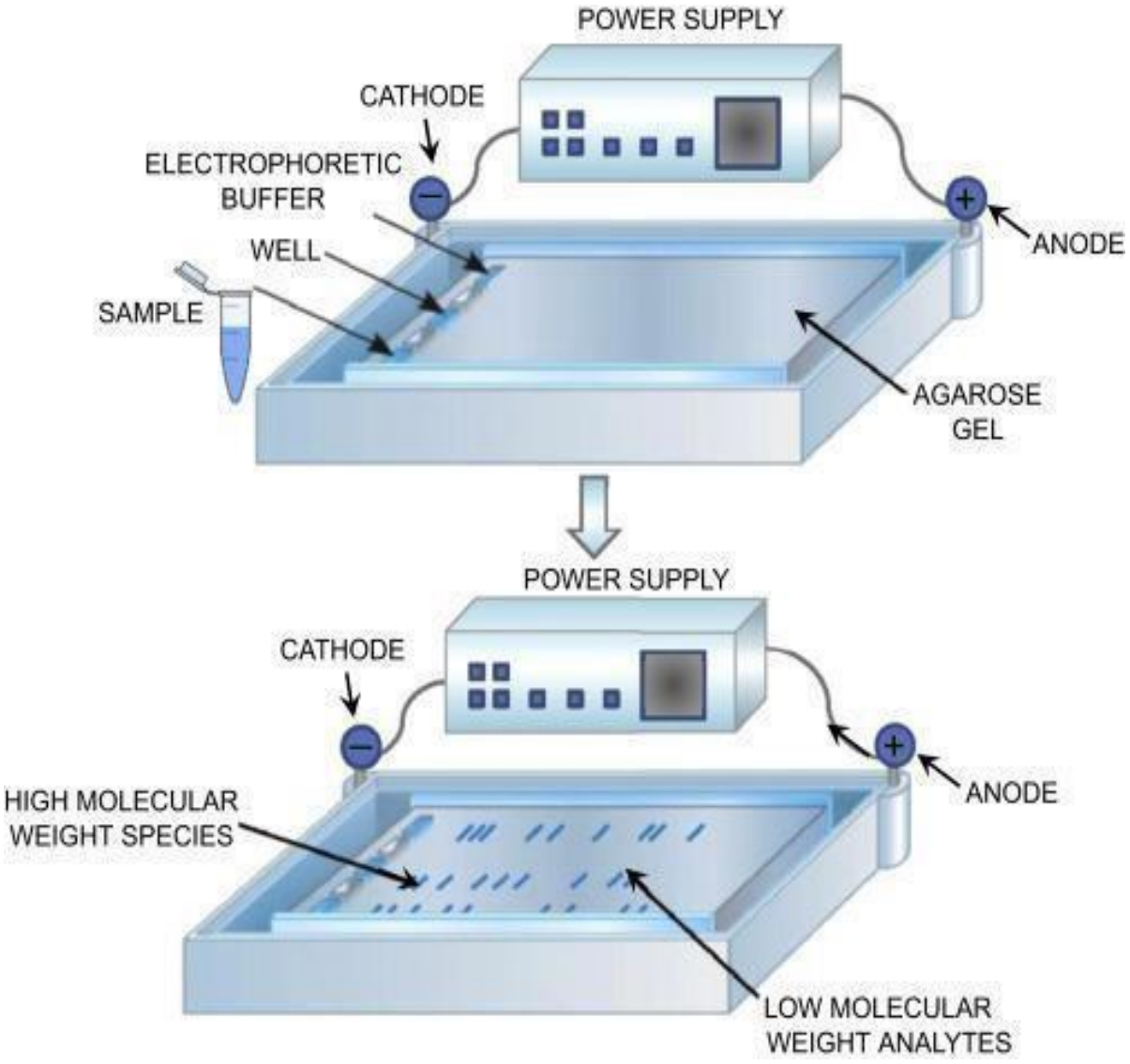

Fig. (2.7): Agarose Gel Electrophoresis for DNA samples (Liang *et al.*, 2018).

2.5. Statistical Analysis

Data (represented as Mean ± SD) were analyzed by using the Statistical Package for the Social Sciences (version 25). An independent t-test was used to evaluate significant differences between patient groups to mention the statistical relationship (association) between any two variables in the present study.

One-way ANOVA test was used to evaluate significant differences among patient groups to mention the statistical relationship in the present study and used an online medical calculator to find odd ratios in genetic analysis.

The levels of significance of 5% ($p \leq 0.05$) and 1% ($p \leq 0.01$) were obtained to represent the strength of evidence in support of significant differences between variables.

Chapter Three

Results and Discussion

3. Results and Discussion

3.1. Demographic Characteristics of Patients Groups

The current cross-sectional study included 160 women participants (40 of them were Osteopenia, 40 were Osteoporosis with T2DM, 40 participants were Osteoporosis with Hypertension, and 40 were Osteoporosis without (T2DM/Hypertension)). Some anthropometric parameters including body mass index and age of the selected groups were illustrated in table (3-1).

Table (3-1): Comparison of age and body mass index between PMOP with T2DM, PMOP with HTN, and PMOP groups as compared with the Osteopenia group.

Parameter	Osteopenia (Mean ± SD) N = 40	OP (Mean ±SD) N= 40	P. value
Age, Year	56.4 ±7.37	62.25 ± 7.25	< 0.01
BMI, kg/m^2	30.34 ± 4.64	28.57 ± 4.51	0.06
Parameter	**Osteopenia (Mean ± SD) N = 40**	**OP with HTN (Mean ± SD), N = 40**	**P. value**
Age, Year	56.4 ± 7.37	66.7 ±7.8	< 0.01
BMI, kg/m^2	30.34 ± 4.64	30.02 ± 4.23	0.74
Parameter	**Osteopenia (Mean ± SD) N = 40**	**OP with T2DM (Mean ± SD) N = 40**	**P. value**
Age, Year	56.4 ±7.37	62.8 ± 6.8	< 0.01
BMI, kg/m^2	30.34 ± 4.64	28.98 ± 4.7	0.2

OP: osteoporosis, N:number, SD:stander deviation, T2DM: type 2 diabetes mellitus, BMI:Body mass index , HTN: hypertension ,Significant P < 0.05.

The comparison of some anthropometric characteristics indicated in table (3-1) showed significant differences between each PMOP with T2DM, PMOP with HTN, and PMOP without (T2DM, HTN) group as compared with the osteopenia group with respect to the age (P<0.01). Age was considered a risk factor for osteoporosis, elderly people were at higher risk for developing

osteoporosis, especially women that were agreed with other investigations (**Cannarella *et al.*, 2019 and Xu *et al.*, 2020**) Satoshi et al. found in his studythat age was significantly higher in osteoporosis patients and that female patient had a higher incidence of osteoporosis(**Tanaka *et al.*, 2018**).

The aging bone has reduced mineral content and is prone to osteoporosis a condition in which bones are less dense, more fragile, and prone to fractures (**Chen *et al.*, 2018**). As people age the rate of bone resorption by osteoclast cells exceeds the rate of bone formation so bone weakens (**Owen *et al.*, 2018**), andthis all happens due to an Inactive lifestyle, Hormonal changes, and Loss of calcium and other minerals in bone(**Pinheiro *et al.*, 2020 and Blake *et al.*, 2021**) see fig. (3-1) age categories of study population.

Body Mass index was indicated in the table (3-1) shows non-significant results differences between each of the PMOP with T2DM, PMOP with HTN, and PMOP without (T2DM, HTN) group as compared with the Osteopenia group($P>0.05$). The results showed that women with high BMI have a lower risk of developing osteoporosis than those with low BMI, these results agreed with some previous studies (**Raška *et al.*, 2017 and Hariri *et al.*, 2019**). This can be explained by the favorable effects of greater mechanical loading on bone and increased estradiol levels due to enhanced conversion of androgen precursors to estrogen in the larger adipose tissue volume are thought to be the cause of the higher BMD in overweight/obese patients (**Xu *et al.*, 2020**).

Higher BMI may stimulate new bone formation and limit bone loss; this considers an effective measure for capturing the weight-bearing element of a bigger load on the skeleton. Excess body weight, on the other hand, can alter BMD by affecting the release of hormones from adipose tissue, and estrogen hasa role in bone formation (**Xu *et al.*, 2020**). Increased BMI has been linked to olderage at natural menopause in some studies (**Rödström *et al.*, 2003 and Reynolds**

***et al.*, 2005**), whereas BMI has been found to have no significant relationship with age at natural menopause in others (**Al-Safi *et al.*, 2015**). See fig. (3-2).

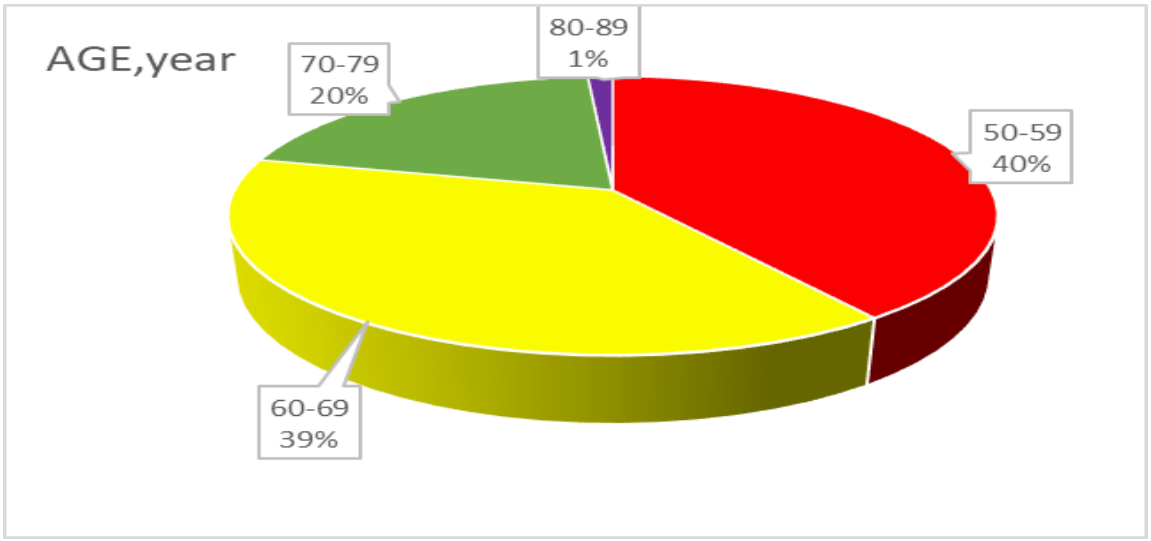

Fig. (3-1): Age categories of study population

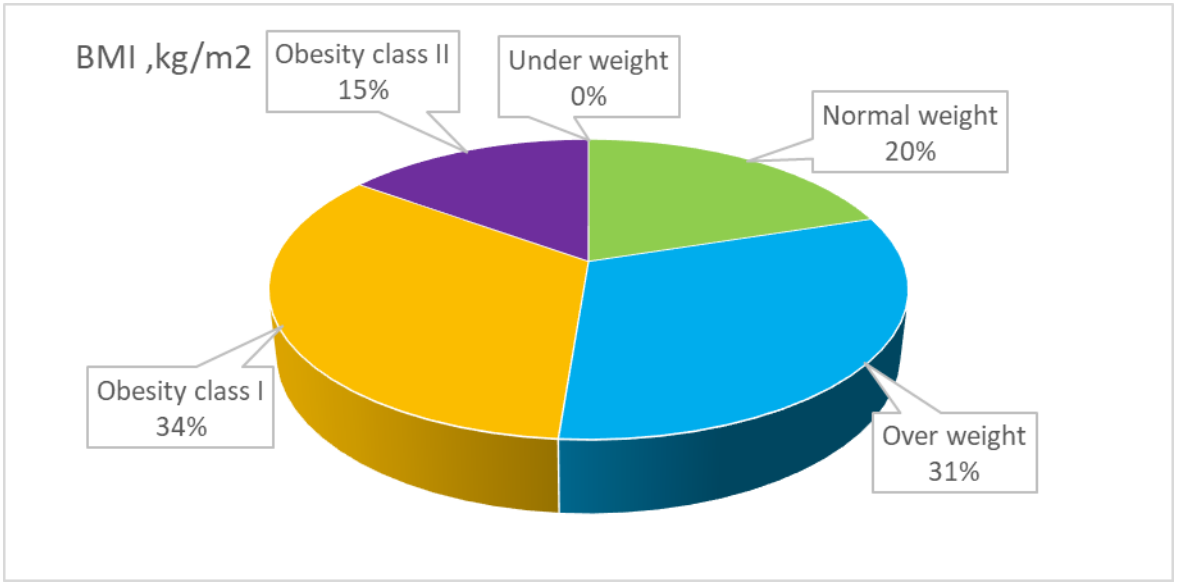

Fig. (3-2): BMI categories of study population

3.2. Bone Mineral Density

The results in table (3-2) show there was a significant difference between each PMOP with T2DM, PMOP with HTN, and PMOP without (T2DM, HTN) groups as compared with the osteopenia group ($P<0.05$) in the BMD and T-score.

Table (3-2): Comparison of Bone mineral density and T-score between PMOP with T2DM, PMOP with HTN, and PMOP groups as compared with the Osteopenia group.

Parameter	Osteopenia (Mean ± SD) N = 40	Osteoporosis (Mean ± SD) N = 40	P. value
BMD, (g/cm^2)	0.86 ± 0.04	0.69 ± 0.05	< 0.01
T.score	(-1.61) ± 0.40	(-3.22) ± 0.41	< 0.01
Parameter	**Osteopenia (Mean ± SD) N = 40**	**OP with HTN (Mean ± SD) N = 40**	**P. value**
BMD, (g/cm^2)	0.86 ± 0.04	0.70 ± 0.1	< 0.01
T.score	(-1.61) ± 0.40	(-3.11) ± 0.5	< 0.01
Parameter	**Osteopenia (Mean ± SD) N = 40**	**OP with T2DM (Mean ± SD) N = 40**	**P. value**
BMD, (g/cm^2)	0.86 ± 0.04	0.72 ± 0.1	< 0.01
T.score	(-1.61) ± 0.40	(-3.04) ± 0.5	< 0.01

OP: osteoporosis, N: number, SD: stander deviation, T2DM: type 2 diabetes mellitus, BMI: Body mass index, HTN: hypertension, Significant P < 0.05, Pvalue: Probability value.

The results were bone mineral density (BMD) and T-score significantly lower in PMOP with T2DM compared with non-diabetic osteopenia and these result agreed with (**Ho-Pham *et al.*, 2018**) and disagreed with (**Akin *et al.*, 2003 and Siddapur *et al.*, 2015**). Most studies indicate less BMD with insulin-dependent diabetes mellitus (**Strotmeyer *et al.*, 2006**) but with type 2 diabetes some authors report increased (**Barrett-Connor *et al.*, 1992**) some report decreased (**Gregorio *et al.*, 1994**) and some others report unaltered BMD (**Sosa *et al.*, 1996**).

Interpretation of fracture data as a measure of bone health is particularly difficult in patients with long-standing diabetes. Visual and neurologic complications could predispose patients to accidents resulting in an increased fracture risk not necessarily dependent on bone density alone. Diabetes could

influence bone through several mechanisms, some of which may have contradictory effects. Obesity, widespread in type 2 diabetes mellitus (T2DM), is strongly associated with higher BMD, probably through mechanical loading and hormonal factors, including insulin and estrogen (**Paul *et al.*, 1996**).

Hyperinsulinemia may promote bone formation (**Yamagishi *et al.*, 2005**). However, low levels of insulin and the progression of T2DM may cause reductions in BMD. Higher glucose levels in the blood interact with several proteins to generate a higher concentration of advanced glycation end-products (AGEs). Yamagishi et al, hypothesized that in collagen may interact with a bone to reduce bone strength, resulting in osteoporosis in patients with diabetes **(Yamagishi *et al.*, 2005 and Alikhani *et al.*, 2007)**.

Accumulated AGEs in the body might stimulate apoptosis of osteoblasts, thereby contributing to the defective bone formation (**Raskin *et al.*, 1978**). Another indirect effect of hyperglycemia is glycosuria, which causes hypercalciuria, leading to decreased levels of calcium in the body and poor bone quality, thus hastening bone loss (**Ishida *et al.*, 1985 and Pittas *et al.*, 2007**). In addition, microvascular complications of diabetes lead to reduced blood flow to bone and may contribute to bone loss and fragility (**Bonjour *et al.*, 2007**).

The results of BMD and T-score were significantly lower in PMOP with hypertension compared with the non- hypertension osteopenia group and these results agreed with (**Chai *et al.*, 2021**) and disagreed with others (**Lidfeldt *et al.*, 2002 and Javed *et al.*, 2012**). In this study, analysis of BMD by DEXA method showed a different result for BMD and T. scor at the lumbar spine in both PMOP with hypertension and postmenopausal osteopenia women.

Tsuda et al. found an inverse relationship between lumbar spine BMD and SBP in Japanese women (**Tsuda *et al.*, 2001**). Cappuccio et al. found an inverse relationship between femoral neck BMD and blood pressure in a study

done on white postmenopausal elderly women (**Cappuccio *et al.*, 1999**). However, the difference to the study, their study was performed only on the femoral neck they could not establish the association at different sites. Lidfeldt et al. found a positive association between BMD and SBP without adjusting confounding variables (**Lidfeldt *et al.*, 2002**).

Yang et al. also found a lower BMD at the femoral neck in hypertensive women compared to normotensive women (**Yang *et al.*, 2014**). The inverse relationship between hypertension and BMD was also evident from the Meta-analysis (**Ye *etal.*, 2017**). Primary hypertension had an association with Ca metabolism, which causes loss of Ca and secondary activation of the parathyroid gland causing increased removal of Ca from bone (**Duque *et al.*, 2020**).

These differences in findings of different studies might be due to differences in patient selections (such as age, ethnicity, and sample size, the inclusion of patients with comorbidities, extent, and etiology of osteoporosis or hypertension) and diagnostic methods or criteria. In this study, only included postmenopausal women, therefore; the findings are not generalizable to men or younger women.

Moreover, the cross-sectional study design of the study made it impossible to find any cause-effect relationship between hypertension and BMD in postmenopausal women. So, a prospectively designed study with a bigger sample size, which incorporates the measurement of biochemical indices of bone mineral metabolism, is required to be done to grasp the relationship between hypertension and BMD.

The presence of hypertension was found to be an independent predictor of low bone density. The long-lasting impairment effect of hypertension on calcium homeostasis may result in age-related excessive reduction of BMD and fracture (**Cappuccio *et al.*, 1999**). Study had found that renin-angiotensin-

aldosterone system(RAAS) activity is associated with osteoporosis (**Chhokar *et al.*, 2005**). The RAAS also plays a crucial part in regulating the remodeling of various organs and tissues, including bone tissues (**Yongtao Zhang *et al.*, 2020**). Some research results suggest that RAAS blockers do not reduce osteoporotic fractures and even increase fracture occurrence (**YanZhang *etal.*, 2017**). Local tissue-specific RAAS could regulate cell growth, inflammation, and angiogenesis (**Queiroz-Junior *et al.*,2019**).

Both osteoporosis and hypertension are known to be related to low calcium uptake, lack of vitamins D, and high consumption of sodium salts (**Rejnmark *et al.*, 2006**; **Ilić *et al.*, 2013**). Specifically, hypercalciuria is a common change that can be observed in women with hypertension and is related to a decrease in bone density, which is more common in women with osteoporosis and hypertension (**Pérez-Castrillón *et al.*, 2003**).

In postmenopausal osteoporosis women without (T2DM, HTN) BMD is one of the factors that influence bone resistance and is associated with the bone mass peak achieved in youth and subsequent bone losses (**Schnatz *etal.*, 2010 and Fistarol *et al.*, 2019**).

The bone mass peak is affected by several factors as genetic predisposition, body mass index, dietary habits, medications, physical activity, and chronic diseases (**Upala *et al.*, 2016; Compston *et al.*, 2017**). In women, the peak bone mass is achieved by the third decade of life, after which a process of slow bone loss begins (**Ma *et al.*, 2013**).

3.3. Clinical and Biochemical Characteristics of Study Subjects.

Table (3-3): characteristics of biochemical markers in PMOP without (T2DM/HTN), PMOP with HTN, and PMOP with T2DM groups as compared with the Osteopenia group.

Parameter	Osteopenia Mean±SD N=40	OP Mean±SD N=40	OP+HTN Mean±SD N=40	OP+T2DM Mean±SD N=40
VIT.D3,ng/ml	31±8.6	26.3±10.9[a]	26.6±7.5[a]	25.9±12.1[a]
Estradiol, Pg/ml	29.5±7.4	24.6±5.7[b]	23.9±6.9[b]	23±9.3[b]
Ca, mg/dl	9.39±0.4	9.28±0.41	9.29±0.42	9.49±0.3
Mg, mg/dl	2.31±0.16	2.23±0.13[a]	2.21±0.2[a]	2.2±0.24[a]
P, mg/dl	3.55±0.65	3.86±0.53[a]	3.91±0.42[b]	3.76±0.66
Zn , µg/dl	83±7.43	82.2±15.6	81.8±13.15	78.9±7.34[a]
TALP, U/L	201.5±41.4	248.3±63.1[b]	224±52.6[a]	227.4±61.4[a]
BALP , ng/l	49.5±7.6	55.6±11.2[b]	55.7±14.2[a]	52.3±11.6
MAEA , Pg/ml	121.8±23.1	105.8±29.5[b]	111.5±29	131±24[a]

OP: osteoporosis, SD: stander deviation, T2DM: Type 2 diabetes mellitus, N: number, HTN: hypertension, (a) Significant P < 0.05, (b) Significant P < 0.01

3.3.1. Levels of Serum Vitamin D3

The results of table (3-3) was showed that observed have decreased serum 25(OH)D3 level in osteoporosis group as compared with the osteopenia group. The mean serum level of Vitamin D3 was statistically significantly lowerin PMOP without (T2DM/HTN) as compared to osteopenia groups (p.value<0.05)and these results are consistent with (**Wang *et al.*, 2019**) and inconsistent with (**Marozik *et al.*, 2021**).

In the present study, a highly significant decrease of 25(OH)D3 level in PMOP without(T2DM/HTN) when compared with the osteopenia group with a significant p.value of <0.05. This study agreed with the works of Alkhenizan et al. who demonstrated a lower level of 25(OH)D3 among osteoporosis patients (**Alkhenizan *et al.*, 2017**). The decrease in serum vitamin D3 levels may be due

to fewer outdoor activities of women also with decrease exposure to sunlight and the habits of wearing long dresses this will prevent the vitamin D3 in the skin to induce and convert to the active form and it is important for calcium and phosphorus absorption when it is level decrease leads to increase parathyroid hormone and then effect on bone health. Normal bone metabolism depends onthe presence of appropriate repletion of vitamin D3.

Vitamin D3 insufficiency has been shown to have adverse effects on calcium metabolism, osteoblastic activity, BMD, and bone remodeling (**Christodoulou *et al.*, 2013**). The fig. (3-3) was clear that the percentage of women who suffer from insufficient vitamin D is 60% of the study population,and this may be a reason for the lack of bone mineral density and the occurrence of osteoporosis. Low serum 25(OH)D3 concentration is associated with secondary hyperparathyroidism, increased bone turnover, reduced BMD, and increased risk of osteoporotic fractures **(Li *et al.*, 2016).**

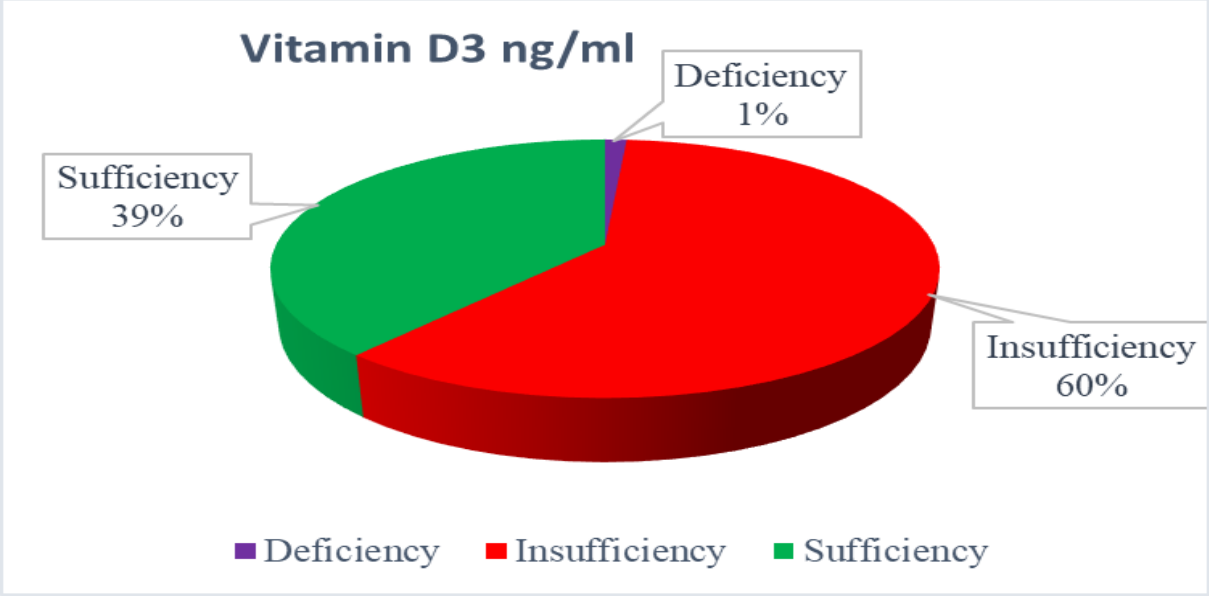

Fig. (3-3): Vitamin D3 categories of study population

The mean serum level of 25-OH Vitamin D was statistically significantly lower in PMOP with HTN when compared with osteopenia group and these result agreed with those (**Chai *et al.*, 2021** and **Mokhtari *et al.*, 2022**). The association between 25(OH)D levels and hypertension had been assessed in several cross-sectional studies (**Wagemaker *et al.*, 2009 and Pasco *et al.*, 2009**).

Most, though not all, of the observational data, support the links between low 25-OH Vitamin D levels and a higher risk of hypertension (**Rueda *et al.*, 2008 and Chan *et al.*, 2012**) and both increased risk of osteoporosis. A large cohort study performed reported an association between a lower risk of hypertension and the highest quintile of 25-OH Vitamin D (**Dorjgochoo *et al.*, 2012**).

The studies showed that vitamin D3 plays a key role in parameters that regulate high blood pressure via the proliferation of vascular smooth muscle cells, endothelial cell function, regulation of renin angiotensin pathway, and in the regulation of blood pressure via increased intracellular calcium leading to decreased renin activity **(Duprez *et al.*, 1994 and Tomaschitz *et al.*, 2010)**.

The mean serum level of 25(OH)D3 was statistically significantly lower in PMOP with T2DM compared with osteopenia groups (p.value<0.05) and these result agreed with (**Saif-Elnasr *et al.*, 2017 and Fondjo *et al.*, 2018**). In contrast, other studies, had not shown any difference in 25(OH)D3 levels between patients with diabetes compared to the normal population (**Payne *et al.*, 2012 and Sheth *et al.*, 2015**).

The decline in vitamin D interfere with the function of pancreatic β cells in insulin secretion and decrease insulin sensitivity due to systemic inflammation (**Harinarayan**, **2014**). Although several investigators have long addressed the question of how DM induces osteopenia and osteoporosis, the exact underlying mechanism is still elusive.

However, it is widely accepted that hyperglycemia is a salient factor that has direct and indirect deleterious effects on osteoblast function and bone formation (**Wang *et al.*, 2010**). T2DM also induces lipid accumulation in the marrow of long bones, thereby leading to the expansion of the marrow cavity and thinning ofthe cortical envelope. The osteoblast to adipocyte shift might also reduce the number of differentiated osteoblasts available for bone formation (**Wongdee *etal.*, 2011**).

Several investigations have demonstrated an increase in AGE or non-enzymatic cross-links within collagen fibers, which, in turn, lead to deterioration in the structural and mechanical properties of bone, and eventually to a decrease in bone strength (**Saito *et al.*, 2016 and Khalid *et al.*, 2022**). Obesity is associated with vitamin D3 deficiency due to excess adiposity, vitamin D3 is known to be sequestrated or stored in adipose tissues and due to the sizeable storage ability of the adipocytes, obese individuals tend to have lower circulating 25(OH)D3 concentrations (**Wortsman *et al.*, 2000 and Bennour *et al.*, 2022**).

Obesity increases the risk for hypovitaminosis D3 due to the deposition of vitamin D3 precursors in body fat stores, reducing its bioavailability to the skin (**Pereira- Santos *et al.*, 2015**). It is noteworthy that most of the respondents had attained overweight and obese see fig.(3-2). The interplay between sunlight exposure, lifestyle habits, and serum vitamin D3 levels cannot be disregarded. Besides, these two factors may not be substantive enough to prevent hypovitaminosis D3 among women with T2DM. Vitamin D3 deficiency among diabetics has been described in different population with varying prevalence (**Fondjo *et al.*, 2018**).

3.3.2. Levels of Serum Estradiol

In comparison to the Osteopenia group, there was a significant difference in (Mean ± SD) of estradiol (E2) between each of the PMOP with T2DM, PMOP with HTN, and PMOP without (T2DM, HTN) groups. The mean serum level of E2 in table (3-3) was statistically significantly lower in PMOP without (T2DM/HTN) when compared to osteopenia groups (p.value<0.01). These resultsare consistent with those (**Pardhe *et al.*, 2017 and Mederle *et al.*, 2018**).

Additionally, the mean of serum level E2 in the same table was statistically significantly lower in PMOP with HTN these results are consistent with **(Yeasmin *et al.*, 2017 and Di Zhao *et al.*, 2018)** and the mean of serum level E2 in the same table was significantly lower in PMOP with T2DM. There results were consistent with (**Mohsen *et al.*, 2018**) when compared to osteopenia groups (p. value<0.01). In contrast, Zhu et al. found an inverted U- shapedassociation between BMD and serum E2 levels. The inverted U-shape indicated that an excessive E2 level may be harmful to BMD at certain E2 levels (**Zhu *et al.*, 2021**).

Low estrogen levels after menopause were believed to cause bone loss and lead to osteoporosis (**Agostini *et al.*, 2018**). A decline in E2 level had been recognized as the most critical hormonal regulator of the menopause-associated decrease in BMD (**Park *et al.*, 2021**). When looking to table (3-2) and table (3-3), it was clear that bone mineral density decreased due tofactors including the low level of estrogen in postmenopausal Osteoporosis when compared to the Osteopenia groups.

These results were supported by what others have found that there is a positive relationship between BMD and estrogen (**Zhu *etal.*, 2021**). A study from Spain reported a positive association between E2 levels and BMD (**Zolfaroli *et al.*, 2021**). Another study found serum concentrations of E2 were significantly

lower in the osteoporosis group, indicative of a positive correlation between E2 and BMD (**Mederle *et al.*, 2018**). When there was an imbalance between therates of bone formation and resorption when Menopause occurs, excessive resorption results in negative remodeling balance leading to osteoporosis and other metabolic bone disease (**Bhattarai *et al.*, 2014**).

The risk factor that was most associated with osteoporosis in postmenopausal women was considered as estrogen deficiency. In addition, age- related factors also contribute to the risk of osteoporotic fracture (**Khosla *et al.*, 1997**). Women reportedly lose 1% of their bone density on average per year during and after menopause (**Suchetha Kumari *et al.*, 2010**). Harlow et al define a period of 5–6 years of menopause as early menopause and of more than 5 years of menopause as postmenopause (**Harlow *et al.*, 2012**).

After menopause, the ovaries cease to produce significant amounts of estradiol; therefore, symptoms and diseases associated with estradiol deficiency are of more importance to women's health. Bones and blood vessels are considered to be important targets for estrogen, which can improve the function of endothelial cells and vascular smooth muscle cells, inhibit platelet aggregation,and affect blood vessel responses to injury **(Knowlton *et al.*, 2012**).

Oestrogen in serum can reduce the number and activity of osteoclasts and inhibit bone resorption. The decrease in estrogen results in increased bone resorption (**Cauley, 2015 and Khosla *et al.*, 2018)**. In addition, the reduction may lead to an increase in proinflammatory cytokines, which are related to bone loss and severe arteriosclerosis (**Pfeilschifter *et al.*, 2002 and Baek et al., 2006)**.

Menopause is the cessation of menstrual cycle due to reduced secretions of estrogen and progesterone and it is defined as 12 months without menses (**Hakim *etal.*, 2022**). Oestrogen increases the activity of 1-α-hydroxylase that responsible for the activation of vitamin D and upregulates the vitamin D receptor (**Fondjo *et***

***al.*,2018**). Aging in women and the subsequent drop in estrogen levels are thus associated with a decline in vitamin D levels. Additionally, the decline in estrogen associated with postmenopausal women decreases the activity of alpha 1-hydroxylase vitamin D responsible for activating vitamin D and its receptors (**Fondjo *et al.*, 2017**).

Postmenopausal osteoporosis is one of the major health problems associated with menopause-related estrogen deprivation (**Bonaccorsi *et al.*, 2018)**. States of estrogen imbalance or deprivation are associated with higher risks of hypertension and cardiovascular disease in women; however, the effects of estrogen on hypertension are multifactorial, complex, and not completely understood (**Srivaratharajah *et al.*, 2019**).

Notably, biological sex has been revealed as a key factor in understanding variation in the development of hypertension (**Fryar *et al.*, 2017**). Therefore, aging was characterized by increases in blood pressure in both men and women, and it is well known that the incidence of hypertension increases after menopausein women (**Hanfy *et al.*, 2019**). Actually, women experience steeper increases in blood pressure than men as they age (**Ji *et al.*, 2020**).

Notably, it is more difficult to achieve Blood Pressure control in elderly women, and women are at a greater risk of developing resistance to antihypertensive treatment than men (**Daugherty *et al.*, 2012**). Hu et al. who found hypertension, coronary heart disease were the main risk factors for osteoporosis in the elderly (**Hu *et al.*, 2019**).

Previous study reported that estrogen lowers glucose level, which is associated with enhanced glucose uptake in muscle through induction of GLUT4 expression (**Barreto-Andrade *et al.*, 2018**). Estrogens deficiency enhances metabolic dysfunction predisposing to T2DM (**Hevener *et al.*, 2020**). Both clinical and animal studies show a strong correlation between estrogen deficiency

and metabolic dysfunction (**Ko *et al.*, 2020**). The reduction of estrogen in postmenopausal women accelerates the development of insulin resistance and T2DM (**Lambrinoudaki *et al.*, 2022**). T2DM may accelerate the onset of menopause (**Brand *et al.*, 2015**) and Menopause predisposes women to osteoporosis due to declining estrogen levels, This results in a decrease in bone mineral density and an increase in fractures (**Levin *et al.*, 2018**). The reduced of estradiol levels in women with type 2 diabetes in this study may due to the weak capacity of ovaries in women with type 2 diabetes to convert androgen toestrogen because the decrease of aromatase activity in the ovaries (**Crespo *et al.*,2018**).

3.3.3. Levels of Serum Minerals

Minerals such as magnesium, zinc, calcium, and phosphorus are all essential for health. They help to promote strong bones and are important for bone metabolism. Studies have demonstrated that calcium, magnesium, and zinc are essential for organic bone matrix synthesis (**Mutlu *et al.*, 2007**). The biochemical and clinical features of the PMOP with T2DM, PMOP with hypertension (HTN), and PMOP without (T2DM/HTN) as compared with the apparently osteopenia group were illustrated in the table (3-3).

The four groups studied were found to be different with respect to calcium, phosphorus, zinc and magnesium levels. Table (3-3) shows significant and non-significant differences between all groups respect to each of calcium, phosphorus, zinc and magnesium levels.

3.3.3.1. Levels of Serum Calcium

The mean ± SD values of the variables in the table (3-3) showed calcium was not significant in PMOP without (T2DM/HTN) and PMOP with T2DM(p.value >0.05) and these results are consistent with those **(Shamsulddin *et al.*, 2020 and Wen *et al.*, 2021)** and no significant in PMOP with HTN groups compared to the Osteopenia group and this results consistent with (**Yazici *et al.*, 2011 and Hazari**

***et al.*, 2012**) within normal range, and disagreed with findings (**Al-Khakani *et al.*, 2018 and Sudjaroen *et al.*, 2022**) that found serum Ca significant decrease in osteoporotic compared to healthy groups, Mohamed who found serum Ca significant decrease in osteoporotic patients compared to non-osteoporotic groups (**Mohammed, 2018**). The decreased serum total calcium might be due to ageing and loss of estrogen which lead to a significant increase in osteoclastic activity.

In addition, a decrease in calcium intake or impaired absorption of calcium from the gut lowers the serum level of calcium. Decrease value could be due to decline in either the active calcium transport or diffusion component of the calcium absorption system, probably a result of loss of direct effect of estrogen oncalcium transport in the gastrointestinal tract. Deficiency of calcium and mal- absorption due to hormonal imbalance may lead to a disorder of bone mainly osteoporosis.

The present study shows no significant difference in serum calcium in a hypertensive group compared with a normotensive group which is consistent with other findings of (**Kosch *et al.*, 2001**). Decreased serum calcium was observed in hypoparathyroidism, vitamin D deficiency, and steatorrhea. Increased calciuria is also a feature of essential hypertensive patients (**Borghi *et al.*, 1999**). Type 2 diabetes has been recognized as an independent risk for fragile fractures (**Neglia *et al.*, 2016**).

The high fracture risk in T2DM patients could be induced by hypoglycemia, muscle weakness and the chronic complications (such asretinopathy, neuropathy, and neuropathy) which usually happen in the patient with a longer duration of T2DM (**Majumdar *et al.*, 2016**). However, hyperglycemia should always be kept in mind because it plays a vital role in impaired bone metabolism in T2DM patients, leading to reduced bone strength (**Romero-Díaz *et al.*, 2021**). In PMOP with T2DM Serum Ca was controlled and

maintained homeostasis between the serum and bone compartment. Increasing serum Ca and BMD reduction in osteoporotic women were implied to have a negative correlation between serum Ca and BMD status (**Hamdi**, **2013**).

3.3.3.2. Levels of Serum Magnesium

In the table (3-3) the results show a significantly lower difference in the mean serum Mg levels in PMOP without (T2DM/HTN) as compared to the osteopenia group(p.value<0.05) and these results are consistent with other study (**Mederle *et al.***, **2018**). In this study, a decreased concentration of Mg was observed in osteoporosis postmenopausal women. Postmenopausal women from the northwest of Iran supported this study by observing the deficiency in energy and loss of micronutrients, i.e. Ca, Vitamin D3, and Mg which can be deleterious to bone health (**Hejazi *et al.***, **2009**).

Similar to study which observed a lower concentration of Mg in osteopenic women and the lowest concentration in PMOP as compared to healthy postmenopausal women (**Mutlu *et al.***, **2007**). In addition, others supported this study by observing the significantly lower level of Mg in red blood cells in postmenopausal women with osteoporosis, further concluding Mg transport mechanisms could be affected in osteoporosis patients (**Odabasi *et al.***, **2008** and **Okyay *et al.***, **2013**). On the contrary to this, in another study of elderly Chinese women observed a higher concentration of Mg in women with osteoporosis and osteopenia compared to normal women, an inverse relationship between serum Mg and Ca was observed (**Wang *et al.***, **2006**).

Magnesium has a close relationship with bone as bone stores comprise about 60% of total Mg. One-third of this skeletal Mg is found in cortical bone andserves as a reservoir for exchangeable Mg, which is beneficial to maintain the extracellular physiological concentrations of cations (**Castiglioni *et al.***, **2013**). Magnesium deficiency can lead to parathyroid hormone secretion disorders and

can affect vitamin D and 1,25(OH)2-vitamin D synthesis. The latter three are important regulators of calcium and bone homeostasis (**Rude *et al.*, 2009**). Besides,Mg is mitogenic for osteoblasts. Low Mg inhibits osteoblast proliferation by increasing the release of nitric oxide through the up-regulation of inducible nitric oxide synthase (**Leidi *et al.*, 2012**), while it increases the number of osteoclasts by promoting osteoclastogenesis (**Belluci *et al.*, 2013**).

Oxidative stress is implicated in postmenopausal osteoporosis by the loss of balance between antioxidative and oxidative markers. Monitoring of oxidative stress-related markers is useful for the diagnosis and prognosis of osteoporosis (**Zhao *et al.*, 2021**). Hence, the reduction of serum Mg and Zn in osteoporosis may affect the antioxidant status, especially antioxidant enzymes.

Maintaining Mg homeostasis can help maintain the homeostasis of osteoblasts and osteoclasts to prevent osteoporosis. The regulation of serum magnesium mainly depends on intestinal absorption and renal excretion (**Ayuk *et al.*, 2014**). In general, Mg deficiency is mainly due to lower consumption, inadequate absorption, and/or increased excretion (**Razzaque, 2018**).

The present study showed a statistically significant lower difference in the mean serum Mg levels in PMOP with HTN compared with the osteopenia group within the normal range($p.value<0.05$) and consistent with others in terms of low level of magnesium in the hypertension group compared to the normotensive group (**Rekha *et al.*, 2019**) and agreed with (**Ferdousi *et al.*, 2012**) and inconsistent with other (**Onor *et al.*, 2021**).

The wide ranging importance of magnesium makes it an important factor in several serious conditions, including a wide range of cardiovascular disorders and diabetes, and other electrolyte imbalances. In addition, the magnesium level is inversely related to blood pressure (**Wu *et al.*, 2017**). Magnesium has strong vasodilator properties, especially in patients receiving calcium-channel blockers

(**Murata *et al.*, 2016**). Others reported in their study that the serum magnesium level was significantly lower in 30 offsprings of essential hypertensive parents when compared to the 30 age and sex matched offsprings of normotensive parents and also who found that the erythrocyte magnesium level was lower in cases when compared to controls (**Ferdousi *et al.*, 2012**).

In other study, there is a negative correlation exists between serum magnesium and BP (**Rekha *et al.*, 2019**). The present study also showed statistical significant lower difference in the mean serum Mg levels in PMOP with T2DM compared with osteopenia group within normal range (p.value <0.05) and this results consistent with **(Ashok *et al*., 2020)**. Diabetes mellitus is the most prevalent endocrine and metabolic condition linked to magnesium deficiency.

Numerous investigations have demonstrated that individuals with type 2 diabetes have lower mean plasma Mg levels than non-diabetic control persons and showed Significant negative correlations between magnesium and fasting plasma glucose **(Li *et al.*, 2022**). The various factors that contribute to low magnesium levels in diabetics include low magnesium diets, Osmotic diuresis, which results in high renal excretion of magnesium, insensitivity to insulin affecting intracellular magnesium transport and thereby causing increased loss of the extracellular magnesium (**Liu *etal.*, 2020**), widespread use of loop and thiazide diuretics, which promotes magnesium wasting (**Singh *et al.*, 2019**), and decreased tubular reabsorption because of insulin resistance (**Barbagallo *et al.*, 2015**).

Imbalances in Mg^{2+} status, more frequently hypomagnesemia, inhibit glucose transporter type 4 translocation, increase insulin resistance (**Feng *et al.*, 2020**). Intracellular magnesium plays a key role in regulating insulin action, insulin-mediated glucose uptake, and vascular tone. Reduced intracellular Mg concentrations result in a defective tyrosine-kinase activity, postreceptorial

impairment in insulin action, and worsening of insulin resistance in diabetic patients. Cellular magnesium is a critical cofactor for the activities of various enzymes involved in glucose transport, glucose oxidation, insulin release, and is a cofactor for ATPase and adenylate cyclase enzymes (**Ruhul- Kabir *et al.*, 2019**). Chronic hyperglycemia causes reduction in the net tubular reabsorption of magnesium leading to hypomagnesaemia (**Kostov**, **2019**).

3.3.3.3. Levels of Serum Phosphorus

In the table (3-3), the current study serum phosphorus showed a statistically significant increase (p.value<0.05) in the Osteoporotic without(T2DM/HTN) group when compared with the Osteopenia group. The values in the present study were consistent with the study of (**Shakoor *et al.*, 2014 and Mohammed**, **2018**), and disagreed with other investigators **(Al- Azzawie *et al.*, 2020**) and Mishra et al who found serum phosphorus showed no significant in postmenopausal osteoporotic compared with osteopenia groups (**Mishra *et al.*, 2015**).

In the same study, serum phosphorus showed a significant increase in an osteoporotic group with HTN when compared with the Osteopenia group (p.value<0.01), and the result was consistent with the work of **(Prabha *et al.*, 2015)** and inconsistent with others (**Al-Hariri *et al.*, 2020**). Phosphorus and calcium are regulated mainly by two hormones PTH and the active form of vitamin D, there for any interference with the action of PTH can lead to a lowering of serum calcium and an increase of serum Phosphorus **(Underland *et al*., 2020)**.

Postmenopause was the most common cause of osteoporosis because of the effects of estrogen deficiency, which increases the rate of bone remodeling, resulting in high turnover bone loss (**Cheng *etal.*, 2022**). Increase serum phosphate levels can result from increased phosphate intake, decreased phosphate

excretion, or a disorder that shifts intracellular phosphate to extracellular space and in postmenopausal osteoporosis women increase phosphate level is related to the loss of estrogen effect is supported by the observation that postmenopausal women taking estrogen replacement therapy have lower serum phosphate than women who are not taking estrogens (**Zhang *et al.*, 2014**). Serum phosphate concentration varies with age, with the highest concentration being in infants,who required more of the mineral for bone growth and soft tissue buildup, and concentrations declining towards adulthood.

Accordingly, in both the intestinal tract and the kidney, there is an age-related decline in phosphate absorption and reabsorption, respectively, that is correlated with decreased gene and protein expression of sodium phosphate co-transporters, The rate of bone remodeling is important in determining the concentration of plasma phosphorus, as disproportionately increased bone resorption will lead to a higher plasma phosphorus concentration whereas increased mineralization will lead to a lower one (**Penido *et al.*, 2012**).

In this study, Phosphorus was nonsignificant increase in PMOP with T2DM (p.value >0.05) when compared to the osteopenia group, and the result was consistent with the work of (**Wen *et al.*, 2021**) and inconsistent with Fang et al who found The serum level of phosphate in type 2 diabetic group was significantly lower than that in the control group (P<0.05)(**Fang *et al*.,2016**), others found serum phosphorus significant higher in non-diabetic compared with diabetic groups within the normal range (**Zeid *et al.*, 2020**).

3.3.3.4. Levels of Serum Zinc

In table (3-3), the study showed no significant lower difference in the mean serum zinc(Zn) levels in PMOP without (T2DM/HTN) these results are consistent with those (**Ferdous *et al.*, 2019 and Festus *et al.*, 2020**). Others demonstrated lower levels of zinc in serum samples of postmenopausal women

with osteoporosis and osteopenia than normal women in the normal range. In contrast, others found a significantly lower difference in serum zinc levels in the PMOP group compared non the osteoporotic group (**Okyay *et al.*, 2013**). But the result is different from other result (**Bednarek-Tupikowska *et al.*, 2010**). Increased serum Zn in postmenopausal women may be the effect of increased bone resorption because of estrogen deficiency in the postmenopausal period. While some other study did not find any significant difference in the serum Zn level between pre and postmenopausal women (**Ansar *et al.*, 2015**). This study showed no significant lower difference in the mean serum Zn levels in PMOP with HTN and consistent with (**Ferdous *et al.*, 2019**) as compared to the non- osteoporotic group within the normal range. The researchers indicated a low serum zinc level in hypertensive patients in both men and women (**Rahman *et al.*, 2021**), This finding was in agreement with previous studies (**Tiwari *et al.*, 2019 and Li *et al.*, 2019**).

In contrast, some researchers indicated a high serum zinc level in hypertensive patients when compared with the control group (**Ekun *et al.*, 2021**). This disparity could be due to variations in sample size and study population. Zinc was significantly lower in PMOP with T2DM as compared to the nondiabetic osteopenia group within the normal range($p.value<0.05$). There results were consistent with Farooq et al **(Farooq *et al.*, 2020)**. These results are consistent with others who found no significantly lower serum zinc levels in diabetic compared to nondiabetic groups (**Siddapur *et al.*, 2015**). This is in accordance with others who similarly found a difference in the serum Zn levels between type 2 diabetics and controls, while others found significantly higher serum Zn levels in type 2 diabetics compared to controls which they thought could be due to the imbalanced rate of absorption to excretion of Zn (**Diwan*et al.*,2006 and Emeribe *et al.*, 2012**). some causes that caused loss of zinc in postmenopausal women might be malabsorption, age, some drugs that cause loss

of zinc in urine and some drinks contain phytates that decrease absorption of zinc.

3.3.4. Alkaline Phosphatase Activity Level

In table (3-3) the results showed a significantly higher difference in the mean serum TALP levels in PMOP without (T2DM/HTN) (P. value<0.01), these results are consistent with (**Sudjaroen *et al.*, 2022**) and inconsistent with others (**Hamdi, 2013 and Tariq *et al.*, 2021**). Bone metabolism remains stable when osteoblasts and osteoclasts proliferate in balance. Because ALP stimulates the production of the mineralization matrix proteins of osteoblasts through the hydrolysis of pyrophosphate and inorganic phosphate, osteoblast mineralization is a crucial component in the development of osteoporosis (**Yokoi, 2021**). Also, the study showed a significantly higher difference in the mean serum TALP levels in PMOP with HTN (P.value<0.05) as compared to the non-osteoporotic groupwithin the normal range and consistent with other investigators (**Al-Hariri *et al.*, 2020**).

ALP was raised as a result of increased osteoblastic activity and a condition of accelerated bone turnover, both of which are thought to be necessary for the mineralization and synthesis of new bone (**Arjumand *et al.*, 2016**). Furthermore, the association between high blood pressure and bone loss might contributed to the risk of fractures (**Zhang *et al.*, 2015**). The study showed TALP activity levels was significantly higher in PMOP with T2DM as compared to the osteopenia group within the normal range (P. value<0.05). These results areconsistent with others (**Wan *et al.*, 2022**) and inconsistent with anothers who found nonsignificant in mean serum TALP between women with T2DM and the nondiabetic group (**Hadzibegovic *et al.*, 2008**). The loss of BMD in the osteoporotic group was supported by the increased activity of ALP**(Macdonald*et al.*, 2004).** Increased ALP could be caused by prolonged exposure to PTH which eventually resulted in increased osteoblastic activity **(Botolin *et al.*, 2007)**.

In table (3-3), the study showed a significantly higher difference in the mean serum of BALP levels in PMOP without (T2DM/HTN) (P. value<0.01), these results are consistent with (**Saad *et al.***, **2021**) and inconsistent with Dongfeng et al who found nonsignificant different between osteoporotic and control group in serum BALP (**Dongfeng Zhao *et al.***, **2015**).

BALP has been found at higher levels in osteoporotic postmenopausal women due to a high bone turnover rate (**Cho *et al.***, **2016**). Additionally, thestudy showed a significantly higher difference in the mean serum BALP levels in PMOP with HTN (P. value<0.05) as compared to the non-osteoporotic group within the normal range and consistent with (**Al-Hariri *et al.***, **2020**) and inconsistent with (**Hu *et al.***, **2021**).

Another prominent finding of the study was the loss of bone minerals in osteopenia and osteoporotic groups which was supported by the increased activity of ALP (**Gourlay *et al.***, **2015**). BALP is considered to be a factor required for the mineralization and synthesis of a new bone, and it is elevated as a result of increased osteoblastic activity and a state of increased bone turnover (**Arjumand *et al.***, **2016**). Detection of the early bone changes by ALP holds the key to the management of OP and is an effective indicator to reflect the efficiency of its management (**Kuo *etal.***, **2017**).

This study showed BALP was nonsignificant higher in PMOP with T2DM as compared to the osteopenia group within the normal range (P. value>0.05). The results were consistent with (**Kulkarni *et al.***, **2017**) and inconsistent with (**Wen *et al.***, **2021**) who found significantly higher BALP in T2DM compared to the nondiabetic group but a difference in BMI and sample size with the study results. Also consistent with (**Li *et al.***, **2021**) and inconsistent with (**Oz *et al.***, **2006**) who found significantly lower BALP in T2DM patients compared to the

control. The mean value of BALP is slightly increased in type 2 diabetes mellitus compared to the osteopenic group but statistically not significant. The elevation in BALP could be due to prolonged exposure to the parathyroid hormone which in turn increases osteoblastic activity (**Withold *et al.*, 1996**). Higher levels of ALP were related to lower T-scores, which showed an imbalance between osteoblastic and osteoclastic activity, shifting the equilibrium towards increased osteoclastic activity, and the study found that osteoporotic patients had higher levels of BALP than the osteopenic group, and these high levels may be related to vertebral fractures, which are common and BALP levels in diabetic populations may raise the risk of osteoporosis (**Wen *et al.*,2021**).

3.3.5. Macrophage Erythroblast Attacher Levels

In table (3-3), the study showed a significantly lower difference in the mean serum MAEA levels in PMOP without (T2DM/HTN) (P. value<0.01) and these results were consistent with only one study performed which found that MAEA level protein in patients with PMOP was significantly lower than that in controls and his study difference by sample size, BMI and age (**Cai *et al.*, 2021**).

The study showed a non-significant difference in the mean serum MAEA level in PMOP with HTN (P. value>0.05) and also showed a significantly higher difference in the (mean ± SD) of serum MAEA levels in PMOP with T2DM (P. value<0.05). Studies of this MAEA protein in postmenopausal osteoporosis patients with hypertension and type 2 diabetes are limited.

It seems that other factors confuse the results of protein levels in these patients, as the above compatible result was in patients with osteoporosis only, and it differed for osteoporosis patients who suffer from blood pressure, as it was not significant, as well as the protein levels differed in patients with osteoporosis with diabetes type 2, and the results were significant. There is insufficient information in this study about patients with type 2 diabetes and blood pressure

patients from an immunological point of view, the effect of cytokines, inflammatory status, increase in white cells and thus Macrophage cells, and their effect on the levels of this protein in diabetic or hypertensive patients.

3.4. Molecular Analysis of Macrophage Erythroblast Attacher and Osteoporosis

Isolation of nucleic acids is the first step in most molecular analytical studies and recombinant DNA techniques. A large number of procedures, based on a variety of methods and principles, exist for the extraction and purification of nucleic acid. They all share the common requirements that the biological material needs to be lysed, cellular nucleases must be inactivated, and finally, the desired nucleic acid has to be purified from the cellular debris. Blood samples of apparently healthy and osteoporosis samples were subjected to genomic DNA extraction within 24-48 hours**.**

The DNA extraction method eliminates the need for organic solvent extractions and DNA precipitation, allowing for the rapid purification of many samples simultaneously. The concentration of genomic DNA extracted was determined and the band integrity was found to be different according to the amount of genomic DNA and its purity which depends upon the amount of WBCs in the sample used. In addition, using fresh blood samples was found to be better than that stored at -20 ^{o}C for several days, therefore, the genomic DNA should be applied as early as possible.

3.4.1. Measurement of DNA Concentration and Purity.

DNA concentration and purity were estimated by the measurement of the A260/A280 ratio. Results were clarified in the table (3-4). DNA samples seemed to be pure (**1.90 ± 0.10)** and their concentrations determined as mean ± SD was (**38.93 ± 17.38)** µg/ ml.

Table (3-4): DNA concentration and purity.

DNA Properties	Mean ± SD
DNA concentration (µg/ ml)	38.93 ± 17.38
DNA purity	1.90 ± 0.10

DNA: Deoxyribonucleic acid; SD: Standard deviation

3.4.2. Results of Amplification Reactions among the Genotypes of MAEArs10025665.

Allele-Specific PCR was used to assay genotypes of MAEA rs10025665 SNP for rapid screening of polymorphism in the patients. An amplification product was obtained to have a size of 258 bp for rs10025665. PCR product was electrophoresed and directly visualized with agarose gel which was colored with ethidium bromide under UV light to confirm the presence and integrity of the extracted DNA. The visualization method of DNA extraction was performed by staining with the fluorescent dye ethidium bromide which is the most convenient and commonly used method to visualize DNA in agarose gels.

The results of amplifications were analyzed and three genotypes were obtained for SNP rs10025665, (AA) genotype (homozygous wild type) represented in the sample (1,2) in figure (3-4), (AG) (heterozygous type) insamples (7,8) in figure (3-4) and figure (3-5) the (1,2), (3,4) and (GG) (mutant type) represented in samples (3,4), figure (3-4). Figure (3-4) genotype variation ofthe amplification fraction after a polymerase chain reaction of MAEA gene (rs10025665) of human samples obtained from osteoporosis sera by agarose gel electrophoresis (1.5% agarose) and then stained with ethidium bromide.Individuals with heterozygous (AG) revealed two bands of (258bp), while individuals with abnormal homozygous (GG) revealed one band of (258bp), and the individuals with normal homozygous (AA) revealed one band (258bp) as

shown in figures (3-4; 3-5). Gene macrophage erythroblast attacher (MAEA) is located on chromosome 4p16.3 and is expressed in a wide range of human cells, including osteoblasts and osteoclasts, which are important for bone metabolism. The encoded protein of MAEA is an integral membrane protein that mediates the attachment of erythroblasts to macrophages. One intronic SNP was chosen for genotyping. The genomic DNA of this study subjects was extracted from peripheral blood leukocytes using a DNA extraction kit (Promega Co. Ltd, USA) according to the protocol provided by the manufacturer.

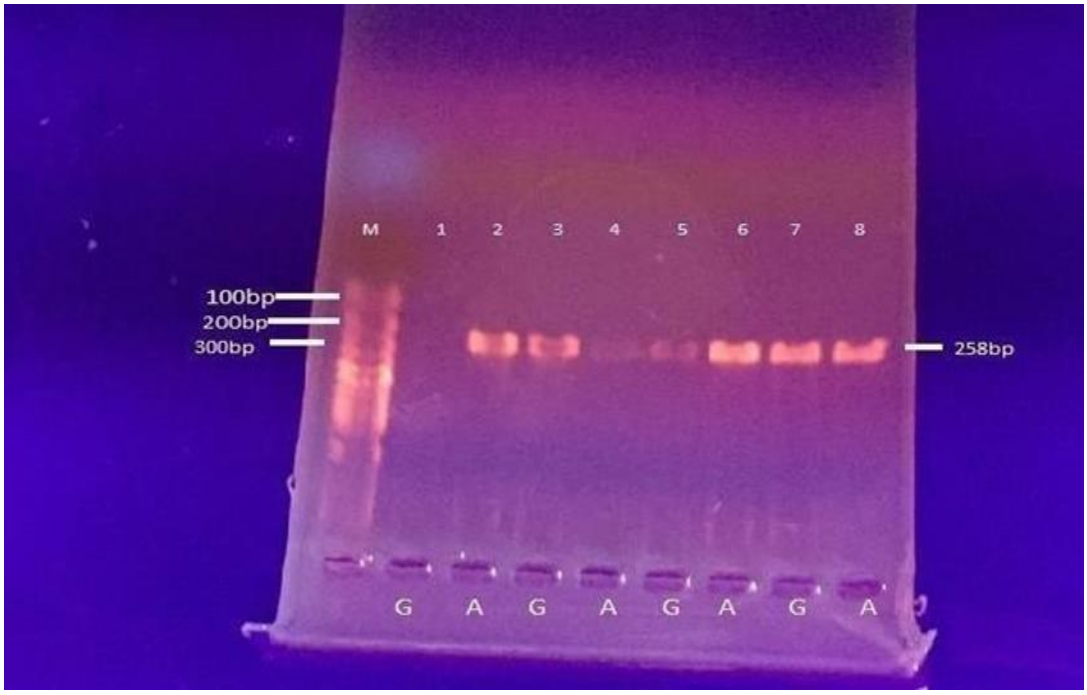

Fig. (3-4): The electrophotic graph of amplification rs10025665 region by Allele Specific PCR of human samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: DNA Marker (Ladder 100bp). Lanes 1-8 resemble 258bp of 5 µL ofPCR product loaded in each well. Lane (7, 8): two bands, the fragment (258bp) represented the heterozygous genotype (AG), lane (1,2): has one band (258bp) is the homozygous genotype (AA) and lane (5,6): has one band (258bp) represented the homozygous genotype (AA) and lane (3,4): has one band (258bp) represented the homozygous genotype (GG).

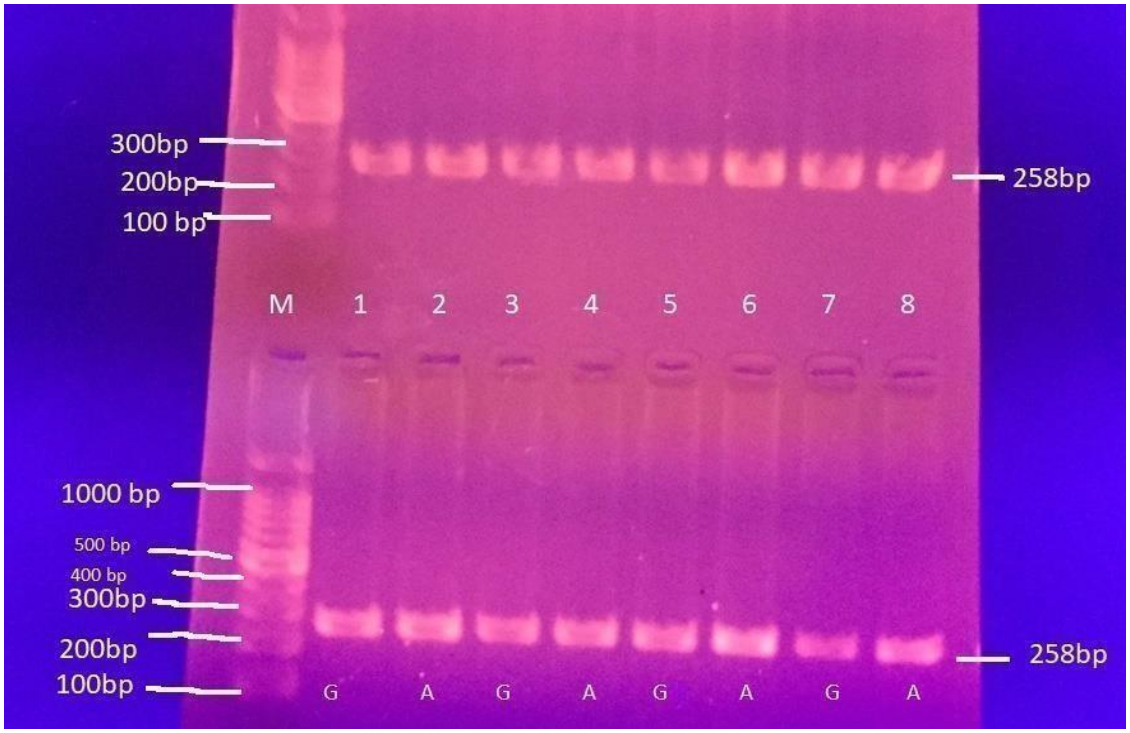

Fig. (3-5): The electrophotic graph of amplification rs10025665 region by Allele Specific PCR of human samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: DNA Marker (Ladder 100bp). Lanes 1-8 resemble 258bp of 5 µL of PCR product loaded in each well. Lanes (1, 2), (3,4), (5,6), and (7,8) each pair has two bands, the fragment (258bp) represented is the heterozygous genotype (AG).

The size of each of the GG, AA, and AG genotypes was indicated in table (3-5).

Table (3-5): Size of bands of MAEA gene polymorphism (rs10025665) obtained from sera of patients.

Genotype	No. of Bands		Size of band bp
Homozygous	GG	1	258
Wild Homozygous	AA	1	258
Heterozygous	AG	2	258

No: Number; bp: Base pair

3.4.3. Analysis of MAEA rs10025665 Polymorphism on MAEA Protein Efficacy with Comparison of some Biochemical Parameters.

The findings in distributions of MAEA protein blood level in the table (3-6) indicated that the genotypes of SNP rs10025665 were significantly associated in patients with osteoporosis and osteopenia in (AA) and (AG) genotypes (p.value <0.05) while (GG) genotype was nonsignificant when compared with osteopenia groups (p.value>0.05). The results of MAEA genotypes(AA, AG) in the osteoporosis with T2DM group are different from the osteoporosis withoutT2DM group, These results might be explained by difference sample size in the groups, where the patients who had AG were more than those who had AA and GG, as well as the higher MAEA protein concentration in the OP groups with T2DM than the rest of the groups, were also involved in showing the results as they are and the (GG) is the minor allele while the (AA) is a major allele. There is a nonsignificant association between these genotypes of SNP rs10025665 and BALP, and also a nonsignificant association between these genotypes and vitamin D in all osteoporosis groups in (AA), (AG) and (GG)genotypes(p.value>0.05) when compared with osteopenia group as shown in table (3-6).

Table (3-6): The distribution of the Allele Genotype in subjects according to the SNP(rs10025665).

Parameter	Genotype	OP&DM	OP&HTN	OP	Osteopenia	P.value
		Mean±SD	Mean±SD	Mean±SD	Mean±SD	
MAEA	AA	144.3±26.8	102.4±26.5	113.3±31.1	126±29	<0.05
	AG	128.3±21.1	107.7±28.4	102.7±28.8	118.5±21.2	<0.05
	GG	114.7±21.3	136.4±23.5	103.8±31.8	124.3±11.6	>0.05
BALP	AA	49.3±12.5	50±11.3	56.6±10.5	49±9.09	>0.05
	AG	53 ±11.8	59.1±15.8	57.2±12.6	50.63±7.3	>0.05
	GG	55±10.4	51.5±7.8	49.3±5.5	47±3.7	>0.05
VIT.D	AA	25.5±14.1	25.7±4.4	27.1±14.3	31.6±9	>0.05
	AG	26.4±12.4	26.9±8.5	26.9±10.5	30.4±7.5	>0.05
	GG	25.2±7.8	27±7.4	23.1±6	32±13.4	>0.05

3.4.4. Relation between MAEA SNP rs10025665 genetic polymorphism and Osteoporosis.

There is a nonsignificant difference between the osteoporosis and osteopenia group in (AA), (AG), and (GG) genotypes in MAEA SNP rs10025665 (p. value >0.05) and also a non-significant difference between osteoporosis with T2DM and osteopenia group in (AA), (AG)and(GG)genotype in MAEA SNP rs10025665(p.value>0.05) and the nonsignificant difference between osteoporosis with HTN and osteopenia group in (AA), (AG)and (GG)genotype in MAEA SNP rs10025665 (p. value >0.05) as shown in table (3-7).

The study showed that there is no significant association between the investigation MAEA SNP rs10025665 and the risk of osteoporosis in the study population.

Table (3-7): Genotype frequency of MAEA SNP(rs10025665) between Osteoporosis and Osteopenia group.

MAEA Genotype	OP+DM (N=40)	Osteopenia(N=40)	OR	95%CI	P.value
AA	11	14	0.70	0.2722 - 1.8228	0.47
AG	23	21	1.22	0.5067 - 2.9574	0.65
GG	6	5	1.23	0.3444 - 4.4307	0.74
TOTAL	40	40			
MAEA Genotype	OP+HTN (N=40)	Osteopenia(N=40)	OR	95%CI	P.value
AA	9	14	0.54	0.2011 - 1.4458	0.22
AG	24	21	1.35	0.5595 - 3.2922	0.5
GG	7	5	1.48	0.4287 - 5.1428	0.53
TOTAL	40	40			
MAEA Genotype	OP(N=40)	Osteopenia(N=40)	OR	95%CI	P.value
AA	11	14	0.7	0.2722 - 1.8228	0.47
AG	22	21	1.1	0.4590 - 2.6641	0.82
GG	7	5	1.48	0.4287 - 5.1428	0.53
TOTAL	40	40			
OR:odds ratio,HTN:hyprtension,OP:osteoporosis,CI:confidence interval					

Table (3-8): Alleles frequency of MAEA SNP (rs10025665) among osteoporosis and osteopenia group.

MAEA allele	OP+DM N=40	Osteopenia(N=40)	OR	95%CI	P.value
A	45	49	0.81	0.4330 - 1.5279	0.52
G	35	31			
MAEA allele	OP+HTN N=40	Osteopenia(N=40)	OR	95%CI	P.value
A	42	49	0.7	0.3730 - 1.3108	0.26
G	38	31			
MAEA Genotype	OP(N=40)	Osteopenia(N=40)	OR	95%CI	P.value
A	44	49	0.77	0.4120 - 1.4512	0.42
G	36	31			

OR:odds ratio,HTN:hyprtension,OP:osteoporosis,CI:confidence interval

There is no relation between alleles of MAEA SNP rs10025665 and the risk of osteoporosis in hypertension and T2DM when compared with osteopenia groups (p. value>0.05). As shown in table (3-8).

Table (3-9): Application of Hardy equilibrium on osteopenia group.

Hardy - Weinberg equilibrium			
Genotype	AA	AG	GG
Observed genotype	14	21	5
Expected genotype	15.01	18.99	6.01
p.value	0.79	Chi-square value	0.45

There is no deviation between observed and expected genotypes because the p.value >0.05 and this result means no difference between values in observed and expected genotypes and is consistent with HWE as shown in table (3-9).

3.4.5. Previous Studies of MAEA Gene

Hundreds of genetic loci have been reported to be associated with low BMD (**Estrada *et al*., 2012**; **Richards *et al*., 2012**; **Kemp *et al*., 2017** and **Sabik *et al*., 2017**). Especially, vitamin D receptor gene is considered to be important because it regulates calcium absorption and metabolism (**Holick *et al*., 2008**). A recent study reported the relationship of the gene MAEA with low bone mineral density in postmenopausal Japanese women. Che et al how found The MAEA gene polymorphism rs6815464 was associated with low hip BMD in postmenopausal women. The percentage of subjects with low BMD in the lumbar spine, total hip, and femoral neck.

After adjusting age, BMI, HbA1c, smoking, and alcohol consumption, the G-allele carriage was found to be associated with low BMD of the total hip but not of the lumbar spine or femoral neck (**Che *et al*., 2019**) and this is the first study to report an association between MAEA gene polymorphism and bone disease. A previous study demonstrated that the allele C of MAEA rs6815464 G/C polymorphism was a significant risk factor for type 2 diabetes in the Japanese population (**Imamura *et al*., 2012**).

Diabetes is one of the known risk factors for bone loss (**Schwartz *et al*., 2011**). Cai et al used Four tag single nucleotide polymorphisms that covered the gene region of MAEA were chosen for genotyping (rs10025665, rs72501966, rs6815464, and rs12641735) to investigate the association of MAEA with postmenopausal osteoporosis in Han Chinese individuals, Cai et al who found SNP rs6815464 was significantly associated with the risk of PMOP and the C allele was positively associated with decreased MAEA protein levels in the blood while SNP rs10025665 was nonsignificant associated with the risk of PMOP(**Cai *et al*., 2021**) and this result agreed with the present study.

Conclusions and Recommendations

Conclusions

1. Age is a risk factor for osteoporosis in postmenopausal women

2. BMI is a protective factor against osteoporosis but other factors that contribute to osteoporosis occur in women such as T2DM and HTN, and age women.

3. No found difference was found in BMI between selected groups and thus it's not regarded risk factor for osteoporosis.

4. Vitamin D is significantly lower in osteoporosis with T2DM and osteoporosis with HTN and a high level of vitamin D is protective against osteoporosis.

5. A significant Physiological decrease in E2 is a risk factor for women after menopause in all groups when compared with the osteopenia group.

6. In postmenopausal women, the serum levels of zinc in osteoporosis and osteoporosis with HTN group were nonsignificant and significantly lower in osteoporosis with T2DM.

7. There is no difference indicated between osteoporosis and osteopenia groups in serum total calcium.

8. In postmenopausal women, high serum levels of phosphorus appear was a development of osteoporosis in Iraqi women.

9. Magnesium is an important mineral for women after menopause and its significantly lower in osteoporosis, osteoporosis with HTN, and osteoporosis with T2DM groups.

10. High levels of TALP and BALP in postmenopausal women are associated with the development of osteoporosis.

11. The study showed significantly lower levels of serum MAEA protein in the

osteoporosis group lead to increase risk development of osteoporosis but osteoporosis in the HTN group was non-significant and osteoporosis in the T2DM group was significant and this variation needs more investigation to evaluate abnormality.

12. The study showed that there is no significant association between the investigation of MAEA SNP rs10025665 and the risk of osteoporosis in the study population.

13. The distribution of MAEA SNP rs10025665 genotypes are all in Hardy Weinberg equilibrium.

14. There is no relation between alleles of MAEA SNP rs10025665 and the risk of osteoporosis in osteoporosis with hypertension and osteoporosis with T2DM when compared with osteopenia groups.

Recommendations

1. Conducting a study using similar biochemical markers but in aged 25 years old (premenopausal women) to women (postmenopausal) age ≥50 years.
2. Conducting a bone scan with DEXA on different other sites in the body such as hip, forearm, and wrist bones and measuring bone mineral density to know the extent of its impact on the selected pathological groups.
3. Conducting the study on an additional group of patients with high blood pressure and type 2 diabetes together, comparing bone mineral density with these study groups, in addition to evaluating the most affected group and considering it as a risk factor for osteoporosis in postmenopausal women.
4. Measurement of some immunological markers to identify infections, especially in patients with type 2 diabetes and blood pressure patients, to explain the abnormality in the levels of MAEA protein.
5. Conducting an assessment of liver and kidney functions to explain any abnormalities in the levels of some markers, especially vitamin D, TALP, calcium, and magnesium.
6. Evaluating some additional hormones such as FSH, LH, and PTH as well as the measurement of some biochemical markers associated with diabetes type 2, such as glycated hemoglobin and it relation with MAEA protein.
7. Estimation of Triacylglycerol and cholesterol in blood pressure patients and their relationship to bone mineral density and MAEA protein.
8. Study of distribution other MAEA SNPs (rs72501966, rs6815464, rs12641735) and its relation with MAEA protein in postmenopausal osteoporosis.
9. Study of MAEA protein in T1DM and T2DM to know the levels of MAEA protein and its relationship to BMD and compare it with healthy subjects.

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ARTICLE ACCEPTANCE LETTER

Article ID: AFR/2022-0229

Article Title: Investigation of Macrophage Erythroblast Attacher as New Protein and Its Association with Vitamin D3 Levels among Hypertensive and Normotensive Osteoporosis Postmenopausal of Iraqi Women

Corresponding Author: Hussein Ali Al-Obaidi

Thank you very much for your submission to our journal.

We are pleased to inform you that your paper has been reviewed, and accepted for publication. Your article will be published in upcoming current issue.
Thank you for making the journal a vehicle for your research interests.

Best wishes, Editor-in-Chief

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LETTER OF ACCEPTANCE

Manuscript Title:

Comparative Study of Bone Specific Alkaline Phosphatase, Bone Mineral Density, and Zinc in Postmenopausal Osteoporosis in Iraqi Type 2 Diabetic Women

By

Hussein Ali Al-Obaidi[1•]; Fadhil J. Al-Tu'ma[1] ; Zena A. M. Al-Jawadi[2] and Tamadhur S. M. Al-Hasnawy[3]

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ACCEPTED	~~REVISIONS REQUIRED~~	~~REJECTED~~

With Warm Regards,

Editor Manager
HIV Nursing

Certificate of Participation

This certificate is proudly presented to:

Hussein Ali Al-Obaidi, Fadhil J. Al-Tu'ma, Zena A. M. Al-Jawadi, Tamadhur S. M. Al-Hasnawy and Amir F. Al-Tu'ma

Who had been participated in the **Scientific Abstract Season** by abstract entitled:

Investigation of Macrophage Erythroblast Attacher as New Protein and its Association with Vitamin D3 Levels Among Hypertensive and Normotensive Osteoporosis Postmenopausal of Iraqi Women

In the: ***"Second Scientific International Conference for Biology and Pure Sciences (SSICBPS2022)"***

Which had been held at the College of Science, University of Mosul,

on October 19-20, 2022 - Mosul, IRAQ

Prof. Dr. Hiyam A. Altai
Conference Chair
Dean of the College of Science

Republic of Iraq
Ministry of Higher Education &
Scientific Research
Al_zahraa University for Women
Continuing Education Department

جمهورية العراق
وزارة التعليم العالي
والبحث العلمي
جامعة الزهراء (ع) للبنات
قسم التعليم المستمر

شهادة مشاركة

نؤيد أن

السيد/ة : حسين علي حسين المحترم/ة

قد حضر/ت المؤتمر العلمي الدولي الأول للتخصّصات الصحية والطبية المقام تحت شعار :

{التقنيات العلمية الحديثة نهجنا للتقدّم والرقي}

في يومي الأحد والأثنين الموافق (٥-٦/٦/٢٠٢٢)

أ. د . زهير محمدعلي جدوع
مساعد رئيس الجامعة للشؤون العلمية

م.د. فرح باقر أحمد الفاضلي
مديرة قسم التعليم المستمر

الخلاصة

تمثل هشاشة العظام مشكلة صحية عالمية متزايدة، مع أعلى معدلات الإصابة عند النساء بعد سن اليأس. هشاشة العظام هي اضطراب هيكلي نموذجي يتم وصفه بانخفاض كتلة العظام، مما يؤدي إلى انخفاض قوة العظام وزيادة خطر الإصابة بالكسور. تم اقتراح جينات عديدة مشاركة في هشاشة العظام بعد سن اليأس، منها تعدد الأشكال النيوكليوتيدي الأحادي rs1002566 لجين مرفق البلاعم بالأرومة وارتباطه بهشاشة العظام .تهدف هذه الدراسة إلى التحقق من ارتباط تعدد الأشكال الجيني لجين مرفق البلاعم بالأرومة بهشاشة العظام لدى النساء بعد سن اليأس في محافظة كربلاء وتحديد انزيم الفوسفاتيز القلوي في مصل هشاشة العظام ومقارنتها بمجموعة غير المصابين بهشاشة العظام.

كان تصميم الدراسة عبارة عن دراسة مقطعية شملت 160 امرأة تم تشخيصهن من قبل الطبيب وتم تقسيمهن إلى أربع مجموعات: كل مجموعة تتكون من أربعين امرأة كما يلي: هشاشة العظام بعد سن اليأس مع داء السكري من النوع 2، هشاشة العظام بعد سن اليأس مع ارتفاع ضغط الدم وهشاشة العظام بعد سن الياس دون (داء السكري / ارتفاع ضغط الدم) ومجموعة غير المصابين بهشاشة العظام بعد سن الياس. تم جمع نساء المجموعات من مركز الحسن للغدد الصماء ومركز هشاشة العظام في مدينة الحسين الطبية في مدينة كربلاء، للفترة من تشرين الثاني 2021 الى آذار 2022.

تم تشخيصهم بمرض هشاشة العظام عن طريق قياس درجة T من خلال فحص الأشعة السينية ثنائي الطاقة في مناطق العمود الفقري القطني المؤكدة لهشاشة العظام. تألفت هشاشة العظام من أربع فئات تم جمعها عند القبول وتم تقييم شدة المرض باستخدام معايير منظمة الصحة العالمية. تم تشخيص المرضى سابقًا بارتفاع ضغط الدم ويستند المرضى الذين يعانون من داء السكري من النوع الثاني إلى تشخيص الأطباء قبل زيارتهم الحالية للعيادات الخارجية. تم تشخيص مرض هشاشة العظام عند زيارتهم لمركز هشاشة العظام لأول مرة.

أظهرت نتائج فيتامين د ٣ فرقا معنويا لوحظ انخفاض مصل فيتامين د ٣ في جميع مجموعات هشاشة العظام بالمقارنة مع مجموعة غير المصابين بهشاشة العظام (قيمة الاحتمالية اقل من 0.05) كما أظهرت نتائج المغنيسيوم انخفاضًا معنويًا في جميع مجموعات هشاشة العظام مقارنة بمجموعة غير المصابين بهشاشة العظام (قيمة الاحتمالية اقل من 0.05).

أظهرت نتائج بروتين مرفق البلاعم بالأرومة انخفاضًا معنويًا في مجموعة هشاشة العظام لغير المصابين (بداء السكري من النوع الثاني وضغط الدم) عند مقارنتها بمجموعة غير المصابين بهشاشة العظام (قيمة الاحتمالية اقل من 0.01)، كما أظهرت نتائج هذا البروتين زيادة معنوية في مجموعة هشاشة العظام المصابين بداء السكري من النوع الثاني مقارنة بمجموعة غير المصابين بهشاشة العظام (قيمة الاحتمالية اقل من 0.05) ، بينما لا يوجد فرق كبير بين هشاشة العظام المصابين بضغط الدم ومجموعة غير المصابين بهشاشة العظام (قيمة الاحتمالية اكبر من 0.05).

أظهرت نتائج انزيم الفوسفاتيز القلوي العظمي زيادة معنوية في مجموعة هشاشة العظام ومجموعة هشاشة العظام المصابين بضغط الدم بالمقارنة مع مجموعة غير المصابين بهشاشة العظام (قيمة الاحتمالية اقل من 0.05) وزيادة غير معنوية في مجموعة هشاشة العظام المصابين بداء السكري من النوع الثاني عند المقارنة مع مجموعة غير المصابين بهشاشة العظام (قيمة الاحتمالية أكبر من 0.05).

ارتبطت الأنماط الجينية لتعدد الاشكال النيوكليوتيدي الاحادي لجين مرفق البلاعم بالأرومة rs10025665بشكل كبير ببروتين مرفق البلاعم بالأرومة في الأنماط الجينية (AA) و) (AG) (قيمة الاحتمالية اقل من 0.05) وكان هناك ارتباط غير مهم بين الأنماط الجينية لـ rs10025665 وانزيم الفوسفاتيز القلوي العظمي في الأنماط الجينية (AA) و (AG) و(GG) (قيمة الاحتمالية أكبر من 0.05). لم يكن هناك ارتباط ذو دلالة احصائية في التحقيق بالأنماط الجينية لجين مرفق البلاعم بالأرومة لتعدد الاشكال الجيني الاحادي rs10025665 وخطر الإصابة بهشاشة العظام في مجتمع الدراسة.

ترتبط المستويات المنخفضة من مصل بروتين مرفق البلاعم بالأرومة في هشاشة العظام لغير المصابين بضغط الدم وداء السكري من النوع الثاني والمستويات العالية من إنزيم الفوسفاتيز القلوي العظمي في النساء بعد سن اليأس بتطور هشاشة العظام في النساء العراقيات.

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دراسة تعدد الاشكال لجين مرفق البلاعم بالأرومة وارتباطه مع بروتين كمؤشر لهشاشة العظام بعد سن اليأس.

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مجلس كلية الطب كجزء من متطلبات نيل درجة
الماجستير في الكيمياء الحياتية السريرية

من قبل

حسين علي حسين علوان

بكالوريوس علوم -2005

وبأشراف

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و

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