Republic of Iraq
Ministry of Higher Education
and Scientific Research
University of Baghdad
College of Medicine

Effects of CD20 Inhibitor Therapy in Comparison to TNF α Inhibitor Therapy on Serum IL-17 in Patients with Active Rheumatoid Arthritis

A thesis

submitted to the Council of College of Medicine / University of Baghdad in a partial fulfillment of the requirements for the degree of Master of science in Clinical Biochemistry

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1434 **2013**

بسم الله الرحمن الرحيم

(وَمَا أُوتِيتُم مِّنَ الْعِلْمِ إِلَّا قَلِيلًا)

صدق الله العلي العظيم

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Dedication

-To my father , my mother and my brother Dr.Ali

-To those who support me all the time

- My brothers : Dr.Adil and Dr.Fadhil
- My sisters
- My special small family
- And my friends.

Mohammed

Certificate

We the examining committee , after reading this thesis

" Effects of CD20 Inhibitor Therapy in Comparison to TNF α Inhibitor Therapy on Serum IL-17 in Patients with Active Rheumatoid Arthritis "

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Acknowledments

First of all I thank ***Allah*** who gave me the support to fulfill these part thesis .

I would like to express my deepest gratitude and thanks to my supervisor ***Assist Professor Dr.Halla Ghazi Mahmood*** for her valuable advices , support and helping me to give opinions good views for the completion of this research.

Special thanks to my supervisor ***Dr.Mohammad Hadi Al-Osami*** for his assistance and patients in choosing samples and advices through out the study in Baghdad Teaching Hospital .

Also I would like to express my special thank and acknowlgement to ***Proff. Dr. Kismah Mohammad Turki*** head of biochemistry department for her great efforts , facilities and support during all the time of the search .

The thanks is also continuous to ***all staff of departments of Teaching laboratories / Medical City.***

My gratitude and thanks to all ***staff of department of Biochemistry*** , college of medicine,university of baghdad.

Finally my gratitude and thanks to ***my family , all of them ,*** for their encouragement , supporting and helping through all period of my study.

Mohammed

Abstract :

Background :

Rheumatoid arthritis (RA) is a chronic , systemic , inflammatory disorder that may affect many tissues and organs ,but principally attacks flexible(synovial) Joints , the pathogenesis of RA is not completely understood .

Objective :

To evaluate the effects of CD20 inhibitor therapy in comparison to effects of TNF α inhibitor therapy on serum IL-17 in patients with active rheumatoid arthritis.

Setting :

This study was performed during the period from October 2012 to May 2013 . The subject were selected from the patients attending the out patients clinic in Medical city /Baghdad Teaching Hospital / Rheumatology unit and the laboratory tests were done in Medical City /Teaching Laboratories .

Subjects :

The study include 70 patients and 20 healthy control individuals , their age range from 20 – 68 years . The patients were divided into three groups:

- Group (1)consist of 20 RA patients received disease modifying anti rheumatic drugs(DMARDs) .
- Group(2) consist of 25 RA patients received biological treatment Etanercept (anti TNFα).
- Group(3) consist of 25 RA patients received biological treatment Rituximab (anti CD20).

Methods :

Enzyme Linkade Immunosorbent Assay (ELISA) test was used for the determination of IL-17 , Leptin and hsCRP , while colorimetric method was used for the determination of uric acid . Rheumatoid factor (RF) was detected by serological investigation . The only anthropometric parameter in this study was body mass index(BMI).

Results :

The current results revealed that serum levels of hsC-RP , ESR and RF were significantly higher in patients than in healthy controls. While serum level of IL-17 was significantly lower in patients who are undertreatment than in healthy control ($P < 0.05$).

There was significant difference among groups : serum level of IL-17 :

- in group 1 higer than in group 2 ($P < 0.05$)
- in group 2 higer than in group 3 ($P < 0.05$)

There was no significant difference between all the groups in this study regarding level of uric acid ($P > 0.05$) and the level of the leptin ($P > 0.05$) , also significant positive correlation among each of ESR , hsC-RP , IL-17 ($P < 0.05$) and ($0 < r < 1+$).

Conclusion :

- IL-17 has a significant effect on the pathogenesis of RA .
- IL-17 level is higher in normal people and people with RA receiving DMARDs compared to RA patients receiving biological treatment.
- In patients with biological treatment , these received TNFα inhibitor (Etanercept) has a high IL-17 level compared to these received CD20 inhibitor (Rituximab) .
- IL-17 can be used as a marker for RA activity.

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List of Abbreviations

Abbrev.	Meaning
ACCP	Anti-Cyclic Citrollinated Peptide
ACPA	Anti-Citrollinated Peptide Antigen
ACR	American College of Rheumatology
ANA	AntiNuclear Antibodies
ARA	American Rheumatisme Association
BMI	Body Mass Index
CCR6	Chemokines (C-C motif) Receptor 6
CD20	Cluster differentiation 20
DAS28	Disease Activity Score 28
DMARDs	Disease modifying Anti-Rheumatic Drugs
EBV	Epstin Barr Virus
ELISA	Enzyme Linkade Immunosorbent Assay
ECT	Electro Convulsive Therapy
ESR	Erythrocyte Sedimentation Rate
EULAR	European League Against Rheumatism
FGF	Fibroblast Growth Factor
hsC-RP	High sensitive C-reactive Protein

HLA	Human Leukocyte Antigen
IFN	Interferon
IL	Interlukin
MHC	Major Histocompatibility Complex
PDGF	Platelet Derived Growth Factor
RF	Rheumatoid Factor
SD	Standar Deviation
SLE	Systemic Lupus Erythmatosus
TFG	Trans Formig Growth factor
Th	T helper

Chapter one

Introduction

and

Literature Review

1. Introduction:

1.1 Rheumatoid Arthritis (RA) :

1.1.1 Definition:

Rheumatoid arthritis (RA) is a chronic , systemic , inflammatory disorder that may affect many tissues and organs . The process involves an inflammatory response of the capsule around the Joints (synovium) secondary to swelling (hyperplasia) of synovial cells , excess synovial fluid and the development of fibrous tissue (pannus) in the synovium. (Jijith .,*et al* , 2011).

About 1 % of the world's population has rheumatoid arthritis, women three times as often as men , onset is most frequent between the age of 40 and 50 , but people of any age can be affected . The name is based on the term "rheumatic fever", an illness which includes joint pain and is derived from the Greek word.(Majithia , Geraci SA,2012).

Rheumatoid arthritis should not be confused with other formes of arthritis such as osteoarthritis or arthritis associated with infection and it is an autoimmune disease which means that the body's immune system mistakenly attaks the tissue. (Lubberts ,2008 ; Annunziato *et al*, 2009).

Rheumatoid arthritis is a chronic illness which can last for years and patients with this disease may experience long periods without symptoms.In some people with rheumatoid arthritis the chronic inflammation leads to the destruction of the cartilage , bone , and ligaments ,causing deformity of the joints . Damage to the joints can occur early disease and be progressive . (William and Meliss Conrad , 2012).

To diagnose RA patients the American College of Rheumatology (ACR) collaborated to create a classification criteria for RA which is the recommendation in 2010 . Patients with RA live 3 to 12 years less than general Population . (Guideline *et al* ,2012). Increased mortality in these patients is mainly due to accelerated cardiovascular disease especially in those with high disease activity and chronic inflammation .The relatively new biologic therapies may reverse progression of atherosclerosis and extend life in those with RA . (Katherine and Herbert , 2013).

1.1.2 History

The first known traces of arthritis date back as far as 4500 BC and a text dated 123 AD first describes symptoms very similar to RA which also noted in skeletal remains of Native Americans found in Tennessee but in old world the disease is vanishingly rare before the 17 th century , and on this basis investigators believe it spread across the Atlantic during the age of exploration while in 1859 the disease acquired its current name .(Aletaha ,*et al* , 2010).

The art of Peter Paul Rubens may possibly depict the effects of rheumatoid arthritis and in his later paintings , rendered hands show in the opinion of some physician increasing deformity consistent with the symptoms of the disease .

RA appears to some to have been depicted in 16 th century painting ,however ,it is generally recognized in art historical circles that the painting of hands in the16 th and 17 th century followed certain stylized conventions , most clearly seen in the mannerist movement . (Hart , *et al* , 2012). Historic treatment for RA have also included : rest , ice , compression and elevation , apple diet , nutmeg , some light exercise every now and then , nettles , bee venom , copper bracelets ,

rhubarb diet , extractions of teeth , fasting , honey , vitamins , insulin, magnets , and electroconvulsive therapy (ECT) . Most of these these treatment have either had no effect at all , or their effects have been modest and transient , while not being generealizable.
(Jijith , *et al* , 2011).

1.1.3 Epidemiology

Worldwide,the annual incidence of RA is approximately 3cases per 10,000 population and the prevalence rate is approximately 1 % increasing with age and peaking between the ages of 35 and 50 year . RA affects all population though it is much more prevalent in some groups and much less prevalent in others. (Prakken and Albani , 2011).

Disease concordance in monozygotic twins is approximately 15-20% , suggesting that non genetic factors play an important role and the worldwide frequency of RA is relatively constant on ubiquitors infectious agent has been postulated to play an etiologic role .

Women are affected by RA approximately 3 times more often than men but sex differences diminish in older age groups. (Ahlmen and Svensson , 2010).

1.1.4 Pathophysiology :

The pathogenesis of RA is not completely understood . An external trigger (eg , cigarette smoking , infection , or trauma) that triggers an autoimmune reaction , leading to synovial hypertrophy and chronic joint inflammation along with the potentiall for extra-articular manifestations , is theorized to occur in genetically susceptible individuals . (Lindhardsen and Ahlehoff , 2012).

Synovial cell hyperplasia and endothelial cell activation are early events in the pathologic process that progresses to uncontrolled inflammation and consequent cartilage and bone destruction .

Genetic factors and immune system abnormalities contribute contribute to disease propagation .(Aletaha and Alasti , 2012).

Clusters of differentiation (CD)4T cells,mononuclear phagocytes , fibroblasts , osteoclasts and neutrophils play major cellular roles in the pathophysiology of RA whereas B cells produce autoantibodies (ie , Rheumatoid Factor : are autoantibodies with specifity for FC fragment of immunoglobuline G ,of which there production is a major immunological abnormalities). Abnormal production of numerous cytokines,chemokines and other inflammatory mediators (e g , tumor necrosis factor alpha TNF α , IL-1 , IL-6 , IL-8 , transforming growth factor beta TFG – beta , fibroblast growth factor FGF , and platelet - derived growth factor PDGF) has been demonstrated in patients with RA.

Ultimately inflammation and exuberant of the synoviam (ie , pannus) leads to destruction of various tissues , including cartilage , bone , tendons , ligaments ,and blood vessels although the articular structures are the primary sites involved by RA , other tissues are also affected. (Katherine and Herbert , 2013).

1.1.5 Etiology

The cause of RA is unknown . Genetic , environmental , hormonal , immunologic ,and infectious factors may play significant role.

Socioeconomical , psychological , and lifestyle factors (eg , tobacco use , the main environmental risk) may influence disease outcome . (Carlens and Hergens , 2010).

1.1.5.1 Genetic factors

Genetic factors account for 50 % of the risk for developing RA. About 60 % of RA patients in the United States carry a shared epitope of the human leukocyte antigen (HLA)-DR4 cluster which constitutes one of the peptide – binding sites of certain HLA-DR molecules molecules associated with RA (eg , HLA-DR beta 0401 , 0404 , 0405) HLA - DR1 (HLA – DR beta 0101) also carries this shared epitope and confers risk .

Other HLA - DR4 molecules (eg , HLA-DR beta 0402) lack this epitope and do not confer this risk. Genes other than those of the major histocompatibility complex (MHC) are also involved and results from sequencing genes of families with RA suggesting the presence of several resistance and susceptibility genes including PTPN22and TRAF. (Potter and Eyre,2007).

1.1.5.2 Infectious agents

For many decades numerous infectious agents have been suggested as potential causes of RA ,including mycoplasma organis , Epstein – Barr virus (EBV) , and rubella virus .

This suggestion is indirectly supported by the following evidence :

- Occasional reports of flu-like disorders preceding the start of arthritis.
- The inducibility of arthritis in experimental animals with different bacteria or bacterial products (eg , streptococcal cell walls).
- The presence of bacterial products , including bacterial RNA , in patient's joints.
- The activity of several agents that have antimicrobial effects as disease modifying drugs (eg , gold salts , antimalarial agents and minocycline). (Routsias and Goules , 2011).

1.1.5.3 Immunologic factors

All of the major immuonologic elements play fundamental roles in initiating , propagating and maintaining the autoimmune process of RA.The exact orchestration of the cellular and cytokine events that lead to pathologic consequences (eg , synovial proliferation and subsequent joint destruction) is complex involving T and B cells , antigen –presenting cells (eg , B cells , macrophages and dendritic cells) and virus cytokines . Aberrant production and regulation of both proinflammatory and anti - inflammatory cytokines and cytokine pathways are also found in RA.(Agrawal and Misra , 2007).

T cells are assumed to play a pivotal role in the initiation of RA ,and the key player in this respect is assumed to be theT helper 1(Th1) CD4 cells. (Th1 cells produce IL-2 and interferon *IFN* gamma) , these cells may

subsequently activate macrophages and other cell populations , including synovial fibroblasts , macrophages and synovial fibroblasts are the main producers of TNF-α and IL-1 .

Experimental models suggest that synovial macrophages and fibroblasts may become autonomus and thus lose responsiveness to T-cell activities in the course of RA.(Thompson , et al , 2009).

B cells are important in the pathologic process and may serve as antigen – presenting cells . B cells also produce numerous autoantibodies (RF and Anti Cyclic Peptide Antibodies ACPA) and secrete cytokines .The hyperactive and hyperplastic synovial membrane is ultimately transformed into pannus tissue and invades cartilage and bone , with the latter being degraded by activated osteoclasts.

The major difference between RA and other forms of inflammatory arthritis such as psoriatic arthritis ,lies not in their respective cytokine patterns but rather in the high destructive potential of the RA synovial membrane and the local and systemic autoimmunity .(Varche and Narbonne , 2011).

1.1.6 Symptoms and signs :

The symptoms of RA can come and go depending on the degree of tissue inflammation : when body tissues are inflamed the disease is active while when tissue inflammation subsides , the disease is inactive (in remission).

Remissions can occur spontaneously or with treatment and can last weeks , months or years . During remession symptoms of the disease disappear and people generally fell well . When the disease becomes active again(relapse) , symptoms return with the return of disease activity and symptoms is called a flare .The course of RA varies among affected individuls and periods of flares and remissions are typical . (William and Melissa , 2012).

When the disease is active symptoms can include fatigue , loss of energy , loss of appetite , low – grade fever , muscle and joint aches and stiffness . Muscle and joint stiffness are usually most notable in the morning and after periods of inactivity . Arthritis is common during disease flares , during which joints frequently become red , swollen , painful , and tender . This occurs because the lining tissue of the joint (synovium) becomes inflamed resulting in the production of excessive joint fluid (synovial fluid) . The synovium also thickens with inflammation (synovitis) . (Seo and Philip , 2009).

The most common symptoms of RA include :

- Swelling , pain and heat in the joints .
- Stiffness in the joints especially in the morning .
- Persistent fatigue .
- Sleeping difficulties because of the pain.
- Weak muscles.
- The same joints on both sides of the body are usually affected . (William and Shiel , 2013).

Other symptoms include :

- Chest pain when taking a breath (pleurisy).
- Dry eyes and mouth (Sjogren syndrome).
- Eye burning , itching , and discharge.
- Nodules under the skin(usually a sign of more severe disease).
- Numbness , tingling , or burning in the hands and feet.
- Sleep difficulties. (Huizinga and Pincus , 2010)

While RA primarily affects joint , problems involving other organs of the body are known to occur.Extra – articula " outside the joints " manifsetations other than anaemia (which is very common) are clinically evident in about15 – 25 % of individuals with RA. It can be difficult to determine whether disease manifestations are directly caused by rheumatoid process itself , or from side effects of medications commonly used to treat it – for example , lung fibrosis from methotrexate or osteoporosis . (Turesson and Fallon,2013).

1.1.7 Diagnosis and Differential diagnosis :

1.1.7.1 Making diagnosis :

If symptoms and physical examination suggest RA , Systemic lupus erythromatosus SLE , Sjögren's syndrome , Lyme disease or one of a few other inflammatory forms of arthritis , the following tests can often confirm the doctor's suspicions :

a. *Antinuclear Antibody (ANA)* : commonly found in the blood of people who have SLE , ANAs (abnormal antibodies directed against the cell's nuclei) can also suggest the presence of polymyositis , scleroderma , Sjögren's syndrome , mixed connective tissue disease or RA . Tests to detect specific subsets of these antibodies can be used to confirm the diagnosis of a particular form of arthritis.

b. *hs-C-Reactive Protein (hs-CRP)*: CRP is an acute phase reactant made mainly in the liver . Its productin is controlled by an inflammatory cytokines , and it is commonly measured to screen for inflammation or infection , it is a marker of a acute inflammation , is elevated 100-1000 fold after infection . (Gremese and Salaffi , 2012).

CRP is one of the acute phase protein ,the serum or plasma levels of which rise during general , nonspecific response to a wide variety of disease . This include acute phase of rheumatoid arthritis . Composed to other indicators , detection of CRP is more reliable and sensitive indicator of the Inflammatory process than the erythrocyte sedimentation rate , as elevated CRP values are always associated with pathological changes .The CRP assay provides useful information for the diagnosis , therapy and monitoring of

inflammatory processes and associated disease also measurement of high - sensitivity CRP provides a quantitative determination of CRP in human serum. (Kushner ,*et al*, 2012).

c. *Rheumatoid Factor (RF)* : Designed to detect and measure the level of an anti – body that acts against the gamma globulin , this test is often positive in people with RA , in it , antibodies that collect in the synovium of the joint are known as rheumatoid factor . In about 80 % of cases of RA blood tests reveal rheumatoid factor , however , when it appears in patients with arthritic pain on both sides of the body , it is a strong indicator of RA . (Smolen and Aletaha , 2007).

d. *Erythrocyte sedimentation rates* : Also called ESR or" sed rate " , this test measures how fast red blood cells cling , fall and settle (like sediment) in the bottom of a glass tube over the course of an hour . The higher the rate , the greater the amount of inflammation .(Kushner , *et al* , 2012).

e. *Lyme serology* : This test detects an immune response to the infectious agent that causes Lyme disease and thus can be caused to confirm a diagnosis of the disease. (Lyngdoh and Marques , 2011).

f.*Skin biopsy* : Taking small samples of skin and examining them under a microscope can help doctors diagnose forms of arthritis that involve the skin , such as lupus , vasculitis (inflammation of the lupus , vasculitis (inflammation of the blood vessels) and psoriatic arthritis . (Lyngdoh and Marques , 2011).

g. *Muscle biopsy* : By going a little deeper into the tissue than with the skin biopsy , the surgeon can take a sample of muscle to be examined for signs of damage to the muscle fibers , findings can confirm a diagnosis of polymyositis or vasculitis. (Lyngdoh and Marques , 2011).

h. *Joint fluid tests* : In this procedure , which is similar to drawing blood , the doctor inserts a needle into a joint space and aspirate fluid . An examination of the fluid may reveal uric acid crystals or bacteria , confirming a diagnosis of gout or septic arthritis, suggesting that the joint inflammation is caused by infection . (Lyngdoh and Marques , 2011).

1.1.7.2 Differential Diagnosis :

A number of different medical conditions may be considered in the differential diagnosis of rheumatoid arthritis , these include :

- Connective tissue disease.
- Fibromyalgia.
- Viral arthritis.
- Thyroid disease .
- Infective endocarditis.
- Sarcoidosis.
- Hemachromatosis.

Early in the course of RA , self-limited viral syndromes should be considered especially hepatitis B and C, parvovirus , rubella (infectious or vaccination) , and Epstein -Barr virus . At any time systemic lupus erythmatosus , psoriatic arthritis , and reactive arthritis may present diagnostic challenge . This requires a targeted history and examination

to elucidate associated clinical symptoms , such as rashes , oral ulcers , nail changes,dactylitis,urethritis , and renal , pulmonary , gastrointestinal complications . (Jijith Krishnan ., *et al* , 2011).

Table 1.1 : Differentiate RA from other inflammatory arthritis.

RA Likely	***Differential Diagnosis***	***Features Suggesting Alternative Diagnosis***
.Morning stiffness > 30 minutes.	.Crystal artropathy .	.Mucosal ulcers,photosensitivity, psoriasis,skin rashes.
.Painful swelling of 3 or more joints.	.Psoriatic arthritis.	.Raynaud's.
.Symmetric involvement of hands and feet (especiallymetacarpoph-alangeal and metatarso-phalange al joints).	.Lupus.	.Ocular inflammationiritis.
.Duration of pain for 4 or more weeks.	.Reactive arthritis. .Spondyloarthropathies . .Polyarticularsepsis.	.Urethritis. .Inflammatory bowel disease. .infectious diarrhea. .Nephritis. .Isolated distal interphalangeal Joint inflammation.

(Semla and Beizer , 2010)

1.1.8 Risk Factor of RA

The etiology of RA is still unknown . Many cases are believed to result from an interaction between genetic factor and environmental exposure . (Jijith Krishnan ., *et al* , 2011).

. Socio-demographic : The incidence of RA is typically two to three times higher in women than men .The onset of RA , in both women and men , is highest among those in their sixties . (Jijith Krishnan , *et al* , 2011).

. Genetics : There is a longstanding evidence that specific HLA class || genotypes are associated with increased risk . Most attention has been given to the DR4 and DRB1 molecules of the major histocompatibility complex HLA class || genes .The strongest associations have been found between RA and the DRB1"0401 and DRB 1 "0404 alleles . Recent investigations indicate that of the more than 30 genes studied , the strongest candidate gene is PTPN22 , a gene that has been linked to several autoimmune conditions . (Gremese and Salaffi , 2012 .)

. Modifiable : Several modifiable risk factors have been studied in association with RA including reproductive hormonal exposure , tobacco use , dietary factors , and microbial exposures(Aletaha , *et al* ,2012).

1.1.9 Preventing symptoms (Reducing Risks) of RA

Studies show a correlation between RA and family genes , smoking , pollution , environmental toxins , lower estrogen levels and a viral infections . Suggesting that factors affecting overall health can also contribute to symptoms of RA , to preventing these must be :

1- Get tested early .

2- Increase estrogen .

3- Don't smoke.

4- Boost vitamin D .

5- Limit caffeine .

6- Eat more fish .

7- Prevent viral infections .

8- Avoid environmental toxins. (Carol , *et al* , 2012).

If the doctor suspects that the patient have RA , usually must be referred to a joint specialist (a rheumatologist) . This is to confirm the diagnosis and to advise on treatment . It is very important to start treatment as early as possible after symptoms begin . This is because any joint damage done by the disease is permanent. Therefore, it is vital to start treatment as early as possible to minimise or even prevent any permanent joint damage . (Aletaha , *et al* ,2012) . These treatments can make a big difference to reduce symptoms and improve the outlook by decreasing the disease activity , preventing joint damage , reduceing pain and stiffness in affected joints and reduceing the risk of developing associated conditions such as cardiovascular disease or osteoporosis. (Gremese and Salaffi , 2012 .)

1.1.10 Treatment and Management

1.1.10.1 Pharmacological Therapy :

Once a diagnosis is made , the main treatment goals are to control disease activity and slow the rate of joint damage ,in addition to minimizing pain , stiffness , inflammation and complications.

Pharmacologic therapies that are used include :

a-nonbiologic and biologic DMARDs .

b-adjunctive agents such as corticosteroids , NSAIDs and Analgesics.

(Anderson and Caplan 2012).

1.1.10.2.1 Early DMARD therapy :

Desease modifying anti rheumatic drugs (DMARDs) can be classified into nonbiologic and biologic agents . The recognition of TNF-α and interlukin (IL-1) as a central pro-inflammatory cytokines has led to the development of biologic agents that block these cytokines or their effects,in addition to improving signs and symptoms and quality of life . All biologic agents significantly retard radiographic progression of joint erosions .DMARDs represent the most important measure in the erosions treatment of RA . These agents can retard or prevent disease progression and joint destruction and subsequent loss of function.
(Gremese and Salaffi 2012).

1.1.10.2.1.1 Nonbiological DMARDs :

Include:

- hydroxyl chloro quine (HCQ)
- azathioprine (AZA)
- sulfasalazine (SSZ)
- methotrexate (MTX)
- leflunomide
- cyclosporine
- gold salts
- pencillamine
- and minocycline .(Lindhardsen and Ahlehoff , 2012)

1.1.10.2.1.2 Biological DMARDs :

Biological agents are expensive.Consensus statement do not recommend their use until at least one nonbiological DMARDs, usually MTX , has been administered without sufficient success.

The biological DMARDs include :

1.1.10.2.1.2.1 Tumor necrosis factor alpha (TNFα) inhibitor:

Tumor necrosis factor alpha (TNF α) is a pro - inflammatory cytokine produced by macrophages and lymphocytes . It is found in large quantities in the rheumatoid joint and is produced locally in the joint by synovial macrophages and lymphocytes infiltrating the joint synovium . TNFα is one of the critical cytokines that mediate joint damage and destruction due to its activities on many cells in the joint as well as effects on other organs and body system.

TNFα antagonists were the first of the biological DMARDs to be approved for the treatment of RA .These drugs began to enter the market for RA in 1999 and are now considered a part the American College of Rheumatology (ACR) recommendation for treatment of RA.(Clifton , *et al* , 2012).

1.1.10.2.1.2.2Non -Tumor necrosis factor alpha agents :

Rituximab (B-Cell Depletion) :

B - cells are an important inflammatory cell with multiple functions in the immune response .They serve as antigen presenting cells , directly interacting with T-cells and others . They can secrete cytokines , and differentiate into antibody - forming plasma cells . The depletion of B cells has been shown to be effective in reducing signs and symptoms of RA and in slowing radiographic progression . (Singh and Furst , 2012).

One B cell depleting agent , Rituximab is currently available for the treatment of RA . Rituximab causes a rapid and sustained depletion of circulating B cells in the circulation with clinical improvement in many patients . Clinical trials have demonstrated that Rituximab is effective in decreasing signs and symptoms and in slowing radiographic progression in RA patients who have failed other DMARDs therapies . The agent is currently approved only in patients who have failed TNFα antagonists .Rituximab is a chimeric monoclonal antibody that binds to the CD20 molecule on the B cell surface leading to the removal of B cells from the circulation . (Clifton , *et al* , 2012).

1.1.10.2.1.3 Combination DMARD therapy :

In clinical trials 30 –70 % of patients using DMARDs either as mono therapy or in combination therapy achieving partial responses . The American College of Rheumatology (ACR) disease activity score indicates that it is not possible to predict which patients will not have a response.

In clinical practice , there are 3 strategies that are employed to reduce disease activity in patients whose disease are not responding or in those with clinical response that are regarded as insufficient :

. Increasing the dose of medication.

. Switching to other DMARDs.

. Initiating combination therapy .(Katherine and Herbert ,2013)

1.2 Inflammatory Mediators in RA

1.2.1 Cytokines

Cytokines are immune mediators that play important roles in the pathogenesis of RA . Cytokines are the most important group of mediators in RA , the most prominent of these are TNFα, IL1 , and IL6. These cytokines released in the synovial microenvironment have : autocrine (activating the same cell) , paracrine (activating near - by cells) and endocrine (acting at distant sites). These effects accounts for many systemic manifestations of the disease. There are many shared functions of TNFα , IL1 ,IL6,and these cytokines in turn upregulate the expression of other cytokines . Among the important effects of these cytokines are :

- Induction of cytokine synthesis.
- Upregulation of adhesion molecules.
- Activation of osteoclasts.
- Induction of other inflammatory mediators including prostglandins , nitric oxide , matrix metalloproteinasees.
- Induction of the acute phase response (eg . C-reactive protein , increased ESR) .
- Systemic features (eg. Fatigue , fever , cachexia).
- Activation of B cells (IL6).(Brian and Erin , 2011).

Other cytokines are increasingly described in RA which include IL8, involved in cellular recruitment ,IL15 involved inTcell proliferation and IL17 which has pleiotropic in T cell proliferation . IL17 has also pleiotropic effects on multiple cell types including osteoblast leading to osteoclast activation .IL-23 is also involved by increasing Th 17 cell differentiation. (Clifton , *et al* ,2012).

1.2.2 IL- 17 :

Interleukin -17 has been implicated in the pathogenesis of a wide range of diseases including RA . IL-17 is best defined as a product of CD4 +Th17 cells but it can be also produced by CD8+Tcells

Interleukin -17 is a proinflammatory cytokine . There are several proposed mechanisms by which IL-17 mediates the pathogenic events in the course of arthritis . Some of them :

-By upregulating the production of proinflammatory cytokines.

-By facilitating cellular infiltration into the synovium.

-By enhancing innate immune response. (Smolen ,*et al* , 2012)

IL-17 response can be modulated by multiple cytokines . A combination treatment of Infliximab , an anti - TNF-α antibody, and methotrexate , an antimetabolite , is shown to significantly reduce disease along with decrease in the frequency of Th-17 cells and the levels of IL-17 in RA patients without significant adverse effects . Clinical trials aimed at inhibiting IL – 17 response show that such an agent holds promise as an efficacious treatment for arthritis . (Brian and Erin , 2011).

1.3 Leptin :

Leptin is one of the adipokines , functions as a hormone and a cytokine . Leptin is a 16 – kDa nonglycosylated protein encoded by the obese gene , which is located on human chromosome 7 . Leptin is synthesized primarily by the white adipose tissue . Increase secretion of leptin is associated with chronic inflammatory conditions . It activates monocytes /macrophage cells and potentiates production of the proinflammatory cytokines ,TNFα , IL-6 and directs T-cell differentiation to Th1 phenotype. It has been suggested that leptin may influence the outcome of RA .(Faten and Hisham ,2011).

There are several studies that showed significantly elevated concentrations of leptin in patients with RA,and noted elevated leptin serum concentrations in patients with higher disease activity evaluated by disease activity score 28 (DAS28) , ESR , and the number of tender joint . It was also noted that plasma concentrations of leptin were significantly higher than synovial fluid leptin,and this difference was particularly evident in non - erosive arthritis.(Robert and Gabriela , 2012)

1.4 Body Mass Index (BMI):

Body mass index (BMI) is a standard way to express a person's weight in proportion to height and is defined by the following equation :

$$BMI = \frac{\text{Mass in kg.}}{\text{Height in m}^2}$$

This single value of BMI gives a much clearer and more immediate indication of how healthy a person's weight is , and using it as "underweight , normal , overweight , obese , and severely obese" as in table 1.2 :

Table 1.2 : Relation between BMI and body weight description

Body Mass Index	*Body Weight Description*
< 20	Underweight
20 - 24	Normal
25 -29	Overweight
30 – 50	Obese
> 50	Severely Obese (also known as Morbidly Obese)

(Flegal , *et al* , 2013).

Aim of the Study :

The aim of the present study to evaluate the effects of CD20 inhibitor therapy in comparison to the effects of TNF α inhibitor therapy on serum level of IL-17 in patients with active rheumatoid arthritis .

Chapter two

Subjects

and

Methods

2.1 Subjects

2.1.1 Patients

Seventy patients were enrolled in this study their age range from 20 – 68 year . They were from attendants seeking treatment in the rheumatology and rehabilitation out patients clinic at Baghdad Teaching Hospital in Medical city in Baghdad from October 2012 to May 2013 .

The diagnosis of each case was established by clinical examination done by specialist rheumatologist and confirmed by laboratory and radiological investigation . The patients were subjected to questionnaire about name , age , gender , weight , hight , disease duration , type of drug used ,drugs duration and Disease Activity Score (DAS 28), (a copy of the data sheet is provided in appendix).

The patients were divided into three groups :

- Group (1)consist of 20 RA patients received disease modifying anti rheumatic drugs(DMARDs) .
- Group(2) consist of 25 RA patients received biological treatment Etanercept (anti TNFα).
- Group(3) consist of 25 RA patients received biological treatment Rituximab (anti CD20).

Criteria of inclusion :

- Known cases of established and confirmed RA by : clinical, laboratory , and radiological diagnosis .
- Non – complicated as well as complicated cases .
- patients on medical treatments that never affect the laboratory tests.

- Have disease activity which is defined by the fulfillment of at least three of the following five criteria:
  - Swelling in at least three joints.
  - Tenderness in at least six joints.
  - An ESR > 28 mm/1hr.
  - Morning stiffness of at least 45 min.
  - Disese activity score DAS 28 > 3.2 (DAS28 value was established by the physician in rheumatology unit. (Christopher,et al , 1999).

- **Criteria of exclusion :**
  - Diabetes mellitus (DM) .
  - Chronic renal failure .
  - Chronic liver disease .

2.1.2 Controls

Apparently healthy volunteers whose their ages and gender were matched with patients group consisted of 20 individuals considered as control . All of them had received no treatment with no complain of other chronic or systemic disease ,and all of them had negative RF test , their age range 21-54 year .Ethical approval informed concernt was obtained from each participant included in this study according to the declaration of Helsinki-ethical approval was obtained from the ethics of colleges of medicine.

2.2 Study Protocols :

The study protocols include :

1- Detrmination of IL-17 , by enzyme - linked immunosorbent assay (ELISA).

2- Detrmination of hs-CRP , by enzyme - linked immunosorbent assay (ELISA).

3- Detrmination of Leptin , by enzyme - linked immunosorbent assay (ELISA)..

4- Qualitative determination of Rheumatoid Factor.

5- determination of uric acid.

2.3 Materials :

2.3.1 Instruments and equipments :

Table 2.1 : Instruments and equipments

Instrument or equipment	Company
Centrifuge	Hettich - Germany
Micropipettes with disposable tips	GILSON-France
ELISA UNIT	Human -Germany
Refrigerator , Freezer	USA
Water bath	Memmert-Germany
Spectrophotometer	Shimadzu 670 A - Japan
Timer wach	England
Eppendrof's and plane tube	Afma – Disposable
Disposable syringe 5 ml	Medeco
Westergren pipette and rack	Arienfei
Stirrers	Sailing boat
Flask 500 ml	Hysil

2.3.2 Kits :

Table 2.2 : Kits

Kit	Company
IL-17 kit	CUSABIO-China
Leptin kit	CUSABIO-China
High sensitive C-reactive protein hsC-RP kit	Germany
Rheumatoid Factor(RF) kit	Spinreact,S.A. , Spain
Uric acid kit	Spinreact,S.A. , Spain

2.4 Methods :

2.4.1 Blood samples collection :

Five ml of venous blood were drawn from patients and controls , blood sample was collected in plastic gell tube and anticoagulate tube for haematologic tests (Hb , WBC , ESR) , and then centrifuged gell tubes for separation of serum , after that serum sample was divided into 5 aliquots in eppendroffs tubes , and kept at - 20˚C till used.

2.4.2 Detrmination of IL-17:

• *Principle of the assay:*

The assay employs the quantitative sandwich enzyme immunoassay technique . Antibody specific for IL-17 has been pre-coated onto a microplate . Standards and samples are pipetted into the wells and any IL-17 present is bound by the immobilized antibody . After removing any unbound substances , a biotin-conjugated antibody specific for IL-17 is added to the wells . After washing , avidin conjugated Horseradish Peroxidase (HRP) is added to the wells . Following a wash to remove any unbound avidin-enzyme reagent , a substrate solution is added to the wells and color develops in proportion to the amount of IL-17 bound in the initial step . The color development is stopped and the intensity of the color is measured.

•*Materials provided :*

Table 2.3 : Material provided with kit of IL-17

Reagents	Quantity
Assay plate (12 X 8 coated micro wells)	1 (96 wells)
Standard (Freeze dried)	2
Biotin – antibody (100 X concentrate)	1 X 120 µl
HRP – avidin (100 X concentrate)	1 X 120 µl
Biotin – antibody diluent	1 X 10 ml
HRP – avidin diluent	1 X 10 ml
Sample diluent	1 X 20 ml
Wash buffer (25 X concentrate)	1 X 20 ml
TMB substrate	1 X 10 ml
Stop solution	1 X 10 ml
Adhesive strip (for 96 wells)	4
Instruction manual	1

•*Assay procedure*

1.All reagents and samples were bring to room temperature (18 – 25 ˚C) before use .

2. Refer to the assay Layout Sheet to determining the number of wells to be used

3. Adding 100 µl of standard and sample per well , covering with the adhesive strip provided , incubating for 2 hrs. at 37˚C , aplte layoute is provided to record standard and samples assayed.

4. Removing the liquid of each well , don't wash .

5. Adding 100 µl of biotin-antibody to each well , covering with a new adhesive Strip , incubating for 1 hr. at 37˚C .

6. Aspirating each well and wash , repeating the process two times for a total of three washes by buffer wash.

7. Adding 100 µl of HRP-avidin to each well , covering the microtiter plate with a new adhesive strip , incubating for 1 hr. at 37˚C.
8. Repeating the aspiration/wash process for five times as in step 6 .
9. Adding 90 µl for TMB substrate to each well , incubating for 15-30 minutes At 37˚C , protecting from light.
10. Adding50 µl of stop solution to each well , gentlying tap the plate to ensuring through mixing.
11. Determining the optical densityof each well within 5 minutes , using a Microplate reader set to 450 nm.
12. The results are calculated by interpolation from standard curve , were constructed in the same assay as the sample , the absorbance for each sample on the vertical axis was located and the corresponding IL-17 concentration on the horizontal axis was read. (Human IL-17 ELISA kit Catalog).

2.4.3 Detrmination of hsC-RP:

• Principle of the assay :

Microtiterstrips coated with anti-CRP antibody are incubated with diluted standard sera and patients sample . During this incubation step CRP is bound specifically to the wells . After removal of the unbound serum proteins by a washing procedure , the antigen –antibody coplex in each well is detected with specific peroxidase – conjugated antibodies , after removal of the unbound conjugate , the strips are incubated with a chromogen solution containing tetramethylbenzidin and hydrogen peroxide : a blue colour develops in proportion to the amount of immunocoplex bound to the wells of the strips. The enzymatic reaction is stopped by the addition of 1N acidic solution and the absorbance value at 450 nm are detrmined . A standard curve is obtained by plotting the

absorbance values versus the corresponding standard values . The concentration of CRP in patient samples is determined by interpolation from the standard curve.

•*Reagents :*

1.Coated microtiterstrip – SORB MT

12 X 8 – well stripes coated with monoclonal antibodies to human CRP.

2. Standard sera – CAL

5 vials , each containing 1/10 prediluted CRP standard solutions (0.2 ml) , N having following values :

CAL 0:0 ; CAL 0.4:0.4 ; CAL 1:1 ; CAL 5:5 ; CAL 10:10 µg/ml. containing 0.09 % NaN_3 . Calibrated against the NIBSC 1st international standard , 85 / 506.

3. Conjugate – CONJ

1vial containing peroxidase conjugated monoclonal anti-human CRP antibodies (12 ml)contains antimicrobial agents and an inert red dye.

4. Specimen dilution buffer-SPEC BUF 5x

1 vial , containing 40 ml dilution buffer 5x concentrated , contain 0.095 NaN_3 and an inert green dye.

5. Washing solution – WASH SOLN 20x

I vial containing 50 ml 20x concentrated phosphate buffered washing solution.

6. Chromogen solution – CHROMO SOLN

1 vial containing 15 ml of a solution containing H_2O_2 and tetramethylbenzidin.

7. Stopping solution – STOP SOLN

1 vial containing 12 ml of 1 N acidic solution.

• *Assay procedure :*

1. The 10 x prediluted standard sera (2) are diluted 1:100 as follows : Pipetting 10 µl of each calibrator , adding 990 µl of diluted specimen dilution buffer and mixing carefully.
2. The samples were diluted 1:1000 in two consecutive steps : pipetting 10µl of each sample and adding 990 µl of diluted specimen dilution buffer, mixing thoroughly, adding450µl of diluted specimen dilution buffer to 50 µl of these 100x prediluted samples , mix thoroughly.
3. Pipetting100 µl of the diluted calibrators and samples into each of a pair of adjacent wells .
4. Incubating the covered microtiterstrips for 30 ± 2 min at room temperature.
5. Washing the microtiterstrips three times with washing solution.
6. Adding 100 µl of conjugate solution and incubating the covered microtiterstrips for 30 ± 2 min. at room temperature.
7. Repeating the washing procedure as described in step 5.
8. Adding 100 µl of chromogen solution to each well.
9. Incubating for 10 ± 2 min at room temperature .
10. Adding 50 µl of stopping solution to each well.
11. Determining the absorbance of each well at 450 nm within 30 min following the addition of acid.
12. The result is obtaind by : the average absorbance value of each calibrator is plotted against the corresponding CRP value and the best calibration curve (e.g. log / linear) is constructed . Use the average absorbance of each sample obtained in the hsCRP– ELISA to determine the corresponding value by simple interpolation from the curve . Depending on the experience and/or availability of computer capability . (hsCRP ELISA , User's Manual , Germany).

2.4.4 Detrmination of Leptin:

• *Principle of the assay :*

The assay employs the quantitative sandwich enzyme immunoassay technique . Antibody specific for LEP has been pre- coated onto a miceoplate . Standards and samples are pipetted into the wells and any LEP present is bound by the immobilized antibody . After removing any unbound substances , a biotin-conjugated antibody specific for LEP is added to the wells . After washing , avidin conjugated Horseradish Peroxidase (HRP) is added to the wells . Following a wash to remove any unbound avidin-enzyme reagent , a substrate solution is added to the wells and color develops in proportion to the amount of LEP bound in the initial step . The color development is stopped and the intensity of the color is measured.

•*Materials provided :*

Table 2.4 : Material provided with kit of Leptin

Reagents	Quantity
Assay plate (12 X 8 coated micro wells)	1 (96 wells)
Standard (Freeze dried)	2
Biotin – antibody (100 X concentrate)	1 X 120 µl
HRP – avidin (100 X concentrate)	1 X 120 µl
Biotin – antibody diluent	1 X 10 ml
HRP – avidin diluent	1 X 10 ml
Sample diluent	1 X 20 ml
Wash buffer (25 X concentrate)	1 X 20 ml
TMB substrate	1 X 10 ml
Stop solution	1 X 10 ml
Adhesive strip (for 96 wells)	4
Instruction manual	1

•*Assay procedure*

1.All reagents and samples were bring to room temperature (18 – 25 ˚C) before use .

2. Refer to the assay Layout Sheet to determining the number of wells to be used .

3. Adding 100 µl of standard and sample per well , covering with the adhesive strip provided , incubate for 2 hrs. at 37˚C , aplte layoute is provided to record standard and samples assayed.

4. Removing the liquid of each well , don't wash .

5. Adding 100 µl of biotin-antibody to each well , covering with a new adhesive Strip , incubating for 1 hr. at 37˚C

6. Aspirating each well and washing , repeating the process two times for a total of three washes by buffer wash.

7. Adding100µl of HRP-avidin to each well , covering the microtiter plate with a new adhesive strip , incubating for 1 hr. at 37˚C.

8. Repeating the aspiration/wash process for five times as in step 6 .

9. Adding 90 µl for TMB substrate to each well , incubating for 15-30 minutes at 37˚C , protecting from light.

10. Adding 50 µl of stop solution to each well , gentlying tap the plate to ensuring through mixing.

11. Determining the optical densityof each well within 5 minutes , using a microplate reader set to 450 nm.

12. The results are calculated by interpolation from standard curve , were constructed in the same assay as the sample , the absorbance for each sample on the vertical axis was located and the corresponding LEP concentration on the horizontal axis was read. (Human LEP , ELISA kit Catalog).

2.4.5Qualitative determination of Rheumatoid Factor (RF):

• *Principle of the method* :

The RF - latex is a slide agglutination test for the qualitative and semi-quantitative detection of RF in human serum.Latex particles coated with human gammaglobulin are agglutinated when mixed with samples containing RF.

• *Assay procedure* :

1- The reagent and controls were allowed to reach room temperature.

2- The reagent was gently shaked to disperse and suspend latex particles in the buffer solution.

3- Fifty µl of the sample and one drop of each positive and negative control were placed into separate circles on the slide test.

4- A drop of reagent was placed next the drop of serum , mix both drops well with a stirrer covering the surface of the slide section.

5- The drop mixed with a stirrer , spreading them over the entire surface of the circle.

6- The slide placed on a mechanical rotator at 80 - 100 rpm for two minutes . False positive results could appear if the test is read later than two minutes.(User ' s Manual RF kit).

2.4.6 Determination of uric acid:

• *Principle of the method* :

Uric acid is oxidized by uricase to allantoine and hydrogen Peroxide ($2H_2O_2$), which under the influence of POD , 4-aminophenazone (4-AP) and 2 – 4 dichlorophenol sulfonate (DCPS) forms a red quinoneimine compound :

$$\text{Uric acid} + 2H_2O + O_2 \xrightarrow{\text{Uricase}} \text{Allantoine} + CO_2 + 2H_2O_2$$

$$2H_2O_2 + \text{4-AP} + \text{DCPS} \xrightarrow{\text{POD}} \text{Quinoneimine} + 4H_2O$$

The intensity of the red color formed is proportional to the uric acid concentration in the sample.

• *Reagents* :

Table 2.5 : Reagents of Kit for Uric acid

R1 Buffer	Phosphate pH7.4	50 mmol/L
	2-4 Dichlorophenol sulfonate(DCPS)	4 mmol/L
R2 Enzymes	Uricase	60 U / L
	Peroxidase(POD)	660 U / L
	Ascorbate oxidase	200 U / L
	4-Aminophenazone (4-AP)	1 mmol/L
Uric acid Cal.	Uric acid aqueous primary standard	6 mg/dl

• *Procedure* :

1- Assay conditions :

Wavelength…… 520 nm (490-550)

Cuvette …….. 1cm light path

Temperature……... 37˚C/15-25˚C

2- Adjusting the instrument to zero with distilled water.

3- Pipetting into a cuvette:

Table 2.6 : Procedure for determine Uric acid

	Blank	Standard	Sample
WR (ml)	1.0	1.0	1.0
Standard (µL)	--	25	--
Sample (µL)	--	--	25

4- Mixing and incubating for 5 min. at 37˚C or 10 min. at 15-25˚C.

5- Reading the absorbance (A)of the samples and standard , against the blank.The colour is stable for at least 30 minutes.

• *Calculation* :

(A)sample ÷ (A)standard x 6 (standard conc.) = *** mg/dl. *uric acid in the sample*

……..

(A) : absorbance.

(Conc.) : concentration.

2.5 Statistical Analysis:

Data analysis was computer assisted using statistical package package for social sciences (SPSS) . Frequency distribution for selected variables was done first . The Statistical significance of difference in mean of a quantitative normally distributed variable between 3 groups was tested by ANOVA test . Whereas non - normally distributed variable as shown by histograms and Smirnove – Kolmogorove test are described by median and the non - parametric tests of significance were advocated for use . The statistical significance of difference in median between 3 groups was tested by Kruskal-Wallis test . P value less than 0.05 level of significance was considered statistically significant. Pearson Correlation(r) : A type of correlation coefficient that represents the relationship between two variables that are measured on the same interval or ratio scale. The Pearson coefficient is represented the same way as a correlation coefficient that is used in linear regression; ranging from -1 to +1. A value of > 0 and $< +1$ is the result of a perfect positive relationship between two or more variables. Conversely, a value of < 0 and < -1 represents a perfect negative relationship while 0 indicate no linear relationship. (Sorli , 1995).

Chapter three

Results

3.1 Demographic and clinical featurs :

The results reppresented in this study were based on the analysis of 70 patients with RA divided into 3 groups:

- Group(1) consist of 20 RA patients received disease modifying anti rheumatic drugs(DMARDs) .
- Group(2) consist of 25 RA patients received biological treatment Etanercept (anti TNFα).
- Group(3) consist of 25 RA patients received biological treatment Rituximab (anti CD20).

compared with 20 apparently healthy individuals considerds as controls .

3.1.1 Distribution of patients according to age and gender:

In the present study the age of RA patients ranged between 20-68 year with mean ± S.E. of (41.266± 2.666) year , table(3.1) . Chi - square test showed that there was no statistical significant difference in the frequency of age groups between RA and control group($P = 0.224 > 0.05$) .

Regarding age groups , the highest rate of incidence of the disease was found in the third and fourth decade of life (24.28% for every decade) , followed by sixth and fifth decades (22.85%) and (17.14%) respectively as clearly shown in figure (3.1).

Furthermore the current results demonstrated that female have a higher ratio of being affected with the disease than males (3:1) , figure (3.2) , on the other hand , there is no statistical significant difference in the frequency of gender types between RA patients and control group ($P =0.193 > 0.05$) .

Table 3.1:Descriptive statistics of age in RA patients and control(in years).

Characteristics	Healthy control	RA patients
Age range (years)	21-54	20-68
Mean	37.55	41.266
S.E.	2.177	2.666

RA : Rheumatoid Arthritis **S.E. :** Standard Error.

P value = 0.224 > 0.05

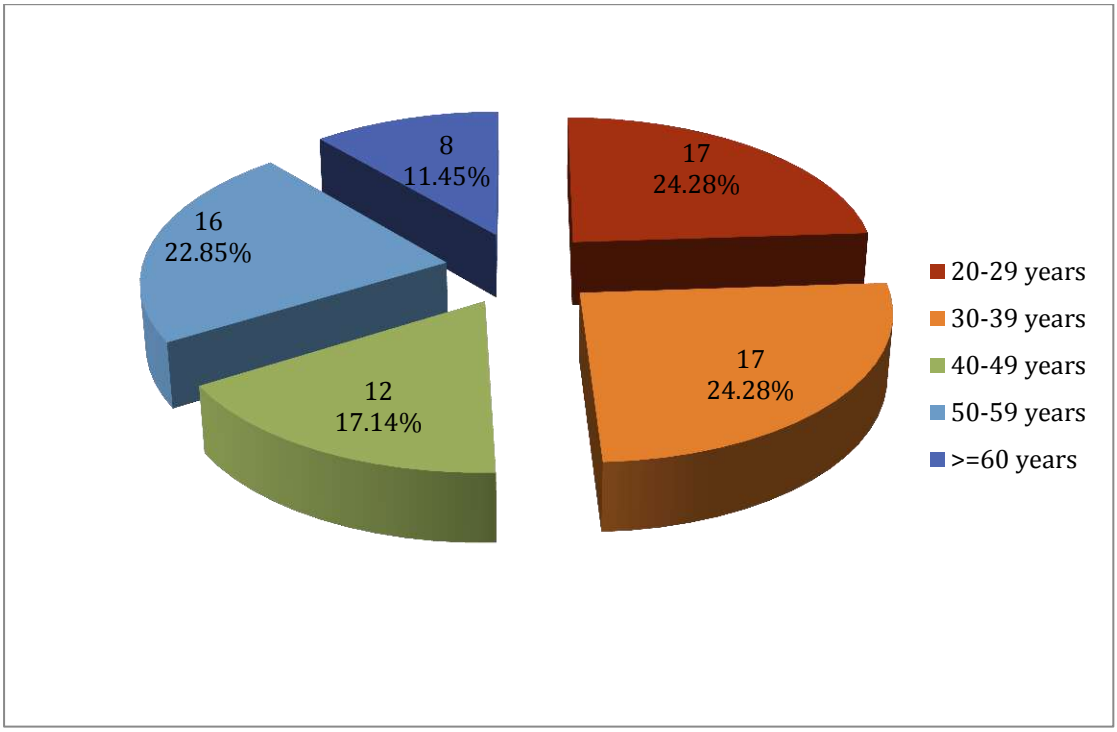

Figure 3.1 : Distribution of RA patients according to age groups

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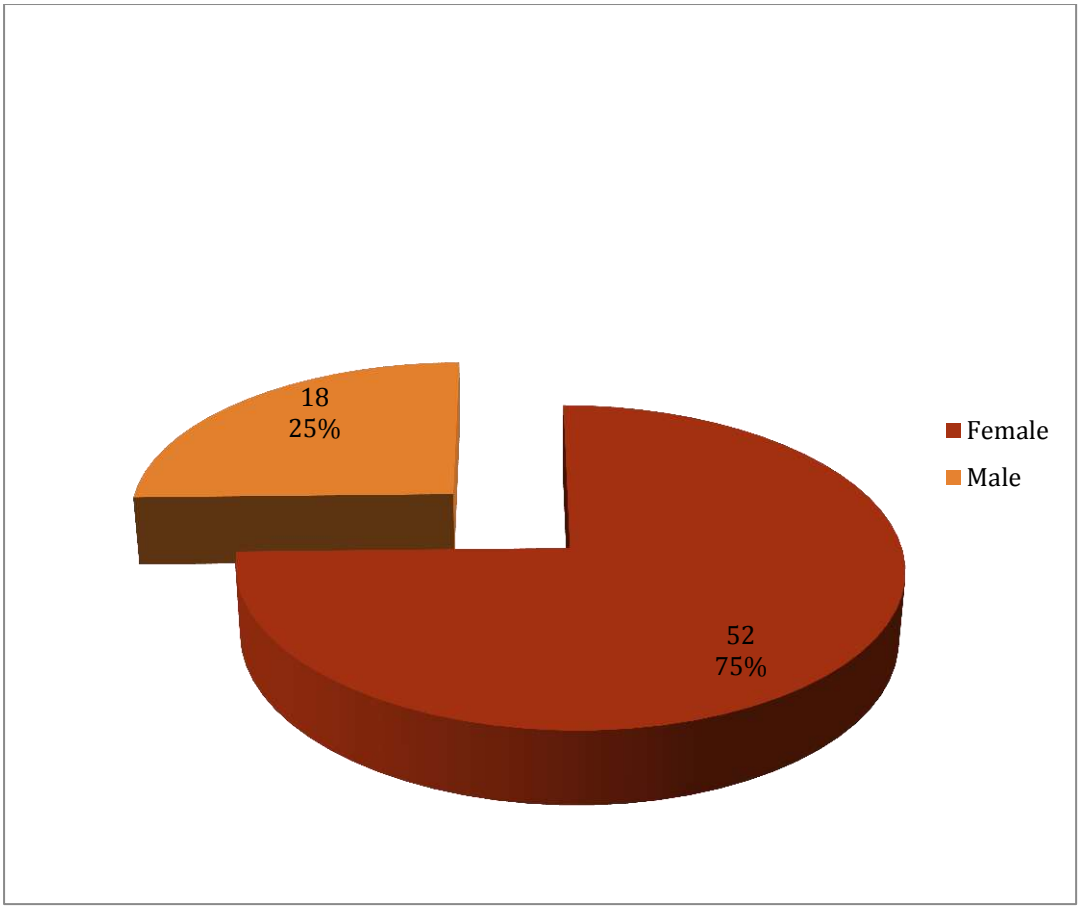

Figure 3.2:Gender type distribution in RA patients.

3.1.2 Body Mass Index(BMI)

BMI in the present study demonstrate that higher percentage of RA were overweight (39.44 %) followed by (32.39 %) were normal , and followed by (19.72 %) were obese, statistically there is no significant difference between healthy control group and RA patients in the frequency of body mass index scores . (P = 0.104 > 0.05) , figure (3.3).

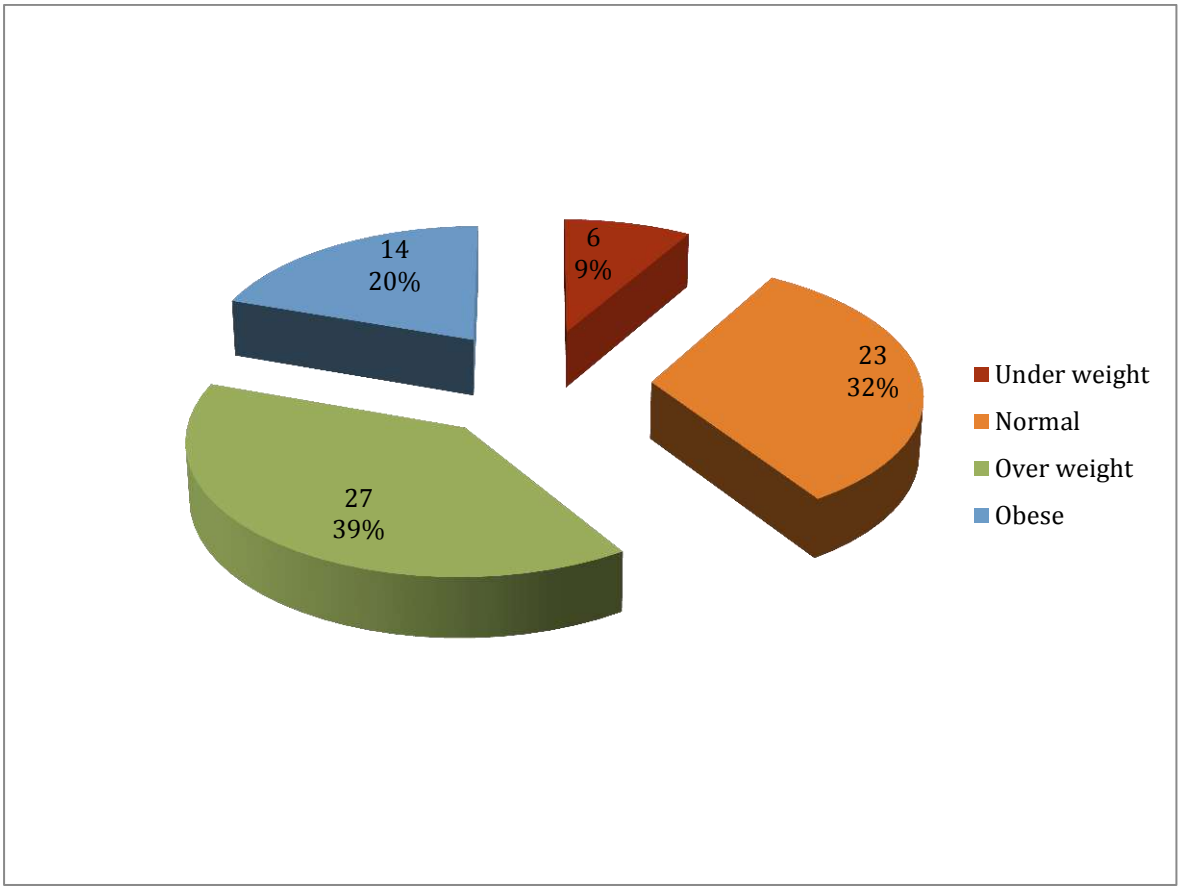

Figure 3.3 : Distribution of BMI score among RA patients.

3.2 Laboratory Findings

3.2.1 Erythrocyte Sedimentation Rate : ESR (mm/1hr)

The laboratory results of ESR showed that there is a higher value of ESR in RA patients than control group with highly statistical significant difference (P=0.01) table(3.2).

Table 3.2 :Descriptive statistics of ESR in healthy control vs RA patients

ESR mm/1hr	Mean ± S.E.	P value
Healthy control	8.4±0.678	P=0.01
RA patients	39.686±4.223	

ESR : Erythrocyte Sedimentation Rate .

S.E. : Standard Error

Within RA patients group , there is a significant difference between group 1 patients (not biological treatment : treated by Disease Modifying Anti Rheumatic Drugs (DMARDs) and other groups of RA patients that were treated with different types of biological treatment. Mean values of ESR were higher in group 1 patients as compared with group 2 patients who treated with Etanercept ($P = 0.026 < 0.05$) and group 3 patients who treated with Rituximab ($P = 0.039 < 0.05$) as demonstrated in figure (3.4).

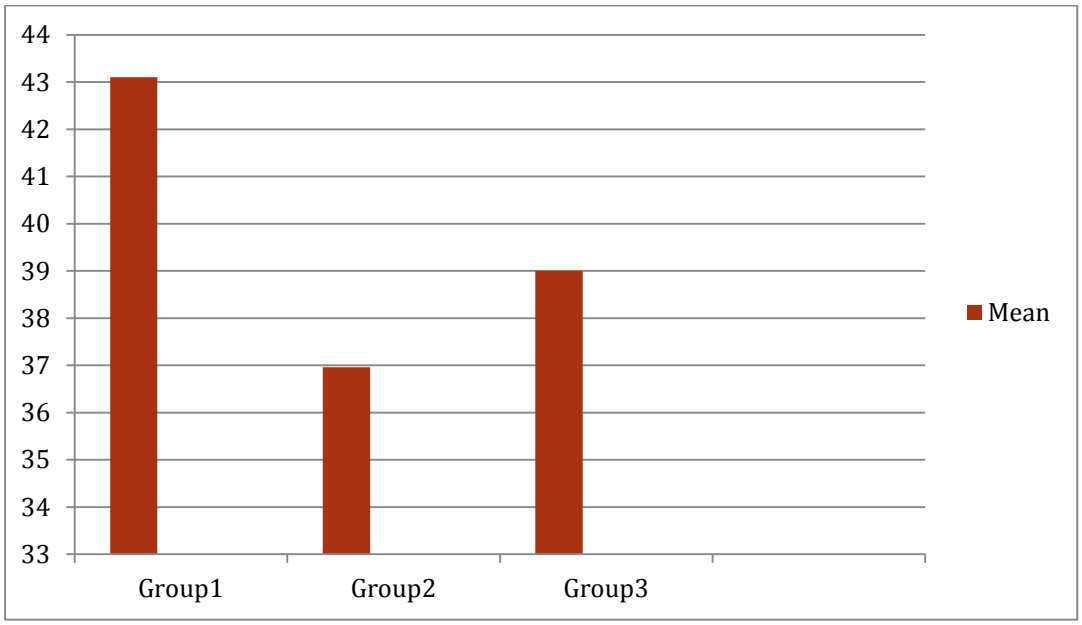

Figure 3.4 : Mean value of ESR in study groups.

3.2.2 High Sensitive C– Reactive Protein : hsC-RP (µg/ml)

In general , the current study showed that RA patients have higher percentage of rising level of hsC-RP than those in control group (8.695±0.953 and 2.078±0.432 respectively) , there is highly statistical significant difference between RA patients and control group (P=0.01), (figure 3.5).

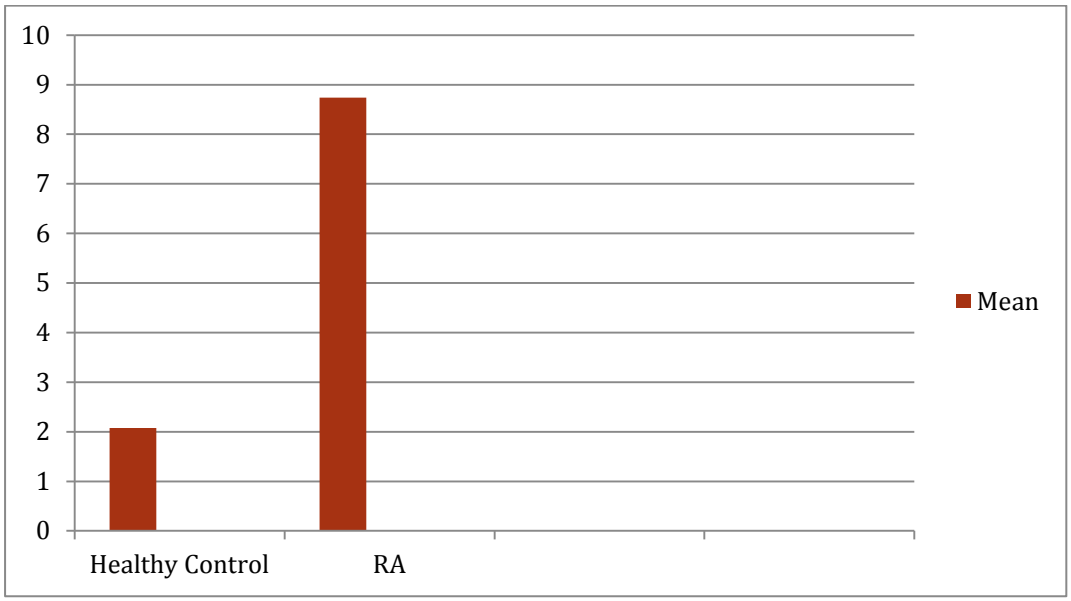

Figure 3.5. : Mean value of hs C-RP in study groups.

Comparison among RA groups revealed that group 1 patients has higher levels of hsC-RP than those of patients in group 2 (9.7±1. 044 and 8.86±0.92 respectively)there is significant difference (P 0.042 < 0.05) , and group 2 patients has higher levels of hs C- RP than those of patients in group 3 (8.86 ±0.92 and 7.52 ± 0.88 respectively) there is significant difference (P 0.034 < 0.05) , figure (3.6).

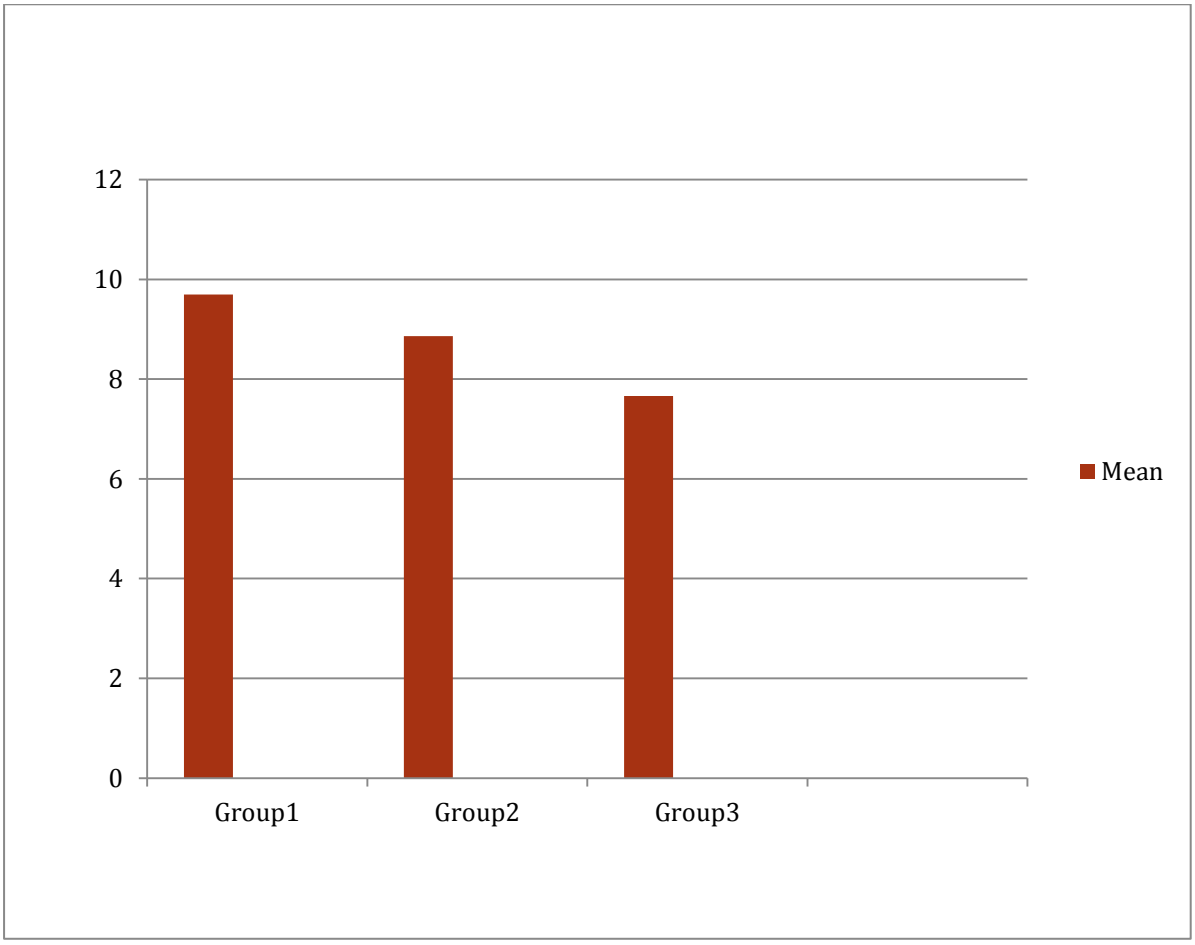

Figure 3.6 : Mean value of hsC-RP in different groups of RA.

3.2.3 Rheumatoid Factor (RF)

As clearly shown in figure (3.7) RA patients have 85.72% RF positive cases, in comparison with healthy control group this percentage is highly statistical significant difference . (P=0.01) .

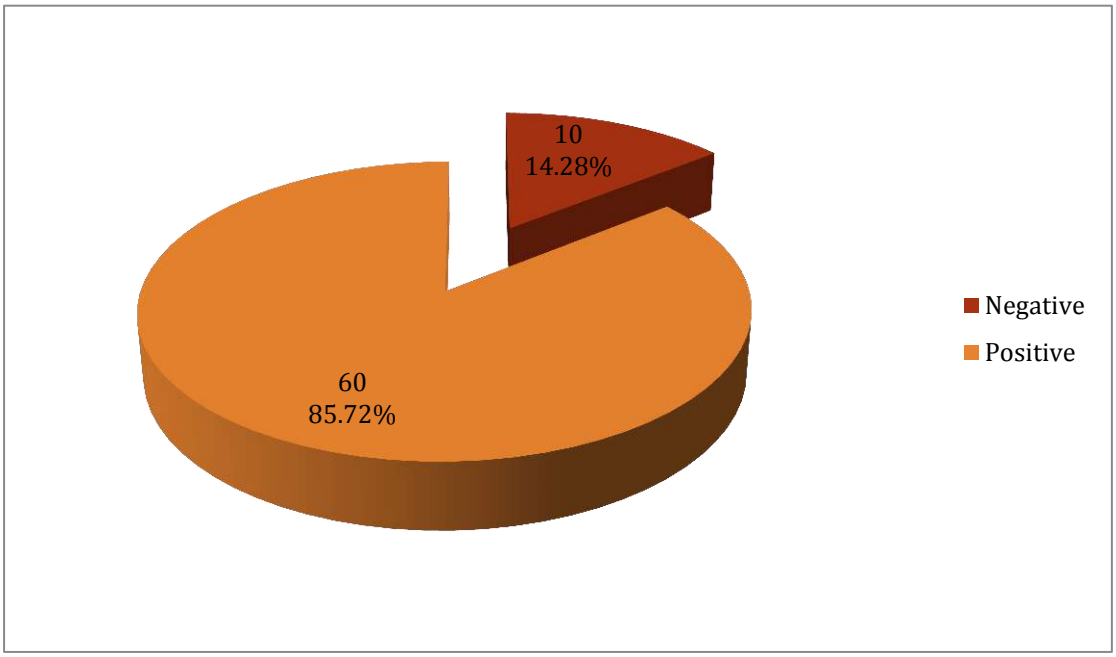

Figure 3.7 : Distribution of RA patients according to RF positivity.

3.2.4. Serum level of Uric acid (mg/dl):

Results in figure 3.8 has shown there is no significant difference btween healthy control group and RA patients (P = 0.28 > 0.05) and among groups of RA in their serum level of uric acid :

- Between 1 and 2 (P = 0.32 > 0.05) .
- Between 1 and 3 (P = 0.38 > 0.05).
- Between 2 and 3 (p = 0.64 > 0.05).

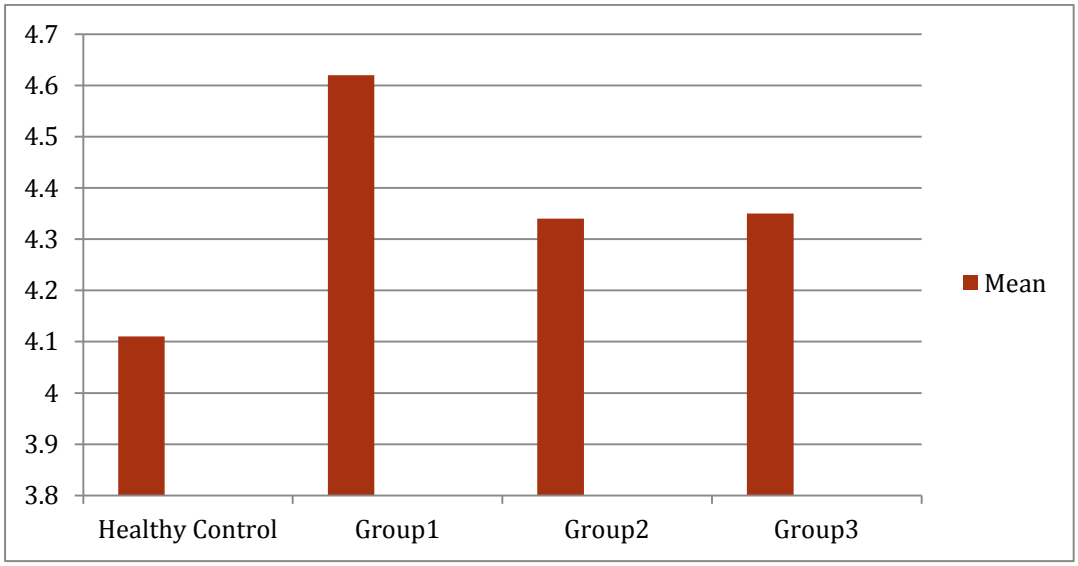

Figure 3.8 : Mean value of Uric acid in study groups.

3.2.5. Serum level of Leptin (ng/ml):

Results in figure (3.9) has shown there is no significant difference btween healthy control group and RA patients (P = 0. 42 > 0.05) and among groups of RA in their serum level of leptin :

- Between 1 and 2 (P = 0.083 > 0.05) .
- Between 1 and 3 (P = 0.074 > 0.05).
- Between 2 and 3 (p = 0.077 > 0.05).

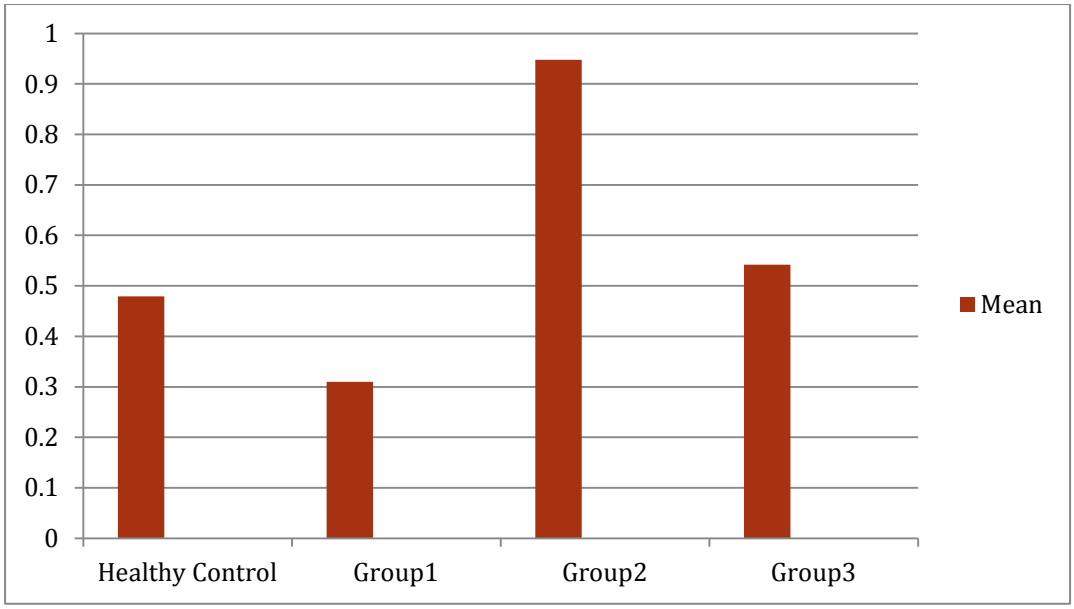

Figure 3.9 : Mean value of Leptin in study groups.

3.2.6. Serum level of IL-17 (Pg/ml):

The results in this study showed that there is a significant elevation in the mean serum level of IL-17 in healty control than those of RA patients figure (3.10) , (P = 0.036 < 0.05).

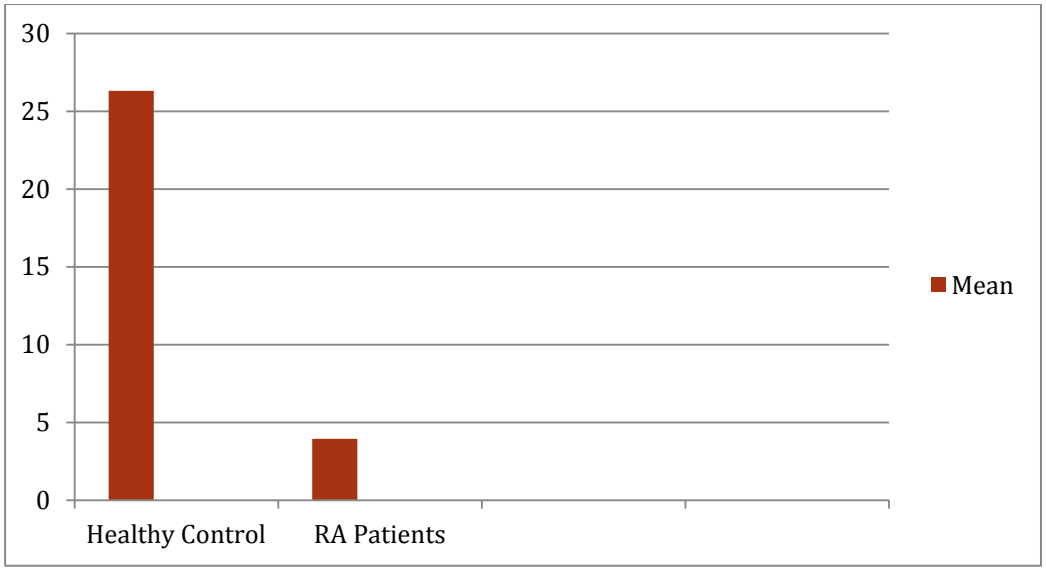

Figure 3.10 : Mean serum level of IL-17 in RA patients and healthy control group

Comparison among RA groups revealed that group 1 patients has higher levels of IL-17 than those of patients in group 2 (5.268±0.69 and 3.811 ± 0.694 respectively), there is significant difference between them (P = 0.047 < 0.05) and group 2 patients has higher levels of IL-17 than those of patients in group 3 (3.811 ± 0.694 and 2.75±0.469 respectively) , there is significant difference between them (P = 0.039 < 0.05) , figure (3.11) .

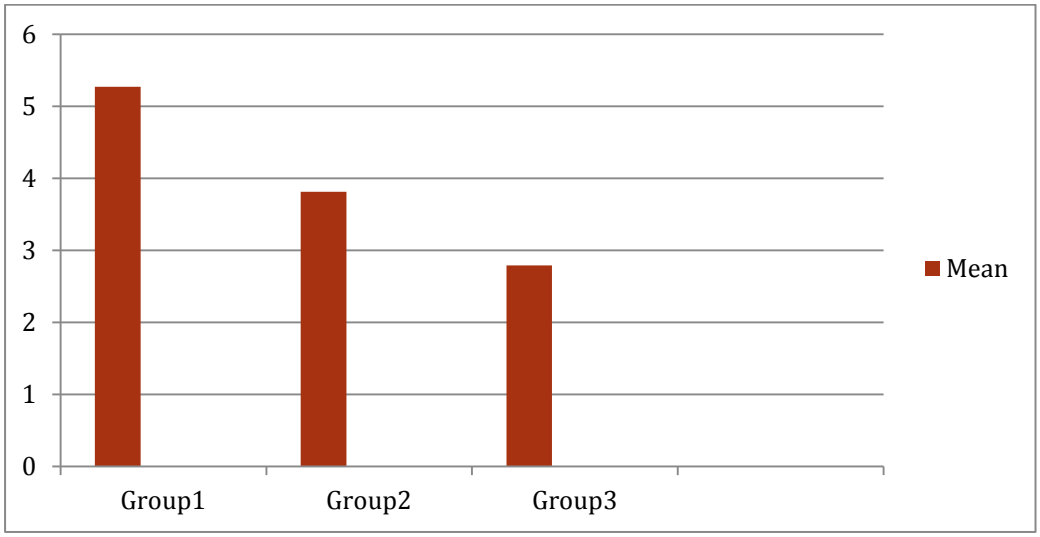

Figure 3.11 : Mean value of IL-17 in different groups of RA.

An anticipated mean serum level of IL-17 were also decrease significantly in patients treated with Rituximab group 3 (2.75) than those of group 2 Etanercept (anti-TNFα) treated group patients (3.81) table(3. 3) and figure (3.12) .

Table 3.3 : Descriptive statistics of IL-17 between group 2 and group 3.

Serum level of IL-17 (Pg/ml)	**Group 2**	**Group 3**
Minimum	0.33	0.18
Maximum	16.6	8.52
Mean	3.811	2.75
S.D.	3.47	2.345
S.E. = S.D. / √N , N : Sample Size.		

Group 2 : Etanercept(anti-TNFα)treated group.

Group 3 : Rituximab (anti-CD20) treated group.

S.D. : Standard Deviation

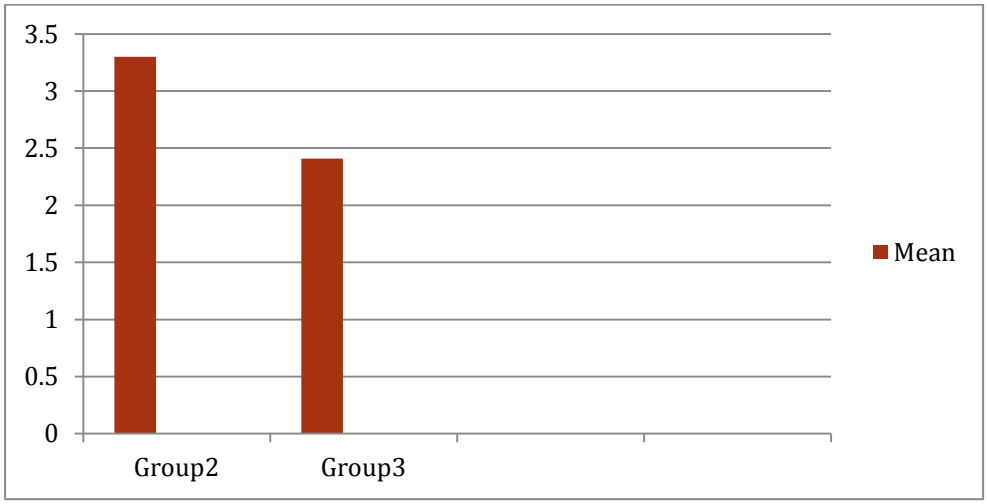

Figure 3.12 : Mean value of Serum IL-17 in group 2 and group 3.

3.3 Correlation among Different Parameters in RA cases:

Table 3.4 reveales the correlation between different parameters studied in this work as seen. The present study showed significant positive correlation among each of :

- ESR
- hsC-RP
- IL-17 , $P < 0.05$ and $0 < r$

while there is no correlation between :

- Age
- BMI
- Uric acid
- Leptin

with other parameters :

- ESR
- hsCRP
- RF
- IL-17 , $P > 0.05$ and $0 = r$

Generally strong positive correlation was found between :

- IL-17

and each of :

- ESR
- hsC-RP , $P < 0.05$ and $0 < r$

Table 3.4 : Correlation Coefficient among Different Parameters in RA cases

		Age	*BMI*	*ESR*	*RF*	*Uric acid*	*hsCRP*	*Leptin*	*IL-17*
Age	**r**	**1**	**-1.034**	**0**	**0**	**-1.329**	**0**	**0.074**	**0**
	p	**0**	**0.78**	**0.066**	**0.386**	**0.105**	**0.123**	**0.543**	**0.936**
BMI	**r**	**-1.034**	**1**	**0**	**0**	**0.138**	**0**	**-1.143**	**0**
	p	**0.78**	**0**	**0.128**	**0.543**	**0.256**	**0.888**	**0.237**	**0.39**
ESR	**r**	**0**	**0**	**1**	**0.631**	**0**	**0.43**	**0**	**0.488**
	p	**0.066**	**0.128**	**0**	**0.038**	**0.06**	**0.004**	**0.898**	**0.049**
RF	**r**	**0**	**0**	**0.631**	**1**	**0.141**	**0.515**	**0**	**1.208**
	p	**0.386**	**0.543**	**0.038**	**0**	**0.386**	**0.003**	**0.78**	**0.256**
Uric acid	**r**	**-1.329**	**0.138**	**0**	**0.141**	**1**	**0**	**-1.059**	**0**
	p	**0.105**	**0.256**	**0.06**	**0.386**	**0**	**0.931**	**0.629**	**0.076**
hsCRP	**r**	**0**	**0**	**0.43**	**0.515**	**0**	**1**	**0**	**0.922**
	p	**0.123**	**0.888**	**0.004**	**0.003**	**0.931**	**0**	**0.386**	**0.-034**
Leptin	**r**	**0.074**	**-1.143**	**0**	**0**	**-1.059**	**0**	**1**	**0**
	p	**0.543**	**0.237**	**0.898**	**0.78**	**0.629**	**0.386**	**0**	**0.616**
IL-17	**r**	**0**	**-1.104**	**0.488**	**1.208**	**0**	**0.922**	**0**	**1**
	p	**0.936**	**0.39**	**0.049**	**0.256**	**0.076**	**0.034**	**0.616**	**0**

r **:** correlation coefficient. **p** **:** p value
BMI **:** Body Mass Index **ESR** **:**Erythrocyte Sedimentation Rate
RF **:** Rheumatoid Factor **hsC-RP:**highsensitive C-Reactive Protein
IL-17 : Interlukin-17

Chapter four

Discussion

Discussion

Rheumatoid arthritis is a complex diseas resulting in localized erosion to the joint and its accessory structurs . Due to the progressive nature of the disease , extra – articular complications will occur in multiple organ system . The identification and diagnosis of RA early in the disease course is becoming increasingly important because early and intensive treatment has been demonstrated to prevent joint damage , to preserve joint function , and to improve work participation of the patient. The prognosis of patients with RA has improved considerably as a result of intensified treatment regimens and the availability of new drugs. (Visser *et al*., 2002 ; Pincus *et al* .,2004).

Over the past decade , the management of RA has evolved with disease modifying anti rheumatic agents with biologic activity targeting specific components of the immune system . With advanced therapy , management includes halting further progression of the disease and maintaining quality of life . The American College of Rheumatology has provided updated guidelines regarding the use of biologic therapies like Rituximab (anti-CD20) and Etanercept (soluble receptor for TNF-α) as monotherapy or in compination with nonbilogic therapy. (Waldburger and Firestein , 2009).

4.1 Demographic and clinical features:

This study included 70 RA patients divided into three groups according to treatment ; group 1 treated with DMARDs , group 2 trated with Etanercept , group 3 treated with Rituximab.

4.2 Age:

In this study , the mean of age of RA patients was 41.266±2.666 years , the range was (20 -68) year and the peak incidence is 24.28 % at the age group (20-29) and age group (30-39) year . This is consistent with other Iraqi studies (Haider , 2006 ; Ahmed , 2011 ; Amar , 2012) and a broad studies (Lipesky , 2001 ; Goronzy and Wayand , 2005) who mentioned that RA affects usually people who are in there three decade of age and starts usually after middle age.

RA starts after 40 years due to the accumulation of many reasons that lead to depression of the immunity as stress , thymic depression , and exposure to different antigens as smoking (tobacco) , drugs and chemicals which leads to activation of auto-reactive lymphocytes that interact with self – antigen . (Kotazin *et al* ., 2000 ; Alamanos *et al* ., 2006).

4.3 Gender:

The current study showed that female to male ratio of 3:1 and this is in agreement with other study conducted by (Amar , 2012) 3.5:1 , (Ahmed , 2011) 4:1 and Khitam that showed a ratio of 5:1 . (Khitam , 2011) . The female predominance may be due to hormonal factors such as estrogen which enhances the function of T- helper cells and inhibits the function of T-suppressor cells . Also estrogen receptors are present on memory T-cells and on synovial cells.(Ansar Ahmed *et al* ., 1985 ; Takagi *et al* ., 2000 ; Nalbandian ., 2005).

4.4 BMI:

Adipose tissue may have immune modulating effects in RA , although its exact role is presently unclear. The present results demonstrate that 39.44% of patients were overweight followed by 32.39% were normal and followed by 19.72 % were obese .

These results argued by Munro , and Capell when reported that 1:8 of patients were under weight (Munro and Caplle, 1997) . However , many studies demonstrate the influence of BMI and/or body fat on RA disease activity . Some studies have shown that high BMI to be associated with RA . (Symmons *et al* ., 1997) and poor disease outcome (Garcia-Poma *et al* ., 2007) , whereas others have found low BMI to be associated with increased erosion in small joints and decreased survival and high BMI being protective (Kaufmann *et al* ., 2003 ; Van Der *et al* ., 2007). But the current study does not observe any relation of BMI with any clinical parameters related to disease activity .

4.5 Laboratory Findings:

4.5.1. ESR (mm/1hr):

ESR measurement in the current studyshowed that RA patients have higher value of ESR than that for healthy control group . The higher rate of sedimentation resulted from the presence of large symmetric molecules in plasma which accelerates the rouleax formation and setting of RBC.The most important of these molecules are fibrinogen (Joseph *et al* ., 1977) . The ESR is increased in nearly all patients with active RA (Dawes *et al* ., 1986 ; Hickling *et al* ., 1983). ESR is the cheapest test , but can easily be influenced by anaemia and hyperglobulinaemia which frequently present in RA (Van Leeuwen *et al*., 1993 and Plant *et al* ., 2000).

A number of studies have suggested that high ESR levels at onset of early RA predicts long-term radiological progression (Morel and Combe ., 2005 ; Combe *et al* ., 2007) . It is , together with CRP , the most frequently used laboratory measure reflecting disease activity , and ESR measurement tend to reflect disease activity of the previous weeks (Van Leeuwen *et al* .,1993) and has been incorporated in several disease activity scoring systems . However ,measurements are influenced by confounding factors including age, sex , fibrinogen levels , RF , hypergammaglobulinaemia , and anaemia(Talstad *et al* ., 1983) .

Nonetheless , the ESR levels has been incorporated into a risk prediction model for rapid radiographic progression that in the future could be used to predict the risk of joint damage progression in RA patients. (Vastesager *et al* ., 2009).

4.5.2 hsC-RP (µg/ml):

Inflammatory processes play a pivotal role in the pathogenesis of RA .The prototypic marker of inflammation is C-RP a member of the pentraxin family . The production of C-RP in the liver is triggered by various proinflammatory cytokines derived either from monocytes and /or macrophages . The proinflammatory response results in the increased secreation of IL-1 and TNF-α which then results in the release of the messenger cytokine , IL-6 which stimulates the liver to secrete C-RP . It was thought as a by stander marker of inflammation , without playing a direct role in the inflammatory process . Several studies suggest that C-RP may also contribute directly to the proinflammatory state . C-RP stimulate monocyte to release of inflammatory cytokines such as IL-1 , IL-6 and TNF-α and may also directly act as a proinflammatory stimulates to phagocytic cells . (Ballore *et al* ., 1992 ; Stankcikova and Rovensky ., 1993 ; Bharadwas *et al* ., 1999) . The detection of C-RP is a more reliable and sensitive indicator of the inflammatory process than the ESR (Shine *et al* ., 1981; Hind and Pepys ., 1984).

In the present study the levels of hsC-RP were significantly high in RA patients compared to healthy control.Similarly also observed high values of C-RP indicative of active inflammation in RA patients .(Yildirim *et al* ., 2004).

Concerning effect of treatment on hsC-RP levels , the current results noticed that the level of hsC-RP decrease in RA patients treated with non biologic drugs DMARDs , biologic drugs : anti-TNF-α , anti-CD20 respectively . Seidman reported that CRP value decrease after treatment in RA patients (Seiman ., 1993) . In contrast , a study of 309 patients with inflammatory polyarthritis on treatment from

the Norfolk Arthritis Register found that clinical and laboratory factors such as age , gender , age at disease onset , baseline RF , and C-RP were poor predictors of treatment response to treatment (Haider .,*et al* ., 2009).

4.5.3. RF:

The present findings revealed that latex test was positive in 60 patients which means that not all the patient were RF positive: in group 1was 80%RF positive, group 2was 76 % , group3was 68 % . These results could lead to a variation proportion of RF positivity in mechanisms not understood and any explanation remains speculative . Succesful therapy has proven to reduce the amount of synovium infiltrating cells , including plasma cells (Smeets., *et al* ., 2003) . Because RF - producing cells are present in inflamed rheumatoid synovium and the local environment may favour synovial RF production , therefore can speculate that the reduction in inflammatory lymphoplasmacytic infiltrate in rheumatoid synovium will lead to a reduced production of RF. (Alarcon., *et al* ., 1990 ; Reparon – Schuijt., *et al* ., 1998 ; Bobbio-Pallavicini., *et al* ., 2004; Chen., *et al* ., 2006 ; Roll., *et al* ., 2012).

4.5.4 Uric acid (mg/dl) :

The present findings observed that these were no significant difference in serum level of uric acid by comparission between RA patients and healthy control and within groups of RA . Uric acid is a major contributer for the total antioxidant capacity in human serum . Contrary to what is traditionally considered as a metabolically inert and waste compound of no physiological significance , uric acid can be oxidized following the nonenzymatic degradation and has been proven to be a selective antioxidant . (Goldstein .,*et al* ., 1979).

4.5.5 Serum level IL-17 (Pg/ml)

Current findings suggest that the management strategy of RA disease status should be improved with an alternative regimen . Inversely , patients treated with biologic therapy (Etanercept and Rituximab) showed lower serum IL-17 level when compared with healthy control or when compared with patients recived DMARDs, $P < 0.05$. These results are in agreement with results reported by Van de Veerdonk *et al* , who stated that Rituximab reduced the local Th 17 response in RA patients , and the decreased Th17 response was associated with strongly reduced IL-17 as well as reduced inflammation and better clinical outcome(Van de Veerdonk., *et al* ., 2011).

These results with current findings support that the IL-17 is highly expressed in the inflammatory joints and drives disease activity , implicating it as a key cytokine and potential therapeutic target . These studies have shown that IL-17 not only drives the proinflammatory response but also enhances the effect of TNF-α promoting increased destruction in the RA joint (Moran, *et al* ., 2009 ; Moran, *et al* ., 2011).

However , Aerts and associates referred that still there is notable values in these groups after biologic therapy is accompanied in Th-17 specific Chemokines C-C motif Receptor 6 (CCR6) expression , which might prevent homing of these potentially proinflammatory cells to the synovium (Aerts *et al* ., 2010).

The current study support that IL-17 implicated in pathology of RA disease especially in active disease rather than remission or milder cases . This statement argued by several researches (McInnes , Schett , 2007 ; Church .,*et al* ., 2010 ; Eggleton .,*et al* ., 2011). However , this cytokine seems to be produced by a distinct lineage of lymphocyte subset in both synovial joint and peripheral blood with different percentage . (Harrington *et al* ., 2005 ; Park., *et al* ., 2005 ; Eggleton .,*et al* ., 2011).

Implication of IL-17 in the RA disease may be explain with different mechanisms . It either by promotes matrix turnover and cartilage destruction , especially in the presence of other cytokines , mimicking the joint environment (Moran., *et al* ., 2009) or stimulate osteoclast increasing of bone erosion (Kotake., *et al* ., 1999) . Or it completes the proinflammatory network IL-1 and TNF-α inducing joint inflammation and pathology by inducing synovium matrix destruction (Chabaud *et al* ., 2000) and inducing cartilage breakdown (Koshy., *et al* ., 2002).

2.4.6 Serum level Leptin (ng/ml):

The current study has shown that there is a no significance difference between healthy control and RA patients or within RA patients groups . These results are in contrast with other studies that showed significantly elevated concentrations of leptin in patients with RA , and noted elevated leptin serum concentrations in patients with higher disease activity evaluated by disease activity score 28 (DAS28) , ESR , and the number of tender joint . It was also noted that plasma concentrations of leptin were significantly higher than that of synovial fluid leptin,and this difference was particularly evident in non - erosive arthritis.(Robert K , Gabriela H 2012).

These differentiation can be explained by the difference in sample population and size.

Conclusions
And
Recommendations

Conclusions

1- The results showed that IL-17 may has a key role in Rheumatoid Arthritis inflammatory process.

2- Patients treated with biological drugs : Etanercept (anti-TNFα) and Rituximab (anti-CD20) showed lower serum levels of IL-17 than patients treated with non biological drugs , and patients treated with Rituximab have lower levels than patients treated with Etanercept.

3- There is no relation between levels of leptin , uric acid and IL-17 in RA patient groups and between that of RA patients and healthy control.

4- hsC-reactive protein is useful to follow up RA patients and to monitor their response to treatment.

Recommendations

1- Molecular technique or any advanced techniques (such as real time polymerase chain reaction) are recommended to detect the gene that is important in clear and specific diagnosis of RA disease and to evaluate the monitoring processes (such as reliable measurement levels of cytokines).

2- Use of serum level of IL-17 and hsC-RP in follow-up of the RA patients under treatment with Etanercept , Rituximab or other biologic agents.

3- Hormonal studies are recommend to investigate the high prevalence of RA in females than males .

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Appendix

Case Sheet

Date

No

Gender

Age

Weight

Height

BMI

Duration of drugs

Duration of disease

Type of drugs

DAS 28

Any complication (DM , Hypertention,others)

الخلاصة

الخلفية :

يعد ألتهاب المفاصل الروماتويدى مرضا مزمنا ، يتعلق بالجسم ككل ، وهو يمثل أضطرابات ألتهابية قد تؤثر على كثير من الانسجة والاعضاء ، غير أنها تـــؤثر بشكل أساسى على الغشاء الزليلى للمفاصل . ان منشأ هذا المرض لم يفهم بشكل كامل .

الهدف :

تقييم تأثير العلاج بمثبطات CD20 المعروف Rituximab مقارنة مع العـلاج البايولوجى الاخر مثبطات TNFα المعروف Etanercept على الانترلوكين 17.

المكان :

أجريت هذه الدراسة خلال الفترة من تشرين الاول 2012 الى مايس 2013 حيث تم أختيار الاشخاص المشاركين فى الدراسة من مراجعى العيادة الاستشارية لمستشـفى بغداد التعليمى فى مدينة الطب وقد أجريت الفحوص المختبرية فى المختبـــــرات التعليمية فى مدينة الطب.

الاشخاص :

تضمنت هذه الدراسة 70 مريضا تتراوح أعمارهم بين 68 – 20 سنة وقد قســـم هؤلاء المرضى الى ثلاث مجاميع :

- **المجموعة الاولى تضمنت 20 مريضا ممن يتعاطون الادوية الغير بايولوجية فى علاج مرض التهاب المفاصل الروماتويدى .**

- المجموعة الثانية تضمنت 25 مريضا ممن يتعاطون العلاج البايولوجى لعلاج هذا المرض وهو Etanercept والمعروف Anti TNF α .
- المجموعة الثالثة تضمنت 25 مريضا ممن يتعاطون العلاج البايولوجى لعلاج هذا المرض وهو Rituximab والمعروف Anti CD20 .

وكذلك تضمنت هذه الدراسة 20 مريضا من المتطوعين الاحصاء.

طرائق العمل :

تم قياس كل من انترلوكين 17 ، لبتين ، البروتين الفعال سى عالى التحسس بطريقة أيلايزا ELISA فى حين أستعملت طريقة القياس بالطيف الضوئى اللونى لقيـــاس تركيز حامض اليوريك فى مصل دم الخاضعين للفحص فى هذه الدراســة فى حين تم الكشف عن وجود عامل الروماتويد فى مصول دمهم بواسطة أختبـــار التلازن وكان القياس الوحيد فى هذه الدراسة بدراسة مقاييس جسم الانسان للمقارنة المعياريـــة لتحديد الـ BMI (معيار كتلة الجسم) .

النتائج :

أظهرت نتائج الدراسة الحالية ان مستويات البروتين الفعال سى العالى التحســس ، معدل ترسب الكريات الحمر ، عامل الروماتويد فى مصل دم المرضى هو عال بشكل معتد به أحصائيا مقارنة بالاصحاء ($P<0.05$) وأن مستوى الانترلـــوكين 17 عند المرضى تحت العلاج أقل منه عند الاصحاء أذا ما أفترضنا مستواه عندهم ضمن حدود طبيعية.

فى هذه الدراسة هنالك أختلاف معتد أحصائيا بين المجاميع الخاضعة للفحص فى هذه الدراسة فى مستوى الانترلوكين17 حيث ان مستواه فى المجمـــوعة الاولى التى لم تتعاطى علاجا بايولوجيا هو أعلى منه فى المجموعة الثانية التى تتعـــــاطى علاجا بايولوجيا Etanercept المعروف Anti-TNFα inhibitors وأن مستواه فى هذه المجموعة أعلى منه فى المجموعة الثالثة التى تتعاطى علاجا بايولوجيا اخر هو Rituximab المعروف Anti-CD20.($P<0.05$) .

لقد أظهرت هذه الدراسة عدم وجود أختلاف ذو أهمية أحصائية بين جميع المجاميـــع الخاضعة للفحص بما يخص حامض اليوريك وكذلك بما يخص اللبتين كما أظهرت هذه الدراسة وجود علاقة أيجابية بين معدل ترسب كريات الدم الحمر،البروتين الفعال سى عالى التحسس ، عامل الروماتويد ، الانترلوكين 17.

الاستنتاجات :

- **للانترلوكين 17 تأثير معتد فى نشوء مرض التهاب المفاصل الروماتويدى .**
- **مستوى الانترلوكين 17 فى مصل دم الاصحاء والذين يتعاطون أدوية غير بايولوجية فى علاج مرض التهاب المفاصل الروماتويدى مقارنه به عند الذين يتعاطون ادويـــة بايولوجية.**
- **فى مرضى التهاب المفاصل الروماتويدى الذين يتعاطـــــون ادوية بايولوجية يكون مستوى الانترلوكين 17 عند الذين يتعاطون Etanercept المعـــــــــــروف Anti-TNFα inhibitors اعلى منـــــــــــــــــــه عند الذين يتعاطون Rituximab المعروف Anti-CD20 .**
- **الانترلوكين 17 ممكن استخدامه كمعلم لفعالية مرض التهاب المفاصل الروماتويدى.**

جمهورية العراق
وزارة التعليم العالى والبحث العلمى
جامعة بغداد
كلية الطب

تأثير علاج CD 20 inhibitors مقارنة بعلاج

TNF α inhibitors

على IL -17

فى مرضى التهاب المفاصل الروماتويدى الفعال

رسالة

مقدمة الى مجلس كلية الطب - جامعة بغداد ضمن متطلبات نيل درجة الماجستير
فى الكيمياء الحياتية السريرية

من قبل

محمد حنون داود

بكالوريوس علوم الكيمياء

بأشراف

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بورد مفاصل وتأهيل طبى

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