Relation between insulin-like growth factor-1 and markers of osteoporosis in postmenopausal women with and without Type 2 diabetes

THESIS

Submitted To the Medical Research Institute
University of Alexandria
In Partial Fulfillment of the Requirements For The

Degree of Ph.D

In

Biochemistry

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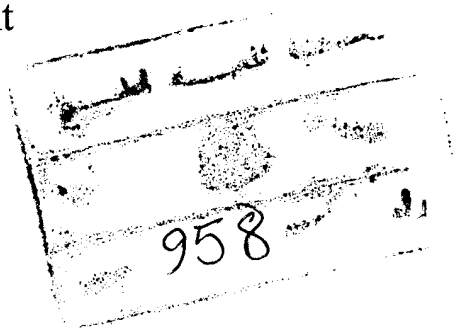

2011

RELATION BETWEEN INSULIN-LIKE GROWTH FACTOR-1 AND MARKERS OF OSTEOPOROSIS IN POSTMENOPAUSAL WOMEN WITH AND WITHOUT TYPE 2 DIABETES

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Acknowledgement

I humbly thank the Almighty God, greatest of all, for giving me the ability and effort to perform this work.

The few words I wrote here can never and can not adequately express the feeling of gratitude; I have for my supervisors and the persons, who helped me to achieve this work. If I vote the heartiest thanks, it is to:

It is with great pleasure, deep satisfaction and gratitude that I acknowledge the supervision and help of ***Prof. Dr. Madiha Hassan Helmy****, Professor of Biochemistry, Medical research Institute, Alexandria University. I would like to express my deep appreciation and sincere thanks for her unlimited effort, unending cooperation and her supervision. I am also indebted to her for her generosity with her time, effort and materials required during this investigation. I will always remain grateful.*

It is a great pleasure to express my deepest gratitude and indebtedness to Prof. Dr. ***Anna Nashaat Abou Rayah,*** *Professor Department of Internal Medicine Faculty of Medicine University of Alexandria for her great effort and long time she invested in this study. I deeply express my appreciation and thanks to her.*

Also I wish to express my deepest thanks and gratitude to ***Dr.Maher Abdel Nabi Kamel****, Assistant Professor of Biochemistry, Medical Research Institute, University of Alexandria, who is really the best friend, for his unlimited effort, continuous encouragement, unending cooperation, his valuable technical help in the work, cooperation, advice and other teaching and research efforts for completing this work.*

I wish to extend my thanks to my family especially my parents and my sister their support and patience.

Dedication

To the person who taught me patience, strife, and pushed me towards success in life and give me all care happiness.

To my father Mahmoud Hania

Mother Amal Hania

Sisters

Hadeel

Hanaa

Heba

Abbreviation

EMF	Electromotive force
AgCl	Silver chloride
ALS	Acid labile subunit
AKT	Serine/threonine protein kinase
BGP	Bone caboxyglutamic acid containing protein
BMD	Bone mineral density
BMI	Body mass index
BMP 2/4	Bone Morphogenic protein 2 / 4
CaCO3	Calcium Carbonate
$CaCl_2$	Calcium chloride
Cbfa-1	Core binding factor alpha-1
cDNA	Complementary DNA
CSF-1	Colony stimulating factor-1
Conj	Conjugative
CT	Calcitonin
Cys	Cysteine
DDH	Death domain-homologous
DISH	Diffuse idiopathic skeletal hyperostosis
DNA	Deoxyribonucleic acid
DPA	Dual-photon absorptiometry
DXA	Dual X-ray absorptiometry
EDTA	Ethylenediaminetetraacetic acid
ETH 1001	Ethoxycarbonylundecyl-N,N′-4,5-tetramethyl-3,6-dioxaoctanediamide
ELISA	Enzyme linked Immunosorbent Assay
FBG	Fasting blood glucose.
FPG	Fasting plasma glucose
GH	Growth hormone
Gla	Glycoprotein
HbA1c	Glycated hemoglobin
HCl	Hydrochloric acid
H_2SO_4	Sulfuric acid
HRP	Horseradish peroxidase
HRT	Hormone replacement therapy
IGF	Insulin-like growth factor
IGF-1	Insulin-like growth factor-I
IGFBP1	IGF binding proteins
IGF1R	IGF-1 receptor
IL-1	Interleukin-1
KCl	Potassium chloride
KDa	kilo Dalton
LIF	Leukemia inhibitory factor
M-CSF	Macrophage colony stimulating factor
mRNA	Messenger RNA
NaCl	Sodium chloride

NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
OCIF	Osteoclastogenesis Inhibiting Factor
OPG	Osteoprotegerin
OC	Osteocalcin
PBM	Peak bone mass
PPAR- gamma	Peroxisome proliferator-activated receptor- gamma
PTH	Parathyroid hormone
PGE2	Prostaglandin E2
PDGF	Platelet-derived growth factor
RANK	Receptor Activator of Nuclear factor Kappa B
RANKL	Receptor Activator of Nuclear factor Kappa B ligand
TGF- beta	Transforming growth factor beta
TMB	3,3,5,5- tetramethylbenzidine
TPO	Thyroid peroxidase
Tris	Tris(hydroxymethyl)methylamine
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor family
UcOC	uncarboxylated osteocalcin

TABLE OF CONTENTS

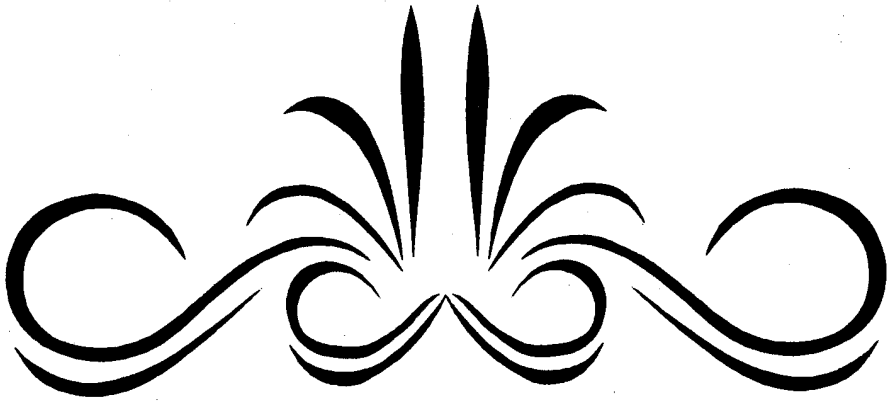
LIST OF TABLES

List of Figures

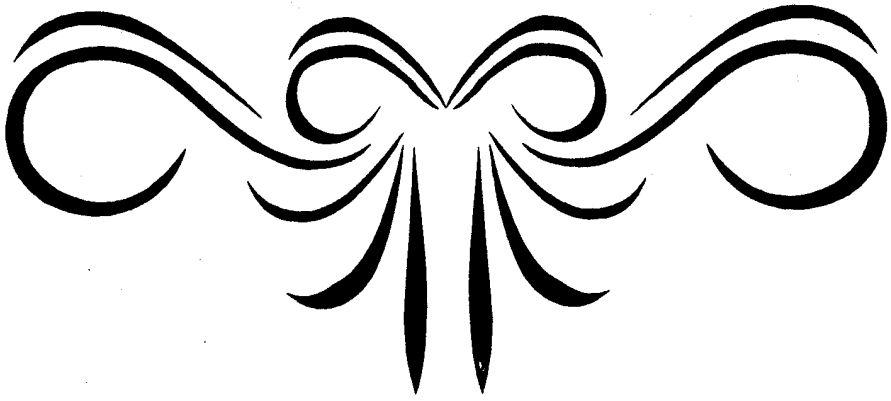
Figure No.

List of Figures

Page



INTRODUCTION



The skeleton serves several important functions, such as structural functions that provide mobility, support for, and protection of the body. It also has an important function as a reservoir for calcium and phosphorus, which, in turn, are influenced by mineral uptake in the intestine and mineral excretion in the urine. The mineral homeostatic mechanisms in the skeleton are controlled by the calcium-regulating hormones; parathyroid hormone (PTH), calcitonin (CT), and 1, 25(OH) 2-vitamin D3 (D3) which regulate the activity of the bone-resorbing cells. Bone tissue is not static, and healthy bones require continuous remodeling and modeling to adapt to their dual roles as a supporting frame and as a regulator of mineral homeostasis [(1)]. Remodeling is a lifelong coordinated and dominant process in the adult skeleton, whereby cortical and trabecular bone is rebuilt, a process initiated by resorption and followed by new bone formation at the same site where the resorption process occurs. If the two processes are quantitatively equal, the remodeling process is balanced [(2)] .

Remodeling is important for the maintenance of bone mass, to repair micro damage of the skeleton, to prevent accumulation of too much old bone, and for mineral homeostasis. Unbalanced remodeling may lead either to loss of bone, as in osteoporosis, or, more rarely, to gain of bone, as in osteopetrosis. Modeling is a process where bone resorption takes place in one site and bone formation at another. Thus, modeling implies that new bone is formed independent of preceding bone resorption at the site of formation. Modeling can lead to a new shape of the skeleton, or to thickening of cortical bone due to periosteal new bone formation [(3)] .

Unbalanced bone remodeling, leading to the loss of bone tissue, is observed in pathological conditions such as osteoporosis, rheumatoid arthritis. Pathological remodeling can also be a consequence of mutations in molecules regulating osteoclasts and osteoblasts differentiation and function [(1)] . In osteoporosis, unbalanced remodeling leads to decreasing amounts of bone tissue in several sites of the skeleton (Figs. 1A, 1B) and, eventually, to skeletal fractures [(2)] .

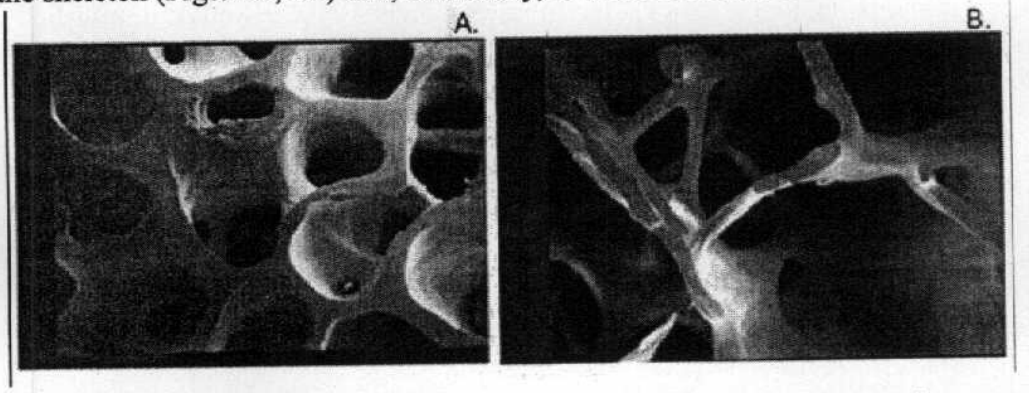


Figure (1). Unbalanced remodeling of the skeleton in post-menopausal osteoporosis because of excessive osteoclastic bone resorption and reduced capacity of osteoblasts to refill the resorption lacunae results in a decreased amount of bone tissue (Quoted from reference 2).

Osteoporosis is defined as a disease characterized by low bone mass and deterioration of bone structure, causing bone fragility and increased risk of fracture (Figs. 1A, 1B) [2] . Osteoporosis is a "silent disease " that millions of people around the world suffer from, and it is important due to its morbidity, mortality, adverse effects on the quality of life and the extra costs imposed on the patient and the society. The increase of life expectancy and so the old age in the society in developing countries such as the Middle East has led to an increase in the prevalence of osteoporosis and its following fractures in the area [3].

Osteoporotic fractures are associated with significant morbidity, mortality, and reduction in quality of life. Known risk factors associated with the development of osteoporosis and fractures include female gender, older age, lower body mass index (BMI), and family history [4] . However, in spite of numerous studies, the relationship between diabetes and osteoporosis remains controversial [5-6]. Type 2 diabetes has also been associated with an increased risk of fractures at any skeletal site. The risk of hip fractures is increased in type 2 diabetes in postmenopausal women, although to a lesser magnitude, with risks varying from 1.5- to 2.8-fold [3]. It seems that the effects of type 2 diabetes on bone metabolism are less clear. There are conflicting study findings; increased, decreased, or similar body mass densities (BMDs) have been reported among type 2 diabetic patients as compared with healthy subjects [7]. Also, it was reported that women with type 2 diabetes had a higher risk of bone fracture than women without diabetes after adjustment for multiple risk factors [8] .

There have been conflicting reports about the skeletal involvement in patients with diabetes mellitus. The alterations of bone mineralization in diabetic subjects by using both radiographic and photonic techniques. This controversy is largely related to the complex pathophysiology of diabetes mellitus characterized by hyperglycemia and concomitant metabolic conditions due to impaired insulin secretion or diminished tissue response to insulin. The endocrine and metabolic alterations in diabetes mellitus can trigger disorders of calcium homeostasis, skeletal metabolism and bone mass [7].

Peak bone mass (PBM) is defined as the amount of bony tissue present at the end of skeletal maturation [9] . Bone strength is mainly determined by volumetric density, i.e., the amount of bony tissue per unit of volume, by outer bone dimensions [10] . It is generally accepted that fractures result from low bone mass. Bone mass accounts for 75-85% of the variance in the ultimate strength of bone tissue, and such measurements also provide an accurate indication of whole bone strength [11]. Figure 2 shows changes of bone mass in both sexes in relation to age.

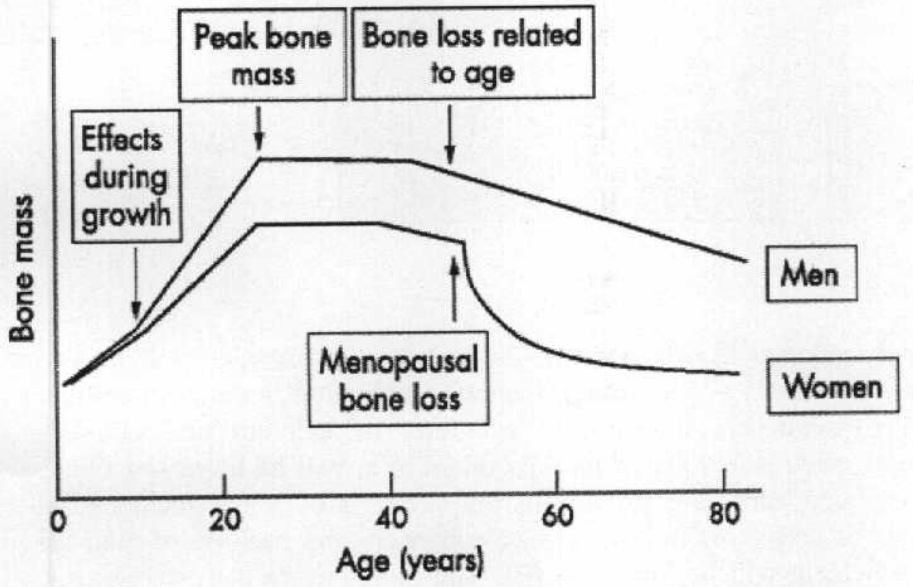


Figure (2). Changes of bone mass in women and men in relation to age **(Quoted from reference 12).**

In women, as shown in figure 2, there tends to be minimal change in total bone mass between age 30 and menopause. But in the first few years after menopause, most women experience rapid bone loss, a "withdrawal" from the bone bank account, which then slows but continues throughout the postmenopausal years. This loss of bone mass can lead to osteoporosis [13] .

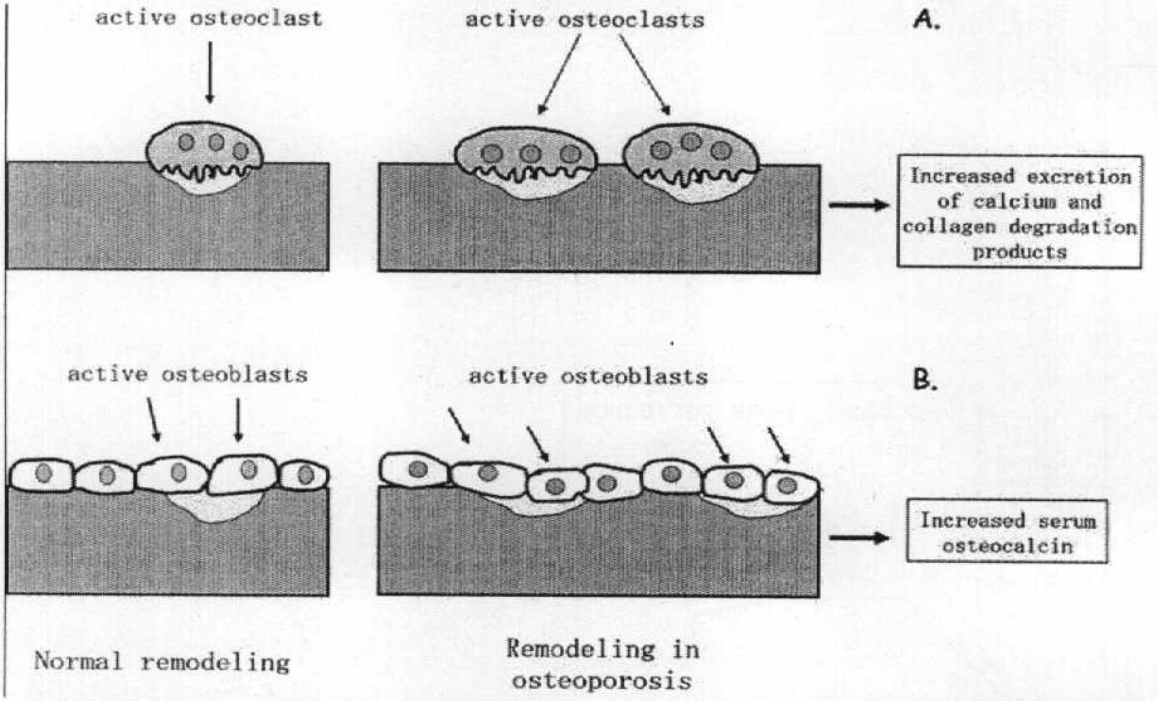


Figure (3). In post-menopausal osteoporosis, the decrease of estrogen will lead to increased numbers of osteoclasts and, thus, enhanced numbers of bone multi-cellular units **(A).** As a consequence, the urinary excretion of calcium and collagen degradation products, such as deoxypyridinoline crosslink's, will be increased. Since more bone multi-cellular units are present in the skeleton of a post-menopausal woman, the number of active osteoblasts will be enhanced, and because of that, the serum level of osteocalcin will be increased **(B)**. The more severe the osteoporosis, the more bone multi-cellular units will be present, and therefore the number of active osteoblasts and serum osteocalcin levels will be an indicator of "high turnover" osteoporosis. However, since the individual osteoblasts are less-well-functioning because of the lack of estrogen, the net effect of resorption and bone formation will be such that the amount of bone tissue will decrease. **(Quoted from reference 14).**

Classification of systemic osteoporosis

Primary osteoporosis is mainly seen in elderly people and is the most common type of osteoporosis. It is more common in women than men. The mechanisms by which elderly people, both men and women, lose bone are not fully understood. Decreased quantities of sex hormones are one important factor causing bone loss. Although most patients suffering from osteoporosis are post-menopausal women exhibiting loss of estrogen, elderly men also develop primary osteoporosis [(15)] . Women exhibit two phases of age-related bone loss: The first starts at menopause, predominantly in trabecular bone, is caused by estrogen deficiency, and results in a disproportionate increase in bone resorption as compared with formation. When this phase peaks after 4-8 years, the second phase starts, exhibiting a persistent, slower loss of both trabecular and cortical bone, and is mainly a result of decreased bone formation. Decreasing levels of estrogen are thought to be responsible for increased resorption, and decreasing testosterone for decreased bone formation [(16)] .Primary osteoporosis can be classified as type I or type II osteoporosis (Table 1)

Table 1. Comparison of type I and type II osteoporosis [17]

factors	Type I postmenopausal women	Type II Age – related senile- osteoporosis
Age	55-75	>70
Sex(F:M ratio)	6:1	2:1
Fractures	Wrist and vertebra	Hip and vertebra

Secondary osteoporosis results from chronic conditions that contribute significantly to accelerated bone loss. These chronic conditions include thyroxin excess, diabetes mellitus, hyperparathyroidism, malignancies, gastrointestinal diseases, medications, renal failure and connective tissue diseases. Osteoporosis is a common complication of long-term glucocorticoid therapy and is responsive to bisphosphonates in this setting. If secondary osteoporosis is suspected, appropriate diagnostic work-up could identify a different management course. For example, if a pituitary tumor is identified; surgical removal could prevent ongoing accelerated bone loss. The bone loss already sustained can be treated. The secondary hyperparathyroidism of renal failure can be ameliorated through dietary modification and calcium supplementation [18] .

Idiopathic juvenile osteoporosis is rare. It occurs in children between the ages of 8 and 14 or during times of rapid growth. There is no known cause for this type of osteoporosis, in which there is too little bone formation or excessive bone loss. This condition increases the risk of fractures [19] .

Bones usually grow weak due to low levels of calcium, phosphorus and other minerals. Lifestyle choices, various diseases and use of some drugs also result in osteoporotic bones. Relative deficit in bone formation could be related at least in part to trabecular perforations that occur during the resorptive phase, leading to the loss of bone surface on which osteoblasts could deposit new bone. Acceleration of the bone remodeling rate, which is common in many metabolic bone diseases including postmenopausal osteoporosis, thereby leads to a greater rate of bone loss [20] .

The osteoporosis condition can operate silently for decades, because osteoporosis doesn't cause symptoms unless bone fractures. Some osteoporosis fractures may escape detection until years later. Therefore, patients may not be aware of their osteoporosis until they suffer a painful fracture. Then the symptoms are related to the location of the fractures.

Early symptoms: in many cases there is no indication of gradual bone loss, many people have no early symptoms until they fracture a bone. Muscular aches and bone tenderness: as the disease progresses to later stages of osteoporosis symptoms may include: neck pain, muscle pain, bone tenderness [21] . Osteoporosis thins and weakens the bones, increasing the risk that a minor injury will result in a bone fracture. Individuals with osteoporosis can suffer from a fracture from something as simple as a cough, sneeze or bumping into a chair. Spinal deformities such as a stooped posture or Kyphosis can be a symptom of osteoporosis. A hunchback and loss of height is the result of multiple vertebral compression fractures in the spine due to loss of bone mass. Loss of height usually will alert healthcare professionals to further evaluate the risk of osteoporosis. Back pain in the lower and upper regions of the back is a common complaint and symptom of many disorders. Risk factors for osteoporosis are strongest risk factor and include: female sex, age > 60 years and family history for osteoporosis in other hand other significant risk factors include ; Caucasian origin , early menopause, low BMI, diabetes, smoking, sedentary lifestyle and long term (>3 months) corticosteroid use [22] .

RANK, RANKL

In the membrane of cells of osteoclast line and in dendritic cells, there is a protein crucial for all calcium-tropic hormones and proresorptive cytokines to increase calcemia and multiplication of osteoclasts in the bone. This protein was identified as RANK (Receptor Activator of Nuclear factor Kappa B). RANK is considered as transmembrance heterotrimer on the surface hematopoietic osteoclast progenitor, mature osteoclast, chondrocytes and mammary gland epithelial cells . Human RANKL is type II transmembrance protein with approximate mass of 45KDa and it's regarded as major regulator of pathological bone resorption .Its located on chromosome 13q14. Sequence variations in the RANKL promoter region may alter the binding of various transcription factors [23] . RANK ligand and antagonist is a protein produced by osteoblasts, cells of bone stroma and by activated T lymphocytes [24] . It was identified as RANKL (RANK ligand); it may promote osteoresorption by induction of cathepsin K gene expression [25] . Osteoblasts and stromal cells produce another protein, which binds RANKL and interferes with its bond on RANK. This protein inhibits differentiation of progenitors into osteoblasts, displays hypocalcemic and antiresorptive effects. It was named osteoprotegerin (OPG) or OCIF (OsteoClastogenesis Inhibiting Factor) [26]. RANKL and its inhibitor osteoprotegerin are decisive for differentiation and osteoresorption function of the osteoclast so that they became the aim of intensive research. Both the molecules occur in a free form, not bound on a membrane; their concentration can be measured and probably applied in diagnostics [27] . RANKL is a novel member of the TNF family of ligands.

There are at least three forms of RANKL, two of which possess a transmembrance domain that positions the biologically active carboxy-terminus to the extracellular domain (i.e., a type II transmembrance protein). One of these forms, RANKL2, is a shorter alternative splicing variant of RANKL1 [28] . Both of these variants can remain on cell surfaces or can be proteolytically cleaved into soluble forms that possess osteoclast-stimulating activities within their TNF-homology domains. RANKL is produced by numerous cell types including cells of the osteoblasts lineage and activated T cells [29] . T Cells express both soluble and membrane-bound forms of RANKL, and both forms are implicated in focal bone erosions associated with inflammatory arthritis. Cells of the osteoblasts lineage can express RANKL on their surface in a manner that facilitates osteoclastogenesis in vitro via cell-to-cell contact with osteoclast precursors [24]. Differentiation of cultured osteoblasts was associated with reduced RANKL expression and decreased ability to support osteoclastogenesis, suggesting that the mature bone-forming osteoblast might not be capable of directing osteoclast activity via RANKL [30] . Membrane RANKL has been suggested to be somewhat more potent than soluble RANKL in stimulating osteoclastogenesis in vitro. However, soluble RANKL is measurable in the circulation, and serum RANKL has been shown in some studies to increase with stimulated bone resorption. Soluble recombinant RANKL is also capable of causing severe skeletal catabolism in mice and in rats. These results indicate that soluble RANKL has the potential to be an important physiological and pathological mediator of bone resorption [31] .

RANKL is involved in numerous aspects of osteoclast differentiation and function. RANKL was implicated in the fusion of osteoclast precursors into multinucleated cells, their differentiation into mature osteoclasts, and their attachment to bone surfaces, their activation to resorb bone, and their continued survival by avoiding apoptosis. In most situations, RANKL probably relies on macrophage colony-stimulated factor (M-CSF, also known as CSF-1) as a cofactor for osteoclast differentiation [32]. Preliminary evidence also suggests that RANKL can stimulate osteoclastogenesis and bone resorption in mice that lack functional M-CSF. These results suggest that RANKL plays a dominant role in the regulation of bone resorption, and no factor or combination of factors have been shown to be capable of restoring bone resorption when RANKL is absent [33] . The receptor that mediates all known activities for RANKL is called receptor activator of nuclear factor B (RANK) [34]. RANK is a homotrimeric TNF receptor family member that was initially discovered from a bone marrow-derived dendritic cell cDNA library. RANK was identified as a receptor that mediated the ability of RANKL to promote the survival of cultured dendritic cells. An important role for RANK in osteoclastogenesis was reported by Nakagawa et al.1998, which referred to this TNF receptor family member as osteoclast differentiation factor receptor [35] . The essential role for RANK in bone resorption was demonstrated soon thereafter by the high bone mass phenotype of RANK knockout mice, which were virtually devoid of osteoclasts. RANK and RANKL knockout mice were virtual phenocopies of each other, which indicated that RANK and RANKL had few if any roles beyond their mutual interactions [36] .

The RANK Ligand Pathway

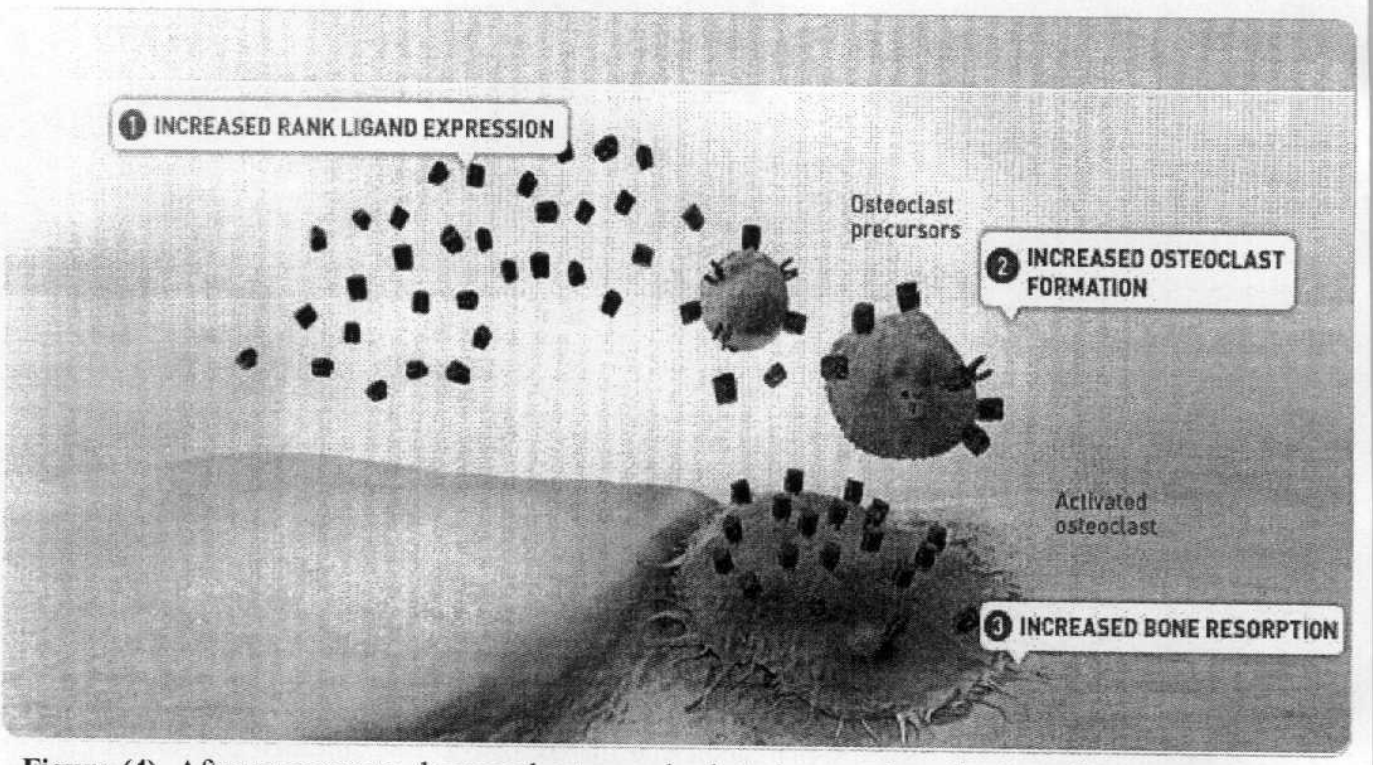


Figure (4). After menopause, decreased estrogen leads to increased production of RANK Ligand (RANKL) an essential mediator of osteoclast-induced bone loss. The resulting increased osteoclast activity leads to increased resorption and decreased bone mass. Over time, this process leads to compromised bone strength and increased risk of fracture throughout the skeleton. (Quoted from reference **37**)

The RANKL pathway mediates osteoclast activity

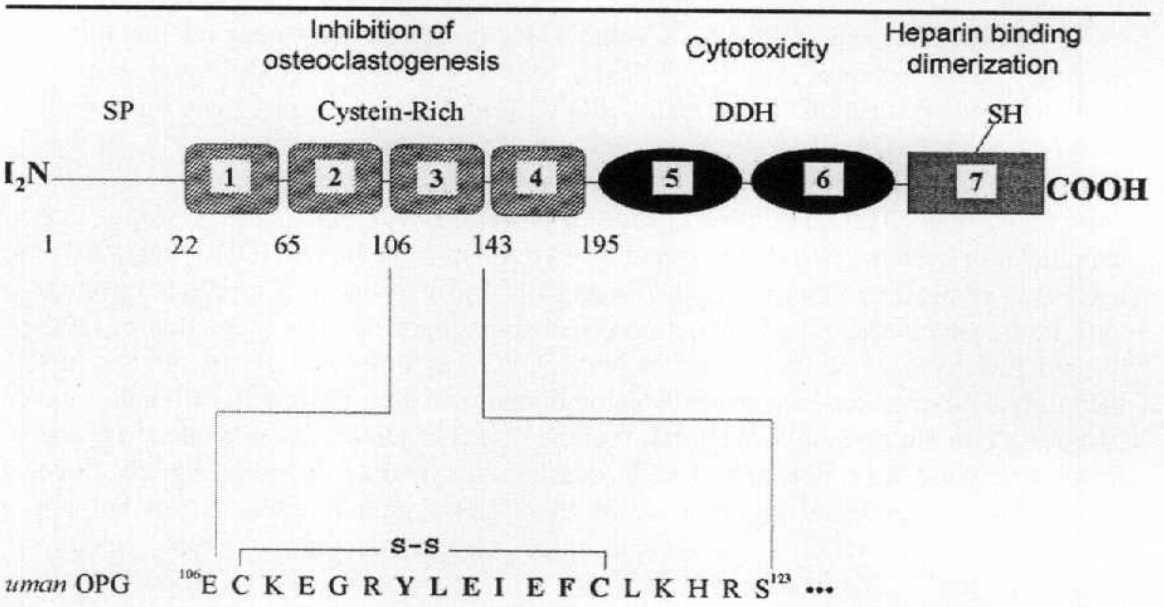
RANKL is a protein that is expressed by various cells including osteoblasts and bone lining cells

RANK is a receptor found on osteoclasts and osteoclast precursors
Binding of RANKL to RANK promotes osteoclast formation, function, and survival several factors, including cytokines and hormones, stimulate the expression of RANKL [37] .

Osteoprotegrin

Osteoprotegerin (OPG) is a soluble decoy receptor member of the tumor necrosis factor receptor family (TNFR), which regulates RANKL and RANK mediated bone resorption. The human OPG represents a single copy gene located on chromosome 8 (8q24). It spans 29 kb and consists of 5 exons, 4 introns [38] . It was evidence that OPG produced locally in bone acts as a paracrine factor to decrease bone resorption .The OPG gene encodes a protein of 401 amino acid residues that contains four cysteine rich domains and death domain-homologous (DDH) regions [39] . OPG is atypical member of the TNF receptor family in that it is a secreted protein with no transmembrane domain and no direct signaling properties. Secretion of OPG is mediated by a 21-residue signal peptide. OPG has three major structural motifs including four cysteine-rich TNF receptor domains, a heparin-binding domain, and two death domain homologous (DDH) regions [40] . The DDH regions share structural features with the death domains of TNF receptors that mediate apoptotic signals. Even though the physiological significance of the DDH region of OPG is not known, administration of OPG to rats and mice rapidly decreases serum calcium concentrations [41] . Expression of OPG mRNA in osteoclastic cells has been shown to regulate the activity of several cytokines including IL-1α, TNFs, and transforming growth factors. Furthermore OPG knockout mice exhibit severe symptoms of osteoporosis, which suggests that OPG is a physiologically important inhibitor of osteoclastic bone resorption [42] . Data from the first clinical treatment using OPG showed successful inhibition of bone resorption in postmenopausal women, suggesting that OPG could be developed as a new therapeutic agent for osteoporosis [43] .

Functional characterization of OPG demonstrated that one to four of its domains were sufficient for osteoclastogenesis inhibitory activity. Therefore, a structure-function analysis of OPG peptides could provide useful information for developing peptide-based drugs that could be delivered more conveniently than the full length protein [38] .



Figure(5). Domain structures and amino-acid sequences of OPG and the OPG peptides Structural domains of native human OPG (Quoted from reference **38**).

Osteoprotegerin (OPG) and Receptor activator of nuclear factor-kB ligand (RANKL) are cytokines essential for the regulation of bone resorption [44]. Both agents are classified into the superfamily of TNF and TNF receptor. RANKL binds on receptors on the surface of preosteoclasts and stimulates their differentiation into active osteoclasts, thus resulting in osteoresorption. It probably acts synergically with TNF-alpha (TNF acts via TNF-1 receptor and leads to a massive osteoclastogenesis after RANKL effect) [45] . OPG inhibits this osteoclastogenesis. It is a receptor for RANKL, which is produced by osteoblasts and binds on RANKL, thus inhibiting maturation of osteoclasts. This may lead even to osteopetrosis, which is caused by and associated with a small amount of osteoclasts and their functional inability [46] . Concentration of OPG and RANKL (osteoclast differentiation) is controlled by many osteotropic hormones and cytokines. Agents reducing OPG/RANKL ratio: glucocorticoid (enhanced osteoclastogenesis by OPG inhibition and osteoprotegerin ligand (OPGL) production via cells of osteoblastic line, which leads to increased osteoresorption), some inflammatory cytokines (HL-1beta , IL-4, IL-6, TNF-alpha, they stimulate osteoclastogenesis by induction of OPGL expression), basic fibroblast growth factor-2 (inhibits OPG production and stimulates RANKL production), PTH (inhibits OPG production and promotes RANKL production), prostaglandins E2, various mesenchymal transcription factors (cbfa-1, PPAR- gamma), 1.25 OH vitamin D3 [47, 48] . Agents enhancing OPG/RANKL ratio: estrogens (enhanced OPG secretion by osteoblastic cells and inhibit RANKL production this effect is supposed to play an important role in the anti- resorption effects of estrogens on the bone), Transforming growth factor beta (induced OPG secretion) [49] .

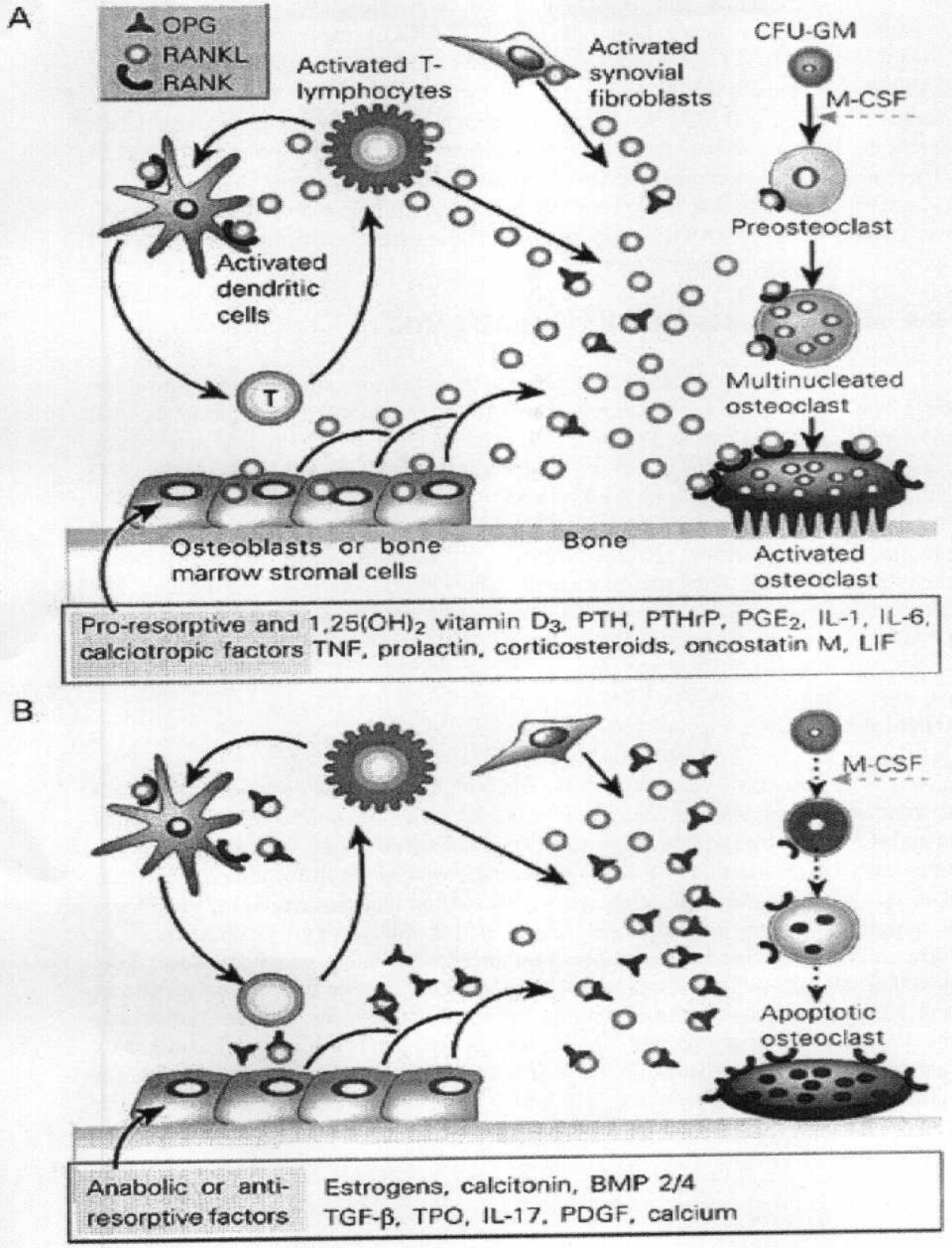


Figure (6) . Osteoclast-osteoblast coupling and osteoclast differentiation. **(Quote from reference 50).**

Figure 6. Schematic representation of the mechanism of action of A, pro-resorptive and calcitropic factors and B, anabolic and anti-osteoclastic factors. Receptor activator of nuclear factor kappa B ligand (RANKL) expression is induced in osteoblasts, activated T-cells, synovial fibroblasts and bone marrow stromal cells, and RANKL subsequently binds to its specific membrane-bound receptor activator of nuclear factor kappa B (RANK), thereby triggering a network of tumor necrosis factor (TNF) receptor-associated factor-mediated kinase cascades that promote osteoclast differentiation, activation and survival. Conversely, osteoprotegerin (OPG) expression is induced by factors that block bone catabolism and promote anabolic effects. OPG binds and neutralizes RANKL, leading to a block in osteoclastogenesis and decreased survival of pre-existing osteoclasts [50].

Mechanisms of action for OPG, RANKL, and RANK.

RANKL is produced by osteoblasts, bone marrow stromal cells, and other cells under the control of various proresorptive growth factors, hormones, and cytokines [51]. Osteoblasts and stromal cells produce OPG, which binds to and thereby inactivates RANKL. The major binding complex is likely to be a single OPG homodimer interacting with high affinity with a single RANKL homotrimer [52] . In the absence of OPG, RANKL activates its receptor RANK, found on osteoclasts and preosteoclast precursors. RANK-RANKL interactions lead to preosteoclast recruitment, fusion into multinucleated osteoclasts, osteoclast activation, and osteoclast survival. Each of these RANK-mediated responses can be fully inhibited by OPG [53] .

Osteocalcin (OC)

Osteocalcin, also known as bone Gla protein, is marker of bone formation. It is vitamin K- and vitamin D-dependent produced by osteoblasts and is the most abundant and most widely studied of non-collagenous proteins in bone [54] . Osteocalcin is 49- residue (5.8 KDa) polypeptide which is highly conserved between species. In humans the osteocalcin gene is located on chromosome 1(1q25-q31) and is regulated at the transcriptional level by 1.25 dihydroxy- vitamin D_3 [55] . Osteocalcin is synthesized as an 11 kDa preproosteocalcin of 98 residues. This molecule consists of three parts, a 23- residue signal peptide that is cleaved during translation, a 26- residue propepitde that targets the protein for Y carboxylation and the 49-residue mature protein [56, 57] . The mature osteocalcin peptide consists of two anti-parallels α – helical domains (residue 16-25 and 30-41) connected by a β turn (residues 26-29). There are two further β turns and β sheet structure at the C- terminal end. The structure is stabilized by a $Cys_{23} – Cys_{29}$ disulphide bond [58, 59] .

Serum osteocalcin reflects the 10-40% of osteocalcin produced that is not incorporated into the bone matrix [60]. Osteocalcin also known as carboxyglutamic acid protein (bone caboxyglutamic acid containing protein, BGP). In the past that is closely related to BGP and osteoporosis, and its main function is to maintain normal bone mineralization, and inhibit osteoblast activity, leading to osteoporosis. However, studies suggest that bone is endocrine organs, the secretion of BGP to negative feedback effect on islet cells, stimulate insulin secretion [61]. So BGP level of the body may be associated with the onset of diabetes, when its lack can result in decreased cell insulin secretion and sensitivity decreased, decreased glucose tolerance, leptin reduced expression and increased incidence of diabetes development. Early studies showed that BGP and osteoporosis-related physiological functions, BGP can inhibit the bone mineralization rate reduction, or by inhibiting cell activity results, leading to osteoporosis [59]. In terms of bone mineralization, BGP and bone mineralization occurs at the same time, bone growth, hydroxyapatite deposition can lead to increased BGP, to maintain normal bone mineralization, inhibition of abnormal formation of light apatite crystals by the chemical structure of BGP interaction with hydroxyapatite and inhibit the rate of growth cartilage mineralization [61]. Osteocalcin (OC) level is inversely related to plasma glucose level and fat mass in elderly non-DM persons [62]. Serum OC level is positively associated with insulin sensitivity in non-DM subjects [63]. Serum OC concentration is inversely associated with fasting plasma glucose (FPG), fasting insulin, homeostasis model assessment for insulin resistance, high-sensitivity C-reactive protein, interleukin-6, body mass index (BMI), and body fat in cross-sectional analyses. They also have found that OC levels are associated with changes infasting plasma glucose (FPG) in prospective analyses [64]. Uncarboxylated osteocalcin (UcOC) is negatively associated with plasma glucose level and fat mass, and positively correlate with adiponectin in diabetes mellitus type 2 (T2DM) patients. These experimental and clinical findings suggest that bone metabolism and glucose/fat metabolism are etiologically related to each other through the action of ucOC or OC [65] (Quoted from reference Figure 7).

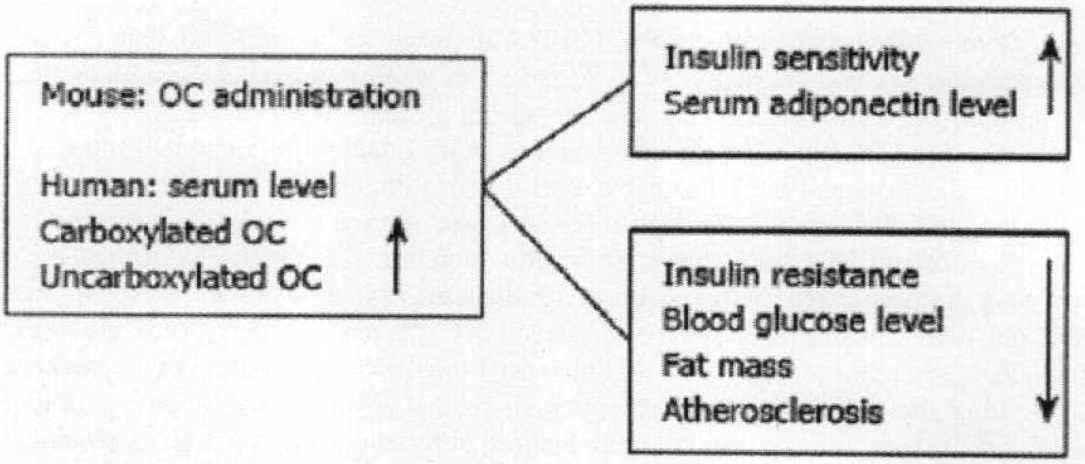


Figure (7) **When osteocalcin was administered to obese mice, it increased insulin secretion, and decreased blood glucose level, fat mass, and triglyceride level. In humans, serum carboxylated and uncarboxylated** osteocalcin levels were positively correlated with insulin sensitivity and adiponectin level, whereas they were negatively correlated with blood glucose level, fat mass, and atherosclerosis index. Thus, osteoporosis and diabetes are pathophysiologically related to each other through osteocalcin (OC) action in mice and humans.

Osteocalcin fragment may be derived from bone resorption and catabolism of the molecule in vivo before clearance by metalloproteases in the kidneys and the liver [66, 67]. Quantitative bone histomorphometry and combined calcium balance/calcium kinetics studies have validated the use of osteocalcin as a marker of bone formation. In postmenopausal women serum osteocalcin levels correlate significantly with both the bone formation rate and the kinetically determined calcium acceralation rate, but not with bone resorption [68, 69].

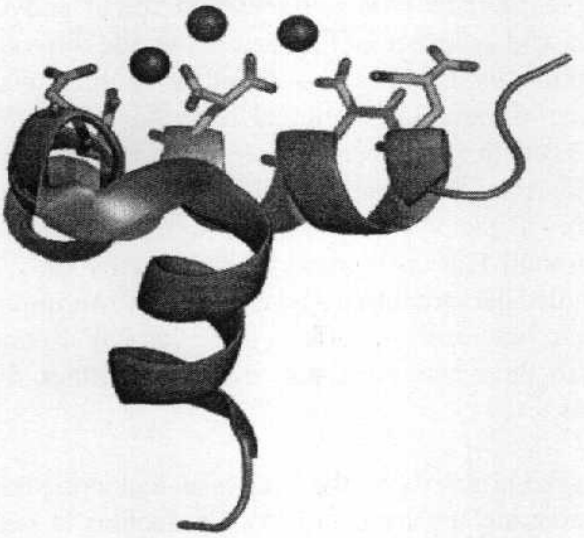


Figure (8). Diagram of the secondary structure of osteocalcin (Quote from reference **70**).

Insulin-like growth factor-I

The IGFs are growth-promoting polypeptides that play essential roles in growth and development. It maps to human chromosome 12q23 in close vicinity of the gene encoding phenylalanine hydroxylase. In vivo, the IGFs are bound to a family of six structurally and evolutionarily related IGF binding proteins (IGFBP1 through IGFPB6) [70] .In serum, the majority (70–80%) of the IGFs exist in a 150-kDa complex comprised of one IGF molecule, IGFBP-3, and the acid labile subunit (ALS). A smaller proportion (20%) of the IGFs are associated with other serum IGFBPs within a 50-kDa complex, and less than 5% of the IGFs are found in the free form of 7.5 kDa. ALS is a protein that binds to the IGF/IGFBP-3 binary complex, primarily in serum. Association with ALS prolongs the $t_{1/2}$ of serum IGFs and facilitates their endocrine actions [71].

IGF-1 controls growth and metabolism in several organs and tissues during both embryonic and post-natal development [72] . At the structural level, IGF-1 is related to insulin with it shares a 50% amino acid identity. Unlike the insulin gene, the IGF-1 gene locus encodes multiple proteins with variable N-terminal and C-terminal amino acid sequences. The amino acid sequence of the mature peptide differs from that of insulin by retention of the C peptide, by a short extension of the A chain to include a novel domain D, and by the presence of variable C-terminal E peptides, Fig. (**9**). Although the IGF-1 gene is highly conserved in numerous species, its relatively large size (over 70 kb), combined with complex transcriptional and splicing patterns, has complicated its analysis [73] . Insulin-like growth factor 1 (IGF-1) also known as somatomedin C or mechano growth factor is a protein that in humans is encoded by the IGF1 gene. IGF-1 is a hormone similar in molecular structure to insulin. It plays an important role in childhood growth and continues to have anabolic effects in adults. IGF-1 consists of 70 amino acids in a single chain with three intramolecular disulfide bridges. IGF-1 has a molecular weight of 7649 daltons [74].

IGF-1 is produced primarily by the liver as an endocrine hormone as well as in target tissues in a paracrine/autocrine fashion. Production is stimulated by growth hormone (GH) and can be retarded by under nutrition, growth hormone insensitivity, and lack of growth hormone receptors. Approximately 98% of IGF-1 is always bound to one of 6 binding proteins (IGF-BP). IGFBP-3, the most abundant protein, accounts for 80% of all IGF binding. IGF-1 binds to IGFBP-3 in a 1:1 molar ratio [75] .

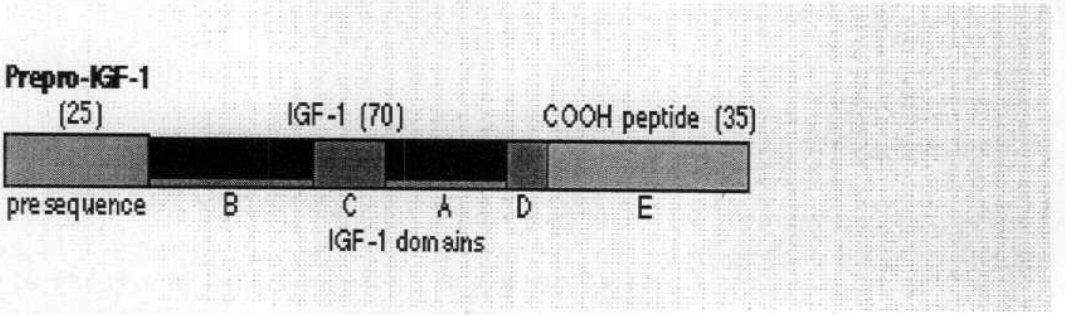


Figure (9). Schematic of structure of isolated mammalian insulin-like growth factor I (IGF-I) cDNAs to illustrate mRNA and precursor heterogeneity. (Quote from reference **71**).

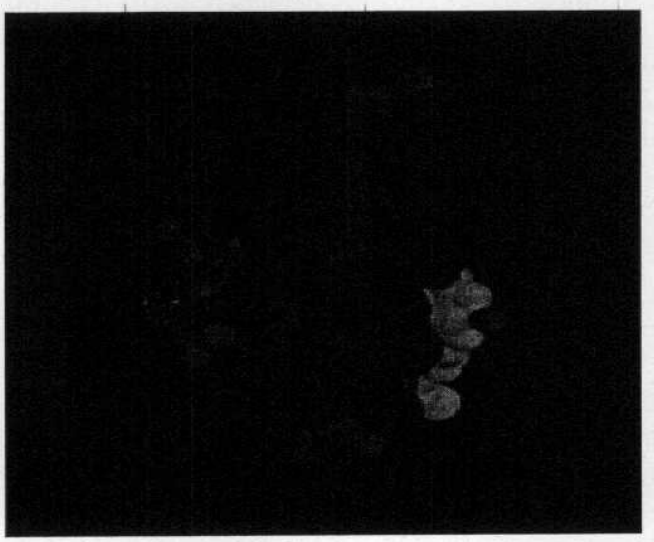


Figure (10). Insulin-like growth factor 1 (somatomedin C) (Quoted from reference **71**).

Mechanism of action

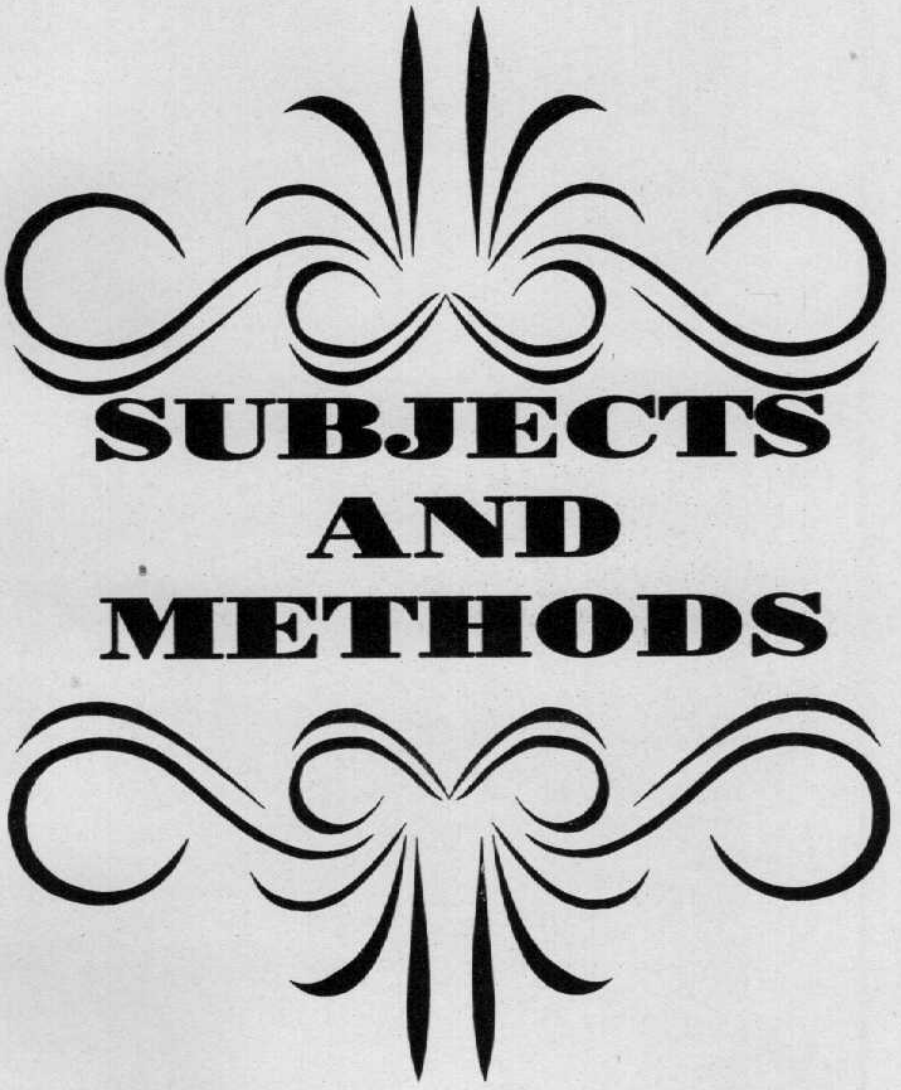
IGF-1 binds to at least two cell surface receptors: the IGF-1 receptor (IGF1R), and the insulin receptor. The IGF-1 receptor seems to be the "physiologic" receptor - it binds IGF-1 at significantly higher affinity than IGF-1 is bound to the insulin receptor [(76)] . Like the insulin receptor, the IGF-1 receptor is a receptor tyrosine kinase - meaning it signals by causing the addition of a phosphate molecule on particular tyrosine. IGF-1 is one of the most potent natural activators of the serine/threonine protein kinase. AKT signaling pathway, a stimulator of cell growth and proliferation, and a potent inhibitor of programmed cell death. IGF-1 is a primary mediator of the effects of growth hormone (GH) [(77)] . Growth hormone is made in the anterior pituitary gland, is released into the blood stream, and then stimulates the liver to produce IGF-1[(78)] . IGF-1 then stimulates systemic body growth, and has growth-promoting effects on almost every cell in the body, especially skeletal muscle, cartilage, bone, liver, kidney, nerves, skin, hematopoietic cell, and lungs [(79)] . In addition to the insulin-like effects, IGF-1 can also regulate cell growth and development, especially in nerve cells, as well as cellular DNA synthesis. IGF-1 is produced throughout life. The highest rates of IGF-1 production occur during the pubertal growth spurt. The lowest levels occur in infancy and old age [(80)] .

The association between diabetes and osteoporosis has been extensively investigated because both diseases are very common and of great socioeconomic relevance. It is now clear that patients with type 1 diabetes, probably as a result of insulin deficiency, have lower bone mineral density (BMD) and higher risk for fractures as compared with subjects without type 1 diabetes [(81)] . Patients with type 2 diabetes often have higher BMI and thus might be expected to be at lower risk for the development of osteoporosis and fracture. Supporting this, several studies have found increased BMD [(82)] in women with diabetes when compared with controls, although other studies have reported no difference. Despite higher BMD, patients with type 2 diabetes appear to have higher rates of foot and ankle, hip and arm fractures .This paradoxical increase in fracture rate may be a result of increased rate of falls among patients with diabetes or lower bone quality [(83)] . Patients with diabetes have many and different forms of skeletal disorders, including osteopenia, osteoporosis and diffuse idiopathic skeletal hyperostosis (DISH). It was demonstrated that diabetes mellitus could be associated with a loss of bone mass leading to osteoporosis [(84)]. This finding in the past 15 years has received a great deal of attention and been investigated [(85)], as osteoporosis is a major health problem, and its occurrence among patients who have diabetes further increases their burden of disease [(86)]. However, in spite of numerous studies, the relationship between diabetes and osteoporosis remains controversial [(87)] . Many other conditions such as Cushing's syndrome, pancreatic insufficiency, polyglandular autoimmune syndrome type 2, post-transplantation state or hereditary hemochromatosis can lead to secondary diabetes. These conditions are also associated with an increased risk of osteoporosis [(88)]. The diagnosis of osteoporosis can be made using conventional radiography and by measuring the bone mineral density (BMD). The most popular method of measuring BMD is dual energy x-ray absorptiometry (DXA or DEXA). In addition to the detection of abnormal BMD, the diagnosis of osteoporosis requires investigations into potentially modifiable underlying causes; this may be done with blood tests [(89)] .

AIM OF THE WORK

AIM OF THE WORK

The present study was aimed to evaluate the relation between insulin-like growth factor-1 and markers of osteoporosis (osteoprotegerin, RANKL, osteocalcin and bone mineral density) in postmenopausal women with and without Type 2 diabetes.



SUBJECTS AND METHODS

Study groups:

After the acceptance of the ethical community of the Medical Research Institute, the study was conducted on 90 female divided into three groups (30 each)

- Group I: Control healthy women with age post menopause and not diagnosed as suffering from osteoporosis or diabetes.
- Group II: Type 2 diabetic postmenopausal women without osteoporosis
- Group III: Type 2 diabetic postmenopausal women with osteoporosis

Exclusion Criteria:

- Steroid users or having disease conditions that could play a significant role in the development of osteoporosis such as hyperparathyroidism, or hyperthyroidism
- Diseases that may affect bone metabolism: chronic gastrointestinal diseases, renal or hepatic impairments.
- Chronic treatment with antiacids, estrogen, adrenal or anabolic steroids, anticonvulsants, anticoagulants, or pharmacologic doses of Vitamin A supplements 6 months prior to the study.
- History of unstable cardiovascular diseases or uncontrolled hypertension

Clinical examination:

An informed concent was taken from all patients included in this study (appendix).
All healthy subjects have no records of osteoporosis and D.M .

Blood sampling and processing

- Blood samples were collected after overnight fasting.
- Five ml venous blood was drawn in tubes for obtaining serum.
- Serum samples were rapidly separated by centrifugation at 3500rpm for 10 min and samples were stored at -20 C.

Determination of soluble RANKL (sRANKL)

Human sRANKL ELISA kit Cat # VBI20452, Germany was used for determination of soluble RANKL (sRANKL).

I. Principle [90] :

sRANKL binds to the pre-coated recombinant Osteoprotegerin (OPG) and forms a sandwich with the detection antibody. sRANKL is quantitatively determined by an enzyme catalyzed color change detectable on a standard ELISA reader. The amount of color developed is directly proportional to the amount of sRANKL present in the sample.

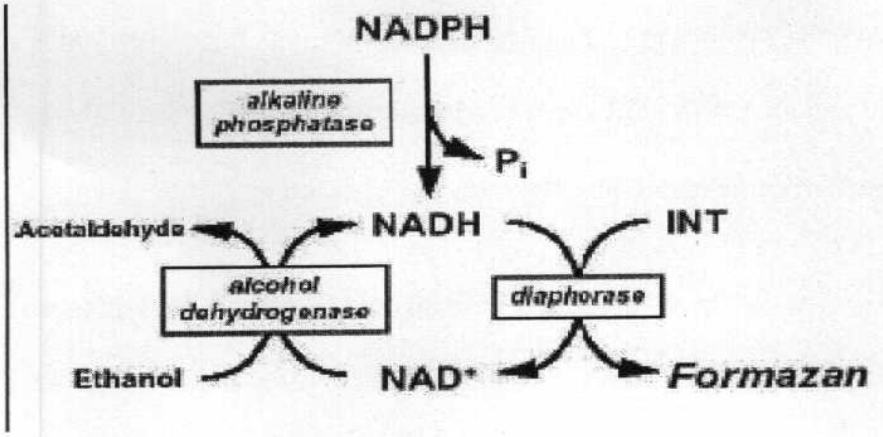


Figure 11 A. Principle of ELISA determination of sRANKL

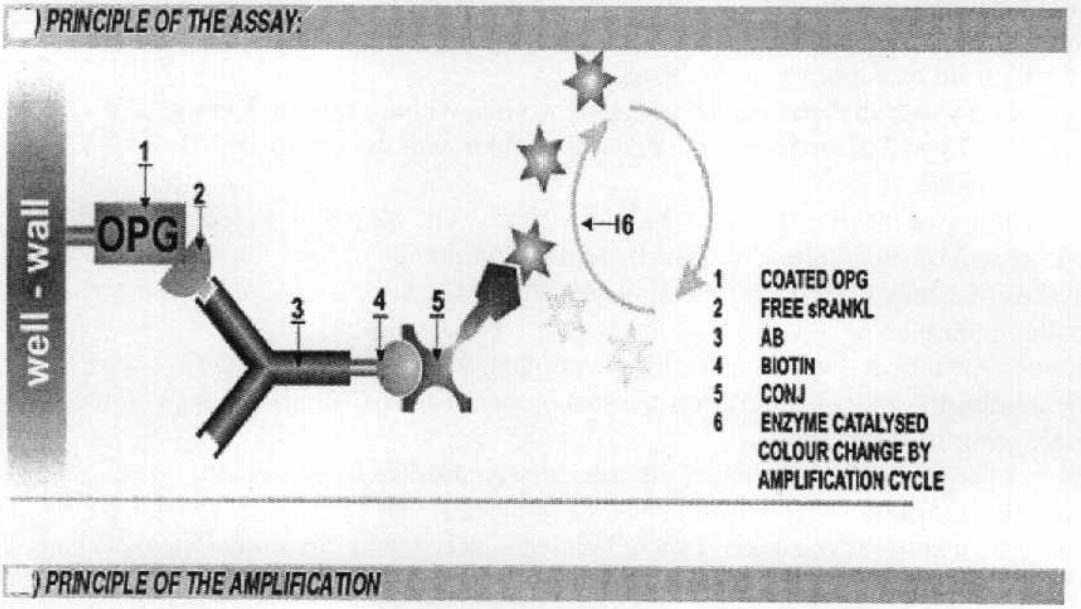


Figure 11 B. Principle of ELISA determination of sRANKL

II. Reagents

The kit contains the following reagents:

1. Antibody: Polyclonal biotinylated anti sRANKL antibody.
2. Standard human sRANKL : (0;0.125; 0.25; 0.5; 1; 2 pmol/l)
3. Conjugate: Streptavidin – alkaline phosphatase.
4. Amplifier A: Inorganic salts and buffered enzyme solution with tertrazoluim violet.
5. Amplifier B: Stabilized NADPH solution.
6. Stop solution: 2M sulfuric acid.

III. Assay Procedure

1- All reagents and samples were allowed to reach to room temperature before use in the assay.
2- 100µl of standard/sample/ control were pipetted in duplicate into respective wells, except blank.
3- 100µl of biotinylated anti sRANKL antibody was added into each well, except blank, and swirled gently.
4- The plate was incubated over night at room temperature with shaking .
5-After aspiration. The plate washed 3 times with diluted wash buffer and blotted after the last wash.
6- 200µl of Conjugate was added to all wells except blank and incubated for 1 hour at room temperature.
7- After aspiration. The plate washed 3 times with diluted wash buffer and blotted after the last wash.

8- 100µl of Amplifier A was added
9- 100µl of Amplifier B was added
10- Then the plate was incubated for 45 minutes at room temperature.
11- 50µl of Stop Solution was added to each well.
12 – The absorbance was immediately measured at 490 nm.

IV. Calculation:

The standard curve was constructed from O.D values of standard, the samples concentration was obtained from this standard curve. The value was presented as pg/ml. **(Figure.12)**

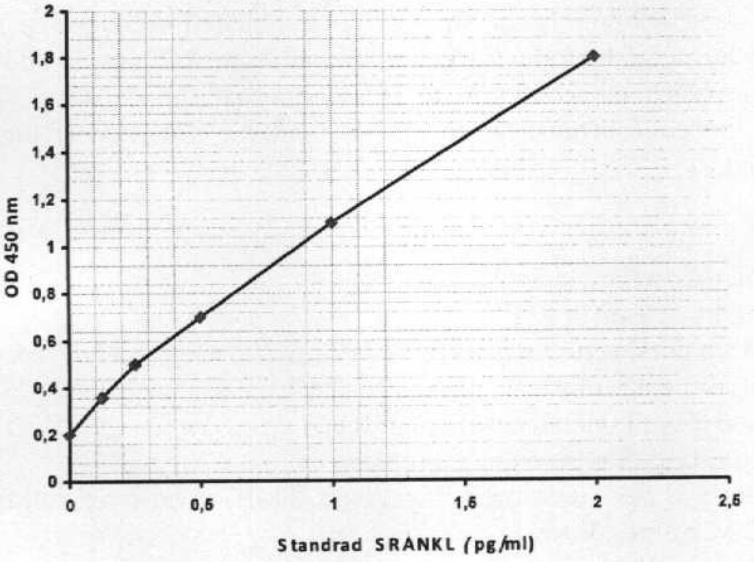


Figure (12): sRANKL strandrad curve

Determination of insulin growth factor -1 (IGF-1)

The RayBio® Human IGF-I ELISA kit (cat#: ELH-IGFI-001), USA was used for the determination of IGF-1.

I. Principle [91] :

This assay employs an antibody specific for human IGF-I coated on a 96-well plate. Standards and samples are pipetted into the wells and IGF-I present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human IGF-I antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a 3,3,5,5- tetramethylbenzidine substrate solution is added to the wells and color develops in proportion to the amount of IGF-I bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

II. Reagents

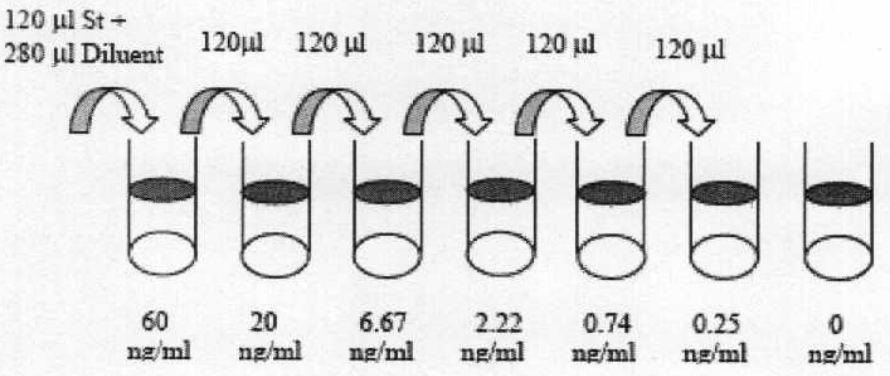
The kit contains the following reagents:

1. Standards recombinant human IGF-I.
2. Assay Diluent A: animal serum with 0.09% sodium azide as preservative.
3. Assay Diluent C: diluent buffer.
4. Detection Antibody: biotinylated anti-human IGF-I
5. Horseradish peroxidase -Streptavidin conjugate.
8. Substrate Reagent 3,3',5,5'- tetramethylbenzidine (TMB) in buffered solution.
9. Stop Solution: 2 M sulfuric acid.

III. Reagent preparation:

1. All reagents and samples were allowed to reach room temperature before use.

2. Preparation of standard: Prepare from the stock standard solution (200ng/ml). a serial dilutions were made using assay diluent as follow.



3- Sample preparation (to release IGF-1 from binding protein)

- **Acid-ethanol extraction solution:** 2.5 ml hydrochloric acid (37% HCl): 10 ml deionized water: 88.5 ml ethanol.
- **Tris buffer (pH=7.6).**

Sample pretreatment

1. 30 µl serum was added to 120 µl acid-thanol extraction solution in a polypropylene tube.
2. The tubes were incubated for 30 min at RT with shaking.
3. The tubes were Centrifuged for 5 min at 10,000 rpm and 100 µl of supernatant was transferred into 200 µl Tris buffer (pH=7.6) in a polypropylene tube. The tubes were mixed thoroughly.
4. 300 µl Assay Diluent A was added mixed well. Assay immediately.

IV. Assay procedure of IGF-1 :

1. All reagents and samples were at room temperature before use.
2. 100µl of standard and sample were added into appropriate wells. The plate was covered and incubated for over night at 4°C.
3. After aspiration, the plate was washed 4 times with washing solution. After the last wash, any remaining wash buffer was removed by decanting. The plate was inverted and blotted against clean paper towels.
4. 100µl of biotinylated antibody was added to each well. The plate was incubated for 1 hour at room temperature with gentle shaking.
5. The solution was discarded and the wash step was repeated as in step 3.
6. 100µl of Streptavidin solution was added to each well and the plate was incubated for 45 minutes at room temperature with gentle shaking.
7. The solution was discarded and the wash step was repeated as in step 3.
8. 100µl of TMB One-Step Substrate Reagent was added to each well and the plate was incubated for 30 minutes at room temperature in the dark with gentle shaking.
9. 50 µl of Stop Solution was added to each well and the absorbance was immediately measured at 450 nm.

V. Calculation: The standard curve was constructed from O.D values of standard, samples concentration was obtained from this standard curve. The value was expressed as ng/ml (**Figure.13**)

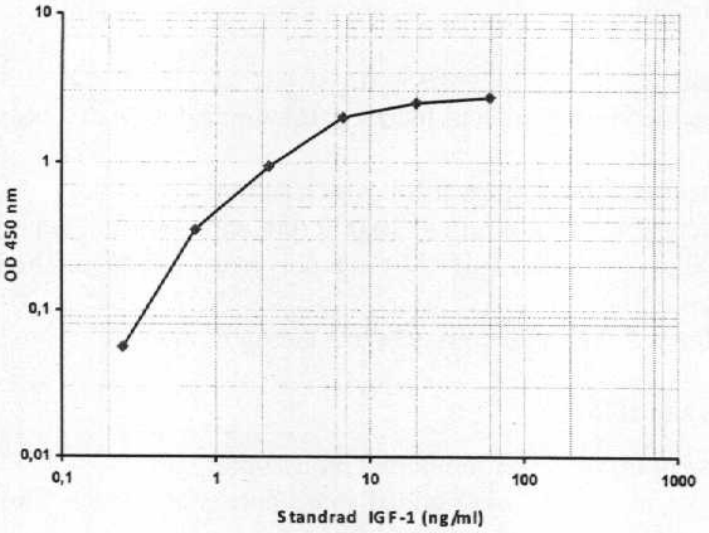


Figure (13): IGF-1 strandrad curve

Determination of Osteoprotegerin

The RayBio® Human Osteoprotegerin ELISA kit (cat#: ELH-OPG-001) USA, was used for determination of Osteoprotegerin.

I. Principle [92] :

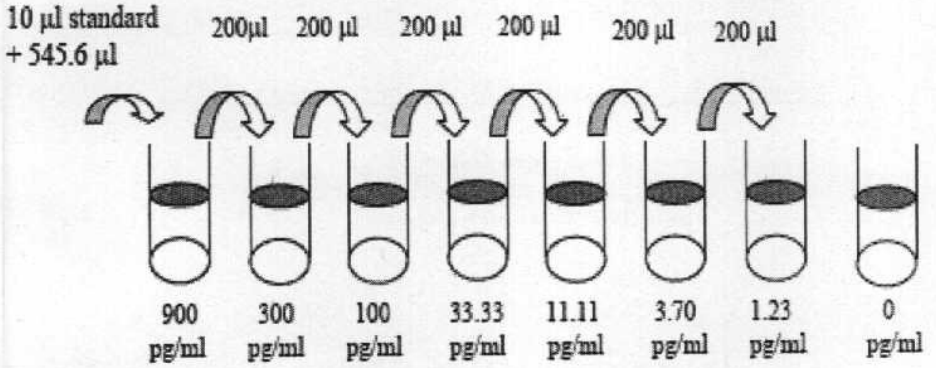
This assay employs an antibody specific for human Osteoprotegerin coated on a 96-well plate. Standards and samples are pipetted into the wells and Osteoprotegerin present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human Osteoprotegerin antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of Osteoprotegerin bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

II. Reagents

Each kit contains the following reagents:
1. Standards recombinant human Osteoprotegerin.
2. Assay Diluent A: 0.09% sodium azide as preservative.
3. Detection Antibody: biotinylated anti-human Osteoprotegerin
4. Conjugate: HRP-conjugated streptavidin.
5. Substrate Reagent 3,3',5,5'- tetramethylbenzidine (TMB) in buffered solution.
6. Stop Solution: 2 M sulfuric acid .

III. Reagent preparation:

1. All reagents and samples were at room temperature before use.
2. Preparation of standard: a stock solution (50ng/ml) was preprared by dissolving the content of the vial in 200 µl Assay Diluent A. 10 µl Osteoprotegerin standard was added into a tube with 545.6 µl Assay Diluent A to prepare a 900 pg/ml stock standard solution. 400 µl Assay Diluent A was pipetted into each tube. The stock standard solution (900pg/ml) was used to produce a dilution series (shown below). Each tube was mixed thoroughly before the next transfer. Assay Diluent A serves as the zero standard (0 pg/ml).



IV. Assay procedure:

1. All reagents and samples were brought to room temperature before use.
2. 100µl of standard and sample were added into appropriate wells. The plate was covered well and incubated for 2.5 hours at room temperature with gentle shaking.
3. The solution was discarded and the plate was washed three times with washing solution. After the last wash, any remaining wash buffer was removed by aspiration or decantation. The plate was inverted and blots it against clean paper towels.
4. 100µl of biotinylated antibody was added and incubated for 1 hour at room temperature with gentle shaking.
5. The solution was discarded and the wash was repeated as in step 3.
6. 100µl of Streptavidin solution was added and incubated for 45 minutes at room temperature with gentle shaking.
7. The solution was discarded and the wash was repeated as in step 3.

8. 100µl of TMB substrate reagent was added and incubated for 30 minutes at room temperature in the dark with gentle shaking.
9. 50µl of stop solution was added then and the absorbance was immediately measured at 450 nm.

V. Calculation:
The standard curve was constructed from O.D values of standard, the samples concentration was obtained from this standard curve. The value was expressed as pg/ml **(Figure.14)**

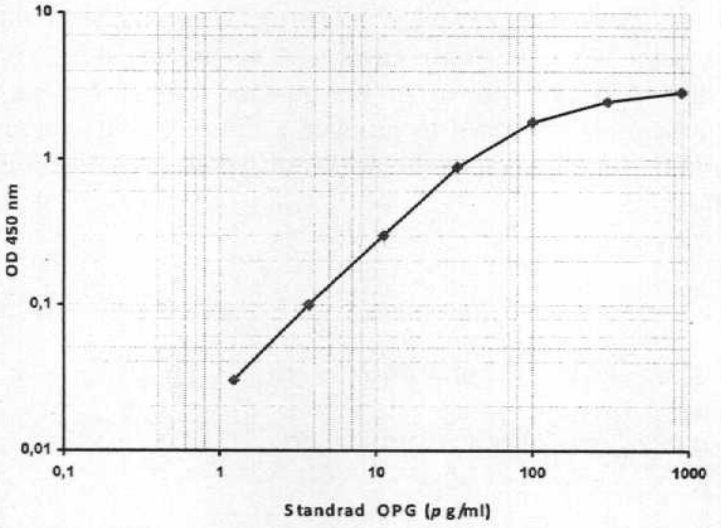


Figure (14): OPG strandrad curve

Determination of Osteocalcin

DSL-10-7600, Germany was used to determination of Osteocalcin

I. Principle [93] **:**
The Osteocalcin assay is an enzymatic amplified "one-step" sandwich –type immunoassay. In the assay standards, controls and unknown diluted serum samples were incubated with anti-osteocalcin polyclonal detection antibody labeled with the enzyme horseradish peroxidase in microtitration wells coated with an affinity purified anti-osteocalcin mouse monoclonal antibody. After incubation and washing, the wells were incubated with the substrate tetramethlybenzidine (TMB). An acidic stopping solution was then added and the degree of enzymatic turnover of the substrate was determined by dual wavelength absorbance measurement at 450 nm. The absorbance was directly proportional to the serum concentration of osteocalcin

II. Reagents

The kit contains the following reagents:

1. Sample diluent: 0 ng/ ml osteocalcin in protein based buffer.
2. Osteocalcin standard: 5, 25, 65, 125 and 250 ng/ml osteocalcin in protein based buffer.
3. Osteocalcin controls: Containing low and high concentration of osteocalcin in protein based buffer.
4. Osteocalcin assay buffer: Containing affinity purified anti-osteocalcin polyclonal antibody conjugated to the enzyme horseradish peroxidase
5. TMB Chromogen Solution.
6. Stopping solution.

III. Assay of procedure:

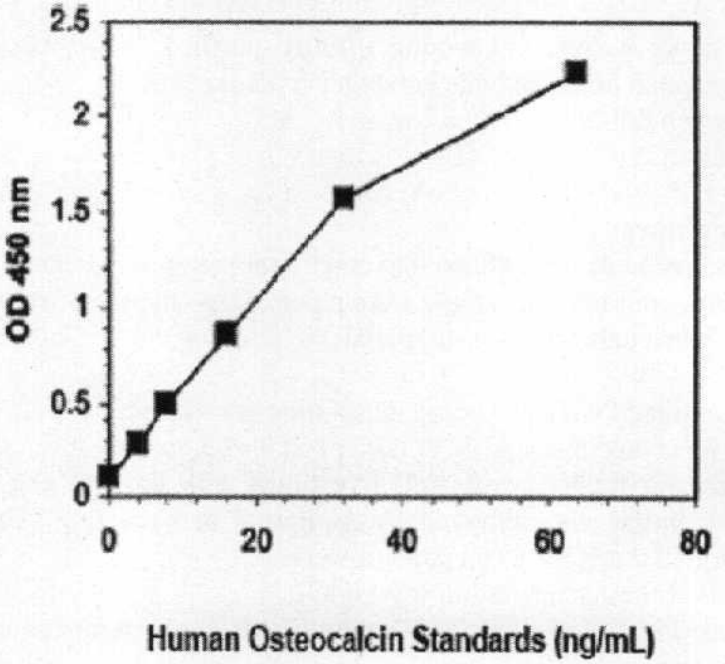
All specimens and reagents were allowed to reach room temperature before use.

1. 100 µL Standard, controls and sample were pipetted into the specified well.
2. The Anti-enzyme conjugate was prepared by diluting the conjugate with assay buffer.
3. 100 µL of the antibody enzyme conjugate solution was added.
4. The plate was incubated and shaked at fast speed for 2 hours at room temperature.
5. Then aspirated and washed each well five times with the washing buffer. Any remaining wash buffer was removed by aspirating or decanting. The plate was inverted and blotted it against clean paper towels.
6. 100 µL of TMB chromogen solution was added.
7. The well was incubated, shaked 10 minutes at room temperature (Avoided exposure to direct sunlight).
9. 50 µL of stop solution was added.
10. The solution in the wells was readed within 30 minutes, using microplate reader set to 450 nm.

V. Calculation:

A set of osteocalicn standards were used to plot a standards curve of absorbance versus osteocalcin concentration from which osteocalcin of the samples were calculated

Figure (15) Standard curve of Osteoclacin



Determination of Calcium

I. Principle [94] :

Calcium ions react with O-cresolphthalaein complexone (O-COC) under alkaline conditions to form a violet colored complex.

Ca^{2+} + O-CPC $\xrightarrow{\text{Alkaline PH}}$ calcium – O- CPC complex

The color intensity of the complex formed is directly proportional to the calcium concentration. It is determined by measuring the increase in absorbance at 578 nm.

II. Reagent:

1- Standard Calcium (ST) 10 mg/dl
2- Reagent 1 (R1 Buffer)
(2- Amino-2-methyl-1- propanol (pH 10.5)
3- Reagent 1(R2 Chromogen)
O-cresolphthalaein complexone
8- hydroxyquinoline

III. Procedure:

- In tube the following reagents were added in 500 µl buffer and 500 µl of chromogen, and then 10 µl of sample or standard were added and mixed. The tube was incubated for 5 min. at R.T
- The absorbance of samples and standard were measured at 578 nm against reagent blank.

IV. Calculation:

$$\frac{A\ sample}{A standard} \times \text{standard conc (10mg/dl)} = \text{Calcium (mg/dl)}.$$

Determination of Phosphorus [95]

I. Principle:
Inorganic Phosphorus + H_2SO_4 + Ammonium Molybdate ---> Unreduced Phosphomolybdate Complex

II. Reagent
Phosphorus reagent contains:
Ammonium molybdate
Sulfuric acid

III. Procedure

1. 50 µl of distilled water, calibrator, or serum was pipetted into separation test tubes to be assayed.
2. 2.5 ml of phosphorus reagent was added and mixed.
3. Following incubation for 5 minutes at room temperature, the absorbance was determine of the calibrator (As) and of each serum (A) were measured at 340 nm against reagent blank.

IV. Calculation of results

The phosphate concentration of sample is calculated as

$$\frac{A_{sample}}{A_{standard}} \text{ standard conc. } \mathbf{(0.28)\ (mg/dl)}$$

Determination of ionized calcium [96]

I. Principle

Instruments have been available for the measurement of free calcium, by ion – selective electrode for more than two decades. Although the reliability and precision of first generation of calcium analyzer 9 electrodes) were unsatisfactory for routine clinical laboratories, improved and reliable second- generation instruments, and electrodes have been available for more than a decade. They provide accurate and precise determinations of free calcium.

Ionized ca (ca^{2+}) in serum samples were determined using ion selective electrodes. Ion selective electrodes by potentiometry is an analytical technique in which the amount of substance in solution is determined, either directly or indirectly, from measurement of electromotive force (emf) between two probes (electrodes) that are dipped into the solution. The emf is measured in units of volt or millivolts. The electrode at which reduction takes place is **cathode** and that at which oxidation occurs is the **anode**. Potentiometric methods of analysis involve the direct measurement electrical potential due to the activity of free ions. Ion selective electrodes are designed to be sensitive toward individual ion.

II. Reagents

1. Calcium standard solution.
2. Calcium carbonate (standard solution).
3. Reference electrode (filling solution) KCl saturated with AgCl.
4. pH adjustment solution:
   1 M sodium hydroxide.
   1 M hydrochloride acid

III. Procedure

1. Electrode preparation

- Soak the cation electrode overnight in standard calcium chloride solution (e.g. 0.1 M or 100 ppm a CaCO3) adjust pH to ~ 8.5- 9 with NaOH.
- Fill the reference electrode by specific filling solution.

2. Direct calibration

- Prepare two standards, which matched the expected sample range and differ in concentration.
- Standards can be prepared in any concentration units to suit the particular analysis requirement.
- All standard should be at same temperature as the samples.
- Measure 100 ml of the more diluted standard (0.001 M) into 150 ml beaker. Stir thoroughly.
- Rinse electrodes with distilled water, blot, dry, and place into the beaker. Adjust the meter to display the value of standard.

- ❖ Measure 100 ml of more concentration standard (0.01 M) into 150 ml beaker. Stir thoroughly.
- ❖ Rinse electrodes with distilled water, dry, and place into beaker. Adjust the meter to display the value of the standard.

3- Analytical procedure

A- Ionized calcium

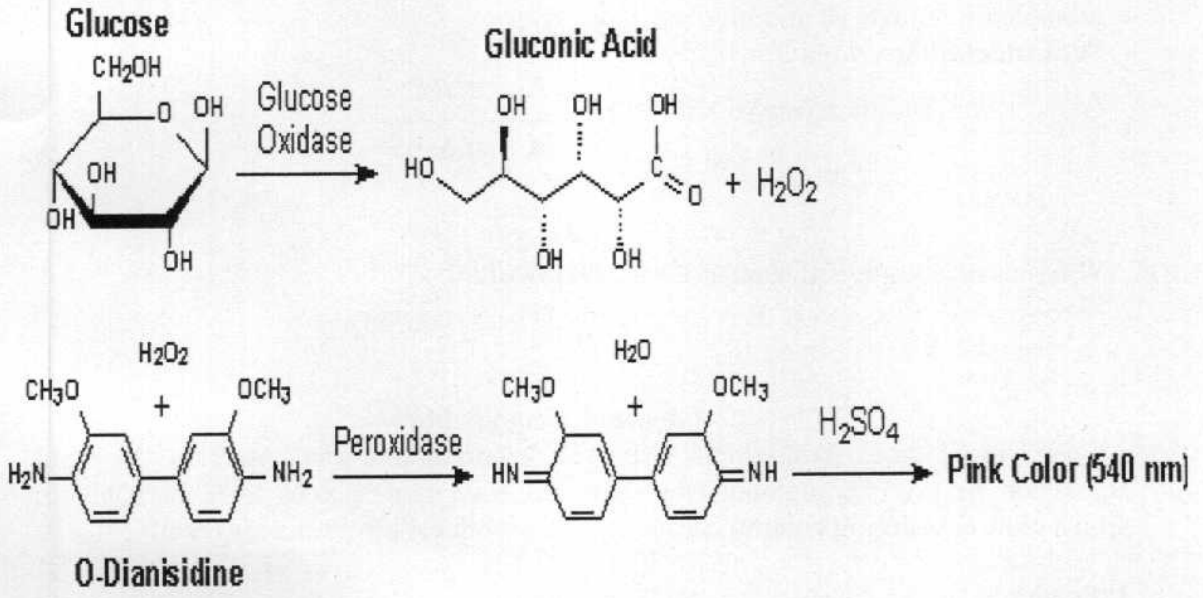
- ❖ Measure 0.3 ml of the sample into a microsample dish. Stir thoroughly
- ❖ Rinse electrodes with distilled water, blot, dry, and place into dish. Record the mV value.

Determination of Serum Glucose [97]

Serum glucose levels were determined according to enzymatic colorimetric method which has been described by Trinder.

I. Principle

Glucose is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase. Hydrogen peroxide reacts with O -dianisidine in the presence of peroxidase to form a colored product. Oxidized O -dianisidine reacts with sulfuric acid to form a more stable colored product. The intensity of the pink color measured at 540 nm is proportional to the original glucose concentration.



II. Working Reagents

Reagent (R1)
Phosphate buffer, pH: 7.5
Phenol

Reagent (R2)
Glucose oxidase.
Peroxidase .
4-Aminoantipyrine.

Reagent (R3) Standard
Glucose.

III. Procedure:

- In tube the following reagents were added 1ml of working reagent and then 10 µL of standard and 10 µL of sample and mixed
- Mix and measure the absorbance (A) after five-minute incubation at 37°C
- Read the absorbance (A) of the samples and standard, against the Blank. The color is stable for at least 30 minutes.

IV. Calculation

$$\text{Glucose concentration (mg/dL)} = 100 \times \frac{\text{A sample}}{\text{A Standard}}$$

Where, concentration of standard was 100 mg/dL.

Glycated Hemoglobin [98]

The Glycated hemoglobin (HbA1c) determination was based on the rapid separation of glycohemoglobin (fast fraction) from non- glycosylated hemoglobin which elute first during column chromatography with cation- exchange resin.

I. Principle

HbA1c reflects the average blood glucose level during the preceding 2 to 3 months. HbA1c is thus suitable to monitor long term blood glucose control individuals with diabetes mellitus. A hemoglobin preparation of the whole blood is mixed continuously for 5 minutes with a weak binding cation – exchange resin. During this time, HbA0 binds to the resin. After the mixing period, a filter is used to separate the supernatant containing the glycohemoglobin from the resin. The percent glycohemoglobin is determined by measuring the absorbance at 415 nm of the glycohemoglobin fraction and the total hemoglobin fraction. The ratio of the two absorbance gives the percent glycohemoglobin.

II. Reagents

1.Resin reagent: 8 mg/mL cation – exchange resin buffered at pH 6.9.
2.Lysine reagent: 10 mM Potassium Cyanide, surfactant added.
3.Glycohemoglobin standard: 10% glycohemoglobin.
4.Separators.

III. Procedure

A. Hemolyzed preparation:

100 µl of EDTA – blood samples, standard and control was mixed with 500 µl of lysing reagent and stands for 5 minutes.

B. Glycohemoglobin preparation:

1. 100 µl of the hemolysate was added to 3.0 mL of glycohemoglobin cation-exchange resin in 13 X 100 mm glass tubes labeled sample, standard and control.
2. A filter separator is positioned in the tubes so that rubber sleeve is approximately 1 cm above the liquid level.
3. The tubes were mixed continuously for 5 minutes.
4. The filter separator pushed into the tubes until the resin was firmly packed.
5. The absorbance of the supernatant was measured at 415 nm against deionized water as a blank for the standard, samples, and control (Glycohemoglobin reading).

C. Total hemoglobin fraction:

1. 20 µl of the hemolysate is added to 5.0 mL deionized water into appropriate labeled tubes.
2. The absorbances were measured for standard, control, and samples at 415 nm against deionized water as a blank. (Total hemoglobin reading).

IV. Calculations:

Results of the unknowns determined in the following manner:

$$\% \text{ of Glycohemoglobin (unknown)} = \frac{R \text{ (unknown)}}{R \text{ (standard)}} \times \text{standard concentration}$$

Where: R is the ratio

$$\text{Ratio (unknown)} = \frac{\text{Abs. of Glyco Hb (unknown)}}{\text{Abs. of Total Hb (unknown)}}$$

$$\text{Ratio (standard)} = \frac{\text{Abs. of Glyco Hb (standard)}}{\text{Abs. of Total Hb (standard)}}$$

Normal reference Values

Normal: < 8.0 %
Good control : < 8.0 – 9.0 %
Fair control : < 9.0 – 10.0 %
Poor control: > 10.0 %

Statistical analysis

The statistical analysis of the results obtained in this work, was performed by statistical package calculated parameters for the Social Sciences (SPSS) program version 14.0 .

RESULTS

This chapter contains a summary of the data on glycemic control and markers of osteoporosis,normal healthy control women and type 2 diabetic postmenopausal women with and without osteoporosis.

N.B. The data of individual experiments are presented in Appendix:

❖ **Clinical data :**

The clinical data of the studied group are summarized in Table (2 and 3) and Figures (18-20) As expected, the fasting blood glucose level in diabetic patients is highly elevated by about 91% in postmenopausal women without osteoporosis and about 161 % in patients with osteoporosis compared to control value (Table 2, Figure 18). Also the result indicated that the control of blood level in those patients over the last two months is not good as evidenced by elevated levels of glycosylated hemoglobin over 46 % in patients without osteoporosis and about 66 % in patients with osteoporosis compared to control value (Table 2, Figure 19).

The result showed that , the diabetic patients without osteoporosis has a decrease in calcium level by about 6 % while the diabetic postmenopausal women with osteoporosis has a significantly decreased level by about 10 % compared to control values (Table 2, Figure 20). The result showed that , the diabetic patients without osteoporosis has a slightly increased level of ionized calcium by about 2.5 % while the diabetic patients with osteoporosis has a slightly decreased level of ionized calcium by about 1.68 % compared to control values (Table 2, Figure 20). The phosphorus level show no significant change in diabetic groups compared to control group (Table 2, Figure 20).

Table (2) Demographic characteristics of the study population (patients n=60 and the control n=30).

	Control (n=30)	Diabetic patients	
		With osteoporosis GroupI (n=30)	Without osteoporosis Group II (n=30)
Age (years)	49.13 ± 5.96	62.66 ± 5.39	50.73 ± 4.86
Height (cm)	165.30 ± 4.43	162.33 ± 3.17	164.03 ± 3.85
Weight (kg)	66.16 ± 3.57	71.36 ± 3.46	68.43 ± 3.05
BMI*	24.64 ± 1.61	26.65 ± 1.64 (+8.15%)	25.07 ± 1.29 (+ 1.74%)

BMI*= weight (kg)/ (height (m))^2.

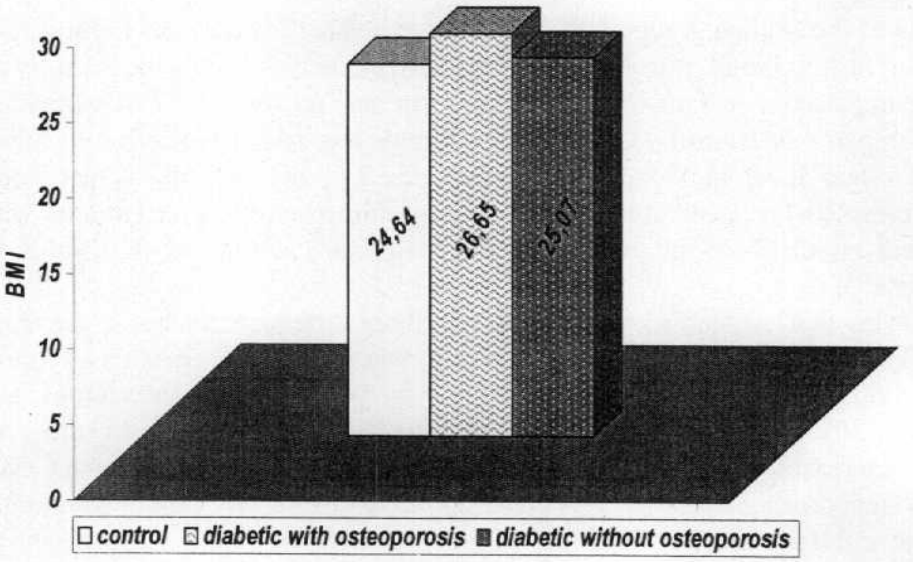


Figure (16). BMI among the studied groups

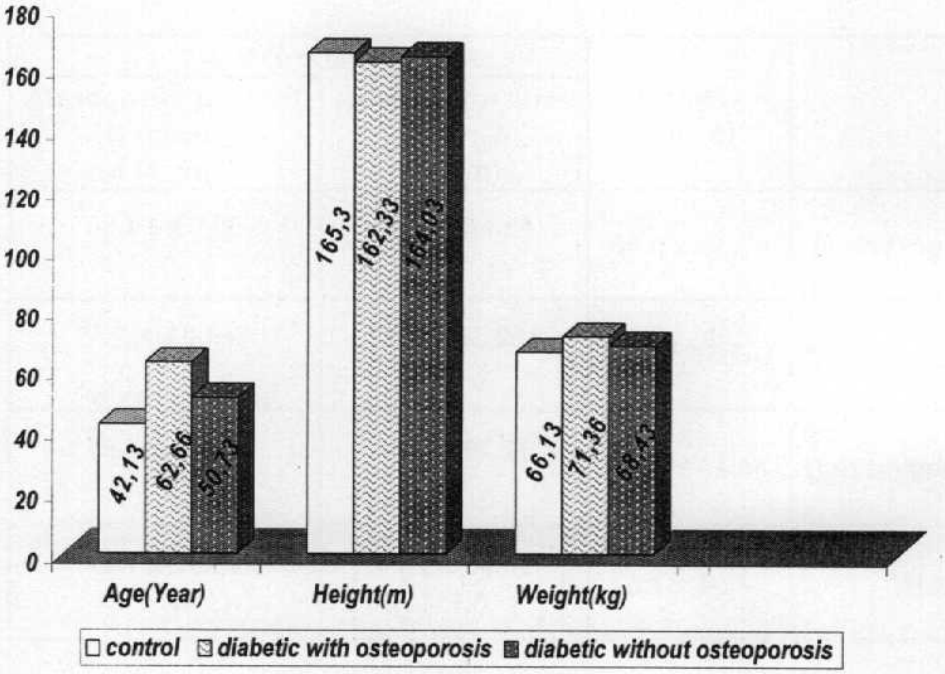


Figure (17). Age, height, weight among studied groups

Table (3). Clinical data of studied groups:

	Control n=30	Diabetic patients	
		With osteoporosis GroupI n=30	With out osteoporosis GroupII n=30
Fasting blood glucose (mg/dl)	**86.37 ± 8.56**	**225.6 ± 25.14[a] (+161.2%)**	**165.29 ± 24.19 [a,b] (+91.37%)**
Hemoglobin A1c	**5.78 ± 1.13**	**9.64 ± 0.71[a] (+66.7%)**	**8.47 ± 0.5 [a,b] (+46.5%)**
Calcium (mg/dl)	**9.32 ± 0.71**	**8.34 ± 0.38 [a] (-10.51%)**	**8.72 ± 0.46 [a,b] (- 6.43%)**
Ionized calcium (mmol/L)	**1.19 ± 0.095**	**1.17 ± 0.11 (-1.68%)**	**1.22 ± 0.09 (+ 2.50%)**
Phosphorus (mg/dl)	**2.1 ± 0.65**	**2.09 ± 0.65 (- 0. 47%)**	**2.11 ± 0.60 (+0.047%)**

a significant different from control by ANOVA ($p<0.05$).
b significant different from diabetic with osteoporosis by ANOVA ($p<0.05$).

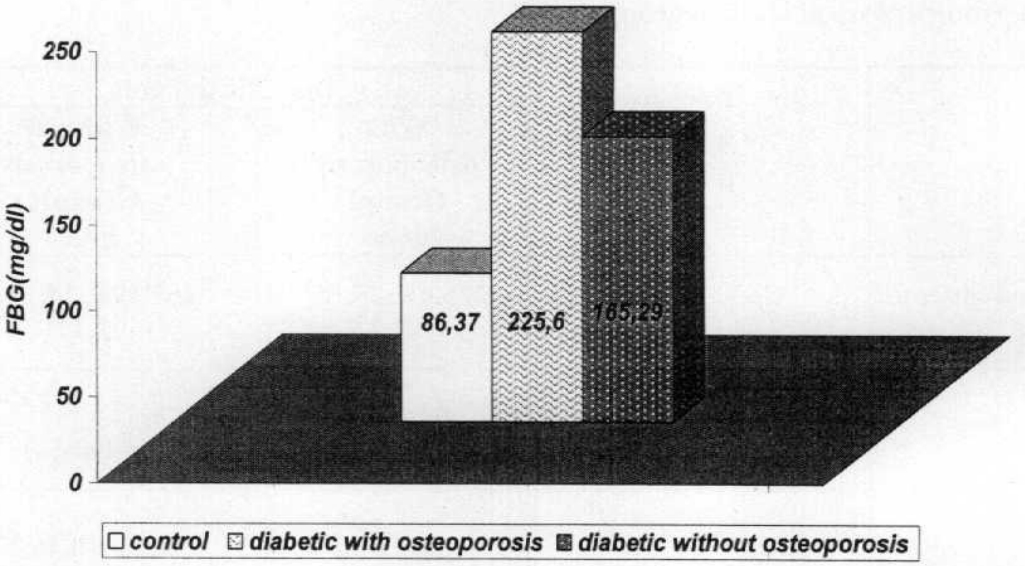


Figure (18). The levels of fasting blood glucose in studied groups

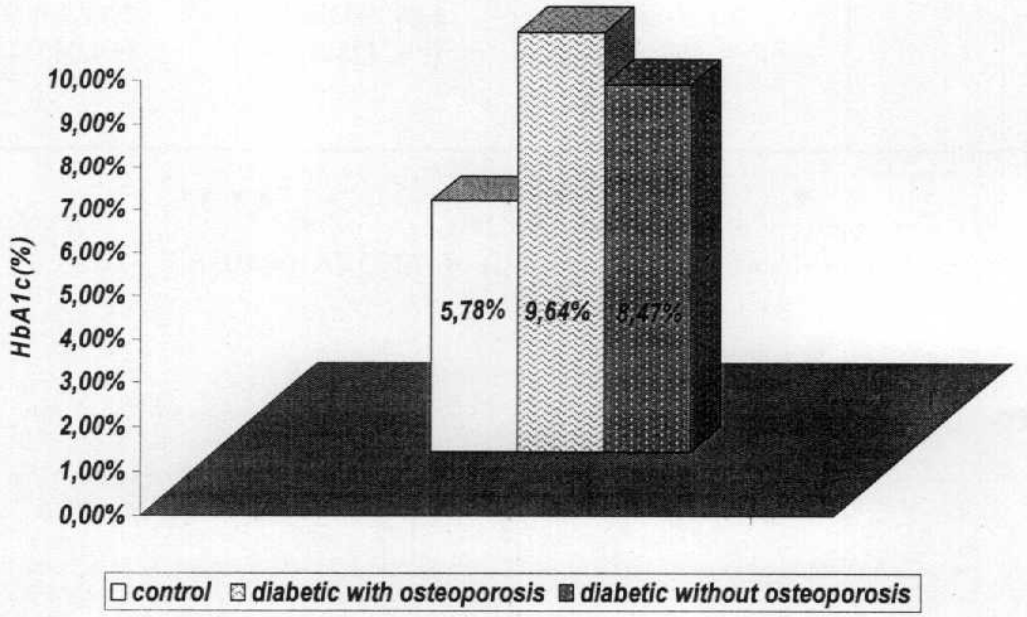


Figure (19). The levels of HbA1c in the studied groups

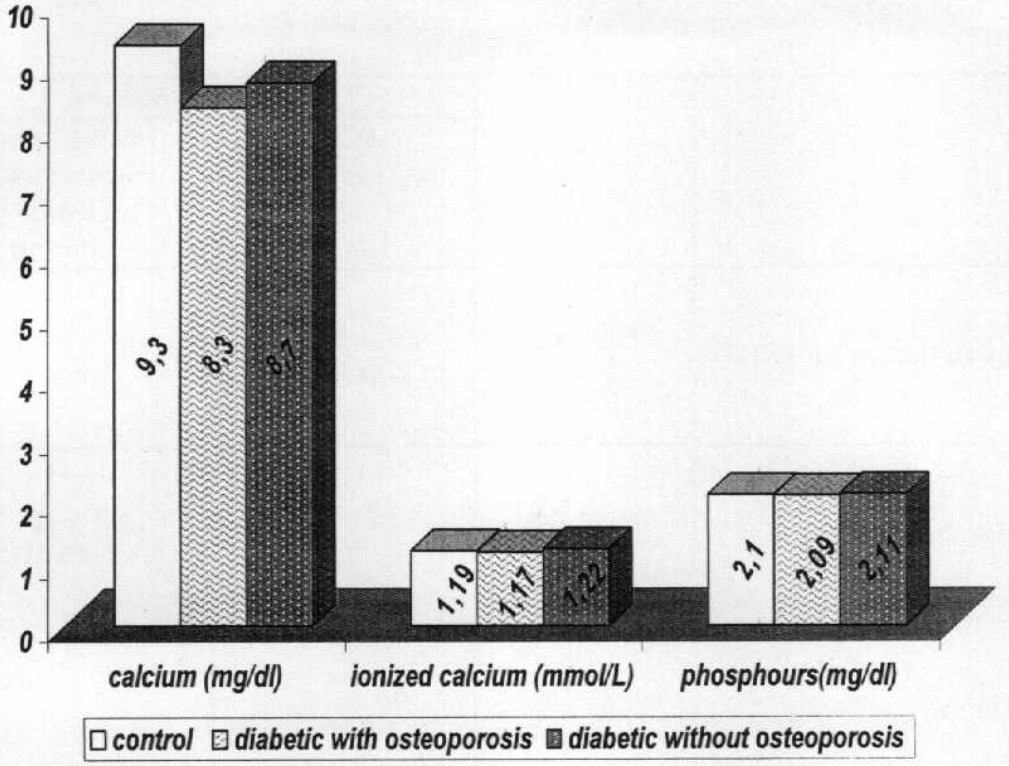


Figure (20).The levels of calcium, ionized calcium and phosphours in the studied groups

Markers of osteoporosis:

Bone turnover parameters of studied groups are summarized in Table (4) and Figures (21-25) the result showed that , the diabetic postmenopausal women with osteoporosis has a significantly decreased IGF-1 level by about 61.6 % while the diabetic patients without osteoporosis has a significant decrease by about 30.4 % compared to control values (Table 3, Figure 21). The osteoprotegerin level in diabetic patients is highly elevated by 95.6% in postmenopausal women with osteoporosis and about 19.4% in patients without osteoporosis compared to control value (Table 4, Figure 22).

The result showed that, the Type2 diabetic postmenopausal women without osteoporosis showed no significant change in sRANKL level while the diabetic postmenopausal women with osteoporosis has a significant decrease by about 7.2 % compared to control group (Table 4, Figure 23). sRANKL/OPG ratio among diabetic osteoporotic women showed a significant decrease by 49 % while the diabetic without osteoporosis showed a slight decrease by about 5.5 % compared to control group (Table 4, Figure 24). Diabetic postmenopausal women who suffer from osteoporosis showed a significant decrease in osteocalcin level by about 27 % while those diabetic women who are not suffering from osteoporosis showed no significant change in osteocalcin level compared to control values (Table 4, Figure 25).

Table (4). Comparison of serum concentrations of IGF-1, OPG, RANKL,

	Controls (n=30)	Diabetic patients	
		With osteoporosis GroupI (n=30)	Without osteoporosis GroupII (n=30)
Insulin growth factor-1 (ng/ml)	98.9 ± 4.20	37.9 ± 7.1 [a] (-61.6 %)	68.9 ± 7.7 [a,b] (-30.4 %)
Osteoprotegerin (pg/ml)	3.6 ± 0.8	7.2 ± 1.1 [a] (+95.6%)	4.3 ± 1.1 [a,b] (+19.4%)
sRANKL(pg/ml)	6.6 ± 3.3	6.1 ± 2.5 [a] (-7.6%)	6.5 ± 3.2 (-1.5%)
sRANKL /OPG ratio	1.8 ± 0.8	0.91 ± 0.51 [a] (- 49.4 %)	1.7± 1.2 [a,b] (-5.5%)
Osteocalcin (ng/ml)	28.38 ± 6.19	20.8±1.7 (-27.4 %)	28.60 ± 8.00 (+0.8 %)

a significant difference from control by ANOVA ($p< 0.05$).
b significant difference from diabetic with osteoporosis by ANOVA ($p<0.05$).

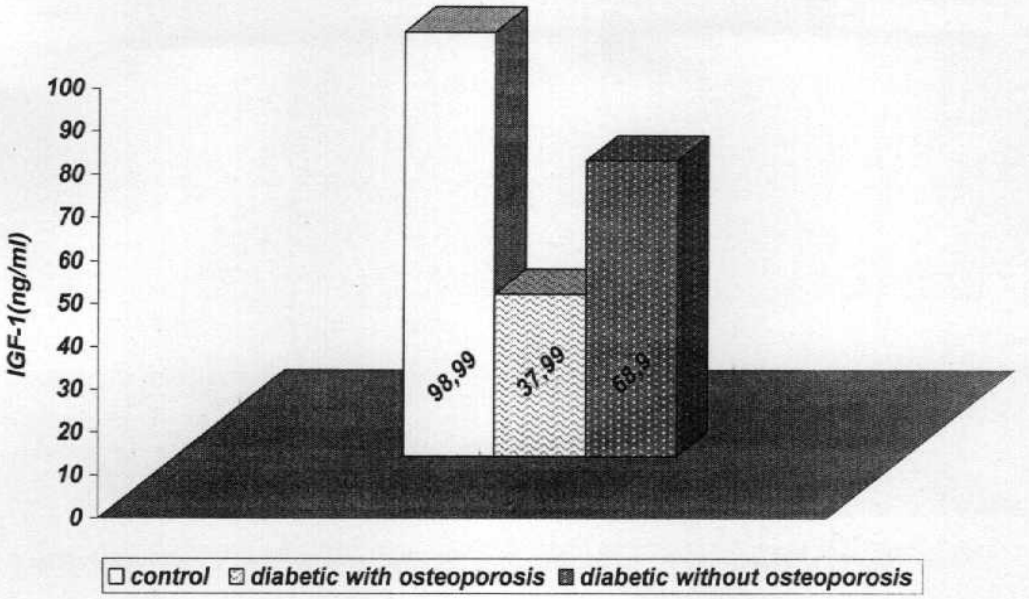


Figure (21). The level of IGF-1 in the studied groups

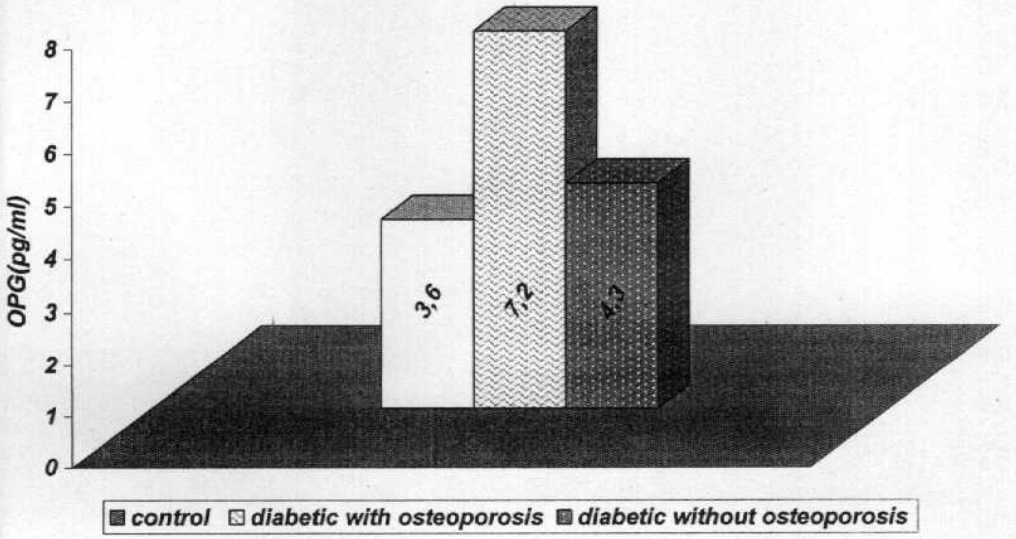


Figure (22). The levels of OPG in the studied groups

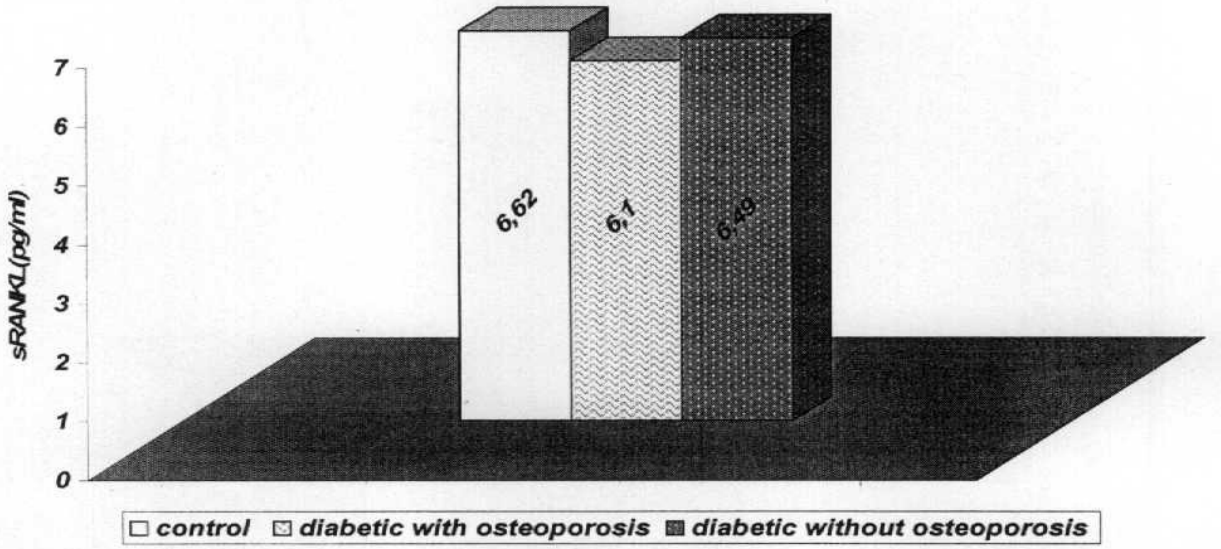


Figure (23). The level of sRANKL in the studied groups

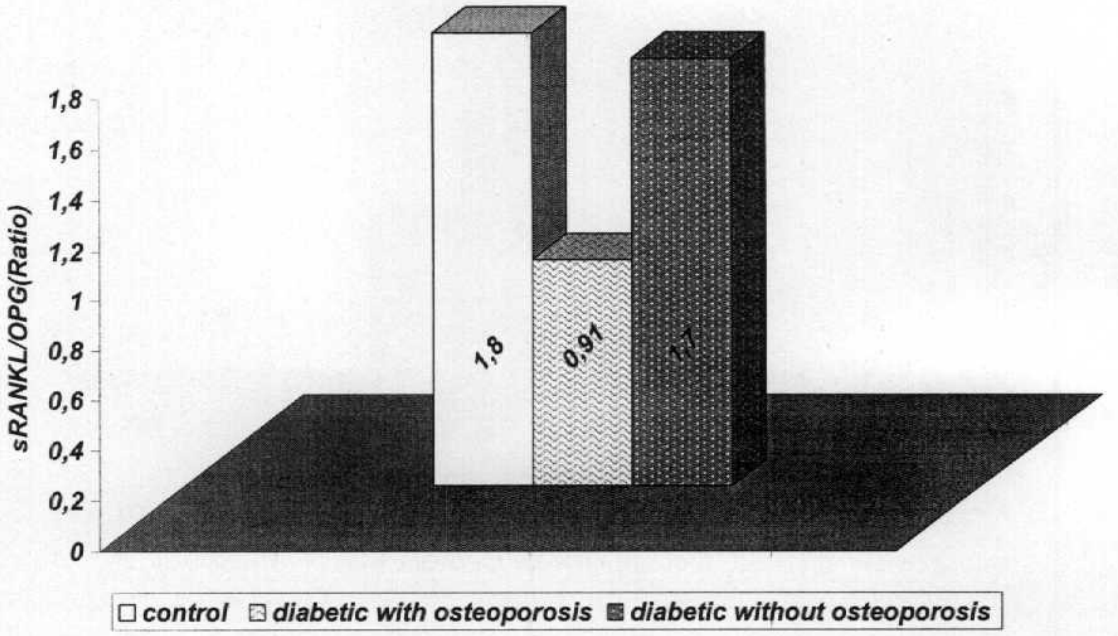


Figure (24). The level of sRANKL/OPG ratio in the studied groups

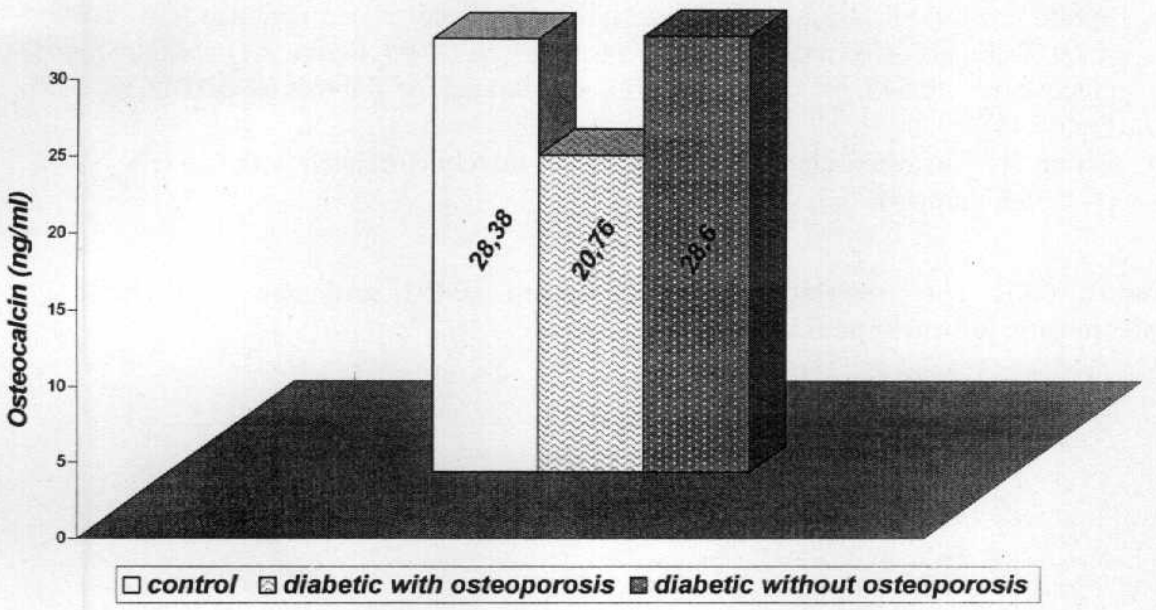


Figure (25). The level of osteocalcin in the studied groups

Correlation studies:

The correlation studies indicated that:

In type 2 postmenopausal women with osteoporosis group we found the following correlation:

1. The serum level of IGF-1 is significantly negatively correlation with; age ($r = -0.55$, $p < 0.0$, Figure 26), osteoprotegerin ($r = -0.887$, $p < 0.01$ Figure 27), fasting blood glucose ($r = -0.58$, $p < 0.01$ Figure 28), osteocalcin ($r = 0.419$, $p < 0.01$ Figure 29), and duration of diabetes ($r = -0.532$, $p < 0.01$ Figure30).

2. The serum of IGF-1 is significantly positively correlated with sRANKL ($r = 0.893$, $p < 0.01$ Figure 31), and sRANKL / OPG ($r = 0.859$, $p < 0.01$ Figure32).

3. The serum level of osteoprotegerin is significantly positively correlated with age ($r = 0.606$, $p < 0.01$ Figure33). Osteocalcin ($r = 0.524$, $p < 0.01$ Figure34), fasting blood glucose ($r = 0.556$, $p < 0.01$ Figure35), and duration of diabetes ($r = 0.485$, $p < 0.01$ Figure 36).

4. Serum level of osteoprotegerin is significantly negatively correlated with; Height ($r = -0.436$, $p < 0.0$ Figure37), and serum level of osteoprotegerin is significantly negatively correlated with; sRANKL ($r = -0.939$, $p < 0.001$ Figure38).

5. Serum level of sRANKL is significantly positively correlated with height (r = 0.415, p< 0.05 Figure39).
6. Serum level of sRANKL is significantly negatively correlated with age (r = - 0.606, p< 0.01 Figure 40), osteocalcin (r = - 0.452, p< 0.05 Figure 41), fasting blood glucose (r = - 0.543, p< 0.05 Figure 42), and duration of diabetes (r = - 0.42, p< 0.05 Figure 43).
7. Serum level of osteocalcin is significantly positively correlated with age (r = 0.379, p< 0.05 Figure 44).

Figure (26). The correlation between serum IGF-1 and age in diabetic osteoporotic postmenopausal women

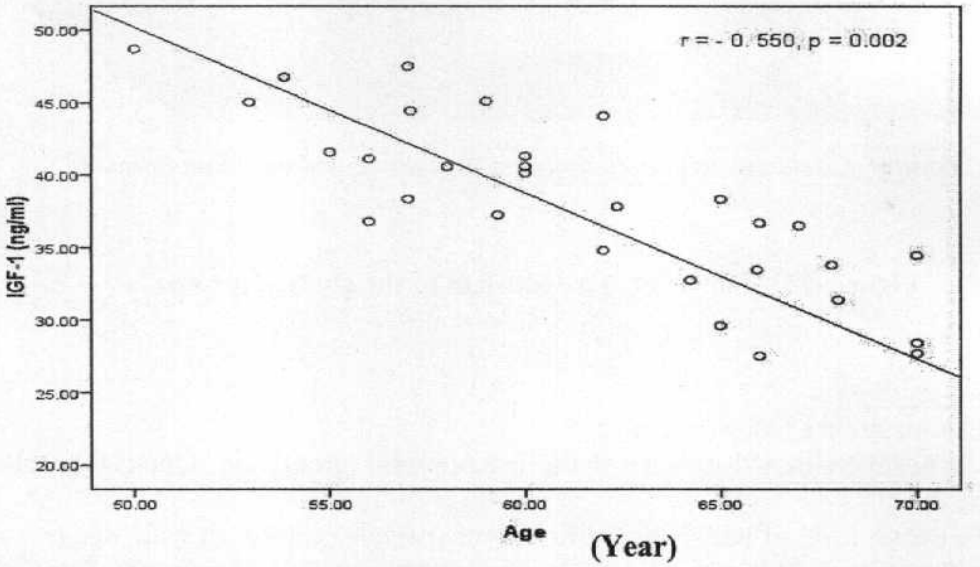


Figure (27). The correlation between serum IGF-1 and OPG in diabetic osteoporotic postmenopausal women

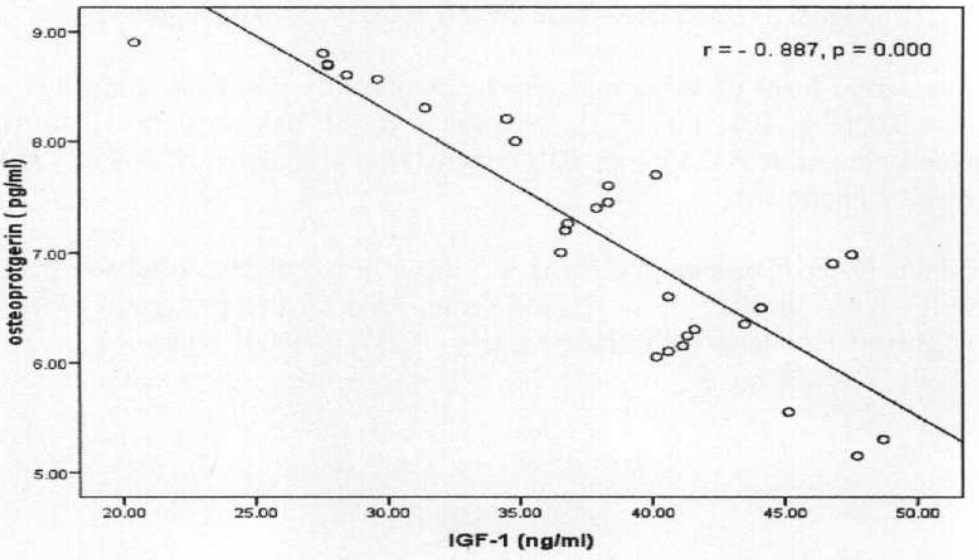


Figure (28). The correlation between serum IGF-1 and FBG in diabetic osteoporotic postmenopausal women

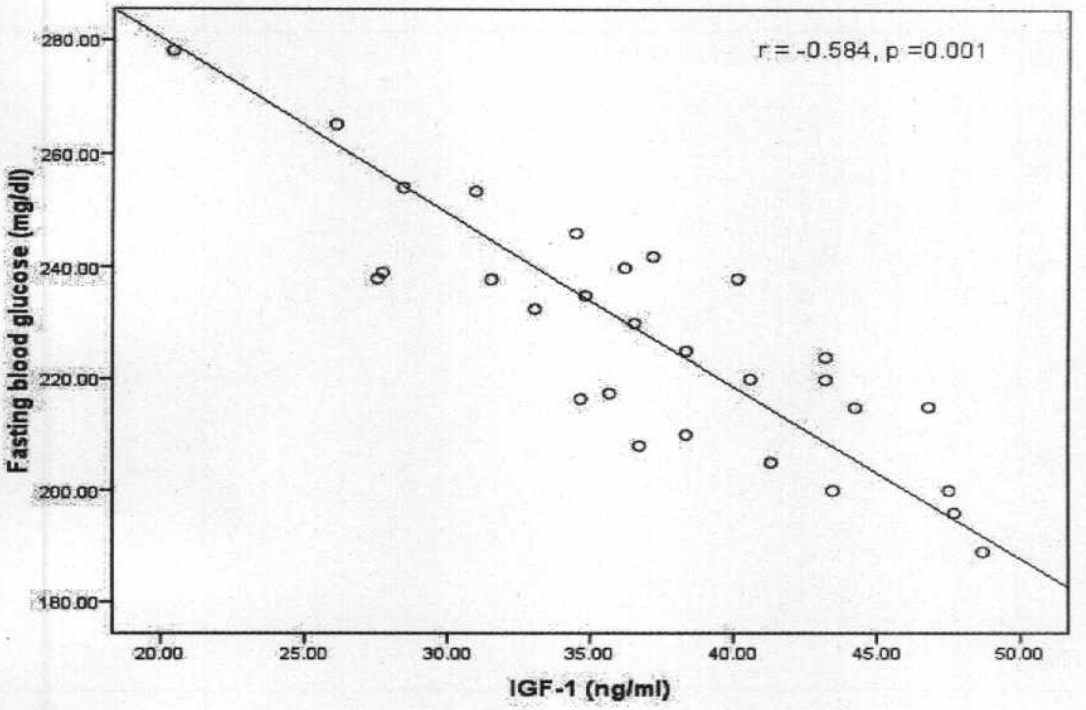


Figure (29). The correlation between serum IGF-1 and osteocalcin in diabetic osteoporotic postmenopausal women

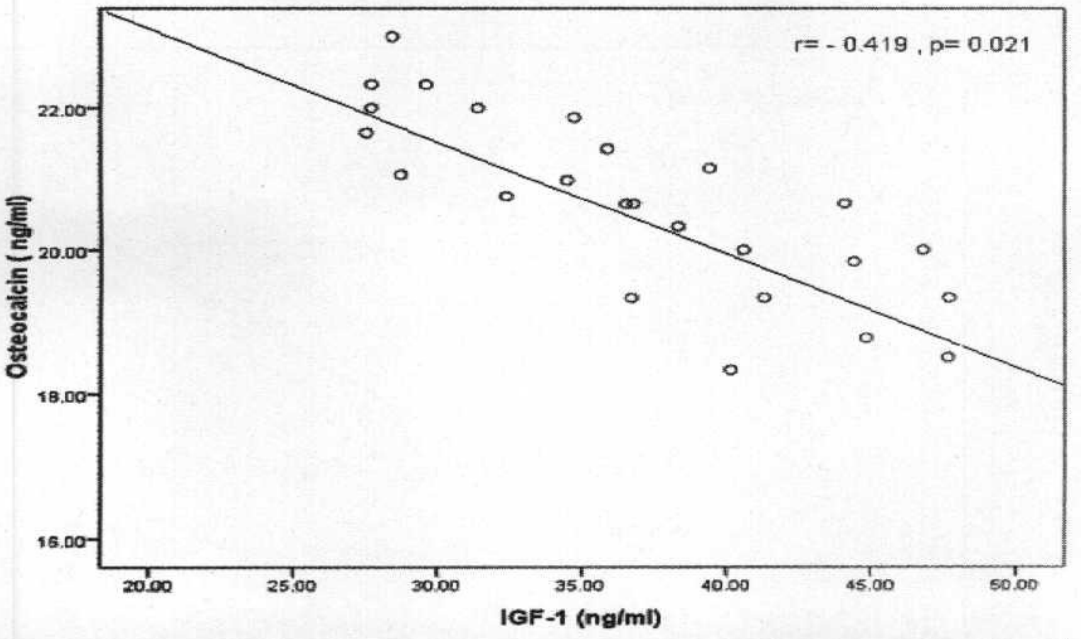


Figure (30). The correlation between duration of D.M and IGF-1 in diabetic osteoporotic group

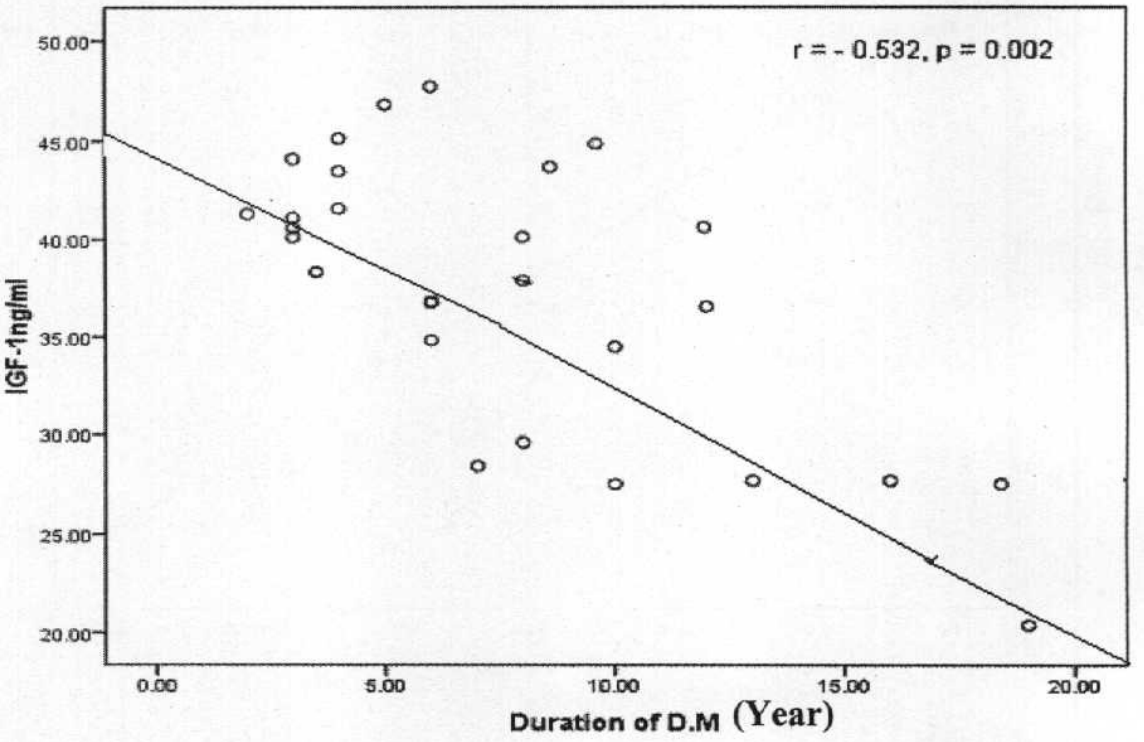


Figure (31). The correlation between serum IGF-1 and sRANKL in diabetic osteoporotic postmenopausal women

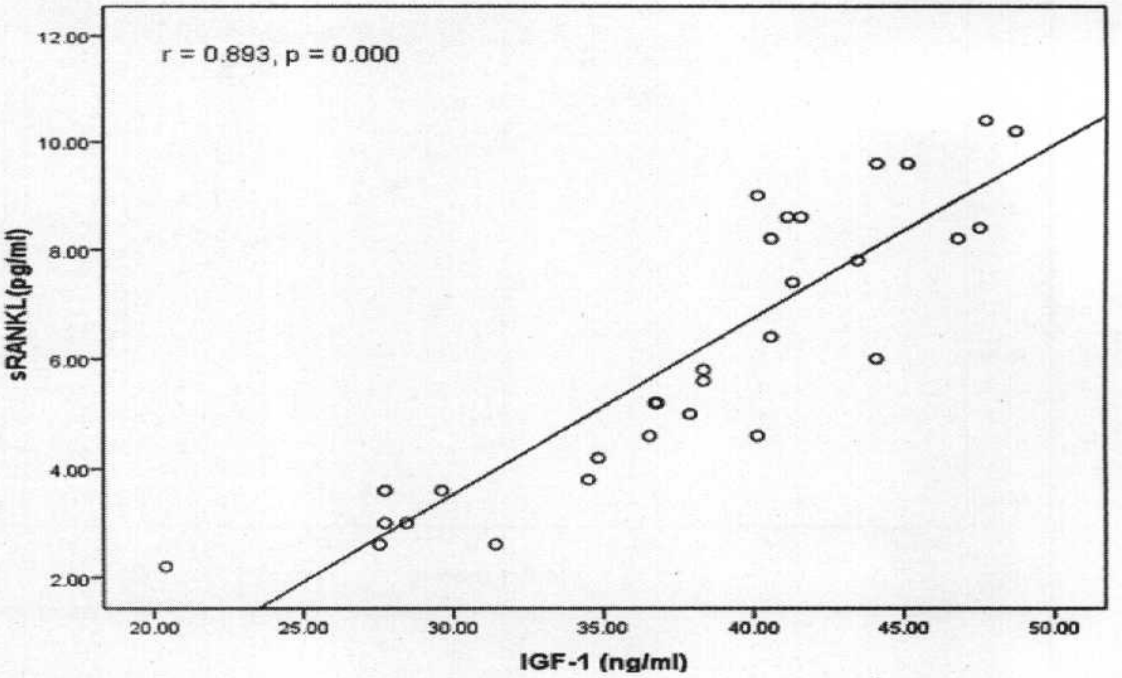


Figure (32). The correlation between serum IGF-1 and sRANKL/OPG in diabetic osteoporotic postmenopausal women

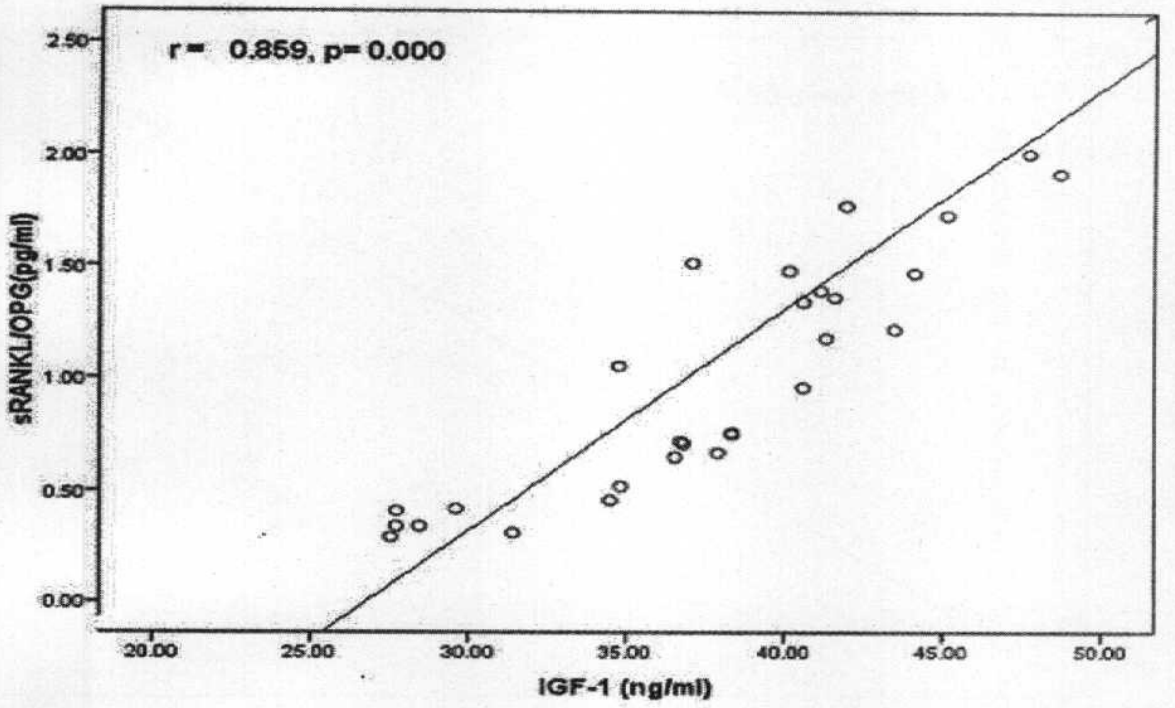


Figure (33). The correlation between serum OPG levels and age in diabetic osteoporotic postmenopausal women

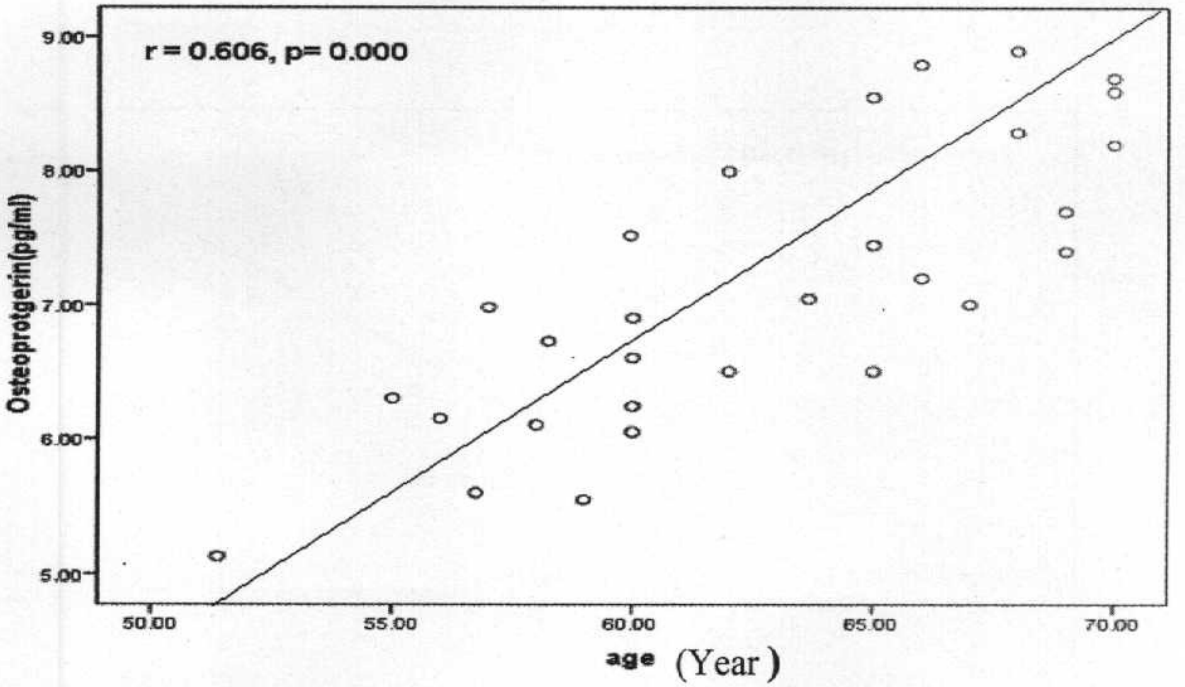


Figure (34). The correlation between serum OPG levels and osteocalcin in diabetic osteoporotic group

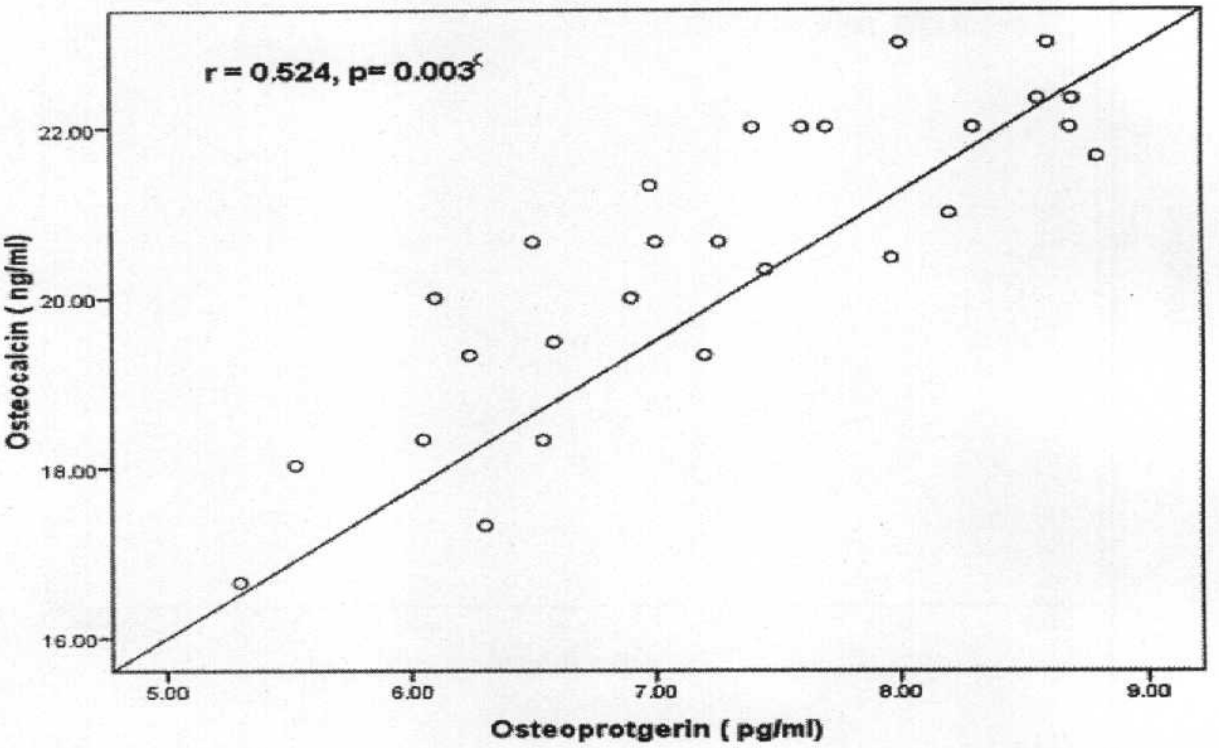


Figure (35). The correlation between serum OPG levels and FBG in diabetic osteoporotic group

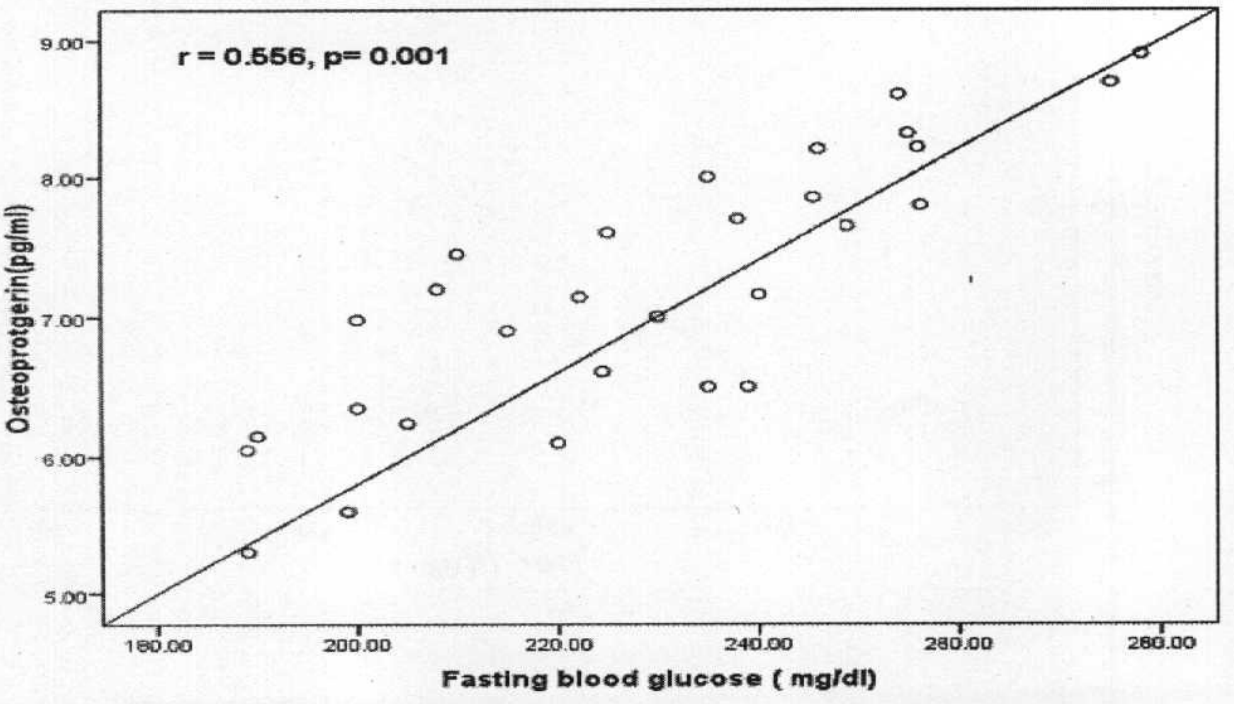


Figure (36).The correlation between duration of DM levels and OPG in diabetic osteoporotic group

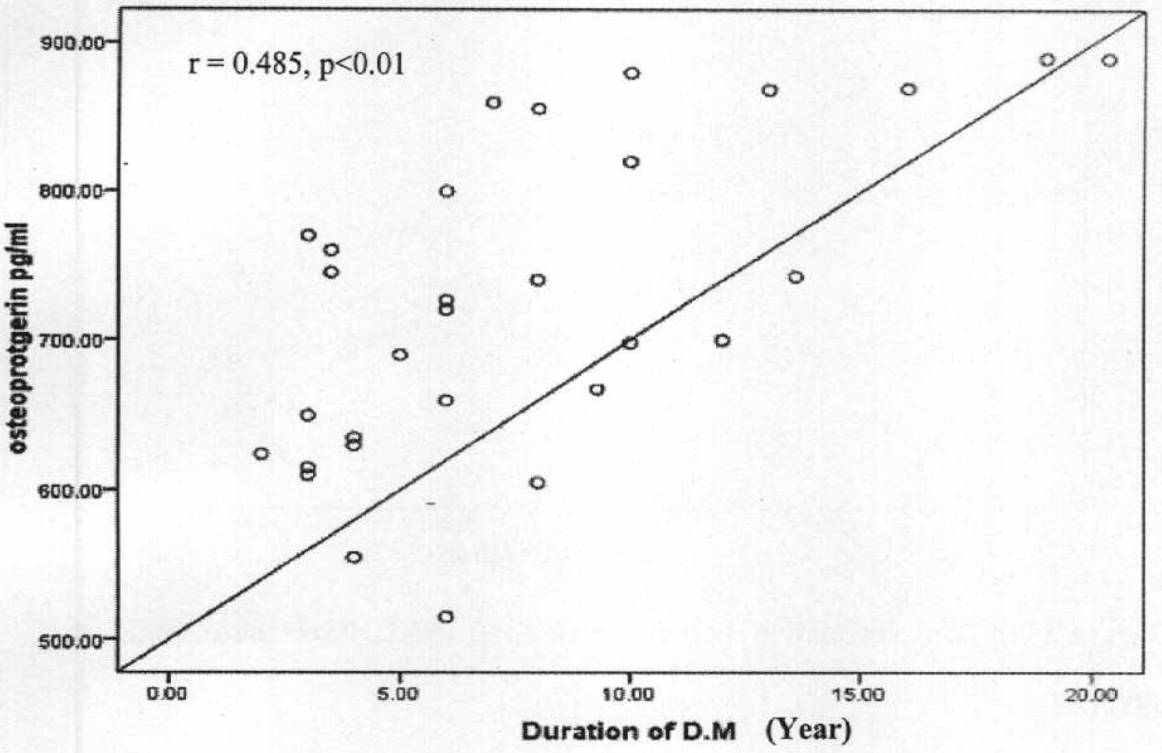


Figure (37). The correlation between serum OPG levels and height in diabetic osteoporotic group

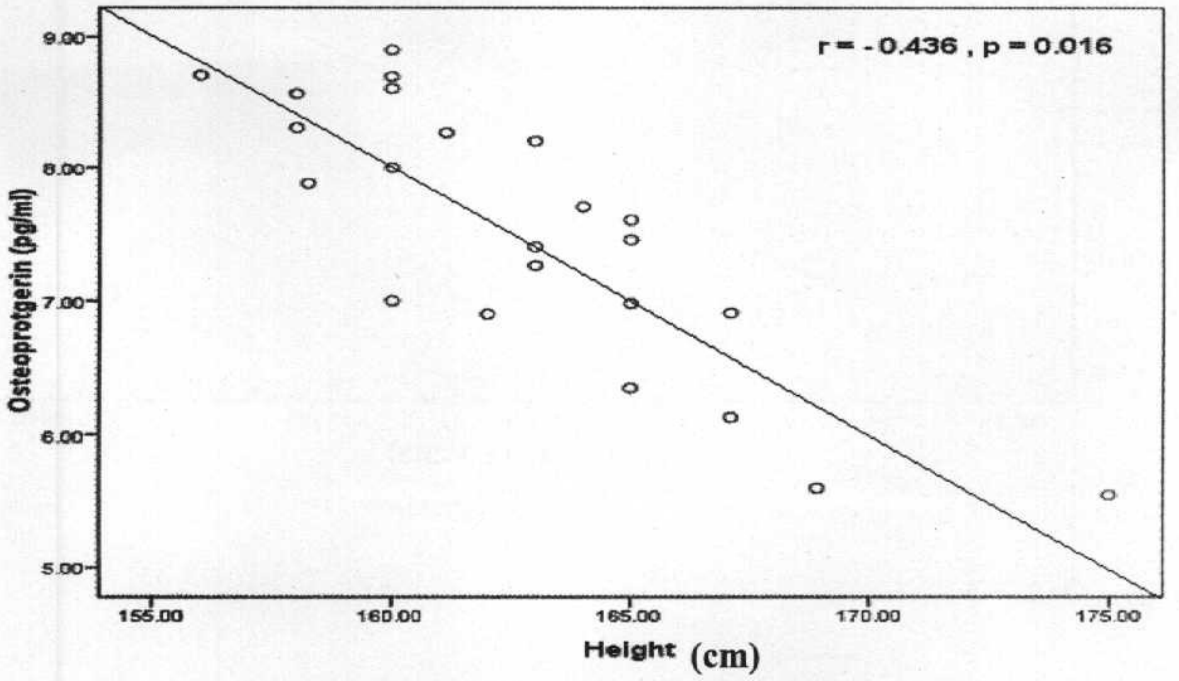


Figure (38). The correlation between serum OPG levels and sRANKL in diabetic osteoporotic group

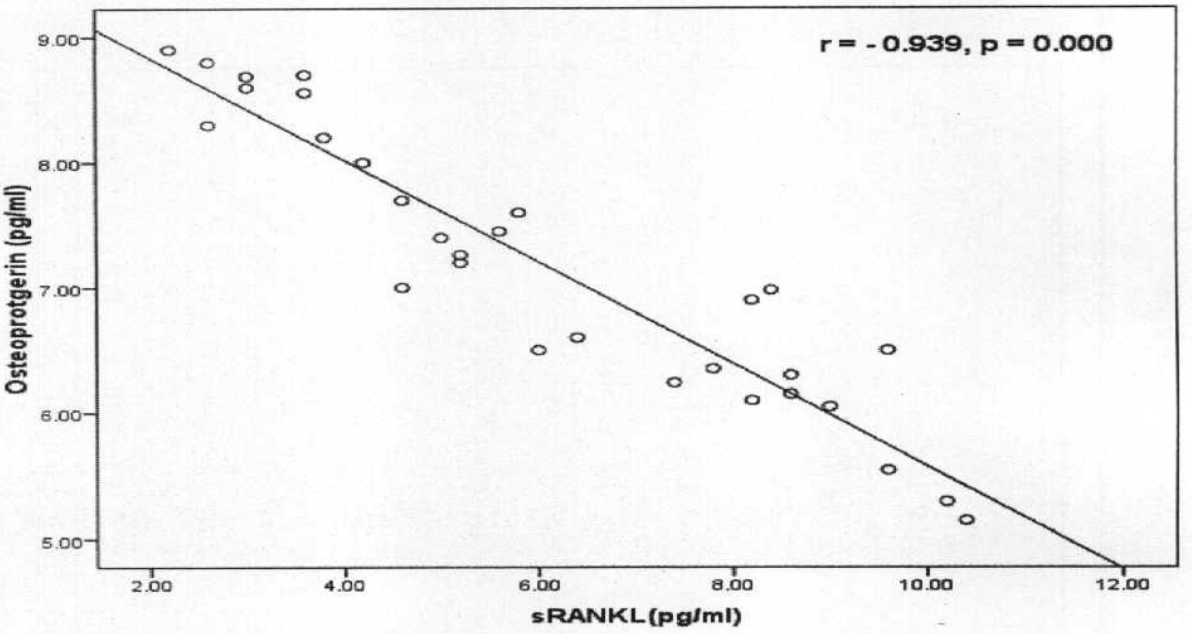


Figure (39). The correlation between serum sRANKL levels and height in diabetic

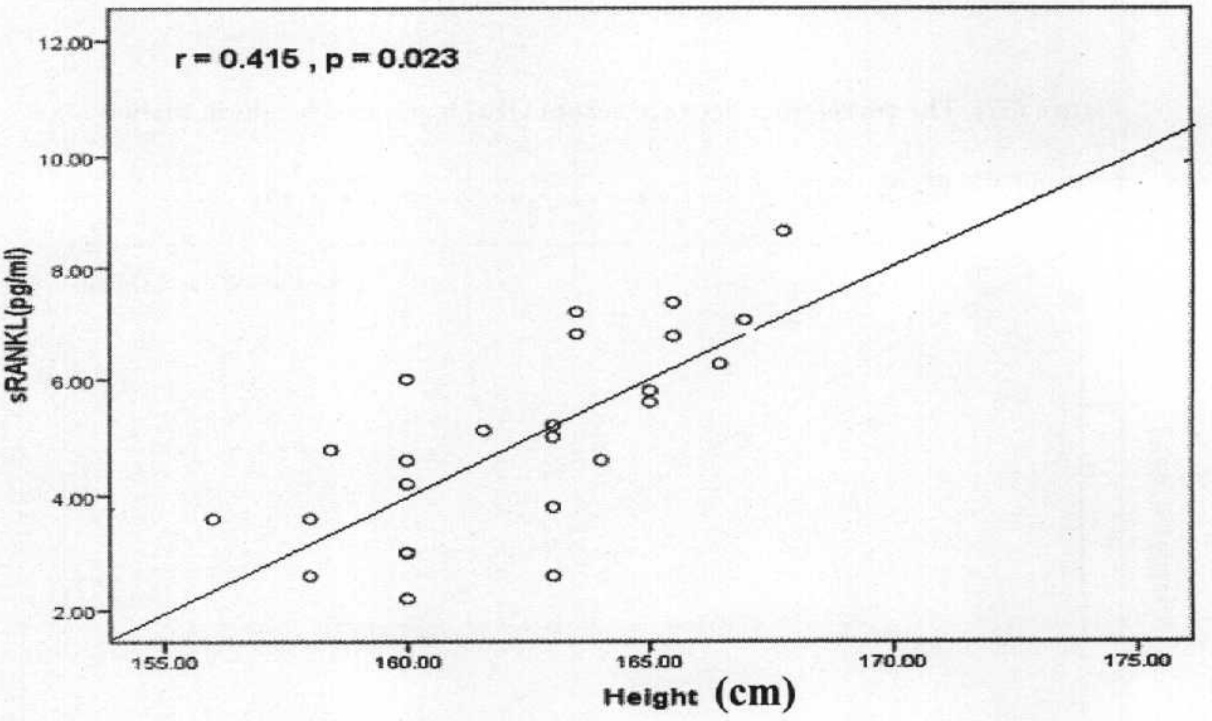


Figure (40). The correlation between serum sRANKL levels and age in diabetic osteoporotic group

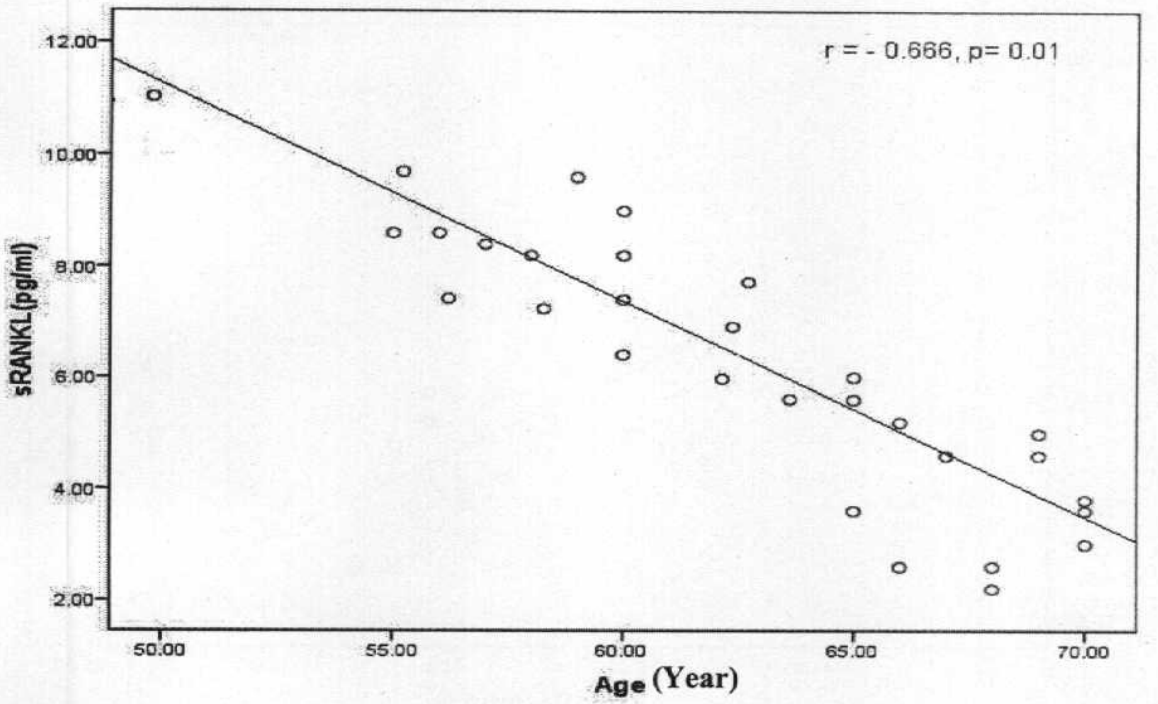


Figure (41). The correlation between serum sRANKL levels and osteocalcin in diabetic osteoporotic group

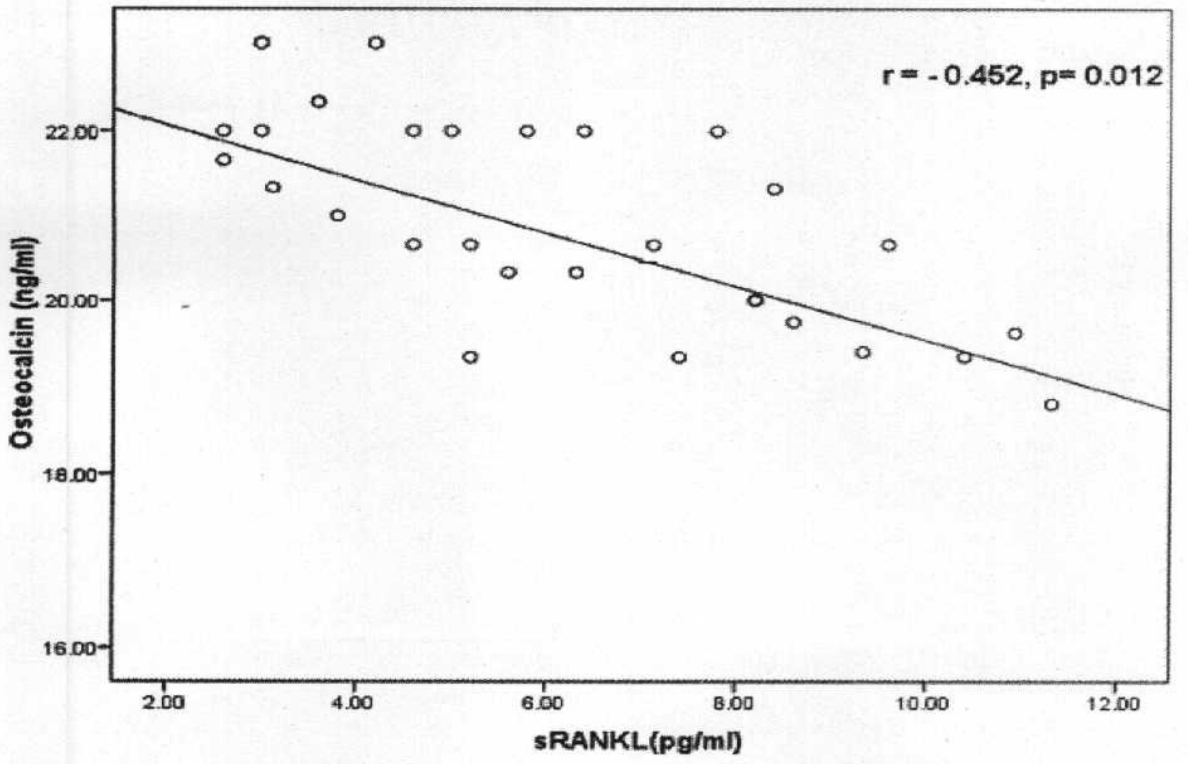


Figure (42). The correlation between sRANKL levels and FBG in diabetic osteoporotic group

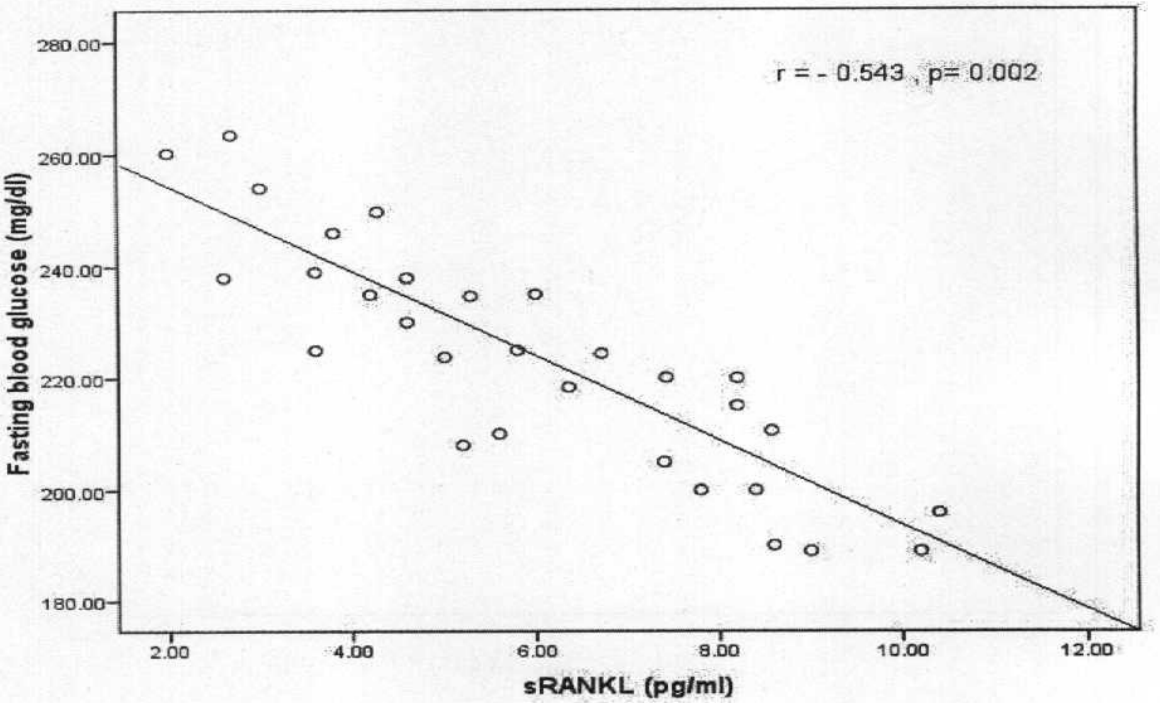
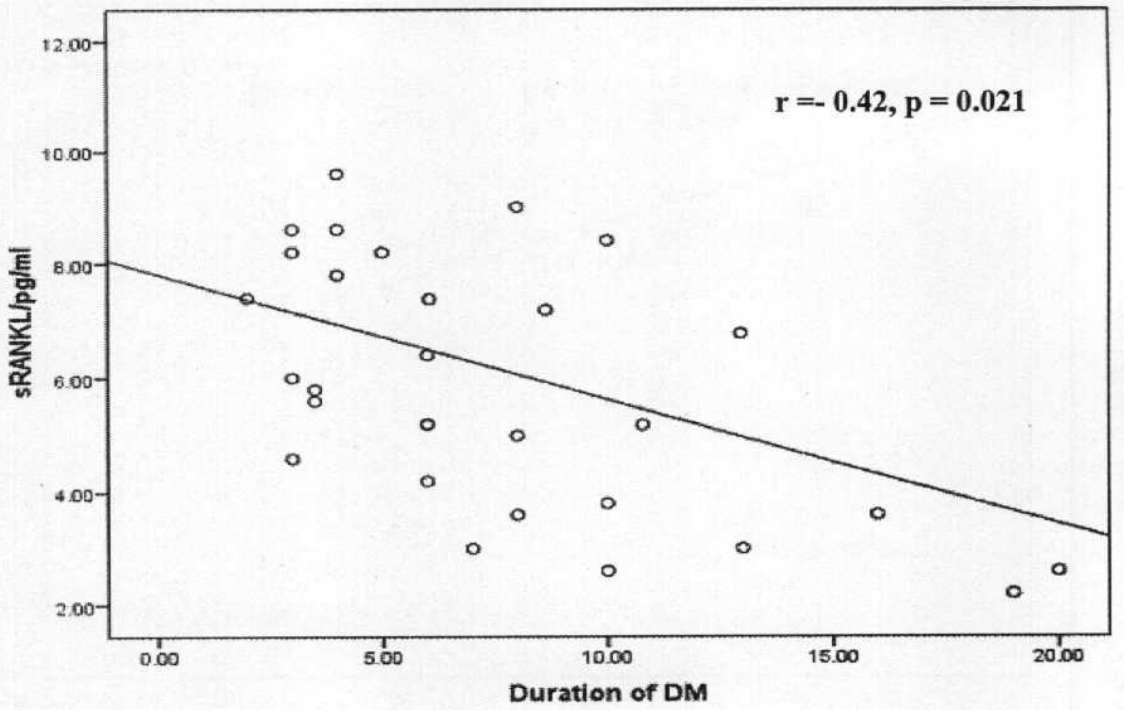
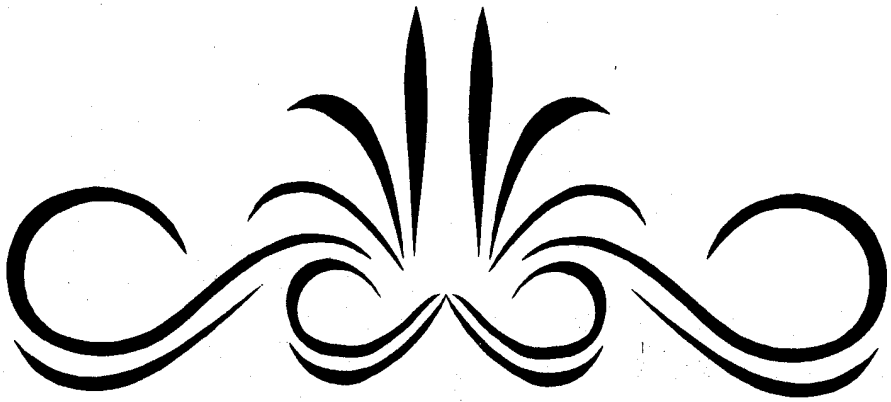
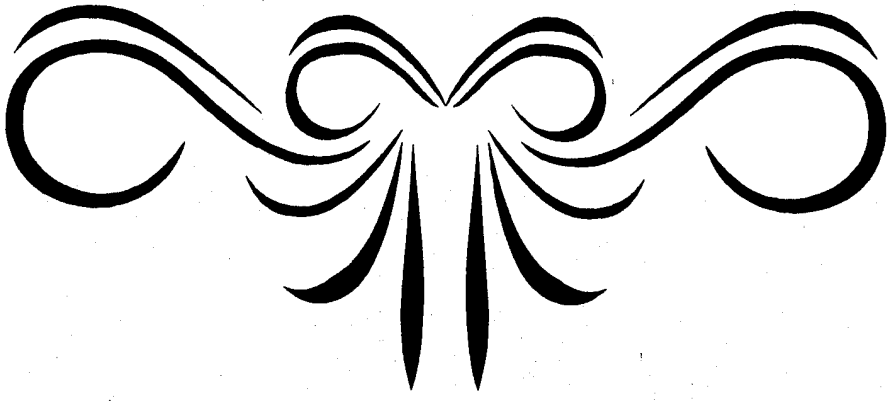


Figure (43).The correlation between duration of D.M levels and sRANKL in diabetic osteoporotic group





DISCUSSION



Osteoporosis and type 2 diabetes mellitus (DM) are traditionally viewed as separate diseases that increase in prevalence with aging. There are accumulating evidences indicate that these diseases are interrelated by different faces [99, 100]. Although, osteoporosis traditionally has not been listed as a complication of diabetes, some studies were suggested a possible increased risk of osteoporosis in patients with either type 1or type 2 DM [101, 86]. But this association remains controversial and conflicting, especially in type 2 [102-104]. Many clinical studies have investigated the association between DM and osteoporosis. Although bone mineral density (BMD) is considered as a gold standard for evaluating fracture risk in non- DM osteoporosis, it is not useful for assessing fracture risks in type 2 DM [99]. Therefore, a diagnostic marker other than BMD needs to be explored. Insulin- like growth factor-1 (IGF-1) and osteocalcin seems to be two of candidate's markers that may replace the insensitivity of BMD in assessing the osteoporosis in type 2 DM patients.

Post- menopause is a strong risk factor for the development of osteoporosis [105]. The osteoporosis in postmenopausal diabetic women is complex process and a matter of debates, so this study was conducted to evaluate osteoporosis in postmenopausal women with type2 DM and to explore IGF-1and osteocalcin as a markers for assessing bone remodeling in those subjects.

IGF-1 is one of the most abundant growth factors present in bone that stimulates osteoblast activity, subsequently leading to bone matrix formation and inhibition of bone collagen degradation [106]. The circulating IGF-1, which is mainly produced in the liver, activates bone remodeling and exerts anabolic effects on bone tissues [107, 108]. Also the locally produced IGF-1 by osteoblasts is among the most important regulators of bone cell function due to its anabolic effects on the skeleton [109].

The key role of the IGF system in the local regulation of bone formation is demonstrated by the finding that about 50% of basal bone cell proliferation can be blocked by inhibiting the action of IGF that produced endogenously by bone cells [110]. Our results indicated that IGF-1 is decreased by about quarter in postmenopausal diabetic women without osteoporosis, while those diabetic women with osteoporosis show about 60% decrease in IGF-1 level compared to control subjects (Table 4, Figure 21). Also, we found age related decrease in serum level of IGF-1 in the diabetic women with osteoporosis ($r = -0.550$, $p = 0.002$, Figure 26). The statistical analysis revealed that serum level of IGF-1 is significantly negatively correlated with fasting blood glucose level ($r = -0.58$, $p = 0.001$, Figure 28) and duration of diabetes ($r = -0.53$, $p = 0.002$, Figure 30) in postmenopausal women with osteoporosis

In accordance with our results it has been demonstrated that the decrease of IGF-1 with age could be partly due to lower protein intake in elderly, as protein intake is one of the major determinants of IGF-1 [111]. Low levels of IGF-1 may increase the risk of fractures, as shown in many longitudinal studies [112-113]. These studies indicated that low level of IGF-1 predicted osteoporotic fractures independently of the BMD.

In line with our results, Zhao et al 2008, demonstrate that serum IGF-1 levels were significantly and negatively associated with age and its level is lower in postmenopausal women than in premenopausal women [114]. Many other previous studies had demonstrated that serum or bone IGF-1 levels decreased with aging [115-116].

It was also found that IGF-1 level decreased after menopause or with estrogen deficiency [117-118]. All age – related decrease of IGF-1 may correlate with age- related bone loss or osteoporosis.

The role of IGF-1 as an important regulator of bone formation is well established, however, its effects on bone resorption is limited and conflicting [119- 120]. Also its impact on the balance of the two peptides produced by osteoblasts; osteoprotegerin (OPG) and receptor activator of nuclear factor – κB ligand (RANKL), is under investigation. Thus we aimed from this study to analyze the relationship of serum concentration of IGF-1 with OPG, RANKL and sRANKL/OPG ratio.

First we studied the change in OPG/sRANKL system in postmenopausal diabetic women with and without osteoporosis. The results of the study indicated that while the serum OPG levels are elevated in diabetic women, especially those with osteoporosis (increased about 95%), the levels of sRANKL tend to decrease insignificantly in diabetic women especially postmenopausal diabetic women with osteoporosis (Table 4, Figure 23). The sRANKL/OPG ratio which represents sRANKL bioactivity index show a significant decrease in diabetic postmenopausal women, the decrease is more prominent in those women with osteoporosis by 49 % (Table 4, Figure 24). We found a strong negative correlation between OPG and sRANKL ($r = -0.939$, $p = 0.000$, Figure 38) this result is confirmed by result of Amore et al 2006 who indicate a negative correlation between OPG and sRANKL in postmenopausal women [121]. In line with our results many studies reported an increased level of OPG in postmenopausal osteoporosis [122-124]. Another studies proved that OPG is increased in diabetic women and its level is correlated with fructosamine concentration [125]. Yano et al. demonstrated that OPG in serum increased with increasing age in both men and women [126] which confirm our results that indicate a significant positive correlation between OPG level and age($r = 0.606$, $p < 0.01$, Figure 33). , fasting blood glucose ($r = 0.556$, $p < 0.01$, Figure 35).

Duration of diabetes ($r = 0.485$, $p < 0.01$, Figure 36) in postmenopausal diabetic women with osteoporosis. Furthermore Yano et al., found that OPG levels were higher in postmenopausal women compared to control [126] .

Elevated levels of plasma OPG were reported in newly diagnosed type 2 diabetic patients these levels were associated with endothelium- dependent arterial dilation [127] . Also in a large study in elderly women, plasma concentrations of OPG were higher in diabetic than non- diabetic subjects [125] . Another recent population – based study found significantly higher levels of serum OPG in postmenopausal Type 2 women than in healthy postmenopausal women [128] .

The study of Abrahamsen et al. 2005, indicate that there was association between OPG and sRANKL levels showed with age and menopause, as sRANKl levels decreased and OPG increased [123] . Also the study confirms our results of decreased sRANKL/OPG ratio. The study indicate that a lower sRANKL bioactivity index (sRANKL/OPG) which suggests that change in the circulating amounts sRANKL with menopause and age to constitute a compensatory mechanism in response to increased bone resorption [123] . The results of increasing levels of OPG and decreasing sRANKL/OPG ratio in osteoporotic women appear to be conflict, as OPG is a decoy receptor for RANKL so it should considered as a protective mechanism so how we can explain these results .

From the available literatures and our study we can explain these results through different approaches:

First: many years ago, the experimental data and clinical observations about OPG-sRANKL system appears however conflicting. OPG-deficient mice develop sever osteoporosis with multiple fractures and calcification of the aorta and renal arteries [129] . On the contrary in patients with osteoporosis, hypertension and cardiovascular diseases the serum levels of OPG are higher [130-134]. These finding suggest that elevated OPG may represent an insufficient compensatory self- defensive mechanism to prevent bone resorption and vascular damage.

Second: The serum levels of OPG dose not necessarily reflect the actual concentration in the bone microenvironment because tissues other than bone (e.g. lung, kidney, endothelial cells and arterial smooth muscle cells) produce OPG [135].

Third : The available ELISA assay used for the measurement of serum OPG detect both monomeric and dimeric OPG as well as OPG bound to circulating sRANKL. It's known that the OPG binding to sRANK reduce its clearance and so results in increased serum levels of OPG [125].

Fourth : sRANKL is membrane anchored molecule which can be cleaved from the cell surface as soluble sRANKL [136] . The RANKL test kits available now detect sRANKL in biological fluid such as serum. Whether the amount and activities of sRANKL are related to their membrane bound form is still uncertain.

Due to those conflict about the level of OPG bone turnover marker in osteoporotic diabetic patients makes them misleading and confusing. So other markers should be explored, in our study we explore the association of these parameter with IGF-1 (as new marker).

The correlation studies indicted a strong significant negative correlation between serum levels of IGF-1 and OPG ($r = -0.887$, $p < 0.01$, Figure 27) in postmenopausal diabetic women with osteoporosis. While its level in the same group show strong positive correlation with sRANKL ($r = 0.893$, $p < 0.01$, Figure 31) and sRANKL/OPG ratio ($r = 0.859$, $p < 0.01$, Figure 32).

In line with our results Rubin et al, 2002 found in vitro study that IGF-1 increased sRANKL and decreased OPG expression in mouse stromal cells [137]. Furthermore, they found that IGF-1 treatment to postmenopausal women decreased OPG expression [137]. Also in accordance with the obtained data Zhao et al, 2008 showed that serum IGF-1 (log transform) is negatively correlated with serum OPG and positively with sRANKL/OPG ratio and sRANKL in postmenopausal women [114].

When they divided the postmenopausal women into normal, osteopenic and osteoporotic groups, it was found that serum IGF-1 levels in osteoporotic women were more than those in normal women but no significant difference was found in OPG and RANKL among the groups [114] . These studies with our study confirmed that the IGF-1 level may act as an indicator of bone remodeling in postmenopausal women with Type2 diabetes.

Another important marker for assessing bone remodeling in diabetic patients is osteocalcin. Osteocalcin, the second most abundant protein in bone after collagen, is secreted by osteoblast and thought to participate in mineralization and calcium ion homeostasis [58] . Also experimental studies revealed for new metabolic function as hormone, being involved in blood glucose regulation, insulin secretion and in adipose tissue structure [61] .

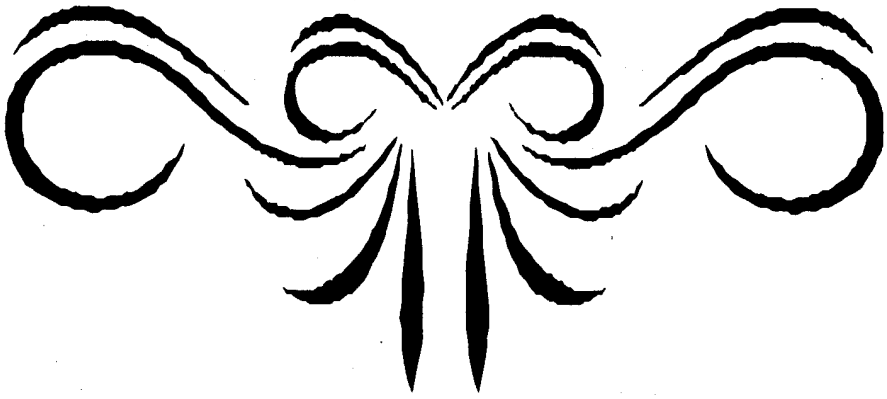
The present study indicated that serum osteocalcin level show no significant change in postmenopausal diabetic women without osteoporosis while those women with osteoporosis show a significant decline compared to healthy control non- diabetic postmenopausal women (Table 4, Figure 25).

In accordance with these results Im et al, 2008 demonstrated that the serum level of osteocalcin is lower and found to be an independent risk factor associated with glucose and Glycated hemoglobin in postmenopausal women with Type 2 diabetes [138]. Also other studies indicated that, Type1 and Type 2 diabetes are associated with lower levels of osteocalcin [138, -140].

Also our correlation studies indicated that the serum level of Osteocalcin is negatively correlated with sRANKL ($r = - 0.452$, $p< 0.012$, Figure 41) and IGF-1 ($r = - 0.419$, $p< 0.021$, Figure 29), while positively correlated with OPG in postmenopausal diabetic women with osteoporosis ($r = 0.524$, $p= 0.003$, Figure 34).

From these result we can suggest that assessing the circulating levels of IGF-1 and osteocalcin may provide a very useful information for diagnosis or even prognosis of osteoporosis in type 2 diabetic postmenopausal women because they are greatly declined with osteoporosis in those patients.

SUMMARY



Summary

Osteoporosis and type 2 diabetes mellitus (DM) are traditionally viewed as separate diseases that increase in prevalence with aging. There are accumulating evidences indicate that these diseases are interrelated by different faces. Although, osteoporosis traditionally has not been listed as a complication of diabetes, some studies were suggested a possible increased risk of osteoporosis in patients with either type 1 or type 2 DM. But this association remains controversial and conflicting, especially in type 2. Many clinical studies have investigated the association between DM and osteoporosis. Although bone mineral density (BMD) is considered as a gold standard for evaluating fracture risk in non- diabetes mellitus osteoporosis, it is not useful for assessing fracture risks in type 2 DM. Therefore, a diagnostic marker other than BMD needs to be explored. Insulin- like growth factor-1 (IGF-1) and osteocalcin seems to be two of candidate's markers. That may replace the insensitivity of BMD in assessing the osteoporosis in type 2 diabetes mellitus patients.

The aim of this study was to evaluate the relation between insulin-like growth factor-1 and markers of bone modulation (osteoprotegerin, Receptor activator nuclear kappa B, osteocalcin and bone mineral density) in postmenopausal women with and without Type 2 diabetes.
The study was conducted on 90 female divided into three groups (30 each)

- Group I: Control healthy women with age post menopause and not diagnosed as suffering from osteoporosis or diabetes.
- Group II: Type 2 diabetic postmenopausal women with osteoporosis
- Group III: Type 2 diabetic postmenopausal women without osteoporosis

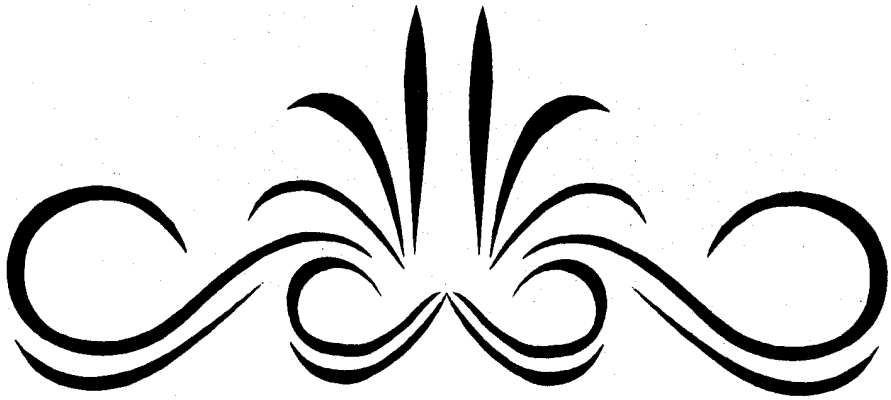
Fasting blood sample was obtained from each participant in the study to obtain plasma used for the following determinations; fasting blood glucose, glycosylated hemoglobin (HBA1C), total and ionized calcium, phosphorus, Insulin- like growth factor-1 by ELISA, OPG, Receptor activator nuclear kappa B , and osteocalcin by ELISA techniques.

The results indicated that, the diabetic patients with osteoporosis has significant decreased in Insulin- like growth factor-1 level about 61 % while the diabetic patient without osteoporosis has decreased significantly about 30 % compared with control values. The osteoprotegerin level in diabetic patients is highly elevated by 95 % in patients with osteoporosis and about 19 % in patients without osteoporosis compared to control value. The result show that, the diabetic patients without osteoporosis has slightly decreased in receptor activator nuclear kappa B level about 2% while the diabetic patients with osteoporosis has decreased significantly about 7 % compared with control values .sRANKL/OPG ratio among diabetic osteoporotic women shows decreased 49 % while the diabetic without osteoporosis shows slightly decreased 5 % compared with control values. Diabetic patients who suffer from osteoporosis shows decreased in osteocalcin level by about 27 % while diabetic patients who are not suffering from osteoporosis shows no significant change compared with control values.

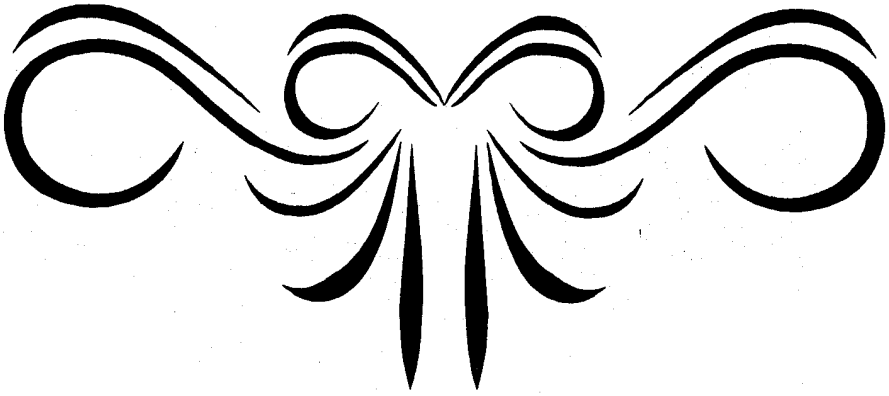
The serum level of Insulin- like growth factor-1is significantly negatively correlation with; age ($r = - 0.55$, $p< 0.0$), osteoprotegerin ($r = -0.887$, $p< 0.01$), fasting blood glucose $r = - 0.58$, $p< 0.01$), osteocalcin ($r = 0.419$, $p< 0.01$), and duration of diabetes ($r = - 0.532$, $p< 0.01$). The serum of Insulin- like growth factor-1 is significantly positively correlated with receptor activator nuclear kappa B ligand ($r = 0.893$, $p< 0.01$), and sRANKL / OPG ($r = 0.859$, $p< 0.01$).

The serum level of osteoprotegerin is significantly positively correlated with age ($r = 0.606$, $p< 0.01$). Osteocalcin ($r = 0.524$, $p< 0.01$), fasting blood glucose ($r = 0.556$, $p< 0.01$), and duration of diabetes ($r = 0.485$, $p< 0.01$). Serum level of osteoprotegerin is significantly negatively correlated with; ($r = - 0.436$, $p< 0.0$), and sRANKL ($r = - 0.939$, $p< 0.001$). Serum level of receptor activator nuclear kappa B ligand is significantly positively correlated with height ($r = - 0.415$, $p< 0.05$). Serum level of receptor activator nuclear kappa B is significantly negatively correlated with age ($r = - 0.606$, $p< 0.01$), osteocalcin ($r = - 0.452$, $p< 0.05$), fasting blood glucose ($r = - 0.543$, $p< 0.05$), and duration of diabetes ($r = - 0.42$, $p< 0.05$). In postmenopausal women type 2 diabetic women, the osteoporosis resulted in derangement in OPG/sRANKL system.

The serum level of OPG is elevated while receptor activator nuclear kappa B is declined in osteoporotic postmenopausal type 2 diabetic women. The circulating levels of osteoprotegerin and sRANKL are not useful markers for bone status in postmenopausal women. The circulating levels of Insulin- like growth factor-1 may consider as new markers for studying osteoporosis in postmenopausal type 2 diabetic women. Insulin- like growth factor-1 level is decreased in diabetic postmenopausal women but those women with osteoporosis show a great decline by about 60 %. Insulin- like growth factor-1 level in osteoporotic diabetic postmenopausal women is correlated with most bone turnover markers (osteoprotegerin, receptor activator nuclear kappa B ligand, OPG/sRANKL). Osteocalcin is another candidate for assessing osteoporosis in diabetic postmenopausal women. Osteocalcin is declined significantly only in those women with osteoporosis not without osteoporosis. Also osteocalcin is correlated with bone turnover markers and Insulin- like growth factor-1. A combination of Insulin- like growth factor-1 and osteocalcin can give a very useful information about bone status in postmenopausal diabetic women.



CONCLUSION



From our study we can conclude that:

- In postmenopausal women type 2 diabetic women, the osteoporosis resulted in derangement in OPG/sRANKL system.
- The serum level of OPG is elevated while sRANKL is declined in osteoporotic postmenopausal type 2 diabetic women.
- The circulating levels of OPG and sRANKL are not useful markers for bone status in postmenopausal women.
- The circulating levels of IGF-1 may consider as new markers for studying osteoporosis in postmenopausal type 2 diabetic women.
- IGF-1 level is decreased in diabetic postmenopausal women but those women with osteoporosis show a great decline by about 60 %.
- IGF-1 level in osteoporotic diabetic postmenopausal women is correlated with most bone turnover markers (OPG, sRANKL, OPG/sRANKL).
- Osteocalcin is another candidate for assessing osteoporosis in diabetic postmenopausal women.
- Osteocalcin is declined significantly only in those women with osteoporosis not without osteoporosis.
- Also osteocalcin is correlated with bone turnover markers and IGF-1.
- A combination of IGF-1 and osteocalcin can give a very useful information about bone status in postmenopausal diabetic women.

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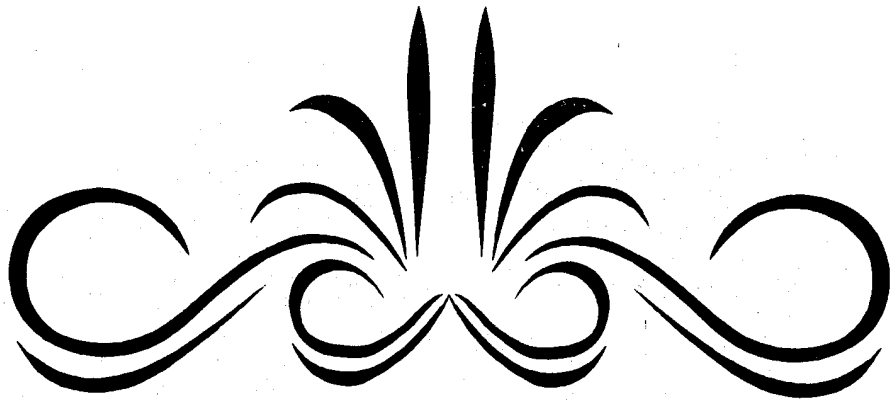
APPENDIX



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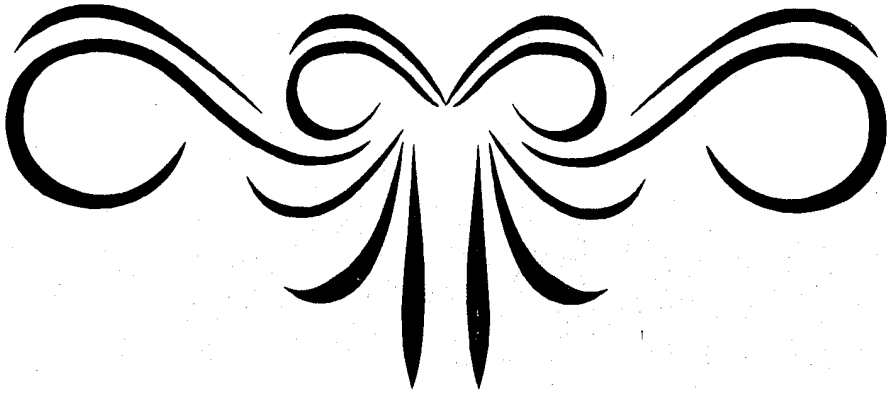

العلاقة بين عامل النمو شبيه الانسولين-١ و علامات هشاشة العظام في السيدات المريضات والغير مريضات بمرض السكر من النوع الثاني بعد سن اليأس

Relation between insulin-like growth factor-1 and markers of osteoporosis in postmenopausal women with and without Type 2 diabetes

Protocol of a thesis submitted to the	خطة بحث مقدمة الى
Medical Research Institute	معهد البحوث الطبية
University of Alexandria	جامعة الاسكندرية
in partial fulfillment of the	إيفاءا جزئيًا لشروط
requirements of the degree of	الحصول على درجة
Ph.D. of Biochemistry	**الدكتوارة فى الكيمياء الحيوية**

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BACKGROUND

Diabetes mellitus is a group of metabolic disorders characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction and failure of various organs especially the eyes, kidneys, nerves, heart and blood vessels. In type 2 diabetes (also referred to as non-insulin dependent diabetes mellitus, NIDDM) patients can still produce insulin, but do so relatively inadequately for their body's needs, particularly in the face of insulin resistance.[1]

Patients with diabetes have many and different forms of skeletal disorders, including osteopenia, osteoporosis and diffuse idiopathic skeletal hyperostosis (DISH) [2]. More than 50 years ago, it was demonstrated that diabetes mellitus could be associated with a loss of bone mass leading to osteoporosis [3].

Osteoporosis is a systemic disease of the skeleton, characterized by low bone mass and alterations in the micro-architecture of the bone tissue that lead to an increase in brittleness with the ensuing predisposition to bone fractures. [4] Osteoporosis is a "silent killer" that millions of people around the world suffer from, and it is important due to its morbidity, mortality, adverse effects on the quality of life and the extra costs imposed on the patient and the society. The increase of life expectancy and so the old age in the society in developing countries such as the Middle East has led to an increase in the prevalence of osteoporosis and its following fractures in the area.[5] However, in spite of numerous studies, the relationship between diabetes and osteoporosis remains controversial [6-7]. Type 2 diabetes has also been associated with an increased risk of fractures at any skeletal site. The risk of hip fractures is increased in type 2 diabetes, although to a lesser magnitude, with risks varying from 1.5- to 2.8-fold [8]. It seems that the effects of type 2 diabetes on bone metabolism are less clear. There are conflicting study findings; increased, decreased, or similar body mass densities (BMDs) have been reported among type 2 diabetic patients as compared with healthy subjects [9]. Also, it was reported that women with type 2 diabetes had a higher risk of bone fracture than women without diabetes after adjustment for multiple risk factors. [10]

Osteoporosis is common disease seen in postmenopausal women, resulting in fragile and weak bones highly susceptible to fractures of hips, spine and wrist [11]. However, the combined effect of menopause and type 2 diabetes on the incidence of osteoporosis and bone density need further study. The main player of bone metabolism and signaling must be involved in any study concerning with osteoporosis.

The insulin like growth factor-1 (IGF-1) provides the main stimulus for bone growth by activating the osteoblast differentiation program, stimulating chondrocyte proliferation at the growth plate, and modulating tubular reabsorption of phosphate and 25-hydroxyvitamin D3–α-hydroxylase activity in the kidney [12]. Studies on human have indicated a correlation between serum IGF-1 levels and bone mineral density [13]. The effect of IGF-1 on bone remodeling may be mediated by the osteoprotegerin/receptor-activator of the nuclear factor-kB ligand (RANKL) system [14].

Osteoprotegerin (OPG) is a member of the tumor necrosis factor receptor (TNFR) family. In mice, OPG mRNA expression has been demonstrated in numerous tissues including calvaria, skin, liver, lung and heart [15]. OPG is a circulating secretory glycoprotein without a transmembrane domain, and it works as a decoy receptor for the receptor-activator of the nuclear factor-kB ligand (RANKL) [16]. RANKL and OPG are a key agonist/antagonist cytokine system, regulating important aspects of osteoclast biology, such as differentiation, fusion, survival, activation and apoptosis [15]. RANKL increases the pool of active osteoclasts by activating its specific receptor RANK located on osteoclastic cells, thus increasing bone resorption, whereas OPG, which neutralizes RANKL, has the opposite effect. Alterations or abnormalities of the RANKL/OPG system have been implicated in different metabolic bone diseases characterized by increased osteoclast differentiation and activation, and by enhanced bone resorption, including glucocorticoid-induced osteoporosis, hyperparathyroidism, Paget's disease, rheumatoid arthritis and bone malignancies [17-18].

Another important player in osteoporosis is osteocalcin which is one of the osteoblast-specific proteins, has several hormonal features and is secreted in the general circulation from osteoblastic cells. Recently it was suggested that osteocalcin is important for not only bone metabolism but also glucose and fat metabolism [19].

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A bone mineral density test (BMD), a non-invasive and painless test, is the best way to determine bone health. BMD tests can identify osteoporosis, determine the risk for fractures and monitor the response to an osteoporosis treatment. Different BMD tests may measure the hip, spine, wrist, finger, shin bone or heel [20].

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AIM OF THE WORK

The present study will aim to evaluate the relation between insulin-like growth factor-1 and markers of bone modulation (osteoprotegerin, RANKL, osteocalcin and bone mineral density) in postmenopausal women with and without Type 2 diabetes.

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Subjects and Methods

Study groups:

After the acceptance of the ethical community of the Medical Research Institute, the studies will be conducted on 90 female divided into three female group (30 each)

- Group I: Control healthy women with age menopause and not diagnosed as suffering from osteoporosis
- Group II: Type 2 diabetic postmenopausal women without osteoporosis
- Group III: Type 2 diabetic postmenopausal women with osteoporosis

Exclusion Criteria:

- ❖ Steroid users or having disease conditions that could play a significant role in the development of osteoporosis such as hyperparathyroidism, or hyperthyroidism
- ❖ Diseases that may affect bone metabolism: chronic drug use, chronic gastrointestinal disease, renal or hepatic impairment
- ❖ Chronic treatment with antacids, estrogen, adrenal or anabolic steroids, anticonvulsants, anticoagulants, or pharmacologic doses of Vitamin A supplements 6 months prior to the study.
- ❖ Pathological fractures of other origin, i.e., tumor, tumor-like lesions as well as focal demineralization visualized on radiographs
- ❖ History of unstable cardiovascular disease or uncontrolled hypertension

Clinical examination:

An informed concent will be taken from all patients included in this study (appendix).

All healthy subjects have no records of osteoporosis and matched the inclusion criteria were set by the researcher in the control group.

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Biochemical examination:

1. Determination of level of fasting blood glucose. [21]
2. Determination of level of calcium (total and ionized). [22]
3. Determination of level of HbA_1c. [23].
4. Determination of level of osteoprotegerin (OPG). [24]
5. Determination of receptor activator of nuclear factor-kappa B ligand (RANKL). [25]
6. Determination of osteocalcin. [26]
7. Determination of bone mineral density (DEXA). [27]
8. Determination of insulin growth factor -1. [28]
9. Determination of organic phosphate. [29]

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ANALYSIS OF RESULTS

The result of this study will be tabulated and statistically analyzed using ANOVA, paired-t test and Chi-square test.

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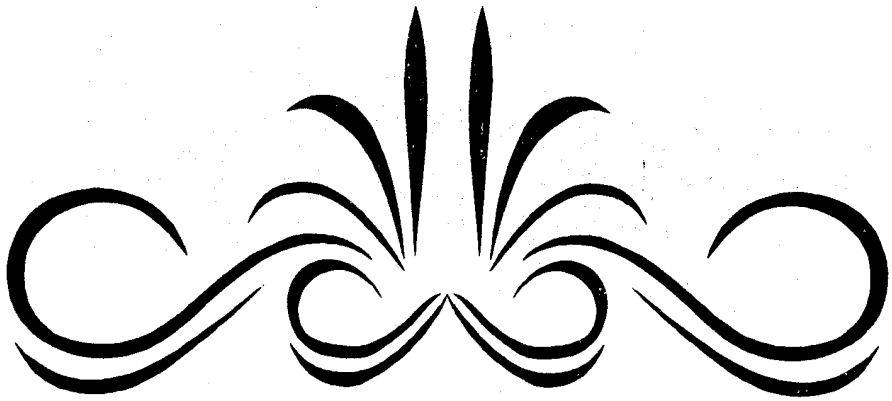
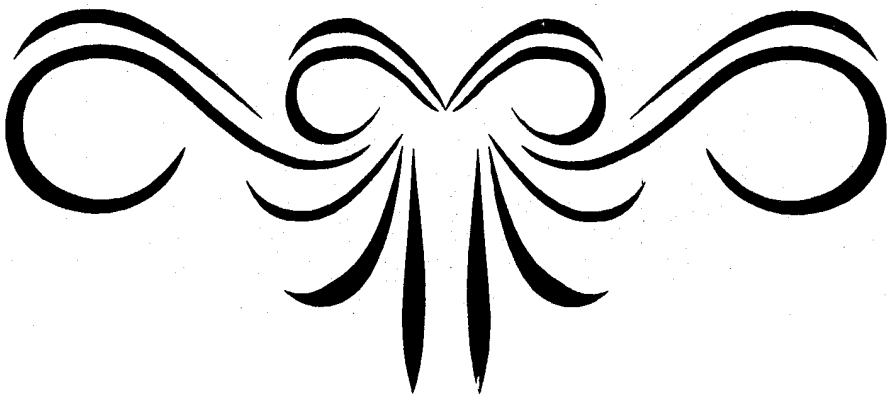
ARABIC SUMMARY



الملخص العربى



الملخص

ينظر عادة الى هشاشة العظام ومرض السكر من النوع الثانى علي انهما مرضين منفصلين تزداد الاصابة بهما مع الشيخوخة . هناك ادلة كثيرة تشير الى ان هذان المرضان مترابطان علي وجوة مختلفة. بعض الدراسات رجحت زيادة مخاطر الإصابة بمسامية العظام في المرضى الذين يعانون من مرض السكر من النوع الاول أو الثانى . لكن هذة العلاقة لا تزال مثيرة للجدل ومتعارضة في بعض الاحيان، وخصوصا في النوع الثانى من الداء السكري . برغم من ان كثافه العظام تعتبر هي المعيار الذهبى لتقيم مخاطر الاصابة بالكسور و بمسامية العظام فى الافراد الذين لا يعانون من مرض السكرالا انة لا يعتبر مفيدا فى تقيم مخاطر الاصابة فى مرضى السكرى من النوع الثانى . ولهذا السبب نحتاج الى استكشاف طرق تشخصية اخرى بدلا من قياس كثافه العظام .عامل النمو شبية الانسولين-١ و الاستيوكالسين كمرشحين لان يكونوا من العلامات التى يمكن ان تشخص المرض .

هذا البحث يهدف إلى دراسة العلاقة بين عامل النمو شبيه الانسولين-١ و دلالات بمسامية العظام م في السيدات المريضات والغير مريضات بمرض السكر من النوع الثاني بعد سن الاياس .

وقد أجريت هذه الدراسة على ٩٠ سيدة مقسمين الى ثلاث مجموعات كل مجموعة تتكون من ثلاثين سيدة وهم:

١- المجموعة الاولى: النساء بعد سن الاياس لا يعانين من مرض سكر ولا من بمسامية العظام (المجموعة الضابطة)

٢- المجموعة الثانية: النساء بعد سن الاياس يعانين من مرض سكر النوع الثانى بمسامية العظام

٣- المجموعة الثالثة :النساء بعد سن اليأس يعانين من مرض سكر النوع الثانى ولا يعانين من بمسامية العظام

تم اخذ عينة دم صائم من كل سيدة للحصول على البلازما لتحديد كلا من (سكر الصائم ، هيموجلوبين متسكر، الكالسيوم كلى و متأين والفوسفات). كذلك تم تعيين كلا من عامل النمو شبيه الأنسولين -١ والاستوبروتجيرن (OPG) و sRANKL و الاستوكالسين و جميعهم تم قياسهم بطريقة ELISA .

اظهرت النتائج فى المجموعة الثانية نقص ذو دلالة احصائية بنسبة ٦١% فى عامل النمو شبية الانسولين -١ بينما فى المجموعة الثالثة كان هناك نقص ذو دلالة احصائية بنسبة ٣٠% بالمقارنة بمجوعة الضابطة . كان مستوى OPG مرتفع جدا بنسبة ٩٥% فى المجموعة الثانية بيمنا كان فى المجموعة الثالثة ١٩% مقارنة بالعينة الضابطة. وايضا اظهرت النتائج ان السيدات اللواتى لايعانين من مسامية العظام لديهن نقص بسيط جدا فى مستوى RANKL بنسبة ١.٥% فى حين السيدات اللواتى يعانين من مسامية العظام كان هناك نقص طفيف بنسبة ٨% فى مستوى sRANKL مقارنة بالعينة الضابطة . اما نسبة sRANKL/OPGفقد اظهرت النتائج ان هناك نقص ذو دالالة احصائية عند السيدات اللواتى يعانين من مسامية عظام بنسبة ٤٩ % بينما السيدات اللواتي لا يعانين من مسامية العظام كان النقص بنسبة ٥% مقارنة بالعينة الضابطة . السيدات اللواتى يعانين من هشاشة العظام كان عندهن نقص فى مستوى الاوستوكالسين بنسبة ٢٧% بينما السيدات اللواتى لا يعانين من مسامية العظام لم يكن هناك اى تغير ملوحوظ مقارنة بالعينة الضابطة. وجد فى المجموعة الثانية علاقة عكسية بين كلا من مستوى النمو شبيه الانسولين – ١ و العمر ، OPG ، سكر صائم ، مستوى الاستوكالسين و فترة المرضية لمرض السكر. بينما كان هناك علاقة طردية بين مستوى النمو شبيه الانسولين – ١ وكلا من sRANKL ، sRANKL/OPG . بينما كان هناك علاقة طردية بين OPG وكلا من العمر ، الاستوكالسين ، سكر صائم و فترة المرضية لمرض السكر . بينما وجد ان هناك علاقة طردية بينOPG مع الطول و عكسية بين sRANKL و كلا من OPG و الطول و العمر و الاستوكالسين و سكر الصائم و الفترة الزمنية لمرض .

من هذة الدراسة يمكننا استنتاج الاتي:

- ان مسامية العظام عند السيدات كانت نتيجة عدم انتظام فى العلاقة بين sRANKL/OPG عند السيدات المصابات بالسكر النوع الثانى بعد سن الاياس حيث وجدنا ارتفاع في مستوى OPG في حين ان مستوى sRANKL قد اخفض طفيفا فى مجموعة السيدات الاتى يعانين من السكر ومسامية العظام .
- لا يعتبر OPG و sRANKL من الدلالات المفيدة فى تقييم حالة العظام عند السيدات بعد سن اليأس بينما يعتبر عامل النمو شبية الانسولين -١ من الدلالات المهمة فى تقيم بالمسامية العظام عند السيدات المصابات بالمسامية العظام والسكر من النوع الثانى حيث ان اظهر نقصان كبير جدا بنسبة ٦٠% عند السيدات المصابات بالمسامية العظام و السكر من النوع الثانى .

- الاستوكالسين من الممكن ان يعتبر من الدلالات المهمة ايضا فى تقيم بالمسامية العظام فقد وجدنا ان مستواة يقل فقط فى مجموعة السيدات المصابات بالمسامية العظام والسكر من النوع الثانى وليس عند مجموعة السيدات غير المصابات بالمسامية العظام .
- وأيضا وجدنا أن هناك علاقة بين عامل النمو شبيه الأنسولين -١ و الاستوكالين .ويعتبر الاثنين معا من الدلالات المفيدة عن حالة العظام في السيدات بعد السن الاياس .

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٢٠١١