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Biochemical Role of Bone Morphogenetic Protein and Oxidative Damage in the Rheumatoid Arthritis

A Thesis

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By

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بسم الله الرحمن الرحيم

(فَفَهَّمْنَاهَا سُلَيْمَانَ وَكُلاًّ آتَيْنَا حُكْماً

وَعِلْماً وَسَخَّرْنَا مَعَ دَاوُودَ الْجِبَالَ

يُسَبِّحْنَ وَ الطَّيْرَ وَ كُنَّا فَاعِلِينَ)

سورة الأنبياء 79

Supervisor Certification

We certify that this thesis was prepared under our supervision at the College of Medicine /University of Kerbala, as a partial fulfillment for the requirement for the degree of Master in Clinical Chemistry

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Dedication

To my family, who always picked me up on time and encouraged me to go on every adventure, especially this one.

Lina abbas

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This Master thesis would not have finished without help of many people within Marjan medical city, Imam Hujja Hospital, and department of Chemistry and Biochemistry - College of Medicine/University of Kerbala.

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Summary

Background: Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammation of the synovial membrane that can lead to joint deformity and physical disability. The biomarkers that studied in RA were vary and they have significant role in the diagnosis, prognosis of treatment, monitorization of the disease activity, and prediction of the response to biologic therapy. Bone morphogenetic proteins (BMPs) are a members of the transforming growth factor beta (TGF-β) superfamily and they induce the formation of cartilage and other connective tissues.it can induce bone formation *in vitro* and at heterotopic sites *in vivo*. It has been reported their largely important role in embryogenesis, early prenatal skeletal formation and development, further to their associated with a number of human skeletal disorders. This study was aimed to investigate the role of bone morphogenetic protein 2 in rheumatoid arthritis patients in relation to the standard and general markers for diagnostic the disease.

Material and method: divided into two group included a case control study of (100) subject: (60) patient withe rheumatoid arthritis and as, (40) healthy control group. patients with Rheumatoid arthritis were selected from the biological treatment centre at Marjan medical city in Babylon. Measurement of serum human bone morphological protein 2, Malondialdehyde level and Superoxide dismutase activity were performed using Enzyme-Linked Immunosorbent Assay Technique. Quantitative measurement of Trace Elements and electrolytes were determined using Flameless Atomic absorption spectrophotometer method by the technique of Graphite Furnace (GFAAS).

Results: Hypernatremia, hypokalemia and hypomagnesemia were presented in RA patients. Decrease level of BMP2 was associated with RA, The median level of BMP2 in patients group was (0.15 ng/ml while in control was 2.95 ng/ml). Furthermore, results were indicated high levels of oxidative stress marker (MDA) and low antioxidant activity. Receiver operating characteristics analysis of BMP2 was also shown a specificity 57% and high sensitivity 95% towards rheumatoid arthritis with Area Under Curve (0.824) at an optimal threshold >0.12 ng/ml.

Conclusion: The study concluded that BMP2 and MDA/SOD ratio have relation with RA. It could provide a view of the complex phenomenon represented by key contributors to the disease outcome, and an increased awareness should be attributed to these biomarkers.

List of Contents

List of Tables

List of Figures

List of Abbreviations:

Abbreviations	Meaning
ACPA	anti-citrullinated protein/peptide antibody
anti-CarP	Anti-carbamylated protein
anti-CCP	anti-cyclic citrullinated peptide
anti-MCV	anti-mutated citrullinated vimentin
AUC	area under the roc curve
AUP	Area Under Curve
B cells	bone marrow cells
BMI	Body mass index
BMPs	bone morphogenetic proteins
CRP	C-reactive protein
ELISA	Enzyme-Linked Immunosorbent Assay
ESR	Erythrocyte sedimentation rate
GDF	growth and differentiation factor
HLA-DRB1	Major Histocompatibility Complex, Class II, DR Beta 1
HRP	horseradish peroxidase
IgA	Immunoglobulin A
IL	Interleukin
MDA	Malondialdehyde
MMP	Matrix metalloproteinases
MOD	Moderate intensity
NOX	NADPH oxidase
RA	Rheumatoid arthritis
RF	Rheumatoid factor
ROC	Receiver operating characteristics
ROS	Reactive oxygen species
SMAD	Small mothers against decapentaplegic
SOD	Superoxide dismutase
T cells	thymus cells
TCA	Trichloroacetic Acid
TGF-β	transforming growth factor β
Th	T helper cells
TNF-RI	Tumor necrosis factor receptor 1
TNF-α	tumor necrosis factor alpha
WHO	World Health Organization

CHAPTER ONE

Introduction and literature review

1. Introduction

1.1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic gradual inflammatory autoimmune disease, It is accomplished by articular, extra-articular, and systemic influences. In developed countries, RA nearly affects 0.5-1% of adult population [(1)]. Some individuals with RA have a moderate self-limited illness, and many others suffer from severe physical impairment, joint destruction, and various co-morbidities [(2)]. Patients with RA have mortality rates that are more than twice as high as the general population [(3)], and this disparity seems to be expanding [(3)]. In the pathogenesis of RA, T cells, B cells, and the coordinated interaction of pro-inflammatory cytokines play significant roles [(4)]. tumor necrosis factor alpha and interleukin 6 (IL-6) are the cytokines most directly involved in this mechanism and IL-1 and IL-17 may also play significant roles [(4)].The assessing of disease severity in terms of structural injury found the percentages of men and women is similar in this erosive disease [(5)] [(6)]. It has been reported that the predictor of inability in females sex and the developed of inability is three times quicker in females [(7)]. Women exhibited a higher Health Assessment Questionnaire for Rheumatoid Arthritis score and a higher prevalence of impairment [(8)], these difference were related to differences in muscular strength and pain score. Due to the fact that women have lower muscle strength than males, RA has a larger influence on functional capacity in this group. Sex hormones also play a significant role in the differences between gender. The severity of RA has negative correlation with androgen levels, which is a potential exegesis for the lower severity of the disease in men [(9)]. Testosterone inhibits the humoral and cellular responses through interacting with the immune system [(10)].

The primary tissue affected by RA is the synovium of the joint. In healthy individuals, the synovial membrane is comprised of a thin cell layer of synovial fibroblasts and macrophages, which maintain homeostasis in the joint. In RA, synovial inflammation leads to the development of a thick cellular layer called the pannus, which begins to grow over the articular surface. Synoviocytes and infiltrating immune cells, most notably neutrophils, macrophages, and T- and B-lymphocytes, fill the inflamed joint with pro-inflammatory cytokines, chemokines, prostaglandins and cartilage-degrading enzymes such as metalloproteinases [(11)]. destroying cartilage as well as driving an inappropriate activation of osteoclasts to resorb bone, ultimately leading to permanent destruction of the joint [(12)]. The disease progresses in a variety of ways, but the majority of patients develop a chronic, progressive disease causing pain, joint destruction, and inability [(13)] [(14)]. A 70% of patients with RA have developed erosive joint damage [(15)].

Table (1-1) summarizes some potential pathological agents. The inflammatory process causes pain, fatigue, a restricted range of motion in joints, and a loss of muscle strength [(16)]. Clinical measures of disease activity and functional ability improve substantially after starting treatment. Although the inflammatory activity improves and the disease stabilizes, joint damage and functional ability deteriorate [(17)].

Table (1-1) Indicators and potential pathological agents

1) Diagnostic Indicators :

1) Rheumatoid factor (RF), anti-mutated citrullinated vimentin (anti-MCV) and anti-cyclic citrullinated peptide (anti-CCP) [18].
2) The 14-3-3 proteins act like chaperonins [19].
3) Carbamylated proteins (anti-CarP) [20] .

2) Prognostic Indicators :

1) High levels of RF were associated with an greater risk of developing RA [21].
2) Extra-articular manifestations are correlated with the existence of the IgA isotype [22].
3) The existence of anti-CCP at diagnosis was correlated with a more significant radiological progression and severe shapes of illness [23].
4) The existence of other indicators was correlated with more severe shapes of RA, such as antiMCV and 14-3-3 eta protein, but these investigations are not commonly recommended and need to be included in health guidelines [24].

3) Indicators for the monitorization of the disease activity [25] :

Parameters	**MBDA: multi biomarker disease activity test**
1) Tender joints	Acute phase reactants: CRP, SAA
2) Swollen joints	Adhesion molecules:VCAM-1
3) Patient global assessment of disease activity	Cytokines and related proteins:IL-6 and TNF-RI
4) Clinician global assessment of disease activity	Matrix metalloproteinases (MMP): MMP-1 and MMP-2
5) ESR or CRP	Human cartilage glycoprotein 39
Other MBDA : Growth factors:EGF and VEGF-A and Hormones: leptin and resistin	

4) Predictive indicators of the response to biologic therapy:

1) Anti-CCP [26]
2) Anti-MCV [27]
3) 14-3-3 eta protein [28]
4) Cartilage oligomeric matrix protein (COMP) [29].
5) Serum calprotectin [30].
6) Survivin [31].

1.1.1. BASIC ASPECTS OF THE SYNOVIAL MEMBRANE

The synovium is the central player in RA pathogenesis. A normal synovium (figure 1-1A) consists of two distinct layers, the lining or intimal layer and the sublining or subintimal layer. The lining layer is the superficial layer that is in contact with the intra-articular cavity and produces lubricious synovial fluid (SF) [32]. It is one to three cell-layers deep, containing macrophage-like type A synoviocytes and fibroblast-like type B synoviocytes. The sublining layer consists of scattered blood vessels, fat cells and fibroblasts residing in a matrix of fibrils and proteoglycans together with occasional mononuclear cells. When inflamed (figure 1-1B) the synovium undergoes profound changes, resulting in an increased volume and surface on macroscopic evaluation with accumulation of an inflammatory SF in the joint space. The lining layer becomes hyperplastic and forms an aggressive front termed “pannus” at the cartilage-bone junction, leading to the characteristic RA bone erosions. Important changes also occur in the sublining layer with massive mononuclear infiltration and blood vessel formation [32] (figure 1-2).

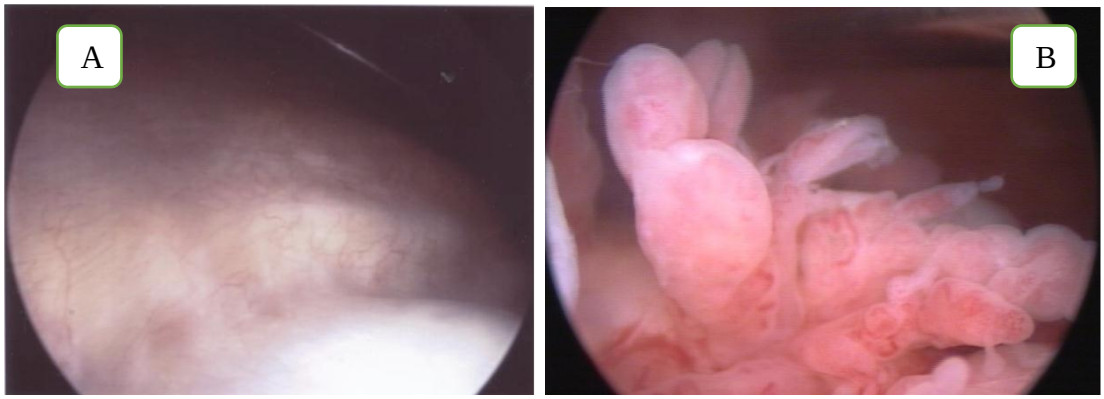


Figure (1-1) A-Normal and B-abnormal synovial membrane distinct layers. [32]

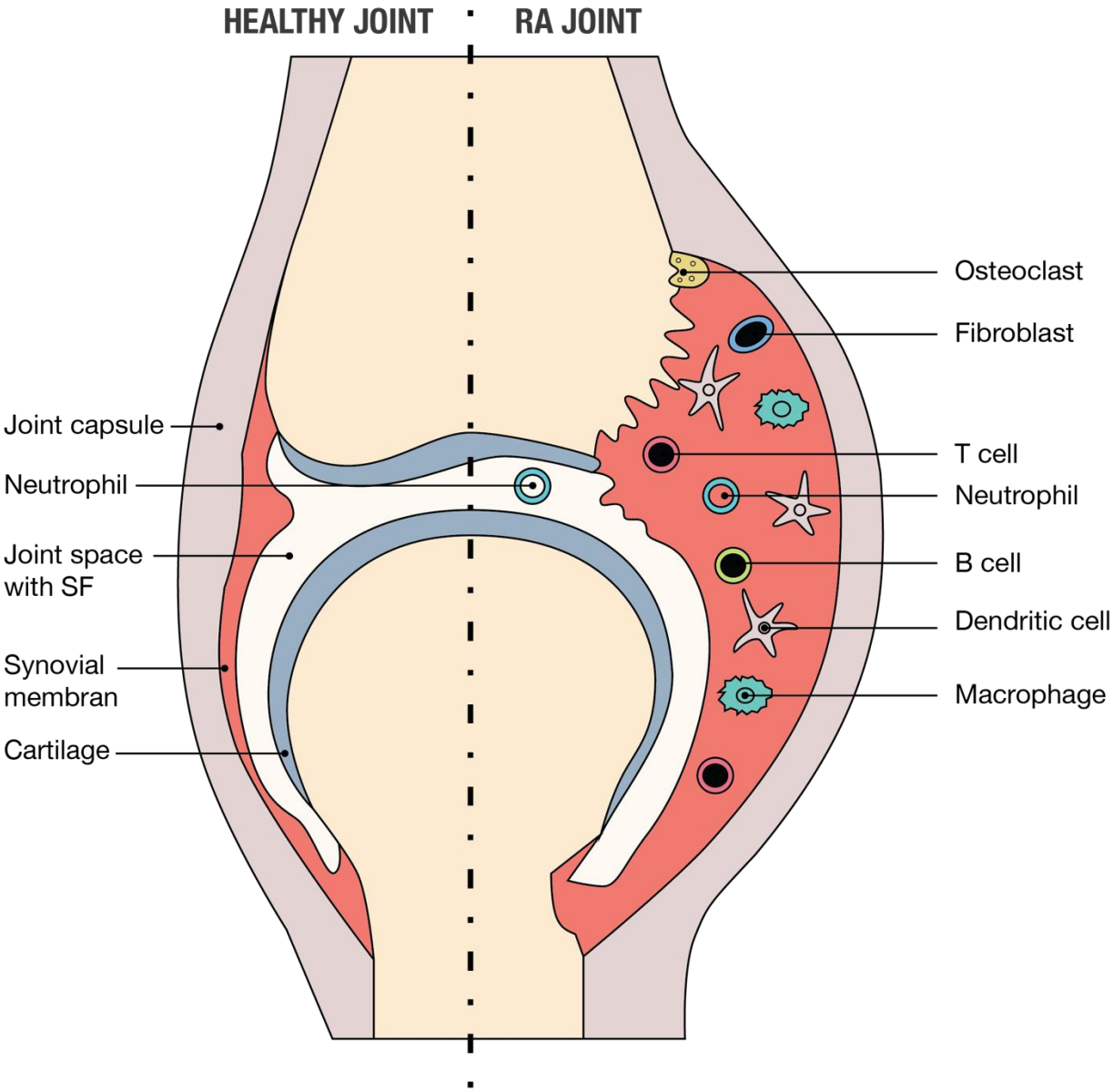


Figure (1-2) schematic representation of a healthy joint & an inflamed RA joint. [32]

Increased joint influx and defective cell death (apoptosis) of resident cells are the mechanisms responsible for these cellular changes [33].And result in local accumulation of pro inflammatory cytokines (such as TNF).These cytokines further contribute to activation of synovial cells and perpetuation of chronic inflammation. The massive hyperplasia of the synovial membrane

during inflammation does not only cause the majority of signs and symtoms of RA but also determines the outcome of the disease.

1.1.2. Pathogenesis and Pathophysiology

The pathogenesis and pathophysiology of RA are a combination of biological markers and environmental factors complicate the understanding of RA and its systemic effect on the body. In adults, RA is typically classified into one of two types: seropositive rheumatoid arthritis or seronegative rheumatoid arthritis. Blood tests identifying the presence of rheumatoid factor (RF) and antibodies to citrullinated protein antigens (ACPAs) indicate seropositive RA [(34)]. Antibodies to citrullinated proteins are pathogenic autoantibodies produced by B memory cells in the body humoral adaptive immune system. The adaptive immune system is characterized by lymphocytes, T and B cells, and dendritic cells that target pathogens in the body. Antibodies to citrullinated proteins are highly specific for RA because they attack extracellular citrullinated protein antigens that are produced in response to inflammation in body tissues and organs [(35)] [(36)] . Recent studies suggest that the presence of these autoantibodies may inadvertently amplify inflammation, directly enhancing arthritis in the body [(37)]. Various sources indicate that these autoantibodies may be present years before the onset of RA-related symptoms occur, signifying the potential for systemic effects of RA that cannot be determined by clinical phenotypes [(38)] [(36)].

In contrast to seropositive RA, seronegative RA patients are ACPA negative. In 2010, the American College of Rheumatology redefined the classification criteria for seronegative patients. The

criteria deemed that seronegative patients must show inflammation in 10 or more joints to meet the criteria for an RA diagnosis. Joint inflammation occurs when the immune system attacks and thickens the synovium lining around the joints. If inflammation around the joints persists, the cartilage and bone within the joint can be permanently damaged.

The second component that adds to the complexity of the development and progression of RA is the interaction between the body immune response to environmental factors. Smoking, infectious agents, periodontal disease, the gastrointestinal microbiome, and adverse life events are all related to the onset of rheumatoid arthritis [(39).] Reports indicate that smoking, one of the strongest known risk factors for RA, may perpetuate the production of citrullinated proteins, resulting in the subsequent development of ACPAs [(40)] .

Synovitis occurs when leukocytes infiltrate the synovial compartment. Leukocyte accumulation primarily reflects migration rather than local proliferation. Cell migration is enabled by endothelial activation in synovial microvessels, which increases the expression of adhesion molecules and chemokines. Accordingly, neoangiogenesis, which is induced by local hypoxic conditions and cytokines, and insufficient lymph angiogenesis, which limits cellular egress, are characteristic features of early and established synovitis [(40)]. These micro environmental changes, combined with profound synovial architectural reorganization and local fibroblast activation, permit the buildup of synovial inflammatory tissue in rheumatoid arthritis (Fig. 1-3).

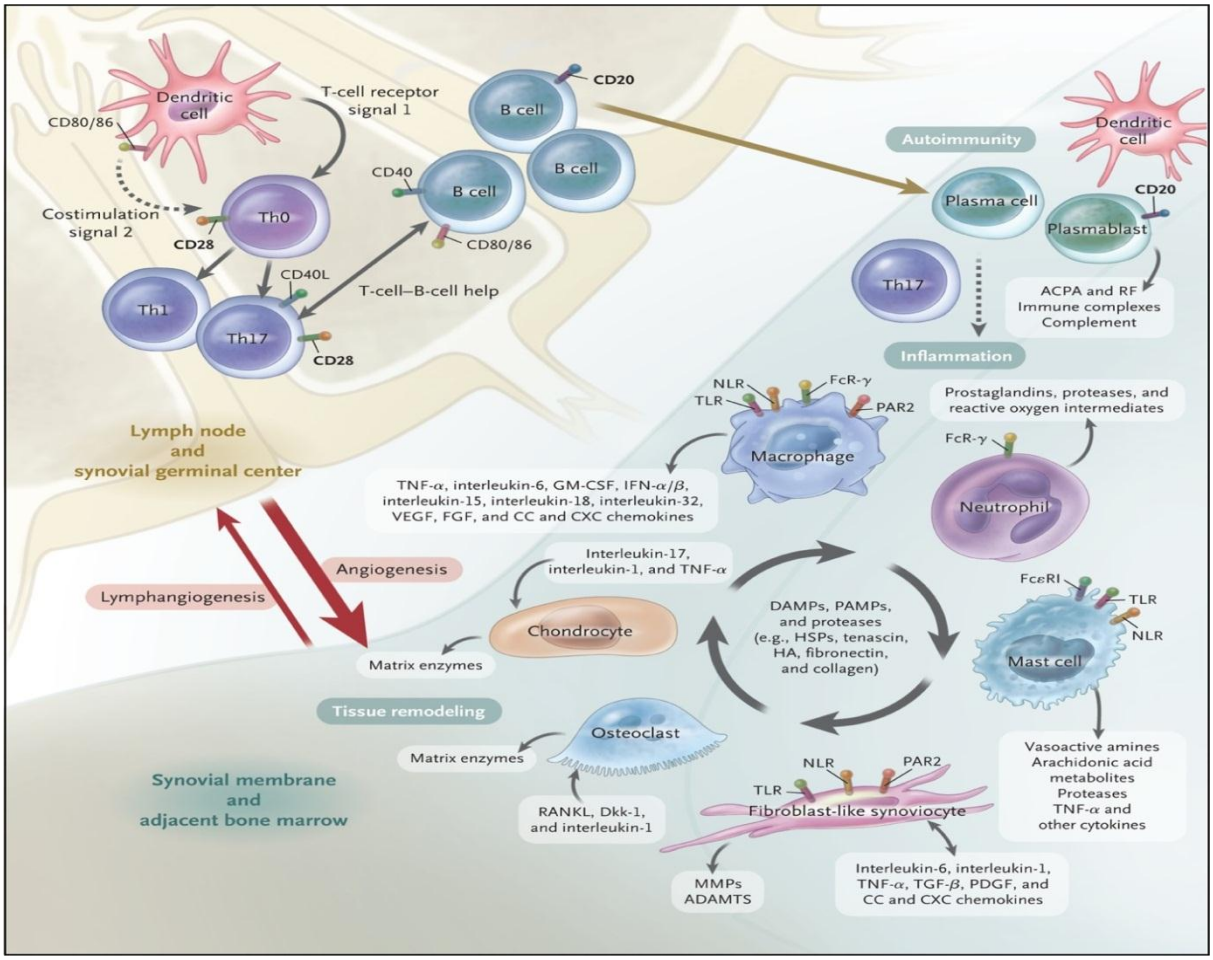


Figure (1-3). Adaptive and Innate Immune Processes within the Joint in Rheumatoid Arthritis. (40)

The co-stimulation-dependent interactions among dendritic cells, T cells, and B cells are shown as occurring primarily in the lymph node; these events generate an autoimmune response to citrulline-containing self-proteins. In the synovial membrane and adjacent bone marrow, adaptive and innate immune pathways integrate to promote tissue remodeling and damage. Positive feedback loops mediated by the interactions shown among leukocytes, synovial fibroblasts, chondrocytes, and osteoclasts, together with the molecular products of damage, drive the chronic phase in the pathogenesis of rheumatoid arthritis (41).

1.1.3. Criteria for rheumatoid arthritis

rheumatoid arthritis criteria was revised and intended for classification purposes rather than diagnostic criteria table(1-2) [42].

Table(1-2): Criteria for rheumatoid arthritis

1. Morning stiffness of • 60 minutes*
2. Arthritis in three or more joint areas*
3. Arthritis in hand joints (wrist, MCP, PIP)*
4. Symmetrical arthritis (wrists, MCPs, PIPs, MTPs)*
5. Rheumatoid nodules
6. Positive rheumatoid factor test
7. Typical X-ray findings in hand- or wrist joints

1.1.4. The Progression of Rheumatoid Arthritis

Table (1-3):The Progression of Rheumatoid Arthritis: As RA progresses, the body changes. Some changes can see and feel, while others cannot. Each stage of RA comes with different treatment goals

Early stage RA stage I:

- It is characterized by synovitis , causing swelling of involved joints and pain upon motion. During this stage, there is a high cell count in synovial fluid as immune cells migrate to the site of inflammation. There is generally no x-ray evidence of joint destruction, with the exception of swelling of soft tissues and possibly evidence of some bone erosion [43] .
- Many people feel joint pain, stiffness, or swelling. There's inflammation inside the joint. The tissue in the joint swells up. There's no damage to the bones, but the joint lining, called the synovium, is inflamed. [44]

Stage II:

- There is a spread of inflammation in synovial tissue, affecting joint cavity space across joint cartilage. This inflammation will gradually result in a destruction of cartilage, accompanied by a narrowing of the joint [43]
- The synovium's inflammation causes damage to the joint cartilage. Cartilage is tissue that covers the end of bones at the site of joints. When cartilage is damaged, people may experience pain and loss of mobility. Range of motion in the joints may become limited. [44]

Stage III:

- It is marked by formation of pannus in the synovium. Loss of joint cartilage exposes bone beneath the cartilage. These changes will become evident on x-ray, along with erosions around the margins of the joint. Joint deformities may also become evident [43].
- Once RA has progressed to stage 3, it's considered severe. At this point, damage extends not only to the cartilage but also to the bones themselves. Since the cushion between bones is worn away, they'll rub together. There may be more pain and swelling. Some people may experience muscle weakness and more mobility loss. The bone can be damaged (erosion), and some deformity may occur. [44]

Stage IV:

- Stage IV is called terminal or end stage RA. The inflammatory process has subsided and formation of fibrous tissue and/or fusing of bone results in ceased joint function. This stage may be associated with formation of subcutaneous nodules [43].
- There's no longer inflammation in the joint. This is end-stage RA, when joints no longer work. People may still experience pain, swelling, stiffness, and mobility loss. There may be reduced muscle strength. The joints may become destroyed, and the bones become fused together (ankylosis). Progression through all four stages can take many years, and some people don't progress through all stages within their lifetime. Some people have periods of no RA activity. In some cases, this may mean that RA has gone into remission [44].

1.1.5. Articular Manifestations of RA

Articular (pertaining to a joint or joints) inflammation and destruction due to RA is generally considered to be the primary symptom indicating disease activity. The destruction of the joints can lead to functional decline and increase the risk of comorbidity in various systems in the body [(38).] Clinicians look for symmetrical inflammation of the small joints in the hands and feet during an initial evaluation of patient symptoms [(45)]. Synovial inflammation can become so severe that the patient experiences a drastic decrease in muscle mass, referred to as rheumatoid cachexia. Studies suggest that rheumatoid cachexia is directly related to the presence of pro-inflammatory cytokines in the joints of patients with RA [(46).]

Cytokines are proteins released by cells that interact with other cells. Cytokines can be either anti-inflammatory or pro-inflammatory, with evidence supporting that specific pro-inflammatory cytokines are directly involved with pathogenic pain [(47).]The elevated presence of cytokines in inflamed joints serve as helpful biomarkers for therapies directed towards mitigating the progressive destruction of the joints and extreme muscle loss seen in patients with rheumatoid cachexia. Understanding the influence of cytokines on the primary articular manifestations of RA is paramount towards formulating therapies that both attack pro-inflammatory cytokines to prevent destruction, and also to limit pathogenic pain [(47)]. Reducing patient pain and fatigue is key to hindering the magnitude of co-morbidities and extra-articular manifestations that tend to develop throughout the disease.

1.1.6. Extra-Articular Manifestations

Extra-articular manifestations are the associated symptoms and conditions of RA that are not related to the articular joints or musculoskeletal system of the body [48]. While these are considered secondary symptoms to the articular manifestations in the synovial linings, they are not to be confused with complications. Approximately 40% of RA patients present extra-articular symptoms, with the onset occurring at any stage in the disease and with the likelihood of occurrence equal amongst both men and women [48] [45]. The extra-articular manifestations in RA patients most commonly and severely impact the heart, lungs, larynx, and vascular systems. Involvement with extra-articular organs positively correlates with the severity of the disease, contributes to increased mortality, and is influenced by genetic and environmental factors [48] [49].The extra-articular involvement within the major organs of the body make these secondary symptoms of RA dangerous and yet are often overlooked.

Recent literature highlights the critical role of inflammation of the vasculature and its correlation with an increased risk of cardiovascular disease and mortality. Vascular leakage and atherosclerosis are examples of two vascular diseases that result from inflammation and a buildup of fatty material on the arterial walls. Both these diseases are associated with RA and contribute to high cardiovascular risk. The inflammatory proteins that cause synovitis, inflammation of the synovial linings of the joints, also attributes to vasodilation of the blood vessels and a decrease in overall blood pressure [50].

1.2. Bone Morphogenetic Protein (BMPs)

Bone Morphogenetic Protein were initially discovered in the pioneering work of Urist in 1965, who discovered that their activity caused ectopic bone formation [51]. Although Urist was able to separate the bone-inducing proteins subsequently [52] , another two decades were taken for these proteins to be separately cloned and characterized [53] .

1.2.1. Classification of BMPs

The transforming growth factor beta (TGF-β) superfamily of secreted growth and differentiation factors has members known as bone morphogenetic proteins (BMPs). BMP-2, BMP-3 (osteogenin), BMP-3β (growth and differentiation factor 10, GDF-10), BMP-4 (BMP-2β), BMP-5, BMP-6, BMP-7 (osteogenic protein-1, OP-1), BMP-8α (OP-2), BMP8β, BMP-9 (GDF-2), BMP-10, BMP-11 (GDF-11 or myostatin), BMP-12 (GDF-7), BMP-13 (GDF-6), BMP-14 (GDF-5), BMP-15 (GDF-9β), BMP-16, BMP-17, and BMP-18 are all members of the BMP family [54].

BMP-1, despite its role as a protease that cleaves procollagen, is not considered a member of the BMP family [55] . The mature proteins of the BMP family can be classified into subgroups based on their amino acid sequence homologies. There are at least four BMP subgroups that are well characterized in figure (1-4) [54]. BMP-2 and BMP-4 have 92 % amino acid sequence homology among the subgroups, while BMP-5, BMP-6, BMP-7, and BMP-8 have 90% sequence homology [56]. The mature proteins in these two categories have a sequence homology of 55-65 % [57].

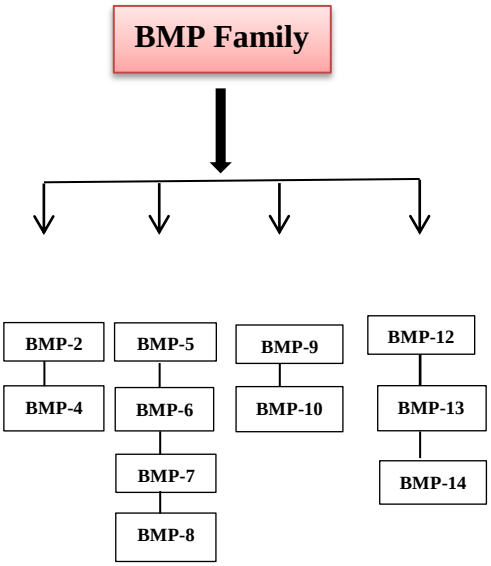


Figure (1-4): BMP subgroup The well-known subgroups within the BMP family, grouped based on the similarity in the amino acid sequence homology of the mature domain (54)

1.2.2. Signaling and regulatory components in the BMP pathway

There are 15 known BMPs are divided into subgroups based on evolutionary analyses of amino acid and nucleotide similarity creates particular subgroups of these ligands. It is vital to remember that BMP-1 is not the same as the rest of the BMP family (58). BMP-1 is not a member of the TGF-β superfamily of proteins, despite its ability to stimulate bone and cartilage growth. Instead, it is a procollagen C-proteinase that aids in the development of collages (59).

The rest of the BMP proteins family act as signaling ligands , binding to cell surface target receptors. BMPs are first generated as precursor proteins in the cytoplasm before being released into the extracellular region, where they become active. These pre-pro-

peptides are made up of an N-terminal signal peptide required for secretion, a prodomain that regulates the folding of the active protein, and a C-terminal part containing the mature peptide capable of binding to and activating its receptors [60].

In the cytoplasm, BMP precursors create dimers, which are then cleaved by convertases to produce the mature BMP ligand, which is secreted into the extracellular space. BMP ligands bind to their receptors on the surface of target cells once they have been secreted, forming a heterotetramic complex with two dimers of type I and type II receptors.

This complex formulation permits the type II receptor to transphosphorylate the type I receptor, and promotion of the type I receptor causes conformational alterations that lead to phosphorylation of downstream R-Smad proteins. R-Smads that have been activated and phosphorylated in the cytoplasm form complexes with co-Smad (Smad4) and then translocate to the nucleus, where they interact with coactivators and corepressors to regulate gene expression [61]. Furthermore , noncanonical BMP signaling pathways that are freelance of Smads have also been determined in addition to this so-called canonical signaling pathway [62].

There is a variation in the interactions between BMP ligands and membrane bound BMP receptors, in addition to the variability in downstream signaling pathways With some type I receptor isoforms [63], both ligand-independent homodimerization and ligand-dependent heterodimerization of BMP with efficient signal transduction have been seen. Furthermore, certain heterodimeric

complexes can only be formed by binding to a specific BMP heterodimeric ligand [64].Such complexities enable an extra layer of signal regulation and coordination.

Bone morphogenetic protein signaling plays critical roles throughout embryogenesis, according to developmental studies in genetic models. Recent researches have investigated the role of BMP signaling in adult pathophysiological circumstances, revealing a wide range of effects on homeostasis and disease states like pulmonary cancer, hypertension, and anemia [65].

1.2.3. Role of BMPs in Bone

Bone has the ability to heal from injury without scar tissue formulation by its ability to repair and regenerate it self . This unique ability of bone tissue led to the detection of bone morphogenetic proteins (BMPs), the growth agents that enhanced bone's distinctive inductive capability [66] .BMPs maintain the normal balance between bone formation and bone remodeling by the paracrine and autocrine growth factors [67].Accordingly, the osteoclastic BMPs might participate to the anabolic pathway of bone remodeling . Monocytes of marrow , which are the precursors of osteoclasts have the ability to stimulate osteoblastic differentiation by marrow stromal osteoprogenitor cells in coculture [68].Two primary cell types are included in bone remodeling Osteoblasts form bone in sites where osteoclasts have already resorbed bone. Many cytokines control bone remodeling, involving BMPs [69].

The types of osteogenic BMPs include BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, and BMP-8. They can induce bone formation in heterotopic positions *in vivo* and at *vitro* [70].

1.2.4. Bone morphogenetic protein-2 (BMP-2)

Bone morphogenetic protein-2 was detected as an osteo-inductive cytokine agent that activates the full cascade of cartilage and bone formulation. It participates in development of several organs including heart, lung, and central nervous system [71].

BMP-2 is a fully characterized regulatory factor in osteoblast cells that can promotes osteoblast differentiation and bone formulation through activation of the intracellular signaling agents such as Runx2, Smad,2 and osterix (Osx/Sp7) [72]. The expression of PI3K/Akt and integrins signaling cascade pathway are regulated by positive regulation of BMP-2 to further promote osteogenic differentiation [73].

For its various biological functions, BMP-2 was studied largely and specifically through osteogenic differentiations [74], Furthermore, it is a critical regulator factor of odontogenic differentiation which is involved in mineralized nodule formulation in vitro as well as dentin formulation *in vivo* [75].The osteoinductive effectiveness of BMP-2 and other osteogenic growth factors is significantly dependent on their concentrations and transmission method . Slow and persistent transmission of a little concentrations of BMP-2 induces bone injury recovery . However, the burst liberation of a high concentrations of BMP-2 not only fails to induce bone recovery but also causes pernicious local and systemic injuries [76].

Phosphates have a higher effect on modulation and stabilization of BMP-2 as they are existent in much greater levels in the bones and they contributes in complex formulation intermediated by bonds as compared to sulphates. The kinetics of BMP-2 in existence of these molecules are due to the reality that phosphates are the great in organic mineral existent in bone matrix and also due to the vital position of magnesium and calcium in bone health maintenance and regeneration [77].

1.2.5. The relationship between Rheumatoid arthritis (RA) and BMPs

BMPs promote Reactive oxygen species (ROS) generation by activating and inducing the expression of NADPH oxidases1-5. NADPH oxidases 4 (NOX4) and NADPH oxidases 2 (NOX2) expression and activity are induced by BMP-2 [78]. In osteoblasts, ROS alternately regulates BMP-2 expression [79] . BMPs and ROS activates a variety of signaling pathways. Uncontrolled alterations in their expression, production and changed signaling could have severe consequences [80]. BMP-2 triggers NOX in osteoblasts, increasing ROS generation [81], and BMP-2, BMP-4, and BMP-7 upregulate NOX2, NOX1, and NOX4 expression in sympathetic neurons, osteoblasts, and monocytes [82]. In oxidative stress-related conditions, BMP-2 and BMP-4 are upregulated [83].

BMP-2 and BMP-4 mRNA are expressed greatly in blood vessel smooth muscle and endothelial cells [84]. In atherosclerotic diseases that associated with oxidative stress, inflammation, and hyperglycemia [85], both BMP-2 and BMP-4 are upregulated. In hyperglycemia-induced vascular calcification, plasma BMP-2

concentrations are high positively-correlated with aggregate calcium density [86].BMP-2 contributes in vascular development as well as pathophysiological actions [87] . Induction of BMP-2 in blood vessels has been linked to vascular inflammation, hyperglycemia, oxidative stress, and hyperlipidemia [88].

BMP-2 also increases leukocyte adherence and upregulates adhesion molecule expression [89]. Diabetes mellitus, which is a common risk factor for atherosclerosis, may enhance some of these pro-inflammatory effects [90].

BMP-2 promotes chemotaxis of monocytes, key immune cells involved in the pathogenesis of atherosclerotic process , and prevents their development into anti-inflammatory M2 macrophages [91]. BMP-2 promotes vascularization, activates proteoglycan production , induces endochondral osteogenesis, and has anabolic effects on chondrocyte function and metabolism [92]. BMP-2 has been linked to the development of vascular inflammation and angiogenesis, also demonstrated to play a detrimental role in both processes [93]. Moreover, high concentration of BMP-2 has complex adverse effects especially in ectopic bone growth, increased bone resorption and carcinogenesis [94].

Vascular calcification and cardiovascular disease, which is the primary cause of death in rheumatoid arthritis, are both caused by systemic inflammation [95]. In RA, elevated concentration of serum IL-6 is important for both local and systemic inflammation. Cardiovascular illness is the major reason of death among RA cases, particularly in young cases [96]. BMP-2 may have a harmful

role in vascular calcification, which is an active process similar to bone formation [97] . The level of BMPs in osteoarthritic cartilage is followed by an upregulation of BMP antagonists such Follistatin and Gremlin. Gremlin1 expression has been raised since the early stages of OA, and its levels have been associated to disease progression [98]. Gremlin1 is also greatly expressed in the synovia and synovial fluids of RA patients, and its concentrations are associated with proinflammatory cytokine levels [99]. When adult synovial cells are inflamed, they can produce morphogenesis genes such BMP-2. Rheumatoid synoviocytes have been reported to produce BMP-2 in vitro after being stimulated with IL-1 [100].

1.2.6. Knowledge gap

Rheumatoid arthritis provides a unique opportunity to explore the involvement of BMPs in inflammatory processes in such system where they are already present. Immune-mediated inflammatory mechanisms are involved in the pathophysiology of RA. Synovial inflammation, increasing joint degeneration, and bone loss which are characteristics signs of RA. In the synovium of RA cases, BMP-2 and BMP-6 are expressed and their expression in patient-derived fibroblast-like synoviocytes in vitro is highly upregulated in response to pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-17 [101]. The pro-inflammatory cytokines TNF-a and IL-17 promote a pro-inflammatory phenotype within synoviocytes which demonstrated by elevated expression of pro-inflammatory cytokines GM-CSF and IL-6 , formation of metalloproteinases MMP2 and MMP3 , and elevated expression of the chemokine IL-8. The activity and expression of these products are significantly involved in the development of RA. Therefore reviewing the

previous studies regarding the BMPs could bring the light to a critical contribution for a crucial improvement in bone regeneration. That could be a part of the knowledge gap which significantly affect some applications in nanomaterial that involve bone tissue engineering (osteoblastic differentiation and bone formation) with multiple proposed functions such as these reported recently about using Nanosilicates [102].

1.2.7. Aim of This Work

- To review the background documents on the state of the art of the scientific literature in this area of work.
- Study the level of BMP2, MDA level , SOD activity, trace elements and electrolytes in Rheumatoid arthritis group.
- To Study the effect of oxidative damage in the skeletal homeostasis.
- To suggest areas where further research is needed, either to deal with gaps in the knowledge related the biochemical role of Bone morphogenetic Protein in the osteoinduction.

CHAPTER TWO

Materials and Method

2. Materials & Methods

2.1. Study Design

The present work included a case control study for a group of (100) samples: (60) patient samples, (40) healthy control samples. The study was conducted from October 2020 to March 2021.Patients with Rheumatoid arthritis were selected from the biological treatment centre at Marjan medical city in Babylon. History of family, smoking state, job, duration of disease also weights and heights were taken from each subject. The sociodemographic aspects of the patients were collected through the self-reported technique (student questionnaire) including age, gender, BMI and having any current chronic diseases. They were also exposed to medical examination for signs and symptoms of rheumatoid arthritis by specialized doctor based on the World Health Organization (WHO) criteria .

2.2 Instruments

In this chapter, materials and instruments were described and listed in Table (2-1) and (2-2)

Table (2-1): The instruments used in the study

NO.	*Instruments*	*Suppliers*
1	*Centrifuge*	*Germany*
2	*Deep freezer*	*Lebanon*
5	*ELISA system*	*Humen germany*
10	*Jell tube*	*China mheco \china*
12	*Atomic absorption system*	*Shimadzo \ japan*

Table (2-2): The materials used in the study

Materials		
1	Human bone morphogenetic protein 2 ELISA kit	Bioassay technology laboratory/china
2	human super oxidase dismutase ELISA kit	Bioassay technology laboratory/china
3	Human malondialchehyche ELISA kit	Bioassay technology laboratory/china

2.3. Inclusion and Exclusion criteria

2.3.1. Patients Criteria

All patients were subjected to the full clinical history, clinical examination, and relevant laboratory investigations. The diagnosis of the arthritis clinical conditions was established according to the latest clinical practice guidelines by the WHO. The degree of rheumatoid was identified based on evaluation of laboratory measurements for the clinical assessment of rheumatoid arthritis, **however** some patient were newly diagnose were not taking therapy(medication and biological therapy)

2.3.2. Patients Exclusion criteria

Patients with Lupus erythematous, Multiple sclerosis and Juvenile Rheumatoid Arthritis were excluded in this study.

2.3.3. Control Criteria

Control group of an apparently healthy 40 subjects (15 male and 25 female) were chosen from well-known volunteers participants. Blood samples were drawn from the volunteers, had no history of rheumatoid arthritis diseases. The percentages of female and male adult individuals were about the same in the patients frame. The ages of the participants were also convergent in the whole study group. Demographic information

of the participants was also collected through the self- reported technique (student questionnaire).

2.4. Study variables

2.4.1. Dependent Variable

Serum BMP2, SOD, MDA, Electrolytes, and lead**.**

2.4.2. Independent Variable

Age, Gender, smoking state, job, duration of disease, geographical distribution.

2.5. Approval of the Ethical Committee

The protocol of the study was approved by Ethical Committee of Kerbala Medical College, and the biological treatment centre at marjan medical city. Samples from serum were obtained after consent from patients or the patients' relatives.

2.6. Measurement and Data collection

2.6.1. Data Collection

A structured questionnaire was specifically design to obtained information which helps to select individuals according to the selection criteria of the study. Sociodemographic aspects of the subjects (patients and control) were also collected through the self-reported technique (student questionnaire) which including: age, gender and BMI, smoking state , chronic disease , duration of disease(for patient) .

2.6.2. Blood Collection and Storage

Blood samples were collected from the biological treatment centre at marjan medical city. Five mL of blood samples were drown by venipuncture

using 5 ml disposable syringes, 4 ml blood was left for (15 min) at room temperature in gel tube. Serums were separated by centrifuging for 10 minutes at approximately 1800 xg . Serum samples were aliquot into two eppendroff and store at -20°C to avoiding multiple freezing-thawing cycles and used for the further measurement.The remaining 1 mL of blood was added to a Blend Tubes containing 1 mL of Trichloroacetic acid, The mixture of blood and acid was left for 10 minutes at room temperature and separated by centrifuge for 15 minutes at 1800 xg, the supernatant were taken into eppendroff tube and store at -20°C . Blood collection tubes were be disposable, non-pyrogenic, and non-endotoxin.

2.7. Methods

2.7.1. Determination of Body Mass Index

The body mass index (BMI) was calculated by the following equation:

$\boldsymbol{BMI}$=W$\boldsymbol{eight}$ ($\boldsymbol{kg}$)/H$\boldsymbol{eight}$2 (meters)

The body mass index (BMI) is the metric currently in use for defining anthropometric height/weight characteristics in adults and for classifying (categorizing) them into groups as shown in Table (2-3) according to WHO.

Table (2-3) Body mass index.

Weight status	*BMI range (kg/m^2)*
Underweight	*15-19.9*
Normal weight	*20-24.9*
Overweight	*25-29.9*
Class I obesity	*30-34.9*
Class II obesity	*35-39.9*
Class III obesity	*≥ 40*

2.7.2. Measurement of serum human bone morphological protein 2 by using ELISA Technique

Enzyme Linked Immunosorbent Assay system (ELISA) was performed using direct method to measure the concentrations of serum BMP2

- **Principle**

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with human BMP-2 antibody. BMP-2 present in the sample is added and binds to antibodies coated on the wells and then biotinylated human BMP-2 Antibody is added and binds to BMP-2 in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated BMP-2 antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of human BMP-2. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

- **Reagents**

The ELISA kit applied for quantitative determination of BMP2 concentration in serum, list of reagents were shown in the Table (2-4).

Table (2-4): Reagents of determination BMP2 concentration.

Components	*Quantity*
Standard Solution (16ng/ml)	*0.5ml x1*
Pre-coated ELISA Plate	*12 * 8 well strips x1*
Standard Diluent	*3ml x1*
Streptavidin-HRP	*6ml x1*
Stop Solution	*6ml x1*
Substrate Solution A	*6ml x1*
Substrate Solution B	*6ml x1*
Wash Buffer Concentrate (25x)	*20ml x1*
Biotinylated human BMP-2 Antibody	*1ml x1*

- **Samples and Reagents Preparation**

  Reagents Preparation

  Stock solutions were prepared based on the procedure of the manufactured kit. All reagents were prepared freshly at room temperature before used.

  Standard Reconstitute the 120µl of the standard (16ng/ml) with 120µl of standard diluent to generate a 8ng/ml standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (8ng/ml) 1:2 with standard diluent to produce 4ng/ml, 2ng/ml, 1ng/ml and 0.5ng/ml solutions. Standard diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20°C and used within one month.

Table (2-5): Dilution of standard solutions suggested are as follows

Standard Concentration	Standard No.	Standard compostion
8ng/ml	Standard No.5	120µl Original Standard + 120µl Standard Diluent
4ng/ml	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
2ng/ml	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
1ng/ml	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
0.5ng/ml	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent

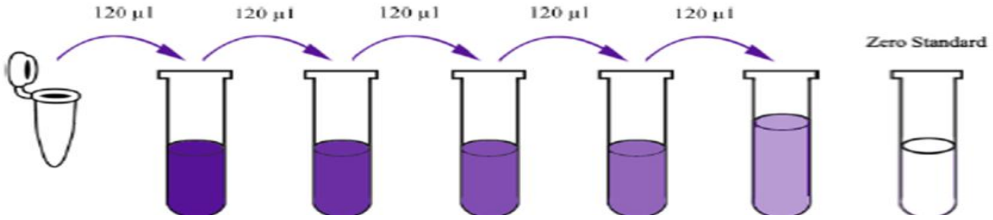


Figure (2-1): Dilution steps of standard preparation of BMP 2

Wash Buffer

Dilute 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

- **Preparing of Standard Curve**

This standard curve is only for demonstration purposes. A standard curve should be generated with each assay.

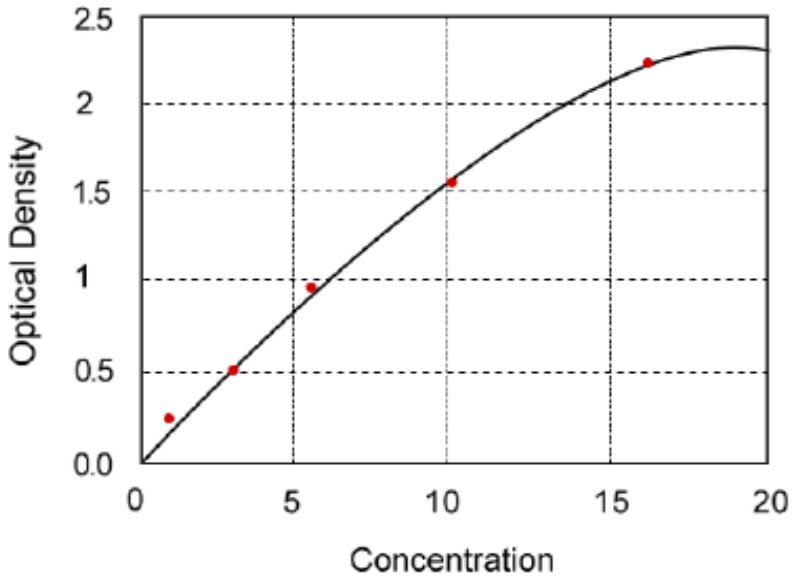


Figure (2-2): Calibration curve of BMP2

Assay procedure

1. All reagents was prepared, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.

2. The number of strips required for the assay were determined. The strips were inserted in the frames for use. The unused strips should be stored at 2-8°C.

3. The standard 50µl was added to standard well. Note: Antibody should not added to standard well because the standard solution contains biotinylated antibody.

4. Sample 40µl was added to sample wells and then 10µl anti-BMP-2 antibody added to sample wells, then 50µl streptavidin-HRP added to sample wells and standard wells (Not blank control well). Then should mix well. the plate covered with a sealer. Incubation performed for 60 minutes at 37°C.

5.The sealer was removed and the plate washed 5 times with wash buffer. Wells soak with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspiration of all wells and washed 5 times with wash buffer, wells overfilling with wash buffer. the plate Blot onto paper towels or other absorbent material.

6. Substrate solution A 50µl was added to each well and then 50µl substrate solution B added to each well. Plate was Incubated and covered with a new sealer for 10 minutes at 37°C in the dark.

7. A 50µl Stop Solution was added to each well and the blue color would change into yellow immediately.

8. The optical density (OD value) was determined of each well immediately using a microplate reader set to 450 nm within 10 minuets after adding the stop solution.

2.7.4. Measurement of serum Human Super Oxidase Dismutase using ELISA Technique

This sandwich kit is for the accurate quantitative detection of human Super Oxidase Dismutase in serum, plasma, cell culture supernates, cell lysates, tissue homogenates.

Assay Principle

The Kit plate has been pre-coated with human SOD antibody. SOD present in the sample is added and binds to antibodies coated on the wells. And then biotinylated human SOD antibody is added and binds to SOD in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated SOD antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of human SOD. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

- **Reagents**

The ELISA kit applied for quantitative determination of SOD concentration in serum, list of reagents were shown in the Table (2-6)

Table (2-6): Reagent Provided

Components	*Quantity*
Standard Solution (960U/L)	*0.5ml x1*
Pre-coated ELISA Plate	*12 * 8 well strips x1*
Standard Diluent	*3ml x1*
Streptavidin-HRP	*6ml x1*
Stop Solution	*6ml x1*
Substrate Solution A	*6ml x1*
Substrate Solution B	*6ml x1*
Wash Buffer Concentrate (25x)	*20ml x1*
Biotinylated human SOD Antibody	*1ml x1*

Samples and Reagents Preparation of measurement SOD activity

Sample Preparation

Serum samples were allowed for 10-20 minutes at room temperature.

Reagents Preparation

All reagents were brought to room temperature before use. Standard Reconstitute the 120µl of the standard (960U/L) with 120µl of standard diluent to generate a 480U/L standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (480U/L) 1:2 with standard diluent to produce 240U/L, 120U/L, 60U/L and 30U/L solutions. Standard diluent serves as the zero standard (0 U/L). Any remaining solution should be frozen at -20°C and used within one month.

Table (2-7): Dilution of standard solutions suggested are as follows

Standard Concentration	*Standard No.*	*Standard composition*
480U/L	*Standard No.5*	*120µl Original Standard + 120µl Standard Diluent*
240U/L	*Standard No.4*	*120µl Standard No.5 + 120µl Standard Diluent*
120U/L	*Standard No.3*	*120µl Standard No.4 + 120µl Standard Diluent*
60U/L	*Standard No.2*	*120µl Standard No.3 + 120µl Standard Diluent*
30U/L	*Standard No.1*	*120µl Standard No.2 + 120µl Standard Diluent*

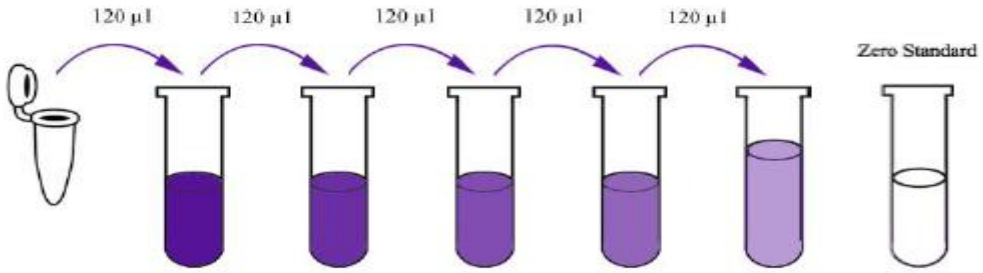


Figure (2-3): dilution step standard of SOD

Preparation of Wash Buffer: 20ml of Wash Buffer Concentrate 25x was diluted into deionized or distilled water to yield 500 ml of 1x Wash Buffer.

Preparing of Standard Curve

This standard curve is only for demonstration purposes Figure (2-4). A standard curve should be generated with each assay.

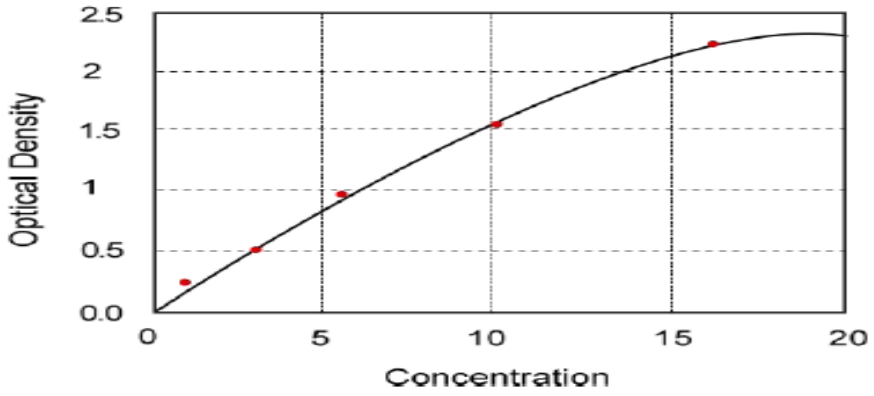


Figure (2-4): calibration curve of SOD

Assay Procedure

1. All reagents were brought to room temperature before use. The assay was performed at room temperature.

2. A 50µl of each standard was added to standard well. Note: antibody shouldn't added to standard well because the standard solution contains biotinylated antibody.

4. A 40µl of each samples were added to sample wells and then add 10µl anti-SOD antibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.

5. Then, sealer was removed and the plate washed 5 times with wash buffer. wells Soak with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspiration of all wells and washed 5 times with wash buffer, wells overfilling with wash buffer. the plate Blot on to paper towels or other absorbent material.

6. Substrate solution A 50µl was added to each well and then 50µl of substrate solution B add to each well. plate Incubated and covered with a new sealer for 10 minutes at 37°C in the dark.

7. Stop Solution 50µl was add to each well, and blue color will change into yellow immediately.

8. The optical density (OD value) was determined of each well immediately using a microplate reader set to 450 nm within 10 minuets after adding the stop solution.

2.7.5. Measurement of serum Human MDA using ELISA Technique

This sandwich kit is for the accurate quantitative detection of human (MDA) in serum, plasma, cell culture supernates, cell lysates, tissue homogenates.

Assay Principle

The plate has been pre-coated with human MDA antibody. MDA present in the sample is added and binds to antibodies coated on the wells, and then biotinylated human MDA Antibody is added and binds to MDA in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated MDA antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of human MDA. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm. Reagents were listed in Table (2-8).

Table (2-8): Reagent Provided

Components	**Quantity**
Standard Solution (80nmol/ml)	0.5ml x1
Pre-coated ELISA Plate	12 * 8 well strips x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate (25x)	20ml x1
Biotinylated human MDA Antibody	1ml x1

Samples and Reagents Preparation

Samples Preparation

Serum samples were allowed for 10-20 minutes at room temperature.

Reagents Preparation

All reagents should be brought to room temperature before use.

Standard Reconstitute the 120µl of the standard (80nmol/ml) with 120µl of standard diluent to generate a 40nmol/ml standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (40nmol/ml) 1:2 with standard diluent to produce 20nmol/ml, 10nmol/ml, 5nmol/ml and 2.5nmol/ml solutions. Standard diluent serves as the zero standard(0 nmol/ml). Any remaining solution should be frozen at -20°C and used within one month.

Table (2-9): Dilution of standard solutions suggested are as follows

Standard Concentration	*Standard No.*	*Standard composition*
40nmol/ml	*Standard No.5*	*120µl Original Standard + 120µl Standard Diluent*
20nmol/ml	*Standard No.4*	*120µl Standard No.5 + 120µl Standard Diluent*
10nmol/ml	*Standard No.3*	*120µl Standard No.4 + 120µl Standard Diluent*
5nmol/ml	*Standard No.2*	*120µl Standard No.3 + 120µl Standard Diluent*
2.5nmol/ml	*Standard No.1*	*120µl Standard No.2 + 120µl Standard Diluent*

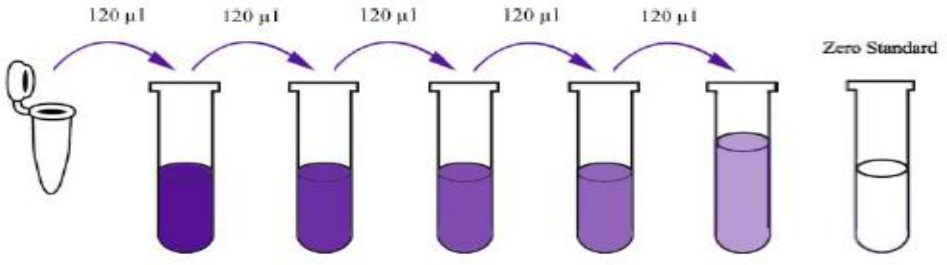


Table (2-5): concentration standard of MOD

Wash Buffer

A 20ml of Wash Buffer Concentrate 25x was diluted into deionized or distilled water to yield 500 ml of 1x Wash Buffer.

Preparing of Standard Curve

This standard curve is only for demonstration purposes Figure (2-6). A standard curve should be generated with each assay.

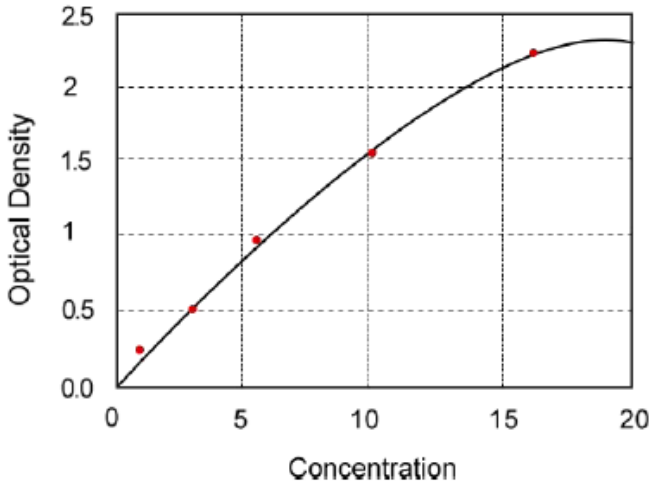


Figure (2-6): Calibration curve of MDA

Assay Procedure

1.All reagents was prepared, standard solutions and samples as instructed. The assay is performed at room temperature.

2. A 50µl of each standard was added to standard well.

3. A 40µl of each samples was added to sample wells and then add 10µl anti-MDA antibody to sample wells, then add 50µl streptavidin-HRP to

sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.

4. The sealer removed and the plate washed 5 times with wash buffer. wells Soak with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspiration of all wells and washed 5 times with wash buffer, wells overfilling with wash buffer. the plate Blot on to paper towels or other absorbent material.

5. Substrate solution A (50µl) was add to each well and then 50µl of substrate solution B add to each well. plate Incubated and covered with a new sealer for 10 minutes at 37°C in the dark.

6.Stop Solution 50µl was Add to each well, and blue color will change into yellow immediately.

7. The optical density (OD value) determined of each well immediately using a microplate reader set to 450 nm within 10 minuets after adding the stop solution.

2.7.7. Quantitative measurement of Trace Elements and electrolytes in serum sample

Principle

Flameless Atomic absorption spectrophotometer method by the technique of Graphite Furnace (GFAAS) was used to determine the trace element. SHIMADZU AA7000 Atomic Absorption Spectrophotometer was used for determination of these elements. GFAAS is one of the most important of the five techniques of the atomic absorption spectrometry in which has the higher sensitivity which can be reach to the low detection limits (in ppb units).

This technique is also called Electro thermal Atomic Absorption Spectrometry (ETAAS) which is a type of spectrometry that uses a graphite furnace tube to vaporize the sample in three stages, drying, ashing, and atomizing.

The fact of this technique is based on that free atoms of element absorb light produced from the specific cathode lamp at specific wavelengths characteristic of the interest element. Within certain limits, the amount of light absorbed reflect the concentration of analyst present and can be linearly correlated to this concentration. Most elements can produce free atoms from samples by the application of high temperatures.

In GFAAS, very small amount of samples (10µL-20µL) is injected in small graphite or paralytic carbon coated graphite tube, which can then be heated by a wide range of temperature to vaporize and atomize the analyst. The atoms absorb the electromagnetic radiation in the ultraviolet or visible region resulting in transitions of electrons to higher electronic energy levels to the excited state and then back to the ground state by emitting it's specific characteristic light which can be measured to determine the samples concentrations. The temperature of the Graphite tube increases over a matter of seconds and can reach up to 3000ºC depending on the element being analyzed.

Preparation of Standard Solutions

Original standard solutions were (1000µg/ml in 2% HNO3) for interested elements. Four standard solutions were prepared by dilution from original standard stock solution using general dilution law ($N_1 V_1 = N_2 V_2$). It must prepare a series of concentrations from the highest one

reaching the values required for calibration curve performs. Series of concentrations which prepared were as follows:-
(1000µg/ml → 100µg/ml → 10µg/ml → 1µg/ml). The value 1µg/ml is equal to 1000ng/ml, so complete preparation from this solution reaching the required concentration of the standard calibration curve as follow:- (1000ng/ml → 100ng/ml → prepare the needed concentration of any element).

Sample Preparation

Samples were digested by transferring 5ml of whole blood (in EDTA Tube) and then addition of 5 ml of (10% TCA). Both volumes mixed well in vortex for (15 min). Centrifugation for 10 min at 1008 xg. Digested sample solutions was filtered and then appropriate solution volume of (20µL) was injected into the graphite furnace tube for reading.

2.7.7.1. Determination of serum Magnesium

Four standard solutions of the element were prepared as mentioned above. The four standards were (5, 10, 20, 30 ng/ml) for calibration curve as shown in figure (2-7). The concentrations of Magnesium in samples were measured directly and continuously beyond measuring of standard solutions depending on the calibration curve.

Conditions for Magnesium Determination: Listed in table (2-10)

Table (2-10): Ideal Conditions for Magnesium Determination

Variable	Ideal condition
Lamp current	8 Ma
Wavelength	285.2nm
Slit width	0.5nm
Lighting mode	BGC-D2
Sample Size	20 µl
Replicates	3

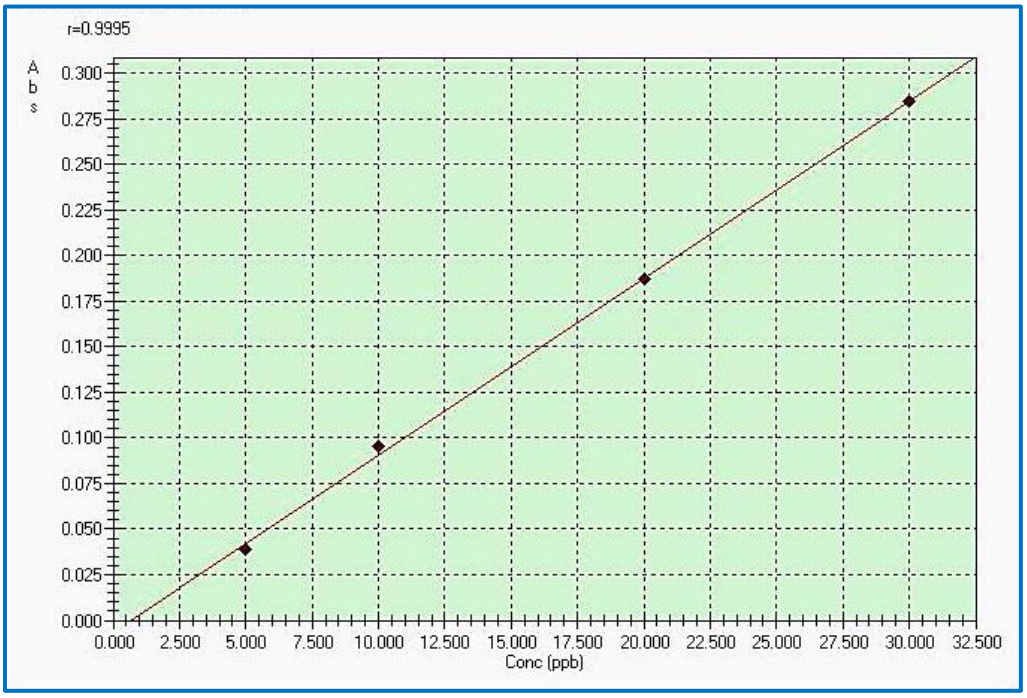


Fig (2-7): Standard curve for Magnesium determination

2.7.7.2. Determination of serum Sodium

Five standard solutions of the element were prepared as previously mentioned above. These standards were (2.5, 5, 10, 15, 20, ng/ml) for calibration curve as shown in figure (2-8). The concentrations of Sodium in

samples were measured directly and continuously beyond measuring of standard solutions depending on the calibration curve.

Conditions for Sodium Determination**:** Listed in table (2-11)

Table (2-11): Ideal Conditions for Sodium Determination

Variable	Ideal condition
Lamp current	10 mA
Wavelength	586.0 nm
Slit width	0.2 nm
Lighting mode	No BG
Sample Size	20 µl
Replicates	3

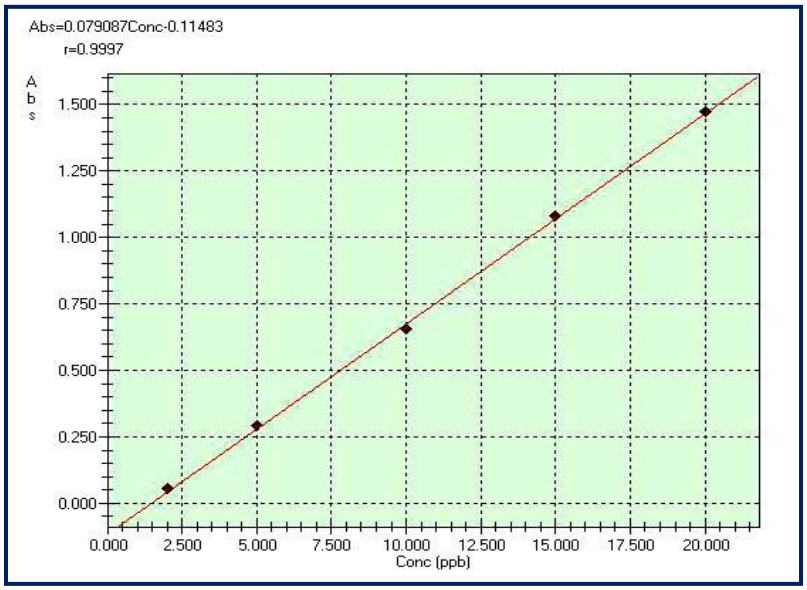


Fig (2-8): Standard curve for Sodium determination

2.7.7.3. Determination of serum Calcium

Four standard solutions of the element were prepared. These standards were (2, 4, 6, 8, 10 ng/ml) for calibration curve as shown in figure

(2-9). The concentrations of calcium in samples were measured directly and continuously beyond measuring of standard solutions depending on the calibration curve.

Conditions for calcium determination were Listed in table (2-12)

Table (2-12): Ideal Conditions for Calcium Determination

Variable	Ideal condition
Lamp current	10 Ma
Wavelength	422.7 nm
Slit width	0.5 nm
Lighting mode	No BG
Sample Size	20 µl
Replicates	3

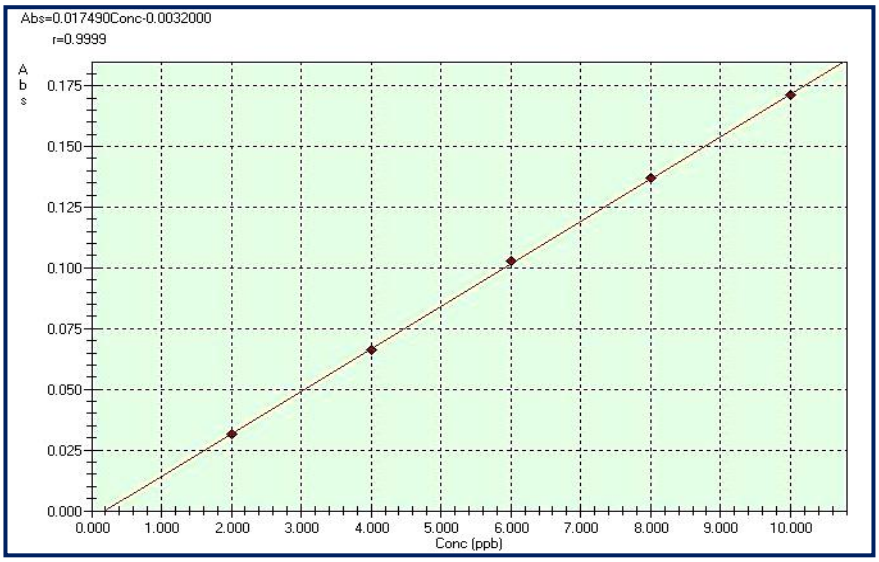


Fig (2-9): Standard curve for Calcium determination

2.7.7.4. Determination of serum Potassium

Four standard solutions of the element were prepared as previously mentioned above . These standards were (5, 10, 15, 20 ng/ml) for calibration curve as shown in figure (2-10). The concentrations of Potassium in samples were measured directly and continuously beyond measuring of standard solutions depending on the calibration curve.

Conditions for Potassium Determination: Listed in table (2-13)

Table (2-13): Ideal Conditions for Potassium Determination

Variable	Ideal condition
Lamp current	10 mA
Wavelength	766.5 nm
Slit width	0.5 nm
Lighting mode	No BG
Sample Size	20 µl
Replicates	3

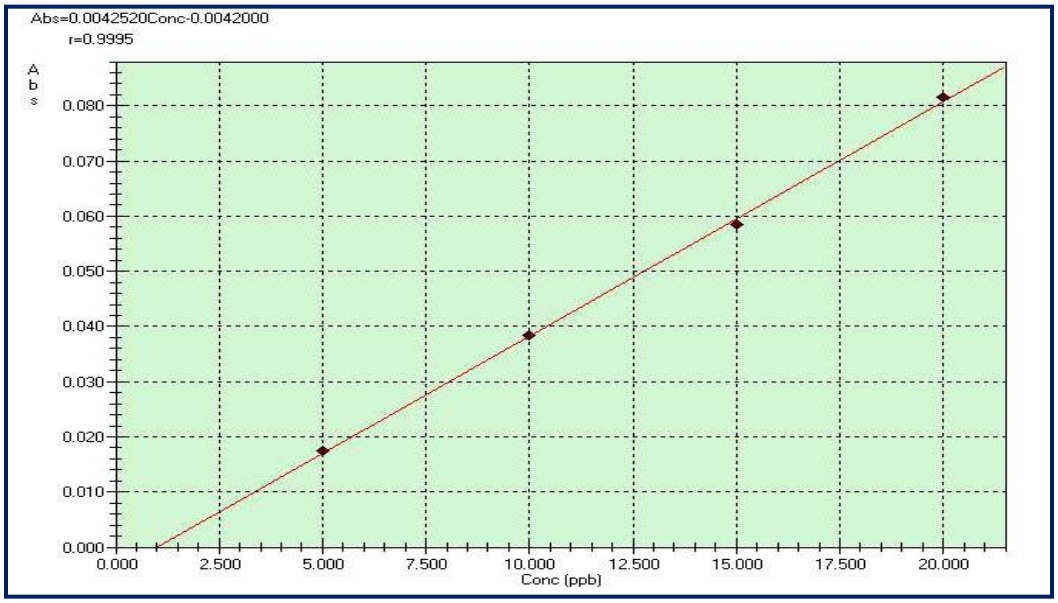


Fig (2-10): Standard curve for Potassium determination

2.7.7.5. Determination of serum lead

Four standard solutions of the element were prepared. These standards were (25, 50, 75, 100 ppb) for calibration curve as shown in figure (2-11). The concentrations of lead in samples were measured directly and continuously beyond measuring of standard solutions depending on the calibration curve.

Conditions for lead Determination: Listed in table (2-14)

Table (2-14): Ideal Conditions for lead Determination

Variable	Ideal condition
Lamp current	10 mA
Wavelength	283.3 nm
Slit width	0.7 nm
Lighting mode	BGC-D2
Sample Size	20 µl
Replicates	1

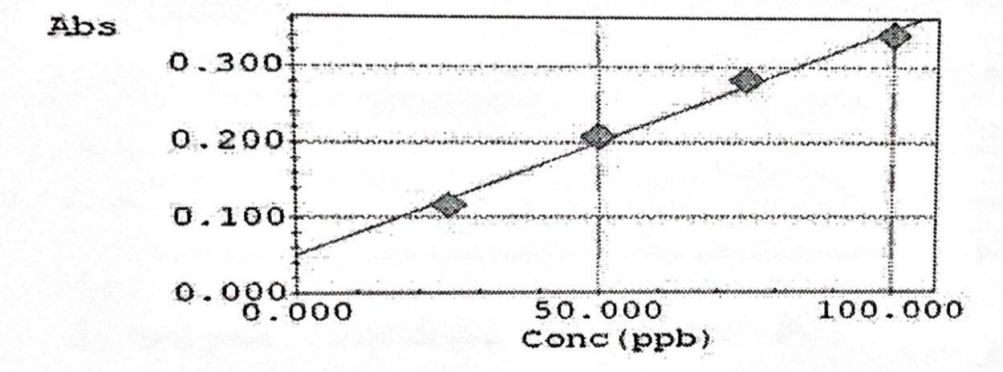


Fig (2-11): Standard curve for lead determination

2.7.7.6.Determination of human serum phosphorus

For measurement of human serum phosphorus, the spectrophotometer device was supplied with the kit provided by Spectrum, (Egypt).

Assay principle

The basic principle is that each compound absorbs or transmits light over a certain range of wavelengths. Phosphate ions react with ammonium molybdate in acidic solution yielding a yellow complex, which by the action of an alkaline buffer is reduced to blue molybedenum that is colorimetric measured as shown in the following equation.

Ammonium molybdate +Sulfuric acid $\xrightarrow{\text{phosphorus}}$ Phosphomolybdic complex

Reagents

Reagents store at 15- 25 °C, table (2-15)

Table (2-15): Standard and reagents supplied with the phosphorus kit.

Standard	5.0mg/dL
Reagents	Catalysator. Polyvinyl pirrolidone and hydroxylamine chloride (2.88 mol/L) yellow to orange
	Molybdate Ammonium molybdate (41mmol/Land sulfuric acid (900 mmol/L)
	Buffer Sodium carbonate (50 mmol/L) and sodium hydroxide (10mol/L)

Procedure

Every reagent were placed at room temperature until use, table (2-16): Include standard, reagents and sample volume used in measure of phosphorus

Table (2-16): Include Standard , Reagents and sample volume used in measure of phosphorus

	Blank	Stander	Sample
Reagent	1000mL	1000mL	1000mL
Stander	-	20ml	-
Sample	-	-	20Ml

All of the previous ingredient were mixed and incubate for 5 minutes and measured the absorbance of standard, sample agents and the reagent blank at 630 nm.

Calculation of Phosphorous Ion concentration

From the following formula the concentration of phosphate calculated:

$$\text{Phosphorous concentration (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 5$$

Reference values

Adults (12-60 years) = 2.8-4.5 mg/dL

Statistical Analysis

Information from the questionnaire and all test results from patients and control samples were entered a data sheet.

The data analysis for this work was generated using the Real Statistics Resource Pack software for Mac (Release 7.2) of the resource pack for Excel 2016. Copy- right (2013 – 2020).

Descriptive statistics was performed on the participants' data of each group. Values were presented as a median for abnormal distribution, and n (%) for categorical variables, respectively. The distribution of the data was checked for normality using the Box plot test.

Receiver operating characteristics (ROC) curves was also used to test the markers' diagnostic performance in both Rheumatoid arthritis and control groups.

CHAPTER THREE

Results and discussion

3. Results and Discussion

Rheumatoid arthritis (RA) is a chronic, progressive, inflammatory autoimmune disease associated with articular, extra-articular and systemic effects. The biomarkers that studied in RA were varied and they have significant role in the diagnosis, prognosis of treatment, monitorization of the disease activity, and prediction of the response to biologic therapy. Bone morphogenetic proteins BMPs are members of the transforming growth factor beta (TGF-β) superfamily and they induce the formation of cartilage and other connective tissues. Also, it can induce bone formation *in* vitro and at heterotopic sites *in vivo*. It has been reported their largely important role in embryogenesis, early prenatal skeletal formation and development, further to their associated with a number of human skeletal disorders. The osteogenic BMPs function was proposed through promoting the differentiation and proliferation of mesenchymal stem cells (MSCs) in the bone marrow into bone-forming osteoblasts and enabling the proliferation of osteogenic cells.

This research was aimed to review the background documents on the state of the art of the scientific literature in this area of work and to study the effect of oxidative damage in the skeletal homeostasis. Furthermore, it was performed to suggest areas where further research is needed, either to deal with gaps in the knowledge related disease or the biochemical role of Bone morphogenetic Protein in the rheumatoid arthritis.

3.1.Demographic and clinical characteristics

The clinical demographic characteristics and laboratory parameters of both patients groups and the healthy control group were summarized in Table (3-1). Table illustrated the mean age of participants which was within the age group of (20 – 75) years . Gender distribution among the studied groups was: 23.3% male, 76.67% female for patients group, while 37.5% male and 62.5% female for control group. The descriptive table also shown an adjustment of other risk factors which were collected through the self-reported technique (student questionnaire), these factors included: age, gender, BMI, history of disease, duration of disease and the site of rheumatoid arthritis.

Table (3-1) The demographic characteristics of the study groups

Characteristics		Study group		Normal values
		Patient	Control	
Demographics	Age, Mean(Median)	48.92(49)	39.18(38)	
	Gender(male/female)	14/46	15/25	
Biochemical Parameters	BMP2 Mean(Median) ng/ml	0.284(0.15)	0.892(2.98)	1.488-3.356ng/ml
	SOD Mean(Median) nmol/ml	0.849(0.843)	44.815(1.619)	31.579-112.007nmol/ml
	MDA Mean(Median) nmol/ml	0.886(1.114)	1.115(0.876)	5.984-14.523nmol/ml
	Lead Mean(Median) µg/dl	2.78(2.56)	6.1(16.72)	>3.5 µg/dl
	Mg Mean(Median) mmol/l	1.52(1.57)	2.044(2.1)	0.85-1.10 mmol/l
	Na Mean(Median) mmol/l	153.8(154.5)	142.6(143)	135-145mmol/l
	Ca Mean(Median) mg/dl	12.47(12.5)	10.304(10.4)	8.6-10.3 mg/dl
	K Mean(Median) mmol/l	3.5(3.2)	4.46(4.6)	3.6-5.2 mmol/l
	CRP(positive/negative) mg/L	45/15		8-10 mg/L
	P Mean(Median) mmol/l	5.2(5.25)	3.62(3.7)	1.12-145mmol/l
	Rf (positive/negative) IU/mL	60/0		0-20 IU/mL
	ESR Mean(Median) mm/hr	44.17(40)		>50y male(0-15mm/hr) <50y female(0-30mm/hr)

Locally, The study was also included examination of the prevalence for the most common site of rheumatoid arthritis, figure (3-1) showed that the highest number of patient having rheumatoid arthritis in Knee joint, wrist joint, metacarpal joint and ankle with mean number of (60, 55, 41 and 31 respectively) while the lowest prevalence type were in elbow joint with mean number of patients (10). The study indicated that the knee joint was the most site of the body could be affected by rheumatoid arthritis. knee is the largest joint in the body and one of the most complicated. However, in the case of rheumatoid arthritis, this inflammation in the joint is unnecessary and causes problems.When the inflammation goes down, the capsule around the synovium remains stretched and can not hold the joint in its proper position. This can cause the joint to become unstable and move into unusual positions.

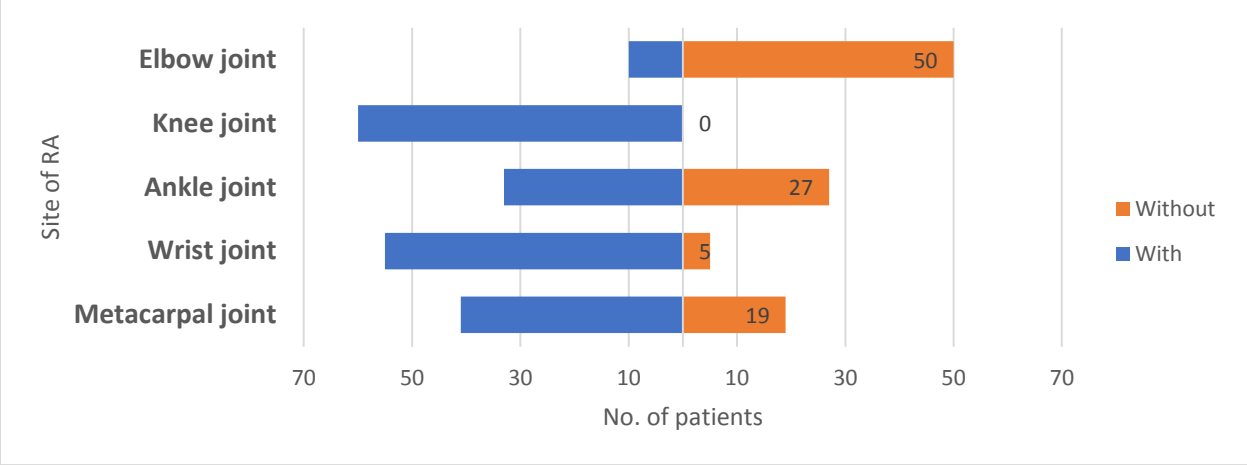


Figure (3-1) The most common site of rheumatoid arthritis in patients group

3.2. Distribution of serum BMP isoform levels in rheumatoid arthritis patients compared to control group

A box plot was used to visually show the distribution of data through displaying the data quartiles (or percentiles) and averages. Box plots show the five-number summary of a set of data: including the minimum score, first (lower) quartile, median, third (upper) quartile, and maximum score. The median is the average value from a set of data and is shown by the line that divides the box into two parts.

In statistics, dispersion (also called variability, scatter, or spread) is the extent to which a distribution is stretched or squeezed. The smallest value and largest value are found at the end of the 'whiskers' and are useful for providing a visual indicator regarding the spread of measurements. On the other hand, figures also indicated that the interquartile ranges of the boxes regarding patients has more dispersion of a data set then health control with indicated more variability.

All control group were demonstrated a symmetric distribution where most of the measurements. Cluster around the central median and the probabilities for values further away from the mean taper off equally in both directions. Extreme values in both tails of the distribution are similarly unlikely.The distribution of serum BMP isoform levels in rheumatoid arthritis patients were decreased markedly compare to control group. The median level of BMP2 in RA patients was (0.15 ng/ml while in control was 2.95 ng/ml) as shown in figure (3-2).

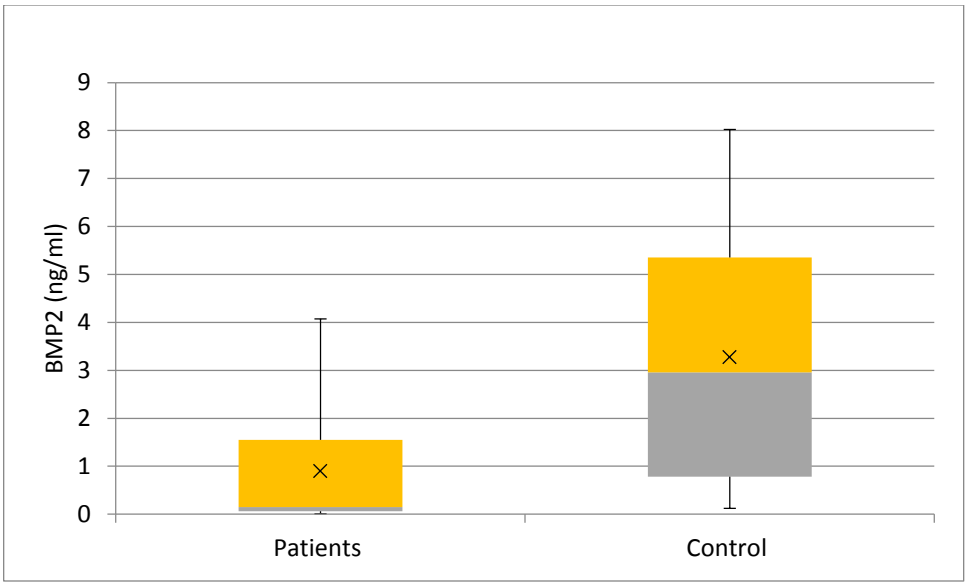


Figure (3-2) Boxplot of the Distribution of serum level BMP2 ng/mL in rheumatoid arthritis group compared to control group

BMPs were negatively correlated with local or systemic parameters of inflammation as well as the duration of the disease. This discrepancy might depend on differences in the biological function and regulation of individual members of the BMP family.

In fact, Lories and colleagues [(103)] reported that BMP-2 and BMP-6, was not increased by stimulation with IL-1β or TNF-α. A similar distribution and predominant expression of different BMPs in fibroblastoid and macrophagocytic cells was also shown by Lories and colleagues [(103)]

The loss of BMP signal might reduce the regenerative capacity of cartilage [104]. loss of BMP expression could be involved in chronic inflammatory and not only degenerative joint diseases [105].Moreover, there are studies suggested that BMP2 and BMP4 might be involved as downstream mediators of the TGF-β effect and that these BMPs might be released by macrophages of the synovial lining layer [106]. Previously reported that the peripheral blood expression profiles of BMPs may act as predictive markers for the development of arthritis, its disease activity, therapeutic responsiveness and overall prognosis [107]. previously suggested that BMPs are beneficial for the repair of joint destruction and tissue responses that may form the basis of chronic arthritis [108].BMP-2 is a member of the BMP family that contributes to bone formation, joint anti-inflammation and synovial repair [109]. Previous research has suggested that recombinant BMP-2may induce bone formation and osteoblastic differentiation by regulating endochondral ossification [110]. In addition, abnormal expression of BMP-2 in mesenchymal cells has been investigated in association with rheumatoid arthritis [111]. BMP-2 expression levels were significantly lower in synovial cells from the mouse model [112].Other studies have reported that BMP-2 may be used to reconstruct segmental mandibular defects and repair ischemic damage by inducing angiogenesis and osteogenesis, and by decreasing osteoclast bone reabsorption activity [113]. However, the half-life of BMP-2 is short *in vivo*, which limits its clinical application [114]. The pro-inflammatory cytokines IL-17 and TNF-α induce a pro-inflammatory phenotype within synoviocytes marked by increased expression of pro-inflammatory cytokines IL-6 and GM-CSF, increased expression of the chemokine IL-8, and increased production of metalloproteinases MMP2

and MMP3. Expression and activity of these products are strongly implicated in the pathogenesis of RA [115] [116].

3.3. Examination the serum bone minerals levels in rheumatoid arthritis group

Magnesium (Mg) is an essential nutrient and fourth most abundant mineral found in the body Mg levels are changed in chronic inflammations and decreased level of Mg has been suggested to be reasonable marker of RA [117]

The distribution of serum electroplates and minerals levels in RA patients group compared to the healthy group was performed. Serum Magnesium levels were decreased significantly in RA group compared to healthy control group, Mean levels was (1.52 mmol/L) compared to control group mean value (2.044 mmol/L) as shown in figure (3-3).

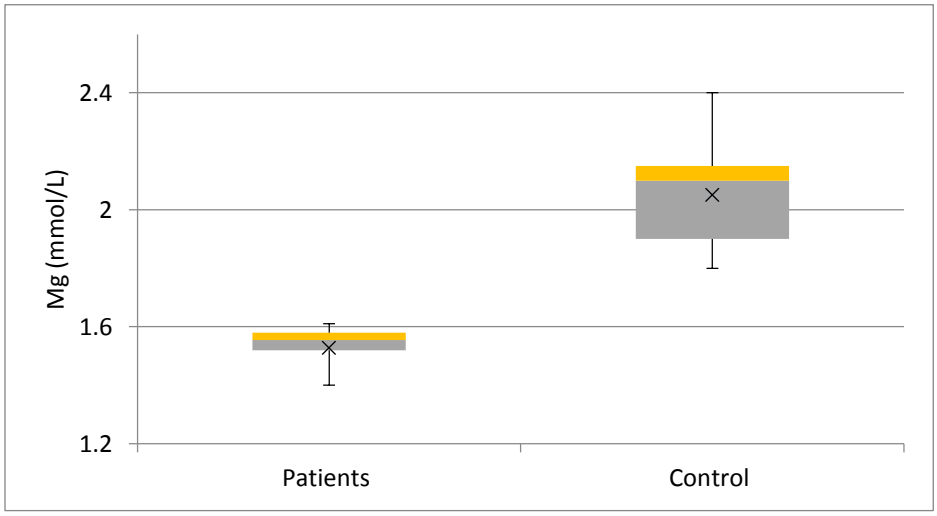


Figure (3-3) Boxplot of the Distribution of serum level Magnesium mmol/L in rheumatoid arthritis group compared to control group

Chronic inflammatory conditions are likely to alter magnesium level and possible mechanism of decrease magnesium in RA is due to chronic inflammation and autoimmune injury [118] [119]. It has been known that RA is associated with serum mineral disturbances and oxidative stress [118]. Decreased levels of serum magnesium and calcium may be due to many reasons. study suggested that hypomagnesemia may be considered as additional nontraditional risk factors of RA patients [120].Inflammation, irrespective of etiology, is capable of inducing marked systemic alterations in trace metal distribution and metabolism. These trace metal alterations appear to be linked to the production of an acute-phase plasma protein response and to be part of a proposed host-defense/ repair system which responds to injury upon activation by factors released from stimulated phagocytes. Arthropathy is caused by Mg deficiency in cartilage. Typical cartilage lesions were found in knee joints of all patients with Mg deficiency. An understanding of Mg's role in cell physiology and metabolism is not readily forthcoming, largely because of the inherent difficulty in measuring free concentration of the ion. Morevere, there has been a renewed interest in the role of Mg in cell function, particularly in rheumatology. With respect to this resurgence in interest in rheumatology, the impetus can be arguably attributed to the ability to readily measure free Mg concentration in the rheumatoid synovial fluid. Other studies have demonstrated the presence of apoptotic synovial cells (Mg-induced) and infiltrating lymphocytes in RA (T cell apoptosis). The presence of these cells suggests that regression of RA may be due to the induction of apoptosis in rheumatoid synovium. Apoptosis serves many functions in the homeostasis of multicellular organisms. Defects in apoptosis may lead to clonal expansion and accumulation of autoreactive lymphocytes, which may result in the rare

human autoimmune lymphoproliferative syndrome, a mild autoimmune reaction against cells in the blood. Defects in the clearance of apoptotic cells lead to accumulation of dying cells, which may enter later stages of cell death and release their contents, thereby critically contributing to the etiopathogenesis of the RA [(121)]. Also, magnesium has been shown to inhibit inflammatory responses , indicates that magnesium has a crucial regulatory role in nuclear factor kappa-B (NF-κB) activation, proinflammatory cytokine production and systemic inflammation, which have been proven to be strongly associated with the pathogenesis of RA [(122)] [(123)]. other were indicates that magnesium has protective effects on articular cartilage because it reportedly enhances chondrocyte proliferation and re-differentiation as well as protects against a substantial proportion of chondrocyte damage [(124)].

In addition, the level of Na in patients with RA increased in an interesting way, As shown in figure (3-4). In patients, the mean level of Na was 153.85 mmol/L) compared to control (142.69 mmol/L).

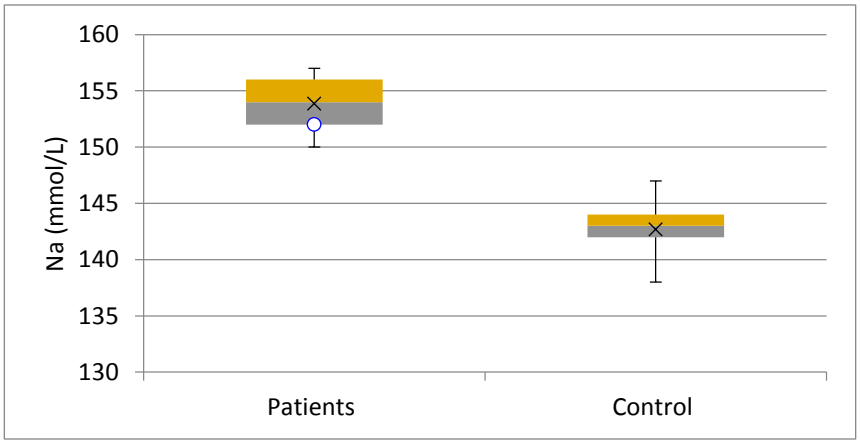


Figure (3-4) Boxplot of the Distribution of serum level of Sodium mmol/L in rheumatoid arthritis group compared to control group

Sodium was associated with T-cell secretion of interleukin 17 (IL-17) and tumor necrosis factor α (TNF-α). However, hypernatremic synovial fluid from RA patients can provide a Th17 polarization. In addition to Th17 differentiation, hypertonicity is essential for lymphocyte proliferation and maturation. Physiologically, lymphoid tissue is hyperosmolar compared with serum [125]. Thus, hypernatremic hypertonicity in RA synovial fluid may increase Th17 differentiation and lymphocyte proliferation, leading to exacerbation of the inflammatory environment [126].

Furthermore, a relative (intracellular due to electrolyte changes) or absolute deficiency of body potassium level has been documented in RA patients. Figure (3-5) was showed a low level of K in RA patient with mean value (3.5mmol/L) compare to (4.5mmol/L) in control group. Overall, the clinical data regarding a correlation between potassium and RA is insufficient and sketchy [127].

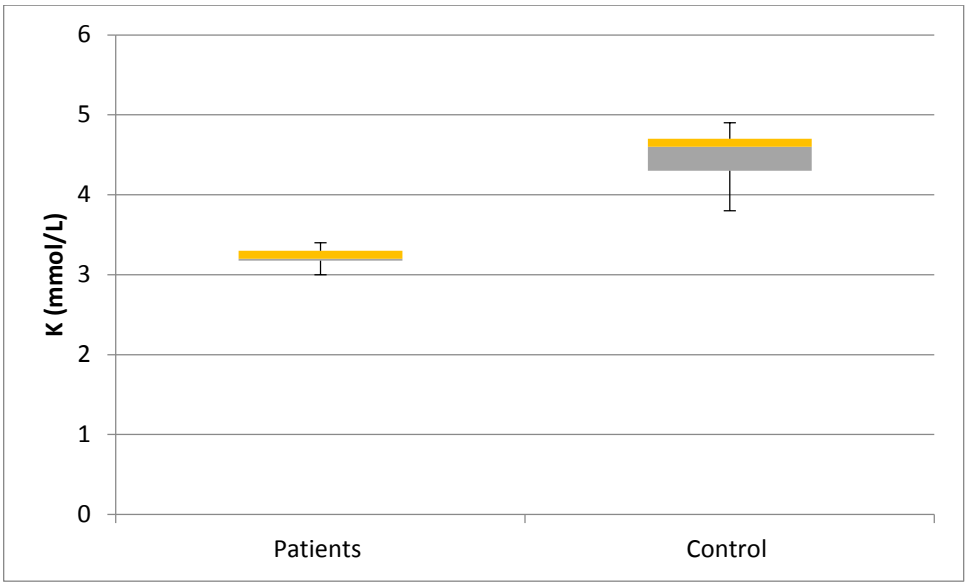


Figure (3-5) Boxplot of the Distribution of serum level of Potassium mmol/L in rheumatoid arthritis group compared to control group

The precise cause of low potassium in RA is unknown. Diet may be an important cause in some patients [128]. However, other causes may be speculated based on the pathophysiology events in RA which may be linked with body K. RA is characterized by a higher than normal metabolic rate and catabolic state which lead to cachexia, low muscle mass, an acid load, and disturbed pH balance [129] . Compensatory changes in cellular K and renal excretion (K) are required to maintain neutral pH. Due to intense immune cellular activity, increased metabolic demand, and energy expenditure, the glycolytic pathways are intensely activated. K is required for several enzymes (glucose phosphate isomerase, enolase, and aldolase) which participate in the glycolytic pathway [130] . It is probable that these hyperactive states require excess K, and thus, there is an overutilization of K. In RA patients, there may be a relative (intracellular due to electrolyte shifts) or absolute deficiency of body K.

3.4. Examination the serum Bone minerals levels in rheumatoid arthritis group

Hypercalcemia in rheumatoid arthritis can be classified as independent of PTH disorder. However, the relationship between rheumatoid arthritis (RA) and hypercalcemia is unclear and conflicting results have been reported.

Serum calcium levels and phosphorus level were increased markedly in RA patients as compared to control group, the mean values in patient were 12.3mmol/L and 5.1mg/dl respectively) while in control group the mean values were (10. 3mmol/L and 3.6 mg/dl) as shown in figure (3-6).

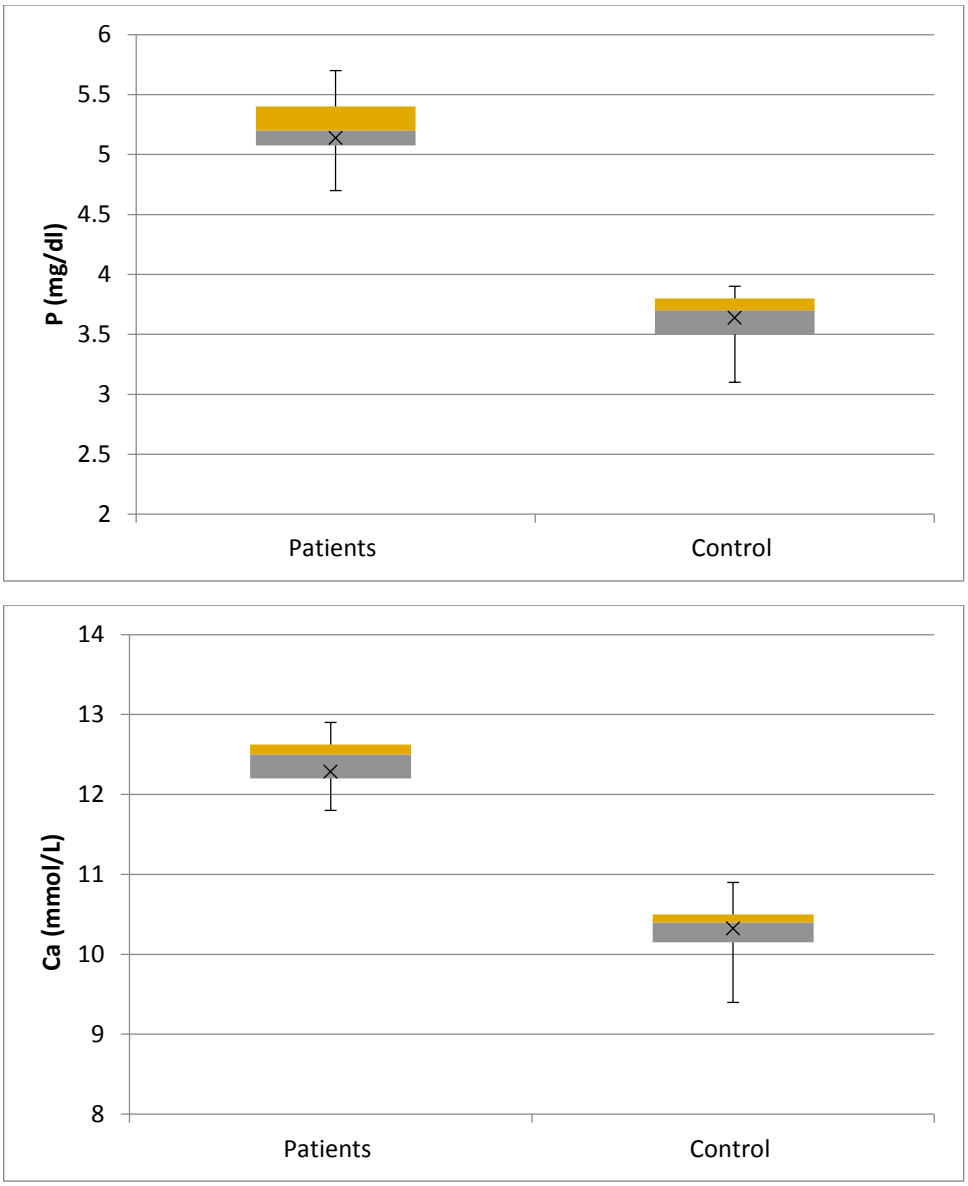


Figure (3-6) Boxplot of the Distribution of serum level of phosphorus and calcium mmol/L in rheumatoid arthritis group compared to control group

It is postulated that the elevation of phosphorous was related to tissue hypoxia with an increase in ATP degradation resulting in the release of inorganic phosphorous from cells. Hypertrophy and hyperplasia creates a hypoxic environment in synovial joints. It is corroborated by the reports of

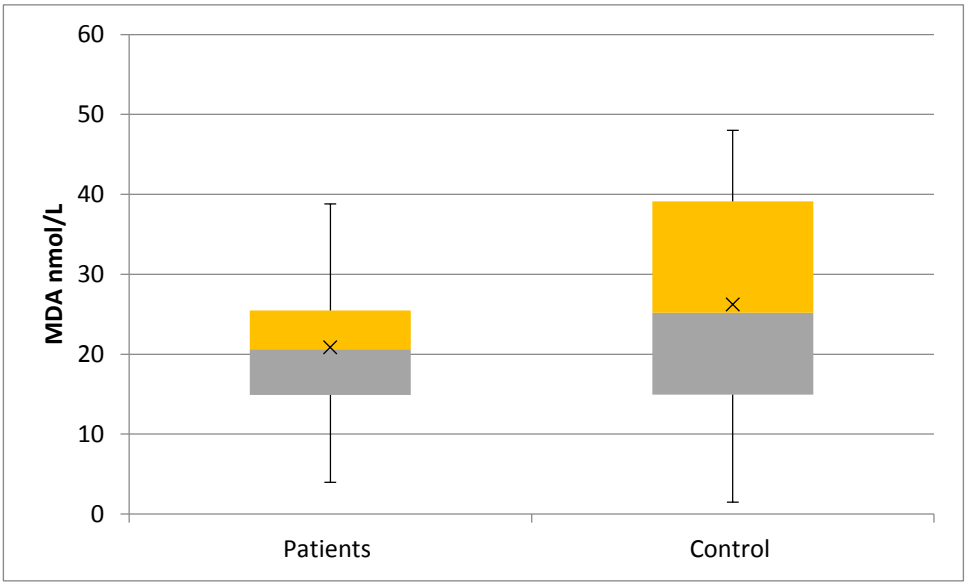
low glucose and high lactate levels in rheumatoid synovial fluid [131]. Acidosis is another factor that may acts to promote shifts of phosphate from the intracellular to extracellular pool. The rise in serum inorganic phosphate may parallel increase in blood lactate levels suggesting that a state of partial anaerobic metabolism may be contributory factor [132]. In RA, hypoxic environment triggers oxygen free radicals generation and alters oxidative metabolism within the cell. It leads to disruption in intracellular ionic environment and altered calcium and phosphorus levels.

3.5. Examination the serum Oxidative stress/ antioxidant levels in rheumatoid arthritis group

Since the pathogenesis of this disease is multifactorial, many compelling evidence suggests that oxidative stress is involved in the onset of RA. The levels of oxidative stress markers and antioxidant enzyme activity in RA patients and the control group were also investigated in this study. In patients with RA, the data indicate high levels of oxidative stress marker (MDA) and low antioxidant activity as shown in figure (3-7), which is reflected by increased lipid peroxidation in peripheral blood of patients with RA. It has been reported by a mechanism in which MDA, the product of lipid peroxidation, reacts with lysine residues in protein to produce immunogenic molecules, which can exacerbate inflammation. The longer chain polyunsaturated fatty acids are especially potent at increasing lipid peroxidation and causing cell damage by oxidative stress [133] Articular cartilage and synovial fibroblasts have been found to synthesize substantial amounts of free radical suggesting joints as a potential source of these product. Affects physiological process within joints would included

modulation of interleukin –1(IL-1) induced bone resorption and cartilage metabolism [134].

Formation of reactive oxygen species and lipid peroxides as a result of disease activity may play an important role in RA. While lowered concentrations of antioxidants in the blood considerably increase the probability of the occurrence of RA [135]. Many investigators have focussed on oxidative stress since last few years and suggest that RA patients are more prone to lipid peroxidation [136]. Generation of reactive oxygen species may be particularly important factor for bone resumption in inflammatory process [137]. Serum antioxidants are low because they have been used in reducing inflammatory products.SOD may affect the immunity of the organism by decreasing H_2O_2 concentration and causing decrease in lymphocyte proliferation. It results in mild anti-inflammatory effect. It may play a role in controlling chronic inflammation of any cause [138].



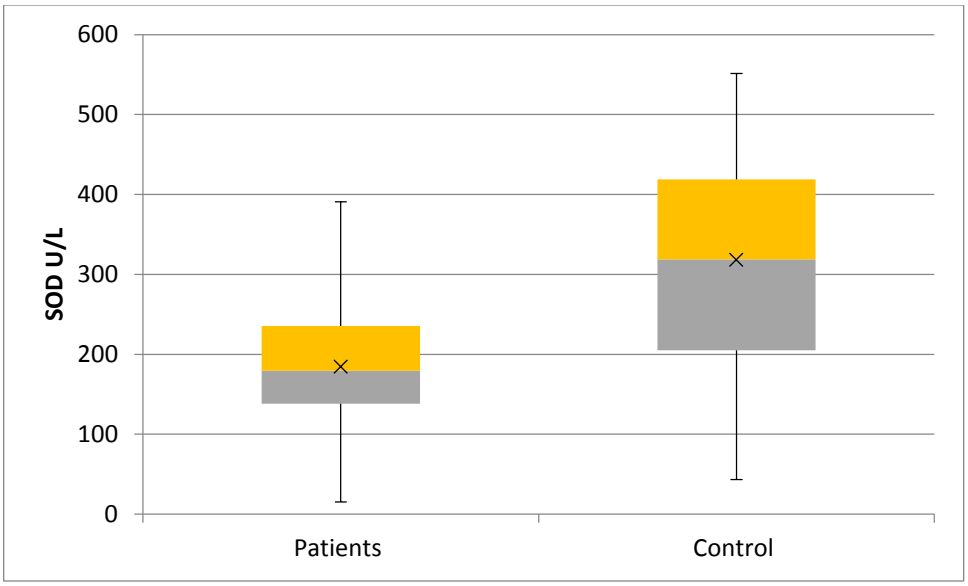


Figure (3-7) serum level of MDA mmol/L and serum activity of SOD U/L in rheumatoid arthritis group compared to control group

3.6. Lead poisoning

It has been reported that Heavy metals might influence markedly the development of autoimmunity disorders such as RA. exposure to such metals like Lead (Pb) has been considered importantly in the development of RA [(139)]. Pb is a most common heavy metal found throughout the environment and it poses a significant health risk if too much enters the body. Many studies showed that Pb is main culprit of oxidative stress as it is shown to deplete antioxidant proteins and induce the production of ROS and reactive nitrogen species (RNS) [(140)].

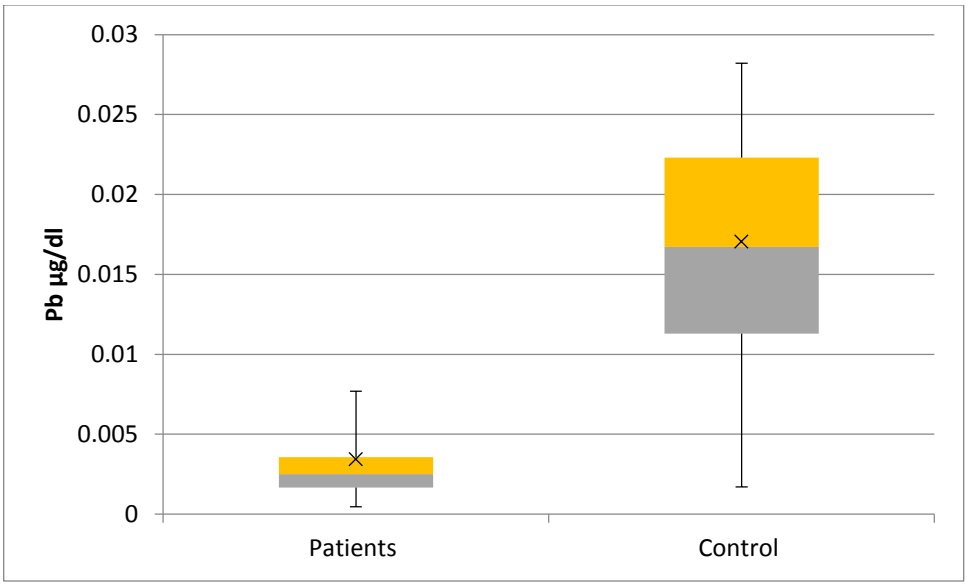


Figure (3-8) the Distribution of serum level Lead (Pb) in rheumatoid arthritis group compared to control group

The present study was compared serum level of heavy metals (pb) in RA and healthy control individuals. Fortunately, serum level of both groups were below the threshold value (3.5µg/dl). Figure (3-8)

3.7. Receiver operating characteristics of biomarkers level in Rheumatoid arthritis patients:

Receiver operating characteristics (ROC) curve analysis of studied markers was performed. The area under the ROC curve (AUC) for the MDA/ SOD ratio was (AUC = 0.72) as shown in Figure(3-9). ROC analysis indicated that MDA/ SOD ratio >0.037 was predictive of increasing oxidative stress at the expense of antioxident with 98% sensitivity, 94% specificity as shown in Table (3-2) .

Table (3-2) AUC, optimal threshold, Sensitivity and specificity of MDA/ SOD ratio obtained by the ROC curves for prediction of Rheumatoid arthritis

RA	AUP	Sensitivity %	Specificity %	P value	Cut-off points	CI (95%)
MDA/ SOD Ratio	0.72	0.98	0.94	>0.001	0.037	0.604-0.833

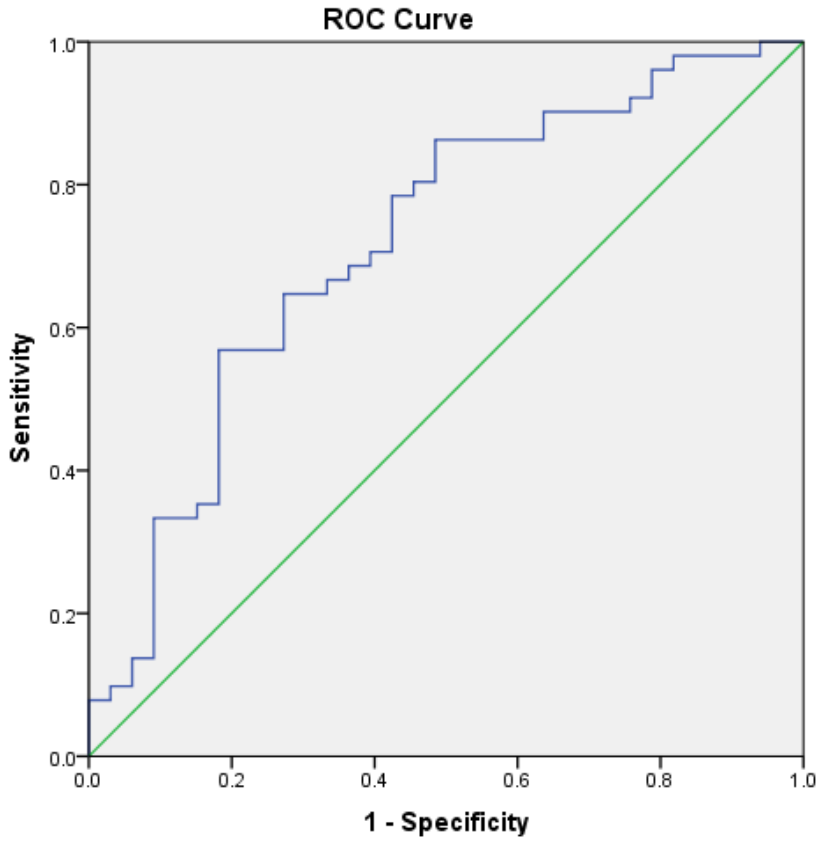


Figure (3-9) Receiver operating characteristics (ROC) curve analysis of MDA/ SOD ratio in the Rheumatoid arthritis patients

Results of the current study was focused on the potential biomarkers associated with an increased pro-inflammatory status and decrease antioxidant activity. Since the RA is autoimmune diseases, pro-inflammatory status could be characterized by an overactivation of the inflammatory cascade which is simply reflected by the MDA/ SOD ratio. Recent investigation by (Mititelu et al; 2020) state that the major need to RA management is to discover noninvasive tests that can be used to monitor the immune status of the body in such patient [(141)]. Roc analysis of MDA/SOD ratio indicated that these biomarkers might be a reliable result for this purpose. In spit of this ratio could be significantly altered in many other pathological conditions, but it might be a tool of inflammation in rheumatoid arthritis along with the previos reported markers such as RF, ESR, and CRP. the inflammasome signaling pathway and are actively involved in RA disease activity. Previous studies showed that the pro-inflammatory agents act as a trigger that switch on the inflammasome and contribute to RA disease progression [(142)]. On the other hand, ROC analysis (figure 3-10) of BMP2 was also shown a specificity 57% and high sensitivity 95% towards Rheumatoid arthritis with AUP 0.824 at an optimal threshold >0.12 ng/ml as shown in Table (3-3) .

Table (3-3) AUC, optimal threshold, Sensitivity and specificity of BMp2 level obtained by the ROC curves for prediction of Rheumatoid arthritis

RA	AUP	Sensitivity %	Specificity %	Cut-off points	P value	CI (95%)
BMP2	0.824	0.95	0.57	0.12	>0.001	0.721-0.927

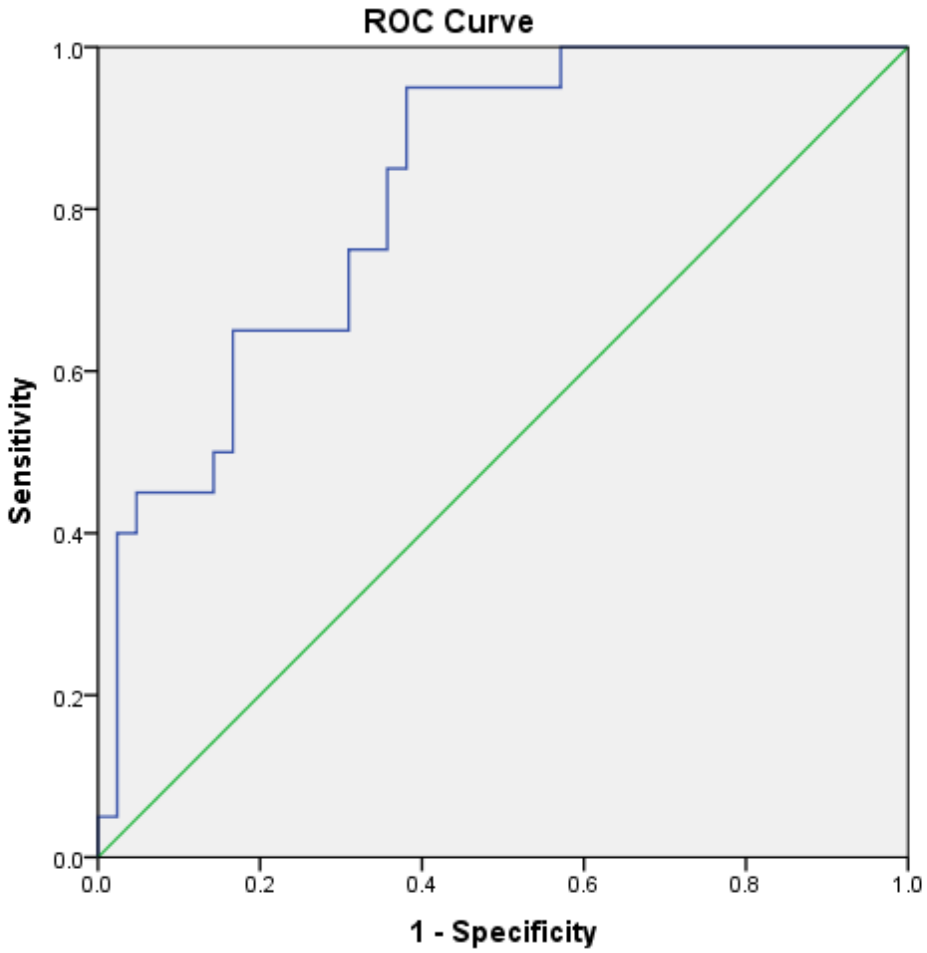


Figure (3-10) Receiver operating characteristics (ROC) curve analysis of BMP2 level in the Rheumatoid arthritis patients

BMP2, 4 and 6 are all thought to play the most important roles in skeletogenesis. Many studies have suggested that BMP2 is a pivotal signal for the regulation of osteoblastogenesis [(143)] . [(144)] also showed that BMP2 promotes and regulating bone formation and the expression of Indian hedgehog (protein) which has a negative feedback loop regulating the onset of hypertrophic differentiation of chondrocytes [(145)]. These findings indicate that BMP6 is an important regulator of bone and cartilage cell

proliferation and also could be used as a good sensitive biomarker of Rheumatoid arthritis.

Conclusion and future work

1. Conclusion and future directions

BMPs are being considered as therapeutic agents to stimulate healing of articular cartilage after damage. Evidence suggests that BMPs are present in adult joints and have roles in healing and maintenance understanding of the role of BMP signaling has expanded beyond its initially discovered role in the skeletal system . Components of BMP signaling are prominently emerging as direct coordinators of inflammatory processes across various cell types from immune cells to endothelial and connective tissue cells. the multifaceted roles of BMP signaling in inflammation will allow greater insight into the molecular mechanisms that drive diseases such as RA.

As a conclusion of this study,

1-BMp2 was associated with RA, Oxidative stress also was contributed. Results were reflected an optimal management of these patients involves determining certain biomarkers, with a high sensitivity and feasibility, which can reflect the complex interrelation between inflammatory status and oxidative stress level.

2- Measurements of serum levels of BMP-2 may possibly serve as a biomarker for determining RA disease and might be categorized into the *Diagnostic of disease* biomarkers. The multifunctional effects of BMPs make them attractive tools for the treatment of cartilage damage in patients with degenerative diseases such as RA.

3-Finally, we conclude that BMP2 and MDA/SOD ratio correlated with RA. It could provide a view of the complex phenomenon represented by key contributors to the disease outcome, and an increased awareness should be attributed to these biomarkers.

2. Future work

However, the biological activity of BMP2 in cartilage cell proliferation and differentiation, as well as related signaling pathways, has remained unclear.

Research and development leading to the successful application of BMPs can initiate a new era in the treatment of cartilage degenerative diseases like RA and OA. Therefore, it might be a good idea for future routine clinical applications to:

1) Looking for further understanding of the molecular mechanism of BMP2 in cartilage which is urgently needed.
2) Study the genes involved in the signaling pathways of expression of BMP2 may related to the regulation by the proliferation and differentiation of cartilage cells, but how the signal such as phosphorylation or other protein modification transmit still need more work to verify.
3) It may be worth considering their pharmaceutical value and investigate the mechanisms that underlying the association between RA and joints remodeling, with the aim to develop effective pharmacological agents to delay the progression of RA.

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Appendix :

RHEUMATIC DISEASE QUESTIONNAIRE:

Patient information:

Name:	Age:	Job:
Height:	Weight:	BMI:
Gender: Male ☐ Female ☐ Menstrual cycle: Yes ☐ No ☐		Duration of diseases:

Chronic diseases:

Diabetes		Hypertension		Smoking	

Required tests:

BMP		
MAD		
SOD		
Lead		
Other investigations		

الخلاصة :
الخلفية: التهاب المفاصل الرثوي هو مرض مناعي ذاتي مزمن يتميز بالتهاب الغشاء الزليلي الذي يمكن أن يؤدي إلى تشوه المفاصل وإعاقة جسدية. تباينت المؤشرات الحيوية التي تمت دراستها في التهاب المفاصل الروماتويدي ولها دور مهم في التشخيص والتنبؤ بالعلاج ومراقبة نشاط المرض والتنبؤ بالاستجابة للعلاج البيولوجي. بروتينات تشكل العظام BMPs هي أعضاء في عائلة عامل النمو المحول بيتا (TGF-β) وهي تحفز تكوين الغضاريف والأنسجة الضامة الأخرى. أيضا ، يمكن أن تحفز تكوين العظام في المختبر والمواقع غير المتجانسة في الجسم الحي. تم التعرف عن دورها المهم إلى حد كبير في تكوين الجنين ، وتكوين الهيكل العظمي المبكر قبل الولادة ، بالإضافة إلى ارتباطها بعدد من اضطرابات الهيكل العظمي البشري. هدفت هذه الدراسة إلى التحقق من دور BMP2 في مرضى التهاب المفاصل الرثوي وربطه بالعلامات القياسية والعامة لأمراض التشخيص.

المواد وطرق العمل: تضمن العمل الحالي دراسة حالة لمجموعة من (100) عينة: (60) عينة مرضى ، (40) عينة اشخاص اصحاء. تم اختيار مرضى التهاب المفاصل الروماتويدي من مركز العلاج البيولوجي في مدينة المرجان الطبية في بابل. تم إجراء قياس البروتين للعظام البشرية في الدم 2 ومستوى Malondialdehyde ونشاط انزيم Superoxide dismutase باستخدام تقنية ELISA. تم تحديد القياس الكمي للعناصر النزرة والإلكتروليتات باستخدام طريقة مطياف الامتصاص الذري عديم اللهب بتقنية فرن الجرافيت (GFAAS).
النتائج:
اظهرت النتائج وجود فرط في صوديوم الدم ونقص البوتاسيوم ونقص مغنسيوم في دم في مرضى التهاب المفاصل الرثوي. ارتبط انخفاض مستوى BMP2 مع التهاب المفاصل الروماتويدي ، وكان متوسط مستوى BMP2 في مجموعة المرضى (0.15 نانوغرام / مل بينما كان التحكم 2.95 نانوغرام / مل). علاوة على ذلك ، أشارت النتائج إلى وجود مستويات عالية من مؤشر الإجهاد التأكسدي (MDA) وانخفاض نشاط مضادات الأكسدة. أظهر تحليل ROC لـ BMP2 أيضًا خصوصية 57 ٪ وحساسية عالية 95 ٪ تجاه التهاب المفاصل الروماتويدي مع (AUP 0.824) عند عتبة مثالية> 0.12 نانوغرام / مل.

الخلاصة: لخصت الدراسة إلى أن نسبة BMP2 و MDA / SOD مرتبطة بـ RA. يمكن أن يوفر رؤية لهذه الظاهرة المعقدة التي تساهم بشكل رئيسي في نتائج المرض ، وينبغي أن يعزى الوعي المتزايد إلى هذه المؤشرات الحيوية.

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الدور البايوكيميائي لبروتينات العظام وجهد الاكسدة في التهاب المفاصل الرثوي

رسالة ماجستير

الى مجلس كلية الطب/ فرع الكيمياء و الكيمياء الحياتية/ جامعة كربلاء كجزء من متطلبات نيل درجة الماجستير في الكيمياء السريرية

من قبل

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بكالوريوس طب وجراحة بيطرية – جامعة القاسم الخضراء – 2017

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