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ANTIOXIDANTS AND INFLAMMATORY BIOMARKERS IN RHEUMATOID ARTHRITIS

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
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AHMED FADHIL YASIR ALZAIDI

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ANTIOXIDANTS AND INFLAMMATORY BIOMARKERS IN RHEUMATOID ARTHRITIS

By Ahmed Fadhil Yasir ALZAIDI

June 2022

We certify that we have read this dissertation and that, in our opinion, it is fully adequate in scope and quality as a thesis for the degree of Doctor of Philosophy.

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Ahmed Fadhil Yasir ALZAIDI

ABSTRACT

ANTIOXIDANTS AND INFLAMMATORY BIOMARKERS IN RHEUMATOID ARTHRITIS

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Doctor of Philosophy in Chemistry

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June 2022

In curent study which aimed to determine if individuals with rheumatoid arthritis (RA) have elevated inflammatory or antioxidant markers. The study was carried out between January to April 2021 in teaching hospitals and private clinics in Iraq. The study included 81 patients previously diagnosed with RA and 50 healthy controls (HC_S). All participants underwent tests for the Biochemical Parameters which are: Adiponectin (ADP), Malondialdehyde (MDA), Protein carbonyls (PC), Catalase (CAT), and Superoxide dismutase (SOD). The results showed that there were significant increased in comparison between RA patients and HC_S in ADP, MDA, and PC. while there were significant decreased in CAT and SOD concentrations. The ROC curve showed that the area under the curve (AUC) of ADP and MDA was very high. while the AUC of PC and SOD was fair and the AUC of CAT was poor. The study concluded that there were no effects of age, gender, and BMI on the studied parameters. Low levels of antioxidants can lead to elevate the levels of oxidative stress and this elevated may be related to an increase in the production of reactive oxygen species (ROS). ADP and MDA is an excellent prognostic indicator for RA. PC and SOD can considered a fair prognostic indicator. CAT is a poor prognostic indicator for RA.

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Keywords: Rheumatoid arthritis, Malondialdehyde, Protien carbonyl, Catalase, Superoxide dismutase, Adiponectin

ÖZET

ROMATOİD ARTRİTTE ANTİOKSİDANLAR VE İNFLAMATUAR BİYOBELİRTEÇLER

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Haziran 2022

Romatoid artritli (RA) bireylerde inflamatuar veya antioksidan belirteçlerin yüksek olup olmadığını belirlemeyi amaçlayan bu çalışmada. Çalışma Ocak-Nisan 2021 tarihleri arasında Irak'ta eğitim hastaneleri ve özel kliniklerde gerçekleştirildi. Çalışmaya daha önce RA tanısı almış 81 hasta ve 50 sağlıklı kontrol (HCS) dahil edildi. Tüm katılımcılara Adiponektin (ADP), Malondialdehit (MDA), Protein karboniller (PC), Katalaz (CAT) ve Süperoksit dismutaz (SOD) olan Biyokimyasal Parametreler için testler yapıldı. Sonuçlar, ADP, MDA ve PC'de RA hastaları ve HCS arasında karşılaştırıldığında önemli bir artış olduğunu gösterdi. CAT ve SOD konsantrasyonlarında ise önemli düşüşler oldu. ROC eğrisi, ADP ve MDA'nın eğrisi altındaki alanın (AUC) çok yüksek olduğunu gösterdi. PC ve SOD'nin AUC'si adil ve CAT'nin AUC'si zayıftı. Çalışma, çalışılan parametreler üzerinde yaş, cinsiyet ve VKİ'nin hiçbir etkisinin olmadığı sonucuna varmıştır. Düşük antioksidan seviyeleri, oksidatif stres seviyelerinin yükselmesine yol açabilir ve bu yükselme, reaktif oksijen türlerinin (ROS) üretimindeki artışla ilişkili olabilir. ADP ve MDA, RA için mükemmel bir prognostik göstergedir. PC ve SOD, makul bir prognostik gösterge olarak kabul edilebilir. CAT, RA için kötü bir prognostik göstergedir.

2022, 151 sayfa

Anahtar Kelimeler: Romatoid artrit, Malondialdehit, Protein karbonil, Katalaz, Süperoksit dismutaz, Adiponektin

PREFACE AND ACKNOWLEDGEMENTS

I dedicate this work to my aunt's memory Dr. Neama Yasser Habil.

First of all I would like to thank ALLAH almighty for giving me the strength to complete this work.

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When you look at your life, the greatest love is the love of the family, I deeply thank my family (my parents, my elder brother) for their financial and moral support and their patience throughout my graduate peroid.

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Ahmed Fadhil Yasir ALZAIDI

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CONTENTS

LIST OF ABBREVIATIONS

ACPA	Anti-citrullinated protein antibody
ACR	American College of Rheumatology
AdipoR1	Adiponectin receptor 1
ADP	Adiponectin
AUC	Area under the curve
BMI	Body mass index
CAT	Catalase
CIA	Collagen-induced arthritis
CKD	Chronic kidney disease
CRP	C-reactive protein
CSF	Colony-stimulating factor
CT	Computed tomography
DAS	Disease activity score
DAS28	Disease Activity Score 28-Joint
DCs	Dendritic cells
DMARD	Disease-modifying anti-rheumatic drug
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ESR	Erythrocyte sedimentation rate
EULAR	European League against Rheumatism
fAPN	Fibrous adiponectin
FGF	Fibroblast growth factor
FPR	False positive rate
gAPN	Globular adiponectin
GM-CSF	Granulocyte macrophage colony-stimulating factor
G1	Patients >50 years
G2	Patients <50 years
HAQ-DI	Health assessment questionnaire Disability Index
HMW	High molecular weight
IBD	Inflammatory bowel disease
IgM	Interferon gamma
IgA	Immunoglobulin A
IFNγ	Immunoglobulin M
IL-10	Interleukin-10
IL-1Ra	Interleukin 1 receptor antagonist
IL-1β	Interleukin one beta
IL-6	Interleukin-6
LMW	Low molecular weight
LPS	Lipopolysaccharide

MCP joints	Metacarpophalangeal joints
M-CSF	Macrophage colony-stimulating factor
MDA	Malondialdehyde
MHC Major	Histocompatibility complex
NET	Neutrophil extracellular traps
MRI	Magnetic resonance imaging
MTP joint	Metatarsophalangeal joint
MMW	Medium molecular weight
NSAIDs	Nonsteroidal anti-inflammatory drugs
OASFs	Osteoblastic fibroblasts
OD	Optical density
PC	Protein carbonyls
PGE2	Prostaglandin E2
PIP joints	Proximal interphalangeal joints
pMRI	Peripheral MRI
PPARs	Peroxisome proliferator-activated receptors
PRO	Patient-reported outcome
PTPN22	Protein tyrosine phosphatase non-receptor type 22
RA	Rheumatoid arthritis
RBC	Red blood cells
RF	Rheumatoid factor
RNS	Reactive nitrogen species
ROC	Receiver Operating Characteristic Curve
ROS	Reactive oxygen species
SCID	Severe combined immunodeficiency
SE	Shared epitope
SFs	Synovial fibroblasts
SOD	Superoxide dismutase
TGF-beta	Transforming growth factor beta
Th	T helper
TLR	Toll-like receptor
TPR	True positive rate
TNF-α	Tumor necrosis factor-alpha
U.A	Uric acid
VAS	Visual analogue scale
VEGF	Vascular endothelial growth factor

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

Rheumatoid arthritis (RA) is a continual systemic inflammatory autoimmune ailment characterised via way of joint infection and structural damage. Recent studies has been focusing at the possible identity of predictor markers of ailment onset and/or progression of joint injury (Van der Woude and van der Helm-van Mil 2018, Narayan *et al.* 2019). The spread of RA is 0.5-1.0 % of the general public worldwide which it influences as a minimum two times as many female as male. Despite the fact that it could appear at any age, the height occurrence is on the age of fifty years (Gabriel *et al.* 1999, Shichikawa *et al.* 1999).

As one of the distinctive lineaments of RA is the permanent inflamed synovial sublining caused by synovial cell infiltration that participate to create a microenvironment wherein stromal cells show a hyper-activated phenotype, liberating numerous pro-inflammatory and mediators that damage the tissue of the joint area (Dakin *et al.* 2018). Although most of the underpinning reasons of RA are nevertheless inconspicuous, systemic and internal immune dysfunction is taken into consideration to coordinate its pathogenesis.

As a matter of fact, that ailment is characterised through the produce of auto antibodies like anti-citrullinated protein antibody (ACPA) and rheumatoid factor (RF), in addition to elevated the levels of e.g., TNF-α, IL-1β and IL-6 which can be recognized as main pro-inflammatory cytokines involved with in chronic synovitis, osteoclast formation and posterior erosive joint damage (McGettrick *et al.* 2012, Firestein and McInnes 2017). Free radicals are extraordinarily reactive molecules with one or more than unpaired electrons within side the outer valence shell (Pruchniak *et al.* 2016).

In biology, the majority significant radicals are reactive oxygen species (ROS), and reactive nitrogen species (RNS) (Len *et al.* 2019). Oxidative stress and Inflammation are two states, but one can affect the other as well as make greater the other (Hui-Ming and Hui Zhou 2014). Oxidative stress can stimulate a group of transcription factors, a number of which cause genes expression of inflammatory cytokines (Reuter *et al.*

2010). Pro-inflammatory cytokines result in the manufacturing of ROS and RNS in immune cells via the protein kinase signalling activation (Liguori *et al.* 2018, Fonseca *et al.* 2019).

The diagnosis of RA relies on a high indicator of doubt based on the medical history of patient and physical examination. In 2010, the American College of Rheumatology (ACR) and the European League against Rheumatism (EULAR) design the most present criteria for the diagnosis of RA (Aletaha *et al.* 2010). These instructions are targeting to distinguish RA among patients recently presenting with synovitis at least in one joint. The 2010 criteria vary from preceding criteria in their ability to differentiate and discover early RA (Arnett *et al.* 1988).

A variety of risk factors are known to be within the development of RA, which includes genetics, epigenetics, female sex and environmental factors. Suggested environmental risk factors contain smoking, silica exposure, infectious agents, vitamin D deficiency, overweight and changes in the microbiota, in spite of the fact that studies for some of these factors are not very enough.

Rheumatoid arthritis is a cumulative and an obstruction autoimmune disease, considered general health problems affecting millions of people worldwide. Therefore, it is essential to increase scientific knowledge to improve our understanding the pathophysiology of inflammation and antioxidant role in RA patients and their relationship with the progress of the disease. To provide new diagnostic biomarker for oxidative damage and identify a novel target for managing RA patients.

The purpose of this research is to evaluate the inflammatory and antioxidant status of patients with early and late rheumatoid arthritis. Additionally, it emphasizes the need of developing more precise and sensitive biomarkers for rheumatoid arthritis.

2. LITERATURE REVIEW

2.1 Rheumatoid Arthritis

RA is term obtained from the Greek origin word ῥεύμα-rheuma (nom.), ῥεύματος-rheumatos (gen.) ("flow, current"). The suffix -oid ("resembling") displays the interpretation as inflamed of joint that look like rheumatic fever. Rhuma this mean that watery discharge may indicate to the reality that the joints are swelling or that the illness perhaps become bad by humid weather (Al-Rubaye *et al.* 2017). RA is the most usual aspect of chronic inflammatory arthritis; characterised by means of joints inflammation, leading in synovial hyperplasia via infiltration of activated immune cells and this leading to cartilage and bone destruction (Shrivastava and Pandey 2013). RA is a systemic chronic inflammatory disorder of unknown etiology, it is characterised through permanent pain and destruction of joint that commonly progresses from far to more nearer joints (Kourilovitch *et al.* 2014). RA usually effects in warm, swollen, and painful joints. Pain and stiffness frequently get worse following rest. The disease may have an effect on different parts of the body, in most cases, the hands and wrist are involved; together with the same joints usually involved on each aspects of the body.This may lead in a lower RBC count, inflamed throughout the lungs, and the heart. Low energy and Fever may possibly already be. Generally, signs and symptoms come on progressively through weeks to months (Geuskens *et al.* 2007).

2.1.1 Stages of RA

Quick identification and treating to management inflammation are of high concern in RA, given the possible for quick demolition of joint and other tissue and impairment of functions. In spite of how RA progression in individuals patients and The kind of joint involvement varies greatly from patient to patient., in the major part of patients with RA (70%) some degree of joint erosion in the feet and hands is an easily detect by x-ray during early stages of the illness. More than 60 % of RA patients may develop considerable functional impairment 20 years after diagnosis if they do not get adequate

therapy (stage III), as well as need of ability to move , loss of capability for self- caring, and require of joint replacement, or loss of independence and need daily care (stage IV) (Suter *et al.* 2011).

Stage I RA:

Early level of RA (stage I) is distinguish by synovitis, or an infection of the synovial membrane, leading swelling of involved joints and pain on the move. During this stage, the number of cells in synovial fluid continues to rise as immune cells migrate towards the infection site. However, with the exception of soft tissue swelling and maybe some bone erosion, there is no sign of joint deterioration on x-rays Figure 2.1 (Al-Rubaye *et al.* 2017).

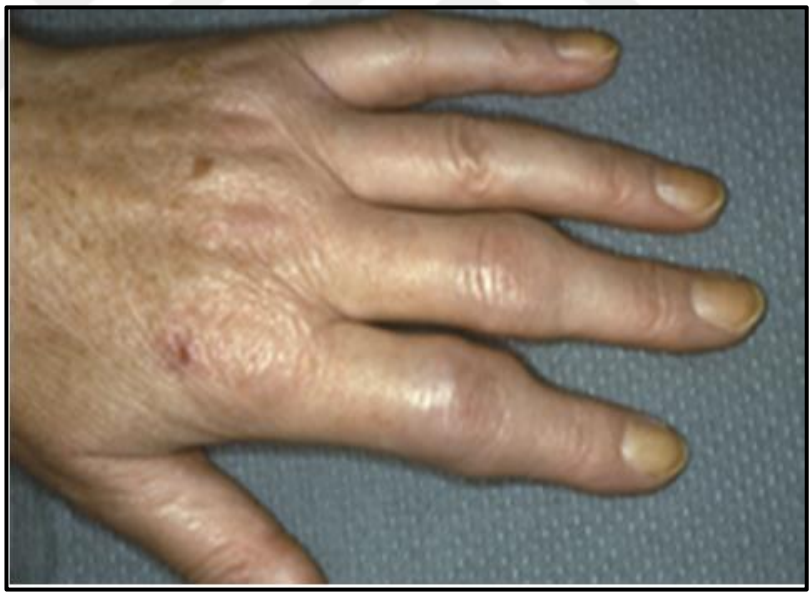

Figure 2.1 Early RA, with fusiform swelling of PIP joints. Adapted from (Littlejohn and Monrad 2018)

Stage II

In moderate RA (stage II), there is increase of infection in synovial tissue, influences joint cavity area over joint cartilage. This inflammation will progressively result in a

destruction of cartilage; go along with by a narrowing of the joint (Al-Rubaye *et al.* 2017).

Stage III

Severe RA (stage III), is noticeable by produce of pannus in the synovium. Losses of joint cartilage uncover bones lower down the cartilage. These changes will appear evident on x-ray, along with erosions on every side on the edge of the joint Figure 2.2 (Al-Rubaye *et al.* 2017).

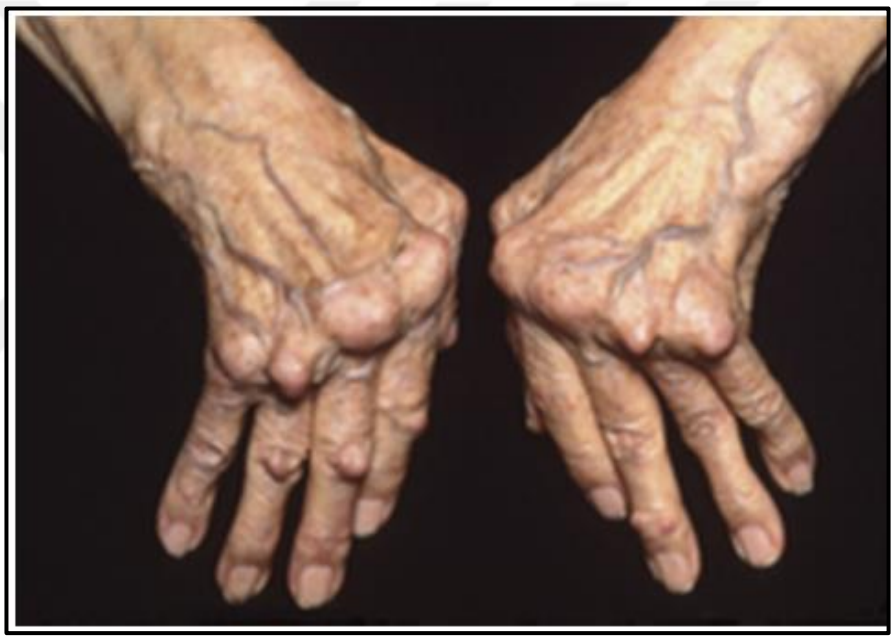

Figure 2.2 Advanced RA with ulnar deflection, MCP subluxation, and interosseous muscle atrophy Adapted from (Littlejohn and Monrad 2018)

Stage IV

Stage IV of RA is referred to be the terminal or last stage. The inflammatory activity has subsided, and the formation of fibrous tissue and/or the fusion of bone have resulted in the cessation of joint function. This stage might be associated with the development of subcutaneous nodules Figure 2.2 (Al-Rubaye *et al.* 2017).

2.2 Epidemiology of RA

RA impact almost 0.5 to 1% of the community wide-ranging, with female 2 to 3 times as likely as male to progress the illness (Tedeschi *et al.* 2013). The prevalence and incidence of RA differs sustainably between geographical districts and this cannot be describe by genetic factors alone, rather than this variation can explained by environmental exposure (Myasoedova *et al.* 2010). The prevalence of RA is among (0,5 to 1)% in North American and European populations, Asia had the lowest rate of disease(0.2 to 0.3)%, and a few Native American residents has unusual high prevalence upto (5%) (Alkazzaz 2013). The aetiology of disease is unclear, individuals who exposed to hard life situations, putting at high risk for stress related medical conditions like area of military conflict e.g. (Iraq) (Boscarino 2004). In Iraq the prevalence scanning for RA was done throughout the summer of 1975 in persons aged 16 and over in areas of Iraq, definite RA was noted in 1% 0f population where in Babylon province was (1.02%), the feet were less effected than hands in that study (Al Rawi *et al.* 1978). Another study that conducted in Iraq Babylon between 2001-2011 by retrospective cohort study in Rheumatology unit, Merjan Teaching Hospital there were1039 patients that diagnosed with RA from 53786 patients in rheumatology unit. The prevalence and incidence of RA was increasing from 1.60% in 2001 to 3.02% in 2011 (Alkazzaz 2013). In turkey the first study on the spread of RA was published in 1968. This study, which was done in Istanbul in rural area, using the 1958 ARA criteria for case definition described a crude spread rate of 0.22%. However, 70% of the specimen peoples in that study were recent newcomer from the Balkans; thus, that study population cannot represent the entire Turkish people (O Yenal and I Lâv 1968). Different epidemiologic study, carried out in region of the Black Sea of Turkey, recorded a very high spread of RA (3.7%) through a total of 1,825 subjects over 20 (Madenci *et al.* 2002). Another study conducted in the region of eastern Black Sea displayed those 59 patients were previously diagnosed with RA according to the ACR criteria, of which 48 female and 11 were male. The spread of RA was 1% in the general people, 0.35% in men and 1.6% in women. The female/male ratio was 4.3: 1.0, showing that RA spread was statistically significantly higher in women (Çapkin *et al.* 2010).

2.3 Aetiopathogenesisof RA

RA is an autoimmune illness, involving both the adaptive and the innate immune system (Smolen *et al.* 2018). In spite of extensive research, it is yet unknown what starting the autoimmune response causing RA, many risk factors have been specified. The synovium becomes hypertrophic, as the result of the synovial inflammation, formation a tumour similar pannus that invade and break down bone and cartilage, resulting to dysfunction and patient pain (Harris 1990). Over time, the majority of patients promote erosions inside the bone and cartilage, a reduction in joint cavity, and per articular decalcification, all of which are usually referred to as radiographic alterations associated with RA Figure 2.3 (Innala 2014). The risk factors specified are of many types genetic, environmental, as well as hormonal - and there are also indications of relationship between them, making the aetiology of RA hard to clarify and till now insufficiently known.

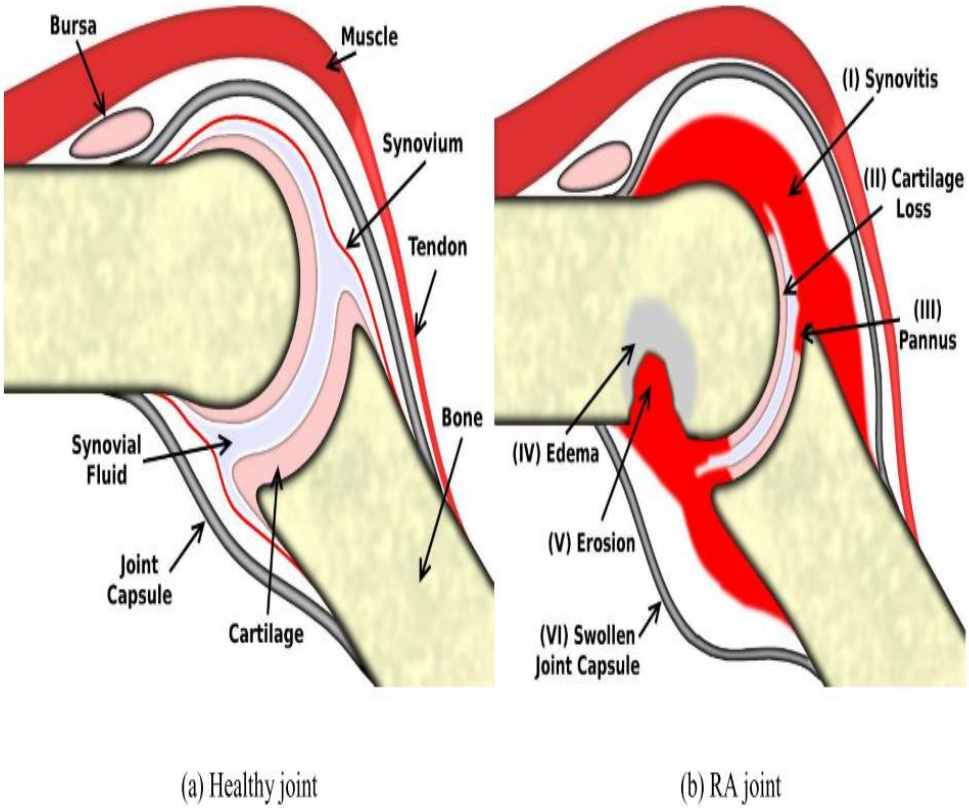

Figure 2.3 A Comparison of a normal joint and a joint affected by rheumatoid arthritis adapted from (Emond 2012)

2.3.1 Genetic risk factors

Several decades ago a hereditary component in the aetiology of RA was identified , when studies observed a high frequency of RA in first degree relatives to patients with RA, than in the common population (Lawrence 1970). Today more than 100 locus have been correlated with risk of RA (Okada *et al.* 2014). The most important inherited factor for develop of RA, and the first one to be recognized, was the HLA DRB1 alleles named shared epitope (SE). The cooperative between RA and SE was later specified to patients with the antibodies against citrullinated peptides (ACPA) and rheumatoid factor (RF) antibodies (Wahlin 2019).

The Most second important risk locus in RA is the protein tyrosine phosphatase non-receptor type 22 (PTPN22) which is one more gene that regulates the immune system outside of the Gene encoding that has been linked with RA (Källberg *et al.* 2007). Working as a negative regulator, this gene regulates the threshold of T-cell activation. T-cells having a lower activation threshold may be produced by the risk allele (Innala 2014). Currently, over a 100 more genetic loci related with RA, and the majority of these risk genes are associated with cell types and pathways that are important in RA pathogenesis, which may provide opportunities for the development of novel RA medicines (Okada *et al.* 2014). According to a current research, the family risk of heritability for ACPA sero-negative RA was 20%, while the risk for ACPA sero-positive RA was 50% Figure 2.4 (Frisell *et al.* 2013).

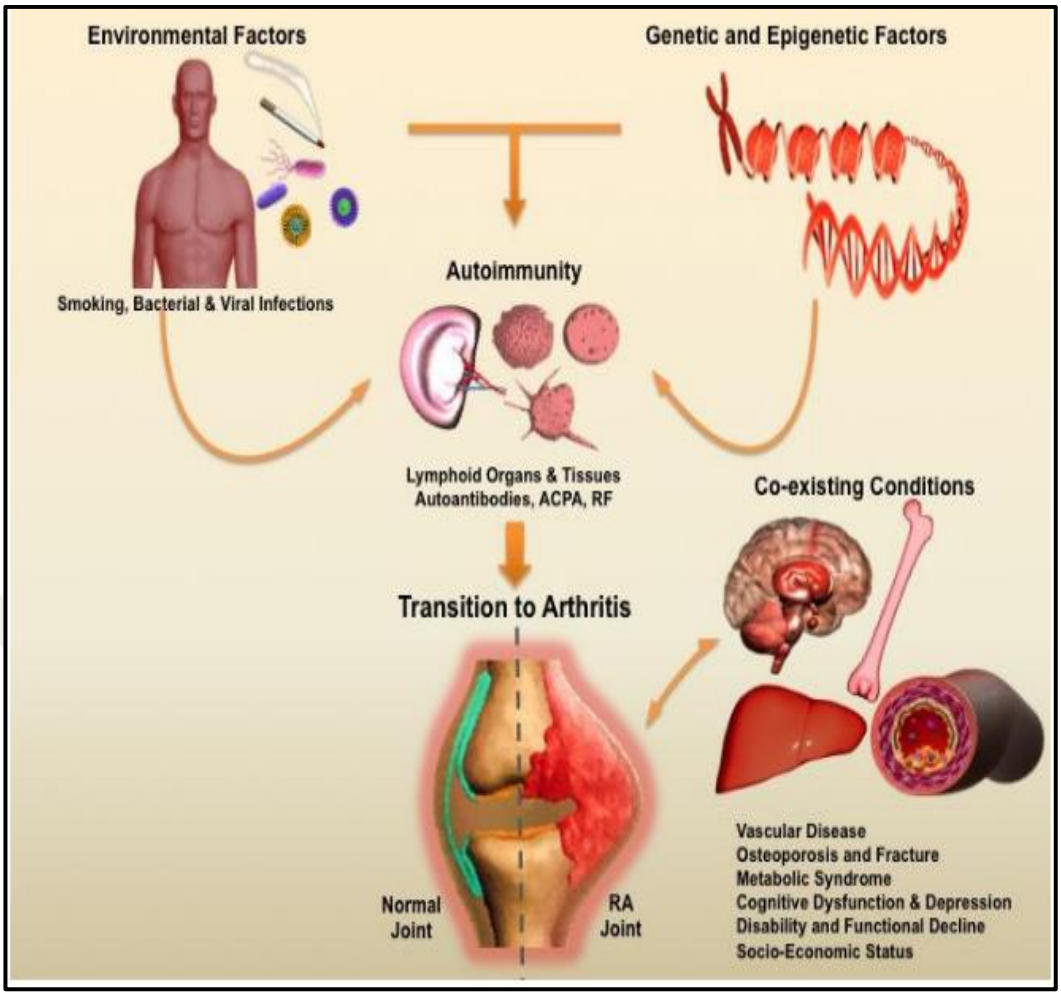

Figure 2.4 Schematic representations of concepts in the pathogenesis of rheumatoid arthritis Interactions between environment and genes lead to autoimmune disorders by a decrease in the body's ability to tolerate its own proteins, and which may be associated with rheumatoid arthritis. For example, smoking lead to the production of (ACPA), which is produced by post-translational modification in persons who have the 'shared epitope'? At this step, serum concentrations of RF/ACPA and acute phase proteins, through with chemokines and cytokines Serum levels of RF/ACPA and acute phase proteins, as well as chemokines and cytokines, begin to rise years before clinical illness begins. Adapted from (McInnes and Schett 2011)

2.3.2 Epigenetics

The previous studies clearly indicate that genetic factors in extensive and HLA-DRβ1 in specific contribute to the development of RA so epigenetic factors are therefore deserving of consideration (Padyukov *et al.* 2011). In recent years, several studies have suggested that epigenetic changes that occur in synovial cells in RA may play a major

role in the pathogenesis of RA. Epigenetic factors also may involve in the lineages of immune cells and may also occur before the clinical disease and thus contribute strongly to the development of RA, for example smoking and exposure to toxins, where these factors induce RA. The term of epigenetics define as the changes that occur in gene expression mediated by causes other than modification of the nucleotide sequence (Holliday 2006). Despite the fact that epigenetic alterations may be reversed, they can highly participate to chromatin structure stabilization, genome integrity, and gene expression modulation, development of embryonic, genomic imprinting and X-chromosome deactivation in females (Turek-Plewa and Jagodzinski 2005). Epigenetic modulation of gene expression is intermediate by a number of mechanisms that consist of DNA acetylation, methylation, sumoylation, and phosphorylation and also through the improvement and post-transcriptional regulation function of microRNAs (miRNAs) (Elmesmari 2014).

2.3.3 Environmental risk factors

The risk of developing RA increases with increasing smoking in a dose-dependent manner, as it has been observed that there is an association mostly between smoking RA for RF-positive RA and ACPA-positive RA (Klareskog *et al.* 2006, Sugiyama *et al.* 2010). Dust inhalation is another risk factor for RA, as is exposure to inhaled silica. There is also an increased risk in individuals exposed to textile dust the increased risk in textile dust exposed individuals is not limited to developing ACPA-positive RA, but also ACPA-negative RA (Too *et al.* 2016).

2.3.4 Hormonal risk factors

RA is more common in females than in males, with a female to male ratio of 4 to 1 in patients under 50 years of age and a ratio of 2 to 1 in patients over 60 years of age. These showed that hormonal factors are in correlate in the pathogenesis of RA, but the mechanisms and role of hormonal factors stay unclear. during the pregnancy the incidence of RA is lower, but regarding for example hormonal replacement therapy,

breast feeding and oral contraceptives, the results are incompatible (Alpízar-Rodríguez *et al.* 2017).

2.3.5 Microbiota

Periodontal disease is related with disease activity in patients with RA and also with an increase incidence of RA (Rodríguez-Lozano *et al.* 2019). It has been recommended that the increased risk at least in one part is mediated by the oral microbiota, in specific Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans, bacteria that are capable to citrullinate peptides. The bacteria in the gut differ in RA patients compared with general population, but with time of disease and treatment of RA the microbiota is changes (Guerreiro *et al.* 2018).

Interactions between risk factors:

There is an evident interaction between environmental and genetic factors on development of RA. Smoking react with PTPN22, as well as shared epitope (SE) , and all three of these risk factors together increase the risk 20-fold for growing of ACPA-positive RA (Källberg *et al.* 2007). There is also an association between SE and textile dust, where dust exposed persons those carrying the SE had a 40-fold increased risk of anti ACPA-positive RA. In respect to interaction between environmental risk factors, persons exposed to both silica dust and smoking have a higher risk of development of RA, than persons exposed to either silica dust or smoking (Stolt *et al.* 2010).

2.3.6 Infectious agents

There is an increased risk of severe infection in people with RA. There are a lot of mechanisms that might explain this occurrence: (i) there is a deficiency in immune cells as well as the disease's underlying causes, (ii) previous diseases or injuries of comorbid conditions, (iii) negative impact of immune inhibitor medication which leads to reduction in immune function, (iv) RA patients lifestyle such as smoking.(Listing *et al.*

2013) In addition to disability and functional limitations, RA patients often have additional significant co-occurring illnesses such as renal and respiratory diseases and diabetes mellitus, All of these factors are also related to raised infection risk in RA (Elmesmari 2014). The majority of infectious illness fatalities, especially in women, are due to respiratory reasons. A dose-dependent increase in the incidence of severe infections has been shown with both long-term glucocorticoid and TNF inhibitor treatment (Bongartz *et al.* 2006, Listing *et al.* 2013).

2.3.7 Antibodies

Many various disease-related autoantibodies have been specified in RA. Rheumatoid The first antibody found was factor (RF), and clinicians are currently using it to diagnosis RA, according the American College of Rheumatology (ACR) 1987 classifications, RF is one of the seven criteria for the categorization of RA (Matsumoto *et al.* 1988). RF is a reacts with the FC component of IgG isotype antibodies, and it may be of the IgA or IgM isotypes, depending on the antibody. 60 - 80 % of established RA patients have RF sensitivity, whereas 95 % of those individuals have a high degree of specificity for RF. Sero-positive old people are becoming more common as the population ages, roughly 17% of healthy elderly adults (Palosuo *et al.* 2003). Additionally, individuals with a variety of autoimmune disorders and infectious illnesses may test positive for RF antibodies. RF's disease specificity is quite limited and cannot explain and drive the inflammatory response that defines RA on its own, as a particular antigen (Newkirk 2002, Palosuo *et al.* 2003). Compared to ACPA, which has been postulated to have a more specialized role in the etiology of RA and bone degradation, this is fundamentally different (Kleyer and Schett 2014). Notwithstanding the above, RF is still highly relevant as an indication marker, especially in the presence of extra-articular symptoms, which are associated with RF but not with ACPA to the same degree (De Rycke *et al.* 2004). While past studies have shown a link between high levels of RF and extra-articular symptoms, high levels of RF seem to be more relevant than high levels of ACPA in individuals with active extra-articular RA, according to the authors of this study (Turesson *et al.* 2007). On other hand, ACPA has been detected at all phases of the beginning of RA (pre-patients), the commencement of the illness (early

RA), and in the midst of the disease (established RA) (Rantapää-Dahlqvist *et al.* 2003). Using citrulline-containing peptides generated from the sequence of filaggrin, the first method of anti-CCP test (anti-CCP1) was developed. This test showed a diagnostic sensitivity of 68% and a disease specificity of 96%. In light of the fact that filaggrin was not present in joints, it was hypothesized that other proteins not linked to filaggrin would possess epitopes more suited for ACPA detection (Van Venrooij and Zendman 2008). To improve the diagnostic sensitivity and specificity of the anti-CCP2 test, a second approach was devised. Sera from RA patients were used as substrates for this test, which included citrullinated peptides that were hand-picked for their ability to distinguish between the two diseases. Anti-CCP2 enzyme-linked immunosorbent assay (ELISA) increased diagnostic sensitivity to 70-75 % with a specificity of 95-99 %, and the anti-CCP2 test is widely used in clinical practice across the world (Van Venrooij and Zendman 2008). A third approach test, the anti-CCP3 test for the detecting of IgG antibodies, was created as an extension of the anti-CCP2 test. This test detects the combination of IgA and IgG antibodies, which is a further development of the anti-CCP2 test. These tests have approximately the same specificity and sensitivity as the anti-CCP2 test for RA in terms of specificity and sensitivity (Enriconi dos Anjos *et al.* 2009). As a result, the presence of ACPA is very valuable as a diagnostic marker in the early stages of illness, with sensitivity comparable to that of RF (70-75 %), but with a greater specificity (about 98 %) (Van Venrooij *et al.* 2008). Because joint destruction occurs early in the disease pathway, the presence of ACPA is also included in the 2010 RA classification criteria for RA. When RF is present at the onset of the illness, ACPA is a more precise diagnostic and prognostication sign than RF alone (Aletaha *et al.* 2010). Currently, the most often tested autoantibodies in clinical settings are ACPA and RF. However, additional antibody tests are now being developed (Shi *et al.* 2013).

2.4 Pathophysiology of RA

2.4.1 Synovitis

This thin membrane that lines the joint capsule acts as lubricant between the stiff tissues of the joint, making active movement possible without the need for additional friction. It

also has an impact on the appropriate feeding of joints. This membrane is composed of two layers (the subintima and the intima), as well as initial cells, which are typically macrophages and fibroblasts in the majority of cases. In RA, joints are found to have an increase in the number of inflammatory cells infiltrating the synovium as well as an increase in synovial lining cell growth with the addition of new blood vessels which yields pannus development (producing synovial cells that infiltrate the cartilage and bone) (Elmesmari 2014). The creation of pannus aids in the breakdown of bone and cartilage near to the surface of the joint, resulting in erosions and the laxity of ligaments and tendons that can be detectable by radiographically (Figure 2.5). The cause of synovitis is still not clear but in RA patients the inflammatory synovitis is distinguished by a quick flowing and reproduction of flaming cells such as T and B-lymphocytes, monocytes together with neutrophils, resulting to an abundance of chemokines, cytokines, and proteases. This causes redness, warmth, discomfort, and swelling in the acute phase reaction, effusion and other appearances of inflammation. Interestingly TNF-, IL-1β, IL-6, and other pro-inflammatory cytokines have been found in higher concentrations in the RA synovial membrane than have the anti-inflammatory mediators (IL-1Ra, IL-10) in various studies, and this cytokine variation likely contributes to the long-term joint inflammation that causes bone erosion and cartilage damage. (Brennan and McInnes 2008, Elmesmari 2014).

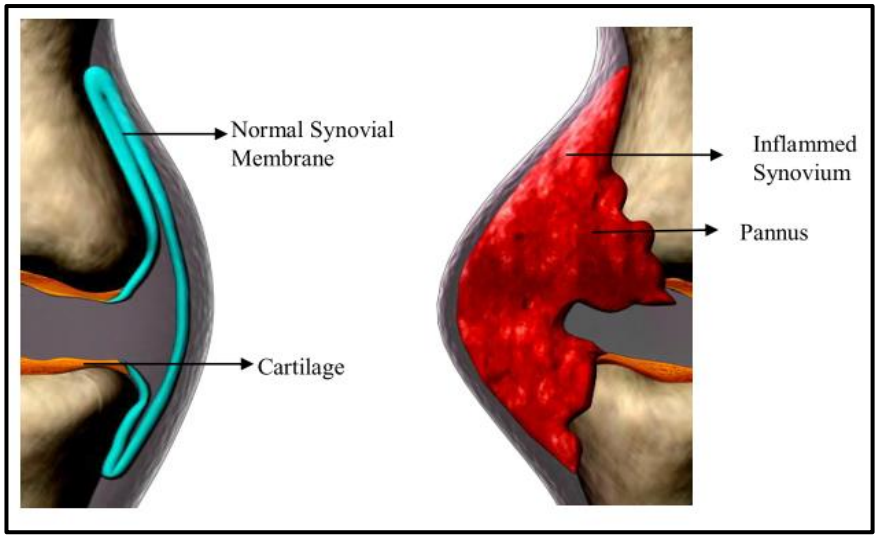

Figure 2.5 Schematic showed rheumatoid arthritis. The figure demonstrates the variation between a healthy joint and an inflammatory synovial joint

2.4.1.1 Synovial T cell subsets in synovitis

The role of T cells in the pathophysiology of RA is a hotly debated question. Although T cells are found in large amount in synovium their derived cytokines like INF-γ and IL-2 are rarely detected in either synovial fluid (SF) or synovium (Fox 1997). Numerous pieces of evidence point to T cells being RA's pathogen, particularly genetic associations in humans, data from mice, and results from targeted therapy. There is a strong association between illness severity and lymphoid-specific PTPN22 and HLA-DR alleles in MHC class II, which supports the idea that T cells play a role in synovitis. Moreover, although cyclosporine or depleting T cells targeting of T cells this process showed no or limited clinical efficiency (Panayi 2006). With the introduction of abatacept, functional improvement and clinical significance have already been noted. Abatacept, a fusion protein copmosed of CTLA-4-immunoglobulin effusion protein, get involved in with T cell-antigen give out cell contact by way of selective costimulation modulator which therefore hinders the activation of T cells (Elmesmari 2014). As a result, it directly included T cells in the pathogenesis of RA, which was a clinical advantage.

The synovium's CD4+ T helper (Th) cells and the cytokines they produce are critical for initiating and maintaining a variety of immunological responses. Naive CD4+ T cells become different into many and various phenotypes of effectors Th subsets, like Th1, Th2, Th17, and regulatory T cells (Tregs) under the effect of a network of inflammatory cytokines signals (Miossec *et al.* 2009). The phenotype of effectors cells in the synovium is traditionally regulated by cytokines; IFN-γ and IL-2 are produced by Th1 cells, which differentiate in the presence of IL-12, while Th2 cells are generated in the presence of IL-4 and are characterized by the production of interleukin IL-4, interleukin IL-5 and interleukin (IL-13).

Recent research shows a previously unknown effectors T cell lineage called Th17, which may produce a diverse array of cytokines including IL-17A, IL-17F, IL-22, and IL-21 (Park *et al.* 2005). In addition to Th1 and Th2 cells, progenitor T cells may develop into Th17 cells when stimulated with growth factor beta (TGF-beta) and other

cytokines such as interleukin-1beta (IL-1β), interleukin-6 (IL-6), interleukin-21 (IL-21), and interleukin-23 (IL-23) (Miossec *et al.* 2009). The idea of Th1 and Th2 cells being disease-associated has developed through time, and presently it is believed that RA is mostly driven by Th17 cells. The initial evidence of the relevance of Th17 cells and IL-17 in RA was provided by the discovery of higher levels of IL-17 in the blood, synovial fluid, and RA patient's synovial tissue (Sue-Yun Hwang and Ho-Youn Kim 2005). Additional evidence to support the theory that a pathogenic function exists for Th17 cells in RA was provided by many studies showings that mice lacking p35 of IL-12 had accelerated collagen-induced arthritis (CIA); this is the primary cytokine responsible for the activation of Th1 differential. A Several inflamm cytokines, like TNF-α, IL-6, IL-1β, and IL-17 are elevated on joints of these mice, leading to severe arthritis (Elmesmari 2014).

The generation of cytokines by T cells, as well as their connection with nearby cells directly or indirectly through cytokines, all contribute to inflammation (synovitis). T cells also stimulate the activation of nearby cells by their connection with them. Once the immune system has been turned on, T cells may produce cytokines such as INF-γ, lymphotoxin-β, TNF-α, and IL-17. Interleukin-17 (IL-17) serves as a molecular signalling intermediary that triggers an inflammation in the joint, in turn resulting in the production of many proinflammatory mediators, including interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), CXCL8/IL-8, colony-stimulating factor (CSF) and prostaglandin E2 (PGE2) (Fossiez *et al.* 1996). Furthermore, monocytes/macrophages and endothelial cells in synovium may be directly stimulated to secrete the following pro-inflammatory cytokines: TNF-α, IL-1β, IL-6, IL-12, IL-15, IL-18, IL-23, and TGFβ, resulting in the additional stimulation of T cell differentiation (McInnes and Schett 2011). Another important factor in creating prolonged inflammation of the joints is the interactions between T cells with fibroblasts (Brennan *et al.* 2002).

2.4.1.2 Monocytes/macrophages in mynovitis

One of the most prominent characteristics of RA is the presence of monocytes and macrophages, may have a significant impact on the development of the disorder by the production of pro-inflammatory cytokines and other inflammatory mediators, among other things. Hematopoietic stem cells (granulocyte-macrophage progenitor) are responsible for the formation of monocytes, and they eventually differentiate into monoblasts and monocytes, which circulate in the circulation and go on to mature and differentiate in peripheral organs. The differentiation and maturation process is regulated by a variety of growth factors and cytokines, such as CSFs, which include macrophage colony-stimulating factor (M-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF) (Hashimoto *et al.* 2011). According to the cytokine milieu, monocytes may undergo additional differentiation in the tissue; for example, interaction to GM-CSF and IL-4 increases DC production regardless of which subset of DCs is being developed (Sallusto and Lanzavecchia 1994). There are two subsets of macrophages identified by their activation state. The first subsets are known as M1 (classical, inflammatory) and the second subset is called M2 (alternative, anti-inflammatory) (classical, inflammatory). This subset of immune cells, which is also referred to as M1, is stimulated by substances such as (IFN-γ), (GM-CSF), and TNF-α then is activated when the Toll-like receptor (TLR) legends are added e.g., LPS. Unlike M2 subset stimulated by substances such as, IL-4, IL-13, M-CSF, immune complexes, and IL-10 (Mantovani *et al.* 2004). There are several categories of macrophage phenotypes, and they are categorized according to their function, such as the defence of the host, wound healing, and immunological control. Large levels of cytokines and chemokines, including TNF-α, IL-1β, IL-6, IL-23, IL-12, type I IFN, CXCL9-11, and reactive nitrogen and oxygen intermediates are all seen in high concentrations in inflamed synovial tissues and are important contributors to the development of RA synovitis (Mosser and Edwards 2008). Although blood monocytes are found in several functional subsets, it is becoming clear that different subsets of monocytes exist and play distinct roles in inflammation and homeostasis (Gordon and Taylor 2005). The presence of infiltrating macrophages in the synovial membrane of inflamed joints serves as sensitive biomarkers of sickness because the level of inflammation in the synovial

membrane is related to synovial destruction and to joint deterioration. Additionally, scores for the local disease activity and clinical improvement whether a therapeutic strategy is used or not (Haringman *et al.* 2005). Furthermore, since macrophages enter the synovial space alongside other cells, it is expected that the interactivity with the diverse cell types will be crucial. Macrophages also participate to synovial inflamed by stimulation of fibroblasts, which results in the release of GM-CSF, IL-6 and CXCL8/IL-8Similarly; they have the ability to activate CD4+ T cells via the MHC class II receptor. Monocytes are not only engaged in the formation of synovitis, but they are also implicated in the stimulation of bone resorption by stimulating the differentiation of osteoclasts at an early stage in the bone resorption process (McInnes and Schett 2007).

2.4.1.3 Synovial fibroblast in synovitis

Synovial fibroblasts (SFs) are present in the synovial membrane of the joint. SFs are responsible for releasing important inflammatory cytokines and proteases, which directly contribute to the development of RA pathogenesis (Kiener *et al.* 2009). It is the active phenotype of RASFs that is distinguished by morphological changes, anti-apoptotic behaviour, and heightened invasion capabilities (Ospelt and Gay 2008). Available findings from severe combined immunodeficiency (SCID) in mice have shown that RASFs exhibit aggressive behaviour and may migrate across joints, hence acting as a pathway for the transmission of the RA-associated phenotype and subsequent cartilage degradation to other joints (Diller *et al.* 2019). This aggressive nature of RASFs seems to be a contributing factor to the cytokine-rich environment, in addition to the RA synovium's characteristic ongoing inflammation and destruction of joints. Although a variety of processes have been linked in the development of the attacker phenotype, epigenetic alterations in RASFs that may affect gene expression, therefore, functions have recently been discovered. RASFs are distinct from other fibroblast types, such osteoblastic fibroblasts (OASFs) and normal fibroblasts (normal-SFs), in that they express high levels of proto-oncogenes and particular metalloproteinase, and also cytokines and adhesion molecules (Rinaldi *et al.* 1997 and Konttinen *et al.* 1999). SFs exist within the joint's tissue spaces, near to T cells and macrophages. The proximity of these cells within tissue spaces and the involvement of

these cells in the amplification of local inflammation are both critical to understanding joint inflammation. TNF-α and IL-1β promote the activation of fibroblasts, which then produce many cytokines and chemokines, which including IL-1, IL-6, TNF-α, and MMPs (Li *et al.* 2012). TLR agonists have the additional effect of amplifying several of these pathways. Additionally, RASFs are involved in angiogenesis because they produce proangiogenic factors including FGF and VEGF. Moreover SFs may inflict harm on joint health by infiltrating the cartilage barrier or via generating matrix-degrading enzymes (Elmesmari 2014).

2.4.1.4 Cytokines in RA

A primary function of cytokines is to contribute to the effectors phase of innate and adaptive immunity. While many cytokines have varied functions, they do share some qualities. They are generated during the phase of host defence, and the majority of them do not contain molecules that have been completely synthesized. However, in fact, some cytokines are pro-molecules that may be cleaved into active forms by biochemical reactions., a number of different cell types are involved in their production. In turn, cytokines operate on a large number of cell types, and on occasion, their actions are many and varied on the same target. While there are many types of cytokines in RA, some of which are pro-inflammatory, TNF-α, IL-1β, and IL-6 are behind the continuous destruction of joints and active inflammation. Several cytokines important in the development of RA are given in the Table 2.1 (McInnes and Schett 2007).

Table 2.1 Cytokines that involved in the pathogenesis of RA adapted from (Elmesmari 2014)

Cytokines	source of cells	Primary influence in the pathogenesis of RA
IL-1	B cells, Monocytes, synovial fibroblasts	Promotes the production of cytokines and chemokines by fibroblasts and monocytes
IL-6	Synovial fibroblasts, B and T cells, Monocytes	Promotes B cell antibody production by inducing Th17 cell differentiation
IL-10	Epithelial cells, B and T cells, DCs, Monocytes	Suppresses inflammation by inhibiting proinflammatory cytokines, as well as inhibiting T cell reproduction and promoting Treg cell development.
IL-12	Dendritic cells (DCs), Macrophages	Proliferation and maturation of Th1 cells, as well as activation of B cells

Table 2.1 (continue)

IL-15	Mast cells, synovial fibroblasts, neutrophils, Monocytes, DCs	It stimulates the activation of T cells, macrophages, fibroblasts, and neutrophils, as well as the differentiation of B cells
IL-17A	Th cells, synovial fibroblast, innate lymphoid cells	Enhances the production of pro-inflammatory cytokines by synovial fibroblasts, monocytes, and T cells
IL-18	Platelets, DCs, Monocytes, endothelial cells	Promotes T cell differentiation and cytokine release, which also increases cytotoxicity
IL-23	DCs, Macrophages	Increase the multiplication and survival of Th17
IL-32	Epithelial cells	Promotes the secretion of TNF, IL-1β, and IL-6, as well as chemokines.
TNF-α	Mast cells, B and T cells, fibroblasts, Monocytes	Increases cytokine release and endothelial activation in monocytes/macrophages, and impairs the regulatory role of regulatory T cells.
INFs	Widespread	Increased MHC expression promotes activation, differentiation, and survival of macrophages and lymphocytes, as well as cytoskeletal alteration.
APRIL	T cells, Monocytes	Enhances B and T cell reproduction
BAFF	T cells, Monocytes, DCs	Proliferation of B cells and co-stimulatory T cells both result in antibody release.
GM-CSF	Fibroblasts, T cells, Monocytes	Regulate the generation, development, and activation of myeloid cells, neutrophils, and macrophages
M-CSF	Fibroblasts, T cells, Monocytes	Encourage the differentiation, proliferation, and survival of monocytes and macrophages.
TGF-β	Monocytes, platelets Synovial fibroblasts, T cells,	Differentiate Th17 and Treg cells
FGF family	Fibroblasts, monocytes	The ability to control the development of mesenchymal epithelial cells
VEGF	Platelets, Monocytes, synovial fibroblasts	development of new blood vessels (Angiogenesis)
PDGF	Platelets, Monocytes, endothelial cells, synovial fibroblasts	Growth factor for a variety of cell types aids in wound healing

2.4.1.5 Chemokines in RA

The infiltration of a combination of inflammatory cells into the sore synovium, synovial fluid, and tissues is remark by the presence of macrophages, neutrophils, mast cells, T cells, B cells, and DCs. In addition, There is a significant rise in vascularity, which includes the development of new blood vessels and the thickness of an intimal layer, where it leads in the degradation of cartilage as well as the underlying bone (Reece *et al.* 1999). It is believed that the invading inflammatory monocytes and other cells inside the synovium of patients with RA are responsible for the production of numerous

inflammatory response including cytokines and chemokines, in addition to matrix destroying enzymes (Harris 1990). Because of this, it should come as no surprise that different chemokines are present in high concentrations in the synovium of RA patients, and that they have been linked to inflammatory cell attraction and angiogenesis in the disease (Miossec 2004). Further this hypothesis has been supported by many investigations that have revealed synovial tissues and fluid and also serum of RA patients to be rich in inflammatory and homeostatic chemokines. These include CCL3/MIP-1α, CCL2/MCP-1, CXCL1/GROα, CCL5/RANTES, 8CXCL5/ENA-78, CXCL8/IL-8, and CXCL12/SDF-1. Moreover, several of animal studies of inflammatory arthritis have shown the significance of these proteins in their functional roles. Other chemokines, such as CXCL4/PF4, CXCL9/MIG, and CXCL10/IP-10, have been suggested to have anti-angiogenic actions (Elmesmari 2014).

2.5 Criteria for RA

Ropes *et al.* established the initial RA classification criteria in 1958, and they were utilized for almost 30 years. As a result of the revisions made by Arnett *et al.* updated these criteria in 1987, publishing them as "The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis" (ARA 1987) (Arnett *et al.* 1988). When new classification criteria were introduced, their primary goal was to classify illness in epidemiological studies and also not to identify individual instances. Despite this, the seven criteria Table 2.2 have shown to be helpful in clinical practice as recommendations for making a detection of RA and distinguishing confirmed RA from another rheumatic diseases. When it comes to early identification of RA, the ARA criteria are problematic because of their poor specificity and sensitivity. They are often used in clinical trials for people with RA throughout the worldwide to guarantee that the participants all have the same diagnosis (Innala 2014).

Studies that have been done over the last decade have shown the criticality of a rapid identification of RA and starting DMARD therapy as soon as feasible for improved patient outcomes in the long term (Finckh *et al.* 2006). In order to identify individuals

with early illness, new criteria were established in 2010, with the goal of allowing physicians to identify the patients sooner (Aletaha *et al.* 2010).

RA is classified using a scoring system developed by the American College of Rheumatology (ACR) and the European League against Rheumatism (EULAR) in 2010. The scoring system evaluates four-point joints (0-5), serology (0-3), symptom duration (0-1), and acute phase reactants measurement (0-1). The revised criteria also include ACPAs and acute-phase reactants such as ESR and CRP. Patients who score 6 or more points out of a potential 10 are considered to have definitive RA Table 2.3 (Aletaha *et al.* 2010). Recent studies indicate that the 2010 ARA criteria for categorization of patients early in the illness course seem to be "superior" to the 1987 ARA criteria (Arnett *et al.* 1988). To better comprehend the long-term consequences, it is also important to get a greater knowledge of the impact of these criteria, especially in terms of disability, radiological damage, and death (Humphreys and Symmons 2013).

Table 2.2 Categorization criteria established by the american rheumatism association (ARA) in 1987. A person is confirmed with RA when four out of the seven criteria are satisfied, and at least six weeks have passed after the onset of criterion (Arnett *et al.* 1988)

Categorization Criteria	**Symptoms**
1. Morning stiffness	Stiffness in or around joints that lasts at least an hour before reducing completely
2. Arthritis affecting three or more joints	Simultaneous swelling or fluid accumulation in at least three joint locations, as assessed by a physician
3. Hand joint arthritis	At least one joint in the wrist, MCP, or PIP is swollen.
4. Arthritis symmetrical	Same joints are afflicted on the both side of the body at the same time. Bilateral involvement of the PIP, MCP, and MTP joints is appropriate in the absence of perfect symmetry.
5. Rheumatoid nodules	Physician-observed subcutaneous nodules on bony prominences, extensor surfaces, or juxta-articular areas.
6. Serum rheumatoid factor	Detection of elevated serum RF levels by any technique that leads to a positive result in 5% of healthy control individuals
7. Radiographic changes	Rheumatoid arthritis radiographic changes in the hands and wrists that should include erosions or unmistakable bone decalcification in and around the affected joints (osteoarthritis changes alone do not qualify)

The 14 regions include the right or left distal interphalangeal (PIP) joint, the metacarpophalangeal (MCP) joint, the elbow, wrist, ankle, and knee, and the metatarsophalangeal (MTP) joint.

Table 2.3 The 2010 classification criteria RA (Aletaha *et al.* 2010)

Criterion	**Score**
A. Joint Involvement	
1large joint	0
2 – 10 large joints	1
1 – 3 Small joints even without inflammation of large joints	2
4 – 10 small joints even without inflammation of large joints	3
> 10 joints (at least 1 small joint)	5
B. Serology (Classification requires at least one test result)	
Negative ACPAand negative RhF	0
Low-positive ACPA or low-positive RhF	2
High-positive ACPA or high-positive RhF	3
C. Acute-phase reactants (Classification requires at least one test result)	
Normal ESR and normal CRP	0
Abnormal ESR or abnormal CRP	1
D. Duration of Symptoms	
< 6 weeks	0
≥ 6 weeks	1

2.6 Symptoms of RA

On the clinical front, the symptoms of RA vary considerably between those of early-stage RA and those of later stage illness that has not received adequate treatment. Early-stage RA is marked by widespread illness symptoms like tiredness, flu-like sensation, swolling and painful joints, and morning stiffness, which are accompanied by higher concentration of C-reactive protein (CRP) as well as an elevated erythrocyte sedimentation rate (ESR)(Brzustewicz *et al.* 2017). While untreated RA may present with a complicated clinical picture that includes lung lesions, effusions, nodule, and an illness of the interstitial lung, cancer of the lymph, the inflammation of the small and middle arteries vasculitis, keratoconjunctivitis, atherosclerosis. Furthermore, there are

hematologic disorders such as anemia, leukopenia, neutropenia, thrombocytopenia. As a result of RA, joints may become misaligned, range of motion may be reduced, bone erosion, cartilage deterioration, and rheumatic nodules may develop (Aletaha and Smolen 2018, Littlejohn and Monrad 2018). All of these systemic symptoms of RA induced by the chronic inflammation state result in an increased mortality in RA patients when considered as a whole.

2.7 Diagnosis of RA

The clinical presentation of RA patients is usually characterized by the recent development of painful and morning joint stiffness, swollen joints , generalized illness symptoms (as described above), as well as abnormalities of laboratory tests (Smolen *et al.* 2016). As a result, timely and accurate diagnosis is critical since early detection may stop disease development in many people, avoiding or significantly delaying disease progression, irreversible joint degeneration, and disability in up to 90 percent of those suffering from the illness (Aletaha and Smolen 2018).

Diagnoses of RA are often made utilizing a mix of patient's symptoms, physical findings, risk factor evaluation, family medical history, joint ultrasonography, and laboratory markers including increased CRP and ESR levels in the blood and the identification of RA- particular antibodies (Burmester and Pope 2017, Aletaha and Smolen 2018).

2.7.1 Blood tests for diagnosis of RA

The use of biological markers in clinical care for individuals with RA helps to better identify and describe the condition, as well as predict and forecast its progression. ESR, CRP, RhF, and anti-cyclic citrullinated peptide (anti-CCP) are the four most frequently utilized biomarkers in the diagnosis of RA. ESR and CRP are indicators of inflammation that are high in the most of patients who are having active illness, but 35 percent to 45 percent of patients may have normal values at the time of presentation

(Olsen *et al.* 1988). It has been discovered that elevated ESR and CRP levels are linked with radiographic development of illness, and that these levels may be reduced during clinical trials using treatment drugs (Olsen *et al.* 1988, Pincus *et al.* 2014). Both RhF and anti-CCP are autoantibodies, unlike ESR and CRP, which are not. RhF is an antibody against the Fc component of immunoglobulin G (IgG), and it is increased in 70 to 85% of RA patients during course of their illness (Song and Kang 2009). The specificity and sensitivity of RhF positivity for the diagnosis of RA are about 82 and 66%, respectively. RhF positivity may also signal a bad prognosis, as shown by severe structural deterioration (Hecht *et al.* 2015). Anti-CCP is a citrullinated protein-specific antibody that is a member of the anti-citrullinated protein antibody family (ACPA). Anti-CCP has a comparable sensitivity (70%) but a greater specificity (95%) unlike RhF in diagnosing rheumatoid arthritis. As with RhF, high anti-CCP levels are linked with a poor prognosis in rheumatoid arthritis. Anti-CCP and RhF may have cumulative effects (Kleyer *et al.* 2014).

2.7.2 Medical imaging in diagnosis of RA

Numerous diagnostic imaging modalities are available for the assessment of the three main physiological processes associated with RA. Synovitis, bone erosions and BME. Erosion detection using medical imaging provides two critical functions. To begin, increased focus is being placed on diagnosing RA early phases of the illness, prior to the onset of significant erosive damage, as evidenced by the 2010 ACR/EULAR RA diagnostic criteria (Aletaha *et al.* 2010). Diagnostic imaging may assist in detecting and assessing erosive overall damage in the illness process, prior to significant development. Second, bone erosions may be tracked in medical practice and clinical studies to determine therapy effectiveness (Tavares 2012). Different imaging techniques are available to aid in the visualization of different illness features, each having its own set of pros and drawbacks to consider. This chapter provides brief overviews of the major imaging modalities that are utilized to scan RA.

2.7.2.1 Radiography

In addition to being easy, cheap, and easily available, radiography, often known as X-ray, may be utilized for diagnostic and therapy monitoring purposes (Boini and Guillemin 2001). Additionally, there is a scoring system that allows for the semi-quantification of bones erosions as well as the narrowing of joint space on an ordinal scale, which is useful in clinical practice. The use of X-rays, on the other hand, has its limits. A 2-dimensional (2-D) image and a 3-dimensional (3-D) anatomy are presented by radiography, which cannot detect immune responses to non-osseous tissue that occur early stages of the disease (such as bone marrow edema and synovitis), nor is it capable of detecting early bone erosions to the same degree of sensitivity that other imaging modalities can (Emond 2012, Tomizza, 2015). In light of the critical significance of detecting and monitoring erosions early in the disease's progression, radiography is a useful tool for RA evaluation, but it is not a complete tool for assessing the disease.

2.7.2.2 Ultrasound

In terms of its features, ultrasound is a helpful modality for studying RA since it has a unique set of characteristics. Due to the fact that ultrasound works by seeing blood flow, it is capable of detecting soft tissue changes, such as the vascular alterations associated with synovitis or tenosynovitis, which is an inflammation of the tendons that is occasionally observed in RA patients, among other things. (Rizzo *et al.* 2013) Ultrasound may also be used to discover bone destroy, and it is has sensitivity greater than radiography (X-ray) in detecting erosions (Baillet *et al.* 2011). However, ultrasonography is mainly operator-dependent, and there are currently no standardized scoring methods for synovitis and bone erosions (Ohrndorf and Backhaus 2013). A further expansion of the use of ultrasound in the patients of RA for assessing erosive damage is required, and it is possible that it will have an impact in both the scientific and clinical contexts in the future.

2.7.2.3 Computed tomography

Computed tomography (CT), which uses a tomographic imaging method, is capable of delineating erosions extremely clearly owing to their decreased signal in comparison to other adjacent sources (e.g. thetrabecular bone and cortex) (Emond 2012). The high level of radiation exposure associated with CT scanning in the clinical environment is the most significant barrier to its adoption. Current CT research is focused on discovering ways to minimize radiation exposure, and peripherals have lately been investigated to see whether they may be used to identify tiny erosions more sensitively in the future (Srikhum *et al.* 2013). Aside from erosion detection, CT does not have the capability of depicting soft tissue changes such as synovitis and bone mineralization. This technological restriction limits the usefulness of CT scans for the assessment of early RA symptoms (Emond 2012).

2.7.2.4 Magnetic resonance imaging

The use of magnetic resonance imaging (MRI) has emerged as a promising tool for the study of RA. Because of its multi-slice imaging technique, MRI gives more visual information than radiography, and it can depict soft tissue changes that are predictive of bone erosions (e.g., synovitis and bone marrow edema). MRI is also more sensitive unlike radiography at identifying erosions early stages of the disease (Hoving *et al.* 2004, Tavares *et al.* 2014). The benefits of MRI make it a desirable tool in the research environment. As a result, as compared to radiography, MRI is preferable since it requires lower sample numbers and shorter study periods to detect and describe treatment effectiveness in terms of the structural changes (Peterfy *et al.* 2013). MRI, likes other imaging technologies, has its own set of limitations. The two most significant drawbacks of using MRI in the therapeutic context are the restricted accessibility and high price (Olech 2009). The development of peripheral MRI (pMRI) scans, typically scan just the anatomy of concern rather than the whole body, may provide a solution to these problems. In comparison to traditional full body scanners, this option has reduced purchase prices, better patient comfort, more flexibility and

convenience, and has been proven to perform well in comparison to conventional full body scanners (Taouli *et al.* 2004, Olech 2009).

2.8 Measurement of Disease Activity, Severity and Functional Ability

In the area of RA, there are numerous clinical and patient-reported outcome (PRO) metrics to choose from. Indicators such as these enable doctors and researchers to assess overall disease progression and treatment effectiveness, as well as whether alterations in status have resulted in observable impacts on the patient's daily activities (ADL) (Rendas-Baum *et al.* 2014). These are some of the most commonly used disease activity score (DAS) and health assessment questionnaire Disability Index (HAQ-DI) measures in both clinical and research settings, and they are among the most widely used in the world.

2.8.1 Disease activity score with 28-Joint count

Individuals with RA must be examined on a regular basis and carefully evaluated if the illness is active, in order to initiate suitable treatment as soon as possible and keep the disease under control (Prevoo *et al.* 1995). (DAS28) is a widely used and adequate measure for assessing disease activity and response to treatment (Haagsma *et al.* 1998). A combination of the DAS28 value and clinical evaluation may assist a physician in making treatment decisions, and it is often used in research to compare outcomes in therapeutic experiments. The DAS28 comprises the numbers of painful and swollen joints, the ESR or CRP, and the patient's overall impression of their health as assessed by a visual analogue scale (VAS). High disease activity is defined as DAS28 >5.1, clinical remission as DAS28 <2.6, low disease activities as DAS28 <3.2, medium disease activity as DAS28 (3.2 to 5.1) (Haagsma *et al.* 1998).

2.8.2 Health assessment questionnaire- disability index

The HAQ-DI is a tool that is used to evaluate disease-related prognoses and is considered the gold standard for assessing functional status in RA. Despite the fact that the HAQ-DI is not disease-specific, the initial study describing the questionnaire is one of the most referenced articles in rheumatology and has been used widely in the area of RA since its introduction (Bruce and Fries 2005). The HAQ-DI is concerned with detecting the constraints associated with doing everyday activities like walking and eating, among other things. Questions are divided into eight categories of disability issues, with scores ranging from 0 (with little trouble) to 3 (with significant difficulty) (unable to do). A final HAQ-DI score is calculated by averaging the item subscores, with 0 being the most favourable and 3 being the least favourable. When evaluating change over time, researchers and clinicians may utilize the least important difference (MID) to identify the lowest variation in the HAQ-DI that an individual would consider to be a significant shift (Crosby *et al.* 2003, Sloan 2005).

2.9 Treatment of RA

The ultimate therapy goal for RA patients is to achieve either successful treatment or at the very least a substantial reduction in disease activity by roughly 6 months so as to avoids joint destruction, immobility, and systemic symptoms of the illness. When it comes to RA therapy, the fact that 80% of patients who are not well treated will had misalignment joints and 40% of RA persons would be incapable to move after 10 years after illness start underscores the significance of timely and focused intervention (Burmester and Pope 2017, Aletaha and Smolen 2018). In order to accomplish the therapeutic objectives, therapy should be started as soon as possible and maintained throughout the course of the illness, with regular evaluation of both the status of the illnesse as well as the efficacy of the medication approach used. Before the soon 1990s, the standard medication approach for RA consisted of resting at home, the prescription of non-steroidal anti-inflamed medicines (NSAIDs), and, if these treatments unsuccessful, the administration of disease-modifying anti-rheumatic drug (DMARD) medication (Burmester and Pope 2017). Nevertheless, the effectiveness of this

treatments approach was restricted, and within a few years, RA often lead to joint damage, impairment, difficulty to work, and an raise risk of death (Fries 2000). Fortunately, during the past 30 years, the number of therapeutic medicines that have been shown to be useful for the treatment of RA has gradually increased. NSAIDs, DMARDs, and immunosuppressive glucocorticoids are currently accessible medication classes. Non-pharmacological treatment, such as physical therapy to maintain joint function and patient counselling to delay the development of the illness, is usually used in conjunction with drug therapy to maximize results (Figure 2.6).

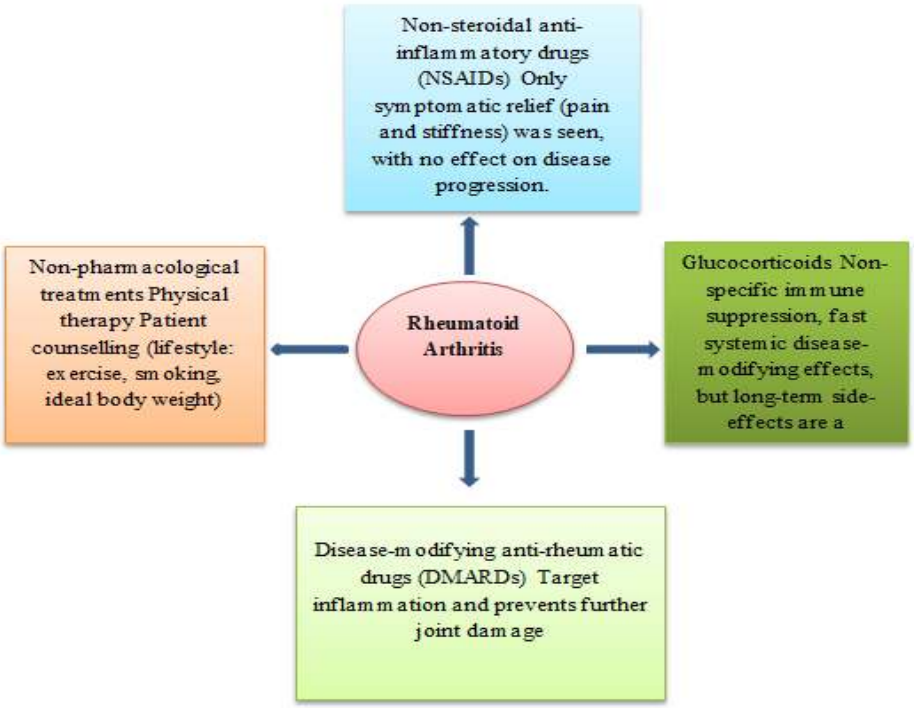

Figure 2.6 An overview of the many therapy options for RA sufferers is provided below. The many therapy options for RA may be classified into four major categories of methods. Nonpharmacological treatments, often known as alternative therapies, First and foremost, there are nonspecific immune system suppression glucocorticoids, then there are disease-modifying anti-rheumatic medications, and lastly there are biologics (DMARDs) adapted from (Lin *et al.* 2020)

Pain and swellem are reduced, and joint mission is improved, using non-steroidal anti-inflamed medications icluding aspirin, diclofenac, and ibuprofen. However, these medications they do not prevent future joint deterioration, hence they are not disease-

modifying (Smolen *et al.* 2016). Physiologically, NSAIDs may be thought of as inhibiting prostaglandin production (Brune and Patrignani 2015). Prostanoids, such as prostaglandin E2, prostaglandin D2, prostaglandin F2α, prostaglandin G2, thromboxane A2, and prostacyclin, are second messengers which respond with and stimulate G-protein coupled receptors on the side of cells, thereby modulating a extensive variety of cellular functions. While NSAIDs are helpful in alleviating RA symptoms, the use of these medications is often associated with adverse effects on the kidneys, liver, gastrointestinal tract, and cardiovascular system (Leslie J Croff ord 2013). Glucocorticoids, such as prednisolone, are very powerful anti-inflammatory medicines that may prevent radiologic development in the early stages of illness by suppressing gene expression throughout the body (Littlejohn and Monrad 2018). Despite these positive benefits, glucocorticoids have been shown to have a limited ability to alter illness, and usage in the long term of glucocorticoids is hindered via significant multi-systemic metabolic side effects such as intestinal bleeding, osteoporosis, and ulcer development (Silvernstein *et al.* 2000, Smolen *et al.* 2016). At last, disease-modifying anti-rheumatic medicines are medications that targeting autoimmune inflammatory and, as a result, stop additional joint deterioration. DMARDs are medicines that, as opposed to treatments that do not stop or prevent ailment development (e.g., NSAIDs or pain relievers), interference together with both the symptoms and signs of RA, enhance functions of the body, and slow the advancement of joint degeneration in the structure, according to their definition (Smolen *et al.* 2016, Aletaha and Smolen 2018). The obtainable DMARDs are then divided into three categories: (1) DMARDs that are conventionally synthesized (hydrochloroquine, methotrexate, and sulfadiazine), (2) Synthetic DMARDs with a specific target (JAK1/2-inhibitors and pan-JAK), and (3) biologic disease-modifying agents (TNF receptor ®inhibitors, TNF-inhibitors, IL-6 receptor inhibitors,IL-6 inhibitors, inhibitors of co-stimulatory chemicals, andB cell depleting antibodies) (Figure 2.7).

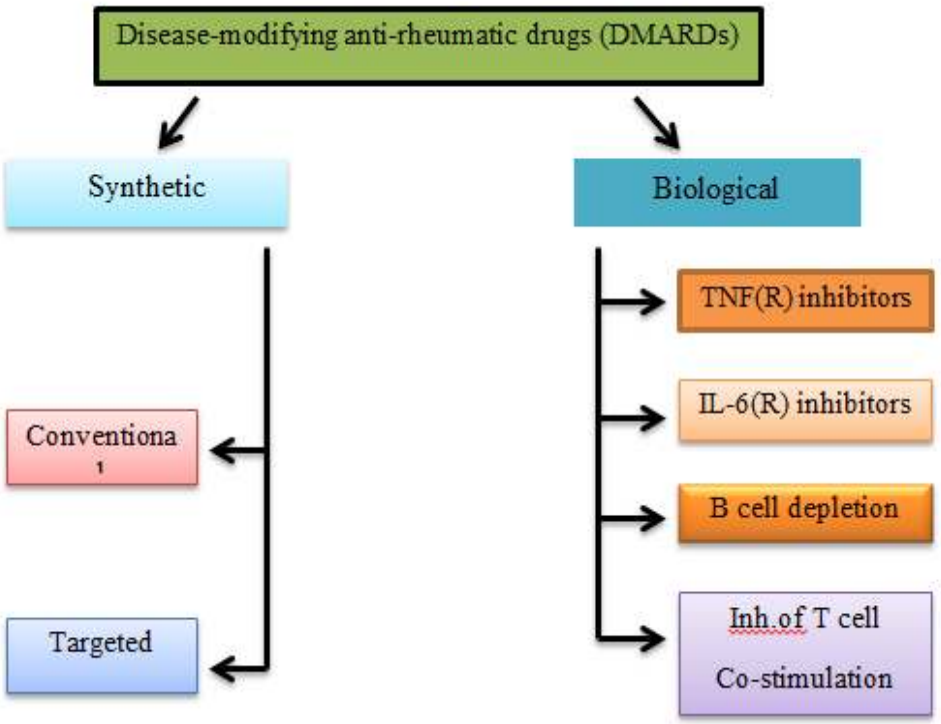

Figure 2.7 Overview of the DMARDs that is presently available. DMARDs are classified into synthetic and biological classes, synthetic class which are further classified into conventional and targeted synthetic DMARDs adapted from (Lin *et al.* 2020)

2.10 The Role of Inflammatory Status in RA

2.10.1 Adiponectin (ADP)

Human adiponectin, expressed via the ADIPOQ gene, is a secreted protein produced mostly by white adipocytes. It belongs to a group of bioactive peptides and proteins, immunological compounds, or mediators of inflammation with a variety of biological functions. Adiponectin was also shown to be expressed in osteoblasts, liver cells, endothelial cells, myocytes, and the placenta (Achari and Jain 2017). Adiponectin, over with other constituents of the adipokine family, is involved in a number of important diseases, including insulin resistance, heart disease, RA, and obesity. Ever from the discovery of the very first adipokine, leptin, in 1994, profiling investigations have discover several of adipokines in the proteome of human fats (adipokinome), which all

have a significant anti-inflammatory effect through autocrine/paracrine and endocrine mechanisms (Mac Donald *et al.* 2019).

Adiponectin, also known as adipoQ, Acrp30, GBP28, andApM1, is a secretory protein produced by adipocytes that has a molecular weight of 28–30 kDa. It was discovered by various researchers using different methods (Choi *et al.* 2020). It is a member of the soluble collagen super family and is structurally composed of a fibrous sub unit at the nitrogen end and a globular sub unit at the carboxyl end. It is homologous to complement factor C1q as well as the tumor necrosis factor (TNF) family of molecules (Guerre-Millo 2002).

One of the most important biological roles of adiponectin is to stimulate fatty acid manufacture in the liver while simultaneously inhibiting glucose production in the liver (gluconeogenesis). Furthermore, it has been shown that adiponectin has antioxidant, anti-inflammatory and anti-atherosclerotic properties as well as other benefits (Choi *et al.* 2020).

There are three distinct isoforms of adiponectin that may be differentiated based on the degree of dimerization of the polymer: Low molecular weight (LMW) with three adiponectin monomers arranged in a trimer, medium molecular weight (MMW) hexamer, and high molecular weight (HMW) multimer comprising 12-32 adiponectin monomers are all possible (Berendoncks *et al.* 2015). Among isoforms, MMW hexamers and HMW multimers are the most abundant forms of adiponectin found in circulation, despite the fact that human plasma contains relatively small amounts of monomers of LMW, and are not usually identified in the circulation (Choi *et al.* 2020). Additionally, the serum includes globular adiponectin (gAPN), a result of proteolytic splitting of fibrous adiponectin (fAPN), which likely has distinct biological functions (Liu *et al.* 2015). Adiponectin acts biologically by binding to two distinct receptors: adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2), each of which has a distinct distribution and features. AdipoR1 was discovered in skeletal muscle for the first time; whereas AdipoR2 was discovered in liver. Adiponectin receptor 1 (AdipoR1) is implicated in the regulation of AMP kinase, whereas AdipoR2 is involved

in the activation of the PPAR receptor. Whether adiponectin receptors are present in various organs, on the other hand, remains a mystery. In addition, both of them function as receptors for globular and full-length adiponectin, which is responsible for the enhanced fatty-acid oxidation and glucose production (Iwabu *et al.* 2019).

Numerous studies have suggested that several adiponectin isoforms play a role with the pathogenesis of a variety of metabolic illnesses, including type 2 diabetes, metabolic syndrome, and its associated problems, particularly cardiovascular illnesses, as well as autoimmune diseases such as RA, systemic lupus erythematosus, and osteoarthritis (Toussirot *et al.* 2012).

Several studies throughout the years have shown adiponectin's multiple roles, which have indeed been the source of contention among different experts. It is well established that the dual function of adiponectin, i.e., anti-inflammatory and pro-inflammatory actions, participate to the development of numerous illnesses (Breitfeld *et al.* 2012, Liu and Liu 2014). When it comes to the cardiovascular system, adiponectin has anti-inflammatory characteristics that are helpful in conditions such as atherosclerosis and metabolic diseases like obesity and insulin resistance. Adiponectin has been shown to have a pro-inflammatory impact in RA, kidney disease, and inflammatory bowel illnesses, which is a collection of intestinal diseases that includes colitis (Van Andel *et al.* 2018).

As stated before, adiponectin, or multifunctional adipokine, has both anti- and pro-inflammatory properties, but its biological activity seems to be regulated by exposure duration, concentration, and environmental factors such as other cytokines and the tissue microenvironment. The above-mentioned disputed proof suggesting adiponectin may have a double anti-inflammatory impact specifies the particular functions of adiponectin in the development and progression of arthritis (Liu *et al.* 2015).

2.10.2 Adiponectin in RA

Dissimilar to other illnesses, systemic autoimmune and chronic inflammatory joint disorders are associated with higher adipokine synthesis, and elevated ADP concentration in serum and synovial fluid has been verified in patients with RA when compared to healthy individuals' plasma (Otero *et al.* 2006, Lee and Bae 2018). In addition, variations in serum adiponectin isoform amounts were found between patients with RA and healthy controls. The level of HMW was greater in RA patients, while there were no alterations in the content of MMW. The level of globular adiponectin (gAPN), which is similar to HMW, was significantly higher in RA patients (Chedid *et al.* 2012, Choi *et al.* 2020). Taking into consideration the above-mentioned dependencies, it has been proposed that the disparities between RA patients and healthy people in terms of HMW and globular adiponectin levels may be considered as possible RA markers to assess the onest stages of ailment development (Chen *et al.* 2013, Toussirot and Dumoulin 2014).

According to a number of studies, adiponectin may have a significant pro-inflammatory function in the pathophysiology of RA, especially in the joints, by promoting production of inflammatory mediators by stimulated synovial fibroblasts that express adiponectin receptors, among other mechanisms (Neumann *et al.* 2010). An in vitro research using cultivated RASFs showed that the RASFs react to adiponectin by elevating pro-inflammatory molecules, such as prostaglandin E2, interleukin-6, interleukin-8, matrix metalloproteinases-1 and -13, (MMPs-1, -13). When HMW isoforms of adiponectin were compare to LMW isoforms in these cells, the HMW isoforms exhibited a greater pro-inflammatory impact (Carrión *et al.* 2019). At the cells another than synovial fibroblasts, such as monocytes, inconsistent effect mediated by different isoforms of adiponectin have been seen. It was determined if different adiponectin isoforms had different impacts on cultured primary monocytes that were obtained from six healthful people. Compared to constitutive secretion, the HWM adiponectin isoform promoted the production of interleukin-6 (IL-6) in monocytes activated with lipopolysaccharide (LPS), whereas the LMW adiponectin isoform inhibited the production of IL-6 and enhanced the production of IL-10 (Neumeier

2006). These findings are particularly significant since IL-6 is often considered as the most pleiotropic cytokine, likely having a major function in the evolution of inflammatory process related with rheumatoid arthritis. Chronic synovitis, FLS proliferation, angiogenesis, and cartilage degradation are only some of the pleiotropic consequences of IL-6 in the synovium (Favalli 2020). All evidence showed unequivocally that adiponectin, which have a pro-inflammatory impact, is a key influence on the development of RA (Figure 2.8, Table 2.4).

Table 2.4 Selected human and animal research examining the relationship between adiponectin and rheumatoid arthritis

Authors	**Study Design**	**Subjects**	**Results/Outcomes**
Su *et al.* 2015 (Su *et al.*, 2015)	Adiponectin's impact on oncostatin M (OSM), a pro-inflammatory cytokine, production in human osteoblastic cells	Human osteoblastic cells	Adiponectin enhanced OSM expression in osteoblastic cells through the PI3K, Akt, and NF-B signaling pathways, suggesting that adiponectin may be a new target for arthritis therapy.
Chennareddy *et al.* (2016)(Chennareddy *et al.*, 2016)	A cross-sectional research was conducted to determine the serum adiponectin concentrations and their relationship to disease activity and radiographic joint damage.	43 RA patients 25 controls	Serum adiponectin conc. are increased in rheumatoid arthritis, however there is no relationship between erosive and non-erosive illness, disease duration, BMI, waist-to-hip ratio, or disease activity.
Liu (2020) (Liu *et al.*, 2020)	The purpose of this study is to determine if AD has an impact on Tfh cells in rheumatoid arthritis.	Human RA FLS Mice	AD-stimulated rheumatoid arthritis FLSs stimulate the development of Tfh cells, primarily via the release of soluble factor IL-6.
Zhang Y *et al.* (2020) (Zhang *et al.*, 2020)	The purpose of this report is to mark the effect of baseline blood adiponectin conc. on the progression of rheumatoid arthritis (RA).	3693 obese individuals were tracked for up to 29 years.	Increased plasma adiponectin levels at baseline were linked with an increased incidence of rheumatoid arthritis in this cohort of obese individuals. Additionally, individuals with both elevated ADP and CRP conc. at baseline were at an increased Possibility of occurrence rheumatoid arthritis.

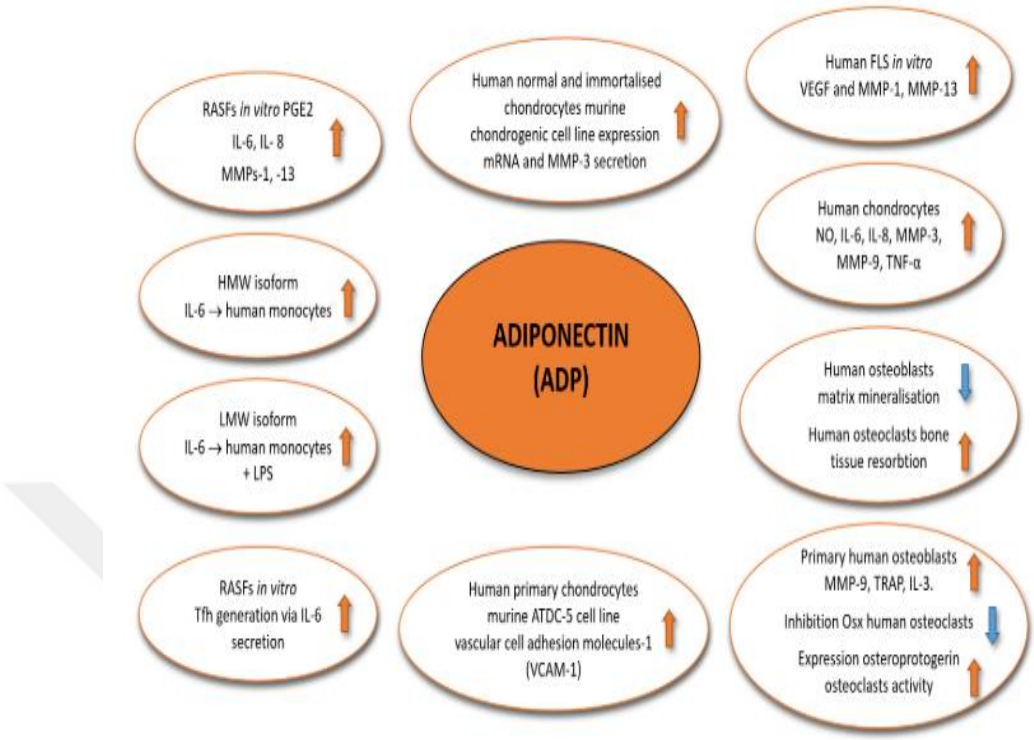

Figure 2.8 The impact of ADP and isoforms on distinct cell types in RA adapted from (Szumilas *et al.* 2020)

2.11 The Role of Oxidative Stress in RA

Elevated oxidative stress, low antioxidant concentrations, and weaken antioxidant defences are all linked with the pathophysiology of RA. Reactive nitrogen species (RNS) and reactive oxygen species (ROS) has been involved in the development of RA as soon as their levels exceed that considered typical, particularly via the degradation of lipids in the plasmatic membranes, proteins, and nucleic acids (Filippin *et al.* 2008). A five time raise in mitochondrial ROS generation have been discovered in the serum and monocytes of patients of RA, indicating that oxidative stress perhaps an important pathogenic feature of the disease in this group (Quinonez-Flores *et al.* 2016).

Inflammatory mediators released by T cells lead to the formation of ROS. Antioxidants, such as glutathione, are essential in reducing ROS levels. Vitamin C and vitamin E are crucial antioxidants that aren't enzymatic that are required of a variety of activities such

as immune system function, bodily tissue repair, and cell membrane protection from oxidative damage. Evidence, on the other hand, indicates that RA's antioxidant defense system has indeed been impaired (Das *et al.* 2021). Patients with RA had substantially decreased vitamin C levels and serum concentrations of vitamin E, confirming the notion that oxidative stress affects the antioxidant system in the disease. Also discovered in RA patients were Tocopherols insufficient, beta-carotene, retinols, SH group amino acids, and the glutathione reductase enzyme and superoxide dismutase enzyme activity, as well as low glutathione reductase and superoxide dismutase activity, according to the study (Hassan *et al.* 2001, Kalpakcioglu and Şenel 2008).

It is possible for the immune response to become inappropriate as a result of T cell exposition to elevated oxidative stress, which may cause growth and death stimulus to be disrupted (Hassan *et al.* 2011). moreover, neutrophils from RA generate greater amounts of neutrophil outside of the cell traps (NET), ROS, and RNS than neutrophils from healthful individuals (Jung *et al.* 2019).

2.11.1 Malondialdehyde (MDA) in RA

Malondialdehyde (MDA) has been utilized as a critical marker for oxidative stress in a variety of illness models, including diabetes, hypertension, heart failure, RA, and cancer, in both in vivo and in vitro studies. MDA, major lipid peroxide and the principal arachidonic acid metabolite with the chemical formula C3H4O2, has been shown to be increased in the serum, plasma, and synovial fluid of RA patients, indicating that it plays a significant role in the pathogenesis of RA. MDA systems and antioxidants work in tandem to control oxidative stress-induced damage and are involved in a variety of biochemical processes occurring inside cells, including covalent protein, RNA, and DNA binding (Jabbari *et al.* 2020).

MDA has been linked to the development of RA in several studies, one of which, conducted by Aljoboury *et al.* in 2020, observed that individuals with RA had greater MDA concentration of than those in the healthfall group, with the difference being highly significant ($P<0.001$) (Aljoboury *et al.* 2020). An additional research discovered

that the serum MDA level in RA patients was 3.85 ± 0.34 nmol/mL which was significantly ($p < 0.01$) higher than the level in control subjects (Das *et al.* 2021).

2.11.2 Protein carbonyls (PC) in RA

Protein oxidation indicators were detected at increased levels in human body fluid of RA persons and were shown to correspond with illness progression and evolution (Datta *et al.* 2014, Ferreira *et al.* 2021). Increased concentration of protein carbonyls and 3-chlorotyrosine was discovered in synovial fluid and samples from different RA patients, as well as elevated levels of myeloperoxidase, which catalyzes the reaction between hydrogen peroxide (H2O2) and chloride anion (Cl-), forming hypochlorous acid (HOCl), which can cause oxidative damage in the living tissues. Thus, a rise in myeloperoxidase activity indicates a high level of oxidative stress (Dalle-Donne *et al.* 2006). HOCl acid may combine with nitrite (NO2-) to form nitrate (NO3+), which has been shown to be elevated in the blood of RA patients (Ersoy *et al.* 2002). Thus, in whatever kind of sample examined, oxidative impairment indicators have consistently been shown to be remarkably greater in RA patients than in controls. In sum, recognized that oxidative stress is a major feature of this disease environment, resulting in the oxidation of biomolecules and activation of inflammatory response in this painful disease.

2.12 The Role of Antioxidants (Superoxide Dismutase, Catalase) in RA

Antioxidants are naturally occurring compounds that are either exogenous or endogenous in nature and which whether prevent the production of harmful oxidants or intercept any that are produced and inactivate them, thereby preventing the propagation of chain reactions caused by these oxidants from occurring (Rangan and Bulkley 1993). The antioxidants can be divided into two categories: enzymatic antioxidants (such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase), non-enzymatic antioxidants such as (nutrient antioxidants) beta-carotene and alpha-tocopherol, ascorbic acid, bioflavonoids, and (metabolic antioxidants) such as glutathione, ceruloplasmin, albumin, bilirubin, ferritin, transferrin, uric acid and

lactoferrn. In recent years, increased clinical and experimental evidence has shown the role of FR/ROS in a variety of pathophysiological conditions, including RA (Mahajan and Tandon 2004).

According to the findings of one research, increasing oxidative stress and/or a deficient antioxidant status contributes to the pathophysiology of RA. Patients with RA had elevated amounts of MDA and low levels of endogenous antioxidants, according to the findings of the research (Surapneni and Gopan 2008). Patients with RA have also been shown to have substantially lower levels of plasma catalase than healthy individuals (Kamanli *et al.* 2004). Another research discovered decreased Superoxide dismutase activity in the synovial fluid of patients with RA (Bazzichi *et al.* 2002). The majority of the studies found that there is an increase in oxidative stress as well as a reduction in the levels of antioxidants.

3. MATERIALS AND METHODS

3.1 Design of Study

In this study (cross sectional control study) that was conducted in teaching hospitals and private clinics in Iraq especially in Al-muthanna city in period from (January, 2021 to April, 2021). Blood samples were collected from all participants (patients and healthy controls). The process of sample collections was randomly assigned, as (81) samples were taken from patients previously identified with RA as stated by the 2010 (ACR) /(EULAR) classification criteria of RA in both gender males and females in different ages (28-75) years, as well as (50) healthy controls (20) males and (30) females, who represent as control subjects for this study.

All patients were classified according to gender, age, and body mass index (BMI) to two categories as shown in (Table 3.1).

Table 3.1 Classification of patients in this study

Total patients	**Classification**	**Categories**	
81 patients	Gender	30 males	51 females
	Age	48 patients <50 years	33 patients >50 years
	BMI	20 normal patients< 25	61 overweight patients> 25

The approval of all study participants was taken; demographic characteristics and basic anthropometric measurements were recorded for each participant as indicated in the Table 3.2 below:

Table 3.2 Approval study form

Participant Profile
Name: -
Age :-( ) Years
gender: -

Table 3.2 (continue)

Weight :-( ) Kg	Length: - ( ) Cm	BMI: -
Patient's medical history: -		
Duration of rheumatoid arthritis: -		
Taken treatments: -		
Biochemical Parameters		
C-reactive protein (C-RP) Rheumatoid factor (RF) Anti-Cyclic Citrullinated Peptides (ACCP) Erythrocyte sedimentation rate (ESR) Uric acid (U.A) Malondialdehyde (MDA) Protein carbonyls concentration (PC) Catalase (CAT) Superoxide dismutase (SOD) Adiponectin (ADP)		

The participant's name, age, gender, weight, Length, body mass index (BMI), medical history, period of infection in RA and treatments of each participant were taken. All participants undergo tests for the Biochemical Parameters as shown in the table above.

3.2 Samples Collection

All blood samples were drawn at random in a private laboratory and laboratory of Al-hussain teaching hospital in Al-muthanna city by venipuncture; in preparation for the drawn operation the participant was seated in a reclining chair. The skin was cleansed and a tourniquet was placed shortly before the process started an anterior cubital fossa vein in the participant's arm was used to collect the blood samples, which were collected by inserting a butterfly needle into the vein. The needle was stabilized in order to reduce participant discomfort while also facilitating the collection of many samples. Seven mL of whole blood were then collected, 2 mL for ESR analysis, and the rest 5 mL into gel tubes for other medical parameters and then left for at room temperature until they coagulate and underwent centrifugation for 15 minutes at a speed of 3000 rpm to separate the serum prior to storage at –20°C.

3.3 Instruments

Table 3.3 shows all the information about the instruments that were used in this study to perform all the required parameters; it shows the country of origin, model and name of the instrument.

Table 3.3 Instruments used in this study

No	Instruments	Manufacture
1	Epithod® 616 analyzer Model Ep.616	Korea
2	ichroma™ Model ichroma II	Korea
3	Mindray Model BS-230	CHINA
4	UV/VIS Spectrophotometer Model 721	CHINA
5	Centrifuge, ALSLAB	CHINA
6	Water bath, SKYTOU Digital Thermostatic	CHINA
7	(ELIZA), Human- Elisys Uno	Germany

3.4 Biochemical Parameters

3.4.1 Estimation the level of adiponectin (ADP) mg/L

Human Adiponectin ELISA Kit (96 wells) was used to determine the level of ADP Cat.No: E1550Hu from Bioassay Technology Laboratory, China b using Human- Elisys Uno device.

Assay Principle

Each kit contains an Enzyme-Linked Immunosorbent Assay (ELISA) method. The platter has previously been coated with a human ADP antibody in order to make the experiment more convenient. The specimen contains ADP, which attaches to the antibodies coating on the holes. Following that, a biotinylated human ADP antibody is

put on, which attaches ADP in the specimen. After that, Streptavidin-HRP is put on, which reacts with the biotinylated ADP antibody. Following incubate, not bound Streptavidin-HRP is removed through a process of wash. After adding the substrate solution, the color will change as a function of the amount of human ADP in the serum. The absorption at 450 nm is measured after an acidic stop solution is added to the process.

Reagent Provided

It is necessary to keep the reagents between 2-8°C. Store at -20°C for more than six months, according to the expiry date Avert repeated freezing and thawing cycles. The kit should be used within 1 month if any of the individual chemicals have been opened (Table 3.4).

Table 3.4 Reagent provided in human adiponectin ELISA kit

Components	**Quantity**
Standard Solution (64mg/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 ADP Antibody	1mL x1
User Instruction	1
Plate Sealer	2 pics
Zipper bag	1 pic

Reagent Preparation

Before using any reagents, it is recommended that they be kept to the temperature in a room. Standard Reconstitute the 120µL of the standard (64 mg/L) with 120µL of the

standard dilute to get a 32 mg/L standard stock liquid by diluting the standard stock solution with the standard diluent. Allow 15 minutes for the standard to rest with moderate agitation before generating dilutions. Prepare duplicates standard solutions by dilute the stock standard liquid (32mg/L) 1:2 together with standard diluent for get solution at 16mg/L, 8mg/L, 4mg/L, and 2mg/L. The standard diluent is used to determine the zero standards (zero mg/L). The following dilutions of standard solutions are recommended (Table 3.5, Figure 3.1).

Table 3.5 Dilutions of standard solutions of ADP

Concentration	Standard no.	Details
32mg/L	Standard No.5	120µL Original Standard + 120µL Standard Diluent
16mg/L	Standard No.4	120µL Standard No.5 + 120µL Standard Diluent
8mg/L	Standard No.3	120µL Standard No.4 + 120µL Standard Diluent
4mg/L	Standard No.2	120µL Standard No.3 + 120µL Standard Diluent
2mg/L	Standard No.1	120µL Standard No.2 + 120µL Standard Diluent

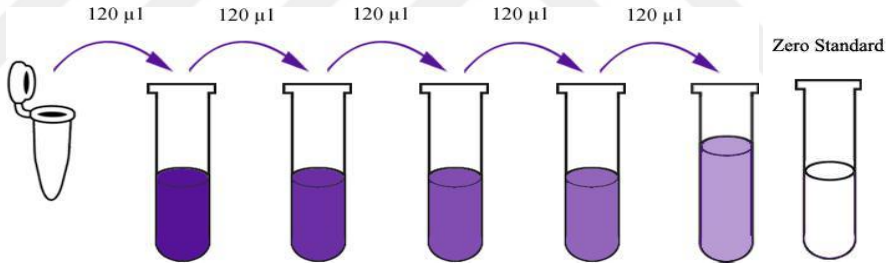

Figure 3.1 Dilutions of standard solutions of ADP

Washing buffer mix 20 mL of buffer solution Concentrate 25x with deionized water to get 500 mL of 1x washing buffer. Mix the solution carefully until all crystals have been totally dissolved, if any crystals have developed in the concentration.

Assay Procedure

Make sure to follow the instructions for preparation of reagents, samples, and standard solutions. First bring the chemicals to ambient temperature before using them. Without

the use of specialized equipment, this test may be performed at ambient temperature without a problem.

Determine how many test strips you'll need to complete the procedure. In order to utilize them, insert the strips into the frames and secure them. It is best to keep strips that haven't been used at 2-8°C.

Fifty µL of standard should be added to the standard well. It is important to note that you should not add antibody to the standard well since the standard solution already includes biotinylated antibody.

When preparing the samples, put 40 µL of each sample to every well, Afterwards, each well received 10 µL of anti-ADP antibody, after that add 50 µL of streptavidin-HRP for both samples and standard (Not blank well). A thorough mixing is required. The plate should be sealed with a sealer. At 37°C, incubate for one hour.

The plate should be washed five times with a wash buffer to clear any leftover sealant once the sealant has been removed. As a general rule of thumb, you should use at least 0.35 mL of wash buffer every wash. Use a wash buffer and aspirate and wash all wells five times, overfilling wells each time. To remove the liquid off the plate, use paper towels or another absorbent material.

Following the addition of 50 µL of substrate solution A to every hole, the second 50 µL of substrate solution B should be add likewise. A new sealer should be applied to the plate, and it should be placed in the dark at 37°C for 10 minutes to incubate the plate.

The blue color in each well becomes yellow as soon as you add 50 µL of Stop Solution. Use a micro plate reader set to 450 nm to measure the optical density (OD value) of each well during the first ten minutes followed the stop solution has been applied.

Calculation of Result

Using the vertical (Y) axis, plot the mean optical density (OD) for each standard vs. the concentration (X) axis, and then draw a best-fit curve across the points on the graph, you will be able to construct your standard curve. It is preferable to conduct these calculations using computer-based curve-fitting tools, and then use regression analysis to determine which line is the best match.

Typical Data

A standard curve should be created for each test since the OD values might vary based on the actual assay performance (ie. the operator's pipetting method, cleaning procedure, or temperature influences). It is purely for comparison and illustrative reasons that the following standard curve and data set is provided (Figure 3.2).

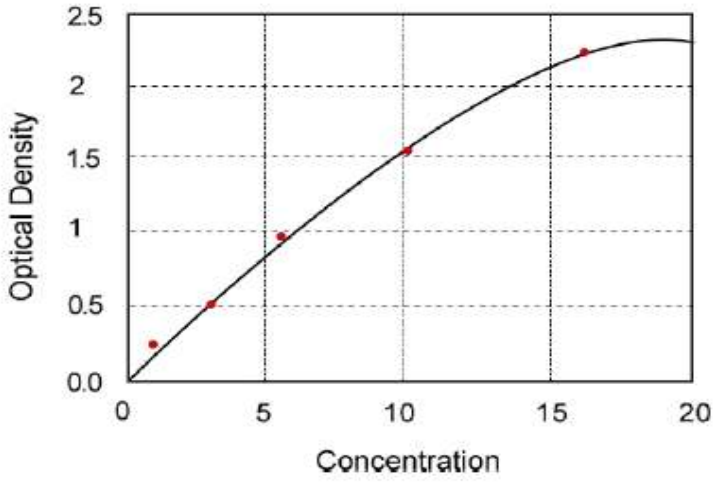

Figure 3.2 Standard curve of ADP

3.4.2 Estimation the level of protein carbonyls (PC) ng/mL

Human Protein carbonyls ELISA Kit (96 wells) was used to determine the level of PC Cat.No: E1426Hu from Bioassay Technology Laboratory, China.

Assay Principle

Each kit contains an Enzyme-Linked Immunosorbent Assay (ELISA) method. The platter has previously been coated with a human PC antibody in order to make the experiment more convenient. The specimen contains PC, which attaches to the antibodies coating on the holes. Following that, a biotinylated human PC antibody is put on, which attaches PC in the specimen. After that, Streptavidin-HRP is put on, which reacts with the biotinylated ADP antibody. Following incubate, not bound Streptavidin-HRP is removed through a process of wash. After adding the substrate solution, the color will change as a function of the amount of human PC in the serum. The absorption at 450 nm is measured after an acidic stop solution is added to the process.

Reagent Provided

It is necessary to keep the reagents between 2-8°C. Store at -20°C for more than six months, according to the expiry date Avert repeated freezing and thawing cycles. The kit should be used within 1 month if any of the individual chemicals have been opened (Table 3.6).

Table 3.6 Reagent provided in human protein carbonyls ELISA kKit

Components	**Quantity**
Standard Solution (640ng/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

Table 3.6 (continue)

Substrate Solution B	6mL x1
Wash Buffer Concentrate (25x)	20mL x1
Biotinylated human PC Antibody	1mL x1
User Instruction	1
Plate Sealer	2 pics
Zipper bag	1 pic

Reagent Preparation

Before using any reagents, it is recommended that they be kept to the temperature in a room. Standard Reconstitute the 120µL of the standard (640ng/mL) with 120µL of the standard dilute to get a 320ng/mL standard stock liquid by diluting the standard stock solution with the standard diluent. Allow 15 minutes for the standard to rest with moderate agitation before generating dilutions. Prepare duplicates standard solutions by mix the standard stock liquid (320ng/mL) 1:2 together with standard dilution for get solutions at 160ng/mL, 80ng/mL, 40ng/mL and 20ng/mL. The standard dilution is used to determine the zero standards (zero ng/mL). The following dilutions of standard solutions are recommended (Table 3.7, Figure 3.3).

Table 3.7 Dilutions of standard solutions of PC

Concentration	**Standard no.**	**Details**
320ng/mL	Standard No.5	120µL Original Standard + 120µL Standard Diluent
160 ng/mL	Standard No.4	120µL Standard No.5 + 120µL Standard Diluent
80 ng/mL	Standard No.3	120µL Standard No.4 + 120µL Standard Diluent
40 ng/mL	Standard No.2	120µL Standard No.3 + 120µL Standard Diluent
20 ng/mL	Standard No.1	120µL Standard No.2 + 120µL Standard Diluent

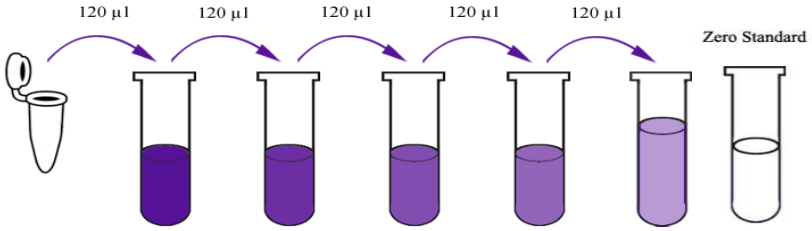

Figure 3.3 Dilutions of standard solutions of PC

Washing buffer mix 20 mL of buffer solution Concentrate 25x with deionized water to get 500 mL of 1x washing buffer. Mix the solution carefully until all crystals have been totally dissolved, if any crystals have developed in the concentration.

Assay Procedure

Make sure to follow the instructions for preparation of reagents, samples, and standard solutions. First bring the chemicals to ambient temperature before using them. Without the use of specialized equipment, this test may be performed at ambient temperature without a problem.

Determine how many test strips you'll need to complete the procedure. In order to utilize them, insert the strips into the frames and secure them. It is best to keep strips that haven't been used at 2-8°C.

Fifty µL of standard should be added to the standard well. It is important to note that you should not add antibody to the standard well since the standard solution already includes biotinylated antibody.

When preparing the samples, put 40 µL of each sample to every well, Afterwards, each well received 10 µL of anti-PC antibody, after that add 50 µL of streptavidin-HRP for both samples and standard (Not blank well). A thorough mixing is required. The plate should be sealed with a sealer. At 37°C, incubate for one hour.

The plate should be washed five times with a wash buffer to clear any leftover sealant once the sealant has been removed. As a general rule of thumb, you should use at least 0.35 mL of wash buffer every wash. Use a wash buffer and aspirate and wash all wells five times, overfilling wells each time. To remove the liquid off the plate, use paper towels or another absorbent material.

Following the addition of 50 µL of substrate solution A to every hole, the second 50 µL of substrate solution B should be add likewise. A new sealer should be applied to the plate, and it should be placed in the dark at 37°C for 10 minutes to incubate the plate.

The blue color in each well becomes yellow as soon as you add 50 µL of Stop Solution. Use a micro plate reader set to 450 nm to measure the optical density (OD value) of each well during the first ten minutes followed the stop solution has been applied.

Calculation of Result

Using the vertical (Y) axis, plot the mean optical density (OD) for each standard vs. the concentration (X) axis, and then draw a best-fit curve across the points on the graph, you will be able to construct your standard curve. It is preferable to conduct these calculations using computer-based curve-fitting tools, and then use regression analysis to determine which line is the best match.

Typical Data

A standard curve should be created for each test since the OD values might vary based on the actual assay performance (ie. the operator's pipetting method, cleaning procedure, or temperature influences). It is purely for comparison and illustrative reasons that the following standard curve and data set is provided (Figure 3.4).

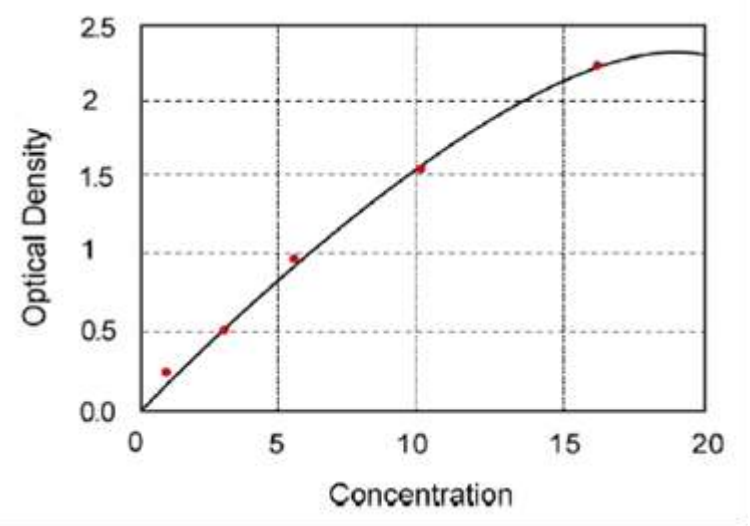

Figure 3.4 Standard curve of PC

3.4.3 Estimation the level of superoxide dismutase (SOD) U/L

Human Superoxide dismutase ELISA Kit (96 wells) was used to determine the level of SOD Cat.No: E0918Hu from Bioassay Technology Laboratory, China.

Assay Principle

Each kit contains an Enzyme-Linked Immunosorbent Assay (ELISA) method. The platter has previously been coated with a human SOD antibody in order to make the experiment more convenient. The specimen contains SOD, which attaches to the antibodies coating on the holes. Following that, a biotinylated human SOD antibody is put on, which attaches SOD in the specimen. After that, Streptavidin-HRP is put on, which reacts with the biotinylated SOD antibody. Following incubate, not bound Streptavidin-HRP is removed through a process of wash. After adding the substrate solution, the color will change as a function of the amount of human SOD in the serum. The absorption at 450 nm is measured after an acidic stop solution is added to the process.

Reagent Provided

Reagents should be kept between 2-8°C. Store at -20°C for more than six months, according to the expiry date Avert repeated freezing and thawing cycles. The kit should be used within 1 month if any of the individual chemicals have been opened (Table 3.8).

Table 3.8 Reagent provided in human superoxide dismutase ELISA kit

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
User Instruction	1
Plate Sealer	2 pics
Zipper bag	1 pic

Reagent Preparation

Before using any reagents, it is recommended that they be kept to the temperature in a room. Use the standard diluent to dilute the 960U/L standard stock solution down to the 480U/L standard stock solution in 120 µL of the standard. Allow 15 minutes for the standard to rest with moderate agitation before generating dilutions. Provide duplicates standard solutions by mixing the standard stock liquid (480U/L) 1:2 with standard diluent for get solutions at 240U/L, 120U/L,60U/L and 30U/L. The standard diluent is used to determine the zero standards (zero U/L). The following dilutions of standard solutions are recommended (Table 3.9, Figure 3.5).

Table 3.9 Dilutions of standard solutions of SOD

Concentration	Standard no.	Details
480 U/L	Standard No.5	120µL Original Standard + 120µL Standard Diluent
240 U/L	Standard No.4	120µL Standard No.5 + 120µL Standard Diluent
120 U/L	Standard No.3	120µL Standard No.4 + 120µL Standard Diluent
60 U/L	Standard No.2	120µL Standard No.3 + 120µL Standard Diluent
30 U/L	Standard No.1	120µL Standard No.2 + 120µL Standard Diluent

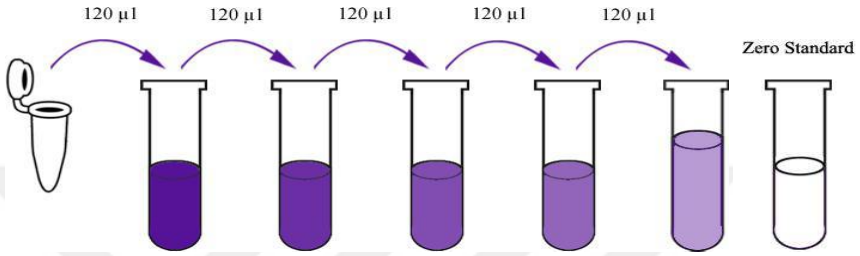

Figure 3.5 Dilutions of standard solutions of SOD

Washing buffer mix 20 mL of buffer solution Concentrate 25x with deionized water to get 500 mL of 1x washing buffer. Mix the solution carefully until all crystals have been totally dissolved, if any crystals have developed in the concentration.

Assay Procedure

Make sure to follow the instructions for preparation of reagents, samples, and standard solutions. First bring the chemicals to ambient temperature before using them. Without the use of specialized equipment, this test may be performed at ambient temperature without a problem.

Determine how many test strips you'll need to complete the procedure. In order to utilize them, insert the strips into the frames and secure them. It is best to keep strips that haven't been used at 2-8°C.

Fifty µL of standard should be added to the standard well. It is important to note that you should not add antibody to the standard well since the standard solution already includes biotinylated antibody.

When preparing the samples, put 40 µL of each sample to every well, Afterwards, each well received 10 µL of anti-SOD antibody, after that add 50 µL of streptavidin-HRP for both samples and standard (Not blank well). A thorough mixing is required. The plate should be sealed with a sealer. At 37°C, incubate for one hour.

The plate should be washed five times with a wash buffer to clear any leftover sealant once the sealant has been removed. As a general rule of thumb, you should use at least 0.35 mL of wash buffer every wash. Use a wash buffer and aspirate and wash all wells five times, overfilling wells each time. To remove the liquid off the plate, use paper towels or another absorbent material.

Following the addition of 50 µL of substrate solution A to every hole, the second 50 µL of substrate solution B should be add likewise. A new sealer should be applied to the plate, and it should be placed in the dark at 37°C for 10 minutes to incubate the plate.

The blue color in each well becomes yellow as soon as you add 50 µL of Stop Solution. Use a micro plate reader set to 450 nm to measure the optical density (OD value) of each well during the first ten minutes followed the stop solution has been applied.

Calculation of Result

Using the vertical (Y) axis, plot the mean optical density (OD) for each standard vs. the concentration (X) axis, and then draw a best-fit curve across the points on the graph, you will be able to construct your standard curve. It is preferable to conduct these calculations using computer-based curve-fitting tools, and then use regression analysis to determine which line is the best match.

Typical Data

A standard curve should be created for each test since the OD values might vary based on the actual assay performance (ie. the operator's pipetting method, cleaning procedure, or temperature influences). It is purely for comparison and illustrative reasons that the following standard curve and data set is provided (Figure 3.6).

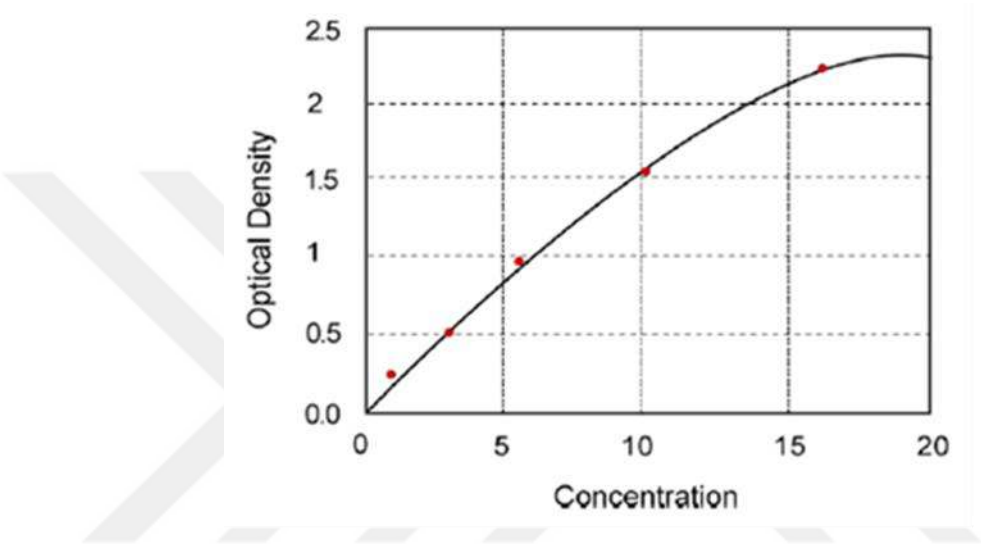

Figure 3.6 Standard curve of SOD

3.4.4 Estimation the level of catalase (CAT) KU/L

Human Catalase ELISA Kit (96 wells) was used to determine the level of CAT Cat.No: E3053Hu from Bioassay Technology Laboratory, China.

Assay Principle

Each kit contains an Enzyme-Linked Immunosorbent Assay (ELISA) method. The platter has previously been coated with a human CAT antibody in order to make the experiment more convenient. The specimen contains CAT, which attaches to the antibodies coating on the holes. Following that, a biotinylated human CAT antibody is put on, which attaches CAT in the specimen. After that, Streptavidin-HRP is put on,

which reacts with the biotinylated CAT antibody. Following incubate, not bound Streptavidin-HRP is removed through a process of wash. After adding the substrate solution, the color will change as a function of the amount of human CAT in the serum. The absorption at 450 nm is measured after an acidic stop solution is added to the process.

Reagent Provided

Reagents should be kept between 2-8°C. Store at -20°C for more than six months, according to the expiry date Avert repeated freezing and thawing cycles. The kit should be used within 1 month if any of the individual chemicals have been opened (Table 3.10).

Table 3.10 Reagent provided in human catalase ELISA kit

Components	**Quantity**
Standard Solution (800KU/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 CAT Antibody	1mL x1
User Instruction	1
Plate Sealer	2 pics
Zipper bag	1 pic

Reagent Preparation

Before using any reagents, it is recommended that they be kept to the temperaturein a room. Standard formation the 120µL of the standard (800KU/L) with 120µL of the standard dilution for get a 400KU/Lstandard stock liquid by diluting the standard stock

solution with the standard diluent. Allow 15 minutes for the standard to rest with moderate agitation before generating dilutions. Prepare duplicates standard solutions by diluting the standard stock solution (400KU/L) 1:2 with standard diluent to get solutions at 200KU/L, 100KU/L, 50KU/L and 25KU/L The standard diluent is used to determine the zero standards (zero KU/L). The following dilutions of standard solutions are recommended (Table 3.11, Figure 3.7).

Table 3.11 Dilutions of standard solutions of CAT

Concentration	Standard No.	Details
400 KU/L	Standard No.5	120µL Original Standard + 120µL Standard Diluent
200 KU/L	Standard No.4	120µL Standard No.5 + 120µL Standard Diluent
100 KU/L	Standard No.3	120µL Standard No.4 + 120µL Standard Diluent
50 KU/L	Standard No.2	120µL Standard No.3 + 120µL Standard Diluent
25 KU/L	Standard No.1	120µL Standard No.2 + 120µL Standard Diluent

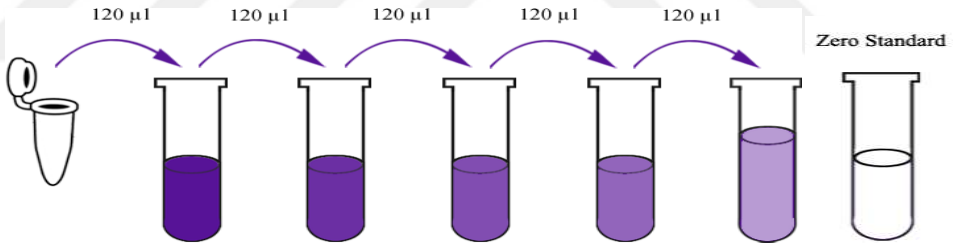

Figure 3.7 Dilutions of standard solutions of CAT

Washing buffer mix 20 mL of buffer solution Concentrate 25x with deionized water to get 500 mL of 1x washing buffer. Mix the solution carefully until all crystals have been totally dissolved, if any crystals have developed in the concentration.

Assay Procedure

Make sure to follow the instructions for preparation of reagents, samples, and standard solutions. Reagents should be brought to room temperature before they are used.

Without the use of specialized equipment, this test may be performed at ambient temperature without a problem.

Determine how many test strips you'll need to complete the procedure. In order to utilize them, insert the strips into the frames and secure them. It is best to keep strips that haven't been used at 2-8°C.

Fifty µL of standard should be added to the standard well. It is important to note that you should not add antibody to the standard well since the standard solution already includes biotinylated antibody.

When preparing the samples, put 40 µL of each sample to every well, Afterwards, each well received 10 µL of anti-CAT antibody, after that add 50 µL of streptavidin-HRP for both samples and standard (Not blank well). A thorough mixing is required. The plate should be sealed with a sealer. At 37°C, incubate for one hour.

The plate should be washed five times with a wash buffer to clear any leftover sealant once the sealant has been removed. As a general rule of thumb, you should use at least 0.35 mL of wash buffer every wash. Use a wash buffer and aspirate and wash all wells five times, overfilling wells each time. To remove the liquid off the plate, use paper towels or another absorbent material.

Following the addition of 50 µL of substrate solution A to every hole, the second 50 µL of substrate solution B should be add likewise. A new sealer should be applied to the plate, and it should be placed in the dark at 37°C for 10 minutes to incubate the plate.

The blue color in each well becomes yellow as soon as you add 50 µL of Stop Solution. Use a micro plate reader set to 450 nm to measure the optical density (OD value) of each well during the first ten minutes followed the stop solution has been applied.

Calculation of Result

Using the vertical (Y) axis, plot the mean optical density (OD) for each standard vs. the concentration (X) axis, and then draw a best-fit curve across the points on the graph, you will be able to construct your standard curve. It is preferable to conduct these calculations using computer-based curve-fitting tools, and then use regression analysis to determine which line is the best match.

Typical Data

A standard curve should be created for each test since the OD values might vary based on the actual assay performance (ie. the operator's pipetting method, cleaning procedure, or temperature influences). It is purely for comparison and illustrative reasons that the following standard curve and data set is provided (Figure 3.8).

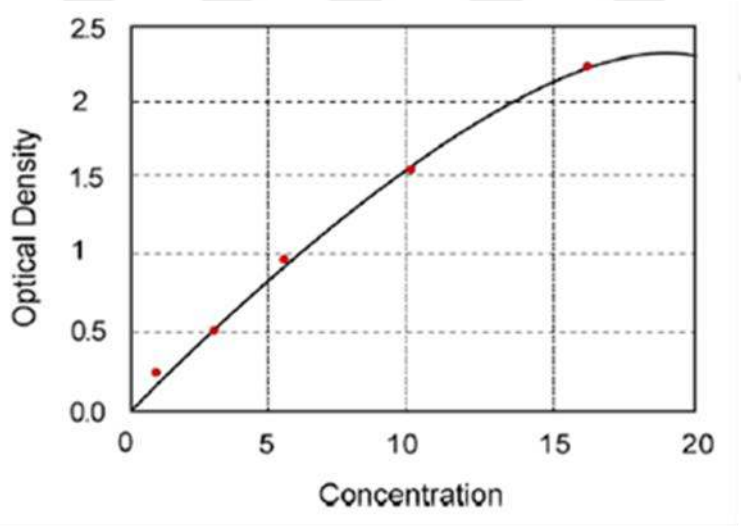

Figure 3.8 Standard curve of CAT

3.4.5 Estimation the level of malondialdehyde (MDA) µmol/L

Human MDA ELISA Kit (96 wells) was used to determine the level of MDA Cat.No: E1371Hu from Bioassay Technology Laboratory, China.

Assay Principle

Each kit contains an Enzyme-Linked Immunosorbent Assay (ELISA) method. The platter has previously been coated with a human MDA antibody in order to make the experiment more convenient. The specimen contains MDA, which attaches to the antibodies coating on the holes. Following that, a biotinylated human MDA antibody is put on, which attaches MDA in the specimen. After that, Streptavidin-HRP is put on, which reacts with the biotinylated MDA antibody. Following incubate, not bound Streptavidin-HRP is removed through a process of wash. After adding the substrate solution, the color will change as a function of the amount of human MDA in the serum. The absorption at 450 nm is measured after an acidic stop solution is added to the process.

Reagent Provided

Reagents should be kept between 2-8°C. Store at -20°C for more than six months, according to the expiry date Avert repeated freezing and thawing cycles. The kit should be used within 1 month if any of the individual chemicals have been opened (Table 3.12).

Table 3.12 Reagent provided in human MDA ELISA kit

Components	**Quantity**
Standard Solution (80 µmol/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

Table 3.12 (continue)

Substrate Solution A	6mL x1
Substrate Solution B	6mL x1
Wash Buffer Concentrate (25x)	20mL x1
Biotinylated human MDA Antibody	1mL x1
User Instruction	1
Plate Sealer	2 pics
Zipper bag	1 pic

Reagent Preparation

Before using any reagents, it is recommended that they be kept to temp of room. Standard formation the 120µL of the standard (80µmol/L) with 120µL of the standard diluent for get a 40µmol/L standard stock solution by diluting the standard stock solution with the standard diluent. Allow 15 minutes for the standard to rest with moderate agitation before generating dilutions. Prepare duplicates standard solutions by dilute the stock standard liquid (40µmol/L) 1:2 together with standard diluent for get solution at 20µmol/L, 10µmol/L, 5µmol/L and 2.5µmol/L The standard diluent is used to determine the zero standards (zero µmol/L). The following dilutions of standard solutions are recommended (Table 3.13, Figure 3.9).

Table 3.13 Dilutions of standard solutions of MDA

Concentration	**Standard No.**	**Details**
40 µmol/L	Standard No.5	120µL Original Standard + 120µL Standard Diluent
20 µmol/L	Standard No.4	120µL Standard No.5 + 120µL Standard Diluent
10 µmol/L	Standard No.3	120µL Standard No.4 + 120µL Standard Diluent
5 µmol/L	Standard No.2	120µL Standard No.3 + 120µL Standard Diluent
2.5 µmol/L	Standard No.1	120µL Standard No.2 + 120µL Standard Diluent

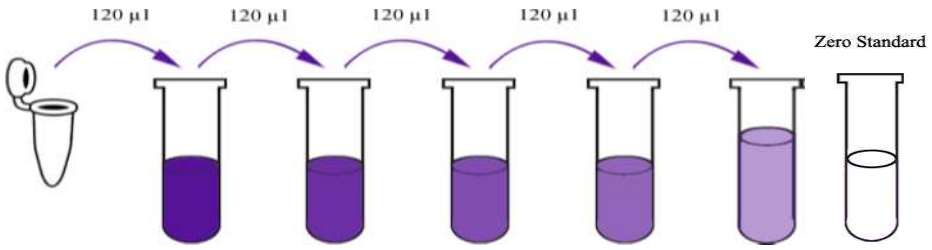

Figure 3.9 Dilutions of standard solutions of MDA

Washing buffer mix 20 mL of buffer solution Concentrate 25x with deionized water to get 500 mL of 1x washing buffer. Mix the solution carefully until all crystals have been totally dissolved, if any crystals have developed in the concentration.

Assay Procedure

Make sure to follow the instructions for preparation of reagents, samples, and standard solutions. First bring the chemicals to ambient temperature before using them. Without the use of specialized equipment, this test may be performed at ambient temperature without a problem.

Determine how many test strips you'll need to complete the procedure. In order to utilize them, insert the strips into the frames and secure them. It is best to keep strips that haven't been used at 2-8°C.

Fifty µL of standard should be added to the standard well. It is important to note that you should not add antibody to the standard well since the standard solution already includes biotinylated antibody.

When preparing the samples, put 40 µL of each sample to every well, Afterwards, each well received 10 µL of anti-MDA antibody, after that add 50 µL of streptavidin-HRP for both samples and standard (Not blank well). A thorough mixing is required. The plate should be sealed with a sealer. At 37°C, incubate for one hour.

The plate should be washed five times with a wash buffer to clear any leftover sealant once the sealant has been removed. As a general rule of thumb, you should use at least 0.35 mL of wash buffer every wash. Use a wash buffer and aspirate and wash all wells five times, overfilling wells each time. To remove the liquid off the plate, use paper towels or another absorbent material.

Following the addition of 50 µL of substrate solution A to every hole, the second 50 µL of substrate solution B should be add likewise. A new sealer should be applied to the plate, and it should be placed in the dark at 37°C for 10 minutes to incubate the plate.

The blue color in each well becomes yellow as soon as you add 50 µL of Stop Solution. Use a micro plate reader set to 450 nm to measure the optical density (OD value) of each well during the first ten minutes followed the stop solution has been applied.

Calculation of Result

Using the vertical (Y) axis, plot the mean optical density (OD) for each standard vs. the concentration (X) axis, and then draw a best-fit curve across the points on the graph, you will be able to construct your standard curve. It is preferable to conduct these calculations using computer-based curve-fitting tools, and then use regression analysis to determine which line is the best match.

Typical Data

A standard curve should be created for each test since the OD values might vary based on the actual assay performance (ie. the operator's pipetting method, cleaning procedure, or temperature influences). It is purely for comparison and illustrative reasons that the following standard curve and data set is provided (Figure 3.10).

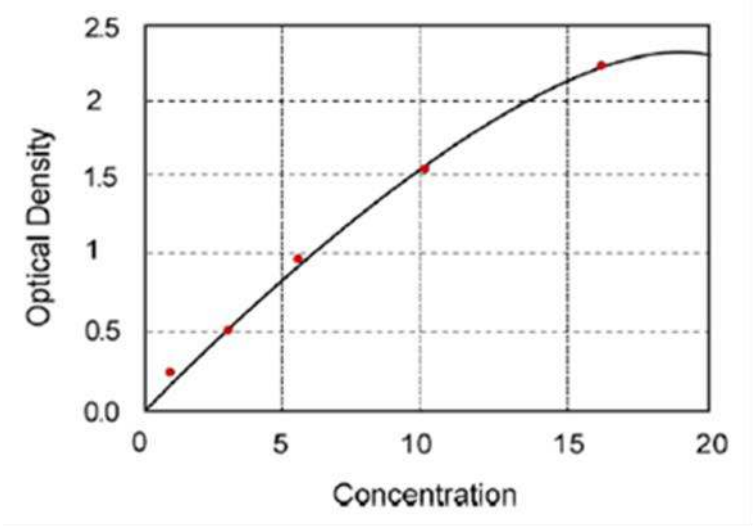

Figure 3.10 Standard curve of MDA

3.4.6 Estimation of ESR by westergren method

At the time of sample collection, 1.6 mL of patient's whole blood was gently mixed with 0.4 mL of sodium citrate (3.8 %). The anticoagulated blood was then suctioned into a glass Westergren pipette, placed on a stand, and held in the head position for one hour. The rate of sedimentation was determined by measuring the column of plasma at the tube's top based on millimeter per hour (Figure 3.11).

Figure 3.11 Estimation of (ESR) by westergren Method

3.4.7 Estimation the level of c-reactive protein (C-RP)

Assay Principle

CRP levels were determined using an Epithod® 616 analyzer. The Epithod 616 CRP test kit is a sandwich immunoassay that measures CRP concentrations in serum. It is comprised of three solutions: dilution solution (R1), a conjugate solution (R2), and washing solution (R3), as well as a cartridge with nitrocellulose membrane covered with CRP-antibodies. The CRP antigen in the diluted sample is caught by antibodies on the membrane of the cartridge, and R2 containing CRP antibodies that have been labeled with an ultra-small gold particle are added after it has been captured. In the sandwich assay, CRP that has been tapping on the membrane binds to the antibody-gold particle. Using a washing solution (R3), excess gold-antibody conjugate is removed from the cartridge. The analyzer analyzes the reflectance colour of the membrane and displays the result in mg / l of solution. Measurement range: 2.5~200 mg/L.

Reagent composition

R1 / reagent: Borate buffer (1.93%), and Detergent (0.10%). R2 / conjugate solution: Antibody 2 (4.00%), AuNP (22.28%), and Buffer (1.95%). R3 / washing solution: Buffer (0.95%), and Detergent (0.10%). Cartridge: Antibody 1 (0.1%), Buffer (0.95%), and Membrane 1 unit.

Assay procedure

Preparation

Switch on the Epithod® 616 analyzer. Before use the content prepares (CRP test kit, capillary tube, pipette, and tips). Warm up a CRP test kit in the room for 20 minutes. Place the item card into the slot. Set the test type and mode on the analyzer's test home screen. Press the next on the Epithod® 616 analyzer.

Diluting sample with R1

Use 5 µL of patient samples. Mix together the collecting tube and flip it about 5-6 times. Set into dilute R1 solution tube. Close and shake well at least 10 times. Press starts on the screen.

Dropping the diluted sample

Pull out 25 µL diluted sample from R1 tube. Drop it over cartridge and wait to absorb for 30 seconds.

Dropping conjugate solution R2

Pull out 25 µL from R2 vial. Drop it over cartridge and wait to absorb for 30 seconds.

Dropping washing solution R3

Pull out 25 µL from R3 vial. Drop it over cartridge and wait to absorb for 30 seconds.

Analysing

Follow the arrow to load the cartridge into the analyser. Press analyses.

3.4.8 Estimation the level of anti-cyclic citrullinated peptides (ACCP)

Assay Principle

The level of serum ACCP was calculated by used ichroma™ device (Ref: CFPC-97) Sandwich immune detection is used in this test, which is carried out with the aid of an ichroma device. The antigen on the detector binds to the sample antibodies, resulting in

the formation of complex of antigen and antibody, which then move on to nitrocellulose matrix, where they are captured by the immobilized-streptavidin on the strip of test.

Extra antibodies present on the sample will result in the formation of more complex of antigen and antibody, which will result in a stronger intensity of fluorescence signal by detection system antigens, which will be processed by the instrument for ichroma tests to determine the concentration of ACCP present in the sample.

Kit components

ichroma™ Anti-CCP consists of cartridges, detector diluent, detector tubes, ID chip,capillary tubes , and instruction for use. The cartridge includes a membrane known as a test strip, which contains streptavidin at the testing line and chicken IgY at the control line. All cartridges are packaged in a box with an aluminum foil bag containing desiccant.

The detector tube contains a granule comprising anti-human IgG-fluorescence conjugate, anti-chicken IgY-fluorescence conjugate, CCP-biotin conjugate, stabilizer bovine serum albumin (BSA), sucrose, and bromophenol blue in phosphate buffered saline.

The detector diluent is pre-dispensed in vials and is packaged in a box. It includes sodium azide as preservatives, bovine serum albumin (BSA) as a stabilizer, and phosphate buffered saline.

Storage and stability

Table 3.14 shows the Storage and stability of anti-cyclic citrullinated peptides.

Table 3.14 Storage and stability of ACCP kit

Components	Storage Temperature	Shelf Life	Note
Cartridge	4-30 C^0	20 months	Disposable
Detector tube	2-8 C^0	20 months	Disposable
Detector diluents	2-8 C^0	20 months	Opened or unopened

Assay procedure

Using a pipette, transfer 150 µL of detector diluent to a detector tube. Five µL of sample (human whole blood, serum, or plasma) should be immediately transferred to the test tube for detection. Ensure that the sample is well mixed by closing the detection tube's lid and shaking it for about 20 times. Seventy-five µL of a sample combination should be pipetted out and loaded into the cartridge's sample well. For 12 minutes, insert and leave the cartridge containing the sample combination in the i-chamber or incubator ($25C^0$). To scan a sample, place the cartridge holding the sample combination in the cartridge holder of the instrument used for ichroma testing. Scanning begins by pressing the "Start" button on the ichroma testing equipment. The results of the test are shown on the device's screen for ichroma testing.

3.4.9 Estimation the level of rheumatoid factor (RF)

Assay Principle

The level of serum RF was calculated by used ichroma™ device (Ref: CFPO-103). Sandwich immune detection is used in this test, which is carried out with the aid of an ichroma device. The antigen on the detector binds to the sample antibodies, resulting in the formation of complex of antigen and antibody, which then move on to nitrocellulose matrix, where they are captured by the immobilized-streptavidin on the strip of test.

Extra antibodies present on the sample will result in the formation of more complex of antigen and antibody, which will result in a stronger intensity of fluorescence signal by

detection system antigens, which will be handle by the equipment for ichroma tests to calculate the concentration of RF present in the sample.

Kit components

Ichroma™ RF IgM composed of cartridges, samples collectors, tubes of buffer detecting, and an ID chip. The cartridge includes a test strip, which is a membrane that contains human immunoglobulin at the test line and streptavidin at the control line.

Each cartridge is individually wrapped in an aluminum foil bag containing a desiccant, which keeps the cartridges from drying out. Twenty-five sealed cartridges are packaged in a box that also includes an identification chip and twenty-five sample collectors.

Anti-human immunoglobulin fluorescence conjugate, BSA-biotin fluorescence conjugate, bovine serum albumin as a stabilizer, and sodium azide in potassium phosphate buffer as a preservative are all included in the detection buffer.

A tube containing the detecting buffer is dispensed. 25 tubes of buffer detecting are bundled in a box and then styrofoam wrapped shipping box with an ice pack.

Storage and stability

If kept between 4-30°C, the cartridge is stable for 20 months (when enclosed in an aluminum foil bag). If storage at a temperature of 2-8°C, the detection buffer dispensed in a tube is shelf stable for 20 months. After opening the cartridge, the test should be conducted immediately.

Assay procedure

Put an empty sample collector into the head of the detection buffer tube to create a puncture. Five μL of sample (human whole blood, serum, or plasma) should be

transferred to the detection tube. Combine the sample collector and tube into a single unit. Shake the sample collector ten times or more until sample is expelled from the sample collector through inversion. The buffer-sample combination must be utilized within 30 seconds. Remove the cap from the assembled tube's top. Before loading, blot two drops of the sample mixture onto a paper towel. Fill the cartridge's sample well with no more than three drops of the mixture. Allow the sample-loaded test cartridge for 5 minutes at room temperature. place the cartridge holding the sample combination in the cartridge holder of the instrument used for ichroma testing. Scanning begins by pressing the "Start" button on the ichroma testing equipment. The test result is shown on the instrument's display screen for ichroma testing.

3.4.10 Estimation the level of uric acid (U.A) by uricase-peroxidase method

Assay Principle

The level of serum U.A was calculated by uricase-peroxidase method (uricase/POD) (cat no UA0103) by use of a mindray device. When ascorbic oxidase is used in conjunction with uric acid to remove ascorbic acid interference, the uric acid is catalyzed to create H2O2, which then oxidizes the 4-AAP to give a coloured dye of quinoneimine. The reduction in absorbency is directly proportional to the concentration of uric acid in the solution. The Kit components shwoed in (Table 3.15).

Table 3.15 Components of UA kit

Reagents	Components	Concentration
R1	Phosphate buffer Peroxidase Ascorbate oxidase TOOS	70 mmol/L 5000 U/L 3000 U/L 0.72 mmol/L
R2	Phosphate buffer Peroxidase 4-Aminoantipyrine Uricase	70 mmol/L 10000 U/L 1.7 mmol/L 1.8 750 U/L

Storage and stability

Store unopened at 2-8°C and away from direct light for up to the expiry date stated on the label. After being opened, the reagents are stable for up to 28 days if kept refrigerated on the analyser or in the refrigerator. Contamination of the reagents must be avoided and do not freeze the reagents.

Assay procedure

The assay procedure to estimation the level of uric acid showed in (Table 3.16).

Table 3.16 Procedure of UA

Reagents	**Blank**	**Sample**
Reagent 1	1200 µL	1200 µL
Dist. Water	25 µL	_
Sample	_	25 µL

Incubate for 5 minutes at 37°C and measure the blank absorbance then add: Reagent 2 (300 µL). Check the absorbance again after mixing well at 37°C for 4-5 minutes. Following calibration, the analyzer determines the concentration of UA in each sample on its own.

3.5 Statistical Analysis

IBM SPSS Statistics version 26, and MedCalc statistical software version 20 was used in this study for the purpose of conducting a biostatistics, the findings are determined as mean ± standard deviation, after the group of patients divided; an Independent T-test was performed in general (patients and control group), and between the categories of patients to show the effect of studied parameters in rheumatoid arthritis, Pearson Correlation was conducted to show the correlation of ADP, MDA, CAT, PC, and SOD with other parameters of this study. Finally ROC Curve was performed on all studied parameters to show the better performance of all parameters.

4. RESULTS

In the present study, total (131) participants, they were chosen in order to the aim of the research. Patients (n=81), and healthy control (n=50). Patients group was subdivided according to age, gender, and BMI into two categories as showed in (Table 3.1).

4.1 General Comparison of Studied Parameters (Independent T-test Analysis)

The clinical features of the subject's work participants are offer in the Table 4.1 showed that there were increased significant variations in comparison between RA patients n= (81) and Healthy controls n= (50) in ADP (P<0.01), MDA (P<0.01), PC (P<0.01), ESR (P<0.01), RF (P<0.01), ACCP (P<0.01), UA (P<0.01), and CRP (P<0.01).whereas CAT (P<0.01), SOD (P<0.01), were found to be significant decreased in the patients as compared to the healthy control.

Table 4.1 General comparison of studied parameters

Parameter	**Healthy control**		**RA patients**		***P* value**
	Mean	**S. D**	**Mean**	**S. D**	
ADP	7.39	0.58	10.65	3.15	*P* <0.001
MDA	1.94	0.51	3.51	0.95	*P* <0.001
PC	57.82	9.16	81.57	37.99	*P* <0.001
CAT	545.70	221.09	202.65	52.24	*P* <0.001
SOD	289.88	147.68	158.58	57.14	*P* <0.001
ESR	8.07	2.99	56.74	22.74	*P* <0.001
RF	3.19	1.39	19.28	10.37	*P* <0.001
ACCP	2.95	1.31	73.49	33.37	*P* <0.001
UA	3.70	0.56	5.29	1.20	*P* <0.001
CRP	2.46	0.89	19.55	11.50	*P* <0.001

4.2 The Effect of Gender, Age, and BMI on Studied Parameters

4.2.1 Adiponectin (ADP) mg/L

The clinical characteristics of the patients group that explain the effect of Gender, Age, and BMI on ADP in Table 4.2 showed that according to the comparison between the gender of the patients group (male n=30) and (female n=51). There was no significant effect between the categories of the gender on ADP (P > 0.05). According to the comparison between the age group of the patients (age <50 years n=33) and (age >50 years n=48). There was no significant effect between the categories of the age on ADP (P > 0.05). Also there was no significant effect between the categories of the BMI (normal weight < 25 n=20) and (overweight > 25 n=61) on ADP (P > 0.05) according to the comparison between these categories.

Table 4.2 Effect of gender, age, and BMI on adiponectin (ADP)

	Gender		**Age**		**BMI**	
	Male n= (30)	Female n= (51)	G1 n= (33)	G2 n= (48)	Normal n= (20)	Overweight n= (61)
Mean	10.24	10.89	10.84	10.52	175.50	153.03
S. D	2.57	3.45	3.00	3.27	61.12	55.16
P value	0.36		0.65		0.57	

4.2.2 Malondialdehyde (MDA) µmol/L

The clinical characteristics of the patients group that explain the effect of Gender, Age, and BMI on MDA in Table 4.3 showed that according to the comparison between the gender of the patients group (male and female). There was no significant effect between the categories of the gender on MDA (P > 0.05). According to the comparison between the age group of the patients (age<50 years and age >50 years) There was no significant effect between the categories of the age on MDA (P > 0.05). Also there was no significant effect between the categories of the BMI (normal weight and overweight) on MDA (P > 0.05) according to the comparison between these categories.

Table 4.3 Effect of gender, age, and BMI on malondialdehyde (MDA)

	Gender		Age		BMI	
	Male n= (30)	Female n= (51)	G1 n= (33)	G2 n= (48)	Normal n= (20)	Overweight n= (61)
Mean	3.47	3.53	3.47	3.54	3.70	3.45
S. D	0.90	0.99	0.98	0.94	1.00	0.94
P value	0.79		0.74		0.31	

4.2.3 Protein carbonyls (PC) ng/mL

The clinical characteristics of the patients group that explain the effect of Gender, Age, and BMI on PC in Table 4.4 showed that according to the comparison between the gender of the patients group (male and female). There was no significant effect between the categories of the gender on PC ($P > 0.05$). According to the comparison between the age group of the patients (age<50 years and age >50 years) There was no significant effect between the categories of the age on PC ($P > 0.05$). Also there was no significant effect between the categories of the BMI (normal weight and overweight) on PC ($P > 0.05$) according to the comparison between these categories.

Table 4.4 Effect of gender, age, and BMI on protein carbonyls (PC)

	Gender		Age		BMI	
	Male n= (30)	Female n= (51)	G1 n= (33)	G2 n= (48)	Normal n= (20)	Overweight n= (61)
Mean	77.37	84.03	77.79	84.16	85.30	80.34
S. D	27.56	43.03	26.92	44.11	25.09	41.46
P value	0.44		0.46		0.61	

4.2.4 Catalase (CAT) ng/L

The clinical characteristics of the patients group that explain the effect of Gender, Age, and BMI on CAT in Table 4.5 showed that according to the comparison between the gender of the patients group (male and female). There was no significant effect between the categories of the gender on CAT ($P > 0.05$). According to the comparison between the age group of the patients (age<50 years and age >50 years) There was no significant

effect between the categories of the age on CAT (P > 0.05). Also there was no significant effect between the categories of the BMI (normal weight and overweight) on CAT (P > 0.05) according to the comparison between these categories.

Table 4.5 Effect of gender, age, and BMI on catalase (CAT)

	Gender		**Age**		**BMI**	
	Male n= (30)	Female n= (51)	G1 n= (33)	G2 n= (48)	Normal n= (20)	Overweight n= (61)
Mean	206.66	200.30	192.12	209.89	212.51	199.42
S. D	54.50	51.26	60.76	44.72	54.89	51.39
P value	0.59		0.13		0.33	

4.2.5 Superoxide dismutase (SOD) U/L

The clinical characteristics of the patients group that explain the effect of Gender, Age, and BMI on SOD in Table 4.6 showed that according to the comparison between the gender of the patients group (male and female). There was no significant effect between the categories of the gender on SOD (P > 0.05). According to the comparison between the age group of the patients (age<50 years and age >50 years) There was no significant effect between the categories of the age on SOD (P > 0.05). Also there was no significant effect between the categories of the BMI (normal weight and overweight) on SOD (P > 0.05) according to the comparison between these categories.

Table 4.6 Effect of gender, age, and BMI on superoxide dismutase (SOD)

	Gender		**Age**		**BMI**	
	Male n= (30)	Female n= (51)	G1 n= (33)	G2 n= (48)	Normal n= (20)	Overweight n= (61)
Mean	168.64	152.67	148.88	165.25	175.50	153.03
S. D	56.08	57.47	61.07	53.90	61.12	55.16
P value	0.22		0.20		0.12	

4.2.6 Erythrocyte sedimentation rate (ESR) mm/hr

The clinical characteristics of the patients group that explain the effect of Gender, Age, and BMI on ESR in Table 4.7 showed that according to the comparison between the gender of the patients group (male and female). There was no significant effect between the categories of the gender on ESR ($P > 0.05$). According to the comparison between the age group of the patients (age<50 years and age >50 years) There was no significant effect between the categories of the age on ESR ($P > 0.05$). Also there was no significant effect between the categories of the BMI (normal weight and overweight) on ESR ($P > 0.05$) according to the comparison between these categories.

Table 4.7 Effect of gender, age, and BMI on erythrocyte sedimentation rate (ESR)

	Gender		**Age**		**BMI**	
	Male n= (30)	Female n= (51)	G1 n= (33)	G2 n= (48)	Normal n= (20)	Overweight n= (61)
Mean	55.16	57.67	55.54	57.56	55.05	57.29
S. D	21.66	23.51	22.21	23.30	21.21	23.36
P value	0.63		0.69		0.70	

4.2.7 Rheumatoid factor (RF) Iu/mL

The clinical characteristics of the patients group that explain the effect of Gender, Age, and BMI on RF in Table 4.8 showed that according to the comparison between the gender of the patients group (male and female). There was no significant effect between the categories of the gender on RF ($P > 0.05$). According to the comparison between the age group of the patients (age<50 years and age >50 years) There was no significant effect between the categories of the age on RF ($P > 0.05$). Also there was no significant effect between the categories of the BMI (normal weight and overweight) on RF ($P > 0.05$) according to the comparison between these categories.

Table 4.8 Effect of gender, age, and BMI on rheumatoid factor (RF)

	Gender		Age		BMI	
	Male n= (30)	Female n= (51)	G1 n= (33)	G2 n= (48)	Normal n= (20)	Overweight n= (61)
Mean	18.87	19.53	18.45	19.86	19.10	19.34
S. D	12.54	8.98	8.80	11.38	9.56	10.70
P value	0.78		0.55		0.92	

4.2.8 Anti-cyclic citrullinated peptides (ACCP) U/mL

The clinical characteristics of the patients group that explain the effect of Gender, Age, and BMI on ACCP in Table 4.9 showed that according to the comparison between the gender of the patients group (male and female). There was no significant effect between the categories of the gender on ACCP (P > 0.05). According to the comparison between the age group of the patients (age<50 years and age >50 years) There was no significant effect between the categories of the age on ACCP (P > 0.05). Also there was no significant effect between the categories of the BMI (normal weight and overweight) on ACCP (P > 0.05) according to the comparison between these categories.

Table 4.9 Effect of gender, age, and BMI on anti-cyclic citrullinated peptides

	Gender		Age		BMI	
	Male n= (30)	Female n= (51)	G1 n= (33)	G2 n= (48)	Normal n= (20)	Overweight n= (61)
Mean	74.46	72.92	76.67	71.30	70.82	74.37
S. D	33.69	33.50	29.60	35.88	31.65	34.12
P value	0.84		0.48		0.68	

4.2.9 Uric acid (UA) mg/dL

The clinical characteristics of the patients group that explain the effect of Gender, Age, and BMI on UA in Table 4.10 showed that according to the comparison between the gender of the patients group (male and female). There was no significant effect between the categories of the gender on UA (P > 0.05). According to the comparison between the age group of the patients (age<50 years and age >50 years) There was significant

effect between the categories of the age on UA (P < 0.05). Also there was no significant effect between the categories of the BMI (normal weight and overweight) on UA (P > 0.05) according to the comparison between these categories.

Table 4.10 Effect of gender, age, and BMI on uric acid (UA)

	Gender		**Age**		**BMI**	
	Male n= (30)	Female n= (51)	G1 n= (33)	G2 n= (48)	Normal n= (20)	Overweight n= (61)
Mean	5.12	5.39	4.92	0.86	5.05	5.37
S. D	0.98	1.32	5.54	1.34	1.13	1.23
P value	0.32		0.02		0.31	

4.2.10 C-reactive protein (CRP) mg/L

The clinical characteristics of the patients group that explain the effect of Gender, Age, and BMI on CRP in Table 4.11 showed that according to the comparison between the gender of the patients group (male and female). There was no significant effect between the categories of the gender on CRP (P > 0.05). According to the comparison between the age group of the patients (age<50 years and age >50 years) There was no significant effect between the categories of the age on CRP (P > 0.05). Also there was no significant effect between the categories of the BMI (normal weight and overweight) on CRP (P > 0.05) according to the comparison between these categories.

Table 4.11 Effect of gender, age, and BMI on c-reactive protein (CRP)

	Gender		**Age**		**BMI**	
	Male n= (30)	Female n= (51)	G1 n= (33)	G2 n= (48)	Normal n= (20)	Overweight n= (61)
Mean	22.72	17.69	20.62	18.82	18.56	19.88
S. D	10.67	11.67	13.59	9.91	11.16	11.69
P value	0.06		0.49		0.66	

4.3 Correlations Between Clinical Parameters in Patients of RA

4.3.1 Correlation of adiponectin (ADP) mg/L with the other parameters

The clinical characteristics of the patients group that interpret the correlation of ADP with the other parameters in the Table 4.12 showed that there was positive correlation significant between ADP with PC r=0.27 Figure 4.4, CAT r=0.34 Figure 4.5, SOD r=0.19 Figure 4.6, and UA r=0.25 Figure 4.10. While there was weak positive correlation between ADP with Age r=0.01 Figure 4.1, BMI r=0.08 Figure 4.2, and ACCP r=0.02 (Figure 4.9). Moreover, weak negative correlation was observed between ADP with MDA r= -0.04 Figure 4.3, ESR r= -.08 Figure 4.7, CRP r= -0.07 Figure 4.11, and RF r= -0.02 (Figure 4.8).

Table 4.12 Correlation of (ADP) with studied parameters

Analyzed parameter	Parameters	Pearson correlation (r)
ADP n=81	Age	0.01
	BMI	0.08
	MDA	-0.04
	PC	0.27
	CAT	0.34
	SOD	0.19
	ESR	-0.08
	RF	-0.02
	ACCP	0.02
	UA	0.25
	CRP	-0.07

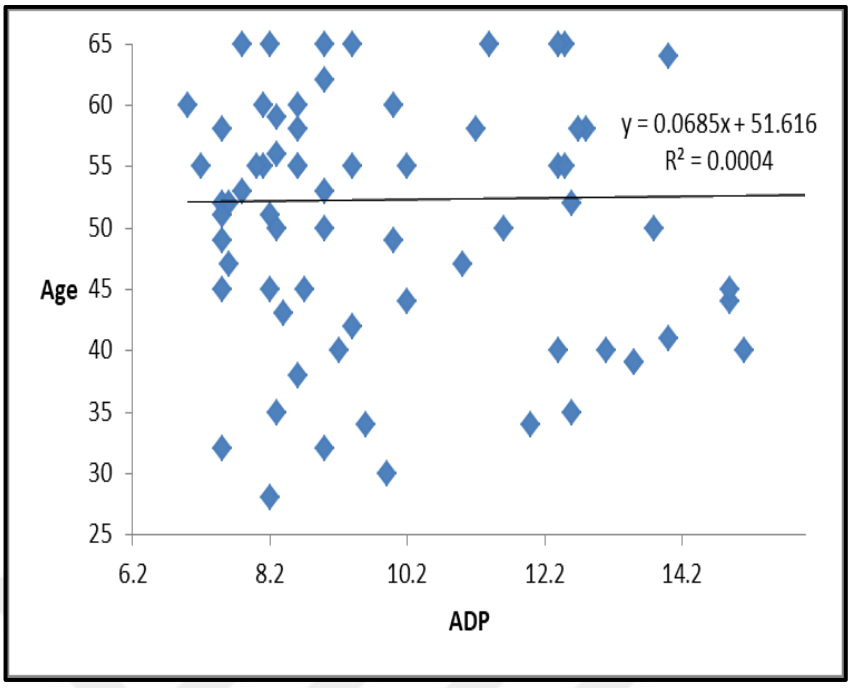

Figure 4.1 Correlation of (ADP) with (Age)

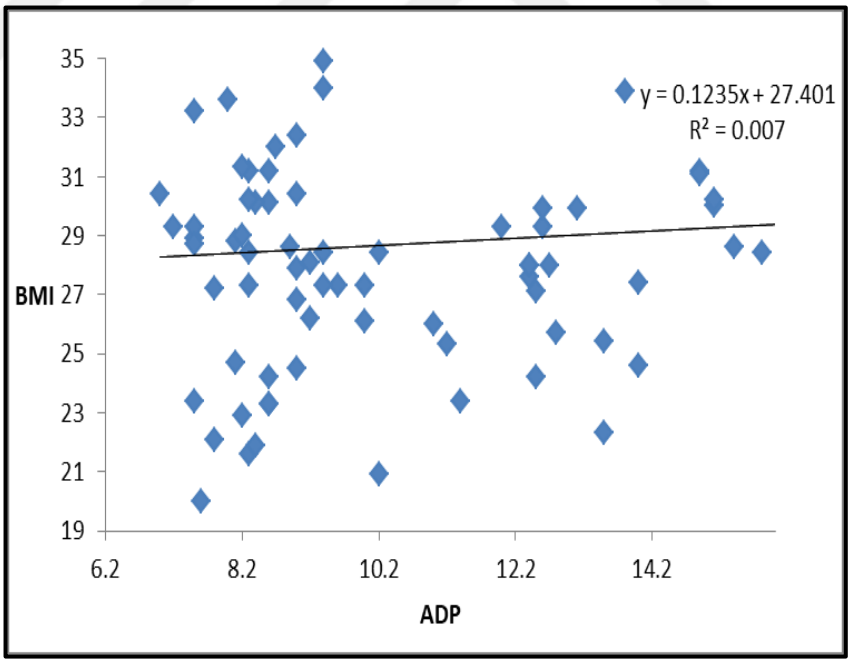

Figure 4.2 Correlation of (ADP) with (BMI)

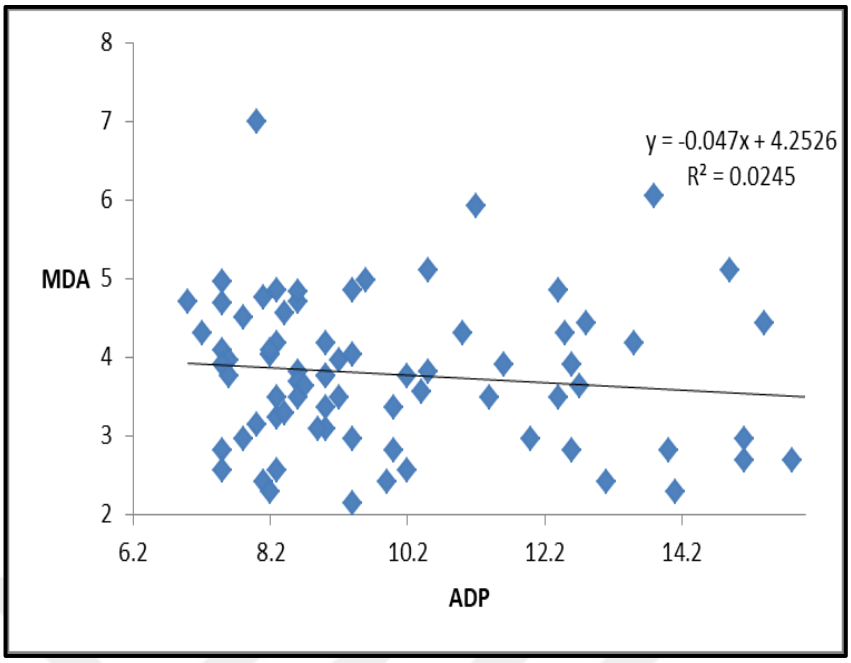

Figure 4.3 Correlation of (ADP) with (MDA)

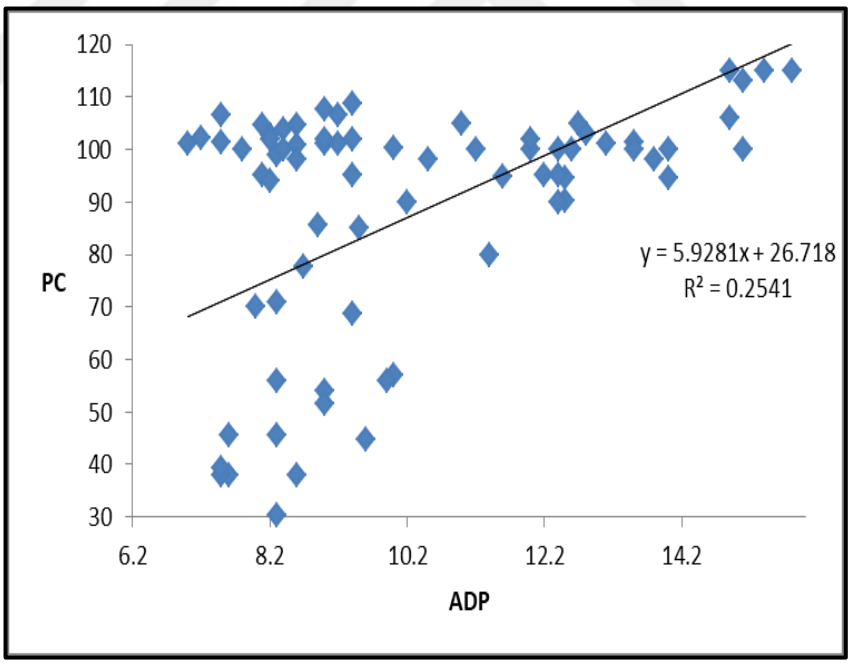

Figure 4.4 Correlation of (ADP) with (PC)

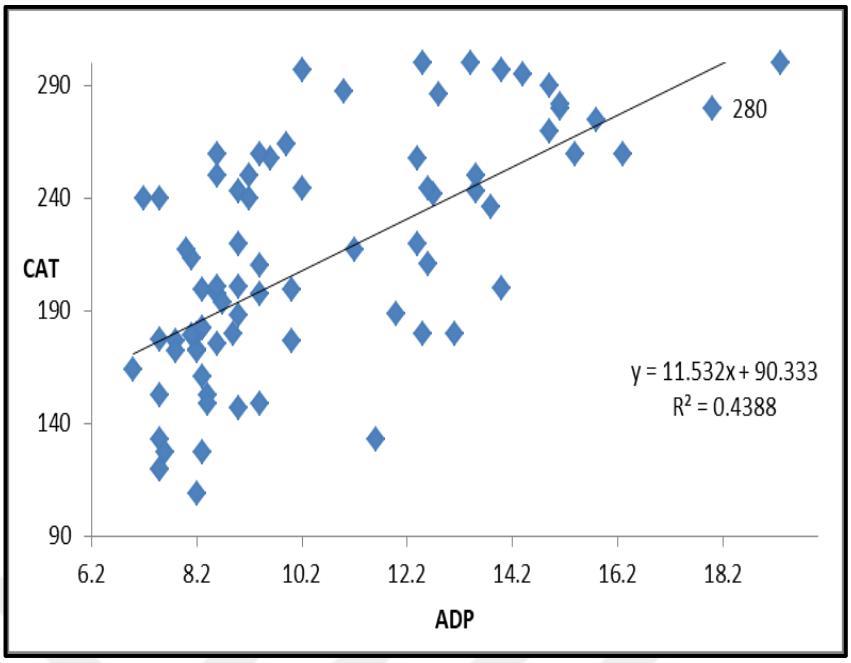

Figure 4.5 Correlation of (ADP) with (CAT)

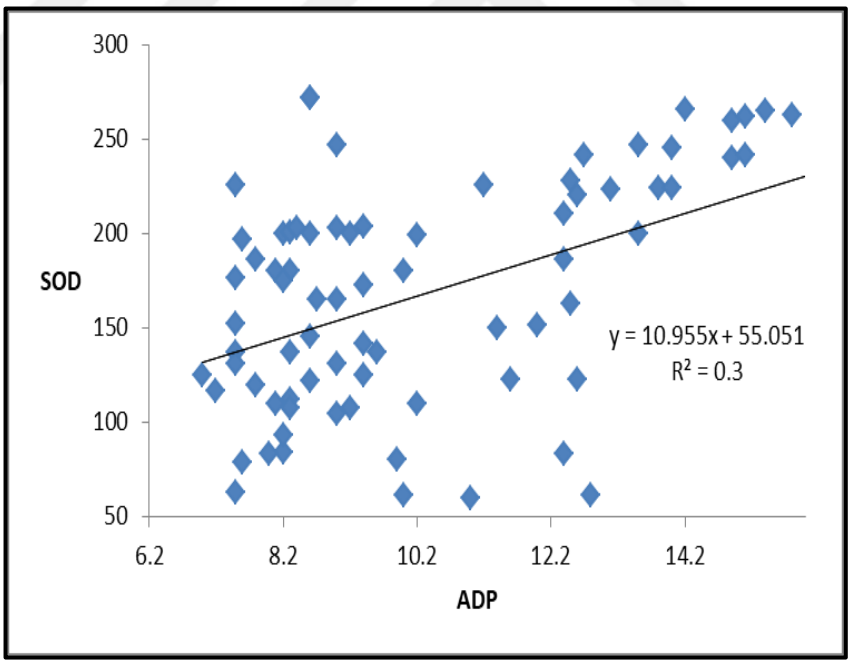

Figure 4.6 Correlation of (ADP) with (SOD)

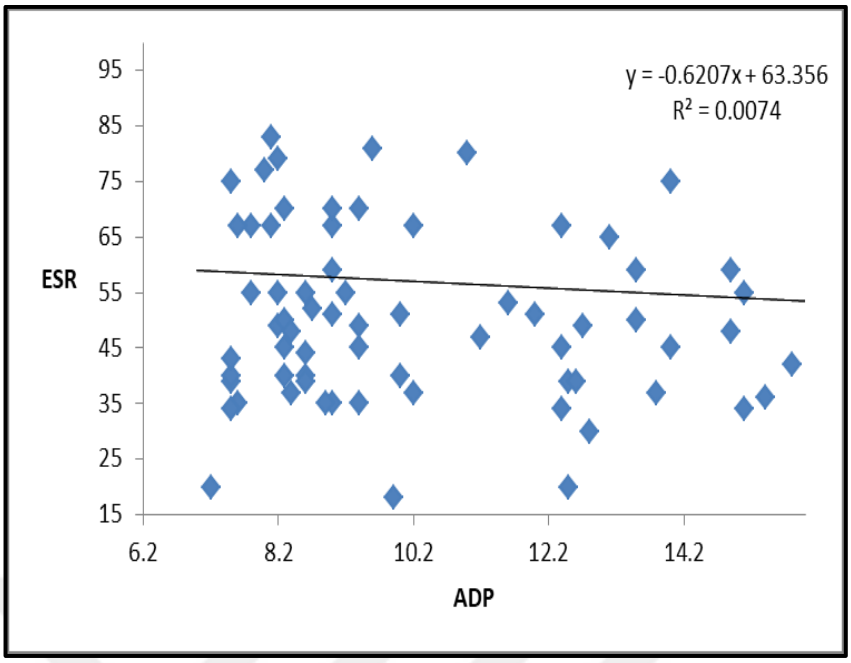

Figure 4.7 Correlation of (ADP) with (ESR)

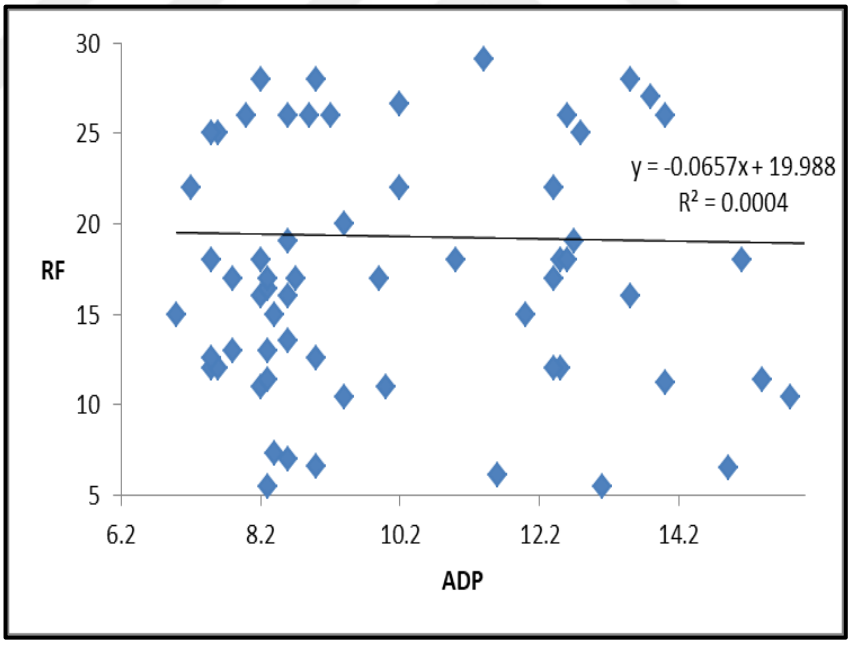

Figure 4.8 Correlation of (ADP) with (RF)

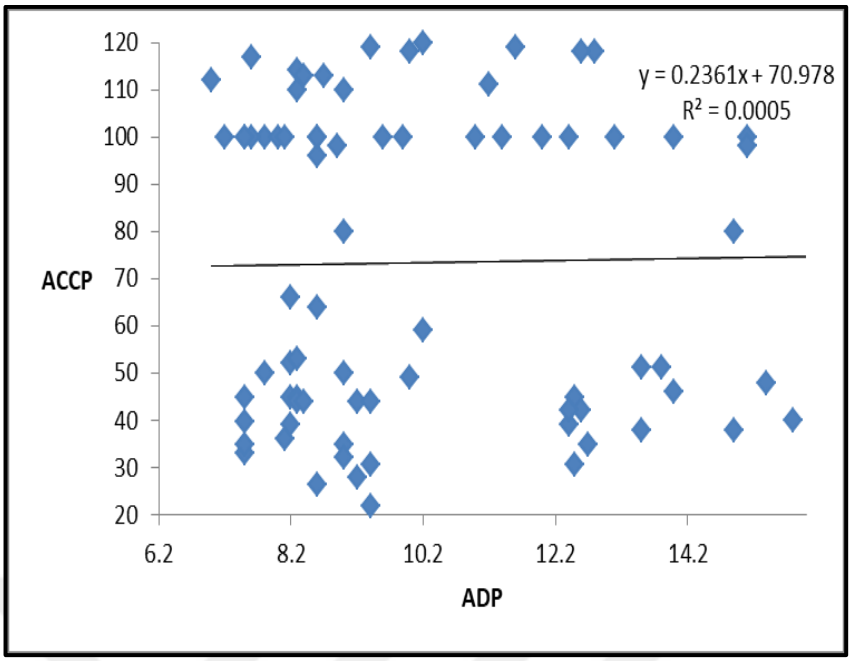

Figure 4.9 Correlation of (ADP) with (ACCP)

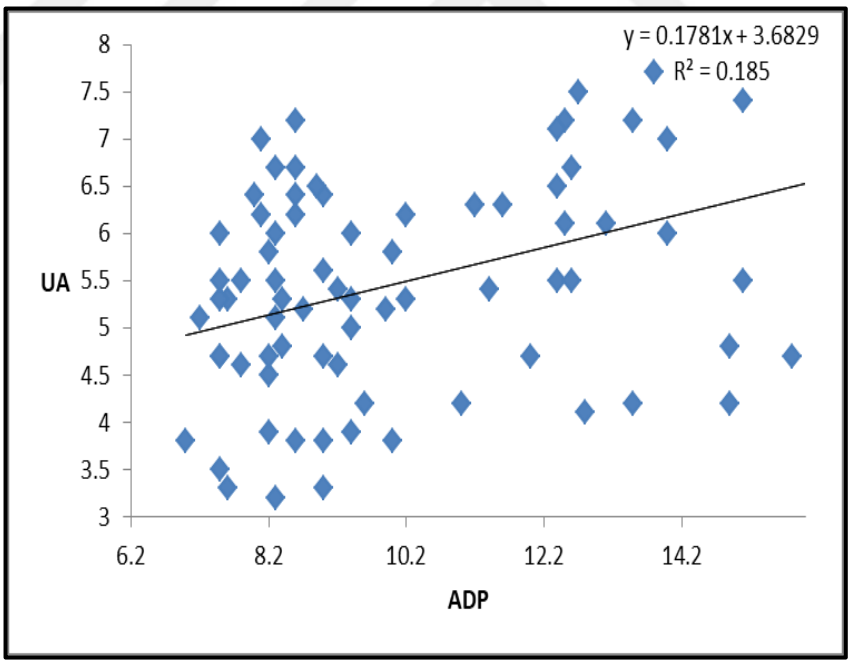

Figure 4.10 Correlation of (ADP) with (UA)

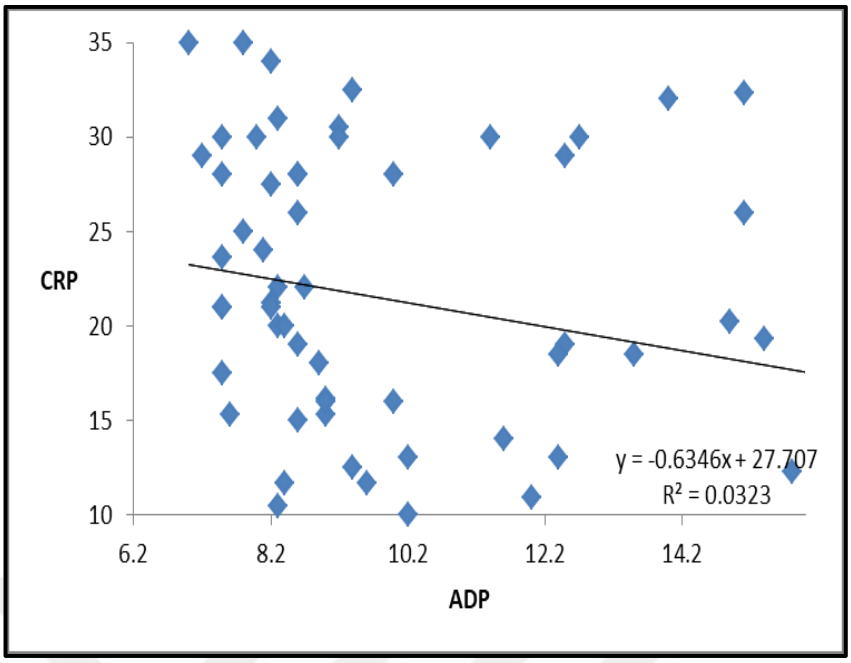

Figure 4.11 Correlation of (ADP) with (CRP)

4.3.2 Correlation of MDA µmol/L with the other parameters

The clinical characteristics of the patients group that interpret the correlation of MDA with the other parameters in the Table 4.13 showed that there was positive correlation significant between MDA with PC r=0.12 Figure 4.14, ACCP r=0.14 Figure 4.19, and CRP r=0.12 (Figure 4.21). While there was weak positive correlation between MDA with CAT r=0.09 Figure 4.15, RF r=0.04 Figure 4.18, and ESR r=0.09 (Figure 4.17). Moreover, weak negative correlation was observed between MDA with Age r= -0.03 Figure 4.12, BMI r= -0.03 Figure 4.13, and UA r= -0.05 Figure 4.20. Also negative correlation significant was observed between MDA and SOD r= -0.15 (Figure 4.16).

Table 4.13 Correlation of (MDA) with studied parameters

Analyzed parameter	Parameters	Pearson correlation (r)
MDA n=81	Age	-0.03
	BMI	-0.05
	ADP	-0.04
	PC	0.12
	CAT	0.09
	SOD	-0.15
	ESR	0.09
	RF	0.04
	ACCP	0.14
	UA	-0.05
	CRP	0.12

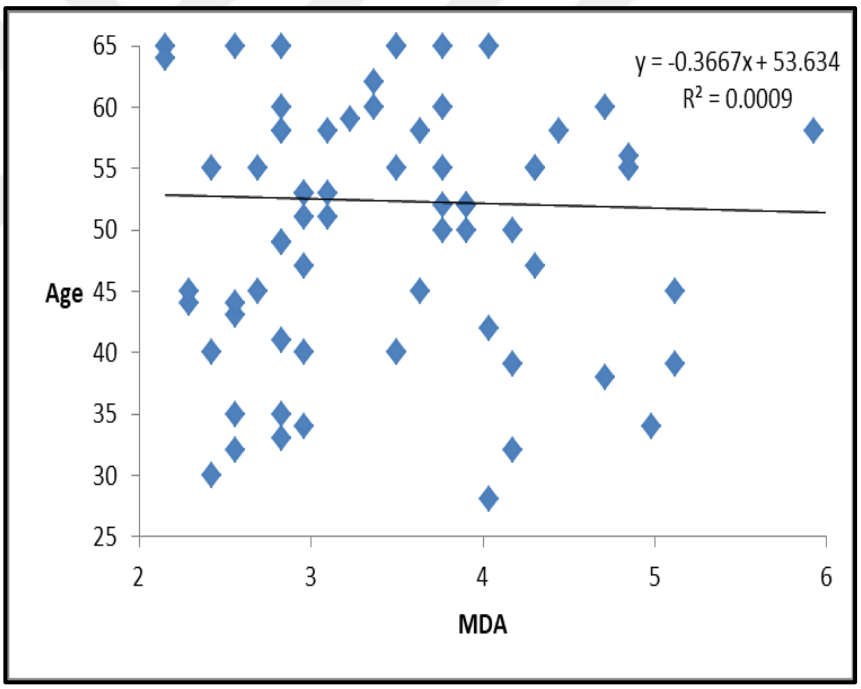

Figure 4.12 Correlation of (MDA) with (Age)

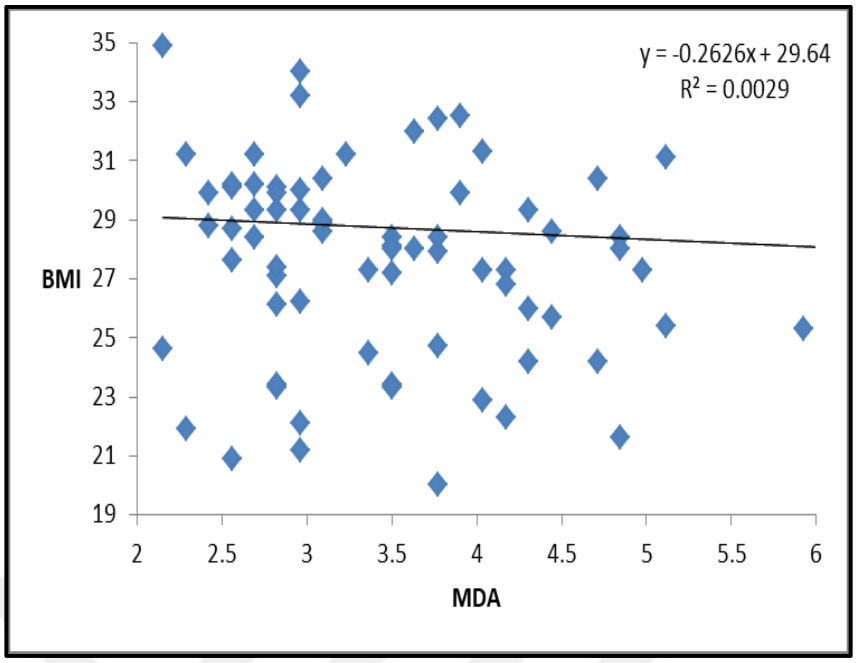

Figure 4.13 Correlation of (MDA) with (BMI)

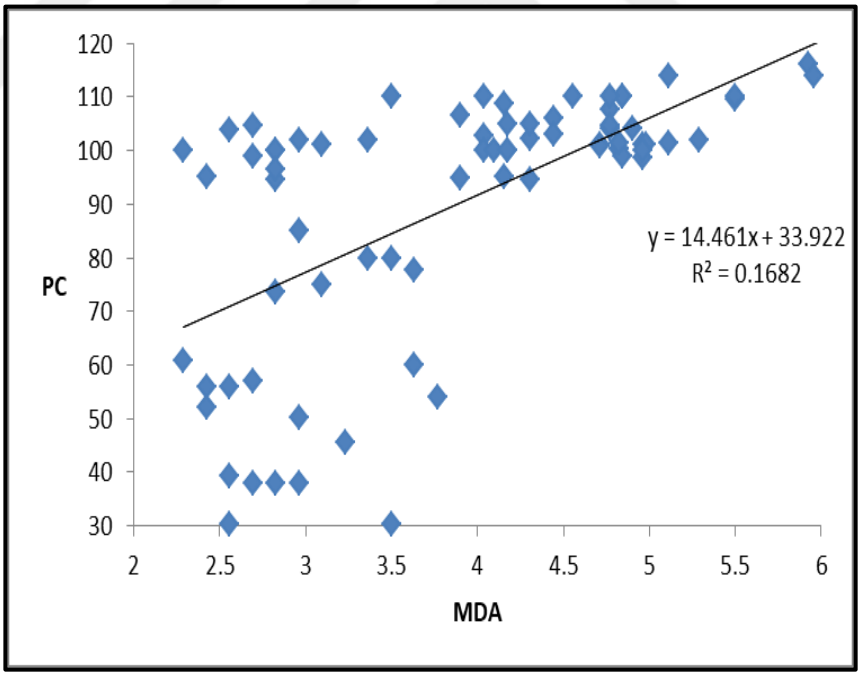

Figure 4.14 Correlation of (MDA) with (PC)

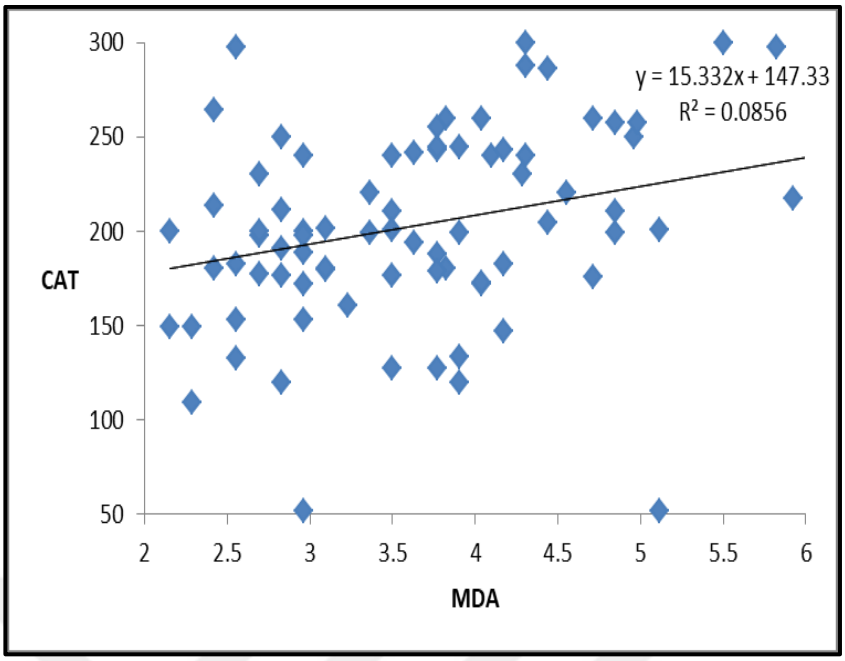

Figure 4.15 Correlation of (MDA) with (CAT)

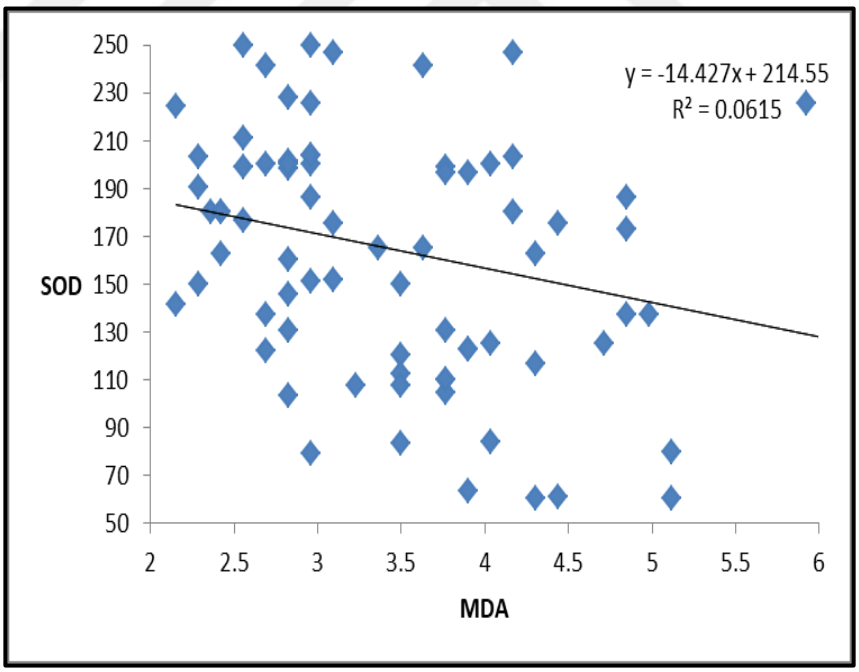

Figure 4.16 Correlation of (MDA) with (SOD)

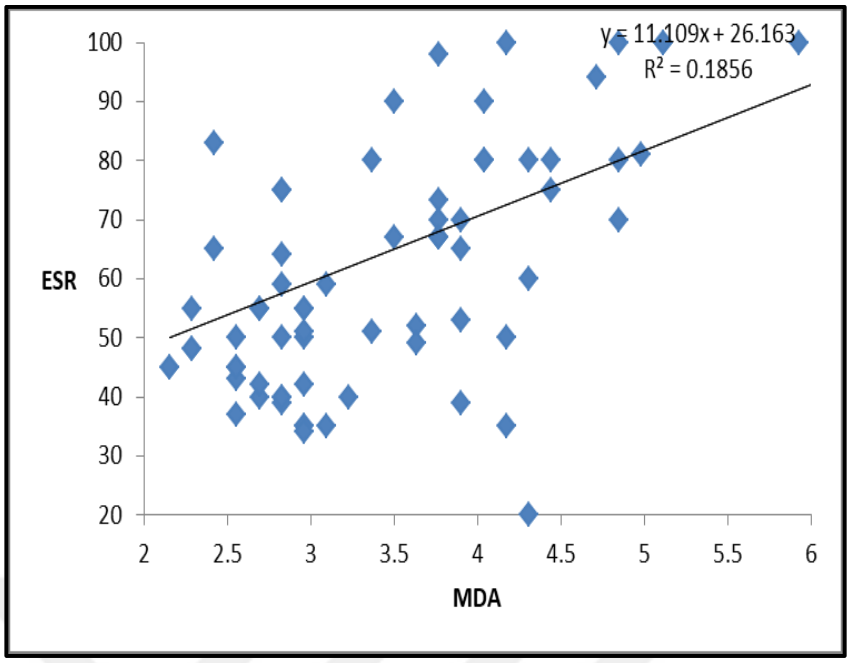

Figure 4.17 Correlation of (MDA) with (ESR)

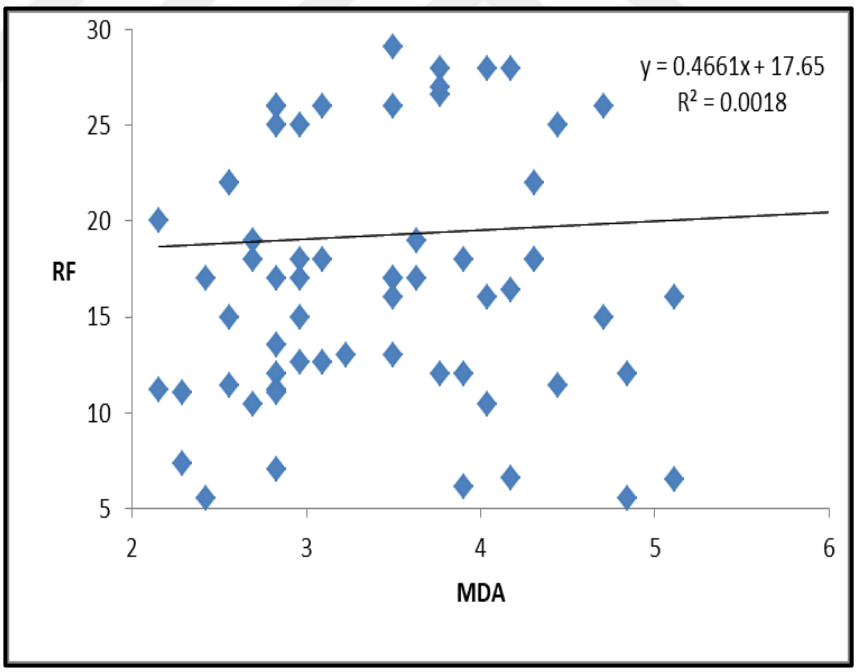

Figure 4.18 Correlation of (MDA) with (RF)

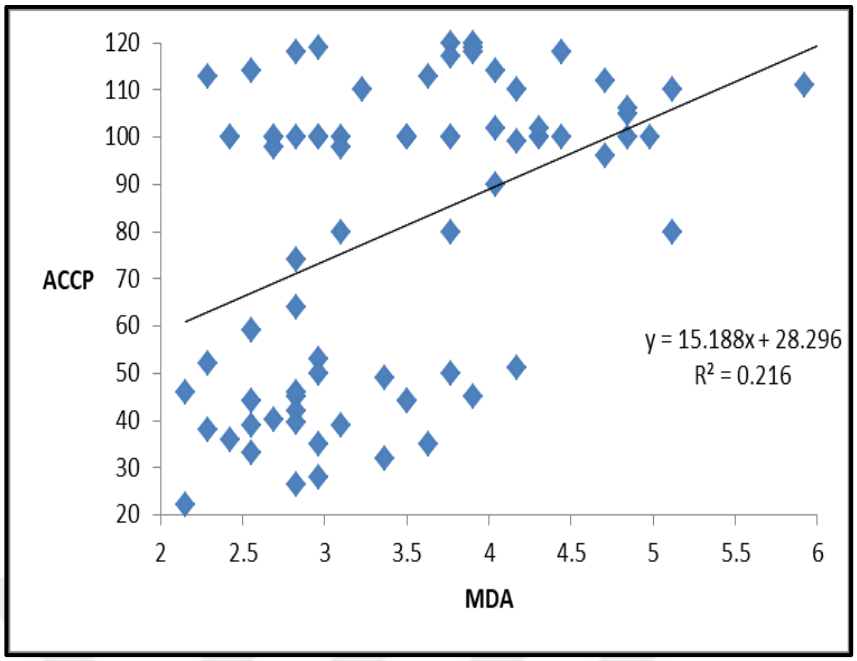

Figure 4.19 Correlation of (MDA) with (ACCP)

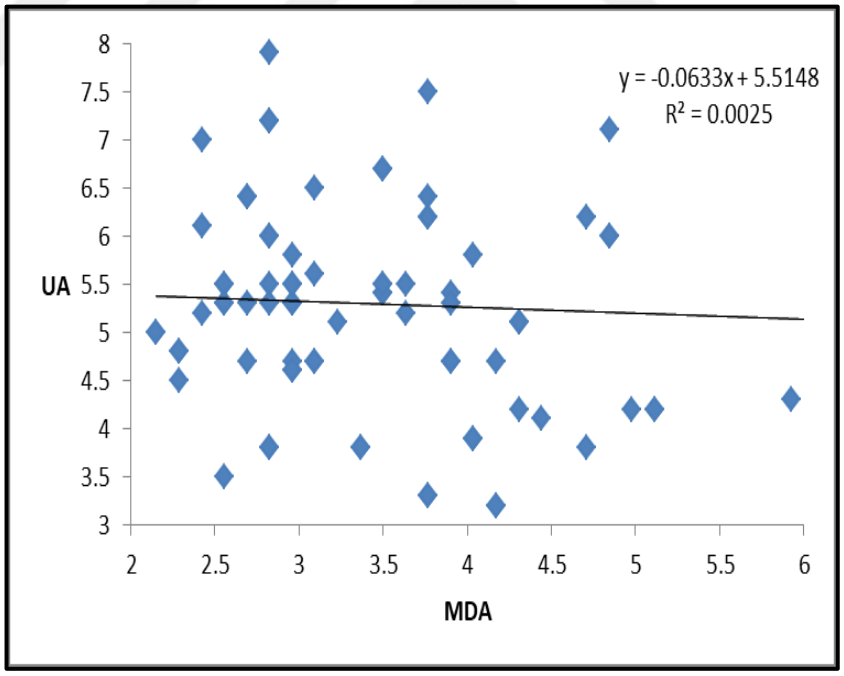

Figure 4.20 Correlation of (MDA) with (UA)

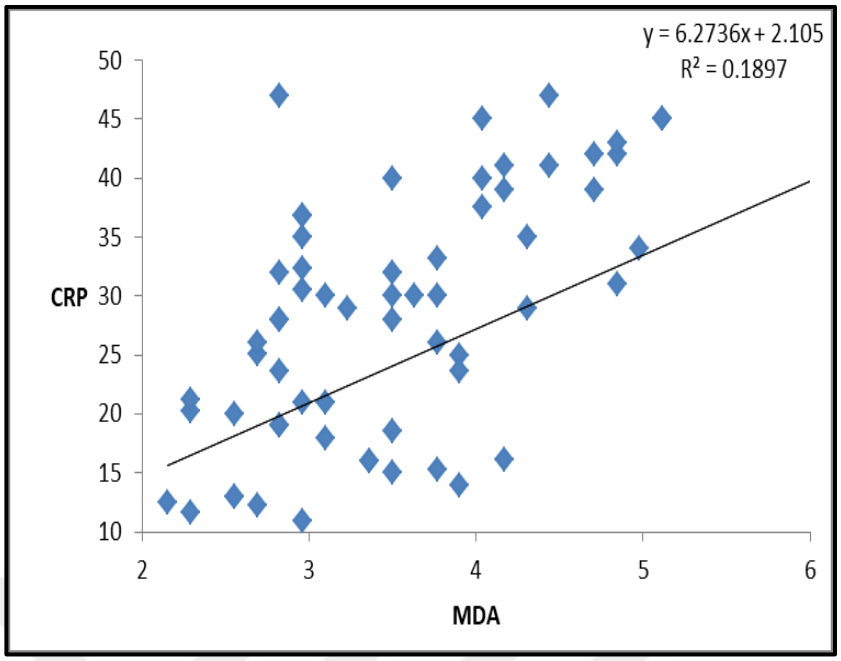

Figure 4.21 Correlation of (MDA) with (CRP)

4.3.3 Correlation of protein carbonyls (PC) ng/mL with the other parameters

The clinical characteristics of the patients group that interpret the correlation of MDA with the other parameters in the Table 4.14 showed that there was positive correlation significant between PC with RF r=0.14 Figure 4.27, and CRP r=0.14 (Figure 4.30). While there was weak positive correlation between PC with CAT r=0.01 Figure 4.24, SOD r=0.03 Figure 4.25, ESR r=0.05 Figure 4.26, ACCP r=0.05 Figure 4.28, UA r= 0.04 Figure 4.29, Age r=0.05 Figure 4.22, and BMI r=0.09 (Figure 4.23).

Table 4.14 Correlation of (PC) with studied parameters

Analyzed parameter	Parameters	Pearson correlation (r)
PC n=81	Age	0.05
	BMI	0.09
	ADP	0.27
	MDA	0.12
	CAT	0.01
	SOD	0.03

Table 4.14 (continue)

	ESR	0.05
	RF	0.14
	ACCP	0.05
	UA	0.04
	CRP	0.14

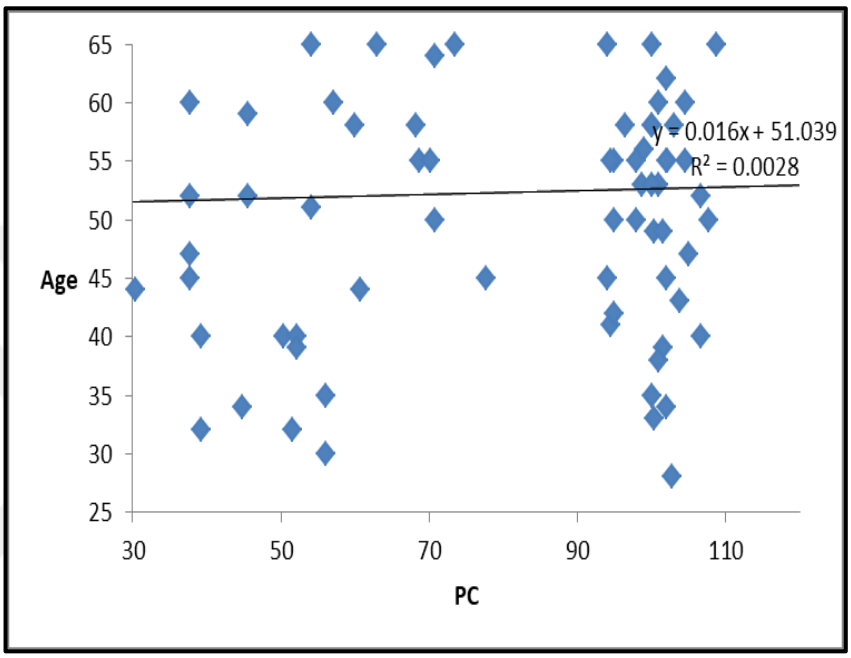

Figure 4.22 Correlation of (PC) with (Age)

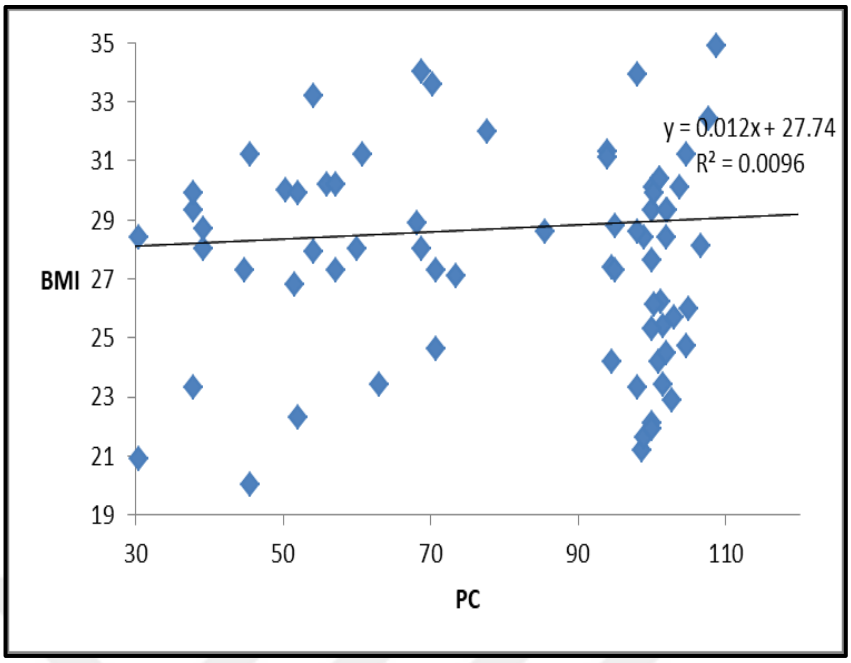

Figure 4.23 Correlation of (PC) with (BMI)

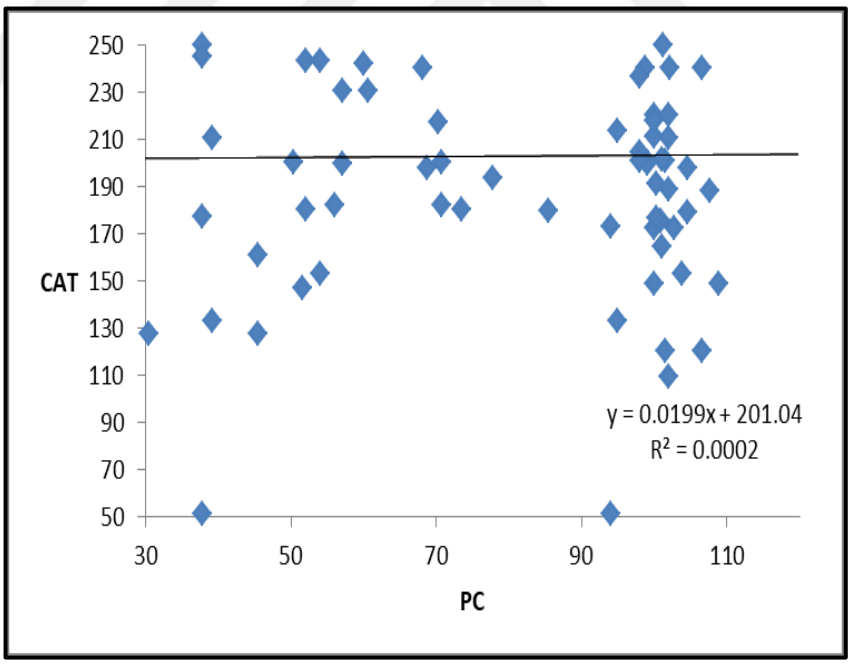

Figure 4.24 Correlation of (PC) with (CAT)

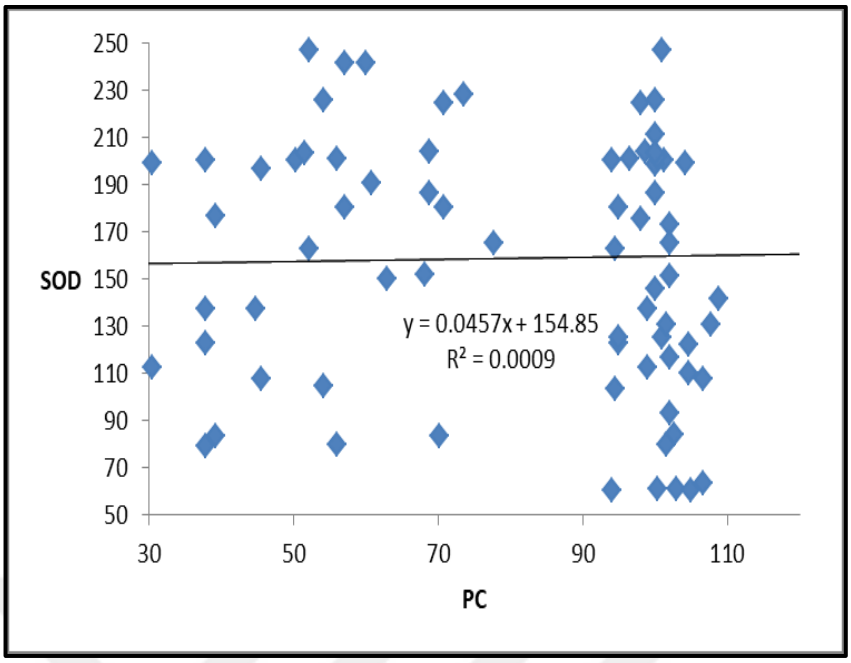

Figure 4.25 Correlation of (PC) with (SOD)

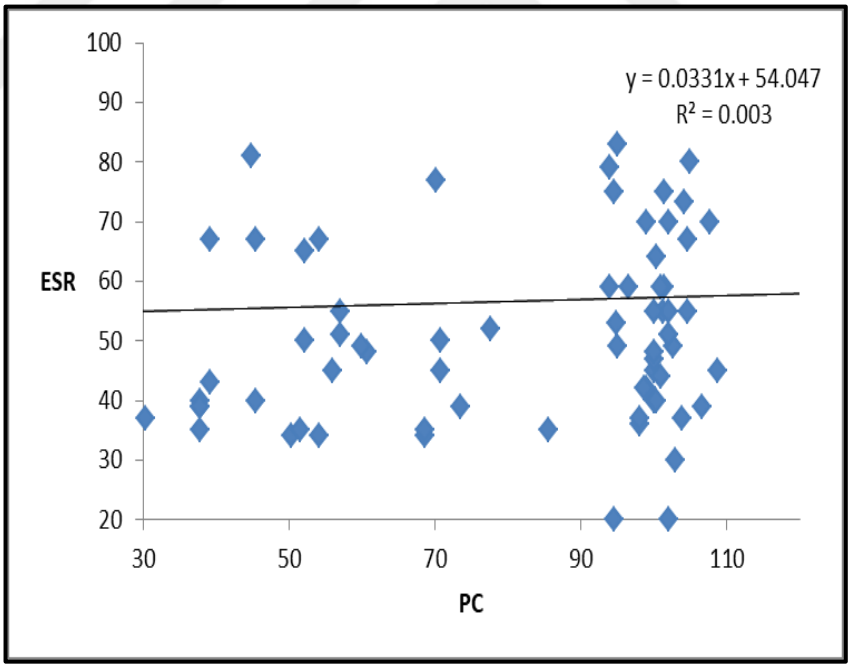

Figure 4.26 Correlation of (PC) with (ESR)

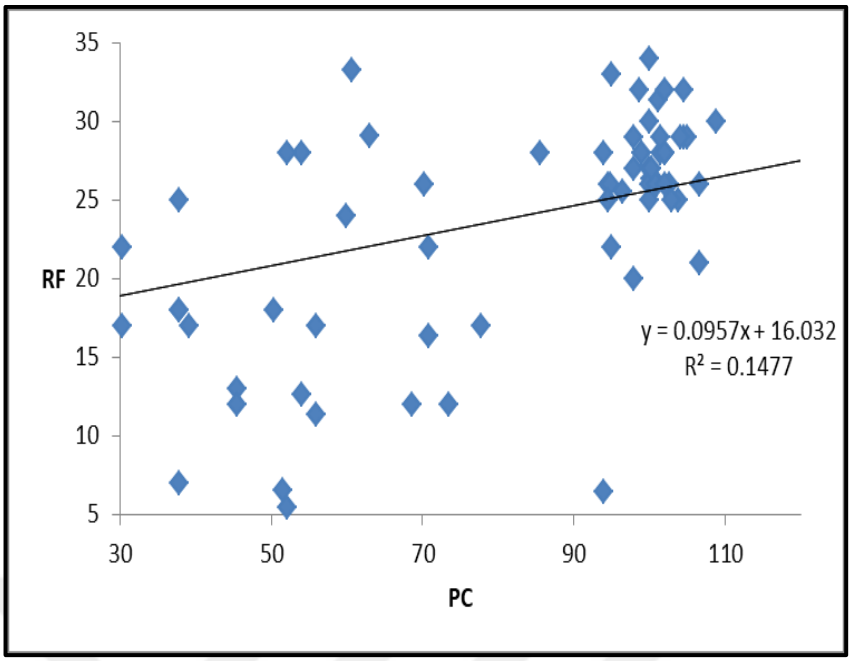

Figure 4.27 Correlation of (PC) with (RF)

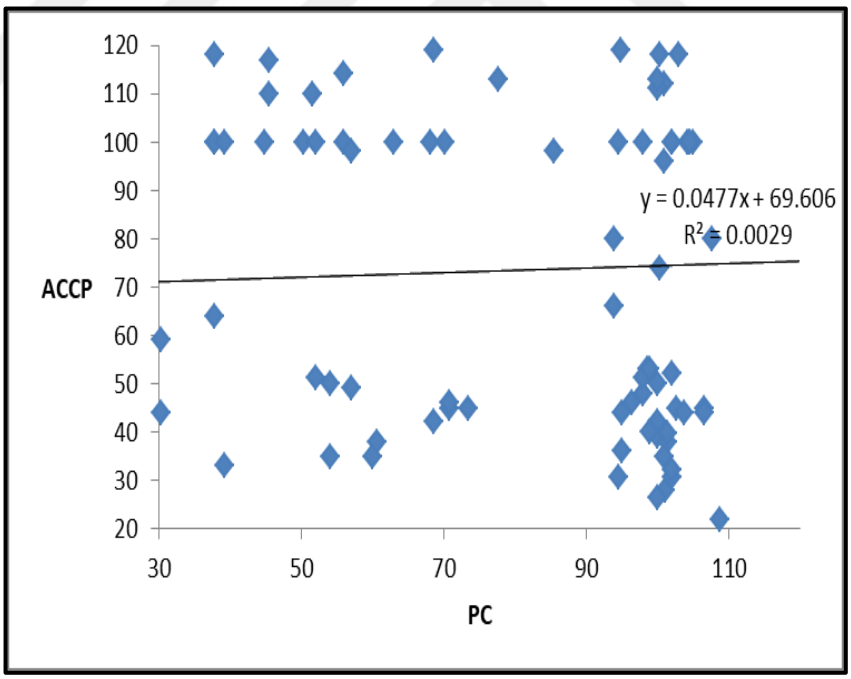

Figure 4.28 Correlation of (PC) with (ACCP)

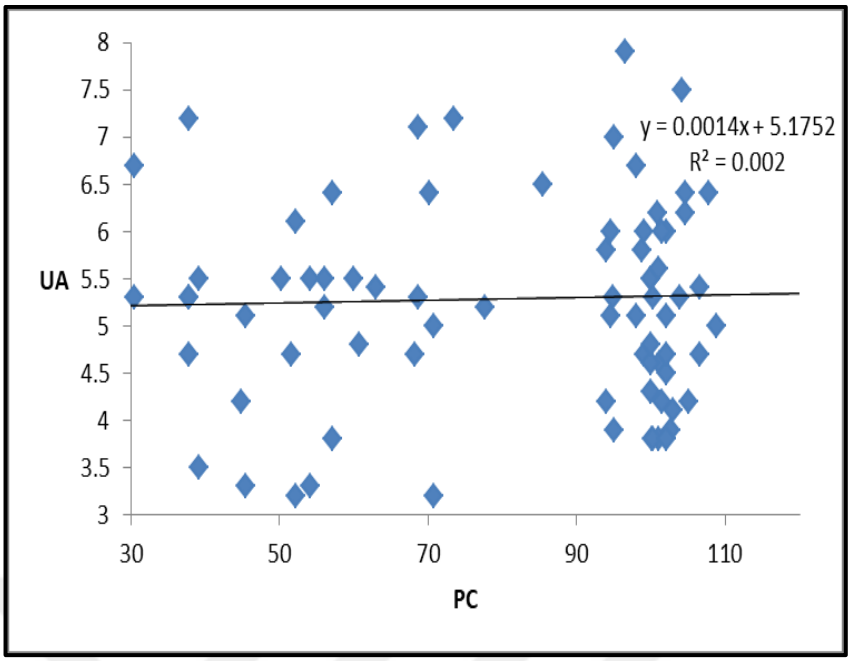

Figure 4.29 Correlation of (PC) with (UA)

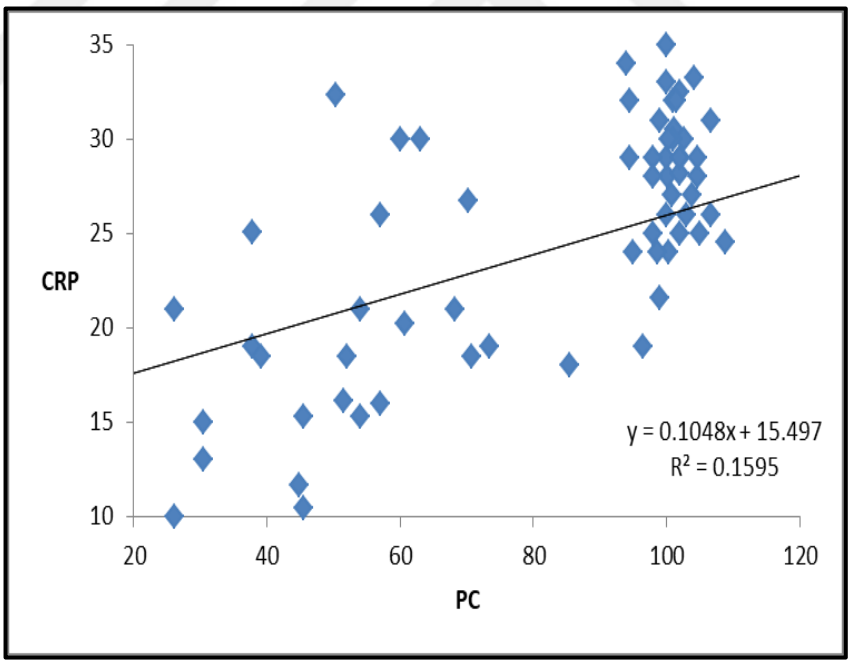

Figure 4.30 Correlation of (PC) with (CRP)

4.3.4 Correlation of catalase (CAT) ng/L with the other parameters

The clinical characteristics of the patients group that interpret the correlation of CAT with the other parameters in the Table 4.15 showed that there was positive correlation significant between CAT with SOD r=0.11 Figure 4.33, RF r=0.16 Figure 4.35, and UA r=0.11 (Figure 4.37). While there was weak positive correlation between CAT with Age r=0.08 Figure 4.31, and ESR r=0.09 (Figure 4.34). Moreover, weak negative correlation was observed between CAT with ACCP r= -0.04 Figure 4.36, and CRP r= -0.08 (Figure 4.38). Also negative correlation significant was observed between CAT and BMI r= -0.22 (Figure 4.32).

Table 4.15 Correlation of (CAT) with studied parameters

Analyzed parameter	Parameters	Pearson correlation (r)
CAT n=81	Age	0.08
	BMI	-0.22
	ADP	0.34
	MDA	0.09
	PC	0.01
	SOD	0.11
	ESR	0.09
	RF	0.16
	ACCP	-0.04
	UA	0.11
	CRP	-0.08

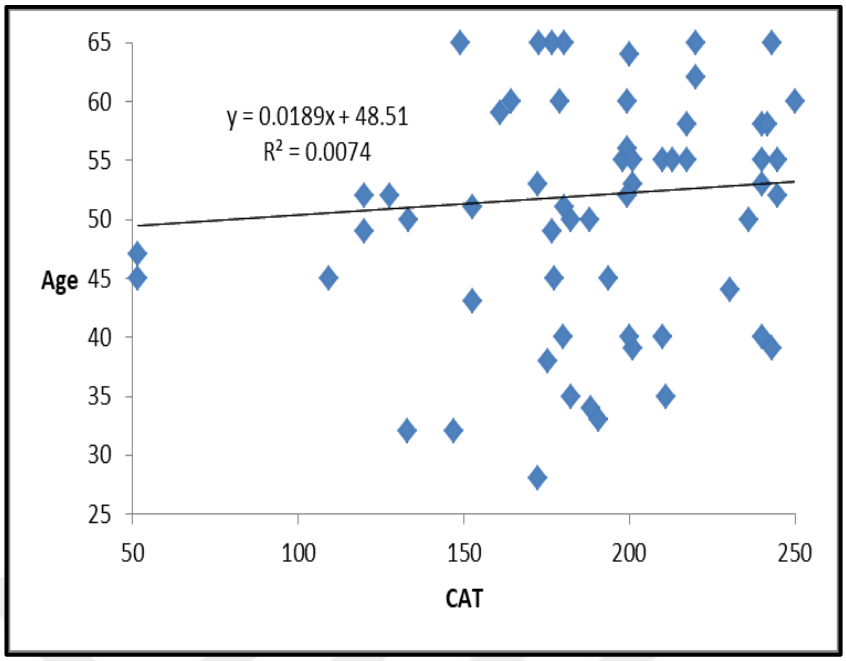

Figure 4.31 Correlation of (CAT) with (Age)

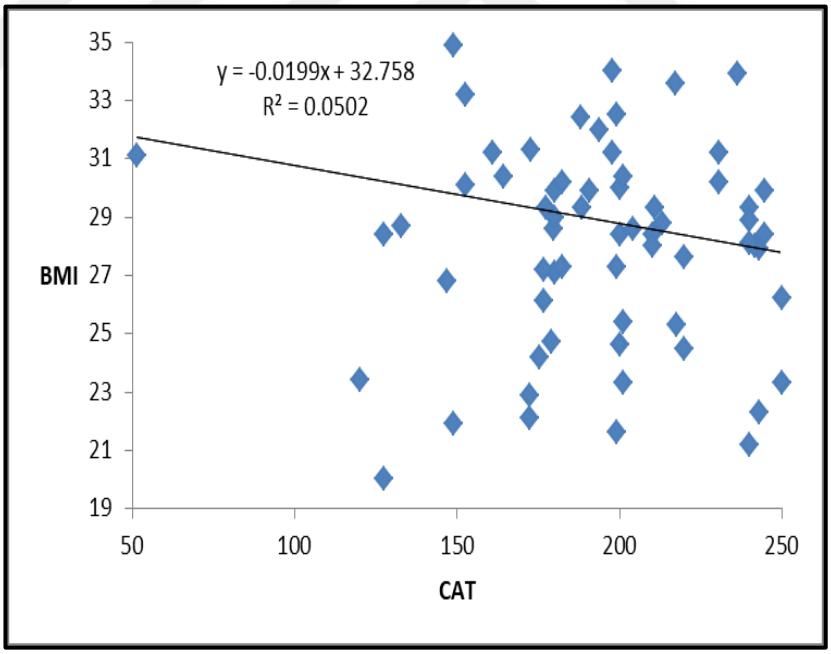

Figure 4.32 Correlation of (CAT) with (BMI)

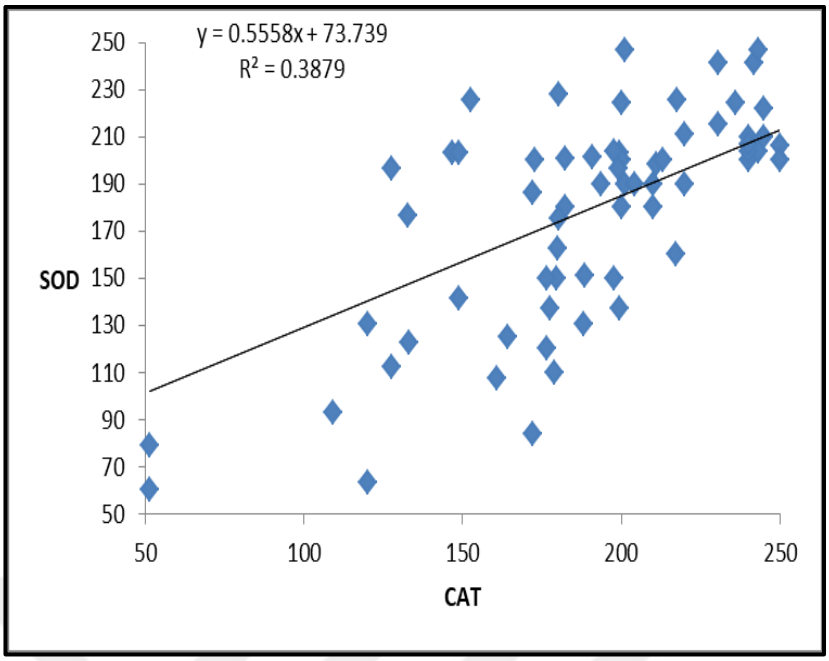

Figure 4.33 Correlation of (CAT) with (SOD)

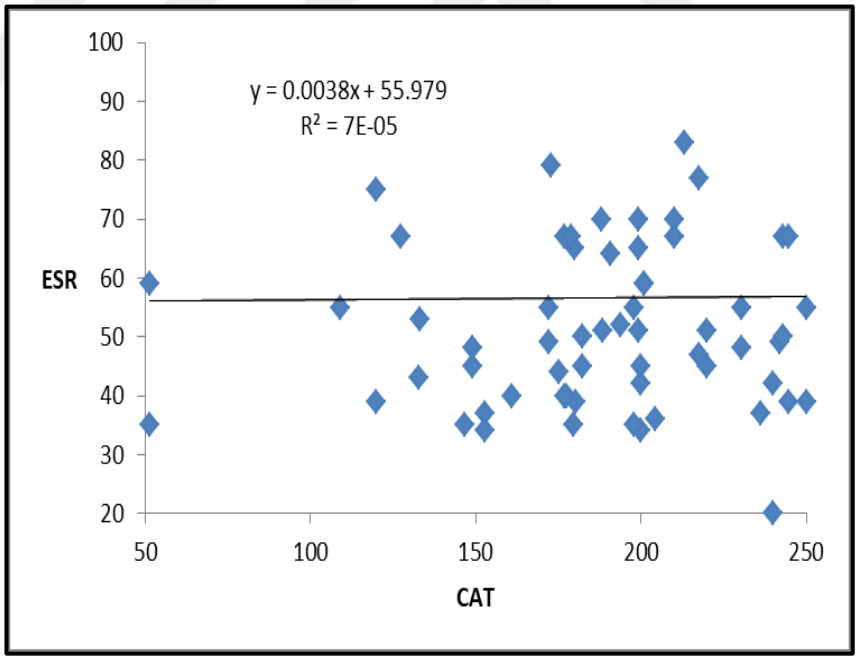

Figure 4.34 Correlation of (CAT) with (ESR)

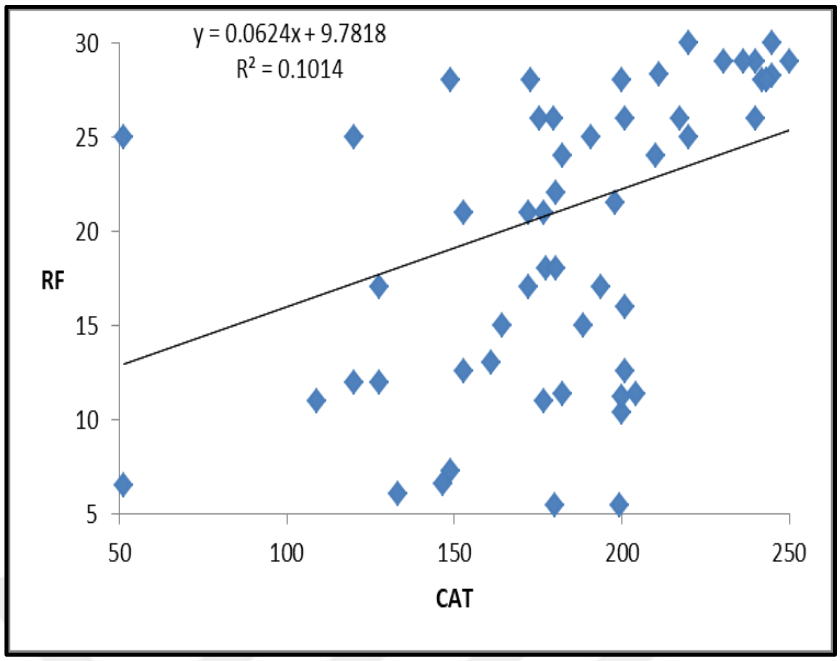

Figure 4.35 Correlation of (CAT) with (RF)

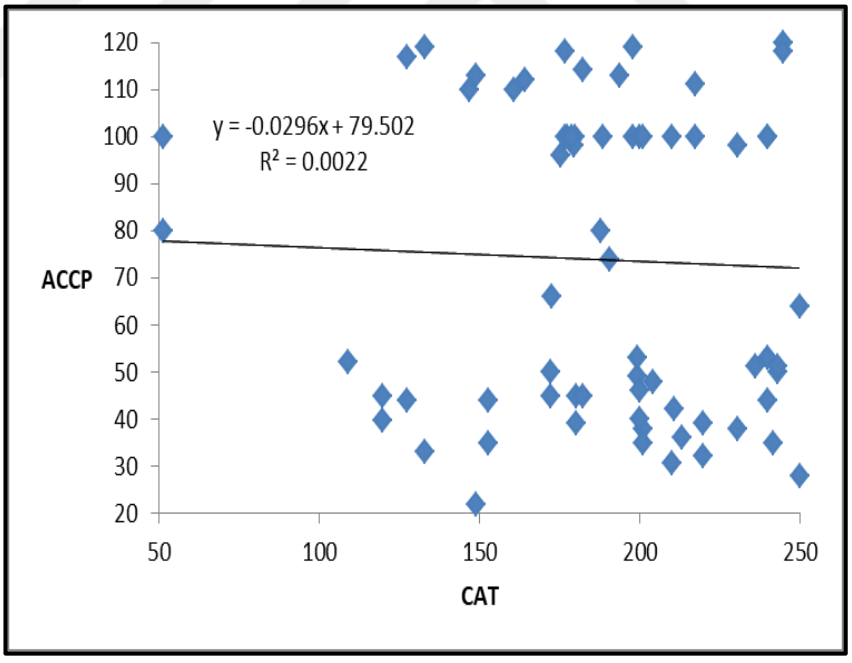

Figure 4.36 Correlation of (CAT) with (ACCP)

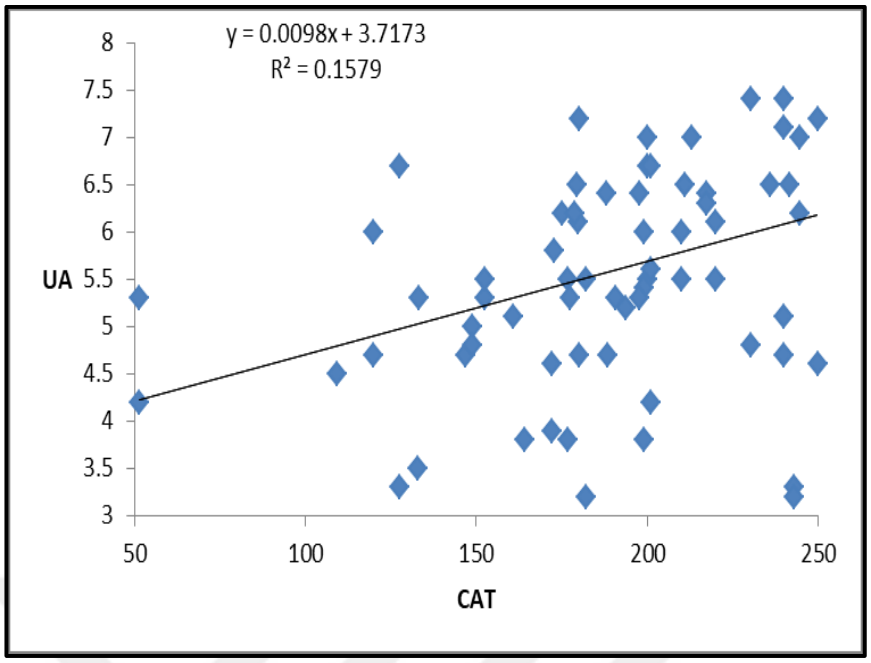

Figure 4.37 Correlation of (CAT) with (UA)

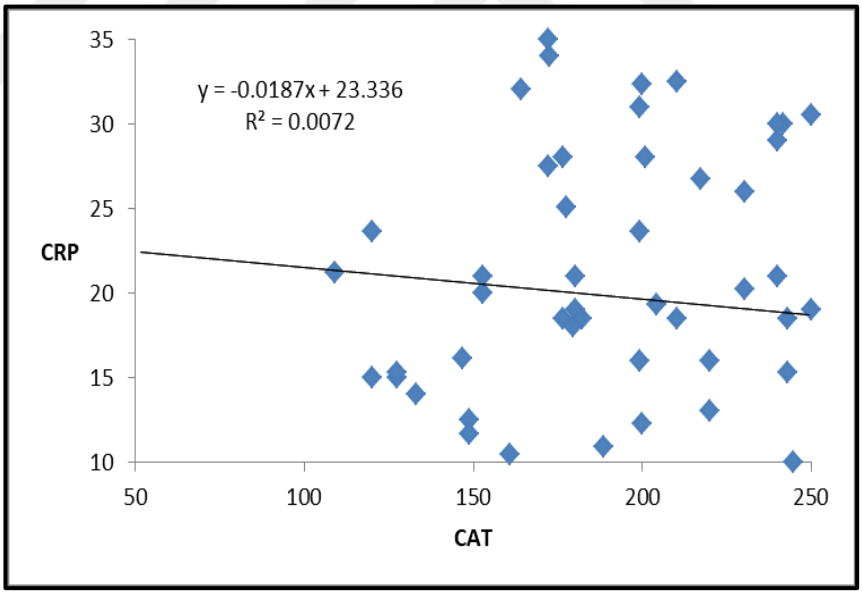

Figure 4.38 Correlation of (CAT) with (CRP)

4.3.5 Correlation of SOD with the other parameters

The clinical characteristics of the patients group that interpret the correlativity between SOD and a number of other variables in the Table 4.16 showed that there was positive correlation significant between SOD with UA r=0.18 (Figure 4.44). While there was weak positive correlation between SOD with Age r=0.07 Figure 4.39, and RF r=0.05 (Figure 4.42). Moreover, weak negative correlation was observed between SOD with ESR r= -0.03 (Figure 4.41). Also negative correlation significant was observed between SOD and BMI r= -0.22 Figure 4.40, ACCP r= -0.20 Figure 4.43, and CRP r= -0.12 (Figure 4.45).

Table 4.16 Correlation of (SOD) with the Studied Parameters

Analyzed parameter	Parameters	Pearson correlation (r)
SOD n=81	Age	0.07
	BMI	-0.22
	ADP	0.19
	MDA	-0.15
	PC	0.03
	CAT	0.11
	ESR	-0.03
	RF	0.05
	ACCP	-0.20
	UA	0.18
	CRP	-0.12

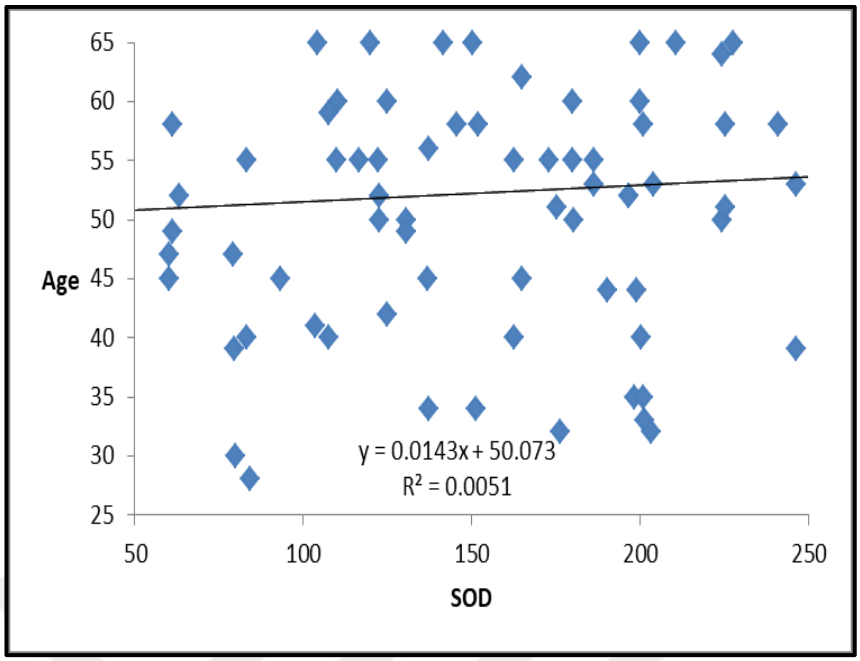

Figure 4.39 Correlation of (SOD) with (Age)

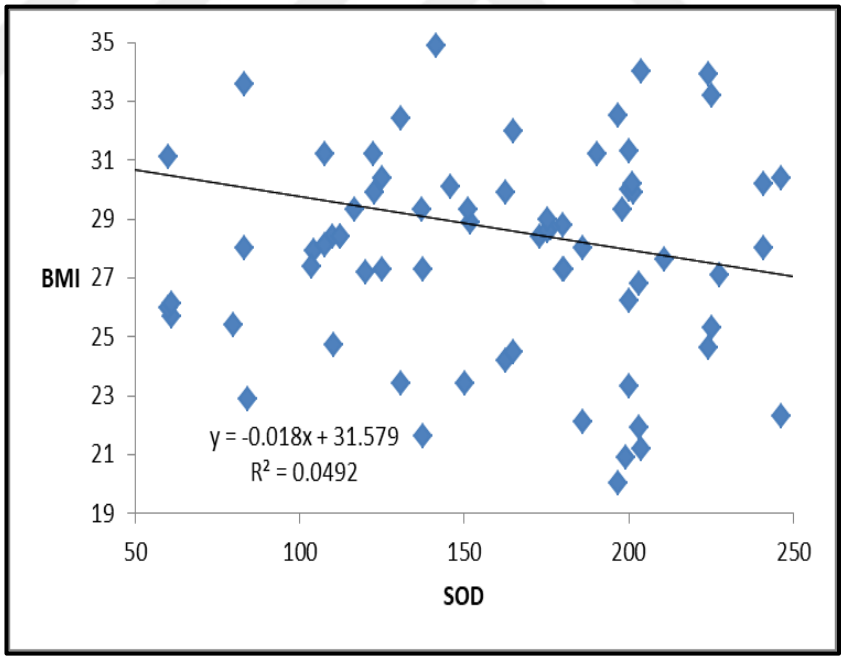

Figure 4.40 Correlation of (SOD) with (BMI)

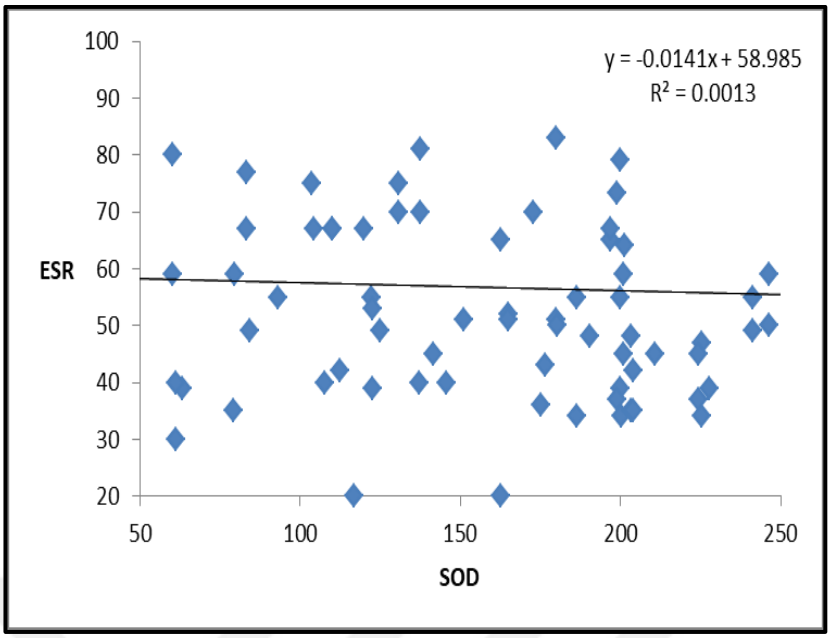

Figure 4.41 Correlation of (SOD) with (ESR)

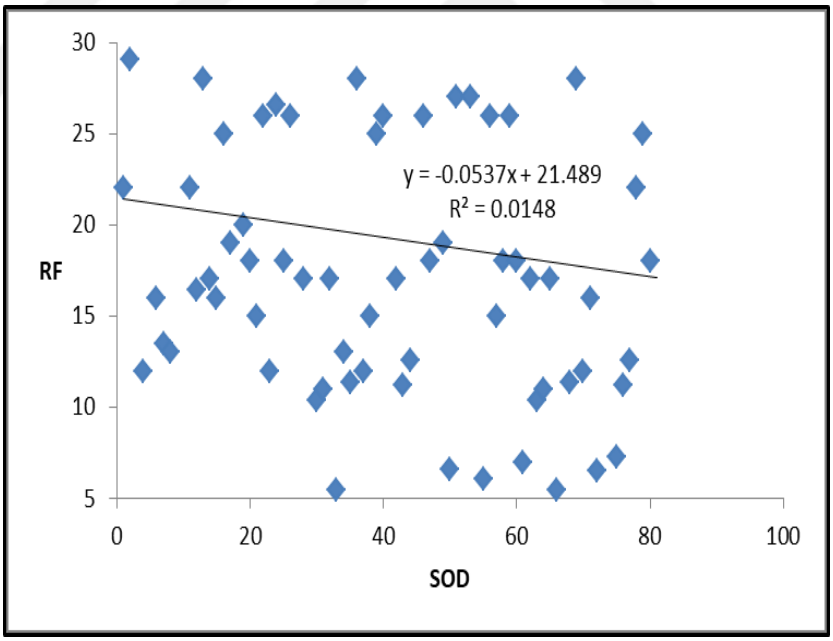

Figure 4.42 Correlation of (SOD) with (RF)

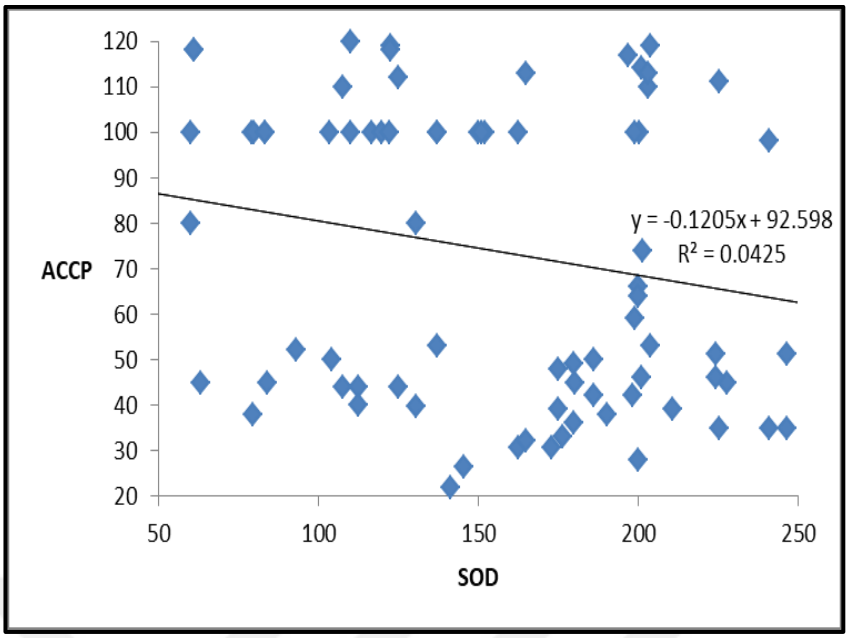

Figure 4.43 Correlation of (SOD) with (ACCP)

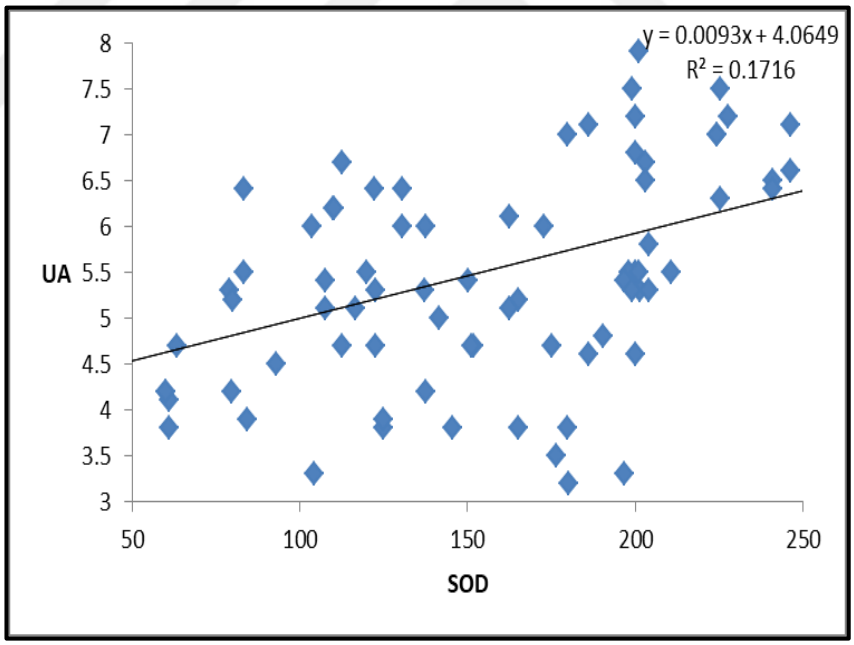

Figure 4.44 Correlation of (SOD) with (UA)

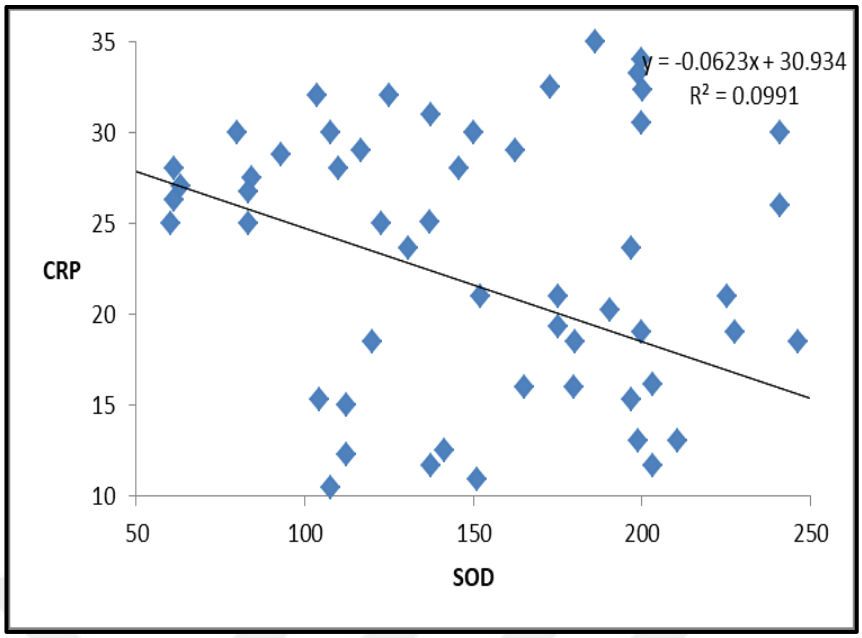

Figure 4.45 Correlation of (SOD) with (CRP)

4.4 Receiver Operating Characteristic Curve (ROC) Analysis

The AUC values, Sensitivity, and Specificity of the all studied parameters that used in this study have been given in the (Table 4.17). The ROC curve of the ADP levels have shown an excellent method for discriminating between patients with RA and healthy controls [AUC=0.918; P < 0.001; 95% confidence interval (CI): 0.857 to 0.959: Standard Error (SE): 0.022; sensitivity = 81.5%; specificity = 88%] (Figure 4.46). Also the MDA values offered very important levels of discrimination [AUC=0.969; P < 0.001; 95% CI: 0.923 to 0.992; SE: 0.012; sensitivity = 90.1%; specificity = 100%] (Figure 4.47).

Our study also found that PC values is a good way for distinguish between RA patients and healthy controls [AUC=0.727; P < 0.001; 95% CI: 0.643 to 0.801; SE: 0.045; sensitivity = 58%; specificity = 100%] (Figure 4.48).While; the ROC curve of CAT levels also demonstrated a poor mark to differentiation [AUC=0.653; P < 0.005; 95% CI: 0.565 to 0.734; SE: 0.053; sensitivity = 100%; specificity = 44%] (Figure 4.49).

The current study gives a demonstration that the values of SOD also a good mark for discrimination [AUC=0.750; P < 0.001; 95% CI: 0.667 to 0.822; SE: 0.051; sensitivity = 96.3%; specificity = 62%] (Figure 4.50). Whereas; ESR and ACCP showed excellent method for discriminatingly between patients with RA and healthy controls [AUC=1.000; P < 0.001; 95% CI: 0.972 to 1.000; SE: 0; sensitivity = 100%; specificity = 100%] Figure 4.51, [AUC=0.960; P < 0.001; 95% CI: 0.911 to 0.987; SE: 0.014; sensitivity = 81.5%; specificity = 100%] Figure 4.53 respectively.

Moreover, RF showed discriminatory efficacy between healthy individuals and patients with RA [AUC=0.966; P < 0.001; 95% CI: 0.919 to 0.990; SE: 0.013; sensitivity = 92.6%; specificity = 94%] (Figure 4.52). CRP is considered as an extremely significant parameter for RA risk [AUC=0.984; P < 0.001; 95% CI: 0.945 to 0.998; SE: 0.007; sensitivity = 91.4%; specificity = 98%] (Figure 4.54).UA values considered as an extremely parameter for RA risk [AUC=0.894; P < 0.001; 95% CI: 0.829 to 0.941; SE: 0.027; sensitivity = 76.5%; specificity = 100%] (Figure 4.55).

Table 4.17 The area under the ROC curve for all analyzed parameters

Parameters	AUC	Std. Error	95% confidence interval	P-value	Sensitivity%	Specificity%
ADP	0.918	0.022	0.857 to 0.959	< 0.001	81.5%	88%
MDA	0.969	0.012	0.923 to 0.992	< 0.001	90.1%	100%
PC	0.727	0.045	0.643 to 0.801	< 0.001	58%	100%
CAT	0.653	0.053	0.565 to 0.734	< 0.005	100%	44%
SOD	0.750	0.051	0.667 to 0.822	< 0.001	96.3%	62%
ESR	1.000	0	0.972 to 1.000	< 0.001	100%	100%
RF	0.966	0.013	0.919 to 0.990	< 0.001	92.6%	94%
ACCP	0.960	0.014	0.911 to 0.987	< 0.001	81.5%	100%
UA	0.894	0.027	0.829 to 0.941	< 0.001	76.5%	100%
CRP	0.984	0.007	0.945 to 0.998	< 0.001	91.4%	98%

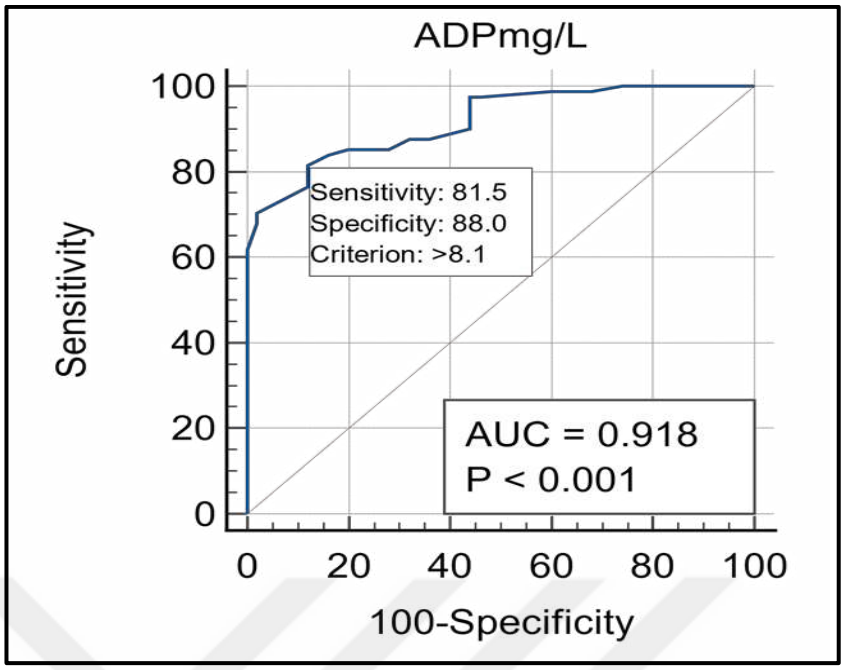

Figure 4.46 ROC curve showing AUC between sensitivity and specificity for ADP

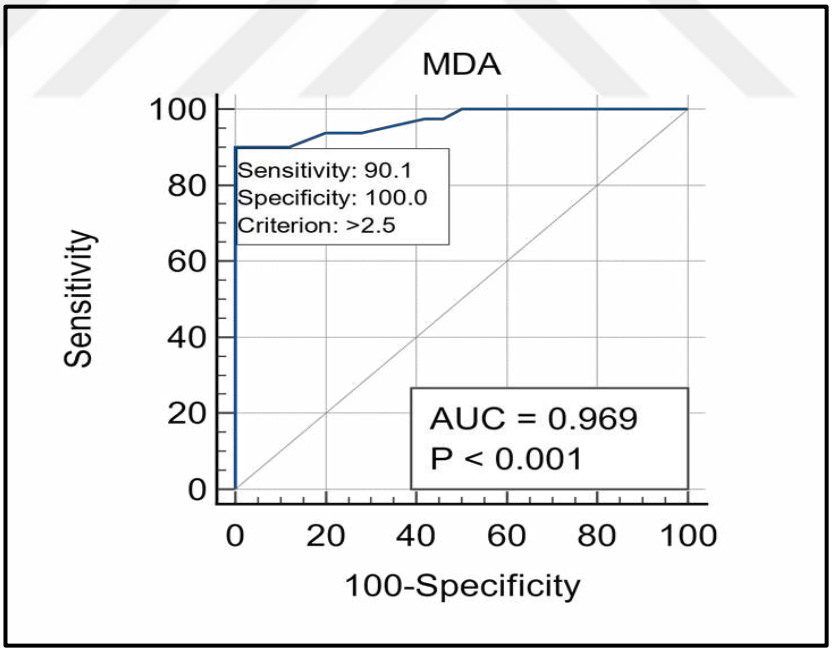

Figure 4.47 ROC curve showing AUC between sensitivity and specificity for MDA

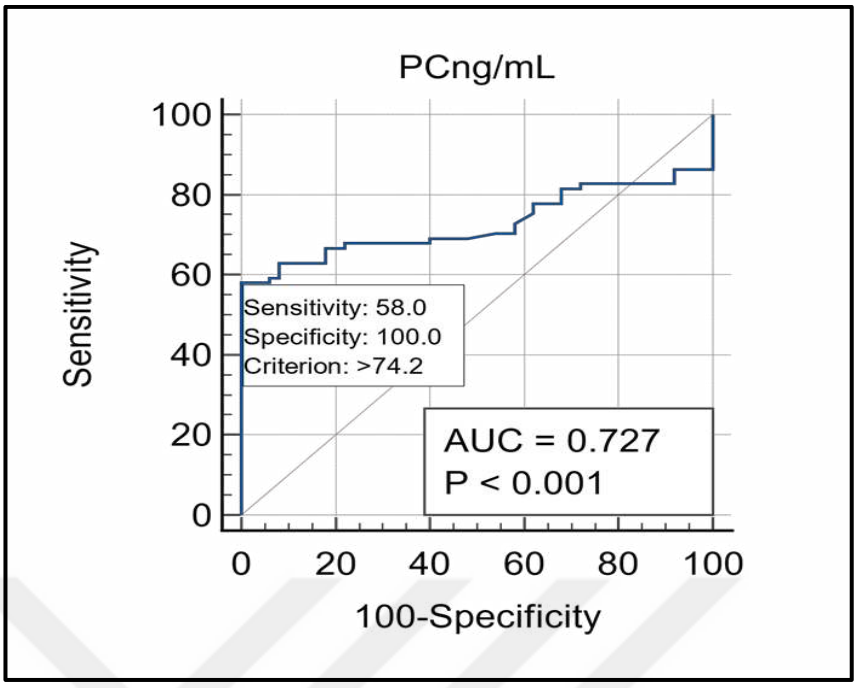

Figure 4.48 ROC curve showing AUC between sensitivity and specificity for PC

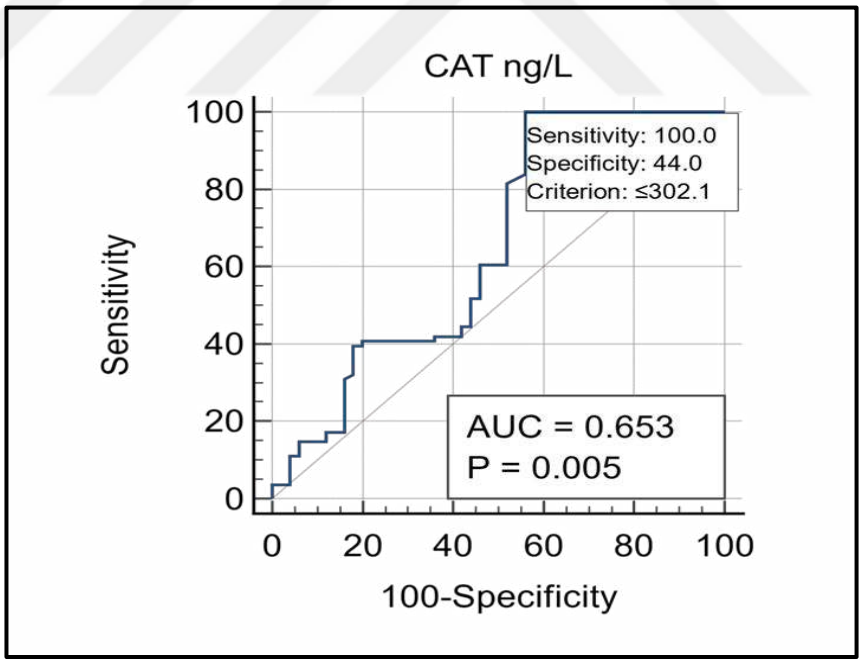

Figure 4.49 ROC curve showing AUC between sensitivity and specificity for CAT

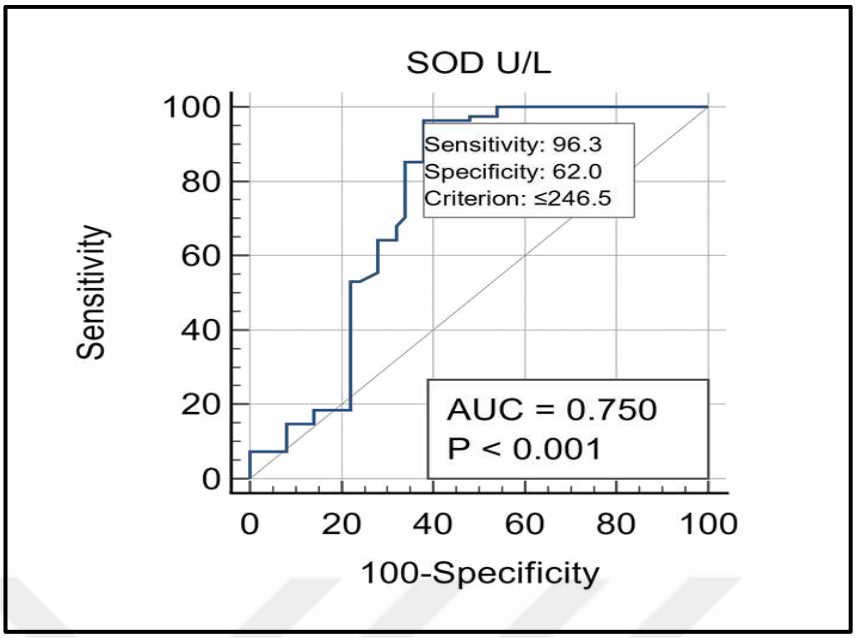

Figure 4.50 ROC curve showing AUC between sensitivity and specificity for SOD

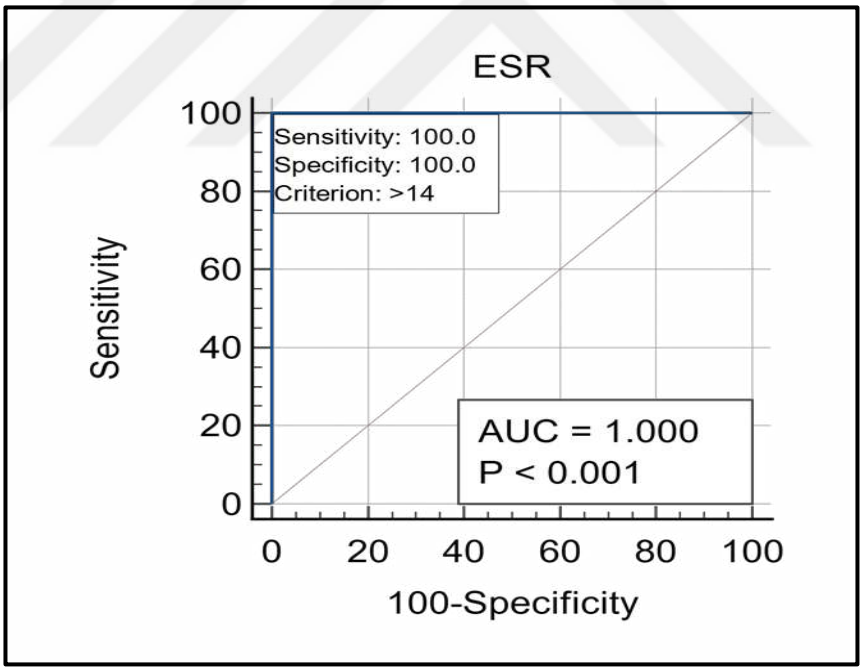

Figure 4.51 ROC curve showing AUC between sensitivity and specificity for ESR

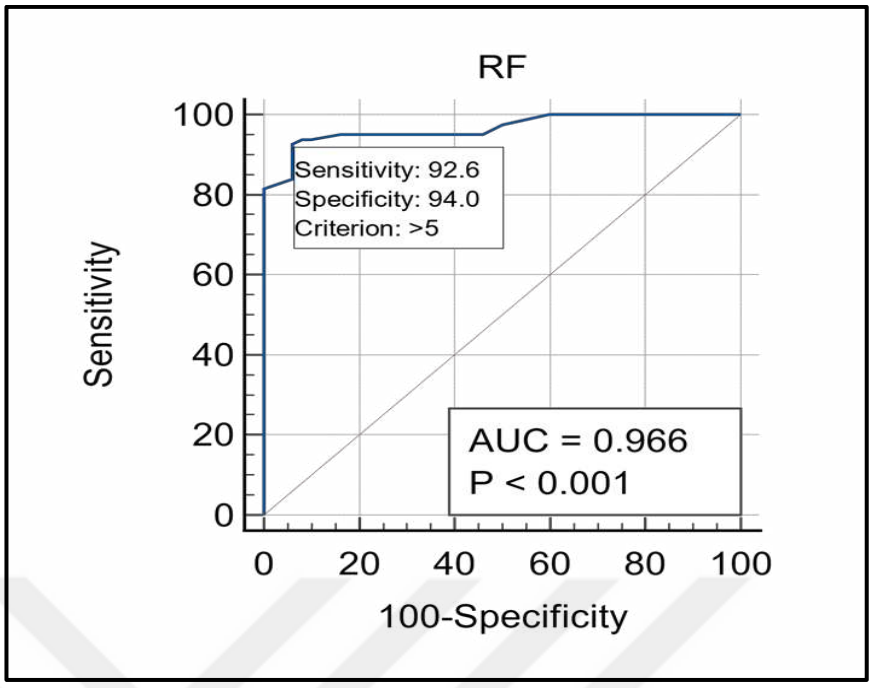

Figure 4.52 ROC curve showing AUC between sensitivity and specificity for RF

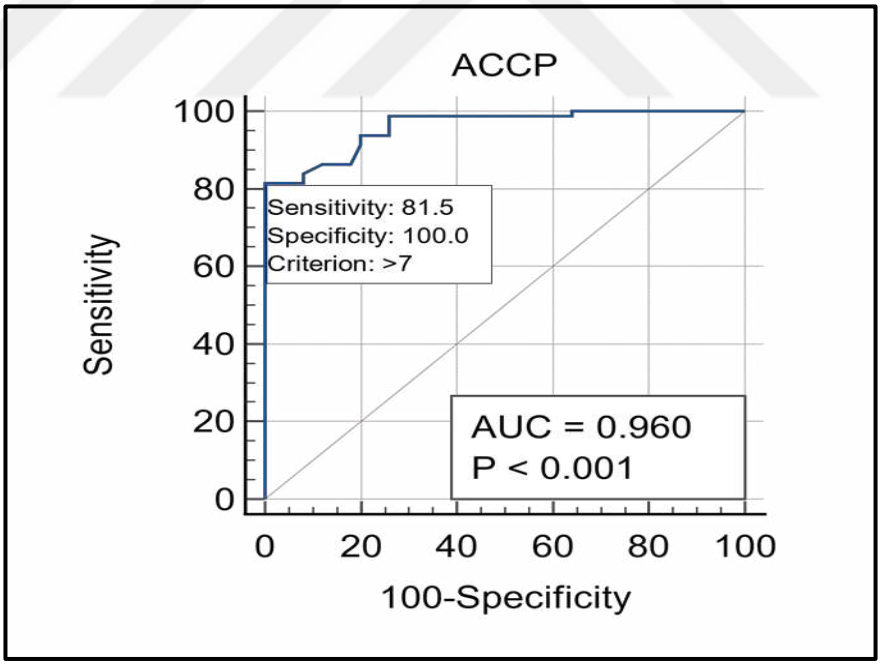

Figure 4.53 ROC curve showing AUC between sensitivity and specificity for ACCP

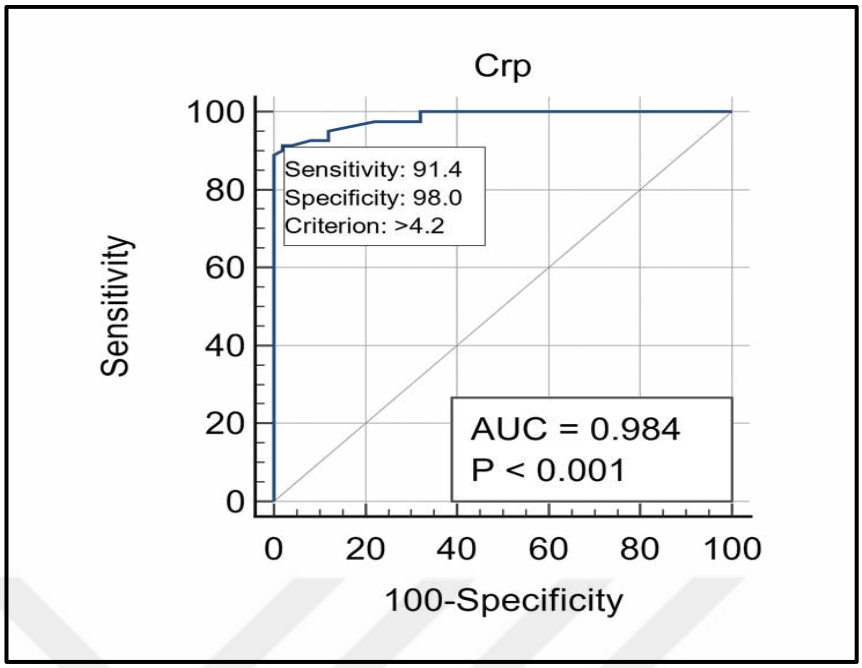

Figure 4.54 ROC curve showing AUC between sensitivity and specificity for CRP

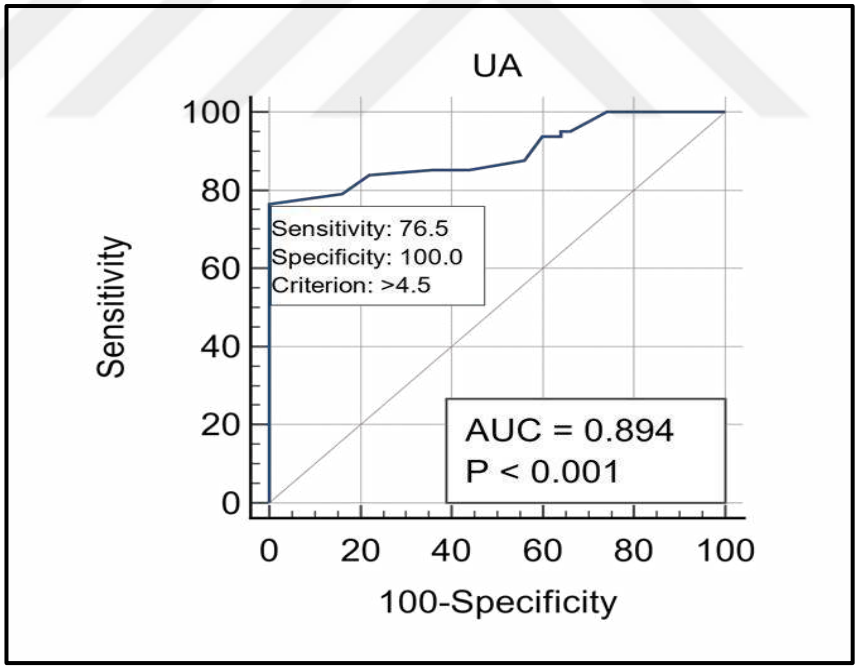

Figure 4.55 ROC curve showing AUC between sensitivity and specificity for UA

5. DISCUSSION

RA is a severe inflammatory illness characterized by persistent inflammation of the joints. RA is the most common kind of arthritis, and it is prevalent in almost all human societies, affecting about 0.5-1 % of the global population where it varies from one place to another. In most cases, females are affected more than males, with an approximately 3:1 female to male ratio, and the disease usually manifests itself later in life. Additionally, these individuals with RA also have more mortality and morbidity, particularly becaose of cardiovascular disease (CVD) (Reed 2017).

Synovial tissue in the joint is mostly impacted by RA. Synovial membrane, seen in healthy people, is made up of macrophages and synovial fibroblasts that regulate joint homeostasis. In RA, as a consequence of synovial inflammation, the pannus (a thick cellular layer) develops, which grows across the articular surface. The inflamed joint is filled with pro-inflammatory cytokines, chemokines, prostaglandins, and cartilage-degrading enzymes like metalloproteinases, in particular, neutrophils, macrophages, and T- and B-lymphocytes (Hitchon 2011).

5.1 General Comparison and the Effect of Gender, Age, and BMI on Studied Parameters

In our study, we made a general comparison of the studied parameters between the patients with RA and the healthy control subjects. In addition, explained the effect of gender, age, and BMI on all parameters that taken in this study.

5.1.1 Adiponectin (ADP)

Adiponectin is a secreted product of adipocytes, a kind of cell found in white adipose tissue that is highly metabolically active. Adipokines, which play a crucial role in both physiological and pathological processes (Szumilas *et al.* 2020).

The data were first analyzed in patients with RA and healthy controls subjects in Table 4.1 showed that serum ADP levels in RA patients were higher than healthy controls. These results leads that ADP levels were significantly increased in RA patients compared with healthy controls (P <0.001). This may be explained by adiponectin's favourable anti-inflammatory impact on inflammatory cytokines. Instead, adiponectin may increase the inflammatory response in RA by promoting inflammatory mediators that are secreted by RA synovial fibroblasts. Contrary to the results that inflammation inhibits adiponectin synthesis (Lee and Bae 2018). Our results came agree with several studies like study conducted by (Khajoei *et al.* 2019, Mohammed *et al.* 2021). That reported there was significant increase of ADP levels in patients with RA compared healthy controls (P>0.001). Another study showed that there was significant increase in ADP levels in RA patients more than healthy controls (P>0.01) (Lee and Bae 2018). In addition, a study conducted by (Šenolt *et al.* 2006). Agreed with our results observed that serum ADP was significantly high in RA patients compared with controls (P<0.02). Moreover, study also showed that ADP levels in patients with RA were higher than in healthy controls ($p \leq 0.003$) (Laurberg *et al.* 2009). Other Previous studies (Ebina *et al.* 2009, Abo-ragab *et al.* 2012). Reported that there were high significant increase of ADP in patients with RA compared with healthy controls (P>0.001).

The current study results in Table 4.2 showed that the effect of gender, age, and BMI on ADP in patients with RA were no significant effect of any of gender, age, and BMI on ADP (P>0.05) according to the comparison between the gender, age, and BMI categories. And these results agree with other studies findings that there was no significant effect of any of gender, age, and BMI on ADP in patients with RA (P>0.05) (Šenolt *et al.* 2006, Ebina *et al.* 2009, Laurberg *et al.* 2009, Yoshino *et al.* 2011, Chennareddy *et al.* 2016, Lei *et al.* 2020).

5.1.2 Malondialdehyde (MDA)

MDA is an indicator of oxidative stress since it is a lipid peroxidation result that is used to quantify the amount of damaged lipids in the cell membrane caused by ROS, elevated MDA levels affect the permeability of cell membranes. Numerous enzymatic and non-

enzymatic antioxidants work to reduce the high levels of MDA by converting superoxide free radicals to hydrogen peroxide or oxygen molecules (Vijayakumar *et al.* 2006, García-González *et al.* 2015).

The data from persons with RA and healthful controls were examined, as demonstrate in Table 4.1 the results showed that serum MDA levels were significantly increased in RA patients compared with healthy controls group (P <0.001). The concentration of MDA present in the blood serum was used to assess lipid peroxidation. The increase in lipid peroxidation product may be related to an increase in the production of ROS, which tend to accumulate in large quantities during chronic inflammation and cause excessive damage to the tissues (Aljoboury *et al.* 2020). The results of our study are in line with results of other studies conducted by (Toosi *et al.* 2020) and (Das *et al.* 2021). That showed there were a considerable elevate in the level of MDA in persons with RA as comparison with healthy groups (P < 0.001), (P<0.01) respectively. In addition, studies by (Ozkan *et al.* 2007, Vasanthi *et al.* 2009, Vyas *et al.* 2016). Showed the level of MDA was high significant increase in RA patients compared with controls (P < 0.03), (P < 0.001), (P < 0.001) respectively.

The results of present study in Table 4.3 showed the effect of gender, age, and BMI on MDA in patients with RA, the results were clarified that there was no significant effect of any of gender, age, and BMI on MDA (P>0.05) according to the comparison between the gender, age, and BMI categories.

5.1.3 Protein carbonyls (PC)

Protein carbonyls are a by-product of the oxidation of proteins by ROS and RNS, most frequently lysine, arginine, and proline. The concentration of PC in blood and tissues is a sensitive biomarker of protein oxidation. It is especially advantageous in this regard due to its long-term stability at appropriate storage temperatures (i.e., -80°C). PC is the most frequently used and most basic biomarker of protein oxidative degradation (Fernando *et al.* 2016).

Following an examination of data from individuals with RA and healthful controls, as indicated in Table 4.1 the results showed that serum PC levels were significantly higher in RA patients compared with healthy controls group (P <0.001). Despite conflicting views on the significance of oxidative stress in the origin and maintenance of the damage seen in RA, there is evidence that it may contribute to the disease's aetiology, it has been observed that oxidative damage in synovial fluid is associated with structural alterations in hyaluronic acid, collagen, and cartilage, as well as increased lipid peroxidation, PC content, and DNA alterations (García-González *et al.* 2015). The results of our study are in line with results of other studies like a study conducted by (Mititelu *et al.* 2020) that showed serum PC levels were significantly high in RA patients compared with healthy controls group (P <0.05). Another study performed by (Mahmoud and Ismail 2011) reported that serum PC levels was high significant in patients with RA more than healthy controls (P < 0.001). More study observed that there was high increase significant in PC levels in patients with RA more than healthy controls (P < 0.001) (Tetik *et al.* 2010). Moreover a research by (García-González *et al.* 2015) recorded a remarkable elevate in PC levels in RA patients as compared with healthy individuals (P<0.05). Finally a study by (Mateen *et al.* 2016) agree with our results that showed there was high increase significant in serum PC levels in RA patients compared with healthy controls group (P < 0.001).

The results of our study in Table 4.4 showed the effect of gender, age, and BMI on MDA in patients with RA the results were clarified that there was no significant effect of any of gender, age, and BMI on MDA (P>0.05) according to the comparison between the gender, age, and BMI categories.

5.1.4 Catalase (CAT)

CAT is enzymatic antioxidants. Antioxidants are exogenous or endogenous chemicals that either prevent the generation of harmful oxidants or bind to and inactivate those that are generated, thus preventing the chain reaction caused by these oxidants from propagating. In recent years, growing experimental and clinical evidence has shown the

role of FR/ROS in a variety of pathophysiological conditions, including RA (Mahajan and Tandon 2004).

As shown in Table 4.1, after an analysis of the data from patients with RA and healthy controls the results showed that serum CAT levels were significantly lower in RA patients compared with healthy controls group (P <0.001). This is because increased oxidative stress and/or a deficient antioxidant state contribute to the pathophysiology of RA, as well as the invasion of ROS (Mahajan and Tandon 2004, Toosi *et al.* 2020). Our findings corroborate those of previous research, such as one performed by (Kamanli *et al.* 2004) that showed serum CAT levels were significantly decreased in RA patients compared with healthy controls group (P <0.001). In addition, a study by (Kerimova *et al.* 2000) reported that serum CAT levels was low significant in patients with RA compared with healthy controls (P < 0.001). Another study performed by (Toosi *et al.* 2020) that showed serum CAT levels were significantly lower in RA patients compared with healthy controls group. and finally two study by (Mahajan and Tandon 2004), and (Mateen *et al.* 2016) also reported that the levels of serum CAT were significantly lower in RA patients compared with healthy controls group.

The results of our study in Table 4.5 showed that the effect of gender, age, and BMI on MDA in patients with RA, the results were clarified that there was no significant effect of any of gender, age, and BMI on MDA (P>0.05) according to the comparison between the gender, age, and BMI categories.

5.1.5 Superoxide dismutase (SOD)

The SODs are a class of enzymes that catalyze the dismutation of the superoxide radical anion very effectively (O2-) (McCord and Edeas 2005). SODs are metalloenzymes that use a redox-active metal to reduce two molecules of superoxide to oxygen and hydrogen peroxide, the latter of which is eliminated by catalase and peroxidase enzymes (Broxton and Culotta 2016).

As shown in Table 4.1, after an analysis of the data from patients with RA and healthy controls the results showed that serum SOD levels were significantly lower in RA patients compared with healthy controls group (P <0.001). Since superoxide is formed as a consequence of the dismutation of superoxide radical, SOD helps the body defend against free radical production by helping the body detoxify superoxide and hydrogen peroxide, which otherwise causes the inactivation of catalase. It is possible that the reduced SOD activity is related to the ROS-mediated destruction of SOD occurring during the detoxification process. Our findings corroborate those of previous research, such as performed by (Akyol *et al.* 2001, García-González *et al.* 2015, Mateen *et al.* 2016) that revealed that serum SOD levels were significantly lower in RA patients compared with healthy controls group (P<0.05). Another studies also reported that there was significant decrease in serum SOD levels in patients with RA compared with healthy controls (P<0.001) (El-barbary *et al.* 2011, Mateen *et al.* 2018).

The results of the current study in Table 4.6 showed that the effect of gender, age, and BMI on SOD in patients with RA, the results were clarified that there was no significant effect of any of gender, age, and BMI on SOD (P>0.05) according to the comparison between the gender, age, and BMI categories.

5.1.6 Erythrocyte sedimentation rate (ESR)

The ESR is a kind of blood test that determines the rate at which erythrocytes settle to the bottom of a test tube containing a blood sample, Red blood cells normally settle rather slowly. A faster-than-normal rate may reflect that the body is inflamed. Inflammation is a natural component of the immune response system. It may occur as a result of an illness or damage. Additionally, inflammation may be a symptom of a chronic illness, an immunological issue, or another medical problem (Suliman 2019).

As shown in Table 4.1, after an analysis of the data from patients with RA and healthy controls, the results showed that the levels of ESR in patients with RA were significantly increased as compared with healthy controls subjects (P<0.001). The recent study's findings indicated that the increased levels of ESR indicate the presence

of inflammation in the body, therefore ESR can be considered as marker of inflammation. The results of our study are in line with results of other studies like a research done by (El-barbary *et al.* 2011) showed that there was high significant of ESR in RA patients than controls group (P<0.001). Another study also revealed that ESR levels was significantly increase in patients with RA as compared with healthy group (P<0.01) (Tetik *et al.* 2010). In addition, a researchs done by (Shen *et al.* 2015, Narayan *et al.* 2019, Reyes-Pérez *et al.* 2019) showed same our results that ESR levels was significantly increase in patients with RA as compared with healthy group (P<0.001).

The results of our study in Table 4.7 showed that the effect of gender, age, and BMI on ESR in patients with RA the results were clarified that there was no significant effect of any of gender, age, and BMI on ESR (P>0.05) according to the comparison between the gender, age, and BMI categories.

5.1.7 Rheumatoid factor (RF)

Rheumatoid factors (RFs), a class of immunoglobulins (Igs) with distinct isotypes and affinities, were discovered more than 70 years ago, but much remains unknown regarding their production, physiological function, and pathological conditions. RF isotypes are beneficial in the management of RA patients from the time of diagnosis to the time of therapeutic strategy selection. RF testing in RA patients has a sensitivity of 60% to 90% and a specificity of 85% (Ingegnoli *et al.* 2013). RF testing is not appropriate in individuals with musculoskeletal pain and no joint swelling for screening for RA. It is important to note that the predictive value of a test for RA relies on the sensitivity and specificity of the test and the pre-test incidence of RA. The sensitivity of a test that measures RF will be decreased since RF is present in many different diseases, and is not present in all individuals with RA (Wilson 2006).

As shown in Table 4.1, after an analysis of the data from patients with RA and healthy controls, the results showed that the levels of RF in patients with RA were significantly increased as compared with healthy controls subjects (P<0.001). RF is a considerable

predictor of poor outcome in individuals with RA. A higher risk of joint erosion, extra-articular manifestations, and greater impairment may be seen at high concentrations. The deposition of RF in tissue promotes the formation of Rheumatoid Nodes (Wilson 2006). The results of our study are in line with results of other studies as in a research study by (Saleh *et al.* 2020) showed that the level of RF was significantly higher in RA patients in compare with healthy controls (P<0.001). Another study performed by (Narayan *et al.* 2019) also showed that the levels of RF in patients with RA was significant increased compared with healthy subjects (P<0.001). (Shen *et al.* 2015) also observed that there was significant increase in levels of RF in patients with RA (P<0.01).

The results of the present study in Table 4.8 showed that the effect of gender, age, and BMI on RF in patients with RA the results were clarified that there was no significant effect of any of gender, age, and BMI on RF (P>0.05) according to the comparison between the gender, age, and BMI categories.

5.1.8 Anti-cyclic citrullinated peptides (ACCP)

Anti-cyclic citrullinated peptide (CCP) detection is a relatively new diagnostic for the diagnosis of RA. The test has a comparable sensitivity as RF but a higher specificity. Sensitivity is between 39 and 94%, while specificity is between 81 and 100% (Abdul Wahab *et al.* 2012). Recently, new criteria for diagnosing RA were established, which include anti-CCP as well as RF (Aletaha *et al.* 2010).

As shown in Table 4.1, after an analysis of the data from patients with RA and healthy controls, the results showed that the levels of ACCP in patients with RA were significantly increased as compared with healthy controls subjects (P<0.001). As a result, individuals with anti-CCP antibodies have a more severe disease course and greater radiological damage than patients with RA who do not have these autoantibodies (Van der Helm-van Mil *et al.* 2005). The results of our study are agreement with a results of many studies conducted by (Reyes- Shen *et al.* 2015, Pérez

et al. 2019, Alwan and Ghali 2021) revealed that there were significant increase in levels of ACCP in patients with RA compared with healthy controls.

The results of our study in Table 4.9 showed that the effect of gender, age, and BMI on ACCP in patients with RA, the results were clarified that there was no significant effect of any of gender, age, and BMI on ACCP (P>0.05) according to the comparison between the gender, age, and BMI categories.

5.1.9 Uric acid (UA)

Uric acid (UA) is formed during the purine metabolism and is further reduced to allantoin in most mammals by the liver enzyme uricase (urate oxidase) (Watanabe *et al.* 2002). As an oxidizable substrate for hemoprotein and hydrogen peroxide, UA has the ability to shield cells from oxidative damage by serving as an electron donor. It may also chelate metal ions, turning them into less reactive forms that can then be used to catalyze free radical processes (Senthilkumar and Reddy 2020).

As shown in Table 4.1, after an analysis of the data from patients with RA and healthy controls, the results revealed that the levels of serum UA in patients with RA were significantly increased as compared with healthy controls subjects (P<0.001). UA's function in oxidative stress-related diseases is not completely understood. Increased blood UA levels are a risk factor for cardiovascular disease, where oxidative stress plays a significant pathophysiological role, according to evidence derived mostly from epidemiological research (Glantzounis *et al.* 2005). As a result, UA is a critical tool for assessing oxidative stress in humans. Additionally, UA is ionized to form urate, which has a low solubility in water and, when produced in excess, crystallizes, resulting in arthritis (Senthilkumar and Reddy 2020). The findings of the current research are in agreement with results of many studies conducted by (Chavan *et al.* 2015, Mohamed 2020) that showed there were a respectable elevate in the levels of UA in persons with RA as comparison with healthy subjects.

The results of our study in Table 4.10 showed that the effect of gender, age, and BMI on UA in patients with RA, the results were clarified that there was no significant effect of any of gender and BMI on UA (P>0.05) according to the comparison between the gender and BMI categories. While there was a significant effect of the age categories on UA in patients with RA (P> 0.05), Where the levels of UA in the first age category (<50 years) was higher than the second age category (>50 years).

5.1.10 C-reactive protein (C-RP)

C-reactive protein (CRP) is a molecule generated by the liver in reaction to inflammation; it is also known as highly sensitive CRP (hs-CRP) and ultra-sensitive C-reactive protein (us CRP). A high CRP level in the blood is a sign of inflammation, which may be produced by a number of diseases ranging from infection to cancer. Increased CRP levels may also suggest the presence of inflammation, such as a heart attack. The CRP level is expressed in milligrams per liter of serum (mg\L). A lower CRP value is preferable to a higher one since it indicates that the body is less inflamed (Suliman 2019).

As shown in Table 4.1, after an analysis of the data from patients with RA and healthy controls, the results showed that the levels of CRP in patients with RA were significantly increased as compared with healthy controls subjects (P<0.001). CRP is the most accurate and objective marker of disease progression, as well as a valuable predictive factor for joint destruction and functional prognosis in patients with RA due to the fact that CRP reflects both local and systemic inflammatory reactions. The CRP level may rise by 1,000 times or more in the presence of infection, inflammation, and tissue damage (Kim *et al.* 2015). The results of our study are in agreement with results of study conducted by (Das *et al.* 2021) displayed that there was a significant increase in serum CRP levels in patients with RA compared with healthy controls (P<0.01). Another study also showed that there was high significant increase in serum CRP levels in RA patients compared with healthy subjects (P<0.001) (El-barbary *et al.* 2011). Another two studies have results in line with our results regarding to the CRP (Tetik *et al.* 2010, Yazici *et al.* 2010).

The results of our study in Table 4.11 showed that the effect of gender, age, and BMI on CRP in patients with RA, the results were clarified that there was no significant effect of any of gender, age, and BMI on CRP (P>0.05) according to the comparison between the gender, age, and BMI categories.

5.2 Correlations Between all Clinical Parameters Studied in Patients of RA

In the current study the results in Table 4.12, that interpret the correlation of ADP with the other studied parameters showed that there were positive significant correlation between ADP with PC, CAT, and UA. While positive correlation but not significant was found between ADP with Age, BMI, SOD, and ACCP. A negative correlation was observed with MDA, ESR, RF, and CRP. The results of our study agree with several studied conducted by (Šenolt *et al.* 2006, Gonzalez-Gay *et al.* 2008, Ebina *et al.* 2009, Klein-Wieringa *et al.* 2011, Yoshino *et al.* 2011, Fioravanti *et al.* 2019, Lei *et al.* 2020).

The results of the current study in Table 4.13, interpret that the correlation of MDA with the other studied parameters showed a positive correlation between MDA with PC, ACCP, and CRP. While a negative correlation was found but not important between MDA with Age, BMI, ADP, SOD, and UA. These results are in agreement with (Bagis *et al.* 2005, Ozkan *et al.* 2007, Aljoboury *et al.* 2020). Positive correlation but not important was observed with CAT, ESR, and RF. Same our results are reported in studies conducted by (Hassan *et al.* 2011, Sinan *et al.* 2016).

In the current data in Table 4.14, interpret that the correlation of PC with the other studied parameters revealed a positive correlation between PC with ADP, MDA, RF, and CRP. While positive correlation but not important was observed with Age, BMI, CAT, SOD, ESR, ACCP, and UA. Some of our results are agree with studies conducted by (Lemarechal *et al.* 2006, García-González *et al.* 2015, Khorasani *et al.* 2020).

According to our data in the Table 4.15, interpret that the correlation of CAT with the other studied parameters the results showed a positive correlation between CAT with

SOD, RF, and UA. While a negative correlation was demonstrated between CAT with BMI. Correlation but not important was found between CAT with MDA, Age, PC, and ESR, ACCP, and CRP. The results of the current study were in line with studies conducted by (El-barbary *et al.* 2011, Veselinovic *et al.* 2014)

Finally depending on our results in Table 4.16, interpret that the correlation of SOD with the other studied parameters. Positive correlation was found between SOD with ADP, CAT, and UA. Negative correlation also was observed between SOD with BMI, MDA, ACCP, and CRP. No important correlation was found with Age, PC, ESR, and RF. The present study's findings are consistent with previous research (El-barbary *et al.* 2011, García-González *et al.* 2015, Kardeş *et al.* 2018).

5.3 Receiver Operating Characteristic Curve (ROC) Analysis for all Clinical Parameters

The receiver operating characteristic curve (ROC) curve is a graphical representation of sensitivity (TPR) on the y-axis and (1 – specificity) (FPR) on the x-axis for various cut-off points of test results. This is often represented in a square box for convenience, and its two axes are numbered from 0 to 1 on both edges. The area under the curve (AUC) is an efficient and integrated measure of sensitivity and specificity for evaluating a diagnostic test's intrinsic validity. Maximum AUC = 1, indicating that the diagnostic test is perfectly capable of discriminating between sick and non-diseased individuals (Kumar and Indrayan 2011).

The results of the current study in Table 4.17 presented that the AUC of ROC curve for Adiponectin (ADP) in patients and healthy controls (AUC=0.918) was very high (Figure 4.6). In this case ADP can be considered an excellent indicator of the onset of the disease and can prognosticate progression of disease and thus disease severity. In addition in the optimal cut-off point the sensitivity and specificity of ADP were (81.5%, 88%) respectively.

our results was agreed with studies conducted by (Toussirot and Dumoulin 2014, Kuryata and Sirenko 2017, Grygiel-Górniak *et al.* 2018, Mohammed *et al.* 2021) indicated that ADP has predictive properties to the RA.

In the same (Table 4.17) the results revealed that the AUC of lipid peroxidation. Malondialdehyde (MDA) in patients with RA and healthy controls (AUC=0.969) was very high (Figure 4.7). These results indicate that MDA can be excellent predictive marker and had an important role in pathophysiology of RA. The sensitivity and specificity of MDA in the optimal cut-off point were (90.1%), (100%) respectively. The findings of our investigation was in line with other studies (Liao *et al.* 2018, Ryndina 2019, Mititelu *et al.* 2020) that indicated that lipid peroxidation MDA has predictive properties to the RA.

Furthermore, our results showed the AUC of PC in patients with RA and healthy controls (AUC=0.727) was moderate (Figure 4.8). These results indicated that PC has a fair predictive marker to the RA and the sensitivity and specificity of PC in the optimal cut-off point were (58%), (100%) respectively. The results of our study was in line with other study (Mititelu *et al.* 2020). Moreover the results of our study demonstrated that the AUC of Catalase (CAT) in patients with RA and healthy controls in ROC curve was poor (AUC=0.653). These results indicate that catalase has a poor predictive ability for RA. The sensitivity and specificity of CAT in the optimal cut-off point were (100%), (44%) respectively. The results of our study was in line with other study (Ryndina 2019). The AUC of ROC curve of SOD in patients with RA and healthy controls was fair (AUC= 0.750). These results leads to that SOD have a fair predictive ability for RA. The sensitivity and specificity of SOD in the optimal cut-off point were (96.3%), (62%) respectively; despite lack of previous reports supporting our finding regarding the ROC curve of serum SOD with predictive of RA.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

According to the findings of this research on antioxidant and inflammatory biomarkers in RA patients, the following conclusions may be reached:

1. The current study concludes that there were increase in the level of serum concentration of ADP, MDA, and PC in RA patients, and also there were decrease in the level of serum concentration of CAT, and SOD in the same group of the study.
2. There were no negative consequences of age, gender, and BMI on the studied parameters in this study.
3. There is a marked elevated in the cnocentration of UA in RA patients. UA function in oxidative stress-related diseases is not completely understood. As a result, UA is a critical tool for assessing oxidative stress in humans.
4. Low levels of antioxidants can lead to elevate the levels of oxidative stress and this elevated may be related to an raise in the production of ROS.
5. A positive significant correlation between ADP with PC, SOD, CAT, and UA. Positive significant correlation between MDA with PC, ACCP, and CRP. Positive significant correlation between PC with RF, and CRP. Positive significant correlation between CAT with SOD, RF, and UA. Positive significant correlation between SOD with UA.
6. By using ROC curve, the study concludes that ADP and MDA is an excellent prognostic indicator for RA. PC and SOD can considered a fair prognostic indicator for RA. CAT is a poor prognostic indicator for RA.
7. The present study demonstrated that RA is associated with oxidative stress and this can see by the increase the level of MDA and PC in RA patients.

6.2 Recommendations

1. An increase in the number of samples in a future study.
2. Study the types of ADP which are: low molecular weight (LMW), medium molecular weight (MMW), and high molecular weight (HMW).
3. Intensive study should be done on the effect of PC, SOD, and CAT on the RA patients.

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