Science Faculty, Charles University, Prague

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DIPLOMA THESIS

The Expression of Novel Cytokines in Rheumatoid Arthritis.

Vyjádření nových cytokinů v Revmatoidní artritida.

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Declaration:

I declare that I worked and wrote this Diploma Thesis personally, only by using cited literatures, under the leadership of the Head of our Department.

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Benjamin Yadollahi

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I would like to acknowledge my appreciation of the nice colleagues who helped me in throughout the process of the work involved in my diploma thesis. Doc. MUDr. Ladislav Šenolt, PhD, Head of our laboratory, gave me useful and comprehensive advice and research strategies in distinguishing suitable signals in research progress. The other colleagues who helped me in different fields were Mrs.Alena Kozáková in preparing tissue and cell cultures and Eng. Hana Hulejova for helping in cytometric measurements.

Abstract

A large number of cytokines are expressed in the feet and hands joints of patients with Rheumatoid arthritis. It is necessary to study the new cytokines which may help in prognosis and diagnosis of this autoimmune disease. Omentin1 and Interleukin 20 are the new cytokines and their expressions may have a role in the expression of proinflammatory cytokines; IL-1, IL-6 and TNFα, in different cell tissues such as synovial fibroblasts, chondrocytes, peripheral blood mononuclear cells and, sera. It is conceivable that these biomarkers may be used in biological therapies.

Key words: Rheumatoid diseases, Cytokines,proinflammatory, Omentin1, Interleukin 21, Interleukin1, Interleukin 6, Tumor necorsis factor- joint damages.

1. Content

2. INTRODUCTION

Rheumatoid Arthritis (RA) is categorized as an inflammatory disease which damages to some parts of joints tissues that are in a line with synovium. The affection symmetrically appears in both hand and feet. (NICE Clinical Guideline 2009)

Evidence shows the abnormalities in components of the immune system which come with an inflammatory reaction: damage to affected tissues and the joints are hot and red, increases in layers of synovial membrane, pain and finally loss of muscles around the joint that decreases the joint function. (MURPHY 2011)

In addition, if the inflammation of this membrane is not suppressed, it releases the enzymes that degrade proteins from the inflamed cells, damaging the bone and the cartilage tissues related to the joints. (MURPHY 2011)

A group of secreted polypeptides identified as Cytokines are the mediators in the inflammation process and are divided according to whether the inflammation is acute or chronic. The profile of cytokines in every process of inflammation is different. The systematic reactions to inflammation, the cellular activation mood and the array of cellular infiltration are mediated and determined by cytokines. Most of them are multifunctional. They are engaged in wide networks with synergistic and antagonistic interactions with both negative and positive effects on target cells. (FEGHALI 1997)

In identifying and separating, prognosis and diagnosis of the diseases and also finding and following its progress, the biomarkers including cytokines have very important roles especially in some chronic diseases like diabetic nephropathy, Osteoporosis (OA) and Rheumatoid Arthritis (RA) (which have the greates number of affected patients worldwide).(HILL 2008)

The aim of my diploma thesis is the study and examination of new cytokines as biomarkers in rheumatoid diseases. It have mainly worked on Rheumatoid arthritis in comparison to Osteoarthritis. This research has been aimed to work and find the molecular biologic markers which are assigned as biomarkers that have prognostic and/or diagnostic roles in inflammatory disease, one of them being Rheumatoid Arthritis. Inflammation is the common specification between oncogenic and inflammatory diseases. So the findings in one can open a window for the other. (HILL 2008)

The samples were removed from patients affected by Rheumatoid Arthritis and Osteoarthritis and are commercial ones. Included were Fibroblasts isolated from the synovial membranes, Peripheral Blood Mononuclear cells (PBMCs), sera and Chondrocytes (commercial).

This work is a part of the grant.....

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3. REVIEW OF LITERATURES

3.1. Rheumatoid diseases

Rheumatoid Arthritis first appeared in the 1850s; its classification was developed over the next 50 years. The main attention on Rheumatoid arthritis started in the beginning of the 20th century. The findings about this disease indicate and illustrate this autoimmune disorder as a complicated long-term disease with extra-articular traits and complexity in treatment. (Scott D.L. et al., 2010)

Rheumatoid arthritis, as a chronic inflammatory disorder, shows joint swelling and tenderness that affects the cartilage and bone of joints (small and middle-sized bones). Destruction of synovial joints results in vigorous disablement and premature mortality. Some other organs may be engaged, such as the vascular system, lungs and larger joints. (FIRESTEIN 2003, BEACKLUND 2006)

During the progress of the disease, inflammatory cells invade the synovium, which in turn results in cartilage damage, bone and other tissue erosions and finally inability in the function of affected joints. Other side effects can be atherosclerosis and lymphoma development. On average, Rheumatoid arthritis reduces life expectancy by seven years, and creates the need for long-term medication; the possibility of psychological disorders because of invalidity and social disability are the next sequences. (FIRESTEIN 2003, BEACKLUND 2006, GREGRO2004)

The clinical appearance of Rheumatoid arthritis usually shows an overlapping with other Rheumatoid diseases such as MCTD (Mixed Connective Tissue Disease), and it may appear with autoimmune diseases, including Hashimoto thyroiditis and or Sjörgen syndrome. (BEACKLUND 2006, GREGRO2004, HILL 2008)

The course of the disease shows different profiles. Cases are between ranges of mild to severe depending on the amount of destruction. There are many causes and effects which are unknown till now. Thus, it is necessary to apply different therapeutic strategies to the subgroups of the disease. The data from the analysis of Genetic risk factors suggests autoantibody reflexes.

This autoimmune disease is disputable in immunological aspects. A disorder in the immune system that results in attacks to self-body cells and its reasons are unknown. When the disease begins, it may be a long time before the beginning of clinical symptoms, with gradual increases of C-reactive protein levels.

RA is a disorder with multifactor causes; genetic and environmental factors, between different ethnic groups, and alternative, clinical, radiological and laboratory detections. So it seems the most studied factors include autoantibodies, hormones and genetic alternations, influence on Rheumatoid arthritis development.

The attendance of ACPA (Anti-Citrullinated Protein Antibody) and RF (Rheumatoid Factor) as autoantibodies in clinical tests of Rheumatoid arthritis put it in the group of autoimmune diseases. Radiography and other visualising techniques show the structural changes, but the only clear determined difference between RA and other Arthritic diseases is the joint damage which may not be distinguishable in the early stage of disease. (GARWAL 2009, HYRICH 2005)

The usage of disease modifying anti-rheumatic drugs (DMARD), mainly methotrexate, and applying some biological agents such as tumor necrosis factor (TNF) inhibitors and other new ones have caused a dramatic revolution in the development of RA management. (VIEIRA 2011, BUKHARI 2003). However it should be noted that using corticosteroids and disease modifying anti-rheumatic drugs (DMARDs) are applied without complete conceptions of the mechanisms of their action. (VIEIRA 2011)

TNF repels inflammation in the synovium and joint destruction by inducing interactions between macrophage, hymphocytes (T and B cells) and SLFs (Synovial- like fibroblasts).

There are proinflammatory cytokines such as interleukin 1(IL-1) and interleukin 6 (IL-6) whose their blockages show different profiles in every related disease. In addition, it has been clearly illustrated that early treatment, which depends on on-time prognosis or diagnosis, amends the clinical outcomes and diminishes the joint damage and disability. There is not any way to properly diagnose in the early stages, thus it is not possible to establish a valid criteria for classification of patients according to the early stages of this disease (ALETAHA 2010).

There was a classification criterion that has roots in RA defining by American College of Rheumatology (ACR). It is used to divide the individuals into groups with or without Rheumatoid Arthritis. However if one considers the patients with a combination of other rheumatologic diagnosis, this criterion shows its disability and limits. (FIRESTEIN 2003)

Characterization of RA is assigned with chronic synovial inflammatory; leukocytes combat against the synovial membrane by inducing cytokines.(KADIOGLU 1998) It is definite that between autoimmune diseases, RA is a chronic one which has an imbalance in pro- and anti-inflammatory cytokines and stimulates the excitation of autoimmunity inflammation and joint destruction.(THALHAMER 2008) Preventing joint destruction and the reduction of synovial inflammation are the main goal in s Rheumatoid Arthritis therapy. (VIEIRA 2011)

3.1.1. Clinical symptoms

Rheumatoid arthritis is defined as tenderness, swelling joints and stiffness, along with synovial tissue destruction, hyperaemia (feeling warmth around joints) and pain at night and early morning. These are the common physical symptoms and the signs that define Rheumatoid Arthritis (RA) as a chronic inflammatory disorder. These symptoms are finally followed by severe disability and premature mortality. The synovio-macrophages and fibroblast-like synoviocytes are the important source of inflammatory mediators. It is included as an autoimmune disease as it means the immune system targets the individual body cells. (ALETAHA 2010, LORENZ 2012, STANICH 2009)

The clinical presentation in diagnosis of RA does not show a similar diagram in affected patients. Diarthrodial joints are affected in chronic symmetric condition. Symmetrical pain and swelling in hands and feet joints, and also knees, commonly appear by different inception in individuals. At the beginning, RA can be marked by systematic symptoms such as fatigue, fever, and weight loss and then shows other classic symptoms in the following weeks and/or months. In some patients it can begin on one side of the body (Monoarthritis) and then progress to other side. Subcutaneous granulomatis lesions, called rheumatoid nodules, are the other symptoms shown by some patients. Parenchymal lung disease, secondary Sjögren's syndrome, cutaneous vasculitis and pericartidis are also extra-articular manifestations of Rheumatoid Arthritis.(MCINNES 2011,STANICH 2009)The characteristics of Rheumatoid arthritis have been indicated and defined in a multifactorial way by Assil et al.2008.(Table 1)

Characteristic of Rheumatoid Arthritis	
Clinical	Female more than male (2 or 3 times more), Morning stiffness (more than 1hour), fatigue, fever.
Laboratory	Rheumatoid factor (RF), Anti-CCP antibodies, inflammatory markers including ESR and CRP level.
Radiographic	Erosions, periarticular osteopenia, joint space loss.

Musculoskeletal	Symmetrical, polyarticular, joint involvement, metacarpophalangeal, Proximal, Interphalangeal joints, metatasophalangeal, and wrists. Affects cervical spine, Spares thoracolumbar spine.
Extra-articular	Cardiovascular disease (atherosclerosis), Rheumatoid nodules, taneousvasculitis, Ophthalmic irtis, pulmonary fibrosis and nodules, renal manifestations (AA amyloidiosis), Sicca symptoms (dry eyes and mouth), hematologic (anaemia of chronic disease and Felty'ssyndrome),Osteoporosis.

Table 1: Characteristic of Rheumatoid Arthritis (ASSIL 2008)

An article written by Scott et al. (2010) illustrated assessments of RA as it progresses as shown below. (Table 2)

Panel: **Assessments in rheumatoid arthritis**

Disease activity

Core assessments

- Joint counts (tender and swollen joint counts)
- Global assessment (doctor and patient) and pain score
- Laboratory (erythrocyte sedimentation rate and C-reactive protein)
- Disability (eg, health assessment questionnaire)

Additional assessment

- Fatigue
- Radiological damage

Combined status indices

- Disease activity score
- Simple disease activity score
- Clinical disease activity score

Change in status (trials only)

- ACR20, ACR50, and ACR70 responders

Extra-articular disease

- Nodules
- Pulmonary
  - Pulmonary nodules
  - Pleural effusion
  - Fibrosing alveolitis
- Ocular
  - Keratoconjunctivitis sicca
  - Episcleritis
  - Scleritis
- Vasculitis
  - Nail fold
  - Systemic
- Cardiac
  - Pericarditis
  - Pericardial effusion
  - Valvular heart disease
  - Conduction defects
- Neurological
  - Nerve entrapment
  - Cervical myelopathy
  - Peripheral neuropathy
  - Mononeuritis multiplex
- Cutaneous
  - Palmar erythema
  - Pyoderma gangrenosum
  - Vasculitic rashes
  - Leg ulceration
- Amyloidosis

Comorbidities*

Cardiovascular

- Myocardial infarction
- Heart failure
- Stroke
- Peripheral vascular disease
- Hypertension

Cancer

- Lymphoma and lymphoproliferative diseases
- Lung cancer
- Skin cancer

Infection

- General
- Bacterial

Other

- Depression
- Gastrointestinal disease
- Osteoporosis
- Psoriasis
- Renal disease

ACR20, ACR50, and ACR70=20%, 50%, and 70% improvements in five of the seven measures of American College of Rheumatology criteria. *Some comorbidities are mainly associated with rheumatoid arthritis (eg, cardiovascular), some with treatment (eg, gastrointestinal disease), and some with both disease and treatment (eg, infection).

Table 2: Clinical assessments in Rheumatoid arthritis(Scott L. D.,et al.2010)

3.1.2. Epidemiology

Nearly 0.5% to 1% of the European and North American populations are affected by Rheumatoid Arthritis. Comparisons of prevalence are shown in table 3, illustrating noticeable regional differences.

Region	**No. of cases per ten thousands**	**Estimate of annual incidence case per million**
Southern European countries	3.3	16.5
Northern European countries	5.0	29
North America	10.7	38
China , as an Asian country	2.8 - 3.5	-

Table 3: The RA prevalence in different regions.

The differences in age groups, strains and regions were investigated and shown to affect the prevalence of the disease. It appears in the population in the age range of 40-70 years, but it can happen in earlier ages (juniors). Furthermore women show a higher amount of

effect, times more than do men. It was estimated that risk of recurrence for siblings is 4%, for first and second degree relatives are respectively around 4.7% and 1.9%. The severity of disease shows a 15% recurrence risk for first degree relatives in comparison with mildly affected groups. (STANICH 2009, KWAN-MORLEY 2007, KVIEN 2009?)

3.1.3 Pathogenesis

The etiology of Rheumatoid Arthritis is nearly unclear and unknown. Pathogenesis starts with inflammation in synoviums in the joints of the hands and feet. In healthy joints, the thin synovial membrane is composed of two layers of cell type's lines, one synovio-macrophage (with the ability of antigen producing) and the second is fibroblast-like synoviocytes (which produce synovial fluids and its own matrix). Decreasing apoptosis and cell proliferation in this layer results in inflammation and swelling (hyperplasia), leading ultimately to Rheumatoid Arthritis. (STANDICH 2009)

Rheumatoid arthritis (RA), as a chronic disease, commonly involves with disability, obstacle and impairment. The average duration of the Disease Repercussion Profile (DRP) is 11 years. Disease activity, the state and quality of therapy, and disease duration are the factors that are important in the improvement of RA treatment. (SHARPE 2001)

Genetics, environmental and immunological factors are involved in its genesis. In normal conditions without infection, regulatory processes restrain immunity responses to antigen. (NAKKEN 2012)

The importance of prognosis and diagnosis of Rheumatoid Arthritis is the reasoning behind numerous researches in this field. (EBERHARDT 1998)Some recent data indicates that in the diagnosis of RA versus other types of arthritis, inhibition of synovial leukocyte apoptosis in early phases of the disease pathogenesis may be enough help. (STANICH 2009)

Neutrophilic inflammation is a character for a prototype of this disease. The amount of neutrophils is high in the synovial fluid of RA patients. (OTTONELLO 2002)

Cytokines

One of the low molecular weight peptides (produced by different cells connected to immunity system, including lymphocytes macrophages, monocytes, eosinophils, mast cells and blood vessel endothelial cells) are cytokines. Pleiotropism and redundancy respectively define the multiple functions of each cytokine in the cell, and depending on the target (Figure 1), different cytokines can show similar biologic function. (CARP 2004)

Increases in the production of cytokines are associated with RA disease. There is a positive correlation between the disease activation and the number of macrophage, interleukin-6 and tumour necrosis factor-α expression in the synovial tissues.

Cytokines as soluble factors have mediatory roles in inflammation, defined as a tissue response to injury. Inflammatory cytokines are divided to two groups according the state of inflammation, i.e. acute and chronic ones, but some are included in both groups.

(FEGHALI 1997) Inflammatory cytokines such as IL-1β, TNFα destroy the tissue structure in RA by inducing the release of tissue-damaging enzymes. (KANEKO 2001)

The important and fundamental roles of this large group of cytokines have shown their effects on inflammation and articular destruction. At present there is a comprehensive analysis on the function of different cytokines in Rheumatoid Arthritis as a chronic inflammatory disease. Another important role of cytokines is involved in differentiation, maturation and activation of cells with important effects on immune-inflammatory responses. Final effect of cytokines is tissue destruction because of deviation in immune regulation and local inflammatory processes. (ARINGER 2005, BRENNAN 2008)

New technologies provide a quick and easier way profile the cytokines in Rheumatoid diseases. The measurement can be done by judging these two areas: protein level (antibody array, bead-based multiplexing) and message level (cDNA and oligoneucleotide micro-arrays). Tissue supernatant, synovial fluids, sera and biopsy tissue are the sources of the comprehensive profile. (BRENNAN 2008)

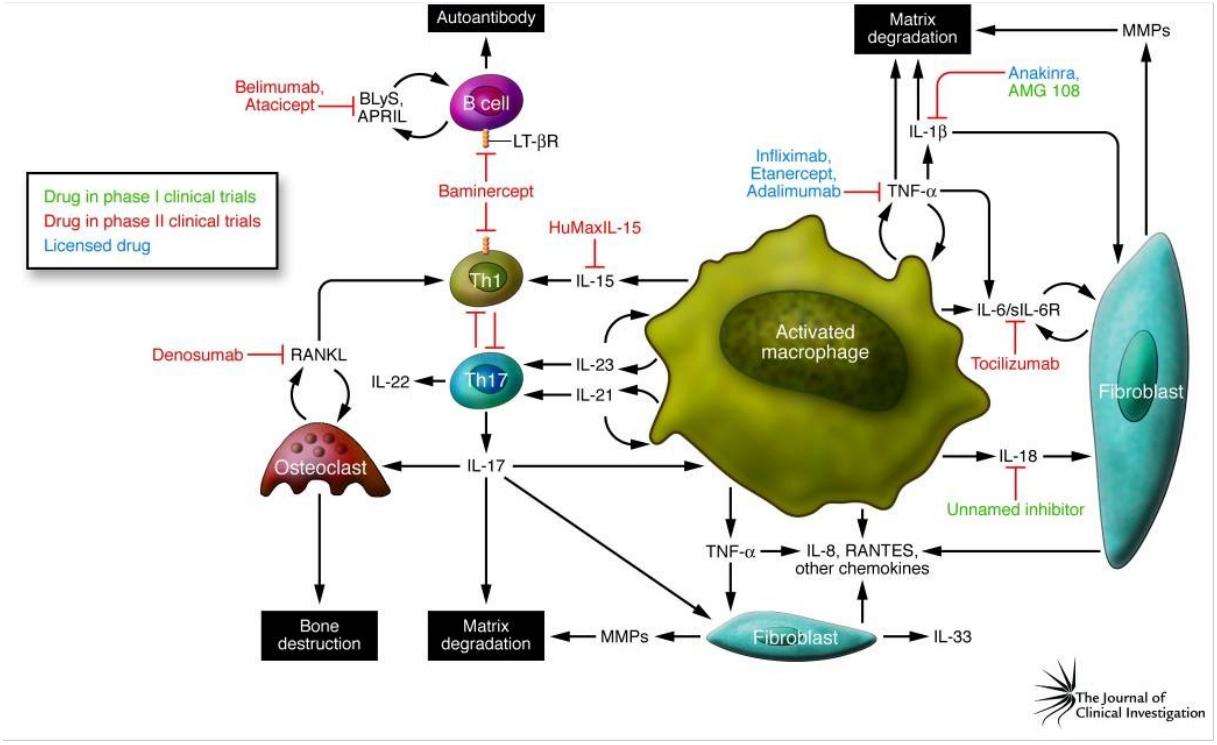

Figure 1: Cytokine targets in RA (BRENNAN 2008)

Because of importance of cytokines in the regulation of the immune and the inflammatory response, especial interests were concentrated on the cytokines in autoimmunity diseases such as Rheumatoid arthritis (RA). The cytokines are produced not only by the cells which are related to the immune system but also have been found in synoviocytes, fibroblasts and even epithelial cells. (STEINER 1999)

In 1997, Carol A. Faghali and his colleague classified the known cytokines according to the inflammation state. (Figure 2)

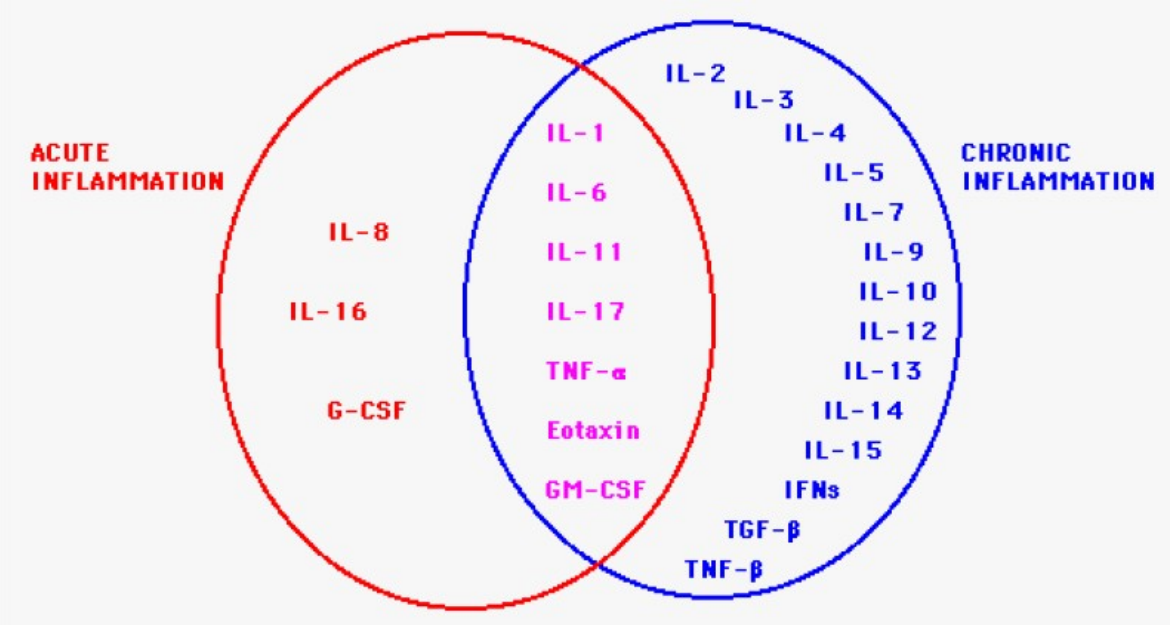

Figure 2: Cytokines involved in acute and chronic inflammation (FEGHALI 1997)

-Interleukin-1

One of the inflammatory cytokine is IL-1, which its cDNA was cloned in 1984. There are two kinds of this cytokine, IL-1α and IL-1β (with 20-25% of homology in amino acids), that are coded by two different genes on the human chromosome 2 with the size range of 22-31 kDa.

The sources of this interleukin are mononuclear phagocytes, fibroblasts, keratinocytes and the lymphocytes T and B. It causes fever by increasing in the level of prostaglandin E_2 (PGE_2). (FEGHALI 1997),

-Interleukin-6

IL-6 is a glycoprotein with 21 to 28 kDa size, and its gene is on the chromosome 7. It inhibits the production of TNF-α, and limits the acute inflammatory reaction by negative feedback. It also acts as a growth factor in the maturation of B cells and is involved in T cell differentiation and activation. When it up-regulates in Rheumatoid arthritis (OGATA 2011), it means that there is a chronic inflammatory. This up-regulation has been reported in different autoimmune diseases such as systemic sclerosis (FEGHALI 1992), renal cell carcinoma and leukaemia. (HIRANO 1992)

-Tumor necrosis factor

Another couple of cytokines are TNF-α and TNF-β with respectively 17 and 25 kDa sizes. These cytokines have a few more biological functions bound to common receptors on the target cell's surface. Their position lie within the MHC region of Chromosome 6 and is produced by fibroblasts, mast cells, macrophages/monocytes, some T cells and NK cells. It can induce fever like IL-1. It shares its inflammatory properties with both IL-1 and IL-6. (Figure 3) (FEGHALI 1997)

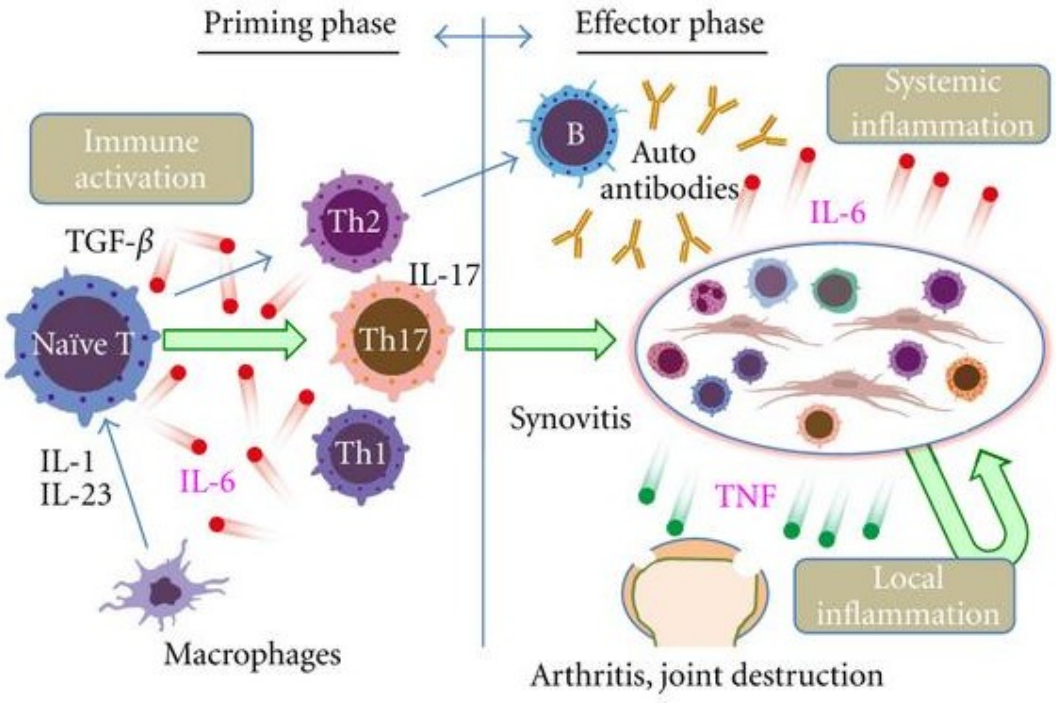

Figure 3. The role of inflammatory cytokines. (OGATA 2011)

3.1.4. Individual risk factors of RA genesis

Genetics, environmental factors (Figure 4) and chance compose the causal reasons of Rheumatoid Arthritis occurrence in affected patients. (MCINNES 2011) According to

main articles, it is observed in the correlation and association of ACPA positive RA phenotype with environmental risk factors. The gene- environment interaction is specially visible and perceivable on the shared epitope of HLA gene and some non-genetic factors.

Different risk factors were investigated in published papers, and these factors can be divided in two groups, including genetic and non-genetic or environmental factors. (LIAO 2009)

-Genetic factors

The comparison of monozygote (identical) and dizygot (non-identical) twins was the base of evaluation of the familial risk which is influenced by inheritable genetic factors and environmental ones. Also this evaluation indicates that by separating the identical twins, it was clear that there may be differences in X- chromosome inactivation, which in turn indicates the probability of alternation genetic factors.

The most important identified genetic factor in RA is the human leucocyte antigen (HLA) class II. There is a correlation between the alleles of HLA-DRB1 and RA susceptibility, and also the severity of RA. The common amino acid sequences between these alleles (QKRAA, QRRAA or RRRAA, shared epitope) are shared at the position 70-74 of the DRβ1 chain.

The main genetic works done on RA in the recent years (2006-2007) resulted in whole-genome identification of this gene. The newly recognized genetic factors related to RA are C1854T SNP in the PTPN22 gene and A49G SNP in the CLTA4 gene (small-size markers). Both of these genes influence T-Cell activation. (BALDING 2006, CHRISTENSEN 2007, BIJLSMA 2009**).**

A joint set of alleles of the Human Leukocyte Antigen (HLA)-DRB1 gene is the main genetic risk factor amongst the RA patient population, common in both Caucasian and

Asian ancestry. Many other genes have been studied, including PTPN22, PADI4, SLC22A4 and FCRL3 and have been shown to have different outbreaks in these two ethnic groups. This means that the probability of expression of PTPN22 in Caucasian populations and PADI4, SLC22A4, FCRL3 in Asian ones is significant to other groups. The heterogeneous susceptibility of RA has been observed in both groups. A similar or equal degree of RA risk has been found in STAT4 haplotype in these groups. (LEE 2009)

In the study on twins, monozygotic and dizygotic ones indicate 15 to 30% and 5%, respectively. HLA –DRB1 locus is the distinguished and proven region in patients with RF and ACPA positive. The shared epitope with QKRAA amino acid motif in HLA-DRB1 evolves the special predisposition. (MCINNES 2011)

Mutation in tumor suppressor genes P53 and expression of stress proteins as HSP70 (Heat-Shock Protein 70) develop fibroblast-like synovial and gives control of the function of ER (Endoplasmic Reticulum) to an E3 ubiquitin ligase (synoviolin), the balance of proliferation –apoptosis equilibrium. P53 expression is negatively adjusted by synoviolin and also by ligation of TNFα receptors. Other important factors considered in recent years have been the roles of methylation and acetylation regulating genes in cell cycle and the expression of microRNA. (McInnes L.B. 2011)

-Environmental factors

The main and well-investigated environmental risk factor in RA is tobacco smoking. There is a positive correlation between tobacco smoking and the RF positive and anti- CCP

positive. The association between RA risk and other factors such as diet, alcohol, and caffeine intake and body mass index are very weak or not proven. Also no evidence has been found to show the role of any bacteria. (LEE 2009)It is hypothesized that sex hormones or reproductive factors can play an important role in the RA formation because of higher (two to three times) prevalence of RA in female than male patients.

Here some environmental factors are reviewed and are mainly studied to know their effects or relations with Rheumatoid Arthritis.

.Tobacco smoking

The important and known environmental risk factor for RA is smoking, as proven by ACPA tests. There is a significant correlation between tobacco smoking and ACPA positive in RA patients. There is a linear relationship between smoking and the risk of RA. The doses of cigarettes per day and the years of smoking have been considered. (MCINNES 2011) an increasing rate of lung cancer in RA patients has been observed and is an explanation of this association between Rheumatoid Arthritis and smoking.

Many articles have illustrated the declining effect on TNF production via smoking induction. In one research it has been indicated that smoking significantly decreases TNFα production after LPS (Lipopolysacharide) stimulation. (BAKA 2009)

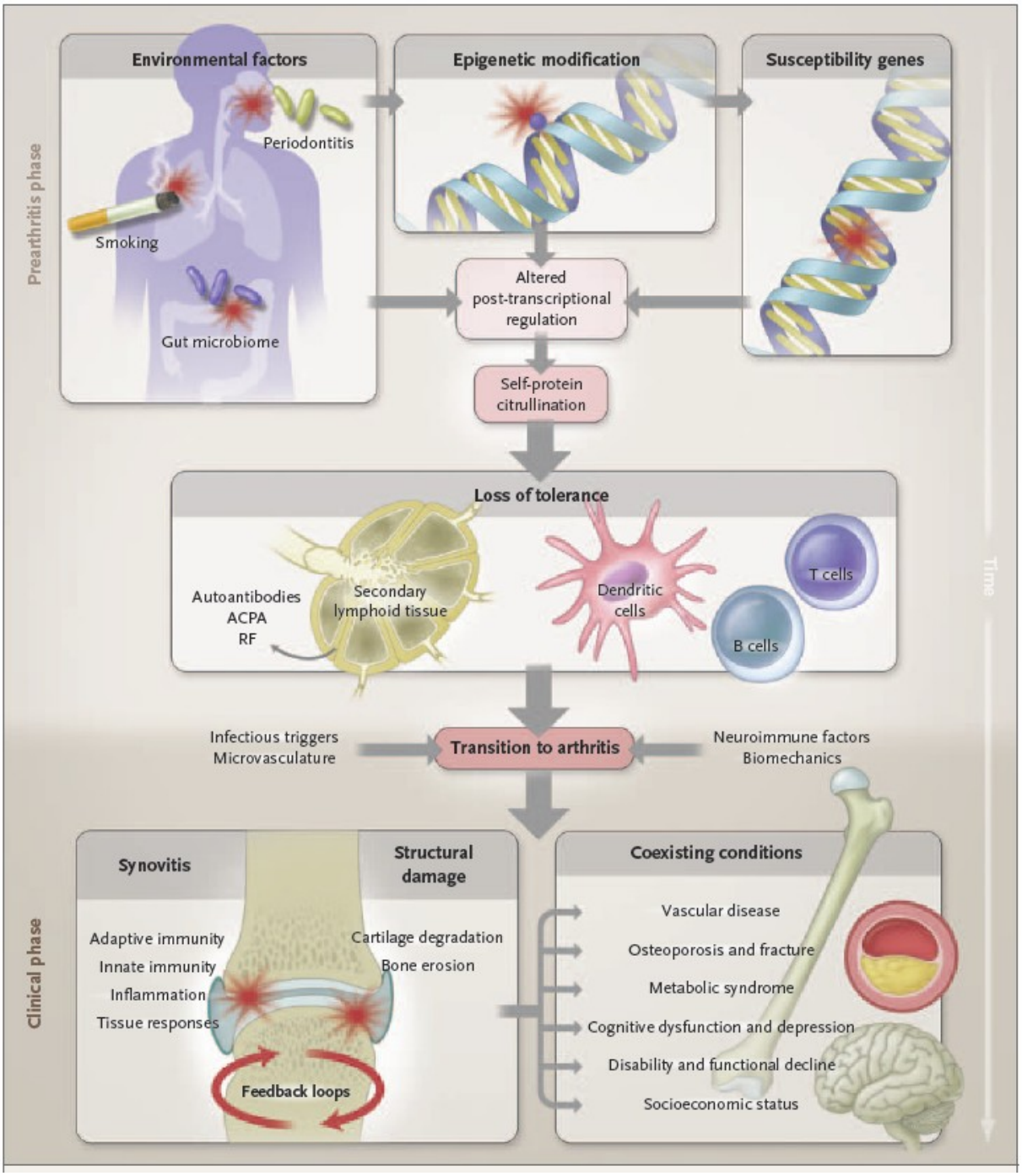

Figure 4: Gene-Environment interactions effect. The process which is completely not known and clear as it follows by diagram, it shows which tissues, cells and organs have a role in affection to Rheumatoid arthritis. (McInnes L.B.2011)

.Alcohol

According to a Danish study, comparison the RA patients who drink alcohol and those who do not drink found that alcohol may decrease the risk of RA, especially in ACPA positive RA ones. (LIAO 2009).

In other research, the protective effect of alcohol consumption has been distinguished significantly, and it is assumed that alcohol may play the protective role in some systemic inflammation. **(**WOUDE 2012**)**

Bing Lu and his colleague in their study in alcohol consumption of RA patients indicated that the high consumption of alcohol results in depressing the immune system and increases the risk of affection to bacterial diseases, but low to moderate drinking of alcohol has a protective effect on some diseases related to RA, such as chronic heart disease. (LU 2010)

.Vitamin D

Vitamin D, an essential hormone for bone and mineral homeostasis, has been hypothesized to have a role in RA risk. It is involved in both innate and adaptive immune systems via VDR (Vitamin D receptor) as a suppressor in pro-inflammatory reactions. It has a significant role in other autoimmune diseases like type1 diabetes and multiple sclerosis. (LIAO 2009) Vitamin D intake is reversely associated with the risk of RA. It has been shown that the higher intake doses of Vitamin D decreases the RA risk in older women. (MERLINO 2004)

3.1.5. Classification and diagnosis of RA

The designed early classification criteria distinguish Rheumatoid arthritis from other joint diseases. ACR and EULAR have established a new classification chart which has combined two older classification criteria. The three phases are included; patients with early arthritis and the identification of factors such as weights in the first step, second phase indicated refining the factors and at last phase summarising all data to arrive in a prediction mode and pluralisation(Figure 5).(SCOTT, 2010)

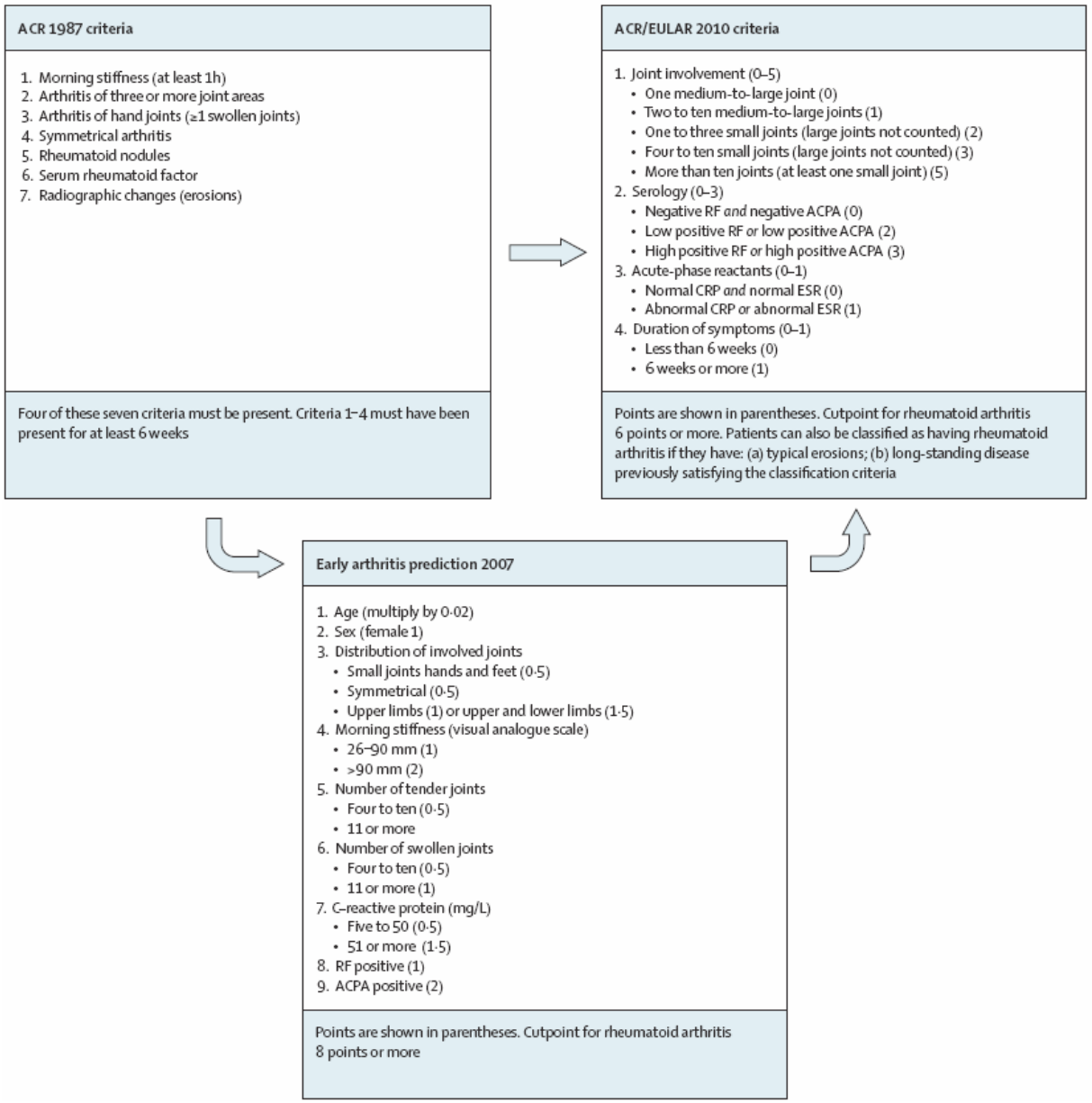

Figure 5: conventional and new classification criteria for rheumatoid arthritis (Scott D.L., 2010)

3.2. Cell culture

3.2.1 Basic guidelines for working in cell and tissue culture

The first and most important point in the work in a laboratory of cell and tissue culture is the hygienic instructions to protect all the laboratory facilities and materials from contamination.

3.2.2. Work environment

It should be very calm, clean and with low personnel traffic. The laboratory should be used only for work in this field. The facilities and materials have to be placed correctly to give suitable accessibility to everything that is necessary during the work with cell and tissue.

-Personal hygiene

Every person who is working in the laboratory has to put on clean working clothes, wash their hands before and after work and use hygienic gloves. If he/she has been infected by a microorganism, they are forbidden to work in the laboratory while they have the disease.

-Materials and equipments

Every laboratory is equipped with the necessary instruments and materials that are used daily and should be kept clean and disinfected. Routinely, every few days it is necessary to disinfect the places including chairs, tables and instruments such as refrigerators, freezers, incubators, centrifuges and so on. The incubators have to disinfect before and after working with them.

-Work technique

There are one-time-use materials including pipettes which should be used according to their instructions. The chemical and biological materials should be labelled completely so that all personnel are able to find and use them easily. The date of the beginning of use should be written on the label. If anything is added to the stock material or if it is diluted, it should be cited. Every person has his/her own material in some cases to decrease the risk of contamination.

-Work in laminar box

Work with the laminar box has its own importance. The UV light should be switched on before and after every time work is done in the box for 20 minutes. It is necessary to turn the ventilation system on for a minimum of 15 minutes before beginning any work under the box. In addition, suitable disinfectant (70% alcohol, 70% benzinalcohol, incidur) should be used to disinfect all the empty spaces in the box and everything used in the box. Once work is completed the laminar box should be cleaned again and everything should be returned to its rightful place.

3.2.3. Contamination

Contamination can happen at any time with only a small mistake. Bacteria, molds, yeasts and mycoplasma are the microbial contamination agents in the laboratory. All the routine aseptic instructions should be observed to prevent any contamination, and if it happens, the contamination should be removed as soon as possible. Contaminated cultures have to be liquidated, and if there is possibility to save some cell lines, they have to be treated by antibiotics such as kanamycin, gentamycin and other strong ones. However work with this kind of cell lines often has a risk factor.

3.2.4. Preparing water, solutions and media for tissue culture

Water

The water used in work with media or dilution of biochemical materials should be selected and provided as appropriate. Water without organic and an-organic substances is suitable and should be autoclaved for 30 minutes in 120°C.

Preparing solutions

There are a few solutions that are used in cell and tissue culture continuously.

PBS

PBS solution is used in different steps of cell and tissue culture. Before using trypsin, PBS is added to the cell culture to rinse grown cells. In preparing the PBS, one must consider the pH value (between 7.3 to 7.5), and the final solution needs to be autoclaved for 30 minutes in 120°C and 1 atm.

Trypsin

One important solution in cell passage is Trypsin, which is applied to separate the cell masses from each other and also from the solid substrate. The employee can order and use sterile one withor without EDTA (EthylenDiaminTetraAcid) in the appropriate concentration (Trypsin-EDTA, Gibco, distributor KRD, Prague). The solutions are prepared in lower amounts, 10ml to 15 ml, and stored in – 20°C.

Media

Different media are applied for culturing the cells and tissues from storing ones in the freezer.

Culturing media

In cell and tissue culturing, DHLBECCO'S MEM NUT MIX F-12 (Gibco, distributor KRD, Prague) medium is used in the laboratory. Preparing the medium depends on the amount of fetal bovine serum (FCB and BOFES, which must be inactivated in 56°C for 30 minutes and shaken every 10 minutes) according to the cell growth conditions. For example, the medium used for Fibroblasts have 10% FCS, HeLa cells (transformed cancer cells) and only needs 5% FCS. Antibiotics are also added to the medium (to keep the cell lines out of any bacterial contamination), L-glutimin (essential aminoacid), HEPES (buffer in stabilizing pH in the solution), and fungicide (to protect the cells against molds).

Freezing media

The suitable media used for storing the cell lines is a mix of FCS and DMSO.

3.2.5. Culture of the adhering cells

Some cells do not grow if there is not a solid substrate to adhere it. The substrate can be glass or plastic material. The cells grow in a thermostat incubator at 37°C and in 5% CO2. As they start to grow, they will cover all the surface of the substrate (90-100% confluence), and the grown cell must then be changed and divided in different substrates (passage). Depending on the kind of cell, this is done 1 to 2 times per week. The passage process is done under the laminar box in aseptic conditions. Some necessary solutions need to have the 37°C temperature before adding to medium. Passage process starts with the aspiration of the old medium to waste container, and then one must rinse with PBS and apply Trypsin. The Flask should be kept in incubator for 10 minutes. In these 10 minutes, the cells will disengage, as observable under a microscope. By adding the medium, the effect of Trypsin is blocked. The cells are divided in other new flasks according to necessity. The specification of the cell, the date and the number of passage are written on every flask and they are then returned to the incubator.

3.2.6. Long- time cell storing, freezing

The cell lines are stored in liquid Nitrogen in reverse. The cells which are in logarithmic or top phase are suitable cells for storing. The medium changes a day before freezing. The cells should be checked to ensure that they are healthy and not contaminated.

The cells are aspirated to falcon tubes and rotated in centrifuge 10 000 rpm for 15 minutes. The supernatant is poured and quickly added to the related medium for freezing. The cells are chilled in cryo-tubes (Nunc. Schöeller, Prague) and must be labelled by the kind, number, and the passage number of cell and the date. Every tube can be used for 1 to 1.5ml media. At first the tubes are put in Cryo 1°C freezing container (NALGENE®), then in – 20°C for 20 minutes. The ideal condition for freezing is when the temperature comes down slowly. Finally the cell tubes are transferred to -80°C. If the cells are to be stored for a long time, the tubes must be moved often.

3.2.7. Defrosting the cells

A lukewarm bath is first prepared with 20-30°C water, and then the chilled tubes are transferred quickly into it. After melting, the cells are dispersed in the separate flasks, and the medium is added so as to be ten times more than the volume of cell solution. The medium is changed the next day.

4. Materials and Methods

4.1. Materials

The synovial tissues, fibroblasts which are used to culture, PBMC (Peripheral Blood Mononuclear Cells) and sera are provided from patients with Rheumatoid arthritis (RA), an autoimmune disease and Osteoarthritis (OA). Also the commercial chondrocytes for related tests have been cultured. The kinds of tissues were selected and used according to the findings in related articles to our research field. The new cytokines has been used in other diseases such as oncologic ones. There are different tissues which are engaged in the Rheumatoid Arthritis affection.

4.1.1. Fibroblast

Fibroblasts, cartilage cells and bone cells (Figure 6) are the members of connective-tissue cells that secrete collagenous extracellular matrix. Fibroblasts are scattered throughout the body and secrete type I and type II collagen. The migration of proliferated fibroblasts to the injured part of the body begins with the secretion of collagenous matrix to separate and repair the injured tissue. It shows the easy growth of fibroblast, used as suitable cells in culturing for biological studies. (NCBI)

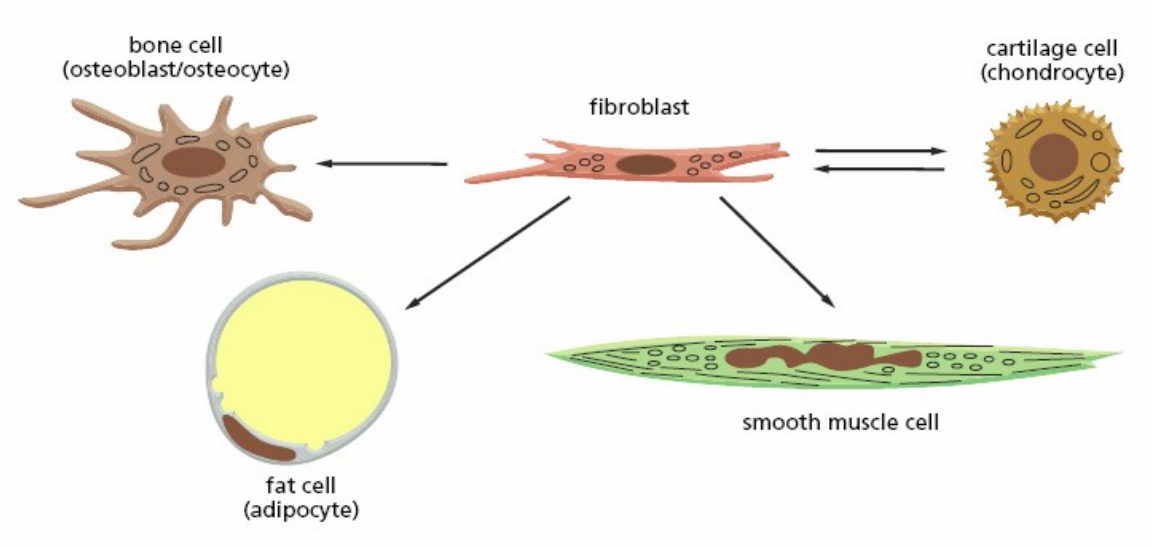

Figure 6: the family of connective tissue. (NCBI)

The fibroblasts (Figure 6) were provided from the RA patients who refer to our institute or other hospitals in the city. They were cultured and used after five or more passages.

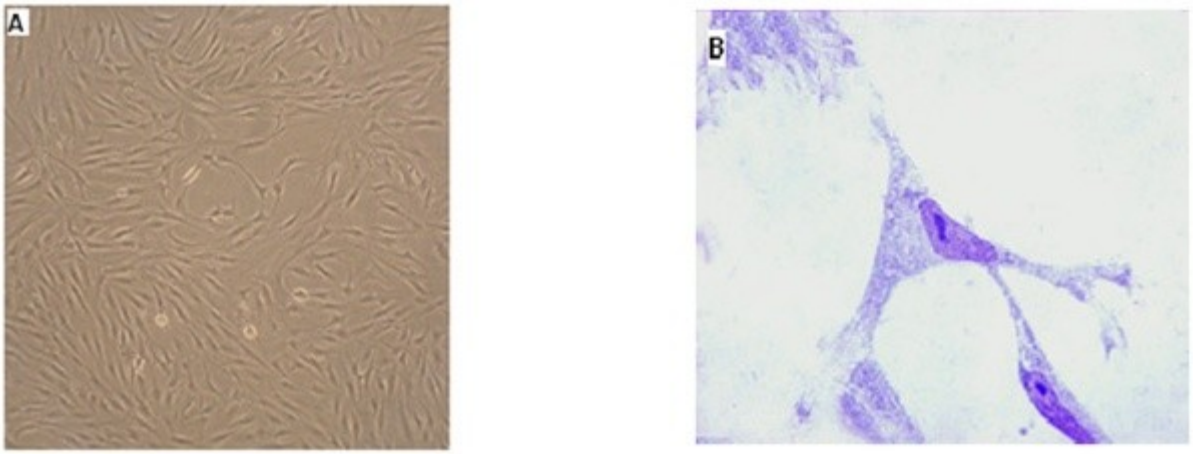

Figure 6: Fibroblast like synovioacytes 10X, the healthy control (A) and a fibroblast cell (B) 100X

4.1.2. Chondrocyte

Chondrocytes (Figure 7) are mature cells found in cartilage. Cartilage cells, or chondrocytes, occupy a small cavity, lacunae, in the matrix (Figure8). Usually there are not any blood vessels in this tissue and their nutrient and gases exchanges are passed through

porous matrix. Hyaline cartilage in the matrix commonly has type II collagen and type I collagen fibres by establishing a dense network appearing in the body regions which bear the mechanic force of weight. This tissue is called fibrocartilage. (JUNQUEIRA 1998, ALBERTS 1989)

For our experiment we provide commercial chondrocytes (Clonetics™ normal human articular chondrocytes, NHAC-kn, Lonza).

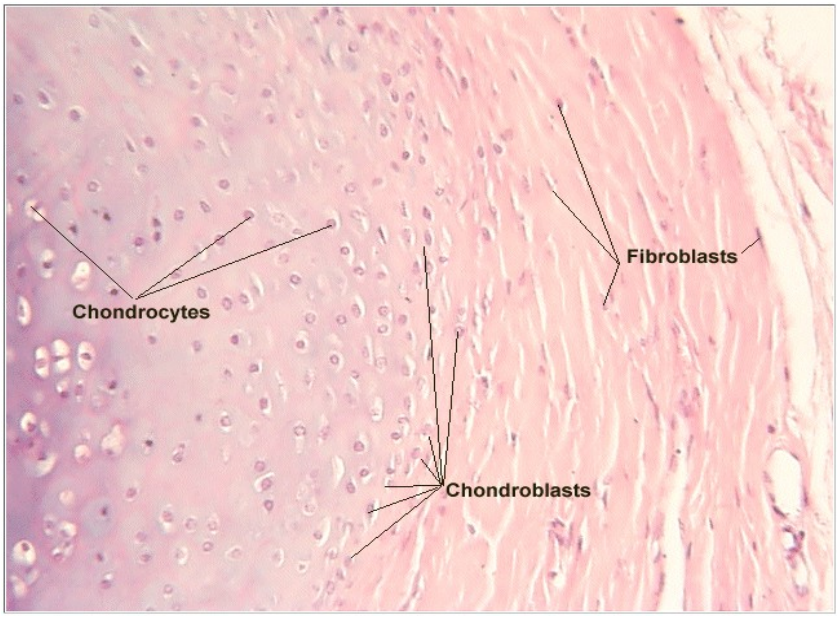

Figure 7: Chondrocytes in cartilage matrix with the mature connective tissue cell.

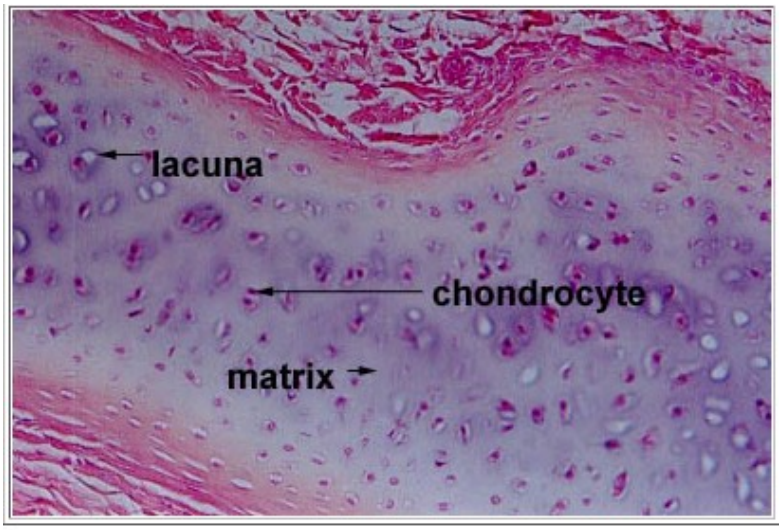

Figure 8: another slide shows chondrocyte along with matrix.

4.1.3. Synovial tissue

At joint Diarthroses of long bones with more mobility like the knee and elbow, there are ligaments and a capsule of connective tissue enclosed by an articular cavity with synovial fluid. The synovial fluid is colourless, transparent and has viscosity. The synovial layer produces a higher dose of hyaluronic acid in comparison with blood plasma. Hyaluronic acid and protein are lubricants in the synovial fluid and also transfer nutrients to joint cartilage surfaces. The capsule has two layers: fibrous, a dense connective tissue, and the synovial layer. The synovial layers are two types, one with fibroblast and the other with macrophage specification. (ALBERTS 1989)

4.1.4. PBMC

In phagocyte system, the monocytes of blood are the precursor of mononuclear cells. PBMCs are included in blood cells with a round nucleus and play a key role in the immunie system. They can be separated from the other blood cells by Ficoll, a hydrophilic

polysaccharide (Fig 9). Peripheral blood mononuclear cells are also used in parts of gene expression studies.

The same experiment was done on another kind of cell, PBMCs. Ten blood samples of patients with RA diagnosis (which were coded by M58 and M59 in the data receipts) were selected and every five samples were mixed and poured in a 50ml tubes. The PBMCs were separated by using the Ficoll. The gathered cells were calculated by a cytometric software and machine. The isolation of PBMCs process from the blood samples was repeated because the amounts of acquired cells were not enough. For every well in the 6-Well plate, it is necessary to have one million live cells. The cell count and related data have been interpolated in the appendix. This experiment was repeated in triplet. The cells were stimulated by proinflammatory cytokines to trace the Omentin-1 expression.

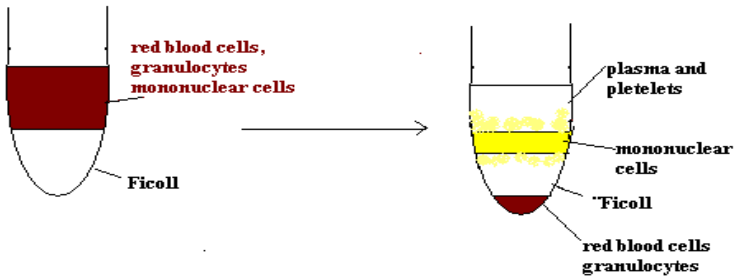

Figure 9: PBMC isolation of Blood samples by Ficoll.

4.1.5. Serum

Blood contains the fluid and cells which are circulated in the veins and vessels. The blood elements are suspended in a liquid part named plasma. One part of a clot is a clear

layer that is called serum. Serum is different from plasma in its lack of fibrinogen. (JUNQUEIRA 1998)

4.1.6. Omentin 1 (Intelectin 1)

Intestinal lactoferrin receptor (Intelectin-1), also called Omentin-1, is coded by the INTL1 gene. (https://en.wikipedia.org/wiki/ITLN1) It is an adipose tissue-derived peptide categorized in adipocytokines (Figure 10). It has an effective role in obesity-related diseases and it can have a significant role in some inflammatory diseases, such as Rheumatoid arthritis. (ŠENOLT 2010)

The human recombinant Omentin-1 (Prospecbio, E.Coli source, Cat.No. CYT-061) was added to selected fibroblast cell lines in different concentrations (10ng/ml, 100ng/ml, and 200ng/ml) to stimulate the cells. Along with this new cytokine, other inflammatory cytokines such as IL-1, IL-6, and TNFα were applied to follow the expression of Omentin-1 under these stimulation factors. There was a positive (LPS stimulated) sample and a negative (only cell lines without adding any cytokine) one. The cell culture was done in a triplet set for statistical reasons.

More than one experiment was done on fibroblast as related to Omentin-1 expression and its relationship with studied and main cytokines.

The first (on fibroblasts) and second (on PBMCs) were done with the determined concentrations (10ηg/µl, 100 ηg/µl and 200 ηg/µl) and the third experiment was done on fibroblasts again with 10ηg/µl, 50ηg/µl, 100ηg/µl concentrations of Omentin-1. In this experiment five RA synovial fibroblast (RASF) samples and one OA synovial fibroblast (OASF) were selected. Six cell lines in double sets were arranged and included the

negative and positive controls. Omentin-1and LPS were separately applied to the cells to stimulate them.

In this experiment Recombinant Human Omentin-1 was used to stimulate chondrocytes. Different concentrations of Omentin-1 were applied to the cells to follow the expression of proinflammatory cytokines, including IL-1β, IL-6 and TNFα.

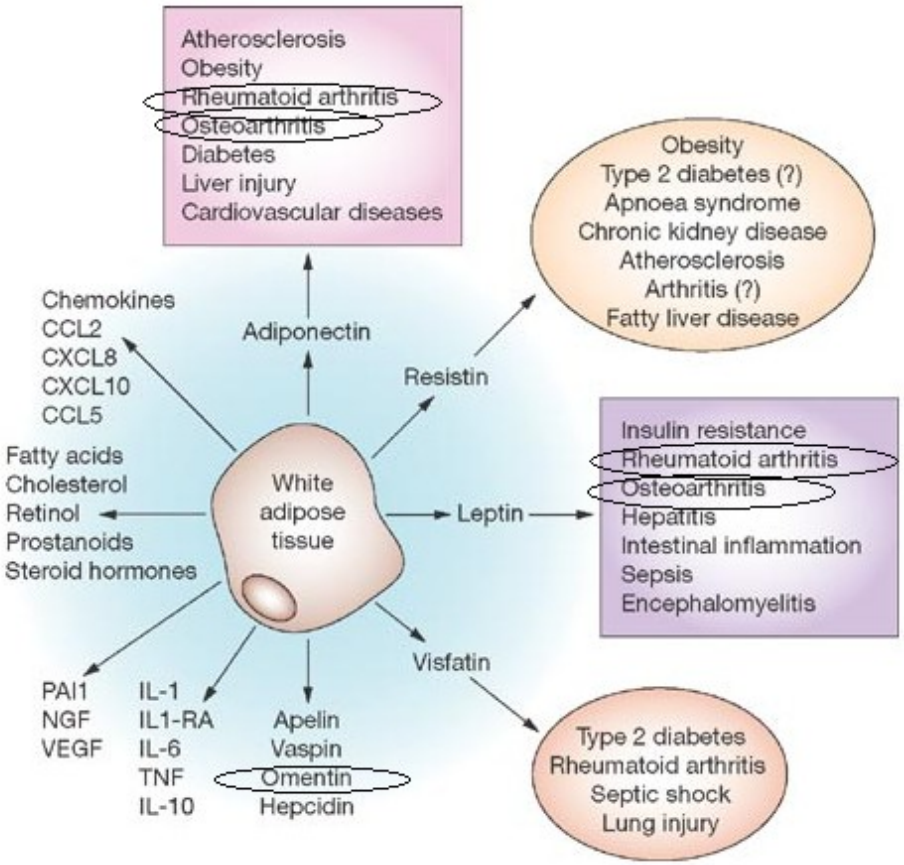

Figure 10 : The multiple functions of Adipose tissue as a source of proinflammatory factors.(LAGO 2007)

4.1.7. Interleukin 20 (IL-20)

One of newest cytokines is Interleukin-20 with pleiotropic inflammatory which belongs to the IL-10 family including IL-19, IL-22, IL-24 and IL-26. IL 20 has two pairs of receptors which are common in this family. (FICKENSCHER 2002, TRITSARIS 2007)

IL-20 is expressed in monocytes, epithelial and endothelial cells. By activation of a few receptor pairs including IL-20R1/IL-20R2 or IL-22R1/IL-20R2 can apply biological functions on different cells. IL-20- induced accumulation of inflammatory cells has been cited as a reason of Rheumatoid Arthritis inception and its progress, an angiogenesis-dependent disorder. (TRITSARIS 2007)

The role of IL-20 antibody has been researched in the therapy of Rheumatoid arthritis. The synthesis of this cytokine is done in synovial fibroblasts (SFs) which have a significant role in RA etiology with other cytokines; IL-1β and TNFα (HSU 2010).In our experiment different concentrations of this cytokine were applied to study the expression of other cytokines such as IL-1β, IL-6 and TNFα.

The human recombinant IL-20 (Life Technologies Company, E.Coli expression, Cat.No.PHC0201) is provided commercially and, according to papers which used expression experiments in cancerous diseases, was designed in the applied concentrations for stimulating different cells.

IL-20, as the second cytokines along with Omentin-1, is used in this research. In the same way it was applied on the chondrocyte cell line, and I tried to distinguish if there was any trace of this interleukin in chondrocytes and then assigned it to other cell and tissue reactions.

From the stock amount of Recombinant Human IL-20 (E. Coli-derived, 17,6 kDa, R&D systems) that we received, 200µg was taken and mixed with 1ml ddH2O to dilute the stock material.

The commercial chondrocytes, which grew in several passages, were cultivated in 6-Well plates to be stimulated by IL-20. The used cell lines passed 6 passages and 10^5cells in every plate's well were needed in every plate's well. The applied concentrations of IL-20 for stimulating the chondrocytes were 100ηg, 500ηg and 1000ηg. The experiment was done in triplet for this cell line for every concentration and also controlled ones.

4.1.8. Polymyxin B

PolymyxinB is commonly used to neutralise the LPS contamination. The proteins expressed in Escherichia coli vectors are besmeared usually with endotoxin. The polymyxin B neutralizes the effect of the LPS that is probably in contaminated recombinant protein. There is a limitation in the capacity of Polymyxin B in blocking these effects, and often it is not considered. When the polymyxin B is added to the culture which is contaminated, the level of cytokines such as TNFα and IL-10 reduce. (CARDOSO 2007)

In one other experiment I tried to find if there was any contamination in the Recombinant Human Omentin-1. Different combinations of stimulators were selected to find any possible contamination of Recombinant Human Omentin-1.The concentration of polymyxin B (Polymyxin Sulfate Salt, SIGMA) used was 10ηg/ml.

4.2. Methods

The following techniques (including molecular biology), were used in this Diploma thesis: cell and tissue cultures, stimulation of the cells of different tissues to trace the expression of studied cytokines, RNA isolation from fibroblasts, chondrocytes and PBMCs, reverse transcriptase to get cDNA, Real-time PCRs (RT-PCR), ELISA and Immunohistochemistry.

4.2.1 Cell and Tissue Cultures

Tissues were provided from the patients' biopsy were cut to small pieces to be cultured in the flasks with suitable medium included. The medium for every kind of cell or tissue was chosen according to the recommendation of provided companies or the articles included in the protocols.

Fibroblasts were cultured in the medium DMEM (5% concentration that should be diluted for use by adding antibiotics and bovine serum).

The chondrocytes were commercial samples, and the recommended media provided by the company were used. The necessary information about the tissue was found according to the received certificate and the recommended medium. The kit used was assigned as CDM Bullet Kit (Cc-3225) and provided by the Lonza Company. Their recommended seeding density was 10,000 cells per cm2 and 1 ml of media per 5 cm2.

The cell cultures were checked every 3 to 4 days and were substituted with a new one if there was a significant change of the medium colour.

4.2.2. Stimulation of the cells by studied molecules

Synovial tissues of patients, chondrocyte commercial sample or PBMCs isolated of RA patients' bloods were calculated to be sure that we had enough number of cells for our experiments. When using the 6-well plate, the correct number of cells which differs for every tissue was added in every well.

After 24 hours the studied cytokines to every cell line were added according to the work plan. It depended on the concentration of stimulators and every cell line was repeated while considering the statistical aspects. The stimulated cells were usually harvested after 6 and 24 hours.

4.2.3. RNA isolation

RNA isolation was done by using MagNA Pure Compact (Roche). In automatic isolation process of RNA, the harvested samples were used from the 6 hours cultures. The volume of samples were 350µl included cells and RTL buffer (Qiagen). The process of isolation is indicated in figure 11.

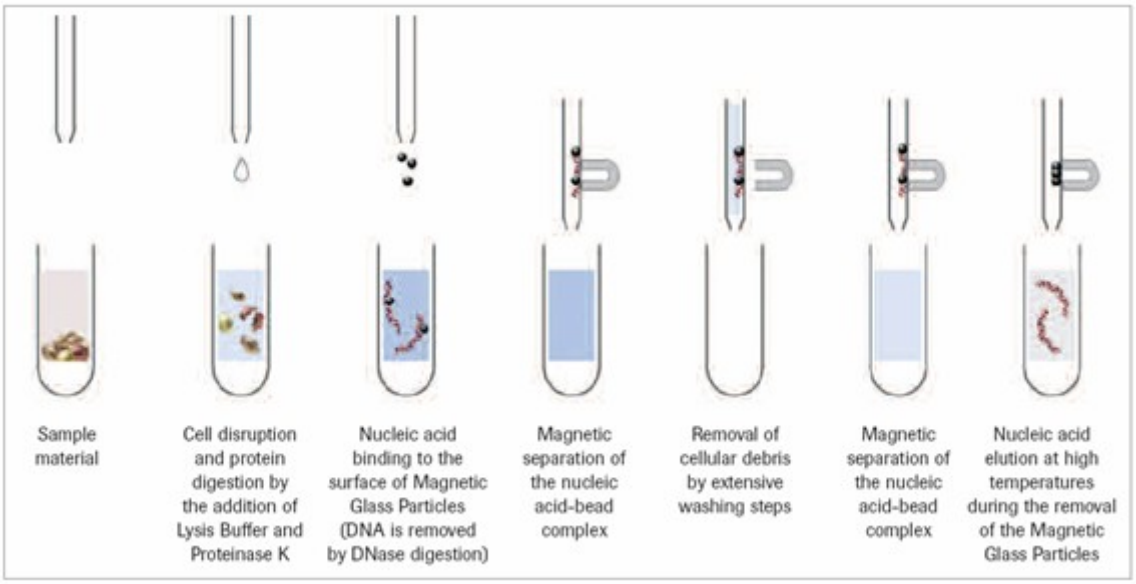

Figure 11: the process of acid nucleic isolation by MagNA Pure Compact machine. (http://www.rochediagnostics.cz/download/la/0105/magna.pdf)

RNA concentration measurement

Exactly after RNA isolation, the RNA concentrations were measured by biophotometer (Eppendorf) according to the work protocol :

1-Add 5 µl of isolated RNA ti 65µl of distilled water (dH2O).

2- Measure the blank at first step.

3- Measure of all samples one by one.

The suitable concentration of RNA is 10µg/ml, and a purity between 1,7-2,1 is acceptable.

4.2.4. Reverse Transcriptase

Reverse transcriptase (RT) was done by The RevertAid™ Premium First Strand cDNA Synthesis Kit (FERMENTAS) in the thermal cycler (BIORAD). The isolated mRNAs were used to get the cDNAs that were then used as templates for the next step, PCR. The reaction was done by RNA dependent- DNA polymerase. All of the processes were completed in a place that was RNase free and on the ice bath. The work protocol was as follows:

1- Write on the microtubes and leave them on ice in the rack.
2- Transcriptase is the inhibitor brings out of freezer before adding to the tubes.
3- 1µl of primers do pipette to every microtubes.
4- Add 11µl of homogenized RNA to related named microtubes.
5- Centrifuge at 3000 rounds per minute, then vortex and again centrifuge at the same speed for a few seconds.
6- At the same time prepare the master mix and include 4µl of buffer 5x conc.,1µl inhibitor and 2µl dNTPs.
7- Add 7µl of master mix to every sample tube.
8- Activate RNase during the 5 minutes incubation of the samples in 37°C.
9- Add 1µl of reverse transcriptase to the samples.
10- Centrifuge at 3000 rounds per minute , vortexand again centrifuge.
11- Transfer the microtubes, including samples, to thermocycler.
12- Check the saved related program and start the process.
13- Store the samples in -80°C.

4.2.5. Quantitative Real Time PCR (qRT-PCR)

Polymerase Chain Reactions (PCR) is a revolutionary method and an alternative for molecular cloning in proliferation of the studied DNA sequences. It is used for cloning, genetic engineering and sequencing. (NCBI)

RT-PCR (called reverse or real time PCR, or in some articles as a quantifying PCR in a real time) can be used in conditions where there is a small amount of sample. In every cycle, the products and their quantity can be detected. The reaction is done in the presence of primers, DNA polymerase and a detectable signal. In our experiments we often used it in gene expression studies. (NCBI)

The stimulated samples were harvested after 6 hrs and 24 hrs. The first group of samples harvested after 6 hours included RNAs that were isolated by the MagNA Pure Compact machine. The isolated RNAs were then processed to get the cDNA. The cDNA of every sample was applied as a template for RT-PCR.

The results taken from the RT-PCR were processed and analyzed by the excel program to calculate the different values including dCt (the difference between the values of 18s gene expression as control and the expression of studied gene), ddCt (relative determination of gene expression in qRT-PCR) and x-fold. The final results of x- fold were used in the GraphPad program (GraphPad Prism 5.00.288, GraphPad Software, Inc.) to draw the graphs according to the x-fold data.

Work protocol:

1- Prepare the probes which have to be traced, premix and cDNA samples.
2- Add 80µl of dH2O (DNase and RNase free, Water PCR-Grade, Bioline) to every 20µl samples of cDNA
3- Prepare the premix including the Master mix, dH2O and probes.
4- Total volume reaction is 25µl that contains 3 µl of cDNA sample and 23 µl of premix (12.5µl Master mix, TaqMan Universal PCR Master Mix (Roche), 1.25µl of probes and finally 8.25µl of dH2O)
5- Prepare the plate; be careful not to touch the plate.
6- Pipette 22µl of premix to every well of plate and add 3µl of the sample to relate well.
7- Wrap by adhesive covers.
8- Shake the plate and centrifuge for a few seconds.
9- Put the plate on the RT-PCR machine and start the related saved program.

4.2.6. Immunohistochemistry

Immunohistochemistry is another protein detecting method that is an alternative for immunofluorscence assay. As usual an antibody is chemically bound to an enzyme, and via this *in situ* reaction, a colourless layer is changed to coloured one. The changed colour regions of substrate can be observed under a light microscope. The studied tissue is fixed by chemical fixation methods and then change colour after adding the antibodies.

(MURPHY 2012)

In this experiment, 5µm frozen sections were fixed by acetone and 4% paraformaldehyde, blocked by 0.3% H2O2. Sections were afterwards incubated in the TBS (Tris Buffered Saline) buffer and incubated with Omentin-1 antibody in a dilution of 1:250 for 1 hour and the, rinsed again in TBS buffer. Antigen-antibody complexes were visualized by Histofine detection system (Nichirei Biosciences Inc.), by using 3, 3´-diaminobensidine as chromogene. Sections were slightly counterstained with Harris´ haematoxylin.

Work protocol:

1. Fix the tissue sample and in and covered with paraffin.
2. Cut the samples into very thin slices.
3. Remove the paraffin by putting the slices in a hot water bath.
4. Capture 3 slices (one of them is control sample) on a clean slide.
5. Deparaffinise slides:

A. Add Xylol I	10 min.
B. Add Xylol II	10 min.
C. Add ethanol absolute I	3-5 min.
D. Add ethanol absolute II	3-5 min.
E. Add ethanol 96%	3-5 min.
F. Add ethanol 80%	3-5 min.
G. Add distilled water	5 min.

6. Pre-treat with Trypsin for 30 minutes.
7. Wash the well in ddH2O for 5 minutes.
8. Wash 2X in buffer1/Tween (PBS) for 5 minutes.

9. Block unspecific binding with 2-5% serum (horse, or goat) in buffer 1 30-40min.

10. Title the slides (do not wash).

11. Incubate primary AB and negative control diluted in buffer 1 at RT for 1 hour (or at 4°C over night).

12. Wash 2X in buffer1/Tween for 5 minutes.

13. Incubate secondary biotinylated AB diluted in buffer 1for 30 minutes.

14. Prepare ABC reagent for alkaline Phosphate in buffer 1

15. Wash in buffer1/ Tween.

16. Incubate with ABC reagent for AP.

17. Wash with TBS.

18. Wash with buffer2.

19. Develop Fast Red, Fast Blue Substrate or any other substrate for alkaline phosphate.

20. Stop colour reaction with ddH2O and rinse well.

21. Cover slides with aqueous mounting medium.

Notes:

- In the case of the frozen slides after the fourth step, the following steps should be taken including: thaw the slides and leave to dry (30 min. to 1 hr.), fixating in 4°C acetone (10min.) and air dry (30min. or longer).

- The primary AB with dilution 1:100.

- The negative control and dilution is (PBS) rabbit IgG 1:4000 (5µg/ml)

- The secondary AB and dilution is according to α rabbit hom. Kit

4.2.7. ELISA (Enzyme-linked Immunosorbent Assay)

An antibody can be bound to an antigen in physiological conditions including salt concentration, pH and temperature and by a covalent.

ELISA is a qualified method in detecting a specific protein in a complex mixture. The detection is done on microplate wells with antibodies related to studied protein. It is commonly used in medical laboratories. There are different types of ELISA, but the basic elements are the same in all of them and are included in capturing antigens in the sample by its fixation in a microplate directly or indirectly, thus blocking unnecessary molecules in the sample by covering all the free sites on the wells. The incubation of the antibody-antigen complex is a part of probing and detection, and finally detection of the signal and measuring it. (www.piercenet.com)

The Omentin-1 ELISA Kit (Enzo, Life Science), a 96 Well Enzyme Immunoassay Kit, is used for measuring *in vitro* the quantity of Omentin-1 in the serum of patients with Rheumatoid Arthritis in comparison with OA samples (Table...).In this experiment, the results were measured and read by Sunrise Tecan microplate reader, and were analyzed by Kim32 program software.

The ELISA test was completed according to the protocol which is in the brochure of every company product.

5. Results

5.1. Omentin1 Stimulation

5.1.1. Immunohistochemical diagnostic method

Tissues belonging to patients affected by RA and OA were provided, and the Omentin-1 antibody was applied according to the immunohistochemical method. The slides were observed under the microscope to find the existence of any reaction and also to compare the reaction of both tissues to the same antibody. The expression of Omentin-1 in RA

samples versus in OA ones is indicated. As observed in the RA pictures with 100X magnifying (Figure 12), we see the cell proliferation in the tissue of this sample in comparison to OA samples. The colour shows the inflammatory cells density. As explained, the synovial fibroblasts are two layers that are easily observable below.

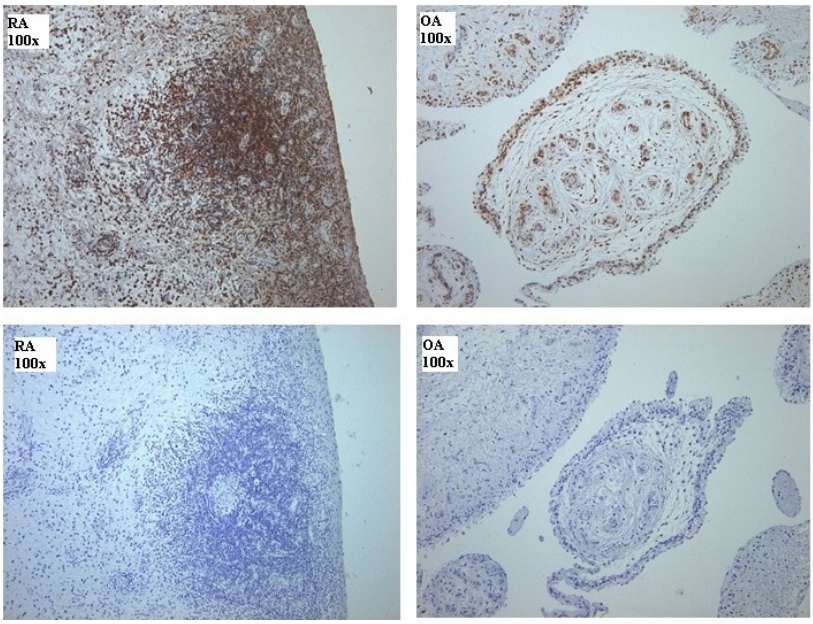

Figure 12: the expression of Omentin-1 in RA fibroblast sample in comparison with an OA one.

5.1.2. Quantitative determination of Human Omenitn1 by using Intelectin-1(Human) ELISA Kit

More of the results of the measurements show low levels and the wells have statistically lower amounts able to be read by the microplate reader machine. It should be noted that the kit of this provider was used for the first time. So the results may not be reliable (Figure 13).

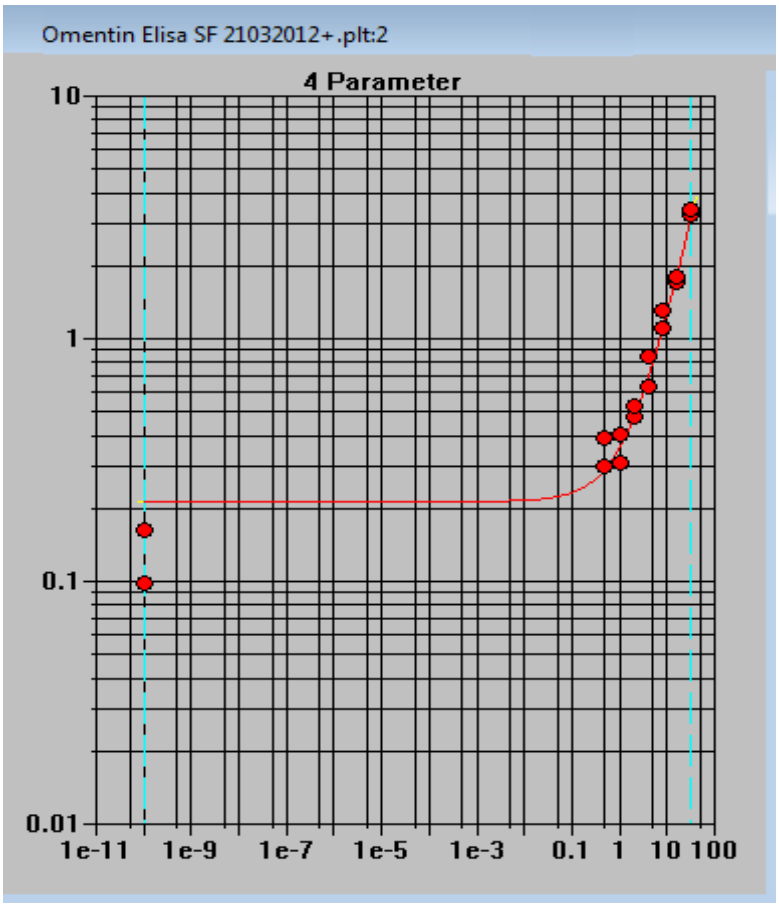

Figure13: Omentin-1 immunoassay result of RA and OA samples(y=(A-D)/(1+(x/C)^B)+D ; A=0.214,B=0.879,C=11157.1, D=527.9)

The complete number of samples and the table of results may be seen in the appendix.

5.1.3. Stimulation of the fibroblast cells by Omentin1

At first experiment, a fibroblast sample of a patient with RA positive was chosen to follow the expression of Omentin-1 in the presence of other cytokines stimulating cells and also the expresses of other cytokines in the cells stimulated by Omentin-1. According to the work plan and the related protocol instructions, the selected cell line was cultured in triplet and then stimulated by different cytokines including Omentin-1, IL-1β, IL-6, TNFα, Matrix Metalloproteinases (MMPs, the proteins which are caused the breakdown of

collagens type I, II and III.) such as MMP1,MMP13 and other factors MCP-1 and RANKL.

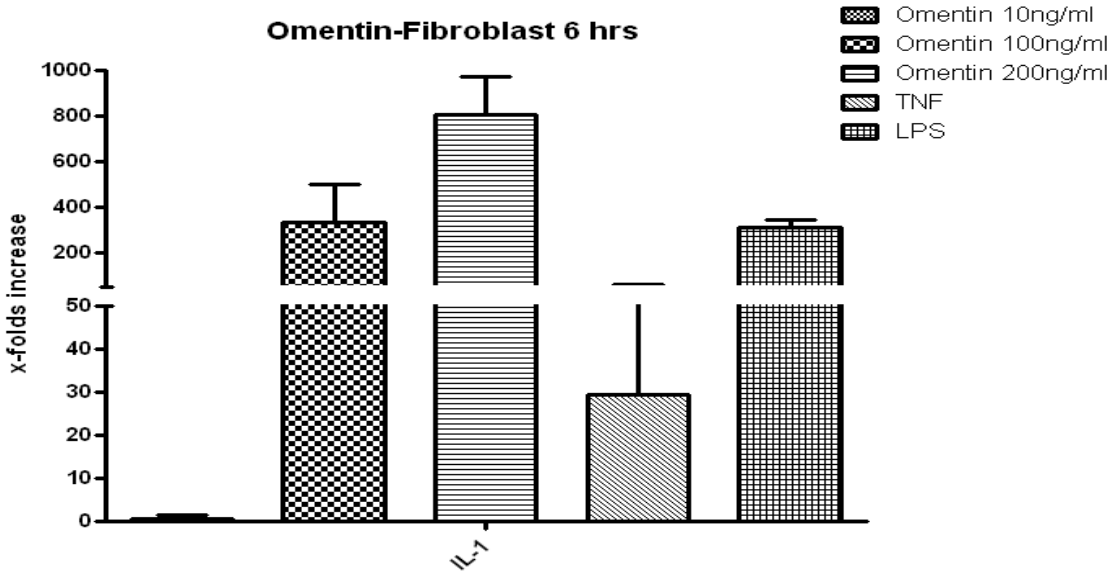

Figure 14: IL-1β induces up-regulation of Omentin-1 mRNA expression in synovial fibroblasts.

The inflammatory cytokines including IL-1β (Figure 14), IL-6, TNFα and RANKL were up regulated with the increase of the Omentin-1 concentration in the fibroblast cells (Figure 15).

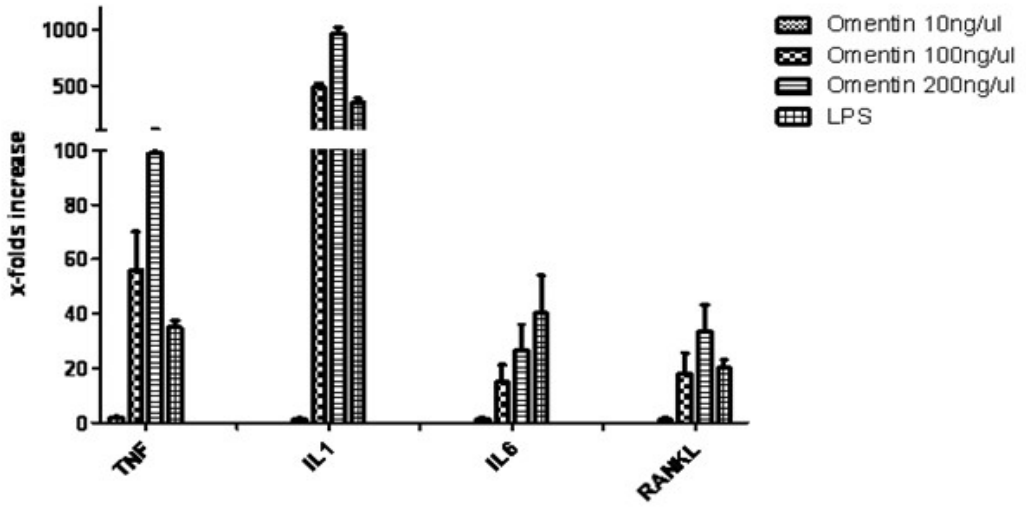

Figure15: the expression of cytokines in the presence of Omentin-1.

In the presence of different concentrations of Omentin-1, the expression of MMPs shows a significant increase in the MMP1. There is not any sensible change in the expression of MP13 and MCP1 (Figure 16).

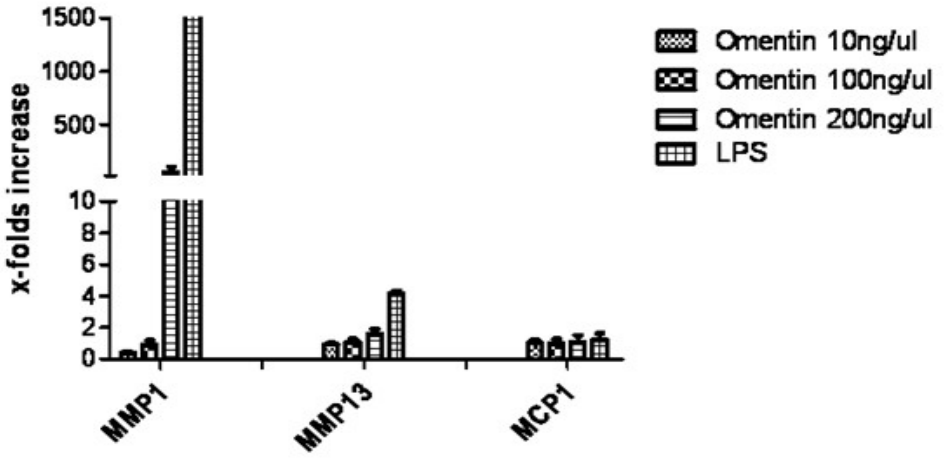

Figure 16: The expression of MMPs and MCP1 in the fibroblasts stimulated by Omentin-1 after 6 hrs.

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The expression of Omentin-1 shows a correlation with the expression of IL-6 and LPS more than TNFα. These results indicate that Omentin-1 increases the expression of pro-inflammatory cytokines in synovial fibroblasts. The effect of this adipocytokine on Matrix Metalloproteinase and RANKL shows interesting results. The positive feedback is observable in the Figure 17.

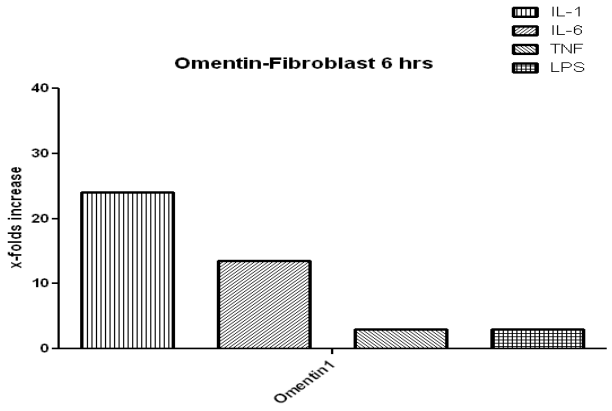

Figure 17: Omentin-1 positive feed back

The results of the work on PBMCs do not meet the expectations indicated in the first experiment. (Figure 18)

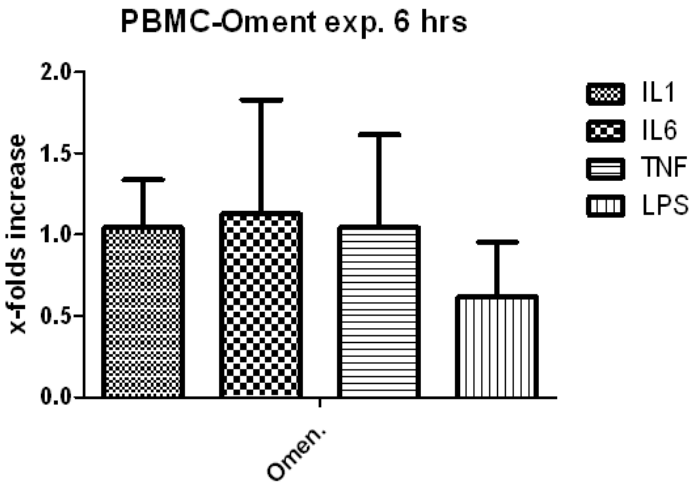

Figure 18: the expression of Omentin-1 in the stimulated cells by proinflammatory cytokines.

The obtained data from this experiment shows differently from the first experiment. As we see in the all the graphs there are differences in different samples. In the first graph we see and trace the expressions of cytokines other than the ones that were used in the first experiment. In the sample RA323 the expression of Omentin-1 is not significant enough to be considered. (Figure 19)

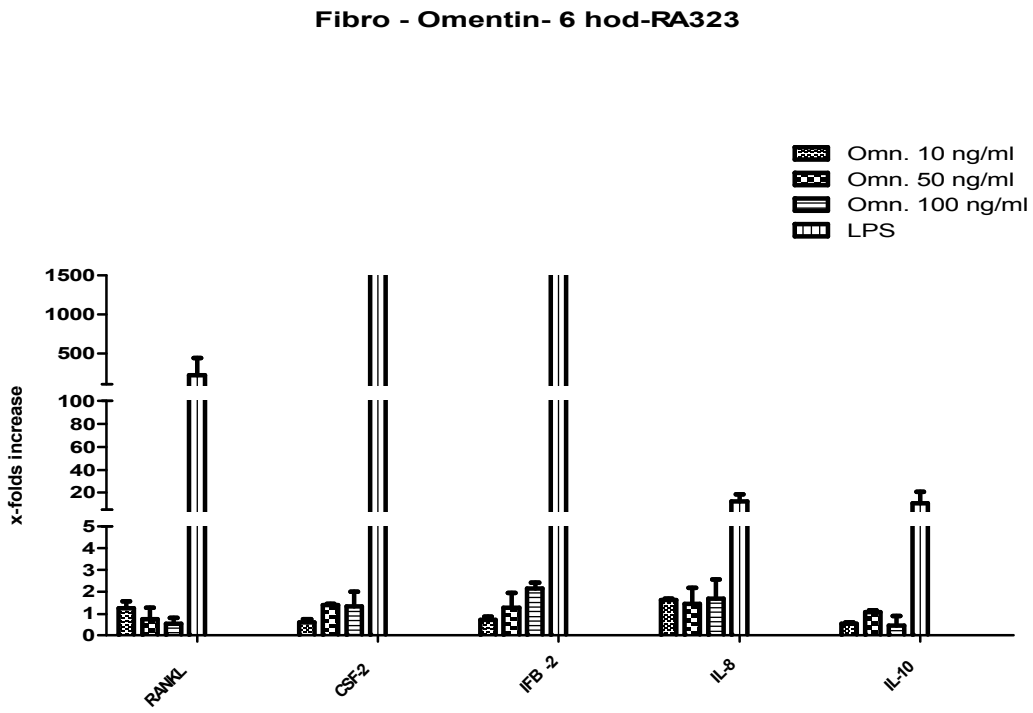

Figure 19: the expression of cytokines in fibroblast stimulated by Omentin-1

The second RA sample does not show the expected results. As seen in different concentrations of Omentin-1, every cytokine expression behaves in different and non-regular ways. (Figure 20)

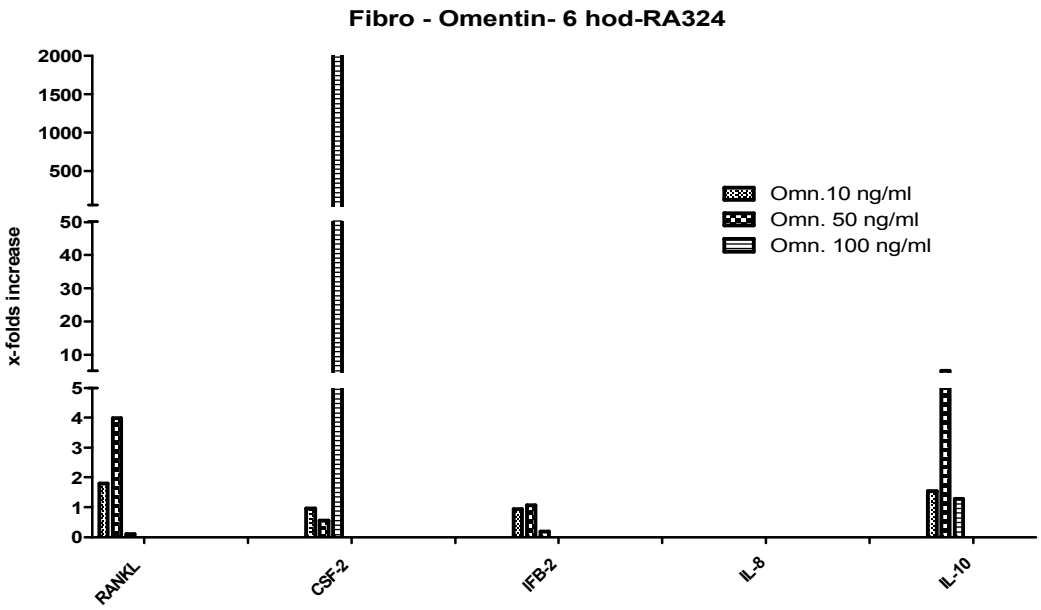

Figure 20: the expression of cytokines in fibroblast stimulated by Omentin-1

Observed (Figure 21) the other RASF sample, RA325, did not give us any definable and acceptable result, too.

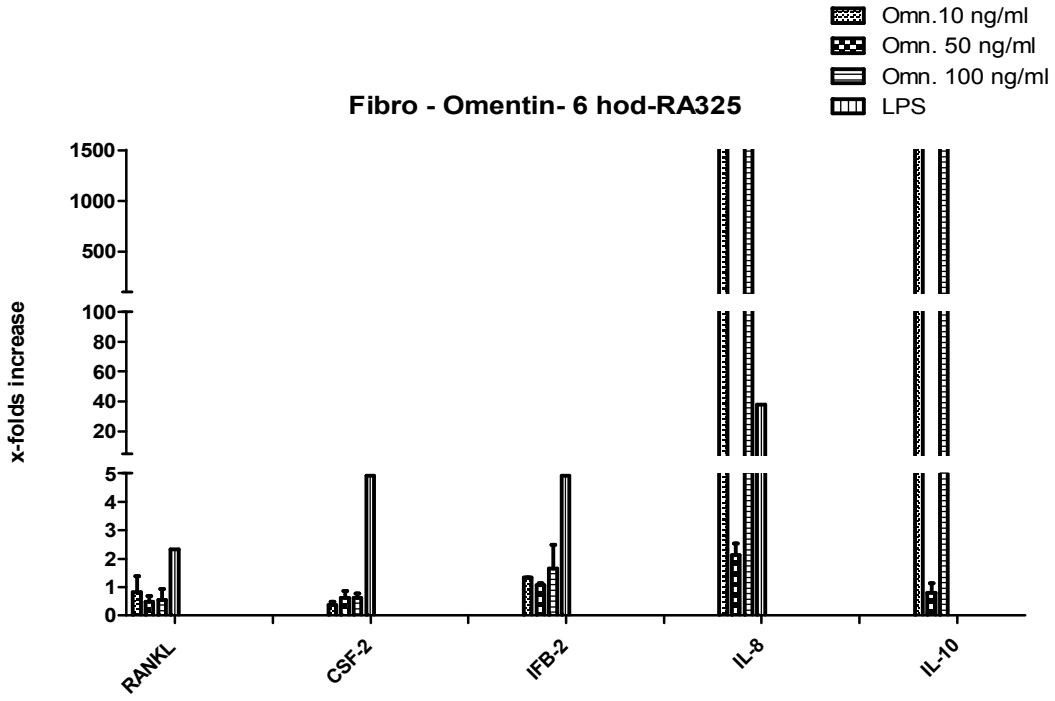

Figure21: the expression of cytokines in fibroblast stimulated by Omentin-1

The results are the same as others in the samples RA329 and RA351. The graph does not appear to interpret any interesting explanation for the behaviour of this new cytokine (Figures 22 and 23).

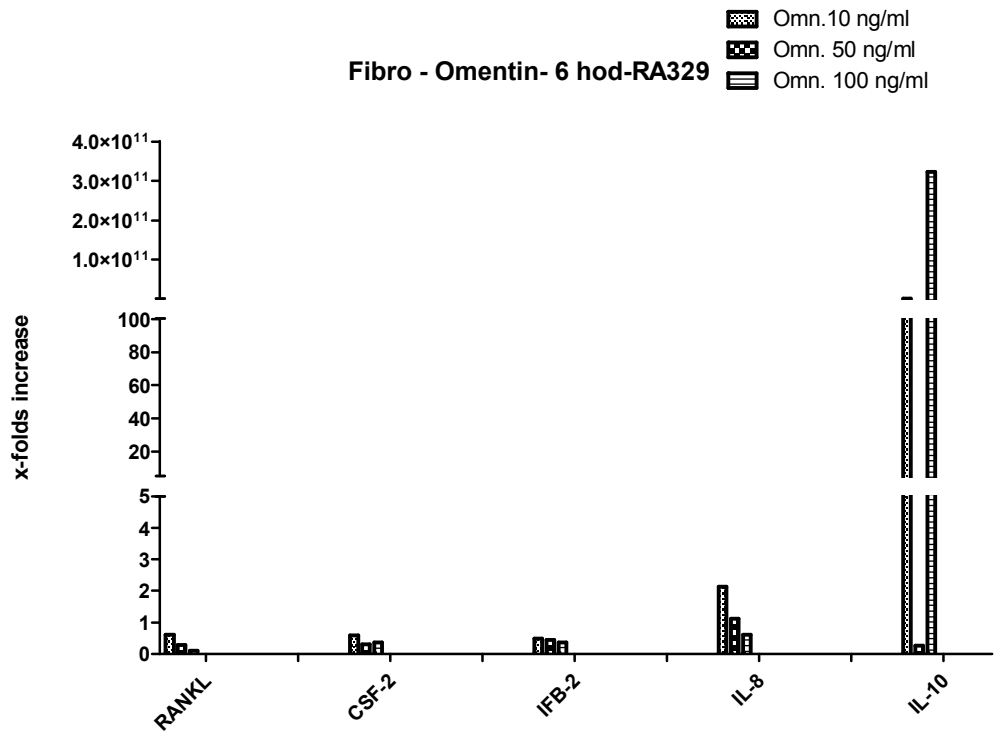

Figure 22: the expression of cytokines in fibroblast stimulated by Omentin-1

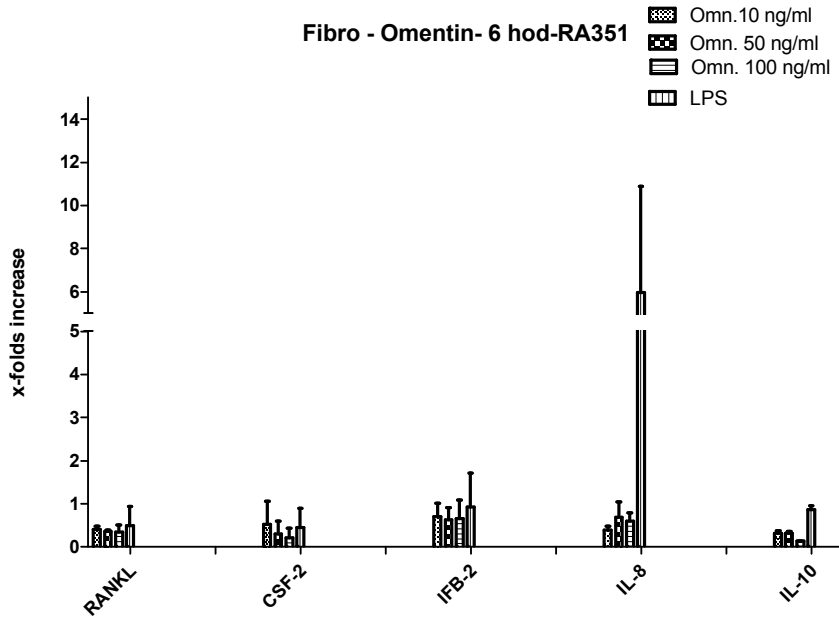

Figure 23: the expression of cytokines in fibroblast stimulated by Omentin-1

Finally we see the only OASF sample which shows low an increase in the examined biomarkers in the low x-fold. (Figure 24)

This means that there does not appear to be any differences between RA and OA samples. An explainable reason to illustrate the behaviours between all the studied cytokines and their interactions should be found.

However, in comparison with OA sample, a significant difference is observed between the RASF samples and OASF sample. The difference can be indicated in the values of x-fold increases in these samples.

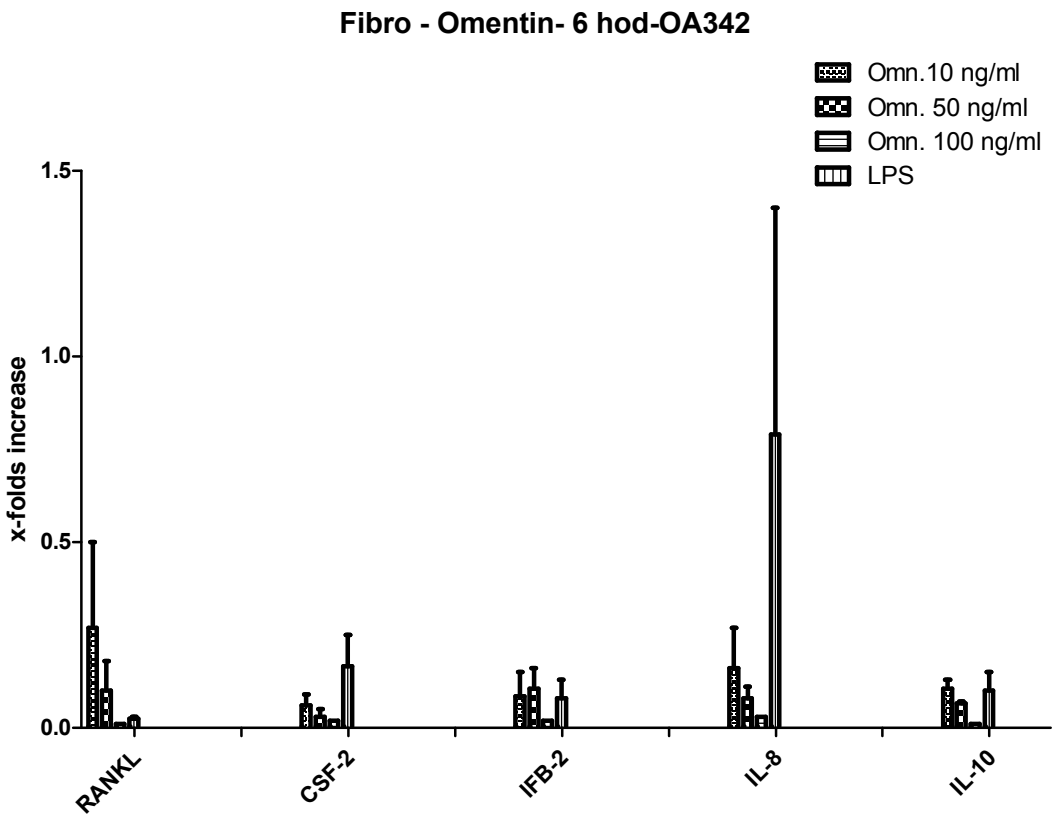

Figure 24: the expression of cytokines in fibroblast stimulated by Omentin-1

There were three samples (RA323, RA325 and RA351) used to find if there were any contamination of LPS. The results in the following graphs show nothing useful, and it may be necessary to repeat it again (Figure 25 and 26).

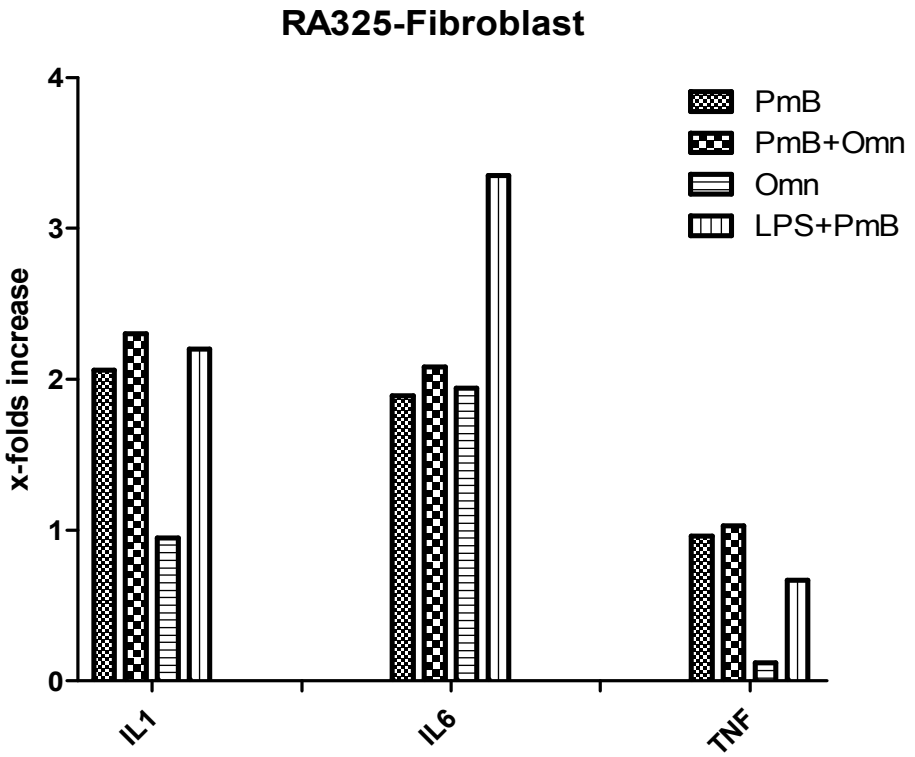

Figure 25: the expression of cytokines to find any contamination in Recombinant Omentin-1

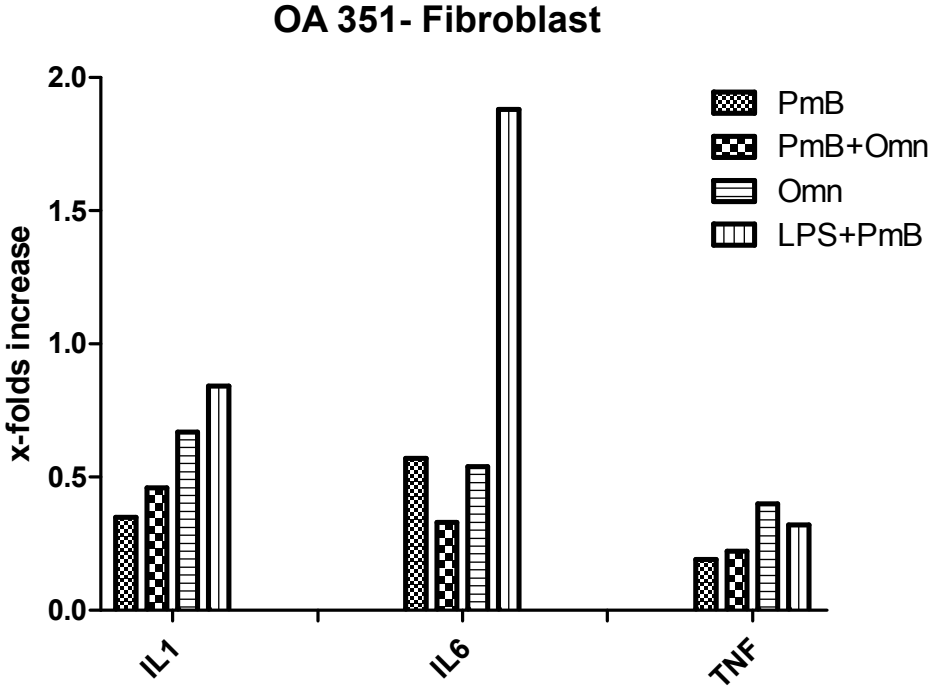

Figure 26: the expression of cytokines to find any contamination in Recombinant Omentin-1

During our work on stimulation of synovial fibroblasts by Omentin-1, we tried this Recombinant factor on another kind of tissue, chondrocyte.

The study of possible role of Omentin-1in chondrocytes

The results show that the values of x-fold are not high enough to interpret any effect of Omentin-1 in prognosis or diagnosis of RA in this kind of tissue (Figure 27).

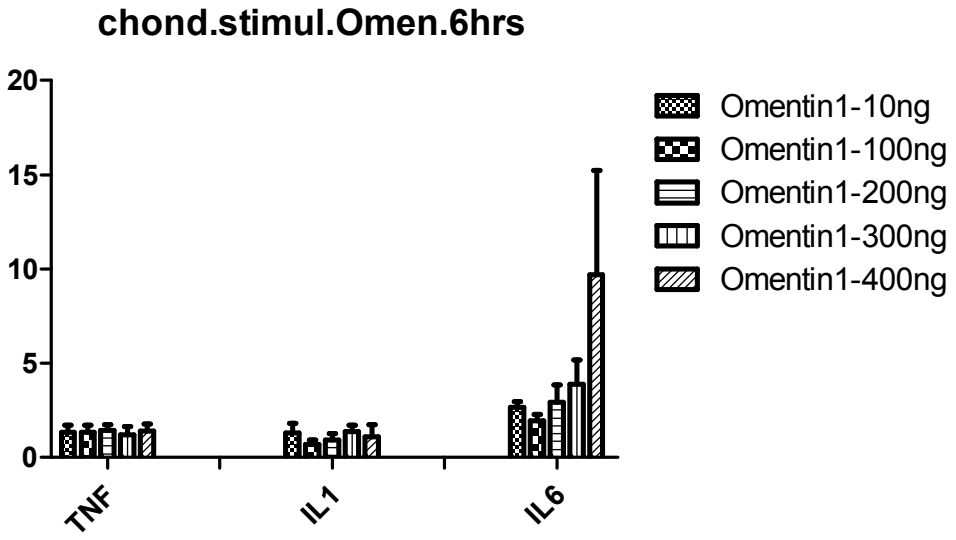

Figure 27: the expression of proinflammatory cytokines in chondrocytes stimulated by Omentin-1

5.1.4. The stimulation of Chondrocytes by IL-20

As shown in figure 28, there is not any significant increase in the expression of pro-inflammatory cytokines in chondrocytes after 6 hrs.

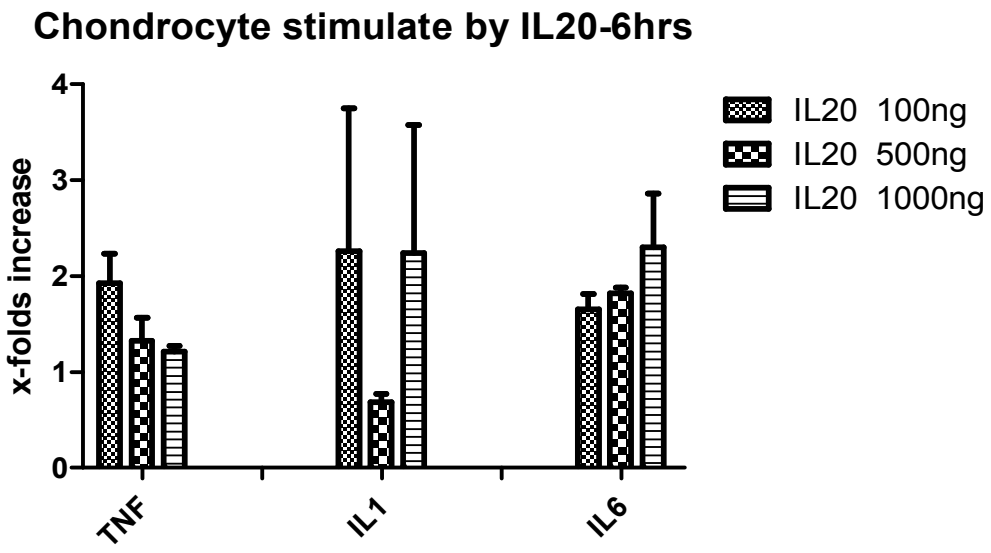

Figure 28: the expression of proinflammatory cytokines in chondrocytes stimulated by IL-20

6. Discussion

The expression of Omentin-1 in human adipose tissues was found in both protein and mRNA levels. The decrease of Omentin-1 was indicated in women affected to PCOS with higher weight and also affected by Metabolic Syndrome. (TAN 2008, AUGUET 2011) (In other research Jose Maria Moreno-Navarrete (2010) and his colleagues found an increase in the level of circulating Omentin-1 concentration in a group of women who lost weight. There was a correlation between the serum Omentin-1 value and arterial stiffness.

The findings gathered from this study show that a relationship can be found in the level of Omentin-1 in synovial fibroblasts in patients with Rheumatoid arthritis and other pro-inflammatory cytokines that it may help with prognosis and diagnosis of RA. The immunohistochemical study shows that the expression of Omentin-1 mRNA in RA is different in comparison to Osteoarthritis.

In a study published recently by Lin Xu and his colleague (2012), it was indicated that there were not any differences in the Omentin1 level of serum between the OA patients and a healthy control group.

Omentin-1 is significantly up-regulated in RA as compared to OA synovial tissues. The gene expression of Omentin-1 is induced by pro-inflammatory cytokines, (e.g. IL-1) and Omentin-1 also stimulates synovial fibroblasts to increase the expression of IL-1 and IL-6 mRNAs in a dose-dependent manner. Furthermore it enhances its own activity by a positive feedback.

In this research, fibroblasts, PBMCs and Chondrocytes were used at the beginning. Between these tissues, the effect of Omentin-1 expression is noteworthy only in synovial

fibroblasts at this time. However more experiments must be repeated to get a solid result in the role of Omentin-1 and its relationship to this autoimmune disease.

We tried to trace the expression of this adipocytokine in other tissues, but the results were not reliable at this time.

At the same time, the expression of another new cytokine thought to have a role in this autoimmune disease was followed. There is some information about the IL-20 receptors in comparison with Omentin-1. To start the chondrocytes were stimulated with Recombinant Human IL-20 to indicate the expression of proinflammatory biomarkers that are important in the disease progress. The data from the qRT-PCR did not indicate any significant increases in the expression of these cytokines.

As observed in this research, the expression of some biomarkers in the presence of cell stimulations by these new cytokines may be distinguished as prognostic or diagnostic biomarkers in Rheumatoid arthritis. We know that the proinflammatory cytokines such as IL-1β, IL-6 and TNFα at the beginning can show the next steps of inflammatory process if any change appears in their expression. The synovial cytokines along with RANKL have a known role in osteoclast differentiation and activation that, in turn, can destroy mineralized tissues such as cartilage. Also we know that the inhibition of TNFα, IL-6 and RANKL postpones the erosion in RA. The blocking of RANKL acts on bone but does not have any effect on inflammation and cartilage degradation. So in our research we followed any relationship between these cytokines and our new ones. In another way we tried to find a connection between these two cytokines and Matrix Methaloproteinases. As discovered, fibroblasts synthesize with some members of the MMP family including MMP1, MMP3, MMP8, MMP13, MMP14, and MMP16 as they have a role in the degradation of collagenous cartilage matrix. So we tried to check the expression of these proteins by helping qRT-PCR.

The survey in this field needs more work and experiments in DNA, RNA and protein levels as is the main aim of this research in the next steps and is running.

7. APPENDIXES

7.1. Fibroblast stimulation

10ng/µl	100ng/µl	200ng/µl
10ng/µl	100ng/µl	200ng/µl

10ng/µl	100ng/µl	200ng/µl
K	K	K

10ng/µl	100ng/µl	200ng/µl
10ng/µl	100ng/µl	200ng/µl

10ng/µl	100ng/µl	200ng/µl
K	K	K

10ng/µl	100ng/µl	200ng/µl
10ng/µl	100ng/µl	200ng/µl

10ng/µl	100ng/µl	200ng/µl
K	K	K

IL-1	IL-6	TNF
IL-1	IL-6	TNF

IL-1	IL-6	TNF
LPS	LPS	LPS

IL-1	IL-6	TNF
IL-1	IL-6	TNF

IL-1	IL-6	TNF
LPS	LPS	LPS

7.2. RT-PCR plates –sample arrangement.

RT PCR Datum: **Jméno:**

		1	2	3	4	5	6	7	8	9	10	11	12
A	MMP1	1	1	2	2	3	3	4	4	5	5	6	6
B		7	7	8	8	9	9	10	10	11	11	12	12
C		22	22	23	23	24	24						
D	MMP3	1	1	2	2	3	3	4	4	5	5	6	6
E		7	7	8	8	9	9	10	10	11	11	12	12
F		22	22	23	23	24	24						
G	omentin	13	13	14	14	15	15	16	16	17	11 7	18	18
H		19	19	20	20	21	21	22	22	23	23	24	24

		1	2	3	4	5	6	7	8	9	10	11	12
A	MMP13	1	1	2	2	3	3	4	4	5	5	6	6
B		7	7	8	8	9	9	10	10	11	11	12	12
C		22	22	23	23	24	24						
D	RANKL	1	1	2	2	3	3	4	4	5	5	6	6
E		7	7	8	8	9	9	10	10	11	11	12	12
F		22	22	23	23	24	24						
G	visfatin	13	13	14	14	15	15	16	16	17	11 7	18	18
H		19	19	20	20	21	21	22	22	23	23	24	24

7. 3. The RT-PCR data analysis

Fibroblast – Omn.1expression.							
after 6 hours stimulation							
	sample	**18S**	**studied gene**		**dCt**	**ddCt**	**x-fold**
I.		14,24758	TNF	36,159372	21,91		
	Omen. 10 ng/ml	14,44725		35,7467425	21,30	0,61	1,53
			IL1	37,947635	23,70		
				37,38101	22,93	0,77	1,70
			IL6	30,922964	45,17		
				30,9686155	45,42	-0,25	0,84
			MMP1	35,183522	49,43		
				37,530668	51,98	-2,55	0,17
			MMP13	29,424144	43,67		
				29,6084465	44,06	-0,38	0,77
			MCP1	33,6937295	47,94		
				33,6788515	48,13	-0,18	0,88
			RANKL	28,855852	43,10		
				29,038171	43,49	-0,38	0,77
I.	k	14,24758	TNF	36,159372	21,91		
	Omen. 100 ng/ml	14,34778		31,2439395	16,90	5,02	32,35
			IL1	37,947635	23,70		
				29,239854	14,89	8,81	448,19
			IL6	30,922964	45,17		
				28,352421	42,70	2,47	5,54
			MMP1	35,183522	49,43		
				36,7352525	51,08	-1,65	0,32
			MMP13	29,424144	43,67		
				29,825879	44,17	-0,50	0,71
			MCP1	33,6937295	47,94		
				34,86713	49,21	-1,27	0,41
			RANKL	28,855852	43,10		
				25,6722915	40,02	3,08	8,48
I.	k	14,24758	TNF	36,159372	21,91		
	Omen.200ng/ml	14,48081		30,1331745	15,65	6,26	76,61

			IL1	37,947635	23,70		
				28,401301	13,92	9,78	878,90
			IL6	30,922964	45,17		
				27,579631	42,06	3,11	8,63
			MMP1	35,183522	49,43		
				27,655754	42,14	7,29	156,99
			MMP13	29,424144	43,67		
				28,8723185	43,35	0,32	1,25
			MCP1	33,6937295	47,94		
				34,2013735	48,68	-0,74	0,60
			RANKL	28,855852	43,10		
				24,263162	38,74	4,36	20,53
I.	k	14,24758	TNF	36,159372	21,91		
	IL1	14,20468		33,580038	19,38	2,54	5,80
			IL1	37,947635	23,70		
				26,0417395	11,84	11,86	3724,93
			IL6	30,922964	45,17		
				23,672475	37,88	7,29	156,87
			MMP1	35,183522	49,43		
				35,8064385	50,01	-0,58	0,67
			visfatin	Undetermined			
				Undetermined			
			Omentin1	28,957328	14,71		
				24,8705405	10,67	4,04	16,49
I.	k	14,24758	TNF	36,159372	21,91		
	IL6	14,13215		36,9764565	22,84	-0,93	0,52
			IL1	37,947635	23,70		
				37,867651	23,74	-0,04	0,98
			IL6	30,922964	45,17		
				33,20073	47,33	-2,16	0,22
			visfatin	Undetermined			
				Undetermined			
			Omentin1	28,957328	14,71		
				29,5986935	15,47	-0,76	0,59
I.	k	14,24758	TNF	36,159372	21,91		
	TNF	14,23004		32,028005	17,80	4,11	17,31
			IL1	37,947635	23,70		
				31,537562	17,31	6,39	84,01
			IL6	30,922964	45,17		
				27,0674835	41,30	3,87	14,65
			visfatin	Undetermined			
				Undetermined			
			Omentin1	28,957328	14,71		
				27,151988	12,92	1,79	3,45
I.	k	14,24758	TNF	36,159372	21,91		
	LPS	14,21363		31,0111005	16,80	5,11	34,64
			IL1	37,947635	23,70		
				29,373122	15,16	8,54	372,36

			IL6	30,922964	45,17		
				26,9217835	41,14	4,04	16,39
			MMP1	35,183522	49,43		
				33,866608	48,08	1,35	2,55
			MMP13	29,424144	43,67		
				27,4750945	41,69	1,98	3,95
			MCP1	33,6937295	47,94		
				33,870754	48,08	-0,14	0,91
			RANKL	28,855852	43,10		
				25,10958	39,32	3,78	13,74
			visfatin	Undetermined			
				Undetermined			
			Omentin1	28,957328	14,71		
				27,2934705	13,08	1,63	3,09

7.4. Fibroblast- Omentin1 Contamination

Sample RA323

Fibroblast, sample RA323							Table A
Stimulation in 6 hours							
Sample		**18S**	**studied gene**		**dCt**	**ddCt**	**x-fold**
I.	k	16,34113	IL1	39,5011385	23,16		
	pMb	16,02022		39,57023	23,55	-0,39	0,76
			IL6	33,618367	17,28		
				33,67384	17,65	-0,38	0,77
			TNF	38,6119615	22,27		
				38,1636605	22,14	0,13	1,09
I.	k	16,34113	IL1	39,5011385	23,16		
	pMbOm3	15,73498		39,30023	23,57	-0,41	0,76
			IL6	33,618367	17,28		
				32,966841	17,23	0,05	1,03
			TNF	38,6119615	22,27		
				37,9270825	22,19	0,08	1,06
I.	k	16,34113	IL1	39,5011385	23,16		
	Omn	14,77363		37,575823	22,80	0,36	1,28
			IL6	33,618367	17,28		
				33,485926	18,71	-1,44	0,37
			TNF	38,6119615	22,27		
				37,398167	22,62	-0,35	0,78
I.	k	16,34113	IL1	39,5011385	23,16		
	pMb,Om	28,81194		Undetermined	-28,81	51,97	2701,08
			IL6	33,618367	17,28		
				Undetermined	-28,81	46,09	2124,21
			TNF	38,6119615	22,27		

				Undetermined	-28,81	51,08	2609,45

Sample RA325

Fibroblast, sample RA325							Table B
Stimulation in 6 hours							
Sample		**18S**	**studied gene**		**dCt**	**ddCt**	**x-fold**
I.	k	15,59334	IL1	39,37638	23,78		
	pMb	15,71888		38,460392	22,74	1,04	2,06
			IL6	35,20353	19,61		
				34,410553	18,69	0,92	1,89
			TNF	38,326458	22,73		
				38,503826	22,78	-0,05	0,96
I.	k	15,59334	IL1	39,37638	23,78		
	pMbOm3	15,9035		38,48747	22,58	1,20	2,30
			IL6	35,20353	19,61		
				34,459312	18,56	1,05	2,08
			TNF	38,326458	22,42		
				38,28948	22,39	0,04	1,03
I.	k	15,59334	IL1	39,37638	23,78		
	Omn	15,47498		39,33792	23,86	-0,08	0,95
			IL6	35,20353	19,61		
				34,129257	18,65	0,96	1,94
			TNF	38,326458	22,73		
				41,257263	25,78	-3,05	0,12
I.	k	15,59334	IL1	39,37638	23,78		
	lpsPMB	15,65803		38,305843	22,65	1,14	2,20
			IL6	35,20353	19,61		
				33,52477	17,87	1,74	3,35
			TNF	38,326458	22,73		

				38,964993	23,31	-0,57	0,67

Sample OA351

Fibroblast, sample OA351							Table C
Stimulation in 6 hours							
Sample		**18S**	**studied gene**		**dCt**	**ddCt**	**x-fold**
I.	k	14.947857	IL1	41.332	10,28		
	pMb	14.999819		Undetermined	11,78	-1,50	0,35
			IL6	34.654747	10,87		
				34.76218	11,69	-0,82	0,57
			TNF	38.110798	14,93		
				37.9637	17,31	-2,38	0,19
I.	k	14.947857	IL1	41.332	10,28		
	pMbOm3	15.201188		41.336483	11,41	-1,12	0,46
			IL6	34.654747	10,87		
				34.18717	12,48	-1,61	0,33
			TNF	38.110798	14,93		
				37.74146	17,12	-2,19	0,22
I.	k	14.947857	IL1	41.332	10,28		
	Omn	14.8482485		41.164524	10,86	-0,58	0,67
			IL6	34.654747	10,87		
				33.691147	11,76	-0,90	0,54
			TNF	38.110798	14,93		
				38.226818	16,24	-1,31	0,40
I.	k	14.947857	IL1	41.332	10,28		
	pMb,Om	14.860284		34.710255	10,53	-0,25	0,84
			IL6	34.654747	10,87		
				37.965073	9,96	0,91	1,88
			TNF	38.110798	14,93		
				36.316284	16,59	-1,65	0,32

8. ABBREVIATIONS

AA	Amyloid A
ABC	Avidin-Biotin Complex
ACPA	Anti-Citullinated Protein Antibody
ACR	American College of Rheumatology
AP	Alkaline Phosphate
CDM	Chondrocyte Differentiation Media
cDNA	complementary Deoxyribonucleic Acid
CRP	C - reactive protein
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
ddH2O	Double-Distilled Water
dH2O	Distilled Water
DMARD	Disease Modifying Antirheumatic Drugs
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
dNTP	deoxy Nucleutide Triphosphate
DRP	Disease Repercussion Profile
EDTA	Ethylene Diamine Tetra Acid

EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ESR	Erythrocyte Sedimentation Rate
FCRL	Fc Receptor-Like protein
HeLa	A cell line of cervical cancer cells of Henrietta Lacks
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human Leukocyte Antigen
HLA-DRB	Human Leukocyte Antigen complex on Chromosome 6
IgG	Immunoglobin G
IL	Interleukin
INTL1	Intelectin-1, the other name of Omentin1
kDa	KiloDalton, unified atomic mass unit
LPS	Lipopolysacharide
MCP	Monocyte Chemotactic Protein
MCTD	Mixed Connective Tissue Disease
MHC	Major Histocompatibility Complex
miRNA	Micro Ribonucleic Acid
MMP	Matrix Metalloproteinase

NCBI	National Center for Biotechnology Information
NK	Natural Killer (cells)
OA	Osteoarthritis
OASF	Osteoarthritis Synovial Fibroblast
PAD4	Peptydil Arginine Deiminase type 4
PBMC	Peripheral Blood Mononuclear Cell
PBS	Basic cell culture media contains phosphate and NaCl
PCOS	Polycystic Ovary Syndrome
PCR	Polymerase Chain Reaction
PGE2	Prostaglandin E2
PTPN22	Protein Tyrosine Phosphate Non-receptor type 22
qRT-PCR	quantitative Real Time- Polymerase Chain Reaction
RA	Rheumatoid Arthritis
RANKL	Receptor Activator of Nuclear factor Kappa-β Ligand
RASF	Rheumatoid Arthritis Synovial Fibroblast
RF	Rheumatoid Factor
RTL	a lysis buffer
SLC22A4	Solute Carrier Family 22, member 4
SLF	Synovial-Like Fibroblast

SNP	Single Nucleotide Polymorphism
STAT	Signal Transducer and Activator of Transcription protein
TBS	Tris-Buffered Saline
TNF	Tumour Necrosis Factor
UELAR	European League Against Rheumatism
VDR	Vitamin D Receptor

9. USED SOLUTIONS

PBS

NaCl	8 gr.
KCl	0.2 gr.
Na_2HPO_4	2.9 gr.

KH_2PO_4	0.2 gr.

Adding 1 ltr.ddH_2O , pH 7.3- 7.5, autoclave (120^0C, 30 minutes)

Medium for fibroblast culture

DMEM	500 ml
FCS	50ml
Penicilin(500IU/ml)	5ml
L- Glutamin (200mM)	5ml
Herpes Buffer (1M)	5ml
Fungicid	1ml

11. LITERATURES

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