Study of Interleukin-6, Parathyroid Hormone and Estradiol in Relation to Bone Changes in Postmenopausal Females

THESIS

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To my parents

My husband

&

My child

CONTENTS

List of Abbreviations

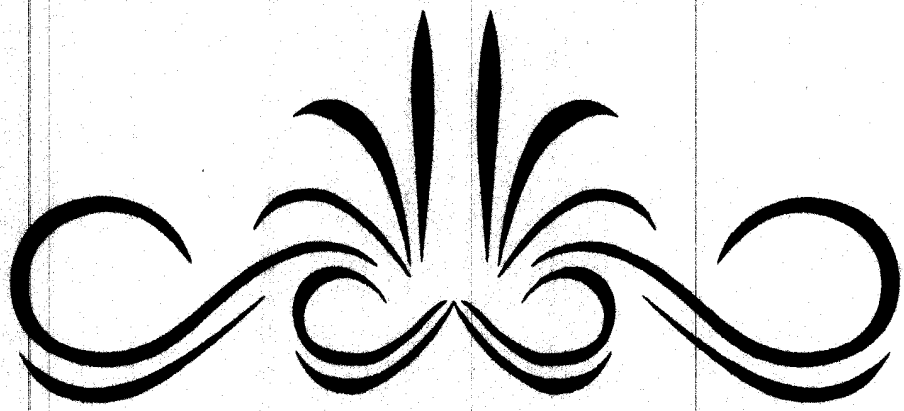
Δ A / min	:	Change in absorbance per minute
a.a.	:	Amino acids
ACTH	:	Adrenocorticotropic hormone
ALP	:	Total alkaline phosphatase
ALT	:	Alanine aminotransferase
BMD	:	Bone mineral density
BMI	:	Body mass index
BMP-2	:	Bone morphogenic protein
CAMP	:	Cyclic adenosine monophosphate
CD	:	Cluster of differentiation
CNTF	:	Ciliary neurotropic factor
CRP	:	C-reactive protein
CSF-1	:	Colony stimulating factor-1
$1,25(OH)_2D_3$	:	1,25 dihydroxycholecalciferol
DNA	:	Deoxy-ribonucleic acid
DXA	:	Dual x-ray absorptiometry
E_3	:	Estriol
E_1	:	Estrone
E_1S	:	Estrone sulfate
E_2	:	17-β estradiol
ECF	:	Extracellular fluid
ER	:	Estrogen receptor
FGF	:	Fibroblast growth factor
FSH	:	Follicle-stimulating hormone
GH	:	Growth hormone

GM-CSF	:	Granulocyte-macrophage colony stimulating factor
gp	:	Glycoprotein
HRPO	:	Streptavidin-horseradish peroxidase
IFNα	:	Interferon α
IFNβ	:	Interferon β
IGF	:	Insulin-like growth factor
IgG	:	Immunoglobulin G
Igs	:	Immunoglobulins
IL	:	Interleukin
IL-6Rα	:	Interleukin-6 receptor α
iPTH	:	Intact parathyroid hormone
ISE	:	Ion selective electrode
JAKs	:	Janus kinases
KD	:	Kelodalton
LD	:	Lactate dehydrogenase
LH	:	Luteinizing hormone
LIF	:	Leukemia inhibitory factor
M-CSF	:	Macrophage-colony stimulating factor
NAD	:	Nicotinamide adenine dinucleotide
NADH	:	Reduced form of nicotinamide adenine dinucleotide
NFκB	:	Nuclear factor kappa-B
NF-IL-6	:	Nuclear factor of interleukin-6
ODF	:	Osteoclast differentiation factor
OPG	:	Osteoprotegerin
OSM	:	Oncostatin M
PDGF	:	Platelet-derived growth factor
PE_2	:	Prostaglandins E_2

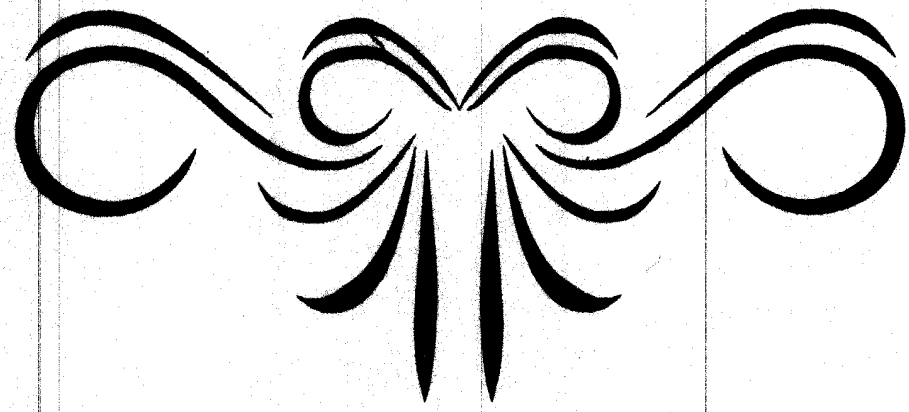
PGI	:	Biglycan
PTH	:	Parathyroid hormone
PTHrP	:	Parathyroid hormone related protein
RANK	:	Receptor activator of nuclear factor
RANKL	:	Ligand of receptor activator of nuclear factor
RMAPK	:	Ras-mitogen activated protein kinase
SHP_2	:	SH_2-domain-containing tyrosine phosphatase
STATS	:	Signal transducers and activators of transcription
TGF-β	:	Transforming growth factor beta
TNF	:	Tumor necrosis factor
TRANCE	:	TNF-related activation induced cytokine

List of Tables

List of Figures



INTRODUCTION



INTRODUCTION

Bone Structure and Remodeling

Bone structure

Bone is a special form of connective tissue. It consists of cells and an extracellular mineralized matrix (35% organic and 65% inorganic). Ninety percent of the organic component is type I collagen. The remainder includes many non-collagen products of the osteoblasts, such as osteocalcin, osteonectin, and proteoglycans. The mineral is present mainly as a complex mixture of calcium and phosphate in the form of hydroxyapatite.[1,2]

Two anatomical types of bone may be defined: the trabecular or cancellous type and the cortical or compact type.[2] The proportion of these types differs from one bone to another; for example, vertebral bodies are predominantly trabecular, and the shafts of the long bones are cortical. Trabecular bone contains more metabolically active surfaces in a given volume than cortical bone. Cellular activities take place on the surfaces of trabecular bone and through resorbing channels (cutting cones) in cortical bone.[1]

Most bones are made up of an outer layer of compact bone surrounding trabecular bone and, in many instances, a bone marrow cavity. Trabecular or spongy bone is made up of bone spicules separated by spaces. In spongy bone, nutrients diffuse from bone extracellular fluid (ECF) into the trabeculae, but in compact bone, nutrients are provided via

Haversian canals, which contain blood vessels. Around each Haversian canal, collagen is arranged in concentric layers, forming cylinders called osteons or Haversian systems.[2]

Bone cells

Conventional histological sections of bone demonstrate four types of bone cells which are clearly different: osteoblasts, osteocytes, lining cells and osteoclasts.[1]

(A) Osteoblasts

Osteoblasts are bone-forming cells derived from stromal cell precursors in the bone marrow. They secrete large quantities of type I collagen, other bone matrix proteins, and alkaline phosphatase.[2]

This bone forming osteoblasts synthesize a number of other proteins that are incorporated into the bone matrix, including osteocalcin and osteonectin, which constitute 40% to 50% of the noncollagenous proteins of bone. Other osteoblast-derived proteins include glycosaminoglycans, which are attached to one of two small core proteins: PGI (or biglycan) and decorin; the latter has been implicated in the regulation of collagen fibrillogenesis. A number of other minor proteins such as osteopontin, bone sialoprotein, fibronectin, vitronectin, and thrombospondin serve as attachment factors that interact with integrins.[3]

Osteoblasts are thought to regulate the local concentrations of calcium and phosphate in such a way as to promote the formation of hydroxyapatite.[4]

Osteoblasts express relatively high amounts of alkaline phosphatase, which is anchored to the external surface of the plasma membrane. Alkaline phosphatase has been long thought to play a role in bone mineralization.[5]

(B) Osteocytes

Osteocytes are the most abundant cell type in bone. There are 10 times as many osteocytes as osteoblasts. Osteocytes are regularly spaced throughout the mineralized matrix and communicate with each other and with cells on the bone surface via multiple extensions of their plasma membrane that run along the canaliculi.[6]

Osteocytes evidently sense changes in interstitial fluid flow through canaliculi (produced by mechanical forces) and detect changes in the levels of hormones, such as estrogen and glucocorticoids, that influence their survival and that circulate in the same fluid.[7,8] Therefore, disruption of the osteocyte network is likely to increase bone fragility.[3]

(C) Lining cells

The surface of normal quiescent bone (i.e. bone that is not undergoing remodeling) is covered by a 1-2 μm thick layer of unmineralized collagen matrix on top of which there is a layer of flat and elongated cells. These cells are called lining cells and are descendents of osteoblasts.[9] Osteoclasts can not attach to the unmineralized collagenous layer that covers the surface of normal bone. Therefore, other cells, perhaps the lining cells, secrete collagenase, which removes this matrix before

osteoclasts can attach to bone. It has been proposed that targeting of osteoclast precursors to a specific location on bone depends on a "homing" signal given by lining cells; and that lining cells are instructed to do so by osteocytes, the only bone cells that can sense the need for remodeling at a specific time and place.[10]

(D) Osteoclasts

Osteoclasts are multinuclear cells that erode and resorb previously formed bone. They are derived from hematopoietic stem cells via monocytes. They become attached to bone via integrins in a membrane extension called the sealing zone. This creates an isolated area between the bone and a portion of the osteoclast.[2]

Proton pumps, which are H^+ dependent ATpase, then move from endosomes into the cell membrane apposed to the isolated area, and they acidify the area to pH 4. The acidic pH dissolves hydroxyapatite, and acid proteases secreted by the cell dissolve collagen, forming a shallow depression in the bone.[2]

Osteoclasts are controlled by systemic and local hormones. Calcitonin directly inhibits the osteoclasts, and suppresses the generation of new osteoclasts. Bone resorption is increased by parathyroid hormone (PTH) and 1,25 dihydroxycholecalciferol [1,25-$(OH)_2D_3$]. Since the osteoclast contains no receptors to either of these hormones it is proposed that their resorbing effect is mediated via the osteoblast. Figure (1)[1]

The molecular mechanism of the dependency of osteoclastogenesis on cells of the mesenchymal lineage has been elucidated with the discovery of three proteins. Two of these proteins are membrane bound cytokine-like molecules: the receptor activator of nuclear factor-$_k$B (NF_kB) (RANK) and the RANK-ligand (RANK-L). Other names for RANK-L are osteoprotegerin ligand (OPG-L) and TNF-related activation induced cytokine (TRANCE). RANK is expressed in hematopoietic osteoclast progenitors, while RANK-ligand is expressed in committed preosteoblastic cells and T-lymphocytes.[11,12]

RANK-ligand binds to RANK with high affinity. This interaction is essential and, together with M-CSF (macrophage colony stimulating factor), is sufficient for osteoclastogenesis. 1,25-$(OH)_2D_3$, PTH, parathyroid hormone related protein (PTHrP), glycoprotein 130 (gp130), activating cytokines (e.g. IL_6-IL_{11}) and IL-1 induce the expression of the RANK-ligand in stromal / osteoblastic cells.[13,14] Osteoprotegerin (OPG), the third of the three proteins, unlike the other two, is a secreted disulfide-linked dimeric glycoprotein.[15] OPG has an inhibitory effect on osteoclastogenesis by competing with the binding of RANK / RANK-L.[3]

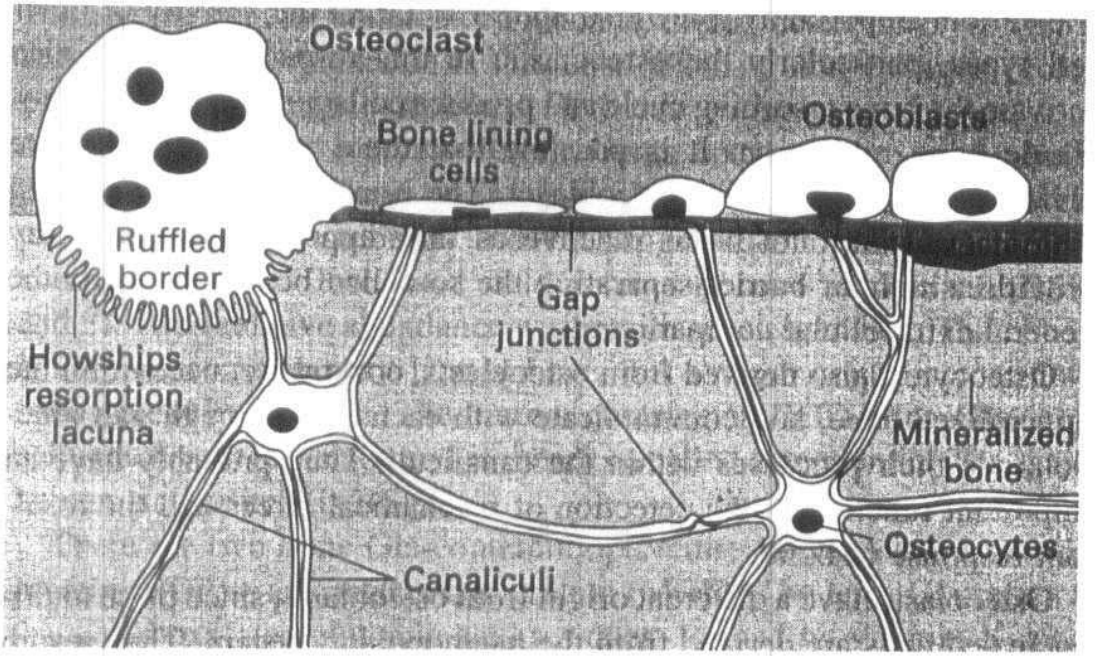


Fig. (1): A diagram showing the structure of bone and the relationship of the different cell types.[1]

Bone Modeling and Remodeling

The skeleton is a metabolically active organ that undergoes continuous remodeling throughout life. This remodeling is necessary both to maintain the structural integrity of the skeleton and to subserve its metabolic functions as a storehouse of calcium and phosphorus.[16]

Throughout life, physiological remodeling, removal, and replacement of bone occurs roughly at the same location without affecting the shape or the density of the bone.[17]

Skeletal remodeling can be triggered by changes in mechanical forces or microdamage and by hormonal responses to changes in calcium and phosphorus supplies.[18]

Modeling and remodeling do not result from the activity of a single type of cell (osteoclast or osteoblast) or a single cell function (resorption or formation of bone). Instead, they result from coordinated resorption and formation of bone over extensive regions of bone and prolonged periods.[19]

Bone is initially formed by "modeling", that is, the deposition of mineralized tissue at developmentally determined sites.[16]

"Remodeling" of bone begins early in fetal life, and once the skeleton is fully formed in young adults almost all of the metabolic activity is in this form. The bone remodeling cycle[20] involves a series of highly regulated steps that depend on the interactions of two cell lineages, the

mesenchymal osteoblastic lineage and the hematopoietic osteoclastic lineage. The initial "activation" stage involves the interaction of osteoclast and osteoblast precursor cells. This leads to the differentiation, migration, and fusion of the large multinucleated osteoclasts. These cells attach to the mineralized bone surface and initiate resorption by the secretion of hydrogen ions and lysosomal enzymes, particularly cathepsin K, which can degrade all the components of bone matrix, including collagen, at low pH. The attachment of osteoclasts to bone may require specific changes in the so-called "lining cells" on the bone surface, which can contract and release proteolytic enzymes to uncover a mineralized surface.[16]

Osteoclastic resorption produces irregular scalloped cavities on the trabecular bone surface, called Howship lacunae, or cylindrical Haversian canals in cortical bone. Once the osteoclasts have completed their work of bone removal, there is a " reversal" phase during which mononuclear cells, which may be of the macrophage lineage, are seen on the bone surface. The events during this stage are not well understood, but they may involve further degradation of collagen, deposition of proteoglycans to form the so-called cement line, and release of growth factors to initiate the formation phase. During the final "formation" phase of the remodeling cycle, the cavity created by resorption can be completely filled in by successive layers of osteoblasts, which differentiate from their mesenchymal precursors and deposit a mineralizable matrix.[16]

Regulation of Bone Remodeling

Bone-cell function is regulated at both the systemic (Table 1)[16] and the local levels (Table 2).[16] The genome and systemic hormones control bone cell function throughout the skeleton. Exercise can have both systemic and local effects on bone, and molecules released from bone matrix and bone cells, including cytokines and prostaglandins, exert local effects on bone cells.[17]

A- Systemic regulators of bone

The metabolic functions of the skeleton are served in large part by three major calcium regulating hormones, parathyroid hormone (PTH), 1,25-dihydroxyvitamin D and calcitonin.[18]

Parathyroid hormone (PTH) regulates serum calcium concentration. It is a potent stimulator of bone resorption and has biphasic effects on bone formation. There is an acute inhibition of collagen synthesis with high concentrations of PTH, but prolonged intermittent administration of this hormone produces increased bone formation, a property for which it is being explored clinically as an anabolic agent.[21]

Within bone, PTH promotes osteoclastic resorption of bone by stimulating osteoclast precursors to differentiate into osteoclasts and then resorb bone actively.[22,23] It also may stimulate osteoclasts to increase the size and volume of their ruffled border; causing bone-lining cells to retract, allowing osteoclasts to gain access to the mineralized surface of the bone, and have inhibitory effects on osteoblasts.[23,24]

Plasma PTH tends to increase with age, and this may produce an increase in bone turnover and a loss of bone mass, particularly of cortical bone.[24]

1,25-dihydroxyvitamin D has its greatest effect on intestinal calcium and phosphate absorption, but it may also have direct effects on bone by influencing both resorption of bone and mineralization of matrix.[25-29]

Other systemic hormones are important in regulating skeletal growth:

Growth hormone, acting through both systemic and local insulin-like growth factor (IGF) production, can stimulate bone formation and resorption.[30]

Glucocorticoids are necessary for bone cell differentiation during development, but their greatest postnatal effect is to inhibit bone formation.[31] This is the major pathogenetic mechanism in glucocorticoid-induced osteoporosis. Indirect effects of glucocorticoids on calcium absorption and sex hormone production may, however, increase bone resorption.[16]

Thyroid hormones: Thyroxine and triiodothyronine can also stimulate bone resorption and formation and are critical for maintenance of normal bone remodeling.[32]

Sex steroids (androgens and estrogen): Sex hormones are important in maintaining normal bone turnover. Androgens accelerate growth and somatic development including skeletal maturation.[33] Probably the most important systemic hormone in maintaining normal bone turnover is estrogen.[34] Estrogen has been shown to prevent parathyroid hormone mediated bone resorption and to stimulate renal 1-α hydroxylase activity. Estrogen deficiency leads to an increase in bone turnover in which resorption outstrips formation, with a resultant decrease in bone mass.[35-38]

Calcitonin lowers the levels of serum calcium by inhibiting osteoclastic resorption of bone.[39-41] Calcitonin may also cause osteoclasts to withdraw from the surface of the bone and to divide into mononuclear cells. The effects of calcitonin appear to be short-term; thus its role in the physiology of normal bone remains unclear.[17]

Table (1): Systemic regulation of bone remodeling[16]

	Bone resorption	Bone formation
PTH	↑[a]	↑(↓)[b]
1,25$(OH)_2$ vitamin D	↑	↑ (↓)[b]
Calcitonin	↓	?
Estrogen	↓	(↓)[c]
Androgen	?	↑
Growth hormone/IGF	↑	↑
Thyroid hormone	↑	↑
Glucocorticoids	↑[d]	↓

[a]↑ increase; ↓ decrease; ? not known

[b]PTH and vitamin D decrease collagen synthesis in high doses.

[c]Estrogen decreases bone formation by decreasing remodeling, but formation is decreased less than resorption and bone mass increases.

[d]Glucocorticoids may increase resorption indirectly by inhibiting intestinal calcium absorption and sex hormone production.

B- Local bone regulators

Bone is a storehouse for local growth regulatory factors known as cytokines. They are termed bone remodeling units as they control bone formation and resorption through their effects on osteoblasts and osteoclasts respectively.[42-47]

Among the various types of cytokines, four main categories are mainly involved with bone modeling and remodeling, namely interleukins, tumour necrosis factor family, colony stimulating factors and growth factors. The cytokines that are involved in bone resorption include interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-11 (IL-11), colony stimulating factors (CSFs) and tumour necrosis factor (TNF) family.[42-50] On the other hand, insulin like growth factor (IGF) system, transforming growth factor (TGF) family, fibroblast growth factor (FGF) and platelet derived growth factor (PDGF) stimulate mainly osteoblastic activity, resulting in enhanced bone formation.[42-51]

In general, most of the hormones and cytokines that inhibit osteoclastic activity act directly on osteoclasts. In contrast, most of the hormones and cytokines that stimulate osteoclastic activity act indirectly through osteoblasts and stromal cells.[51,52]

Prostaglandins, like cytokines, may influence local bone-cell activity.[23,28,53,54] These fatty acids can modulate a variety of processes including inflammation, blood flow, and ion transport across cell

membrane. They may have an initial inhibitory effect on osteoclasts, but their predominant long-term effect is to stimulate the resorption of bone by increasing the proliferation and formation of osteoclasts.[53,54] Figure (2)[19]

Table (2): Local factors acting on the skeleton[16]

• Cytokines that may cause bone loss: IL-1, TNF, [a]IL-6, IL-11, and ODF.
• Cytokines that may prevent bone loss: IL-4, IL-13, IL-18, IFN, OPG, and IL-1ra.
• Colony-stimulating factors: M-CSF and GM-CSF.
• Prostaglandins, leukotrienes, and nitric oxide.
• Growth factors: IGF, TGFβ, FGF, PDGF, and PTHrP

[a]TNF, tumor necrosis factor; ODF, osteoclast differentiation factor; IFN, interferon; M-CSF, macrophage colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; TGFβ, transforming growth factor-β; FGF, fibroblast growth factor; PDGF, platelet derived growth factor; PTHrP, PTH-related protein; IL-1ra, Interleukin-1 receptor antagonist.

It was reported that down-regulation of OPG expression and upregulation of RANKL expression may be one of the mechanisms for the stimulatory effects of glucocorticoids, PTH, 1,25-$(OH)_2D_3$, prostaglandins and interleukin-1 on osteoclastogenesis. Figure (3)[54]

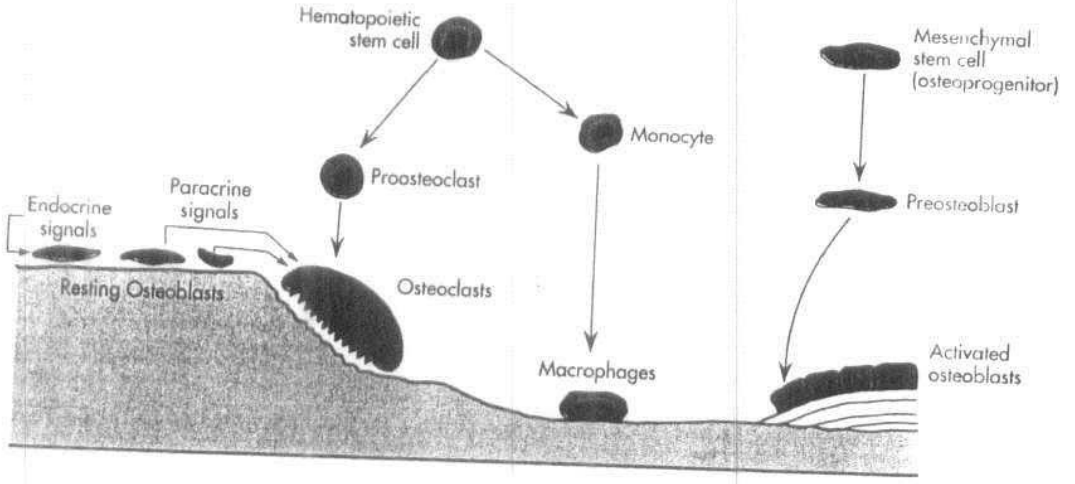


Fig. (2): Process of bone remodeling. Endocrine signal to resting osteoblasts generate local paracrine signals to nearby osteoclasts and osteoclast precursors. The osteoclasts resorb an area of mineralized bone, and local macrophages complete the clean-up of dissolved elements. The process then reverse to formation as osteoblast precursors are recruited to the site and differentiate into active osteoblasts. These lay down new organic matrix and mineralize it. Thus, new bone replaces the previously resorbed mature bone.[19]

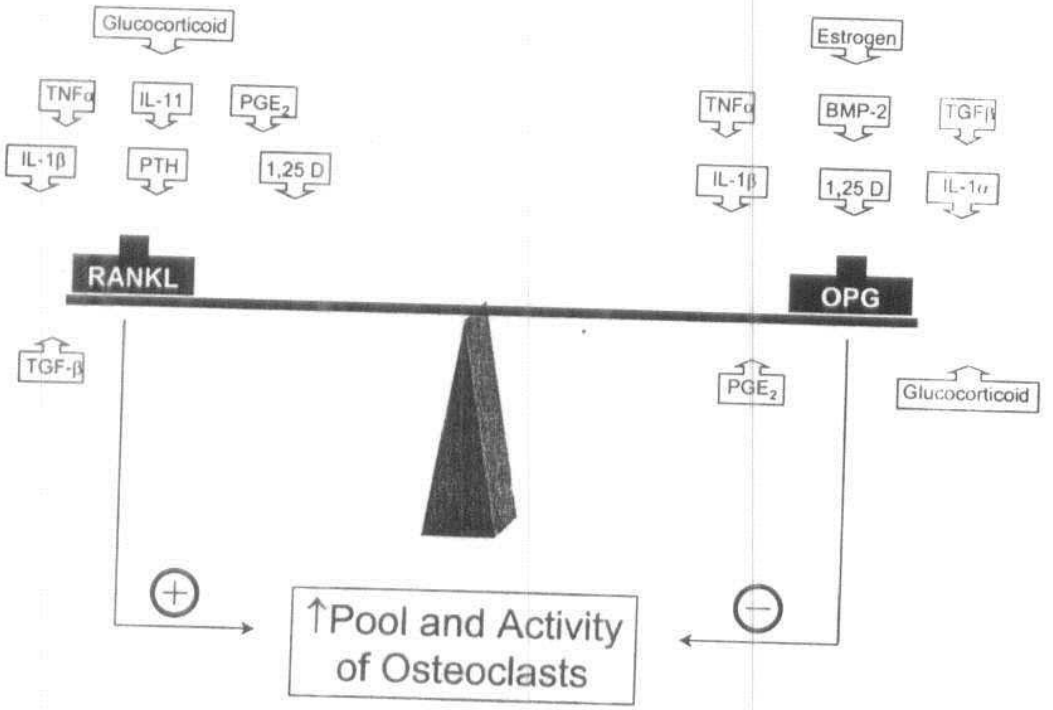


Figure (3): Diagramatic representation of the influence of RANKL and OPG on osteoclast number and activity. RANKL will tip the balance towards increased osteoclast number and activity whereas increase in OPG will oppose this effect. The hormones and cytokines scattered around the ends of the balance beam will tip the balance in the direction indicated by the arrows.[54]

Parathyroid Hormone (PTH)

PTH is secreted by chief cells of the parathyroid glands which are responsible for its synthesis, storage and secretion.[17]

Synthesis

Pre-pro PTH is the first molecule produced in the biosynthesis of PTH.[55] It is a peptide that is 115 amino acid (a.a.) in length, with "pre", "pro" and PTH segments of the molecule containing 25, 6, and 84 a.a, respectively. The N-terminal, "pre" segment is involved in the binding of polyribosomal complex to the endoplasmic reticulum and in the transporting of 'pre-pro PTH molecule across the membrane in the cisternae. This hydrophobic leader sequence is enzymatically cleaved, yielding pro PTH with 90 a.a. The N-terminal hexapeptide is promptly enzymatically removed producing intact PTH. After packaging into secretory granules by the golgi apparatus, PTH can be secreted, stored or degraded intracellularly.[56]

Biological activity resides in the first third or N-terminal region of intact PTH. The middle portion of the molecule is quite immunogenic because of its hydrophobicity and species specificity. Figure (4)[56]

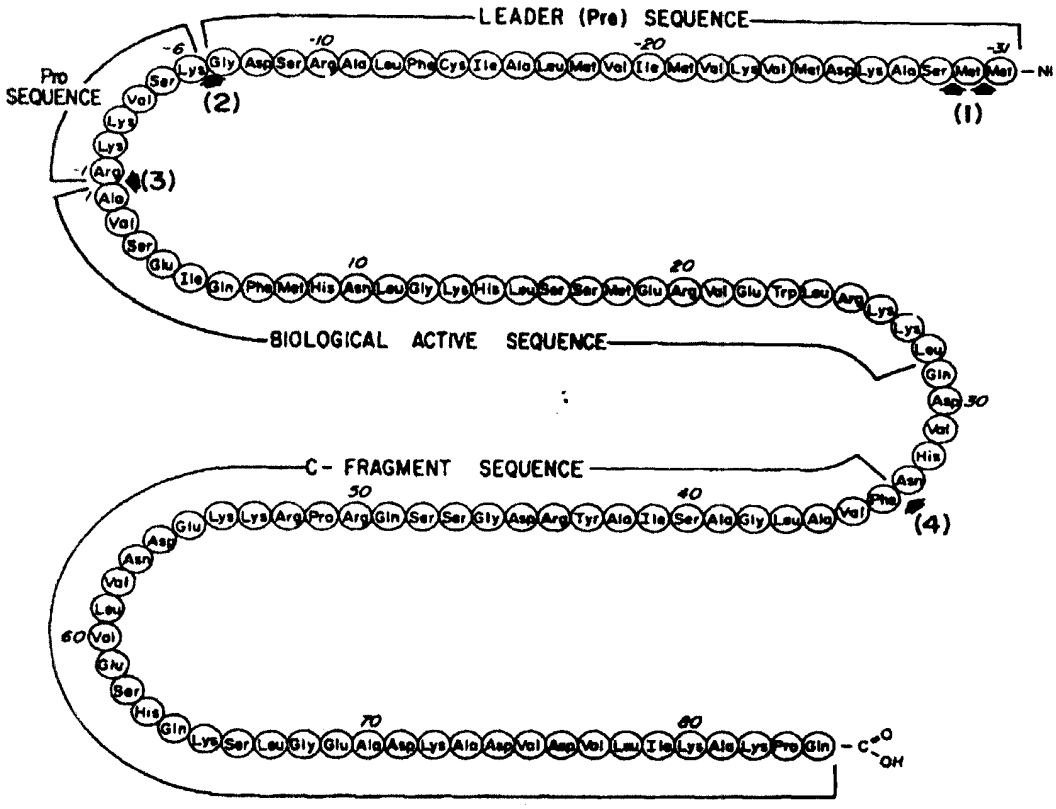


Fig. (4): Amino acid sequence of preproparathyroid hormone. Arrows indicate the sites of cleavage by proteases to remove the N-terminal methionines (1) the leader (pre) sequence (2), and the pro sequence (3), producing intact PTH (1-84). Cleavage at position 4 produces inactive carboxyl (C)-terminal fragments.[56]

Secretion

The concentration of free calcium in blood or extracellular fluid is the primary regulator of PTH secretion.[57,58] The effect of calcium on PTH secretion is mediated by binding to a free calcium-sensing receptor that activates intracellular events leading to release of free calcium from intracellular stores.[59] This rise in intracellular free calcium modulates PTH secretion and biosynthesis and represents a negative feedback loop.[56]

Magnesium, vitamin D, phosphate, and catecholamines have been reported to influence the secretion of PTH. Acute hypomagnesemia may stimulate PTH secretion.[57,58] Hypermagnesemia suppresses PTH secretion, although not as effectively as calcium. The hormone 1,25-dihydroxy vitamin D interacts with vitamin D receptors in the parathyroid glands to chronically suppress PTH synthesis. Phosphate administration stimulates PTH secretion. Catecholamines increase PTH secretion by interacting with β-adrenergic receptors.[56]

Biological actions

PTH influences both calcium and phosphate homeostasis with its actions on both bone and kidney.[55,60,61,62]

In the kidney, PTH causes stimulation of calcium reabsorption and inhibition of phosphate reabsorption from the renal tubules and stimulation of renal production of 1,25 $(OH)_2D_3$, which increases intestinal absorption

of calcium and phosphate. The amino terminal end of the PTH molecule binds to PTH receptor to elicit this biologic responses.[56,63]

Under physiologic conditions, PTH plays a critical role in calcium homeostasis and in regulating the rate of bone turnover. The initial response in bone to a rise in circulating PTH is an increase in bone resorption. It is currently believed that PTH exerts its effect on bone resorption by inducing osteoblasts and/or stromal cells to produce soluble and cell-surface factors that act on mature osteoclasts to increase their resorptive activity and on osteoclast progenitor cells to increase proliferation.[64-73]

Possible mediators for these actions of PTH include osteoclast differentiation factor (ODF/TRANCE/RANK-L), colony stimulating factor-1 (CSF-1), interleukin (IL)-11, and IL-6.[64,73]

Increasing evidence suggests that IL-6 may be one of the key cytokines mediating PTH's proresorptive effect on bone.[74]

PTH receptor

PTH receptor has been cloned and found to be a member of the large family of receptors that contain a seven transmembrane-spanning domain and work through activation of G-protein.[75]

PTH exerts its actions on target tissues such as bone and kidneys by interacting with PTH receptors located in the plasma membrane of target cells.[55]

The formation of PTH-receptor complexes initiates a cascade of intracellular events, including the generation of cyclic AMP, activation of kinases, phosphorylation of proteins, entry of calcium, increases in free calcium, stimulation of phospholipase C with generation of diacylglycerol and phosphoinosites, activation of enzymes and transport systems, and secretion of lysosomal enzymes.[56]

Metabolism

PTH metabolism is complex and produces several fragments of varying biological and immunological reactivity. The intact and biologically active peptide has a half-life in the circulation of < 4 min.[76]

Intact PTH is cleared rapidly by kidney and liver.[77,78] Hepatic Kupffer cells take up intact PTH and degrade it into very small peptides as well as cleave it into discrete fragments that are released into the circulation.[79,80]

The released inactive carboxy terminal fragments (C-terminal) circulate considerably longer than intact hormone, mainly because they are cleared exclusively by glomerular filtration.[81]

Peripheral metabolism appears to inactivate and degrade intact hormone without releasing measurable concentrations of biologically active N-terminal fragments. N-terminal fragments do not enter the circulation during the peripheral metabolism of intact hormone.[55]

The intact hormone and inactive midregion / C-terminal fragments are the principal circulating forms of PTH.[56]

There is now general agreement that parathyroid hormone concentrations increases with age. A key hypothesis implicating parathyroid hormone in the development of osteoporosis relates either to the increase in parathyroid hormone concentrations with age or to enhanced sensitivity at target organs. As sensitivity to parathyroid hormone is enhanced in postmenopausal females, so the circulating level of PTH could be deleterious. Enhanced sensitivity to PTH has been involved as an explanation for why, in the first decade after menopause, primary hyperparathyroidism frequently occurs. There is a possible relationship between PTH and accelerated bone loss following estrogen withdrawal.[82]

INTERLEUKIN-6 (IL-6)

Interleukin-6 (IL-6) is a member of the family of cytokines collectively termed "the interleukin-6 type cytokines",[83] which belongs to the four α-helical long chain family. It includes IL-12, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotropic factor (CNTF), IL-11, IFNα, IFNβ and IL-10.[84]

It is known to be a pleiotropic cytokine produced by many cell types "lymphoid and non-lymphoid" and affecting diverse biological areas (regulation of T- and B- cell function, Immunoglobulin (Ig) secretion, acute phase inflammatory reactions and haematopoiesis.[84]

The purified product is a glycoprotein with a molecular weight between 21 to 28 KD. It contains 179 a.a. residues processed from a precursor of 212 a.a residues.[83,84] Figure (5)[85]

The chromosomal location of IL-6 and its receptor are as follows:

- 7 p 21-14 (IL-6).
- 1 (IL-6 Rα).
- 5 and 17 (gp-130).[84]

IL-6 is secreted by a wide variety of cells, including T- and B-lymphocytes, monocytes/macrophages, fibroblasts, hepatocytes, endothelial cells, nerve cells,[83] epithelial cells, mast cells, astrocytes, microglia, mesangial cells, osteoblasts, epidermal langerhans cells, dendritic cells and keratinocytes.[86]

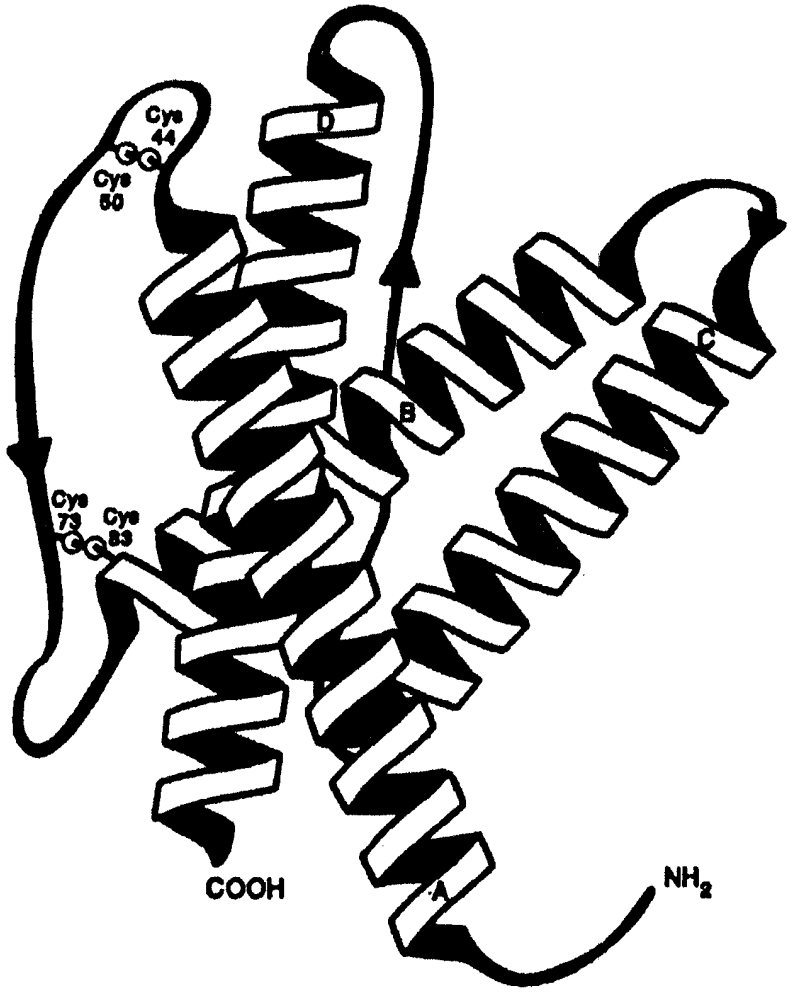


Fig. (5): Schematic representation of IL-6.[85]

IL-6 receptor

IL-6 receptor (IL-6R) has a 339 a.a. extracellular region, 28 a.a transmembrane region, and 82 a.a intracellular region.[83] Figure (6)[85]

It consists of a noncovalent association of a 80-KDa α ligand-binding chain subunit (CD126) and a 130-KDa β signal-transducing subunit (CD130, gp130).[83]

Both soluble and membrane bound form of IL-6R mediate IL-6 activity by stimulating cell signaling through activation of gp130.[84]

IL-6 binds to the IL-6 Rα which is a non signaling component of the receptor. Ligand-induced dimerization of the β-subunits initiates intracellular signaling by activating members of a family of receptor-associated tyrosine kinases. Signal transduction through gp 130 is mediated by two pathways: the JAK-STAT (Janus kinase family tyrosine kinase-signal transducer and activator of transcription) pathway and the Ras-mitogen-activated protein kinase (RMAPK) pathway. The janus kinases (Jaks), phosphorylate several proteins, including the β components of the receptor complex, the kinases, and a series of cytoplasmic proteins termed signal transducers activators of transcription (STATs). STAT phosphorylation causes the formation of protein complexes that migrate to the nucleus and initiate gene transcription. The other pathway includes activation of MAPK cascade which requires SHP_2 (SH_2-domain-containing tyrosine phosphatase)-binding site Tyr^{759} of gp 130, leading to the formation of phosphorylated protein complex that migrate to the nucleus to initiate gene transcription.[87,88] Figure (6)[85]

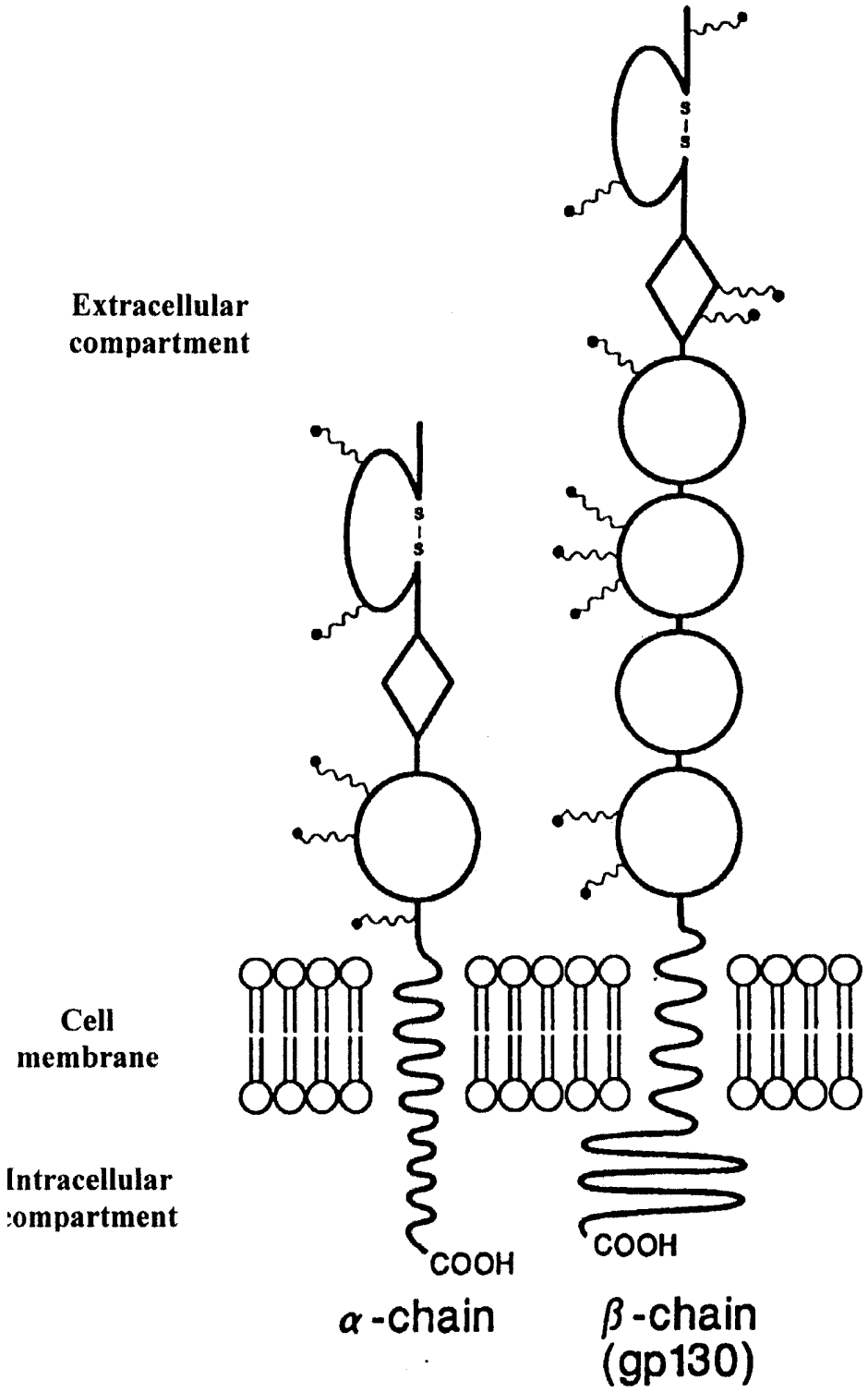


Fig. (6): Diagram of IL-6 receptor.[85]

Biological actions

Table (3): Principal biological activities of IL-6[84]

Target	Action
Immune system	
B-cells	Late growth and differentiation factor (increases Ig_s production)
T-cells	Activation and proliferation (increases IL-2R expression) Differentiation of T cells
Bone marrow	Enhances hematopoiesis
Brain	
Pituitary	Increases ACTH
Hypothalamus	Rise of temperature
Astrocyte	Increases nerve growth factor production
Kidney	Increases mesangial proliferation
Endothelial cells	Secretion of IL-1 and IFNα Increases adherence for lymphocytes
Keratinocytes	Proliferation Secretion of IL-1 and TNFα
Human malignant plasma cells	Chemotaxis inhibitory effect

Action on bone

IL-6 is secreted by stromal cells and mature osteoblasts and acts positively on osteoclast maturation during the first stages of osteoclastogenesis.[89] IL-6 has also been shown to prevent osteoclast apoptosis. Finally IL-6 promotes a maturer osteoblast phenotype through the janus kinase signal transducer and activator of transcription (JAK-STAT) pathway.[90]

The osteoclastogenic property of IL-6 depends not only on its ability to act directly on hematopoietic osteoclast progenitors, but also on the activation of gp 130 signaling in the stromal/osteoblastic cells that provide essential support for osteoclast formation.[13]

IL-6 type cytokines are capable of influencing the differentiation of osteoblasts as well.[3]

Control of IL-6 secretion

I- Glucocorticoids and IL-6 expression

Glucocorticoids repress expression of IL-6 gene. During times of stress or inflammation IL-6 levels are increased. IL-6 in turn, can induce release of corticotropin-releasing factor,[91,92] which results in elevated systemic levels of corticosteroids. Natural and synthetic corticosteroids inhibit IL-6 production from a variety of tissues.[93]

II- Androgen and IL-6 expression

Androgens are capable of mediating repression of IL-6 promoter activation.[83]

Orchiectomy resulted in increased replication of bone marrow osteoclast progenitors and found that this could be prevented by administration of IL-6 neutralizing antibody or implantation of a slow release form of testosterone.[83]

It was observed that 17β-estradiol ($17\beta E_2$) can inhibit bone loss observed in men treated by orchiectomy for prostate cancer.[94]

III- Estrogen and IL-6 expression

Estrogen's ability to repress IL-6 expression was first recognized in human endometrial stromal cells.[95] Additional clues came from the observations that menopause or ovariectomy resulted in increased IL-6 serum levels,[96] increased IL-6 mRNA levels in bone cells,[97] and increased IL-6 secretion by mononuclear cells.[98,99]

Sex steroids inhibit the expression of the genes encoding IL-6, gp 80 and gp 130, most likely by repressing the activity of transcription factors such as NF_{KB} and NF-IL-6.[87]

IV- PTH and IL-6

PTH stimulates the production of IL-6 by stromal/osteoblast cells,[98] as it acts through the rapid stimulation of osteoblastic IL-6 gene expression, whereby the released cytokine stimulates bone resorption.[90,100]

Estrogens

Synthesis of estrogen

The naturally occurring estrogens 17 β-estradiol (E_2), estrone (E_1), and estriol (E_3) are C_{18} steroids derived from cholesterol. After binding to lipoprotein receptors, cholesterol is taken up by steroidogenic cells, stored and moved to the sites of steroid synthesis.[101] Different estrogens are formed by reduction of the number of carbon atoms from 27 to 18 through cleavage enzymes. Aromatization is the last step in estrogen formation. This reaction is catalyzed by P450 aromatase monooxygenase enzyme complex that is present in the smooth endoplasmic reticulum. In three consecutive hydroxylating reactions, estrone and estradiol are formed from their obligatory precursors androstenedione and testosterone, respectively. The final hydroxylating step in aromatization does not require enzymatic action and is not product sensitive.[101]

They are secreted by the theca interna, and granulosa cells of the ovarian follicles, the corpus luteum, and the placenta. Theca interna cells have many luteinizing hormone (LH) receptors, and LH acts via cyclic AMP to increase conversion of cholesterol to androstenedione. Some of the androstenedione is converted to estradiol, which enters the circulation. The granulosa cells make estradiol when provided with androgens, they have many follicle stimulating hormone (FSH) receptors and FSH facilitates their secretion of estradiol by acting via cyclic AMP to increase their aromatase activity. Mature granulosa cells also, acquire LH receptors, and LH also stimulates estradiol production.[101]

Almost all of this estradiol comes from the ovary, and there are two peaks of secretion: one just before ovulation and one during midluteal phase. The estradiol secretion rate is 36 μg/d (133 μmol/d) in the early follicular phase, 380 μg/d just before ovulation, and 250 μg/d during midluteal phase. Estradiol production rate in men is about 50 μg/d (180 μmol/d).[102]

In postmenopausal women, serum estradiol concentrations are often lower than 20 pg per milliliter (73 pmol per liter) and most of estradiol is formed by extragonadal conversion of testosterone. Estrone is the predominant estrogen in these women. Estradiol, not estrone, is believed to be the effector hormone at the nuclear receptor. Estradiol is also 4-10 times more potent than estrone. Estradiol is the major sex steroid hormone that has a strong, consistent, and positive relation to skeletal metabolism.[102,103]

Transport and metabolism of estrogens

In the serum, estradiol reversibly binds to sex-hormone binding globulin,[104] a β-globulin, and binds with less affinity to albumin in a non-saturable manner and about 2-3 percent is free. Estrogens are inactivated by sulfation or glucuronidation, and the conjugates are excreted into the bile or urine. Hydrolysis of these conjugates by the intestinal flora and subsequent reabsorption of the estrogen result in an enterohepatic circulation.[101]

Estrogens are also metabolized by hydroxylation and subsequent methylation to form catechol and methoxylated estrogens.[105] Hydroxylation of estrogens yields 2-hydroxyestrogens, 4-hydroxyestrogens and 16 α-hydroxyestrogens (catechol estrogens), among which 4-hydroxyestrone and 16 α-hydroxyestradiol are considered carcinogenic.[101]

Lipoidal estrogens are fatty esters of estrogens that comprise a separate class of steroid hormones.[106] Lipoidal estrogens are found predominantly in adipose tissue. They are synthesized in blood, where they circulate and bind to lipoproteins. Overall, less than 10 percent of serum estradiol is associated with lipoproteins, mainly high density lipoproteins.[107]

Lipoidal estrogens are more resistant to catabolism than free estrogens and are therefore cleared slowly. Figure (7)[101]

Estrogen receptor

Estrogen receptor (ER) is a member of the nuclear hormone-receptor superfamily. It is composed of 595 a.a. and can be divided into six functional regions (A-F). Region E (a.a. 302-552) is responsible for efficient hormone binding, for dimerization of the receptor, and activation of transcription.[101] Region A/B (a.a. 1-185) is involved in transcription modulation and is the most immunoreactive part of the receptor. Region D (a.a. 250-274) is involved in nuclear localization of the receptor. Region F, the COOH-terminal region (a.a. 552-595) have specific modulatory function that affects the transcriptional activity of the liganded ER and also the agonist/antagonist effectiveness of antiestrogens. Region C (a.a. 185-250) is a highly conserved DNA-binding domain.[108]

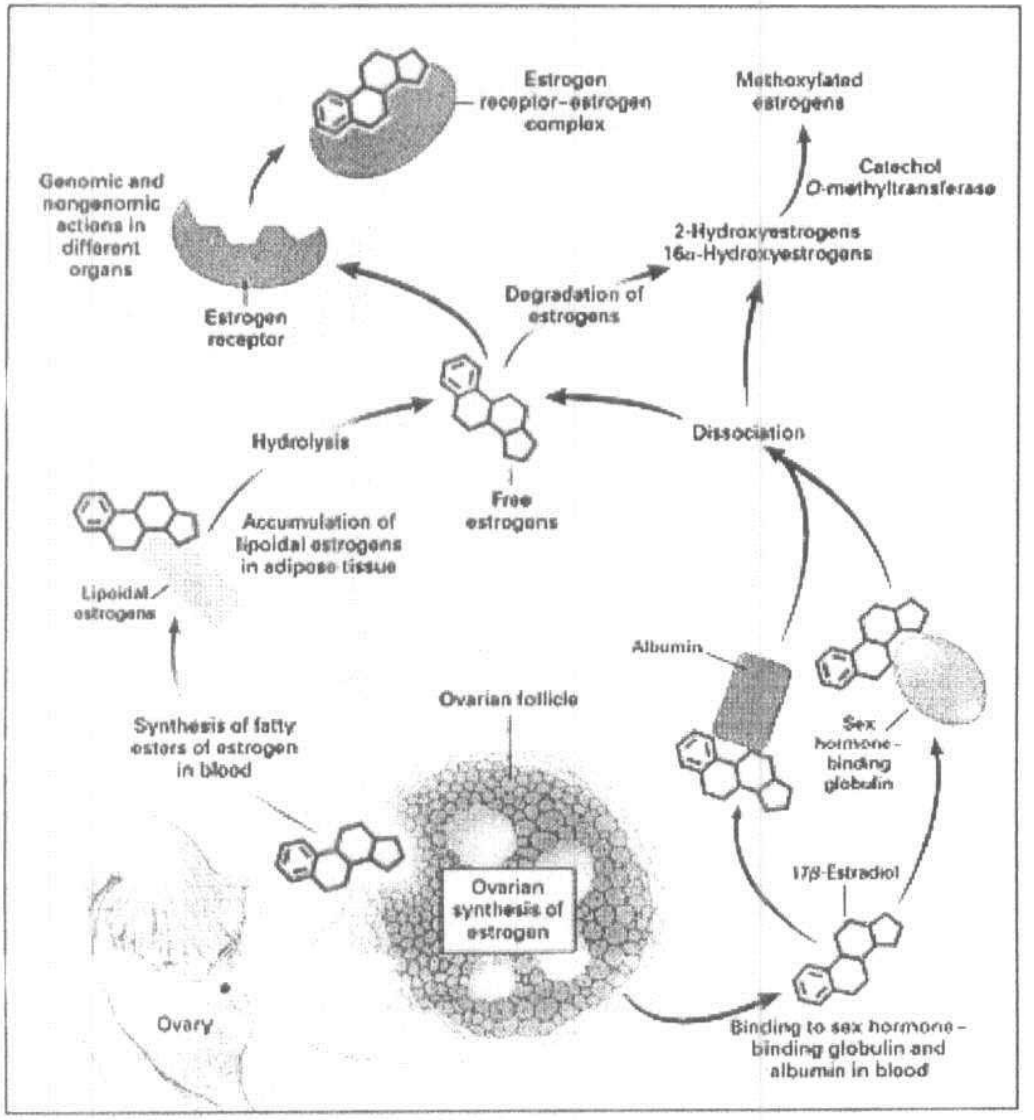


Fig. (7): Ovarian synthesis. Transport, and Metabolism of Estrogens.

After synthesis, mainly in the ovary, 17β–estradiol is secreted into the bloodstream, where it binds to sex-hormone-binding globulin and albumin. Free estrogens diffuse into target tissues to exert their specific genomic or nongenomic effects. Lipoidal estrogens are synthesized in the blood and presumably in other tissues but accumulate predominantly in fat. Enzymatic catabolism of estrogens yields the hydroxyestrogens and methoxyestrogens.[101]

There are two subtypes of estrogen receptor and several isoforms and splice variants of each subtype.[108] The first subtype is the classic estrogen receptor α, the second subtype is estrogen receptor β.[109]

Although the DNA-binding domains of estrogen receptors α and β are very similar, the overall degree of homology of the receptors is low.[110,111]

The tissue distributions of estrogen receptor α and estrogen receptor β differ, although there is some overlap. Granulosa cells and developing spermatids contain mostly estrogen receptor β, and this subtype is present in several nonclassic target tissues, including the kidney, intestinal mucosa, lung parenchyma, bone marrow, bone, brain, endothelial cells, and prostate gland.[112,113] In contrast, endometrium, ovarian stroma and breast cancer cells contain mostly estrogen receptor α.[101]

Some biological actions of estrogens

Estrogens stimulate growth, blood flow, and development of female sex organs and secondary female sex characters. In the liver, estrogens increase lipoprotein receptors, resulting in a decrease in serum concentrations of low density lipoprotein cholesterol.[114]

On the other hand, estrogens increase the potential for coagulation. In the gastrointestinal tract, estrogens may protect against colon cancer.[115] In aging skin, estrogens increase turgor and collagen production and reduce the depth of wrinkles.[116]

Effect on bone

Estrogen has many important actions on skeletal function at molecular, cellular and tissue levels. Even the low levels of endogenous estrogens present in postmenopausal women have recently been noted to be associated with meaningful skeletal effects.[117,118]

Its principal effect is to decrease bone resorption.[119-121] There is considerable evidence that much of this action is mediated by paracrine factors produced by osteoblasts which decrease osteoclast formation or activity. Paracrine mechanisms implicated in the antiresorptive effect of estrogen including decreased production of the pro-resorptive cytokines interleukin (IL)-Iβ, TNF-α, IL-6, macrophage-colony stimulating factor (M-CSF), and prostaglandin E_2 and increased production of the antiresorptive cytokines IL-1 receptor antagonist and transforming growth factor (TGF)-β.[122] Estrogens exert this effect by inhibiting the stimulated expression of the IL-6 gene through an estrogen-receptor mediated action on the transcription factors ($NF\text{-}_k\beta$ and NF-IL-6).[119]

In conclusion estrogen acts on osteoblastic cells to increase the secretion of OPG, a potent inhibitor of bone resportion. Thus a local increase of OPG levels in the bone microenvironment may be an important component of the paracrine mechanisms by which estrogen reduces bone resorption.[119]

Menopause and Bone Changes

Menopause is that point in time when permanent cessation of menstruation occurs following the loss of ovarian activity.[123]

Postmenopause is clinically defined as the absence of menses for 12 months following the onset of menopause.[124]

Menopause is classified as:

1- Natural biological menopause.
2- Induced menopause (by surgical castration or iatrogenic ablation of ovarian function (chemotherapy, radiation treatment).
3- Premature ovarian failure (spontaneous cessation of ovarian function before age 40).[124]

Hormonal changes with established menopause

The most significant findings are the marked reduction in estradiol (E_2) and estrone (E_1). Serum E_2 is reduced to a greater extent than E_1. Serum E_1, on the other hand, is produced primarily by peripheral aromatization from androgens. Estrone sulfate (E_1S) is an estrogen conjugate that serves as a stable circulating reservoir of estrogen, and its levels are the highest of any estrogen. In postmenopausal women, its level average is 350 pg/ml. A part from elevations in FSH and LH levels, pituitary hormones are not affected specifically, growth hormone, thyroid stimulating hormone, and adrenocorticotropic hormone (ACTH).[123]

Serum prolactin levels may be very slightly decreased because prolactin is somewhat influenced by estrogen status. Both the postmenopausal ovary and adrenal gland continue to produce androgens.[123]

The ovaries continue to produce androstenedione and testosterone but not E_2, and this production has been shown to be at least partially dependent on LH.[123]

Effects of declining estrogen

Vasomotor symptoms: such as hot flushes, night sweats and heart palpitations.[125]

Estrogen-dependent target tissue symptoms which include dryness of vulva, vagina and increased frequency and urgency of micturition (due to atrophy of the urethra and trigone of the bladder mucosa) and this is due to large number of estrogen receptors in these organs.[125]

Cardiovascular system: estrogens have direct effects on the arterial wall. They are antioxidants, and appear to protect the endothelial cells from injury due to stress. Estrogens inhibit endothelial cell expression of adhesion molecules, this action may inhibit the platelet aggregation.[126-128]

Collagen: estrogen has a positive effect on collagen that is important for bone, skin and other sites such as the pelvis and urinary system.[123]

Bone changes in postmenopause

It is well established that estrogen deficiency leads to bone loss which leads to osteoporosis.[129]

Osteoporosis is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue [129] with parallel reduction in bone mineral and bone matrix so that bone is decreased in amount but is of normal composition.[129,130]

Osteoporosis, according to a 2000 consensus panel convened by the National Institutes of Health, is "..... a skeletal disorder characterized by compromised bone strength, predisposing a person to an increased risk to fracture".[131] Compromised bone strength may refer to low bone mass, microarchitectural deterioration of bone tissue, or both, resulting in increased bone fragility.[132]

Osteoporosis is one of the major and growing health care problems around the world largely related to the general aging of societies, with improvement in public and preventive health and delay in mortality associated with major osteoporotic fractures.[90]

Etiology and pathogenesis

The amount of bone in the adult depends on the peak bone mass and the rate of its subsequent loss. Peak bone loss depends on the interaction between genetic and mechanical factors, modified by nutritional and

endocrine influences, which together determine the balance between bone resorption (by osteoclasts) and bone formation (by osteoblasts).[1]

Several factors affect the bone architectural structure and bone marrow density such as race, genetics, gender, time of puberty, frequency and intensity of exercise, calcium intake and vitamin D concentration.[124] Figure (8)[130]

After peak bone density is reached at about 30 years of age bone density remains stable for years and then declines. Bone loss begins before menses cease in women, although the precise time of onset is unknown.[130] Once mences ceases, the rate of bone loss is accelerated during the first 5-10 years after menopause, followed by a slower steady decrease thereafter. The latter phase is also seen in men after the age of 40.[131]

According to current concepts of estrogen action on bone, a direct effect of estrogen on bone cell function may be involved as well as an indirect effect on Ca homeostasis.[132,133]

Estrogen deficiency leads to bone loss via an increase in skeletal production of bone resorbing cytokines such as IL-1, IL-6 and TNF, it also decreases production of osteoprotegerin, a soluble member of the tumor necrosis receptor family that normally reduces osteoclastogenesis and bone resorption. Estrogen deficiency may also reduce skeletal production of growth factors that stimulate bone formation, such as insulin-like growth factor-1 and TGF-β.[133]

Estrogen deficiency increases the skeleton's sensitivity to the resorptive effects of PTH so it leads to small increase in calcium level which in turn suppress PTH secretion, thereby decreasing 1,25-dihydroxy vitamin D formation, which limits intestinal absorption of calcium.[130]

Finally, the discovery of estrogen receptors on osteoblasts suggests that estrogen deficiency may also alter bone formation directly.[130]

There are two recognizable forms of osteoporosis. **Type I osteoporosis** is excessive, accelerated, disproportional, menopause-related predominantly with trabecular bone loss (up to 5% yearly). The postmenopausal accelerated phase of trabecular bone loss predisposes women to common site fractures, such as vertebrae (43%), the hip (17%), and the distal part of the forearm (1%).[134]

The osteopenia that results from normal aging and occurs in both women and men is termed **type II osteoporosis** or **"senile osteoporosis"**.[134]

Type II osteoporosis is the loss of both trabecular and cortical bone structure. This type is characterized by skeletal deformity such as dorsal kyphosis, multiple wedge type vertebral fracture, as well as fractures of the hip, pelvis, humerus, and tibia.[135]

Osteoporosis is classified also as either primary or secondary type.[136] **Secondary osteoporosis** is associated with the following medical conditions:[136,137]

- Anticonvulsant therapy.
- Chronic neurologic disease.
- Chronic obstructive lung disease.
- Connective tissue disease.
- Heparin therapy.
- Hypogonadism.
- Hypercortisolism.
- Hyperthyroidism.
- Malabsorption syndrome.
- Primary hyperparathyroidism.
- Rheumatoid arthritis.

Diagnosis of osteoporosis

Osteoporosis is asymptomatic unless it results in a fracture usually vertebral compression fracture or a fracture of the wrist, hip, pelvis or humerus, but the following symptoms may be present as acute pain associated with any fracture and chronic back pain.[130]

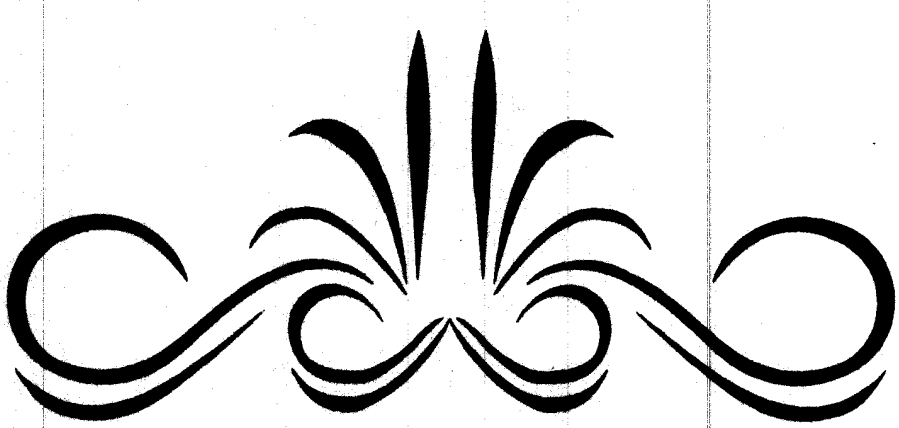
Diagnosis of osteopenia can be made by either documenting a typical fragility fracture or by measuring bone mineral density (BMD). From the world health organization (WHO) definition, a diagnosis of osteoporosis is made if the BMD is 2.5 standard deviations or more below the young adult female mean value. This difference is known as the T-score. Patients are considered to have osteopenia if the T-score is between –1 and –2.5.[138]

In the recent years, there has been considerable interests in tests for biochemical markers of bone formation (serum osteocalcin, bone spesific alkaline phosphatase, or type I procollagen carboxy-terminal propeptide) and bone resorption (urine hydroxyproline, urine pyridinium cross links, or urine cross-linked N-telopeptides of type 1 collagen). Measurement of these markers can predict rates of bone loss in postmenopausal women receiving Ca or other antiresorptive agents when other data do not.[130,138]

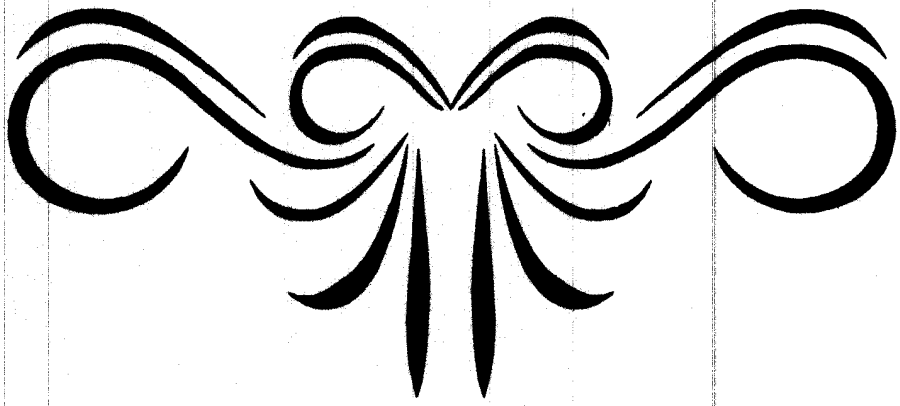
AIM OF THE WORK

AIM OF THE WORK

The aim of the work is to study the serum levels of interleukin-6, parathyroid hormone and estradiol in postmenopausal females and their possible role in bone changes in such cases.



SUBJECTS & METHODS



SUBJECTS

The present study included two groups of subjects:

Group I: It consisted of twenty healthy young, non-pregnant adult females.

Group II: It consisted of thirty postmenopausal females, who were not on estrogen replacement therapy. Most of them had radiological evidence of bone changes and according to their bone mineral density they were subdivided into 20 subjects with osteopenia (T score "-1 to –2.5") and 10 subjects with osteoporosis (T score ≤ -2.5). They were clinically free and had no history of hypertension, diabetes mellitus, renal impairment, nor malignancy and they were not on contraceptive pills and were clinically free from any inflammatory condition. They were inquired about their life style and their diet all of them were non-smokers.

METHODS

To all subjects, the following parameters were carried out:

I- Full clinical examination: Including

1- Detailed history taking with special stress on the presence of bone and joint pain (or), pathological fractures, and intake of contraceptive pills or hormonal therapy. The duration of menopause is recorded in the postmenopausal females group.

2- Thorough physical examination including blood pressure, bone tenderness and skeletal deformities (including lumbar scoliosis, and thoracic kyphosis) or fractures.

II- Investigations: Including

A- Laboratory investigations

Sampling

Five milliliters venous blood samples were taken (filling the syringe completely) from each subject after an overnight fasting. In the control group, the samples were taken immediately (2-5 days) after menstruation. After clotting, the blood samples were centrifuged, and serum was separated. Part of the serum was used for immediate determination of the routine analytes, CRP and PTH (which was transported in ice), while the rest was stored in aliquots in an eppendorf tube for estradiol determination.

Anaerobic conditions were taken into consideration for the sample used for ionized calcium determination.

In addition 1 mL of blood was taken on heparin, immediately centrifged and the plasma was stored in an eppendorf tube at –20°C for the determination of IL-6.

Analytes determined for all subjects included in this study were:

1- Fasting and postprandial serum glucose level[139]

Glucose was determined without deproteinization using an enzymatic method based on the following reaction:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + H_2O$$

The oxidized rose colour product, which is proportionate to the concentration of glucose in the sample (T) was read spectrophotometrically at λ 546 nm, and compared to standard glucose solution (S) of a known concentration (Cs) similarly treated. Serum glucose (C_T) was calculated as follows:

$$C_T = \frac{A_T}{A_S} \times C_S$$

mmol glucose / L = mg/dL × 0.055

2- Serum creatinine concentration[140]

Creatinine was determined without deproteinization using Jaffé reaction in a kinetic manner. The complex formed by creatinine in the sample (T) and alkaline picrate was measured spectrophotometrically at an

interval of 1 minute at λ 492 nm and compared to a standard creatinine solution (S) of a known concentration (C_s) similarly treated.

The difference between the absorbances at 20 and 80 seconds (Δ A/min) was used to determine creatinine concentration in the sample (C_T) as follows:

$$C_T = \Delta A_T / \Delta A_S \times C_S$$

$$\text{mmol Cr / L} = \text{mg/dL} \times 0.0884$$

3- Serum calcium[141]

Total serum calcium was determined without deproteinization using Arsenazo III monoreagent which specifically binds to calcium. The formed complex was read spectrophotometrically at λ 660 nm (T) and compared to a standard calcium solution (S) of a known concentration (C_S) similarly treated. The total calcium concentration (C_T) was calculated as follows:

$$C_T = A_T / A_S \times C_S$$

$$\text{mmol calcium / L} = \text{mg/dL} \times 0.25$$

4- Serum ionized calcium[141]

Ionized calcium was determined using a direct ion selective electrode without sample deproteinization or dilution. The measured potential between the calcium measuring electrode and the reference electrode was the result of changes in potential which developed across the ion selective electrode (ISE) membrane/sample interface which was related to the

natural logarithm of the ionic activity according to Nernst equation. Results were obtained in mmol/L and were converted to mg/dL as follows:

mg/dL = mmol / L × 4.

5- Serum inorganic phosphate[142]

Serum inorganic phosphate was determined without deproteinization using ammonium molybdate in acidic medium. The formed yellow coloured complex (T) was measured at 340 nm, and compared to a standard phosphorous solution (S) of a known concentration (C_S) similarly treated.

The serum inorganic phosphate concentration (C_T) was calculated as follow:

$$C_T = \frac{A_T}{A_S} \times C_S$$

mmol inorganic phosphate / L = mg/dL × 0.323

6- Serum alkaline phosphatase activity[143]

Total alkaline phosphatase (ALP) activity was determined, without deproteinization, using paranitrophenyl phosphate as substrate. ALP catalyzes the hydrolysis of this substrate liberating yellow coloured paranitrophenol in an alkaline solution.

The rate of increase in absorbance (ΔA) due to the formed coloured paranitrophenol product at 37°C was monitored kinetically for 3 minutes at 405 nm. The enzyme activity expressed in Units/L was calculated as follows:

Δ A / min × 2757.

7- Alanine aminotransferase (ALT) activity[144]

ALT activity was determined as follows:

$$\alpha\text{-oxoglutarate} + \text{L-alanine} \xrightarrow{ALT} \text{L-glutamate} + \text{pyruvate}$$

$$\text{pyruvate} + NADH + H^+ \xrightarrow{LD} \text{lactate} + NAD$$

(LD: lactate dehydrogenase)

The decrease that occurs in absorbance at 340 nm (due to NADH + H^+ oxidation) was monitored kinetically for 3 minutes. The enzyme activity expressed in Units / L was calculated as follows:

$\Delta A / min \times 1746$.

8- C-reactive protein (CRP)[145]

The CRP reagent contains latex particles coated with antihuman CRP antibody. The reagent was mixed with serum containing CRP and agglutination was noticed.

9- Intact parathyroid hormone (iPTH): (Immulite)[146]

Serum iPTH was measured using a two site, solid phase chemiluminescent enzyme immunometric assay by immulite automated analyzer Diagnostic Products Corporation. The solid phase was a polystyrene bead enclosed in an immulite test unit, coated with an affinity purified goat polyclonal anti-PTH (44-84) antibody.

After adding the sample or calibrator, together with the alkaline phosphatase conjugated affinity purified goat polyclonal anti-PTH (1-34) antibody in the test unit, a 37°C incubation was done approximately 60

minutes, with intermittent agitation. The iPTH (sample or calibrator) was bound to both anti-PTH antibodies to form a sandwich complex.

Unbound conjugate in the test unit was removed by centrifugal wash, and a luminogenic substrate was added to the test unit, which was then transferred to the luminometer chain. Ten minutes later, the unit arrived in front of the photomultiplier tube (PMT), where the light generated by the luminometric reaction was measured.

In luminogenic reaction, the substrate (adamantyl dioxetane phosphate) was dephosphorylated into an unstable anion (unstable intermediate dioxetane) by the alkaline phosphatase conjugate captured on the bead. The unstable intermediary emitted photons upon decomposition, directly proportional to the amount of bound enzyme, and therefore directly proportional to the concentration of iPTH in the serum sample.

10- Estradiol[147]

Principle of the test

Estradiol was determined using the competitive binding enzyme immunoassay. In the assay, standards, controls and unknowns containing estradiol were incubated with biotin-labeled estradiol and rabbit anti-estradiol antiserum in microtitration wells where the unlabeled and biotin-labeled antigens competed for a limited number of anti-estradiol binding sites. After incubation and washing, the wells were incubated with streptavidin-horseradish peroxidase (HRPO), which was bound to the biotinylated estradiol. The unbound streptavidin-HRPO was washed,

followed by incubation with the substrate tetramethylbenzidine (TMB). An acidic stopping solution was added. The produced colour determined by wavelength absorbance measurement at 450 nm, was inversely proportional to the concentration of estradiol in the samples, standards and controls.

Reagents

A- Anti-estradiol coated microtitration strips

One stripholder containing 96 microtitration wells coated with rabbit anti-estradiol IgG immobilized to the inside wall of each well.

B- Estradiol standards

One vial, labeled A, containing 0 pg/mL, and six vials, labeled B-G, containing concentrations of 20, 50, 250, 750, 2000 and 6000 pg/mL (0.07–22 nmol/L) estradiol in human serum with a non-mercury preservative and were ready to be used.

C- Estradiol controls

Estradiol controls level I and II, containing (250±100 pg/ml), (1000±350 pg/ml) of estradiol in human serum with a non-mercury preservative and were ready to be used.

D- Estradiol-biotin conjugate concentrate

It contained biotinylated estradiol in a protein-based (BSA) buffer with a non-mercury preservative. 220 µL of this conjugate were reconstituted in 11 ml of the estradiol-biotin conjugate diluent to obtain the working reagent.

E- Estradiol-biotin conjugate diluent

Estradiol-Biotin conjugate diluent consisted of a protein-based (BSA) buffer with a non-mercury preservative and was ready to be used.

F- Streptavidin-enzyme conjugate concentrate

It contained streptavidin-HRPO concentrate in a protein-based (BSA) buffer and a non-mercury preservative. 420 µL of streptavidin-enzyme conjugate concentrate were reconstituted in 21 ml of the streptavidin-enzyme conjugate diluent to obtain the working reagent.

G- Streptavidin-enzyme conjugate diluent

Streptavidin-enzyme conjugate diluent contained protein-based (BSA) buffer with EDTA and was ready to be used.

H- TMB chromogen solution

It contained a solution of tetramethylbenzidine (TMB) in citrate buffer with hydrogen peroxide and was ready to be used.

I- Wash concentrate

It contains buffered saline with a non-ionic detergent. It was diluted with 25 fold deionized water prior to use.

J- Stopping solution

0.2 M sulfuric acid and was ready to be used.

N.B.: All regents and samples were allowed to reach room temperature and mixed thoroughly by gentle inversion prior to usage.

Assay procedure

1- The microtitration strips were marked.

2- 50 µL of each standard, control and unknown were pipetted into their designated wells.

3- Estradiol-biotin conjugate solution was prepared.

4- 100 µL of estradiol-biotin conjugate solution were added to each well using a multichannel pipette.

5- The wells were incubated and shaken at a fast speed (500-700 rpm) on an orbital microplate shaker, for 1 hour at room temperature (~ 25°C).

6- Each well was washed five times with the wash solution using automatic microplate washer, then it was blot dry by inverting the plate on absorbent material.

7- The streptavidin-enzyme conjugate solution was prepared.

8- 200 µL of the streptavidin-enzyme conjugate solution were added to each well using a multichannel pipette.

9- The wells were incubated and shaken at a fast speed (500-700 rpm) on an orbital microplate shaker, for 30 minutes at room temperature (~25°C).

10- Each well was washed five times with the wash solution using an automatic microplate washer, then it was blot dry by inverting the plate on absorbent material.

11- 100 µL of the TMB chromogen solution were added to each well using a multichannel pipette.

12- The wells were incubated and were shaken at a fast speed (500-700 rpm) on an orbital microplate shaker, for 30 minutes at room temperature (~ 25°C).

13- 100 µL of the stopping solution were added to each well using a multichannel pipette.

14- The absorbance of the solution in the wells was read within 30 minutes, using a microplate reader set at 450 nm.

Calculation of results

1- The mean absorbances of standards and controls were calculated.

2- On semi-log graph paper a standard curve was plotted with the mean absorbance for each of the standards on the Y-axis versus the estradiol concentrations in pg/ml along the X-axis.

3- The estradiol concentrations of the controls and samples were deduced from the standard curve by matching their mean absorbance readings with the corresponding estradiol concentrations.

4- To convert to nmol/L:

pg/ml × 0.0037 = nmol/L.

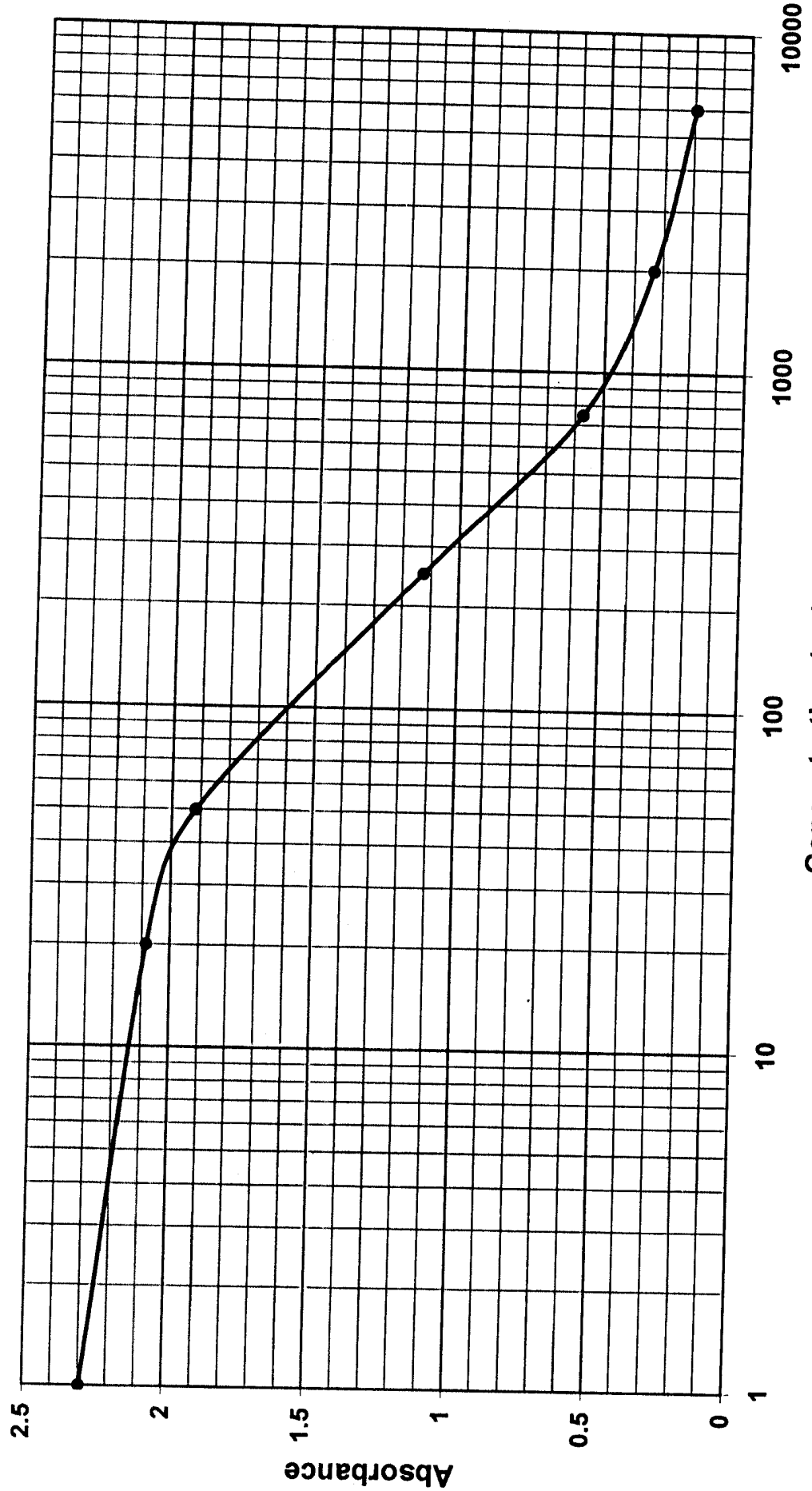


Figure (9) : Standard Curve of Estradiol

11- Interleukin-6[148]

Principle

IL-6 was measured by a competitive enzyme immunoassay (EIA), which measures the natural and recombinant forms of the cytokine interleukin-6 (IL-6).

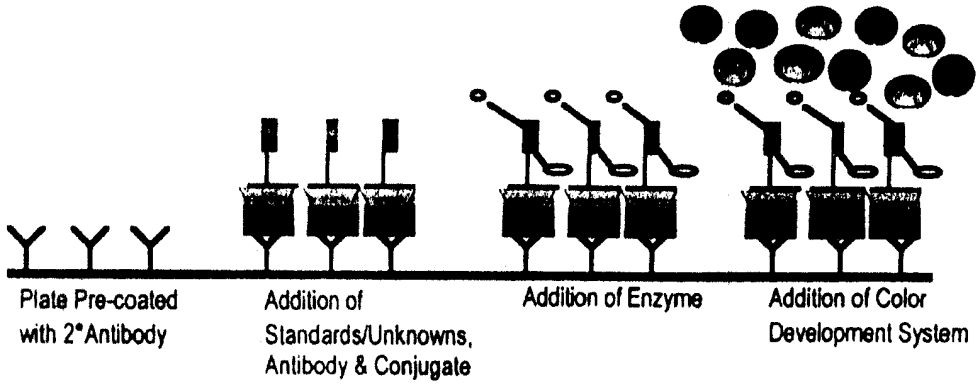


Fig. (10): Schematic representation of ACCUCYTE®'s competitive enzyme immunoassay

In this assay, goat anti-rabbit antibodies were used to capture a specific IL-6 complex in each sample consisting of IL-6 antibody, biotinylated IL-6, and sample or standard.

The biotinylated IL-6 conjugate (competitive ligand) and sample or standard competed for IL-6 specific antibody binding sites.

Therefore, as the concentration of IL-6 in the sample increased, the amount of biotinylated IL-6 captured by the antibody decreased. The assay was visualized using a streptavidin alkaline phosphatase conjugate and an ensuing chromagenic substrate reaction. The amount of IL-6 detected in

each sample was compared to the IL-6 standard curve which demonstrated an inverse relationship between optical density (O.D) and cytokine concentration: i.e. the higher the O.D. the lower the cytokine concentration in the sample.

Reagents

1- Microtiter plate: It consisted of 96 wells coated with goat anti-rabbit antibodies.
2- Sample diluents: two sample diluents (diluent 1 and diluent 2) were used for pretreatment of plasma samples.
3- Rabbit anti-human IL-6 antibody (lyophilized) was reconstituted with the supplied diluent and thoroughly mixed using vortex.
4- Human IL-6 conjugate (lyophilized) was reconstituted with the supplied diluent and thoroughly mixed using vortex.
5- Streptavidin alkaline phosphatase (lyophilized enzyme) was reconstituted with the supplied diluent and thoroughly mixed using vortex.
6- Colour reagents (color reagent A and color reagent B): the supplied colour reagents were just mixed before the step of their addition in the procedure.
7- Washing solution.
8- Recombinant IL-6 standard (lyophilized) was reconstituted with the supplied diluent and thoroughly mixed using vortex (concentration = 200 ng/ml). Serial dilutions were done to construct a standard curve.
9- Stopping solution: 0.5 M sulphuric acid that was ready to use.

Preparation of the serial IL-6 standard dilutions

In polystyrene wassermann tubes, the following was added:

Tube number	Reconstituted standard	Standard concentration
0 Dose test tube	750 µL diluent 1	0 ng/ml
Tube (1)		200 ng/ml
Tube (2)	750 µL diluent + 250 µL of tube (1)	50 ng/ml
Tube (3)	750 µL diluent + 250 µL of tube (2)	12.5 ng/ml
Tube (4)	750 µL diluent + 250 µL of tube (3)	3.125 ng/ml
Tube (5)	750 µL diluent + 250 µL of tube (4)	0.781 ng/ml
Tube (6)	750 µL diluent + 250 µL of tube (5)	0.195 ng/ml

Procedure

1- In duplicate, each 100 µL standard numbered 0-6 were dispensed into their designated wells.

2- For each individual sample, 50 µL of sample + 50 µL of diluent 2 + 100 µL of diluent 1 were added to a 12×75 test tube and were vortexed.

3- 100 µL of each serum preparation were added to their designated wells.

4- 25 µL of reconstituted rabbit anti-human IL-6 polycolonal antibody were added to each well.

The plate was sealed with acetate plate sealer to prevent evaporation and was incubated at room temperature for 3 hours.

5- Carefully the plate sealer was removed and 25 µL of IL-6 conjugate were dispensed into each well and incubated at room temperature for 30 minutes, after covering the plate with another plate sealer.

6- The plate sealer was removed and the plate washed 5 times with washing buffer.

7- 50 µL of reconstituted streptavidin alkaline phosphatase were dispensed into each well. The plate was resealed and incubated at room temperature for 30 minutes.

8- The plate sealer was removed and the plate washed 5 times by washing buffer.

9- 200 µL of the prepared color reagent solution were dispensed into each well and was incubated at room temperature for 15 minutes.

10- After incubation, 50 µl of the stopping solution were dispensed into each well in the same order of addition of the colour reagent.

11- The absorbance of the coloured solution in the plate was read at 490 nm using microplate reader.

NB.: For serum and plasma we used diluent 1.

Calculation of results

- A standard curve was plotted on semilog graph paper with X-axis (log scale) showing IL-6 standard concentrations and Y-axis (linear scale) showing OD.
- The curve was sigmoid in nature.
- After obtaining the sample concentration from the standard curve, the final result was multiplied by a dilution factor of 4 (due to sample pretreatment).

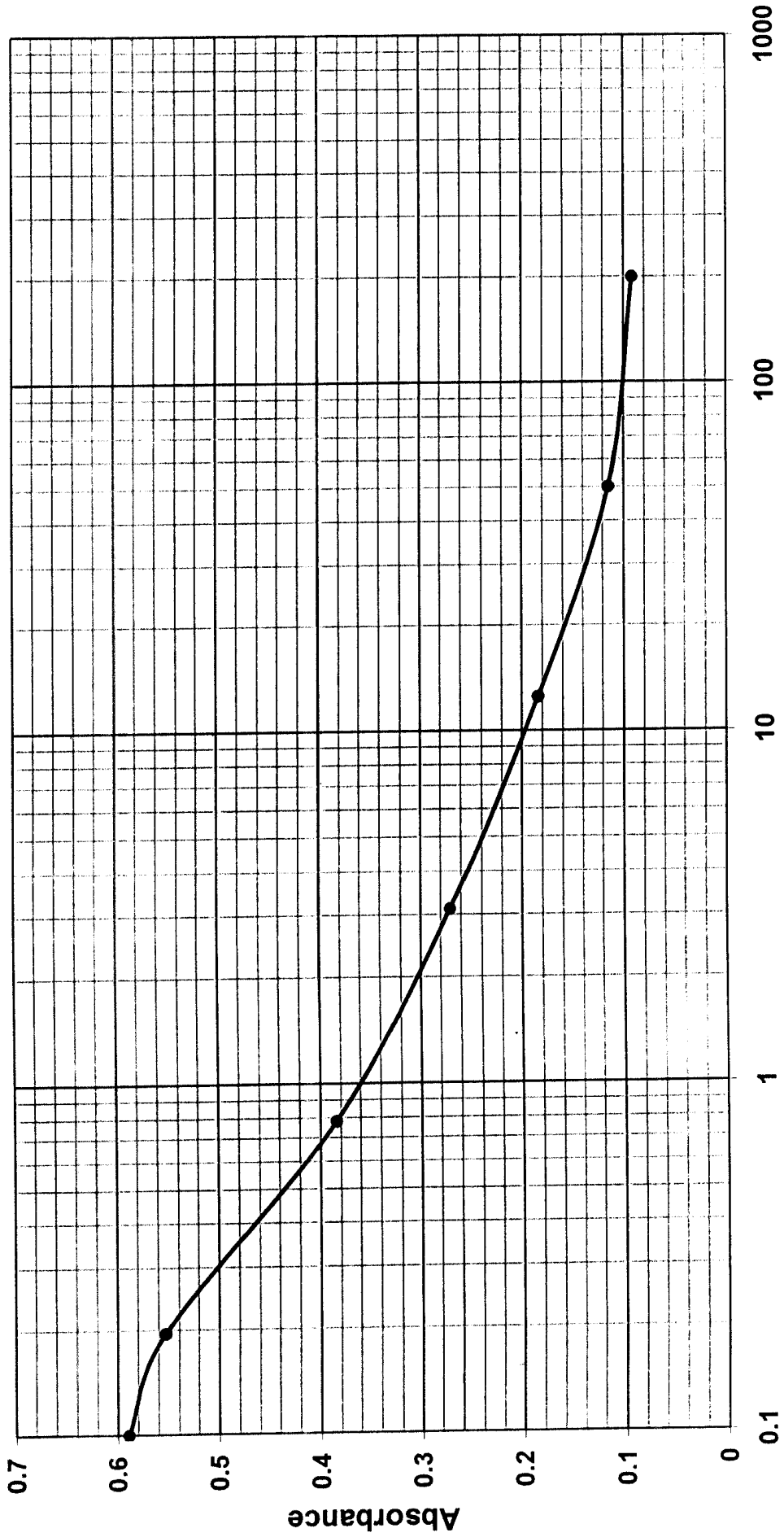


Figure (11) : Standard Curve of IL-6

B- Radiological examination (Densitometery)[149]

Bone densitometry was done using Dual x-ray absorptiometry (DXA) ODR 4500 Elite x-ray Bone Densitometer-USA with switched pulse Dual energy x-ray tube.

Scanning sites include

- **Lumbar spine (in antroposterior and lateral projections):** Where the patient lies supine on the table with large square cushion under the patient's lower legs to make femurs as vertical as possible to reduce lordosis.
- **Proximal femur:** Where the patient lies supine on the table, patient's feet are placed in the hip scan positioning fixture.
- **Forearm:** Where the arm to be examined is placed on the table and the patient faces towards the head of the table.
- **T-score was reported:** subjects with T-score (-1 to –2.5) were classified as osteopenic and subjects with T-score ≤ –2.5 were classified as osteoporotic.

Statistical Analysis

Statistical analysis was done using the SPSS software package.[150]

Statistical analysis was done to obtain the mean, the standard deviation, and for comparison between the different groups involved in this study using student "t" test to compare between independent samples.

1- Arithmetic mean ($\overline{X}$) was calculated as follows:

$$\overline{X} = \frac{\Sigma X}{n}$$

Where ;

$\overline{X}$ = Arithmetic mean

ΣX = Sum of observations

n = number of observations

2- Standard deviation (S.D.) was calculated as follows:

$$S.D. = \sqrt{\frac{\Sigma (X - \overline{X})^2}{n-1}}$$

Where ;

n = number of cases

X = individual values

$\overline{X}$ = Arithmetic mean of the group

3- Standard error:

$$S.E. = \frac{S.D}{\sqrt{n}}$$

Where ;

SD = Standard deviation

n = number of cases

4- Student t-test:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{(S.E_1)^2 + (S.E_2)^2}}$$

Where ;

$\bar{X}_1$ = Arithmetic mean of the 1st group
$\bar{X}_2$ = Arithmetic mean of the 2nd group
$S.E_1$ = Standard error of the 1st group
$S.E_2$ = Standard error of the 2nd group

The probability "P" values, were obtained form the table of "t", where degrees of freedom were taken as $(n_1 + n_2) - 2$ for student "t" test. "P" values of less than 0.05 are considered statistically significant.

5- Coefficient of correlation:

A measure of the strength of the association between 2 variables is calculated by Pearson's product-moment coefficient of correlation "r".

This measure reports the strength of the relationship between dependent and independent variables. For two variables, "r" can have any value from -1.00 to + 1.00. The strength of the relationship is not dependent on the direction of the relationship. It is obtained by:

$$\underline{r =} \frac{n(\Sigma XY) - (\Sigma X)(\Sigma Y)}{\sqrt{[n(\Sigma X^2) - (\Sigma X)^2][n(\Sigma Y^2) - (\Sigma Y)^2]}}$$

Where ;

n = The number of paired observations
ΣX = The sum of the X variable
ΣY = The sum of the Y variable
ΣX^2 = The X – variable squared and the squares summed.
$(\Sigma X)^2$ = The X-variable summed and the sum squared.
ΣY^2 = The Y-variable squared and the squares summed.
$(\Sigma Y)^2$ = The Y-variable summed and the sum squared.


RESULTS

RESULTS

Table (IVa): Some clinical data of the control group (group I). Group I included twenty healthy young nonpregnant females. Their mean age was 30.55±4.36 years. Their mean body mass index was 25.08±3.83 and bone mineral density mean was 0.12±0.53.

Table (IVb): Some clinical data of the postmenopausal females (group II). Group II included thirty postmenopausal females. Their mean age was 54.77±3.58 years and their mean duration of menopause 5.77±2.86 years.

According to their bone mineral density postmenopausal females were divided into 2 subgroups (group II a, group II b).

Group II a: 20 subjects with radiological evidence of osteopenia having a T score from –1 to –2.5.

Group II b: 10 subjects with radiological evidence of osteoporosis having a T score ≤ –2.5 (cases number 1,4,5,8,16,19,23,24,25,29).

Table (IVc): Statistical differences of BMI and BMD in the controls and postmenopausal females. BMI was significantly higher in postmenopausal females than controls. The BMD was significantly lower in postmenopausal females than the corresponding control values.

All the subjects included in both groups were C-reactive protein negative.

Table (IVa): Some clinical data in the control group (group I)

No.	Age (years)	BMI	BMD
1	38	32.88	-0.8
2	30	28.65	+0.3
3	26	22.03	+0.6
4	26	21.48	+0.6
5	26	23.73	+0.5
6	30	28.68	+0.3
7	29	29.38	+0.3
8	28	18.36	+0.5
9	27	21.48	+0.6
10	26	23.73	+0.5
11	25	24.97	+0.6
12	36	24.52	-0.7
13	30	29.03	+0.5
14	27	22.03	+0.6
15	35	25.23	-0.5
16	38	25.71	-0.6
17	36	29.29	-0.7
18	35	28.51	-0.5
19	32	20.20	+0.2
20	31	21.70	+0.1
Mean	30.55	25.08	0.12
SD	4.36	3.83	0.53

BMI: Body mass index.
BMD: Bone mineral density.

Table (IVb): Some clinical data in the postmenopausal females (group II)

No.	Age (years)	Duration of menopause	BMI	BMD
1	58	10	30.08	-4.6
2	59	8	42.45	-2.2
3	53	4	32.00	-1.8
4	47	4	33.78	-2.5
5	51	8	27.05	-2.5
6	51	2	31.25	-1.7
7	54	4	28.76	-1.4
8	56	6	30.08	-3.7
9	55	5	34.19	-1.9
10	52	2	29.13	-0.9
11	56	6	27.68	-1.9
12	54	5	25.39	-1.8
13	56	4	28.88	-1.6
14	51	3	32.87	-0.5
15	58	7	30.38	-1.8
16	55	10	27.55	-3.0
17	56	6	26.31	-1.7
18	48	3	31.11	-1.0
19	54	10	28.25	-2.5
20	54	4	35.18	-2.0
21	55	2	37.57	-1.2
22	58	8	35.79	-0.6
23	55	8	30.91	-2.5
24	63	10	29.75	-3.5
25	58	8	30.91	-2.5
26	59	9	35.37	-2.2
27	52	2	25.71	-1.3
28	55	3	25.39	-1.8
29	60	10	34.96	-2.7
30	50	2	25.71	-1.4
Mean	54.77	5.77	30.82	-2.02
SD	3.58	2.86	4.05	0.90

Table (IVc): Statistical differences of BMI and BMD in the studied groups

	Group I n = 20	Group II n = 30	P. Value
BMI			
Mean	25.08	30.82	0.000
± SD	3.83	4.05	
BMD			
Mean	0.12	-2.02	0.000
±SD	0.53	0.90	

Group I: Controls
Group II: postmenopausal females

Table (Va): Serum levels of some biochemical parameters in the control group. This includes serum levels of fasting and postprandial glucose, creatinine and the alanine aminotransferase activity (ALT).

Table (Vb): The serum levels of some biochemical parameters in the postmenopausal females. This includes serum levels of fasting and postprandial glucose, creatinine and the alanine aminotransferase activity (ALT).

Table (Vc): Statistical differences of fasting and postprandial serum glucose, serum creatinine and alanine aminotransferase activity in the studied groups.

Table (Va): Serum levels of some biochemical parameters in the control group (group I)

No.	FSG (mg/dL)	PPh2 (mg/dL)	Creatinine (mg/dL)	ALT (Units/L)
1	72	100	0.6	20
2	102	100	0.6	16
3	88	100	0.7	15
4	86	90	0.9	8
5	94	100	0.9	10
6	94	102	1.0	26
7	89	104	0.5	15
8	100	99	0.5	20
9	90	100	0.7	19
10	89	95	0.7	14
11	103	100	1.0	11
12	89	90	1.0	16
13	98	100	0.9	14
14	100	99	0.8	16
15	100	89	1.1	20
16	103	105	1.0	39
17	101	105	0.9	16
18	105	100	0.8	20
19	100	89	0.7	19
20	102	105	0.6	18
Mean	95.25	98.60	0.79	17.60
SD	8.16	5.26	0.18	6.48

FSG: fasting serum glucose.
PPh2: postprandial serum glucose after 2 hours.
ALT: alanine aminotransferase.

Table (Vb): Serum levels of some biochemical parameters in the postmenopausal females (group II)

No.	FSG (mg/dL)	PPh2 (mg/dL)	Creatinine (mg/dL)	ALT (Units/L)
1	103	96	0.8	23
2	95	110	0.7	15
3	93	101	0.9	18
4	91	110	0.6	7
5	105	110	0.8	15
6	99	102	0.9	22
7	101	102	0.9	10
8	101	100	0.9	17
9	101	100	0.8	10
10	109	99	0.8	30
11	101	98	0.9	9
12	104	100	0.7	16
13	101	88	0.8	17
14	90	100	0.9	25
15	104	100	0.9	14
16	110	101	0.7	28
17	110	100	0.6	40
18	100	90	0.8	35
19	74	90	0.9	29
20	102	106	0.8	39
21	95	101	0.6	10
22	86	101	0.8	14
23	101	99	0.8	17
24	100	98	1.0	14
25	100	101	0.9	19
26	88	92	0.8	18
27	100	98	0.9	14
28	80	90	0.8	12
29	111	95	0.9	34
30	85	100	0.6	15
Mean	98.00	99.27	0.81	19.53
SD	8.87	5.56	0.11	9.07

Table (Vc): Statistical differences of fasting and postprandial serum glucose, serum creatinine and alanine aminotransferase activity in the studied groups.

	Group I n = 20	Group II no = 30	P. Value
FSG (mg/dL)			
Mean	95.25	98.00	NS
± SD	8.16	8.87	
PPh2 (mg/dL)			
Mean	98.60	99.27	NS
± SD	5.26	5.56	
Cr (mg/dL)			
Mean	0.80	0.81	NS
± SD	0.18	0.11	
ALT (Units/L)			
Mean	17.60	19.53	NS
± SD	6.48	9.07	

NS: No statistical significant difference (P > 0.05)

Table (VI): Serum level of total calcium (mg/dL) in the studied groups.

The mean total serum calcium in the control group was 9.39±0.32 mg/dL, in the postmenopausal females was 9.60±0.50 mg/dL, in the osteopenic group was 9.62±0.51 mg/dL and in the osteoporotic group was 9.57±0.50 mg/dL.

Table (VI): Serum level of total calcium (mg/dl) in the studied groups

No.	Group I (n=20)	Group II (n=30)	Group IIa (n=20)	Group IIb (n=10)
1	8.6	9.9	8.8	9.9
2	9.1	8.8	9.6	9.4
3	9.2	9.6	9.6	9.3
4	9.2	9.4	8.5	10.1
5	9.4	9.3	10.0	9.5
6	9.8	9.6	10.1	10.0
7	9.5	8.5	9.7	8.9
8	9.3	10.1	9.8	10.4
9	9.5	10.0	9.6	9.0
10	9.4	10.1	9.8	9.2
11	9.9	9.7	9.4	
12	9.5	9.8	10.6	
13	9.7	9.6	9.3	
14	9.8	9.8	10.2	
15	9.5	9.4	9.8	
16	9.6	9.5	9.1	
17	9.1	10.6	9.2	
18	9.2	9.3	9.3	
19	9.6	10.0	9.7	
20	8.9	10.2	10.3	
21		9.8		
22		9.1		
23		8.9		
24		10.4		
25		9.0		
26		9.2		
27		9.3		
28		9.7		
29		9.2		
30		10.3		
Mean	9.39	9.60	9.62	9.57
SD	0.32	0.50	0.51	0.50
P		NS	NS	NS
P_1				NS

Group I: control group
Group II: Postmenopausal females.
Group II a: Postmenopausal females with radiological evidence of osteopenia.
Group II b: Postmenopausal females with radiological evidence of osteoporosis.
P: statistical difference between group I (controls), group II (group II a and group II b).
P1: Statistical difference between subjects with osteopenia (group II a) and subjects with osteoporosis (group II b).
NS: No statistical significant difference ($P > 0.05$)

Table (VII): Serum level of ionized calcium (mg/dL) in the studied groups.

The mean serum ionized calcium level in the control group was 4.89±0.22 mg/dL, in the total postmenopausal females was 4.70±0.47 mg/dL, in the osteopenic group was 4.79±0.33 mg/dL and in the osteoporotic group was 4.52±0.64 mg/dL.

Table (VII): Serum level of ionized calcium (mg/dL) in the studied groups

No.	Group I (n=20)	Group II (n=30)	Group IIa (n=20)	Group IIb (n=10)
1	4.95	4.12	4.40	4.12
2	5.16	4.40	4.92	4.76
3	4.64	4.92	4.85	5.36
4	4.65	4.76	4.91	4.13
5	4.73	5.36	4.78	4.68
6	4.99	4.85	5.04	5.75
7	5.12	4.91	4.82	3.64
8	4.80	4.13	5.20	4.08
9	4.84	4.78	4.71	4.22
10	4.66	5.04	4.55	4.41
11	5.13	4.82	4.76	
12	4.70	5.20	4.72	
13	4.77	4.71	4.28	
14	5.24	4.55	4.72	
15	4.84	4.76	4.40	
16	4.79	4.68	4.89	
17	5.16	4.72	5.77	
18	4.50	4.28	4.35	
19	5.12	5.75	4.74	
20	5.00	4.72	4.96	
21		4.40		
22		4.89		
23		3.64		
24		4.08		
25		4.22		
26		5.77		
27		4.35		
28		4.74		
29		4.41		
30		4.96		
Mean	4.89	4.70	4.79	4.52
SD	0.22	0.47	0.33	0.64
P		NS	NS	NS
P_1				NS

Group I: control group
Group II: Postmenopausal females.
Group II a: Postmenopausal females with radiological evidence of osteopenia.
Group II b: Postmenopausal females with radiological evidence of osteoporosis.
P: statistical difference between group I (controls), group II (group II a and group II b).
P1: Statistical difference between subjects with osteopenia (group II a) and subjects with osteoporosis (group II b).
NS: No statistical significant difference ($P > 0.05$)

Table (VIII): Serum level of inorganic phosphate (mg/dL) in the studied groups

The mean inorganic phosphate level in the control group was 3.73±0.41 mg/dL, in the postmenopausal group was 3.72±0.49 mg/dL, in the osteopenic group was 3.79±0.51 mg/dL and in the osteoporotic group was 3.59±0.41 mg/dL.

Table (VIII): Serum level of inorganic phosphate (mg/dL) in the studied groups

No.	Group I (n=20)	Group II (n=30)	Group IIa (n=20)	Group IIb (n=10)
1	2.8	3.3	3.8	3.3
2	3.5	3.8	3.9	4.4
3	3.4	3.9	3.6	3.7
4	3.5	4.4	3.2	3.3
5	4.5	3.7	3.1	4.0
6	3.6	3.6	3.9	3.5
7	3.1	3.2	3.6	3.6
8	3.5	3.3	4.1	3.5
9	3.6	3.1	4.2	3.7
10	4.3	3.9	4.7	2.9
11	4.1	3.6	3.0	
12	3.8	4.1	4.0	
13	3.7	4.2	4.6	
14	4.2	4.7	3.4	
15	3.8	3.0	3.5	
16	3.9	4.0	3.5	
17	3.7	4.0	3.7	
18	3.8	4.6	3.1	
19	3.5	3.5	4.4	
20	4.2	3.4	4.5	
21		3.5		
22		3.5		
23		3.6		
24		3.5		
25		3.7		
26		3.7		
27		3.1		
28		4.4		
29		2.9		
30		4.5		
Mean	3.73	3.72	3.79	3.59
SD	0.41	0.49	0.51	0.41
P		NS	NS	NS
P_1				NS

Group I: control group
Group II: Postmenopausal females.
Group II a: Postmenopausal females with radiological evidence of osteopenia.
Group II b: Postmenopausal females with radiological evidence of osteoporosis.
P: statistical difference between group I (controls), group II (group II a and group II b).
P1: Statistical difference between subjects with osteopenia (group II a) and subjects with osteoporosis (group II b).
NS: No statistical significant difference ($P > 0.05$)

(Table IX): Serum activity of alkaline phosphatase (Units/L) in the studied groups.

The mean alkaline phosphatase activity in the control group was 132.40±14.53 Units/L, in the postmenopausal females group was 197.07±46.14 Units/L, in the osteopenic group was 195.25±46.68 Units/L and in the osteoporotic group was 200.70±47.29 Units/L.

The mean serum activity of alkaline phosphatase was significantly higher in the total postmenopausal females, osteopenic group and osteoporotic group than the corresponding control group but there was no significant difference between osteopenic and osteoporotic groups.

Table (IX): Serum activity of alkaline phosphatase (Units/L) in the studied groups

No.	Group I (n=20)	Group II (n=30)	Group IIa (n=20)	Group IIb (n=10)
1	132	309	186	309
2	151	186	317	200
3	136	317	190	219
4	130	200	128	189
5	103	219	177	192
6	122	190	205	179
7	140	128	249	203
8	140	189	237	190
9	167	177	139	210
10	120	205	183	116
11	145	249	283	
12	134	237	179	
13	125	139	191	
14	150	183	192	
15	140	283	162	
16	117	192	193	
17	118	179	201	
18	120	191	137	
19	130	179	163	
20	128	192	193	
21		162		
22		193		
23		203		
24		190		
25		210		
26		201		
27		137		
28		163		
29		116		
30		193		
Mean	132.40	197.07	195.25	200.70
SD	14.53	46.14	46.68	47.29
P		0.00	0.00	0.00
P_1				NS

Group I: control group
Group II: Postmenopausal females.
Group II a: Postmenopausal females with radiological evidence of osteopenia.
Group II b: Postmenopausal females with radiological evidence of osteoporosis.
P: statistical difference between group I (controls), group II (group II a and group II b).
P1: Statistical difference between subjects with osteopenia (group II a) and subjects with osteoporosis (group II b).
NS: No statistical significant difference ($P > 0.05$)

Table (X): Serum level of intact parathyroid hormone (pg/ml) in the studied groups.

The mean intact parathryoid hormone in the control group was 24.90±2.27 pg/ml, in the postmenopausal females group was 56.13±16.30 pg/ml, in the osteopenic group was 49.69±8.19 pg/ml and in the osteoporotic group was 69.02±20.91 pg/ml.

The mean serum parathyroid hormone level was significantly higher in the total postmenopausal females, osteopenic group and osteoporotic group than the corresponding control group and it was significantly higher in the osteoporotic group than the osteopenic group.

Table (X): Serum level of intact parathyroid hormone (pg/mL) in the studied groups

No.	Group I (n=20)	Group II (n=30)	Group IIa (n=20)	Group IIb (n=10)
1	23.50	68.00	60.00	68.00
2	22.32	60.00	50.00	46.00
3	22.50	50.00	41.00	122.00
4	25.10	46.00	54.00	52.00
5	22.25	122.00	48.00	52.20
6	26.13	41.00	38.00	68.10
7	21.22	54.00	46.00	73.70
8	23.50	52.00	62.00	69.90
9	24.23	48.00	56.00	70.00
10	25.25	38.00	39.00	68.30
11	23.22	46.00	50.20	
12	29.30	62.00	40.10	
13	26.25	56.00	58.60	
14	22.32	39.00	59.20	
15	26.50	50.20	42.20	
16	26.01	52.20	57.00	
17	28.01	40.10	59.00	
18	28.40	58.60	38.00	
19	26.20	68.10	45.30	
20	25.70	59.20	50.20	
21		42.20		
22		57.00		
23		73.70		
24		69.90		
25		70.00		
26		59.00		
27		38.00		
28		45.30		
29		68.30		
30		50.20		
Mean	24.90	56.13	49.69	69.02
SD	2.27	16.30	8.19	20.91
P		0.00	0.00	0.00
P_1				0.01

Group I: control group
Group II: Postmenopausal females.
Group II a: Postmenopausal females with radiological evidence of osteopenia.
Group II b: Postmenopausal females with radiological evidence of osteoporosis.
P: statistical difference between group I (controls), group II (group II a and group II b).
P1: Statistical difference between subjects with osteopenia (group II a) and subjects with osteoporosis (group II b).

Table (XI): Serum level of estradiol (pg/ml) in the studied groups.

The mean estradiol level in the control group was 41.85±26.21 pg/ml, in the postmenopausal females group was 6.85±3.48 pg/ml, in the osteopenic group was 7.88±3.11 pg/ml and in the osteoporotic group was 4.81±3.42 pg/ml.

The mean serum level of estradiol was significantly lower in the total postmenopausal females, osteoporotic group and osteopenic group than the corresponding control group and it was significantly lower in the osteoporotic group than the osteopenic group.

Table (XI): Serum level of estradiol (pg/mL) in the studied groups

No.	Group I (n=20)	Group II (n=30)	Group IIa (n=20)	Group IIb (n=10)
1	50	2.0	5.4	2.0
2	15	5.4	8.0	9.0
3	46	8.0	10.0	2.0
4	56	9.0	9.0	2.0
5	54	2.0	4.5	2.0
6	24	10.0	6.0	3.1
7	20	9.0	10.1	8.0
8	30	2.0	9.8	2.2
9	62	4.5	9.6	7.8
10	60	6.0	9.0	10.0
11	101	10.1	2.9	
12	80	9.8	6.0	
13	9	9.6	3.5	
14	20	9.0	6.9	
15	69	2.9	9.0	
16	20	2.0	5.4	
17	10	6.0	9.6	
18	9	3.5	9.0	
19	40	3.1	6.8	
20	62	6.9	17.0	
21		9.0		
22		5.4		
23		8.0		
24		2.2		
25		7.8		
26		9.6		
27		9.0		
28		6.8		
29		10.0		
30		17.0		
Mean	41.85	6.85	7.88	4.81
SD	26.21	3.48	3.11	3.42
P		0.00	0.00	0.00
P_1				0.05

Group I: control group
Group II: Postmenopausal females.
Group II a: Postmenopausal females with radiological evidence of osteopenia.
Group II b: Postmenopausal females with radiological evidence of osteoporosis.
P: statistical difference between group I (controls), group II (group II a and group II b).
P1: Statistical difference between subjects with osteopenia (group II a) and subjects with osteoporosis (group II b).

Table (XII): Plasma level of interleukin-6 (ng/ml) in the studied groups.

The mean interleukin-6 level in the control group was 2.82±0.66 ng/ml, in the total postmenopausal females group was 8.20±2.23 ng/ml, in the osteopenic group was 7.03±1.46 ng/ml and in the osteoporotic group was 10.54±1.56 ng/ml.

The mean plasma level of interleukin-6 was significantly higher in the total postmenopausal females, osteoporotic group and osteopenic group than the corresponding control group and it was significantly higher in the osteoporotic group than the osteopenic group.

Table (XII): Plasma level of interleukin-6 (ng/mL) in the studied groups

No.	Group I (n=20)	Group II (n=30)	Group IIa (n=20)	Group IIb (n=10)
1	3.5	12.0	8.0	12.0
2	1.7	8.0	7.0	6.9
3	2.6	7.0	6.0	11.0
4	2.2	6.9	6.1	12.0
5	2.6	11.0	8.0	9.5
6	2.5	6.0	5.8	12.0
7	2.4	6.1	8.5	10.0
8	2.0	12.0	5.7	11.0
9	2.6	8.0	5.9	10.0
10	1.8	5.8	6.0	11.0
11	2.4	8.5	10.0	
12	3.3	5.7	8.9	
13	3.1	5.9	6.2	
14	2.4	6.0	5.9	
15	3.5	10.0	5.5	
16	3.5	9.5	9.0	
17	3.5	8.9	9.5	
18	3.3	6.2	6.4	
19	3.7	12.0	5.9	
20	3.8	5.9	6.3	
21		5.5		
22		9.0		
23		10.0		
24		11.0		
25		10.0		
26		9.5		
27		6.4		
28		5.9		
29		11.0		
30		6.3		
Mean	2.82	8.20	7.03	10.54
SD	0.66	2.23	1.46	1.56
P		0.00	0.00	0.00
P_1				0.00

Group I: control group
Group II: Postmenopausal females.
Group II a: Postmenopausal females with radiological evidence of osteopenia.
Group II b: Postmenopausal females with radiological evidence of osteoporosis.
P: statistical difference between group I (controls), group II (group II a and group II b).
P1: Statistical difference between subjects with osteopenia (group II a) and subjects with osteoporosis (group II b).

Table (XIII): Significant correlations in the postmenopausal females (n = 30)

	r	p
Duration of menopause		
- Estradiol	-0.509	0.004
- PTH	0.581	0.001
- IL-6	0.876	0.000
- BMD	-0.672	0.000
IL-6		
- Estradiol	-0.573	0.001
- PTH	0.553	0.002
- BMD	-0.743	0.000
PTH		
- BMD	-0.436	0.016
Estradiol		
- BMD	0.460	0.011

DISCUSSION

DISCUSSION

Throughout recorded history, multiple physical and mental conditions have been attributed to the menopause. Contrary to popular opinion, the menopause is not a signal of impending decline, but rather a wonderful phenomenon that can signal the start of something positive, a good health program.[151]

Since postmenopausal females may live about one third of their lives after ovarian function ceases, that's why a good consideration to this period of life has developed recently in order to provide a better quality of life to this stratum of the community. One of the most important health debilitating hazards is the bone changes that occur in the postmenopausal females.[151]

Osteoporosis is the single most important health hazard associated with the climacteric. It is a disorder characterized by a reduction in the quantity of bone during aging without changes in its chemical composition.[151] It is the most prevalent bone problem in the elderly. Osteopenia is used to indicate low bone mass, whereas osteoporosis is reserved for low bone mass with increased susceptibility to fractures. The osteoporotic disabilities sustained by the postmenopausal women include back pain, decreased height and mobility, and fractures of the vertebral body, humerus, upper femur, distal forearm, and ribs.[152]

Osteoporosis is a common disease affecting the majority of older women and a significant minority of older men. It is one of the major and growing health care problems around the world largely related to the general aging of societies with improvement in public and preventive health and delay in mortality. It is also becoming a major problem even in the developing countries.[153]

Increased bone turnover in women after menopause has been generally explained by two different mechanisms, firstly it has been assumed that there is first a rapid phase of estrogen-dependent bone turnover during early postmenopause, and a second phase of increased bone turnover in older age.[154]

Bone growth and remodeling is a complex dynamic process that achieves a balance between the coupled process of bone formation and resorption. This process is regulated by the interplay of systemic hormones, locally produced cytokines and growth factors.[155]

The aim of this work was to study the serum levels of interleukin-6, parathyroid hormone and estradiol in postmenopausal females and their possible role in bone changes in such cases.

In the present study thirty postmenopausal females with radiological evidence of bone changes were selected and compared to a group of twenty apparently healthy young nonpregnant females. The thirty postmenopausal females were subdivided according to their bone mineral density (T-score) into two subgroups: osteopenic group (N =20) (T score: -1 to –2.5) and the osteoporotic group (N = 10) (T score ≤ -2.5).

Serum estradiol and intact parathyroid hormone (iPTH), total and ionized calcium and inorganic phosphate were measured in the sera of all subjects included in the study. In addition plasma interleukin-6, one of the local bone regulatory cytokines, and total serum alkaline phosphatase activity (a marker of osteoblastic activity) were also estimated.

I- Estradiol

The importance of estrogen in bone and mineral metabolism is incontrovertible. It has many important actions on skeletal function at molecular, cellular and tissue levels, and has been more intensively studied than perhaps any other compound used in skeletal therapeutics. Even the low levels of endogenous estrogens present in postmenopausal women have recently been noted to be associated with meaningful skeletal effects.[117,118]

In the present study, serum estradiol was determined using the competitive binding enzyme immunoassay. The mean estradiol level in the control group was 41.85±26.21 pg/ml, in the total postmenopausal females group was 6.85±3.48 pg/ml, in the osteopenic group was 7.88±3.11 pg/ml and in the osteoporotic group was 4.81±3.42 pg/ml. It was significantly lower in the total postmenopausal females, osteoporotic and osteopenic groups than the corresponding control group. It was also significantly lower in the osteoporotic group than the osteopenic group (Table XI). This was in agreement with the results of other studies.[117,156]

It was reported that in postmenopausal females, serum estradiol is an important determinant of bone loss; when ovarian estrogen production decreases and serum level fall into the postmenopausal range (<30 pg/ml), accelerated bone loss ensues.[157]

In the present work, there was an inverse relation between the serum level of estradiol and the duration of menopause ($r = -0.509$, $p = 0.004$) and a positive correlation between serum estradiol level and bone mineral density in the postmenopausal females group ($r = 0.460$, $p = 0.011$) (Table XIII). In addition, the mean estradiol level in the osteoporotic group was the lowest among the studied groups (Table XI).

Some authors reported a strong inverse relation between endogenous serum estradiol and the risk of hip and vertebral fractures. They also reported that women with undetectable estradiol level (< 5 pg/ml) were about 2.5 times more likely to suffer hip or vertebral fractures than women with detectable levels (5-25 pg/ml).[156]

In the present study 6 osteoporotic females had serum estradiol level below 5 pg/ml and their bone mineral density were ≤ -2.5. None of these patients suffered from osteoporotic fractures.

It was reported that estradiol, even when present in low concentrations, reduces skeletal remodeling, allows for both better quality and mass of bone, and thereby reduces fracture rates.[156]

Estradiol could produce beneficial skeletal effects through several possible mechanisms; it reduces activation of bone metabolic units,[158] it antagonizes PTH's stimulation of bone resorption,[159] it may enhance the survival of osteoblasts via local cytokines or other growth factors,[160] and it improves the efficiency of gastrointestinal calcium absorption and renal calcium conservation.[161] It was also suggested that estrogen prevents bone loss by blocking the production of proinflammatory cytokines by bone marrow and bone cells.[120]

A progress has been made in recent years towards understanding the molecular mechanisms of bone loss associated with estrogen-deficient state.[162] Some studies supported a possible relation between PTH and accelerated bone loss following estrogen withdrawal[74,82,159]

II- PTH

The mechanisms by which PTH regulates bone resorption at cellular level are not fully understood. However, the stimulatory effect of PTH on the development and activity of the bone-resorbing cell, the osteoclast, requires the presence of stromal / osteoblast, suggesting that PTH induces cells of the osteoblast lineage to produce factor(s) that recruit and/or activate osteoclasts.[64-73]

Parathyroid hormone is one of the major factors that plays a key role in bone turnover. In the present study serum intact parathyroid hormone (iPTH) was measured by chemiluminescence and its mean level in the

control group was 24.90±2.27 pg/ml, in the postmenopausal females group was 56.13±16.30 pg/ml, in the osteopenic group was 49.69±8.19 pg/ml and in the osteoporotic group was 69.02±20.91 pg/ml. It was significantly higher in the total postmenopausal females, osteopenic and osteoporotic groups than the corresponding control group. In addition it was significantly higher in the osteoporotic group than the osteopenic group (Table X).

This was in agreement with other studies.[163,164] Orr-Walker et al 2000[163], reported that estrogen-deficient women with primary hyperparathyroidism had accelerated rates of bone loss compared with euparathyroid controls. Masiukiewicz et al 2002[164], reported a possible relationship between PTH and accelerated bone loss following estrogen withdrawal.

In the present work, there is a positive correlation between serum PTH level and the duration of menopause ($r = 0.581$, $p = 0.001$) and a negative correlation with BMD ($r = -0.436$, $p = 0.016$) in the postmenopausal females group (Table XIII).

It was found that postmenopausal estrogen-deficient women demonstrate an age-dependent rise in PTH that is accompanied by increases in the markers of bone resorption.[74,165-167]

The amount of bone at any point of time reflects the balance of the osteoblastic and osteoclastic forces, influenced by a multitude of stimulating and inhibiting agents. Aging and loss of estrogen lead to excessive osteoclastic activity.[168,169]

The highest mean PTH value and the lowest mean estradiol level were observed in the osteoporotic group at the present work. However, the inverse correlation between them did not reach the level of statistical significance.

In the present study serum levels of total calcium, ionized calcium and phosphate were measured and there were no significant differences between these parameters in the postmenopausal females and the control group (Tables VI, VII, VIII). Estrogen deficient women may show increased skeletal sensitivity to the resorbing action of PTH.[164]

In the present study serum alkaline phosphatase activity was taken as a marker of osteoblastic activity. Its mean serum activity was 132.40±14.53 Units/L in the control group, 197.07±46.14 Units/L in the postmenopausal females group, 195.25±46.68 Units/L in the osteopenic group and 200.70±47.29 Units/L in the osteoporotic group. It was significantly higher in the total postmenopausal females, osteopenic and osteoporotic groups than the corresponding control group. There was no significant difference between serum alkaline phosphatase activity in the osteopenic and osteoporotic groups (Table IX).

These findings were in agreement with the results reported by other studies.[132,170] Serum alkaline phosphatase activity can be used as a predictive marker of bone remodeling in postmenopausal females,[132] but it can't be used in the diagnosis of osteoporosis.[170]

III- Interleukin-6

IL-6 has previously been implicated in the increased bone resorption, and bone loss, that accompanies estradiol deficiency.[64,72] It also contributes to the disregulation of skeletal homeostasis and bone loss that accompanies altered circulating levels of a major osteoporotic hormone, PTH.[166] Among the cytokines thought to participate in PTH-induced bone resorption is IL-6.[168]

In the present study plasma IL-6 level was measured by a competitive enzyme immunoassay. The mean level in the control group was 2.82±0.66 ng/mL, in the total postmenopausal females group was 8.20±2.23 ng/mL, in the osteopenic group was 7.03±1.46 ng/mL and in the osteoporotic group was 10.54±1.56 ng/ml. It was significantly higher in the total postmenopausal females group, osteopenic and osteoporotic groups than the corresponding control group. It was also significantly higher in the osteoporotic group than the osteopenic group (Table XII).

In the postmenopausal females group of the present study, interleukin-6 was positively correlated with the duration of menopause ($r = 0.876$, $p = 0.000$) and showed a negative correlation with BMD ($r = -0.743$, $p = 0.000$) (Table XIII).

These findings are consistent with some recent prospective observational studies[87,132,154,171], who concluded that IL-6 is an important mediator of the increased bone resorption after menopause. On the other hand, Nakchbandi *et al* 2002[167] found that IL-6 level did not consistently correlate with changes in BMD.

Scheidt-Nave *et al* 2001[132], reported that IL-6 effect appears to be most relevant through the first postmenopausal decade up to 10 years, and its effect on bone loss is weakened with increasing the distance from menopause and was no longer significant in women more than 10 years after menopause.

All postmenopausal females included in the present study had a menopausal duration ranging from 2 to 10 years with a mean of 5.77±2.86 years. Khosla *et al* 1994[172], have found no differences in circulating levels of IL-6 in osteoporotic and non osteoporotic estrogen-deficient women when studied a group of females with a postmenopausal duration of 16 years. They suggested that IL-6 dysregulation plays a role predominantly in the early postmenopausal period.

In the present work, interleukin-6 was positively correlated with serum PTH level ($r = 0.553$, $p = 0.002$) and negatively correlated with serum estradiol level in the postmenopausal females group ($r = -0.573$, $p = 0.001$) (Table XIII).

The present study supports the conclusion that the IL-6 cytokine system plays a role in increased skeletal sensitivity to PTH seen in estrogen-deficient postmenopausal females as previously reported[164,173], and thus in the estrogen deficient state, there is an exaggerated release of IL-6.[173]

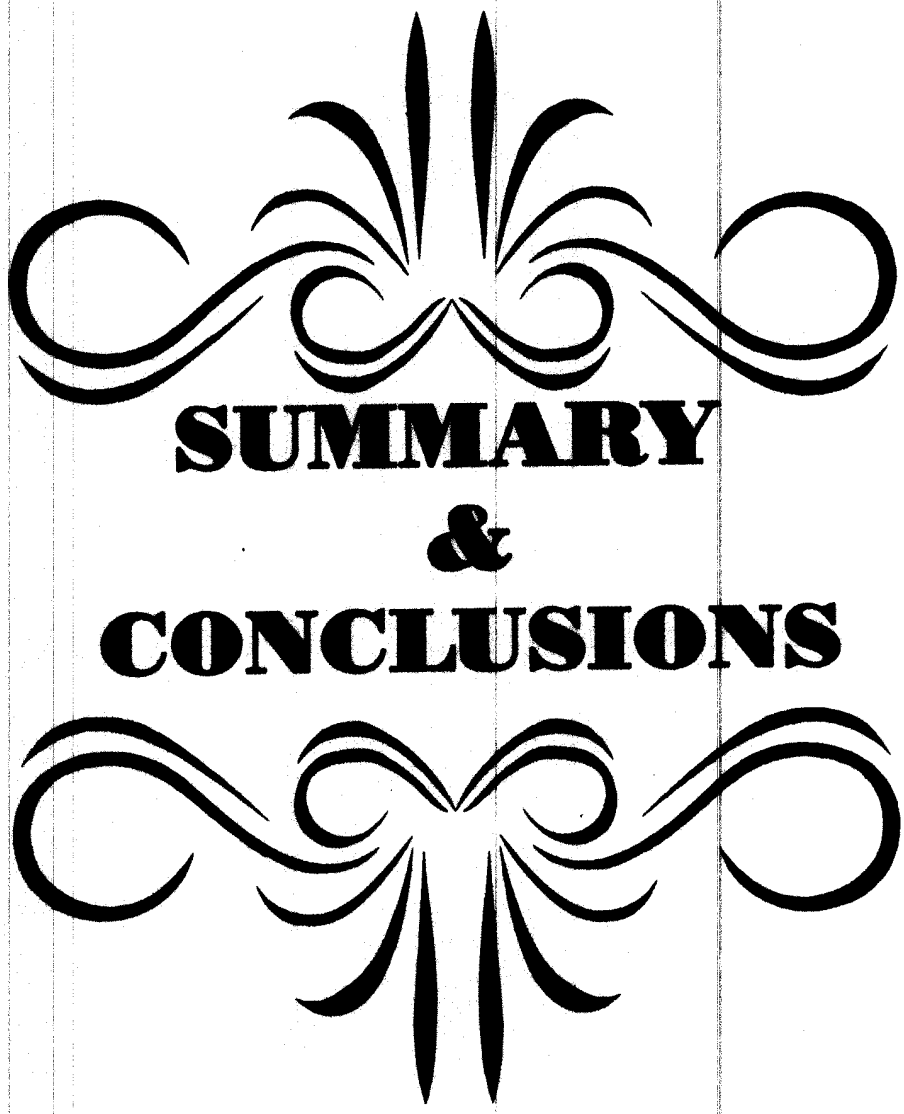
The effects of estrogen on cytokine activity are not confined to cytokine production itself, but are accompanied by complementary changes in the receptors and binding proteins of these cytokines (IL-6 receptor gp 80 and gp 130, soluble IL-6 receptor). These changes may potentially amplify the effects of the cytokine increase.[154]

It has been demonstrated that the cytokine IL-6, which potently induces osteoclastogenesis, is produced by osteoblastic cells in response to PTH. Osteoclasts express IL-6 and IL-6 receptor (IL-6R).[166]

Furthermore, it was reported that circulating levels of IL-6 were elevated in patients with primary hyperparathyroidism and correlate with biochemical markers of bone resorption,[166] as it is a pivotal factor in mediating increased bone turnover associated with the status of PTH excess.[174]

From the previous results, postmenopausal females with high bone turnover (10 subjects with osteoporotic changes) showed the lowest mean estradiol level and the highest PTH and interleukin-6 mean levels. The values observed in osteopenic group were midway between controls and osteoporotic group.

A better understanding of the cascade of cytokine changes that are responsible for postmenopausal bone loss may help to develop new pharmacologic and non-pharmacologic options for the treatment of the manifest postmenopausal osteoporosis with an even better risk-to-benefit ratio than the existing ones.[154]



SUMMARY & CONCLUSIONS

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Osteoporosis is a common disease affecting the majority of older women and a significant minority of older men. It is one of the major and growing health care problems around the world largely related to the general aging of societies with improvement in public and preventive health and delay in mortality. It is also becoming a major problem even in the developing countries.

Osteoporosis is the single most important health hazard associated with the climacteric. It is a disorder characterized by a reduction in the quantity of bone during aging without changes in its chemical composition. Osteopenia is used to indicate low bone mass, whereas osteoporosis is reserved for low bone mass with increased susceptibility to fractures.

Increased bone turnover in women after menopause has been generally explained by two different mechanisms, firstly it has been assumed that there is first a rapid phase of estrogen dependent bone turnover during early postmenopause, and a second phase of increased bone turnover in older age.

Bone growth and remodeling is a complex dynamic process that achieves a balance between the coupled process of bone formation and resorption. This process is regulated by the interplay of systemic hormones, locally produced cytokines and growth factors.

The aim of this work was to study the serum levels of interleukin-6, parathyroid hormone and estradiol in postmenopausal females and their possible role in bone changes in such cases.

Fifty subjects were included in the present study. Thirty postmenopausal females with radiological evidence of bone changes were selected and compared to a group of twenty apparently healthy young nonpregnant females. All subjects had no history of hypertension, diabetes mellitus, renal impairment, any inflammatory condition nor malignancy and they were not on estrogen replacement therapy or contraceptive pills.

To all the studied subjects, thorough history taking and full clinical examination were done. Bone densitometry was done using Dual X-ray absorptiometry (DXA) to three scanning sites: proximal femur, lumbar spine and forearm and according to the T-score level the postmenopausal females group was divided into two subgroups: osteopenic group (T-score: -1 to –2.5) (n = 20) and osteoporotic group (T-score ≤ –2.5) (n = 10).

Laboratory investigations were done to both control and postmenopausal groups. It included determination of serum levels of glucose, creatinine, CRP, total and ionized calcium, inorganic phosphate, intact parathyroid hormone, estradiol and serum activities of alanine aminotransferase (ALT) and alkaline phosphatase. Also, plasma level of IL-6 was estimated as one of the local bone regulatory cytokines.

It was observed that serum estradiol level was significantly lower in the total postmenopausal females, osteoporotic and osteopenic groups than the corresponding control group and it was significantly lower in the osteoporotic group than the osteopenic group. In the postmenopausal group serum estradiol was negatively correlated with the duration of menopause and positively correlated with the bone mineral density.

Estradiol could produce beneficial skeletal effects through several possible mechanisms, it reduces activation of bone metabolic units, it antagonizes PTH's stimulation of bone resorption, it may enhance the survival of osteoblasts via local cytokines or other growth factors, and it improves the efficiency of gastrointestinal calcium absorption and renal calcium conservation.

On the other hand, serum intact parathyroid hormone level was significantly higher in the total postmenopausal females, osteopenic and osteoporotic groups than the corresponding control group and it was significantly higher in the osteoporotic group than the osteopenic group. In the postmenopausal group, serum PTH was positively correlated with the duration of menopause and negatively correlated with the bone mineral density. The present results point to the possible relation between PTH and accelerated bone changes following estrogen withdrawal.

The alkaline phosphatase activity was significantly higher in the total postmenopausal females, osteopenic and osteoporotic groups than the corresponding control group.

The interleukin-6 was significantly higher in the total postmenopausal females, osteopenic and osteoporotic groups than the corresponding control group and it was significantly higher in the osteoporotic group than the osteopenic group. IL-6 was positively correlated with both PTH and duration of menopause and negatively correlated with both estradiol and BMD in the postmenopausal females group.

IL-6 plays a role in increased skeletal sensitivity to PTH serum in estrogen-deficient postmenopausal females. Thus, it is an important mediator of increased bone resorption after menopause especially in the first decade after menopause.

From the previous results, postmenopausal females with high bone turnover (10 subjects with osteoporotic changes) showed the lowest mean estradiol level and the highest PTH and IL-6 mean levels. The values observed in osteopenic group were midway between controls and osteoporotic group.

Conclusions

1- Female subjects included in the present study had a postmenopausal duration ranging from 2-10 years. All showed radiological evidences of bony changes in the form of osteopenia (66.7%) and osteoporosis (33.3%).

2- Postmenopausal females with osteoporotic bone changes had the lowest mean estradiol level and the highest PTH and IL-6 mean levels. The values in osteopenic group were midway between controls and osteoporotic group.

3- BMD was positively correlated with serum estradiol and negatively correlated with PTH and IL-6 in the postmenopausal females group.

4- The duration of menopause showed a positive correlation with PTH and IL-6 and a negative one with estradiol and BMD.

5- IL-6 showed a positive correlation with PTH and a negative correlation with estradiol, BMD.

6- IL-6 could play a role in increased skeletal sensitivity to the resorbing action of PTH seen in estrogen deficient postmenopausal females. A better understanding of IL-6 level changes responsible for postmenopausal bone loss may help to develop new pharmacologic and non pharmacologic options for the treatment of the manifest postmenopausal osteoporosis besides the already available ones such as hormone-replacement therapy.

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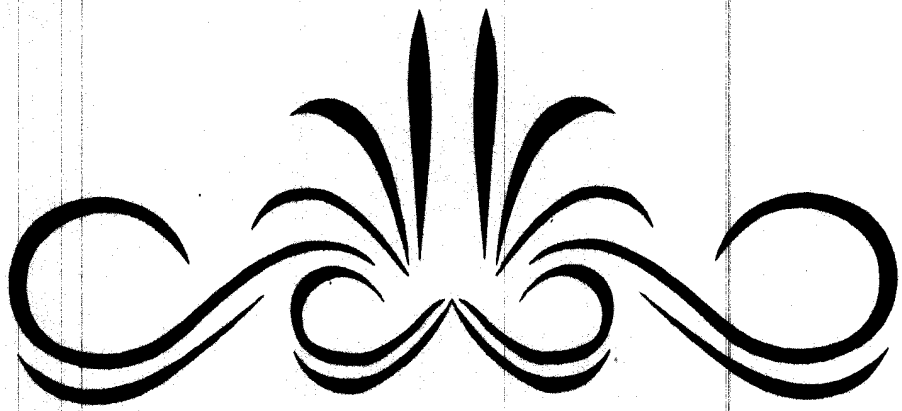

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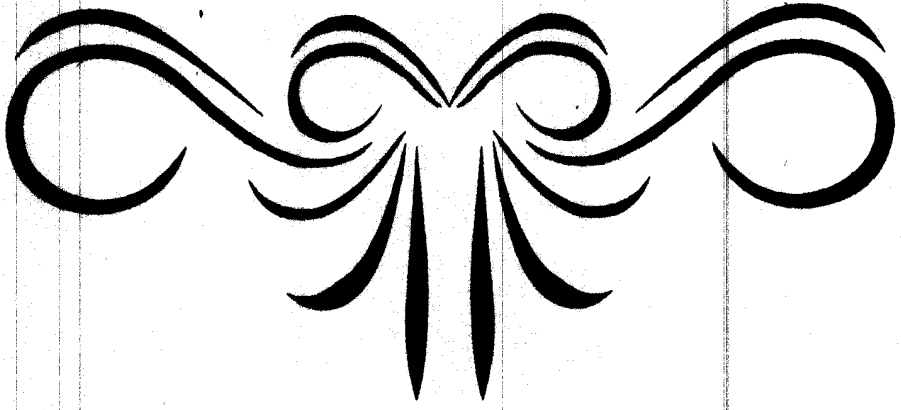

بسم الله الرحمن الرحيم

STUDY OF INTERLEUKIN-6, PARATHYROID HORMONE AND ESTRADIOL IN RELATION TO BONE CHANGES IN POSTMENOPAUSAL FEMALES

دراسة العلاقة بين الإنترلوكين-٦ وهرمونى الغدة الجاردرقية والإستروجين وتغيرات العظام فى السيدات فى سن اليأس

Protocol of a Thesis Submitted to
Medical Research Institute
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for Partial Fulfillment of

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In

Chemical Pathology

By

Rania Mohamed Hassan El-Sharkawy

MBB Ch

University of Alexandria - 1998

خطة بحث مقدمة إلى
معهد البحوث الطبية
جامعة الإسكندرية
إيفاء جزئيا للحصول على
درجـــة الماجستير
فـــى
كيمياء الباثولوجيا
من

الطبيبة/ رانيا محمد حسن الشرقاوى
بكالوريوس الطب والجراحة
جامعة الأسكندرية - ١٩٩٨

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2002

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INTRODUCTION

Throughout life, bone is being constantly resorbed and new bone being formed.[1]

Bone remodeling is mainly a local process carried out in small areas by population of cells called bone remodeling units.[1]

The remodeling is related in part to the stresses and strains imposed on the skeleton by gravity and other factors such as age, sex,[2] nutritional, genetic and endocrinal factors.[3] It is regulated at the systemic and local levels. Systemic regulators include many hormones, while cytokines and growth factors act as local regulators.[1]

Osteoporosis, the most common type of metabolic bone disease, is characterized by parallel reduction in bone minerals and matrix so that bone is decreased in amount but is of normal composition.[4]

Bone density in adults depends on both the peak bone density achieved during development and the subsequent bone loss.[4] Bone loss begins before menses cease in women although the precise time of onset is unknown.[4]

Menopause is defined as cyclic changes of hormones on hypothalamic pituitary ovarian axis and occur usually 30-35 years after menarche.[5] The most significant findings in menopause are the marked reduction in estradiol (E_2) and estrone (E_1) where serum E_2 is reduced to a greater extent than E_1.[4]

Estrogen receptors are abundant throughout the body, and therefore, the absence of estrogen potentially influences virtually all systems especially the bone.[4] Estrogen deficiency increases skeletal production of bone resorbing cytokines such as interleukin-1 (IL_1), interleukin-6 (IL_6) and tumour necrosis factor-α (TNF-α).[4] IL_6 is one of the cytokines that regulates bone density, since IL_6 has some effect-on stimulation of osteoclast resorption and has been implicated in the pathogenesis of bone loss associated with estrogen deficiency.[6,7,8,9]

The effects of parathyroid hormone (PTH) on bone are complex, as evidenced by its stimulation of bone resorption or bone formation, depending on the concentration of PTH and the duration of exposure[10]

PTH acts directly by altering the activity or numbers of osteoblasts (bone-forming cells) and indirectly on osteoclasts (bone-resorbing cells).[10,11] PTH-induced production of cytokines such as IL_6&IL_{11} may play a role in mediating the recruitment and activity of the osteoclasts.[10]

AIM OF THE WORK

The aim of the work is to study the serum levels of interleukin-$_6$, parathyroid hormone and estradiol in postmenopausal females and their possible role in bone changes in such cases.

MATERIAL

The study will include 50 non smoker females divided as follows:

- 20 young non pregnant adult females.
- 30 postmenopausal females.

All the studied subjects should not be receiving any drug (e.g. contraceptive pills in the control group, estrogen replacement therapy in postmenopausal females, cortisol,.....) nor suffering from any clinical condition e.g. (renal disease, inflammation, malignancy,....) that could affect the studied parameters.

METHODS

To all studied subjects the following will be done:

1- Detailed history taking with special stress on menestrual cycle and history of recent bone fractures.

2- Full clinical examination with special stress on skeletal system.

3- Bone Densitometry.[12]

4- Laboratory investigations including:

- Estimation of serum fasting and postprandial glucose & creatinine levels and alanine amino transferase activity.[13]
- Detection of C-reactive protein in serum.[14]
- Estimation of serum total and ionized calcium, inorganic phosphorus and alkaline phosphatase activity.[13]
- Estimation of serum estradiol level by ELISA.[13]
- Estimation of serum parathyroid level by chemiluminescence.[15]
- Estimation of serum Interleukin-6 level by ELISA.[7]

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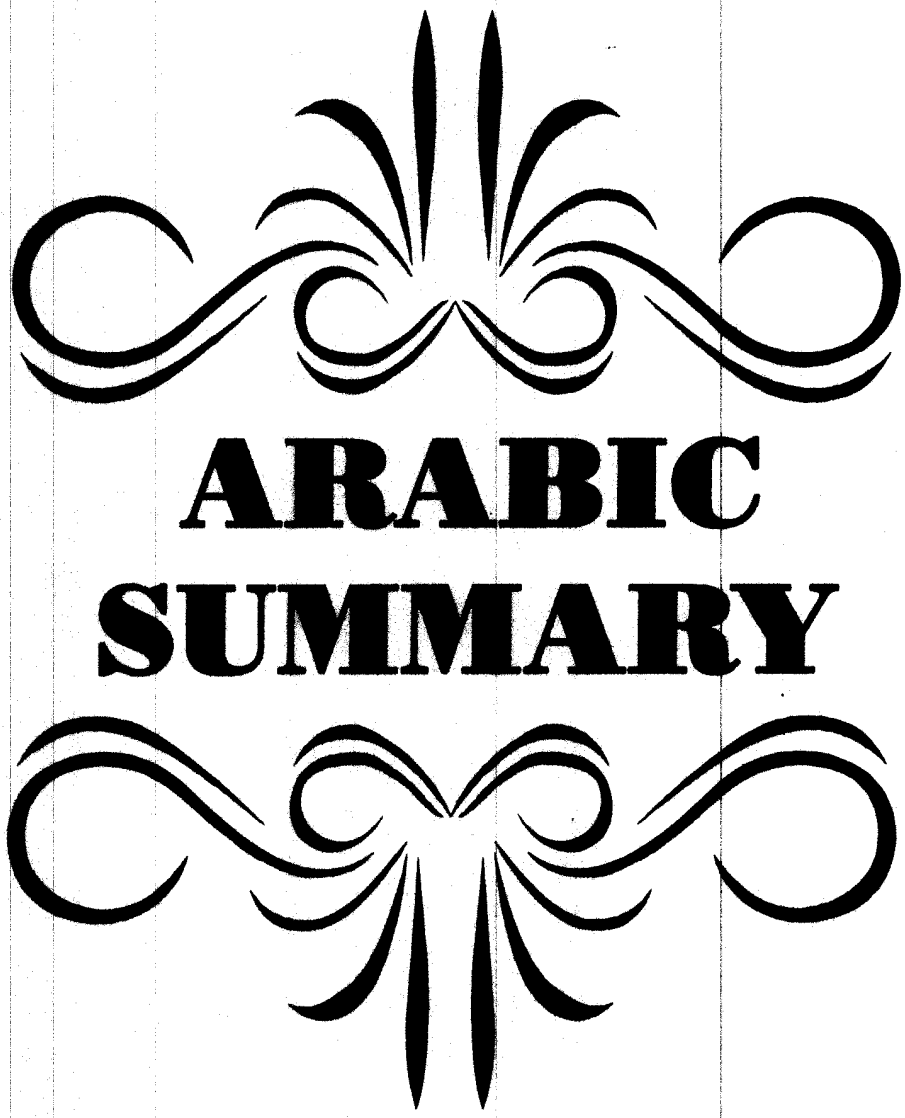

ARABIC SUMMARY

الملخص العربي

الملخص العربى

هشاشة العظام من الأمراض الأكثر شيوعاً بين الغالبية العظمى من النساء وبعض من الرجال فى المراحل العمرية المتقدمة. كما أنها تعد واحدة من أهم المشاكل الصحية فى جميع أنحاء العالم حتى الدول النامية.

إن هشاشة العظام تعتبر واحدة من أهم المشاكل الصحية فى فترة سن اليأس وتتميز بنقص فى كثافة العظم دون أن يحدث تغير فى مكوناته الكيميائية.

وترجع الزيادة فى معدل نقصان كثافة العظام فى السيدات فى سن اليأس إلى عاملين مهمين أساسيين **أولاً**: حدوث نقص فى هرمون الاستروجين فى المرحلة الأولى من سن اليأس مما يؤدى إلى الزيادة فى معدل نقص كثافة العظام. **ثانياً**: نقص كثافة العظام نتيجة تقدم السن (الوهن).

وتعتبر عملية نمو العظام وإعادة تكوينها من العمليات الديناميكية المعقدة التى تتطلب توازناً بين عمليتى تكوين العظام وهدمها وهذا التوازن يتم تحقيقه عن طريق كل من العوامل المركزية والعوامل الموضعية.

ولقد كان الهدف من البحث الحالى هو دراسة العلاقة بين الأنترلوكين-٦ وهرمونى الغدة الجاردرقية والإستراديول وتغيرات العظام فى السيدات فى سن اليأس.

وقد شملت هذه الدراسة خمسون سيدة منهم ثلاثون سيدة فى مرحلة سن اليأس كمجموعة المرضى وعشرون سيدة سليمة كمجموعة ضابطة. وكلا أفراد المجموعتين لا يعانون من ارتفاع فى ضغط الدم أو مرض السكر أو خلل فى وظائف الكلى أو أى أمراض سرطانية كما أنهم لم يكونوا تحت أثر علاج الإستروجين التعويضى أو أقراص منع الحمل. وقد تم عمل فحصاً إكلينيكياً شاملاً وأخذ التاريخ المرضى لكل شخص وتم قياس كثافة العظام فى عظمة الفخد والفقرات القطنية وعظام الرسغ وقد تم تقسيمهم إلى مجموعتين وفقاً لدرجة كثافة العظام. المجموعة الأولى وعددهم عشرة سيدات يعانون من هشاشة العظام والمجموعة الثانية وعددهم عشرون سيدة يعانون فقط من نقص فى كثافة العظام.

وقد شملت الفحوص المعملية على قياس مستوى كل من الجلوكوز والكرياتينين والكالسيوم الكلى والمتأين والفوسفور غير العضوى وهرمونى الغدة الجاردرقية والإستراديول وكذلك نشاط إنزيمى المحولات الأمينية والفوسفاتيز القلوى. كما تم تقييم الإنترلوكين-٦ كواحد من منظمات العظم الموضعية فى مصل الدم.

أظهر مستوى هرمون الإستراديول انخفاضاً ملحوظاً فى المجموعة الكلية للسيدات فى سن اليأس ومجموعة السيدات اللاتى يعانين من هشاشة العظام والمجموعة اللاتى يعانين من نقص فى كثافة العظام فقط عنه فى المجموعة الضابطة. كما وجد أن مستوى هرمون الإستراديول كان منخفضاً فى مجموعة السيدات اللاتى يعانين من هشاشة العظام عن اللاتى يعانين من نقص فى الكثافة فقط. كما لوحظ فى مجموعة السيدات فى سن اليأس ارتباطاً سلبياً بين مستوى هرمون الإستراديول ومدة انقطاع الطمث وارتباطاً إيجابياً مع مستوى كثافة العظام.

هرمون الإستروجين له فوائد عديدة على الهيكل التكوينى العظمى وهذا يحدث من خلال طرق عدة مثل تنشيط الوحدات البنائية للعظم ويضاد عملية النشاط الهدمى الناتجة عن زيادة هرمون الغدة الجاردرقية كما أنه يساعد على زيادة نشاط الخلايا البناءة للعظم من خلال التأثير على العوامل الموضعية. كما أنه يزيد من كفاءة امتصاص الكالسيوم من الجهاز الهضمى ومنع التخلص منه عن طريق الكلى.

كما أظهر مستوى هرمون الغدة الجاردرقية ارتفاعاً ملحوظاً فى المجموعة الكلية للسيدات فى سن اليأس ومجموعة السيدات اللاتى يعانين من هشاشة العظام والمجموعة اللاتى يعانين من نقص فى كثافة العظام فقط عنه فى المجموعة الضابطة. كما وجد أن مستوى هرمون الغدة الجاردرقية كان مرتفعاً فى مجموعة السيدات اللاتى يعانين من هشاشة العظام عن اللاتى يعانين من نقص فى الكثافة فقط كما لوحظ وجود ارتباطاً إيجابياً مع مدة انقطاع الطمث فى المجموعة الكلية للسيدات فى سن اليأس وأيضاً ارتباطاً سلبياً مع نسبة كثافة العظام فى المجموعة نفسها.

أما مستوى الفوسفاتيز القلوى فقد كان مرتفعاً فى المجموعة الكلية للسيدات فى سن اليأس ومجموعة السيدات اللاتى يعانين من هشاشة العظام ومجموعة اللاتى يعانين من نقص فى كثافة العظام فقط عنه فى المجموعة الضابطة.

أما الإنترلوكين-٦ فقد كان مستواه أعلى فى المجموعة الكلية للسيدات فى سن اليأس ومجموعة السيدات اللاتى يعانين من هشاشة العظام ومجموعه السيدات اللاتى يعانين من نقص فى كثافة العظام فقط عنه فى المجموعة الضابطة. كما أن مستواه كان مرتفعاً فى مجموعة هشاشة العظام عن مجموعة نقص كثافة العظام فقط. كما أنه لوحظ وجود ارتباط إيجابى بين مستوى الإنترلوكين-٦ وكلا من هرمون الغدة الجاردرقية ومدة انقطاع الطمث وأيضاً ارتباطاً سلبياً بينه وبين كلاً من مستوى الإستراديول ونسبة كثافة العظام.

الإنترلوكين-٦ له دوراً فعالاً فى زيادة الإحساس العظمى بهرمون الغدة الجاردرقية فى السيدات فى سن اليأس خاصة فى العقد الأول بعد سن اليأس.

ونستخلص من النتائج السابقة ما يلى:

١- مجموعة السيدات المشاركات فى البحث كانت مدة انقطاع الطمث عندهم تتراوح بين ٢-١٠ أعوام وجميعهم أظهروا دلائل إشعاعية على وجود تحلل فى العظام بين نقص فى كثافة العظام (٦٦,٧٪) وهشاشة العظام (٣٣,٣٪).

٢- السيدات اللاتى يعانين من هشاشة العظام وجد عندهم أعلى مستوى لكلاً من هرمون الغدة الجاردرقية والإنترلوكين-٦ أما مستوى هرمون الإستراديول فقد كان أكثر انخفاضاً.

٣- نسبة كثافة العظام ارتبطت ارتباطا إيجابياً مع مستوى هرمون الإستراديول وارتباطاً سلبياً مع كلاً من هرمون الغدة الجاردرقية والإنترلوكين-٦.

٤- مدة انقطاع الطمث ارتبطت ارتباطاً إيجابياً مع كلاً من هرمون الغدة الجاردرقية والإنترلوكين-٦ وارتباطاً سلبياً مع كلاً من مستوى هرمون الإستراديول ونسبة كثافة العظام.

٥- الإنترلوكين-٦ ارتبط ارتباطا إيجابياً مع هرمون الغدة الجاردرقية وارتباطا سلبياً مع هرمون الإستراديول.

٦- الإنترلوكين-٦ يلعب دوراً فعالاً فى زيادة معدل الإحساس العظمى بهرمون الغدة الجاردرقية فى السيدات فى سن اليأس.

٧- الفهم الأمثل للعوامل الموضعية المسئولة عن التحلل العظمى فى السيدات فى سن اليأس يمكن أن يساعد فى التوصل إلى خيارات دوائية وغير دوائية قد تساعد على الحد من عملية هشاشة العظام والتقليل من أعراضها الجانبية.

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يوم الاربعاء ١٩/٥/٢٠٠٤
وقبلت بنجاح بدرجة ممتاز
وتم عمل التعديلات المذكورة

توقيع

جامعة الإسكندرية

معهد البحوث الطبية

الدراسات العليا

موافقة العميد على تشكيل لجنة الحكم بالتفويض فى ١٨/٤/٢٠٠٤

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يعتمد

وكيل المعهد

للدراسات العليا والبحوث

(أ.د. عزت محمد حسن)

دراسة العلاقة بين الإنترلوكين-٦ وهرمونى الغدة الجاردرقية والإستروجين وتغيرات العظام فى السيدات فى سن اليأس

رسالة

مقدمة إلى معهد البحوث الطبية - جامعة الإسكندرية
إيفاءا جزئياً لشروط الحصول على درجة
الماجستير في كيمياء البائولوجيا

مقدمة من

رانيا محمد حسن الشرقاوى

بكالوريوس الطب والجراحة - جامعة الإسكندرية - ١٩٩٨

قسم البائولوجيا الكيميائية
معهد البحوث الطبية
جامعة الإسكندرية
٢٠٠٤