Study of the Serum Level of Receptor Activator of Nuclear Factor-kappa B Ligand (RANKL) and Osteoprotegerin (OPG) in Patients with Rheumatoid Arthritis

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Master of Chemical Pathology, Medical Research Institute, Alexandria University, 2003

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List of Abbreviations

1.25 $(OH)_2$ vit D	:	1.25 dihdroxy cholecalicferol
ALT	:	Alanine amino transferase
AMP	:	Adenosine mono phosphate
ANA	:	Anti nuclear antibodies
Anti-ccp	:	Anti-cyclic citrullinated peptide
Anti-RA 33	:	Anti-rheumatoid arthritis 33
BMPs	:	Bone morphogenetic proteins
BMU	:	Bone modeling unit
Ca	:	Calcium
CBC	:	Complete blood count
Cbfa1	:	Core binding transcription factor-1
CD	:	Cluster of differentiation
CRP	:	C-reactive protein
DAS_{28}	:	Disease activity score 28
DMARD	:	Disease modifying antirheumatic drugs
DNA	:	Deoxy-ribonucleic acid
E_2	:	17-β estradiol
ELISA	:	Enzyme linked immuno sorbent assay
ESR	:	Erythrocyte sedimentation rate
EULAR	:	European league against rheumatism
FGFs	:	Fibroblastic growth factors
FSG	:	Fasting serum glucose
GH	:	General health
GM-CSF	:	Granulocyte – macrophage colony stimulating factor

Hb	:	Haemoglobin
HRP	:	Horseradish peroxidase
IKB	:	Inhibitor of nuclear factor- κB
IL	:	Interleukin
INFs	:	Interferons
ISE	:	Ion selective electrode
JNK	:	Jun-N-terminal Kinase
KD	:	Kelodalton
LD	:	Lactate dehydrogenase
MBP	:	Mean blood pressure
MCP	:	Metacarpophalangeal
M-CSF	:	Macrophage- colong stimulating factor
MTP	:	Metatarsophalangeal
NAD	:	Nicotinamide adenine dinucleotide
NADH	:	Reduced form of Nicotinamide adenine dinucleotide
NF_kB	:	Nuclear factor kappa- B
NSAID	:	Non steroidal antiinflammatory drugs
OCIF	:	Osteoclastogenesis inhibitory factor
ODF	:	Osteoclast differentiating factor
OPG	:	Osteoprotegerin
OPGL	:	Osteoprotegerin ligand
OSF-2	:	Osteoblast stimulating factor-2
Pi	:	Inorganic phosphorus
PIP	:	Proximal inter phalangeal
PTH	:	Parathyroid hormone
RA	:	Rheumatoid arthritis
RANKL	:	Receptor activator of nuclear, factor kappa-B ligand

RANK-m RNA	:	Receptor activator of nuclear factor kappa-B messenger ribonucleic acid
RBC	:	Red blood cells count
RF	:	Rheumatoid factor
RHD	:	Rel homology domain
ROC	:	Receiver operating characteristic
RW	:	Rose waaler test
S	:	Standard
Swelling	:	Number of swollen joints
T	:	Test (sample)
Tend	:	Number of tender joints
TGF-β	:	Transforming growth factor – beta
TMB	:	Tetramethyl benzidine
TNF-α	:	Tumor necrosis factor - α
TR-1	:	Tumor necrosis factor receptor – like molecule-1
TRA P	:	Tartrate-resistant acid phosphatase
TRAFs	:	Tumor necrosis factor receptor – associated factors
TRAIL	:	TNF-related apoptosis inducing ligand
TRANCE	:	Tumor necrosis factor – related activated – induced cytokine
WBCs	:	White blood cells count

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CHAPTER I

Bone biology and remodeling

Bone composition, structure, and function

Bone consists of three cell types, namely, osteoblasts, osteoclasts and osteocytes. However, the close proximity of the bone marrow exposes bone to the influence of other cell types that play a vital role both in the production of osteogenic cells and in the regulation of bone modeling and remodeling.[1]

Bone consists also of an extracellular matrix which is the organic phase. It is composed of type I collagen, proteoglycans and non collagenous proteins including osteoacalcin, bone sialoprotein, osteonectin, thrombospondin and osteopontin. Bone matrix also contains growth factors and cytokines that have an important regulatory role in bone remodeling. The inorganic phase of bone matrix is composed mainly of calcium hydroxy apatite.[1]

1. Osteoblasts

Osteoblasts are responsible for the formation and mineralization of bone. They are derived from pluripotent mesenchymal stem cell, which can also differentiate to condrocytes, adipocytes, myoblasts and fibroblasts.[2,3] (Fig. 1)

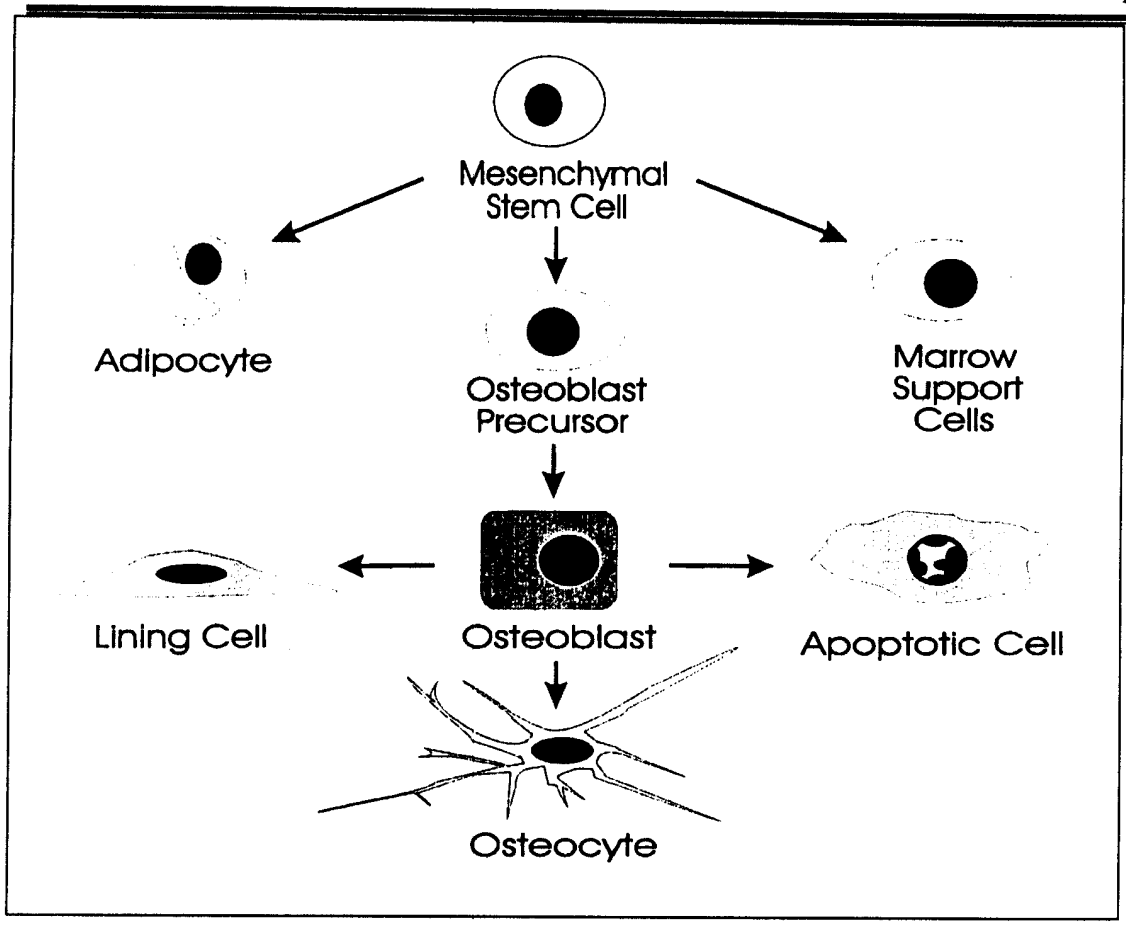


Figure (1): Interaction of hematopoietic and stromal cells.

The cells of the osteoblast lineage can interact with hematopoietic cells to initiate osteoclast formation. The same cells can also differentiate to become matrix synthesizing osteoblasts. The latter pathway may be stimulated by substances released from the osteoclast or from the bone matrix during resorption.[2]

The core binding transcription factor-1 (cbfa1), which is also known as osteoblast stimulating factor 2 (osf2), has been shown to be essential for osteoblast differentiation.[4,5] In addition, a number of other factors are required for normal osteoblast differentiation including fibroblastic growth factors (FGFs), transforming growth factor-β (TGF-β), bone morphogenetic proteins (BMPs), glucocorticoids and 1,25-dihydroxy vitamin D.[6]

Developing and mature osteoblasts express a number of products including type I collagen, alkaline phosphatase, osteopontin and osteocalcin that may be used to identify the osteoblastic phenotype in vivo and in vitro.[1] Osteoblasts may subsequently undergo apoptosis or become bone lining cells or osteocytes.[7]

2. Osteocytes

Osteocytes are small flattened cells within the bone matrix and are connected to one another and to osteoblastic cells on the bone surface by an extensive canalicular network that contains the bone extracellular fluid.[8] Osteocytes are terminally differentiated and may undergo apoptosis or be phagocytosed during the process of osteoclastic resorption.[8]

The function of osteocytes is not well understood, but they may play a role in the activation of bone remodeling.[7] They are believed to play a central role in the response to mechanical stimuli, sensing mechanical strains and initiating an appropriate modeling or remodeling response via a number of chemical messengers including glucose-6-phosphate dehydrogenase, nitric acid, and insulin-like growth factors.[1]

3. Osteoclasts

Osteoclasts are large multinucleated bone-resorbing cells, derived from hematopoietic precursors of the monocyte/macrophage lineage. They are formed by the fusion of mononuclear cells and are characterized by the presence of a ruffled border.[9,10] (Fig. 2)

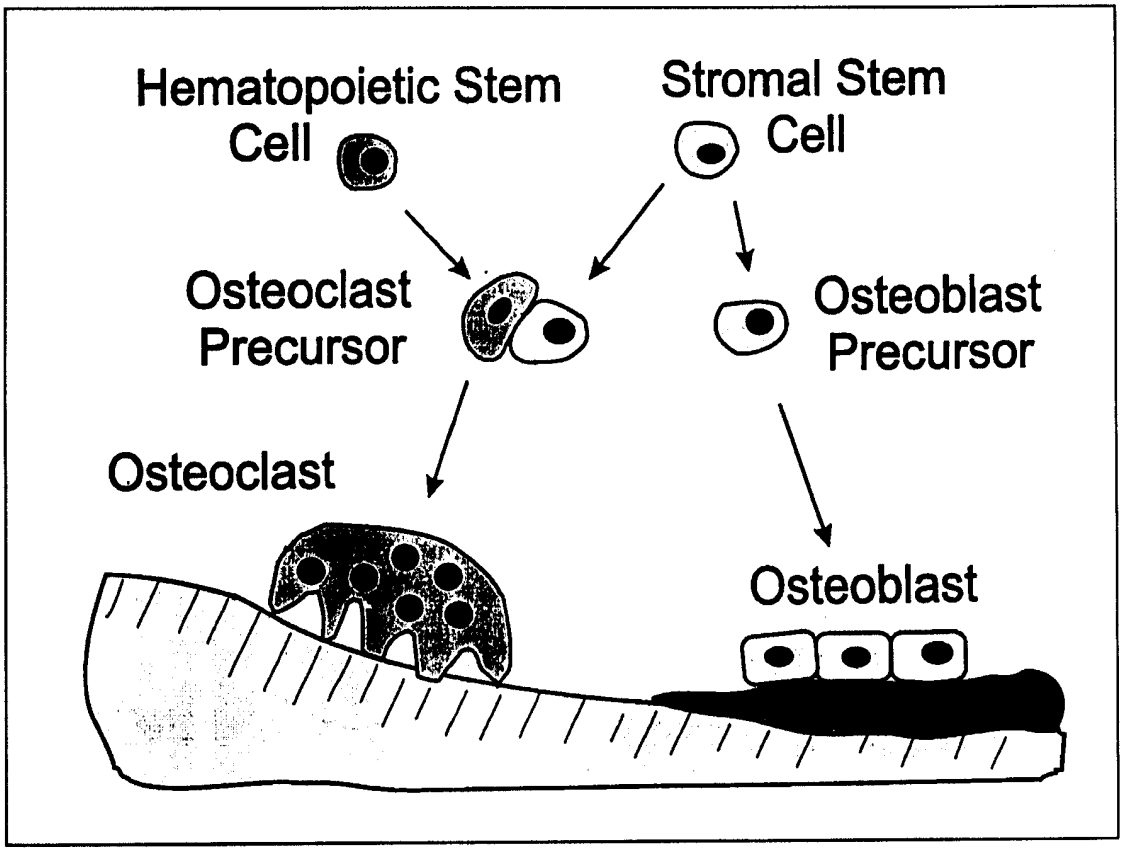


Figure (2): Origin and fate of osteoblasts.
The mesenchymal stem cell that gives rise to osteoblasts can also produce cells of other lineages. It is also possible that osteoblast precursors can differentiate into or derive from adipocytes and marrow support cells. Osteoblasts can be buried as osteocytes, remain in the bone surface as lining cells, or undergo apoptosis. Although this diagram suggests that the fate of the osteoblasts are terminal, reactivation of lining cells and possibly osteocytes back to active osetoblasts has been postulated.[2]

They are rich in lysosomal enzymes, including tartrate-resistant acid phosphatase (TRAP). During the process of bone resorption, hydrogen ions generated by carbonic anhydrase II are delivered across the plasma membrane by a proton pump to dissolve bone mineral. Subsequently, the lysosomal enzymes, collagenase and cathepsins are released and degrade

bone matrix. Attachment of osteoclasts to the bone surface is essential for resorption and is mediated by integrins, and potential ligands.[9,10]

The cytokines interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and macrophage-colony stimulating factor (M-CSF) reduce osteoclast apoptosis,[11] thus prolonging the viability of these cells. In contrast, estrogen increases apoptosis of osteoclasts, an effect which is associated with increased production of transforming growth factor-β (TGF-β) and reduced expression of nuclear factor kappa-B (NFκB) activated genes.[12]

Precursor of osteoblasts and osteoclasts:

Both osteoblasts and osteoclasts are derived from precursors originating in the bone marrow. The precursors of osteoblasts (osteoblast progenitor) are multi-potent mesenchymal stem cells, which also give rise to bone marrow stromal cells, chondrocytes, muscle cells, and adipocytes. [13-15] Osteoblast precursors most likely reach bone by migration of progenitors from neighboring connective tissue, whereas the precursors of osteoclasts are hematopoietic cells of the monocyte/macrophage lineage.[16,17]

Bone remodeling

Removal of bone (resorption) is the task of osteoclasts and formation of new bone is the task of osteoblasts. Bone resorption and bone formation, however are not separate, or independently-regulated processes. In the adult skeleton, all osteoclasts and osteoblasts belong to a unique temporary

structure, known as basic multicellular unit or bone modeling unit (BMU).[18]

The BMU is approximately 1-2 mm long and 0.2-0.4 mm wide. It comprises a team of osteoclasts in the front, a team of osteoblasts in the rear, central vascular capillaries, a nerve supply, and an associated connective tissue.[18] In healthy human adults, 3-4 million BMUs are initiated per year of which about one million that are the region of bone in need of replacement, are operating at any moment.[19]

The cellular components of the BMUs maintain a well orchestrated relationship with each other.[20] Osteoclasts adhere to bone and subsequently remove it by acidification and proteolytic digestion. As the BMU advances, osteoclasts leave the resorption site and osteoblasts move in order to cover the excavated area and begin the process of new bone formation by secreting osteoid, which is eventually mineralized into new bone.[20] (Fig. 2)

The life span of the BMU is 6-9 months; therefore continuous supply of new osteoclasts and osteoblasts, from their progenitors in the bone marrow, is essential for the origination of BMUs and their progression on the bone surface.[20]

The skeleton is a metabolic organ that undergoes continuous remodeling throughout life.[21]

Remodeling of bone begins early in fetal life. Once the skeleton is fully formed in young adults, almost all of the metabolic activity is in the form of bone remodeling cycles.[22] These cycles involve a series of highly regulated steps, which depend on the interactions of two cell lineages; the mesenchymal osteoblastic lineage and the hematopoietic osteoclastic lineage. This remodeling is necessary both to maintain the structural integrity of the skeleton and to subserve its metabolic functions as a storehouse of calcium and phosphorous. The dual function often comes into conflict under conditions of changing mechanical forces or metabolic and nutritional stresses.[1,21]

The bone remodeling cycle involves a complex series of sequential steps that are highly regulated, these steps include activation, resorption, formation and mineralization.[1] (Fig. 3)

The initial activation stage of bone remodeling involves the interaction of osteoclasts and osteoblast precursor cells. This leads to the differentiation, migration and fusion of the large multinucleated osteoclasts. These cells attach to the mineralized bone surface and initiate resorption by the secretion of hydrogen ions and lysosomal enzymes particularly cathepsin K, which can degrade all the components of bone matrix, including collagen.[21]

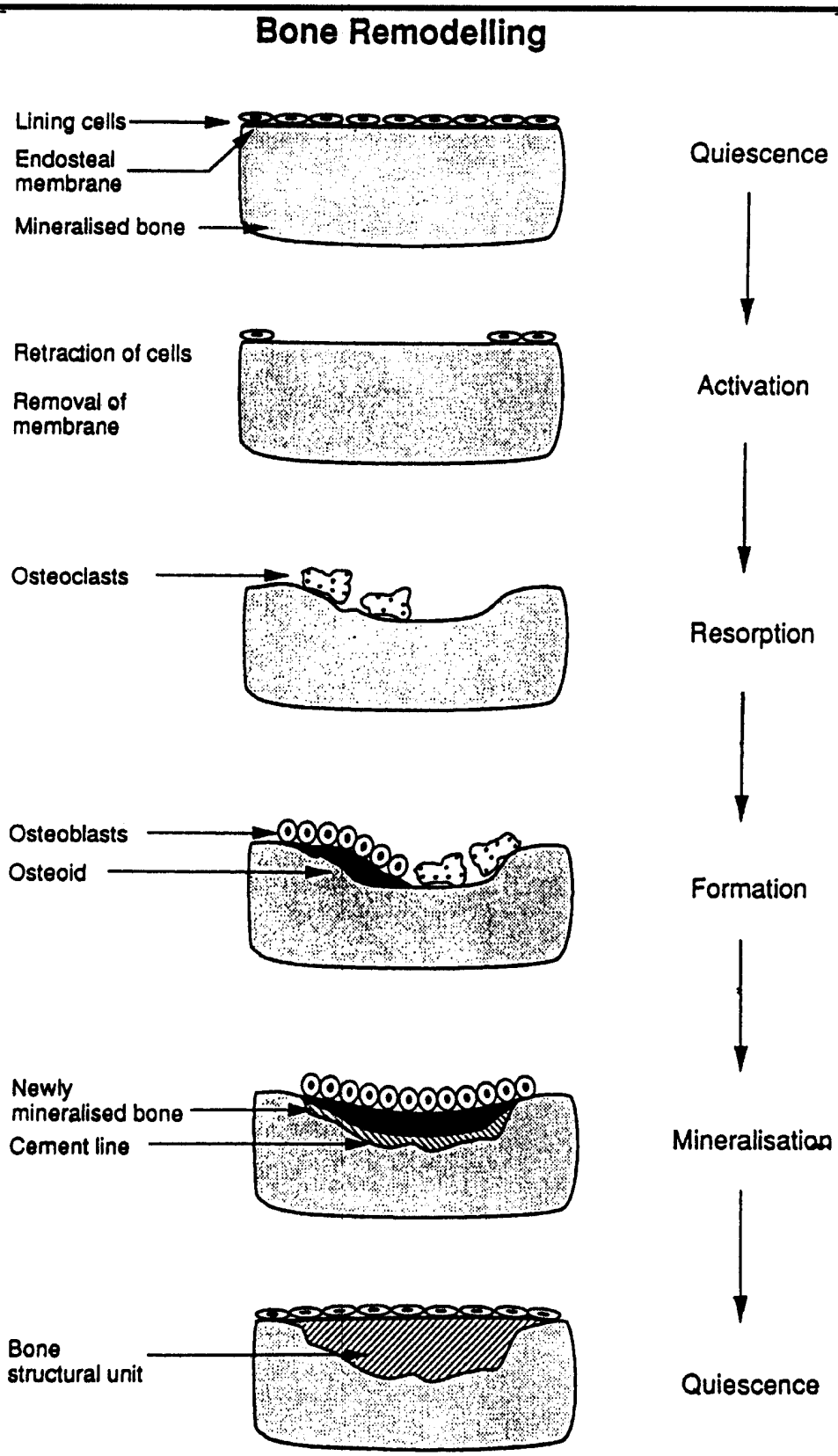


Figure (3): Schematic representation of bone remodeling.[1]

The attachment of osteoclasts to bone may require specific changes in the lining cells on the bone surface, which can contract and release proteolytic enzymes to uncover a mineralized surface.[21] Once the osteoclasts have completed their work of bone removal, there is a "reversal' phase during which mononuclear cells, that may be macrophage lineage, are seen on the bone surface. This stage may involve further degradation of collagen and deposition of proteoglycans to form the so-called cement line, as well as release of growth factors to initiate the bone formation phase. During the final "formation" phase of the remodeling cycle, the cavity created by resorption can be completely filled by successive layers of osteoblasts and deposition of a mineralizable matrix.[21]

Regulation of bone modeling

The development and differentiation of osteoblasts and osteoclasts are controlled by both systemic and local bone regulators (Table I, II).

Table (I): Systematic regulation of bone remodeling[21]

	Bone resorption	Bone formation
PTH	↑[a]	↑ (↓)[b]
1.25$(OH)_2$ vitamin D	↑	↑ (↓)[b]
Calcitonin	↓	?
Estrogen	↓	(↓[c])
Growth hormone/IGF	↑	↑
Thyroid hormone	↑	↑
Glucocorticoids	↑[d]	↓

↑[a]: increase, ↓: decrease, ?: unknown

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

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

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

Table (II): Cytokines and growth factors affecting bone[1]

Cytokines/growth factors	Abbreviation
Stimulators of bone resorption	
Interleukin 1,-6,-8,-11	IL-1, -6, -8, -11
Tumor necrosis factors	TNFs
Epidermal growth factor	EGF
Platelet derived growth factor	PDGF
Fibroblast growth factors	FGFs
Macrophage-colony stimulating factor	M-CSF
Granulocyte/macrophage-colony stimulating factor	GM-CSF
Inhibitors of bone resorption	
Interferon-γ	INF-γ
Interleukin-4	IL-4
Stimulator of bone formation	
Insulin like growth factors	IGFs
Transforming growth factor-β	TGF-β
Bone morphogenetic proteins	BMPs
Fibroblast growth factors	FGFs

Several systemic hormones as well as mechanical signals exert potent effects on bone remodeling, as well as osteoclast or osteoblast development and differentiation. The systemic hormones, e.g. parathyroid hormone (PTH), calcitonin and vitamin D, modulate bone-cell activity throughout the skeleton particularly through their effect on bone cells (Table I).[23,24]

The local bone regulators are growth factors and cytokines, which are produced in the bone marrow microenvironment (Table II), as well as adhesion molecules that mediate cell-cell and cell-matrix interaction.[1]

Although many details remain to be established concerning the operation of this network of these local bone regulators, a few of them have emerged:[25]

- **Firstly:** Several of these growth factors and cytokines control the production of each other in a cascade fashion and, in some instances, form positive and negative feed back loops.
- **Secondly:** There is extensive functional redundancy among them.
- **Thirdly:** Some of these factors are capable of influencing the differentiation of both osteoblasts and osteoclasts.
- **Fourthly:** Systemic hormones influence the process of osteoclast and osteoblast formation, via their ability to control the production and/or the action of local mediators.[20]

Exercise can have both systemic and local effects on bone as well as on the molecules released from bone matrix and bone cells, including cytokines and prostaglandins, which exert local effects on bone cells.[26]

The ability to induce the bone formation or resorption, as needed, would substantially improve the treatment of age-related loss of bone mass, certain bone diseases and skeletal injuries.[26]

CHAPTER II

Receptor-activator of nuclear factor-Kappa B and its ligands

The development of osteoclasts requires close interaction between osteoclast precursors (osteoclast progenitor) and osteoblastic stromal cells. This interaction involves not only hormones, cytokines and growth factors, but also some proteins that are tumor necrosis factor related: the so called RANK/RANKL/OPG system.[27-36] This system consists of: the Receptor Activator of Nuclear factor-Kappa B (RANK), its ligand (RANKL) and its competitor osteoprotegerin (OPG).[27,30-36]

The proteins in this system are responsible for the interaction between cells of the osteoblastic and osteoclastic lineage: the first molecule on the osteoblast precursor, which is called receptor activator of nuclear factor kappa B ligand (RANKL),[37] can activate cells of the osteoclast lineage by interaction with a second molecule on osteoclast progenitor called receptor activator of nuclear factor kappa-B (RANK).[38] A third molecule, is a soluble receptor known as osteoprotegerin (OPG), which is RANK competitor, that is produced by cells of the osteoblast lineage. It acts as a decoy receptor for RANKL, blocking its interaction with RANK and inhibiting osteoclast formation.[21] (Fig. 4)

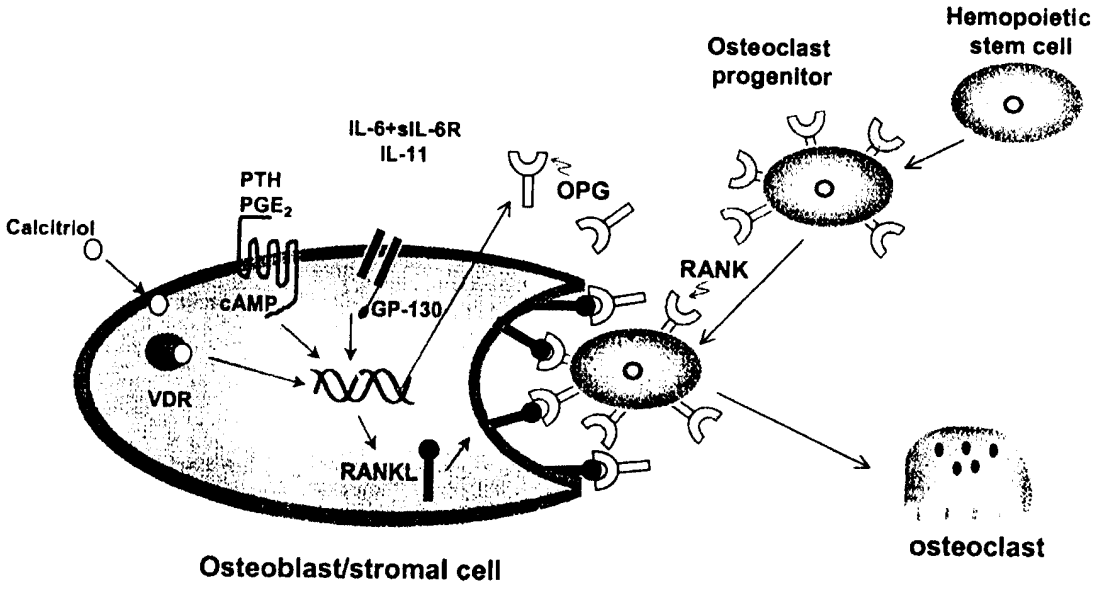


Figure (4): Diagrammatic representation of the role of RANK/RANKL/OPG system in interaction between the osteoblastic stromal cell and osteclast progenitor.[38]

Nuclear factor-Kappa B (NF-KB)

The interaction of RANKL with RANK activates a cascade of interacellular events that involve activation of NF-kB and the protein kinase JNK, and interaction with TNF receptor-associated factors (TRAFs).[39]

Nuclear factor-Kappa B was first identified in 1986 as a factor regulating the expression of kappa light chains in mouse B lymphocytes[40], subsequently it has been identified in most cell types.[41]

The NF-Kappa B is a family of dimeric proteins, which belongs to the Rel family, distinguished by a homologous domain of about 300 amino acids, the Rel homology domain (RHD). NF-Kappa-B is normally held in cytoplasm in an inactive form bound to an inhibitory protein, Inhibitor of nuclear factor-κB IKB, of which several types are recognized (α, β and γ).[42]

Nuclear factor-KB is a transcription factor (DNA-binding protein), that regulates the expression of multiple immune and inflammatory genes.[43] It is activated by many environmental stimuli, leading to the coordinated expression of the inflammatory response.[43]

Many environmental stimuli activate NF-KB, including inflammatory cytokines (interleukin-Iβ, tumour necrosis factor-α, interleukin-11, interleukin-17), G/M-CSF, lipopolysaccharide, enzymes as nitric oxide synthase, protein kinase-C activators, adhesion molecules, receptors, oxidants, ultraviolet light, ionizing radiation and viruses. All, activate NF-KB and utilize it to synthesize proteins and to regulate their own expression.[41]

The biological factor of NF-KB is to induce rapid expression of multiple genes involved in immune and inflammatory response.[44]

It also plays a key regulatory role in host defense and in chronic inflammatory diseases, such as asthma, inflammatory bowel disease and rheumatoid arthritis.[41]

Receptor activator of nuclear factor-kappa B (RANK)

The receptor activator of nuclear factor-kappa-B (RANK) is a receptor present on the osteoclast progenitor.[45] It binds to its ligand (RANKL) on the osteoblastic stromal cell to form the active osteoclast.[27,30-36] It was demonstrated that RANK-m RNA was highly expressed by isolated bone marrow-derived osteoclast progenitors and by mature osteoclast in vivo.[46,47]

Human RANK is a 616-amino acid peptide, with a N-terminal extracellular domain, 28 amino acid signal peptide, a short-transmembrane domain of 21 amino acids, and a large C-terminal cytoplasmic domain.[45] It is expressed primarily on cells of the macrophage/monocytic lineage, including preosteoclastic cells, T and B cells, dentritic cells, and fibroblasts.[48,49] RANK activation by its ligand RNAKL i.e. RANKL/RANK signaling pathway is followed by its interaction with TNF-receptor-associated factor (TRAF) family members, and activation of nuclear factor (NF-KB).[48]

The RANK, initiates osteoclastogenic signal transduction after ligation with RANKL. The proximal RANK-derived signals include binding of TNF-receptor association factor (TRAF) family members such as $TRAF_{2,3,5,6}$, which in turn initiates a cascade of kinases.[50]

Receptor activator of nuclear factor K-B ligand (RANKL)

The receptor activator of nuclear factor kappa-B ligand (RANKL) is also known as osteoclast differentiating factor (ODF), tumor necrosis factor-related activated-induced cytokine (TRACE) and osteoprotegerin ligand (OPGL).[1] RANKL is a membrane protein belongs to TNF family that is expressed on the osteoblastic stromal cell.[28-37,51]

It can activate cells of osteoclastic lineage by interaction with RANK that is expressed on the surface of osteoclast progenitors.[28-37,52-55] (Fig. 4) This takes place in the presence of macrophage colony stimulating factor (M-CSF) resulting in osteoclast maturation.[56-58]

Human RANKL, is a 317 amino acid peptide. It has now been shown to exist in two forms, a 40-45 KDa cellular form i.e. membrane-bound and a 31-KDa soluble form derived by cleavage of the full length form.[35]

RANK and RANKL have been shown to be expressed in dendritic cells and T-lymphocytes, in which they appear to be important regulators of the interaction between these cells.[59] It was suggested that, apart form osteoclast differentiation and activation, RANK and RANKL are involved in the immune system as suggested by a study on the mice RANK-knockout models.[47]

It was reported that RANKL knockout mice have severe osteopetrosis with defects in tooth eruption.[37] They also have a complete absence of osteoclasts. In addition they exhibit defects in early differentiation of T and B cells, lack lymph nodes and have defects in thymic differentiation.[60]

RANKL m-RNA is expressed at highest levels in bone and bone marrow as well as in lymphoid tissues.[28,45] Its major role in bone is the stimulation of osteoclast differentiation.[61] In the presence of low levels of macrophage-colony stimulating factor (M-CSF), RANKL appear to be both necessary and sufficient for complete differentiation of osteoclast precursor cells into mature osteoclast.[29] In addition, it is clear that RANKL has a number of effects on immune cells, including activation of C-jun N-terminal kinase (JNK) in T-cells[59], inhibitor of apoptosis of dentritic cells[62] and induction of cluster formation by dentritic cells, and also has an effect on cytokine-activated T-cell proliferation.[45,63]

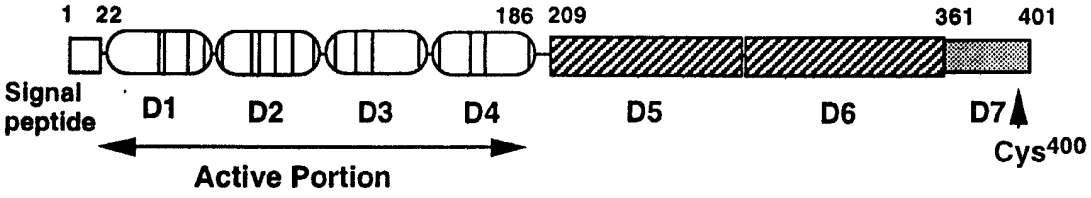
Four independent signals were proposed to enhance RANKL expression: vitamin D receptor, cyclic AMP, glycoprotein 130 and low calcium environment.[64-67]

Osteoprotegerin (OPG)

Simonet and Lacey (1997) eported the discovery of OPG that inhibited bone resorption. Osteoprotegerin (OPG) is also known as osteoclastogenesis inhibitory factor (OCIF) or tumor necrosis factor (TNF) receptor-like molecule-1 (TR-1).[68]

It is a TNF-related protein produced by osteoblastic stromal cells. It is a naturally occurring inhibitor of the RANKL interaction with RANK. It binds to RANKL with high affinity, preventing RANK interaction with RANKL, resulting in inhibition of osteoclast development and maturation. OPG and RANKL are cytokines regulating osteoclastogenesis.[28,68-74]

Osteoprotegerin was found to be initially synthesized as 401 amino acid peptide, with a 21-amino acid propeptide that was cleaved, resulting in a mature protein of 380 amino acids.[68,73] In contrast to all other TNF receptor super-family members, OPG lacks trans-membrane and cytoplasmic domains and was secreted as a soluble protein. The N-terminal region contains four cysteine-rich domains (D_1-D_4) and was most closely related to TNF receptor-2 and CD 40. The C-terminal region contains two death domain homologous regions (D_5 and D_6) as well as a region (D_7) containing a heparin binding site and a cysteine residue necessary for homodimerization.[54,75] (Fig. 5).



D1- D4 : Cysteine-rich domains
D5, D6 : Death domain homologous regions
D7 : Heparin binding domain
Cys^{400} : Essential for dimer formation

Figure (5): A diagrammatic representation of functional domains of OPG.[54]

The OPG –mRNA was found to be expressed in a number of tissues, including lung, heart, kidney, liver, stomach, intestine, brain, spinal cord, thyroid gland and bone.[49] Because the major biologic action of OPG is to inhibit osteoclast differentiation and activity, the potential role of OPG in these other tissues (than bone) remains to be established. However, mice with targeted ablation of OPG not only developed severe osteoporosis due to markedly increased osteoclast formation and subsequent bone resorption,[76,77] but also, have profound calcification of the large arteries, marked intimal and medial proliferation and partial aortic dissection.[78-81]

As soon as OPG was characterized, it was going to be the key for identifying long sought osteoclast differentiation factor expressed on osteoblastic/stromal cells that was essential for osteoclast development.[35]

TNF-related apoptosis-inducing ligand (TRAIL)

TNF-related apoptosis-inducing ligand is another member of the TNF family. It shares homology with RANK and RANKL which are also members of TNF family. TRAIL has been shown to bind to OPG.[71]

RANK/RANKL/OPG system

The identification of the OPG/RANKL/RANK system as the dominant, final mediator of osteoclastogenesis represents a major advance in bone biology.[35]

The osteoblast played a central role in mediating the hormonal control of osteoclastogenesis and bone resorption. Osteoblastic/stromal cells were essential for osteoclastogenesis. These cells regulated osteoclast differentiation both by producing soluble factors and also by signaling to osteoclast progenitors through cell-to cell contact.[82]

Control of the RANK/RANKL/OPG system

The actions of both systemic and local bone regulators, i.e. hormones and cytokines, on the balance between activators and suppressors of osteoclast number and activity might be mediated through the RANK/RANKL/OPG system.[34,83] (Fig. 6)

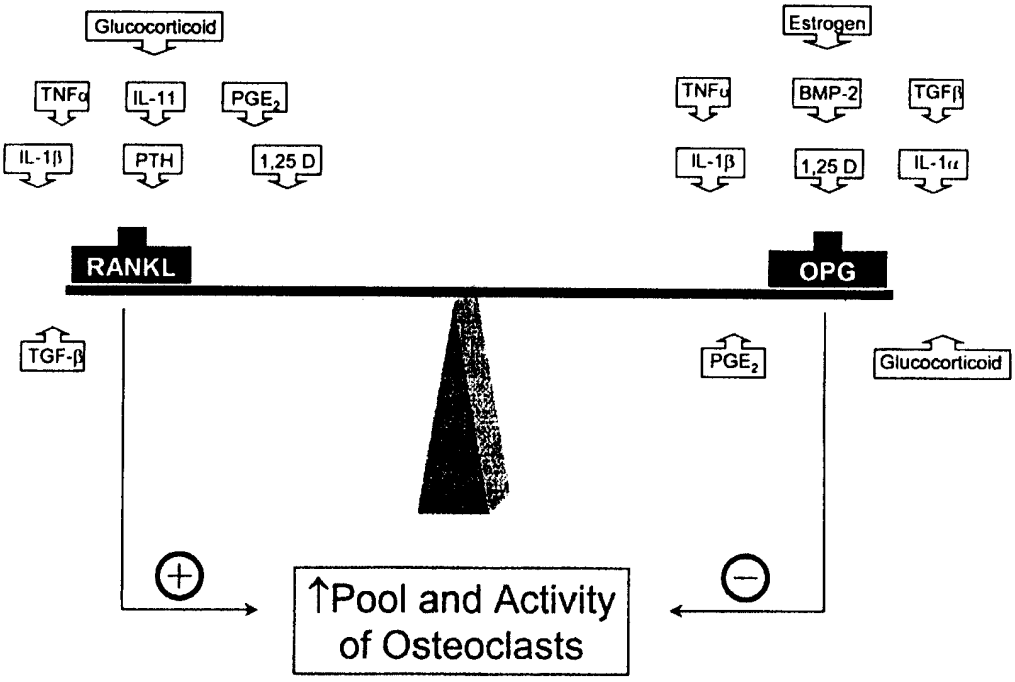


Figure (6): Diagrammatic representation of the influence of RANKL and OPG on osteoclast number and activity. RANKL will tip the balance towards increased osteoclast number and activity whereas increase in OPG will oppose this effect. The hormones and cytokines scattered around the ends of the balance beam will tip the balance in the direction indicated by the arrows.[38]

A- Agents reducing OPG/RANKL ratio

On osteoclastogenesis, the up regulation of RANKL expression and the down-regulating of OPG expression may be one of the mechanisms for the effects of glucocorticoids, PTH, 1,25 $(OH)_2$ D_3 and prostaglandins E_2.[84-90] Some inflammatory cytokines (IL-1β, IL-4, IL-6, IL-11, IL-17 & TNF-alpha) can also stimulate osteoclastogenesis by induction of RANKL expression and basic fibroblast growth factor 2 (which inhibits OPG production and stimulate RANKL production). Also various mesenchymal transcription factors can increase RANKL expression and inhibit OPG expression.[49,91-93]

B- Agents enhancing OPG/RANKL ratio

Estrogens enhance OPG secretion by osteoblastic cells and inhibit RANKL production. This effect is supposed to play an important role in the anti-resorption effect of estrogens on the bone. Transforming growth factor-beta induces also OPG secretion.[94]

There is also a negative regulation of osteoclastogenesis by unique signaling cross talk between RANKL and interferons (IFNs). Series of in vivo experiments revealed that two distinct IFN-mediated regulatory mechanisms are important to maintain homeostasis of bone resorption[95]:

1- The interferone-gamma (IFN-γ) produced by the activated T-cells induces rapid degradation of the RANK-adaptor protein: TRAF6, resulting in strong inhibition of the RANKL-induced activation of NF-KB and JNK (c-jun N-terminal kinase).

2- RANKL induces IFN-β gene in osteoclast precursor cells, which strongly inhibits the osteoclast differentiation by interfering with the RANKL-induced expression of C-fos.[95]

Several groups of investigators have demonstrated that the interaction between RANKL and RANK is essential for development of the mature osteoclast.[37,57] So RANKL, expressed on the surface of presosteoblastic/stromal cells, binds to RANK on the osteoclastic precursor cells. Osteoclasts are formed under the control of the two keys, i.e. RANKL and macrophage-colony stimulating factor.[96,97] (Fig. 7) The Production of macrophage-colony stimulating factor (M-CSF) by

osteoblastic/stromal cells is also essential for osteoclastogenesis,[1] although unlike RANKL, it does not appear to have effects on osteoclast activity.[98] The M-CSF which binds to its receptor C-fms, on preosteoclastic cells (osteoclast progenitor cells), appears to be necessary for osteoclast development because it is the primary determinant of the pool of these precursor cells.[99]

Differentiation along the macrophage/osteoclast lineage requires the transcription factor, c-fos, which activates the c-jun N-terminal kinase (JNK) signaling pathway.[100,101] Thus, exposure of bone marrow cultures to RANKL in combination with M-CSF stimulates osteoclast development.[28] RANKL, however, is critical for the differentiation and fusion into multinucleated cells as well as activation, and survival of osteoclastic cells. OPG puts a brake on the entire system by blocking the effects of RANKL.[86]

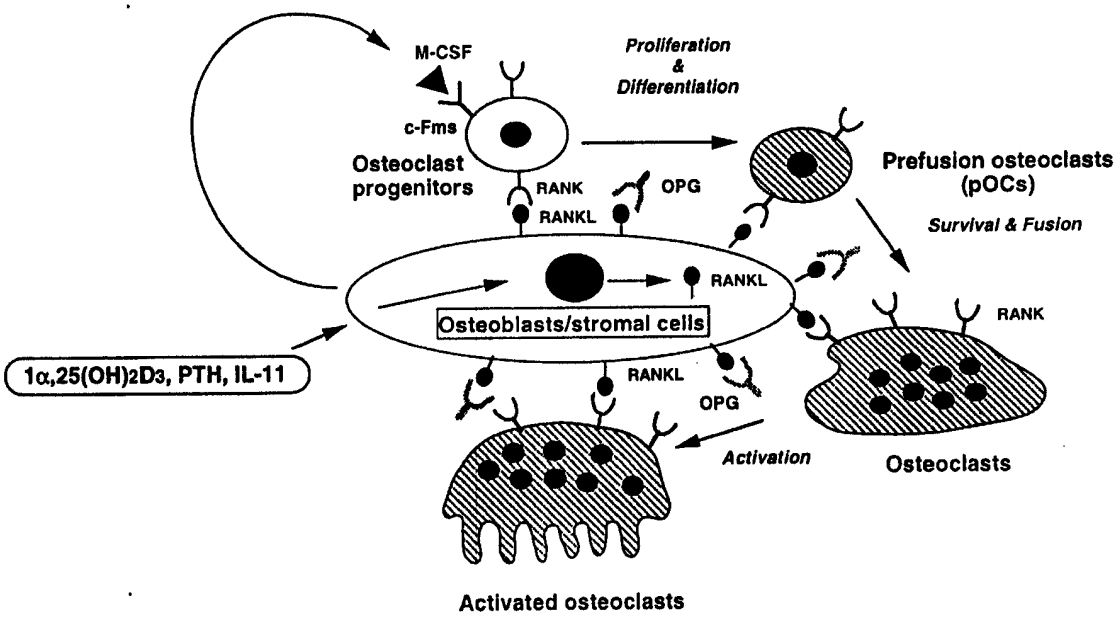


Figure (7): A schematic representation of osteoclast differentiation and function supported by osteoblasts/stromal cells.[54]

It was also found that the ability of preosteoclastic/stromal cells to support osteoclast development is lost rapidly during differentiation down the osteoblast pathway, due principally to down-regulation of RANKl and increased OPG.[35]

As OPG interacts with RANKL and prevents the binding of RANKL to its receptor (RANK) on the osteoclast precursor, therefore, the role of OPG as a decoy receptor for RANKL, leads to failure of osteoblastic stromal cells to communicate with osteoclast precursors, resulting in impaired osteoclastogenesis.[32]

Several evidences have confirmed the ability of OPG to counteract the actions of RANKL. In in vitro study, OPG has been shown to inhibit the increase in osteoclast development and activity induced by RANKL. In another experimental animals study, OPG inhibits the increase in serum calcium caused by administration of RANKL.[28] Other studies in experimental animals showed that the administration of OPG leads to increased bone mineral density. Over expression of OPG in transgenic animals results in osteopetrosis, and targeted disruption of the OPG gene, resulting in osteoporosis.[73,74,76]

Regulation of osteoclast differentiation is a central aspect in understanding the pathogenesis and the treatment of bone disease such as autoimmune arthritis and osteoporosis. In fact, excessive signaling by RANKL, may contribute to such pathological conditions.[102]

OPG/RANKL in certain pathological states

The role of the OPG/RANKL system in the pathogenesis of a common disorder, such as postmenopausal osteoporosis, remains controversial. Although estrogen increases OPG production by osteoblast and marrow stromal cells,[103] serum OPG level is higher in post menopausal women with osteoporosis is increased bone turnover.[104] This increase may be a homeostatic mechanism limiting the more rapid bone loss.[104]

In addition, depressed bone formation is the major abnormality in glucocorticoid-induced osteoporosis[105] and bone resorption is often increased. This is because glucocorticoids are potent inhibitors of OPG production and also can stimulate RANKL levels in osteoblastic cells.[35] This decrease in the OPG/RANKL ratio may account for an enhanced ability of preosteoblastic cells to support osteoclast development, leading to the observed marked imbalance between bone formation and resorption and rapid bone loss occurring in this condition.[105]

A decreased OPG concentration and reduced OPG/RANKL ratio occurs also in patients with multiple myeloma, where OPG concentration correlates negatively with the grade of skeleton destruction.[106,107]

OPG is supposed to be increased in uncontrolled diabetes mellitus. One hypothesis suggests a correlation between increased OPG and vascular calcification, which occurs more frequently in diabetic patients and leads to cardiovascular complications. OPG increases also in patients with severe renal failure and the preliminary results indicate that this is due to impaired OPG clearance.[108]

OPG increases also in patients with metastatic spread of the skeleton in prostatic carcinoma, where the OPG increases both in systemic circulation and metastatic bone focus.[109,110]

The OPG/RANKL system has been studied also in rheumatoid diseases because they are often associated with changes of bone metabolism.[49] It was found that patients with rheumatoid arthritis had intensified osteoresorption and may have a reduced ratio of OPG/RANKL.[92]

Utilization of the OPG/RANKl system in therapy

The application of OPG has been discussed for palliative therapy of metastasizing tumors, rheumatoid arthritis, administration of glucocorticoids, renal failure and patients with paget diseases.[47,54,111] Due to the favorable effect of OPG on bone formation, it can be used as a therapy for individuals with osteoporosis. Animal and human studies were performed with application of OPG to individuals with severe osteoporosis or destructive diseases of the skeleton. Recombinant OPG was administered experimentally in order to reduce or stop osteoresorption and bone invasion in metastasizing mammary, colon and prostate carcinomas. Most intervention studies, proved a significant effect of OPG in reducing the development of osteoporosis.[49]

CHAPTER III

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease of unknown cause that primarily affects peripheral joints in a symmetric pattern. Rheumatoid arthritis is characterized by progressive bone and cartilage destruction as a result of chronic synovitis.[112] The constitutional symptoms, e.g. fatigue, malaise, and morning stiffness are common. Rheumatoid arthritis causes also joint destruction and extra-articular involvement of organs such as the skin, heart, lungs and eyes.[113]

Rheumatoid arthritis has substantial personal, social, and economic costs. It can not be completely cured. The long term prognosis is poor: 80% of affected patients are disabeled after 20 years [114] and life expectancy is reduced by an average of 3 to 18 years.[115]

The worldwide incidence of RA is approximately 3 cases per 10,000 population, and the prevalence rate is approximately 1%. The first-degree relatives of patients with RA have an increased frequency of the disease.[115] Females are 2-3 times more likely to develop RA than males. The frequency of RA increases with age and it peaks in persons aged 35-50 years. Nevertheless, the disease can be observed in both elderly persons and children.[112]

The American College of Rheumatology developed the following criteria for the diagnosis of RA.[116]

1. Morning stiffness: this occurs in and around the joints and lasts at least for one hour before maximal improvement.
2. Arthritis of three or more joint areas: At least 3 joint areas simultaneously have soft tissue swelling or presence of fluid. There are 14 possible joints to be affected: right or left proximal interphalangeal (PIP), meta carpo phalangeal (MCP), wrist, elbow, knee, ankle, and meta tarso phalangeal (MTP) joints.
3. Arthritis of hand joints of at least one area swollen in a wrist, MCP, or PIP joints.
4. Symmetric arthritis with simultaneous involvement of the same joint areas on both sides of the body. Bilateral involvement of PIPs, MCPs and MTPs is acceptable without absolute symmetry.
5. Rheumatoid nodules: Subcutaneous nodules are present over bony prominences, extensor surfaces or in juxta-articular regions.
6. Presence of Rheumatoid factor (RF) as demonstrated by any laboratory method. However, positive results may be found in fewer than 5% of healthy subjects.
7. Typical radiographic changes of RA on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or adjacent to the involved joints. Osteoarthritic changes alone do not qualify.

A patient can be diagnosed as having RA if four of these seven criteria are present. Criteria from one to four must be present for at least six weeks.

Pathophysiology

The precise pathogenic role of RA is unknown. Genetic, environmental, hormonal, immunologic and infectious factors may play significant roles. Socioeconomic, psychological, and life style factors may also influence the disease outcome.[117]

Rheumatoid arthritis is associated with a number of autoimmune responses, but whether autoimmunity is a secondary or a primary event is still unknown.[117]

For many decades, numerous infectious agents have been suggested to induce RA. Among these are *Mycoplasma* organisms, Epstein-Barr virus, rubella virus and others.[117]

Sex hormones may also play a role. This is evidenced by: The disproportionate number of females with RA, its amelioration during pregnancy, its recurrence in the early postpartum period, and its reduced incidence in women using oral contraceptives. In addition, hyperprolactinemia may be a risk factor for RA.[113]

The T-cells are assumed to play a pivotal role in the initiation of RA. The key player in this respect is assumed to be the CD4 cells.[113]

Antigen-activated $CD4^+$ T cells stimulate monocytes, macrophages, and synovial fibroblasts to produce the proinflammatory cytokines [interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor α (TNFα)] and to secrete matrix metalloproteinases. The interleukin-1,

interleukin-6, and TNF-α are the key cytokines that drive inflammation in rheumatoid arthritis. In addition, the activated $CD4^+$ T cells express RANKL that stimulates osteoclastogenesis. Such activated T cells cause joint damage in an animal model of rheumatoid arthritis.[118] The activated $CD4^+$ T cells also stimulate B cells, to produce immunoglobins, including rheumatoid factor. The activation of complement through the formation of immune complexes may also be involved in the pathogenesis of RA.[119]

The activated macrophages, lymphocytes, and fibroblasts, as well as their products, also stimulate angiogenesis which explain the increased vascularity found in the synovium of patients with rheumatoid arthritis.[119]

The hyperactive and hyperplastic synovial membrane is ultimately transformed into pannus tissue and invades cartilage and bone. The later being degraded by activated osteoclasts.[113]

Stages of RA[113]

Four stages are present in RA:

Stage I: The early RA

In this stage, no destructive changes could be observed upon radiographic examination. But the radiographic evidence of osteoporosis is possible.

Stage II (Moderate progression)

There are the following criteria:

⇒ Radiographic evidence of periarticular osteoporosis with or without slight subchondral bone destruction.

⇒ Slight cartilage destruction is possible.

⇒ Joint mobility is possibly limited, but without observed joint deformities.

⇒ Adjacent muscle atrophy.

⇒ Extra-articular soft tissue lesions (e.g. nodules, tenosynovitis) is possible.

Stage III (Severe progression)

There are the following criteria:

⇒ Radiographic evidence of cartilage and bone destruction in addition to periarticular osteoporosis.

⇒ Joint deformity (e.g. subluxation, ulnar deviation or hyperextension) without fibrous or bony ankylosis.

⇒ Extensive muscle atrophy.

⇒ Extra-articular soft tissue lesions is possible.

Stage IV (Terminal progression)

⇒ Fibrous or bony ankylosis.

⇒ Criteria of stage III.

Diagnosis of rheumatoid arthritis[120]

Apart from good clinical examination, laboratory and radiological investigations help in diagnosis of RA.

- Markers of inflammation, such as high erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) are associated with disease

activity. Additionally, the CRP value over time correlates with radiographic progression.[113]

- Anemia, thrombocytosis and mild leucocytosis are frequently encountered in patients with RA.[113]
- Immunologic parameters include the presence of rheumatoid factor, anti nuclear antibodies, and possibly anti-rheumatoid arthritis 33 (anti-RA33), and anti-cyclic citrullinated peptide (anti-ccp):
  - Rheumatoid factor (RF): It is present in approximately 60-80% of patients with RA over the course of their disease.
  - Antinuclear antibodies (ANA): They are present in approximately 40% of patients with RA.
  - Anti-rheumatoid arthritis 33 (anti-RA33), anti-cyclic citrullinated peptide (anti-ccp): They need further validation before their general use can be recommended. It was suggested that the anti-ccp antibodies had a sensitivity and specificity similar to RF but had an increased frequency of positive results in early RA.[113,121]

Imaging studies such as radiographs, MRI, sonography, bone scanning and densitometry can help in diagnosis and staging of RA.[113]

Local bone erosion in rheumatoid arthritis

Rheumatoid arthritis (RA) is a highly osteodestructive process, which leads to local, juxta-articular and systemic bone loss.[122]

Bone is eroded eccentrically starting from the junction zone, where bone, cartilage and synovial membrane are closely attached to each other. Bone is invaded by an inflammatory synovial tissue, known as "pannus" which contains fibroblasts, mononuclear infiltrates, mast cells and numerous blood vessels.[123]

Local bone erosion has become a key monitoring parameter of RA and is associated with unfavorable prognosis, such as functional loss.[124,125] Osteoclasts have a pivotal role in local bone erosions, since: -the bone erosion requires osteoclasts that are preharbored in the inflammatory synovial tissue-[126] Cells in the synovial pannus show all the different maturation steps of the osteoclast lineage.[127] The resorption lacunae were detected at the site of the erosion fronts, where the lacunae are filled with multinucleated mature osteoclasts.[123]

Joint damage in rheumatoid arthritis

Rheumatoid arthritis is characterized by progressive joint damage that is mediated by several mechanisms. Early erosion of cartilage and bone is associated with the formation of a proliferating pannus. The interface between pannus and cartilage is occupied predominantly by activated macrophages and synovial fibroblasts that express matrix metalloproteinases and cathepsins.[128]

Interleukin-1 and TNF-α stimulate the expression of adhesion molecule on endothelial cells and increase the recruitment of neutrophils

into joints. Neutrophils release elastase and proteases, which degrade proteoglycan in the superficial layer of cartilage.[128] The depletion of proteoglycans enables immune complexes to precipitate in the superficial layer of collagen and exposes chondrocytes.[129] Synovial fibroblasts and chondrocytes release matrix metalloproteinases when stimulated by interleukin-1, TNF-α, or activated CD4+ T cells. Matrix metalloproteinases, in particular stromelysin and collagenases, that degrade connective tissue matrix, are thought to be the main mediators of joint damage in rheumatoid arthritis.[129]

Remission of RA

A remission is considered when five or more of the following conditions are present for at least two consecutive months:[113]

⇒ Duration of morning stiffness not exceeding 15 minutes.

⇒ No fatigue.

⇒ No joint pain.

⇒ No joint tenderness or pain with motion.

⇒ No soft tissue swelling in joints or tendon sheaths. ESR of less than 30 mm/h for female or less than 20 mm/h for male.

RANK/RANKL/OPG system and rheumatoid arthritis

Experimental studies suggested that RANKL may have a major pathophysiological importance in the bone and joint destruction observed in inflammatory arthritis, such as RA. The activated T cells, which play a

central role in the pathogenesis of RA, may contribute to the osteoclast mediated-bone resorption via RANKL expression.[130]

Osteoclast formation and activation at the cartilage junction found in RA is an essential step in the destruction of bone matrix in RA patients.[130]

A number of inflammatory cytokines found in RA synovial tissue have the potential to promote osteoclast formation and bone resorption.[131-133]

Recent studies suggested that RANKL-mRNA is highly expressed in synovial tissues from patients with rheumatoid arthritis but not in normal synovial tissues.[134-135]

Moreover, inflamed joint, the end result of the production of inflammatory cytokines, is likely to be due to the up-regulation of RANKL and RANK.[136]

The RANK/RANKL/OPG system has not been well studied in disorders of bone remodeling in humans.[32] Rheumatoid arthritis is an interesting clinical model for the study of the role of this system in bone erosion, and cartilage destruction that is found in this disease.

Aim of the Work

AIM OF THE WORK

The aim of the work is to study the serum levels of receptor activator of nuclear factor-Kappa B ligand and osteoprotegerin in patients with rheumatoid arthritis. Correlations of their levels with the clinical condition and radiological finding are also done in such patients.

Subjects

SUBJECTS

The present study was conducted on 67 subjects. They were divided into two groups:

I- Patients group: It includes 45 patients suffering from Rheumatoid arthritis. Their age ranged from 28-65 years. They included 41 females and 4 males. They were attending the outpatients clinic of the Internal Medicine Department of Medical Research Institute, Alexandria University. The diagnosis was based on history taking and physical examination. The diagnosis was confirmed by laboratory and radiological investigations. The patients were selected according to the criteria of the American collage of Rheumatology for Rheumatoid Arthritis.[116]

II- A control group: 22 healthy volunteer subjects were included as a reference (control) group. They included 20 females and 2 males. They were matched with the patients as regards age, sex, and socio-economic status.

All subjects were selected to be free from liver or renal disease, gout, systemic lupus erythematosis, malignancy or acute infection.

Methods

METHODS

To all subjects the following parameters were carried out:

A- A full history, with special stress on joint pain, stiffness and swelling as well as history of taking non-steroidal anti-inflammatory drugs, corticosteroid therapy or immunosuppressive drugs.

B- Thorough physical examination especially for the joints.

Measurement of blood pressure

The mean arterial blood pressure was calculated as follows:

Diastolic blood pressure + 1/3 pulse pressure.

Pulse pressure = systolic blood pressure-diastolic blood pressure.

C- Calculation of the disease activity score (DAS_{28}).[137]

The following parameters were considered for measuring the disease activity scores (DAS_{28}):

1- 28-joints counts for swelling and tenderness (the joints include 2 shoulder, 2 elbow, 2 wrist, 10 metacarpophalangeal, 10 proximal interphalangeal and 2 knee joints).

2- Patients, and physician global assessment of general health (GH) (disease activity or pain) on visual scale analogue (0 = best possible, 100 = worst possible).

3- Westergren ESR, (≤ 45 mm or > 45 mm).

The disease activity score (DAS_{28}) was measured from the following equation:

$$DAS_{28} = 0.56 \sqrt{tend} + 0.28 \sqrt{swelling} + 0.70 \ln (ESR) + 0.014 (GH)$$

Patients were divided into: low activity (≤ 3.2), and high activity (> 3.2).

D- Plain X-ray for both hands and wrists.

E- Laboratory investigations:

Blood sampling

10 ml venous blood were withdrawn from each subject after 8 hours fasting. One ml was taken on EDTA and used for complete blood count (CBC) examination.[138] Two ml were taken on 3.8% citrate for ESR estimation.[138] The rest of the blood was centrifuged and the separated serum was divided into two parts: the first part was used for determination of fasting serum glucose, creatinine, uric acid, total and ionized calcium, inorganic phosphorus levels, and serum alanine transferase activity, as well as for detection of rheumatoid factor (by Rose Waaler test), C-reactive protein and anti-DNA antibodies. The second part was stored immediately in two Eppendorfs at -70°C until used for estimation of the serum levels of RANKL and OPG when all samples were collected.

1- Estimation of serum glucose level[139]

The glucose was determined without deproteinization using an enzymatic method based on the following reactions:

1st step: (specific for glucose)

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + \text{hydrogen peroxide } (H_2O_2)$$

2nd step: (not specific for glucose)

$$2H_2O_2 + \text{phenol} + 4\text{ aminophenazone} \xrightarrow{\text{peroxidase}} \text{quinoneimine} + 4H_2O$$

(rosy colored product)

The rosy colored product which is proportionate to the concentration of glucose in the sample (T) was measured spectrophotometrically at λ505 nm. The concentration of glucose was determined after comparison with a standard glucose solution (S) of known concentration (CS) similarly treated and glucose was calculated as follows:

$$\text{mg glucose/dl} = \frac{\text{absorbance of sample (T)}}{\text{absorbance of standard (S)}} \times \text{concentration of st. (CS)}$$

(N.B. mmol glucose/L = mg/dL × 0.055)

2- Etimation of serum creatinine[140]

It was determined kinetically without deproteinization. The complex formed by creatinine in the sample with picric acid in an alkaline medium (sodium hydroxide) was measured at an interval of 1 minute at λ500 nm. A standard creatinine of a known concentration (CS) was similarly treated. The difference in optical density at 20 and 80 seconds (Δ) was used to

determine the creatinine in the sample (T) and standard (S) according to the following equations:

$$\text{mg creatinine/dL} = \Delta T/\Delta S \times CS$$

(N.B. mmol creatinine/L = mg/dL × 0.0884)

3- Estimation of serum uric acid[141]

It was determined enzymatically using uricase enzyme according to the following reactions:

1st step (specific for uric acid)

$$\text{Uric acid} + 2H_2O + O_2 \xrightarrow{\text{uricase}} \text{Allantoin} + CO_2 + H2O_2$$

2nd step (not specific)

$2H_2O_2$ + 3,5-dichloro-2-hydroxybenzenesulfonic acid + 4-aminophenazone $\xrightarrow{\text{peroxidase}}$ N-(4-antipyryl)-3-chloro-5-sulfonate-p-benzo-quinoneimine

The rosy color produced (oxidized chromogen), which was proportional to uric acid concentration in the sample (T), was measured spectrophotometrically at λ 520 nm and compared to a known concentration (CS) of standard (S) similarly treated.

Uric acid level was calculated as follows:

$$\text{mg uric acid/dl} = \frac{\text{absorbance of sample (T)}}{\text{absorbance of standard (S)}} \times CS \text{ (mg/dl)}$$

(N.B: mmol uric acid/L = mg/dl × 0.0595)

4- Estimation of serum calcium:[142]

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

$$\text{mg/dL calcium} = \frac{\text{absorbance of sample (T)}}{\text{absorbance of standard (S)}} \times \text{Cs}$$

(N.B: mmol calcium/L = mg/dL × 0.25)

5- Estimation of serum ionized calcium:[142]

Ionized calcium was determined using a direct ion selective electrode without sample deproteinization or dilution. The measured potential between the calcium measuring electrode and the reference electrode was the result of changes in potential which developed across the ion selective electrode (ISE) membrane/sample interface which was related to the natural logarithm of the ionic activity according to Nernst equation. Results were obtained in mmol/L and were converted to mg/dL as follows: mg/dL = mmol/L× 4.

6- Estimation of serum inorganic phosphate:[143]

Serum inorganic phosphate was determined without deproteinization using ammonium molybdate in acidic medium. The formed yellow coloured complex was measured spectrophotometrically (T) and compared to a standard phosphorus solution (S) of a known concentration (Cs) similarly treated. The serum inorganic phosphate concentration was calculated as follows:

$$\text{mg/dL phosphate} = \frac{\text{absorbance of sample (T)}}{\text{absorbance of standard (S)}} \times \text{Cs}$$

(N.B: mmol inorganic phosphate/L = mg/dL × 0.0735)

7- *Determination of alanine amino transferase (ALT) activity:*[144]

ALT activity was determined kinetically as follows:

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

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

(LD: lactate dehydrogenase).

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

Units of ALT activity/L = ΔA/min × 1746

8- *Estimation of C-reactive protein (CRP):*[145]

It was done by immunoturbidimetric assay. Anti-CRP antibodies react with CRP antigen in the samples to form an antigen/antibody complex, which leads to turbidity, this is measured turbidimetrically.

9- *Detection of antideoxyribonucleo protein antibodies (DNA):*[146]

The principle of this test is based on the agglutination reaction that occurs between latex particles coated with DNA being brought into contact with a serum which contains anti-DNA antibodies. Agglutination indicates a positive reaction.

10- Rose-Waaler:[147] test for detection of rheumatoid factor (RF)

The RF-waaler is a rapid agglutination procedure developed for the direct detection and semi-quantitation of rheumatoid factor (RF-waaler) in serum. The determination is made by an agglutination of a stabilizated suspension of sheep red cells, sensibilizated with rabbit gamma-globulins anti-red cell from sheep, against the rheumatoid factor in serum.

11- Estimation of soluble receptor activator or nuclear factor kappa B ligand (s RANKL)[148]

Serum s RANKL was measured by enzyme-linked immunosorbent assay (ELISA) [Biomedical Gruppe].

Principle

Serum RANKL was assayed by an ELISA non competitive sandwich immunoassay. In a first step, human s RANKL present in the samples, standards or control binds to the pre-coated recombinant osteoprotegerin (OPG) and forms a sandwich with the biotinylated anti-sRANKL detection antibody.

Following a wash to remove all unbound materials, streptavidin-HRP conjugate was added to the wells. After removal of unbound conjugate by washing, tetramethyl benzidine (TMB) was added to the wells as substrate. s RANKL was quantitated by an enzyme catalysed colour change. The amount of colour developed was directly proportional to the amount of s RANKL present in the sample. The colour development was stopped and the intensity of the colour was measured with a set of sRANKL standards. A standard curve of absorbance versus concentration was plotted on a linear graph paper from which sRANKL concentration in the unknown samples were calculated.

Reagents

- Human recombinant OPG coated microtiter strips containing 96 polystyrene microtiter wells.
- Washing buffer concentrate, containing 100 ml of washing buffer.
- Four vials of standards containing human s RANKL, standard solutions: 0, 0.5, 2, 8 pmol/L sRANKL in human serum base.
- One vial of human serum base control, lypholizid (4.2 ± 0.5 pmol/L)
- Detection antibody: The vial contains 11 ml biotinylated polyclonal anti-sRANKL antibody, ready to use.
- Conjugate: the vial contains 22 ml streptavidin-HRP conjugate ready to use.
- Tetramethyl benzidine (TMB) chromogen solution, ready to use.
- Stopping solution contains 7 ml of 0.2 sulfuric acid.

Preparation of reagents

1- Wash solution: wash concentrate was diluted 10 folds with deionised water.
2- Standards and control were reconstituted in 700 µl of deionised or distilled water.

Assay procedure

1- Positions for blank, standards, control and samples were selected.
2- 100 µl of standards, control or samples were dispensed into appropriate wells.
3- 100 µl of detection antibody were added to all wells, except that of the blank.
4- Strips were covered with plastic film and incubated overnight (16-24 hours) at 4°C.

5- At the end of incubation time, the plastic film was removed and the contents of the wells were discarded and washed with the washing solution 4 times. This was done by complete aspiration of the liquid from each well and then dispensing 300 µl from diluted washing buffer into the well each time.

6- Then, 200 µl of streptavidin-HRP conjugate were added to all wells.

7- The plate was reincubated for 60 minutes at room temperature.

8- At the end of incubation, the plate was washed 5 times with diluted washing buffer by dispensing 300 µl into each well and aspirated again.

9- Then, 200 µl of substrate (TMB) was added to all wells and incubated for 30 minutes. at room temperature in the dark.

10- At the end of incubation, 50 µl of the stopping solution was added to each well.

11- The absorbance of the solution in the wells was read using microplate reader set to 450 nm and the concentration of samples were read from the standard curve.

Calculation of results

- The extinction of the blank was subtracted form all other values. A calibration curve was constructed from the standard points as follows:
  - ♦ Using a linear graph paper, the mean of absorbance of duplicate readings of the standards were plotted on the Y-axis versus the concentration on the X-axis.

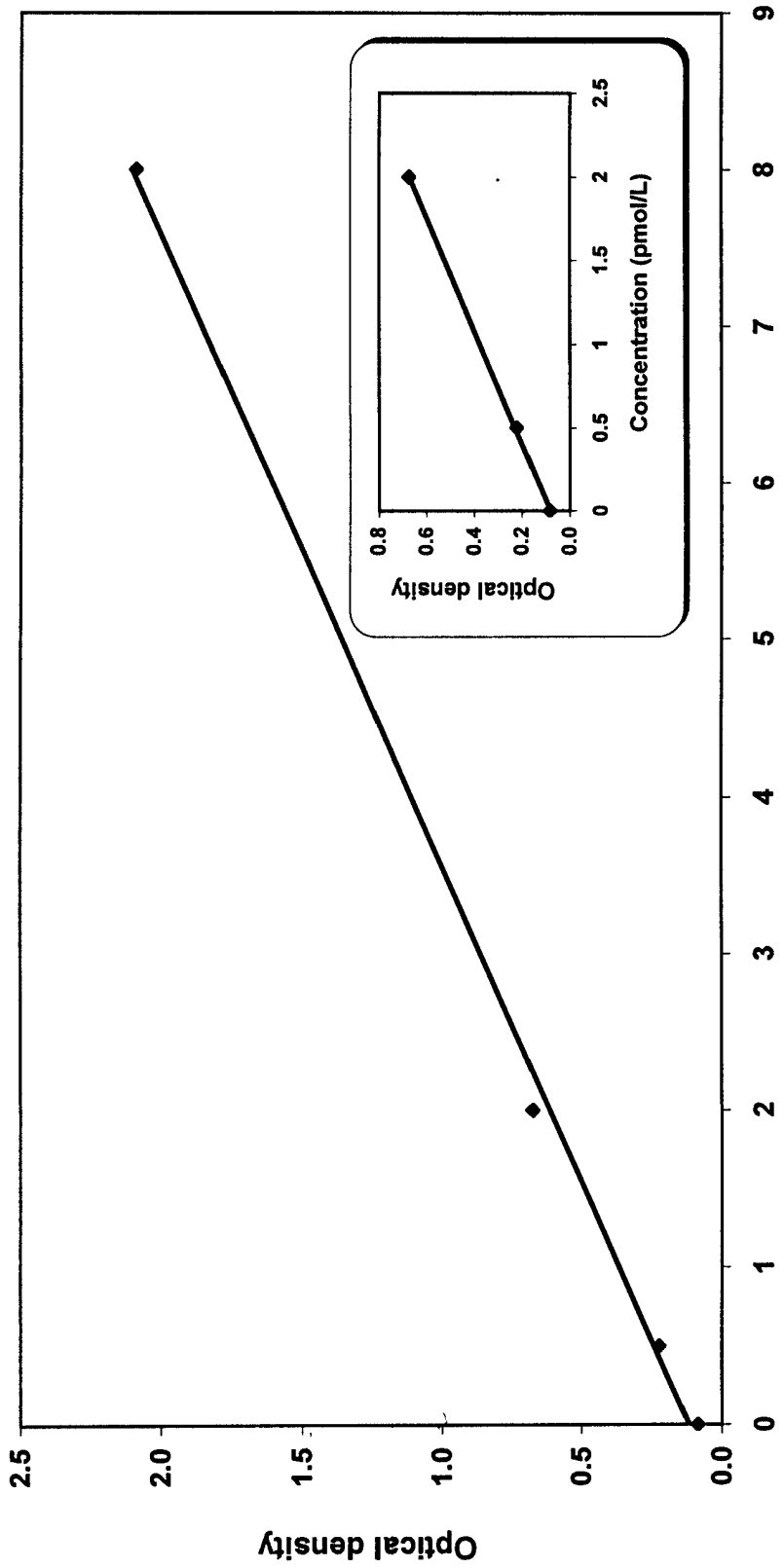


Figure (8) : Standard curve of RANKL

12- Serum osteoprotegerin;[149]

Principle

The osteoprotegerin estimation was done using an enzyme non competitive sandwich immunoassay. Human OPG present in samples, standards or control binds to the precoated capture antibody and forms a sandwich with the biotinylated OPG detection antibody.

After incubation and washing step, which removes all non-specific bound material, steptavidin-HRP conjugate was added to the wells. After removal of unbound conjugate by washing, tetramethyl bezidine (TMB) was added to the wells as substrate. Osteoprotegerin was quantitated by an enzyme catalysed colour change detectable on a standard ELISA reader. The amount of colour developed was directly proportional to the amount of osteoprotegerin present in the sample.

Reagents

- Monoclonal anti-osteoprotegerin antibody coated microtiter strips containing 96 polystyrene wells.
- Washing buffer concentrate, containing 100 ml of buffer.
- Five standards vials containing synthetic human osteoprotegerin. Standards containing 0, 1.1, 3.3, 10, 30 p mol/L in human serum base, ready to use.
- One human serum base control, ready to use (5.6 ± 1.0 p mol/L).

- Detection antibody. The vial contains 7 ml biotinylated polyclonal anti-osteoprotegerin antibody, ready to use.
- Conjugate vial contains 23 ml streptavidin-HRP conjugate, ready to use.
- Substrate vial contains 22 ml TMB solution, ready to use.
- Stopping solution containing 7 ml of 0.2 sulfuric acid, ready to use.
- Assay buffer contains 25 ml, ready to use.

Preparation of reagents

1- Wash solution: wash concentrate was diluted 10 folds with distilled water.

Assay procedure

1- Positions for blank, standards, control and samples were selected.

2- 100 µl of assay buffer were dispensed into each well.

3- 50 µl of standards, control or samples were dispensed into appropriate wells.

4- 50 µl of detection antibody were dispensed to all wells, except blank.

5- Strips were covered with plastic film and incubated overnight (16-24 hours) at 4°C.

6- At the end of incubation time, the contents of the wells were discarded and washed 5 times with 300 µl diluted wash buffer. Any remaining fluid was removed by hitting plate against paper towel after last wash.

7- Then 200 µl of streptavidin-HRP conjugate were added to all wells.

8- The plate was reincubated for 60 minutes at room temperature.

9- At the end of incubation, the contents of the wells were discarded and washed 5 times with 300 µl diluted wash buffer.

10- Then 200µl of substrate (TMB) was added to all wells and incubated for 20 minutes at room temperature in the dark.

11- 50 µl of the stopping solution were added to all wells.

12- The absorbance of the contents in the wells was determined with ELISA reader at 450 nm against 690 ~~or 620~~ nm as reference and the concentration of the samples were read from the constructed standard curve.

Calculation of the results

- The extinction of the blank was subtracted from all other values. A calibration curve was constructed from the standard points as follows:
  - ♦ On a linear graph paper, the mean of absorbance of duplicate reading of the standards were plotted on the Y-axis versus the concentration on the X-axis, using linear curve fit.

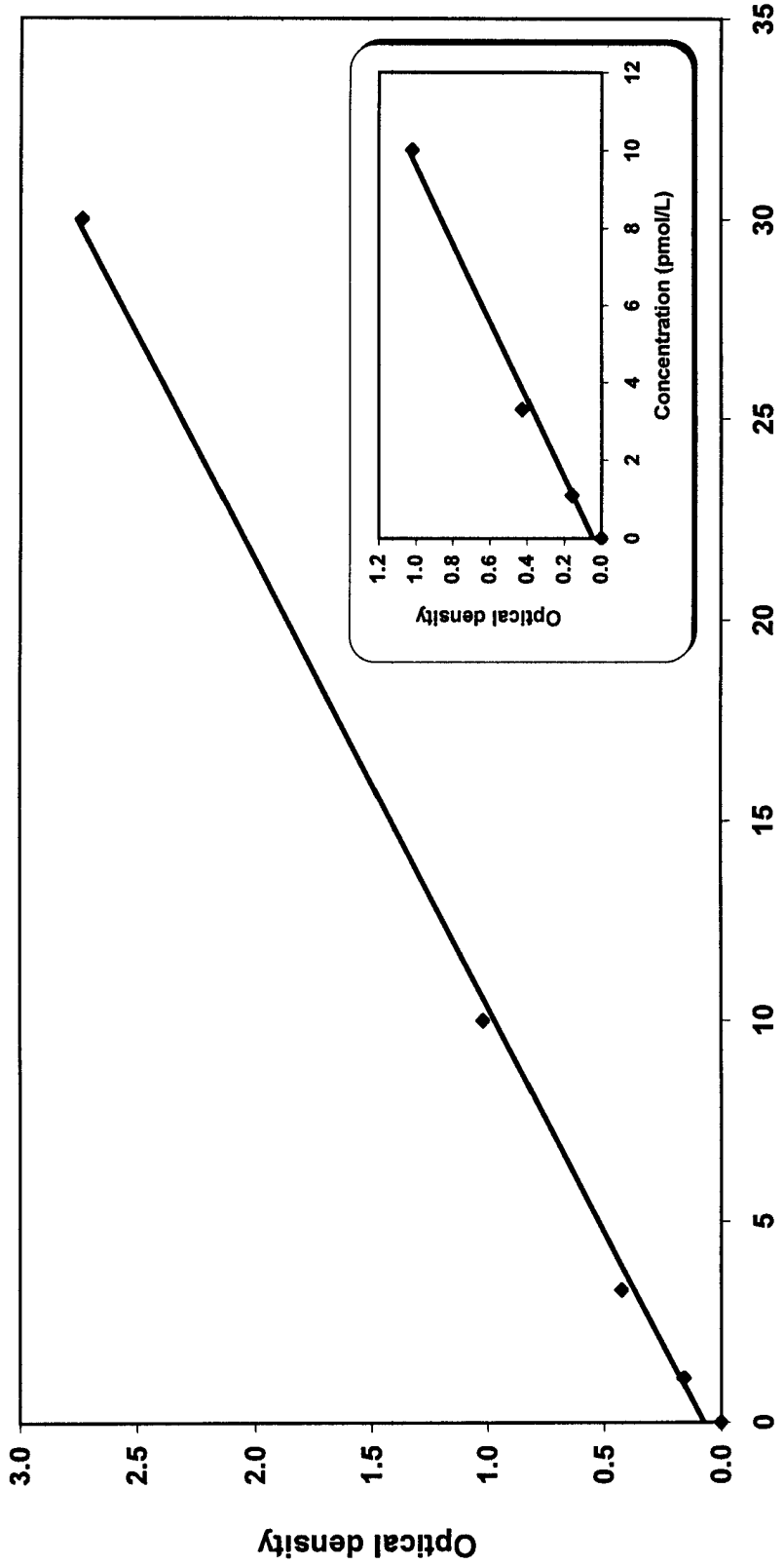


Figure (9) : Standard curve of OPG

Statistical Analysis[150]

Statistical analysis was done using the SPSS software Package version 11.5.

Statistical analysis was done to obtain the mean, the standard deviation, the standard error of each mean and for comparison between the different groups involved in this study using student "t" test to compare between independent samples, one way analysis of variance (ANOVA) for compression between more than 2 samples:

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 observation

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

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

Where n = number of cases.

$\sum X^2$ = the sum of the squares of individual values

$(\sum x)^2$ = the square of the sum of the values

3- Standard Error

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

4- Student "t" test:

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

Where $\overline{X}_1$ = the first mean

$\overline{X}_1$ = the second mean

$S.E_1$ = the standard error of the first sample

$S.E_2$ = the standard error of the second sample

5- One way analysis of variance (ANOVA) was performed for comparison between more than two groups

Variance ratio F was computed by the formula.

$$F_{(r-1),(n-1)} = \frac{\text{Mean square between classes}}{\text{Mean square within classes}}$$

Where r = number of groups

n = total sample size

- The least significant difference (LSD) was calculated at follows:

$$\text{L.S.D. between to group} = t^* \sqrt{\frac{RMS}{n_1} + \frac{RMS}{n_2}}$$

Where t* : critical t value at 5% level

RMS = Residual (within group) mean square

n_1, n_2 = number of observations in two groups

6- Chi-square test:

It is used to test the association between two qualitative variables or to detect difference between two or more groups.

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$

Where;

O is the observed number in each cell table.

E is the expected number in each cell and obtained by multiplying the corresponding row and column totals and dividing by the total sample size (n).

$\sum$ is the summation sign.

7- Coefficient of correlation: A measure of the strength of the association between 2 variables.

Pearson's 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 form -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

$\sum XY$ = the sum of the product of all variables

$\sum X$ = 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

8- Diagnostic performance of both RANKL and OPG:

The chosen cut off value from the ROC curve is that equal to the mean + one standard deviation of the control values.

Positive test results > cut off value

Negative test results < cut off value

	N° with positive test result	**N° with negative test result**	**Totals**
N° of patients with disease	TP	FN	TP + FN
N° of subjects without disease	FP	TN	FP + TN
Totals	TP + FP	FN + TN	TP + FP + TN + FN

Abbreviations:

TP = True positive (number of diseased patients correctly classified by the test)

FP = False positives (number of nondiseased patients misclassified by the test)

FN = False negatives (number of diseased patients misclassified by the test)

TN = True negatives (number of non diseased patients correctly classified by the test)

- Diagnostic Sensitivity = Positivity in diseased patients, expressed as percent = $\frac{TP}{TP+FN} \times 100$

- Diagnostic Specificity = Negativity in non-diseased subjects, expressed as percent = $\frac{TN}{FP+TN} \times 100$

- Predictive value of positive results (PV^+) = Percent of subjects with positive results who are diseased = $\frac{TP}{TP+FP} \times 100$

- Predictive value of negative results (PV) = Percent of subjects with negative test results who are non diseased = $\frac{TN}{TN+FN} \times 100$

- Calculation of diagnostic sensitivity and specificity of both RANKL and OPG were also chosen at the optimal cut off which has a highest positive likelihood ratio (^+LR)

$$\text{where } {}^+LR = = \frac{\text{Sensitivity}}{1-\text{specificity}} = \frac{\text{TP rate}}{\text{FP rate}}$$

N.B. $^+LR > 1$ denotes good performance of a test

- Receiver operating characteristic curve (ROC):

It is generated by plotting sensitivity (TP) on Y axis versus 1-specificity (FP) on X axis at different cut off values. The area under the ROC curve denotes the diagnostic performance of the test. Area more than 50% gives acceptable performance and area about 100% is the best performance for the test. The ROC curve allows also a comparison of performance between two tests.

Results

RESULTS

Table (IIIa): Some clinical data of the control group:

They included twenty two healthy adults (20 females and 2 males). Their mean age was 45.5±8.1 years. Their mean blood pressure (MBP) was 86.9±5.8 mm Hg.

Table (IIIb): Some clinical data of the patients group:

They included forty five patients (41 females and 4 males). Their mean age was 44.7±9.4 years and their mean blood pressure (MBP) was 88.3±11.4 mm Hg.

In this group the following was found:-

- The duration of rheumatoid arthritis ranged from 3 months to 22 years.
- Thirty two patients had fatigue.
- 13 patients had morning stiffness for less than 30 minutes, 18 patients had morning stiffness for 30-60 minutes, 11 patients had morning stiffness for 1-2 hours, and 3 patients had morning stiffness for 2-4 hours.
- 14 patients had bone deformities.
- 16 patients were receiving non steroid anti-inflammatory drugs, 12 patients were receiving corticosteroid, and 16 patients were receiving immuno-suppressive drugs, and 20 patients did not receive any treatment.

Table (IIIc): Statistical differences of age, sex, and mean blood pressure (MBP) in the controls and patients groups:

The two groups were comparable in age, sex and mean blood pressure.

Table (IIIa): Clinical data of control group

Case no.	Age (years)	Sex	M.B.P (mmHg)
1	41	F	93
2	35	M	83
3	55	F	90
4	37	F	93
5	38	F	80
6	40	F	83
7	36	F	93
8	30	F	83
9	47	F	80
10	45	F	93
11	56	F	80
12	52	F	80
13	46	F	90
14	53	F	93
15	60	F	93
16	46	M	83
17	49	F	80
18	39	F	93
19	39	F	90
20	50	F	80
21	53	F	93
22	54	F	83

F : female
M: male
MBP: mean blood pressure

Table (IIIb): Clinical data of the patients group

No.	Age	Sex	Duration of RA (years)	Fatigue	Morning stiffness (minutes)	No. of tender joints	No. of swollen joints	Defor-mities	Drugs			M B.P (mmHg)
									NSIAD	Cortico steroid	Imm-supp	
1	33	F	20	+ve	2	26	23	-	-	-	+	73
2	40	F	3	+ve	3	26	23	-	-	-	-	80
3	29	F	6	+ve	2	28	25	-	-	+	+	73
4	36	F	1	-	1	1	0	-	-	-	-	83
5	55	F	8	+ve	3	28	10	+ve	-	+	-	83
6	36	F	6	+ve	3	27	22	-	-	-	-	120
7	30	F	0.25	+ve	2	27	24	-	-	-	-	87
8	56	F	8	-	1	2	0	-	-	-	-	103
9	55	F	22	+ve	3	28	23	+ve	+	+	-	83
10	53	F	14	+ve	3	26	22	+ve	+	-	+	130
11	51	F	18	+ve	2	26	21	+ve	+	+	+	93
12	38	F	16	+ve	3	28	24	-	-	-	+	83
13	38	F	14	+ve	2	24	12	+ve	-	-	-	90
14	53	F	20	+ve	3	28	22	-	-	-	-	87
15	58	F	4	+ve	2	28	22	-	+	-	-	120
16	65	F	6	+ve	4	27	20	+ve	+	-	-	83
17	43	M	2	-	1	1	0	-	+	+	+	80
18	43	F	0.33	+ve	3	28	24	-	+	-	+	90
19	55	F	3	-	1	1	0	+ve	+	-	+	93
20	41	M	3	+ve	3	28	23	-	+	+	-	83
21	61	M	12	+ve	3	28	24	-	+	+	+	73
22	49	F	2	-	1	1	0	-	+	-	-	87
23	28	F	6	+ve	2	26	24	+ve	-	-	-	93
24	50	F	2	-	1	2	0	-	+	-	+	93

NSAID: Non steroid anti: inflammatory drugs
Imm-supp: Immuno suppressive drugs.
MBP: Mean blood pressure.
Morning stiffness units: 1 (less than 30 minutes) 3 (1-2 hours)
2 (30-60 minutes) 4 (2-4 hours)

Table (IIIb): Clinical data of the patients group (Cont.)

No.	Age	sex	Duration of RA (years)	Fatigue	No. of tender joints	No. of swollen joints	Morning stiffness (minutes)	Defor-mities	Drugs			M. B.P (mmHg)
									NSAID	Cortic steroid	Imm-supp	
25	51	F	10	-	2	0	1	-	+	+	-	87
26	39	F	15	-	1	0	1	+ve	-	+	+	80
27	31	F	10	+ve	28	24	4	+ve	-	+	-	87
28	35	F	1	-	1	1	1	-	-	-	-	83
29	39	F	1	+ve	28	26	2	-	+	-	-	93
30	37	M	10	+ve	28	23	2	-	-	-	-	83
31	32	F	1	+ve	25	22	3	-	-	-	-	80
32	43	F	0.25	+ve	28	13	2	-	-	+	+	83
33	45	F	0.33	+ve	25	13	2	-	-	-	-	87
34	40	F	3	+ve	24	22	2	+ve	-	-	-	93
35	42	F	2	-	1	1	1	-	-	-	-	80
36	55	F	1	-	1	0	1	-	-	-	-	87
37	40	F	2	+ve	28	23	2	-	-	-	-	93
38	45	F	1	+ve	26	23	2	-	-	+	+	93
39	46	F	15	+ve	28	26	2	+ve	-	-	+	83
40	43	F	1	+ve	27	22	2	-	-	-	-	80
41	57	F	3	+ve	27	22	2	-	-	-	-	83
42	41	F	2	+ve	28	24	2	-	+	-	+	93
43	60	F	2	+ve	28	25	4	+ve	+	-	+	80
44	50	F	1	-	2	0	1	-	-	-	-	93
45	45	F	2	-	1	1	1	+ve	-	-	-	83

NSAID: Non steroid anti: inflammatory drugs
Imm-supp: Immuno suppressive drugs.
MBP: Mean blood pressure.
Morning stiffness units: 1 (less than 30 minutes) 3 (1-2 hours)
2 (30-60 minutes) 4 (2-4 hours)

Table (IIIc): Statistical analysis of the clinical data in the studied groups

	Controls n = 22	Patients n = 45
Age (years)		
Mean	45.5	44.7
± SD	± 8.1	± 9.4
P		NS
Sex		
Male	N = 2	N = 4
Female	N = 20	N = 41
P_1		NS
Mean blood pressure (mmHg)		
Mean	86.9	88.3
± SD	± 5.8	± 11.4
P		NS

NS : Not significant

P : Statistical difference between patients and controls

P_1 : Statistical difference between patients and controls (Chi square)

Table IV: X-ray findings on the hands and wrist joints in the patients group:

35 patients had signs of osteoporosis, 21 patients had signs of bone erosion, 14 patients had bone deformities, and 10 patients had normal X-ray.

Table (IV): X-ray findings in the patients group

	Normal	Signs of osteoporosis	Signs of erosion	Bone deformity
1		+ve	+ve	-ve
2		+ve	-ve	-ve
3		+ve	+ve	-ve
4	✓	-ve	-ve	-ve
5		+ve	+ve	+ve
6		+ve	+ve	-ve
7	✓	-ve	-ve	-ve
8		+ve	-ve	-ve
9		+ve	+ve	+ve
10		+ve	+ve	+ve
11		+ve	+ve	+ve
12		+ve	-ve	-ve
13		+ve	+ve	+ve
14		+ve	+ve	-ve
15		+ve	+ve	-ve
16		+ve	+ve	+ve
17	✓	-ve	-ve	-ve
18		+ve	-ve	-ve
19		+ve	+ve	+ve
20	✓	-ve	-ve	-ve
21		+ve	-ve	-ve
22		+ve	+ve	-ve
23		+ve	+ve	+ve

Table (IV): X-ray findings in the patients group (Cont.)

	Normal	Signs of osteoporosis	Signs of erosion	Bone deformity
24		+ve	-ve	-ve
25		+ve	-ve	-ve
26		+ve	+ve	+ve
27		+ve	+ve	+ve
28	✓	-ve	-ve	-ve
29	✓	-ve	-ve	-ve
30		+ve	-ve	-ve
31		+ve	+ve	-ve
32	✓	-ve	-ve	-ve
33		+ve	-ve	-ve
34		+ve	-ve	+ve
35	✓	-ve	-ve	-ve
36		+ve	-ve	-ve
37	✓	-ve	-ve	-ve
38	✓	- ve	- ve	-ve
39		+ve	+ve	+ve
40		+ve	-ve	-ve
41		+ve	-ve	-ve
42		+ve	+ve	-ve
43		+ve	+ve	+ve
44		+ve	-ve	-ve
45		+ve	+ve	+ve

Table (V): Disease activity score (DAS_{28}) in patients group

The mean of DAS_{28} in patients group was 6.65 ± 2.3.

Table (V): Disease activity score (DAS_{28}) in patient group

No.	DAS_{28}	No.	DAS_{28}
1	8.06	25	3.15
2	7.85	26	2.82
3	8.20	27	8.47
4	3.08	28	3.17
5	7.66	29	8.38
6	7.89	30	8.49
7	8.29	31	7.55
8	3.02	32	7.78
9	8.24	33	7.90
10	7.82	34	7.85
11	7.54	35	3.25
12	8.72	36	3.16
13	7.61	37	7.73
14	7.64	38	8.13
15	7.65	39	8.80
16	8.83	40	8.03
17	3.17	41	7.88
18	8.24	42	8.01
19	3.14	43	8.62
20	8.27	44	3.09
21	8.53	45	3.23
22	3.16		
23	8.01		
24	3.10		
Mean ± SD			***6.65 2.30***

Table (VI): Division of the patients according to the degree of disease activity (DAS_{28}):

The patients were divided into two groups:

1- Patients with mild activity ($DAS_{28} \leq 3.2$). They were 11 patients. Their mean DAS_{28} value was 3.1 ± 0.1

2- Patients with high activity ($DAS_{28} > 3.2$). They were 34 patients. Their mean DAS_{28} value was 7.8 ± 1.2.

Table (VI): Division of the patients according to degree of activity (DAS_{28} value ≤ 3.2 or DAS_{28} > 3.2)

Patients with DAS ≤ 3.2		Patients with DAS > 3.2	
Patient's number (n = 11)	DAS_{28}	Patient's number (n = 34)	DAS_{28}
4	3.08	1	8.05
8	3.02	2	7.85
17	3.17	3	8.22
19	3.14	5	7.66
22	3.16	6	7.89
24	3.10	7	8.29
25	3.15	9	8.24
26	2.82	10	7.82
28	3.17	11	7.54
36	3.16	12	8.72
44	3.09	13	7.61
		14	7.64
		15	7.65
		16	8.83
		18	8.24
		20	8.27
		21	8.53
		23	8.01
		27	8.47
		29	8.38
		30	8.49
		31	7.55
		32	7.78
		33	7.90
		34	7.85
		35	3.25
		37	7.73
		38	8.13
		39	8.80
		40	8.03
		41	7.88
		42	8.01
		43	8.62
		45	3.23
Mean	3.10		7.80
± SD	0.10		1.21

Tables (VIIa, b, c): **Haemoglobin (Hb), red blood cells count, (RBCs), white blood cells count (WBCs), and platelets count in controls (a), in patients (b) and their statistical analysis (c):**

- Haemoglobin levels, and red blood cells counts in patients group showed significant decrease than their corresponding values in controls.
- Platelets count in patients group showed significant increase than that in controls.
- White blood cells count did not significantly differ in both groups.

Table (VIIa): Haemoglobin (Hb), red blood cells count (RBCs), white blood cells count (WBCs), and platelets count in control group

Case No.	Hb (gm/dL)	RBCs ($\times 10^6$/cmm)	WBCs ($\times 10^3$/cmm)	Platelets ($\times 10^3$/cmm)
1	12.5	4.33	6.3	254
2	14.0	4.80	4.1	350
3	12.7	4.60	5.4	210
4	13.0	5.20	8.0	190
5	12.2	4.70	5.3	252
6	11.9	3.70	5.9	177
7	12.0	4.50	4.9	317
8	11.8	4.40	6.6	208
9	13.1	4.38	6.0	170
10	13.5	4.37	5.6	195
11	11.8	4.23	5.4	273
12	12.4	4.78	6.3	313
13	11.7	4.61	4.5	211
14	14.2	4.65	6.4	203
15	11.9	4.36	4.9	184
16	13.5	4.5	4.9	210
17	12.0	4.43	6.4	254
18	13.5	4.43	4.5	220
19	12.5	4.31	5.4	215
20	12.5	4.56	6.3	185
21	12.0	5.51	5.1	235
22	13.0	5.07	8.0	230
Mean $\overline{X}$	12.6	4.6	5.7	229.8
± SD	0.75	0.41	1.0	48.0

Table (VIIb): Haemoglobin (Hb), red blood cells count (RBCs), white blood cells count (WBCs), and platelets count in patients group

Case No.	Hb (gm/dL)	RBCs ($\times 10^6$/cmm)	WBCs ($\times 10^3$/cmm)	Platelets ($\times 10^3$/cmm)
1	10.1	4.30	6.8	213
2	10.2	4.31	5.6	287
3	7.9	3.71	5.3	342
4	10.9	5.52	5.7	250
5	10.0	3.50	6.0	211
6	7.9	3.56	9.9	352
7	9.5	2.64	4.8	160
8	11.0	4.50	5.8	310
9	12.2	4.20	4.2	138
10	11.9	5.09	8.2	277
11	11.5	4.60	5.7	288
12	12.5	4.40	7.8	358
13	10.6	3.70	13.3	153
14	11.0	4.90	6.3	227
15	12.4	4.80	4.4	253
16	7.8	3.08	5.1	342
17	8.2	4.04	2.9	282
18	10.5	4.00	5.6	283
19	12.3	4.80	5.4	193
20	9.8	4.03	6.7	212
21	8.2	3.55	5.9	324
22	12.1	4.61	7.5	284
23	11.8	4.52	3.2	128

Table (VIIb): Haemoglobin (Hb), red blood cells count (RBCs), white blood cells count (WBCs), and platelets count in patients group (Cont.)

Case No.	Hb (gm/dL)	RBCs ($\times 10^6$/cmm)	WBCs ($\times 10^3$/cmm)	Platelets ($\times 10^3$/cmm)
24	10.9	4.04	5.3	228
25	12.4	4.14	5.3	186
26	9.6	4.30	15.8	261
27	12.4	4.90	7.9	213
28	11.9	4.30	5.5	345
29	10.2	3.75	6.4	272
30	9.8	4.52	4.8	547
31	9.2	5.20	4.5	516
32	12.7	4.73	4.6	201
33	13.2	4.50	4.3	224
34	11.0	4.50	6.0	230
35	8.8	4.00	3.7	280
36	11.6	4.25	4.9	219
37	11.9	4.50	4.7	242
38	12.4	4.35	10.1	305
39	11.5	4.39	3.9	304
40	8.8	3.69	7.2	150
41	13.2	4.80	9.1	306
42	11.1	3.68	11.6	328
43	9.8	3.50	3.7	357
44	11.3	4.60	3.8	227
45	11.5	4.25	9.5	210
Mean $\overline{X}$	10.78	4.22	6.32	267.1
$\pm$ SD	1.5	0.53	2.6	84.4

Table (VIIc): Statistical differences of haemoglobin (Hb), red blood cells (RBCs), white blood cells (WBCs), and platelets count between the studied groups

	Controls	Patients
Haemoglobin (Hb) (gm/dL)		
Mean	12.6	10.78
± SD	0.75	1.5
P		0.000*
RBCs $\times 10^6$/cmm		
Mean	4.6	4.22
± SD	0.41	0.53
P		0.004*
WBCs $\times 10^3$/cmm		
Mean	5.7	6.32
± SD	1.0	2.6
P		NS
Platelets $\times 10^3$/cmm		
Mean	229.8	267.1
± SD	48.0	84.4
P		0.025*

P: Statistical difference between patients and controls

N.S.: Not significant

*: Significant

Tables (VIIIa, b, c) : <u>Serum levels of fasting serum glucose (FSG), creatinine, uric acid and alanine amino transferase (ALT) in controls (a), in patients (b) and their statistical analysis (c)</u>:

- There was no significant difference in these parameters between both groups.

Table (VIIIa): Fasting serum levels of glucose (FSG), creatinine, uric acid, and alanine amino transferase activity (ALT) in controls

Case No.	FSG (mg/dl)	Creatinine (mg/dl)	Uric acid (mg/dl)	ALT (U/L)
1	105	0.8	4.2	16
2	97	1.2	5.4	33
3	87	1.0	3.6	26
4	110	1.0	4.2	19
5	87	0.9	3.1	10
6	93	0.8	2.9	12
7	99	0.9	2.7	8
8	85	0.8	2.8	10
9	90	0.9	2.2	15
10	101	1.0	3.4	20
11	95	1.0	4.2	25
12	82	1.1	3.9	11
13	94	1.1	2.8	16
14	111	1.0	4.9	24
15	95	1.0	2.1	13
16	86	1.2	5.7	10
17	102	0.9	3.6	21
18	103	1.1	3.7	21
19	105	0.8	3.8	22
20	98	1.1	4.8	15
21	80	0.9	3.8	11
22	102	1.0	4.4	13
Mean $\bar{X}$	95.77	0.97	3.73	16.86
± SD	8.8	0.12	0.95	6.49

Table (VIIIb): Fasting serum levels of glucose (FSG), creatinine, uric acid, and alanine amino transferase activity (ALT) in the patients group

Case No.	FSG (mg/dl)	Creatinine (mg/dl)	Uric acid (mg/dl)	ALT (U/L)
1	92	0.9	4.2	38
2	94	1.0	3.6	15
3	89	0.8	3.5	8
4	79	0.9	2.5	18
5	97	1.0	4.1	24
6	89	0.8	5.3	14
7	88	0.9	7.0	9
8	91	1.2	2.7	17
9	94	0.9	4.6	23
10	104	1.2	4.0	17
11	84	1.2	2.3	11
12	114	1.0	4.4	13
13	91	1.3	3.2	32
14	92	1.0	4.4	32
15	110	1.0	4.0	40
16	81	1.0	5.8	9
17	89	0.9	2.9	16
18	109	1.0	3.4	20
19	96	1.1	4.8	13
20	93	0.9	4.6	22
21	113	1.5	8.0	7
22	101	1.0	3.8	-
23	82	0.8	4.3	18

Table (VIIIb): Fasting serum levels of glucose (FSG), creatinine, uric acid, and alanine amino transferase activity (ALT) in the patients group (Cont.)

Case No.	FSG (mg/dl)	Creatinine (mg/dl)	Uric acid (mg/dl)	ALT (U/L)
24	84	0.9	3.1	25
25	99	1.0	4.4	12
26	114	1.0	3.1	16
27	102	1.0	2.6	12
28	103	1.3	4.5	17
29	94	1.1	3.0	13
30	89	0.9	4.0	11
31	90	0.9	2.6	14
32	116	1.0	6.3	37
33	115	1.0	5.3	26
34	90	0.9	3.0	30
35	92	1.0	2.5	8
36	104	1.0	3.4	21
37	96	1.1	3.4	18
38	88	1.0	5.4	20
39	86	1.0	2.4	5
40	106	1.0	5.2	45
41	102	0.9	4.0	16
42	107	1.0	3.6	22
43	95	1.4	5.2	8
44	89	0.8	2.0	12
45	90	1.1	4.3	25
Mean $\overline{X}$	96.1	1.01	4.01	18.8
± SD	9.8	0.15	1.26	9.4

Table (VIIIc): Statistical differences of fasting serum levels of glucose (FSG), creatinine, uric acid, and alanine amino transferase activity (ALT) between the studied groups

	Controls (n = 22)	Patients (n = 45)
FSG (mg/dl)		
Mean	95.77	96.1
± SD	8.8	9.8
P		NS
Creatinine (mg/dl)		
Mean	0.97	1.01
± SD	0.12	0.15
P		NS
Uric acid (mg/dl)		
Mean	3.73	4.01
± SD	0.95	1.26
P		NS
ALT (U/L)		
Mean	16.86	18.8
± SD	6.49	9.4
P		NS

N.S.: Not significant

Tables (IXa, b, c): Serum levels of total calcium (Ca), ionized Ca (Ca^{++}) and inorganic phosphorus (Pi) in controls (a), in patients (b) and their statistical analysis (c):

- Both total calcium and inorganic phosphorus did not significantly differ in both groups.
- The serum ionized calcium (Ca^{++}) in the patients group showed significant decrease than that in controls.

Table (IXa): Serum levels of total calcium (Ca), ionized Ca (Ca^{++}), and inorganic phosphorus (Pi) in control group

Case no.	Total Ca (mg/dl)	Ca^{++} (mg/dl)	Phosphorus (mg/dl)
1	9.3	3.7	3.4
2	9.3	4.5	3.8
3	9.8	4.1	3.6
4	8.9	4.2	3.0
5	9.1	4.3	-
6	8.9	4.1	2.6
7	9.0	3.8	2.6
8	8.7	4.4	2.6
9	8.8	4.1	3.1
10	9.2	4.0	2.7
11	9.0	4.0	3.1
12	8.6	3.8	3.6
13	8.7	4.0	2.4
14	9.3	4.0	2.9
15	9.1	3.7	4.3
16	10.3	4.0	4.0
17	9.1	3.8	3.5
18	9.3	3.9	3.5
19	9.5	4.3	2.7
20	9.4	3.6	3.1
21	9.6	3.7	3.1
22	8.9	4.2	3.1
Mean	9.17	4.01	3.17
± SD	0.39	0.23	0.51

Table (IXb): Serum levels of total calcium (Ca), ionized Ca (Ca^{++}), and inorganic phosphorus (Pi) in patients group

Case no.	Total Ca (mg/dL)	Ca^{++} (mg/dL)	Phosphorus (mg/dL)
1	8.7	3.2	3.2
2	9.5	4.5	4.5
3	9.9	3.9	3.8
4	8.2	3.8	3.2
5	9.1	3.3	3.4
6	9.0	3.9	3.3
7	8.9	3.7	3.7
8	10.3	3.7	4.0
9	9.1	3.7	3.7
10	9.7	3.5	4.0
11	9.5	3.6	3.7
12	9.2	3.9	2.5
13	8.1	4.2	3.0
14	9.8	3.9	3.3
15	9.8	3.7	4.0
16	9.2	4.3	3.4
17	8.6	3.8	3.3
18	9.2	4.2	3.0
19	9.7	3.7	3.2
20	9.1	3.2	3.4
21	9.7	3.2	2.9
22	9.3	3.6	4.3
23	9.0	4.1	2.2

Table (IXb): Serum levels of total calcium (Ca), ionized Ca (Ca^{++}), and inorganic phosphorus (Pi) in patients group (Cont.)

Case no.	Total Ca (mg/dl)	Ca^{++} (mg/dl)	Phosphorus (mg/dl)
24	9.0	3.9	2.5
25	9.4	3.5	3.4
26	9.3	4.2	2.4
27	9.2	3.4	4.0
28	9.1	4.2	2.6
29	9.1	4.1	2.6
30	9.2	3.7	3.3
31	8.9	4.0	3.0
32	9.0	4.1	2.3
33	9.7	4.1	2.9
34	9.2	3.9	3.5
35	8.9	3.4	3.9
36	8.9	3.2	3.2
37	8.2	3.3	3.0
38	9.7	4.0	3.1
39	8.9	3.5	3.7
40	8.8	3.5	3.8
41	9.0	4.1	2.4
42	9.2	3.6	3.0
43	9.8	4.7	5.1
44	9.7	3.3	3.7
45	9.2	4.2	3.1
Mean	9.2	3.97	3.3
± SD	0.45	0.36	0.61

Table (IXc): Statistical differences of total calcium, ionized Ca (Ca^{++}), and inorganic phosphorus between the studied groups

	Controls	Patients
Total calcium (mg/dl)		
Mean	9.17	9.2
± SD	0.39	0.45
P		NS
Ca^{++} (mg/dl)		
Mean	4.01	3.79
± SD	0.23	0.36
P		0.03*
Phosphorus (mg/dl)		
Mean	3.17	3.3
± SD	0.51	0.61
P		NS

N.S.: Not significant

*: Significant

P: Statistical significance between control and patients groups

Tables (Xa, b, c): **Erythrocyte sedimentation rate (ESR), Serum Rose Waaler test (RW) and C-reactive protein (CRP) in controls (a), in patients (b) and their statistical analysis (c):**

- Erythrocyte sedimentation rate, Rose Waaler and C-reactive protein in the patients group showed significant increase than that in controls.

Table (Xa): Erythrocyte sedimentation rate (ESR), serum Rose Waaler test (R.W), and C-reactive protein (CRP) in controls group

Case no.	ESR (mm)	R.W. (U/ml)	CRP (mg/dl)
1	15	3	0.38
2	10	2	0.26
3	8	1	0.17
4	13	5	0.43
5	7	6	0.12
6	11	3	0.25
7	9	4	0.24
8	12	2	0.38
9	12	1	0.41
10	18	1	0.32
11	15	2	0.36
12	12	2	0.26
13	15	3	0.42
14	9	5	0.19
15	18	5	0.42
16	10	3	0.24
17	15	4	0.32
18	9	4	0.23
19	19	2	0.41
20	9	3	0.31
21	15	2	0.32
22	23	3	0.47
Mean	12.9	3	0.31
± SD	4.1	1.41	0.095

Table (Xb): Erythrocyte sedimentation rate (ESR), serum Rose Waaler (R.W) test, and C-reactive protein (CRP) in patients group

Case no.	ESR (mm)	R.W (U/ml)	CRP (mg/dl)
1	50	8	0.9
2	46	512	1.2
3	50	512	2.4
4	30	256	0.9
5	47	8	1.7
6	47	128	2.5
7	62	64	2.2
8	20	64	0.6
9	46	384	1.7
10	46	384	2.0
11	48	384	1.1
12	88	192	5.2
13	65	48	2.4
14	45	8	1.5
15	46	96	0.9
16	107	192	1.8
17	28	48	0.7
18	66	768	2.3
19	33	8	1.1
20	48	24	1.8
21	100	8	6.4
22	34	48	0.9
23	82	8	2.1

Table (Xb): Erythrocyte sedimentation rate (ESR), serum Rose Waaler (R.W) test, and C-reactive protein (CRP) in patients group (Cont.)

Case no.	ESR (mm)	R.W (U/ml)	CRP (mg/dl)
24	15	8	0.6
25	24	64	0.6
26	17	32	0.8
27	75	128	15.2
28	23	512	0.8
29	50	512	1.6
30	120	1024	10.8
31	50	512	2.9
32	47	8	0.9
33	47	128	0.9
34	46	512	1.5
35	21	64	0.1
36	34	16	0.6
37	47	32	1.2
38	68	512	1.0
39	90	256	3.2
40	85	1024	1.3
41	46	32	1.3
42	47	1024	3.1
43	60	1024	12.1
44	18	32	0.1
45	25	64	0.7
Mean	50.8	259.4	2.34
± SD	24.5	313.7	3.1

Table (Xc): Statistical difference of erythrocyte sedimentation rate (ESR), Rose Waaler (R.W) test, and C-reactive protein between the studied groups

	Controls	Patients
ESR (mm)		
Mean	12.9	50.8
± SD	4.1	24.5
P		0.000*
R.W. (U/ml)		
Mean	3	259.4
± SD	1.41	313.7
P		0.000*
CRP (mg/dL)		
Mean	0.31	2.34
± SD	0.095	3.1
P		0.000*

*: Significant

P: Statistical significance between control and patients groups

Tables (XIa, b, c): Serum levels of receptor activator of N-factor kappa-B ligand (RANKL), osteoprotegerin (OPG) and RANKL/OPG ratio in controls (a), in patients (b) and their statistical analysis (c):

- RANKL, OPG and RANKL/OPG ratio in the patients showed significant increase than their corresponding levels in controls.

Table (XIa): Serum levels of receptor activator of Nuclear factor kappa-B ligand (RANKL), osteoprotegerin (OPG) and RANKL/OPG ratio in controls group

Case no.	RANKL (pmol/L)	OPG (pmol/L)	RANKL/OPG ratio
1	0.24	4.25	0.06
2	0.27	2.40	0.11
3	0.17	1.70	0.10
4	0.64	4.25	0.15
5	0.17	1.25	0.14
6	0.30	2.40	0.13
7	0.14	1.70	0.08
8	0.57	3.55	0.16
9	0.45	1.55	0.29
10	0.20	1.75	0.11
11	0.24	2.25	0.11
12	0.24	1.45	0.17
13	0.34	1.51	0.23
14	0.34	-	-
15	0.14	2.65	0.05
16	0.20	2.65	0.08
17	0.34	1.90	0.18
18	0.51	2.55	0.20
19	0.17	2.10	0.08
20	0.14	3.25	0.04
21	0.27	3.65	0.07
22	0.16	4.90	0.03
Mean	0.28	2.56	0.12
± SD	0.14	1.05	0.065

Table (XIb): Serum levels of receptor activator of Nuclear factor kappa-B ligand (RANKL), osteoprotegerin (OPG) and RANKL/OPG in patients group

Case no.	RANKL (pmol/L)	OPG (pmol/L)	RANKL/OPG ratio
1	1.00	3.50	0.29
2	1.25	2.25	0.56
3	1.25	3.80	0.33
4	0.51	1.50	0.34
5	0.27	9.90	0.03
6	1.30	2.25	0.58
7	1.10	1.85	0.59
8	0.21	3.00	0.07
9	1.10	4.90	0.22
10	1.85	3.30	0.56
11	1.00	3.40	0.29
12	0.27	4.00	0.07
13	0.61	5.40	0.11
14	2.10	2.50	0.84
15	0.80	5.10	0.16
16	0.68	3.55	0.19
17	0.40	1.00	0.40
18	1.34	3.20	0.42
19	0.34	1.70	0.20
20	0.95	1.50	0.63
21	1.28	5.35	0.24
22	0.21	2.85	0.07

Table (XIb): Serum levels of receptor activator of Nuclear factor kappa-B ligand (RANKL), osteoprotegerin (OPG) and RANKL/OPG in patients group (Cont.)

Case no.	RANKL (pmol/L)	OPG (pmol/L)	RANKL/OPG ratio
23	0.41	2.80	0.15
24	0.74	2.80	0.26
25	0.40	4.20	0.10
26	0.14	3.55	0.04
27	0.10	2.40	0.04
28	0.48	2.35	0.20
29	1.55	2.25	0.69
30	2.00	4.00	0.50
31	0.75	3.50	0.21
32	0.64	2.50	0.26
33	0.95	2.80	0.34
34	0.84	2.80	0.30
35	0.31	2.70	0.11
36	0.48	4.10	0.12
37	0.71	2.40	0.30
38	0.81	3.40	0.24
39	0.80	3.90	0.21
40	0.91	4.00	0.23
41	0.75	2.80	0.27
42	1.88	2.40	0.78
43	1.00	3.20	0.31
44	0.73	3.10	0.23
45	0.31	3.60	0.09
Mean	0.83	3.27	0.292
± SD	0.5	1.4	0.20

Table (XIc): Statistical difference of receptor activator of nuclear factor kappa-B ligand (RANKL), osteoprotegerin (OPG) and RANKL/OPG ratio between the studied groups

	Controls	Patients
RANKL (pmol/L)		
Mean	0.28	0.83
± SD	0.14	0.5
P		0.000*
OPG (pmol/L)		
Mean	2.56	3.27
± SD	1.05	1.4
P		0.04*
RANKL/OPG		
Mean	0.122	0.29
± SD	0.065	0.20
P		0.000*

*: Significant

P: Statistical significance between controls and patients groups

Table XII: Some parameters in relation to the duration of Rheumatoid arthritis:

The patients were divided according to the duration of rheumatoid arthritis into two subgroups: those with duration < 5 years and those with duration ≥ 5 years.

1- The levels of Ca^{++}, RW, DAS_{28}, RANKL, and RANKL/OPG ratio showed no significant difference between the two subgroups of patients, while the levels of ESR, CRP and OPG showed significant increase in patients with duration ≥ 5 years than their corresponding values in patients with duration < 5 years.

2- In both subgroup of patients, the levels of ESR, RW, and RANKL showed significant increase than their corresponding levels in controls.

3- In the subgroup of patients with duration ≥ 5 years, the levels of Ca^{++} showed significant decrease than that in controls, while the levels of CRP and OPG showed significant increase than their corresponding levels in controls.

Table (XII): Some parameters in relation to the duration of rheumatoid arthritis

Item	Controls (n = 22)	Patients with duration < 5 y (n = 26)	Patients with duration ≥ 5 y (n = 19)
Ca^{++} (mg/dl)			
$\overline{X}$ ± SD	4 ± 0.23	3.83 ± 0.38	3.73 ± 0.34
P	-	NS	0.007*
P_1			NS
RW (U/ml)			
$\overline{X}$ ± SD	3 ± 1.41	301.5 ± 349.6	201.6 ± 254.3
P		0.000*	0.01*
P_1			NS
CRP (mg/dl)			
$\overline{X}$ ± SD	0.31 ± 0.095	1.64 ± 2.25	3.31 ± 3.78
P		NS	0.000*
P_1			0.02*
ESR (mm)			
$\overline{X}$ ± SD	12.9 ± 4.1	43.2 ± 16.8	61.4 ± 29.65
P		0.000*	0.000*
P_1			0.002*
RBCs×10^6/cmm			
$\overline{X}$ ± SD	4.61 ± 0.41	4.24 ± 0.52	4.2 ± 0.55
P		0.013*	0.011*
P_1			NS
Hb (gm/dl)			
$\overline{X}$ ± SD	12.6 ± 0.75	10.97 ± 1.4	10.53 ± 1.6
P		0.000*	0.000*
P_1			NS
WBCs×10^3/cmm			
$\overline{X}$ ± SD	5.73 ± 1.01	5.9 ± 2.14	6.9 ± 3.16
P		NS	NS
P_1			NS

P: Significant difference between controls and each subgroup of patients
P_1: Significant difference between the two subgroups of patients
NS: Not significant
*: Significant

Table (XII): Some parameters in relation to the duration of rheumatoid arthritis (Cont.)

Item	Controls (n = 22)	Patients with duration < 5 y (n = 26)	Patients with duration ≥ 5 y (n = 19)
DAS_{28}			
$\overline{X} \pm SD$		6.14 ± 2.41	7.34 ± 1.97
P			
P_1			NS
RANKL			
$\overline{X} \pm SD$	0.28 ± 0.14	0.79 ± 0.39	0.88 ± 0.63
P		0.000*	0.000*
P_1			NS
OPG			
$\overline{X} \pm SD$	2.56 ± 1.05	2.75 ± 0.89	3.98 ± 1.68
P		NS	0.000*
P_1			0.001*
RANKL/OPG			
$\overline{X} \pm SD$	0.122 ± 0.065	0.319 ± 0.189	0.255 ± 0.221
P		0.000*	0.017*
P_1			NS
Patients with +ve X-ray finding			
+ve finding	n = 0	n = 16	n = 19
-ve finding	n = 22	n = 29	n = 26
P_2			0.002*

P: Significant difference between controls and each subgroup of patients
P_1: Significant difference between the two subgroups of patients
NS: Not significant
*: Significant
P_2: Significant difference between the two subgroups of patients (Qui square)

Table XIII: Some parameter in relation to patients disease activity score (DAS_{28}):

The patients were divided according to the disease activity score into two subgroups: those with $DAS_{28} \leq 3.2$ (mild activity) and those with $DAS_{28} > 3.2$ (high activity).

1- The mean serum levels of Ca^{++} and OPG showed no significant difference between the two subgroups of patients, while RW, CRP, ESR, RANKL, and RANKL/OPG ratio showed significant increase in patients with $DAS_{28} > 3.2$ than their corresponding levels in patients with $DAS_{28} \leq 3.2$. All patients with high activity had morning stiffiness more than 30 minutes and 79.4% of them had positive X-ray finding.

2- In both subgroups of patients, the level of Ca^{++} showed significant decrease than that in controls, while ESR showed significant increase than the corresponding levels in controls.

3- In the subgroup of patients with $DAS_{28} > 3.2$ the levels of RW, CRP, RNAKL, OPG, and RANKL/OPG ratio showed significant increase than their corresponding levels in controls. But these parameters dis not show significant difference between patients with $DAS_{28} \leq 3.2$ and the controls.

Table (XIII): Some parameters in relation to disease activity score of rheumatoid arthritis (DAS_{28})

Item	Controls (n = 22)	$DAS_{28} \leq 3.2$ (n = 11)	$DAS_{28} > 3.2$ (n = 34)
Ca^{++} (mg/dL)			
$\bar{X} \pm SD$	4.01 ± 0.23	3.72 ± 0.31	3.81 ± 0.38
P		0.018*	0.029*
P_1			NS
RW (U/mL)			
$\bar{X} \pm SD$	3 ± 1.41	98.9 ± 153.3	311.2 ± 335.7
P		NS	0.000*
P_1			0.016*
CRP (mg/dL)			
$\bar{X} \pm SD$	0.31 ± 0.095	0.7 ± 0.256	2.87 ± 3.37
P		NS	0.000*
P_1			0.012*
ESR (mm)			
$\bar{X} \pm SD$	12.9 ± 4.1	25.1 ± 7.1	59.2 ± 22.2
P		0.04*	0.000*
P_1			0.000*
RANKL (pmol/L)			
$\bar{X} \pm SD$	0.28 ± 0.14	0.42 ± 0.19	0.96 ± 0.5
P		NS	0.000*
P_1			0.000*
OPG (pmol/L)			
$\bar{X} \pm SD$	2.56 ± 1.05	2.74 ± 1	3.4 ± 1.5
P		NS	0.016*
P_1			NS
RANKL/OPG ratio			
$\bar{X} \pm SD$	0.122 ± 0.065	0.184 ± 0.11	0.327 ± 0.21
P		NS	0.000*
P_1			0.042*

P: Statistical significance between controls and each subgroup of patients.
P_1: Statistical significance between both subgroups of patients.
NS: Not significant.
*: Significant.

Table (XIII): Some parameters in relation to disease activity score of rheumatoid arthritis (DAS_{28}) (Cont.)

Item	Controls (n = 22)	$DAS_{28} \leq 3.2$ (n = 11)	$DAS_{28} > 3.2$ (n = 34)
No. of patients with morning stiffness > 30 minutes			
< 30`		n = 11	n = 0
> 30`		n = 0	n = 34
P_2			0.000*
No. of patients taking corticosteroid therapy			
No	n = 22	n = 8	n = 25
Yes	n = 0	n = 3	n = 9
P_2			NS
No. of patients with positive X-ray finding			
-ve	n = 22	n = 3	n = 7
+ve	n = 0	n = 8	n = 27
P_2			NS

P: Statistical significance between controls and each subgroup of patients.

P_1: Statistical significance between both subgroups of patients.

NS: Not significant.

*: Significant.

P_2: Statistical significance difference between both subgroups of patients (qui square).

Table XIV: Some parameters in relation to presence of positive X-ray findings:

The patients were divided according to the presence of positive X-ray findings into two subgroups: those without positive findings and those with positive findings.

1- The mean levels of Ca^{++}, RW, CRP, ESR, DAS_{28}, RANKL and RANKL/OPG ratio showed no significant difference between the two subgroups of patients, while the level of OPG and duration of rheumatoid arthritis showed significant increase in patients with positive X-ray findings than their corresponding values in patients without positive X-ray findings.

2- In both subgroups of patients, the level of Ca^{++} showed significant decrease than that in controls while, the levels of RW, ESR and RANKL and RANKL/OPG ratio showed significant increase in both subgroups than their corresponding levels in controls.

3- In the subgroup of patients with positive X-ray findings the levels of CRP and OPG showed significant increase than that in controls, but these parameters did not significantly differ between patients without X-ray findings and the controls.

Table (XIV): Some parameters in relation to presence of positive X-ray finding

Item	Controls (n = 22)	Patients without X-ray finding (n = 10)	Patients with X-ray finding (n = 35)
Ca^{++} (mg/dL) $\bar{X} \pm SD$ P P_1	4 ± 0.23	3.76 ± 0.35 0.05* 	3.8 ± 0.37 0.019* NS
RW (U/mL) $\bar{X} \pm SD$ P P_1	3 ± 1.41	203.2 ± 223.9 0.04* 	275.4 ± 335.9 0.000* NS
CRP (mg/dL) $\bar{X} \pm SD$ P P_1	0.31 ± 0.95	1.12 ± 0.6 NS 	2.69 ± 3.4 0.001* NS
ESR (mm) $\bar{X} \pm SD$ P P_1	12.9 ± 4.1	42.4 ± 16.2 0.000* 	53.2 ± 26.1 0.000* NS
DAS_{28} $\bar{X} \pm SD$ P P_1		6.1 ± 2.55 	6.8 ± 2.2 NS
RANKL (pmol/L) $\bar{X} \pm SD$ P P_1	0.28 ± 0.14	0.74 ± 0.37 0.006* 	0.86 ± 0.53 0.000* NS
OPG (pmol/L) $\bar{X} \pm SD$ P P_1	2.56 ± 1.05	2.14 ± 0.69 NS 	3.59 ± 1.4 0.003* 0.001*
RANK/OPG ratio $\bar{X} \pm SD$ P P_1	0.122 ± 0.65	0.376 ± 0.197 0.000* 	0.268 ± 0.201 0.003* NS
Duration of rheumatoid arthritis (years) $\bar{X} \pm SD$ P P_1		1.35 ± 0.87 	7.6 ± 6.5 0.019*

NS: Not significant.
*: Significant.
P: Statistical significance between controls and each subgroup of patients.
P_1: Statistical significance between studied subgroups of patients.

Table XV: Some parameters in relation to the cut off value of RANKL (0.42 pmol/L) in the patients group:

Using a cut off value of RANKL = mean in control group + one SD the patients were divided into: those with RANKL level ≤ 0.42 pmol/L and those with RANKL level > 0.42 pmol/L (high RANKL level).

1- The levels of Ca^{++}, CRP and OPG showed no significant difference between the two subgroups of patients, while the levels of RW, ESR, and DAS_{28} and RANKL/OPG ratio showed significant increase in patients with high RANKL (> 0.42 pmol/L than) that in patients with RANKL ≤ 0.42 pmol/L.

2- In both subgroups of patients, the level of Ca^{++} showed significant decrease than that in controls, while the levels of CRP and ESR showed significant increase than their corresponding levels in the control group.

3- In the subgroup of patients with RANKL > 0.42 pmol/L (high RANKL) the level of RW showed significant increase than that in controls. The level of OPG showed significant increase in patients with RANKL ≤ 0.42 than that in controls, while no significant difference in its level was found between patients with high RANKL and the controls.

Table (XV): Some parameters in relation to the cutoff value of RANKL (0.42 pmol/L) in the patients group

Item	Controls (n = 22)	Patients with RANKL ≤ 0.42 (n = 12)	Patients with RANKL > 0.42 (n = 33)
Ca^{++} (mg/dL)			
$\bar{X} \pm SD$	4.01 ± 0.24	3.7 ± 0.32	3.8 ± 0.38
P		0.023*	0.027*
P_1			NS
RW (U/mL)			
$\bar{X} \pm SD$	3.0 ± 1.41	60.7 ± 53.3	331.6 ± 337.8
P		NS	0.000*
P_1			0.001*
CRP (mg/dL)			
$\bar{X} \pm SD$	0.31 ± 0.095	2.5 ± 4.2	2.3 ± 2.6
P		0.021*	0.006*
P_1			NS
ESR (mm)			
$\bar{X} \pm SD$	12.9 ± 4.1	41.2 ± 25.8	54.4 ± 23.5
P		0.000*	0.000*
P_1			0.05*
DAS_{28}			
$\bar{X} \pm SD$	-	4.8 ± 2.5	7.3 ± 1.8
P			
P_1			0.003*
OPG (pmol/L)			
$\bar{X} \pm SD$	2.56 ± 1.05	3.5 ± 2.2	3.2 ± 1.6
P		0.05*	NS
P_1			NS
RANK/OPG ratio			
$\bar{X} \pm SD$	0.122 ± 0.065	0.133 ± 0.102	0.357 ± 0.192
P		NS	0.000*
P_1			0.000*

NS: Not significant.
*: Significant.
P: Statistical significance between controls and each subgroup of patients.
P_1: Statistical significance between both subgroups of patients.

Table XVI: Some parameters in relation to the cut off value of OPG (3.61 pmol/L) in the patients group:

Using a cut off value of OPG = mean in control group + one SD, the patients were divided into: those with OPG level ≤ 3.61 pmol/L and those with OPG level > 3.61 pmol/L (high level).

1- The levels of RW, CRP, RANKL, and DAS_{28} showed no significant difference between the two subgroups of patients, while the levels of Ca^{++} and RANK/OPG ratio showed significant decrease in patients with high OPG than that in patients with OPG (≤ 3.61). The level of ESR showed significant increase in patients with high OPG than in patients with OPG (≤ 3.61).
2- In both subgroups of patients, the levels of RW, CRP, ESR and RANKL showed significant increase than their corresponding levels in controls.
3- In the subgroup with OPG ≤ 3.61, RANKL/OPG ratio showed significant increase than that in controls, but no significant difference in this ratio was observed between patients with high OPG and controls.

Table (XVI): Some parameters in relation to the cut off value of OPG (3.61 pmol/L) in the patients group

Item	Controls (n = 22)	Patients with OPG ≤ 3.61 (n = 33)	Patients with OPG > 3.61 (n = 12)
Ca^{++} (mg/dl)			
$\overline{X} \pm SD$	4.01 ± 0.24	3.9 ± 0.37	3.6 ± 0.32
P		NS	0.001*
P_1			0.03*
RW (U/ml)			
$\overline{X} \pm SD$	3.0 ± 1.41	243.6 ± 294.4	302.7 ± 372.4
P		0.001*	0.002*
P_1			NS
CRP (mg/dL)			
$\overline{X} \pm SD$	0.31 ± 0.095	2.1 ± 3.1	3.1 ± 3.0
P		0.014*	0.003*
P_1			NS
ESR (mm)			
$\overline{X} \pm SD$	12.9 ± 4.1	45.3 ± 20.1	66.3 ± 29.7
P		0.000*	0.000*
P_1			0.002*
DAS_{28}			
$\overline{X} \pm SD$	-	6.39 ± 2.4	7.5 ± 2.0
P			
P_1			NS
RANKL (pmol/L)			
$\overline{X} \pm SD$	0.28 ± 0.14	0.83 ± 0.51	0.85 ± 0.51
P		0.000*	0.000*
P_1			NS
RANKL/OPG ratio			
$\overline{X} \pm SD$	0.122 ± 0.65	0.329 ± 0.214	0.191 ± 0.129
P		0.000*	NS
P_1			0.017*

NS: Not significant.
*: Significant.
P: Statistical significance between controls and each subgroup of patients.
P_1: Statistical significance between both subgroups of patients.

Table XVII: Some parameters in relation to corticosteroid therapy:

On dividing the patients according to the intake of corticosteroid treatment into two subgroups: those not taking corticosteroid therapy and those taking corticosteroid therapy.

All the parameters did not show any significant difference between the two subgroups of patients.

Table (XVII): Some parameters in relation to corticosteroid therapy

Item	Patients not taking corticosteroid (n = 33)	Patients taking corticosteroid (n = 12)
FSG (mg/dl) $\overline{X} \pm SD$ P_1	95.3 ± 9.4	98.2 ± 10.9 NS
Ca^{++} (mg/dl) $\overline{X} \pm SD$ P_1	3.83 ± 0.37	3.67 ± 0.34 NS
RW (U/ml) $\overline{X} \pm SD$ P_1	289.6 ± 342.1	176 ± 207.1 NS
CRP (mg/dl) $\overline{X} \pm SD$ P_1	2.16 ± 2.6	2.85 ± 4.1 NS
ESR (mm) $\overline{X} \pm SD$ P_1	51.2 ± 25.2	49.8 ± 22.8 NS
DAS_{28} $\overline{X} \pm SD$ P_1	6.58 ± 2.3 NS	6.83 ± 2.3 NS
RANKL (pmol/L) $\overline{X} \pm SD$ P_1	0.88 ± 0.52	0.69 ± 0.42 NS
OPG (pmol/L) $\overline{X} \pm SD$ P_1	3.1 ± 0.88	3.8 ± 2.2 NS
No. of patients with positive X-ray finding -ve +ve P_2	n = 6 n = 27 (81.8%)	n = 4 n = 8 (66.6%) NS

P_1: Statistical significance between both subgroups of patients.
P_2: Statistical significance between both subgroup of patients (qui square).
NS: Not significant.
*: Significant.

Table XVIII: Some parameters in relation to immunosuppressive therapy:

The patients were divided according to the intake immunosuppressive treatment into two subgroups: those not taking immunosuppressive therapy and those taking immunosuppressive therapy.

- All the parameters did not show any significant difference between the two subgroup of patients.

Table (XVIII): Some parameters in relation to immunosuppressive therapy

Item	Patients not taking immuno-suppressive therapy (n = 29)	Patients taking immuno-suppressive therapy (n = 16)
Ca^{++} (mg/dl) $\overline{X} \pm SD$ P_1	3.78 ± 0.35	3.81 ± 0.39 NS
RW (U/ml) $\overline{X} \pm SD$ P_1	224 ± 285.9	323.5 ± 359.4 NS
CRP (mg/dl) $\overline{X} \pm SD$ P_1	2.13 ± 3.1	2.73 ± 3.01 NS
ESR (mm) $\overline{X} \pm SD$ P_1	49.5 ± 24.8	53.3 ± 24.7 NS
DAS_{28} $\overline{X} \pm SD$ P_1	6.5 ± 2.3	6.9 ± 2.3 NS
RANKL (pmol/L) $\overline{X} \pm SD$ P_1	0.78 ± 0.49	0.92 ± 0.52 NS
OPG (pmol/L) $\overline{X} \pm SD$ P_1	3.3 ± 1.6	3.2 ± 0.99 NS

NS: Not significant

*: Significant

P_1: Statistical significance between each subgroup of patients.

Table (XIX): Significant correlations of some studied parameters in the patients group

			r	p
RANKL	#	R.W.	0.512	0.000**
	#	ESR	0.321	0.032*
	#	Morning stiffness	0.373	0.012*
	#	DAS_{28}	0.541	0.000**
OPG	#	Age	0.331	0.027*
	#	Duration of RA	0.353	0.017*
	#	X-ray finding	0.431	0.003**
RANK/OPG ratio	#	R.W	0.343	0.021*
	#	RANKL	0.848	0.000*
	#	OPG	- 0.422	0.002*
	#	DAS_{28}	0.369	0.013*
X-ray findings	#	Duration of RA	0.415	0.005**
	#	OPG	0.431	0.003**
DAS_{28}	#	R.W.	0.364	0.014*
	#	CRP	0.417	0.004**
	#	ESR	0.747	0.000*
	#	RANKL	0.541	0.000**
	#	Morning stiffness	0.803	0.000**
CRP	#	ESR	0.560	0.000**
	#	R.W.	0.368	0.013*
	#	Platelets	0.357	0.016*
	#	Morning stiffness	0.571	0.000*
	#	DAS_{28}	0.417	0.004**
ESR	#	R.W.	0.354	0.017*
	#	CRP	0.560	0.000*
	#	RANKL	0.321	0.032*
	#	Morning stiffness	0.604	0.000*
	#	DAS_{28}	0.747	0.000**
R.W.	#	CRP	0.368	0.013*
	#	ESR	0.354	0.017*
	#	Platelets	0.452	0.002*
	#	RANKL	0.512	0.000**
	#	DAS_{28}	0.364	0.014*
Duration of RA	#	OPG	0.353	0.017*
	#	X-ray finding	0.415	0.005**

* Significant correlation.

Table XX: **Diagnostic performance of both RANKL and OPG:**

The diagnostic performance of RANKL showed high diagnostic specificity, sensitivity, efficiency, and positive likelihood ratio when compared to corresponding values of OPG (using optimal cut off for both items as obtained from ROC curve).

Figure 10: The area under the ROC curve of RANKL (87%) showed significant increase when compared to corresponding value of OPG (67%).

Table XX: Diagnostic performance of both RANKL and OPG

	Sensitivity	Specificity	Predictive value of positive	Predictive value of negative	Efficiency	$^{+}$LR
RANKL	73.33%	81.82%	89.19%	60%	76.1%	4.1
OPG	26.67%	77.27%	70.59	34%	66%	1.4

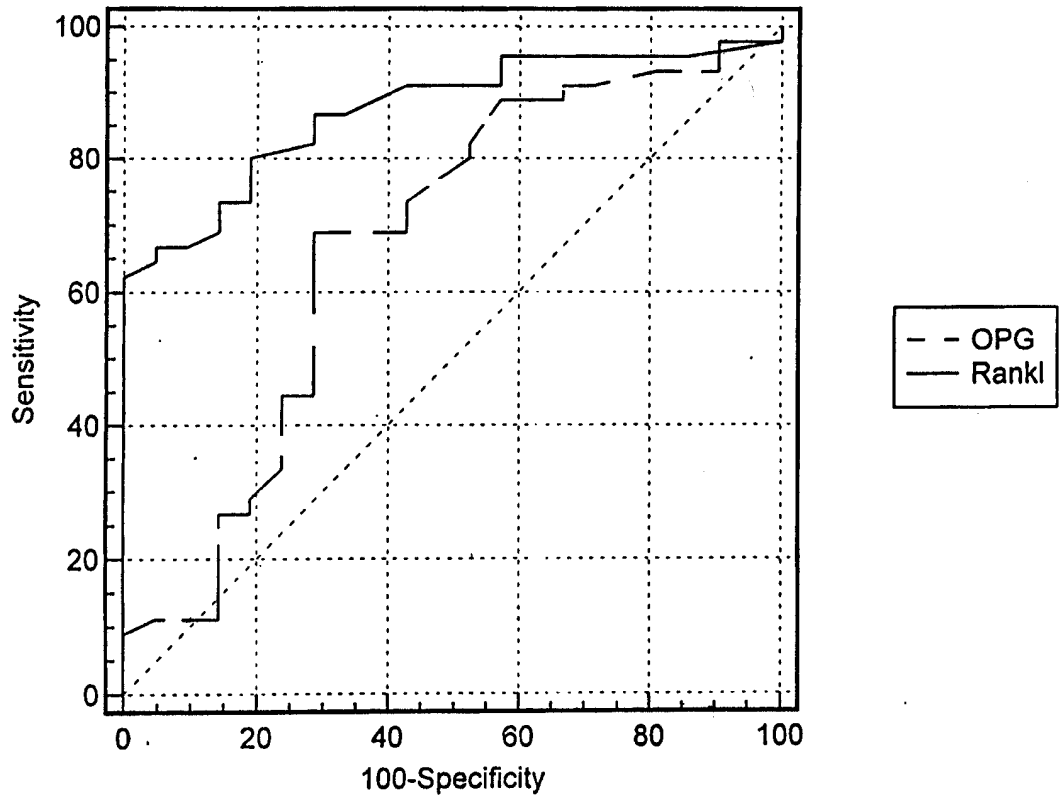


Figure (10): ROC curve of RANKL and OPG

Discussion

DISCUSSION

Rheumatoid arthritis is a common chronic inflammatory and destructive arthropathy that can not be cured. It has substantial personal, social, and economic costs. The long-term prognosis is poor since, 80% of affected patients are disabled after 20 years,[114] and life expectancy is reduced by an average of 3 to 18 years.[115]

Any inflammatory process is usually tightly regulated, involving both mediators that initiate and maintain inflammation and mediators that shut the process down. In states of chronic inflammation, an imbalance between the two mediators leaves inflammation unchecked, resulting in cellular damage. In cases of rheumatoid arthritis, this damage is manifested by the destruction of cartilage and bone.[119]

Three forms of bone disease have been described in rheumatoid arthritis. These include:[151]

1- Focal bone loss affecting the immediate subchondral bones and bones at the joint margins.
2- Periarticular osteopenia adjacent to inflammed joints.
3- Generalized osteoporosis involving the axial and appendicular skeleton.

An understanding of these distinct pathological forms of bone loss has relevance not only with respect to gaining insights into the different pathological mechanisms, but also for developing specific and effective strategies for preventing the different forms of bone loss in rheumatoid arthritis.[151]

Rheumatoid arthritis is a disease characterized by focal bone erosion and systemic bone loss in different regions of the skeleton. The occurrence of bone loss is usually a result from disequilibrium between rate of bone resorption and bone formation.[16]

A general understanding of the cellular and biochemical events associated with skeletal remodeling is essential for defining the pathophysiology of bone loss in RA.[16]

Throughout life the skeleton is in a dynamic state of remodeling, during which the bone remodeling units or the basic multicellular units are resorbed and new bones are formed to replace the resorbed matrix.[18,152] Each remodeling cycle is initiated by the recruitment of osteoclast precursors. These precursor cells subsequently differentiate into active bone resorbing osteoclasts.[16] After cessation of the resorpiton cycle the bone surface is lined by osteoblasts that synthesize new bone matrix. This unmineralized bone matrix, or osteoid, subsequently undergoes mineralization.[16]

Under physiological condition, bone remodeling is a complex process during which osteoblastic bone formation is coupled with osteoclastic bone resorption. It is regulated by systemic hormones and local factors that function together to maintain bone mass.[153]

RANK/RANKL/osteoprotegerin (OPG) system is a novel local cytokine system that is a member of TNF ligand and receptor superfamily.

It plays an important role in osteoclast biology.[34,54] This newly recognized cytokine system consists of:

1- A ligand, RANKL, that exists in both cell-bound and soluble forms.

2- A cell-bound receptor, RANK.

3- A secreted decoy receptor, OPG.[154]

RANKL may promote osteoclast differentiation[155] and activation[156] through RANK binding. Activation of its decoy receptor, OPG, causes opposite effects.[156]

So, osteoprotegerin puts a brake on the entire system by blocking the effects of RANKL and thus inhibits osteoclastogenesis.[86]

RANKL/RANK/OPG system plays an important role in the coupling of osteoblasts with osteoclasts and is under the influence of local factors and hormones. This process provides a mechanism for repair of local microdamage to the skeleton.[156]

RANK/RANKL, and OPG mRNA are expressed in tissues of the RA joint, and the levels of RANKL, RANK and OPG are believed to determine whether osteoclastogenesis will occur or not.[157] The ratio of RANKL to OPG may be a key factor in determining the level of osteoclastic bone resorption.[158,159]

In RA, the infiltration of chronic inflammatory cells, which include macrophages, lymphocytes, and plasma cells is likely to contribute to osteolysis, directly through differentiation of macrophage into osteoclasts

and, indirectly, through release of cytokines that promote osteoclastogenesis and recruitment of macrophage to the joint.[160]

Rheumatoid arthritis is characterized by the presence of inflammatory synovitis and destruction of joint cartilage and bones.[161] The role of the RANKL/RANK/OPG system in cartilage destruction in this disease is an open question. Whether the RANKL/RANK/OPG system has a direct effect on cartilage or whether the changes in the RANKL/OPG ratio directly modulate osteoclastic bone resorption in RA need to be further investigated.[162]

The aim of this study is the estimation of the serum levels of receptor activator of nuclear-factor Kappa β ligand (RANKL) and serum osteoprotegerin (OPG) in rheumatoid arthritis patients and correlation of their levels with the clinical condition and radiological findings.

This study was conducted on 67subjects. They were divided into two groups:

- Control group which included 22 healthy subjects (20 females and 2 males) with a mean age of 45.5 ± 8.1years (Table IIIa). They were selected with matched age, sex, and socioeconomic state as patients and they were selected without history of any arthritic disease.
- Patients group which included 45 rheumatoid arthritis patients (41 females and 4 males) with mean age of 44.7 ± 9.4 years. The duration of RA ranged from 3 months to 22 years. The patients were suffering from fatigue, morning stiffness, and bone deformities. Some patients were

receiving therapy (NSAID, corticosteriods, and immunosuppressive treatment) and other patients did not receive any treatment. (Table IIIb)

The results of the present study revealed that, most of the patients are females and their age ranged from 28 to 60 years, with a mean of 44.7 ± 9.4 year (Table III b, c).

It was reported that female, are more likely to develop RA than males. The frequency of RA increases with age and it peaks in persons aged 35-50 years. However, the disease can be observed in both elderly persons and children.[113]

In the patients group, the levels of haemoglobin and red blood cells count showed a significant decrease when compared with their corresponding levels in the controls. The level of platelets count showed significant increase in patients group than that in controls (Table VII a-c).

Rheumatoid arthritis is a chronic inflammatory disease in which Anaemia of chronic disease is common and correlates with disease activity. It improves with successful therapy. Anaemia may also be related to disease modifying antirheumatic drug (DMARD) therapy. Thrombocytosis is common and is also associated with disease activity.[113]

In present study, the level of ionized Ca (Ca^{++}) showed significant decrease in rheumatoid arthritis patients when compared with corresponding values in the controls (Table IX a-c).

Rheumatoid arthritis is one of the diseases characterized by occurrence of osteoporosis in which bone resorption and turnover are

mediated by activated osteoclasts. Osteoporosis is usually associated with decreased level of total calcium and also ionized Ca (Ca^{++}) which can be controlled by many hormones like parathyroid hormone, and 1, 25 $(OH)_2$ vitamin D_3.[163]

In the patients group of this study, the levels of erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and Rose waaler test showed a significant increase when compared with that in the controls (Table X a-c).

Rheumatoid arthritis disease is usually associated with inflammation especially around the joints.[161] Acute phase reactants usually reflect the condition of inflammation.[164]

Most factors known to stimulate osteoclast formation bind to receptors on stromal cells/osteoblastic cells, rather than binding to receptors on osteoclast progenitors, to induce the release of osteoclast stimulating factors.[165] Two essential factors namely M-CSF and RANKL are supplied by stromal/osteoblastic cells for the differentiation and maturation of osteoclast progenitor.[165]

RANKL, which is expressed on the surface of preosteoblastic/stromal cells, binds to RANK on the osteoclastic precursor cells. RANKL is an essential cytokine in osteobiology, it is critical for the differentiation of bone resorbing osteoclast from monocyte-macrophage precursors and for the survival and function of mature osteoclast.[161,166,167]

The RANKL gene encodes a TNF superfamily molecule. RANKL is formed as a membrane-anchored molecule and can then be released from the cell after proteolytic cleavage by the metalloprotease desintegrin TNF-α convertase.[168]

In the present study, the serum RANKL level was 0.28 ± 0.14 pmol/L in the controls and 0.83 ± 0.5 pmol/L in the patients, showing a significant increase than that in controls (Table XII a-c).

Several studies reported that, serum RANKL level was significantly higher in rheumatoid arthritis patients than that in healthy subjects.[47,51,123,167]

The high levels of serum RANKL in rheumatoid arthritis patients can be explained by the fact that rheumatoid arthritis is a disease with focal and diffuse bone loss. It is a condition of gradual joint destruction related to chronic inflammation with T-cell activation.[161]

The skeletal complications of RA consist of focal bone erosions and periarticular osteoporosis at sites of active inflammation and generalized bone loss with reduced bone mass. In this disease the interaction between the RANKL/RANK/OPG system and T-cells may explain the lesions.[161]

Activated T cells could therefore directly induce osteoclastogenesis through both membrane bound and soluble RANKL.[47,169]

RANKL is a key mediator of joint destruction and bone loss in rheumatoid arthritis[161,170] as well as, binding of RANKL with its receptor RANK results in osteoclastogensis and activation of mature osteoclasts.[48,161]

In present study, there was a positive correlation between serum RANKL and RW, ESR, morning stiffness and disease activity (DAS_{28}) (table XIX), which means that inflammation, disease activity and bone destruction are associated with high level of serum RANKL.

The balance between RANK – RANKL signaling and the levels of biologically active OPG regulates development and activation of osteoclasts and bone metabolism. All factors that inhibit or increase bone resorption via osteoclasts act via regulation of RANKL – RANK and/or OPG – RANKL interaction.[161,167]

OPG functions as a soluble receptor for RANKL and competes with RANK for RANKL binding. Consequently, OPG is an effective inhibitor of osteoclast maturation and osteoclast activation in vitro and in vivo. It has a documented effect on the regulation of bone metabolism and inhibition of bone resorption.[161,171-173]

Osteoprotegerin (OPG) is produced by osteoblastic cells and in the inflammed synovium of RA, it is produced also by dendritic cells, B cells, and other immuno-competent cells.[174] OPG is formed of 401 amino acid but, unlike all other members the family, it lacks a transmembrane domain and represents a secreted TNF receptor.[161]

In the present study, the mean value of serum OPG level was 2.56 ± 1.05 pmol/L in control group and was 3.27 ± 1.4 pmol/L in patients group, showing a significant increase in the patients group (Table XI a-c).

These results are in agreement with some workers who reported an increased level of OPG in rheumatoid arthritis patients. They reported a mean OPG level of 2.2 ± 1.01 pmol/L in controls and 4.2 ± 2.0 pmol/L in patients group.[167]

The presence of raised serum OPG levels in RA patients can be explained as being a protective mechanism against local destructive effects and systemic bone loss caused by RANKL. It could also be explained by activation of osteoclasts.[167] It was found that, serum OPG concentrations are correlated with the severity of bone loss.[175]

In the present study, there was significant positive correlation in the patients group between OPG level and presence of positive X-ray findings (table XIX).

Other workers, reported low level of OPG in rheumatoid arthritis patients than its level in healthy subjects.[51,161,168] This is usually due to excessive bone erosion and cartilage destruction.

A deficiency in OPG expression may have a role in the pathogenesis of the bone erosions which characterizes RA and suggests that OPG may well have a therapeutic role in the future management of RA.[51]

OPG could protect the cartilage by maintaining the underlying subchondral bone, therefore insulating the overlying cartilage from the inflammatory cells infiltrates in the bone marrow. In addition, inhibition of RANKL activity by OPG can prevent cartilage destruction, a critical, irreversible step in the pathogenesis of arthritis.[176]

It was reported that, age affect the inter individual variation of OPG.[177] In the present study, there is a positive correlation between age and the level of serum OPG ($r = 0.331$, $p = 0.02$) (Table XVII). This agreed with other study that reported the same result in both sex.[178]

It was reported that, the longer the duration of the RA disease, the more are the destructive changes that occur. To compensate this high level of destruction, the level of OPG begins to rise.[175]

In the current study, there was a positive correlation between the duration of the disease and the level of serum OPG ($r = 0.35$, $p = 0.02$) (Table XIX). This confirms the role of OPG as a protection mechanism against progression of bone loss.

RANK, RANKL and OPG mRNA are expressed in tissue of the RA joint, and the levels of RANKL, RANK and OPG are believed to determine whether osteoclastogenosis will occur or not.[166]

The ratio of RANKL to OPG may be a key factor in determining the level of osteoclastic bone resorption.[166]

It appears that local alteration rather than systemic changes of RANK/OPG ratio are the critical determinants of bone destruction.[161,179]

In present study ,the level of RANKL/OPG ratio, showed significant increase in patients group(0.292 ± 0.2) than that in control group (0.122 ± 0.06) (Table XI a-c).

The same results were reported by other workers.[161,180] The high ratio of RANKL/OPG in rheumatoid arthritis patients reflect the occurrence of excessive bone resorption and cartilage destruction.[161]

T-cell activation that occurs in rheumatoid arthritis enhances the ratio of RANKL to OPG, hence it promotes osteoclastogenesis and bone loss.[181]

In the present study the level of RANKL/OPG ratio was also increased in subgroups of patients with high disease activity than in those with low activity (Table XIII).

In addition, a significant positive correlation was found in patients group between RANK/OPG ratio and disease activity. ($r = 0.369$, $p = 0.013$) (Table XIX). Also there was positive correlation between this ratio and Rose waaler level ($r = 0.343$, $p = 0.02$). These results reflect the fact that activation of the disease and degree of inflammation are usually associated with high RANKL/OPG ratio, which results from increasing RANKL level with corresponding decrease in OPG level.

Rheumatoid arthritis disease is a chronic disease and subjects suffering from RA cannot be completely cured and their long term prognosis is poor.[114,115]

In the present work, the duration of the disease in the studied patients ranged from 3 months up to 22 years. On classifying these patients into two subgroups according to duration of the disease: one subgroup with duration < 5 years and other subgroup with duration ≥ 5 years (Table XII), there were significant increase in the level of OPG, in addition to the levels of ESR and CRP in patients with long duration (≥ 5) years than patients with duration < 5 years and the controls. At the same time no significant differences could be detected in OPG level between patients with duration < 5 years and the controls (Table XII). This was also proved by the presence of a significant positive correlation between OPG level and the duration of the disease ($r = 0.353$, $p = 0.017$) (Table XIX).

As regard the serum RANKL level, it did not show significant difference between two subgroups of patients in relation to the duration of the disease, but both subgroups showed significantly higher RANKL level than that in controls. This denoted that RANKL level increased markedly from the start of the disease.

The level of ionized Ca (Ca^{++}) showed significant decrease in subgroup with long duration (≥ 5 yrs) than in control group but no significant difference observed between the subgroup with short duration (< 5

yrs) and the controls (Table XII). This means that long duration of the disease affects the status of Ca^{++} and increases the risk of osteoporosis.

It was reported that, in the active osteoclast there is likely a continuous influx of Ca^{++} at the resorptive site, balanced by the its efflux at the basolateral surface. Therefore, an imbalance between this influx and efflux results in a net increase in cytosolic Ca^{++}.[182]

In addition, in the present study, there was a significant positive correlation between duration of the disease and presence of positive X-ray findings ($r = 0.415$, $p = 0.005$) (Table XIX).

All the above data support the concept: the longer the duration of the disease, the greater are the destructive lesions in the bone and joints.

Rheumatoid arthritis (RA) is a chronic, inflammatory, systemic disease, with an unpredictable course, varying from mild to very severe and disabling condition.[137,183] Therefore, to study such a disease, long-term observational studies are needed, with regular assessment of patients using valid and reproducible measures.[137]

Several tools to assess the course of disease in patients with RA were developed and validated. The disease activity score (DAS_{28}) and European league against rheumatism (EULAR) response criteria are examples of these tools. They are widely accepted and used. Using these measurements the course of the disease, prognostic and predictive factors can be studied.[137]

The present study used DAS_{28} score to measure the activity of the rheumatoid arthritis disease. This score is designed as a mathematical formula including number of tender joints, number of swollen joints, ESR levels and state of general health.[137]

Using this score, the patients group was subdivided into two groups, those with low activity ($DAS_{28} \leq 3.2$) and those with high activity ($DAS_{28} > 3.2$).

In the current study, the levels of ESR, CRP and RW showed significant increasing values in patients with high activity than their corresponding levels in those with low activity (Table XIII). In addition, the levels of both RANKL and RANKL/OPG ratio showed also significant increase in patients with high activity than both patients with low activity and control group. As regards the level of OPG, there was significant increase in its level in the group with high activity than that in the controls, but there was no significant difference between the group with low activity and controls or between both subgroups of patients. This means that level of OPG was significantly increased only in active rheumatoid arthritis. These findings were in accordance with other workers.[167,184-186] However, other workers reported a decreased level of OPG in active RA.[51,176] No significant difference could be found in levels of RW, CRP, RANKL, and RANKL/OPG ratio between patients with low activity and controls, while only ESR showed significant increase, denoting that ESR is a sensitive test for RA disease.

These findings were also confirmed by the presence of significant positive correlation between disease activity score (DAS_{28}) and RANKL

(r = 0.541, p = 0.000), ESR (r = 0.747, p = 0.000), CRP (r = 0.417, p = 0.004), RW (r = 0.364, p = 0.014) (Table XIX). This supports the idea that RANKL, CRP, and RW can be used as indicators for disease activity.

A major role in bone erosion has been attributed to the RANKL released by activated lymphocytes and osteoclasts.[161] Haynes *et al* (2001), showed an increased expression of RANKL in tissues surrounding bone erosions from patients with active RA.[157] Other workers reported that, the highest levels of RANKL were detected in patients with RA with active synovitis.[161]

The local bone erosion and systemic bone loss are the hallmarks of rheumatoid arthritis and cause progressive disability. Chronic inflammation and bone loss are closely linked. This is impressively illustrated in RA, a chronic inflammatory disease of the joints, which is characterized by synovial inflammation and cartilage and bone destruction.[187]

Bone erosions are mostly present in the hands and wrists but also knees, feet, elbows, hips and the cervical spine can be also involved in RA.[113]

Radiographs can be used as an outcome measure to evaluate the bony changes that occur in RA and to assess the severity and progression of RA, and to establish the effects of treatment. They also provide a permanent record with which the disease can be serially evaluated.[188]

In the present work, X-ray on both hands and wrists were done for all patients. The positive x-ray findings include signs of osteoporosis, signs of erosions and hand deformities. In present study 10 patients had normal X-ray, 35 patients (77.8%) showed signs of osteoporosis along or associated with

other bone lesions: 21 of them showed signs of bone erosions and 10 patients were presenting with hand deformities (Table IV).

In the current study, the patients were divided according to the presence of positive X-ray findings into two subgroups: those without positive findings and those with positive findings. Only OPG level showed significant increase in patients with positive X-ray finding than patients without positive finding and the controls (Table XIV). Also there was a significant positive correlation between OPG level and the presence of positive X-ray findings ($r = 0.431$, $p = 0.003$) (Table XIX).

However, the ESR, RW and RANKL level showed significant increase and ionized calcium showed a significant decrease in both subgroups of patients (with and without x-ray finding) than their corresponding levels in controls, but no significant difference between both subgroups. (Table XIV).

In the present study, a cut off value of RANKL was calculated from the mean RANKL levels in controls + 1SD (cut off = 0.42 pmol/L). Rheumatoid arthritis patients were subdivided according to this cut off value into two subgroups (Table XV): patients with RANKL level (≤ 0.42) (12 patients) and patients with high RANKL level (> 0.42) (33 patients).

The levels of ESR, RW, disease activity (DAS_{28}) and the RANKL/OPG ratio: showed significantly increased levels in patients with *high* RANKL level than those with RANKL (≤ 0.42) level. This means the

high activity of the disease usually associated with high RANKL level. Whereas the mean value of OPG did not significantly differ between patients with high or low RANKL levels. At the same time OPG level in patients with RANKL (≤ 0.42) showed significant increase than the corresponding values in the controls. This may point to the fact that OPG is a decoy receptor that neutralize the effect of the RANKL.

RANKL/OPG ratio showed significantly increased level in patients with high RANKL rather than those patients with low RANKL. This high ratio is mainly due to the increased production of RANKL, since the level of OPG in these patients did not significantly differ from that in controls.

In addition , a cut off value for OPG level was calculated from the mean of OPG level in the control group + 1SD (cut off = 3.61 pmol/L). The patients were subdivided according to this cut off value into two subgroups (Table XVI): patients with OPG (≤ 3.61 pmol/L) and patients with high OPG (> 3.61 pmol/L).

Only ESR level showed significantly increased level in patients with OPG > 3.61 (high level) than that in those with OPG ≤ 3.61 (Table XVI). Since ESR is the sensitive index of disease activity, this result reflects and supports the concept that increased disease activity in rheumatoid arthritis patients is associated with increased level of OPG to counteract increased level of RANKL. OPG acts as a compensatory mechanism against bone destruction and joint inflammation.

In addition, the level of RANKL/OPG ratio showed significant increase in patients with low OPG level than those with high OPG level. This increase is due to presence of significant increase in RANKL level in addition to the decreased OPG level (Table XVI). While the normal ratio of RANKL/OPG in patients with high OPG level is net result of both increased RANKL and OPG production.

The ionized Ca (Ca^{++}) level showed significantly decreased value in patients with high OPG than in both controls and patients with OPG (≤ 3.61). This can be explained by the protective and compensatory effect of OPG against occurrence of osteoporosis and bone loss.

Glucocorticoids are potent anti-inflammatory drugs and are commonly used in patients with RA to bridge the time until disease modifying anti rheumatic drugs (DMARDS) are effective.[113] It is well known that long-term glucocorticoid therapy causes osteoporosis.[189]

Glucocorticoids decrease bone formation by: inducing osteocyte and osteoblast apoptosis, inhibiting osteoblast bone matrix synthesis, decreasing proliferation and differentiation of periosteal precursor cells. Also glucocorticoid therapy is a potent activator of osteoclasts.[163] It was suggested that glucocorticoids promote osteoclastogenesis by inhibiting OPG production, inducing RANKL expression, and enhancing the ratio of RANKL to OPG and thus accelerate bone resorption and induce bone loss.[176,181,190]

In the current study, some patients were on corticosteroid therapy and they did not stop the treatment at the time of investigation. They received only small doses for a short time. So patients were divided into those taking corticosteroid therapy (n = 12), and those not taking corticosteroid therapy (n = 33) (Table XVII).

In the patients receiving corticosteroid therapy the ESR, R.W and RNAKL levels showed relatively lower values when compared with their corresponding levels in those not receiving it, while the level of serum OPG showed relatively higher levels . However all these changes did not reach the level of significance. The result of increased OPG level confirm its protective mechanism and at same time its counteracting effect on RANKL level which show low values in patients receiving corticosteroid therapy than those not receiving it.

The immunosuppressive drugs or disease modifying antirheumatic drugs (DMARDS) are important measure to successful treatment of RA. It can retard or prevent disease progression and hence, joint destruction and subsequent loss of function.[113]

In present work, 16 patients are not taking immunosuppressive treatment and 29 patients are taking immunosuppressive treatment (Table XVIII). No significant changes could be found in the previous parameters between both subgroups. This may denote that immunosuppressive therapy does not affect the parameters of activity and inflammation which occur in such disease.

Finally, to sum up, the levels of RANKL in RA patients was positively correlated with morning stiffness, ESR (which is the most sensitive marker for disease activity), RW and disease activity score (DAS_{28}). Also patients with high RANKL level showed high RW and ESR levels than the corresponding levels in those with normal RANKL level. In addition, on evaluating the diagnostic performance of both RANKL and OPG as markers of bony lesions in RA, in the current study, RANKL had a diagnostic sensitivity of 73.3%, specificity of 81.8%, its predictive value of positive was 89.19% and its positive likelihood ratio was 4.1 (Table XX). In addition the area under the ROC curve was 87%, therefore, RANKL could be used as both a diagnostic marker for bony lesion in RA and an indicator for disease activity.

The OPG had a diagnostic sensitivity of 26.6%, specificity of 77.3%, its predictive value of positive was 70.6%, its positive likelihood ratio was 1.4 and the area under ROC curve was 67%. So it can not be used as a diagnostic marker for bony lesions in RA.

Therefore, these results could confirm the suggestion that RANKL is the principal mediator of bone destruction in human arthritis. A mechanism between T-cell activation, cytokine production, as well as osteoclast activation and joint destruction, via RANKL/OPG/RANK system, could explain the spectrum of skeletal disorders in RA.[167]

Summary and Conclusions

SUMMARY AND CONCLUSIONS

Rheumatoid arthritis is a common chronic inflammatory and destructive arthropathy that can not be cured. It has substantial personal, social, and economic costs. The long-term prognosis is poor, since 80% of affected patients are disabled after 20 years.

Rheumatoid arthritis disease is characterized by local bone erosion and systemic bone loss in different regions of skeleton. The occurrence of bone loss is usually a result of disequilibrium between rate of bone resorption and bone formation.

Under physiological condition, bone remodeling is a complex process during which osteoblastic bone formation is coupled with osteoclastic bone resorption and is regulated by systemic hormones and local factors that function together to maintain bone mass.

The receptor activator of nuclear factor-κB (RANK)/ its ligand (RANKL)/osteoprotegerin (OPG) system is a novel local cytokine system that is a member of TNF- receptor and ligand superfamily. It plays an important role in osteoclast biology. This newly recognized cytokine system consists of (1) a cell-bound receptor or RANK, (2) a ligand, RANKL, that exists in both cell-bound and soluble forms and (3) a secreted decoy receptor, OPG.

RANKL may promote osteoclast differentiation and activation through RANK. Activation of its decoy receptor, OPG, causes opposite effects i.e. inhibition of osteoclastogenesis.

The RANK/RANKL/ and OPG mRNA expressed in tissues of the rheumatoid arthritis (RA) joints and the levels of RANKL, RANK and OPG are believed to determine whether osteoclastogenesis will occur or not.

Rheumatoid arthritis is characterized by the presence of inflammatory synovitis and destruction of joint cartilage and bones. The role of the RANK/RANKL/OPG system in cartilage destruction in this disease is an open question.

The aim of this work was to estimate the serum levels of receptor activator of nuclear-factor kappa B ligand (RANKL) and serum osteoprotegerin (OPG) in rheumatoid arthritis patients and to correlates their levels with the clinical condition and radiological findings.

Sixty seven subjects were included in the present study. Forty five rheumatoid arthritis patients were selected with duration of the disease ranging from 3 months to 22 years and compared to a group of twenty two healthy subjects with matched age, sex and socioeconomic state. Most of the patients were suffering from fatigue, morning stiffness, joint pain and bone deformities. Some of them were receiving therapy and other patients did not receive any treatment.

To all studied subjects, thorough history taking and clinical examination with special stress on joint pain, swelling and morning stiffness as well as history of taking non steroidal anti-inflammatory drugs, corticosteroid therapy or immunosupressive drugs. Calculation of disease activity was done using disease activity score (DAS_{28}). Also plain X-ray for both hands and wrists was done.

Laboratory investigations were done to both controls and rheumatoid arthritis patients. It included complete blood cells count and determination of serum levels of ESR, glucose, creatinine, uric acid, total and ionized Ca, inorganic phosphate, serum activity of alanine aminotransferase (ALT), CRP, Rose Waaler (RW), and detection of antideoxyribonucleoprotein antibodies (DNA). Also serum levels of receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG) were estimated as two of the local bone regulatory proteins.

The study revealed:

1- That the levels of haemoglobin and red blood cells count were significantly lower in rheumatoid arthritis patients than their corresponding levels in the controls. Anaemia of chronic disease is common in rheumatoid arthritis and usually correlates with disease activity.

2- The level of ionized Ca was significantly lower in rheumatoid arthritis patients when compared to corresponding values in the controls. This is due to the occurrence of osteoporosis which is usually present in such patients.

3- The levels of ESR, CRP and RW were significantly higher in rheumatoid arthritis patients than in controls. These results confirm that rheumatoid arthritis is one of the diseases characterized by inflammation especially around the joints and associated with increased levels of acute phase reactants.

4- The receptor activator of nuclear factor kappa B ligand (RANKL) was significantly higher in the rheumatoid arthritis patients than that in

controls. RANKL level was also positively correlated with RW, ESR, morning stiffness and DAS_{28}. Since RANKL is a key mediator of joint destruction and bone loss in rheumatoid arthritis. Its high level in such patients is a reflection of disease activity, excessive bone resorption and chronic inflammation present around the joints.

5- The serum OPG levels were significantly higher in rheumatoid arthritis patients than their corresponding levels in the control group. Serum OPG level was also positively correlated with the duration of the disease and X-ray findings.

The OPG functions as a soluble receptor for RANKL. It competes with RANK for RANKL binding. The presence of high OPG levels in R.A. patients can be explained as being a protection mechanism against local destructive effects and systemic bone loss of RANKL. The longer the duration of the disease, the more are the radiological findings and the more the increase in OPG level to compensate for the excessive bone destruction.

6- The RANKL/OPG ratio was significantly higher in rheumatoid patients than the controls. This increased ratio in patients with R.A. reflects the high levels of both RANKL (mainly) and OPG in such patients. The ratio was also positively correlated with the levels of disease activity (DAS_{28}) and Rose Waaler. At the same time it was negatively correlated with the OPG level. As this ratio may be a key factor in determining the level of osteoclastic bone resorption, these results point to the relation of this high ratio of RANKL/OPG with the activation of the disease and the degree of inflammation.

7- On sub-grouping the patients:

a- The levels of ESR, CRP, RW, RANKL, and RANKL/OPG ratio were significantly higher in patients with high disease activity

(DAS_{28} > 3.2) than their corresponding levels in those with low disease activity. These findings were confirmed by the presence of significantly positive correlation between disease activity score (DAS_{28}) and RANKL, ESR, CRP and RW. Also the OPG in patients with high activity was significantly increased than that of controls, while its level in patients with low disease activity did not significantly differ from its level in controls. This means that OPG was significantly increased in only active stage of R.A. Therefore, beside the CRP and ESR, RANKL and OPG can be used as indicators for disease activity.

b- In addition, both levels of RANKL and ratio of RANKL/OPG showed significant increase than controls in both subgroups of patients when sub-grouped according to the duration of the disease (< 5 ys and ≥ 5 ys), with no significant difference between the two subgroups. The same results were observed when the patients were sub-grouped according to the presence or absence of positive X-ray findings. This denotes that the increase in both levels of RANKL and RANKL/OPG ratio in R.A. patients occurs regardless the duration of the disease and the X-ray findings.

c- On other hand, the OPG levels showed significant increase in only patients with long duration of the disease and those with positive X-ray findings than the corresponding levels in control group. Also serum OPG showed significant increase in subgroups with longer duration and positive X-ray findings than in those with short duration or those without positive X-ray findings.

d- The levels of ESR, RW, DAS_{28} and RANKL/OPG ratio were significantly higher in patients with high RANKL levels than those with low RANKL levels. While in those with high OPG only the

level of ESR was significantly increased than that in patients with low OPG.

e- On other hands in patients with low OPG levels, RANKL/OPG ratio was significantly higher than those with high OPG, denoting that the increased level of RANKL/OPG ratio is a net result of increased RANKL production and decrease OPG expression.

Finally on evaluating the diagnostic performance of both RANKL and OPG in diagnosing bone insult in RA patients, for RANKL, its diagnostic sensitivity was 73.3%, diagnostic specificity was 81.8%, predictive value of positive results was 89.2% and $^{+}$LR was 4.1. Therefore RANKL could be used as a diagnostic marker for bone lesion in RA patients. While for OPG, its diagnostic sensitivity was 26.6%, diagnostic specificity was 77.3%, predictive value for positive results was 34% and $^{+}$LR was 1.4. So it can not be used as a diagnostic marker for bone lesion in R.A.

In conclusions:

1- RANKL is a key mediator of joint destruction and bone loss in rheumatoid arthritis. It can be used as a marker for bone erosion and disease activity.

2- Adopting the cut off value of RANKL (0.42 pmol/L), RANKL is a convincing diagnostic test for both bone destruction and disease activity of rheumatoid arthritis.

3- OPG plays a role in the protection mechanism against bone erosion and joint destruction which are mediated by high RANKL level. This may help to develop new pharmacologic options for the treatment of the manifested osteoporosis and bone erosion occurring in such disease.

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Protocol

بسم الله الرحمن الرحيم

STUDY OF THE SERUM LEVEL OF RECEPTOR ACTIVATOR OF NUCLEAR FACTOR – KAPPA B LIGAND (RANKL) AND OSTEOPROTEGERIN (OPG) IN PATIENTS WITH RHEUMATOID ARTHRITIS

دراسة مستوى الرابط للعامل النووى المنشط للمستقبلات (ر.ن.ك.ل.) والاستيوبروتيجرين فى مصل الدم لمرضى التهاب المفاصل الروماتويدى

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In

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خطة بحث مقدمة إلى
معهد البحوث الطبية
جامعة الإسكندرية
إيفاءاً جزئياً للحصول على

درجـــــة الدكتوراه

فـــى

كيمياء الباثولوجيا

من

الطبيبة / مروى كمال محمد الغندور
معيدة بقسم الكيمياء الباثولوجية
بكالوريوس الطب والجراحة، جامعة الإسكندرية، ١٩٩٧
ماجستير كيمياء الباثولوجيا ٢٠٠٣
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INTRODUCTION

Bone is a metabolically active organ that undergoes continuous remodeling throughout life.[1,2] Bone remodeling cycle involves a series of highly regulated steps that depend on the interactions of two cell lineages: the mesenchymal osteoblastic and hematopoietic osteoclastic lineage.[1,3]

The development and differentiation of osteoblasts and osteoclasts are controlled by many factors, i.e. systemic hormones and locally produced cytokines, growth factors and some specific proteins.[4,5] These proteins are in the family of tumor necrosis factor (TNF) receptors.[6] They are Receptor Activator of Nuclear Factor-Kappa B (RANK) and its ligand (RANKL) and the osteoprotegerin (OPG).[5,7,8]

The osteoblastic stromal cells produces RANKL in response to a variety of stimuli that are essential for the formation of mature osteoclasts. The development of osteoclasts require close interaction between osteoclast precursor (osteoclast progenitor) which have RANK on their surface and osteoblastic stromal cells which produce RANKL.[9-11] Human RANKL exists in two forms: cellular membrane-bound form and soluble circulating form.[12] RANKL is also known as osteoprotegerin ligand (OPGL) as it can bind with osteoprotegerin.[11,12]

On the osteoclast progenitor, RANK, which is a membrane-bound TNF receptor can recognize RANKL through a direct cell to cell interaction of osteoblastic stromal cells with osteoclast progenitor cells.[8,10] RANK is essential for the transduction of signals that lead to osteoclast differentiation.[13]

Osteoprotegerin (OPG) which is also produced by the osteoblastic stromal cells,[5,10] recognizes RANKL and blocks the interaction between RANK and RANKL, leading to an inhibition of osteoclast differentiation and activation.[14,15] OPG deficiency leads to severe osteoporosis due to increased RANK to RANKL interaction and hence enhanced osteoclastic differentiation and function.[16] Therefore, the RANK/RANKL/OPG systems have an important role in many bone diseases associated with osteoporosis.[17,18] They can be also involved in many immune mechanisms as their interaction was expressed in dendritic cells and T-lymphocytes.[19-21]

Rheumatoid arthritis (RA) is a chronic inflammatory disease that occurs due to genetic factors, immuno-regulatory abnormalities and/or autoimmunity. Its pathologic hallmark is synovial membrane proliferation associated with erosion of the articular cartilage and subchondral bone.[22] Patients with RA develop both generalized and periarticular osteoporosis which are believed to be associated with increased production of inflammatory cytokines and enhanced formation and activation of osteoclasts.[18]

Scanty reports were found regarding the RANKL[23,24] or OPG[25] expression in synovial tissues of patients with RA. However, no available data about serum levels of RANKL or OPG. Therefore, it is worth to study their serum levels in rheumatoid arthritis patients as they can likely play an important role in bone erosion and osteoporosis found in such patients.

AIM OF THE WORK

This study aims to estimate the serum levels of receptor activator of nuclear factor Kappa B ligand and osteoprotegerin in patients with rheumatoid arthritis. Correlations of their levels with the clinical condition and radiological finding will also be done.

SUBJECTS

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

- **Control group:** It will include twenty normal adult apparently healthy volunteers of matched age, sex and socioeconomic state as patients. They will be selected with no history of any form of arthritis.
- **Patients group:** It will include forty adult patients with rheumatoid arthritis. They will be selected according to the criteria of the American Collage of Rheumatology for Rheumatoid Arthritis.[26] At least fifteen of these patients will be chosen with clinical picture of active synovitis (based on joint pain, swelling and presence of synovial effusion).[25]

Patients with liver or renal disease, gout, systemic lupus erythromatosus, malignancy and acute infection will be excluded from the study.

METHODS

To all subjects, the following will be done:

(A) Full history taken, with special stress on joint pain, stiffness and swelling as well as history of taking non-steroidal anti-inflammatory drugs or corticosteroid therapy.

(B) Thorough physical examination especially for the joints.

(C) Calculation of the rheumatoid arthritis disease activity index.[27,28]

(D) Plain X-ray for both hands and wrists.

(E) Laboratory investigations:

1- Prelimenary tests which include complete blood picture,[29] erythrocyte sedimentation rate (ESR),[29] estimation of the fasting serum levels of glucose,[30] creatinine,[31] uric acid,[31] ionized calcium[32] and inorganic phosphorus[33] as well as determination of serum alanine aminotransferase activity.[34]

2- Detection of rheumatoid factor (by Rose Waller test), C-reactive protein and anti-DNA antibodies.[35]

3- Estimation of the serum level of RANKL by ELISA technique.[36]

4- Estimation of the serum OPG level by ELISA technique.[37]

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Arabic Summary

الملخص العربى

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إلتهاب المفاصل الروماتويدى من الأمراض الشائعة التى يصاحبها إلتهاب وتآكل فى المفاصل التى يصعب معالجتها وهو يشكل عبء نفس واجتماعى ومادى وكذلك فإن ٨٠٪ من المرضى يصابوا بالإعاقة بعد عشرين سنة من بداية المرض.

يتسم التهاب المفاصل الروماتويدى بتآكل بؤرى للعظام حول المفاصل ونقص فى كثافة العظام فى أماكن متفرقة من الهيكل العظمى.

غالباً ما يكون النقص فى كثافة العظام نتيجة لخلل فى الإتزان بين معدل تكوين العظام وهدمها. وتعتبر عملية نمو العظام وإعادة تكوينها من العمليات الديناميكية المعقدة التى تتطلب توازناً بين عمليتى تكوين العظام وهدمها وهذا التوازن يتم تحقيقه عن طريق كل من العوامل المركزية والعوامل الموضعية.

يعتبر نظام العامل النووى المنشط للمستقبلات (ر.ن.ك)، والرابط للعامل النووى المنشط للمستقبلات (ر.ن.ك.ل) والاستيوبروتيجرين إفراز خلوى جديد وهو عضو فى عائلة الرابط لعامل تحلل الورم – ألفا.

يتكون هذا الإفراز الخلوى الجديد من:

١- الرابط للعامل النووى المنشط لمستقبلات (ر.ن.ك.ل) الذى يوجد متصلاً بالخلية أو غير متصل بها.

٢- مستقبل متصل بالخلية (ر.ن.ك).

٣- مستقبل مفرز (أوستيوبروتيجرين).

يساعد (ر.ن.ك.ل) على زيادة النشاط الهدمى لخلايا العظام عن طريق (ر.ن.ك). أما تنشيط المستقبلات (الاستيوبروتجرين) فله تأثير عكسى وهى زيادة النشاط البنائى لخلايا العظام.

يفرز الحامض النووى (ر.ن.ك) و(ر.ن.ك.ل) فى أنسجة المفاصل المصابة بالإلتهاب الوماتويدى ومستويات هذه العوامل الموضعية تساعد على معرفة ما إذا كان النشاط الهدمى لخلايا العظام سوف يحدث أم لا.

يتصف الإلتهاب الروماتويدى بوجود التهاب للأغشية المبطنة للمفاصل وتدمير لعظام والغضاريف حول المفاصل. ولذلك فإن دور (ر.ن.ك) و(ر.ن.ك.ل) والأستيو بروتجرين فى تدمير المفاصل فى هذا المرض موضع سؤال.

ولقد كان الهدف من هذا البحث هو دراسة مستويات (ر.ن.ك.ل) والاستيوبروتجرين فى مرضى الالتهاب الروماتويدى وإيجاد علاقة بين مستوياتهم وبين الحالة الإكلينيكية ونتائج الأشعة السينية.

وقد شملت هذه الدراسة سبعة وستون شخصاً منهم إثنين وعشرون شخصاً سليماً كمجموعة ضابطه وخمس وأربعون مريضاً مصابون بالتهاب المفاصل الروماتويدى وكانت كلا المجموعتين متقاربتين فى السن والجنس والمستوى الاجتماعى والاقتصادى. معظم المرضى يعانون من إرهاق وتيبس فى المفاصل خاصة فى فترة الصباح، آلام فى المفاصل وتشوهات عظمية. بعضاً منهم تلقى علاج وآخرون لم يتلقوا أى علاج.

وقد تم عمل فحصاً إكلينيكياً شاملاً التاريخ المرضى لكل شخص مع التركيز على آلام المفاصل وتورمها وحدوث أى تيبس فيها بالإضافة إلى معرفة إذا كان المرضى يأخذون علاج أم لا وكذلك حساب نشاط المرضى باستخدام معامل نشاط المرضى وقد تم عمل أشعة سينية على عظام اليد لإكتشاف أى تغير فى العظام وقد اشتملت الفحوص المعملية على قياس مستوى كل من الجلوكوز والكرياتينين وحامض النقرص والكالسيوم الكلى والمتأين والفسفور غير العضوى وكذلك نشاط إنزيمات المحولات الأمينية بالإضافة إلى قياس مستوى البروتين التفاعلى س، والـ روز–ولر وقياس الأجسام المضادة للحامض النووى.

كما تم تقييم الرابط للمعامل النووى المنشط للمستقبلات (ر.ن.ك.ل) والاستيوبروتيجرين كمنظمات موضعية للعظام.

وقد أسفرت النتائج عن أن مستوى الهيموجلوبين وخلايا الدم الحمراء كان منخفضاً فى مجموعة المرضى عنه فى المجموعة الضابطة وهذا يؤكد أن فقر الدم مصاحب لمرضى التهاب المفاصل الوماتويدى. ويزيد حدوثه مع زيادة نشاط المرض.

وكان مستوى الكالسيوم المتأين منخفضاً فى مجموعة المرضى عنه فى المجموعة الضابطة وهذا غالباً نتيجة لهشاشة العظام المصاحبة لهؤلاء المرضى.

كما أظهرت النتائج أن مستوى سرعة الترسيب والبروتين التفاعلى س والروز ولر إرتفاعاً ملحوظاً فى مجموعة المرضى عنه فى المجموعة الضابطة وهذا لأن مرضى المفاصل الروماتويدى يتميز بالتهابات شديدة حول المفاصل.

أما مستوى الرابط للعامل النوى المنشط للمستقبلات (ر.ن.ك.ل) فقد كان مرتفعاً إرتفاعاً ملحوظاً فى مصل المرضى عنه فى المجموعة الضابطة وكان هناك إرتباطاً بين مستواه ومستوى سرعة الترسيب والروز ولر ونشاط المرض. وهذا يؤكد أن الـ (ر.ن.ك.ل) يعطى صورة واضحة عن الالتهابات المصاحبة للمفاصل بالإضافة إلى تأكل العظام التى تحدث فى هذا المرضى.

وقد أظهر مستوى الأستيوبروتيجرين إرتفاعاً ملحوظاً فى مجموعة المرضى عنه فى المجموعة الضابطة وكان هناك ارتباطاً بين مستواه ومدة المرض وكذلك بين مستواه وتغير العظام الذى يظهر فى الأشعة السينية. زيادة الأوستيوبروتيجين فى هؤلاء المرضى قد يكون من أجل الحماية ضد حدوث تآكل العظام البؤرى ونقصان كثافة العظام. فكلما زادت مدة المرض كلما زاد النقصان فى كثافة العظام وزاد مستوى الأوستيوبروتيجرين ليعادل الزيادة فى النشاط الهدمى لخلايا العظام.

وقد أظهر مستوى نسبة (ر.ن.ك.ل) إلى الأوستيوبروتيجرين إرتفاعاً ملحوظاً فى مجموعة المرضى عنه فى المجموعة الضابطة وكان هناك إرتباط بين هذه النسبة والـ (ر.ن.ك.ل) ونشاط المرض والروز ولر ولكن كان هناك ارتباط عكسى بين هذه النسبة ومستوى الأوستيوبروتيجرين وهذه النتائج توضح أن هذه النسبة هى المفتاح الذى يحدد حدوث تآكل العظام وزيادة نشاط المرض.

وتبعاً لنشاط المرض تم تقسيم مجموعة المرضى إلى مجموعتين الأولى ذات نشاط عالى والثانية ذات نشاط منخفض.

وقد ظهر أن مستوى سرعة الترسيب والبروتين التفاعلى س والروز ولر و(ر.ن.ك.ل) وكذلك نسبة ر.ن.ك.ل إلى الأستيوبروتيجرين أعلى فى المجموعة ذات النشاط العالى عنه فى مجموعة ذات النشاط المنخفض ولكن مستوى الأستيوبروتيجرين كان أعلى فى المجموعة ذات النشاط العالى عنه فى المجموعة الضابطة وفى نفس الوقت لا يوجد اختلاف ملحوظ بين مستواة فى المجموعة ذات النشاط المنخفض و المجموعة الضابطة و هذا يعنى أن مستوى الأستيوبروتيجرين يزيد فقط مع زيادة نشاط المرض وهذا يمكن أن نستخدمه لنجعل من هذه الإختبارات أحد دلالات الزيادة فى نشاط المرض.

بالإضافة إلى هذا فقد ظهر أن مستوى ر. ن. ك. ل ومستوى نسبة ر. ن. ك. ل إلى الأستيوبروتيجرين ليس له علاقة بمدة المرض أو جود دلائل إشعاعية ولكن من جهة أخرى ثبت أن مستوى الأستيوبرتيجرين أعلى فى وجود دلائل إشعاعية أو مع زيادة مدة المرض.

وقد أظهر مستوى سرعة الترسيب والروز ولر ونشاط المرض وكذلك نسبة (ر.ن.ك.ل) إلى الأوستيوبروتيجرين إرتفاعاً ملحوظاً فى المرضى الذين يعانون من نسبة عالية فى (ر.ن.ك.ل) عنه فى المرضى المصابون بنسبة منخفضة فى (ر.ن.ك.ل).

ومن جهة أخرى أظهر مستوى سرعة الترسيب فقط إرتفاعاً ملحوظاً فى المرضى المصاحبون لنسبة عالية من الأوستيوبروتيجرين عنه فى المرضى المصاحبون لنسبة منخفضة من الأستيوبروتيجرين.

ولكن مستوى نسبة ر.ن.ك.ل إلى الأستيوبورتيجرين منخفضاً فى المرضى المصاحبون بنسبة أوستيوبروتيجرين منخفضة عنه فى المرضى المصابون بنسبة أوستيوبروتيجرين عالية.

وهذا يؤكد أن زيادة نسبة ر.ن.ك.ل إلى الأستيوبروتيجرين غالباً بسبب زيادة فى إفراز ر.ن.ك.ل وإنخفاض فى الأوستيوبروتيجرين.

ونستخلص من النتائج السابقة ما يلى:

١- ر.ن.ك.ل يعتبر أحد المسببات لتآكل العظام والمفاصل التى تحدث فى مرضى لتهاب المفاصل الورماتويدى ويمكن أن يستخدم كأحد الدلالات على زيادة تآكل العظام وزيادة نشاط المرض.

٢- الأستيوبروتيجرين يلعب دوراً فعالاً كمعامل حماية ضد حدوث تآكل العظام بسبب زيادة ر.ن.ك.ل وهذا يمكن إستخدامه فى التوصل إلى خيارات دوائية لعلاج هشاشة العظام وتأكلها التى تحدث فى هذا المرض.

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جامعة الإسكندرية

دراسة مستوى الرابط للعامل النووى المنشط للمستقبلات (ر.ن.ك.ل) والاستيوبروتجرين فى مصل الدم لمرضى التهاب المفاصل الروماتويدى

مقدمة من

مروى كمال محمد الغندور
بكالوريوس الطب والجراحة - جامعة الإسكندرية، ١٩٩٧
ماجستير كيمياء الباثولوجيا ، معهد البحوث الطبية، جامعة الإسكندرية، ٢٠٠٣

للحصول على درجة

الدكتوراه

فى

كيمياء الباثولوجيا

لجنة المناقشة والحكم على الرسالة **موافقون**

أ.د / ثناء فتحى مغازى
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.....

الأستاذة الدكتورة/ منى محمد أباظة
أستاذ الباثولوجيا الكيميائية
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.....

الأستاذة الدكتورة/ وفاء سعد رجب
أستاذ الباثولوجيا الكيميائية
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.....

الأستاذة الدكتورة/ إقبال محمد أبو هاشم
أستاذ الباثولوجيا الإكلينيكية
كلية الطب
جامعة المنصورة

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دراسة مستوى الرابط للعامل النووى المنشط للمستقبلات (ر.ن.ك.ل) والاستيوبروتجرين فى مصل الدم لمرضى التهاب المفاصل الروماتويدى

رسالة

مقدمة إلى معهد البحوث الطبية - جامعة الإسكندرية

إيفاءاً جزئياً لشروط الحصول على درجة

الدكتوراه

في

كيمياء الباثولوجيا

مقدمة من

مروى كمال محمد الغندور

بكالوريوس الطب والجراحة - جامعة الإسكندرية، 1997

ماجستير كيمياء الباثولوجيا ، معهد البحوث الطبية، جامعة الإسكندرية، 2003

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جامعة الإسكندرية

2005