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M.Sc. in Biochemistry Science and Technology

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**UNIVERSITY OF GAZIANTEP
GRADUATE SCHOOL OF
NATURAL & APPLIED SCIENCES**

**RELATIONSHIP OF SERUM LEPTIN HORMONE LEVEL AND
DISEASE ACTIVITY IN RHEUMATOID ARTHRITIS PATIENTS**

M.Sc. THESIS

IN

BIOCHEMISTRY SCIENCE AND TECHNOLOGY

BY

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Supervisor

Prof. Dr. Ali AYDENİZ

BY

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January 2018



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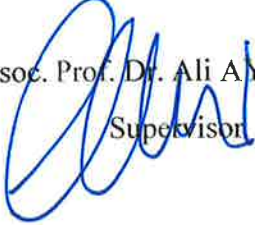

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Ahmed Mohammed Shukur DAWOODI

ABSTRACT

RELATIONSHIP OF SERUM LEPTIN HORMONE LEVEL AND DISEASE ACTIVITY IN RHEUMATOID ARTHRITIS PATIENTS

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M.Sc. in Biochemistry Science and Technology

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This study was set to investigate serum leptin levels in rheumatoid arthritis patients, and to find correlation of serum leptin levels in rheumatoid arthritis patients with disease activity. This study was conducted of 35 adult RA patients and 30 matching age persons were served as the control group. Assessment of disease activity was done using the DAS-28-ESR scoring system. Calculation of body mass index (BMI) was carried out for both RA patients and controls. Leptin ELISA kit was used to measure serum leptin level in both RA patients and control groups. RA patients showed statistically significant higher mean of serum leptin level than control groups (26.65 versus 13.57 ng/ml respectively, $P = 0.01$). In addition, serum leptin level showed a statistically significant positive correlation with body mass index ($P < 0.01$). No significant correlation was found between serum leptin level and patients' age, disease duration and disease activity. Mean serum leptin level was 33.97 ng/ml in seropositive patients and 8.36 ng/ml in seronegative patients, a finding which proved to be statistically significant when comparing the two groups ($P = 0.01$). Even though serum leptin level was significantly higher in the RA patients than control groups, no correlation was found between leptin level and clinical and laboratory parameters of disease activity. However, the serum leptin level positively correlated with BMI in RA patients.

Keyword: Leptin, Rheumatoid arthritis, Inflammation, BMI.

ÖZET

RUMOMATOİD ARTRİT HASTALARINDA SERUM LEPTİN HORMON DÜZEYİ VE HASTALIK ETKİNLİĞİNİN İLİŞKİSİ

DAWOODI, Ahmed Mohammed Shukur

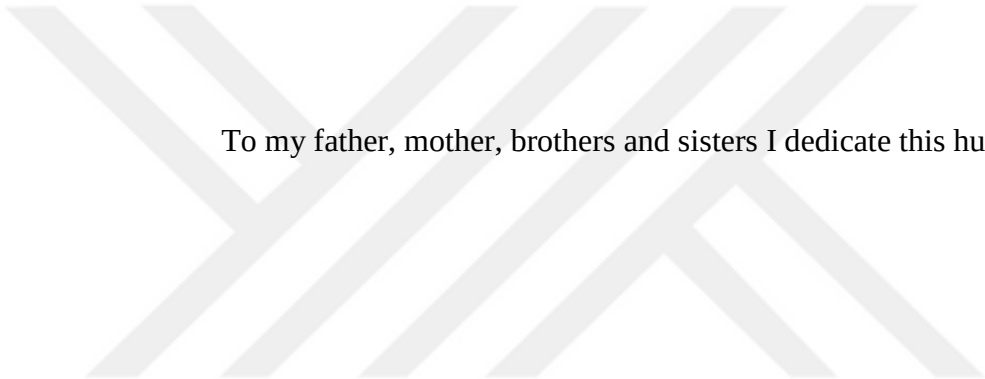
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Bu çalışma, romatoid artritli hastalarda serum leptin düzeylerini araştırmak ve romatoid artritli hastalarda serum leptin düzeylerinin korelasyonunu araştırmak. Bu çalışma otuzbeş yetişkin RA hastası üzerinde yürütülmüş ve 30 uyumlu yaş gurubu kontrol grubu olarak görev yapmıştır. Hastalık aktivitesinin değerlendirilmesi, DAS28-ESR puanlama sistemi kullanılarak yapıldı. Vücut kitle indeksinin (VKİ) hesaplanması, hem RA hastaları hem de kontroller için gerçekleştirildi. Leptin ELISA Kiti hem RA hastalarında hem de kontrol grubunda serum leptin düzeyini ölçmek için kullanıldı. RA hastaları kontrol grubuna göre istatistiksel olarak anlamlı yüksek serum leptin düzeyi gösterdi (Sırasıyla 13.57 ng/ml karşı 26.65, $P = 0.01$). Buna ek olarak, serum leptin düzeyi, vücut kitle indeksi ile istatistiksel olarak anlamlı pozitif korelasyon gösterdi ($P < 0.01$). Serum leptin düzeyi ile hastaların yaşı, hastalık süresi ve hastalık aktivitesi arasında anlamlı korelasyon bulunmadı. Seropozitif hastalarda ortalama serum leptin düzeyi 33.97 ng/ml, seronegatif hastalarda 8.36 ng/ml idi ve bu iki grup karşılaştırıldığında istatistiksel olarak anlamlı bulundu ($p = 0.01$). Serum leptin düzeyi RA hastalarında kontrol grubuna göre anlamlı derecede yüksek olsa da, leptin düzeyi ile hastalık aktivitesinin klinik ve laboratuvar parametreleri arasında korelasyon saptanmadı. Ancak, RA hastalarında serum leptin düzeyi ile VKİ arasında pozitif korelasyon vardı.

Anahtar Kelime: Leptin, Romatoid artrit, Inflamasyon, VKİ



To my father, mother, brothers and sisters I dedicate this humble work.

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LIST OF CONTENT

	Page
ABSTARCT	v
ÖZET	vi
ACKNOWLEDGEMENTS	viii
LIST OF CONTENT	ix
LIST OF TABLE	xii
LIST OF ABBREVIATION	xiii
CHAPTER 1	
1. Introduction.....	1
1.1 RA background	1
1.2 Classification criteria of RA.....	3
1.3 Outline of thesis	7
CHAPTER 2	
2. Literature review	8
1.1 Rheumatoid Arthritis	8
1.2 Epidemiology of Rheumatoid arthritis.....	9
2.3 Etiology pathogenesis of RA.....	9
2.3.1 Genetic factors:	10
2.3.2 Environmental factors	11
2.4 Clinical features of RA.....	13
2.5 Synovitis.....	14
2.6 Joints in RA patients	15
2.7 Innate immune system role in RA.....	17
2.7.1 Innate immune cells in RA	17
2.7.2 Monocytes/macrophages (Mφ)	18
2.7.3 Natural Killer cells (NK).....	19
2.7.4 Dendritic cells (DCs).....	19

2.7.5 Mast cells	20
2.8 Osteoclast and Osteoblast in RA	21
2.9 Adaptive immune system role in RA	22
2.10 Rheumatoid arthritis and T cells role	23
2.11 Rheumatoid arthritis and B cells role	24
2.12 Autoantibodies values in RA pathogenesis	25
2.12.1 Rheumatoid factors (RFs)	26
2.12.2 Citrullinated antigens autoantibodies	27
2.12.3 Some other autoantibodies	28
A. Anti-RA 33	28
B. Anti-collagen antibodies	28
C. Anti-Calpastatin antibodies	28
2.13 RA cytokines in brief	29
2.13.1 IL-1 family	29
2.13.2 Cytokines binding a γ_c -containing receptor	30
2.13.3 IL-17 family	31
2.13.4 TNF family	31
2.13.5 Cytokines binding a gp130-containing receptor	32
2.13.6 Interferons (IFNs)	33
2.13.7 Chemokines	33
2.13.8 Adipokines	34
2.14 Leptin	35
2.14.1 Synthesizing and regulating of leptin 39	37
2.14.2 Leptin receptor and its signaling	38
2.14.3 Leptin action and immune system	39
A. Leptin role in innate immunity	39
B. Leptin role in adaptive immunity	40
2.14.4 Leptin as an inflammation marker	40
2.14.5 Leptin in rheumatoid arthritis	41
CHAPTER 3	
3. Methods and results	44
3.1 Methodology and patients	44

3.2 Used materials and used apparatuses	45
3.3 Statistics	46
3.4 Results	46
CHAPTER 4	
4. Discussion and Conclusion	51
REFERENCES.....	55



LIST OF TABLES

	Page
Table 1.1 1987 ACR criteria for rheumatoid arthritis	5
Table 1.2 2010 ACR/EULAR	6
Table 3.1 Clinical data of RA patients	49
Table 3.2 Laboratory data of RA patients.....	50
Table 3.3 Clinical data of controls.....	50
Table 3.4 Laboratory data of controls.....	51
Table 3.5 RF results of RA patients and Leptin serum levels.....	51
Table 3.6 DAS comparison with serum leptin level in RA patients.....	52
Table 3.7 Comparison serum Leptin levels of RA patients and Controls.....	52

LIST OF ABBREVIATIONS

ACR	American College of Rheumatology
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ACPA	Anti-Citrullinated Protein Antibodies
ANA	Anti-Nuclear Antibodies
Anti-CII	Anti-Collagen type 2
Anti-CCP	Anti-Cyclic Citrullinated Peptides
ALT	Alanine Transaminase
APCs	Antigen-Presenting Cells
ARA	American Rheumatism Association
BMI	Body Mass Index
BMP	Bone Morphogenic Protein
CD	Cluster of Differentiation
CNTF	Ciliary Neurotrophic Factor
CTLA	Cytotoxic T Lymphocyte-Associated Molecule
CRP	C - Reactive Protein
DAS28	Disease Activity Score of 28 joints
DCs	Dendritic Cells
db	Diabetes gene
DNA	Deoxy Ribonucleic Acid
FLS	Fibroblasts-Like Synoviocytes
ELISA	Enzyme-Linked Immunosorbent Assay
ESR	Erythrocytes Sedimentation Rate
EULAR	European League Against Rheumatism

GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
gp130	Protein Glycoprotein 130
GWAS	Genome-Wide Association Studies
Hb	Hemoglobin
hnRNP	Heterogeneous Nuclear Ribonucleoprotein
HLA	Human Leukocyte Antigen
HRP	Horse-Radish Peroxidase
IFN γ	Interferon Gamma
Ig	Immunoglobulin
IL	Interleukin
IL-1Ra	Interleukin -1 Receptor antagonist
IRS1/2	Insulin Receptor Substrate $\frac{1}{2}$
JAK	Janus Kinase
KDa	Kilo Dalton
LIF	Leukemia Inhibitory Factor
MCSF	Macrophage Colony-Stimulating Factor
MCTD	Mixed Connective Tissue Disease
mDC	Myeloid Dendritic Cell
MMP's	Matrix Metalloproteinases
mm/hr	Millimole per Hour
MPS	Mononuclear Phagocyte System
mRNA	Messenger Ribonucleic Acid
MS	Multiple Sclerosis
M ϕ	Monocytes/macrophages
NK	Natural Killer
NOS2	Nitric Oxide Synthase type II
ng/ml	Nanogram/milliliter
ODF	Osteoclast Differentiation Factor
OA	Osteoarthritis

Ob-R	Obese Receptor
OPG	Osteo-Prote-Gerin
PADI	Peptidyl Arginine Deiminases Citrullinating Isoenzyme
pDC	Plasmacytoid Dendritic Cell
PLT	Platelet
PTPN22	Protein Tyrosine Phosphatase, Non-Receptor Type 22
RA	Rheumatoid Arthritis
RANKL	Receptor Activator of Nuclear Factor Kappa-B Ligand
RF	Rheumatoid Factor
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SD	Standard Deviation
SE	Shared Epitope
SF	Synovial Fibroblasts
SLE	Systemic Lupus Erythematosus
SNP	Single Nucleotide Polymorphism
STAT	Signal Transducer and Activator of Transcription
SPSS	Statistical Package for Social Sciences
TNF-R	Tumor Necrosis Factor Receptor
TNF- α	Tumor Necrosis Factor Alpha
TGF- β	Transforming Growth Factor Beta
Th	T helper
TIMP	Tissue Inhibitors Metalloproteinases
WAT	White Adipose Tissues
WBC	White Blood Cell
Wnt	Wingless
γ_c	Gamma chain

CHAPTER 1

Introduction

1.1 RA Background

Rheumatoid arthritis (RA) is perhaps the most common chronic, potentially harmful inflammatory disease that effects primarily the joints distinguished by both tenderness and swelling in joints and destructing of synovial joints, over time resulting, disability and premature mortality [1]. Disregarding of race and geographic location, but abnormal systemic immune response is an evidence and could cause a variety of extra-articular manifestations [2].

RA is affecting from 0.5 % to 1% of general worldwide population and RA is more occurring in females than males, this contrast difference is maybe due to the sex steroid hormone that in female sex predominance in RA possibly linked to low levels of androgen that is prior to disease onset [3]. This type of arthritis is can be defined by the presence of four out of the seven criteria that were developed by the American College of Rheumatology (ACR) in 1987 [4]. That is going to be mentioned in detail in this context, the criteria where been formulated by experts based on the characteristics of the patients with long-term standing of RA (mean disease duration of 8 years) [5]. Chronic inflammation is a process of accumulation of the activating lymphocytes and macrophages at the infection site or injury, once macrophages get activated by interferon gamma ($\text{IFN}\gamma$), tumor necrosis factor alpha ($\text{TNF-}\alpha$) will secreted by the macrophages and $\text{IFN}\gamma$ will impact on the macrophages and leads to increase of the $\text{TNF}\alpha$ production [6].

Chronic inflammation is common and is seen very often in several autoimmune diseases such as RA and some cases that a pathogen cannot be cleared enough by immune system, the best example for chronic inflammation that is not cleared is tuberculosis [7]

Intuitively obvious, one understands that the patients who these criteria can be fulfilled on, may have different pathogenetic entities that indirectly lead to fulfillment of this syndrome. Actually, data of studies of negative and positive autoantibody disease suggest that risk factor may differ. So, the issue of RA when is going to be started needs to be considered according to different entities present in the syndrome of RA [8]. Present indication refers that identified genetic risk factor dose not dispose to all RA, but only thing that specify the subset of the disease based on the autoantibodies, and in exact ACPA, which these are the most common specific antibodies for RA [9].

One environment and three genetic risk factors have been identified so far for ACPA-positive disease and one genetic risk factor for ACPA-negative with an evidence that IRF5 gene is also a risk factor for ACPA-negative disease and CTLA4 [10-13].

Beside of its systemic acute phase response, adipocytes have a very important role in local destruction and inflammation, the relationship between acute phase proteins such as CRP and disease activity on the one hand and the radio-graphical progress on other hand has been confirmed and demonstrated [14,15]. Even though CRP appears to be a good biomarker for predictive aims for seeing the effect of treatment, and this is like an indicator of inflammation which influenced by some other stimuli of the acute phase response [16].

The adipocytes mainly produce leptin and then be secreted into the blood stream to act as a very important physiological factor, there is explicit relationship between blood plasma level and body mass index (BMI) with considerable variability in its concentrations among the patients with same BMI [17].

The discovery of leptin, in 1994 tremendously, has reformed idea of the most popular old-style view of white adipose tissue (WAT), from that was well known for its role as a fat storage for decades with a minor or null of endocrine role to one of the core organs with a very vigorous role in

regulation of energy balancing, metabolism and body immune inflammatory processes [18, 19].

Among the different types of endocrine mediators. Leptin likely acts the most vital part in the pathogenesis of the RA [20]. Leptin is a hormone belongs to peptides class of protein, leptin is produced from adipose tissue as we mentioned earlier and regulated in major by innate immune system [21], the increase of leptin level production during inflammation strongly lead to be imagined, that leptin is taking part as a part of the cytokines cascade which coordinate the innate immune response and work like defense mechanism [22]. The decrease of the leptin level leads to enhancement of many clinical manifestation and biological measurements of the disease activity of RA [23].

Cytokines are soluble proteins, small in size about 25 KDa. These proteins take part in transmitting of the information that exchanged between immune system cells. Cytokines are critical mediators of every stage of the development and regulation of an immune response [24]. Cytokines can be classified into 2 types, hematopoietin that includes many growth factors and interleukins and TNF that also includes 19 members for example TNF α , these two types play a big role in adaptive and innate immune response [25]. Cytokines have affecting behavior of lots of cells in the autocrine and paracrine level, they can act like pro-inflammatory such as a process of having a fight to invade the pathogen of some favor that is in one hand, in other hand sometimes work as anti-inflammatory like a promoter to terminate the inflammation and restoring the homeostasis of the tissue [26].

1.2 Classification criteria of RA

Methodology for development of RA classification criteria are paramount in population studies to set up the epidemiology of disease, the methodology for development in RA classification criteria have been refined over time especially the last 50 years [27].

In general, the physician's opinion has been taken as gold standardization verse evaluated criteria. Though the list of the diagnostic tests has been developed, each test should be precisely defined and then be evaluated in a

form of series of cases and controls and the exact purpose of the classification criteria is to optimally classify and differentiate one rheumatic disease one from another and proper clinical and epidemiological researches action to be taken into consideration [28].

Each of these criteria must be easy to measure, precise and reliable. The used cases must be covered a wide spectrum of disease and be plotted from primary, secondary and tertiary [29]. In the testing, the most important step is individual diagnostic tests and then the process of the evaluation to the criteria set is control group selection. Thus, the important chore is to be able to differentiate infected individuals of the RA from those who are not infected with arthritis or with osteoarthritis (OA) [30].

Nevertheless, in early arthritis clinic, the first important step is to identify the individuals with RA from other inflammatory diseases such as reactive arthritis and connective tissue disorders, and it is recommended to be there more than one set of the control. To use these classification criteria for diagnostic purpose should have a good sensitivity and high specificity [31].

Various RA classification criteria have been developed mainly for scientific research purposes, the main goal of these criteria has been to distinguish RA disease from other rheumatic diseases with a high sensitivity and specificity in clinical trial course [32].

During a last 5 decades, the most applied criteria for RA classification were the American College of Rheumatology (ACR), formerly the American Rheumatism Association (ARA) that has been revised in 1987 (Table1-1) [33].

Table 1.1 1987 ACR criteria for rheumatoid arthritis

Criteria	Definition
1 Morning stiffness	In and around the joints, will last at least for an hour before maximal improvement.
2 Arthritis in 3 or more joints	Soft tissue swelling or fluid will last at least for 6 weeks.
3 Hand joints Arthritis	6 weeks swelling of wrist or MCP or PIP
4 Symmetrical arthritis	Symmetrically joint swelling in a simultaneous involvement of the same joint on both side parts of body.
5 Rheumatoid nodules	Subcutaneous nodules over bony prominences, or extensor surface
6 Rheumatoid factor (RF)	Detected by a method positive in less than 5% normal controls.
7 Radiographic changes	Typical of RA on posteroanterior hand and wrist radiographs which should include erosions.

According to these mentioned criteria the disease can be considered as RA if 4 out of the 7 criteria are fulfilled. The 1987 ACR criteria were used particularly valid for distinguishing RA from other rheumatic diseases but these criteria been criticized for not being able to recognize early RA, as evidence has proven the early treatment of the RA critical improved outcomes [34].

As a result of criticism against the 1987 ACR criteria, chiefly because of lack of sensitivity in early disease, it was urgent to new classification be proposed, therefore in 2010 ACR and the European League Against Rheumatism (EULAR) set a new classification criterion to be handled in newly presenting patients with at least one swollen joint, the details of the 2010 ACR/EULAR are mentioned below (Table 1-2) [35].

Table 1.2 2010 ACR/EULAR

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

The new criteria stressed more on serologic tests, including RF in addition to ACPA, ESR and CRP. Articular involvement is qualified by joint swelling and tenderness. Application of these criteria provides a score of 0 to 10, with a score of 6 being indicative of the presence of definite RA [36].

The 2010 criteria render an earlier diagnosis possible, even with a very low number of tender and/or swollen joints. Although the 2010 criteria yield excellent sensitivity (97%) in diagnosing patients with RA, the specificity is substantially lower than that of the 1987 criteria (55% vs. 76%, respectively) [37].

1.3 Outline of Thesis

This thesis is encompassing of five chapters, **Chapter 1** is an Introduction which provides an overview and clarifying and dealing with the aim and objective of the thesis it. **Chapter 2** is a Literature Review of earlier studies and researches which have been done in epidemiology, the burden and clinical trials features of Rheumatoid Arthritis as well as the RA management, this thesis is also providing Leptin role in progression of rheumatoid arthritis disease. **Chapter 3** explains the getting results and its interpretation and the procedures of the study in addition of the number of the patients and control groups and what kind of clinical tests where have been undertaken. **Chapter 4** is Discussing the results that we got from laboratory and clinic tests that we have done, what to be done to improve the life quality of RA patients and what is the lifestyles to be modified to suppress the effect of this disease. As well as the Conclusion of what we came up of solutions during our study? And we will shed the light of prospective treatments of the RA disease.

The study was designed to focus on measurement of serum leptin level in patients with rheumatoid arthritis, and to correlate it with clinical measurements, disease activity and remission in early stage of RA. In addition to evaluate of DAS28 as an index for assessing disease activity in RA, also relationship between leptin and other laboratory bio-markers such as ESR and CRP in RA patients.

CHAPTER 2

Literature review

2.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is systemic and chronic inflammatory disease characterized by far-reaching synovitis developing in erosions of articular cartilage and marginal bone that result a joint demolition [7].

It is like many other chronic inflammatory diseases, introduces a very significance considerable impact on social by effecting the patient functional capability causing an impact on the quality of the life and economic sector outcoming to direct and indirect burden to society and the health care system [38].

RA has a multistep process pathogenesis that comes up with autoimmune development, with continuing local inflammation and then finally triggering the bone destruction [39]. In many cases, RA disease course is unpredictable especially when it comes to the patients who are with an active disease [40]. Usually its progression develops cartilage demolition, joint destruction and then gradually deformity comes up [41]. RA disease progression under normal conditions monitored throughout standard medical, laboratory functional indices, while structural damage of hands and feet can be assessed by serial X-ray [42, 43].

Both genetic and environmental factors are affecting disease course, susceptible individuals cause to grow an implicit autoimmune that obvious as the existence of autoantibodies, such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) [44]. In this point called as pre-articular or lymphoid phase, rheumatoid arthritis disease medical appearance can be anticipated by a decade before the disease onset [45].

Heretofore the reason remains unknown how and why the systemic fact or process of losing of tolerance is connected to a localization beginning of joints inflammation. Various factors might engage among them neurologic, microvascular, micro-trauma and mechanism of related biomechanical [46]. Disease articular phase is characterized by synovitis that results to cartilage and bone damage [47]. Both pathways of adaptive and innate immune are get triggered and influencing the activity of inflammation. A very problematic and complicated cytokines web contributes in inflammation and in continuance of RA disease by positive feedback loops improving systemic disorder [48].

2.2 Epidemiology of Rheumatoid arthritis

In general population worldwide, RA prevalence is approximately calculated to be at 1 percentage of the world inhabitation and the disease occurrence rate is 20 to 50 cases every 100.000 person per year [49]. Epidemiology data showed that high prevalence of RA is recorded in Native-American populations such as Pima Indians folk, meanwhile low incidence rate is recorded in Japan, China and Africa if compared to Caucasian countries [50]. Even though, RA the disease may occur at any time of age, RA disease occurrence is increasing with age and likely to be varied in accordance of the types of the criteria been used in classifying [51]. The onset age peak is risen to 50 years or more, and it is commonly more occurring in females then that in males with a statistic ratio of 3 to 1 [52].

2.3 Etiology pathogenesis of RA

RA is a multifactorial autoimmune disease of elusive, unclear etiology. The “Bermuda triangle” genetic, environment and immune response factors are contributing an important role in influencing the developing of disease course in susceptible individual [53]. Many cases of RA disease are believed it comes as an outcome of combining both factors [54].

Upcoming sections will provide great detail overview of genetic and environmental risk factors that engaged in the etiology of RA.

2.3.1 Genetic factors: RA has a very extremely intricate genetic basis, twin studies and family studies have had demonstrated the genetic participation in RA susceptibility in humans [55, 56].

Genetic of RA is a very complex with the prevalence among the sibling's increases from < 1% in worldwide general population 2 to 4 percentage [57]. Twin studies have displayed a concordance average for RA of 12-15% for mono-zygotic twins if be compared to 3.5% for di-zygotic twins [55]. Familia clustering evidence has verified the occurrence from 2 to 12 percentage in RA patients of first-degree relatives [58].

The hallmark risk of genetic factor in RA has been discovered in the human leukocyte antigen (HLA) locus [59]. exactly and particularly the sequence of amino acid QKRAA, QRRAA or RRRAA at the position site 70-74 of the DRβ1 chain, called shared epitope (SE), which relates to disease and also connected with producing of ACPA, by the way the connection of these alleles and RA has been noticed only in case of ACPA positive patients [60, 61]. DRβ1 gene shares the SE such as HLA-DRβ1 *0404, *0405, *0408, *0101, *0101, *0102, *1001 and *1402 [62].

Ultimately Genome-Wide Association Studies (GWAS) is an introduction of new techniques that have led to identifying of much more than 30 alleles outside of HLA genes boundaries that contributing of susceptibilities in RA patients [63]. The second cornerstone genetic association in Caucasian population is in gene protein tyrosine phosphate non-receptor type 22 (PTPN22) [64].

A single nucleotide polymorphism (SNP) encodes an arginine to tryptophan substitution increasing the peril in patients of RA by 40-80% [12]. The gene PTPN22 is encoding for tyrosine phosphate, Lyp, an effective inhibitor to activate T cell. What has been hypothesized is that the disease-associated alleles producing a protein affecting threshold for both B cell and T cell receptors signaling [65].

In Asian population, the gene peptidyl arginine deiminases citrullinating isoenzyme 4 (PADI4) looks like to be second hallmark susceptibility locus after HLA-DRB1 [66]. PADI4 is one of the multiple isoenzymes that converting residues of arginine the post-translation to citrulline, so this might be related to ACPA production [67].

Signal transducer and activator of transcription 4 (STAT4), is a member, belongs to STAT family of transcription factors. The molecules that play a pivotal function for IL-12 signaling in natural killer (NK) cells and T cells that resulting to the manufacturing of interferon (IFN)- γ and differentiation of Th1 and Th17 cells. In Europe population, a SNP haplotype in third intron of STAT4 is connected in susceptibility of both RA and Systemic Lupus Erythematosus (SLE) [68]. Some other genes also have been demonstrated their implications in RA etiology, such as the genes that encode tumor necrosis factor (TNF)- α and interleukins (IL) [69]. Twin studies estimated the genetic factor in RA is to about 50% while the rest is environmental factors [70].

2.3.2 Environmental factors: Several studies have been taken place on environmental factors that trigger the onset of RA disease and the link connection of genetic and environmental factors have confirmed in RA. Smoking, exposure to mineral oils, infections by viruses and bacterial agents, hormonal factors, dietary factors, socioeconomic, consumption and birth weight [71].

Normally environmental agents are considered to act as a trigger rather than as directly contributed in disease course and intricate interaction between environmental and genetic factors are likely needed to initiate the disease onset in the susceptible patients. The approach of immune response to these agents probably release pro-inflammatory cytokines resulting in an immune mediated inflammatory [72]. Smoking is the strongest recognized environmental risk factor in RA disease, and this relationship was not known for many decades until the year 1987 when the risk of RA has been observed in smokers in accidental discovery study with unrelated objectives [73].

So, since that time huge numbers of epidemiology studies have been done confirming the increasing risk of RA in smokers [74]. The risk is increasing with number of cigarettes and duration of smoking and also risk is greater in men rather than in women [75]. Shared Epitope (SE) can modify risk factor, for example RA patients who are smoking and do not have SE have like 1.5-fold higher risk of RA progression and ACPA as compared as to those who are not smoking, while the risk is increased by 21-fold in smoker patients who are carrying 2 copies of SE if it is compared with non-smoker patients who are carrying 2 copies of SE [76].

Hormonal factors probably functioning a key role in etiology of RA disease. RA is much more well-established in women than men, so, the gender association looks like linked to sex hormones, the fact is supported by that RA risk and aggravation of disease increasing post-partum period and amelioration of the- disease is very popular during the pregnancy [77,78]. Furthermore, protective effect role of oral contraceptive pills in development of RA disease, this may be interplayed to content of progesterone in these pills and this issues still in need of much more debates and researches [79, 80]. Breast feeding has showed, protecting the Mums against RA in two big case studies [81, 82].

Diet factors and stress considered to play a possible influencing in RA course progression and expression [83]. D vitamin and its metabolites might be having opposite connection with disease activity in inflammatory RA, due to the immunomodulatory actions that these substances may have [84].

Studies showed that large amount of olive oil, fish oil, vegetables and fruits and beta-cryptoxanthin might act as a protective on the development of RA disease. Meanwhile having low mount of antioxidant enriched foods could be resulting in increasing of RA development [85].

High consumption of red meat and low mount of C vitamin might activate development of inflammatory disease [86]. Large prospective studies have showed that a high birth weight is increasing RA risk by 2-fold [81].

Socioeconomic factors are suspected to be linked with risk development of RA course, as a result of many different types of chronic disease. The data of RA might be conflicting, in one major study in Norway and United Kingdom, no influence of socioeconomic on RA [87, 88].

In contrast, a Swedish case-control study has found mild to moderate effect of education level on RA. An inverse associating between socioeconomic factors can be measured by occupational class and education levels [89].

2.4 Clinical features of RA

Clinically, RA is showing a symmetric polyarthritis disease characterized by morning stiffness lasts for more than an hour, pain, swelling and loss of function. The synovitis beginning is involving the metacarpophalangeal, the proximal interphalangeal, the wrist the metatarsophalangeal joints, meanwhile it might other joints be affected [90].

The hallmark symptoms of the onset in RA is such as fatigue, malaise, weight loss, fever and stress [91]. Regardless of the importance of articular and periarticular manifestations, RA might influence some other tissues and organs and time to time the extra-articular manifestations probably come related with a disease by itself or the manifestations come as a byproduct of the treatment [92]. In other hand, sometimes but always, the autoimmune diseases are overlapping to each other [93].

Despite of the presence of the link with male, severe arthritis, high level of inflammatory biomarkers, smokers, existence of rheumatoid factor (RF), ACPA, HLA-related shared epitope (SE) and anti-nuclear antibodies (ANA) [94], RA nodule is cornerstone for extra-articular manifestation, that makes up to 30% of the cases, meanwhile several of these appearances occur in 1% in RA patients [79]. Anemia disease, Sjögren syndrome and lung manifestation are common in RA, RA can be affected through RF-positive by rheumatoid vasculitis, which might affect any organ [95].

Among these manifestations some cases such as amyloid, neuromyopathies, fibrosis, interstitial pulmonary, systemic vasculitis and Felty's syndrome that can be very difficult to be treated. Also, RA can be symbolized such as an

independent risk factor for cardiovascular diseases like heart failure, myocardial infarction and cerebrovascular [96, 97].

Irremediable inflammation, immune-complexed and altered particles of lipid are making up a substrate for speeding up atherosclerosis in rheumatoid disease [98].

RA is accompanied with high level of risk of non-Hodgkin's lymphoma and this risk can lead to more disease progression by the immunosuppressive treatment [99]. The development of lung cancer linkage and smoking is extensively studied, it is found to be confined to the onset of RA and it is an indicator of RA [100].

Finally, manifestations of extra-articular and high degree of disease activity can affect the disease period outcome and can lead to mortality and morbidity in RA patient [101].

2.5 Synovitis

The first RA inflammatory site that can be affected, is synovium, infiltration of synovial membrane is like a disease hallmarks of RA [102]. Synovial membrane is belonging to connective tissue that made up mainly from two sub-layers, lining and sub-lining synovial. Synovial lining layer is made up of two synoviocytes called fibroblasts-like synoviocytes (FLS) (synovial fibroblasts (SF)) and macrophage-like [103].

About one-third of lining layers of synovial membrane are FLS, FLS have unique appearance and a specific amorphous surface marker. Synovial membrane lacks basement membrane the lining layers has to perform this function [104].

FLS are responsible in production of synovial matrix component such as different types of collagen, heparin sulfate and chondroitin [105], synovial fibroblasts are functioning as a barrier and supporting joint cavity and neighboring cartilage by producing lubrication substances like hyaluronic acid and nutritive plasma, and they are reacting to environmental triggers. During inflammation FLS are becoming more effective by targeting the injury tissue through response regulation [106].

Synovial fibroblasts contributing and mediating in neoangiogenic processes and also, within the joints, they are taking part to the resolution of the inflammation by secreting chemokines to the inflammation site in injured cell [107].

Huge numbers of studies have showed that synovial fibroblasts are acting like cornerstone in immune system and integrating signals from many resources into tissue response in the most frequent chronic joint disease [108]. The composition of sub-lining synovial membrane is a set of elastic fibers network, different types of collagen and laminin, it is a loose and soft connective tissues that smoothening the joints movement [109]. It is containing blood, lymphatics vessels, nerve fibers and several cells including macrophages, fibroblasts and adipocytes [106].

Cellular hyperplasia, increased vascularity and cells inflammation infiltratory grow and destroy the neighbor cartilages and bones are the vast major characteristics of synovial membrane in rheumatoid arthritis [109]. Synovial angiogenesis comes a result of improper oxygenation process, the synovial tissue mass increasing is known as pannus that comes up with increasing oxygen demand that leads to hypoxia [110]. Macrophages are responsible of producing proangiogenic factors, like platelet-derived growth factor, interleukin-8, transformation growth factor- β , fibroblast growth factor and vascular endothelial growth factor [111].

RA synovitis infiltration process is collected of mast cells, CD4⁺ T cells, B cells, plasma cell, natural killer cells and dendritic cells [112,113]. To consolidate the approach of RA and the idea to be more understandable, each one these cells in rheumatoid arthritis course disease are going to be explained in detail in upcoming pages.

2.6 Joints in RA patients

RA is a destructible disease of articular cartilage and subchondral bone, this can be happened by ectopic and hyperplastic synovitis process. Synovial membrane and synovial fluid are together involving in synovial joints in RA [114]. With polymorphous predominance, both cellularity and synovial fluid level volumes are increased [115].

Large number of plasma cells, macrophages, dendritic cells, T lymphocytes and B lymphocytes are detected in synovial membrane and synovial fluid, so synovial membrane lining layer getting thick with increasing number of macrophages-like and fibroblast-like [116,117]. Synovium in RA, turns into a very high vascular in angiogenesis process [angiogenesis: is a process of new blood vessels forming]. As the disease is progressing and invading adjoining cartilages the synoviocytes propagate then cytokines secretion and bone-degradation and cartilage enzymes that comes up with rheumatoid arthritis disease alterations [118].

In synovium of RA, synovium has a vast number of both anti and pro-inflammatory cytokines, mainly, these cytokines are originally come from T-cells and macrophages [119]. The prominent of anti-inflammatory cytokines are include IL-4,10,11,13 and TNF- α receptor and IL-1 receptor antagonist while major cytokines of pro-inflammatory are TNF- α and interleukin-1,6,8,12,15,18 and (IFN- γ). Any lacking balance in anti and pro-inflammatory cytokines might result in RA pathogenic mechanism [120]. Most of systemic manifestation and articular of RA come from some pro-inflammatory mediators such as TNF- α and IL-1 [121].

There is no comprehensible explanation mechanism of the destruction of cartilage and bone, so it may go back to pannus due to it is secreting various types of destructive enzyme like [122]. The most important part among these destructive enzymes are matrix metalloproteinases (MMP's) which comprise stromelysins, gelatinases and collagenase that work on collagen and proteoglycan matrix destroying and these enzymes are governed by some physiological retardants (inhibitors) such as tissue inhibitors metalloproteinases (TIMP) [123,124]. Any misbalance of regulation of inhibitors and destructive enzymes can result in RA or take part in the disease course [125].

Some other factors are involving in destruction of cartilage and bone, including the cytokines TNF- α and IL-1 by activating osteoclast pathways resulting in resorption process of bone [126].

Destruction also can be mediated via many other factors such as osteoclast differentiation factor (ODF) and the integration of TNF- α and IL-1 and ODF together might facilitate periarticular and furthermore can lead to systemic osteoporosis in rheumatoid arthritis disorder [127, 128]. RA not affects only the joints it is also targeting numerous other body structure resulting systemic manifestation, among these organs that may be targeted the rheumatoid nodules, interstitial lung disease and serositis [91].

Again, the mechanism of the disease is not elucidated and one of the hypotheses is explaining the involvement of the rheumatoid factor (RF) which triggers the macrophages to release Fc- γ receptors then the chemokines and pro-inflammatory cytokines are get produced which lead to series of influxes of inflammatory cells. RF also triggers complement pathway leading to immune complex deposition in the perivascular tissues coming up with inflammation and vasculitis, severe disease and extra articular manifestations might be linked with a double dose of SE specifically in DR4/DR15 [129,130].

2.7 Innate immune system role in RA

Many inflammatory processes are getting involve in the development of RA course disease, so in the upcoming sections the involved cells in RA will be outlined with stressing mainly on the response of innate immunity action in case of the disease.

2.7.1 Innate immune cells in RA

With taking into the consideration of both functional and therapeutic role of innate immune cells, they have a great role in preclinical stage of RA, it has demonstrated that innate immunity system activation can be as bench mark of the sign of RA, the activation of innate immune system is outcoming to the outset of inflammatory processes via triggering pro-inflammatory cytokines and chemokines [131]. Many cell types are engaging in disease like monocytes/ macrophages, dendritic cells, natural killer cells, mast cells and osteoclasts [132].

2.7.2 Monocytes/macrophages (M Φ)

Monocytes/macrophages own a very wide pro-inflammatory, destructive and remodeling potential. These make macrophages are taking part in both acute and chronic RA [133].

In RA, the role of monocytes/ M Φ ensue from the integration stimulatory effects. At the tissue level, there will be an influx of pre-activated monocytes, maturation of M Φ , interplay and activate with other synovial cells. Sophistication of interlink is product of paracrine activation mechanisms that produced throughout sheer cell-cell contact [134]. Macrophages are extending from the circulating monocytes that positioning throughout the human body and the other cells of mononuclear phagocyte system (MPS). Among them the precursors of bone marrow of myelomonocytic lineage [135].

They have primary key role in tissues where they engulf and degrade debris, dead cells, and foreign substances, and coordinating the inflammatory processes and activation of the macrophages will come up of releasing chemokines and cytokines that attracting other cells to the site of the inflammation so they shall be distinguished components in the membrane which has get inflamed [136,137].

The dogma role of macrophages in RA is enhanced by the verity that the treatments are acting to suppress and minimize the level of chemokines and cytokines that secreted by the macrophages [138]. For sure the link between macrophages infiltration and tissue damage in the synovial membrane has been confirmed [133,139].

Macrophages action in RA is to produce large quantity of pro-inflammatory cytokines as TNF- α which increases the cytokines expression, adhesion of molecules, collagen and collagenase by the synovial cells [140,141], it is also producing large amount of IL-1, IL-6, IL-18, IL-23 and IL-27 [142-144]. Macrophages are activated via cell interaction with T cells, resulting to produce IL-1 α and IL-1 β [145,146]. Similar way has been demonstrated for

natural killer cells which triggers monocytes/macrophages to produce TNF- α when cell-contact interlink [147].

2.7.3 Natural Killer cells (NK)

Natural Killer cells are belonging to white blood cells types called lymphocytes, they are also apart of innate immune system, defending the body against the cancer cells and the microbial agents, they are large and have granularly shape [7,148]. Recently they have been considered as response agents and acting in adaptive immune system [149].

Many search studies have been made and they have confirmed the role of NK cells as participants in a pathogenesis of several immune-mediated diseases, so it is also being approved in many researches that NK cells are showing impaired activity role in progression of RA disease meanwhile other researchers have showed opposed result [150,151]. Beside of the complex cytokines milieu, cell-cell interaction between different cell types in RA synovial membrane may also exist in case of inflammation, NK cells can react topically and/or many different types of cells get activated and the best example for this is B cells signaling inducing [152].

Dendritic cells (DCs) response can be suppressed or enhanced by NK cells, so the consideration data results on experimental arthritis have demonstrated that NK cells role is not clear yet whether NK cells involve in the trial course of RA disease development [153,154].

2.7.4 Dendritic cells (DCs)

DCs are a special type of cell that playing as cornerstone in development of both adaptive and innate immune response systems to foreign antigens, these cells are acting as a present antigen to T cells, they are heterogeneous, sparsely but widely distributed in different location [7,155].

DCs can be distinguished from other antigen-presenting cells (APCs) throughout their ability that they show towards prime naïve T cells for supporting and cytotoxic role in the body [156]. They are also the crucial in generating the primary antibody response, also DCs are engaging into the

promotion of peripheral tolerance, and they are good promoters of NK cell cytotoxicity [157].

In human, there are two major subsets of DCs, both known as myeloid DC (mDC), the major subsets of mDC involves the tissue of epithelial best-known as Langerhans cells and some other tissues known as interstitial DCs, and plasmacytoid DC (pDC) is recognized to represent APCs, these two subtypes are capacitated to activate the response to specific T-cell ligands that effects on antigen-present and cytokine secretion like TNF and IFN- α [158,159]. It is supposed that DCs are involving in several ways in pathogenesis of RA, DCs coordinate both chronic inflammatory and autoantibody pathology developing which the clinical manifestations of RA are governed by [160].

Both synovial membrane and synovial fluid can be infiltrated by DCs action so they are capable to take up process and present antigen locally contributing to prolongation of disease [161,162]. Innate immune inflammatory mediators that affecting RA pathology are produced along in case of DCs, M ϕ and synovocytes combination [7.163].

2.7.5 Mast cells

Mast cells are a tissue cell in innate immune system, embroiled in several autoimmune disorders pathogenesis among them rheumatoid arthritis which show very active role in initiation phase of the disease [164]. These cells are tissue-resident, long-lived normally it is found in connective tissues as intestinal submucosa, muscle, skin and it is also available in a joint synovium with small numbers while it is increased in number in inflammatory situation [165,166].

In RA, mast cells get accumulate in the synovial membrane markedly with average of 5% of synovial cells and this accumulation can be different from patients to others, this process of accumulation mast cells are called “mastocytosis”, this difference in patients is depending highly on the degree of inflamed joints [167]. Mastocytosis not only affects RA but it is also affecting other arthropathies among them psoriatic, OA and traumatic arthritis [168].

Apart of mast cell contribution in inflammation mediating, many evidences are hypothesized that mast cells also are acting in inflammation resolution, so mast cells having ability to downgrade pro-inflammatory cytokines and the way that mast cells are counterbalancing inflammation begun to merge [166,169]. Mast cells looks like contributing in inflammation bone resorption in RA, and it is believed mast cells do so throughout several mediators such as IL-6, TNF and heparin due to these mediators are acting to promote osteoclasts processes that finalized to bone resorption [170,171].

2.8 Osteoclast and Osteoblast in RA

Bone is specialized connective tissue supports locomotion, prevention or protection and balancing calcium contents in the body via metabolic function [172]. Chronic inflammation alters the physiological process of bone equilibrium. Two types of cells are controlling bone remodeling, osteoclasts cells and osteoblasts cells. The process of bone resorption is called osteoclasts cells, this resorption process has a key role in bone remodeling through their ability to contribute in bone growth, damage and repair [173,174].

Osteoclasts are multinucleated cells; these cells are coming from hematopoietic cell lineage that are related directly to DCs and monocytes/macrophages [175]. At first Osteoclasts starts by demineralizing process of inorganic molecules of bone (decalcifies), throughout hydrochloric acid secretion that provides acidic milieu to solubilize bone calcium. Then second extracts organic component of bone also osteoclasts have ability to secrete some enzymes that effect on bone matrix proteins component and degradation called matrix-degrading enzymes such as cathepsins [176].

Osteoclasts not take place until the proper environment factors for their development are being ready to growth and differentiate them from monocytes precursor, these factors are macrophage colony-stimulating factor (MCSF) and the Receptor activator of nuclear factor kappa-B ligand (RANKL) that enhancing Osteoclastogenesis [177, 178].

In addition, the activated T cells can produce IL-17 which is considered to show as a key role in osteoclasts formation. Some certain cytokines such as TNF, IL-1, IL-6 and IL-17 augment the regulation of the expression of RANKL that in its turn found in synovial membrane in RA [179,180].

Many therapeutic administration studies of bisphosphonates and osteoprotegerin have shed the light on the osteoclasts in RA, abundantly suggested that bone damage is related to RA and not related to the chronic inflammation [181].

Osteoblasts are cuboid-shaped, bone forming cells that stemming from bone marrow mesenchymal stem cells, these cells are synthesizing bone matrix protein components such as both collagenous and non-collagenous, and their action is opposing osteoclasts cells by forming bone and supporting bone growth. To protect and prevent against further bone resorption, osteoblasts are a good producer of osteoprotegerin (OPG), a soluble decoy receptor that binds RANKL to be inhibited and osteoclast differentiation factors to be suppressed and stopped [182]. Bone remodeling depends on key physio-pathways that up-regulate osteoblast differentiation and function, the Wntless (Wnt) and bone morphogenic protein (BMP) signaling pathways [183].

In addition to the Wnt signaling pathway, the BMP pathway also shows a key role in both processes osteoblast differentiation and bone remodeling. BMPs made up of a large group of growth factors that belong to the transforming growth factor beta superfamily (TGF- β) [184].

2.9 Adaptive immune system role in RA

To date, the scenario of rheumatoid arthritis pathogenesis is intricate and not fully understandable. Many factors are contributing in its progression among these factors adaptive immune system [185].

Both autoantibodies and genetics of RA clearly clarify involvement role of adaptive immunity system in the early pathogenesis. Nevertheless, despite T-cells are considerably available in the synovial milieu, their role is not fully elucidated but up-to-date therapeutic methods that targeting both T and

be cells such as biologic treatments that strongly shows the hallmark role of adaptive immune system [186].

The adaptive immune system comprises of T and B cells that express clonally distributed antigen receptors. Adaptive immune spreads its responses throughout the clonal expansion of T lymphocytes and antigen-specific B, antigen-specific antibodies after differentiation of B-cells into the plasma cells releases extracellular antigen eliminator while T-cells are upholding other cells in immune response to get rid of infected cells, or act on minimizing undesirable responses of immune system [187,188]. The overview of T-cells and B-cells and their engagements in rheumatoid arthritis will be addressed in the next two sections.

2.10 Rheumatoid arthritis and T cells role

T cells is a type white blood cell, an organ contributing in immune system. It is also called T lymphocyte, it is the first primary type of lymphocytes [189]. Broadly speaking, $CD4^+$ T cells function is either considered as supporter to regulate T lymphocytes and in some others $CD4^+$ T cells act on exhibiting cytotoxic activity or directly act on cell-cell contact that is called T helper (Th) to secrete cytokines. While $CD8^+$ T cells works on secreting cytokines that act on immune response generation to kill infected target cell [190,191].

A couple of studies have showed $CD4^+$ T cells involvement in RA developing pathogenesis. Adaptive immunity role has been confirmed throughout the genetic predisposition act in the development of RA such as HLADR β 1 and most lately PTPN22, CD28, CTLA4 and CD40 [192]. For example, HLADR β 1 gene is made up of amino acids sequence in peptide-binding pocket, the SE that able be as the antigen to T-cell receptor on $CD4^+$ T cells [193].

T cells are get activated and secrete pro-inflammatory cytokines such as that IFN- γ , TNF- α , IL-2, IL-12, IL-18 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Specially Th1 cytokines that are secreted in synovial fluid and expressed in synovial membrane [194]. Macrophages will be activated by these cytokines to excrete some other pro-inflammatory cytokines such as IL-12, TNF- α , IL-1 β and IL-6 that in turn activate B cells

differentiation and stimulate the secretion of matrix MMPs that triggering the cartilage degradation and at the end lead to bone resorption due to the osteoclast have been activated [195,196]. Many studies have suggested an alternative route for producing pro-inflammatory cytokines because of cell-cell contact, between synovial T cell and neighboring macrophages or fibroblasts [197].

The proof that shows the involvement of these cells in the pathogenesis of RA is their presence as an activated form of CD4⁺ T cells in synovial fluid, synovial membrane and peripheral blood [198,199]. Many immunosuppressive drugs are regulating T cells to improve RA disease such as Methotrexate, corticosteroid, Sulfasalazine and Leflunomide. These drugs reduce pro-inflammatory cytokines [200].

2.11 Rheumatoid arthritis and B cells role

Historically, it was not thought that B cells contributing in the immunopathogenesis of inflammatory joint disease specially RA, regardless of this, it is agreed on that autoantibodies show active role in joint inflammation. Beside this, these autoantibodies can also serve as a diagnostic, prognostic and classification criteria marker [201].

The development of B cells triggers in the bone marrow that various differentiation stages are taking place to come up with immature form of B cells from stem cells [202]. Finally, in spleen the maturation of B cells is taking place [203]. In normal situations, B cells are considered to be a very needful part of immune response and are responsible for maintenance of humoral protective memory [204,205]. B cells yields pro-inflammatory cytokines such as TNF and IL-6 and some other immunoregulatory cytokines such as IL-10 in turn these cytokines may trigger macrophages, intensify the stimulation of pro-inflammatory signaling that leading to increase of the level of IL-6, IL-1 and TNF. Meanwhile IL-6 is a good cytokines source to B cells get growth [206]. Also, B cells can produce IL-10 that activate DCs that is going to be much more effective antigen-presenting cell. The rest of cytokines that produced by B cells contributing

in some certain steps of immune activation in general and RA particularly are not fully lucid [207].

B cells are involving in RA in certain steps of regulation of immune activation. They differentiate into autoantibodies cell known as rheumatoid factor (RF) and antibodies to citrullinated peptides (anti-CCP) in 60 % to 80% in the patients. The higher level of RF and anti-CCP in synovial fluid is highly suggesting that these autoantibodies are produced in large mount in synovial membrane. RF and anti-CCP are remarkably found in the patient of RA. Serologically, RF has been considered to be a decent marker to diagnosis the disease [208,209].

The most conspicuously deliberated aspect of B cells in RA is antibodies against exogenous and self -antigens as well as cyclic citrullinated peptides. So, the earliest manifestations proof role of B cells was serum autoantibodies discovery [210]. No doubtfully, the early pinpointing RA disease and starting proper treatment will lead to desirable outcomes and to do so specific and sensitive serologic tests should be done [211].

2.12 Autoantibodies values in RA pathogenesis

Studies shown that there are many autoantibodies contributing in RA pathogenesis. These autoantibodies categorized into two groups, those can be present in inflammation diseases such as rheumatoid factor (RF), anti-collagen type 2 antibodies, anti-RA 33 antibodies and anti-calpastatin antibodies and some other that can be present in disease that relatively specific to RA such as anti-heavy chain binding protein and the most of antibodies that directed against cyclic citrullinated peptides (anti-CCP) [212].

RF testing is widely well practiced in clinical laboratories to diagnosis RA with a very low specific specificity somehow around (40-60) %, this low diagnostic specificity is due to RF also occurs in a wide range of other diseases that cannot dogmatize RA disease exactly [213].

To increase RA disease diagnostic specificity to approximately 98% anti-CCP testing is used as a back up to RF testing but the thing that should be kept in mind is these tests cannot be enough to diagnosing RA in the absence of other diagnostic criteria [214].

The presence of RF and/or anti-CCP can be determined in some patient's couple years before the onset of the disease showing clear break down of immune tolerance in B-cells in disease pathogenesis but there is insufficient documentation to make proper clinical decision before the onset of the first symptoms is appeared [215,216]. We will outline the role of RF and anti-CCP antibodies in the pathogenesis, diagnosis and prognosis of RA with swift alluding of other autoantibodies in such disease.

2.12.1 Rheumatoid factors (RFs)

Rheumatoid factors are defined as a family of autoantibody that directly binds to the Fc portion of normal human IgG, they were first described by Waaler in 1940 [217].

RFs can be classified as multiple immunoglobulin isotypes of IgE, IgM, IgA, and IgG. IgM RFs are most activated form of RFs in RA and are detected in 50 – 80% of RA patients and locally produced in RA disease by B cells present in lymphoid follicles and germinal center-like structures that develop in inflamed synovium [218-220]. Classically, RFs are not found only in the susceptible individuals but also are in healthy ones, and to differentiate between these two groups, RFs in RA patients are featured from RFs in healthy individuals in that they exhibit affinity maturation, whereas those in healthy individuals are poly-reactive and are of low affinity [221].

RF specificity in RA is increased at high titers (e.g. IgM RF > 50 IU/ml) and with IgA isotypes. High titer RF and IgA isotypes are also related with radiologic erosion, extra-articular manifestations and thus, undesirable results [222,223].

The connection of high titer RFs status and a poor diagnosis refer that RFs possibly will show a key role in RA pathogenesis. Furthermore, RFs have recognized to be the utmost suitable disease indicator of RA, as counted in the ACR classification criteria for RA [224].

Under normal situation RFs support immune complex clearance, and uphold B cells to uptake immune complex, they are facilitating complement fixation throughout binding to IgG containing immune complexes [225].

2.12.2 Citrullinated antigens autoantibodies

In the last decades, the researches on RA patient's sera, unusual autoantibodies amino acid citrulline was found as one of the contributors directed to epitopes in RA disease [226]. In general, it is known as anti-citrullinated protein antibodies (ACPAs) [227].

ACPAs are produced by synovial B cells, the same cells that produce RF, this made the believe that these 2 antibodies are participating together in causing inflammation in joints of patients and then starting destructive processes of them [228]. Citrullination is a certain step to identify the different types of proteins that are expressed during the inflammation by ACPAs such as collagen type 2, fibrin, fibronectin and vimentin [229].

ACPAs are used as pretty good markers in diagnostic and prognostic of RA patients since it is clear that the early treatment of RA suppresses the disease progression, and to do identification many commercial assays are used as an antigen [230].

ACPAs can be found in early disease course up to 10 years before RA disease onset, as similar as RF [231]. ACPAs show a very good diagnostic sensitivity ability that might reach up to 75% and show specificity to 95% in established RA patients but this sensitivity is found less than 50% in very short disease duration with increasing until reaches 64 % in patients that have less 1 year of disease duration [232].

Recent studies demonstrated prognostic role of ACPAs specially in the association with bone damage, moreover their association with RA-associated shared epitope (SE) (HLA-DR alleles) particularly positive ACPAs patients of (SE) showed high development level of risk to develop sever forms of RA [233,234].

2.12.3 Some other autoantibodies

A. Anti-RA 33

Anti-RA 33 are a heterogeneous nuclear ribonucleoprotein A2 (hnRNP) A2 antigen, these RNA and DNA 36-kDa binding protein [235] were described in 1989 and they have introduced as markers in early arthritis symptoms [236] which have relevance in both pathogenesis and diagnosis of RA with incidence (20-30) %. Furthermore, anti-RA 33 is not specified to RA but it is also occurring in some other diseases such as SLE or mixed connective tissue disease (MCTD) [237].

Anti-RA 33 can show a high specificity to (90-96) % with a low sensitivity to 32 % in RA patients [238].

B. Anti-collagen antibodies

Although there are 14 different types of collagen, but the most utmost joint species antigen is collagen type 2 (anti-CII). But it is not specific for RA due to it is found in a pathologic of a couple of different kinds of abnormal condition [239].

It was found that the level of anti-CII is getting high in the early of disease course but again runs down with progression disease, anti-collagen antibodies cannot be considered as a good diagnostic marker due to lack of specificity towards RA disease [240,241].

C. Anti-Calpastatin antibodies

Calpastatin is a nature inhibitor of calpains which are composed of calcium-ion-dependent neutral cysteine protease [242], and these proteins are found in RA patients with elevated concentration in synovial fluid with relevance detection in sera of RA of 40 % to 60%. Noteworthy also, this detection is not limited to RA only because they are found in many other diseases such as SLE and myositis as well as they can be found in healthy individual [243].

2.13 RA cytokines in brief

RA disease has a multifactorial and an intricate pathogenesis, many factors are contributing in disease course, the human immune system involves highly complex and well-coordinated processes in which small proteins named cytokines play a key role [31].

Cytokines are low weight proteins molecule which function as a mediator inside biology cellular, they show pleiotropic key role in regulation, development, activation, differentiation and growth of the cell thus they contribute nearly a vital role in all biological processes [244].

It has become clear that there are pro- and anti-inflammatory cytokines, derived mainly from cells of macrophage lineage, play hallmark role in the initiation and perpetuation of the chronic inflammatory process in the RA synovial membrane [245].

Non-attendance of unified classification of cytokines have given functional activity and structural homologies names of them in various inflammatory responses by primary cell of origin for example early or late inflammatory, pro- or anti-inflammatory, cytokines have capacity to secret on spot responses by stimulating direct signaling that swiftly changes gene transcription [246].

Anyway, cytokines can be divided into super-families, this dividing either established on structural homologies and shared functional properties or based on the type of the receptor that they get engage [247]. Below is the brief overview of cytokines families.

2.13.1 IL-1 family

IL-1 family is consisting of 11 structural homologies cytokines, and they are IL-1 α , IL-1 β , IL-1 receptor antagonist (IL-1Ra) and IL-18 [248]. A new terminology system proposed that IL-1 α , IL-1 β , IL-1Ra and IL-18 become IL-F1, IL-F2, IL-F3, and IL-F4 respectively [249].

Noteworthy, all IL-1 cytokines family members are extracellular except IL-1Ra is produced by Golgi apparatus and endoplasmic reticulum but the nature of how IL-1 family members are get secreted is still not lucid. The

mechanism that governs signaling in IL-1 family members is taken place throughout a bunch of closely related receptors [250].

IL-1 family is exercising a wide range of pro-inflammatory effects throughout inducing of mononuclear and stromal cells to secret the mediators of pro-inflammatory such as cytokines, chemokines degradative enzymes, adhesion molecules and promoting of articular destruction by affecting on the maturation of osteoclasts and chondrocytes activating, also it is observed that IL-1 is involving in differentiation of Th17 and producing of IL-17 [251,252].

IL-18 is secreted by some cells such as macrophages, DCs, osteoblasts and synovial fibroblasts. IL-18 is controlled via soluble IL-18-binding protein by inhabiting interaction with their receptors [253]. IL-18 is embroiled in RA pathogenesis and it is found with high level in serum of RA patients and also in RA synovium [254].

Ultimately another family member of the IL-1 has been described and named to interleukin 33 (IL-33). This cytokine is accepted to function in inflammatory diseases throughout exerting profound effects in human RA and preventing its receptors showed powerful potential therapeutic approach [255]. Mainly, it is produced by synovial fibroblast, in RA, it is found predominantly in synovial membrane. The effect of IL-33 is to become visible vastly by mast cells activation [256].

2.13.2 Cytokines binding a γ_c -containing receptor

These cytokines have structural features of four-helix bundle motif. They are grouped on the basis that comprise a gamma chain receptor subunit as signal-transducing chain with a cytokines-specific chain. These cytokines signaling throughout γ_c -containing receptor comprise IL-2, IL-4 (IL-3), IL-9, IL-15 and IL-21. [257,258].

In RA patients, a polymorphism in IL-4 receptor is found that result in reducing responsiveness to IL-4 that associated with erosive disease [259]. IL-4 has been identified in synovial fluid of early stages of RA patients while IL-13 can reduce pro-inflammatory cytokines production and it is detected synovial fluid of RA patients [260].

2.13.3 IL-17 family

This family is comprised of six members as IL-17A to IL-17F that binds to a unique class of cytokines receptors. This family is a number of cytokines that shares little homology with other cytokines [261].

IL-17 family have a critical pathogenic role in the defense against extracellular through inducing the production of pro-inflammatory cytokines such as IL-1, TNF- α and IL-6 by endothelial cells, macrophages and fibroblasts [262]. IL-17 family upregulates RANKL on osteoclasts to promote osteoclastogenesis. Also, this family is inducing some chemokines that stimulate T cells and macrophages to be established to inflammation site [263].

Some studies have showed that IL-17 plays an importance role in RA pathogenesis [264]. IL-17 is found in synovial fluid of RA patients, also Th17 has been detected in synovial tissue [265]. The investigation of RA patients has discovered that IL-17 has a potential therapeutic target and expressed by mast cells in RA synovium [266].

2.13.4 TNF family

TNF family members and their receptors are structurally related group of molecules functioning in a variety of cellular aspects with stressed role in regulation of both adaptive and innate immune response [267].

TNF family cytokines bind to membrane-bound receptors of TNF receptors family which considered typically soluble transmembrane protein. Many family members are there but the most important one is TNF - α [268].

TNF - α is considered as a classical pro-inflammatory cytokine and it is acting importance role in host defense also involve in both development and progression of RA. TNF- α is predominately secreted by active form of macrophages/ monocytes. It can be secreted other leukocytes such as T cells, NK and neutrophils [269].

In order to initiate signaling, TNF- α binds to one of its receptors either TNF receptor 1 (TNF-R1) or TNF receptor 2 (TNF-R2). Soluble form of TNF- α cytokines are binding to TNF-R1 while TNF- α membrane-bound ones are binding to TNF-R2, also the two pathways can be taken place at the same time but, each pathway has its own primary procedure until binds to TNF- α , noteworthy, TNF-R1 is expressed in most tissues [270]. TNF-R2 is known to be expressed on endothelial and immune cells. Apart of TNF-R1, TNF-R2 can be fully activated by membrane-bound TNF- α and cannot be activated by soluble TNF- α due to cytoplasmic death domain in TNF-R2 is absent [271].

The relevance development of active biologic agents that act as TNF- α antagonists in RA remedy is progressing such as infliximab, adalimumab, etanercept, certolizumab (certolizumab with methotrexate [272]) and golimumab. Some studies have recommended that TNF inhibitors can efficiently suppress clinical symptoms of RA and inhibit the development of the structural impairment [273].

2.13.5 Cytokines binding a gp130-containing receptor

Several cytokines depend in signaling on a receptor contain the signal-transducing protein glycoprotein 130 (gp130). These kinds of cytokines include IL-6, IL-11, IL-27, IL-31, ciliary neurotrophic factor, osteopontin and leukemia inhibitory factor [274,275].

IL-6 is a pleiotropic cytokine made by macrophages, fibroblasts, endothelial, B cells and T cells. It exercises in inflammatory cascade and also in immune responses, it takes control on leukocytes activation and production of antibody [276,277]. Also, it stimulates differentiation of osteoclast [278]

In RA patients, it is expressed in synovial fluid and it correlates the relationship between synovial fluid levels and disease activity [279]. Several study lines displayed pathogenic the involvement of IL-6 in RA, and it mediates systemic impacts to promote acute phase response, lipid-metabolism dysregulation, anemia and cognitive dysfunction [280].

Targeting IL-6 with a monoclonal antibody against IL-6 receptor (tocilizumab) is very influential in treatment course of RA, [281]. Tocilizumab is a humanized monoclonal antibody that act to bind soluble and membrane-expressed IL-6 receptor (IL-6R) to reduce inflammation and erosion [282].

2.13.6 Interferons (IFNs)

Interferons are cytokines expressed in a wide range with three main classes, powerfully affecting anti-viral and growth-inhibitory. These classes are classified as type I, type II and type III. With subtypes in human as IFN- α , IFN- β , IFN- ϵ , IFN- κ , and IFN- ω [283]. IFN- α and IFN- β are most highly expressed by many cells in response to infection, and IFN- α and IFN- β activate NK cells to eliminate virus-infected cells, much more IFN- α and IFN- β activate adaptive immune response by expressing co-stimulatory molecules on macrophages and ND cells to initiate them to function as antigen presenting cells. Several lines of evidence showed that type I of IFNs have a crucial role in RA pathogenesis [284,285,286].

RA synoviocytes exposed to IFN- α exhibit increased expressing of anti-inflammatory molecules such as soluble TNF-R and IL-Ra [287]. IFN- β is engaged in RA by reducing of pro-inflammatory cytokines IL-1 β and TNF- α , inducing of anti-inflammatory cytokines IL-Ra, and IL-10 [288].

2.13.7 Chemokines

Chemokines are small peptide molecules (8-12 kDa), they regulate cell migration and also, they contribute in many multiple processes such as lymphocyte trafficking, angiogenesis and inflammation [289].

So far about 50 types of chemokines have been described with shared sequence homology [290]. These chemokines initiate their biological functions throughout binding to their own family chemokines receptors which are G Protein Coupled Receptor, and the ability of chemokines to bind to multiple receptors let they be called or described as “promiscuous” [291].

First, chemokines were named based on their function but with increasing of discovered chemokines number, the systematic naming system was necessary to classify them. They have been classified in according to the spacing and positioning of the first two N-terminal cysteine residue in specific motif, so they are divided into four families (CC, CXC, XC and CX3C) the vast number of CC chemokines are showing a key role in triggering cells to be gone to inflammation site [292-294].

In RA, the level of chemokines is increased notably in the synovium of RA disease. They are involved in the pathology of RA because of the ability that they have to recruit and retain leukocytes in the joints [295]. Chemokines activate the release of inflammatory cytokines by other cell types that induce synovial fibroblasts to escalate the mount of IL-6 and IL-8 and thus enhance cell proliferation of synovial fibroblasts [295-297].

2.13.8 Adipokines

Adipokines are a group of soluble mediators with inflammatory approach that mostly are produced by adipose tissue, some studies suggested that these adipokines might reinforce and preserve inflammatory responses in RA [298]. Several adipokines are there, they comprise of leptin, visfatin, adiponectin, resistin, ghrelin, chemerin, lipocalin 2, Vaspin, omentin and serum amyloid 3 [299].

Leptin was the first adipokine family member described, it is a protein aggregated to the class I cytokines superfamily. Leptin works to suppress the food intake and increase energy expenditure, as well as works a pro-inflammatory cytokine to induce T cells and differentiation of Th1[300].

The evidence showed that there is real link of leptin levels and the RA disease trial [301,302]. The elevation of leptin levels in RA patients, serum levels and synovial fluid mount are interlinked with disease duration and disease activity [303,304]. Since this adipokine is the hub of current search, so it will be discussed in much more detail in next sections.

Adiponectin is a collagen-like protein with structural homology to complement factor C1q. Adipocytes are the main source of producing adiponectin but it can be produced by some other cells. Adiponectin can be found in the blood with different molecular isoforms [305,306].

Adiponectin boosts fatty acid oxidizing process and suppress synthesizing of glucose in liver. Researchers reported adiponectin has ability to act as pro-inflammatory and anti-inflammatory, its levels is correlated with disease severity [307], ESR and RF [308].

Ultimately, two adipokines receptors have been described, the first one is AdipoR1, which is produced widely by skeletal muscles. The second one is AdipoR2, which is produced in liver [305,309]. Finally, the side effect of adiponectin in perpetuating joint inflammation, the use of adiponectin as potential therapeutic target of blocking therapies is proposed [310].

Resistin is a cysteine-rich dimeric protein, it is agreed on that it is mainly expressed by white adipose tissues (WAT), but some studies demonstrated that in human is derived for circulating macrophages/monocytes [311], it induces the production of IL-6 and TNF- α , its levels are higher in RA patient and are correlated with inflammation and joint destruction [312,313]

Visfatin is secreted mainly by visceral fat, it was described as a cytokine that implicates in early B-cells development [314]. Visfatin has pro-inflammatory properties by inducing monocytes for producing IL-6, IL-1 β and TNF- α [315]. In RA, circulating levels of visfatin are increased in serum and synovial of RA patients. Its levels are correlated with disease activity, RF levels, inflammation and radiographic joint damage [316,317].

2.14 Leptin

Leptin is an adipokines which originally described as a cornerstone mediator molecule in food intake regulation and body weight [318]. Biologically, leptin is observed to have both active and inactive circulating forms bounded to blood plasma protein and/or to the soluble leptin receptor [319].

In both mice and human, circulating leptin levels are reflecting the amount of energy stored in adipose tissue and are rational to body adipose mass, so it means that obese individuals have greater levels of leptin than that in leaner individuals [320,321]. Noteworthy, leptin level expression is found to have much higher concentration in females than in males with same body mass and this is due to gender bias characterization. Leptin producing is inhibited by testosterone hormone and is stimulated by ovarian sex steroids [322,323].

Historically, it is interesting to mention quick briefing on how the final form of leptin is identified, in 1950 Jackson laboratory conducted study on mutant obese mice that the effects of leptin were observed [324]. Consecutive studies in same laboratory by Douglas Coleman showed that the ob encoded body weight regulator hormone that a second mutation and causing obesity and also the receptor hormone that encoding it is db/db gene. Finally, at Rockefeller University, in 1994 Jeffrey M. Friedman, Rudolph Leibel, Douglas Coleman and their co-research teams discovered through the study of mice, the final form of leptin [325-327].

The discovery of leptin has changed a lot of the idea of white adipose tissue (WAT). For decades, WAT was known as a triglyceride reservoir with passive to null role in endocrine system but its discovery identified that adipose tissue is one of the active endocrine organ that can contribute in energy homeostasis regulation, metabolism and immune inflammatory processes [328,329].

Structurally, leptin is classified as a type I of cytokines superfamily, it is a 16 kDa non-glycosylated anorexic peptide which known as a modulator of food intake at hypothalamic level, body weight and fat store. Mostly is synthesized by adipocytes with encoding by ob gene. Directly, leptin concentration in blood is correlated to adipose tissue mass, leptin acts at hypothalamic level to decrease food intake and to increase energy expenditure [329-331].

2.14.1 Synthesizing and regulating of leptin

Leptin is mostly produced in adipose tissue, it can be expressed in other organs and tissues such as liver, placenta, muscle, ovaries, lung, stomach, kidney and heart [332]. Leptin is composed of 167 amino acids units with 21 amino-terminal secretory signal sequences. in serum, various factors governing the level of this hormone such as insulin, endotoxin, cytokines, glucocorticoids and obesity [333,334].

It is worthy to be mentioned that leptin levels are influenced by some factors and are suppressed in case of prolonged fasting, environmental factors such as cold and temperature, hormonal status of the individual and heavy sport exercises [335]. Some studies showed that leptin involves in lipid metabolism regulation whether it was supplied in vivo or in vitro. Nevertheless, obesity activates a set of cellular processes that reduce the leptin signaling and leading to leptin resistance [336,337].

The functions of leptin do not stop at controlling the food intake. Leptin is considered not only key player in endocrine system, it is also acting an important role in insulin secretion, bone formation, reproduction, energy homeostasis and angiogenesis [338-341].

Notably, the crucial role of leptin in both of innate and adaptive immune systems is approved in some studies, due to these functions in immune activities the decrease of leptin levels in plasma results in the impairment of immune function. This may be due to the similarities of leptin structural properties with long-chain helical family of cytokines that include IL-6, IL-11 and IL-12. [342,343]. Leptin synthesis is regulated by activity of inflammatory mediators [344].

In general, leptin is considered as a pro-inflammatory adipokines, due to leptin can stimulates the pro-inflammatory cytokines production such as TNF- α , IL-6 and reactive oxygen species (ROS) in monocytes, it is also altering the balancing of Th1/Th2 throughout inducing CC-chemokines production [345,346].

2.14.2 Leptin receptor and its signaling

Leptin receptors (Ob-R), are encoded by diabetes gene (db), these receptors are classified under class I cytokine receptor superfamily which comprised of the receptors for IL-6 leukemia inhibitory factor (LIF), gp130, oncostatin M, granulocytes-colony stimulating factor (G-CSF) and ciliary neurotrophic factor (CNTF) [329,347].

The db gene expression is nearly ubiquitous [348], has six receptor isoforms with cytoplasmic domain of different length, among them one soluble form (Ob-Re), one long-functional isoform (Ob-Rb) and four short forms (Ob-Ra, Ob-Rc, Ob-Rd and Ob-Rf) [349,350].

In recent decades, studies of leptin binding signaling to its receptors have progressed very much to describe the biochemical and molecular mechanism of leptin function [351]. Studies on Ob-R showed a quick recognizing of JAK/STAT pathway as the main cascades of leptin signaling activity [352]. Some other approved the activation role of Ob-Rb including intracellular motifs in JAK/STAT [353]. In JAK/STAT pathway, leptin binding to its receptor to make dimerization to dimer-associated JAK2 closes and results in the phosphorylation. Activated JAK2 phosphorylation of tyrosine 1138 (Tyr1138) binds to SH2 domain of STAT-3 [354,355]. The dimerization is detached and entering the nucleus [356].

MAPK/ERK pathway is initiated after the phosphorylation of Tyr 972 and Tyr 985 of SH2 domain-containing phosphate 2. This activates MAPK pathway throughout connection with growth factor receptor-bound protein 2 [353,357]. Which enter the nucleus and mostly mediate chemokines and cytokines gene [358].

PI3K/Akt pathway is considered one of the important signaling pathway due to this pathway is very necessary to induce the immune and inflammatory responses [359]. Also, this pathway regulates many effectors including anti-apoptotic transcription factor nuclear factor kappa B (NF-kB), Akt decreases the biological function of transcription factor fork-head box protein O1 (FoxO1), which is reported that it can directly impact on regulation of leptin function at hypothalamus [360]. Leptin and Ob-R undergo through

dimerization and stimulate insulin signaling such as insulin receptor substrate $\frac{1}{2}$ (IRS1/2) by phosphorylated JAK2 [361].

2.14.3 Leptin action and immune system

Leptin, adipocytes-derived hormone, has pro-inflammatory activities and some other comparable action to those of acute phase reactants, and upregulating of inflammatory cytokines producing such as TNF- α , IL-6, and IL-12 [362]. In turn, in adipose tissue, TNF- α and IL-1 β are increasing leptin mRNA secretion affecting each other in promoting inflammation [363]. Leptin has ability to sustain low-grade inflammation that might favor a role in the development of chronic disease [364].

Leptin is considered to play pluripotent role in immune homeostasis, many studies showed leptin role is linked to several autoimmune diseases like multiple sclerosis (MS), SLE, type I diabetes and RA [365,366]. Also, it has pleiotropic action in the mediation of both innate and adaptive responses in immunometabolism, infection and autoimmunity [367].

A. Leptin role in innate immunity

The innate branch of immune system status is also altered by leptin, ultimately, research has discovered mechanism and functional role of leptin in several types of innate immunity cells [368].

In DCs, leptin is contributing in maturation and survival of DCs, and when exogenous leptin is supplied as a treatment of DCs, Akt pathway activates to induce CD40 expression [369]. Conversely, in the absence of leptin signaling, DCs show Th2-biased cytokines profile at the same time exogenous leptin stimuli slopes the cytokines balancing to Th1 profile [370].

In monocytes, leptin triggers human monocytes proliferation in vitro, in which it increases the production of CD39, CD69, CD25, CD72 and IL-R α . Also, it upregulates HAL-DR and IL-6 and TNF- α expression [371,372].

Leptin activates macrophages and enhances phagocytic activity via phospholipase activation to generate nitric oxide, eicosanoids, cholesterol acyltransferases-1 and some other pro-inflammatory cytokines [373,374]. Leptin in neutrophils, activates chemotaxis and secretes ROS as well as it protects neutrophils from apoptosis [375,376].

Finally, leptin is also promoting NK cells proliferation, cytotoxicity, differentiation and activation throughout IL-2 upregulation and expression of perforin via STAT3 activation [377].

B. Leptin role in adaptive immunity

Leptin, is playing a hallmark key of thymic homeostasis and thymic maturation. Leptin-deficient mouse has a low thymic cellularity and high thymocytes apoptosis but this situation can be changed or be reversed if exogenous leptin has been administrated [378].

Leptin able to promote T cell activation and skews the T cells cytokines production towards a Th1 response, throughout increasing of (IFN)- γ and IL-2 production, and decreasing of Th2 and IL-4 production [379]. Exogenous leptin administration leads to significant increase of CD4⁺ and CD8⁺ T cells in addition to cytokines responsiveness [379]. In human, CD4⁺ T cells counts are reduced, impaired in cell proliferation and cytokines release [380].

Apart of the finding that showed leptin capacity to promote survival of both T and B lymphocytes by decreasing Fas-mediated apoptosis [381]. Recent researches suggested leptin ability to negatively affect the proliferation on naturally occurring human CD4⁺ CD25⁺ FoxP3⁺ regulatory T cells (Tregs) [382]. Tregs are important mediators of peripheral immune tolerance via proliferation suppression mechanism and generating of inflammatory cytokines in target cells [383].

2.14.4 Leptin as an inflammation marker

Production of leptin is not only regulated via food intake, due to it is can be regulated also by many hormones in addition to the several of inflammatory mediators [384]. Broadly, serum levels of leptin are correlated to insulin

levels and oppositely are correlated with glucocorticoid levels, with elevation in case of acute infection and sepsis [385,386].

Studies proposed the relationship of leptin levels and inflammatory markers like soluble state of TNF receptors or C-reactive protein (CRP). [387,388], while some other studies could not confirm this relationship [389].

Recent studies suggested the correlation between serum leptin levels and inflammation levels, for example leptin levels are raised in case of active disease such as RA, and meanwhile its levels are lowered when the disease is controlled [390]. Taking everything into account, leptin is dimorphic hormone due to in physiologic conditions, in female, its levels are higher than that in male [391]. In this concern, it is noteworthy to mention that many epidemiological studies showed higher prevalence of several autoimmune diseases in female [392].

In chronic inflammation conditions, leptin levels are elevated. Some reports have showed leptin role in the pathogenesis of many autoimmune disease which are classified as chronic inflammation [392,393].

2.14.5 Leptin in rheumatoid arthritis

In general, leptin is associated with autoimmune disease, and particularly with RA. In RA, cytokines play a cornerstone role in inflammation and joint destruction, producing in synoviocytes and leucocytes infiltrating synovial tissue [394]. Adipose tissue originates and secretes highly bioactive substances called adipocytokines that include several members among them Leptin [395].

Conflicting results of leptin circulating levels in RA patients are observed, some studies have correlated its level with disease activity [396,397], while some other studies showed no correlation between serum level of leptin and disease activity in RA patients [398], this conflicting of results clearly indicating the intricate role of leptin in the development of RA disease [399].

But, few reports proposed that leptin levels in RA patient's sera associated with body mass index (BMI), but these levels were as that as in healthy control, in other hand leptin levels do not correlate with RA severity in RA patients [397,400].

Synovial fluid and serum leptin levels are higher in RA patients with moderate disease activity, but the sera levels are much lower in same patients. So, it was suggested by Bokarewa et al. that in joint the leptin consumption likely to prevent or protect erosion of joints. This brings into our minds that leptin can be involved in down-regulating of the erosive course in the joints [371,401]. And the best proof of that is, if RA patients were treated by anakinra (IL-1 receptor antagonist) they can be avoided to joint destructive process [402].

Emerging studies showed that released quantity of leptin from articular adipose tissue was as same as that released from synovial membrane [403], but IL-6 β treated articular adipose tissue generates leptin four times greater than that in synovial tissue. So, it is believed that articular adipose tissue can be a crucial associating factor in RA joint pathology [404].

To keep it brief, several studies proposed to elucidate this conffliction of the results: first, relatively small sample size might not be sufficient to precisely describe this big numbers of RA patients; secondly, basic standards such as disease duration, age, race and BMI that considered to be proper characteristics in identifying of leptin levels can be inconsistent which may have impact on leptin levels; thirdly, RA patients with other co-existing autoimmune diseases cannot be excluded from studies, which might be perplexed factors; fourthly, different leptin levels measurement techniques such as radioimmunoassay and enzyme-linked immunosorbent assay (ELISA) may cause changes and variations of leptin levels. Also, variance in leptin levels in RA patients might come from the different treatments that interfere with the endocrine system [304,317,358,405-407]. This conffliction of results shows that much more studies are crucially needed regarding leptin levels and its role in the development of RA disease.

Leptin plays a crucial role as pro-inflammatory during joint inflammation which, in synergy with INF- γ or IL-1 to activates nitric oxide synthase type II (NOS2) release from mouse chondrocytes [408].

RA patients with erosive joints disease have higher levels of leptin than those patients who did not have erosive joint disease, with possibilities of risk increasing of progressive joint destruction if leptin levels are elevated [303]. Leptin levels were positively correlated to DAS28 values that might be implicated to leptin role as a pro-inflammatory [302].

Leptin as pro-inflammatory cytokines can increase IL-6 production in RA throughout activation JAK2/STAT-3 [409]. Yoshino et al uncovered in one study that leptin can play as a pro-inflammatory cytokine when he found significant correlation between CRP levels and leptin levels in RA patients [410]. Nonetheless, describing leptin as an anti-inflammatory cytokine in joint erosive in RA has been explained. Suppressed level of leptin in synovial fluid is implicated with non-erosive joint disease, referring the protective role of leptin verse RA destruction [401].

Leptin shows radiographic joint damage suppression. It has been noticed that enhancing leptin effects may return to RA patient's benefit, this inverse the involvement between leptin and radiographic damage may lead from the sense that elevated levels of leptin in RA truly worsen joint damage conditions, but this impact is protected by adiponectin, which shelters against joint damage because of the anti-inflammatory characteristics of leptin [317,411].

Leptin has direct influence on cartilage generation, including both growth and development of skeleton. Long run treatment with anti-TNF- α is very active in ameliorating body mass decrease in RA patients, as well as no variation is observed in plasma leptin during etanercept therapy. In RA remission, leptin has an important key role in preserving energy homeostasis in anti-inflammatory responses [412].

CHAPTER 3

Methods and Results

3.1 Methodology and patients

This study is consisting of thirty-five of rheumatoid arthritis patients (30 females and 5 males). The selection of RA patients was made haphazardly, in department of rheumatology and rehabilitation outpatient clinics of Gaziantep education and research hospital. Thirty individuals with matched ages of patients were served as the control group (19 females and 11 males). The study was free of the patients who suffer diabetes mellitus even those who are using the drugs that can leave impact on nutrition state of individuals.

The protocol of study and its design was approved by ethical committee of Gaziantep University, all the subjects were aware of the study objectives and an informed consent of each subjects was obtained in accordance to that in Declaration of Helsinki. To be compatible with diagnosing measurements of rheumatoid arthritis classification criteria of the 1987 American College Rheumatology (ACR) all patients were above 18 years old [4].

Important diagnostic measurements of rheumatoid arthritis patients were taken into the consideration such as clinical examination, laboratory, radiological assessments and fully history of all patients. Mathematical calculations of body mass index (BMI) were done at the beginning of the study in accordance to $\text{weight (kg)/height}^2 (\text{m}^2)$ [413].

Overall examination of detection of both articular and extra-articular involvement were done and as well as both tender joint counts and swollen joint counts were scored, disease activity was calculated with disease activity scores of 28 joints (DAS28) [314]. Routine laboratory data were determined in all subjects such as ESR, CRP, Hb, and both PLT and ALT counts.

Serum leptin levels were determined by using leptin enzyme-linked immunosorbent assay kit (ELISA kit), that principally based on double-antibody sandwich technique to detect human serum leptin. The leaflet was followed strictly. Serum samples were centrifuged at 2000G for 10 minutes and stored at -80 C°.

Leptin was added to monoclonal antibody enzyme wells which pre-coated with human leptin monoclonal. To reaction taken place, the well left in incubation process for 60 minutes at 37 C°; then it was conjugated with horse-radish peroxidase (HRP) to form immune complex; then incubated for 10 minutes at same temperature degree and washed automatically with automatic washing machine to remove any uncombined enzyme. Later chromogens were added then incubated for 10 minutes at 37 C°; the solution color was changed into blue. Finally, the reaction was stopped by adding stop solution because of acid, the color was changed into yellow.

At last the optical density measurements were done within 15 minutes after adding stop solution at wavelength of 450 nm. According to standards' and corresponding optical density values, and by using ELISA reader software human serum leptin levels were calculated.

3.2 Used materials and used apparatuses

ELISA kit

Automatic washing machine

Standard enzyme machine

Distilled water

Absorbent paper

Disposable tube for sample dilution

Micro pipettes

Disposable Latex gloves

Goggles

Wash bottle

3.3 Statistics

Statistical analyses were conducted by Statistical Package for the Social Sciences (SPSS), for windows version 17.0 (SPSS, Chicago, IL). Demographic and clinical data were presented as mean $\pm$ standard deviation (SD). Different among patients and controls were calculated by t-test for continuous data. Statistical significance was set at $P < 0.05$.

3.4 Results

The current study was conducted on 35 adult RA patients (30 females and 5 males) with the age ranges from 23 to 77 years with a mean $\pm$ SD of 52.91 ± 14.38 years (Table 3.1). Disease duration range of the patients was from 9 months to 25 years with a mean $\pm$ SD of 8.08 ± 5.18 . Meanwhile 30 adult persons (19 females and 11 males) served as a control group with the age ranges from 19.00 - 68.00 years with a mean $\pm$ SD of 45.60 ± 12.75 years (Table 3.3).

In our study, we found no mentionable significant statistic correlation of age, sex and BMI of both groups (P values > 0.05). DAS-28 score was calculated as DAS28-ESR ranged from 2.10 to 6.10 with a mean $\pm$ SD 3.79 ± 1.19 . Noteworthy, tender joint count was found ranges from 2 to 28 with a mean $\pm$ SD 12.0 ± 7.0 and also swollen joint count was found ranges from 2 to 26 with a mean $\pm$ SD 5.99 ± 6.20 (Table 3.1).

Six patients were found in remission with a range of 2.10 - 2.40 and DAS28 mean $\pm$ SD is 2.30 ± 0.12 and serum Leptin levels were ranged from 10.10 (ng/ml) to 143.75 (ng/ml) with a mean $\pm$ SD of 47.68 ± 58.54 (ng/mL), eight of RA patients were found in low disease activity with a range of 2.70 - 3.20 and DAS28 mean $\pm$ SD is 2.90 ± 0.17 and serum Leptin levels were ranged from 7.24 (ng/ml) to 53.19 (ng/ml) with a mean $\pm$ SD of 20.64 ± 15.17 (ng/mL), eleven RA patients were found in moderate disease activity ranged 3.30 - 4.70 (DAS28 mean $\pm$ SD 3.95 ± 0.44) and Leptin Range and Leptin Mean $\pm$ SD were 2.88 (ng/ml) - 24.22 (ng/mL), 12.34 ± 6.55 (ng/ml) respectively, Sever disease activity was scored in eight RA patients and it was found DAS28 ranges from 5.20 to 6.10 with a mean $\pm$ SD of 5.56 ± 0.36

and range and Mean $\pm$ SD of Leptin levels were as 7.99 - 143.90 (ng/mL), 40.14 $\pm$ 45.30 (ng/mL). It is interested to say that in current study there was no correlation between DAS28 and leptin levels and correlation was significant at the 0.947 level (Table 3.6).

Rheumatoid factor (RF) results are explained in (Table 3.5) and the correlation was found significant at 0.01, 25 RA patients out of 35 were having positive RF with leptin levels range from 2.88 (ng/ml) to 143.90 (ng/ml) and leptin Mean $\pm$ SD was 33.97 $\pm$ 38.73, and 10 out of 35 patients were having negative RF with range 6.41 (ng/ml) - 10.10 (ng/ml) and mean $\pm$ SD of leptin levels 8.36 $\pm$ 1.09 (ng/ml). correlation between RF and leptin levels was significant at P value = 0.01.

In regard of BMI of RA patients, it was little bit higher than that in controls as a mean but there was no significant correlation between both study group (P value was 0.45), also we uncovered that BMI in RA patients correlated to serum Leptin levels by (P value 0.01). No correlation was found between control BMI and serum leptin levels (P value was 0.540).

Serum Leptin level in RA patients ranged 2.88 (ng/ml) to 143.90 (ng/ml). A mean $\pm$ SD of Serum Leptin level in RA patients 26.65 $\pm$ 34.59 (ng/ml). Serum Leptin level in controls ranged 0.24 (ng/ml) to 56.33 (ng/ml). A mean $\pm$ SD of Serum Leptin level in controls 13.57 $\pm$ 11.84 (ng/ml). The correlation between the study groups was found statistically significant at (P value 0.01).

Table 3.1 Clinical data of RA patients

Clinical data	Range	Mean $\pm$ SD
Age (years)	23 -77	52.91 $\pm$ 14.38
Disease duration (years)	0.9 - 25.00	8.08 $\pm$ 5.18
Tender joint count	2-28	12.0 $\pm$ 7.0
Swollen joint count	2-26	5.99 $\pm$ 6.20
Body mass index (Kg/m ²)	22.2 – 32.39	27.97 $\pm$ 1.92

Table 3.2 Laboratory data of RA patients

Laboratory data	Range	Mean $\pm$ SD
ESR (mm/hr.)	2.00 - 106.00	29.78 $\pm$ 22.89
CRP (g/dl)	0.45 - 68.24	8.87 $\pm$ 13.01
RF	5.80 - 544.50	95.35 $\pm$ 129.90
WBC (count/mm ³)	4.18 - 127.00	40.16 $\pm$ 189.12
ALT (u/dl)	7.00 - 101.00	23.02 $\pm$ 21.32
PLT (count/mm ³)	175.00 - 391.00	289.40 $\pm$ 52.62
Hb (g/dl)	6.10 - 15.70	12.62 $\pm$ 1.97
Creatinine (mg/dl)	0.38 - 1.41	0.75 $\pm$ 0.21
Leptin (ng/ml)	2.88 - 143.90	26.65 $\pm$ 34.59

Table 3.3 Clinical data of controls

Clinical data	Range	Mean $\pm$ SD
Age (years)	19.00 - 68.00	45.60 $\pm$ 12.75
Body mass index (Kg/m ²)	23.40 - 30.10	26.19 $\pm$ 1.48

Table 3.4 Laboratory data of controls

Laboratory data	Range	Mean $\pm$ SD
ESR (mm/hr.)	2.00 - 63.00	19.95 $\pm$ 16.47
CRP (g/dl)	0.29 - 25.04	3.61 $\pm$ 4.79
WBC (count/mm ³)	5.13 - 12.29	7.76 $\pm$ 1.69
ALT (u/dl)	8.00 - 99.00	28.96 $\pm$ 18.43
PLT (count/mm ³)	162.00 - 491.00	297.23 $\pm$ 73.03
Hb (g/dl)	7.80 - 17.10	13.84 $\pm$ 2.12
Creatinine (mg/dl)	0.51 - 3.52	0.89 $\pm$ 0.67
Leptin (ng/mL)	0.24 - 56.33	13.57 $\pm$ 11.84

Table 3.5 RF results of RA patients and leptin serum levels

RF	RA patients no.	Percentage	Range	leptin Mean $\pm$ SD
Positive	25	71.429 %	2.88 - 143.90	33.97 $\pm$ 38.73
Negative	10	28.571 %	6.41 - 10.10	8.36 $\pm$ 1.09

Table 3.6 Disease activity score comparison with serum leptin level in RA patients

DAS2	DAS28 Range	DAS28 Mean $\pm$ SD	Leptin Range	Leptin Mean $\pm$ SD
Remission	2.10 - 2.40	2.30 $\pm$ 0.12	10.10 - 143.75	47.68 $\pm$ 58.54
Low	2.70 - 3.20	2.90 $\pm$ 0.17	7.24 - 53.19	20.64 $\pm$ 15.17
Moderate	3.30 - 4.70	3.95 $\pm$ 0.44	2.88 - 24.22	12.34 $\pm$ 6.55
High	5.20 - 6.10	5.56 $\pm$ 0.36	7.99 - 143.90	40.148 $\pm$ 45.30

Table 3.7 Comparison serum leptin levels of RA patients and controls

Study Groups	Range	Mean $\pm$ SD
RA patients	2.88 - 143.90	26.65 $\pm$ 34.59
Control	0.24 - 56.33	13.57 $\pm$ 11.84

CHAPTER 4

Discussion and Conclusion

Rheumatoid arthritis (RA) is a form of arthritis that brings about pain, swelling, stiffness and loss of function in the joints. It can affect any joint but is commonly seen in fingers and wrist [241].

This form of arthritis is classified as an autoimmune disease due to the arthritis comes from own immune system and attacking the body tissues mistakenly. It is not clear enough the causes that result rheumatoid arthritis (RA), environmental, genes and hormones might contribute in its etiology [7].

Leptin hormone discovery has completely changed the traditional view of white adipose tissue (WAT) from triglyceride storage with a passive or null endocrine system role to one of the main endocrine organs with active and relevant role in regulating in energy homeostasis, metabolism, in addition to immune-inflammatory process [18]. So, the role of leptin has become a subject of various studies in human rheumatic diseases [405].

Several studies found that Th1/Th2 balance considered to show a significant function in RA, with Th1 and Th2 cytokines exerting pro-inflammatory and anti-inflammatory effects [415]

It was observed that Leptin likely plays a crucial role in RA pathogenesis, fasting has led to promote both clinical and biological features in RA patient's due to the leptin levels markedly suppress and disease activity is reduced because of the Th1/Th2 balance shifts toward Th₂ cytokines production [416].

While it was reported that leptin may affect human inflammatory mechanisms of arthritis throughout Th₁ responses induction [348]. Leptin can trigger monocytes/macrophage cells and pushes the secretion of pro-inflammatory cytokines such as TNF- α and IL-6 [417].

The connection between leptin and inflammation has been reported in several inflammatory diseases, in which, direct and indirect effects of leptin have been shown. Obesity is related with metabolic disorders, such as insulin resistance, and non- alcoholic [418] Steatosis and steatohepatitis [419].

It is believed that the elevated leptin levels interlinked with leptin resistance may participate to fat accumulation in the liver, through a reduced hepatic oxidation and an increased synthesis of free fatty acids that lead to liver inflammation and fibrosis through lipid accumulation and peroxidation that trigger the production of reactive oxygen species (ROS). However, type 2 diabetes is the most significant metabolic disorder associated with obesity and their prevalence is increasing in parallel worldwide [420].

Leptin may be a pro-inflammatory factor contributing to insulin resistance. On the other hand, leptin may also release insulin-sensitizing effects, and may also regulate beta cell mass and survival. In any case, leptin administration has not any effect on body weight reduction, probably due to the development of central leptin resistance or tolerance in obese patients with already very high circulating leptin levels [421-423].

Obesity is linked with an increased cardiovascular mortality and morbidity. The pathophysiology of obesity is in part mediated by a state of chronic, low-grade inflammation [424]. Obesity is almost always characterized by elevated plasma leptin levels, reflecting the increase in fat mass as well as the leptin resistance at central level. Moreover, even though more than 30 adipokines may have a role in the adipo-vascular axis, leptin is one of the more studied links between obesity and cardiovascular disease [425].

Leptin is considered as a mediator in many autoimmune diseases, the prevalence of autoimmune diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS), and T1D, is increased in affluent countries and associates with serum leptin levels [426].

Serum leptin levels are higher in RA patients with high disease activity, correlate well with disease activity, and decrease significantly when disease is well controlled [427]. High leptin levels are also related with a higher prevalence of other immune diseases, such as systemic lupus erythematosus (SLE) and with increased susceptibility to the development of osteoarthritis (OA) [428,429]. Recently, leptin has been found to promote SLE by increasing autoantibody production and inhibiting immune regulation [430,431]. And increased leptin expression has also been reported in Behcet's disease, psoriasis, thyroiditis and during the acute phase of ulcerative colitis [432,433].

Role of leptin as an immune modulator and pro-inflammatory cytokines was investigated in several studies. However, the exact biological mechanism of the relationship of leptin and RA still not fully clear yet. Contradictory results were obtained in regard of leptin role and its contribution in RA pathogenesis also some controversial clinical results were reported regarding circulating leptin levels and disease activity.

The main goal of current study was to investigate the serum leptin levels in RA patients and its correlation to disease activity. In our study, RA patient's serum leptin levels mean was higher than that in controls and it was compatible with some other studies [304,317,390,401,405, 434-440]. On contrary, our study of serum leptin levels mean is markedly opposing some others [303, 384, 396, 397, 406, 407].

Recent Studies reported no significant difference between serum leptin levels in RA patients and control group [398,441], Tokarczyk-Knapik et al. demonstrated contrary result which mean of serum leptin concentration in patients with RA was lower than in the control group [406].

In our study, the DAS results were like several studies that reported no significant correlation between RA disease activity and serum leptin level and in our current study P value was 0.947 [384, 396, 397, 398, 401, 405, 441, 439, 441]. But reports found significant correlation between DAS28 and serum leptin levels [303, 390, 398, 400,442].

In our study, serum leptin levels were correlated significantly to BMI at P value of 0.01 and this result is like many studies [397, 405, 435, 438,]. In contrary reports showed no correlation between BMI and serum leptin levels in RA patients [397, 398, 441].

In conclusion, RA is multi-system disease with joints inflammation then can cause chronic pain, disability and boost mortality. Although many aspects linked between leptin role in inflammation and immune system but fully understandable mechanism remains unclear.

Regulation of leptin is intricate and additional studies are needed to elucidate if leptin is a real actor or a simple mediator in the inflammation of these autoimmune diseases. Moreover, the elevated circulating leptin levels in autoimmune diseases may take part to the low-grade inflammation that increases the risk of cardiovascular disease in these patients

Serum leptin levels were detected to be significantly higher in RA patients than control group. This elevation of levels might upregulate joint damage even, there was no significant correlation between serum leptin levels and disease activity measurements. This shows why leptin levels measurement cannot be proper biomarker to monitor RA inflammation, but Serum leptin levels were positively linked to BMI in patients with RA.

We recommend further reports on leptin's role in immune system in term of metabolic state. More studies are needed to clarify leptin's role in RA and its role in RA pathogenesis.

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