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Department of Clinical Laboratories

Evaluation of Some Immunological Parameters and Bacterial Lipopolysaccharide with Rheumatoid Arthritis

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Written by

(Zahraa Falah Mahdi)

B.Sc. in Clinical Laboratories /Applied Medical Sciences/University of Kerbala, 2017

Supervised by

Prof. Dr. Suhad Hadi Mohammed

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بِسۡمِ ٱللَّهِ ٱلرَّحۡمَٰنِ ٱلرَّحِيمِ

(وَقُلْ رَبِّ أَدْخِلْنِي مُدْخَلَ صِدْقٍ وَأَخْرِجْنِي مُخْرَجَ صِدْقٍ وَاجْعَلْ لِي مِنْ لَدُنْكَ سُلْطَانًا نَصِيرًا)

صدق الله العلي العظيم

سورة الإسراء, الآية ٨٠

Dedication

To the great Lord and creator who has help me get to where I am now

To the Prophet of Mercy, Muhammad (peace and blessings be upon him), and his good and honorable family

To the one who will fill the earth with justice just after it was filled with injustice and tyranny... The anticipated Imam Mahdi (AJ)

To the great man who does the impossible to light the way for me... My father

To the priceless and greatest woman who devotes her life to perform her message... My Mother

To my life companions... My brother and sisters

To my life partner… My husband

I dedicate this work

Zahraa, 2023

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Zahraa, 2023

Supervisor's certification

I certify the thesis entitled **(Evaluation of Some Immunological Parameters and Bacterial Lipopolysaccharide with Rheumatoid Arthritis)** was prepared under my supervision by **(Zahraa Falah Mahdi)** at the department of Clinical Laboratories\ College of Applied Medical Sciences\ University of Kerbala, in partial fulfillment of the requirements for the degree of Master in Clinical Laboratories.

Signature

Prof. Dr. Suhad Hadi Mohammed

Supervisor

18/ 9/ 2023

Head of Department Recommendation

In view of the available recommendation, I forward this thesis for debate by the examining committee.

Signature

Assist. Prof. Dr. Linda Hammed Turki

Head of Clinical Laboratories Department

College of Applied Medical Sciences/ University of Kerbala

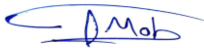
20/ 9/2023

Examining Committee Certification

We, the examining committee, certify that we have read this thesis entitled " **Evaluation of Some Immunological Parameters and Bacterial Lipopolysaccharide with Rheumatoid Arthritis "** and have examined the student (**Zahraa Falah Mahdi)** in its content and that in our opinion it is accepted as a thesis for degree of Master of **Clinical Laboratories**.

Signature:
Prof. Dr. Abeer Thaher Naji

(Chairman)
18/ 9/ 2023



Signature:
Assist. Prof. Dr. Mohanad Jawad Kadhim
(Member)
18/ 9/ 2023

Signature:
Assist. Prof. Dr. Israa Saeed Abass
(Member)
18/ 9/ 2023

Signature:
Prof. Dr. Suhad Hadi Mohammed
(Member / Supervisor)
18/9 / 2023

I have certified upon the discussion of the examining committee .

Signature:
Prof. Dr. Jwadat Noori Ghaaib
Dean of the College of Applied Medical Sciences/ University of Kerbala
21/ 9/ 2023

Approval Certification

We certify that the thesis entitled **"Evaluation of Some Immunological Parameters and Bacterial Lipopolysaccharide with Rheumatoid Arthritis "** fulfills partial requirements of the degree of Master in Clinical Laboratories.

Signature

Head of Clinical Laboratories Department

Assist. Prof. Dr. Linda Hameed Turki

College of Applied Medical Sciences

University of Kerbala

20\9\2023

Signature

Vice Dean Scientific Affairs

Assist. Prof. Dr. Huda Abdalreda Abdullah

College of Applied Medical Sciences

University of Kerbala

21\9\2023

List of Contents

Item No	Subject	Page
	List of Tables	X
	List of Figures	XI
	List of Abbreviations	XII
	Chapter One **Introduction**	
1.1	Introduction	1
	Chapter Two **Literature Review**	
2.1	Definition of Rheumatoid Arthritis	4
2.2	History of Rheumatoid Arthritis	4
2.3	Epidemiology	5
2.4	Pathogenesis	6
2.5	Etiology and Risk Factors	7
2.5.1	Inheritance	7
2.5.2	Environmental factors	8
2.5.2.1	Microbial agents	8
2.5.2.2	Smoking	11
2.5.2.3	Hormonal Factors	12
2.5.2.4	Age	12
2.5.2.5	Diet	13
2.5.2.6	Obesity	13
2.6	Criteria for Rheumatoid Arthritis	14
2.7	Clinical Diagnosis	16
2.7.1	Articular and Peri-articular Manifestations of RA	16
2.7.2	Extra-Articular Manifestations	17
2.7.3	Disease Activity Score	18
2.7.3.1	Erythrocyte Sedimentation Rate (ESR) Test	18
2.7.3.2	Disease Activity Score (DAS28)	19

2.8	Laboratory Diagnosis	19
2.8.1	Immunological Diagnosis	19
2.8.1.1	Rheumatoid Factor (RF) Autoantibodies	19
2.8.1.2	Anti-Cyclic Citrullinated Peptides (ACCP) Autoantibodies	20
2.8.1.3	heterogeneous nuclear Ribonucleic Protein (hnRNP) or RA33 Complex Autoantibodies	21
2.8.1.4	Anti-Sjögren's Syndrome antigen A (Anti-SSA) Autoantibodies	23
2.8.1.5	Anti-double stranded Deoxyribonucleic Acid (Anti-dsDNA) Autoantibodies	23
2.9	Treatment of Rheumatoid Arthritis	24
2.9.1	Analgesics and Non-steroidal Anti -Inflammatory Drugs (NSAIDs)	24
2.9.2	Disease Modifying Anti- Rheumatic Drugs (DMARD)	25
2.9.2.1	conventional Disease Modifying Anti- Rheumatic Drugs (cDMARD)	25
2.9.2.2	biological Disease Modifying Anti- Rheumatic Drugs (bDMARD)	26
2.9.3	Glucocorticoids	26
Chapter Three **Materials and Methods**		
3.1	Subjects	27
3.1.1	Inclusion Criteria and Exclusion Criteria	27
3.1.1.1	Inclusion Criteria	27
3.1.1.2	Exclusion Criteria	28
3.1.2	Questionnaire	28
3.1.3	Ethical Consideration	28
3.1.4	Study Design	28
3.2	Materials	30
3.2.1	Kits	30
3.2.2	Devices, Equipment’s and Apparatuses	30
3.2.3	Tools	31
3.3	Methods	31
3.3.1	Collection of Samples	31
3.3.2	Estimation of Disease Activity Score-28 (DAS-28)	32
3.3.3	Estimation of Body Mass Index (BMI)	32

3.3.4	Estimation of Physiological Parameters	33
3.3.4.1	Estimation of Erythrocyte Sedimentation Rate (ESR)	33
3.3.4.1.1	Principle	33
3.3.4.1.2	Procedure	33
3.3.5	Estimation of Autoantibodies	34
3.3.5.1	Estimation of Rheumatoid Factor (RF) Autoantibodies	34
3.3.5.1.1	Principle	34
3.3.5.1.2	Kit Components	35
3.3.5.1.3	Procedure	35
3.3.5.2	Estimation of Anti-Cyclic Citrullinated Peptide (ACCP) Autoantibodies	36
3.3.5.2.1	Principle	36
3.3.5.2.2	Kit Components	37
3.3.5.2.3	Procedure	37
3.3.5.3	Estimation of Anti-Rheumatoid Arthritis 33 (Anti-RA33) Autoantibodies	38
3.3.5.3.1	Principle	38
3.3.5.3.2	Kit Components	38
3.3.5.3.3	Procedure	39
3.3.5.4	Estimation of Anti-Sjögren's Syndrome antigen A (Anti-SSA) Autoantibodies	40
3.3.5.4.1	Principle	40
3.3.5.4.2	Kit Components	41
3.3.5.4.3	Procedure	41
3.3.5.5	Estimation of Anti-double stranded Deoxyribonucleic Acid (Anti-dsDNA) Autoantibodies	43
3.3.5.5.1	Principle	43
3.3.5.5.2	Kit Components	44
3.3.5.5.3	Procedure	44
3.3.6	Estimation of Bacterial Lipopolysaccharides (LPS)	45
3.3.6.1	Principle	45
3.3.6.2	Kit Components	46
3.3.6.3	Procedure	46

3.4	Statistical Analysis	47
Chapter Four **Results and Discussions**		
4.1	Demographic Data of Patients and Controls	49
4.2	Investigation of Autoantibodies	53
4.2.1	Receiver Operative Characteristic Curve (ROC) for Autoantibodies	53
4.2.2	Comparison of Autoantibodies between Patients and Controls	56
4.2.3	Probability of Autoantibodies Distribution among Patients and Control	59
4.2.4	Distribution of Autoantibodies According to Sex	60
4.2.5	Distribution of Autoantibodies According to Age Groups	64
4.2.6	Distribution of Autoantibodies According to Disease Duration	66
4.2.7	Distribution of Autoantibodies According to Body Mass Index (BMI)	68
4.3	Investigation of Autoantibodies with Disease Activity	71
4.3.1	Distribution of Autoantibodies According to ESR	71
4.3.2	Distribution of Autoantibodies According to DAS28	73
4.4	Investigation The level of Bacterial Lipopolysaccharides (LPS)	76
4.4.1	Receiver Operative Characteristic Curve (ROC) for LPS Titer	76
4.4.2	Comparison of Bacterial LPS between Patients and Controls	77
4.4.3	Distribution of Autoantibodies According to LPS	79
4.4.4	Distribution of LPS According to Sex	82
4.4.5	Distribution of LPS According to Age Groups	84
4.4.6	Distribution of LPS According to Disease Duration	85
4.4.7	Distribution of LPS According to BMI	85
4.4.8	Distribution of LPS According to ESR	87
4.4.9	Distribution of LPS According to DAS28	87
4.5	Correlations	88
4.5.1	Correlation of Autoantibodies, ESR and LPS	88
4.5.2	Correlation of Autoantibodies, DAS28 and LPS in RA Patients Group	90
Conclusions		92
Recommendations		93

References	94
Appendices	118

List of Tables

Table No	Tables	Page
2.1	The 2010 ACR/EULAR Classification Criteria for RA	15
3.1	Kits of the Study	30
3.2	Devices and Apparatuses of The current Study	30
3.3	The tools of The study	31
3.4	DAS28 Cut-off Values	32
3.5	Body Mass Index (BMI)	33
3.6	RF ELISA Kit Components	35
3.7	ACCP ELISA Kit Components	37
3.8	Anti-RA33 ELISA Kit Components	39
3.9	Anti-SSA ELISA Kit Components	41
3.10	Anti-dsDNA ELISA Kit Components	44
3.11	Components of LPS ELISA Kit	46
4.1	Demographic Data of Rheumatoid Arthritis Patients and Controls	51
4.2	The interpretations of Area Under the Curve (AUC)	54
4.3	Receiver Operative Characteristic Curve (ROC) based analysis	55
4.4	Comparison of Autoantibodies between Patients and Controls	58
4.5	Probability of Autoantibodies Distribution among Patients and Control	59
4.6-A	Distribution of Autoantibodies Presence According to Sex	61
4.6-B	Cross-tabulation between Autoantibodies and Sex	62
4.7-A	Distribution of Autoantibodies Presence According to Age Groups	65
4.7-B	Cross-tabulation between Autoantibodies and Age Groups	66
4.8-A	Distribution of Autoantibodies Presence According to Disease Duration	67
4.8-B	Cross-tabulation between Autoantibodies and Disease Duration	68
4.9-A	Distribution of Autoantibodies Presence According to BMI	69

4.9-B	Cross-tabulation between Autoantibodies and BMI	70
4.10-A	Distribution of Autoantibodies Presence According to ESR	72
4.10-B	Cross-tabulation between Autoantibodies and ESR	73
4.11-A	Distribution of Autoantibodies Presence According to DAS28	74
4.11-B	Cross-tabulation between Autoantibodies and DAS28	75
4.12	Receiver Operative Characteristic Curve (ROC) Curve of LPS Titer	76
4.13	Comparison of LPS between Patients and Controls	79
4.14-A	Distribution of Autoantibodies According to LPS	80
4.14-B	Cross-tabulation between LPS and Autoantibodies	81
4.15	Distribution of LPS According to Sex	83
4.16	Distribution of LPS According to Age Groups	84
4.17	Correlation between Autoantibodies, ESR and LPS	89
4.18	Correlation between Autoantibodies, DAS28, and LPS in RA Patients Group	91

List of Figures

Figure No	Figures	Page
2.1	Pathogenic Aspects of Rheumatoid Arthritis	7
2.2	Ebringer's Theory	9
2.3	Lipopolysaccharide Biochemical Structure	10
2.4	RA Patient with Deformity	17
3.1	Study Design	29
4.1	Receiver Operative Characteristic Curve (ROC) Curve Illustrating The sensitivity and 1-specificity Values for RF, ACCP, Anti-RA33, Anti-dsDNA optical density, and Anti-SSA titer	54
4.2	Distribution of ESR between Patient and Control	71
4.3	Receiver Operative Characteristic Curve (ROC) Curve Illustrating The sensitivity and 1-specificity Values for LPS	77
4.4	Distribution of LPS According to Disease Duration	85
4.5	Distribution of LPS According to BMI	86

4.6	Distribution of LPS According to ESR	87
4.7	Distribution of LPS According to DAS28	88

List of Abbreviations

Abbreviations	Description
ACCP	Anti-Cyclic Citrullinated Peptide
ACR	American College of Rheumatology
ANAs	Anti-Nuclear Antibodies
ANOVA	Analysis Of Variance
Anti-dsDNA	Anti-Double Stranded Deoxyribo Nucleic Acid
Anti-SSA	Anti-Sjögren's Syndrome antigen A
AUC	Area Under the Curve
APRs	Acute Phase Reactants
BC	Before Chrisms
bDMARD	biological Disease Modifying Anti- Rheumatic Drugs
BMI	Body Mass Index
Bregs	Regulatory B cells
CDAI	Clinical Disease Activity Index
cDMARD	conventional Disease Modifying Anti- Rheumatic Drugs
CD14	Cluster of Differentiation 14
CMC	Carpal-Meta-Carpal
CRP	C- Reactive Protein
CVDs	Cardiovascular Diseases
DAS28	Disease Activity Score 28
DIP	Distal-Inter- Phalangeal
DM	Diabetes Mellitus
DMARDs	Disease Modifying Anti Rheumatic Drugs
EAMs	Extra-Articular Manifestations
ELISA	Enzyme Linked Immunosorbent Assay

ERs	Estrogen Receptors
ESR	Erythrocyte Sedimentation Rate
EULAR	European League Against Rheumatism
Fc	Fragment crystallizable
FDA	Food and Drug Administration
GI	Gastrointestinal
HAQ	Health Assessment Questionnaire
HCQ	Hydroxychloroquine
HLA	Human Leucocyte Antigen
hnRNP	heterogeneous nuclear Ribonucleic Protein
HRP	Horseradish Peroxidase
HRT	Hormone Replacement Therapy
IFX	Infliximab
IL	Interleukin
ILD	Interstitial Lung Disease
LSD	Least Significant Difference
JRA	Juvenile Rheumatoid Arthritis
Kdo	Keto-deoxy-octulosonic acid
LBP	Lipopolysaccharide Binding Protein
LPS	Lipopolysaccharide
MCP	Metacarpal-Inter- Phalangeal
MHC	Major Histocompatibility
MS	Multiple Sclerosis
MTX	Methotrexate
MD2	Myeloid Differentiation 2
NF	Nuclear Factor
N.S	Non-Significant
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
OCs	Oral Contraceptives
OD	Optical density

PAD	Peptidyl Arginine Deiminase
PBS	Phosphate Buffer Saline
PIP	Proximal-Inter- Phalangeal
PRL	Prolactin
RA	Rheumatoid Arthritis
RA-33	Rheumatoid Arthritis-33
RF	Rheumatoid Factor
RNA	Ribo Nuclic Acid
Ro	Ribonucleoproteins
ROC	Receiver Operating Characteristics
Rpm	round per minute
SD	Standard Deviation
SDAI	Simplified Disease Activity Index
SE	Shared Epitope
SF	Synovial Fluid
SLE	Systemic Lupus Erythematosus
SPSS	Statistical Package for the Social Sciences.
SS	Sjögren's syndrome
TBS	Tris-Buffered Saline
T-h	T-helper
TLR 4	Toll-Like Receptor 4
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor Necrosis Factor
T-regs	Regulatory T cells
VAS	Visual Analog Scale
VGLL3	Vestigial Like Family Member 3
WHO	World Health Organization

Summary

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease with a progressive course that may result in joint degeneration and eventual disability if left untreated.

The current study aimed to investigate disease specific and disease-associated autoantibodies profile in RA patients and their association with the bacterial component. The current study is the first one that deals with characterization of disease specific and disease-associated autoantibodies profile in patients with RA and their relationship with the LPS level and/or with disease activity.

A case-control study was conducted at the College of Applied Medical Sciences/University of Kerbala. Eighty-two subjects (41 RA patients and 41 apparently healthy individuals) were enrolled in the current study. Patients were diagnosed by rheumatologists in Al-Hindiya Teaching Hospital and Al-Imam Al-Hassan Al-Mujtaba Hospital/Karbala Health Directorate in Karbala/Iraq during the period from November 2022 to February 2023. Blood samples were drawn from RA patients and healthy volunteers to be used for erythrocyte sedimentation rate (ESR) estimation, autoantibody detection, and bacterial LPS determination.

The current study revealed that the ages of participants ranged from 27 to 77 years. The Male/Female ratio was 1:4.8. Based on age groups there were significant differences in all tested autoantibodies between RA patients and controls ($P < 0.05$).

Receiver Operative Characteristic Curve (ROC) analysis revealed the presence of high area under the curve (AUC), sensitivity, and specificity rates for all tested autoantibodies, and LPS. This study showed the presence of significant differences between the patient and control groups in all tested autoantibodies and

that 25 (60.97%) of patients had all tested autoantibodies, whereas 27 (65.85%) of the control group were negative.

Regarding LPS, the results showed a highly significant increase in the mean level of LPS between patients and controls (P = 0.000). The current study revealed a significant positive correlation between ESR and all tested autoantibodies and with LPS titers and a negative non-significant correlation between DAS28 and LPS and autoantibodies levels, except in the case of anti-RA33 autoantibodies.

The current study concluded that there were significant differences in all tested autoantibodies and LPS between patients and controls, and this result might reflect the importance of the diagnosis of disease specific and disease-associated autoantibodies during the diagnosis of RA. LPS findings might possibly reflect the role of LPS in RA disease prognosis or development. For disease activity, autoantibodies and LPS were significantly different with ESR but not with DAS28, and this result might reveal the association of autoantibodies and LPS with disease progression. Also, there was a significant positive correlation among ESR with all tested autoantibodies titers and with LPS titers and autoantibodies titers with LPS and a negative correlation between DAS28 with autoantibodies and with LPS levels, but not at significant levels except in the case of anti-RA33 autoantibodies.

Chapter One

Introduction

1.1 Introduction:

The Rheumatoid Arthritis (RA) is a systemic inflammatory autoimmune disease characterized by pain, swelling, stiffness, and deformity in several joints. In the majority of cases, the inflammation reaction begins with the small joints (Ohno *et al*., 2020) of the hands and feet, namely the Proximal-Inter-Phalangeal (PIP), Meta-Carpal-Inter-phalangeal (MCP), and Carpal-Meta-Carpal (CMC) joints, with obvious sparing of the Distal-Inter-Phalangeal (DIP) joints, before attacking the larger joints such as the knees and shoulders (Weissman *et al.,* 2018). The systemic manifestations affect patients in a heterogeneous way. The most common extra-articular sign is the presence of rheumatoid nodules in the juxta-articular region and the lungs. RA is associated with an increased risk for both cancer (lymphomas) and co-morbidities [cardiovascular disease (CVD) and interstitial lung disease (ILD)], (Cerqueira, 2016).

The Rheumatoid Arthritis is a chronic disease affecting approximately 0.5–1% of populations worldwide (Wu *et al*., 2021). It was observed in 1 % of population samples in Iraq (Abdul-qaharr *et al*., 2013). Within the first three years following initial diagnosis, 20 to 30 % of RA patients become severely affected and become permanently disabled (Taylor *et al*., 2011).

Females are affected by RA two to three times more than males (Yousif & Ibraheem, 2020). Although people of any age are susceptible to RA disease, it is most common among people aged 40–50 years (Moeez *et al*., 2013).

The Rheumatoid Arthritis diagnosis is based on clinical signs. Early diagnosis is very important in RA to prevent the reduction of functional capacity and associated morbidity and mortality. Serological tests are routinely used for the detection of autoantibodies in RA patients (Shakiba *et al*., 2014). Autoantibodies are the hallmark of RA, and rheumatoid factor (RF) antibodies and anti-cyclic

citrullinated peptide (ACCP) antibodies are routinely used to confirm the diagnosis. They are also recommended by the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR), the criteria of which are used to diagnose RA (Li *et al.,* 2021). Based on the presence of RF and/or ACCP, RA can be categorized as either seropositive or seronegative (Grönwall *et al.,* 2021).

Erythrocyte sedimentation rate (ESR) indicators can suggest early disease activity and have been included in the assessment system for disease activity observations, but their values are influenced by a variety of factors such as sex, age, fibrinogen levels, RF, hyperglobulinemia and anemia (Yuan *et al.,* 2020). Disease activity score 28 (DAS28) is one of the standard methods to measure disease activity in patients with RA. DAS28 use in clinical practice is recommended by the EULAR (Gorial, 2012).

Lipopolysaccharide (LPS) is a molecule that is found in the bacterial cell wall of gram-negative bacteria and helps to stabilize it. Gram-negative bacteria are the only organisms in nature that have LPS and Lipid A, which is the "endotoxic" component. The molecular weight and structure of LPS vary depending on the species of bacteria, as does its actual pathogenicity (Schultz, 2018).

The presence of gram-negative bacteria in the bloodstream results in the release of LPS from the bacterial cell wall. The presence of enterobacterial LPS might lead to the high and non-specific activation of the human immune system in RA patients. The final outcome of inflammatory reactions is the release of cytokines and antibodies against LPS, which are related to pathogen-recognition mechanisms, including toll-like receptors (TLR4), (Arabski *et al.*, 2012).

The identification of a wide range of both disease specific and disease-associated autoantibodies in RA patients will provide novel pathogenic insights important for the development of methods for diagnosis and prognosis compatible with effective personalized medicine. On the other hand, recent studies documented that lipopolysaccharide (LPS) could play an important role in tracking the progression of the disease in RA patients.

Aim of the study:

The current study aimed to characterize disease specific and disease associated autoantibodies in RA patients and their relationship with the bacterial components, LPS and/or disease severity.

Objectives:

1. Determination the presence or absence of some disease specific and disease associated autoantibodies (RF, ACCP, anti-RA33, anti-SSA, and anti-dsDNA) in RA patients and control groups.
2. Investigation the possible association between these autoantibodies and disease activity.
3. Investigate the presence of a bacterial components (LPS) in the blood of patients and controls.
4. Investigation the association between the presence of LPS with the presence of certain types of autoantibodies.
5. Investigation the association of LPS presence with disease severity.

Chapter Two

Literature Review

Literature Review

2.1 Definition of Rheumatoid Arthritis

The Rheumatoid Arthritis (RA) is an autoimmune, symmetrical, chronic inflammatory disease that begins with small joints (hand and foot) and then progresses to the large joints, causing damage to bone and cartilage. Its common symptoms are morning stiffness that lasts more than 1 hour, joint pain, swelling, immobility, erosion, and deformity (Mohammed *et al*., 2022).

The RA is traditionally regarded as a chronic disease that cannot be cured, and patients require lifelong immune modulation to control the inflammatory process effectively and prevent damage. Such a concept of disease control, but not a cure, is well established in other fields of medicine for conditions such as diabetes, hypertension, human immunodeficiency virus infection, and certain forms of cancer (Schett *et al.*, 2016).

Based on the presence of autoantibodies, mainly RF and ACCP, RA can be subdivided into two main categories: seropositive and seronegative RA. These autoantibodies are seen in 50% of early RA patients and up to 80% of individuals with established RA (Volkov *et al.,* 2020). Seronegative individuals must have more clinical symptoms to be diagnosed with RA according to the 2010 ACR/EULAR criteria, despite often being thought to have a less inflammatory and less destructive form of RA (Reed *et al.,* 2020).

2.2 History of Rheumatoid Arthritis

The history of RA is a long and complex one. There is evidence of rheumatological disease in the Ebers Papyrus, a historically significant medical text from ancient Egypt dating back to 1500 Before Chrisms (BC). The papyrus describes what is probably RA, and examination of Egyptian mummies has pointed toward the existence of RA at this time (Hyndman, 2017).

Rheumatology has rapidly advanced during the last 50 years due to improved diagnosis as a result of progress in immunology, molecular biology, genetics, and imaging. Disease-specific criteria have been developed for the majority of rheumatic diseases to maintain uniformity in diagnosis and classification (Deshpande, 2014).

In 1987, ACR published a new list of classification criteria. The ACR 1987 criteria have been increasingly used as a "gold standard" for the diagnosis of RA, although they were developed as a tool for classifying patients who had been diagnosed by a rheumatologist as having RA (Saraux *et al.,* 2001). The 1987 ACR criteria for the classification of RA proved not to be sensitive enough to recognize RA in the initial stages of disease evolution (Olivieri *et al.,* 2012).

A group of American and European rheumatologists who were experts in the diagnosis and treatment of RA met in Zu¨ rich in 2007 to discuss the limitations of the existing 1987 ACR criteria and to plan the development of a new set of criteria to diagnose and classify patients with RA early in the course of the disease. This meeting resulted in the formation of a joint ACR/EULAR working group that was charged with developing these new criteria using an approach that combined data analysis with a Delphi (Kay & Upchurch, 2012).

2.3 Epidemiology

The RA affects around 23.7 million people worldwide. The prevalence of RA varies greatly among nations, with rates ranging from 0.2 to 1.1%, with greater rates in high-income countries compared to low- and middle-income countries (Ohno *et al.*, 2020).

The total average prevalence of the disease in the world is about 1% (Hassanzadeh & Gholamnezhad, 2020). The prevalence of RA is between 0.5% and 1% in European and North American populations; Asia had the lower rate of the disease at 0.2–0.3%. Some Native American populations had a

remarkably high prevalence, more than 5% (Alkazzaz, 2013). In Iraq, the prevalence of RA is 1% (Al-Ghazaly & Jassim, 2022).

RA is more common in those aged 40 to 50 years (de Brito Rocha *et al.,* 2019). Females have a higher incidence of RA than males, with an incidence ratio of about 2:1 to 3:1 (van Delft & Huizinga, 2020).

2.4 Pathogenesis

In RA patients, immune cells such as T cells, B cells, and macrophages infiltrate into the joint tissue, producing a variety of pro-inflammatory cytokines that promote inflammation and eventually tissue damage (Volkov *et al.,* 2020). As a result, synovial hypertrophy and angiogenesis develop, resulting in the activation of osteoclasts and subsequent bone degradation. T cells and synovial fibroblasts also produce inflammatory cytokines that cause monocyte-macrophage lineage cells to mature into osteoclasts, resulting in bone resorption and erosion (Moeez *et al.*, 2013), as shown in Figure (2.1), (Aletaha & Smolen, 2018).

Immune complexes containing RF are thought to contribute to vasculitis manifestations of RA, such as rheumatoid nodules and rheumatoid vasculitis. However, there is little evidence that RF is involved in initiating the early events in RA and thus may be secondary to chronic inflammation (Allard-Chamard & Boire, 2019).

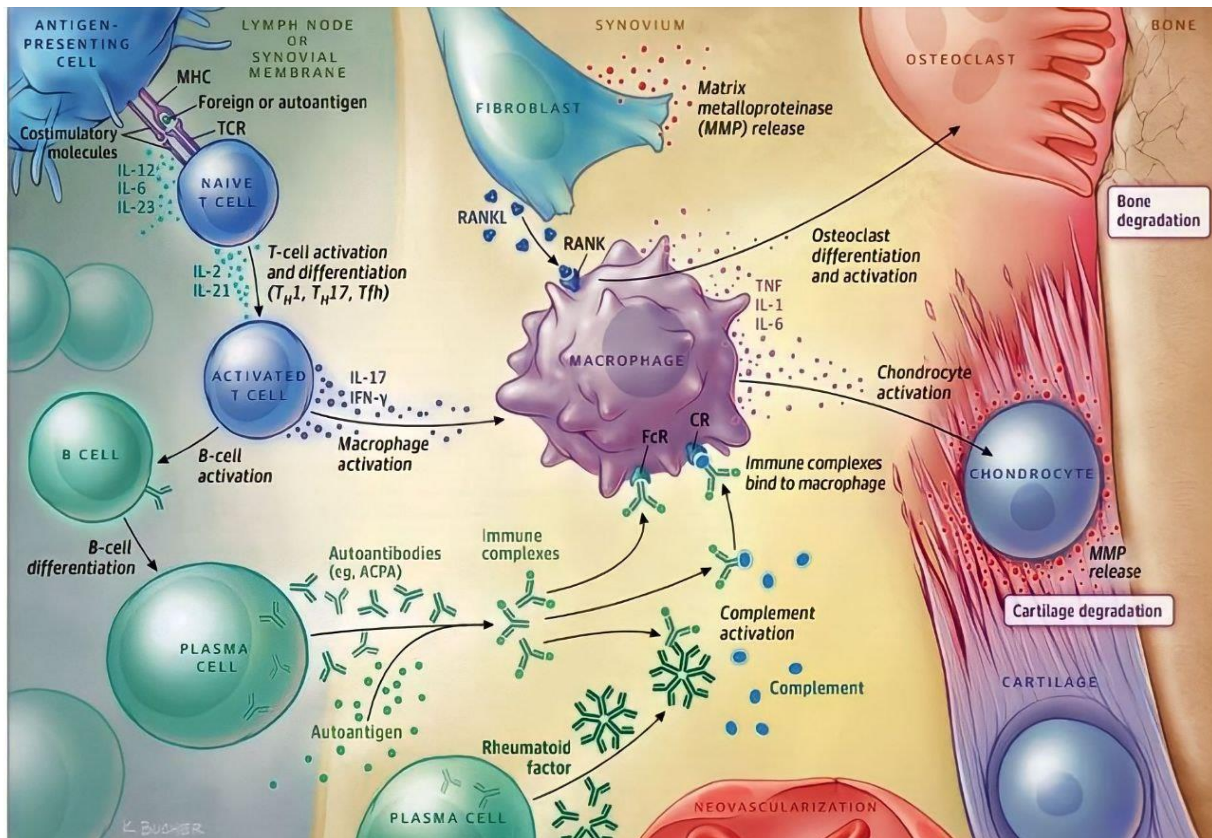


Figure 2.1: Pathogenic Aspects of Rheumatoid Arthritis (Aletaha & Smolen, 2018), RANKL: Receptor Activator Nuclear Factor Kappa B Ligand

2.5 Etiology and Risk Factors

2.5.1 Inheritance

The genetic contribution is thought to be between 50 and 60%, implying that genetic factors have an important effect on RA susceptibility (de Brito Rocha *et al.*, 2019). The concordance of the disease is only about 15% in identical twins, but its overall heritability (a quantitative measure of the amount of variation in disease susceptibility that can be explained by genetic factors) has been estimated to reach 66% (Scherer *et al.,* 2020).

The most important genetic risk allele for RA has been located in the class II major histocompatibility (MHC) locus, accounting for approximately 40% of the genetic effect. Individuals with MHC class II human leukocyte antigens-DR4

(HLA-DR4) alleles had a 5:1 risk of having RA. The association between HLA-DR and RA was first established in the 1970s, with the discovery that HLA-DR4 is present in 70% of RA patients, compared to approximately 30% of controls (Firestein & McInnes, 2017).

The RA has been linked to the HLA-DRB1shared epitope' (SE), which involves the DRB1*04 and DRB1*01 alleles (Turesson *et al.,* 2005). When compared to SE -negative individuals, the risk is four times higher in those with a single SE allele and eight times higher in those with two SE alleles (van Delft & Huizinga, 2020).

2.5.2 Environmental Factors

Although this disease's etiology or triggering factor is unknown, it is believed that interactions between genetic and environmental variables contribute to its incidence (Sulaiman *et al*., 2019).

Nevertheless, individual environmental factors, such as smoking, have generally demonstrated only weak associations with RA. Over the last years, striking gene-environment interactions have been discovered, which have changed the concepts of RA etiopathogenesis. The importance of gene-environment interactions is growing because it opens opportunities for potential interventions and disease prevention (Alpízar-Rodríguez & Finckh, 2017).

2.5.2.1 Microbial Agents

Several pieces of evidence from epidemiological and translational research suggest that interactions between mucosal sites and dysbiotic microbiota might have a causal role in the development of RA (Zaiss *et al.,* 2021).

The presence of a variety of detritus produced by Gram-negative and other bacteria (including wall-less forms) walls and membranes may play an important

role in the course of RA, in addition to the associated CVD and systemic inflammation found in RA disease (Pretorius *et al.,* 2017).

The Ebringer's theory, which states that genetically predisposed individuals are exposed to environmental factors that act as triggers, causing an immunological reaction followed by an autoimmune response that may develop into RA. Ebringer's theory suggests that microbes are the cause of the trigger and the role of LPS, which may result in an imbalance between pro- and anti-inflammatory cytokines, followed by systemic inflammation and the effect on the cardiovascular and hematological health of RA patients, as shown in Figure (2.2), (Pretorius *et al.,* 2017).

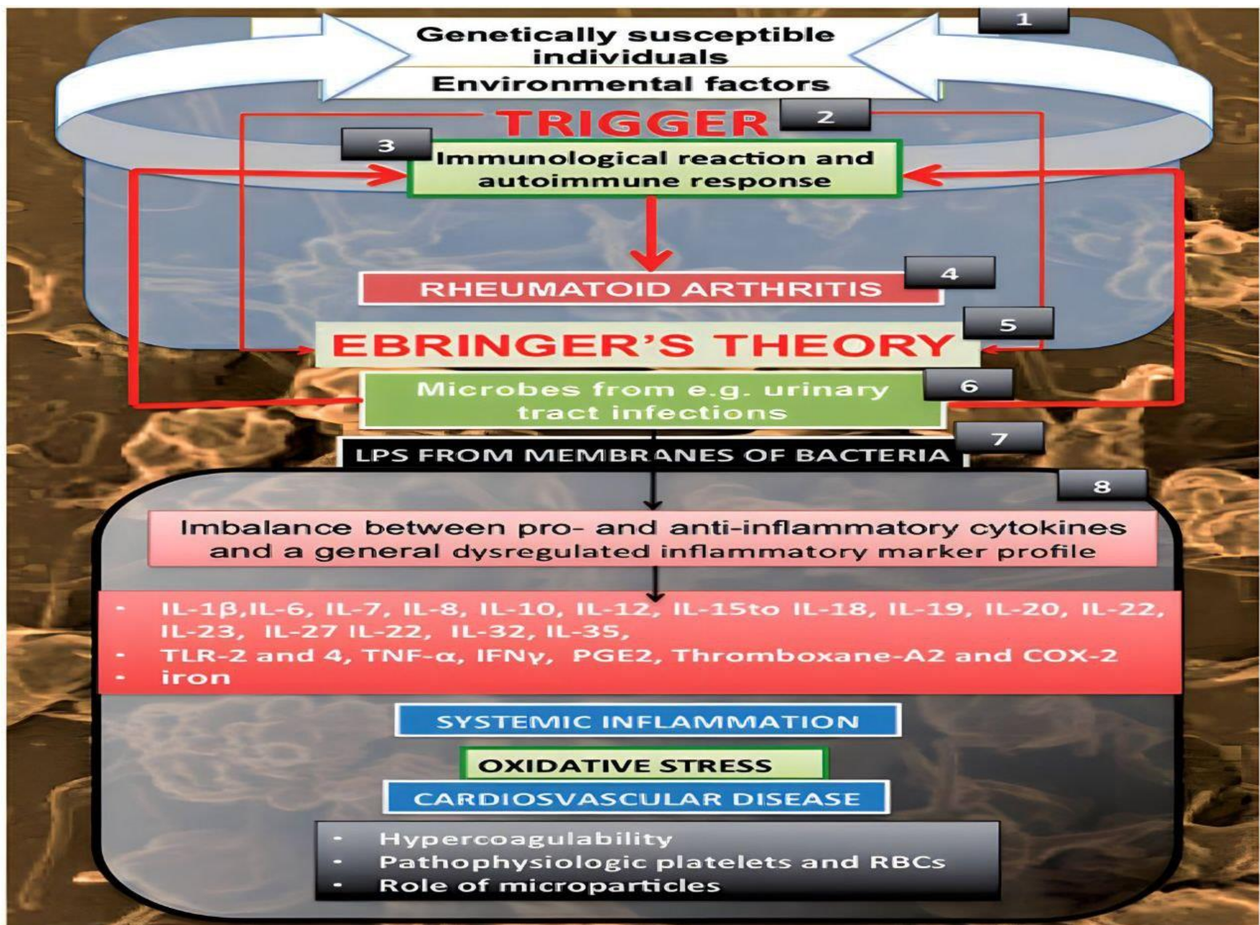


Figure 2.2: Ebringer's Theory (Pretorius *et al.,* 2017)

The LPS is composed of three different regions, as shown in Figure (2.3), (Ding & Su., 2015). The first one, a glycophospholipid moiety known as lipid A, anchors LPS in the bacterial membrane and is responsible for the majority of the biological effects of these potent molecules. Typically, lipid A moieties are connected to a core oligosaccharide through an acidic deoxysugar called 2-keto-3-deoxy-octulosonic acid (Kdo). The third component of LPS molecules called the O-chain, is composed of oligosaccharide repeating units that extend beyond the bacteria. These O-specific chain structures, being unique to a given bacterium, are at the origin of the serotyping, historically utilized to identify gram-negative bacteria (Caroff & Novikov, 2020).

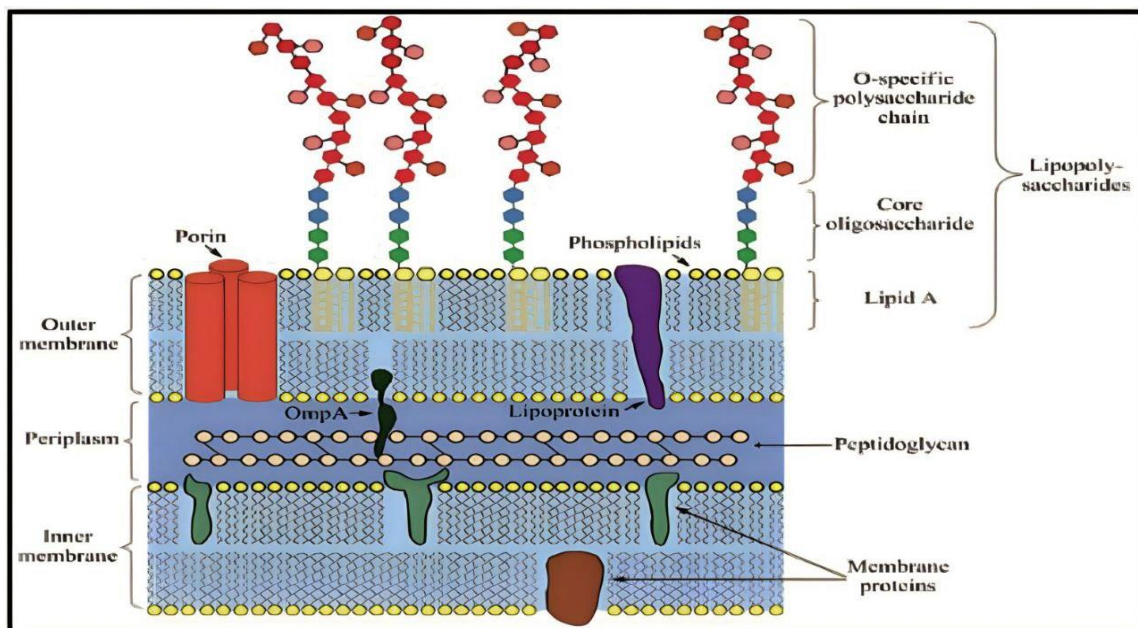


Figure 2.3: Lipopolysaccharide Biochemical Structure (Ding & Su., 2015)

LPS is the main endotoxin component found in the cell walls of gram-negative bacteria. They activate the host's immune cells, including neutrophils and macrophages. The activated cells produce pro-inflammatory factors, such as tumor necrosis factor-alpha (TNF-α), Interleukin-1β (IL-1β), matrix proteases, and free

radicals, which result in severe secondary inflammation in tissues (Lorenz *et al.,* 2013). LPS's interaction with the cell surface TLR4 initiates a chain of signals that leads to the stimulation of nuclear factor (NF)-κB signaling and the subsequent numerous pro-inflammatory mediators' expression. TLR4 recognition of LPS is facilitated by the LPS-binding protein (LBP) and co-receptors cluster of differentiation 14 (CD14) and Myeloid Differentiation 2 (MD2). LBP is an acute-phase reaction protein. It plays a key role in promoting innate immunity against Gram-negative bacteria by transferring LPS to both membrane-bound CD14 and soluble CD14 (Wen *et al*., 2018). LBP is primarily secreted by intestinal cells and hepatocytes, and it is commonly used as an alternate indicator for LPS levels in the serum (Parantainen *et al.,* 2022).

2.5.2.2 Smoking

Personal (active) smoking is the most well-established environmental risk factor for development of RA as demonstrated in multiple epidemiological studies (Yoshida *et al.,* 2021). Smoking increases the risk of developing ACCP-positive RA, particularly in individuals with high-risk RA-susceptibility alleles (Bergot *et al.,* 2020). There are no data on the relationship between early tobacco exposure (i.e., during childhood) and the risk of developing RA. Also, few studies examine the possible role of passive smoking in triggering RA (Seror *et al.,* 2019).

Smoking may increase the incidence of seropositive RA by generating local tissue inflammation, promoting citrullination, and producing neoepitopes that result in autoimmunity. Smoking also triggers immune cells to release pro-inflammatory cytokines, resulting in a systemic inflammatory state (Liu *et al.,* 2019).

2.5.2.3 Hormonal Factors

Steroid hormones influence autoimmunity and may include a further balance between T-helper1 (Th1) and T-helper2 (Th2) cell responses. Th1 lymphocytes generate mostly IL-2 and interferon-gamma and are largely responsible for cell-mediated immunity, whereas Th2 lymphocytes produce mainly IL-4, IL-5, IL-13, and IL-10 and are primarily responsible for humoral immunity. These findings show that cytokine secretion abnormalities may play a critical role in the triggering or maintenance of distinct pathophysiological mechanisms and clinical manifestations of RA and Systemic Lupus Erythematosus (SLE), (Salih *et al.,* 2008).

Female hormonal factors play a vital role in the development of RA. Changes in the hormonal milieu are caused by events such as pregnancy, post-partum, breastfeeding, menopause, or the use of exogenous sex steroids [such as oral contraceptives (OCs) or hormone replacement therapy (HRT)]. In general, estrogen has been shown to be pro-inflammatory, whereas androgens are anti-inflammatory. More specifically, depending on serum levels, reproductive stage or ovarian aging phase (reproductive, menopausal transition, or post-menopausal), expression of estrogen receptors (ERs), or intracellular metabolism, estrogens may have different effects on different immune cells (Alpízar-Rodríguez *et al.*, 2017).

Pregnancy may be associated with a decrease in disease activity in half of the females, but the postpartum period is related to an increase in disease activity in up to 90% of cases (Yousif & Ibraheem, 2020).

2.5.2.4 Age

Aging is related to an increased prevalence of cardiovascular diseases (CVD) and osteoporosis, both of which have been identified as RA comorbidities in RA patients compared with the general population (Nilsson *et al.,* 2021).

The age of the disease's beginning has been related to disease activity, severity, comorbidity, and the efficacy of pharmaceutical therapy. Previous research in this field has revealed individuals with late-onset RA have a more benign form of the disease than those with young-onset RA (Innala *et al.,* 2014).

2.5.2.5 Diet

The RA patients frequently ask their doctors for specific dietary recommendations, and many reports that various foods either improve or worsen the symptoms of the disease. Foods such as red meat, soft beverages, and wine have been reported to worsen symptoms (Vadell *et al.,* 2020).

It has been suggested that a Mediterranean diet rich in fish, olive oil, cooked vegetables, and fruit has a protective effect against RA, which could be due to the high content of omega-3 in such a diet (Ruiz-Esquide & Sanmartí, 2012).

Gluten, an antigen derived from the gut, is an immunological trigger in celiac disease and RA. In celiac disease, the systemic immune response may be directed to sites other than the gut. In RA, gut-derived antigens are the primary initiators and drivers of immune system disorders. These shared immunological mechanisms explain the coexistence of celiac disease and RA (Badsha, 2018).

Fasting for 7–10 days with a partial nutrient intake of vegetable broth, herbal teas, parsley, garlic, and potato decoction; juice extracts from carrots, beets, and celery; and a controlled daily energy intake followed by 1 year of a vegan diet compared to an omnivorous diet were studied in various trials. These trials found a significant reduction in swollen and tender joints, pain, ESR, and C-reactive protein (CRP), (Khanna *et al.,* 2017).

2.5.2.6 Obesity

Obesity, defined as an increase in fat at a sufficient level to cause adverse health consequences, is usually diagnosed by anthropometric measurements of body mass index (BMI), which is calculated as weight in kilograms divided by the

square of height in meters (kg/m^2). As a pandemic public health issue in the western world, overweight/obesity is associated with a high incidence of chronic autoimmune and inflammatory pathologies, such as type 2 diabetes mellitus (DM) and RA, thus resulting in a large social and economic impact (Feng *et al*., 2019).

Numerous studies have connected obesity and overweight to an increased risk of RA in seropositive and seronegative individuals, some of which only applied to females. BMIs of 25 kg/m^2 (overweight) and 30 kg/m^2 (obesity) increase the risk of RA progression by 15% and 21-31%, respectively, and by 60% when paired with smoking (Petrovská *et al*., 2021).

Obesity has been linked to a decreased probability of achieving and sustaining remission in RA. Obese people with RA have worse DAS28 joints, tender joint counts, inflammatory markers, patient global evaluation scores, pain scores, and physical function scores when compared to non-obese patients. Obesity has not been linked to increased mortality in RA, but interventions to prevent and treat obesity can assist in improving RA outcomes and quality of life. Obesity-related foods, such as sweetened soda, have been linked with a higher risk of RA (Badsha, 2018).

Generally, more adipose tissue is linked with an increase in the production of pro-inflammatory molecules; likewise, decreased adiposity is related to a reduction in the level of pro-inflammatory molecules as well as an increase in the concentration of anti-inflammatory molecules, thereby making obesity now presumed to be a pro-inflammatory state (Younis & Al-bustany, 2017).

2.6 Criteria for Rheumatoid Arthritis

The ACR/EULAR RA classification criteria were established in 2010 to aid in diagnosing early RA. RA is now defined by the ACR/EULAR criteria at a very early stage of diagnosis, compared to the 1987 ACR criteria, which primarily predicted erosive disease (Sulaiman *et al*., 2019). Applying these criteria produces

a score from 0 to 10, with a score of ≥6 being sufficient for the diagnosis of definite RA(Jang *et al.,* 2022), as shown in Table (2.1), (Aletaha et al., 2010).

Table 2.1: The 2010 ACR/EULAR Classification Criteria for RA

Criteria	Score
A. Joint involvement	
1 large joint.	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)	2
4-10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint)	5
B. Serology (at least 1 test result is needed for classification)	
Negative RF and negative ACPA	0
Low-positive RF or low-positive ACPA	2
High-positive RF or high-positive ACPA	3
C. Acute-phase reactants (at least 1 test result is needed for classification)	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
D. Duration of symptoms	
< 6 weeks	0
≥ 6 weeks	1

ACPA, Anti-Citrullinated Protein Antibodies; ACR, American College of Rheumatology; CRP, C-Reactive Protein; ESR, Erythrocyte Sedimentation Rate; EULAR, European League Against Rheumatism; RF, Rheumatoid Factor

2.7 Clinical Diagnosis

2.7.1 Articular and Peri-articular RA Manifestations

Early identification of RA articular and peri-articular manifestations that may predict disease severity and require on-referral is essential for the primary care clinician since they have implications for patient safety and best-practice management. A typical course of RA includes chronic low-grade inflammation with periodic flares that might appear as articular or peri-articular manifestations (Briggs *et al.,* 2013).

The synovium is the main target of RA; when antigens in the synovial membrane trigger an immune response in cells, it results in synovitis. The synovium-lined tendon sheaths, bursae, and entheses where ligaments and tendons adhere to bone are all affected by RA (Suh *et al.*, 2021).

Joints that are swollen, painful, and hot become inflexible, restricting movement. Over time, multiple joints are damaged (polyarthritis). The hands, feet, and spine in the cervical parts are the most commonly damaged joints, but larger joints like the shoulder and knee can also be impacted (Hasan *et al*., 2022)**.** Bony erosions, thinning articular cartilage, weakening of peri-articular structures, joint deformities, and peri-articular osteopenia are the hallmarks of joint damage, all of which contribute to increased disability and loss of function (Allard-Chamard & Boire, 2019) , as shown in Figure (2.4)**.**

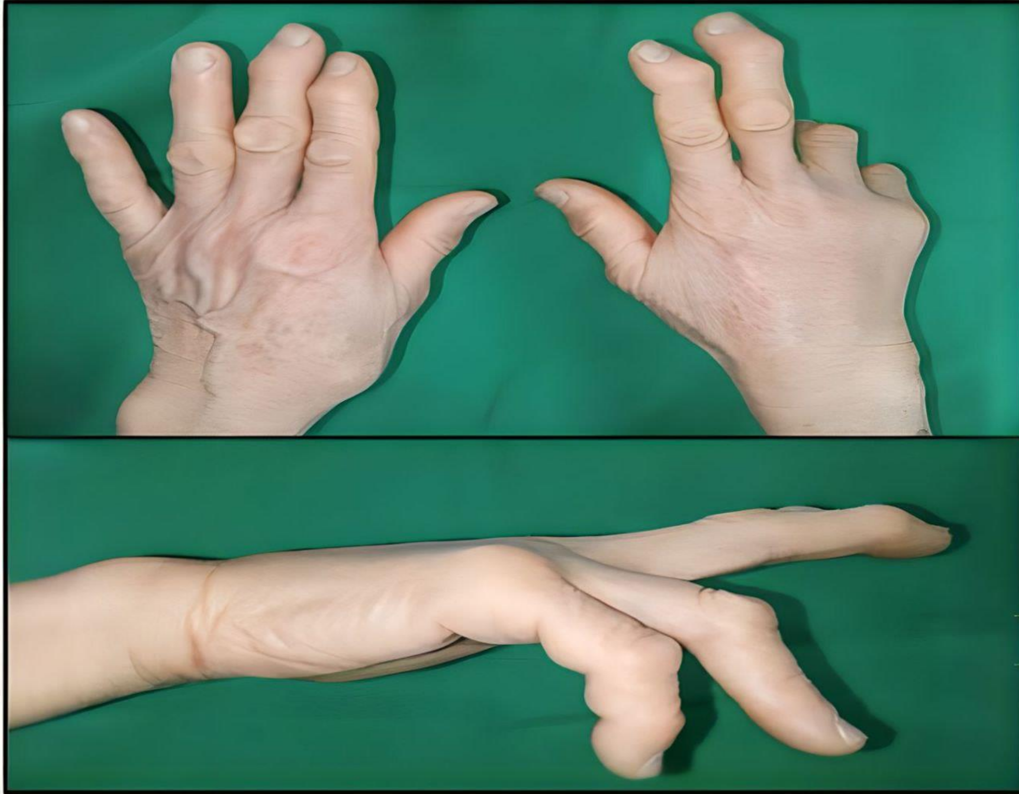


Figure 2.4: RA patient with deformity (Suh *et al.,* 2021)

2.7.2 Extra-Articular Manifestations (EAMs)

Approximately 40–50% of RA patients may have some form of EAM at the beginning of the disease or during the duration of the disease (Chen *et al.,* 2020). The skin, eye, heart, lung, renal, neurological, and gastrointestinal systems, as well as tendons, ligaments, and fascia, are all involved in EAMs. Identification of EAMs is important since treatment is often aimed at controlling the underlying RA disease (Littlejohn & Monrad, 2018).

Weight loss, fever, prolonged early morning stiffness, fatigue, generalized muscle weakness, low mood, and depression are often responsible for a significant loss in the quality of life of patients. Fatigue is reported in 40–80% of RA patients as their most disabling symptom (Vela, 2014).

The main reason for the increased morbidity and preterm mortality in RA patients is CVD (Mantel *et al.*, 2015). RA increases the risk of CV mortality by up

to 50% compared with the general population (Choy *et al.,* 2014). This risk is associated with both classic CV risk factors and inflammatory indicators such as ESR (Solomon *et al.*, 2015).

Thirty to forty percent of RA patients have pulmonary disease, which is the second largest cause of mortality for RA patients and most frequently manifests as primary and secondary interstitial lung disease (Anyfanti *et al.,* 2022).

Gastrointestinal (GI) symptoms may arise as a result of direct involvement by RA, a secondary autoimmune process, or even a treatment adverse reaction. Symptoms and severity also depend on the site of the GI tract involved and the degree of inflammation (Kröner *et al.,* 2019). Liver dysfunction is observed in 18–50% of RA patients, and in most cases, liver dysfunction is induced by anti-rheumatic drugs used for the treatment of RA (Ohira *et al.,* 2012).

RA can affect the kidneys directly by the effect of the disease itself or indirectly by the effects of the drugs used for treatment, such as biological and conventional disease-modifying anti-rheumatic drugs (DMARDs) or analgesics like non-steroidal anti-inflammatory drugs (NSAIDs), (Oweis *et al.*, 2020). It has been reported that a considerable number of RA patients also have proteinuria, hematuria, and renal dysfunction (Mori *et al.,* 2017).

According to studies, RA has a moderately higher overall incidence of malignancy than the general population (Simon *et al.,* 2015). The prevalence of eye involvement was 18% in RA patients (Turk *et al.,* 2021).

Subcutaneous nodules are the most prevalent EAMs, affecting 20 –30% of patients, although nodule prevalence may be decreasing (Turk *et al.,* 2021).

2.7.3 Disease Activity

2.7.3.1 Erythrocyte Sedimentation Rate (ESR) Test

The ESR, discovered in 1897 and widely accepted in 1918, is a simple clinical test commonly used as a sickness index for monitoring several

inflammatory diseases such as temporal arteritis and polymyalgia rheumatic. it is also accepted as a critical prognostic factor in some non-inflammatory conditions, including stroke, heart attack, and prostatic cancer (Isiksacan *et al.*, 2016).

2.7.3.2 Disease Activity Score 28 (DAS28)

The RA advances relatively slowly but progressively, and usually periods of worsening and remission alternate with one another. Every attack causes rapid joint destruction, degeneration of the functional capacity of the musculoskeletal system, and a different degree of incapacity. Measurement of the DAS is one of the main thoughts in the choice of a therapeutic method to prevent the disability of RA patients (Boyadzhieva *et al.,* 2018).

The DAS is a method used to monitor RA activity. It combines patients to assess their general health using a visual analog scale (VAS), tender joints, swollen joints, and acute phase reactants (APR): CRP or ESR (Brzustewicz *et al.,* 2017).

2.8 Laboratory Diagnosis

2.8.1 Immunological Diagnosis

Assessments of autoantibodies have been long-term companions to physicians' managing RA patients, and their importance has increased over the last decades. As guidelines and criteria tend to put increasing emphasis on autoantibody analyses, and because the field is highly dynamic, it becomes even more important for physicians to be aware of the drawbacks and advantages of such analysis (Rönnelid *et al.*, 2021). These autoantibodies are most commonly found in RA patients' serum and synovial fluid (SF), (van Delft & Huizinga, 2020).

2.8.1.1 Rheumatoid Factor (RF) Autoantibodies

The RF was the first autoantibody found in patients with RA. It is an antibody directed against serum gamma-globulins and promotes the agglutination of sheep red blood cells sensitized by sub-agglutinating doses of rabbit antibodies.

It was first described in 1940. These antibodies were first identified in 1948 in patients with RA, and because of their close association with RA, they were given the name RF in 1952. However, although they owe their name to their first detection in RA patients, RFs are found in patients with other autoimmune and non-autoimmune diseases as well as in healthy subjects (Ingegnoli *et al.*, 2013).

The RF is an antibody to the IgG fragment crystallizable (Fc) region; it has been linked to different autoimmune diseases, including RA and SLE. Additionally, IgM and IgA RFs, which are pathogenic biomarkers, were employed to support the diagnosis of RA (Aiman *et al.,* 2020).

Immune complexes and polyclonal B-cell activators, such as bacterial LPS and Epstein-Barr virus, are frequently responsible for the intermittent production of low-affinity IgM RF. It has been shown that RFs have physiological functions that improve the clearance of the immune complex by increasing its avidity and size, aid B cells in absorbing the immune complex, effectively present antigens to T cells, and facilitate complement fixation by binding to immune complexes containing IgG (Song & Kang, 2009). B cells in lymphoid follicles and germinal center-like structures from the inflamed synovial tissue generate RFs locally in RA (Kang *et al.*, 2020).

According to several studies, RF tests in RA patients have a specificity ranging from 48 to 92% and a sensitivity ranging from 60 to 90% (de Brito Rocha *et al*., 2019). In the healthy population, the frequency of RF ranges from 1.3–4% in Caucasians to 30% in some groups of North American Indians (van Delft & Huizinga, 2020).

2.8.1.2 Anti-Cyclic Citrullinated Peptides (ACCP) Autoantibodies

The discovery of ACCP has had a major impact on the understanding of RA. In the late 1990s, it was originally described that RA patients develop autoantibodies that target peptides and proteins containing citrulline, a modified

form of the amino acid arginine. Citrullination is a posttranslational modification of protein-bound arginine into citrulline residues that is mediated by peptidyl arginine deiminase (PAD) enzymes (Dekkers *et al.,* 2016*)*. Citrullination could create particular neoantigens that activate T cells, which in turn will provide antigen-specific help to B cells to produce ACCP. Indeed, citrullination increases the affinity of peptides for HLA-DRB1SE alleles. However, T cells may recognize PAD instead of citrullinated peptides and then help B cells produce antibodies against citrullinated proteins bound to PAD as a hapten/carrier case (Sakkas & Bogdanos, 2018).

The ACCP autoantibodies are present in 70%–90% of patients with RA disease. ACCP autoantibodies show higher disease specificity than RFs (90%–95% versus 70%–80%), (Kang *et al.,* 2020). As a result, ACCP could be an accurate marker for the early identification of RA. In addition, they should be aware that RA patients may experience bone erosion. Since ACCP autoantibodies are mainly seen in RA, while citrullination is seen in many diseases (Liu *et al.,* 2022). Only 1–3% of healthy people have ACCP, and most often at low levels (Volkov *et al.,* 2020).

Further studies established that ACCP antibodies can be recognized years before the onset of RA and are also related to higher disease activity and joint damage in patients with RA (Shakiba *et al*., 2014).

When considering factors that could influence ACCP, age is of interest because several autoantibodies are associated with increasing age, and ACCP positivity is higher in older RA-free women (Berens *et al.,* 2019).

2.8.1.3 heterogeneous nuclear Ribonucleic Protein (hnRNP) or RA33 Complex Autoantibodies

Since 1989, anti-RA33 autoantibodies, which are directed against the RA33 complex or hnRNP, have been identified in RA patients' serum (Lee & Bae, 2016).

Anti-RA33 is present in about 15–35% of RA patients. The main restricting factor in utilizing anti-RA33 in the criteria for RA diagnosis is the extended range of sensitivity between 6% and 75%. The use of anti-RA33 autoantibody in RA diagnosis is a matter of doubt because it was detected in a lower percentage of RA patients (Alattabi *et al*., 2020).

The A2/RA33 is a common mRNA-binding protein that, like other hnRNP proteins, has a modular structure consisting of two conserved RNA-binding domains and a glycine-rich auxiliary domain assumed to be involved in interactions with other proteins. It has a predominantly nuclear localization and exerts multiple functions, including regulating alternative splicing and transporting mRNA. A2/RA33 seems to be expressed everywhere, although the level of expression may change significantly between different tissues (Fritsch *et al.,* 2016).

However, only one epitope of RA33 has been identified as possibly autoantigenic in RA patients, hnRNP-A2 (and its alternatively spliced forms B1 and B2). Actually, about 30 different epitopes of hnRNP have been identified, with each epitope referring to the specific protein sequence that combines with pre-mRNA to produce the hnRNP complex. In addition to hnRNPA2, a few other members of the large family of hnRNPs have demonstrated auto-antigenic participation in systemic autoimmune rheumatoid diseases with more or less affinity (Al-mughales, 2015).

In recent research, antibodies against citrullinated RA33 were mainly observed in individuals with a longer duration of the disease and more erosive disease, whereas antibodies against natural hnRNP A2/B1 (RA33) were found in SF from patients with RA, especially those with early RA (Poulsen *et al.,* 2020).

2.8.1.4 Anti-Sjögren's Syndrome Antigen A (Anti-SSA) Autoantibodies

Anti-Ro, typically seen in primary SS, has been linked to symptoms of oral and eye dryness and secondary SS in RA, SLE, scleroderma, and primary biliary cirrhosis (Zanlorenzi *et al*., 2012).

While SS can co-occur with another autoimmune disease, the accompanying disease is often the main determinant of the phenotype and the therapy. The SS individuals, however, displayed different phenotypes aside from joint involvement when consistent with RA. Risk factors for RA/SS have not yet been identified. Additionally, most SS occurs after RA; however, occasionally RA and SS occur simultaneously or RA begins to occur after SS (Yang *et al*., 2018).

Ro antigens consist of two different proteins, Ro60 and Ro52. In 1984, the target antigen for anti-Ro autoantibodies was first identified as a 60 kDa protein that exists as RNP complexes with small cytoplasmic RNAs (hY-RNA). Subsequently, the complementary deoxyribonucleic acid (cDNA) of Ro60 was cloned. In 1988, the 52 kDa protein, called Ro52, was a component of the Ro antigens, and three years later, the cDNA of human Ro52 was cloned. In humans, the Ro60 gene is around 32 kb in size, located on chromosome 19, whereas the Ro52 gene is 8.8 kb in size, located on chromosome 11 (Yoshimi *et al.*, 2012).

Anti-Ro/SSA antibodies, consisting of anti-Ro/SSA-52kD and anti-Ro/SSA-60kD sub-specificities, are produced as a result of an autoimmune response towards the two intracellular Ro subunits (Ro52-kD and Ro60-kD). They are polyclonal antibodies, often belonging to the IgG class (Lazzerini *et al.,* 2021).

2.8.1.5 Anti-double stranded Deoxyribonucleic Acid (Anti-dsDNA) Autoantibodies

The presence of these antibodies is highly suggestive of SLE. As more sensitive tests have been developed, anti-dsDNA antibodies have been found

occasionally in patients with other diseases. Such antibodies have been observed in patients with RA and juvenile rheumatoid arthritis (JRA), (Bell *et al.,* 1975).

Although they react with DNA, pathogenic anti-dsDNA autoantibodies are not entirely specific to it. Anti-dsDNA antibodies can recognize a variety of self-antigens, which trigger apoptosis, inflammatory responses, and tissue fibrosis. It was reported that synthetic peptides that mimic a molecular DNA structure interact and specifically recognize anti-dsDNA antibodies, suggesting a novel therapeutic opportunity through inhibition of the antigen recognition of dsDNA autoantibodies (Wang & Xia, 2019).

The induction of anti-dsDNA and anti-nuclear antibodies (ANAs) during Infliximab (IFX) therapy is an established phenomenon that has already been observed in earlier clinical studies (Yukawa *et al.*, 2011).

2.9 Treatment of Rheumatoid Arthritis

The aim of treatment in any disease state is to achieve remission or low disease activity if remission is not possible due to chronic disease or comorbidities. Treatment should aim to avoid joint degeneration and disability, as well as systemic manifestations such as CVD, which is best achieved by a treated-target strategy that includes tight disease management (Burmester & Pope, 2017).

2.9.1 Analgesics and Non-steroidal Anti -Inflammatory Drugs (NSAIDs)

In the treatment of RA disease, analgesics, and NSAIDs are mostly utilized on a temporary basis prior to the DMARDs taking action, as well as during flares. The most popular analgesic is acetaminophen (paracetamol), even though any analgesic can be utilized because of its few adverse effects. At least 20 distinct NSAIDs have been utilized, with diclofenac, ibuprofen, ketoprofen, and indomethacin among the popular short-acting NSAIDs. Long-acting NSAIDs include naproxen, celecoxib, meloxicam, nabumetone, and piroxicam (Kumar & Banik, 2013).

2.9.2 Disease Modifying Anti- Rheumatic Drugs (DMARD)

DMARDs are currently the first line of treatment for RA. DMARDs function by modulating various aspects of immune and inflammatory responses that cause clinical manifestations of RA (Yap *et al.,* 2018). DMARDs were classified into biologic (b) DMARDs and conventional (c) DMARDs (Alemao *et al.,* 2020).

2.9.2.1 conventional Disease Modifying Anti- Rheumatic Drugs (cDMARD)

The DMARDs have an influence on the disease's natural course and, in most cases, continue to inhibit its inflammatory activity (Boyadzhieva *et al.*, 2018).

Methotrexate (MTX) is an anti-folate metabolite that prevents DNA synthesis, repair, and cellular replication. It has anti-inflammatory and immune-modulating characteristics, and it was originally used for the treatment of RA and psoriasis in 1951. However, MTX therapy didn't receive more attention for it use in treating RA until the early 1980s. A few years later, studies reported its high efficacy and marked superiority to placebo in chronic and severe RA. Soon after, in 1988, the Food and Drug Administration (FDA) approved MTX as a treatment for RA (Wang *et al*., 2018).

Sulfasalazine (salazosulfapyridine) is a well-known DMARD used to treat patients with RA (Plosker & Croom, 2005).

Hydroxychloroquine (HCQ), which is most frequently utilized as the first-line treatment in individuals with autoimmune disorders including RA and SLE (Lane *et al.,* 2020). HCQ has different immunomodulatory effects, which include inhibiting phagocytosis and chemotaxis, TLR signaling, calcium signaling in lymphocytes, macrophage-mediated cytokine release, and matrix metalloproteinases. It has been found not only to aid in the treatment of RA but also has the potential to help lessen its EAM (Nazir *et al.*, 2021).

Leflunomide is a novel isoxazole derivative that has been shown to be effective in patients with RA. It has both anti-inflammatory and immune-modulatory properties, primarily by inhibiting the *de novo* creation of pyrimidine nucleotides (and therefore DNA and RNA) in immune response cells (Alldred & Emery, 2001).

2.9.2.2 biological Disease Modifying Anti- Rheumatic Drugs (bDMARD)

bDMARDs are large proteins, and those approved to date for the treatment of RA do not have the capability of crossing the cell's plasma membrane. As a result, bDMARDs target extracellular mediators of inflammation with high specificity. These include pro-inflammatory cytokines and cell membrane-associated immune proteins. The management of RA has been altered over the last 20 years by bDMARDs such as TNF inhibitors (Law & Taylor, 2019).

Biologic agents block certain chemicals in the blood from activating the immune system and hence protect patients' joints. A joint treatment regime of both biologic and DMARD therapy is recommended (Tvete *et al.,* 2015).

2.9.3 Glucocorticoids

Glucocorticoids are extremely potent anti-inflammatory agents, and in addition to controlling inflammatory symptoms, there is evidence that early initiation of low-dose glucocorticoids (i.e., ≤ 10 mg of prednisone equivalent) can delay radiologic progression within the first 6 months (Littlejohn & Monrad, 2018).

Chapter Three

Subjects, Materials and Methods

Subjects, Materials and Methods

3.1 Subjects

A case-control study was conducted at the College of Applied Medical Sciences/ University of Kerbala. Forty-one patients with RA were enrolled in this study and diagnosed with RA by rheumatologists in Al-Hindiya Teaching Hospital and Al-Imam Al-Hassan Al-Mujtaba Hospital/ Karbala Health Directorate during the period from November 2022 to February 2023. The age range of the patients was from 24 to 77 years from both sexes. The control group was composed of 41 apparently healthy subjects with no history of RA disease. The matching criteria between patients and controls were sex, age, and residency. Thus, the Male/ Female ratio and the age range were the same in both the patient and control groups. Patients and control subjects were selected randomly according to inclusion and exclusion criteria. Demographic information about the participants was also collected according to the questionnaire, as presented in Appendix (1). Blood samples were drawn from both RA patients and healthy volunteers to be used for investigation.

3.1.1 Inclusion Criteria and Exclusion Criteria

3.1.1.1 Inclusion Criteria

The following inclusion criteria were followed to include patients and control groups in the current study:

1. Patients with RA whom they were clinically diagnosed by physicians.
2. Both sexes, male and female.
3. The ages of the patients were above 18 years.
4. Healthy subjects with no history of RA disease whom they match the same age, sex, and residency as the patients' group

3.1.1.2 Exclusion Criteria: Include

1. Individuals aged less than 18 years
2. Patients with osteoarthritis, psoriatic arthritis, gout, and fibromyalgia were excluded.
3. Pregnant female.

3.1.2 Questionnaire

Information from both patients and control groups were collected, which includes names, age, sex, occupation, family history, BMI, symptoms of SLE, symptoms of SS, CVD, DAS28, and duration of RA, in addition to other questions, as shown in Appendix (1).

3.1.3 Ethical Considerations

This study was approved by the Ethical Committee at the College of Applied Medical Science/University of Kerbala and the Ethical Committee at Al-Hindiya Teaching Hospital and Al-Imam Al-Hassan Al-Mujtaba Hospital. All subjects involved in this work were informed, and agreement was obtained verbally from each one before the samples' collection.

3.1.4 Study Design

A case-control study design was conducted, as shown in Figure (3.1).

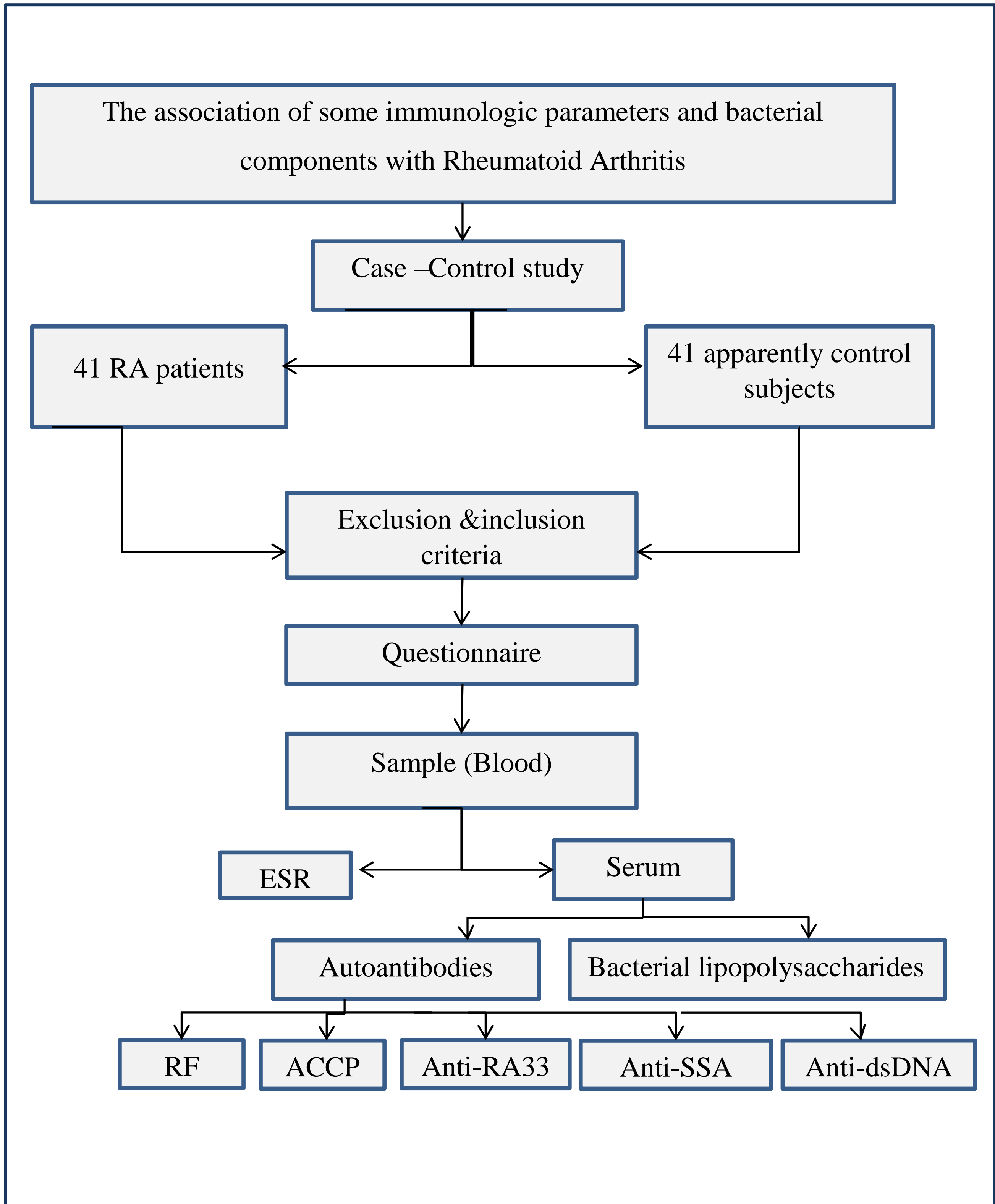


Figure 3.1: Study Design

3.2 Materials

3.2.1 Kits

The kits used in the current study were presented in Table (3.1).

Table 3.1: Kits of The study

No	Kits	Company	Origin
1.	Human Rheumatoid Factor (RF) IgG ELISA Kit	SUNLONG	China
2.	Human anti-cyclic citrullinated peptide antibody (anti-CCP antibody) IgG ELISA Kit	Mybiosource	USA
3.	Qualitative Human Heterogeneous Nuclear Ribonucleoprotein Compies/Anti-RA33-Antibody (hnRNP/RA33) ELISA Kit	Mybiosource	USA
4.	Human Anti-SSA/Ro Antibody (Anti-Ro) ELISA Kit	Mybiosource	USA
5.	Double Stranded DNA (dsDNA) IgG ELISA kit	Mybiosource	USA
6.	Bacterial Lipopolysaccharides (LPS) ELISA Kit	Mybiosource	USA

3.2.2 Devices, Equipment's and Apparatuses

Table (3.2) showed the instruments and apparatuses that were used in this study.

Table 3.2: Devices and Apparatuses of The current Study

No	Devices, Equipment's and Apparatuses	Company	Origin
1.	Centrifuge	ROTOFIX 32 A (Hettich)	Germany
2.	ELISA reader HS	Human	USA
3.	ESR fast detector	Shanghai	China
4.	Refrigerator	LG	Korea

3.2.3 Tools

Table (3.3) showed the tools that were used in this study.

Table 3.3: The tools of The study

No	Tools	Company	Origin
1.	Disposable syringe 5 ml	AL-Shaghaf	China
2.	Eppendorf tube	TRUST LAB	China
3.	ESR tube ,9*120mm	ALS Laboratory supplies	China
4.	Gel tube	TRUST LAB	China
5.	Gloves	KINGFA/MEDICAL	China
6.	Mask	TKMD	Germany
7.	Micro plate	Mybiosource	USA
8.	Multi-channel pipette	CappAero 96	Germany
9.	Pipette Tips	CITOTEST	China
10.	Single-channel Micropipette	Dragon Laboratory	China
11.	Tourniquet	Voltaren	China

3.3 Methods

3.3.1 Collection of Sample

Using a disposable syringe, five milliliters of venous blood were drawn from each participant. About 1.5 ml of blood was collected in the ESR tube for ESR detection, and 3.5 ml of blood was drawn in a gel tube and left to sit at room temperature for 15 minutes. Serum was separated by using a centrifuge for 10 minutes at about 3000 round per minute (rpm). The serum sample was transferred into two eppendorf tubes, stored at -20°C to avoid repeated freezing-thawing cycles, and used for the further measurement of the autoantibodies and bacterial LPS.

3.3.2 Estimation of Disease Activity Score 28 (DAS28)

The DAS 28 is based on the calculated number of tender and swollen joints (28-joint count). The 28-joint count included the shoulders, elbows, wrists, first to fifth MCP joints, first to fifth PIP joints, and knees on both sides of the body, and ESR. DAS 28-ESR (DAS 28-ESR) was calculated using an online calculator by entering the data of tender joint count, swollen joint count, global assessment, and ESR level for each patient (Hammodat & Mustafa, 2018).

The cut-off values for DAS28 were shown in Table (3.4), (Canhão *et al.*, 2018).

Table 3.4: DAS28 Cut-Off Values

DAS 28 status	DAS28 range
Remission	< 2.6
Low activity	≥ 2.6 - ≤ 3.2
Moderate activity	> 3.2 - ≤ 5.1
High activity	> 5.1

3.3.3 Estimation of Body Mass Index (BMI)

BMI is the index currently in use for identifying adult anthropometric height/weight characteristics and grouping (categorizing) them according to WHO, as shown in Table (3.5), (Nuttall, 2015). The following equation was used to calculate BMI:

BMI= Weight (kg)/ Height^2 (meters), (Dang *et al.*, 2022).

Table 3.5: Body Mass Index (BMI)

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

3.3.4 Estimation of physiological parameters

3.3.4.1 Estimation of Erythrocyte Sedimentation Rate (ESR)

3.3.4.1.1 Principle

The ESR test is based on the principle of sedimentation. When the blood in an anticoagulant is left undisturbed, red blood cells will settle down in the container. Then the height of the column of clear plasma was measured after 30 minutes.

3.3.4.1.2 Procedure

1. Venous blood (1.2 ml) was drowned in the ESR tube (9*120 mm), and then immediately, the tube was inverted at 180 degrees 6–8 times to achieve good mixing, which will avoid hemolysis, clotting, and blood bubbles.
2. The ESR fast detector was put on a steady surface, and the bubble level was adjusted with the knob below.
3. At room temperature, the ESR tube was put upright on the ESR detector, and the starting time was documented.
4. After 30 minutes, the plasma concave was aligned in the ESR tube to the zero scale of the ESR detector, and the data was read by aligning the upper surface of the erythrocyte to the scale on the ESR detector.

3.3.5 Estimation of Autoantibodies

All autoantibodies were tested by enzyme-linked immunosorbent assay (ELISA).

3.3.5.1 Estimation of Rheumatoid Factor (RF) Autoantibodies

3.3.5.1.1 Principle

The assay is based on the qualitative enzyme immunoassay technique. The microplate included in this kit has been pre-coated with an antibody specific to RF, making it a solid-phase antibody. Samples were put into the microplate wells and combined with the corresponding antigens. A horseradish peroxidase (HRP)-conjugated antigen specific for RF was then added to each microplate well and incubated. As a result, the antibody-antigen-enzyme-labeled antibody complex is formed. Any unbound reagents will be removed by washing. In each well, 3,3', 5,5'-Tetramethylbenzidine (TMB) substrate solution was then added. Only the wells containing RF and HRP-conjugated RF antibodies will turn blue and then turn yellow after the stop solution was added. Then a spectrophotometer is used to measure the OD at a wavelength of 450 nm.

3.3.5.1.2 Kit Components

RF ELISA kit components were presented in Table (3.6).

Table 3.6: RF ELISA Kit Components

Materials provided with the kit	96 determinations	Storage
User manual	1	Room temperature
Closure plate membrane	2	Room temperature
Sealed bags	1	Room temperature
Microelisa stripplate	1	2-8℃
Negative control	0.5ml×1 bottle	2-8℃
Positive control	0.5ml×1 bottle	2-8℃
HRP-Conjugate reagent	6ml×1 bottle	2-8℃
Sample diluent	6ml×1 bottle	2-8℃
Chromogen Solution A	6ml×1 bottle	2-8℃
Chromogen Solution B	6ml×1 bottle	2-8℃
Stop Solution	6ml×1 bottle	2-8℃
Wash solution	20ml (30X)×1bottle	2-8℃

3.3.5.1.3 Procedure

1. The sample's micropores in the microplate were numbered sequentially. Two wells were left empty as the blank control, two wells served as the positive control, and two wells served as the negative control. (The blank control hole was without samples and HRP-conjugate reagent; the rest of the steps of the operation were the same).

2. Fifty microliters of the negative and positive controls were added to the corresponding control wells, respectively. Forty microliters of sample dilution buffer and ten µl of the sample (the dilution factor was 5) were added to the sample wells. The samples had been loaded onto the bottom without touching the wall of the wells and gently shaken to mix them.

3. The microplate was sealed with a closure plate membrane and incubated at 37 °C for 30 minutes.
4. Distilled water was used to dilute the concentrated washing buffer 30 times.
5. The membrane covering the plate was gently removed, aspirated, and refilled with the wash solution. The wash solution was discarded after resting for 30 seconds. The washing procedure was repeated five times.
6. Except for the blank control well, 50 microliters of HRP-conjugate reagent were added to each well.
7. The microplate was incubated as described in step 3.
8. The microplate was washed as described in step 5.
9. Fifty microliters of chromogen solution A and 50 µl chromogen solution B were added to each well, gently mixed, and incubated at 37 °C for 15 minutes. The coloring step was done without exposure to light.
10. Fifty microliters of stop solution were added to each well to stop the reaction. The color of the wells was altered from blue to yellow.
11. A microtiter plate reader was used to read OD at 450nm. The OD value of the blank control well was set as zero. Assay was carried out within 15 minutes after adding the stop solution.

3.3.5.2 Estimation of Anti-Cyclic Citrullinated Peptide (ACCP) Autoantibodies

3.3.5.2.1 Principle

This assay is based on the qualitative enzyme immunoassay technique. The microtiter plate provided in this kit has been pre-coated with CCP antigen. Samples are pipetted into the wells with anti-human IgG-conjugated HRP. Any CCP-specific antibodies present will bind to the pre-coated antigen. Then, after washing to remove any unbound reagent, a substrate solution is added to the wells, and color develops in proportion to the quantity of human ACCP antibody bound in the

initial step. The development of the color is stopped, and the color intensity is measured.

3.3.5.2.1.1 Kit Components

ACCP ELISA kit components were presented in Table (3.7).

Table 3.7: ACCP ELISA Kit Components

Reagents	Quantity
Assay plate	1(96 wells)
Negative Control	1 x 0.8 ml
Positive Control	1 x 0.8 ml
HRP-conjugate	1 x 10 ml
Sample Diluent	1 x 20 ml
Wash Buffer (25 x concentrate)	1 x 20 ml
Substrate A	1 x 5 ml
Substrate B	1 x 5 ml
Stop Solution	1 x 5 ml
Adhesive Strip (For 96 wells)	4
Instruction manual	1

3.3.5.2.3 Procedure

1. All reagents and samples were kept at room temperature before use.
2. The number of wells used was determined, and the remaining unused wells were stored at 4 °C.
3. A blank well was left empty.
4. One hundred microliters of diluted samples, a positive control, and a negative control were put into each well.
5. The wells were sealed with the supplied adhesive strip and incubated for 30 minutes at 37 °C.

6. Each well was aspirated and washed; the process was repeated four times for a total of five washes. Each well was filled with wash buffer (200–300 µl) using a multichannel pipette, and it was left to stand for 30 seconds. Complete removal of the liquid at each step was crucial for effective performance. Any remaining wash buffer was removed by aspirating or decanting after the last wash. The plate was inverted and blotted against clean paper towels.

7. One hundred microliters of HRP-conjugate were added to each well except the blank wells. It was mixed well and then incubated at 37°C for 30 minutes.

8. The aspiration and washing processes were repeated five times, as done in the sixth step.

9. Fifty microliters of substrate A and 50µl of substrate B were added to each well, mixed well, and incubated at 37 °C for 10 minutes. The plate was maintained in the dark, away from drafts and other temperature fluctuations.

10. Fifty microliters of stop solution were added to each well, and the plate was tapped gently to ensure good mixing.

11. The blank well was taken as the reference (zero), and the OD of each well was determined using a microplate reader set to 450 nm within ten minutes.

3.3.5.3 Estimation of Anti-Rheumatoid Arthritis 33 (RA33) Autoantibodies

3.3.5.3.1 Principle

The kit uses a sandwich ELISA to qualitatively analyze human hnRNP/RA33 antibodies in human serum, plasma, or other biological fluids.

3.3.5.3.2 Kit Components

All reagents provided were stored at 2–8 °C. Anti-RA33 ELISA kit components were presented in Table (3.8).

Table 3.8: Anti-RA33 ELISA Kit Components

Materials	96 Tests
Microelisa stripplate	12wells*8strips
Positive Control	0.5ml/vial
Negative Control	0.5ml/vial
Sample diluent	6.0ml
HRP-Conjugate reagent	10ml
20X Wash solution	25ml
Chromogen Solution A	6.0ml
Chromogen Solution B	6.0ml
Stop Solution	6.0ml
Closure plate membrane	2
User manual	1
Sealed bags	1

3.3.5.3.3 Procedure

1. First, all reagents and samples were naturally brought to room temperature (18–25 °C) for 30 minutes before starting assay procedures.

2. Positive control wells, negative control wells, and sample wells were set. Fifty microliters of positive and negative control were added to each positive and negative control well, respectively. Ten microliters of sample and 40µl of sample diluent were added to each sample's well.

3. One hundred microliters of HRP-conjugate reagent were added to negative control wells, positive control wells, and sample wells. A tape strip was used to cover the wells, and the plate was incubated at 37°C for 60 minutes.

4. The microtiter plate was washed four times.

5. Chromogen Solution A 50 µl and Chromogen Solution B 50 µl were added to each well successively. And it was mixed gently, and then the plate was incubated for 15 minutes at 37°C without light.
6. Fifty microliters of stop solution were added to each well. The color of the wells was changed from blue to yellow.
7. The OD was read at 450 nm using an ELISA reader within 15 minutes after adding the stop solution (around 5 minutes was the best time).

3.3.5.4 Estimation of Anti-Sjögren's syndrome antigen A (Anti-SSA) Autoantibodies

3.3.5.4.1 Principle

The double antigen sandwich ELISA technique is used in this kit. The pre-coated antigen is a related human anti-Ro antigen, whereas the detection antigen is different from it. Samples are added into ELISA plate wells and washed out with phosphate buffer saline (PBS) or tris-buffered saline (TBS) after their respective additions to the wells. Following that, avidin-peroxidase conjugates are added to the wells. After the enzyme conjugate has been completely removed from the wells by PBS or TBS, TMB substrate is added for coloring. TMB reacts to form a blue product from the peroxidase activity and eventually becomes yellow after the addition of the stop solution (Color Reagent C). The concentration of the target analyte in the sample and color intensity are positively correlated.

3.3.5.4.2 Kit Components

Anti-SSA ELISA kit components were presented in Table (3.9).

Table 3.9: Anti-SSA ELISA Kit Components

Name	96 Tests	Storage
Antigen pre-coated plate	8×12	4/-20℃
Human Anti-Ro Standards	2 vial	4/-20℃
Antigen (1:100)	1vial	4/-20℃
Enzyme conjugate (1:100)	1vial	4/-20℃
Enzyme diluent	1vial	4/-20℃
Antigen diluent	1vial	4/-20℃
Standard diluent	1vial	4/-20℃
Sample diluent	1vial	4/-20℃
Washing buffer (1:25)	1vial	4/-20℃
Color Reagent A	1vial	4/-20℃
Color Reagent B	1vial	4/-20℃
Color Reagent C	1vial	4/-20℃
Manual	1 set	Room temperature

3.3.5.4.3 Procedure

1. The ELISA kit was removed from the refrigerator 20 minutes in advance.

2. Test preparation:

a. The concentrated washing buffer was diluted with double-distilled water (1:25).

b. Standard: The lyophilized standard vial was diluted with 1 ml of standard diluent and allowed to rest for 30 minutes. After the standard had been completely dissolved, it was mixed slightly, and the tube was labeled. The standard curve's concentration values were 200, 100, 50, 25, 12.5, 6.25, and 3.12 U/ml.

c. Legend of the standard sample dilution method: seven clean tubes were taken and labeled with their predicted concentrations of 100, 50, 25, 12.5, 6.25, 3.12, and

0 units per milliliter. Three hundred microliters of standard diluent were added to each tube. Three hundred microliters of diluent were pipetted from the reconstituted standard, and it was added to the tube labeled 100 U/mL and mixed well. Three hundred microliters of diluent were pipetted out from the 100 U/mL tube and added to the 50 U/mL, and it was mixed well. These steps were repeated until the 3.12 U/mL standard was reached. The standard diluent in the 0 U/mL tube served as the negative control.

d. Antigen: A quantity of antigen solution adequate for the number of wells being tested was drawn out, and it was diluted with antigen diluent in a ratio of 1:100. This was made 30 minutes in advance.

e. Enzyme Conjugate: the necessary amount of enzyme conjugate solution for the number of wells intended to be tested was taken out. And it was diluted with the enzyme diluent in a ratio of 1:100. This was prepared 30 minutes in advance.

f. Color Reagent: A solution of color reagents A and B was prepared 30 minutes in advance by mixing them in a 9:1 ratio.

g. Manual washing of plates: 350 µl wash buffer was added to each well, and it was allowed to stand for 30 seconds. The plate was dapped with absorbent paper after being sacked to get rid of as much liquid as possible.

3. Steps:

a. The appropriate number of strips were taken out and allowed to warm at room temperature.

b. One hundred microliters of standards and samples were added to their corresponding wells. One hundred microliters of standard diluent have been added to the 0 U/mL well. The adhesive tape strip was used to seal the plate, which was then incubated for 90 minutes at 37°C.

c. The required amount of antigen was prepared 30 minutes in advance.

d. The plate was washed twice.

e. One hundred microliters of the prepared antigen were added to each well. Adhesive strips were used to close the reaction wells, which were then incubated for 60 minutes at 37 °C.

f. The required amount of enzyme conjugate was prepared 30 minutes in advance.

g. The plate was washed three times.

h. One hundred microliters of the prepared enzyme conjugate were added to each well except the blank wells. Adhesive strips were used to close the wells, which were then incubated for 30 minutes at 37 °C.

i. The plate was washed five times.

j. One hundred microliters of the prepared color reagent were added to individual wells and blank wells, which were then incubated in the dark at 37 °C. The incubation stopped when the coloring of the highest standards became darker and the color gradient appeared. The chromogenic reaction has been controlled to within 30 minutes.

k. One hundred microliters of color reagent C were added to each well (also into the blank well). And it was mixed well.

l. The OD was read at 450 nm within 10 minutes.

3.3.5.5 Estimation of Anti-double stranded Deoxyribonucleic Acid (Anti-dsDNA) Autoantibodies

3.3.5.5.1 Principle

The qualitative enzyme immunoassay technique is used in this assay. The microtiter plate of this kit has been pre-coated with dsDNA antigen. Then anti-human IgG-conjugated HRP is added to the wells with the samples. Any antibodies that are specific to dsDNA present in the samples will bind to the pre-coated antigen (dsDNA). A substrate solution is then added to the wells after a wash to remove any unbound reagents, and color develops according to the quantity of

human anti-dsDNA antibodies bound in the first stage. Then the development of the color is stopped by adding a stop solution, and the color's intensity is measured.

3.3.5.5.2 Kit Components

Anti-dsDNA ELISA kit components were presented in Table (3.10).

Table 3.10: Anti-dsDNA ELISA Kit Components

Materials provided	96 Test
Microwells coated with dsDNA antigen	12x8x1
Sample Diluent: 1 bottle (ready to use)	22 ml
Enzyme conjugate: 1 bottle (ready to use)	12ml
TMB Substrate: 1 bottle (ready to use)	12ml
Positive Control: 1 vial (ready to use)	1ml
Negative Control: 1 vial (ready to use	1ml
Stop Solution: 1 bottle (ready to use)	12ml
Wash concentrate 20X: 1 bottle	25ml

3.3.5.4.3 Procedure

1. All specimens and kit reagents have been brought to room temperature (20–25 °C) and mixed gently.
2. Test samples were diluted by adding 10 µl of sample to 200 µl of sample diluent (11:20), mixing well, and then gently shaking the mixture.
3. One hundred microliters of controls, and diluted serum were dispensed into the appropriate wells. For the reagent blank, 100 µl of sample diluent was dispensed in the 1A well position. The holder was tapped to remove air bubbles from the liquid and mixed well. It was incubated for 20 minutes at room temperature.
4. The liquid was removed from all wells, and wells were washed three times with 300 µl of 1X wash buffer. It was dried with absorbent paper or a paper towel.
5. One hundred microliters of enzyme-conjugate were added to each well, and it was incubated for 20 minutes at room temperature.

6. The enzyme-conjugate was removed from all wells. Wells were washed three times with 300 µl of 1X wash buffer. Then it was dried with absorbent paper or a paper towel.
7. One hundred microliters of TMB substrate were dispensed into the well, and the plate was incubated for 10 minutes at room temperature.
8. One hundred microliters of stop solution were added.
9.The OD was read at 450 nm using an ELISA reader within 15 minutes.

3.3.6 Estimation of Bacterial Lipopolysaccharides (LPS)

3.3.6.1 Principle

The quantitative sandwich enzyme immunoassay technique is used in this assay. A microplate has been pre-coated with an LPS-specific antibody. Standards and samples are pipetted into the wells, and the immobilized antibody binds to any LPS antigen that may be present in the samples. After the removal of any unbound substances, a biotin-conjugated antibody specific to LPS is added to the wells. Avidin-conjugated HRP is added to the wells after washing. A substrate solution is then added to the wells after a wash to remove any unbound avidin-enzyme reagent, and color develops in proportion to the amount of LPS antigen bound in the first phase. The development of the color is stooped, and the color's intensity is measured.

3.3.6.2 Kit Components

LPS ELISA kit components were presented in Table (3.11).

Table 3.11: Components of LPS ELISA Kit

Reagents	Quantity
Assay plate (12 x 8 coated Microwells)	1(96 wells)
Standard (Freeze dried)	2
Biotin-antibody (100 x concentrate)	1 x 120 µl
HRP-avidin (100 x concentrate)	1 x 120 µl
Biotin-antibody Diluent	1 x 15 ml
HRP-avidin Diluent	1 x 15 ml
Sample Diluent	1 x 50 ml
Wash Buffer (25 x concentrate)	1 x 20 ml
TMB Substrate	1 x 10 ml
Stop Solution	1 x 10 ml
Adhesive Strip (For 96 wells)	4
Instruction manual	1

3.3.6.3 Procedure

1. All samples and reagents were brought to room temperature before use.
2. One hundred microliters of standard and sample were added per well. Wells were covered with the adhesive strip provided. It was incubated at 37°C for 2 hours. A plate plan has been provided to record standards and samples examined.
3. From each well, the liquid was removed.
4. One hundred microliters of biotin antibody (1x) were added to each well. A new adhesive strip was used to cover the plate, which was then incubated at 37 °C for an hour.
5. Each well was aspirated and washed twice, for a total of three washes. Each well was washed with 200 µl of wash buffer using a multi-channel pipette, and it was

allowed to stand for two minutes. Complete removal of the liquid at each step was essential to good performance. After the last wash, any remaining buffer was aspirated or decanted. The plate was inverted and blotted against clean paper towels.

6. One hundred microliters of HRP-avidin (1x) were added to each well. A new adhesive strip was used to cover the microtiter plate, which was then incubated at 37 °C for an hour.

7. The aspiration and washing processes were repeated five times, as in step 5.

8. Ninety microliters of TMB substrate were added to each well. The microtiter plate was incubated at 37 °C in the dark for 15–30 minutes.

9. Fifty microliters of stop solution were added to each well, and the plate was gently tapped to ensure well mixing.

11. A microplate reader set at 450 nm wavelength was used to calculate the OD of each well within 5 minutes.

3.4 Statistical Analysis:

The Statistical Package for the Social Sciences (SPSS), version 22 software (IBM Corp., NY, and USA), was used to analyze data. Descriptive statistics were used to determine frequencies, the mean, standard deviation, median, range, and cross-tabulation. Bivariate correlations were analyzed to determine significant positive and negative correlations between variables if they were present. An independent sample T-test and the Analysis of Variance (ANOVA) test were used to compare means. The Least Significant Difference (LSD) was also determined. The categorical variables were tested by the chi-square test. Receiver Operating Characteristics (ROC) curve analysis was used to determine the cut-off values of autoantibodies and LPS titers. The statistical significance level was established at $P < 0.05$.

This study was approved by the Ethical Committee at the College of Applied Medical Science/University of Kerbala and the Ethical Committee at Al-Hindiya Teaching Hospital and Al-Imam Al-Hassan Al-Mujtaba Hospital. All subjects involved in this work were informed, and agreement was obtained verbally from each one before the samples' collection.

Chapter Four

Results and Discussion

Results and Discussion

4.1 Demographic Data of Patients and Controls

The blood sample was collected from eighty-two subjects (41 patients and 41 controls) from November 2022 to February 2023 in Karbala Governorate, Iraq. The ages of participants ranged from 27 to 77 years, and the mean±SD of age for patients with RA and control were 48.29±11.22 and 48.37±11.12, respectively. They were divided into two groups: those under 45 years old 18 (43.90%), and those over 45 years old 23 (56.09%). The median age for both groups was 49 years, as shown in Table (4.1).

Seven participants from each group were men (17.07%), and 34 (82.92%) were women. The Female /Male ratio was 34:7 (4.8:1), as shown in Table (4.1). This finding was in agreement with other previous studies in which the Female /Male ratios were 4.5:1 (Hussein *et al.*, 2018), 4.52:1(Mathkhor *et al.*, 2021),and 4.2:1 (Jwad *et al*., 2022)**.** The ratio is also increasing for more than 4 in females in premenopausal age (Kvien *et al*., 2006). A previous study indicated that the reason why the incidence of autoimmune diseases in females is higher than that in males is due to several factors, including environmental exposure, e.g., microbiome, behavior, hormones, and genetics, including inactivation of genes in the X chromosome (Henze *et al*., 2020). Additionally, female patients might possibly didn’t realize that the pain which they experience is not caused by heavy workload which may results in the delay in diagnosis and treatment of the disease.

This phenomenon is widely known, especially with autoimmune diseases caused by autoantibodies. The female-to-male ratios in autoimmune diseases such as SS, SLE, Grave’s disease, and Hashimoto’s thyroiditis were 16:1, 7:1, 7:1, and 19:1, respectively, in which about 80% of the patient population was female. In the middle tier of diseases, which includes RA and multiple sclerosis (MS), the female-

to-male ratios were 3:1 and 2:1, respectively, and the sex distribution has been 60–75% female relative to male (Lasrado *et al*., 2020).

Regarding the BMI of participants, the mean±SD of patients and controls was (30.88±5.86) and (29.82±5.45) respectively. There was no significant difference in the mean level of BMI between patients and control in present study and this might possibly due to distribution of obesity worldwide. World Health Organization (WHO) estimated that more than 650 million adults worldwide were obese and this number is still increasing (Boutari & Mantzoros, 2022). Also, it has been reported that RA onset and/or outcome are affected by obesity (George & Baker, 2016). Furthermore, it has been documented that about two third of RA patients were overweight and obese (Linda Rath, 2022).

The current study revealed that the mean±SD of DAS28 in patients was (4.31±1.16). This finding agreed with a previous study which revealed that the mean±SD of DAS28 in patients was (4.75±1.18), (Jwad *et al.,* 2022*)*.

Eleven of the patients (26.82%) had a family history of RA. This finding was in agreement with other previous study which revealed that 20.2% of the patients had family history (Mathkhor *et al*., 2021). Another previous study found that 18% of patients had family history (Abdulameer *et al*., 2022).

The mean±SD of disease duration was (7.65±8.36) years. Similar findings were reported in previous study which reported that the mean±SD of disease duration was (8.67 ± 6.30), (Al-Rawi *et al*., 2018). Twenty-one of the patients (51.21%) had the disease for less than five years, whereas 10 (24.39%) of them had the disease between 5and 10 years, and 10 (24.39%) had the disease for more than ten years. A similar finding was reported in a previous study (Al-Shaibani, 2021).

This study reported a non-significant difference in the age of disease onset between males and females. However, the age of disease onset in female is lower

than that in males, as shown in Table (4.1). The mean±SD for the disease's onset age was (40.63±14.22). This finding was in agreement with a previous study that showed the mean±SD for the disease's onset age was (41±2.1), (Mathkhor *et al.*, 2021).

Concerning clinical findings, thirty-three of the patients (80.48%) had morning stiffness, 39 (95.12%) had intermittent symptoms, 11 (26.82%) had DM, and 14 (34.14%) had hypertension. None of the patients had SLE, and only three patients (7.31%) had SS symptoms, as shown in Table (4.1).

Table 4.1: Demographic Data of Rheumatoid Arthritis Patients and Controls

	RA patients (Total N=41)	Control (Total N=41)
Age in years, Median (Range) **Mean±SD**	49 (27-77) 48.29±11.22	49 (27-75) 48.37±11.12
Age groups N (%) **≤45 years** **>45 years**	 18 (43.90) 23 (56.09)	 18 (43.90) 23 (56.09)
Sex N (%) **Male** **Female**	 7 (17.07) 34 (82.92)	 7 (17.07) 34 (82.92)
BMI kg/m^2, Median (Range) **Mean±SD**	30.11(19.44-43.10) 30.8871±5.86486	29.4 (20.70-42.90) 29.8244±5.45590
Obesity N (%) **Normal** **Over weight** **Obese**	 7 (17.07) 13 (31.70) 21 (51.21)	 8 (19.51) 14 (34.14) 19 (46.34)
DAS 28, Mean ±SD	4.31±1.16	---
Family history N (%)	11(26.82)	0 (0)

Disease duration in year , Mean ±SD	7.65±8.36	---
Disease duration groups N (%) **< 5 years** **5-10 years** **>10 years**	 21 (51.21) 10 (24.39) 10 (24.39)	---
The disease's onset age in years, Mean±SD	40.63±14.22	---
The disease's onset age for female in years, Mean±SD **The disease's onset age for male in years, Mean±SD** **ANOVA test (*P* value)**	38.98±13.32 48.63±16.79 0.103	---
Swollen joint count, Mean±SD **≤ 7 joints N (%)** **> 7 joints N (%)**	3.05±2.88 36(87.80) 5(12.19)	---
Tender joint count, Mean±SD **≤ 7 joints N (%)** **> 7 joints N (%)**	7.93±7.75 25(60.97) 16(39.02)	---
Global assessment , Mean±SD	4.98±2.83	---
Morning stiffness N (%)	33 (80.48)	---
Intermittent symptoms N (%)	39 (95.12)	---
RA comorbidities **Diabetes mellitus N (%)** **Hypertension N (%)** **Cardiovascular disease N (%)**	 11 (26.82) 14 (34.14) 6 (14.63)	 5 (12.19) 11 (26.82) 0 (0)
Systemic Lupus Erythematosus symptoms N (%)	0 (0)	0 (0)
Sjögren's syndrome symptoms N (%)	3 (7.31)	0 (0)
RA, Rheumatoid Arthritis; N, Number; SD, Standard Deviation; DAS 28, Disease Activity Score 28; BMI, Body Mass Index; ANOVA, Analysis of Variance		

4.2 Investigation of Autoantibodies

4.2.1 Receiver Operative Characteristic Curve (ROC) for Autoantibodies

The concept of ROC originates from the employment of radar during World War II. The ROC curve is used to show the overall efficacy of the test of diagnosis [through connecting the coordinate points with the false-positive rate (1-specificity) as the X-axis and the true-positive rate (sensitivity) as the Y-axis for all cut-off points at which the test results are measured] and to compare the performance of two or more diagnostic tests. It is also used to determine the optimal cut-off value for assessing whether a disease is present or absent. The average value of sensitivities for all possible specificities may be calculated from the Area Under Curve (AUC). The AUC ranges from 0 to 1, but only values greater than 0.5 are considered diagnostic. The test's overall efficacy is improved with a higher value (Nahm, 2022).

The diagnostic test is perfect for differentiating between the diseased and non-diseased when the maximal AUC is 1. This happens when the distribution of test results for the diseased and non-diseased does not overlap. AUC = 0.5 refers to the chance discrimination curve in ROC space that is situated on a diagonal line. The minimum AUC should be regarded as a level of chance, i.e., AUC = 0.5, while AUC = 0 indicates that the test incorrectly classifies all diseased subjects as negative and all healthy subjects as positive, which is extremely unlikely to occur in clinical practice (Hajian-Tilaki, 2013).

In general, Table (4.2) provides interpretations of the AUC values (Nahm, 2022):

Table 4.2: The Interpretations of Area Under Curve (AUC)

AUC	Interpretations
$0.5 \leq AUC < 0.6$	Fail
$0.6 \leq AUC < 0.7$	Poor
$0.7 \leq AUC < 0.8$	Fair
$0.8 \leq AUC < 0.9$	Good
$0.9 \leq AUC$	Excellent

The overall AUC, sensitivity, and specificity for RF, ACCP, anti-RA33, anti-SSA, and anti-dsDNA were as follows: (0.979, 0.976, 100), (0.987, 0.976, 0.902), (0.832, 0.829, 0.707), (0.995, 0.951, 0.976), and (0.938, 0.829, 0.854), respectively, as presented in Figure (4.1) and Table (4.3).

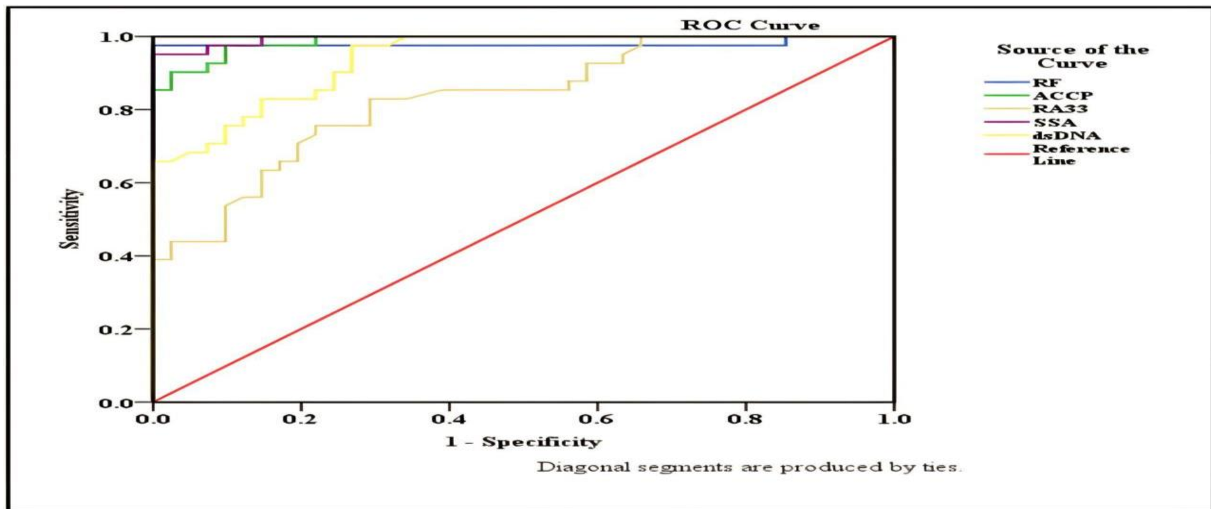


Figure 4.1: Receiver Operative Characteristic Curve (ROC) Curve Illustrating The sensitivity and 1-specificity Values for RF, ACCP, Anti-RA33, Anti-dsDNA optical density, and Anti-SSA autoantibody titer.

Abbreviations: RF, Rheumatoid Factor; ACCP, Anti-Cyclic-Citrullinated Protein; Anti-RA33, Anti Rheumatoid Arthritis 33; Anti-SSA, Anti-Sjögren's Syndrome antigen A ; Anti-dsDNA, Anti-double stranded-Deoxyribonucleic Acid.

Table 4.3: Receiver Operative Characteristic Curve (ROC) based analysis

Test Result Variable(s)	Cut off point OD\ titer (U/mL)	Area	Std. Error	Asymptotic Sig.	Asymptotic 95% Confidence Interval		Sensitivity	Specificity
					Lower Bound	Upper Bound		
RF	0.90150	0.979	0.021	0.000	0.939	1.000	0.976	100
ACCP	0.32800	0.987	0.008	0.000	0.970	1.000	0.976	0.902
Anti-RA33	0.32500	0.832	0.044	0.000	0.746	0.918	0.829	0.707
Anti-SSA	20.9500	0.995	0.005	0.000	0.985	1.000	0.951	0.976
Anti-dsDNA	0.29800	0.938	0.024	0.000	0.891	0.984	0.829	0 .854

RF, Rheumatoid Factor; ACCP, Anti-Cyclic-Citrullinated Protein; Anti-RA33, Anti Rheumatoid Arthritis 33; Anti-SSA, Anti-Sjögren's Syndrome antigen A ; Anti-dsDNA, Anti-double stranded-Deoxyribonucleic Acid

Comparable results were documented in previous studies that showed that the AUC, sensitivity, and specificity of both RF and ACCP were (100%, 100%, and 1.00), (Aiman *et al*., 2020). Another previous study showed that the sensitivity and specificity for RF, ACCP, and anti-RA33 were (84.0% and 80%), (68% and 97.5%), and (58% and 92.5%), respectively (Al-Ani, 2013). Another previous study showed that the sensitivity and specificity for RF and ACCP were (70% and 95%) and (78.8% and 100%), respectively (Ali *et al*., 2013). Another previous study showed the sensitivity and specificity of RF and ACCP were (86.9% and 96%), (73% and 100%) respectively (Al-Attabi, 2016). Additionally, a previous study revealed that the sensitivity and specificity of ACCP and anti-RA33 were (72.7%, 98.8%) and (76.1%, 96.1%) respectively (Alattabi *et al.*, 2020). The differences in sensitivity and specificity values could be due to patients and control subjects chosen and differences in kits and techniques used in these studies.

4.2.2 Comparison of Autoantibodies between Patients and Controls

The current study revealed that 40 (97.56%) of patients had positive RF autoantibodies OD values higher than the cut-off value, and all the subjects in the control group were negative (RF OD lower than the cut-off value), as shown in Table (4.4). This result was in agreement with a previous study in which the author reported that RF was found in all RA patients' serum samples (100% positive results) with 100% negative results in healthy controls (Al-taee *et al.,* 2019). Additionally, another study recorded negative detection (100%) of antibodies for RF in the control group (Ali & Jasim, 2011)**.** It has been documented that RF lab results is not specific for RA and there were different factors (e.g. some conditions and medical procedures that can raise RF level. Includes other autoimmune diseases, certain chronic infection, DM, bacterial endocarditis, cancer, normal aging, vaccination, and transfusion) that can affect lab results. About 20% of RA confirmed patients have no abnormal RF test results whereas, 5% of normal population have abnormal test results (Monica Richey, 2020).

The study also revealed that 40 (97.56%) and 4 (9.75%) of patients and controls, respectively, had ACCP OD values higher than the cut-off value, as shown in Table (4.4). Similar findings were reported in other previous studies (Abd-ali *et al*, 2018; Taha & Moustafa, 2019; Khater & Al Sheik, 2022). A lower percentage was found in another study in which the ACCP was positive in 53.1% of RA patients and 4.7% of controls (Shakiba *et al.*, 2014). The presence of high frequency of patients included in the current study whom they have positive RF and ACCP autoantibodies might be explained by that the patients had the RA for at least 5 years and no newly diagnosed patients were involved.

Regarding anti-RA33, 34 (82.92%) and 12 (29.26%) patients and controls, respectively, had positive anti-RA33 autoantibodies OD value, as shown in Table (4.4). This result was in agreement with a previous study in which the author reported that anti-RA33 autoantibodies were positive in 137 patients (76.1%), (Alattabi *et al*., 2020).

Concerning anti-SSA, 39 (95.12%) and 1 (2.43%) patients and controls, respectively, had anti-SSA titers higher than the cut-off value, as shown in Table (4.4). Lower percentages were reported in previous studies, ranging from (3 – 15.23%), (Yang *et al*., 2018; Kim *et al*., 2020; Khater & Al Sheik, 2022; Waki *et al*., 2022).

Regarding anti-dsDNA, the current study also revealed that 34 (82.92%) and 6 (14.63%) patients and controls, respectively, had Anti-dsDNA autoantibodies OD values higher than the cut-off value, as shown in Table (4.4). Little studies focused on the level of Anti-dsDNA autoantibodies in RA patients. However, one of the previous studies showed that 66.1% of RA patients had anti-dsDNA autoantibodies (Abid Fatehi, 2007). And another study showed that 51% of RA patients had ANA autoantibodies (Yang *et al*., 2018). A lower percentage (6%) was reported in a previous study (Damián-abrego *et al.,* 2015).

This study showed the presence of a highly significant difference between patients and control groups in the OD\ titers of autoantibodies (p =0.000), as found in Table (4.4).

Table 4.4: Comparison of Autoantibodies between Patients and Controls

Autoantibodies	Patients			Control			Chi-square (*P* Value)		T test (*P* Value)
	Positive N (%)	Negative N (%)	Mean ±SD OD\ titer (U/mL)	Positive N (%)	Negative N (%)	Mean ±SD OD\ titer (U/mL)	Positive	Negative	Total
RF	40 (97.56)	1 (2.43)	2.08±1.09	0 (0)	41 (100)	0.74±0.08	0.000*	0.000*	0.000*
ACCP	40 (97.56)	1 (2.43)	0.52±0.18	4 (9.75)	37 (90.24)	0.26±0.04	0.000*	0.000*	0.000*
Anti-RA33	34 (82.92)	7 (17.07)	0.51±0.22	12 (29.26)	29 (70.73)	0.31±0.07	0.000*	0.000*	0.000*
Anti-SSA	39 (95.12)	2 (4.87)	35.52±12.12	1 (2.43)	40 (97.56)	14.21±3.26	0.000*	0.000*	0.000*
Anti-dsDNA	34 (82.92)	7 (17.07)	0.51±0.21	6 (14.63)	35 (85.36)	0.26±0.04	0.000*	0.000*	0.000*

* Statistically significant at 0.05 level; N, Number; RF, Rheumatoid Factor; ACCP, Anti-Cyclic Citrullinated Protein; Anti-RA33, Anti-Rheumatoid Arthritis 33; Anti-SSA, Sjögren's Syndrome antigen A; Anti-dsDNA, Anti-double-stranded Deoxyribonucleic Acid; SD, Standard Deviation

Similarly, the results of previous studies showed that there were significant differences between patients and controls at the ACCP level (Aiman *et al.*, 2020; Hassoon *et al.*, 2020; Abdulameer *et al.*, 2022), in the RF and ACCP levels (Ali *et al.*, 2018; Hassoon *et al.*, 2020; Khater & Al Sheik, 2022), in the anti-RA33 level (Alattabi *et al.*, 2020; Hassoon *et al.*, 2020), and in the anti-dsDNA level (Abid Fatehi, 2007).

4.2.3 Probability of Autoantibodies Distribution among Patients and Control

The current study revealed that 25 (60.97%) of patients had all tested autoantibodies whereas 27 (65.85%) of the control group were negative, as demonstrated in Table (4.5).

Table 4.5: Probability of Autoantibodies Distribution among Patients and Control

Probability	Patient		Control	
	N	%	N	%
RF^{+}, ACCP^{+}, Anti -RA33^{+}, Anti -SSA^{+}, Anti -dsDNA^{+}	25	60.97	0	0
RF^{-}, ACCP^{+}, Anti -RA33^{+}, Anti -SSA^{+}, Anti -dsDNA^{+}	1	2.43	0	0
RF^{+}, ACCP^{-}, Anti -RA33^{+}, Anti -SSA^{+}, Anti -dsDNA^{-}	1	2.43	0	0
RF^{+}, ACCP^{+}, Anti -RA33^{-}, Anti -SSA^{+}, Anti -dsDNA^{+}	6	14.6	0	0
RF^{+}, ACCP^{+}, Anti -RA33^{+}, Anti -SSA^{-}, Anti -dsDNA^{+}	2	4.87	0	0
RF^{+}, ACCP^{+}, Anti -RA33^{+}, Anti - SSA^{+}, Anti -dsDNA^{-}	5	12.19	0	0
RF^{+}, ACCP^{+}, Anti -RA33^{-}, Anti $^{-}$SSA^{+}, Anti -dsDNA^{-}	1	2.43	0	0
RF^{-}, ACCP^{+}, Anti -RA33^{+}, Anti - SSA^{+}, Anti -dsDNA^{-}	0	0	1	2.43
RF^{-}, ACCP^{+}, Anti -RA33^{+}, Anti -SSA^{-}, Anti -dsDNA^{+}	0	0	1	2.43
RF^{-}, ACCP^{+}, Anti -RA33^{-}, Anti -SSA^{-}, Anti -dsDNA^{-}	0	0	2	4.87
RF^{-}, ACCP^{-}, Anti -RA33^{+}, Anti -SSA^{-}, Anti -dsDNA^{-}	0	0	5	12.19
RF^{-}, ACCP^{-}, Anti -RA33^{+}, Anti -SSA^{-}, Anti -dsDNA^{+}	0	0	5	12.19
RF^{-}, ACCP^{-}, Anti -RA33^{-}, Anti -SSA^{-}, Anti -dsDNA^{-}	0	0	27	65.85

N, Number; RF, Rheumatoid Factor; ACCP, Anti-Cyclic Citrullinated Protein; Anti-RA33, Anti-Rheumatoid Arthritis 33; Anti-SSA, Anti-Sjögren's Syndrome antigen A; Anti-dsDNA, Anti-double-stranded Deoxyribonucleic Acid

In a previous study, Al-Attabi (2016) found that 82% of RA patients had RF^{+}, ACCP^{+}, 41% had RF^{-}, ACCP^{+}, 18% had RF^{+}, ACCP^{-}, and 59% had RF^{-}, ACCP^{-}. In another previous study, 38.4% of RA patients had RF^{+}, ACCP^{+}, 13.8% had RF^{-},

ACCP^{+}, 23.9% had RF^{+}, ACCP^{-}, and 23.9% had RF^{-}, ACCP^{-} (Sulaiman *et al*., 2019).

Additionally, in a previous study, 4.1% of RA patients had RF^{+}, ACCP^{+}, 25.65% had RF^{-}, ACCP^{+}, 5.24 % had RF^{+}, ACCP^{-}, and 64.98% had RF^{-}, ACCP^{-} (Reed *et al*., 2020).

4.2.4 Distribution of Autoantibodies According to Sex

The current study revealed significant differences in OD/titers between males and females of both groups regarding all tested autoantibodies, as shown in Table (4.6-A).

Additionally, there were no significant differences in sex within both patients and control groups except in the case of RF in the control group (p =0.02). However, the means of OD/titer were slightly higher in female than male patients, except in the case of anti-RA33 and anti-dsDNA autoantibodies. Similar findings were reported in previous studies that showed no significant differences in RF and ACCP autoantibodies according to sex (Abd-ali *et al*, 2018). In the general population, it has been reported that females are more likely to be seropositive for various autoantibodies including RF and ACCP. However different studies documented that higher rates of sero-positivity in male in comparison to females which could be influenced by smoking. A meta-analysis conducted in 2010 found that smoking influence RF positive disease in male more than female (Sugiyama *et al*., 2010).

Table 4.6-A: Distribution of Autoantibodies Presence According to Sex

Sample	Sex	N	RF	ACCP	Anti-RA33	Anti-SSA	Anti-dsDNA
			Mean ±SD OD	Mean ±SD OD	Mean ±SD OD	Mean ±SD titer (U/mL)	Mean ±SD OD
Control	Male	7	0.68±0.10	0.29±0.06	0.33±0.10	15.98±4.39	0.28±0.04
	Female	34	0.76±0.08	0.26±0.04	0.31±0.07	13.85±2.93	0.26±0.04
	P value		0.02*	0.08	0.61	0.12	0.28
Patient	Male	7	1.54±0.61	0.48±0.16	0.65±0.33	33.81±10.70	0.56±0.25
	Female	34	2.19±1.15	0.53±0.19	0.48±0.19	35.88±12.51	0.50±0.21
	P value		0.15	0.49	0.23	0.69	0.55
T test (*P* value)	Male		0.003*	0.013*	0.026*	0.002*	0.012*
T test (*P* value)	Female		0.000*	0.000*	0.000*	0.000*	0.000*

* Statistically significant at 0.05 level; N, Number; RF, Rheumatoid Factor; ACCP, anti-Cyclic Citrullinated Protein; Anti-RA33, Anti-Rheumatoid Arthritis 33; Anti-SSA, Anti-Sjögren's Syndrome antigen A; Anti-dsDNA, Anti-double stranded Deoxyribonucleic Acid; SD, Standard Deviation

There were positive associations between sex and all tested autoantibodies except for RF, as shown in Table (4.6-B).

Regarding RF, six male patients (6/7, 85.71%) and all (34/34, 100%) female patients were positive. Whereas all the males and females in control group were negative, as shown in Table (4.6-B).

Concerning ACCP, three healthy males (3/7,42.85%) and one female (1/34, 2.94%) were positive. Whereas six (6/7, 85.71%) and 34 (34/34, 100%) of male and female patients were positive, as shown in Table (4.6-B).

Regarding anti-RA33, ten healthy females (10/34, 29.41%) and 27 (27/34, 79.41%) of female patients were positive, as shown in Table (4.6-B).

Out of 34 female patients, 32 females (94.11%) were positive for anti-SSA autoantibodies. Additionally, one healthy male (1/7, 14.28%) were positive, as shown in Table (4.6-B).

This study also showed 28 female patients (28/34,82.35%) and five healthy females (5/34, 14.70%) were positive for anti-dsDNA autoantibodies, as presented in Table (4.6-B).

Table 4.6-B: Cross-tabulation between Autoantibodies and Sex

Sample	Sex	RF		ACCP		Anti-RA33		Anti-SSA		Anti-dsDNA	
		+ N (%)	- N (%)	+ N (%)	- N (%)	+ N (%)	- N (%)	+ N (%)	- N (%)	+ N (%)	- N (%)
Control	Male	0 (0)	7 (100)	3 (42.85)	4 (57.14)	2 (2.57)	5 (71.42)	1 (14.28)	6 (85.71)	1 (14.28)	6 (85.71)
	Female	0 (0)	34 (100)	1(2.94)	33 (97.05)	10 (29.41)	24 (70.58)	0 (0)	34 (100)	5 (14.70)	29 (85.29)
Patient	Male	6 (85.71)	1 (14.28)	6 (85.71)	1 (14.28)	7 (100)	0 (0)	7 (100)	0 (0)	6 (85.71)	1 (14.28)
	Female	34 (100)	0 (0)	34 (100)	0 (0)	27 (79.41)	7 (20.58)	32 (94.11)	2 (6.25)	28 (82.35)	6 (17.64)
Odds Ratio		0.75 (0.235-2.393)		1.697 (0.515-5.591)		1.508 (0.457-4.971)		1.5 (0.47-4.788)		1.06 (0.336-3.351)	

* Statistically significant at 0.05 level; RF, Rheumatoid Factor; ACCP, anti-Cyclic Citrullinated Protein; Anti-RA33, Anti-Rheumatoid Arthritis 33; Anti-SSA, Anti-Sjögren's Syndrome antigen A; Anti-dsDNA, Anti-double stranded Deoxyribonucleic Acid. Odds Ratio at 95% confidence interval.

The relationship between antibodies and autoimmune disease might occur through several potential biological mechanisms, especially those involving B-cell activation. One of them, CD40, has been demonstrated to have a pathogenic role in a wide variety of different autoimmune disorders, such as type 1 DM, inflammatory bowel disease, MS, and RA. A T-cell-driven mechanism for the autoimmune sex bias is further supported by the fact that males may have fewer T lymphocytes in the blood than females. Recently, studies have also shown that females have skin-targeted overexpression of vestigial like family member 3 (VGLL3), which encodes a transcription factor that drives pro-inflammatory genes, including the B-cell activating factor. This finding was independent of age and sex hormone regulation. This promoted B-cell activation explains how women may have had higher total serum antibodies without having more B cells (Kronzer *et al.*, 2021).

Other previous studies revealed the vital role of female hormones, especially estrogen, in inducing RA. According to certain theories, androgens have an anti-inflammatory impact, while estrogens are pro-inflammatory. However, estrogens can have both stimulatory and inhibitory effects on the immune system. Estrogen levels during peri-ovulatory and pregnancy periods enhance B cells and the Th2 response, aiding in the survival of auto-reactive T and B cell clones. On the other hand, estrogens may inhibit cell-mediated responses, including the proliferation of Th17 cells (Alpízar-Rodríguez & Finckh, 2017).

Another previous study showed the role of the prolactin (PRL) hormone in the increased development of RA in females since the level of serum prolactin is higher in the female group than in males due to female prolactin secretion being higher. In humans, rheumatoid synovial T-cells produce PRL. Additionally, PRL receptors are found on T cells, B cells, fibroblasts, and synovial cells. The addition of PRL to rheumatoid synovial cells in rats causes increased production of

proteolytic enzymes, causing cartilage destruction and increased production of cytokines, which suggests that PRL injection in joints causes inflammation (Awad, 2021).

4.2.5 Distribution of Autoantibodies According to Age Groups

This study found that there were significant differences with $p < 0.05$ between patients and controls according to age groups, as shown in Table (4.7-A). This finding was in agreement with the findings reported in previous study ($p=0.018$) (Al-akhras & Al-Nahi, 2019).

Additionally, there were no significant differences between ≤ 45 and > 45 years in both patients and the control except for anti-RA33 in the patients' group, as presented in Table (4.7-A). This finding agreed with a previous study that showed no significant effects of age on RF and ACCP autoantibodies (Abd-ali *et al*, 2018).

Table 4.7-A: Distribution of Autoantibodies Presence According to Age Groups

Sample	Age groups	N	RF OD	ACCP OD	Anti-RA33 OD	Anti-SSA OD	Anti-dsDNA titer (U/mL)
			Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
Control	**≤45 year**	**18**	0.76±0.09	0.27±0.05	0.32±0.09	14.77±3.46	0.26±0.05
	>45 year	**23**	0.74±0.08	0.27±0.04	0.30±0.06	13.77±3.10	0.26±0.04
	***P* value**		0.57	0.97	0.51	0.34	0.96
Patient	**≤45 year**	**18**	2.06±1.16	0.55±0.20	0.59±0.25	35.83±12.70	0.53±0.23
	>45 year	**23**	2.10±1.07	0.50±0.18	0.45±0.19	35.29±11.93	0.49±0.21
	***P* value**		0.92	0.44	0.05*	0.89	0.6
T test (*P* value)	**≤45 year**		0.000*	0.000*	0.000*	0.000*	0.000*
T test (*P* value)	**>45 year**		0.000*	0.000*	0.001*	0.000*	0.000*

* Statistically significant at 0.05 level; N, Number; RF, Rheumatoid Factor; ACCP, Anti-Citrullinated Protein; Anti-RA33, Anti-Rheumatoid Arthritis 33; Anti-SSA, Anti-Sjögren's Syndrome antigen A; Anti-dsDNA, Anti-double stranded Deoxyribonucleic Acid; SD, Standard Deviation

Higher positivity rates were found in patients with more than 45 years old for all of the tested autoantibodies except for anti-RA33, as shown in Table (4.7-B).

There were negative associations between age groups and all tested autoantibodies except for RF regarding patients and control, as shown in Table (4.7-B).

Table 4.7-B: Cross-tabulation between Autoantibodies and Age Groups

Sample	Age Groups	RF		ACCP		Anti-RA33		Anti-SSA		Anti-dsDNA	
		+ N (%)	- N (%)	+ N (%)	- N (%)	+ N (%)	- N (%)	+ N (%)	- N (%)	+ N (%)	- N (%)
Control	≤ 45 year	0 (0)	18 (43.90)	2 (4.87)	16 (39.02)	7 (17.07)	11 (26.82)	1 (2.43)	17 (41.46)	3 (7.31)	15 (36.58)
	> 45 year	0 (0)	23 (56.09)	2 (4.87)	21 (51.21)	5 (12.19)	18 (43.90)	0 (0)	23 (56.09)	3 (7.31)	20 (48.78)
Patient	≤ 45 year	17 (41.46)	1 (2.43)	18 (43.90)	0 (0)	17 (41.46)	1 (2.43)	17 (41.46)	1 (2.43)	16 (39.02)	2 (4.87)
	> 45 year	23 (56.09)	0 (0)	22 (53.65)	1 (2.43)	17 (41.46)	6 (14.63)	22 (53.65)	1 (2.43)	18 (43.90)	5 (12.19)
Odds Ratio		1.11 (0.457-2.676)		0.873 (0.364-2.095)		0.458 (0.186-1.130)		0.917 (0.383-2.194)		0.752 (0.313-1.802)	

RF, Rheumatoid Factor; ACCP, Anti-Citrullinated Protein; Anti-RA33, Anti-Rheumatoid Arthritis 33; Anti-SSA, Anti-Sjögren's Syndrome antigen A; Anti-dsDNA, Anti-double stranded Deoxyribonucleic Acid. Odds Ratio at 95% confidence Interval.

4.2.6 Distribution of Autoantibodies According to Disease Duration

The current study revealed the presence of significant differences with $p = 0.05$ and $p = 0.03$ in RF and anti-RA33 autoantibodies respectively with disease duration, and non-significant differences were found regarding ACCP, anti-SSA, and anti-dsDNA autoantibodies, as found in Table (4.8-A).

Table 4.8-A: Distribution of Autoantibodies Presence According to Disease Duration

Autoantibodies Mean± SD OD\ titer (U/mL)	Disease duration			ANOVA (*P* value)	LSD
	<5 year N=21	5-10 year N=10	>10 year N=10		
RF	2.08±1.09	1.49±0.37	2.67±1.34	0.05*	0.91
ACCP	0.52±0.18	0.50±0.23	0.56±0.17	0.73	N.S
Anti-RA33	0.60±0.25	0.46±0.17	0.39±0.15	0.03*	N.S
Anti-SSA	36.46±12.10	33.78±12.82	35.32±12.58	0.85	N.S
Anti-dsDNA	0.51±0.21	0.61±0.28	0.42±0.13	0.16	N.S

* Statistically significant at 0.05 level; N, Number; RF, Rheumatoid Factor; ACCP, Anti-Cyclic Citrullinated Protein; Anti-RA33, Anti-Rheumatoid Arthritis 33; Anti-SSA, Anti-Sjögren's Syndrome antigen A; Anti-dsDNA, Anti-double stranded Deoxyribonucleic Acid; SD, Standard Deviation ANOVA, Analysis of Variance, LSD, Least Significant Difference; N.S, Non. Significant

Regarding disease duration, Al-ubaidi *et al* (2013) reported no significant difference in ACCP and anti-RA33 autoantibodies; also, Badran, (2014) reported in his study that there was no significant difference in RF and ACCP. Additionally, Abd-ali *et al* (2018) reported no significant difference in RF and ACCP autoantibodies.

The difference in anti-RA33 and RF between the current study and Al-ubaidi *et al.*, Badran, and Abd-ali *et al.* studies might possibly result from differences in the number of patients and differences in disease duration categories between the studies.

Table 4.8-B showed the distribution of positive and negative percentages of the tested autoantibodies according to disease duration.

Table 4.8-B: Cross-tabulation between Autoantibodies and Disease Duration

Autoantibodies	Disease duration					
	<5 year N=21		5-10 year N=10		>10 year N=10	
	+ N (%)	- N (%)	+ N (%)	- N (%)	+ N (%)	- N (%)
RF	20 (48.78)	1 (2.43)	10 (24.39)	0 (0)	10 (24.39)	0 (0)
ACCP	21 (51.21)	0 (0)	9 (21.95)	1 (2.43)	10 (24.39)	0 (0)
Anti-RA33	20 (48.78)	1 (2.43)	9 (21.95)	1 (2.43)	5 (12.19)	5 (12.19)
Anti-SSA	20 (48.78)	1 (2.43)	9 (21.95)	1 (2.43)	10 (24.39)	0 (0)
Anti-dsDNA	16 (39.02)	5 (12.19)	9 (21.95)	1 (2.43)	9 (21.95)	1 (2.43)

* Statistically significant at 0.05 level; RF, Rheumatoid Factor; ACCP, Anti-Cyclic Citrullinated Protein; Anti-RA33, Anti-Rheumatoid Arthritis 33; Anti-SSA, Anti-Sjögren's Syndrome antigen A; Anti-dsDNA, Anti-double stranded Deoxyribonucleic Acid

4.2.7 Distribution of Autoantibodies According to Body Mass Index(BMI)

Regarding BMI, 7 patients (17.07%) and 8 control (19.51%) were normal-weight, whereas 13 patients (31.70%) and 14 controls (34.14%) were overweight, and 21 patients (51.21%) and 19 controls (46.34%) were obese. Similarly, the majority of RA patients were found to be in overweight and obese groups, which were documented in previous studies (Zaccardelli *et al*., 2019; Yousif & Ibraheem, 2020).

There were significant differences in autoantibodies between patients and controls ($p < 0.05$) according to BMI categories. The study also showed that there were no significant differences in autoantibodies among BMI categories in the patients' group, whereas, there were significant differences among BMI categories

in control group for anti-RA33, Anti-SSA, and Anti-dsDNA autoantibodies with p = 0.03, 0.04, and 0.01, as shown in Table (4.9-A).

Table 4.9-A: Distribution of Autoantibodies Presence According to BMI

Sample	BMI group	N (%)	RF	ACCP	Anti-RA33	Anti-SSA	Anti-dsDNA
			Mean ±SD OD	Mean ±SD OD	Mean ±SD OD	Mean±SD titer (U/mL)	Mean ±SD OD
Control	Normal	8 (19.51)	0.70±0.08	0.27±0.02	0.25±0.04	11.81±.36	0.24±0.02
	Obese	19 46.34)	0.75±0.08	0.26±0.05	0.33±0.09	15.19±3.37	0.28±0.05
	Overweight	14 (34.14)	0.77±0.08	0.28±0.04	0.32±0.06	14.253.47	0.25±0.03
	***P* value**		0.18	0.59	0.03*	0.04*	0.01*
Patient	Normal	7 (17.07)	1.81±0.39	0.48±0.10	0.41±0.07	34.09±9.49	0.62±0.30
	Obese	21 (52.21)	2.09±1.10	0.54±0.21	0.51±0.21	36.04±13.47	0.46±0.17
	Overweight	13 (31.70)	2.21±1.36	0.51±0.19	0.57±0.29	35.46±11.86	0.54±0.22
	***P* value**		0.75	0.71	0.31	0.94	0.19
T test (*P* value)	Normal		0.000*	0.000*	0.000*	0.000*	0.003*
	Obese		0.000*	0.000*	0.000*	0.000*	0.000*
	Overweight		0.000*	0.001*	0.004*	0.000*	0.000*

* Statistically significant at 0.05 level; N, Number; RF, Rheumatoid Factor; ACCP, Anti-Cyclic Citrullinated Protein; Anti-RA33, Anti-Rheumatoid Arthritis 33; Anti-SSA, Sjögren's Syndrome antigen A; Anti-dsDNA, Anti-double-stranded Deoxyribonucleic Acid; SD, Standard Deviation; BMI, Body Mass Index

Interestingly, higher positivity rates were found in obese patients for all of the tested autoantibodies, as shown in Table (4.9-B).

Table 4.9-B: Cross-tabulation between Autoantibodies and BMI

Sample	BMI group	RF		ACCP		Anti-RA33		Anti-SSA		Anti-dsDNA	
		+ N (%)	- N (%)	+ N (%)	- N (%)	+ N (%)	- N (%)	+ N (%)	- N (%)	+ N (%)	- N (%)
Control	Normal	0 (0)	8 (19.51)	0 (0)	8 (19.51)	0 (0)	8 (19.51)	0 (0)	8 (19.51)	0 (0)	8 (19.51)
	Obese	0 (0)	19 (46.34)	2 (4.87)	17 (41.46)	9 (21.95)	10 (24.39)	0 (0)	19 (46.34)	6 (14.63)	13 (31.70)
	Over weight	0 (0)	14 (34.14)	2 (4.87)	12 (29.26)	3 (7.31)	11 (26.82)	1 (2.43)	13 (31.70)	0 (0)	14 (34.14)
Patient	Normal	7 (17.07)	0 (0)	6 (14.63)	1 (2.43)	7 (17.07)	0 (0)	7 (17.07)	0 (0)	6 (14.63)	1 (2.43)
	Obese	21 (51.21)	0 (0)	21 (51.21)	0 (0)	17 (41.46)	4 (9.75)	19 (46.34)	2 (4.87)	17 (41.46)	4 (9.75)
	Over weight	12 (29.26)	1 (2.43)	13 (31.70)	0 (0)	10 (24.39)	3 (7.31)	13 (31.70)	0 (0)	11 (26.82)	2 (4.87)

* Statistically significant at 0.05 level; RF, Rheumatoid Factor; ACCP, Anti-Cyclic Citrullinated Protein; Anti-RA33, Anti-Rheumatoid Arthritis 33; Anti-SSA, Sjögren's Syndrome antigen A; Anti-dsDNA, Anti-double-stranded Deoxyribonucleic Acid; BMI, Body Mass Index

Obesity impairs human self-tolerance mechanisms by promoting pro-inflammatory processes, reducing B regs and T regs, and increasing Th17 cells as a result of the latter, which provides the ideal environment for the emergence of autoimmune disorders. This is the reason behind the prevalence of RA was higher in obese and overweight individuals (Tsigalou *et al.*, 2020).

4.3 Investigation of Autoantibodies with Disease Activity

4.3.1 Distribution of Autoantibodies According to ESR

This study showed that there was a highly significant increase in ESR mean (p = 0.000) between patients and control groups with significantly less than 0.05, as shown in Figure (4.2). Previous studies reported similar findings (Ahmad & Zgair, 2021; Abdulameer *et al.*, 2022). Elevation of ESR is an indication for inflammation or tissue damage in the body. ESR is typically elevated in RA patients (Saptarini *et al.*, 2016) .

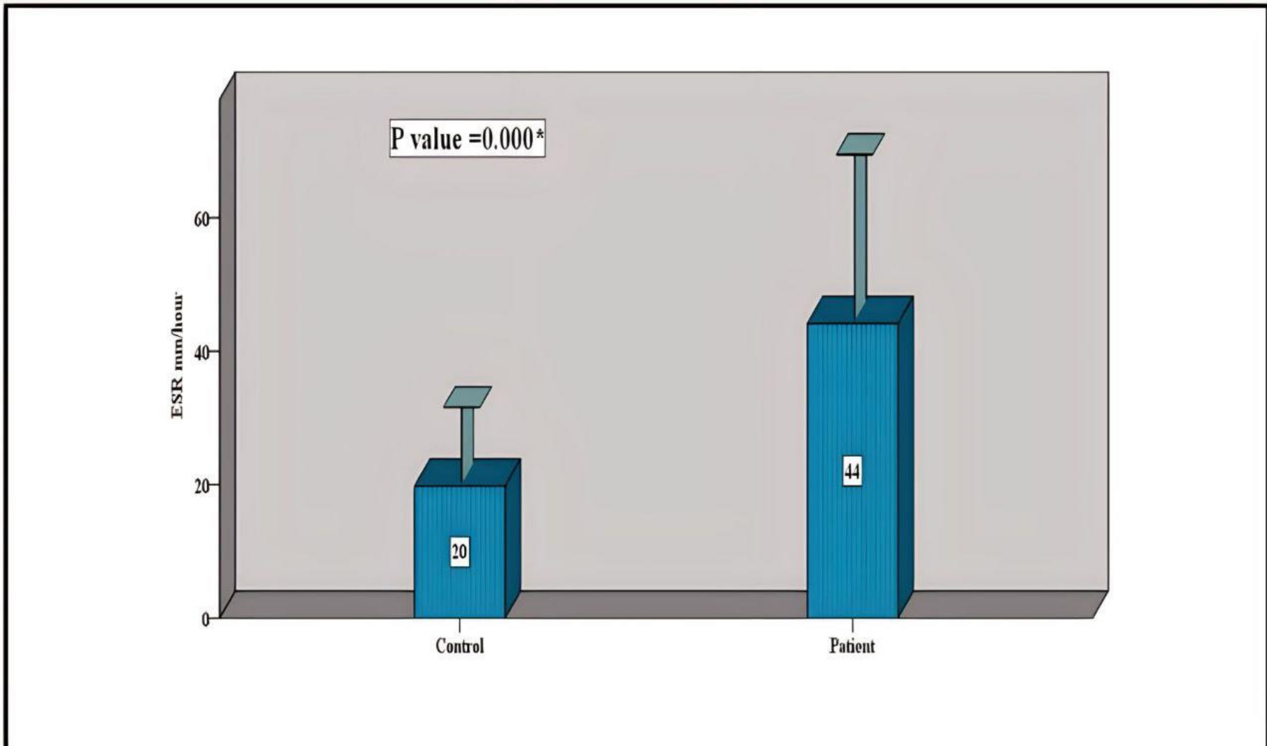


Figure 4.2: Distribution of ESR between Patient and Control

As shown in Table (4.10-A), significant differences were found between patients and controls in both normal and high ESR groups regarding all tested autoantibodies, except in the case of anti-RA33 in high ESR groups.

The study also showed no significant differences in autoantibodies levels between patients whose ESR was normal and those whose ESR was high in all studied autoantibodies. A similar finding was observed in the control group.

Table 4.10-A: Distribution of Autoantibodies Presence According to ESR

Sample	ESR group	N	RF	ACCP	Anti-RA33	Anti-SSA	Anti-dsDNA
			Mean± SD OD	Mean± SD OD	Mean± SD OD	Mean± SD titer (U/mL)	Mean± SD OD
Control	Normal	36	0.75±0.08	0.27±0.04	0.31±0.07	14.08±3.23	0.26±0.04
	High	5	0.77±0.12	0.25±0.05	0.32±0.10	15.18±3.73	0.27±0.06
	Total	41	0.75±0.08	0.27±0.04	0.31±0.08	14.21±3.26	0.26±0.04
	P value		0.61	0.21	0.76	0.49	0.72
Patient	Normal	13	1.71±0.54	0.47±0.14	0.58±0.27	33.46±11.97	0.52±0.20
	High	28	2.25±1.25	0.55±0.20	0.48±0.20	36.49±12.29	0.51±0.23
	Total	41	2.08±1.10	0.52±0.19	0.51±0.23	35.53±12.12	0.51±0.22
	P value		0.06	0.23	0.2	0.46	0.82
T test\ (*P* value)	Normal		0.000*	0.000*	0.000*	0.000*	0.000*
T test (*P* value)	High		0.013*	0.003*	0.088	0.001*	0.031*
ANOVA (*P* value)	Total		0.000*	0.000*	0.000*	0.000*	0.000*

* Statistically significant at 0.05 level; N, Number; RF, Rheumatoid Factor; ACCP, Anti-Cyclic Citrullinated Protein; Anti-RA33, Anti-Rheumatoid Arthritis 33; Anti-SSA, Sjögren's Syndrome antigen A; Anti-dsDNA, Anti-double-stranded Deoxyribonucleic Acid; SD, Standard Deviation; ANOVA, Analysis of Variance; ESR, Erythrocyte Sedimentation Rate

High positivity rates were observed in patients with high ESR levels in all tested autoantibodies. Inversely, high positivity rates were observed in the healthy control group with normal ESR levels, as shown in Table (4.10-B).

Table 4.10-B: Cross-tabulation between Autoantibodies and ESR

Sample	ESR	RF		ACCP		Anti-RA33		Anti-SSA		Anti-dsDNA	
		+ N (%)	- N (%)	+ N (%)	- N (%)	+ N (%)	- N (%)	+ N (%)	- N (%)	+ N (%)	- N (%)
Control	Normal	0 (0)	36 (87.80)	4 (9.75)	32 (78.04)	11 (26.82)	25 (60.97)	1 (2.43)	35 (85.36)	5 (12.19)	31 (75.60)
	High	0 (0)	5 (12.19)	0 (0)	5 (12.19)	1 (2.43)	4 (9.75)	0 (0)	5 (12.19)	1 (2.43)	4 (9.75)
Patient	Normal	13 (31.70)	0 (0)	13 (31.70)	0 (0)	11 (26.82)	2 (4.87)	12 (29.26)	1 (2.43)	12 (29.26)	1 (2.43)
	High	27 (65.85)	1 (2.43)	27 (65.85)	1 (2.43)	23 (56.09)	5 (12.19)	27 (65.85)	1 (2.43)	22 (53.65)	6 (14.63)

RF, Rheumatoid Factor; ACCP, Anti-Cyclic Citrullinated Protein; Anti-RA33, Anti-Rheumatoid Arthritis 33; Anti-SSA, Sjögren's Syndrome antigen A; Anti-dsDNA, Anti-double-stranded Deoxyribonucleic Acid; ESR, Erythrocyte Sedimentation Rate

4.3.2 Distribution of Autoantibodies According to DAS28

There was a non-significant difference in autoantibodies levels according to DAS28 for all tested autoantibodies, as shown in Tables (4.11-A).

Table 4.11-A: Distribution of Autoantibodies Presence According to DAS28

Autoantibodies Mean± SD OD\ titer (U/mL)	DAS28					ANOVA test (*P* value)
	Remission N=5	Low activity N=1	Moderate activity N=23	High activity N=23	Total N=41	
RF	1.85±0.48	0.94±0	2.20±1.21	2.06±1.08	2.08±1.10	0.680
ACCP	0.51±0.14	0.38±0	0.56±0.22	0.47±0.12	0.52±0.19	0.457
Anti-RA33	0.64±0.30	0.33±0	0.56±0.24	0.38±0.09	0.51±0.23	0.059
Anti-SSA	33.65±11.02	31.65±0	36.62±12.63	34.53±12.83	35.53±12.12	0.927
Anti-dsDNA	0.51±0.20	0.46±0	0.52±0.22	0.49±0.25	0.51±0.22	0. 977

N, Number; RF, Rheumatoid Factor; ACCP, Anti-Cyclic Citrullinated Protein; Anti-RA33, Anti-Rheumatoid Arthritis 33; Anti-SSA, Sjögren's Syndrome antigen A; Anti-dsDNA, Anti-double-stranded Deoxyribonucleic Acid; SD, Standard Deviation; ANOVA, Analysis of Variance; DAS28,Disease Activity Score 28

A similar finding was reported in a previous study in which the author reported no significant difference in RF titer according to disease activity (Fawzy *et al.*, 2017).

Theoretically, it is assumed that the DAS is increased by increasing the mean of autoantibodies, but the current study showed inconsistent results. Perhaps the reason is due to the inaccurate measurement of the DAS28 as a result of the bias that results from the wrong global assessment of patients to their health conditions.

Higher positivity rates of autoantibodies were observed in patients with moderate activity and high activity as compared to low activity and remission, as shown in Table (4.11-B).

Table 4.11-B: Cross-tabulation between Autoantibodies and DAS28

Autoantibodies	DAS28							
	Remission		Low activity		Moderate activity		High activity	
	+ N(%)	- N(%)	+ N(%)	- N(%)	+ N(%)	- N(%)	+ N(%)	- N(%)
RF	5 (12.19)	0 (0)	1 (2.43)	0 (0)	22 (53.65)	1 (2.43)	12 (29.26)	0 (0)
ACCP	5 (12.19)	0 (0)	1 (2.43)	0 (0)	23 (56.09)	0 (0)	11 (26.82)	1 (2.43)
Anti-RA33	4 (9.75)	1 (2.43)	1 (2.43)	0 (0)	20 (48.78)	3 (7.31)	9 (21.95)	3 (7.31)
Anti-SSA	5 (12.19)	0 (0)	1 (2.43)	0 (0)	23 (56.09)	0 (0)	10 (24.39)	2 (4.78)
Anti-dsDNA	4 (9.75)	1 (2.43)	1 (2.43)	0 (0)	18 (43.90)	5 (12.19)	11 (26.82)	1 (2.43)

N, Number; RF, Rheumatoid Factor; ACCP, Anti-Cyclic Citrullinated Protein; Anti-RA33, Anti-Rheumatoid Arthritis 33; Anti-SSA, Sjögren's Syndrome antigen A; Anti-dsDNA, Anti-double-stranded Deoxyribonucleic Acid; DAS28,Disease Activity Score 28

It has been documented that both RF and ACCP are not used to monitor disease activity, because they both tend to remain positive despite remission. Once the diagnosis of RA has been made and confirmed, these tests are not repeated (Monica Richey, 2020).

4.4 Investigation The level of Bacterial Lipopolysaccharides (LPS)

4.4.1 Receiver Operative Characteristic Curve (ROC) for LPS Titer

The current study revealed that the AUC, sensitivity, and specificity for LPS were 0.738, 0.634, and 0.61, respectively, as shown in Table (4.12) and Figure (4.3). This results means that the level of LPS is an fair biomarker in discrimination of the diseased patients from healthy individuals.

Table 4.12: Receiver Operative Characteristic Curve (ROC) Curve of LPS Titer

Test Result Variable(s)	Cut off point titer (U/mL)	Area	Std. Error[a]	Asymptotic Sig.	Asymptotic 95% Confidence Interval		Sensitivity	Specificity
					Lower Bound	Upper Bound		
LPS	44.61850	0.738	0.056	0.000	0.627	0.848	0.634	0.61
LPS, Lipopolysaccharides								

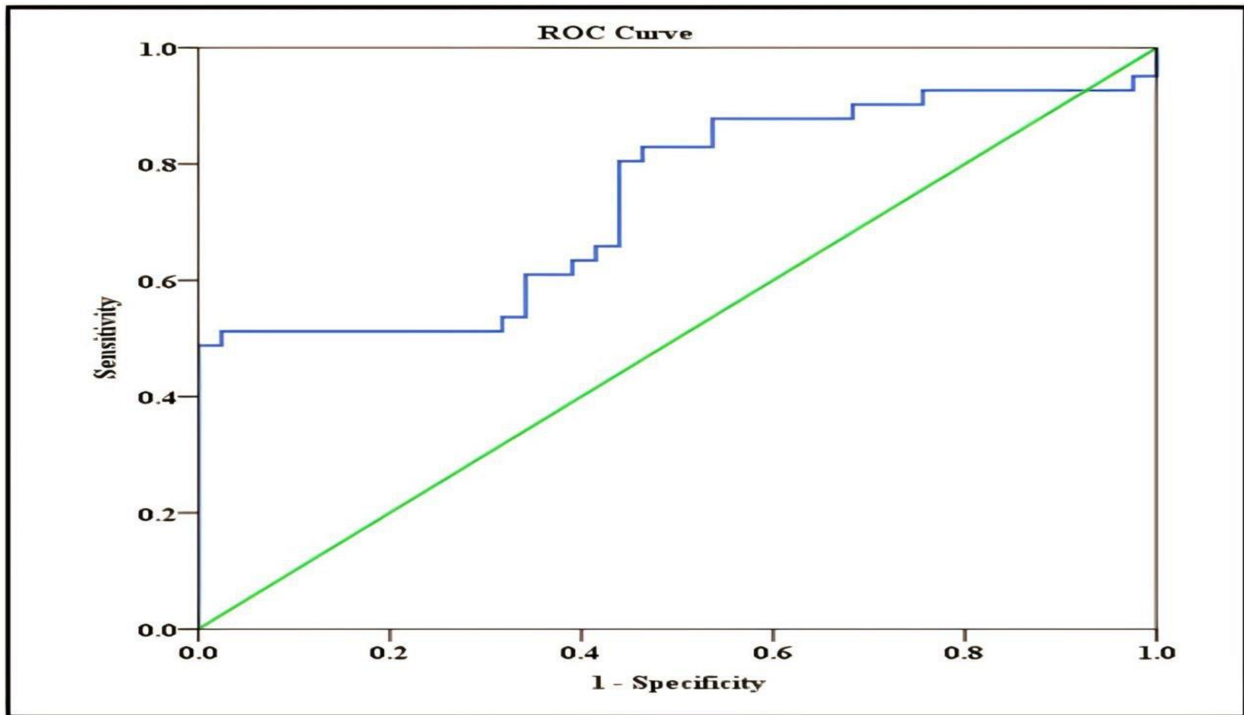


Figure 4.3: Receiver Operative Characteristic Curve (ROC) Curve Illustrating The sensitivity and 1-specificity Values for LPS

4.4.2 Comparison of Bacterial LPS between Patients and Controls

This study showed a significant difference in the mean±SD of LPS between patients and controls, as shown in Table (4.13). The presence of significant higher mean of LPS might possibly result from bacterial leakage or dysbiosis of normal flora that result in the initiation of inflammation.

According to the cut-off value, the current study demonstrated that 26/41 (63.41%) of the patients' group had LPS titer higher than cut-off value, whereas 16/41(39.02%) of control had LPS titers higher than the cut-off value.

Additionally, comparing the mean titer of positive LPS cases between patients and controls revealed highly significant differences with p = 0.000, as shown in Table (4.13). Inversely, no significant difference was observed between the two studied groups in LPS-negative cases with p = 0.134.

No previous studies that measured LPS titers in RA patients or control groups were found. However, there were previous studies that showed the role of the immune response to LPS or detection of LBP level in serum of RA patients (Parantainen *et al*., 2022). It has been documented that RA patients had higher LBP concentration in serum than controls which could be explained that RA patients have increase intestinal permeability which could lead to synthesize translocation of bacterial LPS to the blood circulation (Audo *et al*., 2023).

A gram-negative bacterial cell wall component, LPS serves as a biomarker for bacterial translocation and the resulting host responses. In the bloodstream, LPS binds to LBP, which enables its binding to a monocyte activation marker, soluble CD14 (sCD14). sCD14 transfers the LPS-LBP complex to TLR4 or membrane-bound CD14, inducing the release of pro-inflammatory cytokines: IL-1, IL-6, TNF-α, and type 1 interferon (Moon *et al*., 2019).

Multiple supporting lines suggest a role for LPS in RA pathophysiology. In patients with RA, TLR4 is abundantly expressed in the inflamed synovium, and levels of antimicrobial response factors are higher in the patient's serum (Parantainen *et al.*, 2022). Additionally, a previous study revealed that the immune response against *P. mirabilis* LPS may contribute to RA (Arabski *et al*., 2012).

Table 4.13: Comparison of LPS between Patients and Controls

Subjects	LPS titer (U/mL)					
	Positive		Negative		Total	
	N (%)	Mean ±SD	N (%)	Mean ±SD	N (%)	Mean ±SD
Control	16(39.02)	49.57±2.72	25(60.97)	39.57±2.00	41(100)	43.47±5.43
Patients	26(63.41)	79.31±25.86	15(36.58)	40.79±3.05	41(100)	65.22±27.82
T test (*P* Value)	0.000*		0.134		0.000*	

* Statistically significant at 0.05 level; LPS, Lipopolysaccharides; N, Number; SD, Standard Deviation

4.4.3 Distribution of Autoantibodies According to LPS

The results presented in Table (4.14-A) showed significant differences in autoantibodies OD or titers according to LPS between patients and control groups. Additionally, there were significant differences between patients and controls that had positive and negative LPS titers.

Table 4.14-A: Distribution of Autoantibodies According to LPS

Sample	LPS	N	RF	ACCP	Anti-RA33	Anti-SSA	Anti-dsDNA
			Mean± SD OD	Mean± SD OD	Mean± SD OD	Mean± SD titer (U/mL)	Mean± SD OD
Control	Positive	16	0.79±0.07	0.29±0.04	0.35±0.07	15.57±3.86	0.27±0.05
	Negative	25	0.72±0.08	0.26±0.04	0.29±0.07	13.34±2.53	0.26±0.04
	Total	41	0.75±0.08	0.27±0.04	0.31±0.08	14.21±3.26	0.26±0.04
Patient	Positive	26	2.44±1.20	0.55±0.22	0.55±0.24	36.57±13.42	0.48±0.21
	Negative	15	1.45±0.42	0.47±0.10	0.45±0.20	33.72±9.63	0.57±0.23
	Total	41	2.08±1.10	0.52±0.19	0.51±0.23	35.53±12.12	0.51±0.22
T test (*P* value) positive			0.000*	0.000*	0.002*	0.000*	0.000*
T test (*P* value) negative			0.000*	0.000*	0.001*	0.000*	0.000*
ANOVA test (*P* value) total			0.000*	0.000*	0.000*	0.000*	0.000*

* Statistically significant at 0.05 level; N, Number; RF, Rheumatoid Factor; ACCP, Anti-Cyclic Citrullinated Protein; Anti-RA33, Anti-Rheumatoid Arthritis 33; Anti-SSA, Sjögren's Syndrome antigen A; Anti-dsDNA, Anti-double-stranded Deoxyribonucleic Acid; SD, Standard Deviation; ANOVA, Analysis of Variance; LPS; Lipopolysaccharides

Interestingly, the study showed that patients with positive LPS titer had higher positive rates of autoantibodies. Inversely, subjects with negative LPS titer had autoantibodies titer lower than the cut off value, as shown in Table (4.14-B).

Table 4.14-B: Cross-tabulation between LPS and Autoantibodies

Sample	LPS	RF		ACCP		Anti-RA33		Anti-SSA		Anti-dsDNA	
		+ N (%)	- N (%)	+ N (%)	- N (%)	+ N (%)	- N (%)	+ N (%)	- N (%)	+ N (%)	- N (%)
Control	Positive	0 (0)	16 (39.02)	3 (7.31)	13 (31.70)	8 (19.51)	8 (19.51)	1 (2.43)	15 (36.58)	3 (7.31)	13 (31.70)
	Negative	0 (0)	25 (60.97)	1 (2.43)	24 (58.53)	4 (9.75)	21 (51.21)	0 (0)	25 (60.97)	3 (7.31)	22 (53.65)
Patient	Positive	26 (63.41)	0 (0)	25 (60.97)	1 (2.43)	20 (48.78)	6 (14.63)	25 (60.97)	1 (2.43)	19 (46.34)	7 (17.07)
	Negative	14 (34.14)	1 (2.43)	15 (36.58)	0 (0)	14 (34.14)	1 (2.43)	14 (34.14)	1 (2.43)	15 (36.58)	0 (0)

* Statistically significant at 0.05 level; RF, Rheumatoid Factor; ACCP, Anti-Cyclic Citrullinated Protein; Anti-RA33, Anti-Rheumatoid Arthritis 33; Anti-SSA, Sjögren's Syndrome antigen A; Anti-dsDNA, Anti-double-stranded Deoxyribonucleic Acid; LPS; Lipopolysaccharides

Autoimmune diseases can result from changes in the regulation of B cell responses, leading to the development of high-affinity auto-reactive B cells, the creation of autoantibodies, and tissue damage. Effective immunity depends on B lymphocytes because they produce antibodies and cytokines, present antigens to T lymphocytes, and regulate immune responses (Taher *et al.*, 2017). Because B cells may generate pro-inflammatory cytokines that can promote inflammation and affect the development of other immune cells like T cells, they play a role in autoimmunity and alloimmunity. B cells can release anti-inflammatory cytokines. These so-called regulatory B cells (B regs), which function earlier than regulatory T cells (T regs), are triggered by TLRs rather than antigen receptors. Furthermore, LPS binds the TLR4 receptor (Busse & Zenclussen, 2022).

Understanding the role of LPS in both the toxicity of bacterial infections and its effects on immune regulation has emerged as a critical objective due to the worldwide importance of sepsis (Mbongue *et al.*, 2022). LPS is the classical murine B lymphocyte mitogen, capable of stimulating polyclonal B cells to proliferate, differentiate into plasma cells and secret innate antibodies *in vitro*. Whether accessory cells, such as macrophages and dendritic cells, are required for LPS-induced B-cell activation and antibody production has been debated for many years. Some researchers have argued that direct LPS-B cell contact is required to trigger antibody production (‘one nonspecific signal’) and provided evidence that macrophages suppress direct LPS activation of B-cells. However, other researchers reported that accessory cells and/or their products are required for LPS to cause murine B cell proliferation and/or antibody production (Lu & Munford, 2016).

4.4.4 Distribution of LPS According to Sex

As shown in Table (4.15), there was a highly significant difference ($p = 0.000$) in the titer of LPS between females from both groups. Whereas there was no significant difference ($p = 0.179$) between males from both groups. Also, there was no significant difference between males and females in both studied groups with $p = 0.99$ and $p = 0.45$ respectively. Additionally, negative association between sex and LPS was found (odds ratio 0.66).

This result was consistent with previous study revealed that intravenous administration of LPS provoked in both men and women an acute systemic inflammatory response that was characterized by a transient rise in plasma concentrations of pro-inflammatory cytokines. Importantly, female participants mounted a substantially stronger in vivo cytokine response, with significantly greater increases in TNF-a and IL-6 (Wegner *et al*., 2017).

Table 4.15: Distribution of LPS According to Sex

Sample	Sex	N	LPS titer (U/mL)
			Mean ±SD
Control	**Male**	7	43.47±6.26
	Female	34	43.48±5.36
	***P* value**		0.99
Patient	**Male**	7	57.89±25.94
	Female	34	66.74±28.33
	***P* value**		0.45
T test (*P* value)	**Male**		0.179
T test (*P* value)	**Female**		0.000*
Odds Ratio	0.66 (95% confidence Interval lower 0.209 and upper 2.128)		
* Statistically significant at 0.05 level; LPS, Lipopolysaccharides; N, Number; SD, Standard Deviation			

Responses in inflammatory cells, strongly depend on sex. For instance, LPS-triggered TNF-α release is higher in human monocyte-derived macrophages in females compared to males. Estrogens contribute to the sex differences in immunological responses. It has been shown that LPS and estrogen receptors (ERs) interact in human monocyte-derived macrophages. In both sexes, LPS up-regulates the expression and activity of ER- α with the effect being more obvious in male cells. LPS, on the other hand, only inhibits the expression of ER-ß in female cells. LPS binds the TLR4 receptor, which can, for example, affect the monocyte's chemotaxis (Campesi *et al*., 2022).

4.4.5 Distribution of LPS According to Age Groups

The results showed there were significant differences in LPS titers between patients and control groups according to age groups (≤45 years and >45 years). Additionally, the results showed there were no significant differences between both age groups in patients and control groups, and negative association was found between age and LPS titer, as shown in Table (4.16). Higher mean of LPS was observed in younger patients and healthy subjects. This may be due to the changes in food intake behavior among the elderly age group which might have impact on bacterial translocation and level of LPS in blood.

Table 4.16: Distribution of LPS According to Age Groups

Sample	**Age groups**	**LPS titer (U/mL)**	
		N	**Mean ± SD**
Control	**≤45 year**	18	44.23±6.20
	>45 year	23	42.89±4.81
	***P* value**		0.44
Patient	**≤45 year**	18	70.08±29.37
	>45 year	23	61.42±26.58
	***P* value**		0.33
T test (*P* value)	**≤45 year**		0.000*
T test (*P* value)	**>45 year**		0.003*
Odds Ratio	0.733 (95% confidence interval lower 0.305 and upper 1.76)		
* Statistically significant at 0.05 level; N, Number; LPS, Lipopolysaccharides; SD, Standard Deviation			

4.4.6 Distribution of LPS According to Disease Duration

The results revealed no significant difference (p = 0.482) in LPS titer according to disease duration, as shown in Figure (4.4). However, the mean level of LPS was higher in patients with disease duration for more than 10 years. This might reflect the accumulation and continuous translocation of LPS to the circulation.

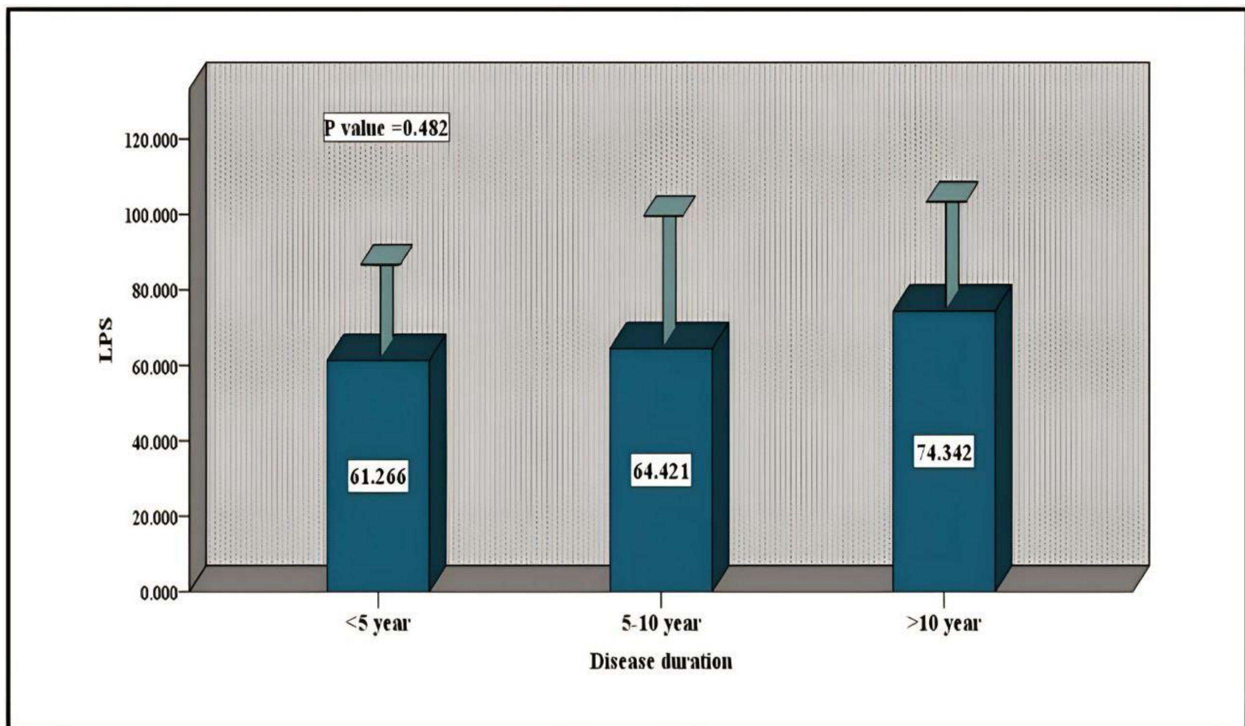


Figure 4.4: Distribution of LPS According to Disease Duration

4.4.7 Distribution of LPS According to BMI

The current study revealed that there were highly significant differences in LPS titers between patients and control groups regarding the obese (p = 0.000) and overweight (p = 0.007) groups of BMI, while there were no significant differences in LPS titers regarding the normal group of BMI. A higher mean±SD was observed in the obese and overweight groups in both studied groups. Additionally, the study

showed that there were no significant differences between BMI categories in patients and control groups, as shown in Figure (4.5).

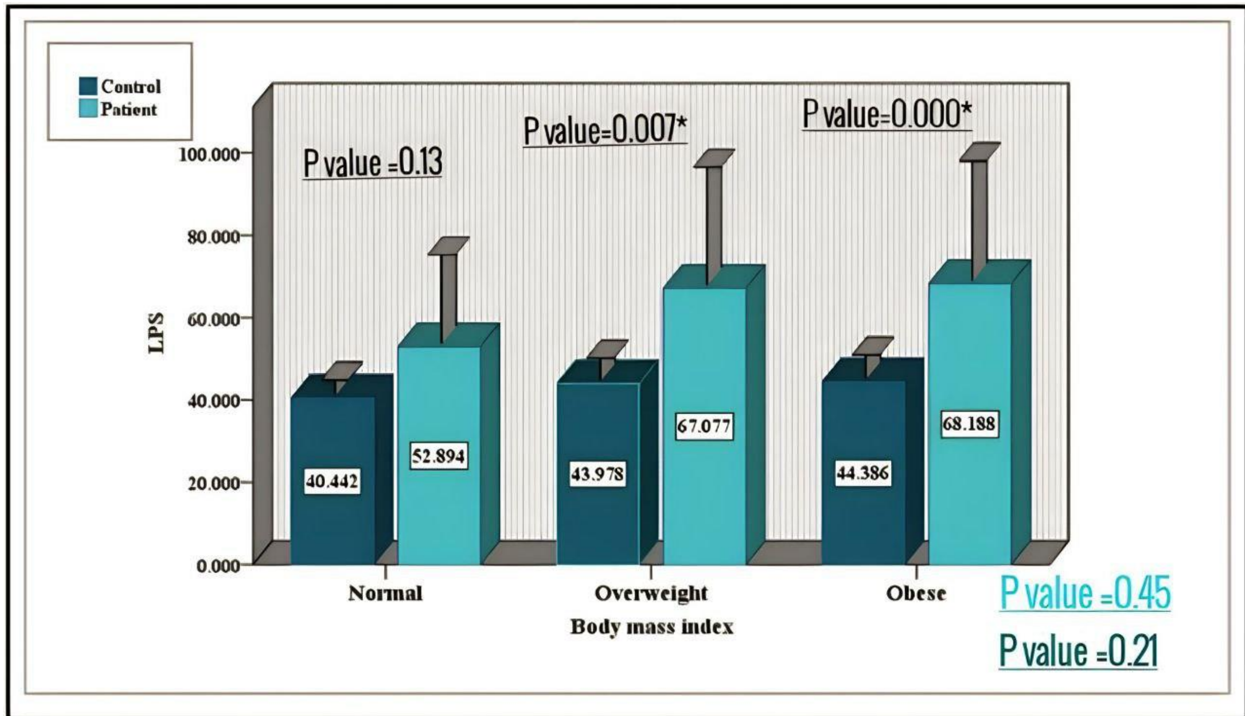


Figure 4.5 Distribution of LPS According to BMI

It has been documented that eating of high-fat foods increases the levels of blood LPS from gram-negative bacteria in the gastrointestinal tract through the LPS translocation from the intestinal microbiome to the blood. LPS from the gut may increase the lipids transport to the adipose tissue through the capillary endothelium by facilitating transcytosis (Hersoug *et al.*, 2018). This might be an indication of how LPS affects RA. LPS, an intestinal bacterial component, has the capacity to drive both the inflammatory and autoimmune processes by providing the second signal for the activation of T cells (Parantainen *et al.*, 2022).

4.4.8 Distribution of LPS According to ESR

The current study revealed significant differences in the mean of LPS between patients and control groups according to ESR groups, as shown in Figure (4.6). This may be because RA disease is an inflammatory disease, and ESR is a non-specific marker of inflammation (Calderon & Wener, 2012).

Additionally, a comparison of LPS titers in both normal and high ESR categories between the studied groups showed no significant differences.

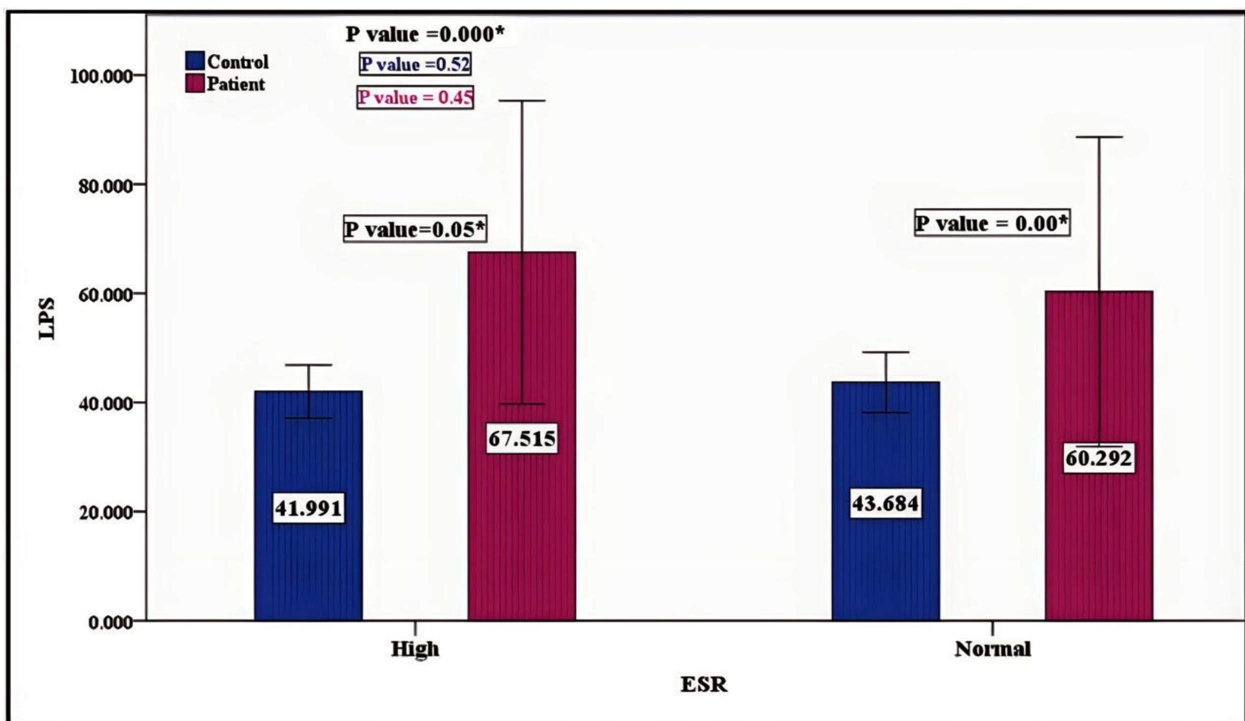


Figure 4.6: Distribution of LPS According to ESR

4.4.9 Distribution of LPS According to DAS28

The result showed that there was no significant difference in LPS titer (p = 0.627) according to DAS28. The higher mean±SD was found in patients with moderate activity, as shown in Figure (4.7). It has been documented that the

majority of patients present with a moderate form of RA, in which episodes of exacerbation occur (Scott *et al*., 2005).

Another previous study showed that serum LBP levels were significantly higher in RA patients with high disease activity than in those with moderate or low activity (Wen *et al*., 2018).

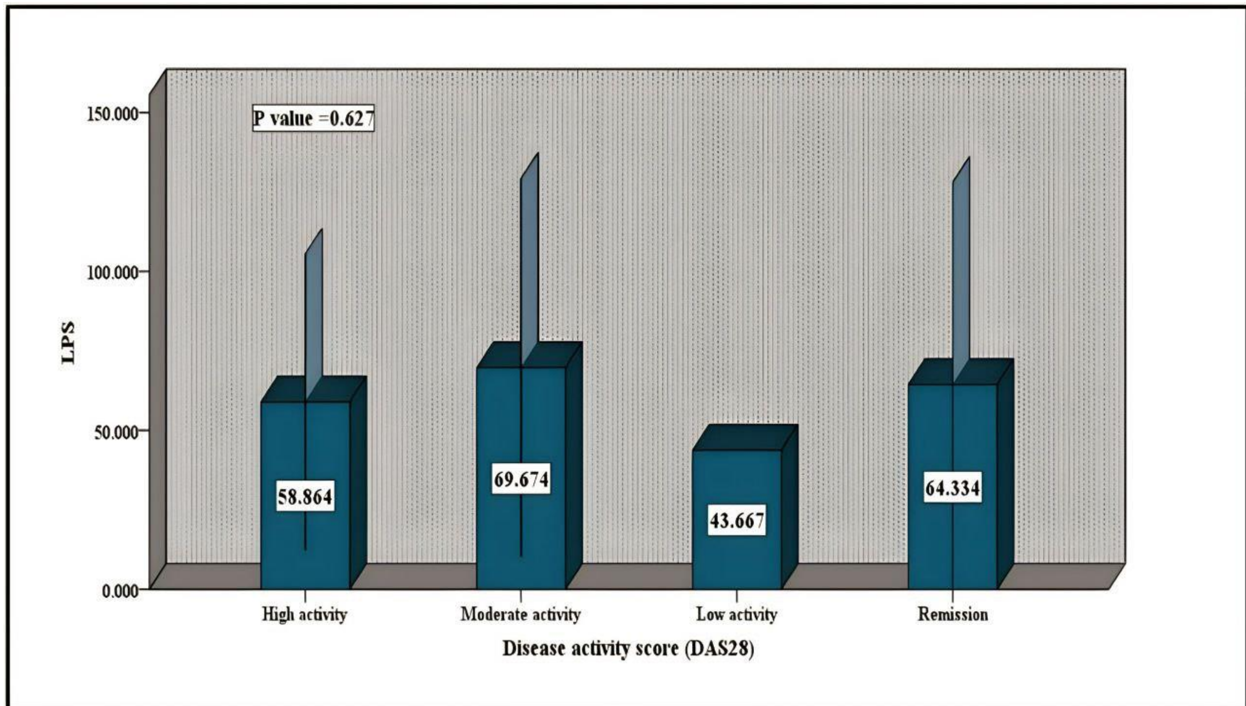


Figure 4.7: Distribution of LPS According to DAS28

4.5 Correlations

4.5.1 Correlation of Autoantibodies, ESR and LPS

The current study revealed a significant positive correlation between ESR and autoantibodies OD or titers, ESR and LPS titers, and between autoantibodies OD or titers and LPS titers, as shown in Table (4.17). This might reflect the association of LPS with the inflammatory process during RA pathogenesis.

Table 4.17: Correlation between Autoantibodies, ESR and LPS

		ESR	RF	ACCP	Anti-RA33	Anti-SSA	Anti-dsDNA	LPS
ESR	**Pearson Correlation**	1	0.467**	0.414**	0.083	0.396**	0.258*	0.278*
	Sig. (2-tailed)		0.000	0.000	0.456	0.000	0.020	0.012
RF	**Pearson Correlation**		1	0.438**	0.299**	0.500**	0.364**	0.536**
	Sig. (2-tailed)			0.000	0.006	0.000	0.001	0.000
ACCP	**Pearson Correlation**			1	0.401**	0.717**	0.443**	0.553**
	Sig. (2-tailed)				0.000	0.000	0.000	0.000
Anti-RA33	**Pearson Correlation**				1	0.504**	0.516**	0.403**
	Sig. (2-tailed)					0.000	0.000	0.000
Anti-SSA	**Pearson Correlation**					1	0.507**	0.457**
	Sig. (2-tailed)						0.000	0.000
Anti-dsDNA	**Pearson Correlation**						1	0.343**
	Sig. (2-tailed)							0.002
LPS	**Pearson Correlation**							1
	Sig. (2-tailed)							

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

RF, Rheumatoid Factor; ACCP, Anti-Cyclic Citrullinated Protein; Anti-RA33, Anti-Rheumatoid Arthritis 33; Anti-SSA, Sjögren's Syndrome antigen A; Anti-dsDNA, Anti-double-stranded Deoxyribonucleic Acid; LPS; Lipopolysaccharides; ESR; Erythrocyte Sedimentation Rate

Similar findings were observed in other previous studies that found a significant correlation between RF and ACCP (Gassid *et al.*, 2012; Abd-ali *et al,* 2018; Aiman *et al.*, 2020), between ESR and both RF and ACCP (Abd-ali *et al,* 2018), between anti-Ro/SSA with both RF and ANA (Khater & Al Sheik, 2022). And between ACCP and ESR (Abdulameer *et al.*, 2022).

Inversely, this result was inconsistent with other previous studies that showed no significant correlation between anti-Ro with both RF and ACCP (Zanlorenzi *et al.*, 2012), between anti-RA33 and RF, also, ACCP and RF (Al-mughales, 2015), between ACCP and ESR (Gassid *et al.,* 2012), between anti-RA33 and ESR (Suhail *et al.*, 2019), and between anti-Ro/SSA with both ACCP and ESR (Khater & Al Sheik, 2022).

It has been documented that LPS bioactivity correlated with the ESR (Parantainen *et al.*, 2022).

4.5.2 Correlation of Autoantibodies, DAS28 and LPS in RA Patients Group

The study revealed a negative correlation between DAS28 and autoantibodies and with LPS titer levels, but not for the significant levels except in the case of anti-RA33 autoantibodies, as shown in Table (4.18).

Table 4.18: Correlation between Autoantibodies, DAS28, and LPS in RA Patients Group

		DAS28	RF	ACCP	Anti-RA33	Anti-SSA	Anti-dsDNA	LPS
DAS28	**Pearson Correlation**	1	-0.080	-0.098	-0.371*	-0.123	-0.073	-0.198
	Sig. (2-tailed)		0.621	0.542	0.017	0.443	0.652	0.214

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

RF, Rheumatoid Factor; ACCP, Anti-Cyclic Citrullinated Protein; Anti-RA33, Anti-Rheumatoid Arthritis 33; Anti-SSA, Sjögren's Syndrome antigen A; Anti-dsDNA, Anti-double-stranded Deoxyribonucleic Acid; LPS; Lipopolysaccharides; DAS28; Disease Activity Score 28

Similar findings were reported in prior studies that showed no correlation between ACCP titers and activity of disease (Gassid *et al*., 2012; Shpatz *et al*., 2021).

Inversely, this result was inconsistent with another previous study that showed a statistically significant strong positive linear correlation between DAS28 and serum ACCP (Al-Derzi, 2017).

Conclusions and Recommendations

Conclusions

The current study concludes the following:

1. The detection of disease specific and disease associated autoantibodies may enhance the early diagnosis of RA patients since there were significant differences between patients and control groups in the level of autoantibodies and more than 60% of RA patients were positive for all tested (specific and non-specific) autoantibodies.

2. All tested autoantibodies differ significantly according to sex, and females have more autoantibodies OD or titer than males. Also, there were significant differences between patients and controls in all tested autoantibodies.

3. All tested autoantibodies were significantly differ between high ESR levels and normal ESR, whereas no significant differences were found according to DAS28.

4. Bacterial translocation which is presented by serum LPS level might have an impact on RA patients since there were significant increase in the mean level of LPS between patients and controls. And there were significant differences in LPS according to ESR but not in DAS28.

5. The presence of significant positive correlations between autoantibodies and LPS levels might reflect the importance of LPS in the inflammatory process and their detection might possibly help in RA patients diagnosis.

Recommendations

The current study recommends the following:

1. Design a cohort study with follow-up of RA patients aiming to determine the concentration of disease-specific and disease associated autoantibodies at different periods of disease to show the extent to which autoantibodies levels are affected by disease duration and treatment.

2. Study the presence of disease specific and disease-associated autoantibodies in patients with other rheumatic diseases like SLE and SS and study their importance in the diagnosis of rheumatic diseases.

3. Make a study to compare the disease activity states using different measurements like DAS28, Health Assessment Questionnaire (HAQ), VAS, Clinical Disease Activity Index (CDAI), and Simplified Disease Activity Index (SDAI), and determine which one of them correlates with disease-specific and disease-associated autoantibodies presence and with LPS levels in RA patients.

4. Study the effect of bacterial translocation on subsequent induced immune response in RA patients.

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The Appendices

Appendix 1: Questionnaire of Rheumatoid Arthritis Patients

Baseline Characteristics						
Name		Code		Age or date of birth		
Gender	Male		Female			
Local address		Phone				
Profile of Other Factors						
Family history		Yes		No		
Weight		Height		BMI		
Hypertension	Yes			No		
DM	Yes		No		Type	
CVD	Yes		No		Type	
Symptoms of SLE	Yes		No			
Symptoms of SS	Yes		No			
Bacterial infection signs						
Profile of Rheumatoid Arthritis						
Disease duration	Acute < 6 month		Chronic > 6 month			
Number of swollen joints		Number of tender joints				
Global assessment						
Morning stiffness	Yes		No			
Extra articular manifestation	Yes		No			
Intermittent symptoms	Yes		No			
ESR						
DAS28						
RF	Positive		Negative		Unknown	
ACCP	Positive		Negative		Unknown	
Anti-SSA	Positive		Negative		Unknown	
Anti-dsDNA	Positive		Negative		Unknown	

الخلاصة

التهاب المفاصل الرثوي هو مرض التهابي مزمن من أمراض المناعة الذاتية تؤدي بالجهاز المناعي لمهاجمة المفاصل، مسببة التهابات وتدميرًا لها ومن ثم الإعاقة وفقدان القدرة على الحركة اذا لم يعالج بصورة فعالة.

هدفت الدراسة الحالية لتقييم الأجسام المضادة الخاصة والمرتبطة بالتهاب المفاصل الرثوي وعلاقتها بالمكون البكتيري .الدراسة الحالية هي الأولى التي تتعلق بالبحث عن أجسام مضادة ذاتية خاصة ومرتبطة بالتهاب المفاصل الرثوي وعلاقتهم بمستوى عديد السكارايد الدهني (LPS) وفعالية المرض.

أجريت الدراسة بين المرضى والأصحاء في كلية العلوم الطبية التطبيقية –جامعة كربلاء. كان العدد الاجمالي للمشاركين في هذه الدراسة 82 (41 مريض بالتهاب المفاصل الرثوي و41 اشخاص أصحاء) خضعوا جميعهم للتشخيص السريري بواسطة اطباء اختصاص المفاصل والروماتيزم في مستشفيات الهندية التعليمي والإمام الحسن المجتبى (ع) \دائرة صحة كربلاء \العراق. بين الفترة من تشرين الاول\ 2022 الى شباط \2023. تم جمع العينات المأخوذة من المرضى والاصحاء وتم استخدامها بتقدير معدل ترسيب كريات الدم الحمراء و الأجسام المضادة الذاتية وعديد السكارايد الدهني.

كشفت الدراسة الحالية أن أعمار المشاركين تراوحت من 27 إلى 77 عامًا. كانت نسبة الذكور / الإناث 1: 4.8. حسب الفئات العمرية كان هناك فرق معنوي كبير في جميع الأجسام المضادة الذاتية بين المرضى والاصحاء ($P < 0.05$).

كشف تحليل منحنى الخصائص التشغيلية للمستقبل (ROC) عن وجود معدلات عالية من منطقة تحت المنحنى، ومعدلات الحساسية والخصوصية لجميع الأضداد الذاتية التي تم اختبارها ، وتم العثور على معدلات أقل لـعديد السكارايد الدهني. أظهرت هذه الدراسة فرق معنوي بين مجموعتي المرضى، والاصحاء في مستوى جميع الاجسام المضادة الذاتية التي تم اختبارها وأن 25 (60.97٪) من المرضى لديهم جميع الأجسام المضادة الذاتية المختبرة ، بينما 27 (65.85٪) من مجموعة السيطرة كانت سلبية.

فيما يتعلق بـعديد السكارايد الدهني (LPS) ، أظهرت النتائج زيادة معنوية في متوسط مستوى عديد السكارايد الدهني بين مجموعتي المرضى والاصحاء ($P = 0.000$). كشفت الدراسة الحالية عن وجود علاقة إيجابية معنوية بين مستوى سرعة ترسيب كريات الدم الحمراء مع جميع مستويات الأجسام المضادة الذاتية المختبرة ومع مستوى عديد السكارايد الدهني وبين مستوى الأجسام المضادة الذاتية مع مستوى عديد

السكارايد الدهني ، ووجود علاقة سلبية بين معدل فعالية المرض (DAS28) مع الأجسام المضادة الذاتية ومستويات عديد السكارايد الدهني ولكن ليس ذات دلالة احصائية باستثناء حالة الأجسام المضادة Anti-RA33.

استنتجت الدراسة الحالية وجود فروق معنوية في جميع الأجسام المضادة التي تم اختبارها وعديد السكارايد الدهني (LPS) بين مجموعتي المرضى والاصحاء ، وقد تعكس هذه النتيجة أهمية تشخيص الأجسام المضادة الخاصة و المرتبطة بالتهاب المفاصل الرثوي في تشخيص التهاب المفاصل الرثوي. قد تعكس نتائج عديد السكارايد الدهني (LPS) دور ال (LPS) في تشخيص التهاب المفاصل الرثوي وتطوره. بالنسبة لنشاط المرض ، كانت الأجسام المضادة الذاتية و عديد السكارايد الدهني مختلفة بشكل معنوي مع معدل سرعة ترسيب كريات الدم الحمراء ولكن ليس مع معدل فعالية المرض (DAS28) ، وقد تكشف هذه النتيجة عن ارتباط الأجسام المضادة الذاتية و عديد السكارايد الدهني بتطور المرض. أيضًا ، كان هناك ارتباط إيجابي كبير بين معدل سرعة ترسيب كريات الدم الحمراء مع جميع الأجسام المضادة التي تم اختبارها ومع عديد السكارايد الدهني كذلك بين جميع الأجسام المضادة الذاتية التي تم اختبارها مع عديد السكارايد الدهني ، وايضا علاقة سالبة بين معدل فعالية المرض (DAS28) مع الأجسام المضادة الذاتية ومع عديد السكارايد الدهني ولكن ليس بمستوى معنوي باستثناء حالة الأجسام المضادة Anti-RA33.

جامعة كربلاء

كلية العلوم الطبية التطبيقية

قسم التحليلات المرضية

تقييم بعض المعايير المناعية وعديد السكر البكتيري بالتهاب المفاصل الرثوي

رسالة مقدمة

الى مجلس كلية العلوم الطبية التطبيقية – جامعة كربلاء

وهي جزء من متطلبات نيل شهادة الماجستير في التحليلات المرضية

كتبت بواسطة

زهـراء فـلاح مـهدي

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بأشراف

أ.د. سهاد هـادي محمـد

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