Sudan University of Science and Technology
College of Graduate Studies

Association of Osteopontin, Interleukin10 and Interleukin17 Levels with Liver Function Tests in Sudanese Patients with Rheumatoid Arthritis

علاقة مستويات الاوستيوبونتين وانترليوكين 10 وانترليوكين 17 مع اختبارات وظائف الكبد في المرضي السودانيين المصابين بالتهاب المفاصل الروماتويدي

A thesis submitted for the requirement of the Ph.D. degree in Medical Laboratory Science (Clinical Chemistry)

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قال تعالي

قَالَ إِنَّكَ لَنْ تَسْتَطِيعَ مَعِيَ صَبْرًا ﴿67﴾ وَكَيْفَ تَصْبِرُ عَلَىٰ مَا لَمْ تُحِطْ بِهِ خُبْرًا ﴿68﴾ قَالَ سَتَجِدُنِي إِنْ شَاءَ اللَّهُ صَابِرًا وَلَا أَعْصِي لَكَ أَمْرًا ﴿69﴾ قَالَ فَإِنِ اتَّبَعْتَنِي فَلَا تَسْأَلْنِي عَنْ شَيْءٍ حَتَّىٰ أُحْدِثَ لَكَ مِنْهُ ذِكْرًا ﴿70﴾

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Dedications

First of all this study to my almighty Allah, who gave his strength and knowledge for me everyday life.
To my father soul who inspired me to be strong despite of many obstacles in life.

This study is wholeheartedly dedicated to my beloved mother, she has been my source of inspiration and continually provide her overwhelming moral and emotional support.

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Abstract

Background: rheumatoid arthritis (RA) is worldwide prevalent autoimmune disease. Associated with abnormal liver functions, and the medications used for RA are often hepatotoxic. Furthermore non treated RA patients also have liver problem. Therefore, this study aimed to investigate the association between pro-inflammatory and anti-inflammatory cytokines and liver function tests in RA patients.

Materials and methods: this is a case-control hospital-based study conducted from December 2017 to August 2020 in Khartoum State in Sudan. Eighty eight Sudanese patients diagnosed with rheumatoid arthritis according to American Criteria for Rheumatology (ACR) at three different hospitals (Alamal, Military and Zain hospitals), patients with age range 28-90 years old were enrolled, 84 of them were women and 4 men. And 88 apparently health control matched (age and sex). Osteopontin (OPN), interleukin17 (IL-17) and interleukin10 (IL-10) were measured using the ELISA technique. Aspartate transaminase (AST), alanine transaminase (ALT), gammaglutamyl transferase (GGT), alkaline phosphatase (ALP), total protein and albumin were estimated using full automation Mindray analyzer. Data were analyzed by using SPSS version 14 and bioinformatics tools.

Results: The frequency of RA was higher among adults aged >41 years 72 (81.8%) than young adults aged ≤41 years 16 (18.2%). RA was more common in women 84 (95.5%) than in men 4 (4.5%) – approximately 21:1-fold. Mean age was (41±11.7) years old. As well as 59 (67%) were anti-CCP positive and other 29 (33%) were negative. There were significant increases in mean level of OPN and IL-10 (38.3±29.6 ng/mL and 45.9±42.9 pg/mL) when compared to control (10.1±10.6 ng/mL and 8.48±7.36 pg/mL), with (*p*-value 0.000 and *p*-value 0.000) respectively,

while IL-17 exhibited insignificant difference (6.55±1.17 pg/mL), in comparing to control (10.3±8.04 pg/mL); with (*p*-value 0.123). As well as mean liver enzymes activities (AST, ALT and GGT) were significantly increased in RA patients (16.6±6.98 U/L, 5.62±2.59 U/L and 27.3±23.1 U/L) than control group (7.86±7.86 U/L, 2.76±3.15 U/L and 20.9±13.4 U/L) with (*p*-value 0.000, *p*-value 0.000 and *p*-value 0.026) respectively. Whereas ALP showed insignificant difference (70.6±21.6 U/L) compared to control (66.8±19.6 U/L) with (*p*-value 0.225). Total protein and albumin were significantly decreased in RA patients (6.68±0.61 g/dL and 3.91±0.40 g/dL), than control group (7.18±0.695 g/dL and 4.22±0.460 g/dL) with (*p*-value 0.000 and *p*-value 0.000) respectively. Young adults had higher abnormal IL-10 than adult RA patients (OR = 3.72, *p*-value 0.044). Abnormal IL-17 (OR = 5.67, *p*-value 0.034) was found to be increased in young-adult RA patients. No association was observed between age and OPN, and between the duration of disease and IL-10, IL-17, and OPN. Similarly, no association was noted between the types of treatment and IL-10, IL-17, and OPN, nor between IL-10, IL-17, OPN and liver parameters (AST, ALT, ALP, ALB, TP, and GGT).

Conclusion: Liver enzymes are higher in RA patients, while liver proteins are lower than control. RA patients had higher OPN and IL-10. No association are observed between Pro-inflammatory, anti-inflammatory cytokines and liver function parameters in RA patients.

المستخلص

الخلفية التهاب المفاصل الروماتويدي (RA) هو مرض مناعي ذاتي منتشر في جميع أنحاء العالم, وله علاقه بحدوث خلل في وظائف الكبد ، وغالبًا ما تكون الأدوية المستخدمة لعلاج التهاب المفاصل الروماتويدي سامة للكبد. علاوة على ذلك ، يعاني مرضى التهاب المفاصل الروماتيزمي غير الخاضعين للعلاج من مشاكل في الكبد. لذلك ، هدفت هذه الدراسة إلى التحقق من وجود علاقة بين السايتوكاينات المؤيدة للالتهابات والمضادة للالتهابات واختبارات وظائف الكبد في مرضى التهاب المفاصل الروماتويدي.

المواد والطرق: هذه الدراسة عبارة عن دراسة تحليليه اجريت في الفترة من ديسمبر 2017 إلى أغسطس 2020 في ولاية الخرطوم في السودان. ثمانية وثمانين مريضًا سودانيًا مصابين بالتهاب المفاصل الروماتويدي تم تشخيصهم وفقًا للمعايير الأمريكية لأمراض الروماتيزم تم جمعه كمجموعة اختبار من ثلاثة مستشفيات مختلفة (مستشفى الأمل و ام درمان العسكري ومستشفى زين) ، تتراوح أعمارهم بين 28-90 عامًا ، 84 منهم نساء و 4 رجال. و 88 من الاشخاص اصحاء مطابقين للمرضي في العمر والجنس تم اختيارهم كمجموعة مكافئة ضابطة. تمت اجازة خطة البحث من كلية المختبرات الطبية بجامعة السودان, وتم اخذ موافقة المرضاء الذين لهم رغبة في المشاركة في هذه الدراسة.

بعد اخذ عينات دم من كلتا المجموعتين (المرضاء و المجموعة الضابطة), تم قياس الاوستيوبونتين (OPN) وانترليوكين 17 (IL-17) و انترليوكين 10(IL-10) باستخدام جهاز الاليزا . وتم قياس انزيم اسبارتيت امينوترانسفيريز (AST) ، الانين امينو ترانسفيريز (ALT) ، جاماجلوتاميل ترانسفيراز (GGT) ، الكالاين فوسفاتيز (ALP) ، البروتين الكلي والألبومين باستخدام جهاز ميندري الآلي. كما تم حساب و تحليل البيانات باستخدام برنامج الحزمة الاحصائية للعلوم الاجتماعيه SPSS النسخة 14

النتائج: اظهرت الدراسة ان معدل التهاب المفاصل الروماتويدي أعلى بين البالغين الذين تزيد أعمارهم عن 41 عامًا من الشباب الذين تقل أعمارهم عن 41 عامًا. بالإضافة إلى ان 59 من المرضاء بنسبة (67٪) كانو انتي سي سي بي موجب و 29 بنسبة (33٪) كانو سلبي. ايضا كانت هناك زيادة كبيرة في متوسط مستوى الاوستيوبونتين و انترليوكين 10, مع (القيمة الاحتماليه 0.000 و0.000) علي التوالي ، بينما أظهر انترليوكين 17 فرقًا ضئيلًا، مع (القيمة الحتماليه 0.123). بالإضافة إلى ذلك اظهر معدل نشاط إنزيمات الكبد اسبارتيت امينوترانسفيريز، الانين امينو ترانسفيريزو جاماجلوتاميل ترانسفيراز زيادة كبيرة في مرضى

الروماتيزم مع ، مع (القيمة الاحتمالية 0.00, القيمة الاحتمالية 0.00 و القيمة الاحتمالية 0.026) علي التوالي، بينما أظهر إنزيم الكالاين فوسفاتيز فرقًا ضئيلًا، مع (القيمة الاحتمالية 0.225). كما ان نسبة البروتين الكامل والألبومين انخفضت بشكل كبير في مرضى التهاب المفاصل الروماتويدي مع (القيمة الاحتمالية 0.000 و 0.000) علي التوالي. كان لدى الشباب البالغين من المرضاء انترليوكين 10 و انترليوكين 17غير طبيعين أعلى من البالغين مع (القيمة الاحتمالية 0.044 و 0.034) بالتتابع. لم يلاحظ أي ارتباط بين العمر و الاوستيوبونتين ، ايضا ليس هنالك ارتباط بين مدة المرض و انترليوكين 10 , انترليوكين 17 و الاوستيوبونتين. وبالمثل ، لم يلاحظ أي ارتباط بين أنواع العلاج و انترليوكين 10 , انترليوكين 17 و الاوستيوبونتين ، بالاضافه الي انه لم يوجد ارتباط بين انترليوكين 10 , انترليوكين 17 و الاوستيوبونتين ووظائف الكبد (اسبارتيت امينوترانسفيريز، الانين امينو ترانسفيريز، جاماجلوتاميل ترانسفيراز, الكالاين فوسفاتيز, البروتين الكامل والألبومين).

الخلاصة: اختبارات وظائف الكبد أعلى في مرضى التهاب المفاصل الروماتويدي مقارنة بالاصحاء. لم يلاحظ أي ارتباط بين السيتوكينات المؤيدة للالتهابات والمضادة للالتهابات و وظائف الكبد في مرضى التهاب المفاصل الروماتويدي

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List of Abbreviation

Abbreviation	Full name
ACPA	Anti-cyclic citrullinated protein/peptide antibodies
ACR	American criteria for rheumatology
ALF	Acute liver failure
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
Anti-CCP	Anti-cyclic citrullinated peptide
AST	Aspartate aminotransferase
BSPI	Bone sialoprotein I
CSFs	Colony-stimulating factors
CT	Computed tomography
DC	Dendritic cells
DMARDs	Disease-modifying anti-rheumatic drugs
ELISA	Enzyme linked immune sorbent assay
ETA-I	Early T-lymphocyte activation
Fc	Fragment of crystallization
FLS	Fibroblast-like synoviocytes
GGT	Gamma-glutamyltransferase
HIV	Human immune deficiency virus
HLA	Human leukocyte antigen
HRP	Horse reddish peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-10	Interleukine 10
IL-10R	IL-10 receptor
IL-17	Interleukine 17

LDH	Lactate dehydrogenase
MMPs	Matrix metalloproteinases
MRIs	Magnetic resonance imaging scan
MTX	Methotrexate
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide H
NAFLD	Non-alcoholic fatty liver disease
NK	Natural killer cells
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
OPN	Osteopontin
RA	Rheumatoid arthritis
RF	Rheumatoid factors
Ric	Rickettsia resistance
SPP 1	Secreted phosphoprotein 1
SPSS	Statistical Package for Social Sciences
T3	Triiodothyronine
T4	Thyroxine
TGF-b	Transforming growth factor-b
Th1	T helper1
Th17	T helper17
Th2	T helper2
TMB	Tetramethyl-benzidine
Treg	T regulatory cells

CHAPTER I

INTRODUCTION

1.1 Introduction

Rheumatoid arthritis (RA) is one of the most prevalent chronic inflammatory autoimmune diseases, with unknown etiology. It is a public health problem, worldwide distributed disease affects roughly 0.5-1% of the population, and is associated with progressive disability, premature death, and social-economic burden. The incidence begins to increase as age increases, and most commonly occurs in older women, estrogen may control this fact. The prevalence of RA varies in different ethnic groups. Researchers postulated that the increased prevalence of RA in Africa and South Africa, is due to the low numbers of rheumatologists and specialist physicians who have to care for patients with RA (Radis, 2012; David *et al*., 2013; Bester *et al*., 2016). There was no scientific publication yet about the prevalence of RA in Sudan (Hyder and Ahmad, 2017). RA is a complicated immune-mediated disease with predisposing of multiple genetic and environmental factors, the interaction between various cells such as fibroblast-like synoviocytes (FLS), cells of the innate immunity (e.g. macrophages, dendritic cells, mast cells, and natural killer cells (NK), neutrophils), and adaptive immune system (e.g. B and T lymphocytes), adhesion molecules and signal transduction pathways are all involved at different stages of the diseases, in addition to autoantibodies, these immunologic and metabolic events precede the onset of clinical disease, that can last many years (Angelotti *et al*., 2017; Firestein and McInnes, 2017). There are two major subtypes of RA according to the presence or absence of anticitrullinated protein antibodies, clinically the positive subset of RA has a more aggressive and more effective treatment response compared to the negative subset. Both anti-cyclic citrullinated protein/peptide antibodies (ACPA) and rheumatoid factors (RF) were included as serological markers associated

with RA (Elshafie *et al*., 2019; Oweis *et al*., 2020). While patients are often missed and misdiagnosed due to the diverse clinical manifestations of RA, lack of typical symptoms, and negative serology in the early stage (Qu *et al*., 2019). Investigators reported that the clinical course of disease is less favorable state in men, while other authors claim the opposite, and when measuring the disease severity in terms of structural damage the results found that there was no difference in radiographic scores between genders (Intriago *et al*., 2019). Variety of cytokines and matrix metalloproteinases (MMPs) are up-regulated and intimately involved in the pathogenesis of RA, high levels of pro-inflammatory cytokines and low levels of cytokines that suppress the immune-inflammatory response, such as interleukin-10 (IL-10) and transforming growth factor-b (TGF-b), have been detected in RA patients (Abdelsalam *et al*., 2011), which may reveal disease severity and lead to extra-articular multisystem immune complications (Pretorius *et al*., 2017; Nemtsova *et al*., 2019). Systemic rheumatologic diseases may be associated with liver abnormalities secondary to the presence of coexisting autoimmune liver disease among patients with arthritis (Selmi *et al*., 2011; Dehestani *et al*., 2015), besides, other studies demonstrated that developed treatment for RA administrated improved outcome as management of disease, but also account as a risk for hepatic complication (Sundbaum *et al*., 2019). Studies using liver biopsies have shown that the pathological feature of liver injury during methotrexate treatment resembled those of nonalcoholic steatohepatitis. The mechanism of liver injury with low-dose methotrexate is incompletely understood, obesity and type 2 diabetes have been influenced the development of liver injury during methotrexate treatment (Mori *et al*., 2018). Therefore RA patients should continuously monitor for liver functions for course management (Conway *et al*., 2017).

It is unclear how the liver problem and hepatic enzymes are elevated in patients with RA patients. Controversial findings were reported in the association between liver functions, RA and treatment of RA, this event prompted us to investigate whether the Osteopontin (OPN), interleukin-17 (IL-17), and interleukin-10 (IL-10) cytokines associated with abnormal liver function tests in RA patients.

1.2 Rationale

The epidemiological evidence emphasized that the prevalence of RA is high and rising in all societies around the world. There are no disseminated data on the prevalence of RA among Sudanese population. In recent years, there was a greater concern about RA and it is association with liver abnormalities. Several interleukins such as Osteopontin and IL-17 approved as chemotaxis and pro-inflammatory cytokines, IL-10 known as anti-disease through containment of immune response, in RA patients Osteopontin up-regulated which recruit and trigger macrophage to secrete pro-inflammatory IL-17 and anti-inflammatory IL-10 cytokines, aggregation of these immune complex cause destruction of cells including hepatocyte. These events suggest or account for the most liver problem associated with RA disease. Assessment of these cytokines associated with RA in Sudanese may play an important role in the prevention of liver problem and come on the line of treatment. Furthermore no powered study has been performed to determine the role of Osteopontin, IL-10, and IL-17 in liver damage and exaggeration among RA patients. Therefore the research hypothesis of the present study is that, there is an association between inflammatory cytokines and liver function tests in RA patients.

1.3 The null hypothesis

Inflammatory cytokines are not associate with liver function tests and study variables in rheumatoid arthritis patients.

1.4 Objectives

1.4.1 General objective

To find out the association between Osteopontin, IL-10, IL-17 level, liver function tests, and study variables in RA Sudanese patients.

1.4.2 Specific objectives

To measure and compare the mean concentration of cytokines (OPN, IL-10, and IL-17), ALT, AST, ALP, GGT, total protein and albumin level in both study groups

To compare mean levels of study parameters of positive anti-CCP versus negative anti-CCP group.

To correlate between Osteopontin, IL-10, and IL-17 cytokines with study variables (duration of disease, gender, age, type of treatment, and liver parameters).

To investigate the risk factors (age and type of treatment) associated with abnormal cytokines and study group

CHAPTER II

LITERATURE REVIEW

2.1 Epidemiology of rheumatoid arthritis

Rheumatoid arthritis RA is a chronic, progressive, inflammatory autoimmune disease, that affected small joint in the hand and feet causing swelling that can result in bone erosion and joint deformity leading to reduced physical function and impaired quality of life (Mursal *et al*., 2016; Xu Bei and Lin Jin, 2017; Liu *et al*., 2018). Characterized by synovial inflammation and hyperplasia, RA with extra-joint involvement, associated with high inflammation Physiologically such as keratitis, pulmonary granulomas (rheumatoid nodules), pericarditis/pleuritis, small vessel vasculitis (Bazzichi *et al*., 2009 ; Stocki *et al*., 2010; Choy, 2012; Demirdal *et al*., 2013; Rudan *et al*., 2015; Guo *et al*., 2018). RA occurs worldwide, it affects around 1% of the general population worldwide and occurs in all races and ethnic groups (Yap *et al*., 2018; Nemtsova *et al*., 2019), affect 42.7 million Americans, with a prevalence of 0.5 to 1% in the Western population (Chen *et al*., 2010; Gomes *et al*., 2011; ELsedig *et al*., 2014 Guo *et al*., 2018; Iqbal *et al*., 2019). Other studies showed that RA affects approximately 1-2% of the world's population (Mursal *et al*., 2016 Xu and Lin, 2017; Liu *et al*., 2018)

To date, the exact prevalence of RA in Africa is not well known, one reason might be that, reports from different parts of Africa have used different criteria for diagnosis and that the age structure differs considerably. Some researchers have reported the disease prevalence in Africa to be lower than that in Europe (Elshafie *et al*., 2016). But others report an expected increase in disease prevalence in Africa and South Africa, due to low numbers of rheumatologists in South Africa and specialist physicians who care for patients with RA (Radis, 2012 David *et al*., 2013; Bester *et al*., 2016).

Despite there have no scientific research published yet investigated the prevalence of RA in Sudan (ELsedig *et al*., 2014; Elshafie *et al*., 2016;

Hyder and Ahmad, 2017). Few studies carried out by (Elshafie *et al.*, 2016; Elshafie *et al.*, 2019), which report that IgA seems to be the diagnostically most sensitive autoantibody marker for RA in Sudan, IgG rheumatoid factor (RF) is the marker most strongly associated with the young age of disease onset and with the occurrence of classical hand deformities, antibodies against the cyclic citrullinated peptide (Anti-CCP2) was also found to be associated with severe disease.

2.1.1 Pathogenesis of rheumatoid arthritis

The pathological process of RA is due to an autoimmune inflammation of the synovial membrane of joints with synovial cells proliferation, consequently, osteoclast and chondrocyte activation and pannus formation, in other word is (tumor-like aggressive granulation tissue) that, promotes articular cartilage erosion, bones destruction, and Synovial tissue dysfunction. Therefore, enhance macrophages, fibroblasts, and activated lymphocytes penetrate to the site of infection. T-lymphocytes produce a variety of pro-inflammatory cytokines, and interleukin super-families as well as growth factors. B-lymphocytes are involved in the production of autoantibodies such as RF and anti-CCP (Firestein and McInnes, 2017; Calabresi *et al.*, 2018; Liu *et al.*, 2018; Yap *et al.*, 2018 Nemtsova *et al.*, 2019). Anti citrullinated peptide antibodies positive subjects exhibit reduce frequency of naive and regulatory T cells and an increased population of atypical T cells. The subset imbalance was predictive of arthritis progression. The reduced regulatory activity may be an early event in the pathogenesis of the disease (Lucchino *et al.*, 2019). The cartilage matrix within joints is eventually degraded by metalloproteinases and other enzymes (Iqbal *et al.*, 2019). Tissue damage in RA is mostly caused by type III hypersensitivity, with the participation of antibodies, complement, antigen-antibody complex, macrophages, T and B cells (Zou, 2017). Although RA is not a fatal disease in general, a

complication associated with a disease may lead to increased mortality (Yap *et al*., 2018).

2.1.2 Rheumatoid Factor and anti-Citrullinated Cyclic Peptide

RF and anti-CCP autoantibodies are common in patients with RA (Gomes *et al*., 2011). RFs are auto-antibodies that, react with the Fc-portion of IgG. The classic RF is an IgM antibody with reactivity against IgG-Fc, but IgA and IgG RF can also be found. RFs are detected in sera of most patients with RA. Although the presence of RFs in RA patients correlates with more active disease, RF has a low specificity as a disease marker for RA when compared to controls with other rheumatic diseases and infections. Anti-citrullinated proteins and/or peptides antibodies have come forward as specific serological markers for RA, with higher diagnostic specificity and positive predictive value. But with similar sensitivity to RF. The event of citrullination of proteins and peptides occurs naturally during inflammation and is a post-translational modification of arginine by deamination. Antibodies against several different citrullinated proteins have been associated with RA, and anti-CCP positive RA patients develop more severe clinical manifestations than anti-CCP negative patients (Ahlin *et al*., 2011; Choy Ernest, 2012; ELshafie, 2013; Etekharian, 2013; Khudair *et al*., 2015).

Sudanese patients with RA have more widespread joint involvement and stronger laboratory signs of inflammation compared with Swedish patients with RA. As well Sudanese patients also have radiological erosions and RA-associated hand deformities to a large extent, although only half of the patients are IgM-RF seropositive (Elshafie *et al*., 2016).

2.1.3 Etiology of Rheumatoid Arthritis

The etiology of RA is not fully understood, since several initiating factors play a role in the etiology of RA. Such as immunological, genetics, and environmental. Moreover, activated immune reactions potentially

contribute to disease development (Klareskog *et al*., 2006; David *et al*., 2013; ELsedig *et al*., 2014; Mohamed, 2015; Xu and Lin, 2017)

2.1.4 Risk factors for rheumatoid arthritis

Researchers studied several factors that have been associated with increased risk of RA include the following:

2.1.4.1 Genetic factors

Genetic factors contribute about 50% to 60% of the risk of developing RA, disease severity, and progression. The gene most strongly associated with RA is the HLA-DRB1 gene in the major histocompatibility complex (Tobon *et al*., 2009; Lee *et al*., 2009; Choy, 2012).

2.1.4.2 Age and gender

RA is far more common in women than in men, with the female-to-male ratio being 3:1. However, the mechanism by which gender influences the susceptibility to RA remains unclear. Studies attribute these to sex hormones. The disease generally is more frequent in older people (peak age at RA onset is the fifth decade) and this does not mean the impossibility of its occurrence in children; when it in children is called juvenile RA (Wasserman, 2011; Sharma *et al*., 2011; Demirdal *et al*., 2013; Eftekharian, 2013; ELsedig *et al*., 2014; Mursal *et al*., 2016).

2.1.4.3 Socioeconomic factors

Socioeconomic factors affect the course and outcome of RA, but do not seem to influence the risk of disease development (Kourilovitch *et al*., 2014; Rudan *et al*., 2015; Yoo *et al*., 2017).

2.1.4.4 Hormonal factors

The predominance of RA in females suggests a role for hormonal factors. Also, estrogens stimulate the immune system. Low testosterone levels have been reported in men with RA and oral contraceptive use may be

protective and reducing the risk for RA development (Tobon *et al*., 2009; Wasserman, 2011; Kanecki and Tyszko, 2015).

2.1.4.5 Ethnicity

Some ethnic and racial groups are at higher risk for RA than others. This high risk may be related to differences in the distribution and interactions of genetic and environmental factors. Comparisons of the populations with similar genetic backgrounds but different lifestyles (Tobon *et al*., 2009; Wasserman, 2011).

2.1.4.6 Environmental factors

Several environmental factors that could increase a person chance of having RA include: firstly smoking: among environmental factors, the smoking strongest association with RA. Smoking increases susceptibility to RA and adversely affects the clinical course of the disease. The association is stronger for men than for women (Tobon *et al*., 2009; Wasserman, 2011; Elshafie, 2013; Kanecki and Tyszko, 2015).

Secondly the Infections: several microorganisms have been implicated in the development of RA, include Proteus spp, Campylobacter, Chlamydia trachomatis, Escherichia coli, Mycoplasmas, and Staphylococcus aures (Radis, 2012; Pretorius *et al*., 2017; Mobini *et al*., 2017). Also, the immunological response to bacterial components results in the production of ACPA that, closely mimic host cell receptors, which are identified as potential risk factors for RA development e.g. of bacteria: Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans (Abdul Sultan *et al*., 2019). Also, alterations in mucosal immunity are supposed to play a central role in the pathogenic events preceding RA (Lucchino *et al*., 2019).

Tertiary the dietary factors and obesity: many types of food such as a diet rich in fish, olive oil, and cooked vegetables have been shown to protect against RA, but a recent study report that obesity was associated with a

modest risk for developing RA (Tobon *et al*., 2009; Kanecki and Tyszko, 2015).

Fourthly Vitamin D: excessive intake of vitamin D may be associated with a lower risk of RA in older women. It was also indicated that patients with more active RA have a lower serum vitamin D level (Kanecki and Tyszko, 2015).

Fifthly Periodontal diseases**:** recent data has been reported that there was a relation between established RA and periodontal disease. The current hypothesis is that the Porphyromonas gingivalis, responsible for the citrullination of human peptides, these consequences may result in the initiation and development of RA-related autoimmunity (Kanecki and Tyszko, 2015).

2.1.5 Signs and symptoms of rheumatoid arthritis

The signs and symptoms of RA include pain, stiffness, swelling and functional impairment, general malaise, and profound fatigue. Also, progressive joint destruction is common. Moreover, observational studies report that RA patients have poor nutrient status, with reduced energy intake from carbohydrates, high consumption of saturated fat, and poor intake of micronutrients. RA consequences are associated with significant increase levels of morbidity and mortality and have a significant impact on total health care costs (Rennie *et al*., 2003 ; Luqmani *et al*., 2009; Stocki *et al*., 2010; Sharma *et al*., 2011; Pasma *et al*., 2013; Mohamed, 2015; Babikir *et al*., 2017; BM *et al*., 2018).

2.1.6 Management of Rheumatoid Arthritis

Many patients undergo delays in diagnosis and initiation of treatment, these event leads to worsening of the condition and poor prognosis (Barhamain *et al*., 2017).

Several new developmental guides' which improve the management and outcome of RA have taken place in recent years, including classification criteria, assessment, and follow-up tools, exercise prescription, and therefore it is imperative for specialist physicians to update themselves with the newest (Nolte and Jansevanrensburg, 2013; Bester *et al*., 2016). Rheumatology nursing also practices specially and contributes significantly to the management of patients with rheumatic musculoskeletal diseases (Kelly *et al*., 2015).

RA is commonly treated with disease-modifying anti-rheumatic drugs (DMARDs), corticosteroids, and non-steroidal anti-inflammatory drugs (NSAIDs) (Pasma *et al*., 2013; Meier *et al*., 2013; Mohamed, 2015)

2.2 Liver and immunity

The liver is the largest solid, most vital organ, it has not only been considered as a reserve but also the ability to regenerate itself, and the symptoms of liver damage may not appear until damage to the organ is quite extensive. A total loss of liver function could lead to death within minutes (Maher, 1997; Maronpot *et al*., 2010; Ozougwu and Jevas, 2017). Moreover, it is a major source of many components of the innate immune response including acute phase and complement proteins as well as inflammatory cytokines and chemokine's (Ishibashi, 2009).

2.2.1 Liver functions

The liver perform a numbers of crucial and vital functions for life such as metabolic detoxification of (drugs, toxins, and steroid hormones), Secretion of bile, metabolism of bilirubin, vascular and hematologic functions, important blood reservoir, metabolism of nutrients, fat metabolism, synthesis of cholesterol/lipoprotein and production of ketoacidosis, protein, amino acid production, gluconeogenesis (turnover of proteins to glucose and converts galactose/fructose to glucose), storage of (glycogen, iron, minerals, and vitamins), endocrine functions

(activation of vitamin D, convert of thyroxine (T4) to triiodothyronine (T3), secretes angiotensinogen, metabolites hormones) and immunological and protective functions(reticuloendothelial component, filters the portal blood from bacteria, important in antigen presentation, phagocytosis via kupffer cells, remove hemolysis products, inactivation of toxins and drugs) (Giannini *et al*., 2005; Ozougwu and Jevas C, 2017; Saranya and Seenuvasan, 2017; Keiding *et al*., 2018).

2.2.2 Liver diseases

Liver disease is one of the most global health burdens and it is a significant cause of morbidity and mortality (Al Ghamdi and Shah, 2018; Hong *et al*., 2015). There are many types of liver diseases that, can be caused by various etiology such as a virus (Hepatitis A, B, and C), damage from drugs or chemicals (alcoholic liver disease and hepatotoxicity), obesity (fatty liver), diabetes, or an attack from own immune system (autoimmune liver disease), liver cirrhosis, in addition to Liver cancer as the primary tumor or metastasis (Sivakrishnan, 2019). The liver cells under normal circumstances produce only minimal levels of cytokines, when liver cells, particularly immune cells (Kupffer cells) persist in the production of cytokines in response to stimuli, these cytokines can have both beneficial and harmful effects, depending on the amount and duration of cytokine release (Mannaa and Abdel-Wahhab, 2016).

2.2.3 Diagnosis of Liver diseases

Diagnosis of liver disease based on initial history and physical examination. A group of tests called liver function tests are commonly ordered as a panel of blood tests that evaluate liver function, liver damage, and biliary system function including intra and extra hepatic bile ducts and gall bladder. As well as complete blood count, CT scan, MRIs or ultrasounds, and liver biopsy to check liver damage or tumors. Serum

enzymes such AST, ALT are raised when liver injury or inflammation. Gamma-glutamyl transferase and alkaline phosphatase are increased in obstructive liver disease. The level of conjugated and unconjugated bilirubin indicates different liver diseases. A blood level of protein and albumin are indicative of the healthy functioning of the liver (Thapa and Walia, 2007; Mutua *et al*., 2018).

2.2.4 Association between liver functions and rheumatoid arthritis

Recent studies have demonstrated that the liver problems in people with RA may be due to underlying disease itself or be the manifestation of an associated autoimmune disease, also treatments used for RA improved outcome, on the other hand, account as a risk for hepatic complications. The adverse effects of RA treatments include asymptomatic elevations of liver enzyme, fibrosis and may be fatal hepatic necrosis. On the other hand liver disorders were noted in untreated RA patients (Lazrak *et al*., 2013; Conway and Carey, 2017; Rakuomi *et al*., 2017; Sundbaum *et al*., 2019).

2.3 Cytokines

Cytokines are soluble in small molecular weight proteins or peptides, messengers between tissues and the immune system, and participate in many physiological processes (Shaikh, 2011; Mannaa and Abdel-Wahhab, 2016).

2.3.1 Cytokines classifications

Cytokines can be either poor anti-inflammatory which suppresses the activity and production of pro-inflammatory signals limiting inflammation and host damage. Or pro-inflammatory cytokines that, induce inflammation as a result of infection or injury. Moreover, inflammatory cytokines can be divided into two groups: one is involved in acute inflammation and others responsible for chronic inflammation (Shaikh, 2011; Mannaa and Abdel-Wahhab, 2016).

Also, the term 'cytokine' comprises monokines, lymphokines, colony-stimulating factors (CSFs), and chemokine's (Holdsworth and Yi, 2015; Yap *et al*., 2018).

Other classifications of cytokines according to the basis from which they are produced either from T helper1 (Th1) cells or T helper2 (Th2) cells. Recently a third subset of T helper (Th) cells T helper17 (Th17) and T regulatory cells (Treg) are categorized (Gulati *et al*., 2016).

2.3.2 Cytokines functions

Cytokines are important in initiating, amplifying, directing, mediating, and regulating adaptive immunity. Unfortunately, they may also direct tissue damage if excessive responses occur or if they are involved in directing and mediating autoimmunity. Under these circumstances, cytokines are potential therapeutic targets (Holdsworth and Yi, 2015; Trifunovic *et al*., 2015).

2.3.3 Association between cytokines and rheumatoid arthritis

The expression of pro-and anti-inflammatory cytokines in the synovial membrane of the inflamed joint is become altered. According to that pro-inflammatory cytokines are overproduced, and fluid from patients with RA also contains detectable levels of anti-inflammatory cytokines (IL-10), but they are insufficient to counterbalance the effect of pro-inflammatory cytokines (Trifunovic *et al*., 2015; Yap *et al*., 2018).

2.3.4 Interleukin-10 (IL-10)

IL-10 also called (cytokine synthesis inhibitory factor), it's a fascinating anti-inflammatory cytokine. Firstly identified by its ability to stop immune response by inhibiting the production number of cytokines. Its mechanisms of action remain poorly understood, and some report shows that IL-10 opposes the switch to the metabolic program induced by inflammatory stimuli in macrophages (Zhdanov, 2004; Tao *et al*., 2011; IP Eddie *et al*., 2017; Fathy *et al*., 217; Sheikhpour *et al*., 2018). It is

activity mediated by the IL-10 receptor (IL-10R) which is a member of the class II cytokine receptor family (Trifunovic *et al*., 2015; Zhang *et al*., 2016).

IL-10 is produced by T helper 2 cell clones, also various cell populations produce IL-10 in the body, including T cell subsets (Th2, Tc2, Tr1, etc), monocytes, and macrophages. As well as IL-10 produced by different cell types in other organs, including the liver, IL-10 producing liver cells has been documented within hepatocytes, sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells, and liver-associated lymphocytes (Zhang and Wang, 2006).

2.3.4.1 Functions of Interleukin-10

The function of IL-10 in regulating the immune system by promoting the suppression of immune responses also plays a role in the proliferation and differentiation of B cells, T cells, and mast cells. As well as IL-10 inhibits the release of various chemokine's by neutrophils. Based on its immune-modulating functions, IL-10 has been considered an attractive candidate for therapeutic applications for the treatment of acute and chronic inflammation, autoimmunity, cancer, and infectious disease (Zhang and Wang, 2006; Bijjiga and Martino, 2013).

Both T regulatory and IL-10 is involved in the suppression of IL-17A production, however, the role of IL-10 signaling in the differentiation of Th17 cells is less clear (Tao *et al*., 2011; Kurata *et al*., 2014).

2.3.4.2 The role of Interleukin-10 in rheumatoid arthritis

IL-10 is a prominent participant in human inflammatory diseases and contributes to the pathogenesis of RA, however, its mechanisms of action are poorly understood. While significant amounts can be measured in the synovium of patients with RA. Previous research reported that IL-10 can effectively block the production of the pro-inflammatory cytokines by snivel macrophages and synoviocytes and also correlated with increased

autoantibody production and B cell activation in RA patients. Administration of IL-10 did not attenuate RA activity (Zhdanov, 2004; Tao *et al*., 2011; Holdsworth and Yi, 2015; Fathy *et al*., 217; Shikhpour *et al*., 2018).

2.3.4.3 Interleukin-10 and liver diseases

Numerous investigations suggest that IL-10 plays a major role in chronic and autoimmune liver diseases, and play a critical role in inducing the acute phase response in the liver. IL-10 deficient mice show more susceptibility to liver fibrosis with inflammatory infiltrate. It is production up-regulated upon liver inflammation under various conditions. (Zhang and Wang, 2006; Hammerich and Tacke, 2014)

2.3.5 Interleukin-17 (IL-17)

IL-17 is a potent pro-inflammatory cytokine. IL-17 and its receptor are members of emerging cytokine. Which has a total of six family members (IL-17A to IL-17F) (Zhou *et al*., 2009; Park and Lee, 2010; Miletic *et al*., 2012; Tan *et al*., 2013; Hassan *et al*., 2014; Blauvelt and Chiricozzi, 2018).

IL-17 produced primarily byTh17 CD4 T cells specifically memory CD4+ T cells, also produced by a wide variety of cell types, including neutrophils, CD8+T cells, γδ T cells, NKT cells, lymphoid tissue, dendritic cells (DC), and macrophages (Wilson *et al*., 2007; Du *et al*., 2013; Hassan *et al*., 2014; Blauvelt and Chiricozzi, 2018; Gouda and Bhandary, 2018). The IL-17 receptor is broadly expressed on several epithelial cells, immune cells, and stromal cells (endothelial, fibroblastic) (Moseley *et al*., 2003; Lemmers *et al*., 2009; Mengesha and Conti, 2017).

2.3.5.1 Functions of Interleukin-17

The major function of IL-17 is a powerful chemo-attractant for neutrophils and has been reported to be involved in many immune processes. most notably in inducing and mediating pro-inflammatory

responses and cause organ-specific disease which recruits Th1 cells to the target tissue, Th17 cells play an active role in shaping the local inflammatory response in the liver, also have a role in the regulation of granulopoiesis and erythropoiesis (Miletic *et al.*, 2012; Hassan *et al.*, 2014; Jian *et al.*, 2015).

2.3.5.2 The role of Interleukin-17 in rheumatoid arthritis

As a pro-inflammatory cytokine, IL-17 contributes to the inflammation of many autoimmune diseases and is up-regulated in the lesions of patients with various chronic inflammatory diseases, such as rheumatoid arthritis. And produced at high levels by many RA samples, although the role of IL-17 in the pathogenesis of the autoimmune diseases is still unclear (Wilson *et al.*, 2007; Sarkar *et al.*, 2007; Du *et al.*, 2013; Ruderman, 2015; Mengesha and Conti, 2017; Miossec, 2017; Lockshin *et al.*, 2018; Elvira *et al.*, 2018). Some researchers suggested that IL-17 is an important mediator between RA inflammation and joint injury. Also, IL-17 stimulates the production and interacts with other proinflammatory cytokines, chemokine's and biologically affect stromal cell activation, angiogenesis, and osteoclastogenesis to promote inflammatory responses which leading finally to cartilage loss (Qu *et al.*, 2019).

Neutralizing anti–IL-17 antibody reduces the severity of arthritis (Chabaud *et al.*, 1999; Sarkar *et al.*, 2007; Park and Lee, 2010; Miletic *et al.*, 2012; Tan *et al.*, 2013; Ruderman, 2015).

2.3.5.3 Interleukin-17 and liver diseases

The conclusion of some studies suggests that IL-17 plays an important role in many liver diseases, including alcoholic liver disease, hepatocellular carcinoma, and autoimmune liver disease, acute and chronic hepatitis B. And it is also associated with disease progression (Du *et al.*, 2013; Tan *et al.*, 2013; Zheng *et al.*, 2013). The receptors of IL-17 have been expressed on the surface of all types of liver cells,

including hepatocytes, Kupffer cells, stellate cells, biliary epithelial cells, and sinusoidal endothelial cells (Abe *et al*., 2013; Peverill *et al*., 2014).

2.3.6 Osteopontin (OPN)

OPN is an intracellular, phosphorylated glycoprotein, silica acid-rich, negatively charge hydrophilic protein. Expressed in mineralized tissues (bone and teeth) and damaged renal tissues (Mazzali *et al*., 2002; Chellaiah *et al*., 2003; Afify *et al*., 2009; Yilmaz *et al*., 2013; Yang *et al*., 2014; Kusuyama *et al*., 2017; Gonzalez *et al*., 2017; Shi *et al*., 2018; Dimitra *et al*., 2018). Osteopontin is also known as bone sialoprotein I (BSPI), early T-lymphocyte activation (ETA-I), Urinary stone protein, Nephropontin, Uropontin secreted phosphoprotein 1(SPP 1), Rickettsia resistance (Ric), and (bone phosphoprotein) (Ramaiah and Rittling, 2008; Sase *et al*., 2012).

OPN is expressed by macrophage, T and B lymphocytes, neutrophils, and eosinophils, and it is a major noncollagenous component of bone matrix (Yang *et al*., 2014; Kusuyama *et al*., 2017; Gonzalez *et al*., 2017; Shi *et al*., 2018; Dimitra *et al*., 2018). Also produced in a variety of tissues: brain, liver, gastrointestinal tract, lung, bone, cardiac tissues, joints, and kidney, and appears in several biological fluids including human plasma, serum, breast milk, and urine (Ramaiah and Rittling, 2008; Elsebaie *et al*., 2012; Sase *et al*., 2012).

2.3.6.1 Functions of OPN

OPN plays several roles in promoting and activation of T lymphocyte, also affecting the differentiation of T lymphocyte into Th1 and Th2 type cell, regulating the balance between Th1 and Th2 (OPN polarizes the early Th1 cytokine response and inhibits Th2 cytokines expression), moreover it promotes efficient type-1 immune responses by regulating macrophage differentiation and participating in the cell-induced immunologic response. At the same time, OPN can stimulate B

lymphocytes to express multi-clone antibodies (Yumoto *et al*., 2002; Gattorno *et al*., 2004; Afify *et al*., 2009; Sase *et al*., 2012; Zhao *et al*., 2018). Besides, OPN as a pro-inflammatory cytokine plays a critical role in the regulation of tissue repair and remodeling (Honsawek *et al*., 2009; Yang *et al*., 2014; Shi *et al*., 2018; Rentsendorj *et al*., 2018). As well as in bone promotes adhesion of osteoclasts to the mineralized matrix regulating bone resorption and formation (Yumoto *et al*., 2002; Iwadate *et al*., 2014).

2.3.6.2 The role of Osteopontin in rheumatoid arthritis

The results of the previous study reported that the production of OPN increases in both RA and osteoarthritis (OA), in both plasma and synovial fluid (Tikkanen *et al*., 2017). However, the putative role of Osteopontin in chronic inflammatory diseases such as rheumatoid arthritis is unclear (Gravallese, 2003). Another research on the function of OPN in arthritis has revealed that OPN stimulates the production of several pro-inflammatory cytokines by mononuclear cells in patients with RA (Take *et al*., 2009). It is deficiency has been shown to protect joints against destruction in arthritis (Bernardini *et al*., 2009).

2.3.6.3 Osteopontin and liver diseases

Because of its pro-inflammatory actions and its effects on macrophages, OPN has been also implicated in the pathogenesis of different physiologic and pathologic events in the liver such as acute liver failure (ALF), non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease, chronic hepatitis B, chronic Hepatitis C, primary biliary cirrhosis and liver fibrosis. However, the roles of OPN in such liver diseases are still controversial (Zeyda *et al*., 2011; Elsebaie *et al*., 2012; Yilmaz *et al*., 2013; Iida *et al*., 2018).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Study designs

This is an analytical case-control hospital-based study.

3.1.2 Study area and duration

The study was conducted in Alamal National Hospital, Omdurman Military Hospital and Zain Clinic, Khartoum State, Sudan. During the period from 2017 to 2020.

3.1.3 Ethical Considerations

The study was approved by research committee of Medical Laboratory Science at Sudan University of Science and Technology. An informed consent was obtained from each participants (appendix I). And data were collected through questionnaire (appendix II). Collected data were secured using a password to protect the patient information and used only for research purposes.

3.1.4 Study population

One hundred seventy sixth subject were enrolled in this study classified as 88 clinically diagnosed as RA patients, according to American criteria for rheumatology (ACR) were randomly selected, 84 were female and 4 were male, age ranged from 28-90 years old. And 88 apparently health subjects were included as control group, age ranged from 30-85 and sex were matched.

3.1.5 Sample Size

The sample size is calculated according to the known formula, which is used to reach a certain desired margin of error in the results.

The sample size in this study is calculated for each category (on average) to give a maximum of error (0.05) with a probability of ($\alpha = 0.05$). Plus the 10% of nonresponsive sample size is as follow:

$$n = \frac{z^2.p.q}{d^2}$$

$$= \frac{(1.96)^2 \times (0.06) \times (0.94)}{(0.05)^2} = 86.6 \sim 88$$

z = the value in normal curve corresponding to level of confidence 95% = 1.96

p = probability prevalence in the community is (highest prevalence reported in Khartoum 6%) or 0.06 (Ministry of health-Khartoum state)

q = (1-p) = 1-0.06= 0.94

d = margin of error = 0.05

= 88

Eighty-eight patients were recruited, and equal for control

Another method used is the EPI INFO software sample size calculator; total sample size equal 178 (with two-sided confidence level (1-alpha 95), power (% chance of detecting 80), the ratio of control to cases 1:1, the hypothetical proportion of control with exposure 40, the hypothetical proportion of cases with exposure 62 and last extreme odd ratio to be detected 2.45).

3.1.6 Inclusion criteria

All patients who clinically diagnosed with RA and referral to Alamal National Hospital, Omdurman Military Hospital and Zain Clinic, were included.

3.1.7 Exclusion Criteria

HIV, Diabetic, hypertensive, smokers, autoimmune disease, viral hepatitis, pregnant women, and patients who refuse to participate in this study were excluded.

3.1.8 Collection and Sampling Technique

Whole blood samples were collected by using dry, plastic syringes; a tourniquet was used to make the veins more prominent, 5ml were collected in plain containers from each volunteer under aseptic condition. The serum was obtained by centrifugation at 4000 rpm, the specimen was kept at -20° till used.

3.2 Methods

3.2.1 Osteopontin Estimation

Human Osteopontin present in a sample or standard bind to antibodies pre-coated in a plate, after the formation of sandwich, streptavidin-HRP bind to biotinylated antibodies, then substrate react to give blue color which changed to yellow color after stop solution was added, the density of color proportion to the concentration appendix(III).

3.2.1.1 Procedure

Micro well strips were washed a twice with working wash buffer 400 μL per well, and the excess of wash buffer were removed by tap micro well stripes on towel paper, then 100 μL of prepared standard were added to all wells labelled as standard. The samples were diluted (80 μL of sample diluent were added and then 20 μL were added) and added to all wells labelled as samples. The plate was covered with adhesive film and incubated at room temperature for 2 hours. The adhesive film was removed, the wells were emptied, and micro-well strips were washed 6 times. Working biotin conjugate 100 μL were added to all wells, and the plate was covered with adhesive media. The plate was incubated at room temperature for 1 hour, then washed 6 times. Working streptavidin-HRP 100 μL were added to all wells, then the plate was covered and incubated for 1 hour and washed 6 times. Tetramethyl-benzidine (TMB) substrate solution 100 μL were added to all wells, plat incubated for 30minutes in dark, finally 100 μL of stop solution were added into each well, then

color read immediately at 450nm and results were calculated from standard curve.

The concentration measured using a microplate ELISA reader (Stat Fax 4200- American) see (appendix IV).

3.2.2 Interleukin-10 Estimation

Human IL10 present in a sample or standard bind to antibodies pre-coated in a plate, after the formation of sandwich, streptavidin-HRP bind to biotinylated antibodies, then substrate react to give blue color which changed to yellow color after stop solution was added, the density of color proportion to the concentration (appendix V).

3.2.2.1 Procedure

The samples or working standards 50 µL were added in to wells, and standard diluent 50 µL was added to the blank well, then biotinylated antibody 50 µL was added to all wells-containing samples and standards. The plate covered with adhesive cover and incubated at room temperature for 2 hours, then the plate was washed with working wash buffer. The recently prepared streptavidin-HRP 100 µL were added to each well. Then plate covered and incubated for 30 minutes at room temperature. Plate washed, and TMB substrate 100 µL were added to all wells. Plate incubated for 30minutes. Finally, stop solution 100 µL was added into each well. Then color read immediately at 450nm and the results were calculated from a standard curve.

The concentration measured using a microplate ELISA reader (Stat Fax 4200- American).

3.2.3 IL-17 Estimation

Sample or standard containing IL-17 react with capture monoclonal antibody coated on the micro-plate well and with biotinylated detection antibodies. After the incubation period allowing the formation of the sandwich, streptavidin-HRP added and bind to biotinylated antibodies

when the substrate was added blue color produced a change to yellow after stop solution was added, the density of color proportion to concentration (appendix VI).

3.2.3.1 Procedure

The samples and reagents were brought to equilibrate with room temperature. Then solution contains (buffer and preservative) 50 μL were pipetted to each well. And working standards or samples 50 μL were added to wells. Biotin conjugate 50 μL were added into all wells, then plate incubated for 2 hours at room temperature, and washed with working wash buffer. Streptavidin-HRP 100 μL was pipetted to each well, then plate covered and incubated for 30 minutes at room temperature. And then plate washed, and TMB substrate 100 μL was added to all wells. Plate incubated for 15minutes in dark. Finally, stop solution 100 μL was added into each well, then color read immediately at 450nm and the results were calculated from a standard curve.

The concentration measured using a microplate ELISA reader (Stat Fax 4200- American).

3.2.4 Estimation of Alkaline phosphatase

By the action of ALP and magnesium ions, p-Nitrophenyl phosphate is catalyzed to p-Nitrophenol, and the absorbency increase is directly proportional to the activity of ALP (appendix VII).

The activity measured by using a fully automated chemistry analyzer (Mindray BS-200, China) see (appendix VIII).

3.2.5 Estimation of Gamma-glutamyltransferase

Gamma-glutamyltransferase transfers the gamma-glutamyl group of gamma-glutamyl-3-carboxy-4-nitroanilide to glycyl-glycine with the production of p-nitroaniline. The amount of 5-amino-2-nitrobenzoate results in elevated absorbance which is directly proportional to the activity of GGT in the sample (appendix IX).

The activity measured by using a fully automated chemistry analyzer (Mindray BS-200, China).

3.2.6 Estimation of aspartate aminotransferase

AST catalyzes the reversible transamination of L-aspartate and α-oxoglutarate to oxaloacetate and L-glutamate. The oxaloacetate is then reduced to malate in the presence of malate dehydrogenase with NADH being oxidized to NAD+. The rate of the photometrically determined NADH decrease is directly proportional to the rate of formation of oxaloacetate and thus the AST activity (appendix X).

The activity measured by using a fully automated chemistry analyzer (Mindray Bs-200, China).

3.2.7 Estimation of Alanine aminotransferase

Alanine aminotransferase catalyzes the reversible transamination of L-alanine and α-oxoglutarate to pyruvate and L-glutamate. The pyruvate is then reduced to lactate in the presence of lactate dehydrogenase (LDH) with the concurrent oxidation of reduced β-nicotinamide adenine dinucleotide (NADH) to β-nicotinamide adenine dinucleotide (NAD). This change in absorbance is directly proportional to the activity of ALT in the sample (XI).

The activity measured by using a fully automated chemistry analyzer (Mindray Bs-200, China).

3.2.8 Estimation of Total Protein

At an alkaline solution (pH>12) copper ions combine with protein to produce a blue-violet color complex. The absorbency increase is directly proportional to the concentration of protein (appendix XII).

The concentration measured by using a fully automated chemistry analyzer (Mindray Bs-200, China).

3.2.9 Estimation of Albumin

At a slightly acid pH (pH=4.2), serum albumin combines with bromcresol green to produce a glaucous complex. The absorbency increase is directly proportional to the concentration of albumin (appendix XIII).

The concentration measured by using a fully automated chemistry analyzer (Mindray Bs-200, China).

3.2.10 Quality control

The reliability and validity of methods used in this study were checked, by commercially prepared control sera and calibrations were performed in all batch of analyses. The validity of ELISA technique for IL-17, IL-10 and OPN were compared with standards optical density (O.D).

3.2.11 Statistical analysis

Statistical Package for Social Sciences (SPSS) version 14 was used. Descriptive statistics presented as Mean±SD and percentages. T-test was used to compare two means of groups. Chi-square was used to correlate between groups (Nominal data) and Pearson's correlation was employed for the association between parameters and study variables (Numerical data), the significant difference considers as P-value < 0.05.

CHAPTER IV
RESULTS

4. Results

Table (4.1) the results of demographic and clinical data demonstrated that, the mean age of RA patient's 51±11.3 years. RA is more common in adults 72(81.8%) than young adults 16(18.2%), the frequency of the RA was found to be higher in female 84(95.5%) than male 4(4.50%). Most RA patients had anti-CCP positive 59(67%), while fewer were negative 29(33%). The number of patients receiving steroids was 52(59.1%) and the rest 36(40.9%) was on non-steroid treatment. Moreover, 62(70.5%) was a duration of disease ≤6, and others 26(29.5%) were>6 Years old. Abnormal IL-10 was found in 63(71.6%), was 25(28.4%) have a normal percentage. The results of the characteristic data shows, 80(91.0%) of RA patients had normal IL-17 whereas 8(9.00%) had abnormal. Normal OPN was observed in 76(86.4%) RA patients while 12(13.6%) were abnormal.

Table (4.2) independent sample t-test analysis observed that serum OPN and IL-10 levels were significantly increased in RA patients (38.3±29.6 ng/mL and 45.9±42.9 pg/mL) when compared to control (10.1±10.6 ng/mL and 8.48±7.36 pg/mL) with (*p*-value 0.000 and *p*-value 0.000) respectively, while IL-17 exhibited insignificant difference (6.55±1.17 pg/mL), in comparing to control (10.3±8.04 pg/mL); with (*p*-value 0.123). As well as liver enzymes AST, ALT, GGT were significantly increased in RA patients (16.6±6.98 U/L, 5.62±2.59 U/L and 27.3±23.1 U/L) than controls (7.86±7.86 U/L, 2.76±3.15 U/L, 20.9±13.4 U/L) with (*p*-value 0.000, *p*-value 0.000 and *p*-value 0.026), whereas ALP enzyme showed no statistic significant (70.6±21.6 U/L) in comparing to control (66.8±19.6 U/L) with (*p*-value 0.225). The result of liver protein (TP and albumin) shows a significant decreased in RA patients (6.68±0.61 g/dL and 3.91±0.40 g/dL) compared to control (7.18±0.695 g/dL and 4.22±0.460 g/dL) with (*p*-value 0.000 and *p*-value 0.000).

Moreover Table (4.3) shows there were insignificant differences in IL-10, IL-17, and OPN among patients' gender with *p*-value (0.543, 0.218 and 0.955) respectively. in addition there were insignificant difference in liver function tests AST, ALT, ALP, GGT, TP and albumin among gender *p*-value respectively (0.744, 0.624, 0.117, 0.490, 0.634 and 0.936).

Table (4.4) revealed that there were statistical insignificant for IL-10, IL-17, and OPN According to anti-CCP respectively *p*-value (0.522, 0.285 and 0.945). On top of that liver function tests AST, ALT, ALP, GGT and TP, exhibited insignificant statistic difference *p*-value (0.481, 0.291, 0.692, 0.325 and 0.574) respectively, while albumin show significant increase in anti cc-p positive compared with negative *p*-value (0.039).

Table (4.5) found that, there were no statistic difference in cytokines (OPN, IL-17 and IL-10) and liver function tests AST, ALT, ALP, GGT and albumin among type of treatment with *p*-value (0.483, 0.316, 0.099, 0.890, 0.967, 0 0.770, 0.406 and 0.988), whereas TP showed significantly difference *p*-value (0.010).

Table (4.6) Chi-square analysis revealed that the young adult group had higher abnormal IL-10 than adult RA patients (OR = 3.72, *p*-value 0.044). Furthermore, abnormal IL-17 (OR= 5.67, *p*-value 0.034) was found to be increased in young adult RA patients whereas no association was observed between age group and OPN (OR= 2.67, *p*-value 0.144).

Furthermore, table (4.7) demonstrated that no association was reported between the duration of disease and IL-10, IL-17, and OPN with *p*-value (0.410, 0.176 and 0.502) and OR= (0.77, 0.37 and 1.30) respectively.

Besides, table (4.8) found that no association between types of treatment and IL-10, IL-17, and OPN with *p*-value (0.246, 0.286 and 0.351) and OR= (1.53, 2.21 and 0.65) respectively.

Table (4.9) result of person correlation analysis revealed that there was no association observed between IL-10, IL-17, OPN, and liver parameters (AST, ALT, ALP, ALB, TP, and GGT).

Table (4.1): Demographic and baseline characteristics of RA patients

Variables	Frequency (%) or Mean ± SD
Age	51.8±11.3
Age groups	
≤41 Years	16 (18.2)
>41 Years	72 (81.8)
Sex	
Male	4 (4.60)
Female	84(95.4)
Anti-CCP	
Positive	59(67.0)
Negative	29(33.0)
Type of treatment	
Steroid	52(59.0)
Non-steroid	36(41.0)
Duration of disease	
≤6 Years	62(70.0)
>6 Years	26(30.0)
Cut off IL-10	
Abnormal	63(71.6)
Normal	25(28.4)
Cut off IL-17	
Abnormal	8(9.00)

Normal	80(91.0)
Cut off OPN	
Abnormal	12(13.6)
Normal	76(86.4)
Total	**88 (100%)**

Table (4.2): Comparison between study parameters of case versus control group

Parameters	Case Mean ±SD	Control Mean ±SD	*p*-value
IL-10 pg/mL	45.9±42.9	8.48±7.36	0.000
IL-17 pg/mL	12.4±9.71	10.3±8.04	0.123
OPN ng/mL	38.3±29.6	10.1±10.6	0.000
ALT U/L	5.62±2.59	2.76±3.15	0.000
AST U/L	16.6±6.98	7.86±7.86	0.000
ALP U/L	70.6±21.6	66.8±19.6	0.225
GGT U/L	27.3±23.1	20.9±13.4	0.026
TP g/Dl	6.68±0.611	7.18±0.695	0.000
ALB g/dL	3.91±0.404	4.22±0.460	0.000

AST= aspartate amino transferase, ALT= alanine amino transferase, ALP= alkaline phosphatase, GGT= gamma glutamyl transferase, TP= total protein and ALB= albumin

Table (4.3): Comparison of study parameters among gender

Parameters	**Male** **Mean ± SD**	**Female** **mean± SD**	***p*-value**
IL-10 pg/mL	58.8±60.7	45.3±42.3	0.543
IL-17 pg/mL	6.55±1.17	12.9±9.85	0.218
OPN ng/mL	39.2±20.4	38.3±30.0	0.955
AST U/L	15.5±5.25	16.6±7.07	0.744
ALT U/L	5.00±1.41	5.65±2.63	0.624
ALP U/L	54.0±15.2	71.4±21.6	0.117
GGT U/L	19.5±7.72	27.7±23.6	0.490
TP g/dl	6.82±.150	6.67±.624	0.634
ALB g/dL	3.92±.359	3.90±.407	0.936

AST= aspartate amino transferase, ALT= alanine amino transferase, ALP= alkaline phosphatase, GGT= gamma glutamyl transferase, TP= total protein and ALB= albumin

Table (4.4): Comparison of study parameters among positive and negative anti-CCP

Parameters	**Positive anti-CCP** **mean± SD**	**Negative anti-CCP** **mean± SD**	***p*-value**
IL-10 pg/mL	43.9±42.6	50.1±44.0	0.522
IL-17 pg/mL	13.1±9.98	10.8±9.10	0.285
OPN ng/mL	38.5±28.8	38.0±31.7	0.945
AST U/L	16.2±5.29	17.3±9.63	0.481
ALT U/L	5.83±2.83	5.20±1.98	0.291
ALP U/L	71.2±19.7	69.3±25.3	0.692
GGT U/L	25.6±21.3	30.8±26.5	0.325
TP g/dL	6.65±.622	6.73±.594	0.574
ALB g/dL	3.97±.372	3.78±.441	0.039

AST= aspartate amino transferase, ALT= alanine amino transferase, ALP= alkaline phosphatase, GGT= gamma glutamyl transferase, TP= total protein and ALB= albumin

Table (4.5): Comparison of study parameters among the type of treatments

Parameters	Steroid mean± SD	Non-steroid mean± SD	*p*-value
IL-10 pg/mL	52.2±45.2	36.8±38.0	0.099
IL-17 pg/mL	13.2±10.5	11.1±8.30	0.316
OPN ng/mL	36.5±29.7	41.0±29.6	0.483
AST U/L	16.5±5.43	16.7±8.84	0.890
ALT U/L	5.63±2.76	5.61±2.35	0.967
ALP U/L	70.0±21.9	71.4±21.5	0.770
GGT U/L	29.0±26.7	24.8±16.9	0.406
TP g/dL	6.45±0.662	6.88±.469	0.010
ALB g/dL	3.91±.386	3.90±.433	0.988

AST= aspartate amino transferase, ALT= alanine amino transferase, ALP= alkaline phosphatase, GGT= gamma glutamyl transferase, TP= total protein and ALB= albumin

Table (4.6): Association of IL-10, IL-17 and OPN with age groups

Variables	Age (yr)		OR	CI-Lower CI-Upper	*p*-value
	≤41 Years	>41 Years			
IL-10					
Abnormal	14 (23.0%)	47(77.0%)	3.72	(0.78-17.7)	0.044
Normal	2 (7.4%)	25(92.6%)			
IL-17					
Abnormal	2 (40.0%)	6 (60.0%)	5.67	(1.24-25.7)	0.034
Normal	12 (15.0%)	68 (85.0%)			
OPN					
Abnormal	4 (33.3%)	8 (66.7%)	2.67	(0.69-10.2)	0.144
Normal	12 (15.8%)	64 (84.2%)			

Table (4.7): Association of IL-10, IL-17 and OPN with duration of disease

Variables	Duration (yr)		OR	CI-Lower CI-Upper	*p*-value
	≤6 Years	>6 Years			
IL-10					
Abnormal	42 (68.9%)	19 (31.1%)	0.77	(0.28-2.13)	0.410
Normal	20 (74.1%)	7 (25.9%)			
IL-17					
Abnormal	4 (50.0%)	4 (50.0%)	0.37	(0.08-1.65)	0.176
Normal	58 (72.5%)	22 (27.5%)			
OPN					
Abnormal	9 (75.0%)	3 (25.0%)	1.30	(0.32-5.25)	0.502
Normal	53 (69.7%)	23 (30.3%)			

Table (4.8): Association of IL-10, IL-17 and OPN with types of treatment

Variables	Treatment		OR	CI-Lower CI-Upper	*p*-value
	Steroid	Non-steroid			
IL-10					
Abnormal	38 (62.3%)	23 (37.7%)	1.53	(0.61-3.83)	0.246
Normal	14 (51.9%)	13 (48.1%)			
IL-17					
Abnormal	6 (75.0%)	2 (25.0%)	2.21	(0.42-11.6)	0.286
Normal	46 (57.5%)	34 (42.5%)			
OPN					
Abnormal	6 (50.0%)	6 (50.0%)	0.65	(0.19-2.21)	0.351
Normal	46 (60.5%)	30 (39.5%)			

Table (4.9): Association between cytokines and liver functions parameters (Pearson's correlation results)

Parameters		*p*-value	R
IL-10	AST	0.626	0.12
	ALT	0.201	0.66
	ALP	0.803	0.05
	ALB	0.161	-0.13
	TP	0.562	0.02
	GGT	0.259	0.15
IL-17	AST	0.182	-0.15
	ALT	0.825	0.02
	ALP	0.826	-0.02
	ALB	0.234	0.12
	TP	0.597	0.05
	GGT	0.172	-0.14
OPN	AST	0.508	0.07
	ALT	0.253	0.12
	ALP	0.895	0.01
	ALB	0.294	-0.11
	TP	0.496	0.07
	GGT	0.982	-0.02

R= Regression coefficient (strength of correlation)

CHAPTER V

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussions

The researchers observed abnormal liver functions in RA patients, further the abnormality were attributed to immune aggregations and/or to the toxicity resulting from using of rheumatic modifying drugs. Accordingly, the present study carried out to assess whether the pro-inflammatory, anti-inflammatory cytokines are associated with liver functions in RA patients. Furthermore, to determine their associations with study variables such as age, type of treatment, and duration of disease.

The difficulty in the diagnosis and studying the prevalence of RA is first due to different selection criteria, and second challenge is due to overlapping with signs and symptoms of other inflammatory autoimmune diseases. The age and sex were also considered as confounding factors for measuring of the prevalence. In addition low numbers of rheumatologists and specialist physicians in Africa who care for RA patients are also might be an obstacles. Therefore, the variations in the prevalence of RA were attributed to the geographical, life style and regional variations (Radis, 2012; David *et al*., 2013; Elshafie *et al*., 2016; Bester *et al*., 2016).

The current results are concurrent with many previous studies reported that, the frequency of RA is higher in elderly subjects, (Eftekharian, 2013; ELsedig *et al*., 2014; Mursal *et al*., 2016). A possible explanation is justified by the protective mechanisms in alder are decreased, which resulting in decreased immunotolerance, cytokines synthesis, and T cell proliferation (Kobak and Bes, 2018). The demographic data indicated that the prevalence of RA was found to be 21 fold higher in females than males. In contrast with a previous study in Sudan, the ratio was found to be 9:1 females to males (Abdelsalam *et al*., 2011). The main factors that impact the higher RA ratio in females are the sex hormones after puberty and the female's immune system, which potentially more reactive than

males. Other predisposing factors such as delaying in diagnosis, lifestyle, abuse of cosmetics and steroids might increase the frequency of RA.

Moreover, 2-fold of RA patients were positive anti-CCP antibodies, since anti-CCP is a hallmark of RA classification, which elevated before the onset of RA symptoms, patients tend to have aggressive disease phenotype, more bone erosion, and worse disease outcome disease severity (Yang *et al*., 2018; Burbano *et al*., 2018), our result come online with that autoimmune antibodies can be detected in around two-thirds of RA patients (Elshafie *et al*., 2019; Zeng, 2017). Therefore periodical screening program for anti-CCP is recommended for elderly, since RA is asymptomatic in early stage (Kanecki and Tyszko, 2015). Other observation demonstrated that, anti-ccp not useful for monitoring disease progression (Fouda *et al*., 2017).

Our results similar to previous reports which stated that, IL-10 and OPN show a significant increase between Rheumatoid Arthritis patients and the control group (Holdsworth and Yi, 2015; kkonen *et al*., 2017). Emphasized the crucial role of this cytokines in the pathogenesis of RA, the experimental evidence on mice study reported that, OPN- deficient mice were found to attenuate articular cartilage erosion and reduce the arthritic score, therefore has been explored as a therapeutic target in many preclinical studies and clinical trials (Farrokhi *et al*., 2018). Significantly increased IL-10 accounts as evidence of enhanced immunopathology and increases the risk of disease progression and severity (Zhdanov, 2004; Tao *et al*., 2011; Holdsworth and Yi, 2015; Fathy *et al*., 217; Shikhpour *et al*., 2018). Despite significant higher mean concentration of IL-17 was observed in RA patients (Miletic *et al*., 2012; Tan *et al*., 2013; Ruderman, 2015), our results found that there was insignificant difference in mean level of IL-17 of case versus control group. Indeed all patients in this study received different RA treatments, therefore might be due to

treatments action, which leads to a significant decline in IL-17 level after therapy (Nasef *et al*., 2019). Moreover other studies reported that, IL-10 inhibits the production of Th17 cells to reduce the development and severity of the disease (Tao *et al*., 2011; Kurata *et al*., 2014). Contradict finding concluded that, RA patients had significantly lower IL-10 and higher in IL-17 level (Qu *et al*., 2019).

In fact that albumin is the most abundant plasma protein (60% of total protein), and used to measure biosynthetic activity of liver (Chien *et al*., 2017). Result of the present study showed that, there were significant statistical decrease in mean serum total protein and albumin concentration in RA patients compared to the control subjects. Matched with previous studies found that, mean albumin level was significantly decreased in RA patients (Mohamed *et al*., 2017; Ganeb *et al*., 2020). Suggesting that, the decrease albumin level due to suppressed hepatic synthetic by inflammatory cytokines, treatment as well as malnutrition status of RA patients (Mohamed *et al*., 2017; Ganeb *et al*., 2020).

To the best of our knowledge liver damage during RA is most common in the form of asymptomatic abnormal liver tests, and patients with RA are more susceptible to develop autoimmune liver diseases (Dinic *et al*., 2018). The present study results found that Liver enzymes activity (ALT, AST, and GGT) were significantly increased in RA patients than in control subjects. These findings agreed with previous study found that, the liver transaminases enzymes activity were significantly increased in RA patients. (khadim and Al-Fartuise, 2020). Other study reported that GGT may be higher in patients with RA (Abraham *et al*., 2004). Contradiction study found normal activity of serum transaminases in RA patients (Thompson *et al*., 1990). Another inconsistent previous study concluded that ALP enzyme may be higher in RA patients (Abraham *et al*., 2004). The possible explanation for increased liver enzymes activity

during RA disease might be a result from aggregation of immune complex and/or using of anti-rheumatic treatments (Lazrak *et al*., 2013; Conway and Carey, 2017; Rakuomi *et al*., 2017; Sundbaum *et al*., 2019).

Insignificant differences in mean level of OPN, IL-10, and IL-17 was noted among gender in this study. This finding comes in line with previous studies conclude that, IL-10, IL-17, and OPN were statistical insignificantly differ when compared males with females (Aulock *et al*., 2006 Akdeniz *et al*., 2018; Azab *et al*., 2020). In contrast with other study, the production of cytokines and chemokine's differs between the sexes, in human male results in greater production of IL-10 compared to female, due to induction of IL-10 by androgen, while the expression of IL-17 depending on the stimulation and purity of T cells therefore insignificant result may appear (Klein and Flanagan, 2016).

Although a significant decrease in mean albumin level in men was reported by previous study (Denko and Gabriel, 1981), our results found that, there were insignificant differences in the mean level total protein and albumin of males in comparison with females. Other study found that, females had significantly lower in AST activity and albumin level than males, whereas insignificant findings were observed in mean activity of ALT, ALP and TP level, variation in the results attributed to age-related gonadal hormones. (Adiga, 2016). Another previous study found that mean total protein level, GGT and AST activity were insignificantly differences among gender, while mean level of albumin and ALT activity revealed significant higher in males than females (Ohawada *et al*., 2017).

Anti-ccp positive group showed insignificantly differences in the mean levels of OPN, IL-10, and IL-17 when compared with negative anti-ccp. Contradict previous finding that, seropositive patients had higher levels of pro-inflammatory cytokines than those of seronegative patients and healthy controls (Burbano *et al*., 2018). Other studies found that anti cc-p

positive had higher IL-10, and IL-17 than synovial fluid seronegative patients (Gómez *et al*., 2015; Abd Elazeem *et al*., 2018).

No available previous studies reported the difference in liver functions among anti-ccp groups. As known as the abnormal liver functions correlate with disease severity of RA, the previous study reported that there was a significant increase disease progression in anti-ccp positive compared to anti-ccp negative rheumatoid arthritis patients (Eltokhy *et al*., 2011). Our study revealed insignificant difference in liver functions test between groups of anti-ccp, while mean albumin level was significantly increased in seropositive group.

The current study reported that young adults were more likely to have higher IL-10 and IL-17. These results disagreed with previous studies conducted by (Abd Elazeem *et al*., 2018; Akdeniz *et al*., 2018). Meanwhile other previous study found a relationship between age and OPN (Iwadate *et al*., 2013). Whereas our study stated no association between OPN and age groups.

Concurrent with previous studies our results revealed that there were no associations between IL-10, IL-17, OPN, and duration of disease groups (Al Zifzaf *et al*., 2015; Abd Elazeem *et al*., 2018; Akdeniz *et al*., 2018).

The result of present study showed that insignificant differences in AST, ALT, GGT, ALP, and Albumin among RA treated groups. Other study showed liver transaminase enzyme were significantly increase after MTX treatment (Dehestani *et al*., 2015).

In an experimental study, animal receiving different doses of glucocorticoids were more likely to have lower protein levels, since glucocorticoids treatment inhibit protein synthesis by liver (Kim and Kim, 1975). Following corticosteroids treatment, the protein breakdown increased reflected by significant increase in amino acid level (Steiner *et al*., 2011).

In fact steroid anti-rheumatic drug more effect on cytokines production compared to non steroids. Moreover steroid inhibit pro-inflammatory cytokines production while the effect of methotrexate (MTX) is more obscure (Noack and Miossec, 2019). The steroids action either directly modulate the pro-inflammatory cytokine or through suppression of cytokines producing cells (Noack *et al*., 2016; Negera *et al*., 2018).

The current study shows that there were insignificant difference in mean levels of IL-10 and IL-17 between types of treatment. Previous study found that IL-17 significantly decreased in response to steroid and non steroid anti-rheumatic drug, whereas IL-10 showed insignificant decrease with non steroid drug and significantly decrease when using steroid as a treatment for RA (Noack *et al*., 2016; Noack and Miossec, 2019). While other study reported that, no change in IL-10 expression was detected during treatment with steroid (Andersson *et al*., 2005). Meanwhile the comparison study showed that OPN level was insignificantly differ among groups of treatment. Concurrent with study reported insignificant difference in OPN level in patients who received steroids and those non-received steroids (Samitas *et al*., 2011). Another studies reported that OPN level was significantly decrease in patients treated with steroid and non-steroid (Medrek *et al*., 2013; Xiao *et al*., 2015).

From the evidence that, IL-17 was negatively correlated with steroid and non steroid treatment, whereas IL-10 was positively related to methotrexate, the different association is due to different drugs action mode (Negera *et al*., 2018; Noack and Miossec, 2019). In contrast, the results of the present study presented no correlation between OPN, IL-10, IL-17 and types of RA treatment.

The current study revealed no association between interleukins and liver function tests. In fact that, liver abnormalities were noted in patients with RA when compared with control group (Dinic *et al*., 2018). Meanwhile

another study in forced the previous finding that long-term using of methotrexate treatment account for a risk for the elevation of liver enzymes as a negative effect (khadim and Al-Fartuise, 2020).

5.2 Conclusion

The frequency of RA is higher in the elderly than in young adults, furthermore, RA patients had higher OPN and IL-10. Liver enzymes (AST, ALT and GGT) are increased, while total protein and albumin are decreased in RA patients. Additionally, young adult RA patients are more likely to have abnormal IL-10 and IL-17. Finally no associations are observed between pro-inflammatory, anti-inflammatory cytokines and liver functions parameters.

5.3 Recommendations

From the results of this study, it is recommended that:

Auto antibodies screening program for a suspected group such as (females, elder individuals) is recommended.

Specify the triggering factors to prevent RA disease and severity.

Periodical monitoring of liver functions among RA patients, and intervention if necessary.

Further epidemiological study is recommended to find out the prevalence of the RA among Sudanese populations.

Further cross-section studies to determine the ratio of disease between females to males.

Further cross section studies to find out the association between body mass index and RA.

Further cohort study is needed to explore the relationship between types of drugs, liver functions and risk factors (type of treatment and age) associated with RA.

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Sudan University of Science and Technology	جامعة السودان للعلوم والتكنولوجيا
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College of Medical Laboratory Science	كلية علوم المختبرات الطبيه
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researcher name: Mohamed Abdelrhman Eltahir	اسم الباحث: محمد عبدالرحمن الطاهر

informed consent form	استمارة موافقه علي المشاركة في بحث

هذا البحث سيقدم ك بحث تكميلي لنيل درجة الدكتوراة

THIS RESEARCH WILL BE SUBMITTED IN A PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF Ph.D. DEGREE

عنوان البحث: علاقة مستويات الاوستيوبونتين و انترليوكين 10 وانترليوكين 17 مع اختبارات وظائف الكبد في المرضي السودانين المصابين بالتهاب المفاصل الروماتويدي

Research title: Association of Osteopontin, Interleukin10 and Interleukin17 Levels with Liver Functions Tests in Sudanese Patients with Rheumatoid Arthritis

Introduction about the research: the liver problems observed during rheumatoid arthritis disease. Controversial findings were reported in the association between liver functions, RA and treatment of RA, this event prompted us to investigate whether the Osteopontin (OPN), interleukin-17 (IL-17), and interleukin-10 (IL-10) cytokines associated with liver function tests in RA patients.

مقدمة البحث: مرض الروماتيزم من الامراض التي تؤثر علي الكبد, الدراسات السابقه وضحت عدة اسباب عن تاثر الكبد اثناء مرض الروماتيزم, وهو ما حفزنا للقيام بهذه الدراسه والتي تقوم بدراسة علاقة السايتوكاينات مع امراض الكبد اثناء مرض الروماتيزم.

Research purpose: to associate between inflammatory cytokines and liver functions in rheumatoid arthritis patients

الغرض من البحث: دراسة علاقة السايتوكاينات مع امراض الكبد اثناء مرض الروماتيزم

The participation Risk and Discomfort: there is no known risk for participation in this study and participant will not suffer anything's rather than the little pain will feel from venipuncture while collecting the blood sample and usually there is no complication following this process

مخاطر المشاركة: لا توجد مخاطر معلومة للمشاركة في الدراسة ولن يعاني المشارك الا من الالم القليل اثناء اخذ عينة الدم من الوريد و عادة لا توجد مضاعفات لهذه العملية

The anticipated benefits: this study will provide significant information about liver problem during RA. The participant will get a copy of blood tests results will be done without payment if he/she want

فوائد المشاركة: ستوفر الدراسة معلومات ذات قيمة عن سبب اصابة الكبد اثناء مرض الروماتيزم,سيتحصل المشارك علي نسخة من نتيجة الفحص بدون مقابل في حال اراد ذلك

The confidentiality and privacy: privacy and confidentiality of all participant, personal information will be maintained, only the researcher and research work team can reach the record that identifying the participants and if the results of the study will publish, the study participant identity will remain confidential

خصوصية وسرية المعلومات: سيتم المحافظة علي خصوصية وسرية كل المعلومات الشخصية للمشاركين, يسمح للباحث وفريق العمل فقط بالوصول اليللسجلات التعريفيه بالمشتركين وسيتم المحافظة علي سرية هويتهم حتي في حالة نشر نتائج الدراسة.

Compensation for participation: there will be no payment or medical compensation anticipated for participation in this study.

التعويض عن المشاركة: لن يكون هنالك اي تعويض مادي او طبي مقابل المشاركة في هذه الدراسة

The contact with the researcher: the participant have right to contact the researcher for answers related questions about research or to inform him in case of withdrawing using this phone number 0121415075

التواصل مع الباحث: يحق للمشاركين التواصل مع الباحث للاجابة علي اسئلتهم المتعلقه بالبحث او اعلامه في حالة الانسحاب عن طريق الاتصال بالهاتف0121415075

Voluntary participation: participant will voluntary participate, willing to collect blood sample from him and provide information needs for the study by filling the given questionnaire. He/she can withdraw from the study at any time with no loss of benefit.

التطوع بالمشاركة: سيوافق المشارك علي المشاركة طواعية مع موافقه علي اخذ عينة من الدم واعطاء المعلومات المطلوبه للدراسة بواسطة ملاالاستبيان المصاحب و من حقه الانسحاب من المشاركة في اي وقت من غير فقدان الفائدة.

I have read and understand all aspects of research, and I willingly agree to participate in the research entitled above with my signature.

Participant name………………………………… Signature…………………………

Researcher signature…………………… Date……………………..

لقد قرات وفهمت كل جوانب البحث و عليه اوافق علي المشاركة بالبحث المعنون اعلاه وعلي ذلك اوقع

اسم المشارك في البحث .... المضاء..........................

التاريخ.......................... امضاء الباحث.......................

Sudan University of science & Technology

College of Graduate Studies

Questionnaire

Association of Osteopontin, IL10 and IL17 level with liver Function Tests in Rheumatoid Arthritis Sudanese Patients

Date:………./……../20… **Sample serial No:**

Patient files No: ☐

Age: ………………..years

Gender: Male: ☐ **Female:** ☐

Duration of disease: …………………………..

Treatment:

Type: steroid: ☐ **non steroid** ☐

Research parameters:

Osteopontin level…………..pg/ml

IL-10 level…………….pg/ml

IL-17 level…………..pg/ml

Liver function tests:

ALP………………U/L

AST………………U/L

ALT………………U/L

GGT………………U/L

Total protein………g/dl

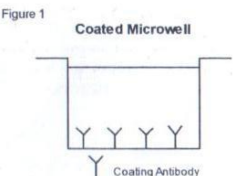
Albumin…………..g/dl

3 Principles of the Test

An anti-human Osteopontin coating antibody is adsorbed onto microwells.

Figure 1

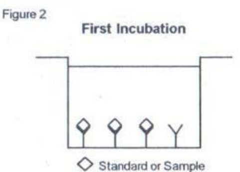
Coated Microwell



Human Osteopontin present in the sample or standard binds to antibodies adsorbed to the microwells.

Figure 2

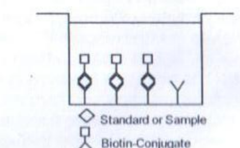
First Incubation



Following incubation unbound biological components are removed during a wash step and a biotin-conjugated anti-human Osteopontin antibody is added and binds to human Osteopontin captured by the first antibody.

Figure 3

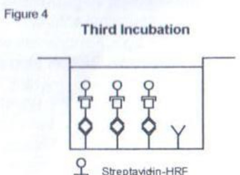
Second Incubation



Following incubation unbound biotin-conjugated anti-human Osteopontin antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human Osteopontin antibody.

Figure 4

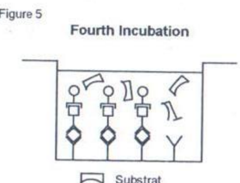
Third Incubation



Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

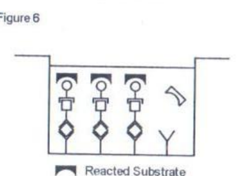
Figure 5

Fourth Incubation



A coloured product is formed in proportion to the amount of human Osteopontin present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human Osteopontin standard dilutions and human Osteopontin sample concentration determined.

Figure 6



4 Reagents Provided

4.1 Reagents for human Osteopontin ELISA BMS2066 (96 tests)

1 aluminium pouch with a **Microwell Plate coated** with monoclonal antibody to human Osteopontin

1 vial (120 µl) **Biotin-Conjugate** anti-human Osteopontin polyclonal antibody

1 vial (150 µl) **Streptavidin-HRP**

2 vials human Osteopontin **Standard** lyophilized, 60 ng/ml upon reconstitution

1 bottle (12 ml) **Sample Diluent**

1 vial (5 ml) **Assay Buffer Concentrate** 20x (PBS with 1% Tween 20, 10% BSA)

1 bottle (50 ml) **Wash Buffer Concentrate** 20x (PBS with 1% Tween 20)

1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)

1 vial (15 ml) **Stop Solution** (1M Phosphoric acid)

6 **Adhesive Films**

4.2 Reagents for human Osteopontin ELISA BMS2066TEN(10x96 tests)

10 aluminium pouches with a **Microwell Plate coated** with monoclonal antibody to human Osteopontin

10 vials (120 µl) **Biotin-Conjugate** anti-human Osteopontin polyclonal antibody

10 vials (150 µl) **Streptavidin-HRP**

10 vials human Osteopontin **Standard** lyophilized, 60 ng/ml upon reconstitution

10 bottles (12 ml) **Sample Diluent**

3 vials (5 ml) **Assay Buffer Concentrate** 20x (PBS with 1% Tween 20, 10% BSA)

5 bottles (50 ml) **Wash Buffer Concentrate** 20x (PBS with 1% Tween 20)

10 vials (15 ml) **Substrate Solution** (tetramethyl-benzidine)

1 vial (100 ml) **Stop Solution** (1M Phosphoric acid)

30 **Adhesive Films**

5 Storage Instructions – ELISA Kit

Store kit reagents between 2°C and 8°C. Immediately after use remaining reagents should be returned to cold storage (2°C to 8°C). Expiry of the kit and reagents is stated on labels.

Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, this reagent is not contaminated by the first handling.

6 Specimen Collection and Storage Instructions

Cell culture supernatant, serum and plasma (EDTA, citrate, heparin) were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or cells as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human Osteopontin. If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for stability refer to 13.5).
Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

7 Materials Required But Not Provided

- 5 ml and 10 ml graduated pipettes
- 5 µl to 1000 µl adjustable single channel micropipettes with disposable tips
- 50 µl to 300 µl adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

8 Precautions for Use

- All reagents should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.

- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

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Procedure Summary

1. Add 50µL of Standards or samples to each well in duplicate.
2. Add 50µL of Biotinylated Antibody Reagent to each well.
3. Cover plate and incubate at room temperature (20-25°C) for 2 hours.
4. Wash plate THREE times.
5. Add 100µL of prepared Streptavidin-HRP Solution to each well.
6. Cover plate and incubate at room temperature for 30 minutes.
7. Wash plate THREE times.
8. Add 100µL of TMB Substrate to each well.
9. Develop plate in the dark at room temperature for 30 minutes.
10. Stop reaction by adding 100µL of Stop Solution to each well.
11. Measure absorbance on a plate reader at 450nm or 450 minus 550nm.
12. Calculate the results using graph paper or curve-fitting statistical software.

Additional Materials Required

- Precision pipettors with disposable plastic tips to deliver 5-1000µL and plastic pipettes to deliver 5-15mL.
- A glass or plastic 2L container to prepare Wash Buffer
- A squirt wash bottle or an automated 96-well plate washer
- 1.5mL polypropylene or polyethylene tubes to prepare standards – do not use polystyrene, polycarbonate or glass tubes
- Disposable reagent reservoirs
- 15mL plastic tube to prepare Streptavidin-HRP Solution
- Standard ELISA reader for measuring absorbance at 450nm and 550nm. If a 550nm filter is not available, the absorbance may be measured at 450nm only. Refer to the instruction manual supplied with the instrument being used.

Precautions

- **All samples and reagents must be at room temperature (20-25°C) before use in the ELISA.**
- Review all instructions carefully and verify components against the Kit Contents list (page 1) before beginning the assay.
- Do not use a water bath to thaw samples. Thaw samples at room temperature.
- When preparing standard curve and sample dilution in culture medium, use the same medium used to culture the cells. For example, if RPMI with 10% fetal calf serum (FCS) was used to culture cells, then use RPMI with 10% FCS to dilute the standard and samples. Do NOT use RPMI without serum supplement.
- To avoid cross-contamination, use new disposable pipette tips for each transfer and a new adhesive plate cover for each incubation step. If using a multichannel pipettor, always use a new disposable reagent reservoir.
- Once reagents have been added to the plate, take care NOT to let plate DRY at any time during the assay.
- Avoid microbial contamination of reagents. Avoid exposing reagents to excessive heat or light during storage and incubation.
- Vigorous plate washing is essential.

B. Plate Washing

1. Gently squeeze the long sides of plate frame before washing to ensure all strips securely remain in the frame.
2. Empty plate contents. Use a squirt bottle to vigorously fill each well completely with Wash Buffer, then empty plate contents. Repeat procedure two additional times for a total of THREE washes. Blot plate onto paper towels or other absorbent material.

Note: For automated washing, aspirate all wells and wash THREE times with Wash Buffer, overfilling wells with Wash Buffer. Blot plate onto paper towels or other absorbent material.

C. Streptavidin-HRP Solution Preparation and Incubation

- Prepare Streptavidin-HRP Solution immediately before use. Do not prepare more Streptavidin-HRP Solution than required.
- Use a 15mL plastic tube to prepare Streptavidin-HRP Solution.
- If using a multichannel pipettor, **use new reagent reservoir and pipette tips** when adding the prepared Streptavidin-HRP Solution.

1. Centrifuge Streptavidin-HRP Concentrate to force entire vial contents to the bottom of the vial.
2. **(PP)** Use only enough of the Streptavidin-HRP Solution required for the number of strips being used. For each strip, mix 2.5µL of Streptavidin-HRP Concentrate with 1mL of Streptavidin-HRP Dilution Buffer. Store Streptavidin-HRP Concentrate reserved for additional strips at 2-8°C.

   For one complete 96-well plate, add 30µL of Streptavidin-HRP Concentrate to 12mL of Streptavidin-HRP Dilution Buffer and mix gently.
3. Add 100µL of prepared Streptavidin-HRP Solution to each well.
4. Carefully attach a new adhesive plate cover, ensuring all edges and strips are tightly sealed. Incubate plate for 30 minutes at room temperature, 20-25°C.
5. Carefully remove the plate cover and discard plate contents. Wash plate as described in the Plate Washing Section.

D. Substrate Incubation and Stop Step

- Use new disposable reagent reservoirs when adding TMB Substrate Solution and Stop Solution.
- Dispense from bottle ONLY the amount of reagent required for the number of wells being used (i.e., 100µL per well). Do not use a glass pipette to measure the TMB Substrate Solution.
- **(PP)** Do not combine leftover substrate with that reserved for the second partial plate. Take care not to contaminate remaining TMB Substrate Solution.

1. Pipette 100µL of TMB Substrate Solution into each well.
2. Allow enzymatic color reaction to develop at room temperature in the dark for 30 minutes. Do not cover plate with aluminum foil or a plate sealer. The reaction yields a blue solution that turns yellow when Stop Solution is added.
3. After 30 minutes, stop the reaction by adding 100µL of Stop Solution to each well.

E. Absorbance Measurement

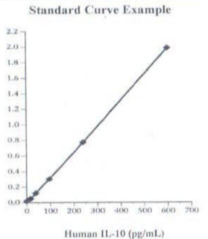
- Evaluate the plate within 30 minutes of stopping the reaction.
- Measure absorbance on an ELISA plate reader set at 450nm and 550nm. Subtract 550nm values from 450nm values to correct for optical imperfections in the microplate. If 550nm is not available, measure absorbance at 450nm only.

Note: Omitting the 550nm measurement will result in higher absorbance values.

F. Calculation of Results

- Use the standard curve to determine IL-10 amount in an unknown sample. Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the vertical (Y) axis vs. the corresponding IL-10 concentration (pg/mL) on the horizontal (X) axis.
- Calculate results using graph paper or curve-fitting statistical software. The IL-10 amount in each sample is determined by interpolating from the absorbance value (Y axis) to IL-10 concentration (X axis) using the standard curve.
- If the test sample was diluted, multiply the interpolated value obtained from the standard curve by the dilution factor to determine amount of IL-10 in the sample.
- Absorbance values obtained for duplicates should be within 10% of the mean value. Carefully consider duplicate values that differ from the mean by greater than 10%.

Standard Curve Example

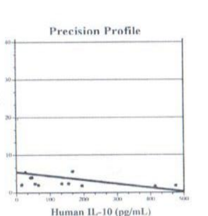


Performance Characteristics

Sensitivity: < 3pg/mL
The Lower Limit of Detection (LLD)[1] was determined by assaying replicates of zero and the standard curve. The mean signal of zero + 2 standard deviations read in dose from the standard curve is the LLD. This value is the smallest dose that is not zero with 95% confidence.

Precision: The inter-assay coefficient of variation is plotted against IL-10 concentration (pg/ml). The points represent samples reevaluated in replicates of four in three different kit lots.

Precision Profile



Assay Range: 15.36-600pg/mL.
Suggested standard curve points are 600, 240, 96, 38.4, 15.36 and 0pg/mL.

Reproducibility:
Intra-assay CV: < 10%
Inter-assay CV: < 10%

Specificity: This ELISA is specific for the measurement of natural and recombinant human IL-10. It does not cross-react with human IL-1α, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, TNFα, TNFβ, or IFNγ. There is ~2% cross-reactivity with recombinant mouse IL-10.

Calibration: Standards in this assay have been calibrated to the NIBSC reference standard lot 93/722. One (1) pg of standard = 1.4 NIBSC pg =0.007 NIBSC units.

Expected Values: Serum, plasma and urine samples were collected from apparently healthy individuals and evaluated in this assay. The levels of human IL-10 detected in each sample type are as follows:

Sample	n	Average (pg/mL)	Range (pg/mL)
serum	15	3.6	0-14.1
plasma	14	4.5	1.3-15.6
urine	9	0.7	0-2.7

- Do not mix reagents from different kit lots. Discard unused ELISA components after assay completion.
- Do not use glass pipettes to measure TMB Substrate. Take care not to contaminate the solution. If solution is blue before use, DO NOT USE IT.
- Individual components may contain antibiotics and preservatives. Wear gloves while performing the assay to avoid contact with samples and reagents. Please follow proper disposal procedures.

Additional Precaution for the 5-plate Kit

- Dispense, pool, and equilibrate to room temperature only the reagent volumes required for the number of plates being used. Do not combine leftover reagents with those reserved for additional plates.

Sample Preparation

- Serum; EDTA, heparin and sodium citrate plasma; urine and culture supernatants may be tested in this ELISA.
- 50µL per well of serum, plasma, urine or culture supernatant are required.
- Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -70°C. Avoid repeated freeze-thaw cycles when storing samples.
- Gradually equilibrate samples to room temperature before beginning assay. Do not use a heated water bath to thaw or warm samples. Mix samples by gently inverting tubes.
- If samples are clotted, grossly hemolyzed, lipemic or contaminated, or if there is any question about the integrity of a sample, make a note on the template and interpret results with caution.
- Samples and standards must be assayed in duplicate each time the assay is performed.
- If the IL-10 concentration possibly exceeds the highest point of the standard curve (i.e., 600pg/mL), prepare one or more 10-fold dilutions of the test sample. When testing culture supernatants, prepare the serial dilutions using your culture medium. When testing serum, plasma, or urine prepare the serial dilutions using the Standard Diluent provided. A 10-fold dilution is prepared by adding 50µL of sample to 450µL of appropriate diluent. Mix thoroughly between dilutions.

Reagent Preparation

For procedural differences when using partial plates, look for **(PP)** throughout these instructions.

Note: When using the 5-plate kit, only one Standard per plate is supplied, therefore, partial plates cannot be used.

Wash Buffer

Note: Wash Buffer must be at room temperature before use in the assay. Do not use Wash Buffer if it becomes visibly contaminated during storage.

1. Label a clean glass or plastic two-liter container "Wash Buffer." The 30X Wash Buffer may have a cloudy appearance.
2. Add the entire contents of the Wash Buffer (50mL) bottle to the container. Dilute buffer to 1.5L with ultrapure water and mix thoroughly.

(PP) When using partial plates, store the reconstituted Wash Buffer at 2-8°C.

Standards

- **(PP)** Reconstitute and use one vial of the lyophilized Standard per partial plate.
- Prepare Standards just before use and use within one hour of reconstitution. Do not store reconstituted standards.

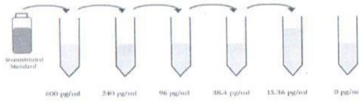
1. When testing **culture supernatant samples,** reconstitute standard with ultrapure water. Reconstitution volume is stated on the standard vial label. The standard will dissolve in approximately 1 minute. Mix by gently inverting vial. Use the sample culture medium to prepare Standard Curve dilutions.

When testing **serum, plasma, or urine samples,** reconstitute standard with ultrapure water. Reconstitution volume is stated on the standard vial label. The standard will dissolve in approximately 1 minute. Mix by gently inverting vial. Use the Standard Diluent provided to prepare standard curve serial dilutions.

- When testing **serum, plasma, or urine and cell culture supernatant samples on the same plate,** validate the media to establish if the same standard curve can be used for both sample types. Prepare a standard curve (including a zero/blank) using culture medium to reconstitute and dilute the standard. Use medium containing serum or other protein to maximize IL-10 stability. Perform this curve in parallel with a standard curve prepared with Standard Diluent. If OD values are within 10% of the mean for both curves, then the assay may be performed with Standard Diluent, whether testing culture supernatant, urine, plasma or serum samples.

2. Label six tubes, one for each standard curve point: 600, 240, 96, 38.4, 15.36 and 0pg/mL, then prepare 1:2.5 serial dilutions for the standard curve as follows:
3. Pipette 240µL of appropriate diluent into each tube.
4. Pipette 160µL of the reconstituted standard into the first tube (i.e., 600pg/mL) and mix.
5. Pipette 160µL of this dilution into the second tube labeled (i.e., 240pg/mL) and mix.
6. Repeat the serial dilutions (using 160µL) three more times to complete the standard curve points. These concentrations, 600pg/mL, 240pg/mL, 96pg/mL, 38.4pg/mL, 15.36pg/mL and 0pg/mL are the standard curve.

Serial Dilutions using 160µL



Assay Procedure

A. Sample and Biotinylated Antibody Reagent Incubation

- **(PP)** Determine number of strips required and leave these strips in the plate frame. Place remaining unused strips in the provided foil pouch with desiccant and store at 2-8°C. Make sure foil pouch is sealed tightly. After completing assay, retain plate frame for second partial plate. When using the second partial plate, place strips securely in the plate frame.
- Use the Data Template provided to record locations of the zero standard (blank or negative control), standards and samples. Perform three standard points and one blank in duplicate with each series of unknown samples.
- If using a multichannel pipettor, use a new reagent reservoir to add the Biotinylated Antibody Reagent. The Reagent may have a cloudy appearance. Remove from the vial only the amount required for the number of strips being used. Take care not to touch the samples in wells with the pipette tip when adding the Biotinylated Antibody Reagent.

1. Add 50µL of reconstituted standards or test samples in duplicate to each well.

   Note: If the IL-10 concentration in any sample possibly exceeds the highest point on the standard curve, 600pg/mL, see Sample Preparation Section.
2. Add 50µL of Standard Diluent to all wells that do not contain standards or samples.
3. Add 50µL of Biotinylated Antibody Reagent to all wells containing standards or samples.
4. Carefully cover plate with an adhesive plate cover. Ensure all edges and strips are tightly sealed by running your thumb over edges and down each strip. Incubate for two (2) hours at room temperature, 20-25°C.
5. Carefully remove adhesive plate cover. Wash plate as described in the Plate Washing section below.

Recovery: Three different levels of recombinant human IL-10 were spiked into human serum, plasma, and urine samples collected from apparently healthy individuals, and a control buffer. Mean recoveries are as follows:

Control Level (pg/mL)	32	110	276
Mean Serum Recovery	89%	93%	80%
Control Level (pg/mL)	20	86	264
Mean Plasma Recovery	94 %	88 %	84 %
Control Level (pg/mL)	38	123	358
Mean Urine Recovery	77%	78%	75%

Cited Reference

1. *Immunoassay: A Practical Guide*, ed. Chan and Perlstein, 1987, Academic Press, p.71.

Limited product warranty

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Product label explanation of symbols and warnings

REF	Catalog Number	LOT	Batch code		Temperature limitation		Use by		Manufacturer		Consult instructions for use		Caution, consult accompanying documents

Manufacturer's address: Bender MedSystems GmbH | Campus Vienna Biocenter 2 | 1030 Vienna, Austria

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X SPECIMEN COLLECTION, PREPARATION, STORAGE AND DILUTION

A. Specimen Collection and preparation

1. The BIOSOURCE IL-17 Cytoscreen kit may be used to measure IL-17 in serum, plasma and cell culture supernatant .Isolation and culture of peripheral blood mononuclear cells may be realized by usual methods. However, one should avoid an unintentional stimulation of the cells by the procedure. The use of pyrogen-free reagents and adequate controls are mandatory.
2. Sampling conditions can affect values measured in serum or plasma, therefore, strict precautions have to be taken during sampling to avoid impurities contained in sampling materials that would stimulate IL-17 production by blood cells and thus falsely increase plasma IL-17 values.
3. Serum must be removed as soon as possible from the clot of red cells after clotting and centrifugation, and kept at 4°C for maximum one day.
4. Collection tubes must be pyrogen-free. Plasma can be collected on sterile EDTA or heparin tubes (at 4°C) and rapidly separated after centrifugation. However, as batches of heparin are often contaminated with pyrogen, it is recommended to test each batch of heparin to avoid unintentional stimulation of blood cells. Other substances in the tube must be also pyrogen-free.

B. Storage

Serum/plasma samples must be kept at -20°C for maximum 2 months, and for longer storage (maximum one year) at - 70°C. Samples with low protein levels (e.g. cell culture medium,) should be stored at -70°C (maximum one year).

C. Sample Dilution

If samples generate values higher than the last standard point, dilute the sample with the appropriate solution (see below).

1. **Serum and plasma** : dilute with Solution A.
2. **Cell culture supernatant** : dilute with Solution B or corresponding medium.

X BIOSOURCE IL-17 CYTOSCREEN PROCEDURE

The instructions of the assay procedure must be followed to obtain reliable results.

A. Procedural notes

1. Allow the samples and reagents to equilibrate to room temperature (18°C to 25°C) before starting the assay. Thoroughly mix the reagents and samples before use by gentle agitation or swirling.
2. Do not use kit components beyond the expiration date.
3. Do not mix materials from different kit lots.
4. Do not mix strips from different plates.
5. Perform Standards, Controls and Unknowns in duplicate. Vertical alignment is recommended.
6. A standard curve should be run with each assay run or each plate run.
7. To avoid drift, the time between pipetting of the first standard and the last sample must be no longer than 30 minutes. Otherwise, results will be affected.
8. Use a clean disposable plastic pipette for each reagent, standard, control or specimen addition in order to avoid cross contamination .
9. For the dispensing of the Chromogenic Solution and Stop Solution avoid pipettes with metal parts.
10. Use a clean plastic container to prepare the Wash Solution.
11. During incubation with Chromogenic Solution, avoid direct sunlight on the microtiter plate.
12. Respect the incubation times described in the assay procedure.

B. Assay Procedure

1. **Select the required number of strips for the run.** The unused strips should be resealed in the bag with dessicant and stored at 2-8°C.
2. Secure the strips into the holding frame.
3. **Pipette 50 µl of Solution B** into the appropriate wells for the : Standards, Controls, and Serum/plasma samples
4. **Pipette 50 µl of Solution A** into the appropriate wells for **cell culture supernatant**
5. **Pipette 100 µl of each Standard, Control, or Sample** into the appropriate wells.
6. **Pipette 50 µl of Biotin conjugate** into all the wells.
7. **Incubate** for **2 hours** at room temperature on a horizontal shaker set at 700 ± 100 rpm.
8. **Aspirate** the liquid from each well ;
9. **Wash** the plate three times by :
   a) dispensing of 0.4 ml of BioSource Wash Solution into each well ;
   b) aspirating the content of each well.
10. **Pipette 100 µl of diluted Streptavidin-HRP conjugate** into all the wells.
11. **Incubate** for **30 min.** at room temperature on a horizontal shaker set at 700 ± 100 rpm.
12. **Aspirate** the liquid from each well ;
13. **Wash** the plate four times by :
   a) dispensing of 0.4 ml of BioSource Wash Solution into each well ;
   b) aspirating the content of each well.
14. **Pipette 100 µl of Chromogenic Solution (TMB)** into all the wells.
15. **Incubate** the plate for **15 min.** at room temperature on an horizontal shaker set at 700 ± 100 rpm, avoiding direct sunlight.
16. **Pipette 100 µl of Stop Solution** into each well.
17. **Read** the absorbance within 1 hour and calculate the results as described in section XI.

XI CALCULATION OF ANALYTICAL RESULTS

A. Reading the plate with an equipment capable to record an optical density of 3.0 or more

- Read the microtiter plate at 450 nm (reference filter : 630 or 650 nm).
- Construct a standard curve by plotting the OD on the ordinate against the standard concentrations on the abscissa using either linear or semi-log graph paper and draw the curve by connecting the plotted points with straight ligne.
- Determine IL-17 concentrations of Samples or Controls.

B. Reading the plate with an equipment capable to record an optical density lower than 3

- Read the microtiter plate at 405 nm (reference filter : 630 or 650 nm).
- It will result in a decrease of the OD units when compared to ODs read at 450 (as shown on the table hereafter). Nevertheless, results remain quite similar.

C. Example of a typical reference curve

The following data are for demonstration purpose only and can not be used in place of data generated at the time of assay.

IL-17 CytoScreen			Reading 450 nm (OD Units)	Reading 405 nm (OD Units)
Standard	0	pg/ml	0.026	0.020
	15.6	pg/ml	0.093	0.039
	31.3	pg/ml	0.160	0.059
	62.5	pg/ml	0.279	0.094
	125	pg/ml	0.521	0.166
	250	pg/ml	0.984	0.302
	500	pg/ml	1.934	0.576
	1000	pg/ml	3.632	1.130

XII QUALITY CONTROL

- The **two Controls** provided in the kit can be used as internal laboratory controls.
- Serum or heparin plasma pools as well as stimulated cell culture supernatants can be collected and frozen immediately in aliquot to serve as controls. Repeated freezing and thawing are not permitted.
- **Record keeping** : it is good laboratory practice to record the kit lot numbers and date of reconstitution for the reagents in use.
- **Controls** : it is recommended that Controls be routinely assayed as unknown samples to measure assay variability. It is recommended that quality controls charts be maintained to monitor the performance of the kits. Control ranges are indicated on vial labels. Out of range control results indicate the assay must be repeated. Repeat patient samples may also be used to measure interassay precision.
- **Sample handling** : strictly adhere to the instruction for handling and storage of samples. Standards, Controls, and Unknowns should be run in duplicate. A clean disposable tip should always be used to avoid carryover contamination.
- **Data reduction** : it is good practice to construct a standard curve for each run to check visually the curve fit selected by the computer program.

III PRINCIPLES OF THE BIOSOURCE IL-17 CYTOSCREEN ASSAY

The BIOSOURCE IL-17 Cytoscreen is a solid phase Enzyme Amplified Sensitivity Immunoassay performed on microtiter plate. Standards or samples containing IL-17 react with capture monoclonal antibody (Mab 1) coated on the microtiter well and with a biotinylated monoclonal antibody (Mab 2). After an incubation period allowing the formation of a sandwich : coated Mab 1 - IL-17 - Mab 2 - Biotin, the microtiter plate is washed to remove unbound biotinylated antibodies. Streptavidin-Peroxidase is added and this binds to the biotinylated antibody. After incubation, the unbound enzyme is removed by washing and a substrate solution is added. The reaction is stopped with the addition of Stop Solution and the microtiter plate is then read at the appropriate wavelength. The amount of substrate turnover is determined colorimetrically by measuring the absorbance which is proportional to the IL-17 concentration. A standard curve is plotted and IL-17 concentrations in a sample is determined by interpolation from the standard curve.

IV REAGENTS PROVIDED

Reagents	96 tests Kit	192 tests Kit	Reconstitution
Microtiter plate with 96 anti-IL-17 coated wells	1 x 96 wells	2 x 96 wells	**Ready** for use
Standard in bovine plasma with preservatives: see vial label for exact concentration	3 vials lyophil.	3 vials lyophil.	**Reconstitute** with distilled water to the volume specified on the vial label
Solution B (buffer with preservatives)	1 vial 22 ml	1 vial 22 ml	**Ready** for use
Solution A (human plasma with preservatives)	3 vials lyophil.	4 vials lyophil.	**Add** distilled water (see the volume on the vial label)
Anti-IL-17-Biotin Conjugate in buffered solution with proteins and preservatives	1 vial 6 ml	2 vials 6 ml	**Ready** for use
Streptavidin-HRP diluent with preservatives	1 vial 22 ml	1 vial 22 ml	**Ready** for use
Controls 1 and 2 in human plasma with preservatives	2 vials lyophil.	2 vials lyophil.	**Add** 2 ml distilled water
Washing Solution Concentrate (buffer with preservatives)	1 vial 10 ml	1 vial 10 ml	**Dilute** 2 ml in 400 ml distilled water or the vial content in 2000 ml distilled water
Concentrated Streptavidin-HRP	1 vial 0.3 ml	2 vials 0.3 ml	**See** the table (VII.3)
Chromogen : TMB	1 vial 25 ml	1 vial 25 ml	**Ready** for use
Stop Solution	1 vial 25 ml	1 vial 25 ml	**Ready** for use

V PRECAUTIONS AND WARNINGS

1. The human blood components included in this kit have been tested by European approved and USA FDA approved methods and found negative for HBsAg, anti-HCV and anti-HIV-1 and 2. No known method can offer complete assurance that human blood derivatives will not transmit hepatitis, AIDS or other infections. Therefore, handling of reagents, serum, or plasma specimens should be in accordance with local safety procedures.
2. Avoid any skin contact with Stop Solution and Chromogen (TMB). In case of contact wash thoroughly with water.
3. Do not eat, drink, smoke or apply cosmetics where kit reagents are used.
4. Do not pipet liquids by mouth.
5. Bovine material used in this kit originates from animals coming from countries where BSE has not been reported.

I EQUIPMENT AND SUPPLIES REQUIRED BUT NOT PROVIDED

1. High quality distilled water.
2. Precision pipette : 50 µl, 100 µl, 250 µl, 1 ml and 10 ml.
3. Vortex mixer and magnetic stirrer.
4. Horizontal microtiter plate shaker capable of 700 rpm ± 100 rpm, microtiter plate reader fitted out with 450, 405 and 650 or 630 nm filter, microtiter plate washer.

VII REAGENT PREPARATION

1. **Solution A and Controls** : Reconstitute the lyophilized Solution A, and Controls to the volume specified on the vial label with distilled water. Allow them to remain undisturbed until completely dissolved, then mix well by gentle inversion.
2. **Standard**: Reconstitute the lyophilized standard to 2500 pg/ml with distilled water. Refer to standard vial label for instructions. Swirl or mix gently to ensure complete reconstitution. Make serial dilutions of the standard as described in the following table

Standard	Add	Into Solution A
1000 pg/ml	200 µl of std 2500 pg/ml	300 µl
500 pg/ml	250 µl of std 1000 pg/ml	250 µl
250 pg/ml	250 µl of std 500 pg/ml	250 µl
125 pg/ml	250 µl of std 250 pg/ml	250 µl
62.5 pg/ml	250 µl of std 125 pg/ml	250 µl
31.3 pg/ml	250 µl of std 62.5 pg/ml	250 µl
15.6 pg/ml	250 µl of std 31.3 pg/ml	250 µl
0 pg/ml		250 µl

3. **Streptavidin-HRP Dilution (51 x concentrated)** : Following the number of wells to be used, dilute the concentrated conjugate with the Streptavidin-HRP diluent in a clean glass vial: see below table for the volumes to pipette. Extemporaneous preparation is recommended.

TABLE STREPTAVIDIN-HRP DILUTION

Number of wells	Streptavidin-HRP	Streptavidin conjugate diluent
16	40 µl	2 ml
32	80 µl	4 ml
48	120 µl	6 ml
96	220 µl	11 ml
192	440 µl	22 ml

4. **Wash Solution** : Dilute 2 ml of Washing Solution Concentrate in 400 ml distilled water or all the content of the Washing Solution Concentrate vial in 2000 ml distilled water (use a magnetic stirrer).

VIII STORAGE AND SHELF LIFE OF REAGENTS

A. UNOPENED vials

Store the unopened vials at 2°C to 8°C. All kit components are stable until the expiry date printed on the labels.

B. OPENED vials

1. The Biotin Conjugate vial must be stored at 2° to 8°C.
2. The reconstituted Standards, and Solution A are stable for 4 days at 2°C to 8°C. Aliquots held for longer periods of time should be frozen, a maximum of two times, at -20°C (maximum 2 months) or at -70°C for longer storage (until expiration date).
3. The reconstituted controls must be frozen immediately after use. They can be frozen a maximum of three times and must be stored at -20°C (maximum 1 month) or at -70°C for longer storage.
4. Store the unused Streptavidin-HRP at 4°C until expiration date of the kit.
5. Store the unused strips at 2°C to 8°C in the sealed bag containing the desiccant until expiration date.
6. The Washing Solution Concentrate is stable at room temperature until expiration date. In order to avoid washerhead obstructions, it is recommended to prepare a fresh diluted Wash Solution each day.

XIII EXPECTED RANGE (Reference Interval)

In process

XIV PERFORMANCE CHARACTERISTICS

1. **Minimum Detectable Concentration (MDC).**
The MDC is estimated to be 2 pg/ml and is defined as the IL-17 concentration corresponding to the average OD of 20 replicates of the zero standard + 2 standard deviations.

2. **Precision** (inter-assay in process)

INTRA-ASSAY				INTER-ASSAY (day-to-day)			
Sample	**n**	**<X> SD (pg/ml)**	**CV (%)**	**Sample**	**n**	**<X> ± SD (pg/ml)**	**CV (%)**
Serum 1	19	169 ± 6	3.7	Serum 1		±	
2	19	456 ± 17	3.7	2		±	

3. **Specificity**
No significant cross-reaction was observed in presence of 300 ng of IL-1ra, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-16, TNF-α, IFN-γ, GM-CSF, OSM, MIP-1α, MIP-1β, LIF, MCP-1, G-CSF,GRO, IP-10, SCF, MCP-3, NAP-2 and RANTES. This IL-17 assay is specific for human natural and recombinant IL-17.

4. **Accuracy**

RECOVERY				DILUTION TEST			
Sample	**Added IL-17 (pg/ml)**	**Recovered IL-17 (pg/ml)**	**Recovery (%)**	**Sample**	**Dilution**	**Theor Conc (pg/ml)**	**Meas. Conc. (pg/ml)**
Plasma	0	0		Activated Plasma	1/2	597	597
	187	175	94		1/4	299	256
	375	323	86		1/8	149	130
	882	741	84		1/16	75	60
					1/32	37	33
High rheumat. Factor Sample	0	0		cell cult.1	1/1	450	450
	187	242	129		1/2	225	232
	375	351	94		1/4	113	120
	882	702	80		1/8	56	54
					1/16	28	25
Cell Cult. Med.	0	0		cell cult.2	1/1	630	630
	187	217	116		1/2	315	326
	375	413	110		1/4	158	156
	882	883	100		1/8	79	85
					1/16	40	36

5. **High dose hook-effect**
A sample spiked with IL-17 up to 0.2 µg/ml gives a response higher than that obtained for the last standard point.

XV LITERATURE REFERENCES

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T Cell IL-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines.
J. of Exp.Med.183(6):2411-2415

XVI SUMMARY OF ASSAY PROCEDURE

	Standards (µl)	**Serum/ plasma samples (µl)**	**Culture Supernatant urine (µl)**
Solution B	50	50	-
Solution A	-	-	50
Standards (0-7), Controls	100	-	-
Serum/plasma samples	-	100	-
Culture supernatant/urines	-	-	100
Biotin-conjugate	50	50	50
Incubate for 2 hours at R.T. with continuous shaking (700 RPM) **Aspirate** the contents of each well **Wash** 3 times with 0.4 ml of Wash Solution and aspirate			
Streptavidin-HRP	100	100	100
Incubate for 30 min. at R.T. with continuous shaking (700 RPM) **Aspirate** the contents of each well **Wash** 4 times with 0.4 ml of Wash Solution and aspirate			
Chromogenic Solution	100	100	100
Incubate 15 min. at R.T. with continuous shaking			
Stop Solution	100	100	100
Read on a microtiter plate reader and record the absorbance of each well at 450 nm (versus 630 or 650 nm).			

BioSource Catalogue Nr : KAC1591 / KAC1592	P.I. Number : 1700568	Date of issue : 09 July 2004

Analyzer: **Mindray** **BS-120**
BS-200
BS-300
BS-380
BS-400

Application: **ALKALINE PHOSPHATASE FL IFCC** - Codes AF F080 / F245 / F400 / F600 CH
Preparation: R1 - INSTALL LIQUID READY TO USE REAGENT A
R2 - INSTALL LIQUID READY TO USE REAGENT B
Storage: REFRIGERATE AT 2-8°C
Stability: AS INDICATED IN THE LABEL

<table>
<tr><th colspan="2">PARAMETER</th><th>BS-120</th><th>BS-200</th><th>BS-300</th><th>BS-380</th><th>BS-400</th></tr>
<tr><td colspan="2">TEST NAME:</td><td colspan="5">ALP</td></tr>
<tr><td colspan="2">No.:</td><td colspan="5">*</td></tr>
<tr><td colspan="2">FULL NAME:</td><td colspan="5">ALP IFCC</td></tr>
<tr><td colspan="2">STANDARD No.:</td><td colspan="5">2</td></tr>
<tr><td colspan="2">REAC. TYPE:</td><td colspan="5">KINETIC</td></tr>
<tr><td colspan="2">DIRECTION:</td><td colspan="5">INCREASE</td></tr>
<tr><td colspan="2">UNIT:</td><td colspan="5">U/L</td></tr>
<tr><td colspan="2">PRECISION:</td><td colspan="5">1</td></tr>
<tr><td colspan="2">PRI. WAVE:</td><td colspan="3">405</td><td colspan="2">412</td></tr>
<tr><td colspan="2">SEC. WAVE:</td><td colspan="3">700</td><td colspan="2">700</td></tr>
<tr><td colspan="2">SAMPLE VOLUME (μL):</td><td colspan="3">5</td><td colspan="2">5</td></tr>
<tr><td colspan="2">R1 (μL):</td><td colspan="3">200</td><td colspan="2">200</td></tr>
<tr><td colspan="2">R2 (μL):</td><td colspan="3">50</td><td colspan="2">50</td></tr>
<tr><td>REAC. TIME</td><td>FROM:</td><td>3</td><td>4</td><td>5</td><td>41</td><td>50</td></tr>
<tr><td>REAC. TIME</td><td>TO:</td><td>13</td><td>15</td><td>20</td><td>56</td><td>69</td></tr>
<tr><td colspan="2">INCUBATION TIME:</td><td>16</td><td>8</td><td>10</td><td colspan="2">-</td></tr>
<tr><td colspan="2">BLANKING TIME:</td><td colspan="3">-</td><td>-</td><td>-</td></tr>
<tr><td rowspan="2">LINEARITY RANGE</td><td>FROM:</td><td colspan="5">5</td></tr>
<tr><td>TO:</td><td colspan="5">2500</td></tr>
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<tr><td colspan="2">FACTOR:</td><td colspan="5">-</td></tr>
<tr><th colspan="7">CALIBRATION</th></tr>
<tr><td colspan="2">RULE:</td><td colspan="5">two-point linear</td></tr>
<tr><td colspan="2">REPLICATES:</td><td colspan="5">3</td></tr>
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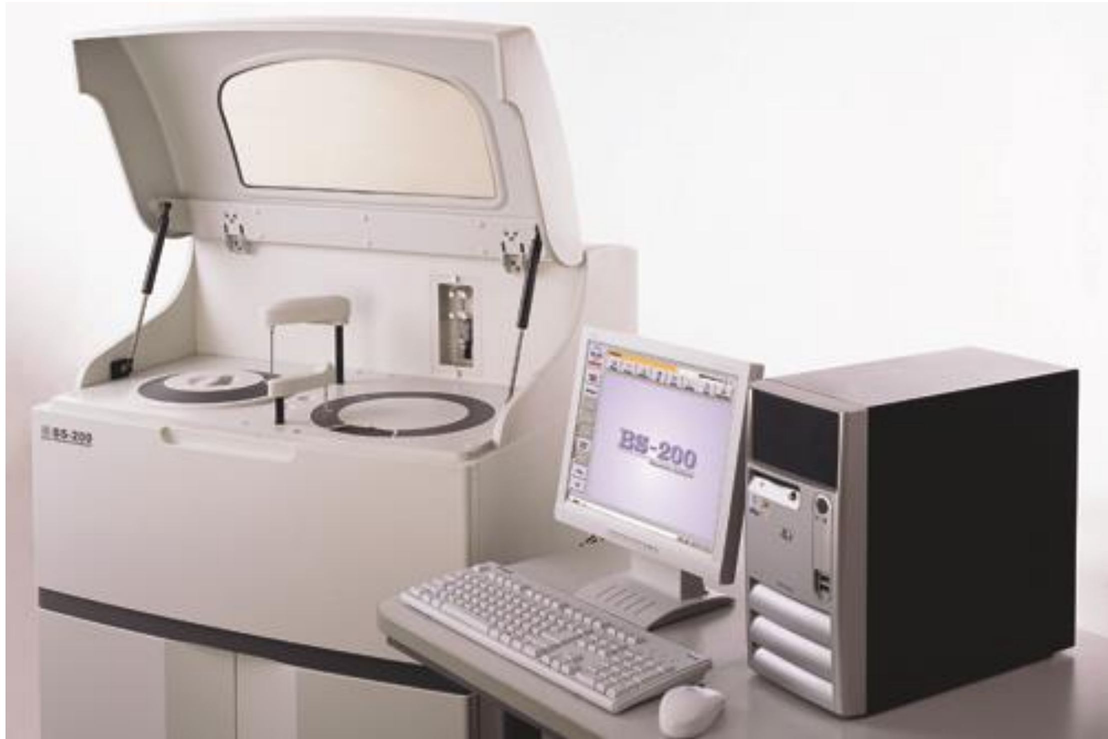
BS-200

Analyzer: **Mindray** **BS-120**
BS-200
BS-300
BS-380
BS-400

Application: **GAMMA-GT FL** - Codes GT F080 / F245 / F400 / F600 CH
Preparation: R1 - INSTALL LIQUID READY TO USE REAGENT A
R2 - INSTALL LIQUID READY TO USE REAGENT B
Storage: REFRIGERATE AT 2-8°C
Stability: AS INDICATED IN THE LABEL

PARAMETER		BS-120	BS-200	BS-300	BS-380	BS-400
TEST NAME:		GGT				
No.:		*				
FULL NAME:		GAMMA GT				
STANDARD No.:		2				
REAC. TYPE:		KINETIC				
DIRECTION:		INCREASE				
UNIT:		U/L				
PRECISION:		1				
PRI. WAVE:		405			412	
SEC. WAVE:		670			700	
SAMPLE VOLUME (µL):		20			20	
R1 (µL):		200			200	
R2 (µL):		50			50	
REAC. TIME	FROM:	3	4	5	41	50
REAC. TIME	TO:	13	15	20	56	69
INCUBATION TIME:		16	8	10	-	
BLANKING TIME:		-			-	-
LINEARITY RANGE	FROM:	3				
	TO:	800				
SUBSTRATE LIMIT:		-				
FACTOR:		-				
CALIBRATION						
RULE:		two-point linear				
REPLICATES:		3				

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Analyzer: **Mindray** **BS-120**
BS-200
BS-300
BS-380
BS-400

Application: **GOT/AST FL** - Codes GO F080 / F245 / F400 / F600 CH
Preparation: R1 - INSTALL LIQUID READY TO USE REAGENT A
R2 - INSTALL LIQUID READY TO USE REAGENT B
Storage: REFRIGERATE AT 2-8°C
Stability: AS INDICATED IN THE LABEL

PARAMETER		BS-120	BS-200	BS-300	BS-380	BS-400
TEST NAME:		AST				
No.:		*				
FULL NAME:		GOT-AST				
STANDARD No.:		2				
REAC. TYPE:		KINETIC				
DIRECTION:		DECREASE				
UNIT:		U/L				
PRECISION:		1				
PRI. WAVE:		340			340	
SEC. WAVE:		405			412	
SAMPLE VOLUME (µL):		20			20	
R1 (µL):		200			200	
R2 (µL):		50			50	
REAC. TIME	FROM:	3	4	5	41	50
REAC. TIME	TO:	13	15	20	56	69
INCUBATION TIME:		16	8	10	-	
BLANKING TIME:		-			-	-
LINEARITY RANGE	FROM:	3				
	TO:	500				
SUBSTRATE LIMIT:		-				
FACTOR:		-				
CALIBRATION						
RULE:		two-point linear				
REPLICATES:		3				

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Analyzer: Mindray BS-120 BS-200 BS-300 BS-380 BS-400

Application: **GPT/ALT FL** - Codes GP F080 / F245 / F400 / F600 CH
Preparation: R1 - INSTALL LIQUID READY TO USE REAGENT A
R2 - INSTALL LIQUID READY TO USE REAGENT B
Storage: REFRIGERATE AT 2-8°C
Stability: AS INDICATED IN THE LABEL

PARAMETER		BS-120	BS-200	BS-300	BS-380	BS-400
TEST NAME:		ALT				
No.:		*				
FULL NAME:		GPT-ALT				
STANDARD No.:		2				
REAC. TYPE:		KINETIC				
DIRECTION:		DECREASE				
UNIT:		U/L				
PRECISION:		1				
PRI. WAVE:		340			340	
SEC. WAVE:		405			412	
SAMPLE VOLUME (µL):		20			20	
R1 (µL):		200			200	
R2 (µL):		50			50	
REAC. TIME	FROM:	3	4	5	41	50
REAC. TIME	TO:	13	15	20	56	69
INCUBATION TIME:		16	8	10	-	
BLANKING TIME:		-			-	-
LINEARITY RANGE	FROM:	3				
	TO:	500				
SUBSTRATE LIMIT:		-				
FACTOR:		-				
CALIBRATION						
RULE:		two-point linear				
REPLICATES:		3				

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Analyzer: **Mindray** **BS-120**
BS-200
BS-300
BS-380
BS-400

Application: **TOTAL PROTEINS** - Codes TP 0100 / 0500 / 1000 / 1500 CH
Preparation: R1 - LIQUID READY TO USE SINGLE REAGENT
Storage: ROOM TEMPERATURE OR REFRIGERATE (2-30°C)
Stability: AS INDICATED IN THE LABEL

PARAMETER		BS-120	BS-200	BS-300	BS-380	BS-400
TEST NAME:		TP				
No.:		*				
FULL NAME:		PROTEINS TOTAL				
STANDARD No.:		2				
REAC. TYPE:		ENDPOINT				
DIRECTION:		INCREASE				
UNIT:		g/dl				
PRECISION:		0.1				
PRI. WAVE:		546			546	
SEC. WAVE:		700			700	
SAMPLE VOLUME (µL):		3			3	
R1 (µL):		300			300	
R2 (µL):		-			-	
REAC. TIME	FROM:	0	0	0	58	77
REAC. TIME	TO:	33	38	50	60	79
INCUBATION TIME:		-	-	-	-	
BLANKING TIME:		-			8-10	10-12
LINEARITY RANGE	FROM:	1.0				
	TO:	12.0				
SUBSTRATE LIMIT:		-				
FACTOR:		-				
CALIBRATION						
RULE:		two-point linear				
REPLICATES:		3				

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Chema Diagnostica - Guidelines for automatic analyzers - Mindray BS series

Analyzer: **Mindray** **BS-120**
BS-200
BS-300
BS-380
BS-400

Application: **ALBUMIN** - Code BC 0100 - 0500 - 1000 - 1500 CH
Preparation: R1 - LIQUID READY TO USE SINGLE REAGENT
Storage: ROOM TEMPERATURE OR REFRIGERATE (2-30°C)
Stability: AS INDICATED IN THE LABEL

PARAMETER		BS-120	BS-200	BS-300	BS-380	BS-400
TEST NAME:		ALB				
No.:		*				
FULL NAME:		ALBUMIN				
STANDARD No.:		2				
REAC. TYPE:		ENDPOINT				
DIRECTION:		INCREASE				
UNIT:		g/dl				
PRECISION:		0.1				
PRI. WAVE:		630			605	
SEC. WAVE:		-			700	
SAMPLE VOLUME (µL):		3			2	
R1 (µL):		400			300	
R2 (µL):		-			-	
REAC. TIME	FROM:	0	0	0	34	44
REAC. TIME	TO:	17	19	25	36	36
INCUBATION TIME:		-	-	-	-	
BLANKING TIME:		-			8-10	10-12
LINEARITY RANGE	FROM:	0.1				
	TO:	6.0				
SUBSTRATE LIMIT:		-				
FACTOR:		-				
CALIBRATION						
RULE:		two-point linear				
REPLICATES:		3				

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Research Article

Association between Inflammatory Cytokines and Liver Functions in Rheumatoid Arthritis Patients

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Editor-in-Chief:
Prof. Mohammad A. M. Ibnouf

Abstract

Background: Rheumatoid arthritis (RA) is associated with abnormal liver tests, and the medications used for RA are often hepatotoxic. Therefore, this study aimed to investigate an association between pro-inflammatory and anti-inflammatory cytokines and liver function tests in RA patients.

Methods: In this descriptive cross-sectional study, 88 RA patients were included, 84 of them were women and 4 men, aged 21–81 years. Serum interleukin-10 (IL-10), interleukin-17 (IL-17), Osteopontin (OPN) were measured and liver function tests were conducted.

Results: The frequency of RA was higher among adults aged >41 years (72 [81.8%]) than young adults aged ≤41 years (16 [18.2%]). RA was more common in women (84 [95.5%]) than in men (4 [4.5%]) – approximately 21:1-fold. Young adults had higher abnormal IL-10 than adult RA patients (OR = 3.72, *p*-value 0.044). Abnormal IL-17 (OR = 5.67, *p*-value 0.034) was found to be increased in young-adult RA patients. No association was observed between age and OPN and between the duration of disease and IL-10, IL-17, and OPN. Similarly, no association was noted between the types of treatment and IL-10, IL-17, and OPN, nor between IL-10, IL-17, OPN and liver parameters (AST, ALT, ALP, ALB, TP, and GGT).

Conclusion: Pro-inflammatory and anti-inflammatory cytokines are not associated with abnormal liver functions, as has been demonstrated in RA patients.

Keywords: rheumatoid arthritis, interleukin, liver function tests, cytokines

1. Introduction

1. Introduction

Rheumatoid arthritis (RA) is a common autoimmune inflammatory disease. Although the prevalence of RA is lower globally (0.5–1%), it is associated with socioeconomic burden and higher risk of mortality rate [1]. Recent studies have demonstrated that the treatments used for RA improved outcome, and also accounts as a risk for hepatic complications [2]. The adverse effects of RA treatments include asymptomatic elevations of liver enzyme, fibrosis, and sometimes fatal hepatic necrosis [3]. On the other hand, liver disorders have been noted in untreated RA patients [4].

Increasing amounts of interleukin-10 (IL-10), a potent anti-inflammatory cytokine [5], can be detected in the synovium of RA patients. Additionally, considering that the activity of RA cannot be attenuated by IL-10 administration [6], many researchers suggest that IL-10 plays an important role in chronic liver diseases [7]. Interleukin-17 (IL-17), a pro-inflammatory cytokine, is upregulated in many autoimmune diseases such as RA; high levels of IL-17 have been reported to be produced in different samples of RA [8, 9]. Some investigators suggest that IL-17 plays a key role in many liver diseases and is also associated with the progress of the disease [10–12]. Osteopontin (OPN) is a pro-inflammatory cytokine that induces RA [13–15], and included in many liver diseases, despite its role in liver problems are still controversial [16]. Therefore, this study was carried out to find out the association between pro-inflammatory, anti-inflammatory cytokines and liver function tests among RA patients.

2. Materials and Methods

This descriptive cross-sectional hospital-based study was conducted on 88 RA patients who were clinically diagnosed according to the criteria of the American College of Rheumatology (ACR) 1987 and were examined at the common RA clinics in Khartoum State (military, Alamal hospital, and Zain clinic). All patients received treatment; the demographic data, type of treatment, and duration of disease for each patient were recorded – 4 men and 84 women aged 28–90 years. Non-Sudanese patients with RA and those with unclear diagnosis were excluded. Serum from each subject were centrifuged at 3000 *g* for 10 min after clotting for 30 min at room temperature and stored at –40°C until analysis. All samples were investigated for OPN, IL-17, and IL-10 by sandwich enzyme-linked immune sorbent assay (ELISA) (ELISA Development; Thermo Fisher scientific Systems, USA) according to the manufacturer's instructions. In addition, liver functions tests (TP, Albumin, AST, ALT, GGT, and ALP) were done using

fully automated Mindray chemistry analyzer (BS 200). Data were statistically analyzed by statistical software package, version 16. Results were expressed as numbers and percentages. Chi-square test was used to determine the level of significance (*P*-value of 0.05 was considered to be statistically significant).

3. Results

RA is more common in adults (72 [81.8%]) than young adults (16 [18.2%]), the frequency of RA was found to be higher in women (84 [95.5%]) than in men (4 [4.5%]). Moreover, 52

3. Results

RA is more common in adults (72 [81.8%]) than young adults (16 [18.2%]), the frequency of RA was found to be higher in women (84 [95.5%]) than in men (4 [4.5%]). Moreover, 52 (59.1%) patients were receiving steroids while 36 (40.9%) were on non-steroid treatment. The duration of disease for 62 (70.5%) patients was ≤6 years and for 26 (29.5%) was >6 years. Abnormal IL-10 was found in 63 (71.6%) patients, while 25 (28.4%) had a normal percentage. The results of characteristic data show that while 80 (91%) RA patients had normal IL-17, 8 (9%) had abnormal. Normal OPN was observed in 76 (86.4%) RA patients and abnormal OPN in 12 (13.6%) (Table 1). Chi-square analysis revealed that young adults group had a higher abnormal IL-10 than adult RA patients (OR = 3.72, p-value 0.044). Also, abnormal IL-17 (OR = 5.67, p-value 0.034) was found to be increased in young adult RA patients while no association was seen between age and OPN (OR = 2.67, p-value 0.144; Table 2). Furthermore, no association was reported between the duration of the disease and IL-10, IL-17, and OPN with p-values 0.410, 0.176, and 0.502 and OR 0.77, 0.37, and 1.30, respectively (Table 3). Similarly, no association could be derived between the types of treatment and IL-10, IL-17, and OPN with p-value 0.246, 0.286, and 0.351 and OR 1.53, 2.21, and 0.65, respectively (Table 4). Pearson's correlation analysis revealed that there were no association between IL-10, IL-17, OPN and liver parameters (AST, ALT, ALP, ALB, TP, and GGT; Table 5).

4. Discussion

Abnormal liver functions were observed in RA patients. The researchers further attributed the abnormality to immune aggregations and others justified it by drugs toxicity. Accordingly, this study was carried out to assess whether the pro-inflammatory or anti-inflammatory cytokines are associated with liver functions in RA patients.

The current study revealed that there is no association between interleukins and liver function tests. In fact, abnormal liver tests were noted in patients with RA [17]. Concurrent with many previous studies, the frequency of RA is higher in elderly subjects [18, 19]. A possible explanation might be that the protective mechanisms in elderly population are

Table 1: Demographic and baseline characteristics of RA Patients.

Variables	Frequency (%)
Age (yr)	
≤41	16 (18.2%)
>41	72 (81.8%)
Sex	
Male	4 (4.5%)
Female	84 (95.5%)
Treatment	
Steroid	52 (59.1%)
Non-steroid	36 (40.9%)
Duration (yr)	
≤6	62 (70.5%)
>6	26 (29.5%)
Cut-off IL-10	
Abnormal	63 (71.6%)
Normal	25 (28.4%)
Cut-off IL-17	
Abnormal	8 (9%)
Normal	80 (91%)
Cut-off OPN	
Abnormal	12 (13.6%)
Normal	76 (86.4%)
Total	**88 (100%)**

Table 2: Association between interleukins IL10, IL17, OPN and age groups.

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Table 2: Association between interleukins IL10, IL17, OPN and age groups.

Variables	Age (yr)		OR	CI-Lower CI-Upper	P-value
	≤41	>41			
IL-10					
Abnormal	14 (23.0%)	47 (77.0%)	3.72	(0.78–17.7)	0.04
Normal	2 (7.4%)	25 (92.6%)			
IL-17					
Abnormal	2 (40.0%)	6 (60.0%)	5.67	(1.24–25.7)	0.03
Normal	12 (15.0%)	68 (85.0%)			
OPN					
Abnormal	4 (33.3%)	8 (66.7%)	2.67	(0.69–10.2)	0.14
Normal	12 (15.8%)	64 (84.2%)			

decreased, resulting in decreased immunotolerance and decreased cytokines synthesis and T cells proliferation [20]. The demographic data indicated that the prevalence of RA was found to be 21-fold higher in women than in men. In contrast to a previous study in Sudan, the female-to-male ratio was 9:1 [21]. Since the change in sex hormones after

Table 3: Association between interleukins IL10, IL17, OPN and duration of RA.

Variables	Duration (yr)		OR	CI-Lower CI-Upper	P-value
	≤6	>6			
IL-10					
Abnormal	42 (68.9%)	19 (31.1%)	0.77	(0.28–2.13)	0.41
Normal	20 (74.1%)	7 (25.9%)			
IL-17					
Abnormal	4 (50.0%)	4 (50.0%)	0.37	(0.08–1.65)	0.17
Normal	58 (72.5%)	22 (27.5%)			
OPN					
Abnormal	9 (75.0%)	3 (25.0%)	1.30	(0.32–5.25)	0.50
Normal	53 (69.7%)	23 (30.3%)			

Table 4: Association between interleukins IL10, IL17, OPN and types of treatment.

Variables	Treatment		OR	CI-Lower CI-Upper	P-value
	Steroid	Non-steroid			
IL-10					
Abnormal	38 (62.3%)	23 (37.7%)	1.53	(0.61–3.83)	0.24
Normal	14 (51.9%)	13 (48.1%)			
IL-17					
Abnormal	6 (75.0%)	2 (25.0%)	2.21	(0.42–11.6)	0.28
Normal	46 (57.5%)	34 (42.5%)			
OPN					
Abnormal	6 (50.0%)	6 (50.0%)	0.65	(0.19–2.21)	0.35
Normal	46 (60.5%)	30 (39.5%)			

puberty is associated with high prevalence of RA in women, a woman's immune system is potentially more reactive than that of a man. The current study reports that young adults are more likely to have abnormal IL-10 and IL-17. However, these results disagree with previous studies [22, 23]. No association was found between age and OPN level. Concurrent with this finding, a relationship between age and OPN has been previously

puberty is associated with high prevalence of RA in women, a woman's immune system is potentially more reactive than that of a man. The current study reports that young adults are more likely to have abnormal IL-10 and IL-17. However, these results disagree with previous studies [22, 23]. No association was found between age and OPN level. Concurrent with this finding, a relationship between age and OPN has been previously reported [24]. Similar to other results, no associations between IL10, IL17, OPN levels and the duration of disease have been demonstrated [22, 23, 25]. Despite reducing IL-17 after the use of steroids therapy, IL-10 was increased [26]. The present study revealed no associations between IL-10, IL-17, OPN levels and the types of treatment. It has become clear that steroids directly modulate the pro-inflammatory cytokine or suppress cytokines-producing cells [26, 27].

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Table 5: Association between cytokines and liver function parameters (Pearson's correlation results).

Parameters		P-value	R^2
IL-10	AST	0.62	0.12
	ALT	0.20	0.66
	ALP	0.80	0.05
	ALB	0.16	–0.13
	TP	0.56	0.02
	GGT	0.25	0.15
IL-17	AST	0.18	–0.15
	ALT	0.82	0.02
	ALP	0.82	–0.02
	ALB	0.23	0.12
	TP	0.59	0.05
	GGT	0.17	–0.14
OPN	AST	0.50	0.07
	ALT	0.25	0.12
	ALP	0.89	0.01
	ALB	0.29	–0.11
	TP	0.49	0.07
	GGT	0.98	–0.02

5. Conclusion

The data of present study shows that women are at a higher risk to have RA. Moreover, young adult RA patients are more likely to have abnormal IL-10 and IL-17. Furthermore, pro-inflammatory and anti-inflammatory cytokines are not associated with abnormal liver functions as has been demonstrated in RA patients.

Acknowledgements

The authors would like to express their sincere gratitude to the Dr. Mariam Abbas for her consultation and wisdom. May God bless her.

Ethical Considerations

Ethical permits for the study were obtained from the ethical review committees at the sites where patients were recruited, and all patients gave informed consent for their participation in the study.

DOI 10.18502/sjms.v16i2.9294

Ethical Considerations

Ethical permits for the study were obtained from the ethical review committees at the sites where patients were recruited, and all patients gave informed consent for their participation in the study.

Competing Interests

The authors declare no known conflicts of interest in relation to this paper.

Availability of Data and Material

The study data are available with the author upon reasonable request

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Original Article

Expression of Osteopontin, Interleukin 17 and Interleukin 10 among Rheumatoid Arthritis Sudanese Patients

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Abstract

Background: Rheumatoid arthritis (RA) is the most common inflammatory arthritis in the world. Its exact cause is unknown; proinflammatory and anti inflammatory cytokines play key roles in the pathophysiology of disease.

Objectives: This study aimed to measure the level of pro inflammatory cytokines Osteopontin (OPN) and interleukin (IL-17) and anti-inflammatory cytokine interleukin 10 (IL-10) in study volunteers.

Materials and Methods: A case control hospital based study for groups of 88 rheumatoid arthritis patients was included from Outpatient Clinic. The control groups matched in numbers, age and sex with case. After inform consent, Serum from Both patients and controls were examined for OPN, IL-17 and IL-10.

Results: The mean age of RA patients was 41.0 ±11.7 years. 84 (95.5%) were females and 4 (4.5%) males. As well as 59 (67%) were anti CCP positive and others 29 (33%) were negative. No significant

difference in OPN, IL-10 and IL-17 among patients gender. No statistic significant for OPN, IL-17 and IL-10. According to anti CCP. There were significant differences in OPN and IL-10, with mean (38.3±29.6, 45.9±42.9); respectively and (p-

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Original Article

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Conclusion: The result indicate that there, is a significant increase of OPN and IL-10 in Rheumatoid arthritis Sudanese patients, moreover no significant in IL-17.

Keywords: Rheumatoid arthritis (RA); Osteopontin (OPN); Interleukin (IL-17) and interleukin 10 (IL-10)

Introduction

Rheumatoid Arthritis (RA) is an autoimmune chronic inflammatory disease, of unknown etiology, with production of several autoantibodies (anti citrullinated cyclic peptide Anti CCP), which correlate with disease severity and used as diagnostic tool [1,2,3,4]. It is public health problem, worldwide distributed disease affect roughly 0.5-1% of population, and most common in females. No previous studies declared official statistics to show the Prevalence of RA in Sudan [4,5,6]. Numerous cytokines are associated with the pathogenesis RA [7].

Osteopontin (OPN) is a pro-inflammatory cytokine, stimulates production of several other pro inflammatory cytokines in patients with RA [8]. A deficiency of level has been shown to protect joints against destruction in arthritis [8,9].

Interleukin 17 (IL-17A) is a potent pro-inflammatory cytokine play role in inflammation of many autoimmune diseases, attributed and produced at high levels during various chronic inflammatory diseases, such as rheumatoid arthritis, although it is role in the pathogenesis of the autoimmune diseases is still unclear [10,11,12]. Neutralizing anti-IL-17 antibody reduces the severity of arthritis [13,14,15].

Interleukin-10 (IL-10) it is fascinating immunomodulatory cytokine, contribute to the pathogenesis of RA, however its mechanisms of action poorly understood [16,17,18]. Administration of IL-10 did not attenuate RA activity [19,20].

Therefore the present study carried out to determine whether the Osteopontin (OPN), IL-17 and IL-10 cytokines associated with rheumatoid arthritis among Sudanese populations.

Materials and methods

Patients and Controls: This was a case control hospital based study carried out in Khartoum State, from December 2017 to October 2018. Local Scientific Committee of Sudan University of Science and Technology approved this study. After obtaining patients verbal informed consent, 88 patients with RA who fulfilled the American criteria of rheumatology (ACR), 4 men and 84 women, with age ranged from 28 to 90 years and 88 matched age and gender healthy controls were recruited from different hospitals (Military Hospital, Alamal Hospital and Zain clinic). Blood from patients and controls, were centrifuged at 3000 rpm for 10 minutes after clotting for 30 minutes at room temperature and samples stored at -40°C until analysis. Demographic and clinical data were collected by structured questionnaire and evaluated, Patients with acute or chronic infectious disease or malignant disease were excluded.

Estimations of cytokines: All patients and controls sera were investigated for OPN, IL-17 and IL-10 by Enzyme linked Immune Sorbent Assay (ELISA) (ELISA Development; Thermo Fisher

Scientific Systems) according to the manufacturer's instructions.

Statistical analysis

Results obtained were analyzed using SPSS software (version 16). Results were expressed as frequency and mean ± standard deviation. Independent sample *t*-test was used to determine the level of significance (*P*-value of 0.05 was considered to be statistically significant).

Result

Results of demographic and clinical data demonstrate that, the mean age of RA patient's 51±11.3 years. Frequency of the RA was found 29/88 (33%), Table-1. Independent t test analysis observed that, serum OPN and IL-10 levels were statistically significant in RA patients (38.3±29.6, 45.9±42.9) respectively; and (*p*.value 0.00) for both cytokines, while IL-17 exhibited no significant difference (6.55±1.17); (*p*.value 0.12), Table-2. Moreover no significant difference in IL-10, IL-17 and OPN among patients gender with (*p*.value 0.54, 0.21, 0.95) respectively, Table-3. The results revealed that, there were no statistical significant for IL-10, IL-17 and OPN According to anti CCP respectively (*p*.value 0.52, 0.28, 0.94), Table-4. Person correlation analysis observed that, there were no association between age, IL-10 and OPN with (r=0.00, 0.333, p. value 0.55, 0.69)

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Results obtained were analyzed using SPSS software (version 16). Results were expressed as frequency and mean ± standard deviation. Independent sample *t*-test was used to determine the level of significance (*P*-value of 0.05 was considered to be statistically significant).

Result

Results of demographic and clinical data demonstrate that, the mean age of RA patient's 51±11.3 years. Frequency of the RA was found more common in female (84/88; 95.5%) than male (4/88; 4.5%). Most of RA patients had anti CCP positive (59/88; 67%), while fewer were negative 29/88 (33%), Table-1. Independent t test analysis observed that, serum OPN and IL-10 levels were statistically significant in RA patients (38.3±29.6, 45.9±42.9) respectively; and (*p*.value 0.00) for both cytokines, while IL-17 exhibited no significant difference (6.55±1.17); (*p*.value 0.12), Table-2. Moreover no significant difference in IL-10, IL-17 and OPN among patients gender with (*p*.value 0.54, 0.21, 0.95) respectively, Table-3. The results revealed that, there were no statistical significant for IL-10, IL-17 and OPN According to anti CCP respectively (*p*.value 0.52, 0.28, 0.94), Table-4. Person correlation analysis observed that, there were no association between age, IL-10 and OPN with (r=0.00, 0.333, *p*. value 0.55, 0.69) respectively, whereas there was association between age and IL-17 level (r=0.02, *p*. value 0.00), figure-1,2,3.

Table 1: Demographic and clinical data

Variables	**Frequency (percentage) Mean ±SD**
Age	51.8±11.3
Sex	
Male	4 (4.5%)
Female	84 (95.5%)
Anti ccp	
Positive	59 (67%)
Negative	29 (33%)
Total	**88 (100%)**

Table 2: Comparison of study parameters of case versus control

Parameters	**Case Mean ±SD**	**Control Mean ±SD**	***p*.value**
IL-10	45.9±42.9	8.48±7.36	0.00
IL-17	12.4±9.71	10.3±8.04	0.12
OPN	38.3±29.6	10.1±10.6	0.00

Table 3: Comparison of study parameters among gender

Parameters	**Male mean±SD**	**Female mean±SD**	***p*.value**
IL-10	58.8±60.7	45.3±42.3	0.54
IL-17	6.55±1.17	12.9±9.85	0.21
OPN	39.2±20.4	38.3±30.0	0.95

Table 4: Comparison of study parameters among anti CCP

Parameters	**Positive anti CCP mean±SD**	**Negative anti CCP mean±SD**	***p*. value**
IL-10	43.9±42.6	50.1±44.0	0.522
IL-17	13.1±9.98	10.8±9.10	0.285
OPN	38.5±28.8	38.0±31.7	0.945

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Table 4: Comparison of study parameters among anti CCP

Parameters	Positive anti CCP mean±SD	Negative anti CCP mean±SD	*p*. value
IL-10	43.9±42.6	50.1±44.0	0.522
IL-17	13.1±9.98	10.8±9.10	0.285
OPN	38.5±28.8	38.0±31.7	0.945

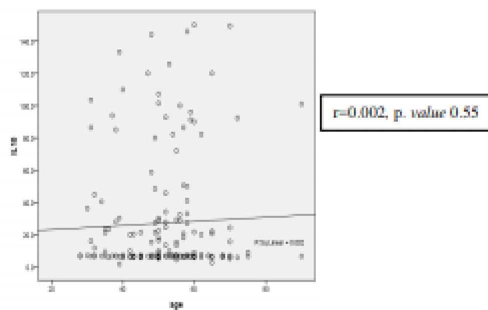


Figure 1: Correlation between IL-10 and patients age

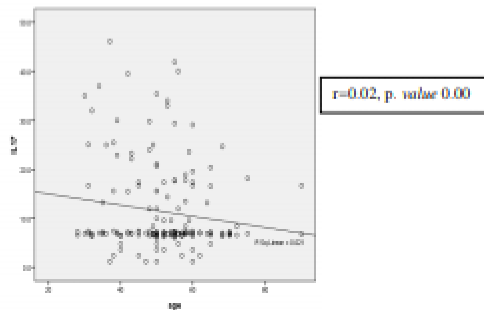


Figure 2: Correlation between IL-17 and patients age

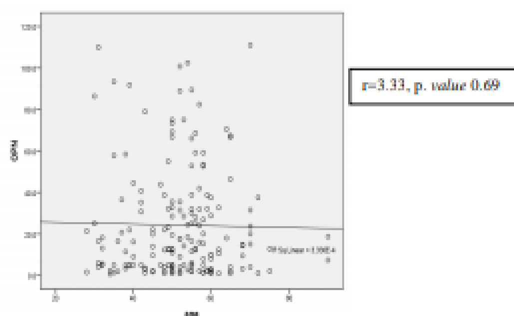
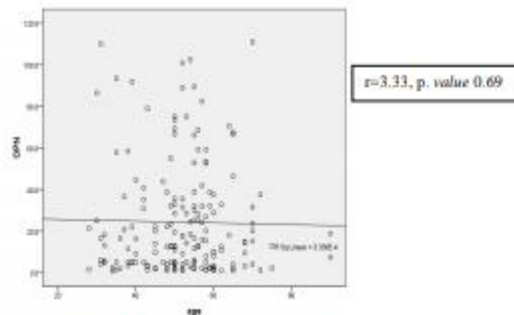


Figure 3: Correlation between OPN and patients age



r=3.33, p. value 0.69

Figure 3: Correlation between OPN and patients age

Discussion

The difficulty in diagnosis of RA is due to overlapping of signs and symptoms with others inflammatory autoimmune diseases. Although unknown causes of disease, cytokines play critical role in pathophysiology and tissue damage, and may be target for therapeutic agents. Accordingly the present study conducted to assess expression level of OPN, IL-17 and IL-10 cytokines among rheumatoid arthritis. Concurrent with previous studies [20,21], indicated that IL-10 and OPN show significantly difference between Rheumatoid Arthritis patients and the control group. In fact [13,14,15], noted that significant level of IL-17 was observed in RA patients, Our result found that, there is no significant difference in IL-17 between case and control. The possible explanation may be due to action of treatment which lead to significant decline of IL-17 level after therapy [22]. The current study revealed that, RA more common in females than males with ratio (21:1). This agree with previous studies which showed that, females more likely to develop RA three time than males [4,23,24] and greater than (9:1) ratio which observed in Sudan [6]. Menopausal sex hormones changes account main reason for this phenomena. Current study found that no significant difference has been found in OPN, IL-10 and IL-17 among gender. This result comes on line to report showed that IL-10, IL-17 and OPN not statistically differ between male and female [25,26,27]. Contraindication finding observed that, OPN is higher in men with SLE and only young women [28] and disagreed to other report which demonstrated that, the IL-17 differ and higher in men than female in patients with multiple sclerosis [29]. The present study observed that, there were no statistical difference in OPN, IL-10 and IL-17 in base of anti-CCP. Contradict with study found that, Seropositive patients had higher levels of pro inflammatory cytokines than those of sero negative patients and healthy controls [30]. Other previous studies focused on the evaluating cytokines in Synovial fluid found that, higher levels of cytokines IL-1β, IL-10, and IL-17 and the CCL20 chemokine in anti-CCP positive patients than those in sero negative patients [31,32]. United in opinion with previous study [32], our study reported that no significant correlation between IL-10 level and age. Although researcher noted that IL-17 not significantly correlate with age [26], present study found that there was positive significant correlation between IL-17 with age. The present study revealed no significant association was found in IL-17 with age. Inconsistency with observation which

showed that plasma OPN positively correlate with age [33].

Conclusion

The current result conclude that significant increase of OPN and IL-10 which may contribute to several aspect in RA Sudanese patients, whereas no increase detected in IL-17.

Ethical Approval: Ethical clearance was obtained from Sudan University of Science and Technology. All patients consented by verbal inform consent. Patient's information's and data

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