Study of Some Local Bone Regulators in Patients with Secondary Hyperparathyroidism under Maintenance Haemodialysis

THESIS

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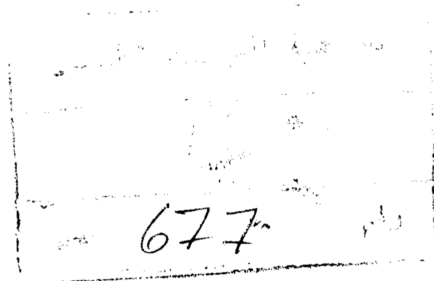

By

Moyassar Ahmad Mohamad Zaki

M.B.B.Ch. Faculty of Medicine. Alexandria University. 1995

Master of Chemical Pathology. Medical Research Institute. Alexandria University 2000

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Medical Research Institute
Alexandria University

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SUPERVISORS

Prof. Dr. Safaa A. El-Hefni

Professor of Clinical Pathology
Medical Research Institute
Alexandria University

Prof. Dr. Thanaa F. Moghazi

Professor and Head of Clinical Pathology Department
Medical Research Institute
Alexandria University

Prof. Dr. Mona H. Kandil

Professor of Clinical Pathology
Medical Research Institute
Alexandria University

Dr. Iman Salah El-Din Khalil

Lecturer of Internal Medicine
Medical Research Institute
Alexandria University

Dr. Tarek Y. Aref

Lecturer of Diagnostic Radiology
Medical Research Institute
Alexandria University

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List of Abbreviations

λ	:	Wavelength
$\Delta A/min$	:	Change in absorbance
$1,25\ (OH)_2D_3$	:	1,25 dihydroxy cholecalciferol
A	:	Absorbance
a.a.	:	Amino acids
ABD	:	Adynamic (Aplastic) bone disease
ACP	:	Acid phosphatase enzyme
Al	:	Aluminium
ALP	:	Alkaline phosphatase enzyme
$B/B_o.100$	:	Percent bound/total bound
BGP	:	Bone Gla protein
BMP	:	Bone morphogenetic proteins
BMSCs	:	Bone marrow stromal cells
BMU	:	Bone metabolic unit (or basic multicellular unit)
BSA	:	Bovine serum albumin
C.T.	:	Connective tissue
C/EBP delta	:	CCAAT /enhancer binding protein delta
CAE	:	Carbonic anhydrase enzyme
cAMP	:	Cyclic adenosine monophosphate
CaR	:	Calcium receptors
$CBFa_1$	:	Core binding factor a_1
CD	:	Cluster of differentiation
CRF	:	Chronic renal failure
Cs	:	Concentration of standard
CSFs	:	Colony stimulating factors
Cys	:	Cysteine

ESRD	:	End stage renal disease
FGF	:	Fibroblast growth factor
GFR	:	Glomerular filtration rate
GH	:	Growth hormone
gp 130	:	Glycoprotein 130
H_2O	:	Water
HD	:	Haemodialysis
HRP	:	Horseradish peroxidase
HSPG	:	Heparan sulphate proteoglycans
HTBD	:	High turnover bone disease
IGF	:	Insulin-like growth factor
IL	:	Interleukin
IL-1Ra	:	Interleukin-1 receptor antagonist
iNOS	:	Inducible nitric oxide synthase
iPTH	:	Intact parathyroid hormone
IRAP	:	Interleukin-1 receptor antagonist protein
IRSPs	:	Insulin receptor substrate proteins
KDa	:	Kilodaltons
LAP	:	Latency associated peptide
LTBD	:	Low turnover bone disease
M-CSF	:	Monocyte/macrophage-colony stimulating factor
MGP	:	Matrix Gla protein
MPS	:	Mononuclear phagocyte system
mRNA	:	Messenger ribonucleic acid
MUOD	:	Mixed uraemic osteodystrophy
NAD	:	Nicotinamide adenine dinucleotide
NADH	:	Reduced form of nicotinamide adenine dinucleotide

O_2	:	Oxygen
OAF	:	Osteoclast activating factor
OCIF	:	Osteoclastogenesis inhibitory factor
ODF	:	Osteoclast differentiating factor
OPG	:	Osteoprotegerin
OPGL	:	Osteoprotegerin ligand
PDGF	:	Platelet derived growth factor
PG	:	Prostaglandins
PKC	:	Protein kinase-C
PTH	:	Parathyroid hormone (parathormone)
PTHrP	:	Parathyroid hormone related peptide
RANK	:	Receptor activator of nuclear factor-kappa B
RANKL	:	Receptor activator of nuclear factor-kappa B ligand
ROD	:	Renal osteodystrophy
S	:	Standard
SHPT	:	Secondary hyperparathyroidism
sIL-6R	:	Soluble interleukin-6 receptors
SMADS	:	Homologues derived from SMA genes (present in caenorhabditis elegans) and MAD genes (present in drosophila and termed mothers against dorsophila decapentaplegic gene product)
T	:	Test (sample)
TGF	:	Transforming growth factor
TGFBP	:	Transforming growth factor β binding protein
TMB	:	Tetramethylbenzidine
TNF	:	Tumour necrosis factor
TR-1	:	Tumour necrosis factor receptor like molecule
TRANCE	:	Tumour necrosis factor-related activation-induced cytokine
TRAP	:	Tartarate resistant acid phosphatase
VDR	:	Vitamin D receptors

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INTRODUCTION

Chapter (I)

Bone Structure and Bone Remodeling

The bone is a special form of connective tissue (C.T.), made up of bone cells and a mineralized collagenous matrix.[1-5] The four principal differentiated cell types found in bone are osteoblasts, osteocytes, osteoclasts and lining cells.[1-5]

The osteoblasts are bone forming mononuclear cells derived from marrow stromal fibroblastic system. They are characterized by their location and morphology, the presence of a specific skeletal isoform of alkaline phosphatase enzyme (ALP) and receptors for parathyroid hormone (PTH) and vitamin D_3 (1,25 $(OH)_2D_3$). They are responsible for secretion of the organic bone matrix, or osteoid which is subsequently mineralized. When an osteoblast becomes embedded in the matrix and stops secreting protein, it is then termed an osteocyte. The osteocyte is thought to be concerned with preservation of bone matrix and mineral content.[1-6]

Osteoclasts are the multinucleated bone resorbing cells of apparently mononuclear phagocytic origin. They are involved in the transport of lysosomal enzymes (e.g. tartarate resistant acid phosphatase (TRAP), arylsulphatase, β-glycerophosphatase, β-glucuronidase, cathepsins B&C and other cysteine proteases). They are also involved in the transport of hydrogen ions which promote solubilization of the mineral phase of bone resulting in calcium release toward the interface of the cells with the mineral bone.[1-5,7,8] A high concentration of carbonic anhydrase enzyme II

(CAE-II) helps in acidification of the extracellular pocket between osteoclast's ruffled border and skeletal resorption surfaces.[9]

The lining cells line the majority of trabecular bone surfaces and may play a role in separating the bone from the marrow space.[3,10]

The extracellular matrix is formed of organic (35%) and inorganic (65%) parts.[1-5,11] The organic part is composed mainly of type I collagen (90%). The remainder includes many non-collagenous products of osteoblasts such as osteocalcin (bone Gla protein = BGP), osteonectin (matrix Gla protein = MGP), bone morphogenitic proteins (BMPs), proteoglycans, phosphoproteins (osteopontin), sialoproteins, thrombospondins and bone derived cytokines and growth factors.[1-5,11-13] Some of these proteins may function in initiating mineralization and in binding of the mineral phase to the organic matrix.

The inorganic (mineral) part which makes up approximately 2/3 of the weight of mature bone exists as a complex mixture of calcium and phosphate in the form of hydroxyapatite crystals, in addition to small amounts of non-phosphate compounds such as sodium, magnesium, potassium and calcium carbonates.[1-13]

The bone is synthesized by secretion of bone collagen (type I) in a highly organized manner, resulting in the formation of layers of bone matrix called lamellae. The organic part in the newly deposited unmineralized state is termed osteoid. Mineralization of the osteoid begins with deposition of amorphous calcium and phosphate at the interface

between osteoid and mineralized matrix, that subsequently mature into hydroxyapatite crystals; the mineral phase characteristic of adult bone.[12,14]

Although the mechanism of bone formation is the same in all bones, it may occur within a cartilage (endochondral), within an organic matrix membrane (intramembranous) or by deposition of new bone on pre-existing one (appositional).[1,15]

The skeleton is a metabolically active organ that undergoes modeling and continuous remodeling throughout life. Bone modeling refers to alterations in the bone shape, whereas remodeling refers to bone turnover that does not alter the shape, however the two processes often occur simultaneously.[15,16]

Modeling and remodeling do not result simply from the activity of a single cell type (osteoblast or osteoclast) or a single cell function (formation or resorption). Instead they may result from co-ordinated resorption and formation of bone over extensive regions of bone and for prolonged periods.[15]

Bone is initially formed by modeling, that is the deposition of mineralized tissue at developmentally determined sites, generally preceded by a cartilage analog.[16] Much of the turnover of bone during growth results from bone modeling but at least some remodeling also occurs.[15]

Remodeling of bone begins early in foetal life, and once the skeleton is fully formed in young adults, almost all the metabolic activity is in this form. The bone remodeling cycle depends on interaction of two cell

lineages, the mesenchymal osteoblastic lineage and the haematopoietic osteoclastic lineage. The cells involved in a particular remodeling event are termed basic multicellular unit or bone metabolic unit (BMU).[3,12,15-18].

Bone remodeling involves three phases or stages.[3,12,15-19] The initial activation phase includes activation of osteoclasts at mineralized bone surface with subsequent bone resorption. It takes about 7-10 days. This is followed by reversal phase where the mononuclear cells prepare the resorption lacunae for subsequent formation. Finally, the formation phase, which requires 2-3 months, is characterized by layering of osteoblasts into the resorbed lacunae with deposition of mineralized matrix.[3,13,17-20] (Figure 1)

The rate of skeletal turnover approaches 100% per year in the first year of life. The rate then declines to about 10% per year in late childhood and continues at this rate or a little bit slower throughout life.[3,6,16-18] This process is integral in mineral homeostasis, particularly calcium, and allows continuous renewal and strengthening of old bone.[3]

The control of bone cell composition and bone cell function is a complex phenomenon, that involves both systemic and local levels of regulation, and is monitored by biochemical markers.[5,21]

The bicohemcial markers of bone formation (osteoblastic activity) include osteocalcin, bone isoenzyme of alkaline phosphatase and type I procollagen propeptide, while the markers of bone resorption include tartarate resistant acid phosphatase, collagen cross links (pyridinoline and deoxypyridinoline), urinary hydroxy proline and galactosyl hydroxylysine.[13,21-24]

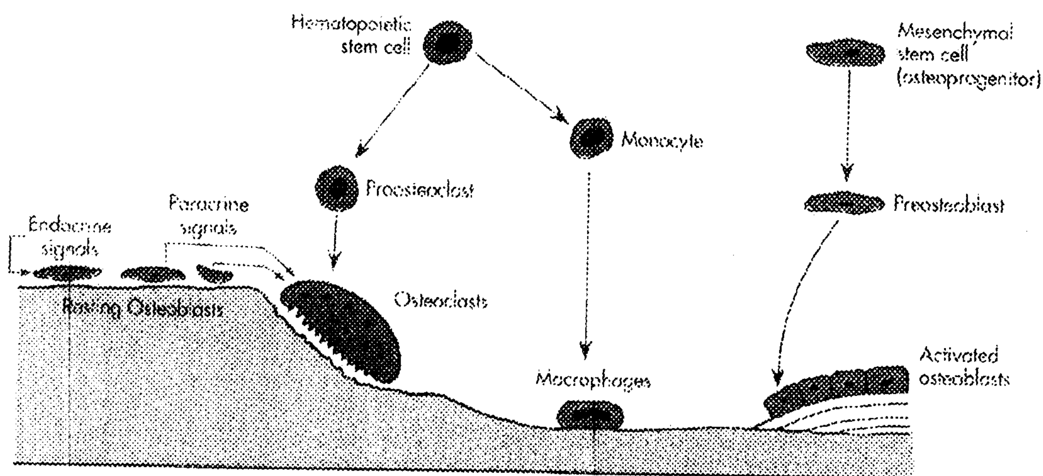


Figure (1): Process of bone remodeling. Endocrine signal to resting osteoblasts generate local paracrine signals to nearby osteoclasts and osteoclast precursors. The osteoclasts resorb an area of mineralized bone, and local macrophages complete the clean-up of dissolved elements. The process then reverse 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.[20]

Chapter (II)

Systemic and Local Bone Regulation

The major regulators of bone growth and remodeling include systemic and local bone regulators.

A- Systemic bone regulators

Parathyroid hormone (parathormone = PTH)

It is an 84 amino acids (a.a.) peptide, secreted by chief cells of parathyroid glands in response to a fall in plasma ionized calcium level.[1,3,17,24-28]

The effects of parathormone on bone are complex and biphasic. With high PTH concentration, there is an acute inhibition of collagen synthesis and osteoblast maturation. However, prolonged intermittent secretion of PTH results in increased bone formation via a direct action on osteoblasts.[24,25,27-30]

Parathormone is also a potent stimulator of bone resorption through increasing osteoclast activity and maturation. The action of PTH on osteoblasts mediates the subsequent activation of osteoclasts. Osteoblasts appear to be the primary target for action of PTH.[3,24-31] Protein kinase-C (PKC) has been shown to be activated by PTH in osteoblasts, suggesting that this activation is a component of the signaling pathway that mediates PTH-stimulated bone resorption.[32]

The plasma PTH level tends to increase with age, resulting in increased bone turnover and loss of bone mass.[17,24]

1,25 dihydroxycholecalciferol: (1,25 $(OH)_2$ D_3)

The chief effects of vitamin D are directed towards intestinal calcium and phosphate absorption. The active form of vitamin D_3 (1,25 $(OH)_2$ D_3) affects the bone by increasing the number and activity of osteoclasts with subsequent bone resorption. It also stimulates osteoblasts to synthesize osteocalcin and bone alkaline phosphatase.[1-5,17,24,33-38]

In addition, there are other hormones and vitamins that play a role in regulating skeletal growth and remodeling which include:

Calcitonin (Thyrocalcitonin)

Calcitonin is a potent inhibitor of osteoclastic bone resorption, that appears to play a minor role in calcium regulation in adult human bone.[1,3,17,23]

Growth hormone (GH)

Growth hormone acts through both systemic and local production of IGF-I to stimulate bone formation and resorption.[1-3,16,17,39-41]

Thyroid hormone

Triiodothyronine, in particular, can stimulate the coupled processes of bone formation and resorption, therefore it is important in skeletal maturation.[1-3,16,17,42,43]

Adrenal glucocorticoids

Glucocorticoids inhibit matrix synthesis and osteoblastic activity. They also increase osteoclastic activity resulting in a net effect of bone resorption.[1-3,16,17,44,45]

Sex steroids (Androgens and estrogen)

Sex hormones are important in maintaining normal bone turnover. Androgens accelerate growth and somatic development including skeletal maturation.[1]

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.[17,37,46,47]

Insulin

Insulin is necessary for normal skeletal growth and bone composition. Diabetes mellitus is associated with osteoporosis.[1]

Vitamin C (Ascorbic acid)

Ascorbic acid is essential for the maintenance of the integrity of all connective tissues (CT) including bone, being an essential co-factor in the hydroxylation of proline and lysine in collagen synthesis. It is also important in the synthesis of matrix glycosaminoglycans.[1]

Vitamin A (Retinol)

Retinol stimulates osteoclastic bone resorption. In vivo hypervitaminosis A is associated with hypercalcemia, excessive bone resorption and periosteal calcification.[1]

Most of the systemic regulators exert their effect on bone through alteration in either production or activity of local growth factors or cytokines that regulate osteoblast and osteoclast precursors.[48,49]

B- Local Bone Regulators

Bone is a storehouse for local growth regulatory factors known as cytokines.(1-5,17,49,50) They are termed bone remodeling units as they control bone formation and resorption through their effects on osteoblasts and osteoclasts respectively.(51,52)

Cytokines are a diverse group of intracellular signaling proteins, produced and secreted by many and perhaps all cell types in response to appropriate stimuli.(53,54) Over hundred, structurally dissimilar and genetically unrelated cytokines have been identified and are responsible for regulation of many physiological processes in our body including bone growth and remodeling.(55-57)

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.(1,3,4,5,16,50,52,58)

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.(1,3,4,5,16,17,50,52,58,59)

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.[59,60]

Local bone regulatory cytokines, in general, are low molecular weight (6-60 KDa) hormone like polypeptides and glycoproteins synthesized as premature inactive precursors. Structurally, they are divided into those with β-sheet structure and those with α-helical structure.[55,57] Most of them consist of a single polypeptide chain , with the exception of TGF-β that consists of two chains.[55] Most of them contain an additional amino acid terminal sequence (signal peptide) to assist in transmembrane transport, except basic-FGF (b-FGF) and IL-1β that lack the signal peptides. This suggested that basic-FGF and IL-1β secretion may be mediated via specific proteases. The active mature polypeptide results from cleavage of the signal peptide from the precursor. [55,61,62]

The production of such cytokines by most cells is temporary. Their transient nature (half life from less than 30 minutes up to 2 hours) has been shown to correlate with the presence of an adenine-uracil (AU)-rich sequence in the 3` untranslated region of their m RNA.[54,55, 63]

Like peptide hormones and other cytokines, the local bone regulatory cytokines and growth factors exert their effects by binding to cell surface receptors, which then transduce intracellular signals. This results in modification of the target cell ultimately by changes in the pattern of gene transcription. The intracellular secondary messenger system used varies

depending on the particular cytokine and its receptor (s). Dimerization of receptors upon binding of the cytokine may be an important step in signal transduction.[55,56]

Except for few cytokines that act in an endocrine manner (on distant target cells) such as TGF-β, monocyte/macrophage- CSF (M-CSF) and stem cell factor, most of these bone regulatory cytokines act locally in either a paracrine (on adjacent cell) or autocrine (on the cell producing it) fashion.[53-55]

The complex and integrated relationships between the different cytokines are mediated through cellular events. Interactions may occur through a cascade in which one cytokine induces the production of another, through transmodulation of the receptor for another cytokine, through synergism or antagonism of two or more cytokines acting on the same cell, and/or through their interaction with systemic bone regulators.[53,63] Some cytokines have different effects on different target cells (pleiotropic). Conversely, the same effect may be mediated by more than one cytokine (redundancy).[53,63]

The process of osteoclastogenesis has been recently studied in details by some workers. The development of osteoclasts in vitro requires close interaction between osteoclast precursors and osteoblastic stromal cells. This interaction involves not only cytokines and growth factors but also some proteins that are TNF related, the so called RANK/ RANKL/ OPG system.[16,64-74]

This system consists of the Receptor Activator of Nuclear factor Kappa-B (RANK), its Ligand (RANKL) and its competitor Osteoprotegerin (OPG).[16,64-71] The RANKL, also known as osteoclast differentiating factor (ODF), tumour necrosis factor-related activation-induced cytokine (TRANCE), and Osteoprotegerin Ligand (OPGL), is a membrane protein expressed on the osteoblastic stromal cells and belongs to TNF family.[16,65-76] It can activate cells of osteoclastic lineage by interacting with RANK expressed on the surface of osteoclast progenitors.[16,65-79] This takes place in the presence of M-CSF resulting in osteoclast maturation.[69,72,80-82] The four independent signals proposed to enhance RANKL expression, are vitamin D receptor, cAMP, glycoprotein 130 and low calcium environment.[65,83-86]

Osteoprotegerin (OPG), also known as osteoclastogenesis inhibitory factor (OCIF) or tumuor necrosis factor receptor like molecule-1 (TR-1), is a TNF related protein produced by osteoblastic stromal cells. It binds to RANK, thus preventing RANKL interaction with RANK, resulting in inhibition of osteoclast development and maturation.[65-70, 85,87-93] (Figure 2)

The actions of many cytokines and hormones on the balance between activators and suppressors of osteoclast number and activity might be mediated through this system.[69,94,95] 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.[68,69,71,96-102] (Figure 3).

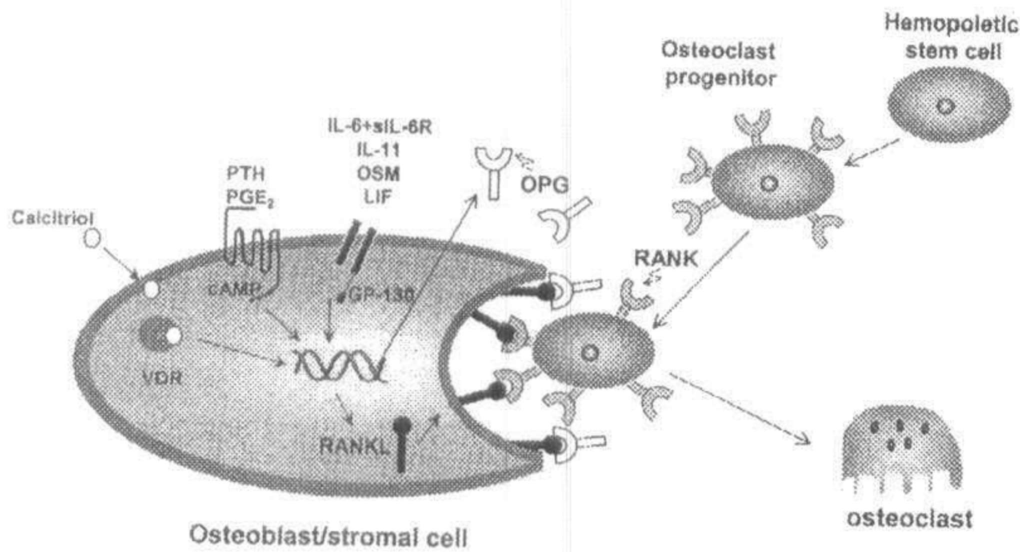


Figure (2): Diagrammatic representation of the role RANK/RANKL/OPG system in interaction between the osteoblastic stromal cell and osteoclast progenitor.[68]

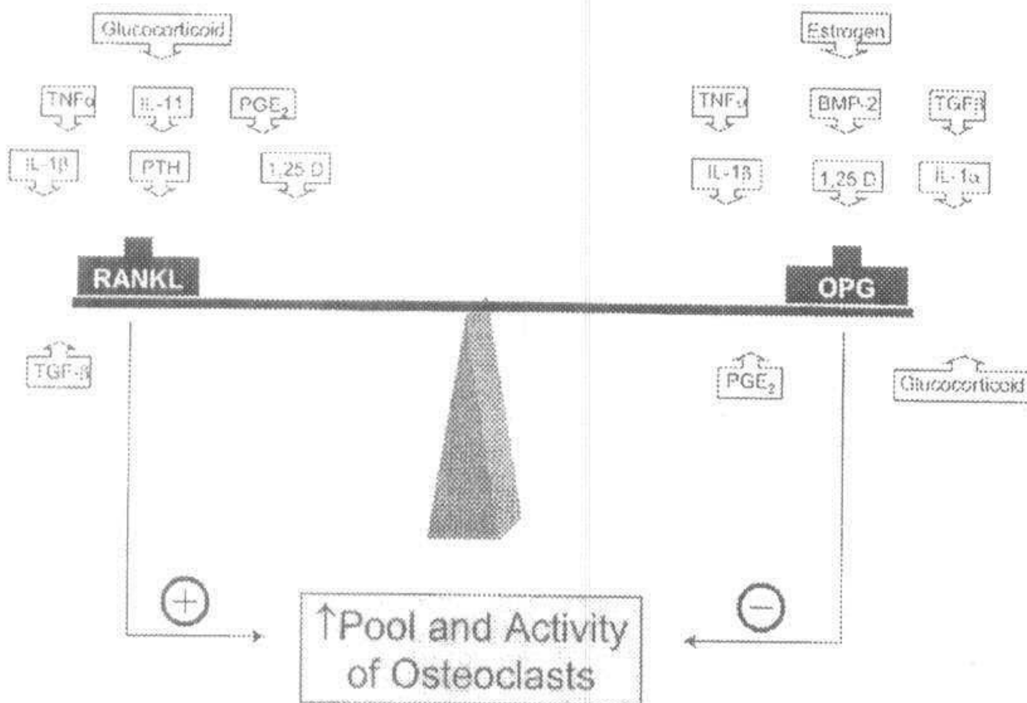


Figure (3): Diagrammatic representation of the influence of RANKL and OPG on osteoclast number and activity. RANKL will tip the balance towards increased osteoclast number and activity whereas increase in OPG will oppose this effect. The hormones and cytokines scattered around the ends of the balance beam will tip the balance in the direction indicated by the arrows.[68]

Although the role of RANK/RANKL/OPG system in disorders of human bone remodeling is under study, the disturbances of this system may impact on renal bone disease.[69] In fact, preliminary studies reported high levels of circulating OPG in uremia,[69] and could be of use in diagnosis of low turnover bone disease, at least in association with PTH levels $\leq$ 300 pg/ml.[103]

Interleukin-1 beta (IL-1β)

It is also known as osteoclast activating factor (OAF). It is one of three members of interleukin-1 family, that include also interleukin-1α (IL-1α) and interleukin- 1 receptor antagonist protein (IL-1Ra or IRAP).[63,104]

Members of the IL-1 family are produced mainly by T-lymphocytes and cells of mononuclear phagocyte system (macrophages), fibroblasts and osteoclasts.[53,55,62-64, 104,105]

IL-1β and IL-1α are synthesized as 31-33 KDa precursors that have a three dimensional, open barrel β-pleated sheet structure and lack a signal peptide (Figure 4).[106] Cleavage of IL-1β by specific proteases (IL-1β converting enzyme) results in the mature form (15-17 KDa).[53,55,62-64,104,105,107]

The mature IL-1β is composed of 12 β-strands held together by hydrogen bonds, with a tertiary structure resembling a tetrahedron, the interior of which is filled with hydrophobic side chains.[53,55,62-64,104]

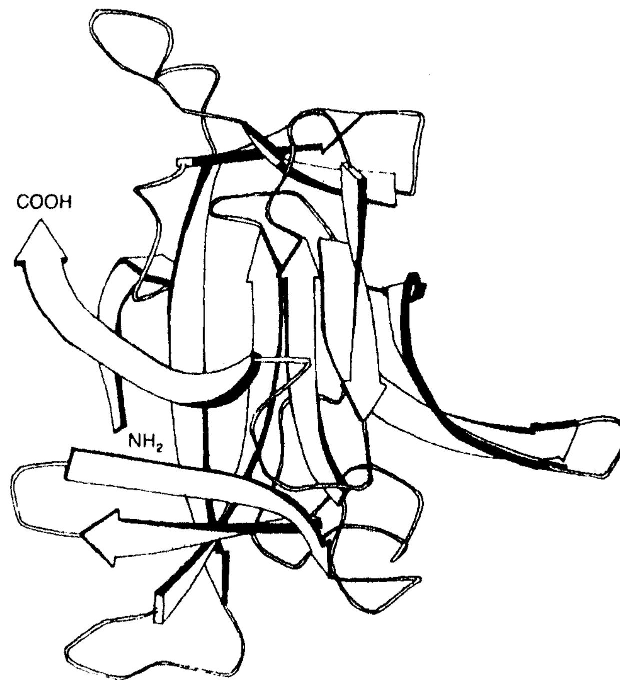


Figure (4): Human IL-1α backbone structure. Resolution 2.3 angstroms [106]

Despite minimal (22-26%) amino acids sequence homology, both IL-1β and IL-1α share similar biologic activities.[62,63,164,105]

The cellular effects of IL-1β are initiated by its binding to two specific high affinity cellular receptors located at the cell membrane. Their extracellular domains are members of the immunoglobulin (Ig) superfamily. Each comprises three IgG like domains, and share a significant(28%) homology to each others.[62,63,104,106] (Figure 5)

Type I receptor (IL-IRI) (80KDa) is found in almost all cells. It is composed of an extracellular portion, of 319a.a., a single hydrophobic transmembrane segment of 21 a.a. and a signal transducing cytosolic domain of 217 a.a. It has a higher affinity for IL-1α than IL-1β.[63,104]

Type II receptor (IL-1RII) (67 KDa) has a much smaller intracellular chain, and on cell activation, becomes shed from the cell to exist as a soluble receptor. It has a higher affinity for IL-1β. The tight binding of IL-1RII to IL-1β makes it a functionally negative "decoy" receptor, as it prevents binding of IL-1β to the signal transducing type I receptor .[63]

IL-1β exerts its bone resorptive effects by stimulating the release of soluble factors (CSF, IL-6 and IL-11) that increase proliferation of osteoclast precursors and activation of mature osteoclasts.[52] The release of soluble factors, particularly IL-6, has been attributed to protein kinase-C (PKC) beta, which is a component of the signaling pathway that mediates IL-1β stimulated IL-6 expression.[32]

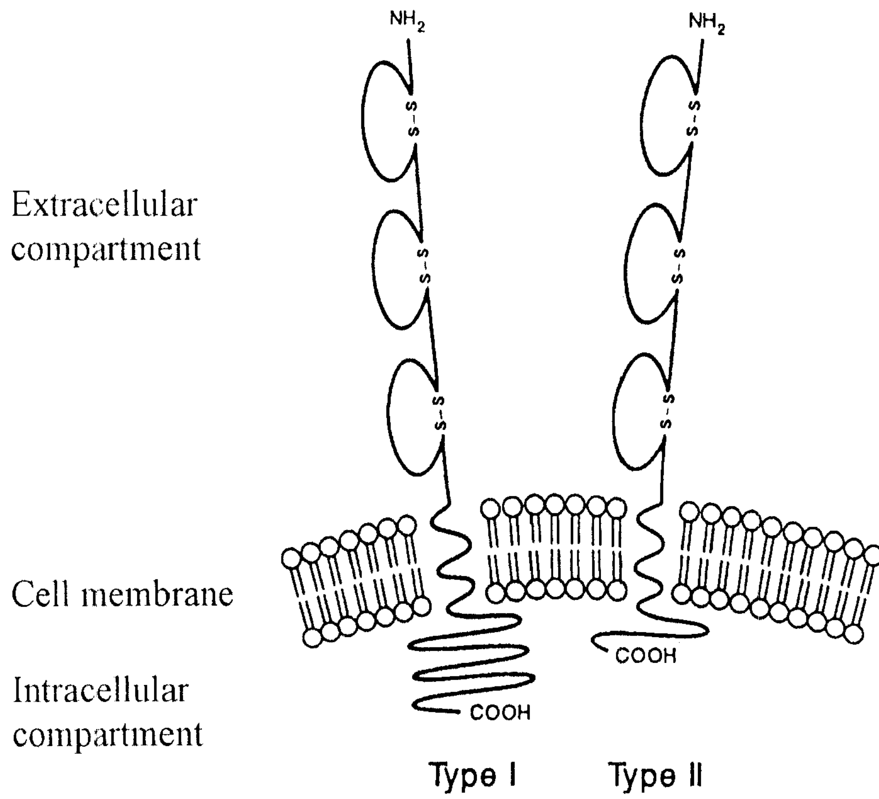


Figure (5): IL-1 receptors, type I and type II[106]

Tsai JA *et al* (2000) reported that actions of IL-1β on bone may be mediated by involving both local increase of parathyroid hormone related peptide (PTHrP) via upregulating its mRNA expression, together with a decrease in transforming growth factor β (TGF-β) synthesis. [108]

IL-1β is a potent stimulator of inducible nitric oxide synthase (iNOS) expression in bone cells. Nitric oxide has been suggested to be involved in regulation of bone turnover particularly in conditions characterized by release of bone resorbing cytokines. The effects of nitric oxide may be mediated by modulating IL-1 induced nuclear activation of nuclear factor Kappa B (NF-κB) in osteoclast precursors.[109,110]

The bone resorbing effects of IL-1β are 13 folds more potent than IL-1α and 1000 folds more potent than tumour necrosis factor-α (TNF-α).[111-113] The bone resorptive effects are associated with increased prostaglandins (PGs) synthesis, particularly the E series (PGE_2).[1,5,16,58,101,114-117] The prostaglandins may have an initial inhibitory effect on osteoclasts, but their predominant long term effect is to stimulate bone resorption via a cAMP-dependent protein kinase-A mediated mechanism and RANKL induction, resulting in osteoclast maturation.[73,118-122] Furthermore, the effects of other agents on bone may be mediated through their effects on synthesis of prostaglandins.[123-126]

As regards its effect on human osteoblasts, IL-1 inhibits phosphorylation of specific proteins induced by growth factors such as

platelet derived growth factor (PDGF) and insulin like growth factor-1 (IGF-I), via a mechanism independent of protein kinase A and C or PGs synthesis.[127]

Tumour necrosis factor-alpha (TNF-α)

Tumour necrosis factor-α is also called cachectin (being responsible for cashexia due to malignant tumours). It is a powerful stimulator of osteoclastic bone resorption in vivo.[53,104,113,128,129]

TNF-α is one of ten members of the TNF family. The TNF family consists of two secretable isoforms, α (referred to as just TNF or cachectin) and β (known as lymphotoxin-α). The other eight members are transmembrane proteins that act chiefly through cell to cell contact.[63,104,128,130]

TNF-α is a 157a.a homotrimer that consists of 3 identical polypeptide chains, having a β-jelly rolls conformation (Figure 6).[131] Both TNF-α and TNF-β share about 30% amino acid homology, and may bind to the same receptor and mediate some shared biological effects.[55,63,104]

TNF-α is produced by many cell types including cells of the mononuclear phagocyte system (activated monocytes and macrophages).[63,64] TNF-α exists in 2 forms, a membrane bound 26KDa precursor that has an extracellular carboxyl terminus and an intracytoplasmic amino terminus, and a 17 KDa mature (secretable) biologically active form that results from proteolytic cleavage of the precursor.[63]

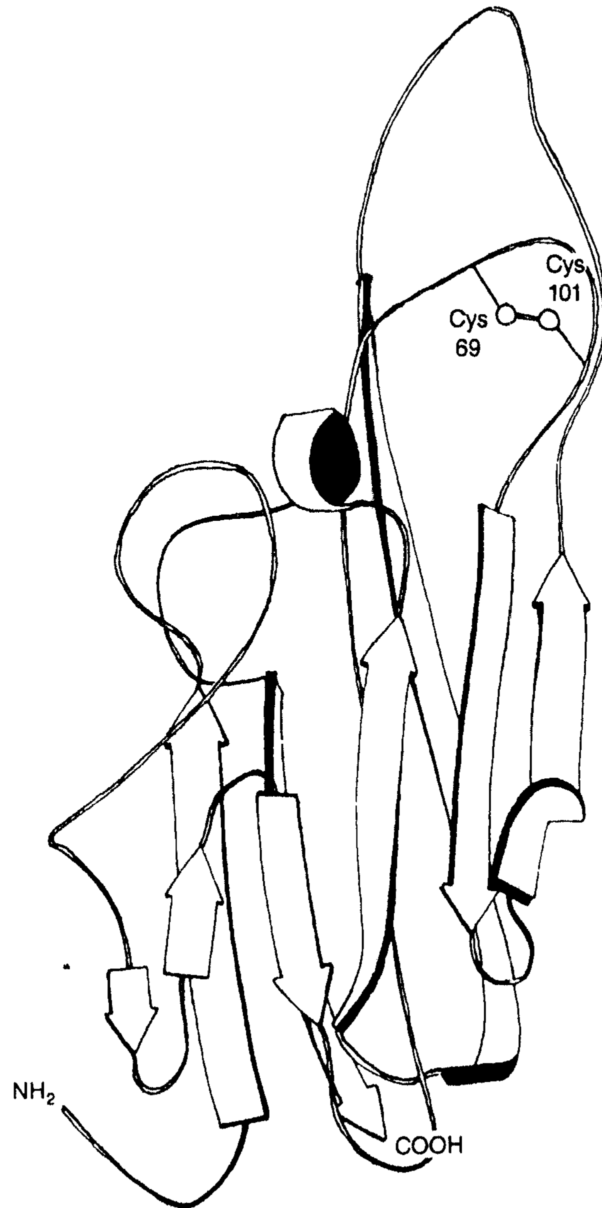


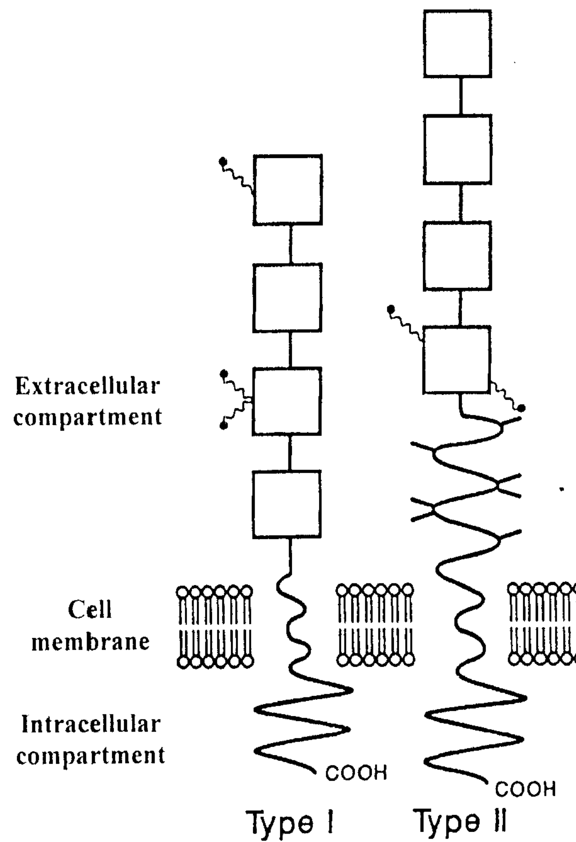
Figure (6): Molecular model of tumour necrosis factor-α (cachectin) (human recombinant form). Resolution 2.6 angstroms.[131]

TNF-α exerts its biological actions by binding to two transmembrane glycoprotein receptors, characterized by cysteine rich amino acid motif in their extracellular domains (Figure 7).[63,104,132] The two receptor types are the 55KDa TNF-receptor I (also known as CD 120 a or p55) and the 75 KDa TNF-receptor II (also known as CD 120b or p75), which are expressed on nearly all nucleated cells and are shed from the cell surface to exist as soluble receptors.[132] The TNF receptor I has been thought to be the major biologically active form, yet recent evidence indicates that TNF receptor II is also functional.[133-135]

TNF-α induces bone resorption through a primary effect on osteoblasts and an indirect effect on osteoclasts. Suppression of osteoblast differentiation is likely to be an important mechanism of decreased bone formation in many circumstances, where excess TNF-α is produced in the bone microenvironment. TNF-α inhibits osteoblast differentiation from precursor cells through inhibiting IGF-I expression. Furthermore, expression of, or response to osteogenic transcription factors induced by bone morphogenetic proteins 2,4 and 6 is inhibited by TNF-α.[136-138] TNF-α was reported to induce osteoblast resistance to vitamin D.[138-140]

TNF-α has been suggested to regulate apoptosis of osteoblasts, a mechanism that could accelerate the exit of osteoblasts or their precursors from the functional pool.[138,141-144]

Resolution 2.85angstroms



Schematic representation

The two receptors for TNF are designated type I (CD120a) and type II (CD120b). Both the 55 KDa type I receptor and the 75 KDa type II receptor bind TNF-α and TNF-β. There are three potential N-linked glycosylation sites in human p55 type I receptor and 2 glycosylation sites in the p75 type II receptors

Figure (7): TNF receptors. [132]

Considering the effect of TNF-α on osteoclast differentiation and activation, it has been demonstrated that TNF-α, indirectly stimulates proliferation of osteoclast haematopoietic precursors (mediated by type II receptor) and activation of mature osteoclasts (mediated by type I receptor), through involvement of protein kinase (C) beta-1 and the release of soluble factors (IL-6 and M-CSF) from nearby osteoclasts.[32,52,112,145,146] The synergistic effect of IL-1β in stimulating the bone resorptive activity of TNF-α has been reported, and has been suggested that TNF-α mediates its bone resorbing effects through PGE_2.[122,144,147,148]

Considering the overlapping in their signaling pathways, it was suggested that TNF-α and RANKL might synergistically orchestrate enhanced osteoclastogenesis via co-operative mechanisms. In fact it has been reported that TNF-RI is required for both basal RANK expression and signaling and for basal osteoclast formation by RANKL. Both are reduced in the absence of TNF-RI.[144,149] Conversely, RANKL increases TNF-α mRNA level and induces TNF-α release from osteoclast progenitors, thus TNF-α mediates, at least in part, RANKL's induction of osteoclastogenesis.[150]

As regards effects of TNF-α on bone matrix, TNF-α has been reported to inhibit osteocalcin and type I collagen synthesis by osteoblasts and stimulates osteoblastic synthesis of proteolytic enzymes such as plasminogen activators and matrix metalloproteinases, which are responsible for degradation of the bone matrix .[46] Moreover, matrix attachment of osteoclast precursors and mature osteoclasts are governed by distinct α_v integrins which are differentially regulated by specific cytokines including TNF-α, whose effect on the β_5 integrin is mediated through the type I receptor.[151]

Interleukin-6 (IL-6)

Interleukin-6 is a 26KDa cytokine, secreted by many cells including mononuclear phagocyte system (MPS) cells, endothelial cells and activated T-helper cells (Figure 8).[152] The major stimuli for its secretion are IL-1 & TNF-α. The half life of IL-6 is 1 hour.[153]

IL-6 exerts its biological activity via interaction with a cell surface receptor, that consists of 2 glycoproteins : a ligand binding 82KDa glycoprotein and a signal transducing 130KDa glycoprotein (gp 130).[154-156] (Figure 9)

IL-6 regulates pleiotropic functions of cells and tissues. In bone, the IL-6 produced by osteoblastic and osteoclastic cell lineages stimulates osteoclastic recruitment and differentiation.[157-160] Its signaling is mediated by a soluble form of receptor (sIL-6R), detected in urine and sera of healthy subjects,[161-163] and depends on signal transducing IL-6R expressed on bone marrow stromal cells (BMSCs) and osteoblasts but not osteoclast progenitors.[164]

IL-6 together with its soluble receptor play an important role in extracellular matrix degradation through enhancing collagenases and gelatinases expression via a transcriptional mechanism.[165]

High IL-6 and sIL-6R levels were reported in haemodialysis (HD) patients together with enhanced IL-6R mRNA expression in osteoclasts that parallels their bone resorbing activity.[69,166-170]

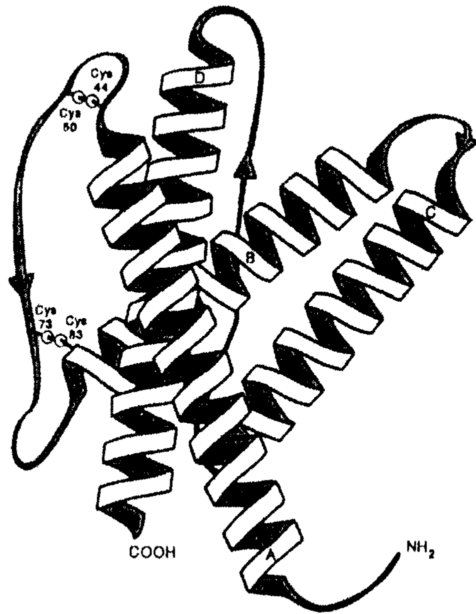


Figure (8): Schematic representation of IL-6[152]

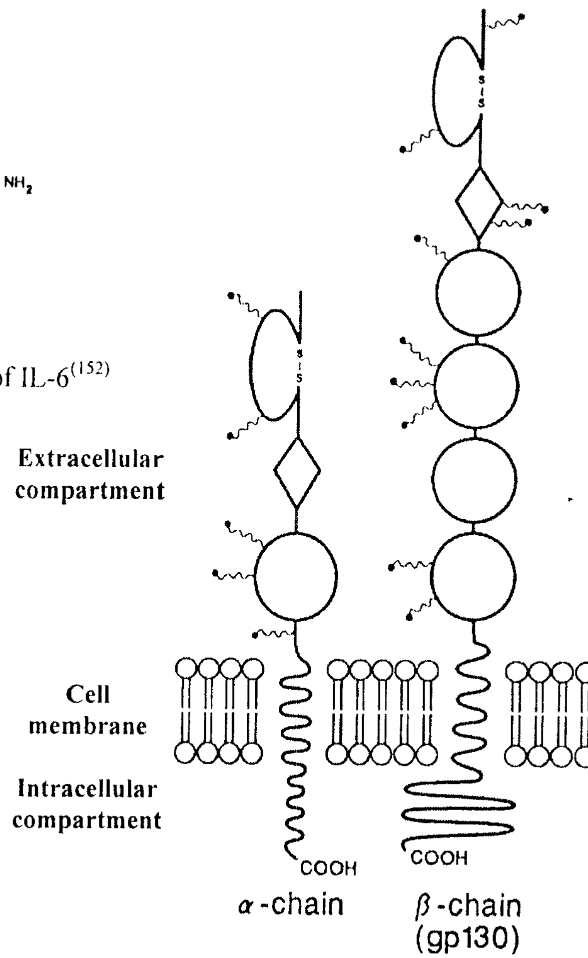


Figure (9): Schematic representation of IL-6 receptor. There are five potential N- linked glycosylation sites in the human IL-6 Rα chain and ten in the gp130.[152]

IL-6 affects mature osteoclastic function through its interaction with extracellular calcium sensing. A high calcium enhances IL-6 secretion. The released IL-6 attenuates calcium denoting a sustained osteoclastic activity in the face of an inhibitory calcium level locally generated by resorption.[171]

IL-6 appears to mediate the effect of selected hormones on bone such as growth hormone that increases IL-6 mRNA and protein levels in osteoblasts.[172] Parathormone also shares a common signal transduction pathway with IL-6 mediated by adenyl cyclase,[173] and glucocorticoids induce osteoclastic IL-6R expression.[164,170]

Monocyte/macrophage-colony stimulating factor

Colony stimulating factors (CSFs) have been so named because of their capacity to stimulate haematopoietic stem cell or osteoprogenitor cells to form colonies in vitro. Some of them have retained their "colony stimulating" names, and include granulocyte-CSF (G-CSF), granulocyte/macrophage-CSF (GM-CSF) and monocyte/macrophage-CSF (M-CSF).[63,64,104,155,174]

Monocyte/macrophage-CSF is found in 2 disulphide bonded homodimeric forms (i.e. peptides of 70-90 and 40-50 KDa respectively). It is also known as CSF-1. It is produced by many cell types which include BMSCs.[63,104]

In addition to its role in stimulating the proliferation, differentiation and activation of the monocyte/macrophage lineage of haematopoietic cells, M-

CSF is essential for osteoclast formation from its progenitor cells.[80-82,145,175-182] Paradoxically, this cytokine inhibits the activity of mature osteoclasts.[178]

The mechanism by which M-CSF exerts its action in this aspect might be directly on osteoclast precursors or indirectly on accessory cells influencing osteoclast generation such as BMSCs of osteoblastic lineage and is mediated by the so-called RANK/RANKL/OPG system.[80-82,175-182]

Transforming growth factor-beta (TGF-β)

Transforming growth factor-beta is thought to play an important role in human bone remodeling, being a central component in the coupling of bone formation to resorption.[16,49,58,183-186]

Transforming growth factor-beta (TGF-β) is a member of a family of dimeric polypeptides that include inhibin, activin, bone morphogenetic proteins (BMPs), Vg-I protein (oncogene product), Müllerian inhibitory substance and gene product of Dorsophilia. TGF-β is not related to TGF-α.[187-189]

Transforming growth factor-beta (TGF-β) is virtually produced by all body cells, but the richest sources are activated macrophages and platelets.[187,188]

The 25KDa polypeptide TGF-β is a disulphide linked homodimer formed of two identical polypeptide chains (12.5KDa each) of 112 amino acids. In humans, three TGF-β isoforms are recognized (TGFβ1,2 and 3), which are structurally similar in their C-terminal region and have the same functions in respect to their regulation of cellular growth and

proliferation.[190,191] They differ in their binding capacities for TGF-β receptors.[192]

TGF-β is synthesized as biologically inactive high molecular weight pre-pro-form of 390-412 amino acids. The cleavage of the 29 amino acids signal peptide and N-glycosylation yield a pro-TGF-β monomer. The cleavage of pro-TGF-β is mediated by endopeptidase to yield the 12.5 KDa monomer.[193] The cleaved fragment dimerizes to form a 75KDa latency binding peptide (TGF-BP) which assists in folding of the 25KDa dimer and remains associated to form the latent complex (Figure 10).[194] A 135 KDa latency associated peptide (LAP) bearing mannose rich carbohydrate may attach to the complexed latency peptide dimer. The latent complex may be stored in platelets (in α granules) or secreted. Secretion is enhanced by the carbohydrate moiety.[194-196] Most of the secreted TGF-β exists in the extra cellular matrix as latent complex (TGF-β and TGF-β latent binding protein held together by disulphide bonds) that prevents TGF-β binding to its receptor. Release of TGF-β peptide from the complex is either mediated by the multifunctional matrix glycoprotein thrombospondin-1 or plasmin, resulting in the mature TGF-β isoform.[194-197]

TGF-β has 3 major biological effects: growth inhibition, stimulation of extracellular matrix formation and immunosuppression.[188,194,197,198] TGF-β_1 exerts its biological actions by binding to three high affinity cell surface receptors (transmembrane serine / threonine kinases) known as type I, II and III. The type III receptor is the most abundant type.[187,188,194,196-198]

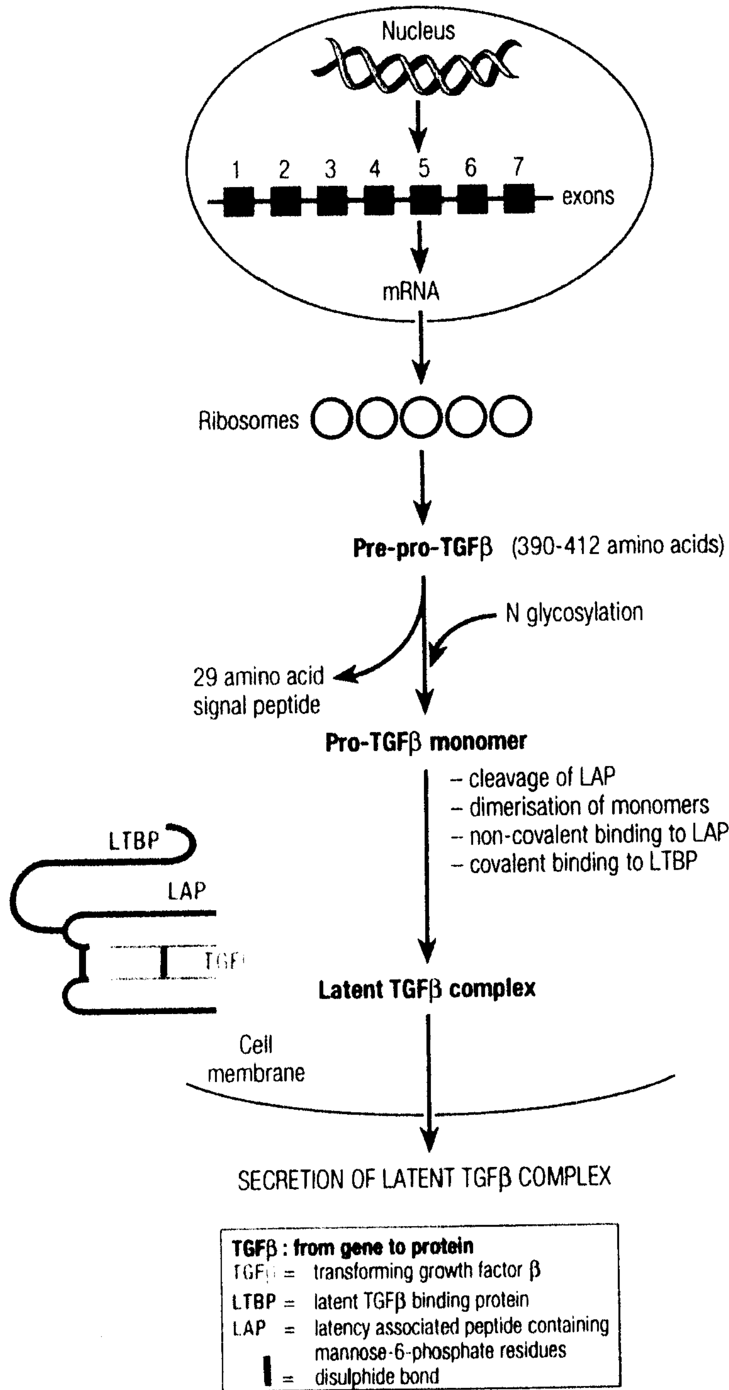


Figure (10): Synthesis and secretion of TGF-β from gene to released product. Details of intracellular processing is shown to TGF-β_1 for which most information is available. Other TGF-β isoforms are believed to follow a similar sequence.[194]

A general mechanism for TGF-β signaling starts when TGF-β binds to the non signal transducing type III receptor that presents TGF-β to type II receptor or directly to type II receptor on cell membrane[196,199]. Once activated, type II receptor recruits, binds and phosphorylates type I receptor, stimulating its protein kinase activity. The activated type I receptor phosphorylates some transcription factors known as SMADs (derived from SMA genes present in C. Elegans and MAD genes = Mothers Against Dorsophila decapentaplegic gene product), particularly SMAD 2 or SMAD 3, that bind to SMAD 4. The resulting SMAD complex moves towards the nucleus, interacts in a cell specific manner with various transcription factors to regulate the transcription of many genes.[194,196,198,200-206] (Figure 11)

The bioactive TGF-β may bind to α_2 macroglobulins forming a complex that is taken up via hepatic mannose-6-phosphate/insulin like growth factor II receptors and possibly catabolized. Bioactive TGF-β may also be degraded by proteases and elastases released at sites of inflammation or may be excreted in urine.[194]

TGF-β_1 is the major isoform produced in human bone cells. The constitutive secretion of TGF-β by bone cells does not vary with age, although it was reported that aging may be associated with a declining capacity of TGF-β to enlarge the pool of bone cells.[206,207]

Both osteoblasts and osteoclasts synthesize and respond to TGF-β yet the exact nature of the response appears to depend on the physiological conditions present.[208-213]

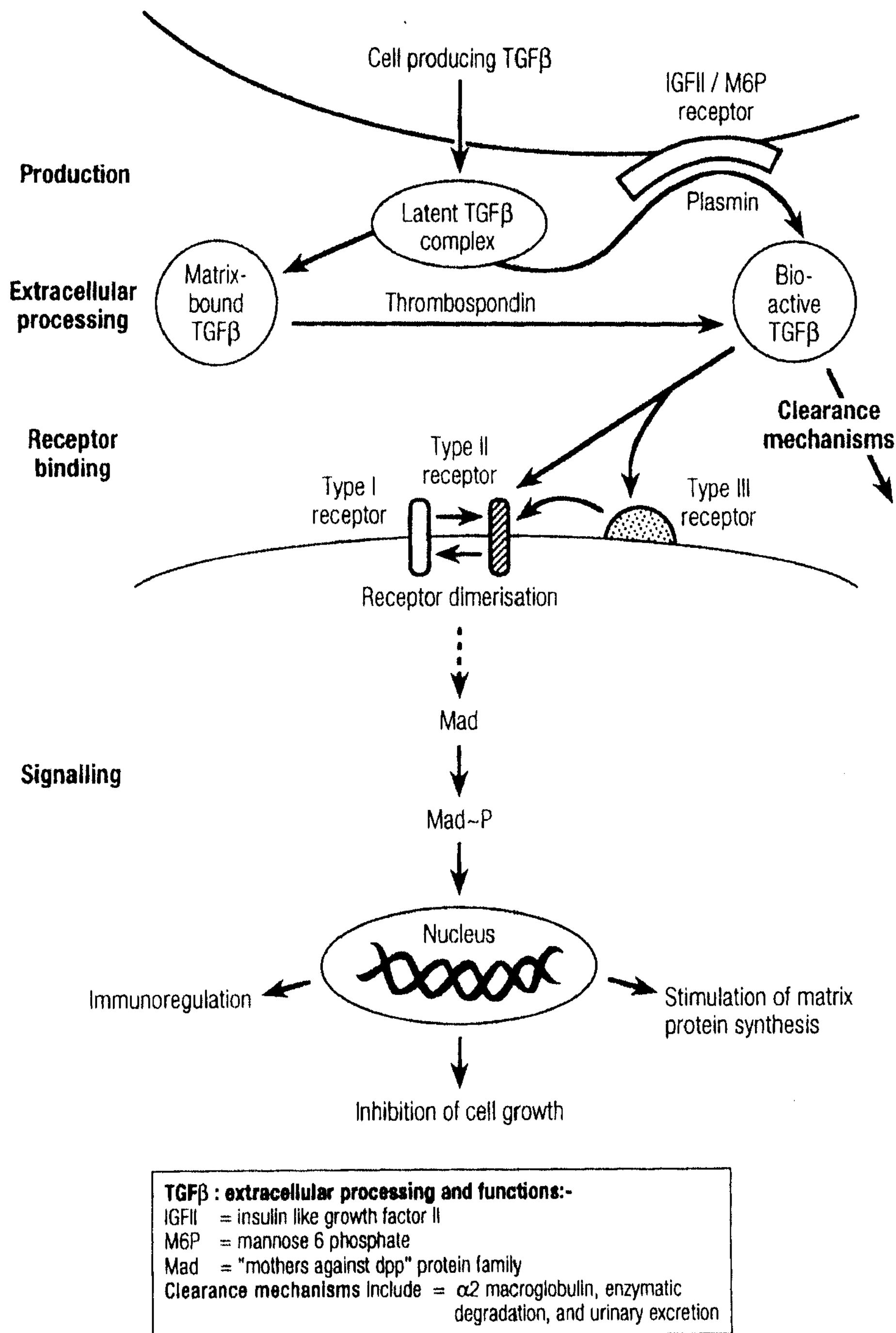


Figure (11): Extracellular pathways of TGf-β: from release of latent form to action on receptors and catabolism.[194]

TGF-β is present abundantly in the extracellular bone matrix. There are several lines of evidence that suggest that osteoclastic resorption of bone releases the stored TGF-β from the latent complex in the bone matrix. This release is activated by the acidic microenvironment created by the vacuolar type proton pump of osteoclasts.[16,183-186,213-218] The activated factor may inhibit the formation of more osteoclasts and promotes both osteoblastic proliferation and differentiation, together with cartilage formation.[16,183-186,213,216-218]

Besides the role of TGF-β in stimulating synthesis and secretion of matrix proteins mainly osteonectin and pro alpha I collagen, it also acts, synergistically with 1,25 $(OH)_2D_3$, in stimulating recruitment of bone marrow stromal cells (BMSCs) to the osteoblast lineage, that play a major role in bone formation.[16,185,186-216-219] Furthermore, the chemotactic activity of TGF-β for osteoblast like cells may be important for their recruitment at sites of bone remodeling.[220,221]

The role of TGF-β in bone resorption is variable and complex, depending on the differentiating stage of the cells. TGF-β inhibits early osteoclastic differentiation from bone marrow monocytes. However, it stimulates bone resorption by differentiated mature osteoclasts.[124,184,213,222-224] This may be through upregulating IL-6 mRNA expression resulting in increased IL-6 level and consequent bone resorption.[225] Its bone resorptive effects may be mediated also through its effects on local prostaglandins synthesis.[124,226]

Moreover, TGF-β stimulation of osteoblasts may lead to a secondary signal, which induces osteoclast differentiation and/or activity similar to the indirect mechanism of action of parathyroid hormone on bone resorption.(227-229) In fact, TGF-β induces osteoblastic secretion of macrophage/monocyte-colony stimulating factor, a regulator of osteoclast maturation and activity.(230)

The chief TGF-β effects, however, lie in inhibition of osteoclastogenesis, mainly through enhancing OPG mRNA expression by BMSCs and primary osteoblasts, via a transcriptional and a post transcriptional mechanism.(100,231-234) It was reported that both TGF-β_1 and OPG not only inhibited osteoclast formation but also impaired their survival by inducing apoptosis in vitro.(233,234)

The other mechanisms postulated to be involved in the suppressive effects of TGF-β on osteoclastogenesis, included suppression of RANKL mRNA expression and a direct inhibitory effect on osteoclast precursors as well as inhibition of growth and differentiation of osteoclastogenesis supporting stromal cells.(233,234) In fact, in vitro studies reported that TGF-β_1 markedly inhibited tratarate resistant acid phosphatase (TRAP) positive multinucleated osteoclast like cells formation in the presence of 1,25 $(OH)_2D_3$.(233) The stimulatory and inhibitory biphasic effects in vitro have been observed at low (10-100 pg/ml) and high (4ng/ml) concentrations respectively.(226)

TGF-β is tightly regulated by a complex set of mechanisms including latency of the molecule, production of various latent forms, its targeting to cells for activation or to matrix for storage and the means of activation of the latent forms. The TGF-β isoforms and the receptor types, affinities and signaling functions add to the complexity of regulation.[198]

Multiple factors, other than TGF-β and its receptors, regulate TGF-β expression including systemic hormones (e.g. PTH, calcitriol, glucocorticoids, androgens, retinoids), cytokines and growth factors (e.g. IL-1, IL-6, fibroblast growth factor, epidermal growth factor) and mechanical loading.[234,235]

The regulation of TGF-β_1 expression by the cytokines IL-1β and IL-6 acts as a protective mechanism against cytokine induced connective tissue catabolism. IL-1 was found to inhibit TGF-β_1 mRNA expression, while IL-6 was reported to induce TGF-β_1 gene expression resulting in a five fold increase in TGF-β_1 secretion.[236] Conversely, TGF-β_1 was reported to induce the denovo synthesis of IL-1Ra,through upregulating its mRNA expression, suggesting a potential mechanism by which TGF-β1 inhibits IL-1 activity.[237]

Bone morphogenetic proteins (BMPs) are members of TGF-β superfamily. About 20 BMPs were discovered, based on sequence homology. BMPs induce both bone and cartilage formation, thus creating an environment that leads to the development of a functional bone marrow.[238-240]

In this respect, BMP-1 (osteogenic protein-1), a procollagen 14 a.a. C-proteinase, is a growth regulatory peptide that enhances bone formation and trabecular bone density. Marrow stromal cells serve as targets for osteogenic protein-1. This is achieved via a marked stimulation of alkaline phosphatase activity and matrix mineralization,[241-245] that is potentiated by GH and basic fibroblast growth factor.[244]

In addition, it was reported that BMP-2 and 4 stimulated osteoclastic bone resorption, through increasing mRNA expression of cathepsin K and carbonic anhydrase II which are the key enzymes for the degradation of organic and inorganic matrices respectively. Their actions were mediated by BMP receptors type IA and II and their downstream signal transduction molecules, SMAD 1 and SMAD 5, that were expressed in isolated osteoclasts as well as osteoblasts.[246-248]

On the other hand, osteogenin (BMP-3) inhibits DNA synthesis and cell proliferation, and stimulates type I collagen synthesis and cAMP production, with an increase in intracellular ALP activity and osteocalcin synthesis.[249]

Insulin like growth factor-I : (IGF-I)

Insulin like growth factors (IGFs) or somatomedins are polypeptide growth factors secreted by the liver, bones, cartilages and other tissues in response to stimulation by a variety of hormones of which growth hormone is the most important. The term somatomedins was given to them because of their growth promoting properties in numerous tissues and the inability to suppress their bioactivity with autoinsulin antibodies.[41,250-253]

The IGF regulatory system consists of IGF ligands, IGF binding proteins and their specific proteases and IGF receptors.[41,250-253] The IGF ligands may act locally in tissues (autocrine/paracrine) or pass into the general circulation. Beside insulin hormone, there are two forms of IGF ligands, in the general circulation; IGF-I (or somatomedin-C) and IGF-II (or somatomedin-A).[41,59,250-256]

The plasma IGF-I level is low in early childhood, increases gradually reaching its peak in adolescence and declines after 50 years of age because GH secretion declines approximately 14% per decade of life.[41,251-253, 256-258] Males exhibit a 10-15% higher serum IGF-I concentration than females across all ages after puberty.[253,259] Plasma IGF-II level is constant from first year of life to beyond eighth decade.[260]

Somatomedins (or IGFs), structurally, consist of three short α-helices, linked together by a set of three disulphide bonds. These factors are closely related to insulin except their C-chains are not separated and they have an extension of the A-chain called the D-domain .[41,59,250-254, 260-262] Both IGFs have 50% structural homology with insulin, and 70% homology with each others.[260]

The 70 a.a. peptide IGF-I (M.W 7.6 KDa) is structurally similar to proinsulin (Figure 12).[251] It exerts some insulin like action. It inhibits lipolysis and increases glucose oxidation in adipose tissue.[263-266] Its level is elevated in pregnancy and periods of pubertal growth spurt. Its level is also correlated well with body size and is dependent on nutritional status of the individual.[41, 253,263,267-269]

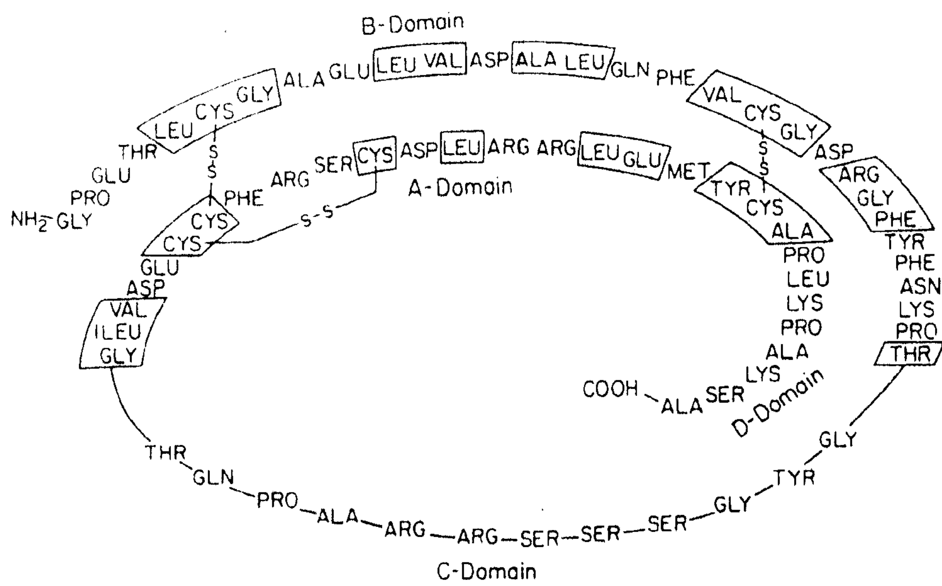


Figure (12) : The structure of insulin like growth factor – I[(251)]

IGF-I is the mediator for action of growth hormone in various tissues including bone. In the skeleton, GH stimulates osteoblast and chondrocyte IGF-I production, and both induce BMP 2 and 4 expression.[49,270-276] Many factors, other than growth hormone, can also regulate IGF-I expression: parathyroid hormone and PGE_2 stimulate IGF-I expression through a cAMP-mediated protein kinase dependent transcriptional mechanism.[229,277-280] Thyroid hormones, particularly tri-iodothyronine increase skeletal IGF-I production,[281,282] while a high cortisol level decreases skeletal IGF-I transcription, which probably contributes to the inhibitory influence of cortisol on bone formation.[283] Estrogen and androgens have been reported to increase IGF-I mRNA expression, probably via a cAMP mediated mechanism.[284,285]

More recent studies point to two nuclear transcription factors termed, core binding factor α_1 (CBFα_1) and CCAAT/enhancer binding protein delta (C/EBP delta) as significant regulators of expression or activity of specific bone growth factors including IGF-I, TGF-β and BMPs.[286-289] Such transcription factors activate cAMP mediated mechanisms in response to glucocorticoids, sex steroids, parathyroid hormone and prostaglandins.[285-288]

As regards the effect of other cytokines and growth factors on IGF-I expression or activity, TGF-β, basic FGF and PDGF decrease IGF-I transcript levels and cause dephosphorylation of IGF receptors.[290-293]

On the contrary, BMPs, particularly BMP-2, enhance IGF-I and II synthesis through both transcriptional (up to 2 folds) and polypeptide levels

(up to 4 folds).[294] In addition, IL-1β increases IGF-I mRNA transcript levels via a PG-dependent mechanism, suggesting a possible role for IL-1β in regulation of bone remodeling.[295]

Moreover, a positive feedback mechanism was suggested to exist between IGF-I and IL-6, since IL-6 decreases IGF-I production by osteoclasts and IGF-I has been shown to increase osteoblastic IL-6 production. [296-298]

IGF-I is a key regulator of bone formation being capable of stimulating both bone cell replication and differentiation. The released IGF-I from stromal cells and osteoblasts during bone resorption enhances bone matrix synthesis through upregulating mRNA levels of pro alpha-1 collagen, osteonectin, osteopontin and bone sialoproteins from osteoblasts and their precursors. It also stimulates alkaline phosphatase activity, suggesting a possible role in bone matrix mineralization.[39,59,299-317]

The 67 a.a. peptide, IGF-II (M.W = 7.5 KDa), is much less affected by growth hormone and plays a role in foetal growth before birth. Relaxin is a member of this family and exists in two forms both resembling IGF-II.[255,262,318-320]

The circulating IGFs concentrations are approximately thousands folds higher than insulin concentration, yet in contrast to insulin only 10% or less of IGF-I exists in the free form.[321]

More than 90% of IGFs are kept inactive in plasma by binding to a family of at least 6 specific binding proteins (IGFBPs), all sharing common

cysteine residues.(322-328) The most important is IGFBP-3, which is a large M.W. (42-49 KDa) glycosylated protein that binds to more than 75% of circulating IGF-I. IGFBP-3 is positively regulated by growth hormone, and potentiates its action in most conditions.(322-330)

IGFBP-3 has the unique property of being able to associate with an acid labile subunit (ALS) after binding to IGF-I or II, forming a large (150 KDa) complex.(322-332) Recent data suggests that IGFBP-5 is capable also of forming a similar complex with IGFs and ALS.(333-335) This large complex limits IGFs to intravascular space, sparing it from proteolytic cleavage, thus raising the plasma half life of IGF-I from 20-30 minutes to 3-18 hours.(256,263,321)

About 20-30% of serum IGFs are found in small (45KDa) complexes containing the other low M.W (< 32 KDa) IGFBPs with no acid labile subunit, allowing IGFs to reach extravascular tissue binding sites.(326)

The IGF activity is further regulated by specific proteases that proteolyse IGFBPs, thereby releasing IGFs from the small complexes.(336-338)

Most target tissues for IGF action, including bone, express IGFBPs that further modulate local IGF action both in a positive (e.g. IGFBP-5) and a negative (e.g. IGFBP-2 and 4) manner.(339-344) Recent studies reported that IGFBP-5 further stimulates bone formation, via an IGF independent mechanism.(52, 345-349) (Figure 13)

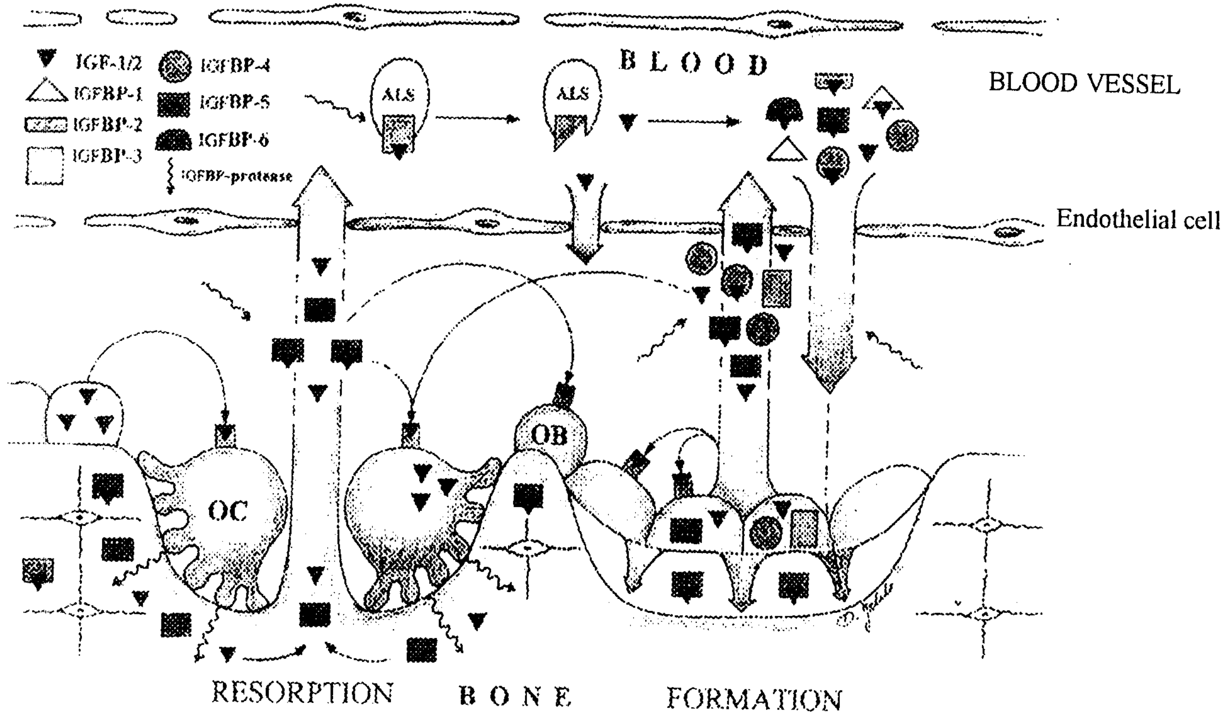


Figure (13): Model demonstrating circulating and locally produced IGF system components and the relationship between IGF system and the coupling of bone formation to resorption (bone remodeling).[52]

ALS = Acid labile subunit

OB = Osteoblast

OC = Osteoclast

The biological actions of IGFs are exerted, through their interaction with specific receptors. The IGF-I receptor is closely related to insulin receptor in both structure (about 60% identity in overall amino acids and 85% homology at the tyrosine kinase domain) and biochemcial properties.[350-355]

The IGF-I receptor is a tetramer, composed of 2 α and 2β subunits, linked by disulphide bonds. The α subunits are extarcellular and contain the IGF-I binding site. The β subunits are mainly intracellular and exhibit tyrosine kinase activity. Autophosphorylation of tyrosine residues on the receptor stimulates tyrosine kinase to phosphorylate other proteins, such as insulin receptor substrate proteins (IRSPs), that are components of the downstream signaling pathway.[41,350-358] IGF-II/ mannose-6-phosphate receptor lacks tyrosine kinase activity, and thus is not considered to have any major role in IGF signal transduction.[41,359]

The IGF-I receptor expresses a high affinity for both somatomedins and a low affinity for insulin. The IGF-II/mannose-6-phosphate receptor has a high affinity for IGF-II, low affinity for IGF-I and no affinity for insulin. The insulin receptor has a high affinity for insulin and a low one for both somatomedins.[41,321]

Platelet derived growth factor (PDGF)

Platelet derived growth factor (PDGF) is a homo or heterodimeric 30KDa molecule composed of A or B chains assembled in different combinations, creating PDGF-AA, PDGF-BB or PDGF-AB.[360-362] The three isoforms interact to different extents with two structurally similar receptor tyrosine kinases, denoted α and β.[362-366]

Besides the systemic form produced by megakaryocytes and stored in platelets, PDGF is produced locally by many cells including osteoblastic cells.[363, 367, 368]

PDGFs, particularly PDGF-AA, are potent cell mitogens that stimulate osteoblast proliferation and protein synthesis by differentiated cells, being detected at sites of bone formation or remodeling sites.[369-371]

Basic fibroblast growth factor (b-FGF) (FGF-2)

FGF-2 (b-FGF) is a member of the FGF family that comprises nine members.[372,373] There are 2 forms of b-FGF, the 18KDa form (146a.a protein) mainly cytoplasmic, and the high molecular weight (HMW) (24KDa) form (210 a.a) mainly nuclear.[374-379]

FGF-2 interacts with specific cell surface receptors of which 4 are identifiable (FGFR 1-4).[373,380] The extracellular heparan sulphate proteoglycans (HSPG) increase FGF-2 surface concentrations hence enhancing FGF-2/FGF-2R interaction.[373,381-384]

In bone, FGF-2 is synthesized by BMSCs and osteoblasts and is stored in the bone matrix.[373,385] It stimulates osteoblastic proliferation and differentiation and induces osteoblastic TGF-β expression.[373,386-395] It inhibits osteoclast like cell formation induced by $1,25(OH)_2 D_3$, PGE_2 and IL-11.[396] The target cells for its action are not osteoclast progenitors but stromal cells and osteoblasts.[373,391-394]

The role of FGF in bone remodelling could be denoted by its ability to enhance osteoblastic mRNA expression of collagenases and stimulation of calcium release from foetal long bone cultures.[396-399]

Chapter (III)

Renal Osteodystrophy

The term renal osteodystrophy (ROD) was first used in 1943 although the association between renal disease and bone abnormalities was reported 60 years earlier.[400] Renal osteodystrophy means abnormalities of bone and mineral metabolism which occur due to impaired renal function. Hence, it is explained as bone (osteo) which is poorly (dys) nourished (trophy) due to chronic renal failure.[401-409]

The frequency of ROD is still unknown, although it is well documented that even minor changes in renal function cause biochemical and histological signs of metabolic bone disturbances.[401-405,409] With a glomerular filtration rate (GFR) of 60-90 ml/min, reduced vitamin D concentration and increased parathyroid hormone concentration in blood can be detected, while a GFR of 10-20 ml/min almost always causes ROD signs.[401-405]

Patients with ROD present very late with a wide variety of non-specific signs and symptoms, usually related to the musculoskeletal system.[3,401-409] A common one is bone pain of gradual onset, in back, hips and legs. Proximal muscle aching and weakness usually occur too. Children with renal insufficiency usually suffer from retarded linear growth and often exhibit skeletal deformities.[3,401-409] By the time symptoms

appear, the patient usually has significant biochemical abnormalities and histologic evidence of bone disease.

Renal osteodystrophy comprises three types. The first type is a high turnover bone disease (HTBD), that occurs due to secondary hyperparathyroidism (sHPT). The second type is a low turnover bone disease (LTBD) that includes osteomalacia and adynamic (aplastic) bone disease. The third type is mixed uraemic osteodystrophy(MUOD) where overlap between the first two types occur, with features of the predominant type.[3,401-409]

High turnover bone disease (HTBD)

In 1933 Langmead and Orr suggested that the parathyroid gland hyperplasia was secondary to advanced CRF.[410] Hyperparathyroidism can occur when the GFR falls below 60-80 ml/min.[52,406,411-413]

The first stimulus for increased parathormone synthesis and secretion is renal phosphate retention. In early renal failure, plasma inorganic phosphate is generally normal or even decreased.[3,52,406,412-415] Only in more advanced stages, hyperphosphatemia develops which in turn lowers serum ionized calcium by exceeding the (calcium × phosphate) solubility product. The high plasma inorganic phosphate inhibits renal 1α hydroxylase activity, added to the diminished number of functioning proximal tubular cells, resulting in reduced calcitriol production, thereby inhibiting intestinal calcium absorption.[52,406,416-418] Recent studies suggest that phosphate has a

direct stimulatory effect on the parathyroid gland independent of calcium and calcitriol levels.(52,406)

The second major stimulus for enhanced parathormone secretion is hypocalcaemia, especially the ionized fraction.(52,406,419,420) It promotes the release of stored hormone within few minutes and enhances parathyroid hormone gene transcription. These actions are mediated by the so called "calcium sensing receptor" within the membrane of the parathyroid cell.(421,422)

Parathyroid cells have specific receptors for vitamin D_3 (VDR). When vitamin D_3 binds to them, it results in suppression of parathyroid hormone gene expression and diminished parathormone secretion.(423-425) During renal failure, the combination of decreased plasma vitamin D_3 level and decreased binding of available vitamin D_3 to the parathyroid cell receptors results in a marked increase in parathormone secretion at all levels of ionized calcium. This is known as altered set point for parathyroid hormone secretion and can be reversed by giving calcitriol.(426-429)

Several other factors modify parathyroid glands function and parathormone action on target organs, such as uraemic toxins, aluminium toxicity, glucocorticoids, reduced degradation of carboxy terminal fragments of parathormone and target cell resistance to parathormone.(419,430, 431)

In addition, prolonged metabolic acidosis causes growth retardation and muscle wasting, in part because of reduced food intake and decreased

growth hormone and IGF-I secretion, resulting in increased serum intact parathormone level and metastatic calcifications.(432-434)

The high parathyroid hormone level adversely affects the mineral content of bone. Parathormone stimulates BMSCs and osteoblasts to release cytokines and factors (e.g. IL-6, M-CSF) that induce proliferation and differentiation of osteoclast precursors and activation of mature osteoclasts.(52,69,165,166,168) Metabolic acidosis resulting from impaired hydrogen ion excretion also stimulates osteoclastic bone resorption and physicochemical bone dissolution.(3,435-437)

On the other hand, the bone forming actions of parathormone are mediated in part, through many cytokines and growth factors , particularly, IGF-1 and TGF-β.(52,227,229)

The clinical picture of hyperparathyroid bone disease (HTBD) is only found in advanced stages of renal failure and usually not before maintenance dialysis. In addition to the manifestations of hypercalcaemia (e.g. soft tissue calcification), there is a broad spectrum of disorders caused by extraskeletal actions of parathormone including haematological, immunological, nervous, cardiac and pulmonary problems. That is why parathormone in ROD patients may be known as a “uraemic toxin” because CRF patients with secondary hyperparathyroidism (sHPT) appear severely ill.(52,406,438)

The radiographic features of sHPT combine manifestations of accelerated bone resorption as well as bone formation, resulting in cysts or

osteoclastomas (brown tumours) which are more frequent in primary type of hyperparathyroidism.[3,52,406,407,439] The most sensitive radiographic sign of sHPT is the presence of subperiosteal erosions of the phalanges and eroded phalangeal tufts.[3,52,406,407,439,440] Osteosclerosis is characterized by an increase in the thickness and number of trabeculae in spongy bone such as increased density of vertebral ground plates (Rugger Jersy spine). In the skull, resorption and osteosclerosis create the salt and pepper or ground glass appearance.[52,406,439,441] In addition to skeletal lesions, soft tissue calcifications including vascular calcification may exist.[52,406,442]

The bone biopsy reveals osteofibrosis (osteitis fibrosa), that is characterized by increased number and activity of osteoblasts with no mineralization defect. Fibrous tissue is found next to, and sometimes replacing the bone trabeculae and even in the marrow spaces.[3,52,406,407]

Low turnover bone disease (LTBD)

Low turnover uraemic osteodystrophy is the other end of the spectrum of ROD. The main histologic bone features include a profound decrease in bone turnover (i.e. low number of active remodeling sites) resulting in bone resorption and suppressed bone formation, with or without a mineralization defect. Two histologic subgroups can be identified in this type of ROD, depending on the sequence of events leading to a decline in the number and/or activity of osteoclasts: low turnover osteomalacia and adynamic (aplastic) bone disease (ABD).[3,52,406,409,443]

Adynamic bone disease (ABD) has been described in end-stage renal disease (ESRD) patients under chronic dialysis. It has been termed aplastic bone disease, but because the main diagnostic criterion is an abnormally low bone formation rate, the term adynamic is preferred.[3,52,406,409,444-446] It was reported that the incidence of ABD in patients undergoing peritoneal and haemodialysis was 60% and 36%; respectively.[403]

Adynamic bone disease (ABD) was originally thought to be caused by aluminium (Al) overload, yet the exact pathophysiological mechanisms behind ABD are not yet elucidated.[447,448] A number of epidemiological and experimental data suggest a multifactorial process, in which hypoparathyroidism and suppression of osteoblasts are the main actors.[449] These findings are associated with supraphysiological dialysate calcium concentration, the use of oral calcium carbonate or acetate as phosphate binders and the excessive use of calcitriol to suppress parathormone secretion, resulting in lowering parathormone to levels which are inadequate to maintain normal bone turnover.[3,406,450-452] It was reported that an intact parathormone plasma level of less than 65 pg/ml had a positive prediction value of 78% for the occurrence of ABD.[406,453,454]

Other factors, beside hypoparathyroidism, considered in playing a role in the pathogenesis of ABD include age,[52,406,445,449] hypothyroidism,[406,455] Cushing's disease and excessive use of steroids,[406,456] hypophosphatemia,[406,457] metabolic acidosis,[406,458] diabetes mellitus,[406,459-461] and toxic doses of fluoride.[406,462]

Patients with idiopathic ABD are often asymptomatic and have no radiologic abnormalities at the time of diagnosis, thus idiopathic ABD at present is a histologic finding rather than a disease state.[406]

The bone biopsy in ABD reveals a decrease in osteoid volume with few osteoclasts and osteoblasts present, resulting in both defective mineralization and collagen synthesis. [3,406]

Compared to ABD, osteomalacia has now become a much rarer disease (about 4%), at least in western countries, though it might still regularly occur in less developed ones.[443]

Earlier, it was thought that osteomalacia resulted from altered vitamin D metabolism. Later other pathogenic factors, including Al accumulation, have been implicated, although refractoriness to treatment with active vitamin D sterols is another feature of Al related osteomalacia.[463-466]

The sources of Al toxicity include ingestion of Al as a phosphate binder, use of Al contaminated dialysate, and infusion of albumin products containing Al.[3,52,406,467,468] The incidence of Al intoxication among dialyzed patients has declined with elimination of its causes. Today aluminium toxicity is mainly found after long term (5-10 years) dialysis.[469,470]

In uraemic patients, osteoidosis and adynamic bone disease are the two major types of Al related osteopathy as evidenced by histological

studies.[403,468,471-473] However, Al overload may simultaneously be found in the presence of osteitis fibrosa or a mixed type of renal osteodystrophy.[473]

Al deposition within the mineralization front results in excessive osteoid formation and impaired bone mineralization.[3,52,406,474-477] In addition, Al is cytotoxic to osteoblasts, resulting in reduction in their number and activity , as evidenced by in vitro studies.[475-478] This leads to an adynamic bone disease.[477,478] Al suppresses PTH secretion,[3,52,406,479,480] and can also induce resistance to PTH and vitamin D.[3,52,406,480]

Clinical features of Al-related osteomalacia include progressive bone pain, affecting mainly the axial skeleton. Proximal muscle weakness and fractures of ribs, pelvis, and vertebrae are common.[52,406]

Radiological findings include the presence of pseudofracture or "looser zones" appearing as radiolucent cortical bands, perpendicular to the long axis of the bone. Other signs include true fractures of ribs and hips and compression fractures of vertebral bodies. Features such as increased haziness or coarsening of trabeculae, biconcavity of vertebral bodies and bending deformities of long bones are said to be typical of osteomalacia. Superimposed radiological picture of sHPT can be seen in these patients.[3,52,406,465,481] The diagnosis can be verified by bone biopsy. The bone histology reveals Al deposits detected along the trabecular bone surfaces and on the cement lines, together with impaired mineralization.[3,406,481]

Recently, it has been found that bone strontium levels were increased in HD patients with osteomalacia, compared to all other types of ROD. The source of strontium is contaminated dialysate resulting from addition of strontium-containing acetate-based concentrates.[443,482] (Figure 14)

Mixed uraemic osteodystrophy

It is mainly caused by hyperparathyroidism and defective mineralization with or without increased bone formation. These features may co-exist in varying degrees in different patients, with an increase in the number and activity of osteoclasts.[3,409]

In ROD patients, there is accumulating evidence suggesting that alterations of the various cytokine systems, involved in the regulation of different stages of the bone remodeling cycle, contribute in the pathogenesis of the remodeling abnormalities of ROD.[52,69,483-488]

Therefore, its is worthy to study some of these local bone regulatory cytokines in ESRD patients with secondary hyperparathyroidism under maintenance haemodialysis.

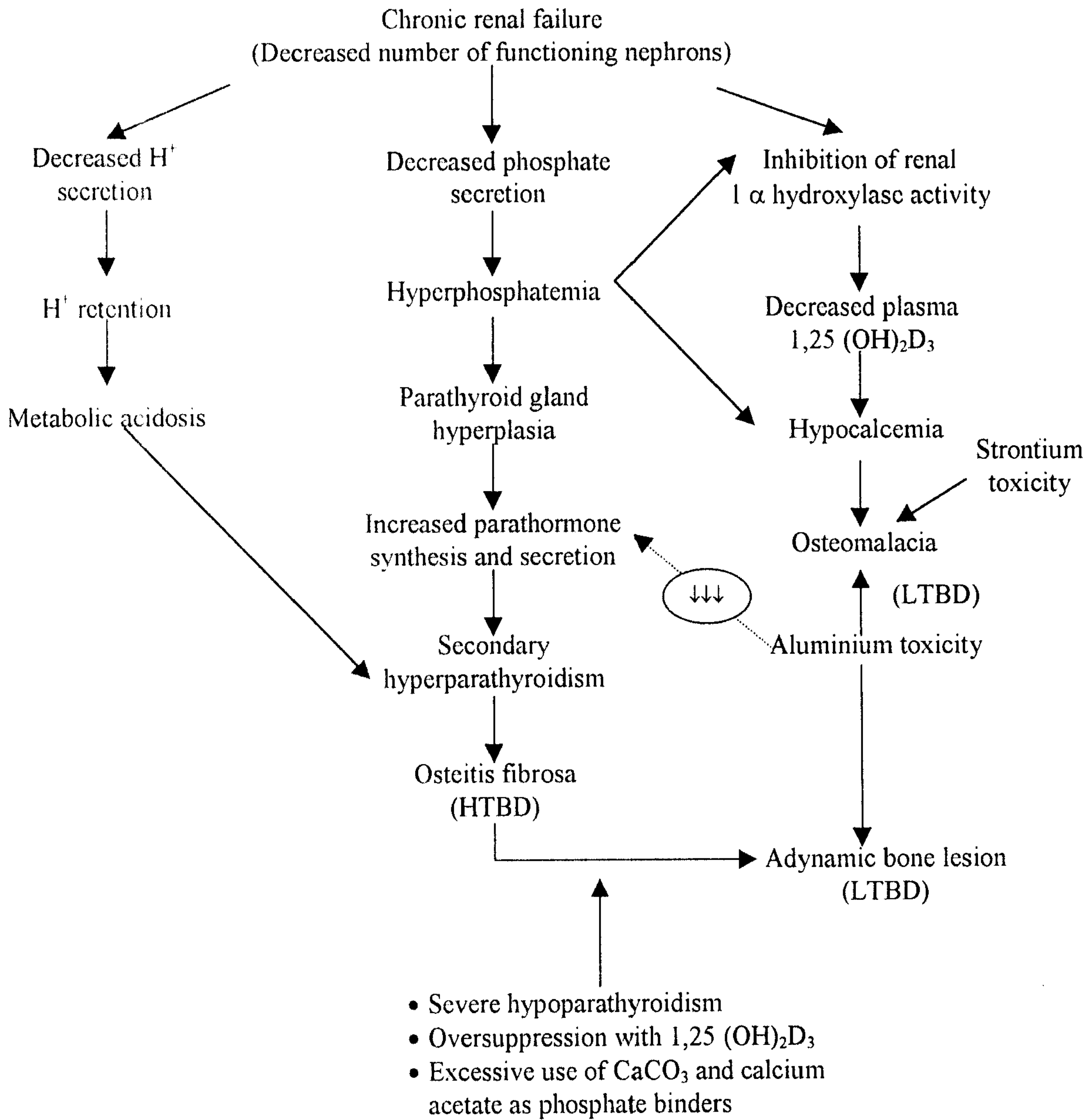


Figure (14): Some important pathogenetic mechanisms involved in the development of renal osteodystrophy.[482]

AIM OF THE WORK

AIM OF THE WORK

This work aims at studying some local bone regulatory cytokines in end stage renal disease patients with secondary hyperparathyroidism under maintenance haemodialysis.

MATERIAL

MATERIAL

The present study included two groups of subjects:

Group I: It consisted of twenty healthy volunteers of matched age, sex and socioeconomic status as the patients. They were clinically free and had no history of hypertension, diabetes mellitus, skeletal, cardiac, hepatic, renal or autoimmune diseases.

Group II: It consisted of forty one patients with end stage renal disease under maintenance haemodialysis. They were selected from the Nephrology Unit of Medical Research Institute, Alexandria University. They had clinical, radiological and/or laboratory evidence of secondary hyperparathyroidism. The dialysis sessions for these patients were three times weekly, with the duration range of each session from four to six hours. A standard dialysis prescription using a cuprophane hollow fiber dialyzer was adopted.

METHODS

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 or joint pain, proximal muscle weakness and pathological fractures. The duration of dialysis in months was recorded in the patients' group.

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

II- Investigations: including:

A- Radiological investigation

Plain X-ray skull, spine and hands were done to detect radiological evidence of renal osteodystrophy.

B- laboratory investigations

Sampling

Ten milliliters venous blood samples were taken from each subject after an overnight fasting. In the patients group, the samples were taken immediately before the dialysis session.

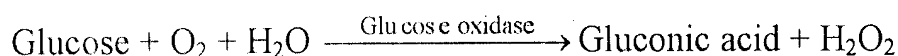
After clotting, the blood samples were centrifuged, and serum was separated. Part of the serum was used for immediate determination of the

routine analytes (including parathormone which was transported in ice), while the rest was stored in aliquots in four eppendorf tubes at –20°C for cytokine determination.

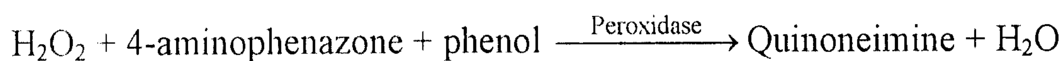
Analytes determined for all subjects included in this study were:

1- Fasting serum glucose level[489]

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

$$C_T = {}^{A_T}\!/_{A_S} \times Cs$$

mmol glucose/L = mg/dl × 0.055

2- Serum creatinine concentration[490]

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 (Cs) similarly treated.

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

$$C_T = \frac{\Delta A_T}{\Delta A_S} \times Cs$$

mmol creatinine/L = mg/dl x 0.0884

3- Serum calcium[491]

Total serum calcium was determined without deproteinization using Arsenazo III monoreagent. Arsenazo III 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 (Cs) similarly treated. The total calcium concentration (C_T) was calculated as follows:

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

mmol calcium/L = mg/dl x 0.25

4- Serum ionized calcium[491]

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 x 4.

5- Serum inorganic phosphate:[492]

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

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

$$C_T = {}^{A_T}\!/_{A_S} \times Cs$$

mmol inorganic phosphate/L = mg/dl x 0.0735

6- Serum alkaline phosphatase activity[493]

Total alkaline phosphatase (ALP) activity was determined, without deproteinization, using para-nitro phenyl phosphate as substrate. ALP catalyzed its hydrolysis liberating yellow coloured p-nitrophenol in 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 405nm. The enzyme activity-expressed in units/L-was calculated as follows: ΔA/min x 2757.

7- Serum acid phosphatase activity[494]

Total acid phosphatase (ACP) activity was determined without deproteinization according to the following equation:

$$\alpha\text{-Naphthyl phosphate} + H_2O \xrightarrow{ACP} \alpha\text{-Naphthol} + \text{phosphate}$$

$$\alpha\text{-Naphthol} + \text{fast red TR salt} \longrightarrow \text{Azo dye}$$

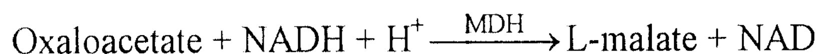
(*N.B.* Fast red TR salt: 4-chloro-2-methyl phenyl diazonium salt)

The increase in absorbance of the resulting azo dye at 37°C was monitored kinetically for 3 minutes at 405nm. The enzyme activity-expressed in units/L-was calculated as follows: ΔA/min x 730.

8- Aspartate aminotransferase (AST) activity: [495]

AST activity was determined as follows:

$$\alpha\text{-oxoglutarate} + \text{L-aspartate} \xrightarrow{AST} \text{L-glutamate} + \text{oxaloacetate}$$



$$\text{Oxaloacetate} + \text{NADH} + H^+ \xrightarrow{MDH} \text{L-malate} + \text{NAD}$$

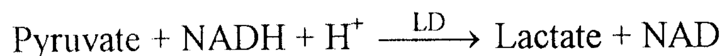
(MDH = malate dehydrogenase)

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: ΔA/min x 1746.

9- Alanine aminotransferase (ALT) activity[496]

ALT activity was determined as follows:

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



$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{LD} \text{Lactate} + \text{NAD}$$

(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: ΔA/min x 1746.

10- Serum albumin:[497]

Albumin was determined using bromocresol green dye that gave a green coloured product with albumin. The resulting colour was read spectrophotometrically at λ 600 nm (T), and compared to a standard albumin solution (S) of a known concentration (Cs) similarly treated. The albumin concentration (C_T) was calculated as follows:

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

gm albumin/L = gm/dl × 10

11- Intact parathyroid hormone (iPTH): (Immulite)[498]

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 (1-38) antibody in the test unit, a 37°C incubation was done for 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.

12- Estimation of insulin like growth factor-I (IGF-I)[499]

Principle

IGF-I was determined using an active non-extraction IGF-I enzymatically amplified "two-step" sandwich enzyme linked immunosorbant assay (ELISA) (using kit from Diagnostic Systems Laboratories Inc. 445 Medical Center Blvd, Webster, Texas 77598-4217 USA Cat No DSL-10-2800. Active Non-Extraction).

In this assay, the antigen (IGF-I) in the standards, controls and pretreated samples, was incubated for 2 hours at room temperature with continuous shaking, in microtitration wells coated with IGF-I antibodies. At the end of incubation, a five wash cycle was performed, followed by the addition of horseradish peroxidase enzyme labeled anti IGF-1 antibody.

Then incubation for half an hour at room temperature with continuous shaking was done. This was followed by a second wash cycle. After the addition of an appropriate substrate (tetramethylbenzidine = TMB), a third incubation was done, at room temperature, with shaking in the dark for ten minutes. An acidic stopping solution was added and the absorbance of the resultant colour, measured at λ450 nm, was directly proportional to the IGF-1 concentration in the wells. Results were deduced from a standard curve of absorbance versus IGF-1 concentration.

Reagents

1- Anti IGF-I coated microtitration strips

A strip holder containing 96 microtitration wells coated with IGF-I antibody.

2- IGF-I standards

Five standard concentrations of 0, 10, 45, 250 and 600 ng/ml IGF-I (synthetic) in a protein based buffer (BSA) with a non mercury preservative. The zero ng/ml concentration was ready to use, while the other 4 concentrations were reconstituted each with 1ml of deionized water.

3- IGF-I controls: (lyophilized)

Two levels I and II containing low (190ng/ml) and high (400 ng/ml) IGF-I concentrations respectively in a protein based buffer (BSA) with non mercury preservative. Each control was reconstituted with 1 ml of deionized water.

4- Assay buffer

A protein based buffer (BSA) with a non mercury preservative.

5- IGF-I antibody enzyme mercury conjugate concentrate

Anti IGF-I antibody conjugated to the enzyme horseradish peroxidase (HRP) in a protein-based buffer (BSA) with a non mercury preservative was diluted with the assay buffer in a ratio of 1:50.

6- TMB chromogen (substrate) solution

A solution of tetramethylbenzidine (TMB) in buffer with hydrogen peroxide.

7- Stopping solution

0.2 M sulphuric acid.

8- IGF-I Sample buffers

Two different sample buffers with non mercury preservatives were used according to the manufacturer's instructions.

9- Wash concentrate

A concentrate containing buffered saline with a non-ionic detergent, was diluted 25 folds with deionized water prior to usage.

Note: All reagents and samples were allowed to reach room temperature and mixed thoroughly by gentle inversion prior to usage.

Sample pretreatment: (Done for samples only)

Procedure

1- Polypropylene 12 × 75mm wassermann tubes were labelled for samples and 20 μl of serum from each sample was pipetted into each.

2- 990 μl of the first sample buffer were added to each tube then mixed by vortex and incubated at room temperature for 30 minutes.

3- 990 μl of the second sample buffer were then added to each tube and mixed as instructed by the manufacturer.

Note: The sample pretreatment was done in the same day prior to analysis.

Assay procedure

1- The microtitration strips to be used were marked.

2- 20 μl of each standard, control and pretreated serum samples were pipetted into their appropriate locations in the antibody coated wells .

3- 100 μl of assay buffer were added to each well.

4- The wells were incubated at room temperature for 2 hours, with shaking at a speed of 500-600 rpm.

5- Using an automatic microplate washer, the contents of each well were aspirated and washed for 5 times with the washing solution . Blot drying of the plate by inverting it on absorbent material was then done.

N.B. At this step, the antibody enzyme conjugate was prepared by its dilution with the appropriate amount of assay buffer.

6- 100 μl of antibody enzyme conjugate were added to each well.

7- The wells were incubated for 30 minutes at room temperature, with shaking at 500-600 rpm.

8- A second wash cycle was done (like the first one).

9- Then 100 μl of TMB chromogen solution were added to each well.

10- The wells were incubated for 10 minutes at room temperature, with shaking at 500-600 rpm. Exposure to direct sunlight was avoided.

11- This was followed by addition of 100 μl of stopping solution to each well.

12- Using a microplate reader set at λ450 nm, the absorbance of the solution in each well was read.

Results

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

2- On a log-log graph paper a standard curve was plotted with the mean absorbance for each of the standards on the Y-axis versus the IGF-I standards' concentrations in ng/ml along the X-axis. (Figure 15)

3- The IGF-1 concentrations of the controls and samples were deduced from the standard curve.

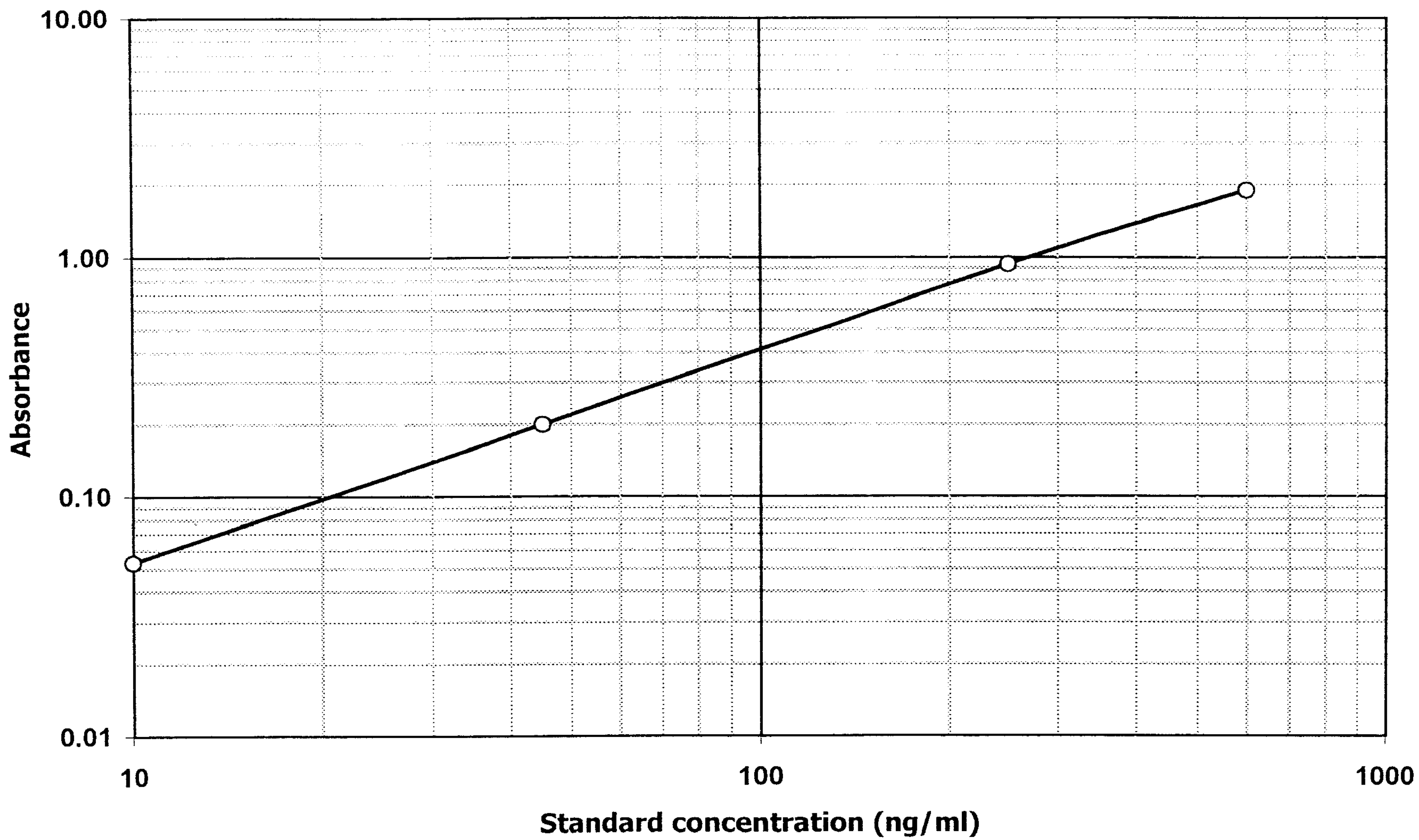


Figure (15) : Standard curve of IGF-I

13- Estimation of transforming growth factor-beta1 (TGF-β_1)[500]

Principle

TGF-β_1 was determined by a competitive enzyme linked immunosorbant assay (ELISA) (using kit from Biosource Europe S.A: Rue de L'industrie, 8 B-1400 Nivelles Belgium. Biosource TGF-β_1 EASIA kit, KAC 1681).

The antigen (TGF-β_1) present in the standards, pretreated controls and serum samples was incubated with a horseradish peroxidase labelled TGF-β_1 (conjugate), for two hours at room temperature with continuous shaking, in microtiter wells coated with TGF-β_1 antibodies. After a three wash cycle, the substrate (tetramethylbenzidine) was added and incubation for half an hour in the dark with continuous shaking was performed. A stopping solution was added, and the absorbance of the resultant colour, measured at λ450 nm, was inversely proportional to the TGF-β_1 concentration in each well. Results were deduced from a standard curve of the percent bound ($B/B_0.100$) TGF-β_1 standard versus TGF-β_1 standard concentration.

Reagents

1- Microtiter plate: It consisted of 96 wells coated with anti TGF-β_1.

2- TGF-β_1 standard: A stock standard of lyophilized TGF-β_1 from human platelets in acetate buffer, from which serial dilutions were done using the dilution buffer.

3- TGF-β_1 control: It was reconstituted with 0.5 ml distilled water.

4- TGF-β_1 horseradish peroxidase conjugate.

5- Dilution buffer.

6- Extraction solution.

7- Acetic acid 2.5 M.

8- Tetramethylbenzidine (TMB) chromogenic solution.

9- Stopping solution.

10- Washing solution.

Sample extraction

This step was done to release TGF-β_1 from latent complexes making it accessible for measurement. This was done only for serum samples and controls:

1- Polypropylene wassermann tubes were prepared for each sample and control.

2- In each tube 100 µl of sample/control were added followed by addition of 100 µl of 2.5 M acetic acid.

3- After good mixing (vortex), the tubes were incubated for 15 minutes at room temperature RT.

4- In another set of polypropylene wassermann tubes: 20 µl of each sample/control mixture were added to 500 µl of the dilution buffer.

5- At this step, samples / control were diluted 1/52.

Standard curve preparation

- The lyophilized TGF-β_1 standard was reconstituted with the appropriate amount of dilution buffer.

- In polypropylene wassermann tubes: 5 fold serial dilutions of the reconstituted calibrator, using the dilution buffer, were done to obtain serial standard concentrations which were multiplied by a factor of 52 to account for sample dilution in the pretreatment step as follows:

Calibrator dilution	ml of calibrator	ml of dilution buffer	Concentration of calibrator (ng/ml)
1/1 (undiluted)	-	-	52
1/5	0.2	0.8	10.4
1/25	0.2	0.8	2.08
1/125	0.2	0.8	0.416

Assay procedure

1- The microtitration strips to be used were marked.

2- Into the appropriate wells, 100 µl of each standard or extracted control or sample were pipetted.

3- 50 µl of TGF-β_1 HRP conjugate were pipetted, into each well.

4- The wells were incubated at room temperature for 2 hours on a horizontal shaker set at 700 rpm.

5- Using an automatic microplate washer, each well contents were aspirated and washed 3 times using the diluted wash solution, followed by blot drying.

6- 100 µl of TMB chromogen solution were pipetted, into each well.

7- The plate was incubated at room temperature in the dark for 30 minutes on a horizontal shaker set at 700 rpm.

8- 100 µl of stop solution were pipetted into each well.

9- The absorbance was read on a microplate reader set at 450 nm (reference filter: 650 nm).

Results and calculation

1- The mean absorbance (A) of duplicate standard and control readings was calculated.

2- For each standard/control/sample. The $B/B_0.100$ was calculated:

$$\frac{A\ (standard/sample)}{A\ (zero\ standard)} x100$$

3- using a semilog graph paper, with Y-axis (linear scale) showing the percent bound TGF-β_1 standard and X-axis (logarithmic scale) showing TGF-$\beta_1$concentrations, the TGF-β_1 concentration of extracted samples/control were deduced. (Figure 16)

N.B. For samples with values higher than the highest standard, the extracted samples were diluted with the dilution buffer.

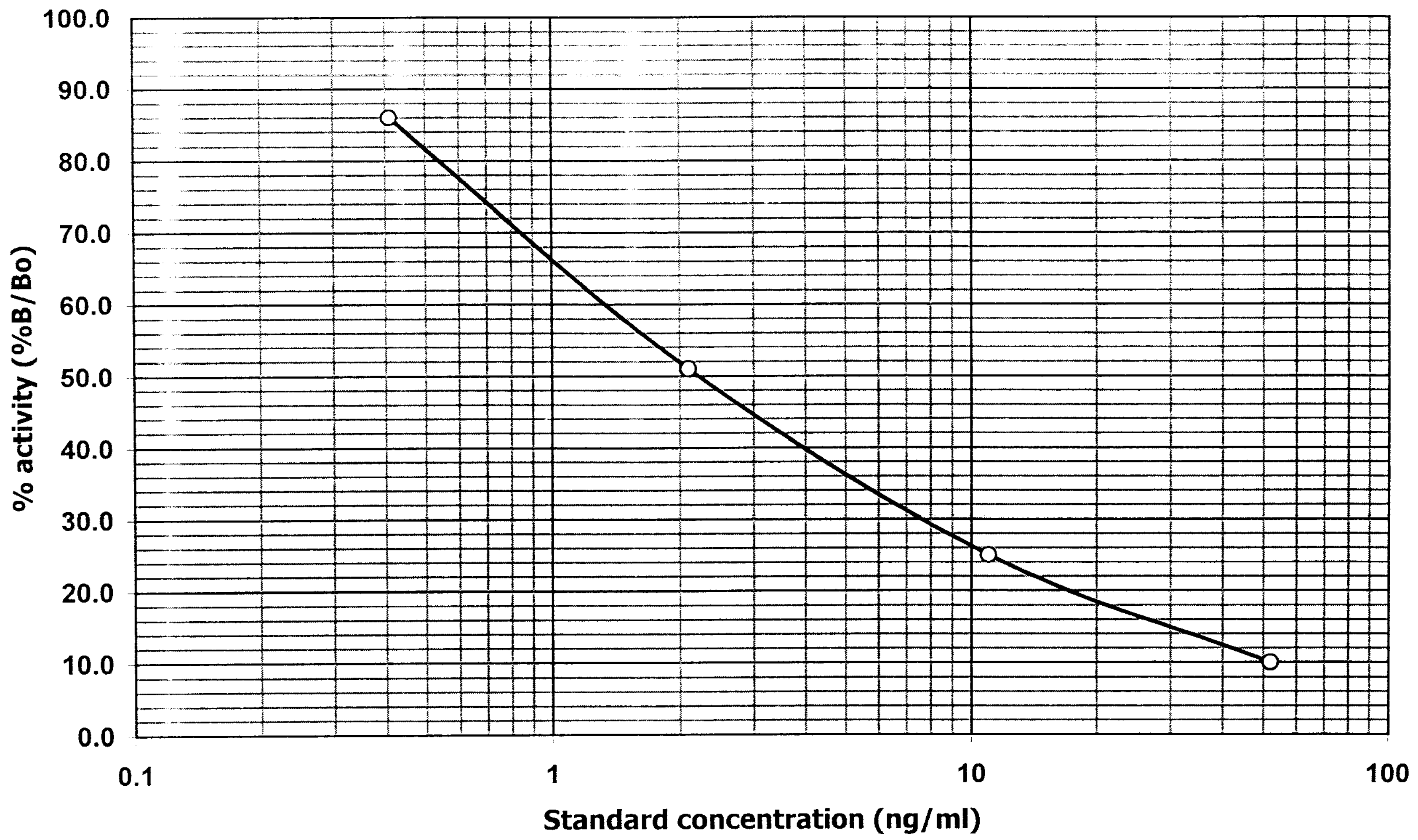


Figure (16) : Standard curve of TGF-β1

14- Estimation of interleukin –1beta (IL-1β)[501]

Principle

IL-1β was determined using a sequential competitive enzyme immunoassay (EIA) (using Accucyte Human IL-1β kit from Cytimmune Sciences Inc 8075 Greenmead Drive, College Park, Maryland 20740).

The IL-1β in the standards or pretreated samples was incubated in wells precoated with goat antirabbit IL-1β-polyclonal antibodies for 3 hours at room temperature. At the end of incubation, the biotinylated IL-1β conjugate was added, and a second incubation time (30 minutes at room temperature) was allowed for binding of the conjugate to the rest of the free polyclonal antibody binding sites.

After a five wash cycle, the streptavidin alkaline phosphatase enzyme was added, followed by a 30 minutes incubation at room temperature to allow enzyme binding to the conjugate (biotin-streptavidin binding).

After another five wash cycle, the substrate (two step colour generating system) was added, followed by a 15 minutes incubation at room temperature, and a final addition of an acidic stopping solution. A two step colour generating system was used. The alkaline phosphatase dephosphorylated NADPH (substrate) to NADH. The NADH then served as a cofactor that activated a cycling redox reaction driven by alcohol dehydrogenase and diaphorase. The latter reaction formed a deep red coloured product (formazan)

The absorbance of the final red colour, read at λ492 nm, was inversely proportional to the concentration of IL-1β in both standards and pretreated samples.

Sample results were deduced from a standard curve of percent bound IL-1β standard (Y-axis) versus IL-1β standard concentrations (X-axis).

Reagents

1- 96 well microtiter plate: precoated with goat antirabbit antibodies (secondary antibodies).

2- Sample diluents: two sample diluents were used for pretreatment of serum sample as instructed by the manufacturer.

3- Rabbit antihuman IL-1β polyclonal antibody: reconstituted with the supplied diluent and thoroughly mixed using vortex (primary antibody).

4- Human biotinylated IL-1β conjugate: reconstituted with the supplied diluent and thoroughly mixed using vortex.

5- Streptavidin alkaline phosphatase: reconstituted with the supplied diluent and thoroughly mixed using vortex.

6- Colour reagents: the supplied colour reagents were just mixed before the step of their addition in the procedure. The colour reagents consisted of NADPH, alcohol dehydrogenase and diaphorase.

7- Washing solution.

8- Recombinant IL-1β standard: 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-1β standard dilution

Serial dilutions of the prepared IL-1β standard with the appropriate amount of diluent were done in wassemann tubes, as follows:

Tube number	Reconstituted standard	Standard concentration
Tube (1)		200 ng/ml
Tube (2)	600 μl diluent + 200 μl of tube (1)	50 ng/ml
Tube (3)	600 μl diluent + 200 μl of tube (2)	12.5 ng/ml
Tube (4)	600 μl diluent + 200 μl of tube (3)	3.125 ng/ml
Tube (5)	600 μl diluent + 200 μl of tube (4)	0.781 ng/ml
Tube (6)	600 μl diluent + 200 μl of tube (5)	0.195 ng/ml

As regards the zero standard: 200 μl of diluent was put in its designated well directly.

Sample pretreatment:

In polystyrene wassermann tubes: 50 μl of the sample were added to a mixture of the two supplied diluents as instructed by the manufacturer (50 μl of the second diluent and 100 μl of the first diluent) with good mixing using vortex.

Procedure

1- Marking of the designated wells was done.

2- Into the designated wells, 100 μl of serial standard dilutions/ pretreated serum sample were dispensed.

3- 25 μl of diluted rabbit antihuman IL-1β polyclonal antibody were dispensed into each well. The plate was sealed with acetate plate sealer (to prevent evaporation), and incubated at room temperature for 3 hours.

N.B. At this step, colour reagents A & B were allowed to come to room temperature.

4- At the end of incubation and following removal of the sealer, 25 μl of biotinylated IL-1β conjugate were dispensed into each well and incubated at room temperature for 30 minutes after sealing with plate sealer.

5- the sealer was removed, and the first washing step was done, in the form of five washing cycles, using an automatic plate washer, followed by aspiration of the washing solution and blot drying of the plate.

6- Into each well 50 μl of reconstituted streptavidin alkaline phosphatase were dispensed, plate was sealed and incubated at room temperature for 30 minutes.

7- The sealer was removed, and the second washing step was done in the form of five washing cycles, using an automatic plate washer, followed by aspiration of washing solution and blot drying of the plate.

8- The colour reagent was prepared then 200 μl of the colour reagent were dispensed into each well. The plate was sealed, and incubated at room temperature for 15 minutes.

N.B. A period of three seconds were allowed for gentle plate shaking using a plate shaker.

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

10- The absorbance of the coloured solution in the plate was read at λ492 nm using a microplate reader.

Calculation

- A standard curve was plot on a semilog graph paper with X-axis (log scale) showing IL-1β standard concentrations and Y-axis (linear scale) showing corresponding % activity which was expressed as $B/B_o.100$).
- The curve was sigmoid in nature. (Figure 17)
- After obtaining the sample concentration from the standard curve, the final result was multiplied by a dilution factor of 4 (due to sample pretreatment)

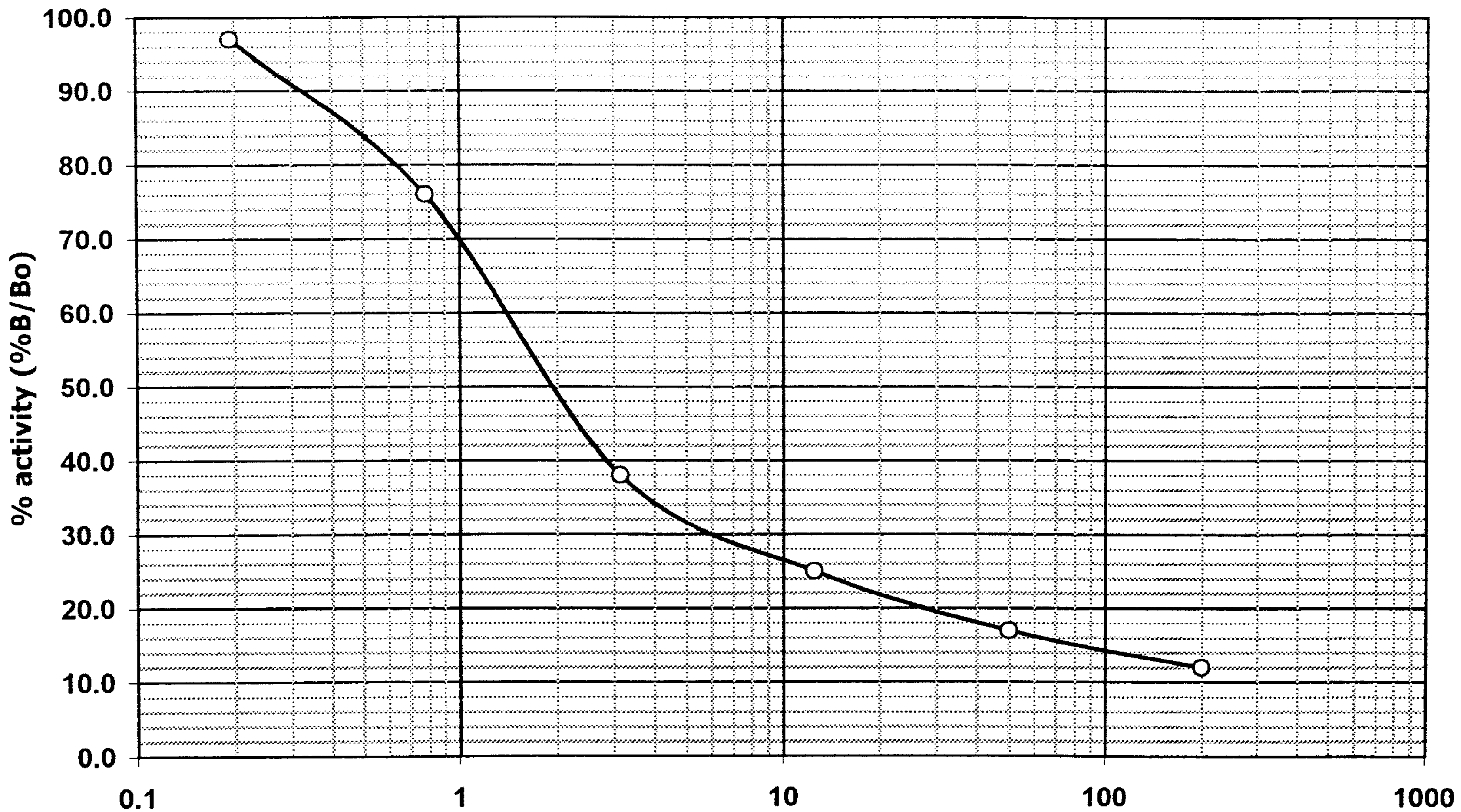


Figure (17) : Standard curve of IL-1β

15- Estimation of tumour necrosis factor-alpha (TNF-α)[502]

Principle

TNF-α was determined using a sequential competitive enzyme immunoasay (EIA) (using Accucyte human TNF-α kit from Cytimmune Sciences Inc. 8075 Greenmead Drive, College Park, Maryland 20740).

The TNF-α (antigen) in the standards or pretreated samples was incubated in microtiter wells precoated with goat antirabbit TNF-α-polyclonal antibodies for 3 hours at room temperature. At the end of incubation, the biotinylated TNF-α conjugate was added, and a second incubation (30 minutes a room temperature) was allowed, in order for the conjugate to bind to the rest of the free antibody binding sites.

A five wash cycle was done, followed by addition of streptavidin alkaline phosphatase enzyme, and a 30 minutes incubation at room temperature, to allow enzyme binding to the conjugate.

A second five wash cycle was done, and the substrate (two step colour generating system) was added, followed by a 15 minutes incubation at room temperature, and finally addition of an acidic stopping solution. A two step colour generating system was used. The alkaline phosphatase dephosphorylated NADPH (substrate) to NADH. The NADH then served as a cofactor that activated a cycling redox reaction driven by alcohol dehydrogenase and diaphorase. The latter reaction formed a deep red coloured product (formazan).

The absorbance of the resulted red colour, read at λ492 nm, was inversely proportional to the concentration of TNF-α in both standards and pretreated samples.

Results were deduced from a standard curve of percent activity (Y-axis) versus TNF-α standard concentrations (X-axis).

Reagents

1- 96 will microtiter plate: precoated with goat antirabbit antibodies
2- Sample diluents: two sample diluents were used for pretreatment of serum sample as instructed by the manufacturer.
3- Rabbit antihuman TNF-α polyclonal antibody: reconstituted with the supplied diluent and thoroughly mixed using vortex (primary antibody).
4- Human TNF-α conjugate: reconstituted with the supplied diluent and thoroughly mixed using vortex.
5- Streptavidin alkaline phosphatase: reconstituted with the supplied diluent and thoroughly mixed using vortex.
6- Colour reagents: the supplied colour reagents were just mixed before the step of their addition in the procedure. The colour reagents consisted of NADPH, alcohol dehydrogenase and diaphorase.
7- Washing solution.
8- Recombinant TNF-α standard: 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 TNF-α standard dilutions

In polystyrene wassermann tubes the following was added:

Tube number	Reconstituted standard	Standard concentration
Tube (1)		200 ng/ml
Tube (2)	600 μl diluent + 200 μl of tube (1)	50 ng/ml
Tube (3)	600 μl diluent + 200 μl of tube (2)	12.5 ng/ml
Tube (4)	600 μl diluent + 200 μl of tube (3)	3.125 ng/ml
Tube (5)	600 μl diluent + 200 μl of tube (4)	0.781 ng/ml
Tube (6)	600 μl diluent + 200 μl of tube (5)	0.195 ng/ml

As regards the zero dose: 200 μl of the supplied diluent were put directing in the designated well.

Sample pretreatment:

In polystyrene wassermann tubes: 50 μl of the sample were added to a mixture of the two supplied diluents as instructed by the manufacturer (50 μl of the second diluent and 100 μl of the first diluent) with good mixing using vortex.

Procedure

1- Marking of the designated wells was done.

2- Into the designated wells, 100 μl of duplicate serial standard dilutions/ pretreated serum samples were dispensed.

3- 25 μl of diluted rabbit antihuman TNF-α polyclonal antibody were dispensed into each well. The plate was sealed using a plate sealer (to prevent evaporation) and incubated at room temperature for 3 hours.

N.B: At this step, colour reagents were allowed to come to room temperature.

4- At the end of incubation, 25μl of biotinylated TNF-α conjugate were dispensed into each well, sealed with a plate sealer and incubated for 30 minutes at room temperature.

5- The first washing step was done, using an automatic plate washer , in the form of five washing cycles, followed by final aspiration of the washing solution and blot drying of the plate.

6- Then 50 μl of reconstituted streptavidin-alkaline phosphatase enzyme were dispensed into each well. The plate was sealed and incubated at room temperature for 30 minutes.

7- The second washing step was performed using an automatic plate washer in the form of five washing cycles, followed by final aspiration of washing solution and blot drying of the plate.

8- Equal volumes of the colour reagents were mixed, and 200 μl of this mixture were dispensed into each well. The plate was sealed and incubated for 15 minutes at room temperature.

N.B. A period of 3 seconds was allowed for gentle plate shaking following addition of the colour reagents using a plate shaker

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

10- The absorbance of coloured solution in the plate was read at wavelength 492 nm using a microplate reader.

Calculation

- A standard curve was plotted on semilog graph paper with X-axis (log scale) showing TNF-α concentration and Y-axis (linear scale) showing

corresponding % activity which was expressed as B/B_o. 100 for standards and samples.

- The resulting curve was sigmoid in nature. (Figure 18)
- After obtaining the sample concentration from the standard curve, the final result was multiplied by a dilution factor of 4 (due to sample pre treatment).

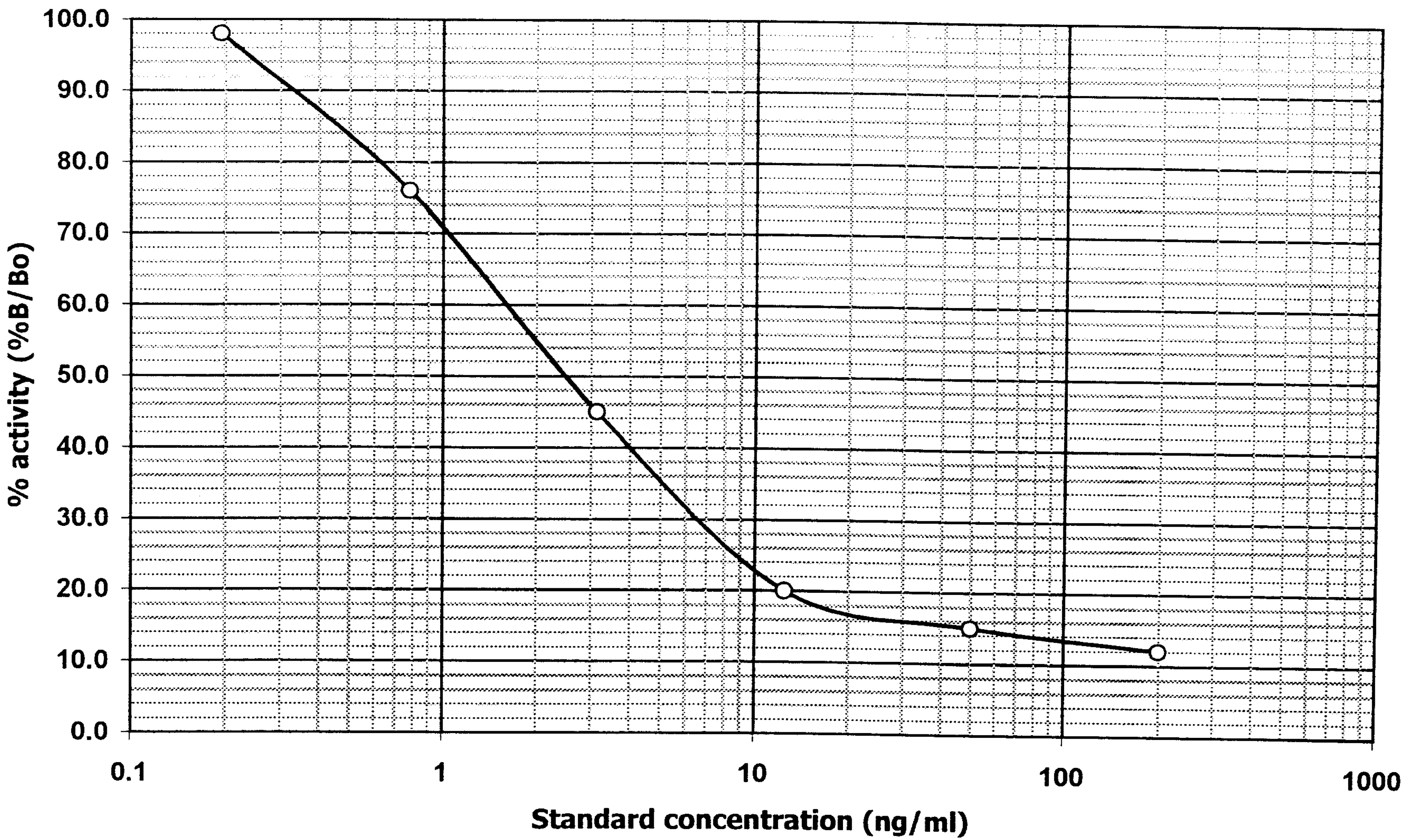


Figure (18) : Standard curve of TNF-α

Statistical Analysis

Statistical analysis was done using the SPSS software package.[503]

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

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

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

Where ;

$\overline{X}$ = Arithmetic mean

$\sum X$ = Sum of observations

n = number of observations

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

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

Where ;

n = number of cases

X = individual values

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

3- Standard error: (SE)

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

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- Wilcoxon test:

It is a non parametric test (distribution free method), used to test the significance of difference between 2 groups, when one or both groups are abnormally distributed.

The following equation was used:

$$SND = \frac{T_1 - \frac{1}{2} n_1 (n+1)}{\sqrt{\frac{n_1 n_2}{12 n (n-1)} \left[n^3 - n - \sum (t^3 - t)\right]}}$$

Where;

SND = Standard normal deviate

T_1 = Sum of ranks of the 1st group

n_1 = Sample size of the 1st group

n_2 = Sample size of the 2nd group

n = $n_1 + n_2$

t = ties between ranks

6- 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:

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

Where ;

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

RESULTS

RESULTS

Table I: Age and sex of the control group (Group I):

Twenty healthy volunteers were included in this study (10 males and 10 females). Their mean age was 36 ± 10.27 years.

Table I: Age and sex of the control group

No	Age (years)	Sex
1	29	F
2	40	F
3	36	F
4	22	F
5	20	F
6	40	F
7	28	F
8	32	F
9	31	F
10	45	F
11	30	M
12	25	M
13	35	M
14	32	M
15	52	M
16	28	M
17	54	M
18	55	M
19	41	M
20	45	M
Mean ± SD	36.0 ± 10.27	F: n = 10 M : n = 10

F = Female

M = Male

Table II: Some clinical and radiological data of the haemodialyzed patients' group (Group II).

Forty one haemodialyzed patients were involved in this study (22 males and 19 females). Their mean age was 45.36 ± 10.03 years, and their mean duration of dialysis was 62.51±62.46 months.

The mean blood pressure of this group was 130.98/79.51 ± 24.37/10.24 mm Hg.

29 subjects of the patient group had clinical evidence of secondary hyperparathyroidism, while 36 had radiological evidence of secondary hyperparathyroidism.

Table II: Some clinical data of the studied haemodiolyzed patients' group

No	Age (years)	Sex	Dur. Dial (months)	B.P. (mmHg)	Evidence of secondary hyperparathyroidism	
					Clinical evidence	Radiological evidence
1	50	M	105	150/90	+	+
2	29	M	91	130/80	+	+
3	45	M	127	120/80	+	+
4	36	M	94	110/70	+	+
5	46	M	36	100/70	+	+
6	55	M	35	100/70	+	-
7	44	M	36	110/70	+	-
8	48	M	60	140/90	-	-
9	42	M	260	110/70	+	-
10	35	M	106	100/70	-	-
11	50	M	36	160/70	-	+
12	21	M	36	150/90	+	+
13	64	M	8	100/70	+	+
14	60	M	33	140/90	+	+
15	45	M	108	120/80	-	+
16	27	M	14	150/90	-	+
17	34	M	54	120/80	+	+
18	45	M	88	110/70	+	+
19	48	M	12	170/110	-	+
20	50	M	13	140/80	+	+
21	35	M	147	100/70	+	+
22	50	M	4	160/100	+	+

F: Female
M: Male
No: Number
B.P.: Blood pressure

+ : Present
- : Absent
Dur. Dial: Duration of dialysis

Table II: Continued

No	Age (years)	Sex	Dur. Dial (months)	B.P. (mmHg)	Evidence of secondary hyperparathyroidism	
					Clinical evidence	Radiological evidence
23	40	F	1	120/70	-	+
24	46	F	1	120/70	-	+
25	38	F	72	110/70	+	+
26	52	F	83	190/90	+	+
27	33	F	172	110/70	+	+
28	54	F	21	120/70	+	+
29	56	F	9	130/80	+	+
30	44	F	9	140/80	-	+
31	30	F	118	130/80	+	+
32	37	F	241	140/90	+	+
33	52	F	83	120/80	+	+
34	60	F	56	110/70	+	+
35	60	F	100	140/80	+	+
36	48	F	4	180/80	-	+
37	60	F	52	190/100	-	+
38	52	F	19	140/80	-	+
39	50	F	8	150/90	+	+
40	45	F	2	110/70	+	+
41	55	F	9	130/80	+	+
Mean	45.36		62.51	$\frac{130.98}{79.51}$		
± S.D	±10.03		±62.46	$\pm\frac{24.37}{10.24}$		

F: Female
M: Male
No: Number
B.P.: Blood pressure

+ : Present
- : Absent
Dur. Dial: Duration of dialysis

Table III a: Some biochemical data in the control group:

Serum levels of glucose, creatinine and albumin and activities of aspartate and alanine aminotransferases (AST & ALT).

Table III b: Some biochemical data in the haemodialyzed patients' group:

Serum levels of glucose, creatinine and albumin and serum activities of aspartate and alanine aminotransferases.

Table III c: Statistical differences of the biochemical data between control group and patients' group.

Serum creatinine level was significantly higher in the patients' group than the corresponding control group.

Serum albumin level was significantly lower in the patients' group than the corresponding control group.

Table IIIa : Some biochemical data in the control group

No	Glucose mg/dl	Creatinine mg/dl	AST (GOT) Units/L	ALT (GPT) Units/L	Albumin gm/dl
1	108	0.6	20	18	4.7
2	98	0.8	14	15	4.4
3	100	0.6	22	20	4.2
4	93	0.5	18	11	4.2
5	80	0.5	23	18	4.5
6	93	0.5	29	24	4.1
7	95	0.5	22	35	4.7
8	93	0.8	19	25	4.3
9	81	0.4	20	11	4.5
10	105	1.1	15	11	4.6
11	104	0.8	9	8	4.6
12	101	0.7	38	22	4.7
13	105	0.7	22	39	4.9
14	87	0.7	25	36	4.2
15	99	0.7	30	40	4.1
16	110	0.6	26	20	4.5
17	86	1.0	17	15	4.4
18	67	1.0	22	24	4.4
19	72	0.8	26	24	4.4
20	69	1.3	20	17	4.0
Mean ± SD	92.30 ± 12.94	0.73 ± 0.23	21.85 ± 6.30	21.65 ± 9.51	4.42 ± 0.24

Table IIIb: Some biochemical data in the haemodialyzed patients' group

No	Glucose mg/dl	Creatinine mg/dl	AST (GOT) Units/L	ALT (GPT) Units/L	Albumin gm/dl
1	71	12.4	29	33	3.7
2	90	14.7	26	40	4.0
3	116	12.3	11	7	3.9
4	79	14.2	34	80	3.9
5	87	11.5	19	14	3.6
6	104	11.0	8	3	3.1
7	83	14.9	15	8	3.9
8	104	18.0	6	9	4.1
9	84	12.5	28	47	3.7
10	82	14.0	13	5	4.0
11	194	12.6	12	19	3.5
12	72	14.6	22	27	4.2
13	99	12.1	7	6	3.8
14	70	13.5	10	13	3.7
15	125	12.0	56	80	3.8
16	83	11.0	17	16	4.1
17	97	16.9	16	13	4.2
18	76	9.0	23	14	4.0
19	103	12.3	9	5	4.0
20	119	7.2	13	31	3.5
21	100	15.8	20	28	3.7
22	123	12.8	11	9	3.7

Table IIIb: Continued

No	Glucose mg/dl	Creatinine mg/dl	AST (GOT) Units/L	ALT (GPT) Units/L	Albumin gm/dl
23	114	7.8	8	6	4.2
24	116	11.9	8	10	3.7
25	86	9.8	72	88	3.7
26	79	12.5	16	14	4.2
27	94	16.8	18	10	4.3
28	70	14.2	33	50	3.7
29	124	9.2	11	9	3.9
30	99	11.9	12	10	3.8
31	75	9.7	22	24	4.2
32	67	10.3	14	16	3.8
33	81	10.8	25	18	3.4
34	87	11.4	10	8	3.8
35	92	9.3	14	10	3.3
36	136	5.6	15	24	3.3
37	95	14.2	8	7	4.3
38	70	10.3	18	20	3.6
39	74	9.1	22	8	4.0
40	109	10.5	12	9	3.2
41	96	11.7	14	12	3.7
Mean ± SD	95.73 ± 23.87	12.01 ± 2.64	18.46 ± 12.75	20.98 ± 20.82	3.81 ± 0.30

Table IIIc : Statistical differences of the biochemical data between control and patients' groups

Analytes	Controls	Patients	P. value
Glucose (mg/dl)			
Mean ± S.D	92.30 ± 12.94	95.73 ± 23.87	0.551
Creatinine (mg/dl)			
Mean ± S.D	0.73 ± 0.23	12.01 ± 2.64	0.000*
AST (GOT) (Units/L)			
Mean ± S.D	21.85 ± 6.30	18.46 ± 12.75	0.267
ALT (GPT) (Units/L)			
Mean ± S.D	21.65 ± 9.51	20.98 ± 20.82	0.891
Albumin (gm/dL)			
Mean ± S.D	4.42 ± 0.24	3.81 ± 0.30	0.000*

* Statistically significant if p value is < 0.05

Table IV a: Serum levels of total calcium, ionized calcium, inorganic phosphate and serum activities of acid and alkaline phosphatases in the control group.

Table IV b: Serum levels of total calcium, ionized calcium, inorganic phosphate and serum activities of acid and alkaline phosphatases in the haemodialyzed patients' group.

Table IV c: Statistical differences of serum total calcium, ionized calcium, inorganic phosphate levels, and serum activities of acid and alkaline phosphatases between control group and haemodialyzed patients' group.

Total and ionized serum calcium levels were significantly lower in patients' group than their corresponding control values.

Serum inorganic phosphate level was significantly higher in patients' group than their corresponding control values.

Serum acid and alkaline phosphatases activities were significantly higher in patients' group than their corresponding control values.

Table IVa: Serum levels of total calcium, ionized calcium, inorganic phosphate, and serum activities of acid and alkaline phosphatases in the control group

No	Total calcium mg/dl	Ionized Calcium mg/dl	Inorganic Phosphate mg/dl	Acid phosphatase Units/L	Alkaline phosphatase Units/L
1	9.9	4.84	3.4	4.1	170
2	8.8	4.70	3.3	3.2	183
3	8.5	4.53	2.9	4.2	237
4	8.9	4.52	4.2	4.3	212
5	8.8	4.43	3.4	4.6	239
6	9.5	4.68	3.6	3.8	190
7	9.8	4.64	3.5	4.5	250
8	9.0	5.22	3.7	4.5	98
9	9.4	4.87	3.5	4.5	96
10	8.8	4.64	3.0	4.7	121
11	8.9	4.85	3.8	3.2	189
12	9.8	4.34	2.7	3.4	156
13	10.2	4.35	3.1	3.6	176
14	10.0	4.50	3.8	3.9	269
15	9.9	5.08	4.0	3.6	67
16	9.2	4.48	3.5	4.2	80
17	9.7	4.89	3.9	4.6	168
18	8.9	5.75	3.3	3.8	157
19	9.0	5.01	3.0	3.8	131
20	10.0	5.08	3.8	3.7	184
Mean	9.35	4.77	3.47	4.01	168.65
± SD	± 0.525	± 0.344	± 0.395	± 0.477	± 57.124

Table IV b : Serum levels of total calcium, ionized calcium, inorganic phosphate and serum activities of acid and alkaline phosphatases in the haemodialyzed patients

No	Total calcium mg/dl	Ionized calcium mg/dl	inorganic phosphate mg/dl	Acid phosphatase Units/L	Alkaline phosphatase Units/L
1	9.8	4.32	7.1	15.0	1414
2	8.1	3.64	5.6	12.3	2472
3	9.8	4.68	7.2	10.4	698
4	7.4	3.90	5.5	11.4	2144
5	10.4	5.46	7.7	12.2	1100
6	8.7	4.04	5.8	12.4	1521
7	10.0	4.36	8.3	12.1	319
8	8.9	3.76	9.0	13.5	377
9	8.6	4.88	4.7	12.1	967
10	8.0	3.79	5.3	12.1	1150
11	6.6	2.52	8.2	11.3	309
12	6.9	3.44	7.7	12.9	534
13	7.7	3.12	7.9	11.3	523
14	10.8	4.90	6.6	10.4	295
15	7.2	3.12	4.9	13.7	1974
16	8.6	4.91	6.1	12.1	186
17	5.6	2.36	4.9	12.7	796
18	6.7	5.09	5.9	13.8	1437
19	9.3	3.28	7.6	9.3	95
20	10.2	5.17	3.6	17.2	64
21	4.7	1.72	7.5	11.4	653
22	7.7	3.75	8.7	8.5	130

Table IVb: Continued

No	Total calcium mg/dl	Ionized calcium mg/dl	inorganic phosphate mg/dl	Acid phosphatase Units/L	Alkaline phosphatase Units/L
23	12.0	5.59	5.6	8.4	1686
24	8.7	4.74	8.6	9.2	125
25	10.1	4.92	6.9	14.3	1327
26	9.0	3.92	5.8	10.7	2457
27	8.0	3.20	4.9	13.5	848
28	8.7	3.24	7.4	10.6	756
29	10.0	4.04	8.2	11.4	525
30	7.3	4.49	9.7	11.7	166
31	7.9	3.61	4.6	11.3	1191
32	8.8	3.32	9.7	12.7	276
33	7.5	4.50	6.3	9.0	239
34	8.5	3.92	8.1	13.3	274
35	7.2	3.85	3.4	12.0	560
36	8.4	5.15	3.8	8.2	720
37	7.7	3.96	8.8	10.9	235
38	8.7	4.31	4.9	10.3	468
39	8.4	3.95	7.6	9.4	164
40	9.5	4.52	8.4	8.2	126
41	9.8	4.78	5.8	8.2	166
Mean ± SD	8.49 ± 1.421	4.13 ± 0.846	6.69 ± 1.685	11.50 ± 2.002	767.49 ± 669.87

Table IVc: Statistical differences of serum levels of total calcium, ionized calcium, inorganic phosphate, and serum activities of acid and alkaline phosphatases between controls and haemodiolyzed patients

Analytes	Controls	Patients	P. value
Total calcium (mg/dL)			
Mean ± S.D	9.35 ± 0.525	8.49 ± 1.421	0.011*
Ionized calcium (mg/dL)			
Mean ± S.D	4.77 ± 0.344	4.13 ± 0.846	0.002*
Inorganic phosphate (mg/dL)			
Mean ± S.D	3.47 ± 0.395	6.69 ± 1.685	0.000*
Acid phosphatase (Units/ L)			
Mean ± S.D	4.01 ± 0.477	11.50 ± 2.002	0.000*
Alkaline phosphatase (Units/ L)			
Mean ± S.D	168.65 ± 57.124	767.49 ± 669.87	0.000*

(NB: Significant difference if the p value is < 0.05)

Table V: Serum intact parathyroid hormone level in both controls and haemodialyzed patients.

Serum intact parathormone level was significantly higher in the patients group ($P < 0.001$) than in the control group.

Table V: Serum intact parathyroid hormone level(pg/ml) in both studied groups

	Controls		Haemodialyzed patients			
	No	Concentration	No	Concentration	No	Concentration
	1	14	1	1624	22	-
	2	53	2	2430	23	2173
	3	37	3	2500	24	2500
	4	38	4	2195	25	2500
	5	21	5	2500	26	1757
	6	21	6	1193	27	1428
	7	47	7	1130	28	1662
	8	26	8	1126	29	1751
	9	48	9	1248	30	1067
	10	59	10	1382	31	1178
	11	15	11	789	32	612
	12	21	12	804	33	460
	13	39	13	612	34	311
	14	18	14	545	35	817
	15	46	15	352	36	344
	16	45	16	330	37	285
	17	33	17	300	38	282
	18	54	18	919	39	199
	19	38	19	143	40	-
	20	48	20	172	41	-
			21	187		
Mean ± SD	36.05 ± 14.11		1100.18 ± 786.11			
P	0.000					

(NB: Significant difference if the p value is < 0.05)

Table VI: Serum transforming growth factor-β_1 levels (ng/ml) in both controls and haemodialyzed patients.

The serum transforming growth factor-beta1, was higher in the patients' group, though statistically not significant, than that in the control group.

Table VI : Serum transforming growth factor- beta1 level (ng/ml) in the studied groups

Controls		Haemodialyzed patients			
No	Concentration	No	Concentration	No	Concentration
1	16	1	47	22	38
2	38	2	30	23	15
3	19	3	18	24	38
4	17	4	38	25	16
5	18	5	31	26	34
6	30	6	60	27	60
7	14	7	52	28	20
8	36	8	60	29	22
9	52	9	47	30	52
10	42	10	47	31	32
11	12	11	46	32	24
12	10	12	21	33	25
13	16	13	47	34	60
14	47	14	28	35	16
15	60	15	20	36	18
16	37	16	22	37	47
17	60	17	47	38	42
18	58	18	38	39	22
19	47	19	46	40	47
20	46	20	21	41	30
		21	15		

	Controls	Haemodialyzed patients
Mean ± SD	33.75 ± 17.35	35.10 ± 14.32
P	0.749	

(NB: Significant difference if the p value is < 0.05)

Table VII: Serum insulin-like growth factor-I levels (ng/ml) in both controls and haemodialyzed patients:

Serum insulin-like growth factor-I level was higher in the control group, though statistically insignificant, than that in the patients' group.

Table VII : Serum insulin like growth factor-I level (ng/ml) in the studied groups

Controls		Haemodialyzed patients			
No	Concentration	No	Concentration	No	Concentration
1	194	1	70	22	195
2	140	2	120	23	170
3	129	3	120	24	205
4	310	4	165	25	140
5	250	5	90	26	100
6	160	6	60	27	135
7	65	7	250	28	230
8	168	8	290	29	145
9	168	9	140	30	250
10	210	10	235	31	225
11	170	11	230	32	160
12	250	12	180	33	49
13	129	13	240	34	150
14	200	14	109	35	95
15	119	15	190	36	59
16	169	16	280	37	170
17	53	17	170	38	90
18	65	18	120	39	105
19	115	19	150	40	165
20	193	20	135	41	164
		21	195		

	Controls	Haemodialyzed patients
Mean ± SD	162.85 ± 65.18	159.54 ± 61.41
P	0.847	

(NB: Significant difference if the p value is < 0.05)

Table VIII: Serum tumour necrosis factor-alpha levels (ng/ml) in both controls and haemodialyzed patients:

Serum tumour necrosis factor-α level was significantly higher ($p = 0.031$) in the patients group than that in the control group.

Table VIII : Serum Tumour necrosis factor- alpha level (ng/ml) in the studied groups

	Controls		Haemodialyzed patients			
	No	Concentration	No	Concentration	No	Concentration
	1	3.5	1	3.2	22	3.6
	2	2.4	2	1.2	23	3.2
	3	0.8	3	2.6	24	1.8
	4	1.0	4	1.8	25	6.8
	5	0.8	5	2.8	26	2.1
	6	0.8	6	8.0	27	1.7
	7	3.6	7	4.4	28	1.1
	8	2.1	8	6.4	29	4.0
	9	3.2	9	3.6	30	3.5
	10	3.8	10	2.1	31	2.2
	11	0.8	11	0.8	32	4.8
	12	0.8	12	1.8	33	0.9
	13	2.1	13	1.7	34	4.8
	14	1.7	14	1.1	35	1.1
	15	2.8	15	1.3	36	3.2
	16	1.1	16	9.2	37	1.7
	17	0.9	17	3.2	38	1.2
	18	1.2	18	3.2	39	0.8
	19	0.8	19	1.3	40	1.7
	20	1.6	20	2.8	41	0.9
			21	2.4		
Mean ± SD	1.79 ± 1.076		2.82 ± 1.965			
P	0.031*					

(NB: Significant difference if the p value is < 0.05)

Table IX: Serum interleukin-1 beta levels (ng/ml) in both controls and haemodialyzed patients:

Serum interleukin-1 beta level in the patients' group was higher, though statistically insignificant, than that in the control group.

Table IX: Serum Interleukin1- beta level (ng/ml) in the studied groups

Controls		Haemodialyzed patients			
No	Concentration	No	Concentration	No	Concentration
1	0.9	1	2.8	22	0.8
2	1.8	2	0.8	23	3.8
3	0.8	3	0.8	24	1.5
4	0.8	4	0.8	25	5.0
5	0.8	5	3.0	26	4.4
6	1.3	6	1.6	27	0.8
7	2.2	7	2.0	28	0.8
8	3.7	8	2.7	29	3.3
9	0.8	9	3.0	30	0.8
10	2.5	10	2.7	31	0.8
11	0.8	11	3.1	32	7.2
12	1.9	12	5.6	33	1.1
13	5.2	13	0.8	34	5.6
14	2.4	14	3.5	35	1.5
15	0.8	15	3.4	36	5.6
16	2.4	16	3.2	37	2.5
17	1.1	17	3.7	38	1.5
18	3.3	18	3.6	39	1.6
19	0.9	19	3.3	40	1.2
20	3.8	20	4.0	41	0.8
		21	1.6		
Mean ± SD	1.91 1.276	2.59 ± 1.657			
P	0.107				

(NB: Significant difference if the p value is < 0.05)

Table X: Some studied items in patients with serum intact parathyroid hormone level < 300 pg/ml and those with serum intact parathyroid hormone level ≥ 300 pg/ml.

The serum alkaline phosphatase activity and tumour necrosis factor-α level were significantly higher in the group of patients with serum intact parathyroid hormone level ≥ 300 pg/ml, than their corresponding levels in those with serum intact parathyroid hormone level < 300 pg/ml.

Table X: Some studied items in patients with serum intact parathyroid hormone level(iPTH) < 300 pg/ml and ≥300 pg/ml

	iPTH<300 (n = 6)	iPTH ≥300 (n = 32)	P. value
Age (years) Mean ± S.D	49.17 ± 8.11	44.56 ± 10.59	0.201
Duration of dialysis (month) Mean ± S.D	29.56 ± 46.49	71.78 ± 63.81	0.073
Total calcium (mg/dl) Mean ± S.D	8.17 ± 1.90	8.50 ± 1.38	0.614
Ionized calcium (mg/dl) Mean ± S.D	3.73 ± 1.16	4.18 ± 0.81	0.250
Inorganic phosphate (mg/dl) Mean ± S.D	6.67 ± 1.98	6.61 ± 1.67	0.937
Acid phosphatase (Units/L) Mean ± S.D	10.38 ± 2.8	11.81 ± 1.64	0.056
Alkaline phosphatase (Units/L) Mean ± S.D	233.44 ± 197.02	917.69 ± 680.37	0.005*
Insulin like growth factor-I (ng/ml) Mean ± S.D	140.83 ± 39.42	161.63 ± 67.09	0.470
Transforming growth factor -β_1 (ng/ml) Mean ± S.D	32.17 ± 14.36	35.34 ± 14.97	0.634
Interleukin-1 beta (ng/ml) Mean ± S.D	2.42 ± 1.05	2.80 ± 1.75	0.617
Tumour necrosis factor-α (ng/ml) Mean ± S.D	1.70 ± 0.76	3.11 ± 2.09	0.010*

* : Statistically significant p value

n = number of studied cases

Table XI: Some studied items in the group of patients with clinical evidence of secondary hyperparathyroidism and the group without clinical evidence of secondary hyperparathyroidism.

The serum IGF-I level was significantly higher in patients with clinical evidence of secondary hyperparathyroidism than in those without.

However, none of the other items were significantly altered between both groups of patients.

Table XI: Some studied items in the patients with clinical evidence of secondary hyperparathyroidism and those without clinical evidence of secondary hyperparathyroidism

	Yes (n =29)	No (n = 12)	P. value
Age (years) Mean ± S.D	45.79 ± 10.77	45.25 ± 8.40	0.877
Duration of dialysis (months) Mean ± S.D	73.83 ± 67.32	35.17 ± 38.68	0.071
Total calcium (mg/dl) Mean ± S.D	8.50 ± 1.46	8.45 ± 1.37	0.920
Ionized calcium (mg/dl) Mean ± S.D	4.02 ± 0.85	4.39 ± 0.83	0.214
Inorganic phosphate (mg/dl) Mean ± S.D	6.61 ± 1.58	6.88 ± 1.99	0.657
Acid phosphotose (units/L) Mean ± S.D	11.75 ± 2.04	10.89 ± 1.85	0.217
Alkaline phosphatase (units/L) Mean ± S.D	826.76 ± 683.96	624.25 ± 639.79	0.385
Insulin like growth factor-I (ng/ml) Mean ± S.D	145.59 ± 52.29	193.25 ± 70.70	0.022*
Transforming growth factor -β_1 (ng/ml) Mean ± S.D	34.00 ± 14.14	37.75 ± 15.06	0.453
Interleukin-1 beta (ng/ml) Mean ± S.D	2.50 ± 1.81	2.84 ± 1.25	0.555
Tumour necrosis factor-α (ng/ml) Mean ± S.D	2.77 ± 1.75	2.98 ± 2.49	0.764

* : Statistically significant p value

n = number of studied cases

Table XII a: Significant correlations in the whole group of haemodialyzed patients.

Table XII b: Significant correlations in the patients' group with serum intact parathyroid hormone level ≥ 300 pg/ml.

Table XII c: Significant correlations in the patients' group with clinical evidence of secondary hyperparathyroidism.

Table XII a: Significant correlations in the haemodialyzed group of patients

Analytes			r	p
IGF-I	with	- Creatinine	0.4102	0.008
		- Inorganic phosphate	0.3939	0.011
		- Albumin	0.3864	0.016
IL-1β	with	- ACP	0.3195	0.042
		- TNF-α	0.3301	0.035
TNF-α	with	- IL-1β	0.3301	0.035
		- ACP	0.3487	0.025
iPTH	with	- ALP	0.5244	0.001
		- Total calcium	0.4105	0.010

Table XII b: Significant correlations in the patients' group with serum intact parathyroid hormone level ≥ 300 pg/ml.

Analytes			r	p
TGF-β_1	with	- Creatinine	0.5242	0.002
		- ACP	0.3771	0.033
iPTH	with	- ALP	0.4335	0.013
		- Total calcium	0.4895	0.004
IGF-I	with	- Inorganic phosphate	0.3943	0.026
		- Creatinine	0.3747	0.035

Table XII c: Significant correlations in the patients' group with clinical evidence of secondary hyperparathyroidism

Analytes			r	P
TNF-α	with	- IL-1 β	0.4353	0.018
		- ACP	0.4159	0.025
iPTH	with	- ALP	0.6501	0.000
		- Total calcium	0.4127	0.036

DISCUSSION

DISCUSSION

Bone is a special form of connective tissue, made up of bone cells and matrix. Bone growth and remodeling is a complex dynamic process that achieves a balance between the coupled processes of bone formation and resorption. This process is regulated by the interplay of systemic hormones, locally produced cytokines and growth factors.[3,13,17-19,486, 504]

The growth regulatory cytokines are termed bone remodeling units (BRU). Their action may be synergistic or antagonistic with each other. They may also interact with systemic bone regulators. [51-53,63]

Cytokines that induce bone resorption, such as IL-1β and TNF-α stimulate the release of soluble factors that increase proliferation and differentiation of osteoclast precursors as well as activation of mature osteoclasts.[32,52,58,101,114-117,145,146,504-506] On the other hand, the cytokines that induce bone formation include the IGF system and peptides of transforming growth factor β-family (TGF-$\beta_{1,2,3}$ and BMPs). IGF-1 decreases collagen degradation, enhances bone matrix deposition and increases osteoblastic cell recruitment [39, 59, 299-317, 504]

Transforming growth factor-β (TGF-β) is released from collagenous matrix during resorption to inhibit osteoclast formation and promote osteoblast proliferation and differentiation with cartilage formation [16,183-186,216-218,504]

In CRF, the balance between osteoblastic and osteoclastic activities is disturbed with a net effect of predominant bone resoprtion.[486]

Renal Osteodystrophy (ROD) comprises a group of complex metabolic skeletal and extraskeletal disorders that occur as a complication of CRF. Nearly all ESRD patients particularly those on maintenance dialysis, suffer from these disorders (ROD).[3,401-409]

Renal Osteodystrophy (ROD) occurs as a consequence of disruption in the bone remodeling cycle. The patterns of the disease are the result of changes in calcium, phosphate, PTH and vitamin D metabolism and may be due as well to the effects of uraemic toxins.[416,486]

Based on histomorphometric findings, ROD is classified into a high and a low turnover types. Overlap between both types (mixed ROD) may occur according to the predominant lesion.[3,401-409]

There is accumulating evidence suggesting that, besides the disordered calcitropic hormone metabolism, the abnormalities of bone acting cytokines and growth factors as well as their receptors and modulators could be considered as potential contributors to the pathogenesis of ROD.[52,69,482-487,504-506]

The aim of the present work was to study some local bone regulatory cytokines in end stage renal disease patients (ESRD) with secondary hyperparathyroidism (sHPT) under maintenance haemodialysis (HD).

In the present study, 41 ESRD patients on maintenance haemodialysis with clinical and/or radiological evidence of renal bone

disease were selected and compared to a group of 20 apparently healthy volunteers as a control group.

Serum intact parathyroid hormone (iPTH), total and ionized calcium and inorganic phosphate, in addition to some local bone regulatory cytokines and some markers of osteoblastic and osteoclastic activities were estimated. IGF-I and TGF-β_1 were chosen to represent bone forming cytokines and total ALP activity was taken as a marker of osteoblastic activity. On the other hand, IL-1β and TNF-α were chosen to represent bone resorbing cytokines with total ACP activity chosen as a marker of osteoclastic activity.

Parathormone is a major factor that plays a key role in bone turnover and ROD. The mean serum intact PTH (iPTH) level in the whole group of haemodialyzed patients (1100.18±786.11 pg/ml) was significantly higher than its mean serum level in the control group (36.05 ± 14.11 pg/ml). (Table V).

The patients' serum total and ionized calcium levels were significantly lower than their corresponding values in the control group (Tables IV a-c) while, serum inorganic phosphate level in the patients' group was significantly higher than its level in the control group (Tables IV a-c). These findings proved the occurrence of secondary hyperparathyroidism.

High serum iPTH level is a constant finding in ESRD patients. In early renal failure (RF), alteration in vitamin D metabolism, decreased

calcitriol level and moderate decrease in ionized calcium may allow greater synthesis and secretion of PTH. As the disease progresses, there is a decrease in number of vitamin D and calcium receptors, making parathyroid gland more resistant to both. [3,52,406,412,426-429,507-509] On the other hand, inorganic phosphates induce parathyroid gland hyperplasia (independent of calcitriol and calcium) and increase PTH synthesis by a post transcriptional mechanism.[3,52,406,412,510,511]

Convincing data indicate that aluminium (Al) accumulation in the bones of CRF patients particularly those on maintenance dialysis can cause osteomalacia.[463,468,474]

In bone, accumulated Al replaces calcium (Ca) at the mineralization front, disrupting normal osteoid formation.[3,52,406,473-476] It has been shown that Al inhibits osteoblastic activity, thereby contributing to reduced matrix synthesis. This is evidenced by the presence of inclusion bodies within active osteoblasts.[474-477]

Aluminum has been reported to inhibit PTH synthesis and release, thereby decreasing bone turnover and rendering the bone more susceptible to osteomalacia.[3,52,406,479,480,512-516] It has been suggested that high PTH levels may protect against Al induced bone disease.[406,476]

In a study done by Khalil NB (2002)[517] on ESRD patients under maintenance HD (of whom 19 were included in the present study),

mean serum Al level of those patients was significantly higher as compared to the control levels.

However, in the present study, the mean serum iPTH level in these 19 patients was 1221 ± 829.9 pg/ml, supporting the previously reported protective effect of PTH against Al-induced osteomalacia.

The pathogenesis of skeletal resistance to calcaemic action of PTH in ESRD patients remains unclear. Phosphate retention, decreased calcitriol levels and uraemic toxins have been involved.[486,518,519] It has been hypothesized that decreased mRNA expression of PTH receptors results in their downregulation or desensitization. This phenomenon results in an altered threshold for cellular response to PTH so that serum PTH levels 2-4 times normal are required to maintain normal bone turnover.[406,412,486,520-522]

In the present work, a significant relation existed between serum iPTH level and radiological findings in the patients' group, denoting that the bony changes were more marked in those with pure secondary hyperparathyroidism.

In chronic dialysis patients, decision levels of iPTH of 200-300 pg/ml, have been suggested for distinguishing patients with secondary hyperparathyroidism (sHPT).[24,523]

According to the results of serum levels of iPTH in the patients' group, they were further categorized into 2 groups: patients with iPTH ≥ 300 pg/ml (32 patients) and patients with iPTH < 300 pg/ml (6 patients).

The ALP activity was significantly higher in the group with serum iPTH ≥ 300 pg/ml than in the group with serum iPTH < 300 pg/m. (Table X), pointing to an increased osteoblastic activity in patients with severe hyperparathyroidism.

In addition, significant positive correlations were found in the group with serum iPTH ≥ 300 pg/ml between PTH and both total calcium (Ca) ($r = 0.4895$, $p = 0.004$) and ALP activity ($r = 0.4335$, $p = 0.013$). (Table XII).

It could be noticed that the duration of dialysis was higher although statistically insignificant in the group with iPTH ≥ 300 pg/ml than in the group with iPTH < 300 (Table X), suggesting an aggravating effect of dialysis to the secondary hyperparathyroidism state in such patients.

Alterations in PTH and calcitriol production, however, do not completely account for abnormalities in bone turnover. This suggests that other factors or mediators were also involved in such alternations. Among them, local bone regulatory cytokines are of particular interest.[482-486]

I- Cytokines regulating bone formation

Alterations in factors known to regulate osteoblastic growth, differentiation and activity have been demonstrated in uraemia.[69,482-486] Among the various bone growth factors produced by osteoblasts and regulated by PTH, the IGF family and peptides of TGF-β may be important in the altered bone remodeling in patients with ROD.[69,484-487]

A- Transforming growth factor-beta-1 (TGF-β_1)

TGF-β is one of the most abundant growth factors in bone, being synthesized by both osteoblasts and osteoclasts and stored in the bone matrix. Its effects on bone are complex but in general appear to promote bone formation and inhibit bone resorption.[184-186,211,213,216,217,222]

Several workers studied the expression and localization of proteins of TGF-β system in the kidney.[524,525]

In the present study, the serum TGF-β_1 level in the patients' group was 35.10 ± 14.32 ng/ml, which showed no significant difference when compared to its level in the control group (33.75 ± 17.35 ng/ml) (Table VI).

In the 32 patients with iPTH ≥ 300 pg/ml, TGF-β_1 level tended to be higher, though statistically not significant than its level in the 6 patients with iPTH < 300 pg/ml.

Although its role has been extensively investigated in the development of glomerular disease,[526-528] little is known about the role of TGF-β in the pathogenesis of ROD.[69,486]

Some workers found that ROD patients had significantly higher levels of intraplatelet and plasma TGF-β than ESRD patients without ROD. They concluded that ROD may stimulate overproduction of TGF-β in ESRD patients under maintenance haemodialysis.[529,530] Howerver,

Hoyland JA and Picton ML (1999) reported a decrease in TGF-β mRNA expression, suggesting a downregulation of its synthesis.[487]

In uraemia, a deficiency of bone morphogenic protein-1 (BMP-1), which is a member of TGF-β family that is normally expressed by the kidneys could potentially lead to failure of osteoblast formation and contributes to the development of LTBD which is part of the spectrum of skeletal abnormalities of ROD.[69, 531]

In the group with severe hyperparathyroidism in the present study (iPTH $\geq$ 300 pg/ml), a significant positive correlation existed between TGF-β_1 and creatinine ($r = 0.5242$, $p = 0.002$) (Table XIIb) denoting a possible relation between TGF-β_1 production and the extent of uraemia in ROD patients.

Also a significant positive correlation existed between TGF- β_1 nd acid phosphatase activity ($r = 0.3771$, $p = 0.033$) (Table XIIb) implying the role of TGF-β_1 in the coupled processes of bone formation and resorption.

B- Insulin like growth factor-I (IGF-I)

Insulin like growth factor-I (IGF-I) plays a key role in regulation of bone formation. It acts as a mediator for action of growth hormone in various tissues including bone.[41,49,270-276]

The IGF-I level in the haemodialyzed patients group (159.54 ± 61.41 ng/ml) showed no significant difference from its level in the control group (162.85 ± 65.18 ng/ml) (Table VII).

Some workers reported a slight or no increase in serum IGF-I level,[532-535] while others reported a significant increase in its serum level in haemodialyzed patients which was likely related to a reduced renal IGF-1 clearance or possibly increased hepatic IGF-I synthesis by the elevated growth hormone level observed in uraemia.[536]

Both in vitro and in vivo studies suggest that the anabolic effects of intermittent PTH secretion are mediated through locally increased IGF-I expression.[229,277,537]

In the present study, the group with iPTH $\geq$ 300 pg/ml showed a higher serum IGF-I level (161.63 $\pm$ 67.09 ng/ml) than those with iPTH <300 pg/ml (140.83 $\pm$ 39.42 ng/ml), though not statistically significant. (Table X).

On the other hand, the serum IGF-I level in the group without clinical evidence of sHPT (193.25 $\pm$ 70.701 ng/ml) was significantly higher ($p = 0.022$) than its level in those with clinical evidence of sHPT (145.59 $\pm$ 52.291 ng/ml) (Table XI).

Andress DL *et al* (1989) reported a significantly higher serum IGF-I level in patients with higher rates of bone formation compared to those with normal or low rates.[536] Furthermore, it was reported that IGF-I correlates better with parameters of bone mineralization, being able to stimulate collagen synthesis and/or hydroxyapatite crystal formation in vivo.[39,59,299-317,536]

In the present study, serum IGF-I level in both whole patients' group and those with iPTH $\geq$ 300 pg/ml showed significant positive correlations with inorganic phosphate levels (r=0.3939, p=0.011 & r=0.3943, p=0.026 respectively) (Table XII a, b).

There is an increasing evidence that CRF patients have IGF-I resistance, that appears to be multifactorial.[52,486,538-540] It has been suggested that chronic acidosis is responsible, via a peripheral mechanism, for the resistance to the growth promoting actions of GH and IGF-I.[433,541] Some workers reported that despite the presence of a normal serum IGF-1 level, there was an elevation in serum levels of IGF binding proteins 1, 2 and 4 which are inhibitory components for IGF system leading to reduction in IGF-1 bioactivity [52,339-344,486,542-544] A functional IGF-I receptor defect with a reduced tyrosine kinase activity was also postulated.[52,486] In addition, decreased IGF-I production in various tissues, including liver and bone was reported. This was evidenced by decreased IGF-I mRNA expression by osteoblasts in LTBD group compared to normal and that of non renal aetiology.[486,545-547] Philips LS *et al* (1984) have shown that uraemic serum had decreased IGF-I activity, probably due to the presence of a low M.W. (<1,000 Dalton) inhibitor in a high level, attenuating any anabolic effect IGF-1 has on bone.[548]

Considering the effect of nutritional status on serum IGF-I level, Andress DL *et al* (1989) reported no significant effect of nutrition on serum IGF-I level. Nearly all of the patients in their study were well nourished and none had recent weight loss. [536]

In the present study, however, the serum albumin in the patients' group (3.81 ± 0.3 gm/dl) was significantly lower than the corresponding control group (4.42 ± 0.24 gm/dl) although still within the accepted

reference range (Table III c). In addition a significant positive correlation was found in the whole patients group between serum albumin and IGF-I levels ($r = 0.3864$, $p < 0.02$) (Table XIIa). Protein restriction can result in decreased level of serum IGF-I. This potential situation in a dialysis patient could make IGF-1 level a poor predictor of bone formation.[536,549,550]

Heparin is known to prevent IGF binding to its carrier protein resulting in increased free IGF-I level.[536,551] Such effects did not exist in the present study since IGF-I estimations were done on serum samples taken immediately before the dialysis session (i.e. 40 hours after the last heparin infusion).

In the present study, a significant positive correlation was found between serum IGF-I and serum creatinine in both the whole patients group and the patients with iPTH ≥ 300 pg/ml ($r = 0.4102$, $p = 0.008$ & $r = 0.3747$, $p = 0.035$ respectively) (Table XII a, b). These findings suggested a potential role of uraemia in influencing IGF-I production particularly in HTBD group of ROD patients.

II- Cytokines regulating bone resorption

The enhanced effect of some local bone resorption factors could possibly be an additional factor in the pathogenesis of ROD.

A- Tumour necrosis factor-alpha (TNF-α)

Tumour necrosis factor-alpha (TNF-α) is a powerful stimulator of osteoclastic bone resorption in vivo.

In the present study, TNF-α level in the whole patients' group (2.82 ± 1.965 ng/ml) was significantly higher than its level in the corresponding control group (1.79 ± 1.076 ng/ml) (Table VIII).

This agrees with the study performed by Heberlin et al (1990) who reported a significant increase in TNF-α plasma level in haemodialyzed patients.[552]

Deschamps-Latscha B *et al* (1999)[553] reported that CRF patients already had increased TNF-α levels even before they started the dialysis therapy.

The TNF-α level in the group with iPTH ≥ 300 pg/ml (3.11 ± 2.09 ng/ml) was significantly higher than its level in the group with iPTH < 300 pg/ml (1.70 ± 0.76 ng/ml) (Table X).

Significant positive correlations were found in the whole patients' group between TNF-α and both IL-1β (r = 0.3301, p = 0.035) and ACP (r = 0.3487, p = 0.025), (Table XIIa), as well as in the group of patients with clinical evidence of sHPT (r = 0.4353, p = 0.018 and r = 0.4159, p = 0.025 respectively) (Table XIIc) denoting the high bone resorbing effect of TNF-α and its synergism with IL-1β.

Although TNF-α and IL-1β are biochemically and immunologically distinct, yet they share remarkable similarities in their biological properties.[552,554,555]

In fact plasma TNF-α, when present in sufficient concentrations could induce IL-1 secretion by monocytes in long term HD patients.[550]

In the present study the reported increase in TNF-α and IL-1β agrees with the reports showing that uraemia per se and/or dialysis related factors could contribute to monocyte activation and consequent TNF-α and/or IL-1β production.[552,553,556-558] However the biological activity of cytokines has not always been verified by specific immunoassays.[553,559]

B- Interleukin-1beta (IL-1β)

Interlukin-1beta (IL-1β), also known as osteoclast activating factor (OAF), stimulates both osteoclast formation from precursors and osteoclast maturation.

High plasma levels of IL-1 have been reported in dialysis patients.[69,484,552,560,561]

In the present study, the serum IL-1β level in the whole patients' group (2.59 ± 1.657 ng/ml) was higher, though statistically insignificant, than its level in the corresponding control group (1.91 ± 1.276 ng/ml). (Table IX). The lack of significant increase in IL-1 could be attributed to the transient release by the process of dialysis since a large portion of IL-1 remains intracellular, and does not pass into the plasma.[562,563]

In the patients' group, significant positive correlations were found between IL-1β and both TNF-α ($r = 0.3301$, $p = 0.035$) and ACP ($r = 0.3195$, $p = 0.042$) (Table XIIa). These findings suggested that IL-1β has a high bone resorbing power (evidenced by the high ACP activity) and demonstrated its synergistic effect with TNF-α.

In this respect, activation of the bone remodeling cycle, which is influenced by the high IL-1β levels, may contribute to the abnormalities seen in ROD patients.[69,484,552]

High IL-1 receptor antagonist levels have been reported to circulate in plasma of HD patients, that may limit IL-1β activity.[564,565]

In the group with iPTH ≥ 300 pg/ml, IL-1β level was higher, although not statistically significant, compared to its level in those with iPTH < 300 pg/ml (Table X) and was positively correlated with TNF-α ($r = 0.4353$, $p = 0.018$) (Table XIIc) in those with clinical evidence of sHPT, emphasizing the bone resorbing power of IL-1β.

From the previous discussion, it could be noticed that the lack of significant changes in the serum levels of most of the studied bone regulatory cytokines can not exclude their local production by bone cells, in abundant amounts, and their influence on bone remodeling.

The variability of cytokine levels in sera of haemodialyzed patients could be due to several factors:

- Poor nutrition and anaemia, which are common features in haemodialyzed patients, are associated with decreased cytokine production.[553]
- Recombinant erythropoeitin therapy when given to anaemic ESRD patients can also augment the cytokine producing capacity of monocytes.[566]
- Acute and chronic infections, autoimmune disease, immunosuppressive therapy and blood transfusions, could also influence the cytokine concentration.[553]

- A large portion of IL-1 remains inside bone cells and is transiently released into plasma by the process of dialysis.[562, 563]
- The very short half life of cytokines, instability and degradation in the sample tubes, presence of cytokine inhibitors and the wide range for reference values could add to the problem.[567-569]

Thus a wide spectrum, ranging from low to normal or even high, cytokine levels, probably exists among dialyzed patients.[553,569]

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Bone growth and remodeling are complex dynamic processes that require a balance between the mechanisms of bone formation and resorption. This balance is achieved by both systemic and local means.

The local bone regulatory cytokines, interact with each other synergistically and antagonistically as well as with systemic bone regulators mainly parathyroid hormone (PTH).

In chronic renal failure, the balance between osteoblastic and osteoclastic activities is disturbed resulting in renal osteodystrophy (ROD), which is a complex metabolic bone disorder that occurs as a complication of chronic renal failure.

Nearly all individuals with end-stage renal disease (ESRD), especially those on maintenance haemodialysis (HD) suffer from ROD, resulting in significant skeletal and extraskeletal pathology.

Renal osteodystrophy (ROD) is classified into two main groups: high and low turnover bone diseases. The high-turnover bone disease (HTBD) which includes moderate and severe secondary hyperparathyroidism, is characterized by osteitis fibrosa cystica. The low-turnover bone disease (LTBD) includes osteomalacia and adynamic bone lesion. Overlap between HTBD and LTBD may occur (mixed uraemic osteodystrophy = MUOD) according to the predominant lesion.

Resistance to some bone forming cytokines as well as enhanced bone resorbing effect of other cytokines could be considered as a potential contributor to the pathogenesis of ROD.

The aim of the present work was to study some local bone regulatory cytokines in end stage renal disease (ESRD) patients with secondary hyperparathyroidism (sHPT) under maintenance haemodialysis (HD).

Sixty one subjects were included in the study; forty one patients under maintenance HD who had laboratory and/or radiological evidences of sHPT, and twenty apparently normal healthy volunteers of comparable age, sex and socioeconomic status.

To all the studied subjects, thorough history taking and full clinical examination were done. Plain X-ray of hands, skull and spine were done to detect any bony change.

Laboratory investigations done to both control and patient groups included determination of serum levels of glucose, creatinine, total and ionized calcium, inorganic phosphate, albumin, intact parathyroid hormone and serum activities of alanine (ALT) and aspartate (AST) aminotransferases, alkaline (ALP) and acid phoshatases (ACP).

In addition, estimation of some local bone regulatory factors was done. The serum levels of two bone forming cytokines [insulin-like growth factor-I (IGF-I) and transforming growth factor-β_1 (TGF-β_1)] and two bone

resorbing cytokines [interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α)] were determined in both patient and control groups.

According to the intact parathyroid hormone (iPTH) level, the patients group was further categorized into those with parathyroid hormone <300 pg/ml (6 cases) and those with parathyroid hormone of ≥ 300 pg/ml, (32 cases).

The iPTH level was significantly higher in the patients' group than the corresponding controls. Most of the patients showed radiological evidence of R.O.D.

The serum alkaline phosphatase (ALP) activity was significantly higher in the patients group than in the corresponding control group. It was also significantly higher in those with iPTH ≥ 300 pg/ml than those with iPTH < 300 pg/ml. A significant positive correlation existed between iPTH & ALP in the patients' group with PTH ≥ 300 pg/ml.

The serum levels of both total and ionized calcium were significantly lower in the patients group than the corresponding controls. A significant positive correlation between total calcium and iPTH appeared in the patient's group with iPTH ≥ 300.

On the other hand, serum inorganic phosphate level was significantly higher in the patients group compared to the controls.

As regards the studied local bone regulatory factors, although there were no significant differences in the serum levels of bone forming growth factors TGF-β_1 & IGF-I, between patients and controls, yet the relatively low IGF-I and the relatively high TGF-β_1 levels in the patients group point more to defective mineralization of bone rather than defective matrix formation.

On the other hand, in the bone resorbing cytokines, the serum level of IL-1β tended to be higher in the patients group than the corresponding controls. It tended to be higher in the group with iPTH $\geq$ 300 pg/ml than in those with iPTH level < 300 pg/ml. Serum TNF-α level was significantly higher in the patients' group than in the control group and also it was significantly higher in those with iPTH $\geq$ 300 pg/ml. These findings may point to the dominance of bone resorption in the studied patients.

Significant positive correlations between TNF-α, IL-1β and acid phosphatase occured in the whole patients group and in those with clinical evidence of secondary hyperparathyroidism (sHPT), emphasizing the potential role of TNF-α in bone resorption in ESRD patients with sHPT under maintenance HD.

From the previous results it could be concluded that:

1- The prevailing lesion in the studied patients is a high-turnover type characterized by radiologically evidenced manifestation of bone resorption. This can be attributed to the high PTH level and the

increase in bone resorbing power as confirmed by the increase in bone resorbing cytokines particularly TNF-α.

2- The lack of change, particularly the lack of increase in PTH-related bone forming growth factors, indicates a blunted responsiveness of bone cells especially osteoblasts to the stimulus for new bone formation.

3- The serum levels of the studied bone regulatory cytokines do not precisely reflect the bony tissue state or the biological activity of these cytokines, as they may be influenced by many factors, mostly related to the uraemic state.

RECOMMENDATIONS

RECOMMENDATIONS

The following is recommended for haemodialyzed patients :

1- Study of other cytokines, growth factors and related proteins that are involved in the process of bone remodeling at the tissue level.

2- Determination of the state of bone mineralization by performing bone densitometry.

3- Study the local effects of high aluminium level on bone regulatory cytokines.

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PROTOCOL

بسم الله الرحمن الرحيم

STUDY OF SOME LOCAL BONE REGULATORS IN PATIENTS WITH SECONDARY HYPERPARATHYROIDISM UNDER MAINTENANCE HAEMODIALYSIS

دراسة بعض منظمات العظم الموضعية في المرضى الذين يعانون من ارتفاع هرمون الغدة الجار درقية الثانوي ويخضعون للغسيل الدموى المتكرر

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In

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By

Moyassar Ahmad Mohamad Zaki
Master Degree in Chemical Pathology
Medical Research Institute
Alexandria University

Chemical Pathology Department
Medical Research Institute
Alexandria University
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خطة بحث مقدمة إلى
معهد البحوث الطبية
جامعة الإسكندرية
إيفاءً جزئيا للحصول على

درجـــة الدكتوراه

فــى

كيمياء الباثولوجيا

من

الطبيب/ ميسر أحمد محمد زكي
ماجستير في كيمياء الباثولوجيا
معهد البحوث الطبية
جامعـــة الإسكندرية

قسم الباثولوجيا الكيميائية
معهد البحوث الطبية
جامعة الإسكندرية
٢٠٠٠

SUPERVISORS

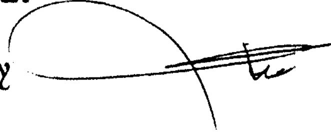
السـادة المشـرفون

Prof. Dr. Safaa A. El-Hefni
Prof. of Clinical Pathology
Medical Research Institute
Alexandria University

أ.د. / صفاء عبد الرحمن الحفني
أستاذ الباثولوجيا الإكلينيكية
معهد البحوث الطبية
جامعة الإسكندرية

Prof. Dr. Thanaa F. Moghazi
Professor of Clinical Pathology
Medical Research Institute
Alexandria University

أ.د. / ثناء فتحي مغازي
أستاذ الباثولوجيا الإكلينيكية
معهد البحوث الطبية
جامعة الإسكندرية

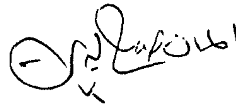


Prof. Dr. Mona H. Kandil
Professor of Clinical Pathology
Medical Research Institute
Alexandria University

أ.د. / منى حسين قنديل
أستاذ الباثولوجيا الإكلينيكية
معهد البحوث الطبية
جامعة الإسكندرية

Dr. Iman Salah El-Din Khalil
Lecturer of Internal Medicine
Medical Research Institute
Alexandria University

د. / إيمان صلاح الدين خليل
مدرس الأمراض الباطنة
معهد البحوث الطبية
جامعة الإسكندرية



Dr. Tarek Y. Aref
Lecturer of Diagnostic Radiology
Medical Research Institute
Alexandria University

د. / طارق يوسف عارف
مدرس الأشعة التشخيصية
معهد البحوث الطبية
جامعة الإسكندرية

INTRODUCTION

Growth and remodeling of bone in non growing individuals require both bone formation and resorption.[1,2] Bone cell function is regulated at both the systemic and local levels.[1] Bone is a storehouse for growth regulatory factors known as cytokines.[3,4] They are termed bone remodeling units as they control bone formation and resorption through their effects on osteoblastic and osteoclastic cells.[4] These cytokines are produced and secreted by bone cells and their action is either autocrine or paracrine.[3,5]

Cytokines that induce bone resorption, i.e. stimulate the osteoclastic activity, such as interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF-α), stimulate the release of soluble factors (colony stimulating factors, IL-6 and IL-11) to increase proliferation and differentiation of osteoclast precursor cells and activate mature osteoclasts.[6,7]

Insulin-like growth factors system components (IGF-1&2) and peptides of the transforming growth factor superfamily (TGF-β 1,2,3 and bone morphogenic proteins) have been characterized as important regulators of osteoblastic activity.[8,9] The IGF system, being a key regulator of bone formation, decreases collagen degradation, enhances bone matrix deposition and increases osteoblastic cell recruitment.[10] TGF-

β decreases the resorptive activity of osteoclasts and promotes both proliferation and differentiation of osteoblasts and cartilage formation.[11]

These local bone regulatory cytokines interact with each others, synergistically and antagonistically, as well as with systemic bone regulatory hormones.[1]

Secondary hyperparathyroidism is initiated in early renal failure[12] and high turnover type of renal osteodystrophy is a common sequel in end stage renal disease (ESRD).[13], Parathyroid hormone stimulates the proliferation and differentiation of osteoclast precursor cells and activates mature osteoclasts. On the other hand, it induces bone formation by increasing the number of osteoblasts.[14]

In chronic renal failure, the balance between osteoblastic and osteoclastic activities is disturbed with a net effect of predominant bone resorption.[7,14]

Resistance to local regulatory factors of bone formation such as IGF-1 has been demonstrated in chronic renal failure patients.[14-17] Enhanced effect of some local bone resorption factors could possibly be an additional factor.[14,18,19]

Therefore, it is worthy to study some of these locally produced cytokines in ESRD patients with secondary hyperparathyroidism.

AIM OF THE WORK

This work aims at studying some local bone regulatory cytokines in end stage renal disease patients with secondary hyperparathyroidism under maintenance haemodialysis.

MATERIAL

Sixty subjects will be included in the study. They will be divided into two groups:

- Forty patients with end stage renal disease under maintenance haemodialysis with laboratory and/or radiological evidence of secondary hyperparathyroidism.
- Twenty normal healthy volunteers of comparable age, sex and socioeconomic state, as a control group.

N.B.: The patients' group will be selected free from any other condition that could affect their bone metabolism.

METHODS

To all subjects, the following will be done:

A- Thorough history taking and full clinical examination.

B- Plain X-ray for hands, feet, skull, vertebrae and/or long bones to detect evidence of any bone lesion (s).

C- Laboratory investigations which include:

1. Estimation of fasting serum levels of glucose, creatinine, calcium (total and ionized) and inorganic phosphorus.[20]
2. Determination of serum activities of acid and alkaline phosphatases, alanine and aspartate aminotransferases.[20]
3. Estimation of serum intact parathyroid hormone level.[21]
4. Estimation of serum levels of the following local bone regulatory factors:
    - interleukin-1.[22]
    - Tumour necrosis factor-alpha.[23]
    - Insulin-like growth factor-1.[24]
    - Transforming growth factor-beta.[25]

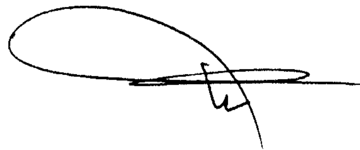
N.B.: For patients' group, all blood samples will be taken immediately before the haemodialysis session.

RESULTS

The results obtained from the study will be tabulated and statistically analyzed. Both groups of the study will be statistically compared and all the studied items will be correlated in each group.

DISCUSSION

The results of each item will be discussed and compared with other available works.



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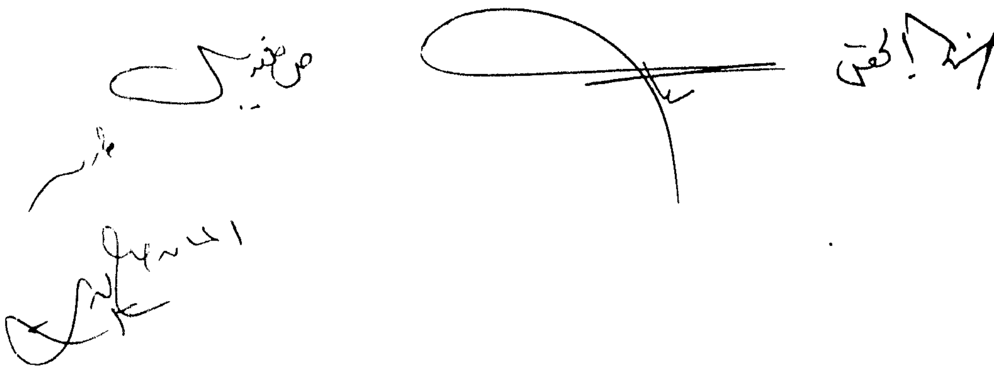

ARABIC SUMMARY

الملخص العربى

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

وتتفاعل منظمات العظم الموضعية بطريقة متلازمة ومتعارضة بالإضافة إلى تفاعلها مع منظمات العظم المركزية وبصفة خاصة هرمون الغدة الجار درقية.

يتميز مرض الفشل الكلوى المزمن باضطراب التوازن بين النشاط البنائى والنشاط الهدمى لخلايا العظام مما ينتج عنه حالة نقص التكوين العظمى الناشئ نتيجة لمرض الكلى والتى تعتبر أحد مضاعفات الفشل الكلوى المزمن ذات التأثير المعقد على أيض العظم.

ويعانى معظم مرضى الكلى فى مراحلها المتأخرة خاصة الذين يعالجون بعملية الغسيل الدموى المتكرر من حالة نقص التكوين العظمى الناشئ نتيجة لمرض الكلى وتأثير هذه الحالة على الجهاز العظمى.

ويمكن تصنيف حالات نقص التكوين العظمى الناشئ نتيجة لمرض الكلى إلى مجموعتين أساسيتين هما مجموعة الإحلال السريع للعظم ومجموعة الإحلال البطئ للعظم؛ وتشتمل المجموعة الأولى على مرضى زيادة نشاط الغدة الجار درقية الثانوى بدرجتيه المتوسطة والشديدة وهذه تتميز بوجود التهاب عظمى متليف ومتحوصل؛ بينما تشتمل المجموعة الثانية على مرضى لين العظام والإصابات اللاديناميكية للعظم مع ملاحظة أن هناك درجة من التداخل بين نوعى الإحلال السريع والبطئ للعظم وهو النوع المختلط اعتمادا على نوع الخلل الغالب.

يمكن أن ينتج مرض نقص التكوين العظمى الناشئ نتيجة لمرض الكلى عن المقاومة لبعض المحفزات الخلوية الخاصة بتكوين العظام أو عن زيادة النشاط الهدمى لبعض المحفزات الخلوية الأخرى.

ولقد كان الهدف من البحث الحالى هو دراسة بعض منظمات العظم الموضعية فى المراحل النهائية لمرض الكلى فى المرضى الذين يعانون من ارتفاع هرمون الغدة الجار درقية الثانوى والخاضعين للغسيل الدموى المتكرر.

وقد شملت هذه الدراسة واحداً وستين شخصاً منهم عشرون شخصاً سليماً كمجموعة ضابطة وواحد وأربعون مريضاً بمرض الكلى فى مراحله المتأخرة ممن يخضعون للغسيل الدموى المتكرر وعندهم دلالات معمليه أو إشعاعية على وجود مرض زيادة نشاط الغدة الجار درقية الثانوى، وكانت كلا المجموعتين متقاربتين فى السن والجنس والمستوى الاجتماعى والاقتصادى وقد تم عمل فحصاً إكلينيكيا شاملاً التاريخ المرضى لكل شخص وبالنسبة لمجموعة المرضى فقد تم عمل أشعة سينية على عظام اليد والجمجمة والعمود الفقرى لاكتشاف أى تغير فى العظام، وقد اشتملت الفحوص المعملية على قياس مستوى كل من الجلوكوز والألبومين، والكرياتينين، والكالسيوم الكلى والمتأين والفوسفور غير العضوى، وهرمون الغدة الجار درقية، وكذلك نشاط إنزيمات المحولات الأمينية وكل من الفوسفاتيز القلوى والحمضى فى مصل الدم.

كما تم تقييم بعض منظمات العظم الموضعية فى مصل الدم، المتمثلة فى منظمات العظم البناءة مثل معامل النمو المشابه للإنسولين-١ ومعامل تحول النمو - بيتا -١، ومنظمات العظم الهدامة مثل الإنترلوكين - ١ - بيتا ومعامل تحلل الورم - ألفا.

وتبعا لمستوى هرمون الغدة الجار درقية، تم تقسيم مجموعة المرض إلى مجموعتين، الأولى ذات مستوى هرمونى أقل من ٣٠٠ بيكوجرام /مل وعددهم ستة مرضى والثانية ذات مستوى هرمون أكثر من ٣٠٠ بيكوجرام/مل وعددهم اثنان وثلاثون مريضاً.

أظهر مستوى هرمون الغدة الجار درقية ارتفاعاً ملحوظاً في مجموعة المرضى عنه في المجموعة الضابطة ، كما أنه كانت هناك علاقة ذات مغزى بين مستوى الهرمون ونتائج الفحص الإشعاعي في مجموعة المرضى، الذين ظهرت عليهم دلالات إشعاعية على وجود مرض نقص التكوين العظمي الناشئ نتيجة لمرض الكلى.

كان نشاط أنزيم الفوسفاتير القلوى في مصل الدم أعلى في مجموعة المرضى عنه في المجموعة الضابطة ، كما أظهر زيادة ملحوظة في مجموعة المرضى ذات مستوى هرمون الغدة الجار درقية ٣٠٠ بيكوجرام /مل فأكثر عنه في مجموعة المرضى ذات المستوى الأقل من ٣٠٠ بيكوجرام/مل مع وجود ارتباط بين نشاط الإنزيم ومستوى الهرمون في المجموعة ذات مستوى الهرمون أكثر من ٣٠٠ بيكوجرام/مل.

أظهر مستوى كل من الكالسيوم الكلى والمتأين في مصل دم المرضى انخفاضاً ملحوظاً عنه في المجموعة الضابطة مع وجود ارتباط بين مستوى الكالسيوم الكلى ومستوى الهرمون في كل مجموعات المرضى.

أما مستوى الفوسفور غير العضوى فقد كان مرتفعاً ارتفاعاً ملحوظاً في مصل المرضى عنه في المجموعة الضابطة، وكان هناك ارتباط بين مستواه ومستوى هرمون الغدة الجار درقية في المجموعة ذات مستوى الهرمون أقل من ٣٠٠ بيكوجرام/مل.

أما فيما يختص بمنظمات العظم الموضعية ، فبالرغم من غياب الفروق ذات الدلالة الإحصائية في مستويات عوامل تكوين العظام (معامل تحول النمو - بيتا -١)، معامل النمو المشابه للإنسولين - ١) بين مجموعة المرضى والمجموعة الضابطة، إلا أن الزيادة النسبية في مستوى معامل تحول النمو - بيتا- ١ والقلة النسبية في مستوى معامل النمو المشابه للأنسولين- ١ في مجموعة المرضى، قد ترجح وجود نقص في الإمداد المعدنى للعظم وليس في تكوين القالب العظمى.

ومن جهة أخرى بالنسبة للمحفزات الخلوية المسئولة عن تحلل العظم، فقد لوحظ أن مستوى مادة الإنترلوكين - ١ - بيتا فى مصل المرضى قد أظهر ميلا للزيادة عنه فى المجموعة الضابطة بجانب وجود علاقة ذات دلالات بين مستواه ومستوى نشاط إنزيم الفوسفاتيز الحمضى. وكذلك فقد كان أعلى فى مجموعة المرضى ذوى التركيز العالى لهرمون الغدة الجار درقية (٣٠٠ بيكوجرام /مل فأكثر) عنه فى المجموعة ذات التركيز الأقل من ٣٠٠ بيكوجرام/مل.

بالنسبة لمعامل تحلل الورم - ألفا، فإن مستواه فى مصل الدم قد أظهر زيادة ذات دلالة إحصائية فى مجموعة المرضى عن المجموعة الضابطة، بالإضافة إلى هذا فإن مستواه فى مجموعة المرضى ذات التركيز العالى فى هرمون الغدة الجار درقية (٣٠٠ بيكوجرام/مل فأكثر) قد أظهر ارتفاعاً ملحوظاً عنه فى المجموعة ذات تركيز الهرمون الأقل من ٣٠٠ بيكوجرام/مل، وتدل هذه النتائج على تغلب تهدم العظام فى مجموعة المرضى.

وقد ثبت وجود علاقة ذات دلالة بين مستوى معامل تحلل الورم -ألفا والإنترلوكين-١ بيتا فى مجموعة المرضى، بالإضافة إلى وجود ارتباط ذو دلالة بين كل منهما وبين مستوى نشاط إنزيم الفوسفاتيز الحمضى، مما يؤكد على الدور المحتمل لمعامل تحلل الورم - ألفا فى عملية تهدم العظام فى مرضى الكلى فى مراحله النهائية الذين يعانون من زيادة مستوى هرمون الغدة الجار درقية الثانوى والخاضعين للغسيل الدموى المتكرر ويستخلص من النتائج السابقة ما يلى:

١- إن الخلل الغالب فى عظام مجموعة المرضى هو من النوع ذو التغير السريع الدرجة، والمميز بوجود دلائل إشعاعية على حدوث تحلل العظام ويمكن إرجاع هذا إلى المستوى العالى لهرمون الغدة الجار درقية والزيادة فى معدل تحلل العظام كما هو مثبت بزيادة المحفزات الخلوية المسئولة عن تهدم العظام وخاصة معامل تحلل الورم - أ.

٢- بالرغم من الزيادة النسبية فى مستوى منظمات بناء العظام المرتبطة بـهرمون الغـدة الجـار درقية، إلا أن ذلك لم يؤدى إلى زيادة فى تكوين العظام، مما يدل على وجود قصـور فـى استجابة الخلايا البناءة المكونة للعظام للأثر التحفيزى لهذه العوامل.

٣- إن مستويات منظمات بناء العظام فى مصل الـدم لا تعكـس الوضـع الحـادث فـى نسـيج العظام، وكذلك لا تعبر عن النشاط الحيوى لهذه المنظمات لأنها قد تكون متـأثرة بعوامـل أخرى مرتبطة بحالة مرض الفشل الكلوى المزمـن المصحـوب بارتفـاع نسـبة البولينـا فـى الدم.

المشـــــرفون

أ.د / صفاء عبد الرحمن الحفنى

أستاذ الباثولوجيا الإكلينيكية

معهد البحوث الطبية

جامعة الإسكندرية

أ.د / ثناء فتحى مغازى

أستاذ ورئيس قسم الباثولوجيا الإكلينيكية

معهد البحوث الطبية

جامعة الإسكندرية

أ.د / منى حسين قنديل

أستاذ الباثولوجيا الإكلينيكية

معهد البحوث الطبية

جامعة الإسكندرية

د / إيمان صلاح الدين خليل

مدرس الأمراض الباطنة

معهد البحوث الطبية

جامعة الإسكندرية

د / طارق يوسف عارف

مدرس الأشعة التشخيصية

معهد البحوث الطبية

جامعة الإسكندرية

سكندرية

البحوث الطبية

لدراسات العليا

موافقة العميد على تشكيل لجنة الحكم بالتفويض فى ١٩ / ٣/١/ ٢٠٠

ماء اللجنة :

أ.د. صفاء عبد الرحمن الحفنى	أستاذ الباثولوجيا الإكلينيكية معهد البحوث الطبية – جامعة الإسكندرية	
٢- أ.د. ثناء فتحى مغازى	أستاذ الباثولوجيا الإكلينيكية معهد البحوث الطبية – جامعة الإسكندرية	(مشرفتان وممتحنتان داخليتان)
٣- أ.د. وفاء سعد رجب	أستاذ الباثولوجيا الإكلينيكية معهد البحوث الطبية – جامعة الإسكندرية	(ممتحن داخلى)
٤- أ.د. محمد حسن القنيشى	أستاذ الباثولوجيا الإكلينيكية كلية الطب – جامعة المنصورة	(ممتحن خارجى)

موافقة مجلس المعهد على منح الدرجة   /   /

يعتمد

وكيل المعهد

للدراسات العليا والبحوث

(أ.د. عزت محمد حسن)

دراسة بعض منظمات العظم الموضعية فى المرضى الذين يعانون من ارتفاع هرمون الغدة الجار درقية الثانوى ويخضعون للغسيل الدموى المتكرر

رسالة

مقدمة إلى معهد البحوث الطبية - جامعة الإسكندرية
إيفاءا جزئياً لشروط الحصول على درجة

الدكتوراه

في

كيمياء الباثولوجيا

مقدمة من

ميسر أحمد محمد زكى

بكالوريوس الطب والجراحة – جامعة الإسكندرية – ١٩٩٥
ماجستير الباثولوجيا الكيميائية – معهد البحوث الطبية – جامعة الإسكندرية ٢٠٠٠

معهد البحوث الطبية
جامعة الإسكندرية
٢٠٠٣