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Inflammatory Anti- Inflammatory and Oxidative Status in Rheumatoid Arthritis Women Patients with and without Type Two Diabetes and the Effect of Nanoformulated Metformin on Isolated Blood Cells

A Thesis

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By

Shatha Jassim Hatem

B.Sc. in Chemistry- College of Science, University of Kerbala (2005-2006)

Supervised by

Prof. Dr. Fadhil Jawad Al-Tu'ma Assist. Prof. Dr. Maher Abbood Mukheef

Department of Chemistry and Biochemistry

College of Medicine – University of Kerbala

2024, A.H. **1446, D.H.**

بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

﴿يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ﴾

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Supervisor Certification

We certify that this thesis entitled" **Inflammatory, Anti- Inflammatory and Oxidative Status in Rheumatoid Arthritis Women Patients with and without Type Two Diabetes and the Effect of Nanoformulated Metformin on Isolated Blood Cells** " was carried under our supervision at the College of Medicine, University of kerbala , as a partial fulfillment for the requirement for the degree of Master of Science in Clinical Biochemistry.

Supervisors

Professor

Dr. Fadhil Jawad Al-Tu'ma

Department of Chemistry and Biochemistry

College of Medicine

University of Kerbala

1 / 9 / 2024

Assistant Professor

Dr Maher Abbood Mukheef

Department of Chemistry and Biochemistry

College of Medicine

University of Kerbala

24 / 9 / 2024

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

Assistant Professor

Dr. Atheer Hameid Al-Ghanimi

Head of chemistry and Biochemistry Department

College of Medicine

University of Kerbala

24 / 9 / 2024

Examination Committee Certification

We the Examining Committee, certify that we have read this M.Sc. thesis entitled:" **Inflammatory, Anti- Inflammatory and Oxidative Status in Rheumatoid Arthritis Women Patients with and without Type Two Diabetes and the Effect of Nanoformulated Metformin on Isolated Blood Cells** " and have examined the student **(Shatha Jassim Hatem)** in its content and our opinion; it meets the standard of thesis for the Master's degree in Clinical Chemistry with an appreciated **"Excellent"**.

Signature
Assist.Prof.Dr.AtheerHameid
College of Medicine
University of Kerbala
24 / 9 / 2024
(Member)

Signature
Prof.Dr.Riyadh Mustafa Murtadha
College of Medicine
University of Kerbala
25 / 9 / 2024
(Member)

Signature
Prof. Dr. Fadhil J. Al-Tu'ma
College of Medicine
University of Kerbala
1 / 10 / 2024
(Member and Supervisor)

Signature
Assist.Prof.Dr Maher Abbood Mukheef
College of Medicine
University of Kerbala
24 / 9 / 2024
(Member and Supervisor)

Signature
Prof. Dr. Abdulsamia Hassan Alta'ee
College of Medicine / University of Babylon
25 / 9 / 2024
(Chairman)

Approved by the College of Medicine/ University of Kerbala

Signature
Prof. Dr. Riyadh Dayhood Mahdi AL-Zubaidi
Dean of Medicine College/ University of Kerbala
/ / 2024

Dedication

To the one who taught me to give without waiting

and I carry his name with pride**.**

My Father

To my love, tenderness, and honesty

Whose connections were the secrets of my success

To the soul of the most precious loved ones.

My dear mother and my dear brother

May God have mercy on them

To my support and strength, my dear husband Ali, and my beloved children, Hassan and Hussein

To my dear sisters

To my extended family, my loved ones, and my friends

and to my dear teachers

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Summary

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovitis, cartilage damage, and bone erosions, which is associated with increased morbidity and mortality compared with the general population. Systemic inflammation associated with RA might contribute to the risk of developing diabetes in the future. RA is associated with chronic inflammation, which might, at least in part, trigger the development of type 2 Diabetes Mellitus (T2DM). Nano-drug delivery systems offer promising solutions to the limitations of conventional treatments for RA and T2DM. Among nanoparticles, niosomes are particularly effective due to their stability, ease of preparation, and ability to reduce systemic toxicity. They provide controlled drug release and enhance the solubility and stability of pharmaceutical compounds. Incorporating hyaluronic acid (HA) into niosomes can further enhance their efficacy by targeting specific cells, improving drug delivery, and increasing therapeutic impact. Metformin, a common T2DM medication was effectively delivered using HA-coated niosomes, demonstrating the potential of this approach in treating RA and T2DM.

This study aims to determine the inflammatory/anti-inflammatory biomarkers to early detection of RA and prognosis to prevent the development of T2DM, association and correlation between variables markers that correlate with disease, and using metformin, a common T2DM medication, effectively delivered using HA-coated niosomes, to demonstrate the potential of this approach in treating for RA and T2DM by using a molecular method of PCR of various factors, such as total oxidant status (TOS), interleukin-23 (IL-23), nuclear factor of activated T-cells cytoplasmic 1 (NFATC1),and receptor

activator of nuclear factor kappa-B ligand (RANKL), was assessed in both treated and untreated Peripheral blood mononuclear cells (PBMCs).

A case-control study was performed on 118 participants divided into 47 as healthy control, 71 as RA patients 33 RA patients with T2DM, and 38 RA without T2DM, all patients with rheumatoid factors positive test. They were all directly interviewed and blood samples were taken. Age at the onset of RA plays an important role in determining the severity of the disease and its development, especially for those between (40-49) years of age (42.3%). This indicates that these age-related factors are crucial for developing effective therapeutic strategies and managing comorbidities in patients with rheumatoid arthritis. Patients suffering from obesity weremore likely to develop RA and their percentage was (43.7%) compared to those from the other groups. This indicates a positive relationship between body mass index (BMI) and the risk of developing RA

In the case of RA with T2DM patients had significantly higher Fasting blood sugar (FBS)(331.30 ± 108.84 mg\dL) levels as compared to RA patients without T2DM(106.92 ± 8.17mg\dL) (P-value < 0.001), indicating impaired blood sugar control, while the glycated hemoglobin HbA1c(5.52 ± 0.64%) a marker for long-term blood sugar control, was significantly elevated in the RA withT2DM group (11.80 ± 1.81%) (P-value < 0.001) this relationship indicates the prognostic importance to monitoring and preventing the development of T2DM in patients of RA. The exogenous antioxidants such as vitamin E related to the reduction of blood glucose as well as glycated hemoglobin as compared with chemical drugs. The result observed that vitamin E and selenium non-significantly decreased RA patients with and without T2DM (P>0.05). Results indicated a highly statistically significant difference

in IL-23 and Transforming Growth Factors - Beta (TGF-β) levels among groups, The mean levels of IL-23, TGF-β in patients in RA (41.32 ± 14.15pg\mL), (40.56 ± 16.35pg\mL) respectively, significantly higher than for the Control group(9.16 ± 1.01 pg\mL),(4.42 ± 2.79 pg\mL) respectively. The results show highlights the potential of hyaluronic acid-coated niosomal nanoparticles as an effective drug delivery system for metformin, targeting RA and T2DM-derived PBMCs. The Hyalo-Nio-met NPs exhibited desirable characteristics, including stability and controlled drug release. The incorporation of HA enhanced the targeting and therapeutic impact of these niosomes. The synthesized Hyalo-Nio-met NPs showed a significant reduction in TOS, the pro-inflammatory cytokine IL-23 inflammation-related genes NFATC1، and RANKL in PBMCs from RA patients. This indicates a promising reduction in inflammation and enhancement of anti-inflammatory and antioxidant defenses. Overall, the Hyalo-Nio-met NPs drug delivery system effectively delivered metformin to PBMCs, showing potential as a novel therapeutic approach for RA and T2DM by reducing systemic toxicity and improving drug delivery to specific cells.

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List of Abbreviations

Abbreviation	Names
AMPK	Activated Protein Kinase
APR	Acute Phase Reactants
AMP	Adenosine Monophosphate
AGE	Advanced Glycation End Products
ACR-EULAR	American College of Rheumatology-European League Against Rheumatis
ANA	Antinuclear Antibodies
AVMs	Arteriovenous Malformations
AFM	Atomic Force Microscopy
CNS	Central Nervous System
CRP	C-Reactive Protein
DNA	Deoxyribonucleic Acid
DCFH-DA	Dichlorofluorescein Diacetate
DEPC	Diethyl Pyro-Carbonate
DMARDs	Disease-Modifying Anti-Rheumatic Drugs
EOS	Early Onset Neonatal Sepsis
ESR	Erythrocyte Sedimentation Rate
ECM	Extracellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assays
FLSs	Fibroblast-Like Synoviocytes
FDRs	First-Degree Relatives

FT-IR	Fourier-Transform Infrared Spectroscopy
HbA1c	Glycated Hemoglobin
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase (Housekeeping genes)
GM-CSF	Granulocyte macrophage Colony-Stimulating Factor
HPLC	High-Performance Liquid Chromatography
MHC	Histocompatibility Complex
HRP	Horry Reaish Peroxidase
HLA	Human Leukocyte Antigen
Hyalo-Nio NPs	Hyaluronic Acid Coated Niosomal Nanoparticles
Ig A	Immunoglobulins Type A
IgG	Immunoglobulins Type G
IgM	Immunoglobulins Type M
IR	Insulin resistance
MSC	Mesenchymal Stromal Cells
mRNA	Messenger Ribose Nucleotide Acid
Hyalo-Nio-met NPs	Metformin-loaded hyaluronic acid-coated niosomal nanoparticles
MENA	Middle East and North Africa
MAPK	Mitogen-Activated Protein Kinase
MS	Multiple Sclerosis.
NK	Natural Killer
non-HLA	Nonhuman Leukocyte Antigen
RANKL	Nuclear Factor Kappa-B Ligand

NFACT1	Nuclear Factor of Activated T-cells Cytoplasmic 1
OSI	Oxidative Stress Index
PBMCs	Peripheral Blood Mononuclear cells
PBS	Phosphate-Buffered Saline
PDI	Poly Dispersity Index
PRSs	Polygenic Risk Scores
PCR	Polymerase Chain Reaction
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
RT-PCR	Real-Time Polymerase Chain Reaction
RORγτ	Retinoid-Related Orphan Receptor Gamma Tau
RA	Rheumatoid Arthritis
RF	Rheumatoid Factor
RPMI	Roswell Park Memorial Institute
SEM	Scanning electron Microscopy
STAT	Signal transducer and Activator of Transcription
Treg	T Cells Regulatory T Cells
TAS	Total Antioxidant Status
TOS	Total Oxidant Status
TCM	Traditional Chinese Medicine
TGF-β	Transforming Growth Factor-beta
TNF	Tumor Necrosis Factor
T2DM	Type 2 Diabetes Mellitus

ZS	Zeta Sizer
IL-23	Interleukin-23
CD4+T	Cluster of Differentiation 4
FBS	Fasting Blood Sugar
NF-Kb	Nuclear Factor Kappa B
VEGF	Vascular endothelial growth factor
FGF	Fibroblast Growth Factors
IL-17	Interleukin-17
DLS	Dynamic Light Scattering
MET	Metformin
ACCP	Anti-Cyclic Citrinullated Peptide
ACPA	Anti-Carbamylated Protein Antibody

Chapter One

Introduction
and
Literature Review

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease resulting from both genetic and ecological factors. It has been hypothesized that genetic predilection, in combination with environmental factors, leads to a cascade of events causing synovitis and eventually destructive arthritis (**Jahid, *et al*., 2023; Babaahmadi, *et al*., 2023)**. Oxidative stress is a condition characterized by an imbalance between the oxidative and antioxidant processes within the human body. As a result, an excess of reactive oxygen species ROS, reactive nitrogen species (RNS), and various other compounds build up, leading to oxidative damage. RA is significantly influenced by the presence of oxidative stress (**da Fonseca, *et al*., 2019; Wang, *et al*., 2024**). The clinical manifestation of the disease is repeated and symmetrical affects the hand, wrist, foot, knee, and other joints. Redness, swelling, heat, pain, and joint dysfunction are in the early phases. In the later phases, rigidity and deformity of the joints are observed **(Fresneda Alarcon, *et al*., 2021)**.

RA is associated with chronic inflammation, which might, at least in part, trigger the development of type 2 diabetes mellitus (T2DM) (**Baghdadi, *et al*., 2020).** Early diagnosis could prevent joint damage; a large body of evidence indicates that statistically significant differences in permanent joint damage can occur within the first two years of disease onset (**Cheng, *et al*., 2021)**. Rheumatoid arthritis is typically diagnosed according to the 2010 ACR-EULAR (American College of Rheumatology-European League Against Rheumatism) **(Ishida, *et al*., 2021).** Routine tests like C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) are used as clinical biomarkers to ascertain the general inflammatory state of RA patients **(He, *et al*., 2020).** RA and other chronic diseases such as (T2DM) with RA are associated with chronic inflammation, **(Baghdadi, *et al*., 2020).**

Inflammation as a driving pathophysiological process, indeed, C-reactive protein (CRP), a marker of inflammation, predicts thefuture development of hypertension and diabetes mellitus better than body mass index **(McCutcheon, K., *et al.*, 2024).** The overproduction of pro-inflammatory cytokines, such as tumor necrosis factor (TNF) and some interleukins, results in the proliferation of synovial cells in joints and the consequent formation of pannus, cartilage destruction, and bone erosions (**Nattagh-Eshtivani, *et al.*, 2021).**

The CD4+ T cells and their cytokines are believed to play major parts in the induction and propagation of pathogenic inflammatory conditions. The T cell-associated cytokines interleukin (IL-23) have recently been proposed as important regulators of this process. This is supported by the recent discovery that IL-23, produced by CD4+ T cells and dendritic cells, respectively, promotes the development of the IL-17A-producing pro-inflammatory CD4+ T cell subset T helper (Th) 17. Angiogenesis is a remarkably active process in RA, especially in its early stages, and is regulated by various pro-angiogenic mediators such as TGF–β, angiopoietin, placenta growth factor, FGF, and vascular growth factor (VEGF) **(Ba, *et al.*, 2021)**. These factors activate endothelial cells and induce the production of proteolytic enzymes that degrade the basement membrane and perivascular extracellular matrix. A gene is defined as the basic physical and functional unit of heredity, composed of a specific sequence of nucleotides in DNA or RNA. Genes are typically located on chromosomes and play a crucial role in controlling the transmission and expression of traits by specifying the structure of proteins or regulating the function of other genetic materials **(Baverstock, K. 2021**). Various genes involved in RA, for instance. Nuclear factor kappa-B ligand (RANKL), which stands for Receptor activator of NF-κB ligand, is a crucial molecule involved in various physiological processes. It is known to play a significant role in bone homeostasis and the formation

of lymphoid tissues (**Mueller, *et al.*, 2012**). That nuclear factor of activated T-cells cytoplasmic 1(NFATC1) is the main regulator of osteoclast generation, has expression is increased in synovial osteoclast precursors of RA patients, indicating a potential link to the enhanced osteoclast differentiation observed in RA (**Niu, *et al.*, 2022).** Metformin (Met) is a flavonoid that has been recognized for its medicinal properties, including anti-inflammatory, anti-cancer, and antioxidant, activities. In terms of inflammation, Met has been shown to possess anti-inflammatory properties. It can inhibit the production of pro-inflammatory cytokines and enzymes which are involved in the inflammatory response **(Gharib, *et al.*, 2021).** Metformin and nicotinamide mononucleotide (NMN) target metabolic and aging processes within the liver **(Hunt, *et al.*, 2021) .**

Niosomes are bilayer nonionic surfactant vesicles with a core-shell assembly that makes them capable of encapsulating hydrophobic drugs into the non-polar region present within the bilayer and hydrophilic drugs inside the core. In structure and drug delivery potential, niosomes are similar to liposomes but have higher chemical stability, making them premiere to liposomes **(Bhardwaj, *et al.*, 2020).** Metformin-loaded nano for oral drug delivery should be attributed to the sustained drug release and enhanced gastrointestinal permeability, resulting in a significant decrease dosage of metformin (**Cui, *et al.*, 2018; Patiño-Herrera, *et al.*, 2019).**

1.1. Literature Review

1.1.1. Rheumatoid Arthritis

1.1.1. 1. Definition

Rheumatoid arthritis (RA) is a common, chronic, inflammatory autoimmune disease that affects and exhibits a variety of extra-articular manifestations **(Cho, *et al.*, 2024).** Rheumatoid arthritis is a heterogeneous

disorder caused by an abnormal autoimmune response initiated by the complex interactions of genetic and environmental factors that contribute to RA etiology **(Zamanpoor, 2019).**

1.1.1.2. Rheumatoid Arthritis Epidemiology

Epidemiologic variations in the incidence and prevalence of RA have been observed based on ethnic and geographic dispersion **(Nair, *et al.*, 2019).** The estimated global prevalence of RA ranges from 0.24 to 1%, although rates differ by region and country **(Almoallim, *et al.*, 2021).** The epidemiology of RA is poorly understood in the Middle East and North Africa (MENA) region, and data on its prevalence and disease activity among Arab populations are rare **(Bedaiwi, *et al.*, 2019)**. A recent global burden study estimated the prevalence of RA in the MENA region to be 0.16 percent, and RA disease severity and management differ geographically within the region **(Yip and Navarro-Millán, 2021).** The incidence of RA in Iraq was 1.1% in 2014 and 2.2% in 2019, compared to 1.6% and 2.1% in 2001 and 2011, respectively. Although this variation is not a statistically significant difference, it may be attributable to disruptions in the healthcare system and immigration during this period **(Al-Badran, *et al.*, 2022).** During the previous decade, it was observed that the prevalence and clinical characteristics of rheumatic diseases varied markedly by region, lifestyle, and social status, indicating that genetic and environmental factors play a substantial role in the onset and progression of rheumatic diseases **(Batko, *et al.*, 2019).**

1.1.1.3. Etiology

The exact cause of RA is unknown, RA may be triggered by exogenous (infective) and/or endogenous stimuli (tissue proteins or immunoglobulin's) in patients with a genetic predisposition. While infectious

agents have been suggested as causing agents for RA or Nonetheless, several agents including Gram-negative anaerobic bacteria like those found in periodontitis have been implemented in the etiology of RA (Demoruelle***,et al.,*** **2011; Yap*, et al.*, 2018).** The etiology of RA is complex and can occur at any age, especially in middle-aged people, with a high disability rate, and there is currently no complete cure **(Guo, *et al.*, 2023).** Psychiatric conditions have a particularly interesting link with RA. An association between post-traumatic stress disorder and an increased risk of RA has been described in both men and women **(Maihofer, *et al.*, 2024)** This has been hypothesized to be related to the previously described dysregulated neuroendocrine-immune mechanisms induced by chronic stress **(Romão, *et al.*, 2021).** Most recently, depression, a well-known common RA comorbidity found in 15–17% of patients, was also proposed as a risk factor for RA, suggesting a bidirectional relationship **(Dougados, *et al.*, 2014; Matcham, *et al.*, 2013).**

1.1.1.4. Rheumatoid Arthritis Risk Factors

Currently, there is an increasing interest in treating patients at risk of RA to prevent the development of this chronic disease. In this sense, research has focused on early identification of predictive factors of this disease **(Novella-Navarro, *et al.*, 2021).** The interaction of environmental factors, epigenetics, and susceptibility genes will lead to changes in the relative levels and expression of encoded proteins, which may contribute to autoimmune tolerance disorders (**Ding, *et al.*, 2023).**

1.1.1.4.1. Genetics of Rheumatoid Arthritis

The importance of inherited risk alleles in the pathogenesis of RA is highlighted by increased concordance for RA in monozygotic compared to dizygotic twins and by the familial clustering. The most important region of the human genome in RA susceptibility is the major histocompatibility

complex (MHC), which encodes for genes that are essential to immune responses, notably the HLA-A, HLA-B, HLA-C, and HLA-D proteins. These structures are expressed on the surface of antigen-presenting cells and are required for recognition of peptide antigens by T lymphocytes, leading to initiation of immune responses (**Fox, *et al.*, 2016; van and Holoshitz, 2017).** More lately polymorphisms that rule additional amino acid differences in HLA-DR that are located outward of the shared epitope have also been strongly associated with susceptibility to RA **(Ding, *et al.*, 2023; Okada, *et al.*, 2014).**

1.1.1.4.2. Environmental Factors

The existence of a genetic component in the onset of RA has long been considered because of the increased risk in first-degree relatives of patients with RA **(Frazzei, et *al.*, 2023; Hensvold, *et al.*, 2015).** However, the overall risk associated with established genetic markers remains limited, and the relatively low penetrance of the disease hints that environmental factors play an important role in the etiology of RA **(Deodhar, *et al.*, 2023).**

A. Smoking: Cigarette smoking is the most known external factor identified as a trigger of RA **(Croia, *et al.*, 2019).** Cigarette smoking has been implicated as an environmental risk factor for seropositive RA, possibly by inducing autoimmunity in the pulmonary mucosa and causing the body to emit inflammatory cytokines that contribute to the joint and organ damage associated with RA, up to 35% of the risk of seropositive RA is attributable to cigarette consumption **(Ren, *et al.*, 2023).** Recent studies have investigated passive cigarette smoking as a possible risk factor for RA in non-smoking patients **(Prisco, *et al.*, 2020).** Passive smokers had a risk of RA that was 12% greater than that of non-exposed individuals; the risk of developing RA was 34% higher in individuals exposed to passive smoking during childhood

compared to those who were not exposed. RA risk may be associated with passive smoking, particularly in childhood exposures **(Zhang, *et al.*, 2023).**

B. Sex: Approximately two-thirds of individuals who develop RA are women **(Hahn, *et al.*, 2023).** The cumulative risk of developing RA in the adult population has been estimated at 3.6% for women and 1.7% for men **(Chiu, *et al.*, 2021).** The role of hormones in the development of RA remains controversial, but this higher frequency of RA in women may be attributed to the stimulatory effects of estrogens on the immune system **(Raine, *et al.*, 2022).** Factors that have been associated with an increased risk for the onset of RA include early menopause **(Raine, *et al.*, 2022).** The presence of polycystic ovary syndrome **(Shah, *et al.*, 2020),** pre-eclampsia **(Jørgensen, *et al.*, 2010),** and post-partum periods **(Peschken, *et al.*, 2012).** Females are more vulnerable to autoimmune diseases due to the skewed inactivation that can occur when half of the x-chromosome genetic material, which is involved in the immune response, is silenced **(Valencia, *et al.*, 2022).** Factors that can protect against the onset of RA include breast-feeding, the use of hormone replacement therapy, and oral contraception (**Eun, *et al.*,2020)** One study showed no increased risk for seropositive RA based on one or more pregnancies, while others, including a meta-analysis, demonstrated that pregnancy confers a protective effect **(De Stefano, L., *et al.*, 2021).**

C. Vitamin D deficiency: Immune cells express vitamin D receptors. Vitamin D is responsible for regulating the innate and adaptive immune responses and for boosting the immune system; a decline in its levels results in autoimmunity. Several autoimmune diseases are linked to vitamin D, including insulin-dependent diabetes mellitus and RA **(Sukharani, *et al.*, 2021) .**Vitamin D deficiency has been found to be a predisposing factor for the development of RA. Various studies from all over the world have shown that vitamin D deficiency is observed in patients with RA and seems to be inversely related to disease activity **(Harrison, *et al.*, 2020)** .Vitamin D may

act on both T and B lymphocyte populations, thus enabling the regulation of the immune response necessary for the prevention or control of the disease **(Athanassiou, *et al.*, 2023).** Activated vitamin D or vitamin D analogs suppress dendritic cell cytokine production, specifically, interleukin (IL)-12, which affects the differentiation of T helper cells into Th1 cells, and IL-23, which affects the differentiation of T helper cells into Th17 cells **(Ao, *et al.*, 2021).**

D. Obesity: Obesity is a controversial risk factor for rheumatoid arthritis (RA). A link between obesity and RA is plausible since biological mechanisms of inflammation are present in adipose tissue, and these may be linked to chronic systemic inflammation. Important advances in understanding of adipokines have elucidated their crucial role as mediators of inflammation and immune response, which are implicated in the pathophysiology of rheumatic diseases, such as RA **(Derdemezis, *et al.*, 2011).** Activated adipocytes in states of hyper adiposity are a source of other pro-inflammatory cytokines such as chemerin, the retinol-4 transporter protein, lipocalin، and, importantly for RA, TNFα, IL-6, and IL-12. Given that IL-1 as well as TNF are fundamental for the creation of chronic inflammation and locally destructive synovial proliferation, which are the etiopathogenic basis of RA, a study of the modulation of the inflammatory activity of RA by the level of body fat is a potentially interesting aspect for the study and clinical treatment of patients with RA **(Alvarez-Nemegyei, *et al.*, 2020)**.

1.1.1.4.3. Family History of Rheumatoid Arthritis

There is an increased prevalence of RA in those families with and ~40–50% risk for seropositive RA, being strongest in first-degree relatives (FDRs) (**Novella-Navarro, *et al.*, 2021).** One study conducted in 1,780 FDRs of RA patients reported that screening for auto-antibodies can identify RA-

related positive individuals; i.e., those who are associated with a higher risk for future onset of RA. In this line, another study observed a greater association between obesity, Anti-Carbamylated Protein Antibody (ACPA) positive status and incidence of periodontitis in FDRs than in healthy controls, suggesting the relevant role of these conditions as a risk of developing RA in FDRs **(Unriza-Puin, *et al.*, 2017).**

1.1.1.5. Symptoms of Rheumatoid Arthritis

Signs and symptoms of RA may be Joint pain, swelling, stiffness, redness around the affected joint morning stiffness that usually lasts longer than 30 minutes fatigue, malaise (a general feeling of un-wellness) low-grade fever, and, occasionally, flu-like symptoms **(Sarazin, *et al.*, 2017; Goodwin, *et al*,2023).** About 40% of people are having RA also experience signs and symptoms that don't involve the joints. Areas that may be affected include (skin, eyes, lungs, heart, kidneys, salivary glands, nerve tissue, bone marrow, and blood vessels, see figure 1-1 **(Amaya-Amaya, *et al.*, 2015).**

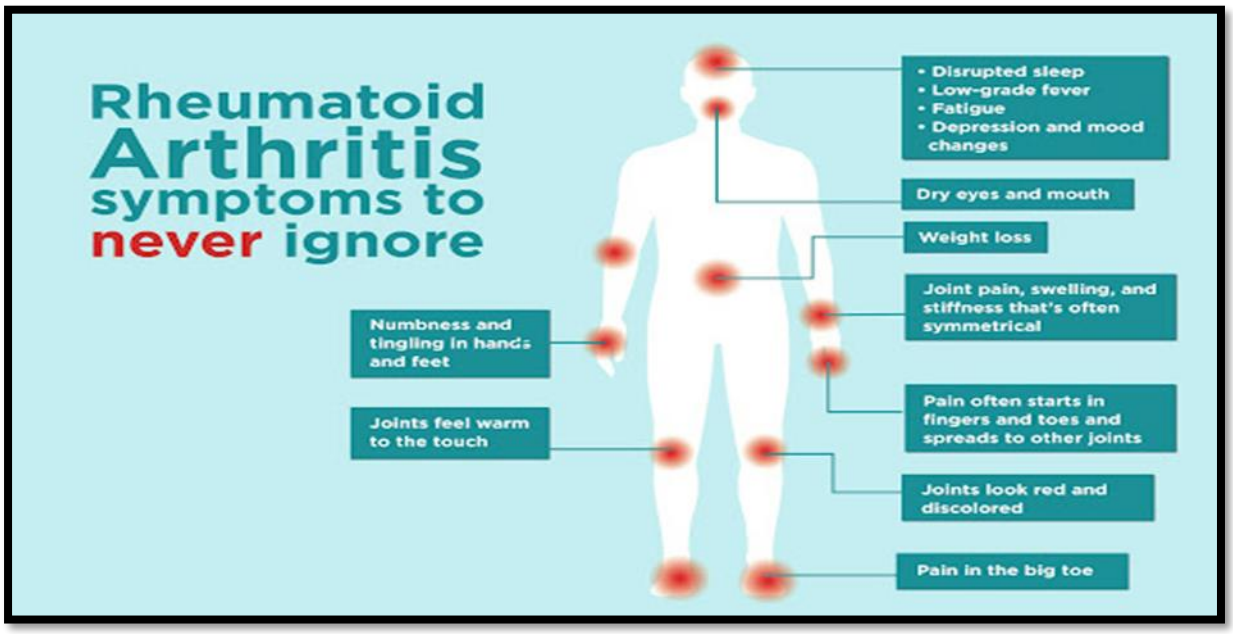


Fig. (1- 1): Rheumatoid arthritis symptoms to never ignore

1.1.1.6. Stages of Rheumatoid Arthritis

Early detection and treatment are crucial in RA, as it can lead to rapid tissue destruction and impairment of functioning. In 70% of patients with RA, joint erosion in the hands and feet is detectable by X-ray within the first two years of the disease (**Zhou, J., *et al.*, 2022**). The pathology of RA has been described in four basic stages:

1. **Stage I (early stage):** An unknown antigen reaches the synovial membrane and initiates a local immune response. Early-stage RA is marked by synovitis, causing joint swelling and pain. High cell count in synovial fluid indicates immune cell migration to the inflammation site. However, no x-ray evidence of joint destruction is found, except for soft tissue swelling and bone erosion **(Moutsopoulos, *et al.*, 2021)**.
2. **Stage II (Moderate stage):** There is a spread of inflammation in synovial tissue, affecting joint cavity space across joint cartilage. This inflammation will gradually destroy cartilage, accompanied by a narrowing of the joints chronic synovial inflammation ensues with numerous cellular infiltrates and cytokines **(Cardoneanu, *et al.*, 2022).**
3. **Stage III:** Severe RA stage III involves synovial pannus formation, joint cartilage loss, bone exposure beneath the cartilage, visible on x-rays, erosions around joint margins, and joint deformities, which may also be evident **(Al-Rubaye, *et al.*, 2017).** Pannus refers to synovial tissue proliferation and has been considered a late, inactive, and irreversible manifestation of RA, contrary to historical findings (**Cajas, *et al.* 2019**).
4. **Stage IV:** The final fourth stage of bone and cartilage destruction leading to irreversible joint damage and deformities the inflammatory process subsides, causing the formation of fibrous tissue and bone fusion, leading to ceased joint function and potential subcutaneous nodules **(Al-Rubaye, *et al.* 2017** ; **Hsieh, *et al.* , 2021).**

1.1.1.7. Diagnosis of Rheumatoid Arthritis

Clinically, RA patients typically present with a recent onset of tender and swollen joints, morning joint stiffness, generalized sickness symptoms, as well as abnormal laboratory tests **(Lin, *et al*., 2020).** Typically, RA is diagnosed by a combination of the patient's symptoms, results of the doctor´s examination, assessment of risk factors, family history, a joint assessment by ultrasound sonography, and assessment of laboratory markers such as elevated levels of CRP and ESR in serum and detection of RA-specific autoantibodies **(Burmester, *et al*., 2017).** RA disorder activity is also assessed by the Disease Activity Score 28. Laboratory assessment of acute inflammation, general health, swollen joints, and tender joints is used to calculate DAS28 scores, which range from 0 to 9.4 **(Greenmyer *et al*., 2020).**

1.1.1.7.1 Acute Phase Reactants of Rheumatoid Arthritis

Acute phase reactants (APRs) are proteins whose serum concentrations increase or diminish by at least 25% during inflammatory states. The effects of cytokines, such as IL6, IL-1 TNF-α, and IFN-ɣ, are primarily responsible for alterations in APR levels **(Almoallim, *et al., 2021*).**

1. Erythrocyte Sedimentation Rate (ESR): The ESR rate increases as a result of any cause or focus of inflammation. When an inflammatory process is present, fibrinogen enters the blood in high amounts and causes red cells to stick to each other, which raises the ESR **(Lapić, *et al*., 2020).** ESR is the elevation is typically 3 to 4 times above normal. ESR also can be very helpful in diagnosing and monitoring chronic pain patients **(Tennant, *et al.*, 2014).**

ESR is the most commonly used laboratory tests for detecting the acute phase response and thus diagnosis and monitoring of inflammatory conditions., Since elevation of ESR results from RBC rouleaux formation, for which fibrinogen, the main inductor, is known to be a slow-reacting positive

acute phase reactant, ESR's response to inflammation is rather slow and insensitive to minor inflammation **(Jishna, *et al.*, 2023).** ESR is widely used as a predictive biomarker in various chronic diseases **(Al-Hadlaq, *et al.*, 2022)**. ESR can be an independent prognostic factor for osteomyelitis recurrence in patients with T2DM **(Guo, *et al.*, 2020).** ESR plays a role in the diagnosis and management of RA **(Alturaiki, *et al.*, 2022).** Elevated ESR is thought to be a better predictor of these outcomes in early RA. ESR is a nonspecific acute phase reactant that may be elevated for a myriad of reasons **(Shapiro, *et al.*, 2021).** ESR is anon-specific laboratory indicator, which is affected by many factors and can only reflect short-term inflammatory status. It is still of great clinical significance to explore new indicators of RA disease activity **(He, *et al.*, 2020).**

2. C - reactive protein: The CRP is synthesized in the liver. Its physiologic role is to bind to phosphocholine expressed on the surface of dead or dying (apoptosis) cells to activate the complement/immune system, which enhances phagocytosis by macrophages **(Olson, *et al.*, 2023)**. Levels of CRP begin to rise within 2 hours of an insult, and have a half-life of about 18 hours **(Malin, *et al.*, 2022**). The rapid action of CRP makes it a participant in the acute or first phase of the inflammatory process, which is why it is often called an "acute-phase protein (**Parry, *et al.*, 2020**). Rapid, marked increases in CRP occur with a wide variety of disorders including infection, trauma, tissue necrosis, malignancies, and autoimmune disorders **(Mouliou, *et al*., 2023).**

CRP is produced in the acute phase of infection by the liver, and an increase in CRP serum levels is a known diagnostic marker for inflammation and infection. Furthermore, the association between platelets and other inflammatory markers, including CRP and IL-6, has been noted during the active phase of infection **(Sherkatolabbasieh, *et al.*, 2020).** CRP may be

superior in later stages of the disease given less susceptibility to other factors like immunoglobulin levels and anemia **(Emery, *et al.*, C 2015).**

The role of CRP in the inflammatory response has been confirmed, and its level is closely related to the health status of the human body **(Nehring, *et al.*, 2022)**. Low-grade inflammation characterized by elevated inflammatory protein levels, including CRP, is linked with T2DM pathogenesis **(Kanmani, *et al.*, 2019; Han, 2021).**

The production of CRP may be triggered by many metabolic and inflammatory factors associated with the development of T2DM, such as increased blood glucose, adipokines, and free fatty acid levels. In addition, an increased level of CRP represents a reliable predictor of vascular complications and progression of cardiovascular disease in diabetic patients **(Esser, *et al.*, 2015; Stanimirovic, 2022).** CRP is a key marker of systemic inflammation in RA **(Pope, *et al*., 2021).** CRP may play a direct role in bone destruction in RA.

Elevated CRP levels are associated with an increased risk for several common comorbidities; CRP is a valuable marker and regulator of systemic inflammation in RA that also appears to play a direct role in bone destruction and radiographic progression. CRP has also been implicated in the etiology of common comorbidities associated with RA. Reducing CRP levels with RA treatment may contribute to reductions in disease activity, although beneficial effects of RA treatment seem to occur irrespective of CRP values **(Pope, *et al*., 2021).**

1.1.1.7.2. Antibody of Rheumatoid Arthritis

A. Rheumatoid Factor:

Rheumatoid factor is the first well-known RA immunologic marker. It is observed in 80-85% of patients with RA. Elevated serum level of RF has been associated with increased disease activity, radiographic progression, and

the presence of extra-articular manifestations. The sensitivity of RF is 50-90%, and specificity is 50-95% **(Matuszewsk, *et al,* 2016).** IgM RF is the major RF species in RA and is found in 60-80% of patients with RA. RF has been observed in many other autoimmune diseases, such as mixed connective tissue disease, primary Sjögren syndrome, and systemic lupus erythematosus, as well as, in non-autoimmune conditions, such as chronic infections and old age **(Radziszewska, *et al.*, 2022).** It is important to note that not all RA cases contain the RF, yet those with RF positive tend to manifest with bone erosion and poorer prognosis. Moreover, RF is not specific to RA as it has been present in patients with other autoimmune diseases and an assortment of infectious diseases **(Sharma, *et al.*, 2024).** Negative RF does not rule out RA as a diagnosis, in some cases we can have seronegative RA. During the first year of the disease, RF is usually negative. However, RF determination is useful for the differential diagnosis of rheumatoid diseases as well as the prognostic factor because its high titer is associated with rapid joint destruction and extra-articular manifestations of rheumatoid nodes, polyneuropathies **(Ziegelasch, *et al*, 2020)**.

B. Anti-Cyclic Citrullinated Peptide (anti-CCP) Antibodies:

Anti-cyclic citrullinated peptide (CCP) antibodies are important serum markers used in the clinical diagnosis of rheumatoid arthritis (RA). However, it has been reported that ACCP antibodies can be positive in various other autoimmune conditions. Multiple studies have investigated previous generations of ACCP assays (CCP 1, CCP 2, CCP 3), and several have shown ACCP to be a highly specific and predictive marker in the diagnosis of RA **(Son, *et al.*, 2021**).

There are differences in reported sensitivity, specificity, and predictive values between the various generations of the ACCP test owing to the specific mixture of cyclic peptides in each. Based on the presence of anti-

CCP antibodies, RA can be classified into two subsets: anti-CCPA positive and ACCP negative RA, having distinct genetic and environmental risk factors and different pathogenesis. ACCP antibodies can be detected at an early stage of RA when symptoms are milder and incomplete or even absent, making these autoantibodies a useful serological marker for early diagnosis. Furthermore, the presence of anti-CCP antibodies can predict the development of joint erosions.

Hence, RA patients with a potentially severe course may be selected for early aggressive treatment, which may reduce structural joint damage **(Rahali, *et al.*, 2023**).

C. Antinuclear antibodies (ANA)

This test looks for the presence of Antinuclear antibodies (ANAs), which are autoantibodies that can attack healthy tissues and cells, indicating the presence of an autoimmune condition such as RA a defining feature of autoimmune connective tissue disease **(Nosal, *et al,* 2022).** ANAs bind to various molecular compounds with the cell's nucleus, including nucleic material and proteins. Antibodies may bind to double-stranded DNA (anti-dsDNA), and studies suggest that antibodies are formed during the incomplete removal of cellular material during apoptosis **(Pisetsky, *et al.*, 2020).** The significance of a positive ANA in people without autoimmune disease is not known, and it is unclear whether they have an altered risk of developing non-autoimmune diseases.

However, ANA + individuals exhibit a unique immunological landscape **(Slight-Webb, 2020).** Characterized by elevated levels of pro-inflammatory mediators and antibody production **(Slight-Webb, *et a.l*,2016) s**uggesting that even in the absence of autoimmune disease, a positive ANA

might alter immune regulation and affect risk of other conditions **(Zanussi, *et al.*,2023).**

D. Anti-Carbamylated Protein Antibody

Antibodies against carbamylated protein antigen (anti-CarP or ACP) antibodies were discovered in RA patients in 2011, subsequent research has demonstrated the predictive and prognostic value of this antibody system and plays a role in the pathogenesis of RA (**Mohamed *et al.*, 2019)** demonstrated the presence of anti-CarP antibodies is predictive for the progression to RA in arthralgia patients as well as increased joint destruction over time, particularly in ACPA negative RA patients **(Van Delft and Huizinga, 2020).**

1.1.1.7.3 Inflammatory Anti-Inflammatory Markers of RA

Cytokines are regulators of host responses to infection, immune responses, inflammation, and trauma. Some cytokines act to make thedisease worse (pro-inflammatory), whereas others serve to reduce inflammation and promote healing (anti-inflammatory).

1. Inflammatory of Rheumatoid Arthritis:

The pathogenesis of RA involves a complex network of various cytokines and cells that trigger synovial cell proliferation and cause damage to both cartilage and bone. Involvement of the cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-6 is central to the pathogenesis of RA, but recent research has revealed that other cytokines such as IL-7, IL-17, IL-21, IL-23, granulocyte macrophage colony-stimulating factor (GM-CSF), IL-1β, IL-18, IL-33, and IL-2 also play a role **(Kondo, *et al.*, 2021).**

A. Interleukin 23: Interleukin -23 is Inflammatory and a member of the IL-12 cytokine family composed of the IL-23 p19 subunit and the IL-12/23 p40 subunit. It is secreted by activated macrophages and dendritic cells

in peripheral tissues such as skin, intestinal mucosa, joints, and lungs **(Schinocca, *et al*., 2021).** IL-23 was associated with disease activity in RA **(Rasmussen, *et al.*, 2020).** IL-23 can induce chronic inflammation through two independent pathways.

The first pathway is *via* enhancing the secretion of IL-17 by non-T cells, and the second pathway is by the activation of Th17 cells. Once activated, Th17 cells produce IL-17, IL-6, IL-22, and TNFα and other factors that are associated with immune-mediated inflammation **(Khantakova, J. N., *et al.*, 2023).** IL-23 is not only a sensitive biomarker but also a key player in the pathogenesis of RA, making it a valuable target for diagnosis and potential therapeutic interventions in rheumatoid arthritis and other autoimmune diseases **(Gremese, *et al.*, 2023).** The direct role of IL-23 in T2DM is involved in inflammation and immune responses suggesting that it may contribute to the pathogenesis of T2DM by promoting inflammatory processes and oxidative stress **(Roohi, 2014).**

Recently, it has been reported that the proportion of receptors with Th17 cells is increased in the peripheral blood of treatment-naïve patients with early RA **(Paulissen, *et al.*, 2015).** In addition, higher frequencies of Th17 cells have been observed in the synovium of RA patients relative to -osteoarthritis (OA) patients **(Penatti, *et al*., 2017).**

2. Anti-inflammatory of Rheumatoid Arthritis

Anti-inflammatory cytokines might effectively inhibit arthritis, either by affecting innate immune cells or by interfering with the activation of B cells or T cells **(Chen, *et al.*, 2019).** Cytokines with anti-inflammatory properties have also been detected in the joints of patients with RA **(Gul, *et al*, 2023).**

A. Transforming growth factor-beta (TGF-β): Transforming growth factor-Beta (TGF-β) is anti-Inflammatory a member of a family of secreted

cytokines with vital biological functions in cells. The abnormal expression of TGF-β signaling is a common finding in pathological conditions. It is considered to be an important immune regulatory cytokine, which is predominantly expressed in the immune system **(Huang, 2022).** Increasing evidence has demonstrated the role of TGF-β signaling in the regulation of many physiological processes, such as wound healing, inflammation, apoptosis, differentiation, and embryogenesis. Furthermore, TGF-β signaling participates in pathological processes including, diabetes, and cancer. **(Kimawaha, *et al.*, 2020)**.

In RA synovial tissues, TGF-β is expressed at high levels and has been linked to synovial hyperplasia, inflammation, and angiogenesis. Inhibition of TGF-β signaling has shown promise in down-regulating rheumatoid synoviocytes and preventing arthritis induction, suggesting a potential therapeutic target for RA treatment (**Michitomo Sakuma, 2007**; **Su, *et al.*, 2024).**

Increased expression of TGF-β and its receptor I in rheumatoid synovial fibroblasts was found to correlate positively with clinical markers of disease activity, pointing to a correlation between TGF-β and inflammation **(Wajda, 2023).** Mechanisms, autophagy, and transforming growth factor–β (TGF-β) signaling pathways may play a causal role in the induction and progression of diabetes. The involvement of dysregulated autophagy and TGF-β signaling pathways in the pathogenesis of diabetes and arising complications including cardiomyopathy, retinopathy, and nephropathy, has been reported in several studies **(Heydarpour, *et al.* 2020).**

1.1.1.7.4. Receptor Activator of Nuclear Factor-Kappa B and Nuclear Factor of Activated T Cells of Osteoclasts in Rheumatoid Arthritis

Osteoclasts, derived from the monocyte/macrophage line of bone marrow hematopoietic stem cell progenitors, are the sole bone-resorbing cells of the body. Receptor Nuclear Factor Kappa-B Ligand (RANKL) and Nuclear Factor of Activated T Cells (NFATC) are critical regulators of osteoclast differentiation and function in RA, and targeting these molecules can be a promising therapeutic approach to mitigate bone destruction in the disease **(Zheng, *et al*., 2024).** Conventional osteoclast differentiation requires a macrophage colony-stimulating factor and receptor activator of RANKL signaling. RA is the most prevalent systemic autoimmune disease and inflammatory arthritis characterized by bone destruction **(Yokota, *et al.* 2024).**

RANKL is a key molecule for the differentiation and activation of osteoclasts **(Takeshita, *et al* 2021),** and is involved in the bone resorption of inflammatory joint diseases, such as RA. A recent study demonstrated that the serum concentrations of RANKL were increased before RA onset **(Messina, *et al*., 2023**). Antibodies against RANKL, a crucial stimulator of osteoclast differentiation, have shown great effectiveness both in laboratory settings and actual patient cases (**Yang, *et al.* 2024**). NFATc1 plays a pivotal role in osteoclast differentiation, and its regulation is crucial for maintaining bone homeostasis **(Omata, *et al*., 2023).**

The role of the Nuclear Factor of Activated T Cells (NFATc1) in both RA and T2DM is involved in the regulation of various cellular processes, including the activation of T cells and the expression of genes involved in the immune response **(Simon, *et al*., 2021).**

1.1.1.7.5. Oxidant and Antioxidant in Rheumatoid Arthritis

Oxidative stress is a pivotal player in the aggravation of chronic inflammatory joint disease. Both experimental models and assessments in patients showed, in addition to elevated Ractive Oxgen Species (ROS) and lipid peroxidation formation, a decreasein antioxidant defenses **(Smallwood, *et al,* 2018).** In this sense, antioxidant therapy may offer novel adjuvant/complementary treatment options aiming at better controlling disease activity **(Batooei, *et al*., 2018).**

1- Oxidant in Rheumatoid Arthritis

The role of oxidative stress is strongly mooted owing to the identification of an enhanced presence of pro-oxidants, pro-inflammatory cytokines (TNF-a, IL-6, IL-17, IL-23, IFN-c, IL-1b), and pro-inflammatory enzymes (NADPH oxidase, myeloperoxidase, xanthine oxidase) **(Ashour and Al-Mashhadani, 2024).** The involvement of oxidative stress in the pathogenesis of inflammatory diseases, such as RA, has been demonstrated in several studies **(Quiñonez, *et al*., 2016).** ROS and Reactive Nitrogen Species (RNS) are important categories of molecules generated in living systems for cellular metabolism**.**

A. Total oxidant species (TOS): Oxidative stress occurs in response to the oxidative damage caused when the body's anti-oxidative and scavenging activities cannot cope with the active oxidants produced by a harmful stimulant **(Chen, *et al.,* 2021).** Reactive oxygen species (ROS) make up the majority of active oxides, and account for more than 95% of total oxides., the total oxidant status (TOS) is usually used to estimate the overall oxidation state of the body **(Klisic, *et al.,*2020).** Patients with T2DM have higher TOS levels compared to healthy controls, indicating increased oxidative stress in the disease. TOS values are elevated in T2DM patients with complications

like neuropathy and retinopathy compared to those without complications **(Al-Kuraishy, 2020).**

TOS plays a significant role in RA by reflecting oxidative stress levels in patients indicating that TOS values are higher in patients with rheumatoid arthritis **(Bozdayi, *et al.,* 2023.** Many antioxidant and oxidant molecules reflect the oxidative stress state in the organism, but measuring their levels separately is both difficult and costly.

Therefore, practically TOS and TAS are measured, and the oxidative stress index (OSI), which reflects the degree of oxidative stress, is calculated by proportioning TOS to TAS **(Cakirca, *et al*, 2020).**

2- Antioxidants in Rheumatoid Arthritis

The increase in ROS and RNS production or the decrease in antioxidant mechanisms generates a condition called oxidative and nitrosative stress, respectively, defined as the imbalance between pro- and antioxidants in favor of the oxidants **(Taysi, *et al*. 2019).** Antioxidants in foods help to prevent oxidative reactions, but their health effect depends on their systemic bioavailability, concentration, and function **(Ali, 2020).** The relationship between serums TAS and the quality and composition of the diet has not yet been studied in RA patients. Recently, it has been demonstrated that diet quality may contribute to the course and activity of RA **(Valencia, 2022).**

A-Selenium: Elemental selenium (Se) is an essential element with a capacity to ameliorate various biological functions, including antioxidant defense, redox homeostasis, growth, reproduction, immunity, and thyroid hormone production **(Mojadadi, *et al*, 2021**). Previous studies have shown that physiological levels outside the recommended range of Se intake are harmful; low dietary Se is linked to thyroid diseases, diabetes, and metabolic disorders while excessive Se causes cytotoxicity **(Au, *et al.,***

2023). Therefore, tight regulation of optimal physiological Se levels is key for metabolic homeostasis and pharmacological safety. Dietary Se is generally obtained from seafood, organs, muscular meat cuts, grains, and seeds **(Mutonhodza, *et al*., 2022).**

Due to the antioxidant and anti-inflammatory effects of selenoproteins, it was at first expected that selenium would be beneficial for diabetes patients, given that T2DM is associated with oxidative stress. Indeed, Se (as selenate) has anti-diabetic, and insulin-mimetic effects, at high supra nutritional doses **(Steinbrenner, *et al.*, 2022).**

The importance of adequate consumption of trace elements selenium (Se), is supported by their effects on the activation of the immune system for the protection of infections caused by bacteria, viruses, and parasites. In addition, these nutrients are cofactors and structural components of important antioxidant enzymes that limit inflammatory activity **(Khanna, *et al*., 2017).** Selenium is another trace element in the human body and showed remarkable anti-inflammation and antioxidant potential in RA. Compared to normal controls, patients with RA presented with significantly lower serum selenium levels **(Ma, *et al.*, 2019).** Moreover, RA patients with higher serum selenium concentration seemed to have milder inflammation, indicated by lower levels of CRP and ESR **(Deyab, *et al.*, 2018).** Additionally, selenium also caused a reduction of ROS and alleviated oxidative stress **(Qamar, *et al*., 2021).**

B-Vitamin E (α-tocopherol)

Vitamin E has multiple important roles within the body and recent studies indicate that γ-tocopherol, δ-tocopherol، and γ-tocotrienol have unique antioxidant and anti-inflammatory properties which are superior to α-tocopherol in the prevention of chronic diseases. In addition, Vitamin E scavenges peroxyl radicals and inhibits pro-inflammatory signaling which

may be useful in the treatment of inflammation-related disorders **(Dasgupta, *et al,* 2024)**.

It has a high antioxidant capacity that can decrease the level of ROS and prevent oxidative damage that can cause cellular senescence and apoptosis. Vitamin E can decrease oxidative stress, inhibit the pathogenesis of the disease, and be used as a therapeutic option **(Handayani, *et al.*, 2024).** Vitamin E has antioxidant properties and may provide some symptomatic relief of pain and reduce the risk of developing RA through antioxidant effects (**Kou, *et al.*, 2023; Karlson, 2008).** Vitamin E may modulate the action of beta-cells by scavenging free radicals and thus delaying T2DM progression. Therefore, vitamin E has been cited as a potential risk factor for T2DM **(Salih, *et al.*, 2021; Aljabri, *et al.*, 2019).**

1.1.1.8. Rheumatoid Arthritis and Diabetes

Systemic inflammation associated with RA might contribute to the risk of developing diabetes in the future. Markers of active inflammation, such as CRP, are associated with an increased risk of diabetes and T2DM in people with RA **(Blum, *et al.*, 2019).** As a result of chronic pain, swelling, and stiffness of the joints, physical inactivity is common in people with RA, which in turn contributes to T2DM **(Chatterjee S, *et al*, 2017)**.

Therefore, the incidence of diabetes could be higher in people with RA **(C.K., *et al.*, 2018)**. We must that more intensive screening and tighter management of DM risk factors should be considered in people with RA **(Tian, *et al*., 2021).** Lifestyle factors such as smoking, alcohol consumption, and high BMI (or obesity) were found to be mainly responsible for developing diabetes among RA patients **(Verma, 2021).** RA is associated with chronic inflammation, which might, at least in part, trigger the development of T2DM **(Baghdadi, 2020).** T2DM It is associated with

significant morbidity and hampered quality of life. Hyperglycemia-induced aberrant levels of insulin or insulin growth factors may lead to neuropathic complications, which enhance pain through central sensitization **(Gupta, *et al,* 2022).**Is also associated with inflammation caused due to pro-inflammatory cytokines like tumor necrosis factor α and interleukin 6. Inflammation encourages insulin resistance and also stimulates other factors like a high level of rheumatoid factor in the blood leading to positivity of rheumatoid factor in RA patients **(Verma, *et al.,* 2021).**

1.1.1.8.1. Role of Sugar in the Rheumatic Diseases

In recent years, as more researchers have explored the relationship between a high-sugar diet and inflammation, they have found that excessive sugar intake is closely associated with the development of low-grade chronic inflammation and autoimmune diseases **(Bodur, *et al.* 2019**). Low-grade chronic inflammation has long been linked to obesity and increased body fat, and excess dietary sugar intake is a key contributor to obesity and weight gain.Has been revealed that dietary sugar is a key factor in inducing low-grade chronic inflammation, autoimmune diseases, and even neuron inflammation **(Ma, *et al.,* 2022).**

1.1.1.8.2. Role of Insulin in the Rheumatic Diseases

Insulin is the main hormone regulating glucose homeostasis and it acts through the transmembrane insulin receptors. The latter is expressed on multiple target cells including hepatocytes, adipocytes, synoviocytes، and muscle cells. Intriguingly, these receptors may also be found on the surface membrane of immune cells **(Haeusler, 2018).** These cells need glucose to produce energy, and through its receptors, insulin exerts its hypoglycaemic function and behaves as a growth-like factor as well as a cytokine regulator **(Hu, et *al.* 2023).**

Therefore, this hormone may also exert immunomodulatory effects on the immune system in addition to well-known metabolic effects **(van Niekerk, *et al.*, 2020).** Insulin signaling, similarly to what is observed in T2DM or metabolic syndrome, may be involved in the dysregulation of immune response in inflammatory diseases. Epidemiological and laboratory studies reported a possible correlation between insulin resistance and RA **(Sánchez-Pérez, *et al*., 2017).** Insulin may decrease levels of CRP reduce the ability of neutrophils to generate ROS and suppress the transcription of different Toll-like receptors (TLRs) on circulating mononuclear cells **(Tripolino, *et al,* 2021).** Insulin induces the upregulation of RANK contributing to the enhancement of osteoclast differentiation by RANKL **(Vachliotis, *et al.*, 2023).**

1.1.1.8.3. Role of insulin resistance in the Rheumatic Diseases

Insulin sensitivity occurs due to biological effects in the insulin-responsive tissue mainly adipose, liver, and striated muscle tissue. Decreased insulin sensitivity is also called insulin resistance (IR) and it is usually classified as decreased suppression of hepatic glucose production, reduced lipolysis rate among adipose or fat tissue, and impaired clearance of glucose in striated muscle **(Roden, 2016).** Insulin resistance in RA partially causes obesity by increasing fat mass, disease activities, and occurrence of RF. Insulin resistance can also be linked significantly with some inflammation markers such as CRP and TNF, insulin resistance is more serious and critical in people experiencing high disease activity than in people experiencing medium disease activity. Insulin is associated with all inflammatory cytokines **(Verma, 2021).**

1.1.1.8.4. Role Glycated Hemoglobin in RA

HbA1c is released into the bloodstream. Apart from indicating long-term blood glucose levels, free HbA1c can increase CRP, oxidative stress, and blood viscosity. These processes collectively contribute to damage to the endothelial cells lining the blood vessels **(Chen, *et al.*, 2023).**

1.2. Metformin

Metformin (MET) is traditionally used for thetreatment of type II diabetes mellitus. MET, is a cationic drug with a daily dose as high as several grams, which demonstrates well-established efficacy and little toxicity **(Wang, *et al.*, 2017; Shurrab, *et al.*, 2020).** Metformin is the most commonly prescribed pharmacotherapy for T2DM. Metformin improves glycemic control while also causing modest weight loss that is clinically relevant **(Kincaid, *et al*, 2024).** Metformin's actions are still under intense scrutiny, with the drug being reported to affect a wide range of biochemical, cellular, and endocrine processes **(Bridges, *et al.*, 2023).** Metformin is abiguanide drug that is widely prescribed as an oral anti-hyperglycemic agent worldwide, and it is recommended as the first-line treatment for T2DM according to recent joint European-American clinical guidelines **(Buse, *et al.*, 2020**).

Preclinical studies have shown that metformin has anti-arthritis and anti-inflammatory effects through several mechanisms including suppression of osteoclasts gene expression, down-regulation of IL-17-producing Th17 cells, up-regulation of Treg cells and lowering the production of pro-inflammatory cytokines **(Gharib, 2021).**Possible use of metformin as adjuvant therapy in RA patients with moderate to high disease activity and its effect on serum adiponectin **(Foretz, *et al.*, 2019).** Treatment is based only on relieving the symptoms and improving the quality of patients' lives.

Metformin, which is used in the treatment of T2DM, has properties that are desirable for autoimmune disease therapy, including anti-inflammatory and antioxidant effects, and the ability to regenerate the endothelium **(Tomczynska, *et al.*, 2016)**.

1.2.1. Chemical Structure of Metformin

Metformin is a white crystalline compound with a molecular formula of $C_4H_{11}N_5$•HCl and a molecular weight of 165.65 g/mol (**Ugwueze, 2020**). Metformin hydrochloride is soluble in water but insoluble in acetone and ether. Belongs to the biguanide class of drugs its molecular formula is $C_4H_{11}N_5$, and its systematic name is a1,1-dimethyl biguanide. Understanding the structure of metformin is crucial for comprehending its pharmacological actions and therapeutic effects (**Rajput, *et al.*, 2023**). Metformin's chemical structure consists of a biguanide moiety, which is characterized by two guanidine groups (-$C(NH_2)_2$) connected by a central carbon atom. The two methyl groups (-CH_3) attached to this central carbon contribute to the molecule's overall structure. This structural arrangement imparts specific properties to metformin, allowing it to interact with various biological targets within the body **(Kupesiz, *et al.*, 2012).**

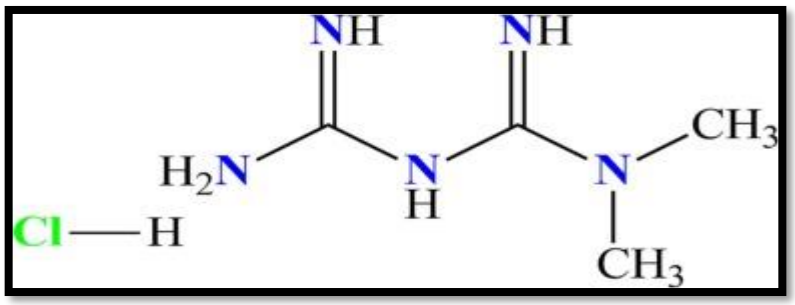


Fig. (1-2): Structure of metformin hydrochloride (Bailey Metformin, 2017)

Metformin has been found to have therapeutic benefits in RA, particularly in patients with T2DM. The anti-inflammatory effects of metformin in RA may be influenced by NFATC1, which is involved in the

regulation of fibroblast like synoviocytes (FLS) proliferation and migration **(Tong, *et al,* 2023)**. Metformin was protective against bone erosion in RA animal models *via* inhibition of osteoclast differentiation and activity.

1.3. Nanotechnology and Nanomedicine

The word "nanometer" was first used in 1914, and in 1959, American physicist and Nobel Prize winner Richard Feynman created the particular notion of "nanotechnology. “There’s Plenty of Room at the Bottom" was the title of the lecture he gave. In 1974, Norio Taniguchi may have been the first to use the phrase "nanotechnology." Nanotechnology, according to Norio Taniguchi, primarily entails the processing, separation, consolidation, and deformation of materials by a single atom or molecule **(Baig, *et al.*, 2021).** Nanotechnology is defined as the understanding and control of matter at dimensions between 1 and 100 nm where unique phenomena enable novel applications **(Litter, *et al.*, 2023).** NPs are small particles with diameters of 1–1000 nm. Their unique physical and chemical characteristics encourage their use to satisfy the demands of a wide range of applications. For instance, they open up opportunities to meet unmet medical needs and present new directions in healthcare diagnostics and therapies (**Gajanan, *et al.*, 2018).**

Recently, polymers have become very popular because of their special qualities, such as strong biocompatibility and simplicity in design and preparation. When developing nanomedicines, it is important to take into account a variety of biological hurdles, such as prolonged circulation, effective accumulation at the target site, efficient penetration into target tissues, and selective uptake of nanoparticles into target cells **(Li., *et al.*, 2022).**

1.3.1. Medical Applications of Nanotechnology

Nanotechnology is significant in nanomedicine; it allows for the use of highly hazardous medications with increased safety. It eliminates the problem of focused therapeutic administration, lowers the risk of side effects, and boosts therapeutic effectiveness. The most exciting application of nanotechnology is in nanomedicine. Its applications span a wide range of industries, including the production of vaccines, medication delivery, imaging and diagnostic tools, and antimicrobial products, as well as the potential creation of specialized drug administration mechanisms, advanced diagnostic techniques, and nonmedical devices **(Haleem. *et al.*, 2023).**

1.3.2. Drug delivery systems

Drug delivery systems based on nanotechnology initially consist of adsorbed polymer matrices and nanoparticles containing one or more therapeutic medicines that can bind or disperse. Significant advancements in Nano-drug manufacture, therapies, and diagnostics have been made in recent years. They are less effective due to many hurdles like their enzymatic degradation or disparity in pH, many mucosal barriers, and off-the-mark effects, and their immediate release enhanced toxicity in blood **(Alshammari, Alshehri, *et al.*, 2023)**.

The advancement of nanomedicine by drug encapsulation is very promising. Modern encapsulation techniques also provide various benefits over traditional medicinal procedures, including the capacity to target and enter into specific tissue at the molecular level and the provision of a wide surface area, due to the medications' or tiny molecules' encapsulation, dose requirements are reduced, and the toxicity of the medication is reduced The science of nanotechnology including the controlling of atoms and molecules

for creating new materials with a different of useful applications in various fields**(Nattah and Mohaisen, 2022).**

1.3.3. Niosomes

Niosomes are vesicles formed by the self-assembly of non-ionic amphiphilic surfactants. Cholesterol, and occasionally, charged molecules, are added to provide rigidity to the bilayers and to enhance the stability of the system. Niosomes share structure similarities with liposomes, and they introduced as an alternative to overcome limitations associated with stability, sterilization, and large-scale production of liposomes **(Thabet, *et al*., 2022)**. The structure of niosomes is shown in figure 1-3 **(Mishra, *et al*., 2020)**.

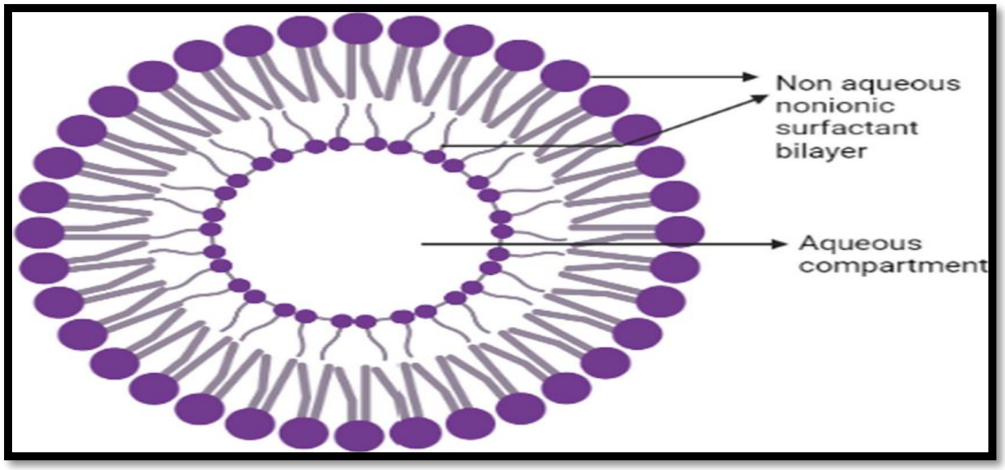


Fig (1-3): structure of niosomes.

1.3.4. Hyaluronic acid

Hyaluronic acid is a polysaccharide with characteristics that play a pivotal role in various fields such as medicine, cosmetics, and materials science for biomedical and cosmetic uses. With its water retention, capacity HA is essential for skin hydration and wound healing **(Papakonstantinou, *et al*., 2012; Li, *et al*., 2022)**.

1.3.5. Nano Formation of Metformin

Nanotechnology-assisted transdermal drug delivery systems offer several beneficial features like enhanced skin permeation, improved bioavailability, controlled release, and targeted drug delivery (**Shariq, *et al*., 2023).** Diabetes requires a long duration of action which can be done by designing a suitable Nano carrier-based formulation. The different pharmaceutical and preclinical characteristics of various Nano formulation-based transdermal drug delivery nanomedicine are devoted to monitoring, repairing, and controlling biological systems via the use of nanoparticles (NPs). As carriers for drugs, nucleic acids, and proteins, NPs have broad applications in disease prevention, diagnosis, and treatment **(Wan, *et al*., 2010).** They are either inorganic or organic. Inorganic NPs contain metals, such as gold, silica, and silver, which allows them to be traced using imaging systems. Organic NPs include liposomes **(Li, *et al*, 2019),** polymeric micelles **(Yokoyama 2014**), dendrimers, fullerenes, and carbon nanotubes.

In recent years, there has been a growing interest among researchers in the field of drug delivery systems regarding the utilization of noisome as a means of targeted drug delivery **(Moghtaderi, 2022**). Noisomes, resembling liposomes in structure are constructed using non-ionic surfactants and can transport drugs that are soluble in water as well as drugs that have low solubility in water **(Zaid Alkilani, 2023**).Nevertheless, noisome exhibit greater stability in both formulation and storage when compared to liposomes **(Marchianò, V., *et al*., 2023).**

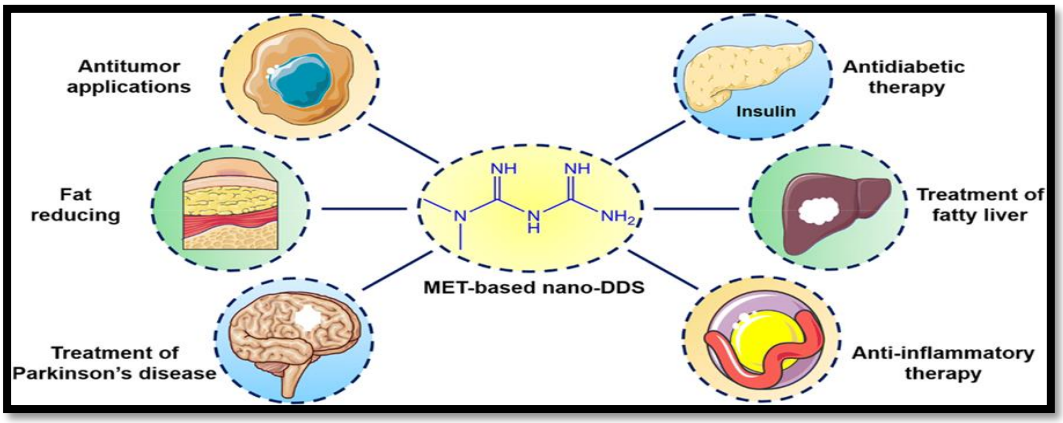


Fig. (1-4*)*: From Emerging Nanoparticulate drug delivery systems of metformin (Chen, *et al.*, 2020).

1.4. Genes

A gene is the basic physical and functional heredity unit passed from parents to offspring. Genes comprise DNA and contain the information needed to specify traits and produce proteins (**Cary *et al.*, 2020**). The exact definition of a gene has evolved: Initially, the term "gene" was coined to denote an abstract "unit of inheritance" without specific material attributes (**Gerstein *et al.*, 2007)**.

In the 1960s, a gene was defined as a continuous segment of DNA sequence specifying a polypeptide chain. However, the discovery of complex patterns of dispersed regulation, pervasive transcription, and non-coding RNAs challenged this notion (**Gerstein *et al.*, 2007**; **Portin *et al.*, 2017**). A more modern definition is: "A gene is a union of genomic sequences encoding a coherent set of potentially overlapping functional products" This definition categorizes genes by their functional products (proteins or RNA) rather than specific DNA loci, with regulatory elements classified as gene-associated regions **(Kuhn and Harold, 2024).** Humans have approximately 19,900 protein-coding genes. Most genes are the same in all people, but a small number (less than 1%) have slight differences called alleles that contribute to each person's unique traits. In summary, a gene is a DNA sequence that provides instructions for making proteins or functional RNA

Molecules and is the basic unit of heredity passed down from parents to offspring **(Gerstein *et al*., 2007).**

1.4.1. Gene Expression

Gene expression refers to the process by which information from a gene is used to synthesize a functional gene product, typically proteins or functional RNA molecules **(Shao *et al*., 2019).** It's crucial in various biological processes like development and response to stimuli (**Oliva *et al*., 2020)**. Factors like copy number variations, gender, and regulatory variants can affect gene expression (**Liu *et al*., 2020**). Gene co-expression networks help understands gene functionality and regulatory pathways. Understanding gene expression dynamics is essential for understanding biological processes and illnesses (Hajieghrari**, et *al*., 2022**).

1.4.2. Receptor Activator of Nuclear Factor-Kappa B (RANKL) and Nuclear Factor of Activated T cell (NFATC1) Gene of Osteoclastgenesis in Rheumatoid Arthritis.

The expression of various genes plays an important role in RA. The NFATC1 gene, encoding the nuclear factor of activated T-cells, plays a crucial role in various biological processes, particularly in bone homeostasis and cancer. It is essential for osteoclast differentiation (**Canalis, *et al*, 2022**). Studies have shown that NFATC1 expression is increased in synovial osteoclast precursors of RA patients, indicating a potential link to the enhanced osteoclast differentiation observed in RA **(Yokota, K. 2024).**

RANKL, which stands for receptor activator of NF-κB ligand, is a crucial molecule involved in various physiological processes. It is known to play a significant role in bone homeostasis and the formation of lymphoid tissues **(Boyce, et al., 2023).** This molecule exists in two forms: membrane-bound RANKL (mRANKL) and soluble RANKL (sRANKL) (**Elango, et *al*.,**

2021). It also plays a crucial role in bone erosion in RA by promoting osteoclast formation, function, and survival **(Feng, 2016)**. Studies have shown that RANKL is highly expressed in synovial fluid B cells of RA patients and is a key cytokine involved in bone destruction **(Fang, *et al.*, 2020).**

Osteoclasts are the major cells responsible for bone destruction in RA. Osteoclast differentiation is stimulated by RANKL, which is produced by activated T cells and Fibroblast-like synoviocytes (FLSs) (**Kim HR, *et al.*, 2019**). Local pro-inflammatory cytokines can also mediate osteoclastogenesis by inducing RANKL or directly stimulating osteoclast precursors (**AlQranei, *et al.*, 2020**). Preclinical studies have found that metformin treatment inhibited osteoclast differentiation and bone-resorbing activity, thereby reducing bone erosions in the joints (**Nandakumar, *et al.*, 2023**). Due to the high energy demands of osteoclast generation and maturation, glycolysis and glutaminolysis are required for osteoclast differentiation and bone resorption (**Peng, *et al*, 2024**).

Aims of the Study

The study aims to explore the relationship between rheumatoid arthritis (RA) and type 2 diabetes mellitus (T2DM) by focusing on several key objectives:

1. To determine the inflammatory and anti-inflammatory biomarkers that can facilitate the early detection of RA and assess the prognosis to prevent its progression to T2DM.
2. To analyze the association and correlation between various biomarkers related to RA and T2DM, highlighting their interrelationships and implications for disease management.
3. To investigate the effectiveness of metformin, a medication commonly used for T2DM, when delivered *via* a hyaluronic acid-coated niosomal nanoparticle system for its potential dual application in treating RA and T2DM.
4. To assess various molecular factors, including total oxidant status (TOS), interleukin-23 (IL-23), nuclear factor of activated T-cells cytoplasmic 1 (NFATC1), and receptor activator of nuclear factor kappa B ligand (RANKL) in both treated and untreated peripheral blood mononuclear cells (PBMCs) from RA patients.
5. To evaluate the therapeutic impact of the synthesized hyaluronic acid-coated niosomal nanoparticles (Hyalo-Nio-met NPs) in reducing inflammation and enhancing antioxidant defenses in RA patients, focusing on their role in delivering metformin effectively.
6. To explore how systemic inflammation associated with RA may contribute to the risk of developing T2DM, emphasizing the need for monitoring and preventative strategies.

7. To investigate the levels of antioxidants such as vitamin E and selenium in RA patients, comparing them to healthy individuals and those with T2DM, to determine nutritional impacts on disease progression.

Chapter Two

Supjuct

Materials and Methods

2. Materials and Methods

2.1. Materials

2.1.1. Study Design

A case-control study was conducted in the Department of Chemistry and Biochemistry, College of Medicine, University of Kerbala. The subjects included were classified as shown in figure 2-1 performed on 118 subjects obtained during October, 2023 to March, 2024.

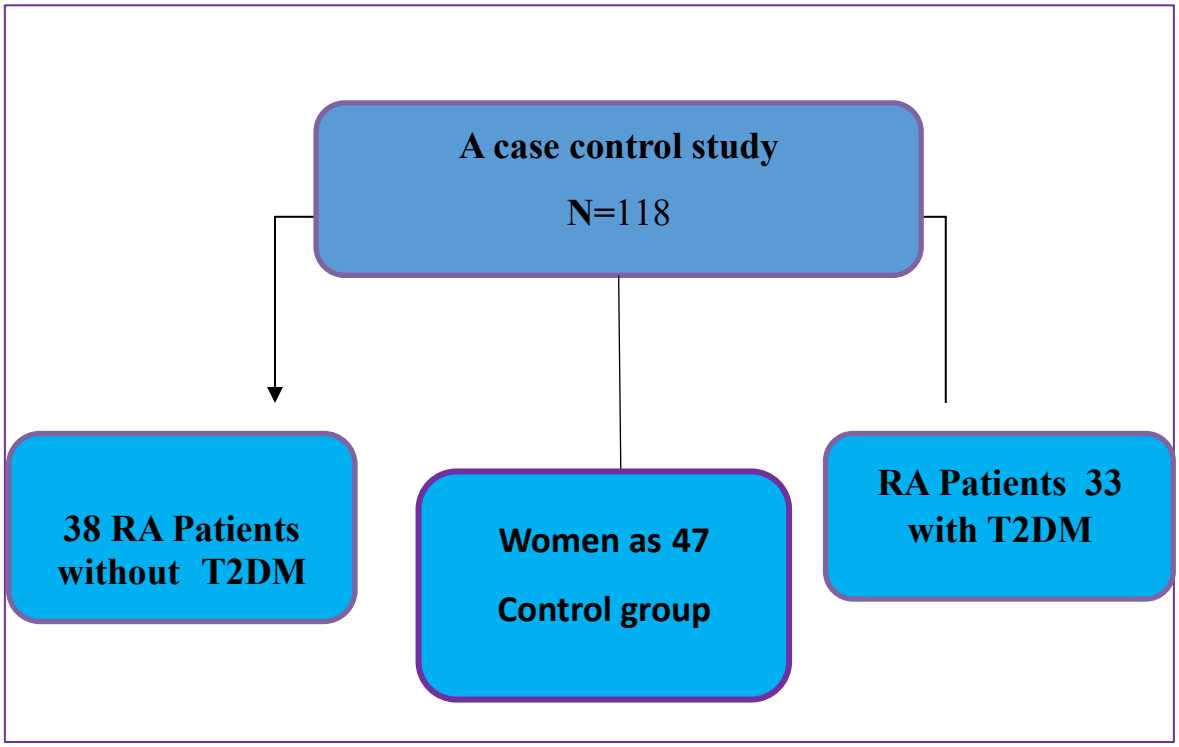


Fig. (2-1) Chart of study design

2.1.2. Patients

All patients who had RA with and without type two diabetes mellitus, and had taken basic information about age, duration of disease, family history, body mass index (BMI) **appendix (I)**.Blood samples taken from 118 women from outpatient clinics attending a joint consultation clinic at Al-Hassan Teaching Hospital, Kerbala Health Directorate / Kerbala – Iraq with

matched ages ranging between (30-70) years. They divided into three groups divided into as patients of RA with T2DM and RA patients without T2DM and subjects as healthy control as follows: (38 patients had only RA; 33 patients had RA with T2DM and the remaining 47 subjects as apparently healthy control). All these patients subjected to full history taking through clinical examination, and laboratory investigations. The diagnosis of arthritis was made based on the European League Against Rheumatism (EULAR) 2010 classification criteria. (**Appendices (II)**)

2.1.3. Blood samples collection

Six milliliters of fasting venous blood are obtained from each participant through the utilization of a sterile disposable syringe for subsequent processing and they are divided into two distinct portions:

A. The first portion, comprising 3ml of blood transferred into gel tubes, followed by an incubation period of 10-15 minutes at ambient temperature. Subsequently, centrifugation was carried out for 10-15 minutes at a speed of 3000 x g to facilitate serum separation. The resulting serum was fractionated into 6 gel tubes, and stored at a temperature of -20 ^{C} until the time of analysis for the determinations of various biomarkers.

B. The remaining fraction of the sample was divided into 2 mL of whole blood in an EDTA tube and stored at -20 °C until they wereused for nanotechnology and molecular analysis. The other 1 mL was preserved in a heparin tube for subsequent Erythrocyte Sedimentation Rate (ESR) analysis and glycated hemoglobin (HbA1c)**.**

2.1.4. Inclusion and Exclusion Criteria

Inclusion Criteria: The samples were taken from the rheumatological consulting unit after being diagnosed with clinical Phusion.

- patient with rheumatoid arthritis whose RF was positive
- patient rheumatoid arthritis with and without T2DM

Exclusion criteria: women with malignant diseases, pregnant, alcoholics, or afflicted with chronic diseases, kidney disease, and liver disease.

2.1.5. Approval of the Ethical Committee

The ethical approvals were obtained from the ethical committee team, the College of Medicine, the University of Kerbela, and the Kerbela Health Directorates / Kerbala – Iraq, see **(Appendices (III A, B & C)**).

2.1.6. Chemicals and Kits: The kits used in the study are listed in Table 2.1

Table (2.1): Chemicals and kits used in the study and their supplies

No.	Chemicals and Kits	Company and country
1	C-reactive protein (CRP) kit	Hipro Biotechnology/China
2	Erythrocyte sedimentation rate (ESR)	AFCCO/Jordan
3	Fasting blood sugar kit	Monarch/British
4	Hemoglobin A1C	Lifotronic H8 /china
5	Insulin kit	Snibe/Germany
6	Interlokine 23 kit	Karmania Pars Gene /Iran
7	Transforming growth factor-bet(TGFβ) kit	Karmania Pars Gene/ Iran
8	Vitamin E (α-tocopherol) kit	Dragon /China

2.1.7. Instruments, Lab. Equipment, Tools used in This Study Listed in Table 2-2

Table (2.2): Instruments and Lab Equipment that used in this study

No.	Instrument	Company supplied
1	Flame Atomic Absorption Spectrometry	SHIMADZU AA6300/Japan
2	Abbott i1000 immunoassay	Architect /USA
3	ELISA reader	ELX800-U.S.A
4	Dynamic light scattering system	UK /ZS 90, Malvern Instruments Ltd Malver
5	Scanning electron microscopy SEM	USA/PerkinElmer, Fremont, CA
6	FT- IR spectrographotometer	Japan/ Shimadzu 8400 S Kyoto
7	Ultraviolet spectrophotometry	USA/PerkinElmer, Fremont, CA
8	Atomic force microscopy AFM	Germany/ Nanowizard II; JPK
9	MTT	Sigma/Germany
10	Deep freezer	COOLTECH/ USA
11	Centrifuge	Kokusan/ Germany
12	Incubator	UKA /Germany
13	Vortex Mixer	Clay Adams/Germany
14	Water bath	Memmert/ Germany
15	Shaker	Taiwan/ Taiwan
16	Micropipettes	Canada/ Bioasic
17	ESR tube class(Westergren)fast detector	Mheco/ China
18	EDTA tube	Mheco/ China
19	Gel tube	Mheco/ China

20	Pipettes	DARWELL/ China
21	Eppendorf tubes	Mheco /China
22	Backer 500 ml	Mheco/ China
23	Cylinder, flask	MEDI/China
24	Syringe (10ml)	Mheco/ China
25	Gloves	DIRUI /china
26	Filter Paper	Slamed/Germany
27	Auto – chemistry analyzer (CS-480)	Geno Lab TEK/USA
28	Lifrotronic H8	MEDI/China

2.2. Methods

2.2.1. Calculation of Body Mass Index

The body mass index (BMI) was calculated in all subjects according to a ratio depending on weight and height obtained by applying a mathematical equation, in which the weight in kilogram was divided by the square height in meters, and the results were considered as the following (**Chang, *et al.*, 2020**).

BMI (kg/m^2) = weight (kg) / height^2 (m^2) the ranges of (BMI) categorized into groups.

Table 2.3: Classification of BMI ranges into groups

Weight status	BMI (kg/m^2)
Underweight	≤ 18.5
Normal weight	18.5 - 24.9
Overweight	25-29.9
Obese	≥ 30

2.2.2. Determination of Erythrocyte Sedimentation Rate

2.2.2.1. Principle of assay

The Erythrocyte Sedimentation Rate (ESR) is a nonspecific assay used to screen for the presence or absence of active disease. The settling of red corpuscles (red blood cells - RBCs) is due to the differential densities of the RBCs and their medium.

2.2.2.2. Procedure of Test

1. The tube was placed upright on the ESR detector. The tubular blue graduation line is consistent with the top of the shelf

2. Waited for 30 minutes, and the concave of the plasma was aligned in the ESR tube with the zero scales of the ESR detector,

3. The data was read by aligning the upper surface of the red blood cells with the scale on the ESR detector.

4. Normal Value: Male: ≤15 mm/hr; Female: ≤ 20 mm/hr; Child: ≤10 mm/hr.

2.2.3. Determination of serum C - reactive protein concentration

2.2.3.1. Principle of CRP Test

Adopted the latex particle that is sensitized by anti-human C - reactive protein antibody.The latex particles react with the C-reactive protein of the sample in the liquid phase and form an insoluble antigen-antibody complex and certain turbidity immediately. The level of turbidity reflects the C-reactive protein level of the sample, and then compared with the same treatment calibrator; after that the C-reactive protein content in the sample was determined.

2.2.3.2. Reagents

Table (2-4) indicates the component of CRP kit.

Reagent-1	R1	Tris buffer	20mmol/L
Reagent-2	R2	Anti-Human CRP AB	Appropriate

2.2.3.3. CRP Assay Procedure

1. Reagent 1 and reagent 2 is a ready-to-use liquid reagent. Gently shake reagent 2 to avoid bubbling.

2. Add 320 μL of R1 to 2 μL of the sample, and then wait for 300 seconds.

3. Then added 80 μL of R2 to the above mixture and left it for 24 seconds, then measured the first absorption at a wavelength of 546 nm, then waited 276 seconds, and measured the second absorption at 546 nm.

2.2.3.4. Calculation:

The calibration curve was formed automatically. Quality control products produced by Dirui are used. According to the calibration concentration and absorbance change of AA, the titration curve was confirmed using the nonlinear function. The concentration value that corresponds to the absorbance change of the tested sample on the titration curve is the test value.

The normal value of CRP Titer = (0-6) mg/L

2.2.4. Determination of Interlaken-23

2.2.4.1. Principle

- Utilized the Sandwich-ELISA principle. The micro-ELISA plate included in this kit has been pre-coated with an antibody specific to IL-23 Samples (or Standards) was added to the micro-ELISA plate wells and mixed with the specific antibody.
- IL-23-specific detection antibodies and avidin Horseradish Peroxidase (HRP) conjugate were added to each microplate well and incubated. Free components washed away. Substrate solution was added to each well. Only wells containing IL-23, detection antibodies, and avidin-HRP conjugate will appear in blue. The reaction of the enzyme with the substrate was stopped by adding a stop solution, causing the color to change to yellow. Optical density (OD) was measured at a wavelength of 450 nm ± 2 nm. The OD value is directly proportional to the IL-23 concentration. The concentration of IL-23 in the samples was determined by comparing the OD of the samples to the standard curve

2.2.4.2. Preparation solution for IL-23

1-Washing buffer: The solution was diluted with distilled water 10 times

2-HRP – Avidin

- The HRP vessel was rotated using a microfuge.
- Added 500 µl from the HRP-Avidin vial to the HRP vial
- overtaxing
- All content added to HRP-Avidin vail
- Added 25 µL of diluted HRP vial to the HRP-Avidin vial
- Shook by hand for 3 minutes until well mixed

Table (2-5): Kit component of IL-23

Item	Catalogue	Volume
Human anti- IL23 coated plate	KPG –IL23P	96 vials
Standards	KPG –IL23SN1- 4	200 µL
HRP-Avidin buffer	KPG- HA	5.5 mL
HRP	HAA	22µL
Substrate	KPG –SU	5.5 mL
Dilution HRP	KPG –DH	100 µL
Stopping	KPG –ST	3.5 mL
10Xwashing bu er	KPG –WB	40 mL
Detection Ab	KPG –IL23D	5.5 mL

Table (2-6): Standard to IL-23

Standard	Pg/mL
Standard 4	200 pg/mL
Standard 3	100 pg/mL
Standard 2	50 pg/mL
Standard 1	5 pg/mL
Blank	0 pg/mL

2.2.4.3. Procedure

1. Taked the dish out of the desired packaging and brought it to room temperature in a dry environment. Added 50 µL of standards 4 to 1 to the first to fourth wells and considered the fifth well to be empty and performed all steps except steps 4 and 6 for the blank.

2. Added 50 µl of the required sample to the rest of the wells and incubated for 60 minutes on a shaker at 200 rpm at room temperature.

3. After proper incubation, the plates were washed 3 times using the wash solution (after adding the wash solution, the plates incubated for approximately 1 minute at room temperature and then filtered) in the HRP vial.

4. Added 50 µl of conjugated antibody (Detection Ab) to all wells (except the blank) and incubated for 50 minutes on a shaker at 200 rpm at room temperature.

5. After proper incubation, the dishes were washed 3 times using a washing solution**.**

6. Added 50 mL of HRP-Avidin solution to all wells (except the blank) and incubated for 30 minutes on a shaker (at least at 200 rpm).

7. After proper incubation, the plates were washed 5 times using washing solution.

8. Added 50 Ml of substrate to all wells. Note that 15 minutes is sufficient for incubation, but if the amount of color produced is high, the time can be reduced to minutes**.**

9. Added 25 µL of stop solution to all wells and the absorbance of the samples were measured in an ELISA reader at a wavelength of 450 nm

2.2.4.4. Calculating the results

The concentration of parameters was placed to the abscissa and the OD value to the abscissa. The standard curve was plotted on the coordinate sheet. According to the OD value of the sample, its corresponding concentration (which is the sample concentration) is located; or calculate the linear

regression equation of the standard curve according to the standard concentration and OD value. The OD value of the sample was then substituted to calculate its concentration.

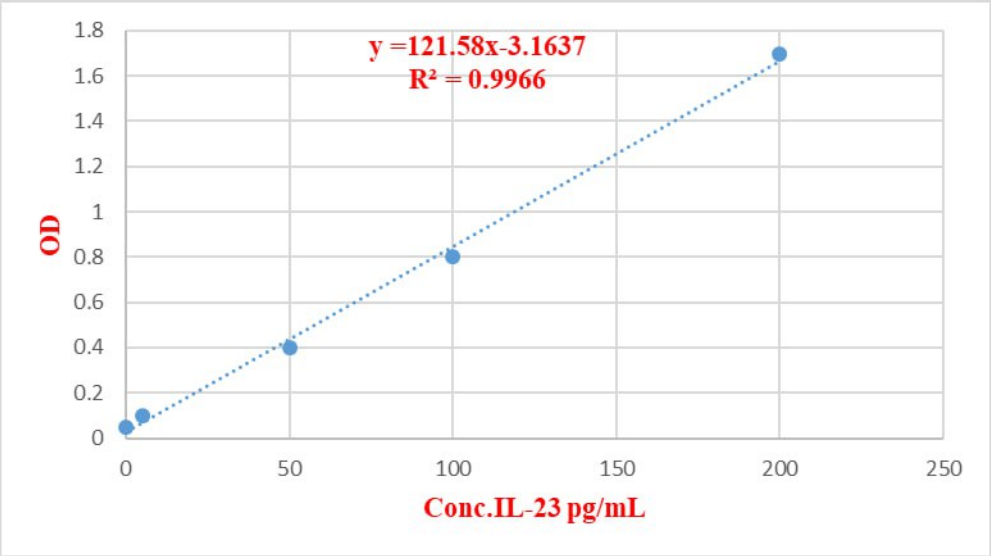


Fig. (2.2): Standard curve for IL-23 determination

2.2.5. Dermentation of Transforming Growth Factor-Beta (TGF-β)

2.2.5.1. Principle

➢ Utilized the Sandwich-ELISA principle. The micro-ELISA plate included in this kit has been pre-coated with an antibody specific to TGF-B Samples (or Standards) was added to the micro-ELISA plate wells and mixed with the specific antibody.

➢ TGF-B-specific detection antibody and Avidin-Horseradish Peroxidase (HRP) conjugate added to each microplate well and incubated. Free components washed away. Substrate solution was added to each well. Only wells containing TGF-B, detection antibodies, and Avidin-HRP conjugate will appear in blue. The reaction of the enzyme with the substrate is stopped by adding a stop solution, causing the color to change to yellow. Optical

density (OD) was measured at a wavelength of 450 ± 2 nm. The OD value is directly proportional to the TGF-B concentration. The concentration of TGF-β in the samples was determined by comparing the OD of the samples to the standard curve.

2.2.5.2. Sample preparation: Serum and human TGF-b activated with acid and then measured by several instruments.

2.2.5.3. Activation of serum TGF-β

Serum was 50 µl incubated with 10 µL of 1 normal NaOH for 10 minutes at room temperature and then with 10 µL of 1 normal NaOH for neutralization. It is ready for examination using the current collection.

Table (2-7): Kit component of TGF-β

Item	Catalogue	Volume
Human anti- TGF coated plate	KPG –TGFP	96 vials
Standards	KPG -TGFSN1- 4	200 µL
HRP-Avidin buffer	KPG- HA	5.5 mL
HRP	HAA	22 µL
Substrate	KPG –SU	5.5 mL
Dilution HRP	KPG –DH	100 µL
Stopping	KPG –ST	3.5 mL
10Xwashing bu er	KPG –WB	40 mL
Detection Ab	KPG –TGFD	5.5 mL
HCL 1N	KPG -HCL 1N	2 mL
NAOH 1N	KPG-NAINE	2 mL

2.2.5.4. Preparation solution for TGF-β

1. Washing buffer: The solution was diluted with distilled water 10 times

2. HRP – Avidin

- The HRP vessel was rotated using a microfuge.
- Added 500 µL from the HRP-Avidin vial to the HRP vial
- overtaxing
- All content added to HRP-Avidin vail
- Added 25 µL of diluted HRP vial to the HRP-Avidin vial
- Shook by hand for 3 minutes until well mixed

Table (2-8): Standard to TGF-β

Standard	Pg\mL
Standard 4	200 pg/mL
Standard 3	100 pg/mL
Standard 2	50 pg/mL
Standard 1	5 pg/mL
Blank	0 pg/mL

2.2.5.5. Procedure

1. The plate has been removed from the desired packaging and brought to room temperature in a dry environment. 50 µL of standards No. 4 to 1 added to the first and fourth wells, the fifth well was considered empty, and all steps performed except the fourth and sixth steps for the blank.

2. Fifty microliters of the required sample added to the rest of the wells and incubated for 60 minutes on a shaker at 200 rpm at room temperature.

3. After appropriate incubation, the plates washed 3 times using the wash solution (after adding the wash solution, the plates were incubated for approximately 1 minute at room temperature and then filtered) in the HRP vial.

4. Then 50 µL of conjugated antibody (Detection Ab) was added to all wells (except the blank) and incubated for 50 minutes on a shaker at 200 rpm at room temperature**.**

5. After proper incubation, the plates were washed 3 times using a washing solution.

6. Then 50 µL of HRP-Avidin solution was added to all wells (except the empty ones) and incubated for 30 minutes on a shaker (at least at 200 rpm).

7. After proper incubation, the dishes washed 5 times using washing solution.

8. Then 50 microliters of substrate added to all wells. We note that 15 minutes is sufficient for incubation, if the amount of color produced is high, the time can be reduced to minutes.

9. Then 25 µLof stop solution added to all wells and the absorbance of the samples was measured in an ELISA reader at a wavelength of 450 nm.

2.2.5.6. Calculation

The concentration of parameters was placed on the abscissa and the OD value to the abscissa. The standard curve was plotted on the coordinate sheet. According to the OD value of the sample, its corresponding concentration (which is the sample concentration) is located; or calculate the linear regression equation of the standard curve according to the standard concentration and OD value. The OD value of the sample was then substituted to calculate its concentration, as shown in figure 2.3

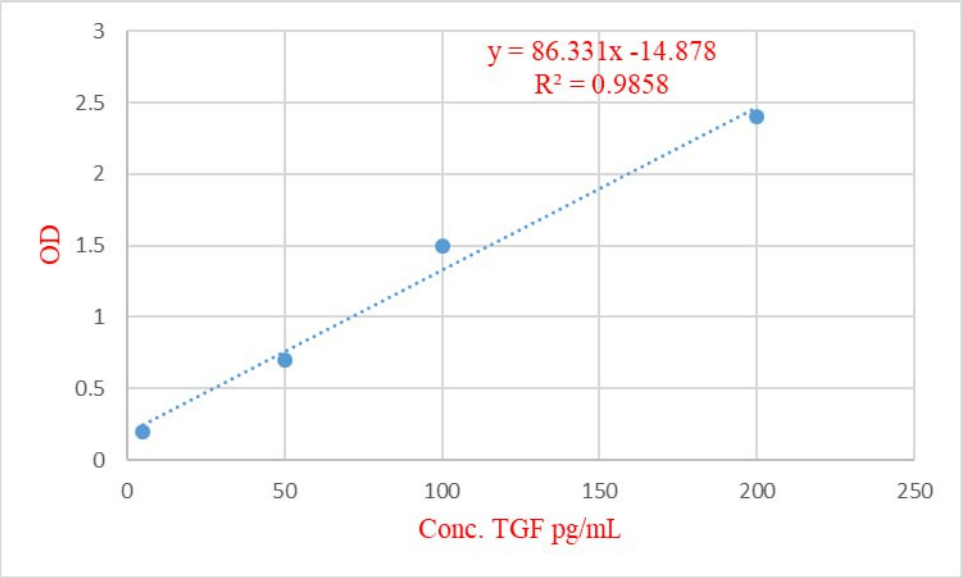


Fig. (2-3): Standard curve for TGF-βdetermination

2.2.6. Determination of Serum Selenium

Four standard solutions (2.5, 5, 7.5, and 10) of the element prepared as mentioned above which used for drawing calibration curves as shown in figure 2-3.

2.2.6.1. Procedures:

The small amount of samples of 20µL injected into a small graphite tube, which can then be heated by a wide range of temperatures to vaporize and atomize the analysis.The concentrations of selenium in samples were measured directly and continuously beyond the measuring of standard solutions depending on the calibration curve. The conditions of selenium determination are listed in Table 2-9.

Table (2.9)*:* Ideal condition for Selenium determination

Variable	Ideal condition
Atomizer	Graphite Furnace
Fuel	Argon gas
Lamp current	35 mA
Wavelength	196 nm
Slit width	0.7 nm
Lighting mode	BGC-D2
Sample size	20 µl

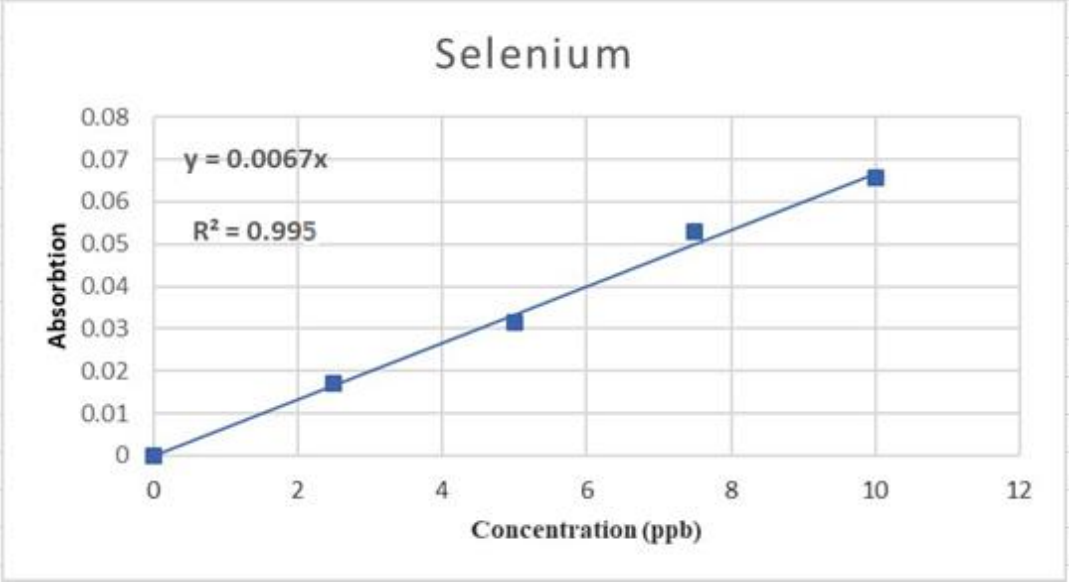


Fig. (2.4): Standard curve for Selenium determination

2.2.7. Determination of Vitamin E (α-Tocopherol)

2.2.7.1. Principle

- Utilized the Sandwich-ELISA principle. The micro-ELISA plate included in this kit was been pre-coated with an antibody specific to Vit. E Samples (or Standards) was added to the micro-ELISA plate wells and mixed with the specific antibody.
- Vit. E detection antibody and Avidin Horseradish Peroxidase (HRP) conjugate added to each microplate well and incubated. Free components

washed away. Substrate solution was added to each well. Only wells containing Vit. E, detection antibodies, and Avidin-HRP conjugate will appear blue. The reaction of the enzyme with the substrate stopped by adding stop solution, causing the color to change to yellow. Optical density (OD) was measured at a wavelength 450 nm. The OD value is directly proportional to the Vit.E concentration. The Vit.E concentration in the samples determined by comparing the OD of the samples to the standard curve.

2.2.7.2. Preparation

1. Standard: The standard prepared with the recommended volume of Standard Diluent Buffer, to make the standard solution. The standard buffer was then used to perform serial dilutions of the standard solution, according to the instructions in the protocol.

2. Wash solution: Concentrated wash: buffer was diluted with distilled water, according to the instructions in the protocol.

3. Preparation of detection reagent: The total volume of working solution required was calculated.Dilute detection reagent A and detection reagent B with diluent A and diluent B, respectively, at 1:100.

All reagents werebrought to room temperature (18–25°C) before use.

2.2.7.3. Procedure for vitamin E determination

1. The standard and the measured sample.

2. Then50 microliters of each standard, control, and sample added to the appropriate wells.

3. The cover was removed and the liquid was disposed of.

4. Then 50 µl of detection reagent was aliquoted into the working solution immediately. The plate was sealed with a lid and incubated for 1 h at 37°C.

5. The cover was removed and the solution was disposed of. The plate was washed 3 times with 1X Wash Buffer.

6. Then 100 µL of working solution of detection reagent B was added to each well closed and incubated at 37 °C for 30 minutes.

7. The solution was discarded and the plate was washed 5 times using the washing solution as described in the previous step.

8. Then 50 µL of stop solution was added to each well. Reading was done at 450 nm immediately.

2.2.7.4. Calculation

The concentration of parameters was placed on the abscissa and the OD value to the abscissa. The standard curve was plotted on the coordinate sheet. According to the OD value of the sample, its corresponding concentration (which is the sample concentration) is located; or calculate the linear regression equation of the standard curve according to the standard concentration and OD value. The OD value of the sample was then substituted to calculate its concentration.

2.2.8. Determination of Fasting Blood Glucose Concentration

2.2.8.1. Principle:

Glucose-oxidase enzyme (GOD) oxidizes glucose to gluconate and hydrogen peroxide, according to the following equation.

$$\text{Glucose} + H_2O + O_2 \xrightarrow{GOD} Gluconic\ acid + H_2O_2$$

$$H_2O_2 + \text{Phenol} + \text{4- Amino-antipyrine} \xrightarrow{POD} \text{Quinonimin} + 4H_2O$$

The absorbance of the color complex was measured at 505 nm.

2.2.8.2. Reagents

- Reagent 1 (Buffer): - Consist of 100 mmol/l of phosphate buffer pH7.5 and 0.75 mmol /L of Phenol.
- Reagent 2 (Enzymes): - Consist of ≥15 KU/Lof glucose oxidase, ≥1.5 KU/L of peroxidase, and 0.25 mmol/l of 4-amino-antipyrine.
- Reagent 3 (Standard): - Consist of 100 mg/dL or 5.55 mmol/L of Glucose.

2.2.8.3. Preparation of the reagents

Working reagents are prepared by adding the substance containing reagent 2 in the vial (enzymes) to the vial of reagent 1 containing reagent 2 in the vial (Buffer). To complete the dissolving of all components, the mixture was mixed gently.

2.2.8.4. Calculation:

Glucose (mg/dL) = Abs (Assay) /Abs (Standard) x Standard concentration

2.2.9. Determination of Serum Fasting Insulin

2.2.9.1. Principle

The insulin test is a chemiluminescence immunoassay. The sample (or calibrator/control), buffer, and magnetic microbeads coated with monoclonal antibody, beta-insulin, and ABEl isbiedanti-Insulin monoclonal antibody mixed well and incubated, forming a sandwich of immune complexes. After sedimentation in a magnetic field, the supernatant was decanted and then a wash cycle was performed. Next, I added Starter1+2 to indicate the chemicals check.

The optical signal was measured by a photomultiplier as relative optical units (RLUs), which are proportional to the insulin concentration present in the sample (or calibrator/control).

2.2.9.2. Preparation of the Reagent

The magnetic microbeads automatically re-suspension when the kit is loaded successfully, ensuring that the magnetic beads are suspended completely homogenously before use.

2.2.9.3. Dilution

Samples diluted to concentrations above the measuring range. The recommended dilution is 1:19. After manual dilution, the result is multiplied by the dilution factor.

2.2.4.4. Calculation: The sample is calculated automatically by calculating the insulin concentration in each sample via a calibration curve generated by two points.

2.2.10. Calculation of Insulin Resistance

Homeostatic model assessment (HOMA) is a technique that calculates insulin resistance and β-cell function. The software program was used to solve the equations so that the estimation of insulin resistance and β-cell function by using fasting glucose and insulin concentration **(Paracha, *et al.*, 2021).**

HOMA- IR = [Glucose (mg/dL) X Insulin (µU/mL)] / 405

2.2.11. Determination of Glycated Hemoglobin

2.2.11.1. Principle

The Fully automated Lifotronic H8 Hemoglobin A1c Analyzer offers a fast throughput of HbA1c results in 13U seconds, with Hb variant detection, providing an outstanding solution for quick and reliable diabetic monitoring. No sample preparation and very little hands-on time by the operator is required for the H8 Analyzer.

2.2.11.2. Sample Volume

5µl; Diluted Blood: 750µl (Dilution Ratio15:1500µL)

2.2.11.3. Reagent Pack

Eluent A, Eluent B, Eluent C, Hemolysin L.

2.2.11.4. HPLC Methodology

High-Performance Liquid Chromatography (HPLC), separates HbA1c directly by measuring the absorbance points continually to form a chromatogram. Using normal distribution curve fitting auto-iterative algorithm to get precise HbA1c testing result, excluding interference of variant and unstable hemoglobin like HbF. Standard Analysis Mode will report HbA1a、HbA1b、HbF、HbA1c、P3、HbA0 peak areas and ratio.

2.2.11.5. Eluents A B and C

Three eluates at different concentrations were used, forming an ascending gradient of ion concentration. Due to the difference in the charges carried by the target proteins, the adsorption capacity against impurities varies. Therefore, when the concentration of the eluent gradually increases,

the target proteins HbA1a, and HbA1b. HbF, LA1c, HbA1c, and HbA0 are removed sequentially.

2.2.11.6. Hemolytic agent

It has been used to dissolve red blood cells and release sugar hemoglobin molecules.

2.2.12. Synthesis of Noisome Nanoparticles

- The fabrication of noisome NPs was conducted using the thin-film hydration method. Initially, a mixture of cholesterol (6 mg) and span 60 (36 mg) dissolved in methanol (6 mL) and chloroform (3 mL) and subjected to a rotary evaporator at 55-60 ◦C and 0.46 atm for 1 hour to form a lipid film and remove solvents. Subsequently, the film was hydrated with 10 mL of Phosphate-Buffered Saline PBS (pH 7.4) under similar conditions for another hour. Ultrasonication for 30 minutes at 24 ◦C was employed to reduce the size of the fabricated niosomal NPs. For the fabrication of metformin-loaded niosomal NPs (Nio-met NPs), metformin (24 mg) was added to the initial mixture. To obtain hyaluronic acid-coated niosomal NPs (Hyalo-Nio NPs) and metformin-loaded hyaluronic acid-coated niosomal NPs (Hyalo-Nio-met NPs), a solution containing 0.1% (w/v) hyaluronic acid in normal saline was gradually added to blank niosomal NPs and Nio-met NPs while stirring, followed by an hour of stirring at ambient temperature to facilitate NP reforming and hyaluronic acid coating.
- Spectral analysis of the compounds before and after nanoparticle fabrication was studied using an FT-IR spectrophotometer (Shimadzu 8400 S, Kyoto, Japan) in the spectral region of 4000-400 cm^{-1}, with a spectra resolution of 4 cm^{-1}. The size, poly disparity index (PDI), and zeta potential of the fabricated niosomal nanoparticles were analyzed using the Zeta sizer dynamic light scattering system (ZS 90, Malvern Instruments Ltd., Malvern,

UK). The surface morphological properties of the fabricated niosomal nanoparticles were examined using scanning electron microscopy (SEM, MIRA3, TESCAN, Czech) and atomic force microscopy (AFM, Nanowizard II; JPK instruments; Germany).

2.2.13. Metformin Release from Niosomal Nanoparticles

To assess the *in vitro* drug release characteristics of Hyalo-Nio-met NPs and Nio-met NPs, the dialysis method was utilized as follows: Initially, 5 mL of nanoparticles were introduced into a dialysis membrane tube (12 kDa) and stirred magnetically at 120 rpm in PBS (pH = 7.4) while incubating at 37 °C. At predetermined intervals, 2 mL of the immersion solution was exchanged with an equal volume of fresh PBS, and the absorbance of the released metformin was measured at 234 nm (the maximum wavelength of metformin) using ultraviolet spectrophotometry (PerkinElmer, Fremont, CA, USA). The process allowed for the monitoring of metformin release kinetics from the niosomal nanoparticles over time, providing insights into their controlled drug-release behavior.

2.2.14. Peripheral Blood Mononuclear Cells Isolation culture

Peripheral blood mononuclear cells (PBMCs) were separated using Histopaque 1077 density gradients by centrifugation (1500rpm, 5 min). The cells were washed three times with phosphate-buffered saline (PBS, pH 7.4) centrifuged, and resuspended in RPMI1640 medium containing 10% bovine serum (FBS, Biochrom, UK) 10 U/mL of penicillin and 10 μg/mL of streptomycin, at a concentration of 1×10^6 cells/mL. These cells were incubated at 37 °C with 5% CO_2 until they reached a confluency of 90% following which they were used in experimental procedures.

2.2.15. MTT Assays

To study the effects of metformin, on PBMCs viability an MTT reduction test was performed using concentrations of pure metformin (2.5, 5, 10, 20, 30 μM) Nio-met NPs (2.5, 5, 10, 20, 30 μM), and Hyalo-Nio-met NPs (2.5, 5, 10, 20, 30 μM). Initially, 1×10^5 cells were seeded in each well of a 96-well plate.Incubated at 37°C with a CO_2 level of around five percent for 24 hours. Subsequently, the cells were exposed to treatment substances at the same temperature and CO_2 level for 48 hours a day. After two days of treatment substances were substituted with a 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma, Germany). Kept in dark conditions at 37°C for 4 hours. Following this incubation period, the MTT solution was removed from each well.Replaced with Dimethyl sulfoxide (DMSO) (Merck, Germany) before shaking the plate for twenty minutes, on a shaker. Finally, the optical density of the wells measured at 570 nm using the EL × 800 Microplate Absorbance Reader (Bio-Tek Instruments), and the proliferation values for pure metformin, Nio-met NPs, and Hyalo-Nio-met NPs determined using GraphPad Prism software.

2.3.16. Evaluation of Total Oxidant Species in PBMCs

Total oxidant species (TOS) in the PBMCs isolated from RA patients, diabetic patients, and healthy individuals' blood determined spectrofluorimetric using 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma, Germany). The treated and untreated PBMCs were washed with PBS and incubated with 30 μM of DCFH-DA at 37°C in the dark condition. After 30 min washed and resuspended in PBS and analyzed using a flow cytometer (ACS Calibur, BD Biosciences, USA).

2.3.17. Determination of Interleukin-23, Transforming Growth Factor-β

PBMCs were seeded in a 6-well plate (1×10^5 cells per well) and allowed to adhere for 24 hours at 37 °C with 5% CO_2. After attachment, the PBMCs were exposed to metformin, Nio-met NPs, and Hyalo-Nio-met NPs for 48 hours at 37 °C with 5% CO_2, while a control group of cells remained untreated. The assays were performed according to the methods outlined in the manufacturer's instructions. Subsequently, the levels of IL-23 and TGF-β in both treated and untreated PBMCs were quantified using an enzyme immunoassay with the human ELISA Kit (Sino Biological Inc., Beijing, China).

2.3.18. Ribose nucleotide Acid Extraction

RNase-free microtubes, tubes, and falcons used for RNA extraction. First, the flask cells were separated by trypsin and counted. About $1*10^6$ cells along with 2 mL of RPMI medium containing 10% FBS were transferred to each well of a 6-well plate and the plate was incubated for 24 hours at 37°C and 5% carbon dioxide for the cells to adhere to the wells. After 24 hours, the medium of the wells was emptied and each well was washed twice with PBS. Then the plates were incubated for 48 hours in an incubator at 37°C and 5% carbon dioxide. After 48 hours, the contents of the wells were emptied and 500 microliters of Trizol were added to each well. After 10 minutes, the triazole was collected from the wells and transferred to a microtube, and 200 µL of chloroform was added to each microtube. Next, the microtubes were transferred to a refrigerated centrifuge and centrifuged for 10 minutes at 12,000 rpm and 4°C. After centrifugation, 3 phases formed inside the microtubes: the upper phase (transparent color) containing RNA, the middle phase (dark and white color) containing DNA, and the lower phase (pink

color) containing protein. Supernatant phase of each microtube was collected and transferred to other microtubes. 200 microliters of isopropanol added to each microtube and the microtubes incubated for 10 minutes at 4°C and centrifuged for 10 minutes at 12000 rpm with a refrigerated centrifuge at 4°C. Next, the supernatant was removed and 1 mL of 75% alcohol was added to each microtube. After centrifugation at 7500 rpm for 5 minutes at 4°C and adding 10 microliters of DEPC water to the samples using a Nano Drop device (ND-1000, Thermo, USA) in the OD260/OD280 ratio, the quality of the extracted RNA samples was measured**.**

2.3.19. Complementary Deoxyribonucleic Acid Synthesis (cDNA)

The synthesis of DNA from the RNA template is called cDNA, which is done by the reverse transcription enzyme. Which requires primers for cDNA synthesis. Since this day, two types of random hexamers and oligo primers have been used to make cDNA. The difference between these two types of primers is that dT oligo is attached to the polyadenine tail of mRNA, while random hexamers are randomly attached to different parts of mRNA. After connecting the primers, the reverse transcription enzyme synthesizes cDNA from template RNA.

2.3.20. Methods of cDNA Detection

Complementary Deoxyribonucleic Acid (cDNA) synthesis was done according to the method mentioned by the kit manufacturer. In this way, 5 microliters of extracted RNA mixed with 1 microliter of random primer and 14 microliters of cDNA master mix and the samples incubated for 30 minutes at 50°C. Also, in order to inactivate the reverse transcriptase enzyme, the

samples incubated at 90°C for 5 minutes. After cDNA synthesis, the samples transferred to a -20-degree refrigerator until use.

2.3.21. Real-Time Polymerase Chain Reaction (RT-PCR)

For the cDNA synthesis, 0.5 µg of total RNA was utilized in the reverse transcription process, employing the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Fermentas) as per the manufacturer's guidelines. The mRNA expression levels of cytokines were quantified using the Rotor-Gene TM 6000 (Corbett). Real-time PCR (RT-PCR) assays were conducted in a 25 µL reaction mixture, comprising 2 µLof the synthesized cDNA, 12.5 µLof 2x Rotor-Gene Probe PCR Master Mix (Qiagen, Germany), 500 nM of each primer, and 250 nM of the TaqMan-probe (FAM, TAMRA). The volume was adjusted to 25 µL with DNase/RNase-free water. The amplification protocol consisted of an initial heating phase (10 min at 94°C), followed by denaturation (94°C for 15 s) and annealing/extension (60°C for 60 s). Cytokine mRNA expressions were standardized to GAPDH (a housekeeping gene), and the relative quantification was determined utilizing the 2-ΔΔCt approach. The sequences of the oligonucleotides for NFACT1, RANKL, and GAPDH primers are detailed in Table 2.

Table2.10. Forward and reverse primers sequences used for real-time PCRreactions.

Genes	Forward	Reverse
NFATC1	AGGCCATCCTCTCCAACACC	GTTCTTCCTCCCGATGTCCGTCT
RANKL	GCTTTTATTACCTGTATGCCAA	CTGCTTATTATTCAAGGCATC
GAPDH	CTTCCAGGAGCGAGATCCCT	CCTGTTGCTGTAGCCAAATTCGT

2.4. Statistical Analyses

Information from the questionnaire and all test results from study group samples were entered into a data sheet. The data analysis for this work was generated using the Statistical Package for the Social Sciences software, version 22.0 (IBM, SPSS, Chicago, Illinois, USA). Values illustrated by n (%) for categorical; Scale variables presented by mean ± standard deviation for normal data. The distribution of the data was checked using the Shapiro-Wilk test as a numerical means of assessing normality. Biomarkers were compared using the one-way analysis of variance (ANOVA) done to compare the means of different groups. Significant differences in categorical variables among the parameters were confirmed through analytical statistical tests. Results of all hypothesis tests with p-values <0.05 (two-tail) are considered to be statistically significant. The optimal threshold with high specificity and sensitivity for critical cases was detected using receiver operating characteristic (ROC) analysis. It found that all the values of P were two-sided, and a $P < 0.05$ was considered to be statistically significant.

Chapter Three

Results

3. Results

3.1 Part I (clinical chemical result)

3.1.1 Demographic Characteristics

The demographic characteristics of a total of 118 participants included in this study of 71 RA patients (with/without T2DM) and 47 controls, divided into subgroups based on age, family history, and. BMI groups. The distribution of age groups is fairly similar across both groups, with a slight trend towards older ages in the patient group. More of the patients (25.4%) fall within this age range of 30-39 years compared to controls (27.7%). Similar distribution for the 40-49 years range, with a slightly higher percentage in the patient group (42.3%) compared to controls (31.9%). A slightly higher percentage of controls (27.7%) are in the age range of 50-59 years as compared with RA patients (14.1%), while about (18.3%) of patients in the age range between 60-70 years as compared to control group (12.7%), as presented in figure 3.1

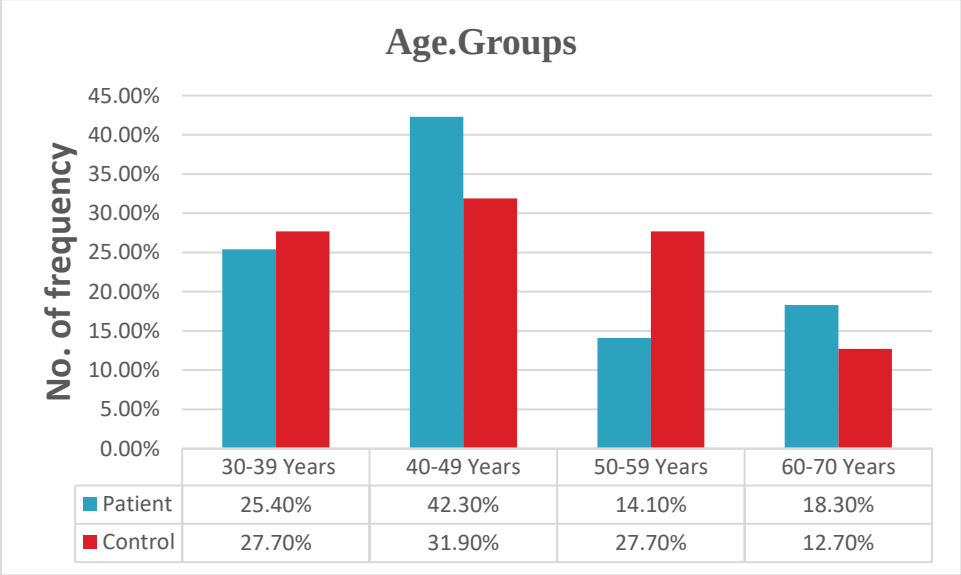


Fig. (3-1): Baseline characteristics and demographic descriptive of the study population in disease and control groups the number of participants for age groups (N= 118).

These results demonstrated that the RA patient group appears to have a higher prevalence of overweight and obesity as compared with the

control group. About (40.8%) patients classified as normal weight as compared to controls (78.7%), whereas a higher percentage of patients (43.7%) overweight as compared to controls (21.3%) whereas About (15.5%) patients the obesity category belongs to the patient group, with none in the control group, as presented in figure 3.2.

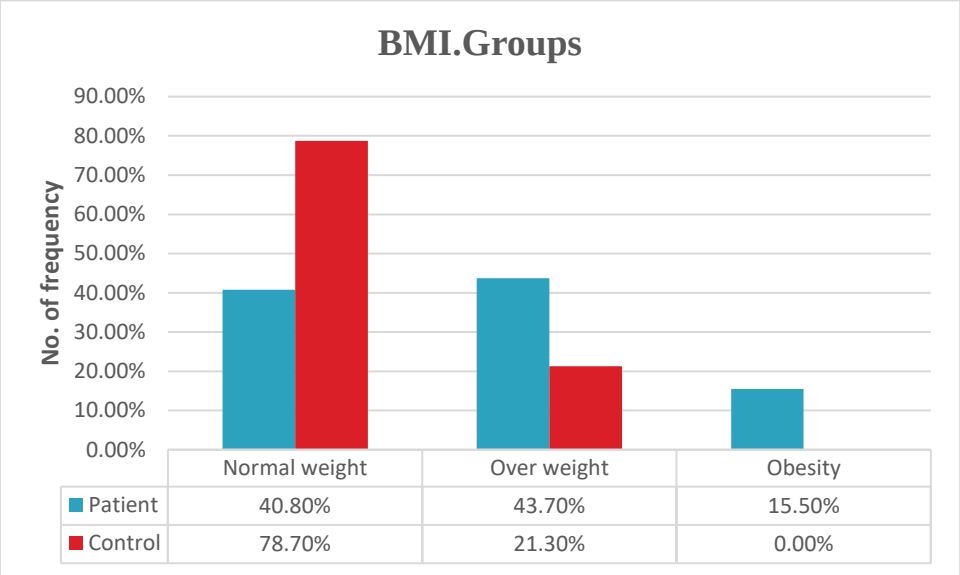


Fig. (3-2): Baseline characteristics and demographic descriptive of the study population in disease and control groups number of participants for BMI groups (N= 118).

3.1.2.1 Inflammatory Biomarkers in Sera of Rheumatoid Arthritis Patients

Table3.1, the mean difference of inflammation Biomarkers for patients with RA Patients (with and without T2DM) showed an increasing range of IL-23, TGF, CRP, and ESR compared to the healthy control groups.

Results indicated a highly statistically significant difference in TGF levels between RA (with and without T2DM) with control groups, (p-value ≤ 0.05), but no significant between RA with T2DM and RA without T2DM, the means and standard deviations are (40.56±16.35pg/mL), (39.20±16.11pg/mL), and (4.42±2.79pg/mL) respectively, presented in Table 3.1.The mean levels of IL-

23 highly statistically significant among the three groups, (p-value ≤ 0.05), the mean and standard deviation (41.32±14.15pg/mL), (57.27±15.86pg/mL), and (9.16±1.01pg/mL) respectively. The mean level of ESR was significant between RA (with and without T2DM) and control groups.But not statistically significant between RA and RA with T2DM.the mean level and standard deviation (50.11±14.18mm/hr), (52.82±12.65mm/hr) and (14.17±4.84mm/hr). Respectively. While CRP was significantly higher in RA (with and without T2DM) (41.32±14.15 mg/L), (57.27±15.86 mg/L) compared with control patient (9.16±1.01 mg/L), (p-value ≤ 0.05).and significantly higher between two group RA (with and without T2DM) patient. These findings suggest that while some inflammatory markers (IL-23 and CRP) may be elevated to a greater extent in RA patients with T2DM, other markers (TGF and ESR) appear to be similar between the two groups as presented in Table 3.1

Table 3.1 The mean ± SD difference of inflammatory marker for level IL23, TGF, ESR, and CRP among patients group of RA, RA with T2DM compared to the control group

Biomarkers	RA Mean±SD N=38	RA±T2DM Mean±SD N=33	Control Mean±SD N=47	P value
TGF pg\mL	40.56±16.35[a]	39.20±16.11[a]	4.42±2.79[b]	<0.001[S]
IL-23 pg\mL	41.32±14.15[b]	57.27±15.86[a]	9.16±1.01[c]	<0.001[S]
CRP mg\L	5.98±1.89[b]	12.09±3.53[a]	0.43±0.22[c]	<0.001[S]
ESR mm\hr	50.11±14.18[a]	52.82±12.65[a]	14.17±4.84[b]	<0.001[S]

ANOVA -test was *: significant at p ≤ 0.05
SD: standard deviation; S: significant; NS= Non-significant.
This means that sharing the same letter in the same row does not find significant differences between them according to the Post Hoc test .

3.1.2.2 Results of other Biomarkers in Sera of Rheumatoid Arthritis Patients

The mean difference of inflammation Biomarkers for patients with RA Patients (with & without T2DM) showed an increasing range of HOMA IR, Insulin, FBS, and HbA1c compared to the healthy control groups.

Results indicated a highly statistically significant difference in HOMAIR was significant between RA with control groups, but not statistically significant between RA (with and without) T2DM with the control group, the mean level and standard deviation (2.96±0.55), (9.80±3.72), and (3.37±0.78).

Results indicated a highly statistically significant difference in Insulin levels between RA (with and without T2DM) with control groups ($p \leq 0.05$), but no significant between RA with T2DM and RA without T2DM, The means and standard deviations are (11.24±1.96μM/mL), (11.87±1.97μM/mL), and (13.04±2.31μM/mL) respectively, presented in Table 3.2. Finally, the mean level of FBS and HbA1c was significant between RA with T2DM and RA without T2DM groups, but not statistically significant between RA (with and without) T2DM with the control group ($p \leq 0.05$), the mean level and standard deviation (106.92±8.17 mg/dL), (331.30±108.84 mg/dL), and (96.98±8.80 mg/dL) respectively for FBS and (5.52±0.64%), (11.80±1.81%), and (5.31±0.68 %) respectively for HbA1c. Their results highlighted a potential link between T2DM and abnormal blood sugar control (FBG and HbA1c) in RA patients.

Table 3.2: The mean ± SD difference of metabolic disorder marker level for HOMO-IR, Insulin, HbA1c, and FBS among patients group of RA,RA with T2DM compared to the control group

Biomarkers	RA without T2DM Mean ± SD N = 38	RA with T2DM Mean ± SD N = 33	Mean±SD N=47	P value
HOMA-IR	2.96±0.55[b]	9.80±3.72[a]	3.37±0.78[b]	<0.001[S]
Insulin,μM/mL	11.24±1.96[b]	11.87±1.97[b]	13.04±2.31[a]	<0.001[S]
FBG, mg/dL	106.92±8.17[b]	331.30±108.84[a]	96.98±8.80[c]	<0.001[S]
HbA1c%	5.52±0.64[b]	11.80±1.81[a]	5.31±0.68[b]	<0.001[S]

ANOVA -test was *: significant at p ≤ 0.05
SD: standard deviation; S: significant; NS= Non-significant.
This means that sharing the same letter in the same row does not find significant differences between them according to the Post Hoc test

3.1.2.3 Oxidative Status in RA Patients with/without T2DM

Table 3.3: Generally, The mean difference of antioxidant

Biomarkers for patients with RA Patients (with and without T2DM) showed an increasing range of Selenium and vitamin E compared to the healthy control groups.

Results indicated a highly statistically significant difference in selenium and Vit.E significant between RA (with and without) T2DM with the control group ($p \leq 0.05$), but not statistically significant between RA with T2DM and RA without T2DM group, the mean level and standard deviation (3.03±0.92 mg/dL), (2.85±1.14 mg/dL), and (10.64±3.60 mg/dL) respectively for Vit. E and (70.54±11.16 mcg/L), (66.62±12.88 mcg/L), and (140.56±15.26 mcg/L) respectively for selenium.

Table 3.3: The mean ± SD difference of antioxidant disorder marker level for Vit. E & Selenium among patients group of RA, RA with T2DM compared to the control group

Antioxidant Biomarkers	RA without T2DM Mean ± SD N=38	RA withT2DM Mean ± SD N=33	Mean±SD N=47	P-value
Vit.E, mg/dL	3.03±0.92[b]	2.85±1.14[b]	10.64±3.60[a]	<0.001[S]
Selenium, mcg/L	70.54±11.16[b]	66.62±12.88[b]	140.56±15.26[a]	<0.001[S]
ANOVA -test was *: significant at p ≤ 0.05 SD: standard deviation; S: significant; NS= Non-significant. This means that sharing the same letter in the same row does not find significant differences between them according to the Post Hoc test				

3.1.3. Correlation Studies

Considering the important role of the measured biomarkers, the Spearman rank test analysis of RA with or without T2DM disease was used to analyze the response relationship between parameters. The correlation study shows many significant correlations among the measured parameters (Table 3.4). The most important correlations between RA with T2DM levels which positively related to the IL-23 pg/mL, TGF-β pg/mL, FBS mg/dL, insulin μM/mL, HOMA-IR, Hba1c%, and Vit. E mg/dL levels. IL-23 had shown a significant positive correlation with CRP ($r = 0.5$, $p < 0.001$) and HOMA-IR ($r = 0.5$, $p < 0.05$), suggesting a potential link between IL-23, inflammation, and IR, while not indicating a significant correlation with other biomarkers (TGF-β, ESR, FBG, insulin, HbA1c%, Vitamin E). TGF-β illustrated a non-significant correlation with most biomarkers, except for a weak positive correlation with Selenium ($r = 0.4$, $p < 0.001$), as indicated in Table (3-4). CRP and HOMA-IR displayed ($r= -0.4$, $p=0.001$), ($r= -0.4$, $p<0.001$), and FBG ($r= -0.4$, $p=0.02$)

significant negative correlations with TGF, suggesting a potential inverse relationship.

Table 3.4*:* The correlation coefficient between (IL-23 and TGF-β) with other biomarkers among Patients group

Biomarkers	IL-23		TGF	
	(r)	*P value*	*(r)*	*P value*
IL-23pg/mL	0.1	-	0.1	0.073[NS]
TGF-β pg/mL	0.1	0.632[NS]	1	-
CRP mg/L	0.5	<0.001[S]	- 0.4	0.001[S]
ESR mm/hr	0.1	0.612[NS]	0.1	0.351[NS]
FBG mg/dL	0.6	0.001 [S]	- 0.4	0.02[S]
Insulin µM/mL	0.1	0.054[NS]	0.1	0.427[NS]
HOMA IR	0.5	0.004 [S]	- 0.4	<0.001[S]
HbA1c%	0.6	0.001 [S]	0.1	0.064[NS]
Vit-E mg/dL	0.1	0.143[NS]	0.1	0.732[NS]
Selenium mcg/L	0.1	0.062[NS]	0.4	<0.001[S]
p<0.05 considered significantly different, [S]= Significant, [NS]= Non significant ®*:Correlation Coefficient*				

3.1.4 Association of Biomarkers with RA (With and Without T2DM) Compared to Controls

Binary logistic regression was performed and forward logistic regression was adopted to analyze the results. The correlation coefficient was used for determining linear relationships among biochemical markers in patient groups. It was found that all biomarkers IL-23, TGF-β, CRP, and ESR HbA1c risk factors and had highly statistically significant difference. An odd ratio of more than one indicates an increased occurrence of an event. An odd ratio of less than one indicates a decreased occurrence of an event (protective exposure), as shown in Table 3.5

Table 3.5 presented the odds ratios (OR) and p-values for the association between various biomarkers and the presence of Rheumatoid Arthritis (RA) with or without (T2DM) compared to a control group. The control group serves as the reference category (OR = 1). Statistically significant associations ($p < 0.05$) are indicated by [S].

Results had shown that IL-23, TGF-β, CRP, and ESR: All biomarkers showed significantly increased odds of having RA compared to controls (p-value < 0.001). This suggests a strong association between elevated levels of these markers (IL-23=6.7, TGF-β=32.2, CRP=4.5, and ESR=133.7) in RA and the OR values that increased in RA with T2DM for (IL-23 = 7.2, TGF-B=32.09, CRP=4.43, and ESR=135.8). Results also showed a significant association between HbA1c=1.63 in RA and 1.5 in RA without T2DM) compared to controls. These findings highlighted the potential role of, HbA1c for RA, and These findings highlighted the potential role of, IL-23, TGF-β, CRP, and ESR as markers for RA, and with presence of T2DM may significantly affect the association of these specific markers.

Table 3.5: **Associated of the analyzed (IL-23, TGF-β, CRP, and ESR) in RA patients (with&withoutT2DM), compared to control group.**

Variable	Groups	OR(Lower–upper)	P value
IL-23 Pg/mL			
	Control	1[a]	-
	RA	6.733 (6.490-6.985)	<0.001[S]
	RA+ T2DM	7.206 (7.202-8.421)	<0.001[S]
TGF-β Pg/mL			
	Control	1[a]	-
	RA	32.261 (31.331-33.218)	<0.001[S]
	RA+ T2DM	32.091 (31.281-34.151)	<0.001[S]
CRP mg/L			
	Control	1[a]	-
	RA	4.565 (1.814-8.308)	<0.001[S]
	RA+ T2DM	4.43 (2.714-5.621)	<0.001[S]
ESR mm/hr			
	Control	1[a]	-
	RA	133.745 (129.109-138.547)	<0.001[S]
	RA+ T2DM	135.804 (135.801-136.403)	<0.001[S]
HbA1c %			
	Control	1[a]	-
	RA	1.632 (0.838-3.178)	<0.001[S]
	RA+ T2DM	1.50 (0.732-2.231)	<0.001[S]
p <0.05 considered significantly different, [S]= Significant, [NS]= Non Significant 1a: reference category is Control			

Table 3.6 presented the odds ratios (OR) and p-values for the association between Vitamin E and Selenium levels and the presence of (RA) with and without (T2DM) compared to a control group. Both RA and RA with T2DM groups showed significantly decreased odds ratios of having RA compared to controls (p-value < 0.001). This translates to an OR of approximately 5.5 for RA and 4.6 for RA with T2DM, indicating that lower Vitamin E levels are associated with an increased risk of RA, regardless of T2DM status. In contrast to vitamin E, both RA and RA with T2DM groups had significantly decreased odds of having RA compared to controls (p-value < 0.001) with OR values around 0.2. This suggests that lower selenium levels are also associated with an increased risk of RA, and the presence of T2DM does not appear to significantly alter this association.

Table 3.6 Associated of the analyzed factors (VIT.E and selenium) in RA patients (with & without T2DM), compared to control group

Variable	Groups	OR (Lower–upper)	P value
VIT.E mg/dL			
	Control	1[a]	-
	RA	5.54 (3.48-8.82)	<0.001[S]
	RA+ T2DM	4.63 (4.63-5.12)	<0.001[S]
Selenium mcg/L			
	Control	1[a]	-
	RA	0.212 (0.204-0.221)	<0.001[S]
	RA+ T2DM	0.206 (0.194-1.54)	<0.001[S]
p <0.05 considered significantly different, [S]= Significant, [NS]= Non Significant 1a: reference category is Control			

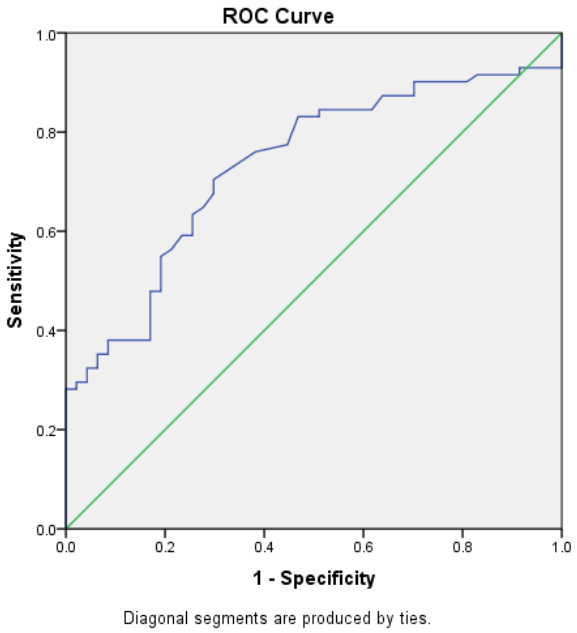
3.1.5. Receiver Operating Curve Analysis

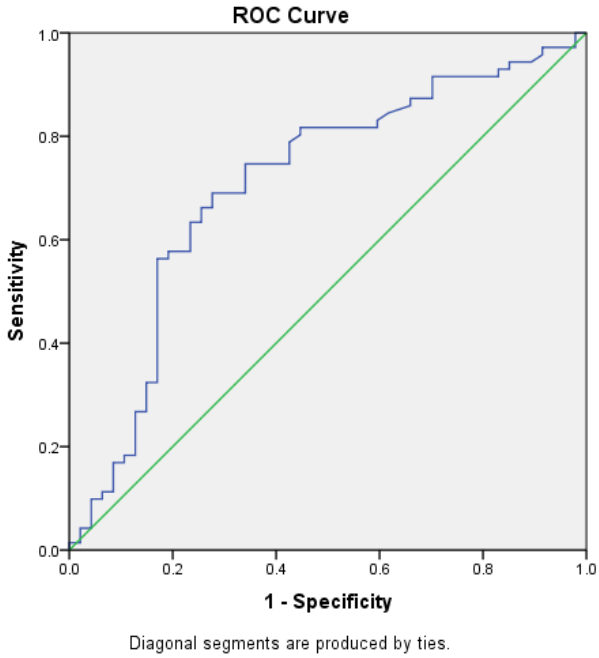
3.1.5.1. ROC curve and AUC analysis for the IL-23 and TGF-β for RA (with T2DM or without T2DM) compared to the healthy groups

Results of the receiver operating curve (ROC) curve and AUC analysis for the insulin besides possible diagnostic parameters.IL-23 and TGF-**β** showed good diagnostic performance for prediction of the RA (with or without T2DM) compared to the control groups between discharge and passed; data are presented in Table 3.7. For IL-23 levels: (sensitivity 70.4%, specificity 70.2%) at a level = 22.465, TGF-β levels: (sensitivity 69%, specificity 72.3%) at a level = 26.291, the p-values of the AUC <0.05 and statistically significant. Youden's J statistics of the parameters in figure 3-3 confirm these results.

Table 3.7: AUC, optimal threshold, Sensitivity, and specificity of proposed marker obtained by the ROC curves RA (with or without T2DM) disease patients

Test Variable	AUC	Sensitivity %	Specificity %	Youden index	Cut-off points Pg/mL	CI (95%)
IL-23 pg/mL	73.40%	70.40%	70.20%	0.406	22.465	0. 643-0.824
TGF pg/mL	70.80%	69%	72.30%	0.413	26.291	0.608-0.807





(A) **(B)**

Fig.(3-3): Receiver operating characteristics (ROC) curve analysis

A- IL-23 in patients RA (with or without T2DM) disease patients compared to control groups.

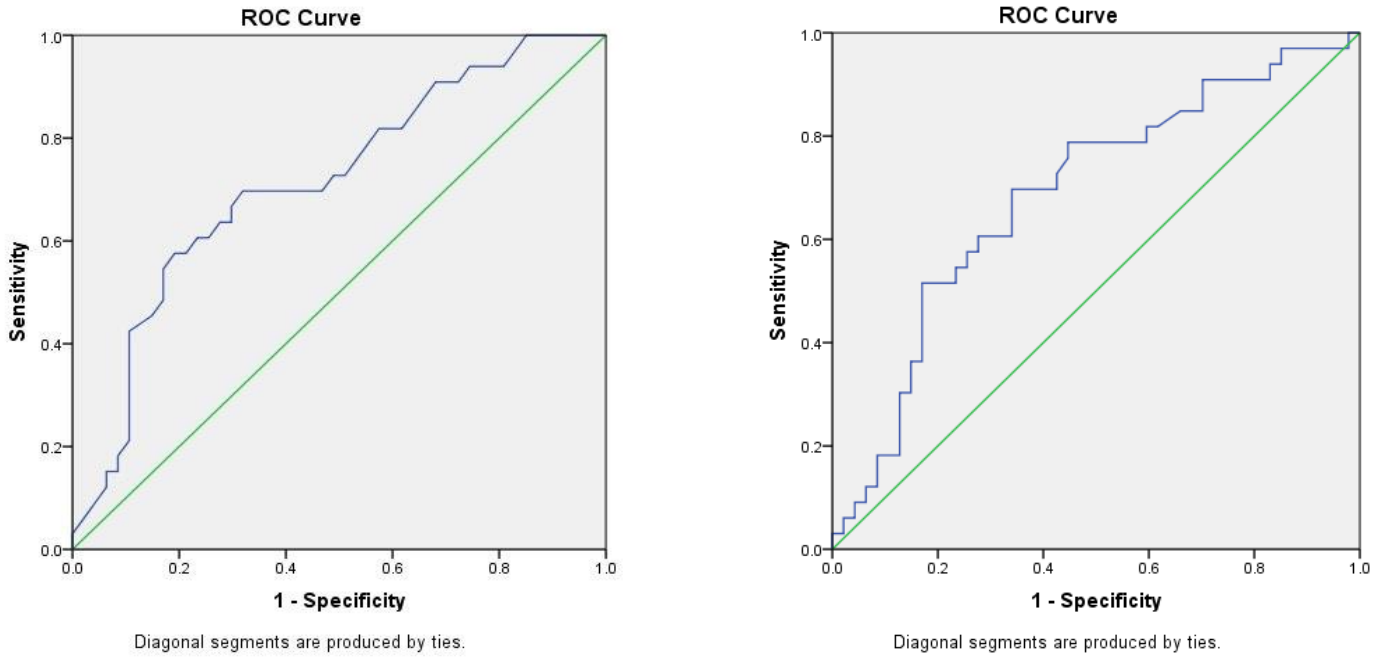
B- TGF-β in patients RA (with or without T2DM) disease patients compared to control groups.

3.1.5.2. ROC curve and AUC analysis for the IL-23 and TGF-β for RA (with T2DM) compared to the healthy groups

Results of the receiver operating curve (ROC) curve and AUC analysis for the insulin besides possible diagnostic parameters.IL-23 and TGF-β showed good diagnostic performance for predication of the RA (with T2DM) compared to the fate groups between discharge and passed; data are presented in Table 3.8. For IL-23 levels: (sensitivity 57.6%, specificity 80.1%) at a level = 29.91, IL-23 levels: (sensitivity 69.7%, specificity 66%) at a level = 25.3, the p-values of the AUC <0.05 and statistically significant. Youden's J statistics of the parameters in figure 3-4 confirm these results.

Table 3.8: AUC, optimal threshold, Sensitivity, and specificity of proposed marker obtained by the ROC curves RA (with T2DM) disease patients

Test Variable	AUC	Sensitivity %	Specificity %	Youden index	Cut-off points Pg/mL	CI (95%)
IL-23 pg/mL	71.40%	57.60%	80.10%	0.384	29.91	0.599-0.829
TGF Pg/mL	61%	69.7	66%	0.357	25.3	0.567-0. 806



(A) **(B)**

Fig. (3-4): Receiver operating characteristics (ROC) curve analysis

A- IL-23 in patient's RA (with T2DM) disease patients compared to control groups.

B- TGF-β in patient's RA (with T2DM) disease patients compared to control groups.

3.1.5.3. ROC curve and AUC analysis for the IL-23 and TGF-β for RA (without T2DM) compared to the healthy groups

Results of the receiver operating curve (ROC) curve and AUC analysis for the Insulin besides as possible diagnostic parameters.IL-23 and TGF-β showed

good diagnostic performance for predication of the RA (without T2DM) compared to the fate groups between discharge and passed; data are presented in Table 3.9.For IL-23 levels: (sensitivity 63.2%, specificity 68.1%) at a level = 21.37, TGF-β levels: (sensitivity 52.6%, specificity 76.6%) at a level = 30.075, the p-values of the AUC <0.05 and statistically significant. Youden's J statistics of the parameters in figure 3-5 confirm these results.

Table 3.9: AUC, optimal threshold, Sensitivity, and specificity of proposed marker obtained by the ROC curves RA (without T2DM) disease patients

Test Variable	AUC	Sensitivity %	Specificity %	Youden index	Cut-off points Pg/mL	CI (95%)
IL-23 Pg/mL	66.20%	63.20%	68.10%	0.312	21.37	0.542-0.781
TGF Pg/mL	64.60%	52.60%	76.60%	0.292	30.075	0.526-0.765

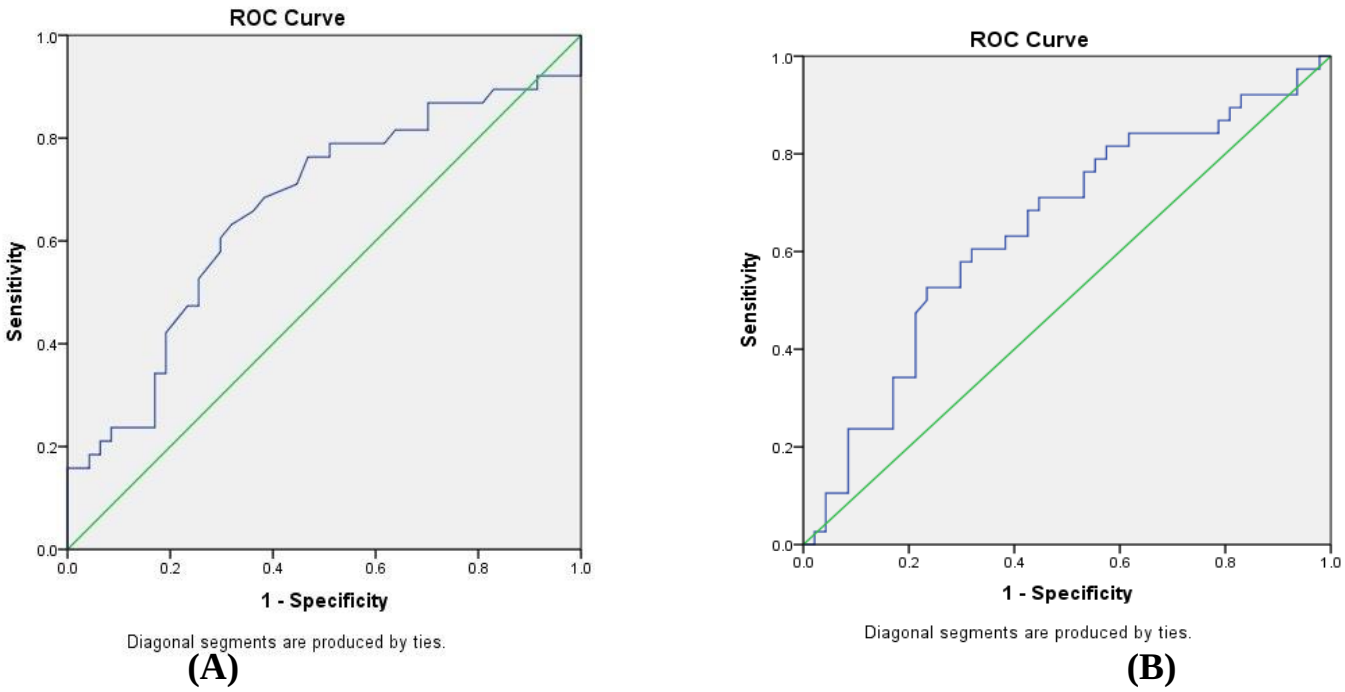


Fig.(3-5): Receiver operating characteristics (ROC) curve analysis

A- IL-23 in patient's RA (without T2DM) disease patients

B- TGF-β in patient's RA (without T2DM) disease patients

3.1.5.7. ROC Analysis for the HbA1c, and FBS, in Sera of RA without T2DM as Compared to the Healthy Groups

Results of the receiver operating curve (ROC) curve and AUC analysis for the HbA1c and FBS besides possible diagnostic parameters also determined. HbA1c and FBS showed good diagnostic performance for predication of the RA without T2DM as compared to the control groups between discharge and passed; data are presented in Table 3.10. For HbA1c levels: (sensitivity 92.2%, specificity 78.7%) at a level = 4.85 For FBS levels: (sensitivity 92-1%, specificity 55.30%) at a level = 96.5. The *P*-values of the AUC <0.05 and statistically significant. Youden's J statistics of the parameters in figure 3-6 confirm these results.

Table 3.10: AUC, optimal threshold, sensitivity, and specificity of proposed marker obtained by the ROC curves RA (without T2DM) disease patients.

Test Variable	AUc	Sensitivity %	Specificity %	Youden index	Cut-off points	CI (95%)
HbA1c mmol/moL	57.80%	92.20%	78.70%	0.708	4.85	0.457-0.700
FBS mg/dL	80%	92.10%	55.30%	0.474	96.5	0.709-0.890

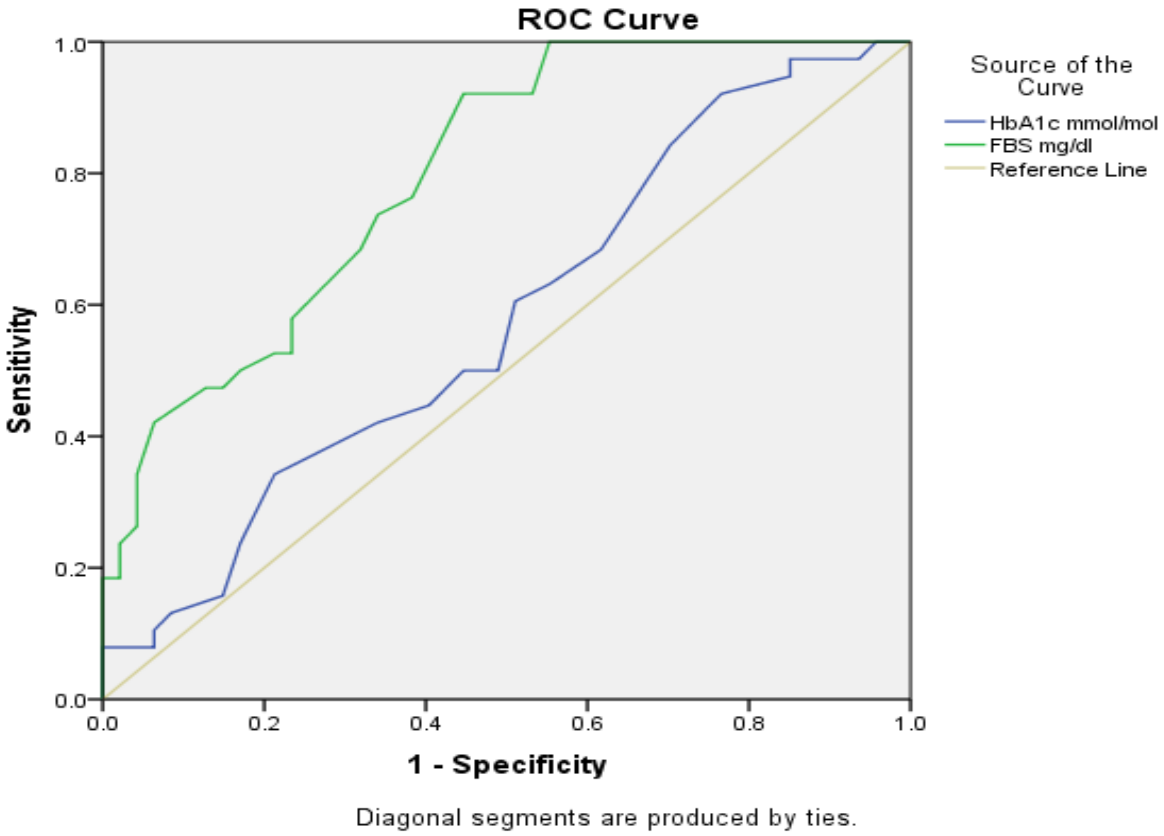


Fig. (3-6): Receiver operating characteristics (ROC) curve analysis HbA1c and FBS in patients RA (without T2DM) disease patients compared to control groups.

3.2. Part II (Nano studies result)

3.2.1. Evaluation of Size, Zeta Potential, and Polydispersity Index (PDI) values of Blank Niosome, Nio-met, and Hyalo-Nio-met NPs using Dynamic Light Scattering (DLS)

The Dynamic Light Scattering (DLS) analysis showed that the blank noisome nanoparticles NPs 151±6.2 nm on average diameter. However, encapsulation of metformin inside these NPs causes an increase in their average diameter to 168±10.2 nm. The highest diameter among fabricated belongs to Hyalo-Nio-met NPs with 179±8.5 nm, this is because of coatation with hyaluronic acid and encapsulation of metformin inside these NPs. Table 3.11 lists the calculated amount of zeta potential for blank niosome, Nio-met, and Hyalo-Nio-met NPs.

Table 3.11: The evaluated size, zeta potential, and PDI values of blank niosome, Nio-met, and Hyalo-Nio-met NPs using DLS.

Groups	Size (nm)	Polydispersity Index	Zeta potential (mV)
Blank noisome	**151 ± 6.2**	**0.426**	**−13.47 ±3.8**
Nio-met	**168 ± 10.2**	**0.453**	**−15.21 ± 2.9**
Hyalo-Nio-met	**179 ± 8.5**	**0.663**	**−9.76 ± 3.4**

Figure 3.7. The highest diameter among fabricated belongs to Hyalo-Nio-met NPs for intravenously administered nanoparticles, diameter is a key factor affecting pharmacokinetics and biodistribution pores in blood vessels.

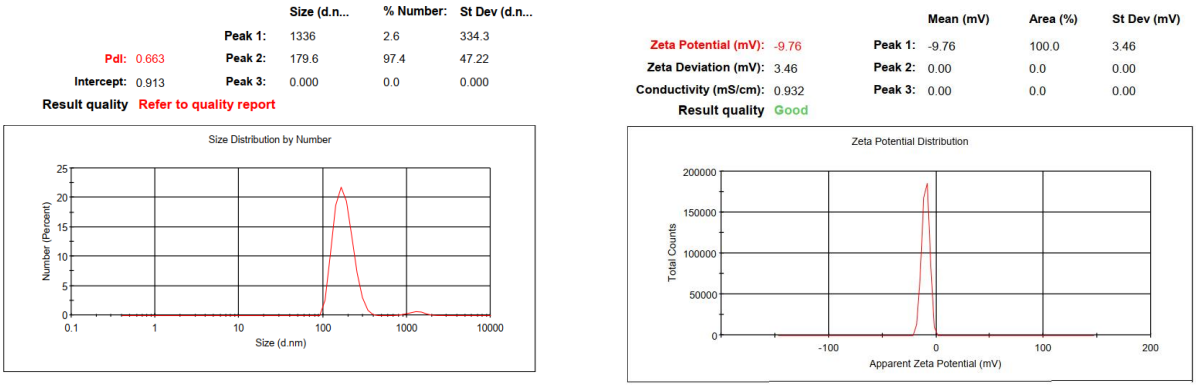


Fig.(3-7) The mean diameter, PDI, and zeta potential value of fabricated Hyalo-Nio-met NPs evaluated by DLS.

Figure 3-8 demonstrated the Scanning Electron Microscopy (SEM) images of fabricated blank noisome and Hyalo-Nio-met NPs. All these NPs showed spherical morphology and the only difference between them is their size.

The Atomic Force Microscopy (AFM) images of NPs are demonstrated in figure 3-8 these images agree with previous Dynamic Light Scattering (DLS) results.

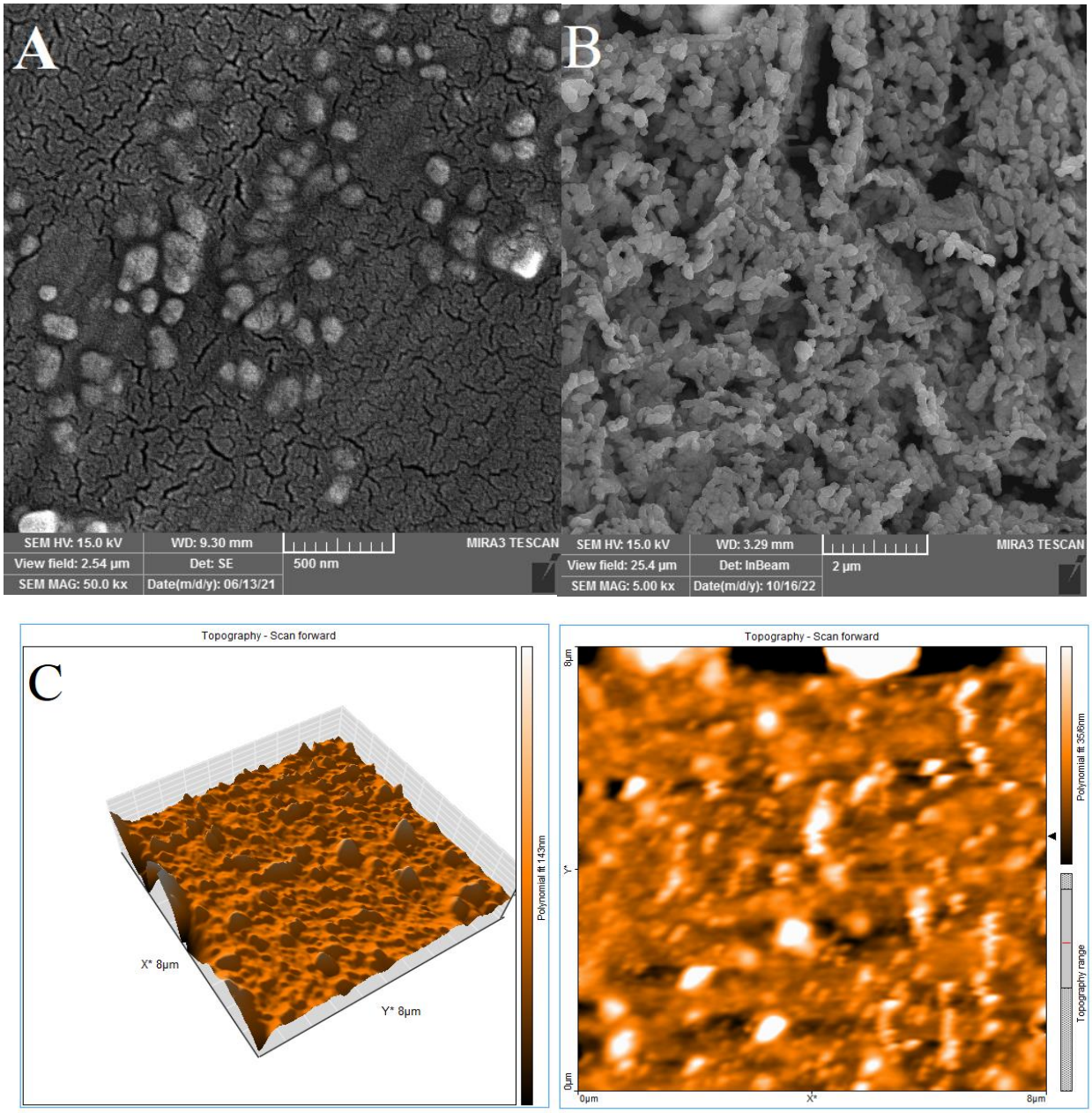


Fig.(3-8) The SEM and AFM images of fabricated blank niosome and Hyalo-Nio-met NPs. A) SEM image of blank niosome NPs, B) SEM image of Hyalo-Nio-met, C) AFM image of Hyalo-Nio-met.

Figure 3-9The characteristic peaks in the Span-60 spectrum appeared at 3389, 2916, and 1736 cm^{-1}, corresponding to OH stretching, carbonyl dimer, and C = O stretching, respectively.

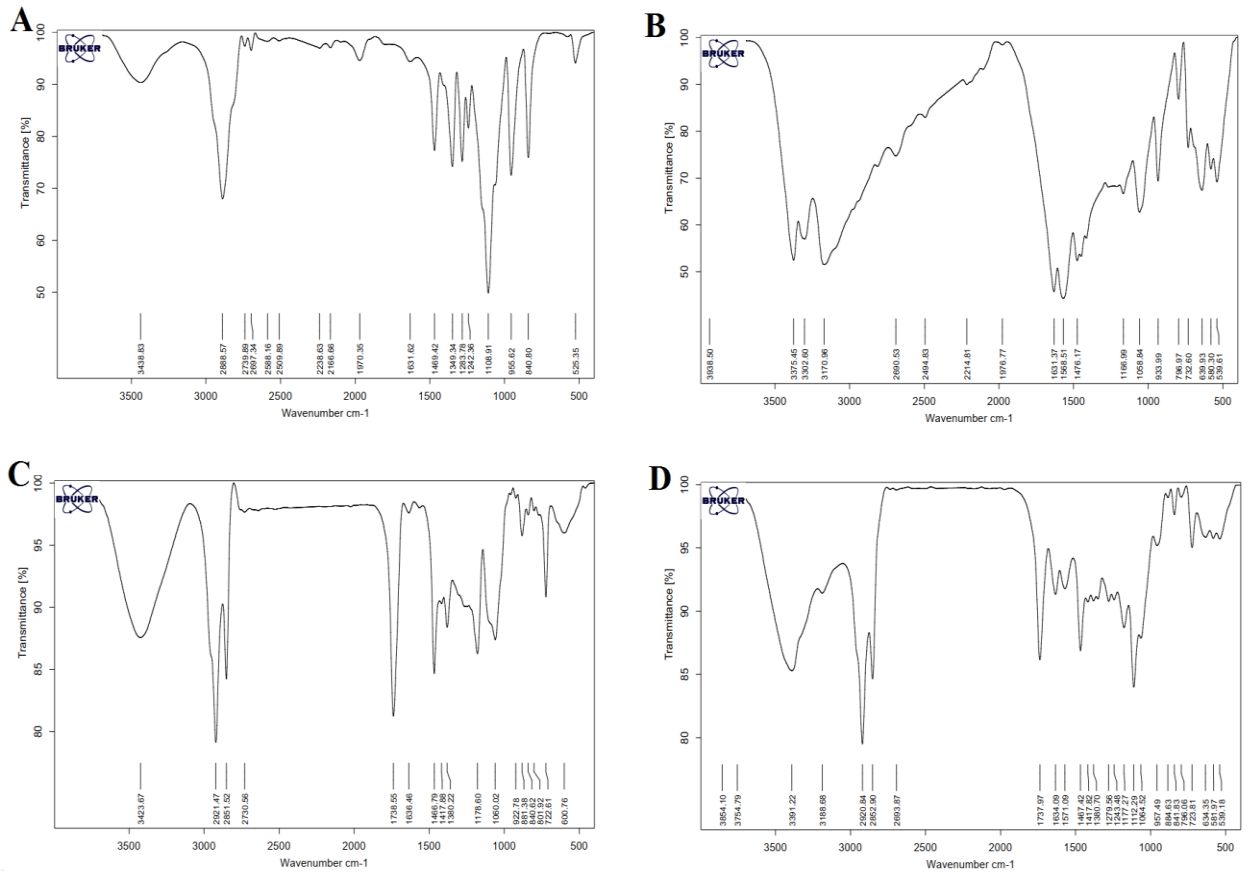


Fig. (3-9)The FTIR spectrum A) Hyaluronic acid, B) Metformin, C) Blank niosome NPs, D) Hyalo-Nio-met NPs.

Figure 3-10 showed the 120 hours' release pattern of metformin from the Nio-met and Hyalo-Nio-met NPs at 37 °C. Both Nio-met and Hyalo-Nio-met NPs show biphasic release pattern. The maximum release rates reached 42% and 47% within the first 12 hours of the experiment, followed by a subsequent decrease.

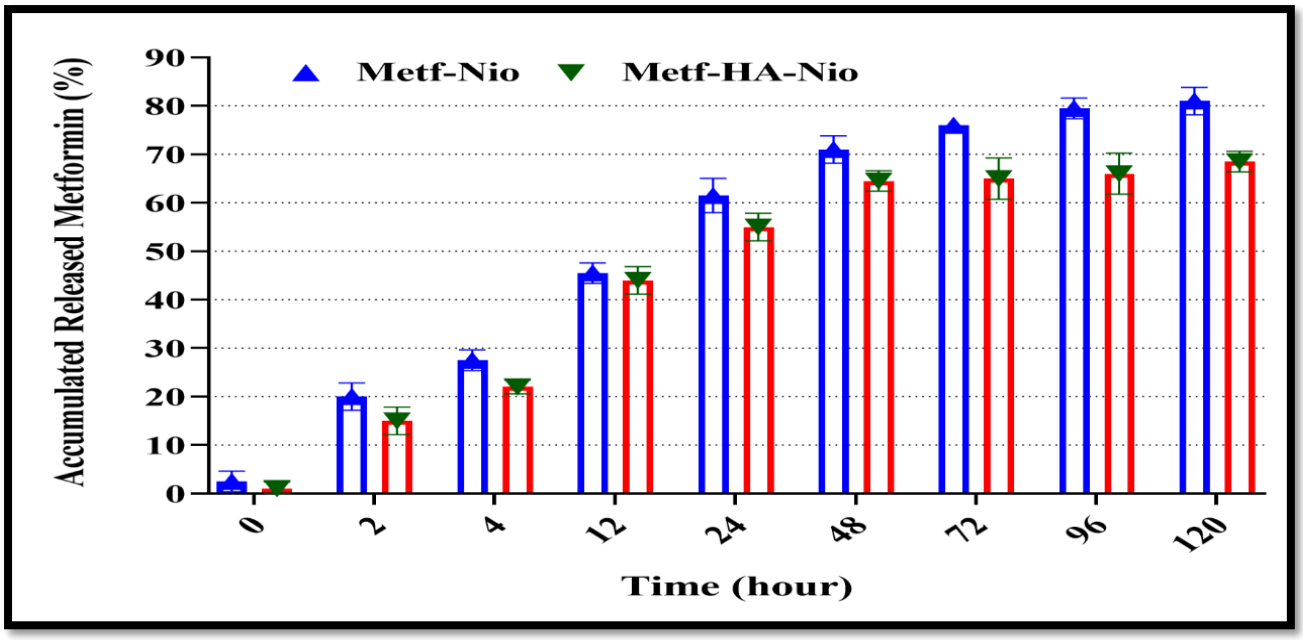


Fig. (3-10): The release pattern of metformin from Nio-met, and Hyalo-Nio-met NPs in physiological condition.

3.2.2. Effect of Metformin, Nio-met, and Hyalo-Nio-met NPs toward PBMCs.

As illustrated in figure 3-11, metformin, Nio-met, and Hyalo-Nio-met NPs exhibit a negligible and insignificant proliferation effect on PBMCs at concentrations of 5 µM. As previously described, niosomal NPs can enhance treatment effectiveness by increasing the solubility and bioavailability of drugs.

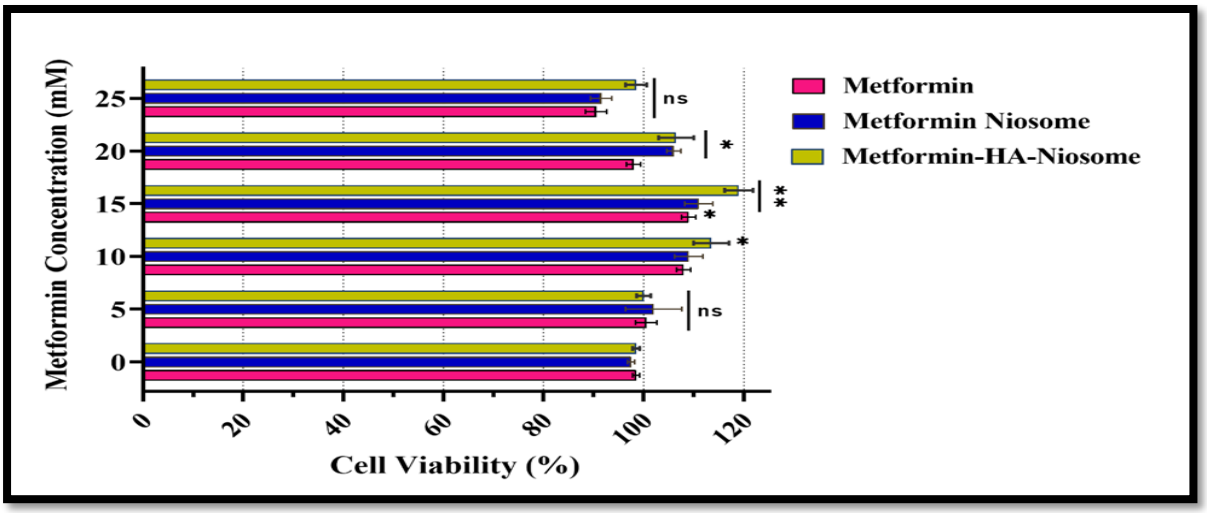


Fig. (3-11): The effect of metformin, Nio-met, and Hyalo-Nio-met NPs toward PBMCs.

3.2.3. Evaluation of Total Oxidant Species in PBMCs

Figure 3-12.TOS levels change in untreated and treated PBMCs of RA and T2DM with metformin, Nio-met NPs, and Hyalo-Nio-met NPs. The Nio-met treated group showed increased reduction compared to the free form of metformin and eventually, the highest reduction belonged to Hyalo-Nio-met treated group.

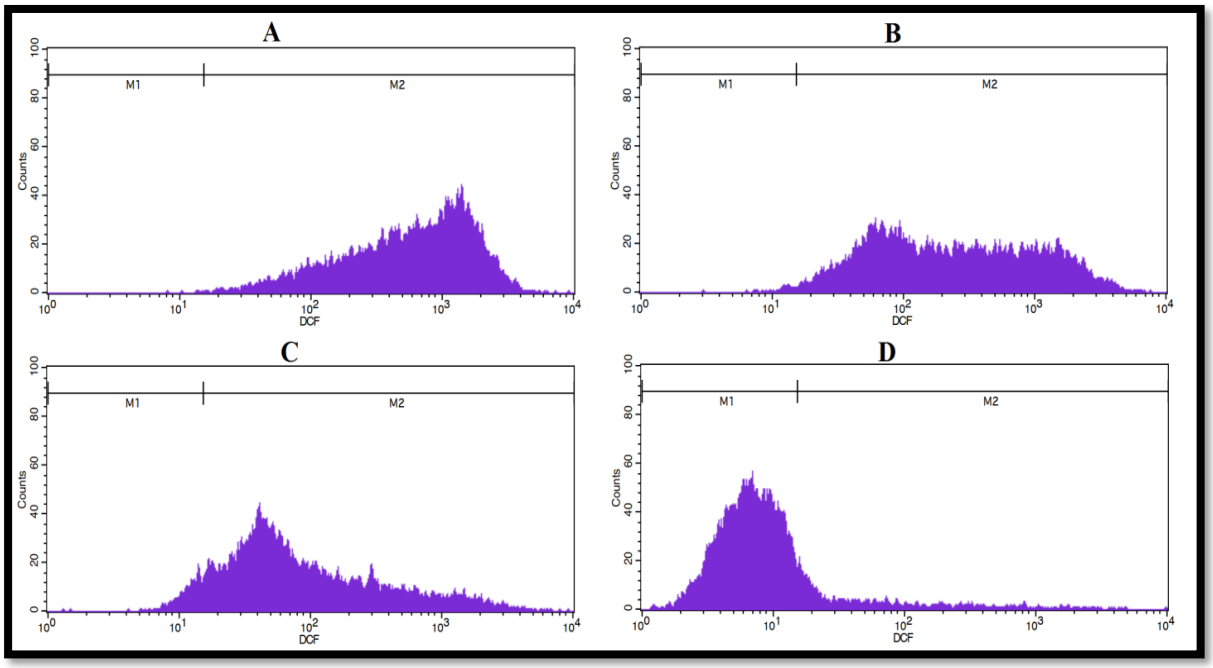


Fig. (3-12): TOS level changes in untreated and treated PBMCs with metformin, Nio-met NPs, and Hyalo-Nio-met NPs. A) Untreated PBMCs, B) Metformin, C) Nio-met NPs, D) Hyalo-Nio-met NPs

The IL-23 and TGF-β levels in PBMCs are illustrated in figure (3-13). These results show a decrease in IL-23 level and an increase in TGF-β level in treated cells.

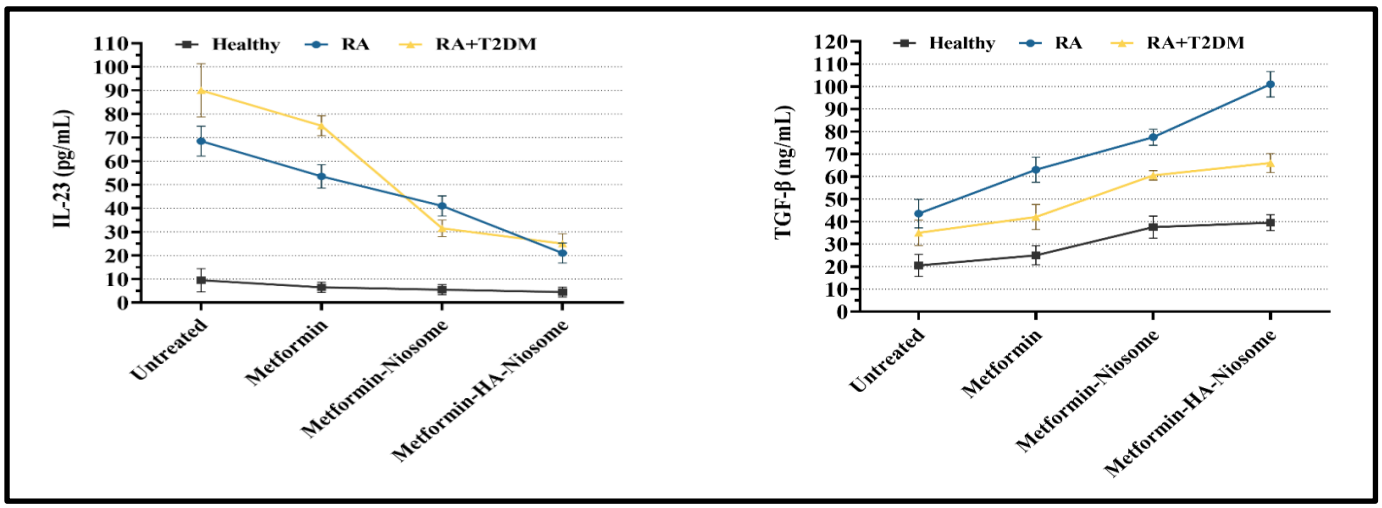


Fig. (3-13): IL-23 and TGF-β levels changes in untreated and treated PBMCs with metformin, Nio-met NPs, and Hyalo-Nio-met NPs.

3.3. Part III (Molecular studies result)

3.3.1 The Effect of the Drugs on the Genes Expression in the PBMCs

Changes in the expression of NFATC1, and RANKL genes in untreated and treated PBMCs with metformin, Nio-met NPs, and Hyalo-Nio-met NPs in RA patients (with and without T2DM).The effect of the drugs on the NFATc1, and RANKL, gene expression in the PBMCs isolated from RA patients (A) and RA patients with T2DM (B). Expressions reduced.in figure 3-14.

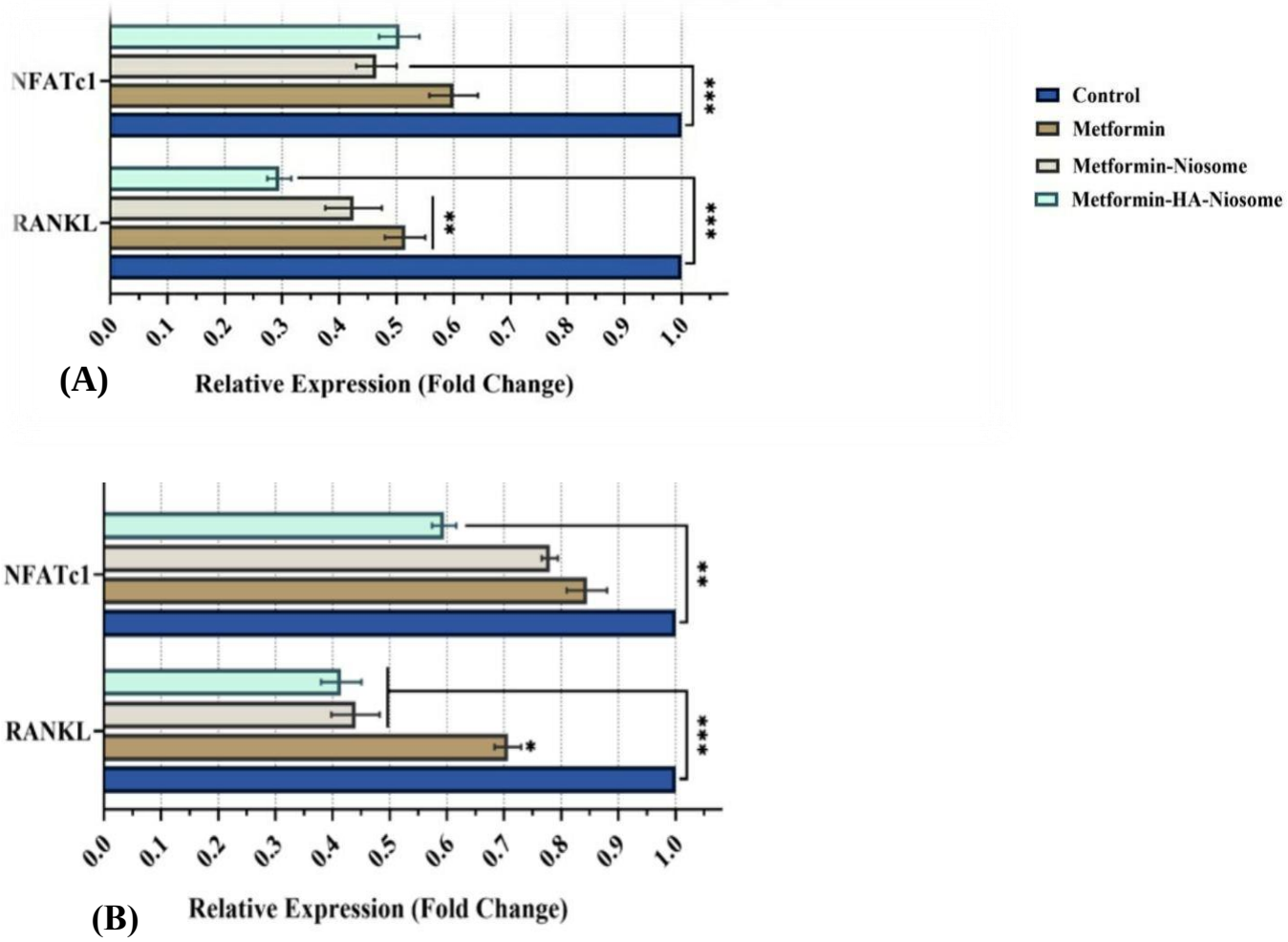


Fig (3-14): The effect of the drugs on the NFATc1, RANKL, and genes expression in the PBMCs isolated from RA patients (A) and RA patients with T2DM (B). p value < 0.001 ***, p value < 0.01 **, and p value < 0.1 *

Chapter Four
Discussion

4. Discussion

Inflammatory processes play a pivotal role in the pathogenesis of RA. Markers of inflammation such as CRP, interleukin (IL-23), ESR, FBS, insulin level, and insulin resistance (HOMA-IR), play an important role in the pathophysiology of RA (**Arias-de la Rosa, I.,** ***et al.,*** **2022; Shrivastava,** ***et al.,*** **2013**). CRP is a general marker of systemic inflammation and is elevated in patients with RA. IL-23 is the key cytokine controlling inflammation in peripheral tissues leading to the development of autoimmune diseases (**Adalwahab, K. E. A. 2023.** ***et al,*** **2023**). TGF-β activation leads to functional immunomodulatory effects according to environmental conditions. The function of TGF-β in the development of arthritis in murine models has been extensively studied with controversial results (**Kwon,** ***et al.,*** **2022)** Some studies reported a higher frequency of increased CRP concentrations in sera of RA patients before the onset of the disease (**Pope,** ***et al.,*** **2021**).

4.1 Part I (Clinical chemistry disscation)

4.1.1. Anthropometric Parameters and Rheumatoid Arthritis

In the current study, the observed results indicated descriptive data between RA patients and control with the age in which the age (40-49) years old highly percentage (42.3%) and more susceptibility to RA, these results agreed with those of an Egyptian study which reported of age between RA patients and control that recorded by the study **(Mazen,** ***et al.,*** **2018**). BMI groups show in higher percentage (43.7%) in overweight patients than other BMI groups, these results agree with that recorded by the study (**Atri, A.,** ***et al.,*** **2020**), (**Vanessa,** ***et al.,*** **2021**).

4.1.2. Biomarkers Studied in Sera of Rheumatoid Arthritis

Different biomarkers in sera of RA patients and control studied and the observed results indicated a highly significant association for more than one parameter (ESR, IL-23, CRP, and TGF-β). The current study showed that the mean ± SD of TGF-β was highly significantly difference between patients and the control group (P<0.001) in which its levels increased and reached (40.56 ± 16.35 pg/mL) in RA patients without T2DM, while in sera of RA patients with T2DM was (39.20 ± 16.11 pg/mL) as compared with the healthy control group (4.42 ± 2.79 pg/mL), therefore, inhibition TGF-β play role in the treatment of RA and T2DM because of it's a multifunctional cytokine that regulates cell growth, inflammation, and angiogenesis by acting on various cell types, and these data agree with result of study **(Ashrafizadeh, M., *et al* ., 2020; Rahim, K., *et al* ., 2024).**

These results are consistent with other investigations that reported the elevation of TGF-β in RA cases. The reason behind that is activated macrophages which are the major producers of inflammatory cytokines, including tumor necrosis factor-alpha (TNF–α). This, in turn, induces the release of other inflammatory cytokines such as interleukin (IL)-1, IL-6, or IL-8, involved in angiogenesis and the proliferation of the synovial fibroblasts and the production of factor platelet-derived growth (PDGF), fibroblast growth factor (FGF) and TGF–β. The sustained activation of these cell types leads to RA's structural alterations **(Cavallo, C., *et al.,* 2023).**

The mean ± SD of the ESR level observed was (50.11 ± 14.18 mm/hr) in RA patients without T2DM and (52.82 ± 12.65 mm/hr) in RA patients with T2DM, while its level in control was (14.17 ± 4.84 mm/hr) which highly significant association in between (p<0.001), this study agreed with that recorded by others study (**Khadim, *et al.,* 2021**).

On the other hand, increasing the inflammatory markers IL-23 and CRP in RA with T2DM was also consistence with other studies (**Jiayu *et al*.,2018**) since both diseases are inflammatory, accumulating evidence corroborates the crucial role of inflammation in T2DM pathologies (**Kanmani, *et al*.,2019)**. Low-grade inflammation characterized by elevated inflammatory protein levels, including C-reactive protein (CRP), is linked with T2DM and RA pathogenesis (**Li, et al., 2023).** The current study showed that the mean ± SD of CRP was highly significant ($p<0.001$) in RA patients as compared with healthy control (5.98±1.89 mg/L) without T2DM as compared with (12.09±3.53 mg/L) in RA patients with T2DM and control (0.431 ± 0.22 mg/L). These data obtained in agreement with others recorded by each of the studies **(Shrivastava, *et al.,* 2015; Khadim, *et al.,* 2021**) respectively. CRP is a well-established marker of systemic inflammation. Higher CRP levels in the RA with T2DM group indicate a more pronounced inflammatory response compared to RA alone **(Wu, et al., 2024).** The production of CRP may be triggered by many metabolic and inflammatory factors associated with the development of T2DM, such as increased blood glucose, adipokines, and free fatty acid levels. In addition, an increased level of CRP represents a reliable predictor of vascular complications and progression of such cases (**Stanimirovic, *et al.,* 2022).**

IL-23 plays a role in the local inflammation of the islets. In both T1DM and T2DM patients, there is an increased production of IL-23 by peripheral blood mononuclear cells (PBMCs) surrounding the islets (**Rezaeepoor, *et al.,* 2020).** Furthermore, an up-regulation of the IL-23 subunit was observed in pancreatic islets from individuals with T2DM **(Mehdi, S. F., *et al.,* 2023).** Mutations in the IL-23 gene have also been linked to the development of T2DM **(Luo, *et al.,* 2024).** A previous study reported an elevated levels of circulating IL-23 in individuals with T2DM, while the current study indicates the mean ± SD of IL-23 was highly significant ($P<0.001$) in RA patients without T2DM

(41.32 ± 14.15 pg/mL) as compared to with RA with T2DM (57.27 ± 15.86 pg/mL) and control (9.16 ± 1.01 pg/mL) and these data agreed with other results performed by another study (**Doaa, *et al.,* 2015**). Elevated IL-23 might be reflected by their central role in the development and maintenance of autoimmune diseases like RA. Increased IL-23 levels might contribute to enhanced activation and proliferation of immune cells that damage joint tissues in RA patients with T2DM **(Xiong, *et al.,* 2022)**, also this finding suggests that circulating levels of IL-23 may be associated with the progression of diabetes. This is consistent with the findings of the study **(von Stebut, et al., 2020**).IL-17A in psoriasis and beyond cardiovascular and metabolic implications.Who reported that genetic variations in IL-23 linked to the severity of T2DM **(Luo, *et al.,* 2024).**

This study shows the mean ± SD of insulin level and HOMA-IR which significantly different between patients and control (P<0.001), insulin level and insulin resistance non-significantly different between RA patients without T2DM (11.24 ± 1.96 µU/mL), and that observed in RA patients withT2DM (11.87 ± 1.97 µU/mL), and both of them significantly lower than that in control (13.04 ± 2.31 µU/mL) for insulin, while the mean ± SD of HOMA-IR in RA patients without T2DM (2.96 ± 0.55 %) and in RA patients with T2DM (9.8 ± 3.72%) as compared with control (3.37 ± 0.78%), these data give a prediction of the possible role of insulin in contributing to the induction of an abnormal level of immune response may be speculated and prognosis during rheumatic diseases, this study agree with (**Claudia Di Muzio, *et al* ., 2022**), also indicate the immune response may be play role of evidence of pathogenesis of Insulin resistance and T2DM. Therefore, common therapeutic targets may exist in patients with RA and concomitant T2DM proposing a possible “bidirectional” treatment, this study agree with the study (**Ruscitti, *et al.*, 2018; Giacomelli, *et al.,* 2016).** For (HOMA-IR**),** both RA groups had higher HOMA-IR values as

compared to controls, indicating greater insulin resistance, while control group shown higher insulin sensitivity levels. These results agreed with other several studies that have reported the presence of IR and impaired b-cell function in RA patients **(Ye, *et al*., 2022**). The potential effects of RA medication on IR may be overlooked in these studies. Indeed, although the effect of non-steroidal anti-inflammatory drugs (NSAIDs) on IR is quite limited **(Hokstad, 2023).** The use of glucocorticoids alters glucose metabolism.(**Erlandsson, *et al, 2024*).**Reported that short-term treatment with low daily dose glucocorticoids resulted in enhanced β-cell function due to its anti-inflammatory effects, while another study indicated that chronic glucocorticoid use might lead to decreased insulin sensitivity, impaired glucose tolerance (IGT), and β-cell function in RA patients**.** Moreover, disease-modifying anti-rheumatic drugs (DMARDs) and anti-TNF-α therapy significantly reduced homeostasis model assessment of IR (HOMA-IR) (**Dessein, *et al*., 2002**). RA with T2DM group displayed significantly elevated fasting blood sugar FBS compared to controls and the RA group. The same group had markedly higher HbA1c% levels, a marker for long-term blood sugar control, compared to both controls and the RA group.

Diabetes mellitus is a long-standing metabolic disease, which signalizes raised sugar levels in the blood. The disease progressively leads to damage to various other organs **(Saeed, *et al*., 2021).** The potential mechanisms might be due to the chronic hyperglycemia which might contribute to increased inflammatory pathways, potentially influencing the production of IL-23 and other pro-inflammatory cytokines. On the other hand, the dysfunction of immune system, T2DM can lead to abnormalities in the immune system, potentially creating an environment that promotes the autoimmune response seen in RA. Also, the increasing level of such markers in RA with T2DM patients could be due to the shared inflammatory pathways. There might be a degree of overlap in the inflammatory mechanisms underlying both T2DM

and RA, leading to a more pronounced inflammatory state when they co-occur (**Rachfal, *et al*., 2021)**

Glycated hemoglobin is considered a reflection of integrated glycemia over the entire 120-day lifespan of the red blood cell; the levels of HbA1c are strongly correlated with FBS. HbA1c provides a reliable measure of chronic glycemia and correlates well with the risk of long-term diabetes, it is an ideal tool for monitoring and chronic management of diabetes **(Mukherjee, S., *et al.,* 2024).** Among diabetics, the blood glucose levels increase in the blood and the glucose attaches to the hemoglobin molecule in a concentration-dependent manner. The glucose-bound HbA1c provides the average glucose levels in an individual's blood as it becomes glycated with the hemoglobin. It is important to note that the HbA1c levels are directly proportional to the blood glucose levels **(Dunn, *et al.,* 2023).** T2DM manifests itself in terms of hyperglycemia due to compromised insulin production (**Moustaki, *et al*., 2023).** The significance of the HbA1c test lies in the diagnosis and the prognosis of diabetes patients, which lends it to a detailed understanding of insulin and insulin resistance. There is a direct correlation between HbA1c and insulin resistance, where HbA1c is more strongly associated with insulin sensitivity in healthy subjects with normal glucose tolerance **(Lin, *et al.,* 2014).**

In the current study, results showed impairment in blood sugar control and insulin resistance in RA with T2DM. These findings suggested a clear link between RA with comorbid T2DM and impaired blood sugar control, along with potential underlying insulin resistance. These results are consistency with other observations of the previous studies. As reported before significantly higher FBG and HbA1c% levels found in the RA with T2DM group as compared to RA alone indicate poorly controlled blood sugar. HbA1c reflects average blood sugar levels over a longer period, confirming chronic hyperglycemia (**Curt and Rohlfing, 2020).** Individuals with rheumatoid

arthritis are at a higher risk for elevated HbA1c levels and the development of T2DM, primarily due to the inflammatory processes associated with RA. Effective management of RA not only alleviates joint symptoms but may also improve glycemic control, highlighting the interconnectedness of these conditions (**Baker, *et al.,* 2021).** The chronic inflammation in RA might contribute to insulin resistance. Inflammatory molecules can interfere with insulin signaling pathways, leading to impaired glucose uptake by cells. Certain factors, like obesity and genetic predisposition, might increase the risk of both RA and T2DM, potentially explaining the observed association (**Rohm, *et al*., 2022).** Despite insulin resistance, similar insulin levels in both groups could be due to several factors such as the pancreas in RA with T2DM patients might be secreting more insulin to compensate for the resistance, resulting in similar measured levels despite dysfunction.

4.1.3. Antioxidant Status and Rheumatoid Arthritis

Antioxidants of vitamins such as Vit. E was a related reduction of blood glucose as well as glycated hemoglobin compared with chemical drugs (**Maria, Balbi, *et al.,* 2018**). Regarding antioxidant biomarkers, vitamin E was to be in both RA groups had lower levels as compared to controls. In this study, the mean ± SD of vitamin E and selenium non-significantly ($P>0.05$), decreased in RA patients with T2DM and reached (2.85 ± 1.14 mg/L), but the level of these was lower than that found in RA with T2DM patients (3.03 ± 0.92 mg/L), and both of them significantly lower than that obtained in healthy control (10.64 ± 3.60 mg/L), ($P<0.001$), although of vitamin E related of reduction of blood sugar and HbA1c% in diabetes patients, therefore, this vitamin is very important to prevent complicated of T2DM to progress to T1DM and very important to prevent development of RA to T2DM (**Maria, *et al* ., 2018).**

Decreased Vit. E and Se level also consistence with previous study; low levels of vitamin E may therefore have a detrimental effect in inflammatory arthritis. Previous results also indicated that the mean synovial fluid concentrations of α-Tocopherol in patients with inflammatory arthritis is significantly lower than that found in controls suggesting that α-Tocopherol is consumed within the inflamed joint. Chronic inflammation can affect serum antioxidant vitamin levels in RA patients elevated risk of RA was seen among adults with low serum levels of selenium and α-Tocopherol at baseline (**Zamani, B., *et al.*, 2024).** Another antioxidant used in the present study was selenium, which was significant when compared with control ($P<0.001$), but showed no significance when compared between RA patients who had and didn't have T2DM ($P>0.05$), mean and SD of selenium in RA with T2DM patient was (66.62 ± 12.88 mgc/L) while the control (140.56±15.26 mgc/L) and mean of RA patient without T2DM was (70.54 ± 11.16mgc/L), many resource shown the selenium protective role against oxidative stress, selenium has drawn increasing attention for preventing T2DM, therefore, the data of this study showed the important role of selenium in a patient with RA to prevent development and progression of RA and in a patient with T2DM to prevent the increase of glucose in blood and development to T2DM. This study agrees with the study **(Rayman, *et al.*, 2012; Xin-liang, *et al.*, 2015).** The decreased level of antioxidant micronutrients might be due to the high consumed and because it acts as a scavenger of reactive O_2 radicals which may protect against free radical-mediated tissue damage in an inflamed joint **(Jena, *et al.*, 2023).** Antioxidants may also protect against the development of RA. Vitamin E (α-Tocopherol) is the major lipid-soluble, chain-breaking antioxidant found in biologic membranes. Lipids are important constituents of normal synovial fluid, and alterations in synovial lipids can occur in RA (**Valgimigli, L., 2023**). Low levels of vitamin E may therefore have a detrimental effect in inflammatory arthritis. Observational case– control and cohort studies suggest an inverse

relationship between vitamin E and the risk of developing RA **(Nikiphorou, E., & Philippou, E. 2023).** Results had shown a decreased level of Vitamin E and Selenium levels in RA with T2DM more than RA without T2DM, but they not significantly affected by the presence of (T2DM) in patients with (RA). Hyperinsulinemia, hyperglycemia, and inflammation that cause oxidative stress all cause decreased level of the antioxidant.

Selenium (Se) is a crucial element for a variety of biological processes, including the protection against inflammation and function as avital enzyme for redox homeostasis (**Zhang, *et al* ., 2017**). A low level of Se impairs important metabolic processes by increasing ROS and decreasing mitochondrial function **(Shimada *et al* ., 2021).** Numerous studies have suggested altered Se concentrations and increased metabolic needs for Se in patients with diabetes, which is characterized by a hyperglycemic state that may lead to inflammatory activation**. (Wang, *et al*., 2020)** These findings might confirm that vitamin E and selenium are generally expected to be protective against inflammatory conditions like RA.

4.1.4. Correlation Coefficient between Biomarkers Various Groups.

The correlation study showed many significant correlations among the measured parameters, the conducting study was to find correlation coefficient to determining linear of different biomarkers between groups of RA without T2DM and RA with T2DM, the study findings highlight a potential link between IL-23, inflammation CRP, and HOMA-IR in patients with RA and comorbid (T2DM). This indicated that higher IL-23 levels coincide with increased inflammation (CRP) and insulin resistance (HOMA-IR). IL-23 is known to play a crucial role in promoting inflammatory responses in autoimmune diseases like RA (**Tian, *et al.*, 2016).** Chronic inflammation can

disrupt insulin signaling pathways, leading to insulin resistance (**Soltani, *et al.*, 2018**). The positive correlation between IL-23, CRP, and HOMA-IR suggests a potential interplay between these factors in RA with T2DM patients. Here's a possible explanation, Increased IL-23 levels might contribute to the inflammatory processes underlying RA. The result of IL-23 levels was highly significant and a positive correlation coefficient (P-value<0.001, r=0.7) between RA without diabetes and with T2DM which is associated with autoimmune diseases, because the IL23 has a central role in autoimmunity disease (**Korta, *et al.*,2023).,** therefore IL-23 levels may be considered as a biomarker used for the diagnosis of RA, and this data is consistent with other studies (**Crînguș, *et al*.,2023; Korta, et al.,2023).**

In the current study, the correlation of insulin levels between RA patients without T2DM and other patients with T2DM was studied which indicates a non-significant and weak correlation coefficient in RA patients with T2DM (p-value>0.05, r=0.13), which may cause a small sample size in this study leading to the weak correlation of insulin between to patients (with and without T2DM), also increasing in risk of developing T2DM may be due to an inflammatory condition associated with RA and this result was inconsistent with other reports of study (**Fabio*, et al* ., 2023; Baker, *et al* ., 2021**).

The result found in glucose level was highly significant in RA patients with T2DM (P-value <0.001, r=0.6) because of a dynamic interaction between glucose and insulin levels to predict insulin sensitivity or β cell production, these results agreed and consistent with another study **(Rasouli, *et al*., 2024).**

Inflammation and insulin resistance chronic inflammation can disrupt insulin signaling, leading to insulin resistance and impaired blood sugar control. This could explain the positive correlation between each other. CRP serves as a marker of systemic inflammation. The correlation between IL-23 and CRP strengthens the notion of an active inflammatory state in RA with

T2DM patients with higher IL-23 levels **(Janet, *et al*., 2020).** This study also shed light on the potential interplay between inflammation, insulin resistance, and blood sugar control in patients with RA and comorbid T2DM. Significant positive correlations were observed between CRP, a marker of inflammation, and Insulin Resistance (HOMA-IR) with Fasting Blood Sugar (FBG) and Hemoglobin A1c (HbA1c). This suggests that higher levels of inflammation and insulin resistance coincide with poorer blood sugar control, as reflected by elevated FBG and HbA1c. Poorly controlled blood sugar (high FBG and HbA1c) can contribute to a pro-inflammatory state, potentially forming a vicious cycle. Interestingly, CRP and HOMA-IR showed negative correlations with Transforming Growth Factor-beta (TGF-β). This suggests that higher levels of inflammation and insulin resistance might be associated with lower levels of TGF-β. That might be due to the complex role of TGF-β as anti-inflammatory properties. A decrease in TGF-β with increasing inflammation (CRP) could be the body's attempt to counteract the ongoing inflammatory process. The relationship between TGF-β and inflammation may vary depending on the disease stage. Higher TGF-β might be present in earlier stages, followed by a decrease as inflammation becomes more established **(Chen, *et al.,* 2023).**

Identifying patient's RA with T2DM who have elevated CRP and HOMA-IR might be crucial for implementing stricter blood sugar control measures and potentially managing inflammation.

All analyzed markers (IL-23, TGF-β, CRP, and ESR) showed significantly increased odds ratios (OR) for RA compared to the control group (p-value < 0.001). This indicates a strong association between elevated levels of these markers and the presence of RA.

Interestingly, the OR values for IL-23, CRP, and ESR nearly identical for both RA (with and without T2DM) groups. Chronic inflammation in synovial

joint leads to systemic complication and destruction of joints **(Pope JE*, et al.*, 2020)**. The disease activity score in RA patients can be measured using both CRP and ESR **(D'Cruz*, et al.*, 2020).**

The possible explanations of elevated IL-23, TGF-β, CRP, and ESR all associated with inflammation. Their increased levels in RA patients suggest a link between ongoing inflammatory processes and the disease. While T2DM is linked to inflammation, it might significantly affect the specific inflammatory pathways reflected by IL-23, CRP, and ESR in the context of RA **(Dessi and, Tadesse, 2021)**. Interestingly, the treatment of RA with biological disease-modifying antirheumatic drugs (bDMARDs) has been shown to improve glycemic control in patients who also have T2DM. For instance, a study found significant reductions in HbA1c levels following the initiation of bDMARD therapy, suggesting that effective management of RA can have a beneficial effect on blood sugar levels **(Lin. *et al.*, 2020**).

These results are in line with the previous study, it has been reported that several cytokines have been implicated in the pathogenesis of RA as they up-regulate the inflammatory cascade, resulting in synovial proliferation, osteoclastic bone resorption, and joint erosion. T cells, particularly T helper 1 cells (Th1) and their associated cytokines play a crucial role in the initiation and progression of RA. More recently, T helper and their cytokine family, including IL-23 and IL-17, have gained attention as a critical pathway in disease development **(Meher, *et al.*, 2023**). They are also associated with disease duration, disease severity, joint erosion, and functional outcomes **(Niu, 2014)**. Another study found that the serum level of IL-23 was higher in early RA compared to chronic RA and healthy **(Meher, *et al.*, 2023).** IL-23 has been implicated in the pathogenesis of erosive arthritis in RA, with serum IL-23 levels directly correlating with radiological severity grading in RA patients (**Selimov, *et al.*, 2023).**

Increasing TGF in such cases has been confirmed before, previous study showed that TGF has been considered an important cytokine in RA with pro- and anti-inflammatory effects **(Klück, *et al.,* 2023).** TGF-b1 is the most abundant isoform in mammals **(Morris-Stiff, 2005**), and it is present in the synovial tissue of RA patients. In addition, it has regulatory effects on lymphocytes, dendritic cells, macrophages, chondrocytes, and osteoblasts, which are important cells in RA pathogenesis. Moreover, the TGF-b has chemotactic properties with the capacity to stimulate cells to produce cytokines **(Pokorny, *et al.,* 2021)**. The association of CRP was consistence with other findings since it has confirmed that higher CRP showed a high grade of systemic inflammation in RA patients. C-reactive protein was elevated in rheumatoid factor-positive patients (**Dessie, *et al.*, 2021)**.

Vitamin E, which is an important lipid-soluble antioxidant, is transported in serum with serum lipids. Previous results had shown that vitamin E levels in the serum of RA patients were significantly lower than the controls. This report was confirmed by other studies. The low concentrations of this antioxidant may in some way relate to the development of RA disease, either directly or as associations with etiologic factors (**Sil, *et al.,* 2014**).Have demonstrated that a low antioxidant level was a risk factor for development of RA disease. It is possible that vitamin E was consumed in the process of counterbalancing the free radicals. A decrease in plasma vitamin E was also observed in juvenile rheumatoid arthritis. Endogenous antioxidants protect cellular systems from the damaging effects of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Vitamin E (α-Tocopherol) has lipid-soluble properties that allow it to act as a chain-breaking reagent in lipid peroxidation. Vitamin E intercalated in cellular membranes plays an important role in reducing lipid hydroperoxides and is probably the best hydrophobic scavenger known **(Vasanthi, *el al.*, 2009).**

The association of Se was also consistence with previous studies, it has been reported that a decrease in Se concentration occurs with a slight increase in the CRP concentration. Likewise, the systemic inflammatory response has been associated with a reduction of up to 48% in plasma Se concentration when increased CRP (**Ghashut, *et al*. 2017)**. The low concentration of Se can deplete circulating antioxidants and thus exacerbate the inflammatory state of the disease through uncontrolled ROS production **(Heyland, *et al. 2017).***

4.1.5. Results of ROC Analysis

The purpose of ROC analysis is relatively better performance (accuracy and reliability) for the diagnosis of study markers. The ROC results for most of the study markers, especially IL-23, TGF-β, HbA1C, and FBS for the group of people with RA and the RA group with T2DM, showed high specificity and sensitivity. Therefore, these signs are considered strong diagnostic markers for both RA patients as well as obese arthritis patients.

Results of the receiver operating curve (ROC) curve and AUC analysis for the Insulin besides as possible diagnostic parameters.IL-23 and TGF showed good diagnostic performance for predication of the RA (with or without T2DM) compared to the control groups between discharges and passed. For IL-23 levels: (sensitivity 70.4%, specificity 70.2%) at a level = 22.465mg\dl, TGF levels: (sensitivity 69%, specificity 72.3%) at a level = 26.291mg\dl, the p-values of the AUC <0.05 and statistically significant. Youden's J statistics of the parameters.

Results of the receiver operating curve (ROC) curve and AUC analysis for IL-23 showed good diagnostic performance for predication of the RA (with T2DM) compared to the control groups between discharges and passed, For IL-23 levels: (sensitivity 57.6%, specificity 80.1%) at a level = 29.91pg\mL.

Results of the receiver operating curve (ROC) curve and AUC analysis for IL-23 showed predication of the RA (without T2DM) compared to the control groups between discharges and passed for IL-23 levels: (sensitivity 63.2%, specificity 68.1%) at a level = 21.37pg\mL.

Results of the receiver operating curve (ROC) curve and AUC analysis for TGF-β showed good diagnostic performance for predication of the RA (with T2DM) compared to the control groups between discharges and passed, For TGF levels: (sensitivity 69.7%, specificity 66%) at a level = 25.3pg\mL.

Results of the receiver operating curve (ROC) curve and AUC analysis for TGF-β showed good diagnostic performance for predication of the RA (without T2DM) compared to the control groups between discharges and passed for TGF levels: (sensitivity 52.6%, specificity 76.6%) at a level = 30.07mg\dL

Results of the receiver operating curve (ROC) curve and AUC analysis for the HbA1c and FBS, showed highly significant (P<0.001) for HbA1c, and FBS, which showed good diagnostic performance for predication of the (RA without T2DM) compared to the control groups between discharges and passed, at a cut-off 4.85 of HbA1c mmol/ml, a cut-off of FBS 110.5 mg/dL, all these markers performed well for current disease.

An HbA1c of 6.5% is recommended as the cut point for diagnosing diabetes. A value less than 6.5% does not exclude diabetes diagnosed using glucose tests. (**WHO, 2011**). Our study revealed preference of results of RA patients predict for diabetes of patients to prevent development to T2DM with highly specificity 78.7% for each of HbA1c while 93.6% for FBS, and sensitivity 69% for FBS, 92.2% for HbA1c, and accuracy was (80% for HbA1c, 85% for FBS, this study agree with (**Pulak Kanti Palit, *et al.*, 2021**) and **(Eun Young Choi, *et al.*, 2011**).

Results of the receiver operating curve (ROC) curve and AUC analysis for TGF-β showed good diagnostic performance for predication of the RA (with

T2DM) compared to the control groups between discharges and passed for TGF-β levels: (sensitivity 72.7%, specificity 61.7%) at a level = 40.344.

4.2. Part II (Nano studies disscation)

4.2.1 Role of Nanoparticles in Rheumatoid Arthritis

Studies have revealed that metformin has an antidiabetic role, by suppressing hepatic glucose production (**Foretz, *et al.*, 2019**). Metformin is widely prescribed for type 2 diabetes as it effectively controls blood sugar levels and provides cardioprotective protection. Moreover, studies suggest that metformin can help manage metabolism and potentially treat conditions like RA by regulating metabolism (**Jiang, *et al.*, 2020**). Enhancing the effects of metformin using NPs for drug delivery shows promise by optimizing controlled release improving drug targeting and reducing effects **(Tran, *et al.*, 2017).**

These carriers enhance drug penetration into tissues, specificity and drug availability making them ideal for targeted delivery (**Upadhyay, *et al.*, 2015**). Nanocarriers also allow drug release boosting treatment effectiveness (**Xiao, *et al.*, 2006**). Niosomes, a type of ionic surfactant vesicles has garnered attention in the pharmaceutical industry due to their ability to encapsulate both hydrophilic and hydrophobic agents **(Ge, *et al.*, 2019)**. These synthetic vesicles, formed by self-assembly of nonionic surfactants, cholesterol, and some other lipids, offer benefits like breakdown in the body and compatibility with living tissue (**Ag Seleci, *et al.*, 2016**). Researchers have utilized these nanoparticles in various applications, including transdermal drug delivery (**Alnaim, *et al.*, 2023**), gene delivery (**Al Qtaish, *et al.*, 2020**), and targeted drug delivery to immune-privileged tissues (**Haddadian, *et al.*, 2022**). Scientists have investigated methods to enhance nanocarriers, for drug delivery purposes. For example, the development of folic acid-modified nano-drug carriers has shown promise in the targeted delivery of specific drugs (**Jing, *et al.*, 2021**). Polydispersity Index (PDI) is recognized as another key factor in assessing the stability and

functionality of NPs for drug delivery applications (**Wilhelm Romero, *et al.*, 2021**). PDI significantly influences various aspects of nanoparticles, including their stability, drug release kinetics, cellular uptake, and biodistribution (**Mato, *et al.*, 2015**). Table (3-11) that maintaining a low PDI (< 0.4) is crucial to achieving a narrow size distribution, which is essential for effective tissue accumulation and renal clearance. According to the DLS results listed in Table 3-11, the blank noisome, Nio-met, and Hyalo-Nio met NPs are all within the acceptable range in terms of size, zeta potential, and PDI values The size of nanoparticles plays a crucial role in their uptake and retention by cells and tissues **(Khan, *et al.*, 2022).** Figure 3-7 for intravenously administered nanoparticles, diameter is a key factor affecting pharmacokinetics and biodistribution of pores in blood vessels (**Albanese, *et al.*, 2012**). The size of nanoparticles influences their ability to overcome transport barriers in biological tissues, affecting their tissue penetration efficacy **(Islam, *et al.*, 2017).**

Additionally, nanoparticle size impacts their biological activity, with factors like concentration and size playing primary roles **(Churilov, *et al.*, 2020).** The interaction of nanoparticles with biological systems is controlled by various factors, including size, shape, and surface properties (**Kenry**, ***et al.*, 2020**). The shape of nanoparticles has been shown to have significant effects on their interactions with biological materials such as cells and tissues (**Liebert, *et al.*, 2011**). Figer 3-8 The morphology of NPs can impact their biological activities, such as cell membrane penetration, wetting, and interactions with proteins (**Chaudhary, *et al.*, (2020); Ge, *et al.*, 2015).** Furthermore, the morphology of biologically synthesized nanoparticles can influence their toxicity, mechanism of action, and applications as antibacterial and antifungal agents **(Guilger-Casagrande, *et al.*,2021**; **Rozhin, *et al.*,2021)** The SEM images of fabricated blank noisome, Nio-met, and Hyalo-Nio-met NPs. All these nanoparticles show a spherical morphology, with the only difference

between them being their size. These images are consistent with previous DLS results.

Fourier Transform Infrared Spectroscopy (FTIR) is an effective analytical method used for detecting functional chemical groups and characterizing covalent bonding information. Figure 3-9 for cholesterol, a notable peak at 3435 cm^{-1} indicated OH stretching. In the blank niosomes' spectrum, the OH stretching peak from Span 60 was seen at 3388 cm^{-1}, while the carbonyl dimer shifted to 2918 cm^{-1} and the C = O stretching peak shifted to 1737 cm^{-1}. These shifts in the carbonyl groups' peaks suggest hydrogen bonding between Span and cholesterol, indicative of noisome formation. In the spectrum of drug-loaded niosomes, significant peaks were observed at 3370, 2920, and 1738 cm^{-1}, likely representing OH stretching, carbonyl dimer, and C = O stretching. These shifts are similar to those seen in blank niosomes and point to interactions that facilitate niosomal formation (**Zaid Alkilani, *et al.*, 2022**). The FTIR spectrum of cholesterol exhibited a broad band at 3432 cm^{-1}, indicating OH stretching vibration. Symmetric and asymmetric stretching vibrations in CH2 groups of alkyl chains were observed at 2989 cm^{-1} and 2882 cm^{-1}, respectively. A strong band at 1716 cm^{-1} was attributed to the double bond in the second ring of the cholesterol structure (**Hanafy, *et al*., 2023)**

Drug release patterns play a key role in the effectiveness of drug therapy. Different controlled release systems have been created to improve the therapeutic performance of drugs (**Bhattacharjee, S. 2021).** Factors, like particle size, surface properties and the porous structure of nanoparticles all affect how drugs are released from nanoparticles **(Ways, *et al.*, 2020; Slowing, *et al.*,2007).** It is highly desirable for drugs to be released steadily from nanoparticles for medical purposes **(Javaid, *et al.*, 2021).**

Noisome, which are composed of biodegradable and non-immunogenic components, can carry both amphiphilic and lipophilic drugs, making them appealing for drug delivery (**Hazira RMN, *et al.*, 2023; Rao NN, *et al.*, 2018**).

Noisome are praised for their ability to offer a controlled release of drugs because of their characteristics **(Jadid MFS, *et al.*, 2023)**. Figure 3-10 the 120-hour release pattern of metformin from the Nio-met, and Hyalo-Nio-met NPs at 37°C and pH 7.4Both Nio-met and Hyalo-Nio-met NPs show a biphasic release pattern. The maximum release rates reached 42% and 47% within the first 12 hours of the experiment, and maximums reached 70% and 85%. followed by a subsequent decrease. This initial high release is attributed to the drug being weakly bound to the surface of the niosomal nanoparticles rather than being encapsulated inside them. MTT is a widely used method for assessing cytotoxicity, viability, and proliferation studies in cell biology (**Pintor, et al., 2020).** This is based on the ability of mitochondrial enzymes in viable cells to reduce the MTT yellow tetrazolium salt to purple formazan crystals (**Hirsch, *et al.*, 2015)**. Figure 3-11 the effect of metformin, Nio-met, and Hyalo-Nio-met NPs exhibit a negligible and insignificant proliferation effect on PBMCs at concentrations of 5 mM. As previously described, niosomal NPs can enhance treatment effectiveness by increasing the solubility and bioavailability of drugs at 10mM. Additionally, the decoration of hyaluronic acid on their surface facilitates the localization of the drug into the PBMCs, thereby enhancing the likelihood of cellular uptake and therapeutic impact. The highly significant result ($p < 0.1^*$) was observed in the Hyalo- Nio-met treated group at a 15 mM concentration. Based on these findings, 15 mM of metformin, Nio-met NPs, and Hyalo-Nio-met NPs have been selected for use in the subsequent experiments of this study **(Wu, *et al.*, 2022**). Metformin, Nio-met, and Hyalo-Nio-met NPs on PBMCs are illustrated. The Hyalo-Nio-met is composed of Span-60, cholesterol, and hyaluronic acid. Hyaluronic acid and cholesterol are both natural components found in the human body and studies confirmed their safety to normal cells. This study, investigated the potential of HA-coated niosomes for targeted delivery of metformin to PBMCs derived from both RA and T2DM patients.

4.2.2. Total oxidant status and NPs in Rheumatoid Arthritis

Total oxidant status (TOS) is highly reactive molecules that can be generated in cells through both enzymatic and non-enzymatic mechanisms (**Villaverde, *et al.*, 2019**). These molecules can interact with active substances, organic compounds, and environmental pollutants, outside the cell (**Wang, *et al.*, 2019**). Elevated levels of TOS can cause stress, a factor associated with aging and the onset of human illnesses (**Ikeda, *et al.*, 2021**). Oxidative stress happens when there is an imbalance, between TOS and antioxidants, leading to tissue damage and the chronicity of diseases (**Mateen, *et al.*, 2016)**. Furthermore, TOS has been linked to the progression and severity of RA, affecting joint tissue injury (**Zhai, *et al.*, 2018).** Figure 3-12 TOS level of untreated and treated PBMCs.The treatment of PBMCs with metformin in free form could successfully reduce the TOS level in these cells. The Nio-met treated group showed increased reduction compared to the free form of metformin and eventually, the highest reduction belonged to Hyalo-Nio-met treated group. These results can be interpreted by the role of niosomes in increasing drug solubility and bioavailability, as well as the role of hyaluronic acid in placing these nanoparticles in the vicinity of cells (**Sadeghi-Ghadi, Z., *et al*., 2021**)

4.2.3. Interleukin-23 and TGF-β of NPs in Rheumatoid Arthritis

IL-23 is a cytokine plays an essential role in different inflammatory and autoimmune conditions. Research indicates that IL 23 can activate shared receptors structurally to induce inflammatory responses (**Bloch, *et al.*, 2018**). It is also recognized as a factor in the development of RA (**Al Sheikh, *et al.*, 2018)**. In RA there is an increased presence of M1 macrophages that produce levels of IL 23 highlighting its involvement in inflammation (**Li, *et al.*, 2013)**. Figure 3-13 Studies have shown elevated levels of IL 23 in the blood of RA patients untreated of 40ng/mL and RA with T2DM was 30ng/mL closely linked

to disease activity, after treatment with metformin of RA 41ng/mL and RA with T2DM 33ng/mL, RA in metformin-HA-Niosome was 98ng/mLand RA with T2DM 60ng/mL (**Al Sheikh, *et al.,* 2018**). Elevated levels of TGF-β with treated with metformin in RA 55ng/ml and RA with T2DM 75ng/mL and in metformin-HA-Niosome of RA 70 ng/mL and RA with T2DM 90 ng/mL. TGF-β serves as a regulator in cellular functions like proliferation, differentiation, migration, cell survival, angiogenesis, and immune surveillance (**Trivedi, *et al.,* 2021).** The TGF beta signaling pathway plays a key role in cancer development and progression by influencing interactions, within the tumor microenvironment (**Gaponova, *et al*.,2020).** TGF-β contributes to increased production of extracellular matrix components and mesenchymal cell activities post inflammatory responses (**Sisto, *et al*.,2021).** It regulates fibroblast function, influences inflammatory responses, and modulates tissue repair processes in RA patients and RA with T2DM (**Li, D. 2020; Cheon, *et al.,* 2019).**

4.3. Part III (Molecular studies discussion).

Gene Expression and NPs in Rheumatoid Arthritis

The expression of various genes plays a role in RA, so the expression of some of these genes was investigated by real-time PCR. The NFATc1 gene, encoding the nuclear factor of activated T-cells c1, plays a crucial role in various biological processes. One such gene is the NFATC1 gene, which encodes the nuclear factor of activated T-cells c1. (**Jiang, *et al.,* 2023; Anwar, *et al.*, 2023).**

Additionally, NFATC1 is activated by the RANKL/RANK signaling pathway, which is regulated by proteins downstream of RANKL, such as TNF receptor-associated factor 6 (TRAF6), and NF-κB. Imbalance in the transcription and negative regulation of NFATC1 after activation is a key factor in osteoclast maturation (**Zheng, et *al.,* 2024**). Figure 3-14 studies

have shown that RANKL is highly expressed in synovial fluid B cells of RA patients and is a key cytokine involved in bone destruction (**Pang, *et al.,* 2019; Park-Min, *et al.,* 2014)**. Anti-RANKL antibody treatment has been proposed as a strategy to protect against joint destruction in RA (**Morita, *et al.*, 2019**). Changes in the expression of NFATc1 and RANKL, genes in untreated and treated PBMCs with metformin. In both groups there is no statistically significant change in NFATc1 of RA patients with 60% of metformin and with treated of metformin-HA-Niosome 50% gene expression following treatment with metformin and Nio-met NPs of RA with T2DM of treated with metformin was 85% and with Nio-met NPs was 58%. In RA patients, there is a significant decrease in RANKL was 55% gene expression following treatment with Hyalo-Nio-met NPs at 45% same results are repeated in RA patients with T2DM was 70%with metformin and treatment with metformin and Nio-met NPs was 42%. The results revealed that when metformin was exposed to PBMCs the NFATc1, and RANKL expressions reduced**.**

Conclusions and Recommendations

1. Conclusion

1. The age of onset of RA plays important role in the severity and progression of the disease, especially between ages 40-49

2. Patients who were overweight were more susceptible to developing rheumatoid arthritis (RA) compared to those who were in other groups.

3. The relationship between family history, genetics, and the pathogenesis of rheumatoid arthritis was discussed in this study, and information was provided about the effect of metformin, which is a treatment for T2DM and which has shown its effectiveness when used in RA patients, which helps to prevent the development, progress, and complication of RA.

4. There is a significant relationship between glycated hemoglobin (HbA1c) and fasting blood sugar (FBS) among patients with rheumatoid arthritis (RA), this mean relationship indicates the prognostic of importance monitoring and preventing the development of type 2 diabetes mellitus (T2DM) in RA patients.

5. IL-23, was significantly compared with all groups of study (patients and healthy control), therefore, these are more important and beneficial in monitors and early detection of disease.

6. The results of this study showed an increase in levels of biomarkers that are important for monitoring and diagnosis of developing disease biomarkers of IL23 may be useful for diagnosis of RA and monitoring to prevent RA from developing to T2DM as a cause of the increase in insulin level that leading to insulin resistance. In addition to anther routine biomarker can be assist to evaluating of progress of disease.

7. The complex role of TGF-β has anti-inflammatory properties. Low TGF-β combined with increased inflammation (CRP) has been the body's attempt to counteract the ongoing inflammatory process.

8. The current study found that the levels of antioxidants, particularly vitamin E and selenium, are significantly lower in patients with RA compared to healthy individuals, and this study indicates more fall of antioxidants in T2DM compared with patients who have only RA, which may be due to inadequate nutrition and compromised immunity in the body.

9. The study showed the highlights of the potential of hyaluronic acid-coated niosomal nanoparticles as an effective drug delivery system for metformin, targeting RA and type 2 diabetes mellitus-derived PBMCs. The Hyalo-Nio-met NPs exhibited desirable characteristics, including stability and controlled drug release. The incorporation of HA enhanced the targeting and therapeutic impact of these niosomes.

10. The synthesized Hyalo-Nio-met NPs demonstrated a significant reduction in Totel oxygen species (TOS), pro-inflammatory cytokine IL-23, and inflammation-related genes (NFATC1 and RANKL) in PBMCs in both RA patients and RA patients with T2DM. This indicates a promising reduction in inflammation and an enhancement of anti-inflammatory and antioxidant defenses. Overall, the Hyalo-Nio-met NPs drug delivery system effectively delivered metformin to PBMCs, showing potential as a novel treatment approach for RA and T2DM by reducing systemic toxicity and improving drug delivery to specific cells.

2. Recommendation

1. Large-Scale Studies: Conduct extensive research to investigate the correlation and association between T2DM and RA to prevent the progression of RA into T2DM.

2. Early detection Implement strategies for the early detection of rheumatoid arthritis to mitigate high levels of inflammation and low antioxidant levels, which contribute to the disease's progression to T2DM.

3. Conduct further studies on metformin to explore its potential as a bidirectional treatment for patients suffering from both RA and T2DM, given its beneficial effects on patient conditions.

4. Investigate the therapeutic potential of the hyaluronic acid-coated niosomal nanoparticles (Hyalo-Nio-met NPs) as a novel drug delivery system. This system has shown effectiveness in targeting peripheral blood mononuclear cells (PBMCs) and reducing inflammation.

5. Perform additional in *vivo* studies and clinical trials with larger sample sizes to validate and extend the promising results observed in preliminary studies.

6. Investigate the roles of inflammatory and anti-inflammatory biomarkers, such as IL-23 and TGF-β, in the early diagnosis and prognosis of RA, which may help in preventing its progression to T2DM.

7. Examine the efficacy of antioxidants like vitamin E and selenium in reducing blood glucose levels and their potential role in managing RA and T2DM.

8. Focus on the development of niosomal formulations that allow for controlled drug release, enhancing treatment efficacy and minimizing systemic toxicity.

9. Study the effects of specific dietary interventions on the management of RA and T2DM, focusing on antioxidants and their role in improving patient outcomes.

10. Conduct studies that assess the impact of RA and T2DM on patients' quality of life, helping to inform treatment approaches that prioritize patient well-being.

11. Explore the comparative effectiveness of traditional treatments versus innovative drug delivery systems in managing RA and T2DM to guide clinical practice.

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Appendices

Appendix I

University of Kerbala – College of Medicine

Department of Chemistry and Biochemistry

Student Name: Shatha Jassim Hatem

Supervisors:

Prof. Dr. Fadhil Jawad Al-Tu'ma - College of Medicine – University of Kerbala

Asst. Prof. Dr. Maher Abbood Mukheef – College of Medicine – University of Karbala

((Experimental Data))

Investigating Serum Levels of Osteoclastic and Inflammatory Anti- Anti-inflammatory biomarkers in Rheumatoid Arthritis Patients (with \ without) T2DM before and after Treatment with Nano-structured Metformin

Sample NO:	
Type of study: a case-control/cross-sectional	
Inclusion Criteria:	
Exclusion Criteria:.	
Rheumatoid arthritis description: severe) , moderate, slightly	
Age:	
Family History:	
Duration of RA : ( ) years	
Type of Treatment:	
Clinical Biochemical Markers :	
C-reactive protein CRP	
Rheumatoid Factor	
ESR	
HbA1C%	
Fasting blood Glucose	
Fasting Insulin	
HOMA-IR	
Total Oxidant Species (TOS)	
Interleukin 23 IL-23	
Selenium	
Vitamin E	
Transforming Growth Factor –β	
Height:	**Weight:**
BMI:	
Nanoparticles Studies	
Synthesis and characterization of hyaluronic acid-decorated loaded NPs and evaluation of RANCAL and NFATC1 gene expression in rheumatoid arthritis patients.	

Appendix II

ACR/EULAR 2010 classification criteria for rheumatoid arthritis (Bullock, *et al.* 2019).

Criteria		
Joint involvement	**1 large**	**0**
	2-10 large	**1**
	1-3 Small	**2**
	4-10 Small	**3**
	>10(at least 1 small)	**5**
Serology	**Negative RF and ACPA**	**0**
	Low positive RF/ACPA	**2**
	High positive RF\ACPA	**3**
Acute phase reactants	**Normal CRP/ESR**	**0**
	abnormal CRP/ESR	**1**
Symptom duration	**< 6 week**	**0**
	> 6 week	**1**
		Total= overall score

Rheumatoid arthritis is diagnosed when a patient's joint score is ≥ 6, indicating clinical synovitis, with symptoms categorized based on regional norms and serology results (Dekkers 2019).

Appendix III

University of Kerbala
College of Medicine
Medical Research Bioethical Committee
No: 32
Date: 2 / 11 / 2023

ETHICAL APPROVAL LETTER

Shatha Jasim Hatem
Biochemistry department\ College of Medicine \ University of Kerbala

Title of Project:
" Investigating Serum Levels of Osteoclastic and Inflammatory- Anti-Inflammatory Biomarkers in Rheumatoid Arthritis Patients (with/without) T2DM before and after Treatmrnt With Nano-Structured Metformin"

This is to certify that proposal provided have satisfactorily addressed the research bioethical guidelines..
Please consider the following requirements of approval:

1. Approval will be valid for one year. By the end of this period, if the project has been completed, abandoned, discontinued or not commenced for any reason, you are required to announce to the Committee. And you should inform the committee if the study extends over one year.
2. Please remember the Committee must be notified of any alteration to the project.
3. You must notify the Committee immediately in the event of any adverse effects on participants or of any unforeseen events that might affect continued ethical acceptability of the project.
4. Always consider the confidentiality of participants/ patients' information and/or opinions. And they must never be obligated to participate in the study and can withdraw at any time.
5. At all times you are responsible for the ethical conduct of your research in accordance with the standard bioethical guidelines.
6. The Committee should be notified if you will be applying for or have applied for internal or external funding for the above project.
7. This document does not compensate administrative or ethical approval might be required from directorate of Education in Karbala or other bodies.
8. All participants must be informed about the research issue and objectives prior to taking blood samples.
9. No cost or extra money for investigation should be charged on patients

Professor Dr. Ali A. Abutiheen
Chair, Medical Research Bioethical Committee
College of Medicine – University of Kerbala
D.r Abdulhussain sahibkadhim

الخلاصة

التهاب المفاصل الرثوي (RA): هو مرض التهابي مزمن يتميز بالتهاب الغشاء المفصلي، وتلف الغضروف، وتآكل العظام، والذي يرتبط بزيادة معدلات الإصابة بالأمراض والوفيات مقارنة مع عامة السكان. قد يساهم الالتهاب الجهازي المرتبط بالتهاب المفاصل الرثوي في خطر الإصابة بمرض السكري في المستقبل. يرتبط التهاب المفاصل الرثوي بالالتهاب المزمن، والذي قد يؤدي، جزئيًا على الأقل، إلى تطور مرض السكري من النوع 2 (T2DM). تقدم أنظمة توصيل الأدوية النانوية حلولاً واعدة لقيود العلاجات التقليدية لالتهاب المفاصل الرثوي (RA) ومرض السكري من النوع 2 (T2DM). من بين الجسيمات النانوية، تعتبر النيوسومات فعالة بشكل خاص بسبب ثباتها وسهولة تحضيرها وقدرتها على تقليل السمية الجهازية. أنها توفر إطلاقًا خاضعًا للرقابة للأدوية وتعزز قابلية ذوبان واستقرار المركبات الصيدلانية. يمكن أن يؤدي دمج حمض الهيالورونيك (HA) في النيوزومات إلى تعزيز فعاليتها من خلال استهداف خلايا معينة، وتحسين توصيل الأدوية، وزيادة التأثير العلاجي. في هذه الدراسة، يتم تقديم الميتفورمين، وهو دواء شائع من T2DM، بشكل فعال باستخدام النيوزومات المغلفة بـ HA، مما يدل على إمكانات هذا النهج في علاج RA وT2DM.

تهدف هذه الدراسة إلى تحديد المؤشرات الحيوية الالتهابية/المضادة للالتهابات للكشف المبكر عن التهاب المفاصل الرثوي والتشخيص لمنع تطور مرض T2DM، والارتباط والارتباط بين علامات المتغيرات التي لها علاقة بالمرض، واستخدام الميتفورمين، وهو دواء شائع لمرض T2DM، يتم تقديمه بفعالية باستخدام HA- النيوسومات المغلفة، لإثبات إمكانات هذا النهج في علاج RA وT2DM باستخدام الطريقة الجزيئية لـ RT-PCR لعوامل مختلفة، مثل حالة الأكسدة الكلية (TOS)، والإنترلوكين 23 (IL-23)، والعامل النووي لـ T المنشط. تم تقييم الخلايا السيتوبلازمية 1 (NFATC1)، ومنشط مستقبلات العامل النووي kappa-B ligand (RANKL) ، في كل من PBMCs المعالجة وغير المعالجة.

أجريت دراسة الحالات والشواهد على 118 مشاركاً مقسمين إلى 47 كمجموعة مراقبة، 71 مريضاً منهم 33 مريضاً مصابين بالتهاب المفاصل الرثوي مع داء السكري من النوع الثاني و38 مريضاً يعانون من التهاب المفاصل الرثوي فقط، وكان جميع المرضى مصابين بعوامل الروماتويد وكان اختبارهم إيجابياً والذين قبلوا المشاركة في الدراسة. وتمت مقابلتهم جميعاً بشكل مباشر وتم أخذ عينات الدم. يلعب العمر عند بداية التهاب المفاصل الرثوي أدوارًا مهمة في تحديد شدة المرض وتطوره، خاصة بين

الأشخاص الذين أعمارهم بين(40-49) عامًا (42.3٪). يشير هذا إلى أن هذه العوامل المرتبطة بالعمر ضرورية لتطوير استراتيجيات علاجية فعالة وإدارة الأمراض المصاحبة لدى مرضى التهاب المفاصل الرثوي.

المرضى الذين يعانون من الوزن الزايد كانوا أكثر عرضة للإصابة بالتهاب المفاصل الرثوي (RA) وكانت نسبتهم (43.7%) مقارنة مع أولئك الذين كانوا من المجموعات الأخرى. ويشير هذا إلى وجود علاقة إيجابية بين مؤشر كتلة الجسم (BMI) وخطر الإصابة بالتهاب المفاصل الرثوي.

أظهر متوسط الفرق في المؤشرات الحيوية للالتهاب لدى مرضى التهاب المفاصل الرثوي (مع وبدون T2DM) مدى متزايد لـ IL-23 وTGF مقارنة بمجموعات المراقبة السليمة.أشارت النتائج إلى وجود فرق ذو دلالة إحصائية عالية في مستويات IL-23 وTGF بين المجموعات، وكان متوسط مستويات IL-23 وTGF في المرضى أعلى بكثير من المجموعة الضابطة. توجد علاقة ذات دلالة إحصائية بين الهيموجلوبين السكري (HbA1c) وسكر الدم الصائم (FBS) لدى مرضى التهاب المفاصل الرثوي، وتشير هذه العلاقة إلى أهمية الانذارفي مراقبة ومنع تطور مرض السكري من النوع الثاني (T2DM). ومن ناحية أخرى أشارت النتائج إلى وجود فروق ذات دلالة إحصائية في مستويات الأنسولين بين المجموعات والمتوسطات والانحرافات المعيارية للأنسولين في السيطرة والتي كانت أعلى بكثير من مجموعة المريض في المرضى الذين يعانون من التهاب المفاصل الرثوي. ووجدت الدراسة الحالية أن مضادات الأكسدة التي تحتوي على فيتامين E لها علاقة بخفض نسبة الجلوكوز في الدم وكذلك الهيموجلوبين السكري مقارنة بالعقار الكيميائي. أظهرت هذه الدراسة عدم وجود معنوية لفيتامين E والسيلينيوم (P>0.05) والمتوسط و SD للفيتامين. كانت النسبة المئوية لفيتامين E لمرضى التهاب المفاصل الرثوي مع مرضى T2DM أقل مقارنة مع مرضى التهاب المفاصل الرثوي فقط ولكنها أظهرت درجات أقل بكثير مقارنة بين المرضى ومتوسط السيطرة الصحية وSD. بالمقارنة مع الأفراد الأصحاء، تشير هذه الدراسة إلى انخفاض أكبر في مضادات الأكسدة في T2DM مقارنة بالمرضى الذين يعانون من التهاب المفاصل الرثوي فقط، والذي قد يكون بسبب عدم كفاية التغذية وضعف مناعة الجسم.

وأظهرت الدراسة تسليط الضوء على إمكانات الجسيمات النانوية النانوية المغلفة بحمض الهيالورونيك كنظام فعال لتوصيل الدواء للميتفورمين، مستهدفًا التهاب المفاصل الرثوي والنوع الثاني من مرض السكري المشتق من PBMCs. أظهرت Hyalo-Nio-met NPs خصائص مرغوبة، بما في ذلك

الاستقرار وإطلاق الدواء الخاضع للرقابة. أدى دمج HA إلى تعزيز الاستهداف والتأثير العلاجي لهذه النيوسومات.

أظهرت Hyalo-Nio-met NPs المُصنَّعة انخفاضًا كبيرًا في أنواع الأكسجين (TOS)، والسيتوكين المؤيد للالتهابات IL-23، والجينات المرتبطة بالالتهاب (NFATC1 وRANKL) في PBMCs من مرضى التهاب المفاصل الرثوي. يشير هذا إلى انخفاض واعد في الالتهاب وتعزيز الدفاعات المضادة للالتهابات ومضادات الأكسدة. بشكل عام، قام نظام توصيل الأدوية Hyalo-Nio-met NPs بتسليم الميتفورمين بشكل فعال إلى PBMCs، مما يظهر إمكانية كنهج علاجي جديد لـ RA وT2DM عن طريق تقليل السمية الجهازية وتحسين توصيل الدواء إلى خلايا معينة.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء ـ كلية الطب

فرع الكيمياء والكيمياء الحياتية

الحالة الالتهابية المضادة للالتهابات والأكسدة لدى النساء المصابات بالتهاب المفاصل الرثوي والمرضى المصابين وغير المصابين بمرض السكري من النوع الثاني وتأثير الميتفورمين النانوي على خلايا الدم المعزولة

رسالة ماجستير

مقدمة إلى مجلس كلية الطب / جامعة كربلاء كجزء من متطلبات نيل درجة الماجستير في (الكيمياء السريرية)

من قبل

شذى جاسم حاتم جاسم

بكالوريوس علوم كيمياء – جامعة كربلاء (2005 – 2006)

إشراف

الأستاذ الدكتور

فاضل جواد آل طعمه

كلية الطب ـ جامعة كربلاء

الأستاذ المساعد الدكتور

ماهر عبود مخيف

كلية الطب ـ جامعة كربلاء

2024 ميلادية

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