Effect of genetic variations of PADI4, PTPN22 and TIMP4 in the susceptibility of Rheumatoid arthritis in North Indian Population

Thesis

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D edicated to

M y L oving parents

B eloved

W ife & L ittle S on

DECLARATION

I hereby declare that thesis entitled **"Effect of genetic variations of PADI4, PTPN22 and TIMP4 in the susceptibility of Rheumatoid arthritis in North Indian Population"** is my own research work carried out under the supervision of **Dr. M.Y. Khan**, Professor, Department of Biotechnology, School of Biosciences and, Biotechnology, Babasaheb Bhimrao Ambedar University, Lucknow (India) and the Co-Supervision of **Dr. Varsha Gupta**, Assistant Professor, Department of Biotechnology, Chhatrapati Sahuji Maharaj, University, Kanpur. The research work is original and no part of this work has been submitted for any other degree or diploma. All the above given information is true the best of my knowledge.

Date : 16/04/18
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CERTIFICATE

This is to certify that the thesis titled **"Effect of genetic variations of PADI4, PTPN22 and TIMP4 in the susceptibility of Rheumatoid arthritis in North Indian Population"** submitted by **Vivek Kumar** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University Lucknow satisfies all the requirements stipulated in the Doctor of Philosophy (Ph.D.) regulation – 1999 as amended in 2010 and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

Date: 16/04/18

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(Vivek Kumar)

ABBREVIATIONS

ACR	American College of Rheumatology
ADAMTS	A Disintegrin And Metalloproteinase
	With Thrombospondin motifs
ALP	Alkaline Phosphatase
Anti CCP	Anti –Cyclic Citrullinated Peptide
ASDs	Autism Spectrum Disorders
BMI	Body Mass index
BP	Blood Pressure
c-DNA	Complementry Deoxy ribonucleic Acid
Cu	Copper
CPMG	Carr-Purcell-Meiboom-Gill
CRP	C-Reactive Protein
CVD	Cardiovascular Disease
Cm	Centimeter
C	Degree Centigrade
Csk	C-terminal Src *kinase*
DAS	Disease Activity Score
DMARDs	Disease-Modifying Antirheumatic Drugs
ECM	Extra Cellular Matrix
ESR	Erythrocyte Sedimentation Rate
Fc	Fragment Crystallizable
GPx	Glutathione Peroxidase

GR	Glutathione Reductase
GWAs	Genome Wide Association Studies
H_2O_2	Hydrogen Peroxide
Hb	Haemoglobulin
HCl	Hydro Chloric Acid
HDL	High Density Lipoprotein
hsCRP	High Sensitive c-Reactive Protein
HLA	Human Leukocyte Antigen
IgA	Immuno Globulin A
ICs	Immuno Complexs
IgG	Immuno Globulin –G
IU	International Unit
IL	Interleukin
Kg	Kilogram
L	Litre
Lck	Lymphocyte-specific protein tyrosine kinase
ln	Log
LYP	Lymphoid Protein Tyrosine Phosphate
LDL	Low density lipoprotein
MHC	Major histocompability constant
MMPs	Matrix mMtalloproteinase
m^2	Metre Square
MCP	Meta carpophalangeal
m-RNA	Messenger Ribonucleic Acid
µ	Micro
ml	Milli litre
MDA	Malondialdehyde

MQ	Milli Que
M	Molar
MVA	Multivariate Analysis
Nm	Nanometer
NMR	Nuclear Magnetic Resonance
NSAIDs	Non Steroidal Anti Inflammatory Drug
NADPH	Nicotinamide adenine di nucleotide phosphate
OA	Osteoarthritis
OD	Optical density
PADI	Peptidyl Arginine Deiminase
PCA	Principal Component Analysis
PIP	Proximal Interphalangeal Joint
PLS	Projection to latent structure
PTPN22	Protein Tyrosine Phosphatase Receptor 22
RA	Rheumatoid Arthritis
RF	Rheumatoid Factor
R	Arginine
Rpm	Revolution per minute
RNS	Reactive Nitrite Species
ROS	Reactive Oxygen Species
SLE	Systemic Lupus Erythematosus
SOD	Super Oxide Dismutase
SGOT	Serum Glutamic Oxaloacetic Transaminase
SFPT	Serum Glutamic Pyruvic Transaminase
SNP	Single Nucleotide Polymorphism
TNF-α	Tumor Nacrosis Factor-alpha
TG	Tri Gyceraldehydes

TBA	Thiobarbutyric acid
TCA	Tri carboxylic acid
TDW	Triple Distilled Water
TIMP	Tissue Inhibitor Matrix Metalloproteinase
TC	Total Cholesterol
U/L	Unit/Litre
VLDL	Very low density lipoprotein
VAS	Visual Analog Scale
V	Volume
W	Tryptophan
Wt	Weight
Zn	Zinc

TABLE OF CONTENTS

1. INTRODUCTION

The immune system protects our body from invading pathogens i.e. it is the defense system of our body. This system does recognition and then initiates the response. It is able to recognize and differentiate the components which belong to our body or self-component from non-self components. However, sometimes this mechanism fails, resulting in inappropriate targeting of self-components leading to a condition called autoimmunity, which activates self-reactive B and T lymphocytes. These abnormal, autoreactive T and B lymphocytes, either target specific organs to cause organ specific autoimmunity as diabetes mellitus (DM), intestinal bowel diseases (IBD) or affects whole body to cause systemic autoimmune diseases as systemic lupus erythematosus (SLE), *Rheumatoid arthritis* (RA), systemic sclerosis (SS) and Sjogren's syndrome (SJS). These diseases are characterized by the failure of immune-tolerance against self-antigens that are controlled by complex genetic factors (mediating central tolerance or peripheral tolerance) or infectious agents (viruses/bacteria) or environmental factors or combination of these.

Rheumatoid arthritis (RA) is a chronic inflammatory disease which results in progressive destruction of synovial joints (Bodman and Roitt, 1994; Yasuto and Toshihide 2016). The worldwide prevalence of RA is 0.8% with an annual incidence of 0.5-1% in both developed and developing countries (O'Dell *et al*., 2007; Scott *et al*., 2010). In India, the affected population due to *Rheumatoid arthritis* is 0.75-1% (Malaviya *et al*., 1993; Akhter *et al*., 2011). The diagnosis of RA is done according to 3/4 findings of American College of Rheumatology criteria (ACR), which involves radiological, clinical and serological parameters. Of all the serological parameters, increase in C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) is

considered as important criteria for the diagnosis of inflammatory changes in RA. Serologically presence of rheumatoid factor or anti-cyclic citrullinated antibody provides important diagnosis along with clinical symptoms.

The redox state inside the cell may be an important factor leading to the progression of the disease (Yuliya *et al.*, 2015). The increase of oxidants and imbalance of antioxidants is enough to induce structural and functional changes in cells and tissues, and this state is termed as oxidative stress. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced in the process of oxidative stress are detrimental for the body (Feldmann *et al.*, 1996; Mateen *et al.*, 2016). These ROS are highly damaging to connective tissue, membrane lipids and nucleic acids of the cell and are essential mediators of inflammation. The synovial cavity is an important target for the attack of immune system in RA. This is due to chemo-attractant nature of the synovial cells, resulting in accumulation of leukocytes inside the synovium causing respiratory burst which is characterized by high oxygen consumption and high anaerobic glycolysis leading to the generation of superoxide, hydroxyl, hypochloric radicals etc. (Marshall and Bangert, 1995).

Blake *et al.*, 1981 showed that enzymatic and non-enzymatic free radical scavenging system or antioxidant systems are highly perturbed in RA. The higher inflammation, oxidative stress and dyslipidemia (Mishra *et al.*, 2012) in RA patients not only affects joints but also affects various other systems leading to important systemic manifestations in the form of cardiovascular disease (CVD). RA patients have high morbidity and mortality due to dyslipidemia and CVD (Wolfe *et al.*, 1994; Erum *et al.*, 2017).

RA has a genetic predisposition and many genetic loci are implicated in the disease. The heritability of RA has been predicted in 60% populations (Mac *et al.*,

2000; Deighton *et al.,* 1989). First-degree relatives of the RA patients are at four to six times higher risk of developing RA (Nakamura, 2000). MHC-II encoding alleles in HLA-DR molecules are found to be associated with more severe disease condition (Mattey *et al.,* 2007; Van *et al.,* 2004; Matzaraki *et al.,* 2017). Linkage disequilibrium studies revealed susceptibility loci for RA within several chromosomes. Many genes are reported which increase the susceptibility to development of RA (Van *et al.,* 2010; Szodoray *et al.,* 2010). Peptidyl arginine deiminase type IV (PADI4) (EC 3.5.5.15) is one of the member of PADI gene family, located on chromosome 1p36. It encodes the enzyme responsible for the posttranslational conversion of arginine residues into citrulline in many mammalian tissues (Zhou and Menard, 2002). Another gene PTPN22 is located on chromosome 1p13 which encodes the lymphoid-specific tyrosine phosphatase (LYP), which is involved in the suppression of T cell activation and thereby in T cell-dependent antibody production (Gregersen *et al.,* 2006). The R620W polymorphism in PTPN22 gene affects a proline-rich motif of LYP that is involved in the protein-protein interactions.

Begovich *et al.,* 2004 first described the association between a functional single-nucleotide polymorphism (SNP) in the coding region of the gene PTPN22 and RA, and the study has been replicated by several other groups in the UK, Spanish and Dutch RA populations (Lee *et al.,* 2005; Hinks *et al.,* 2005; Orozco *et al.,* 2005). Risk of RA is increased 2 fold in the presence of the PTPN22 polymorphism R620W genotype (Harrison *et al.,* 2006).

The actual agents involved in joint degradation are the matrix metalloproteinase (MMPs). The matrix metalloproteinase (MMPs) are zinc-dependent endopeptidases that regulate the breakdown of extracellular matrix (ECM). Extracellularly, the activity of MMPs is regulated by tissue inhibitor of

metalloproteinase (TIMPs) (Visse and Nagase, 2003). The ratio of MMP: TIMP regulate the tissue damage and is associated with RA (Lee *et al.,* 2003).

Analyses of metabolites using NMR allow rapid identification of metabolite and their role in biological systems.

As RA is a complex disease which involves interplay of many factors, therefore this study was planned to analyze i) serological profiling, ii) oxidant and antioxidant status analysis, iii) studies of genetic polymorphism of PADI4, PTPN22, and TIMP4, iv) heavy metal analysis and v) metabolomic analysis of healthy controls and patient. Analysis of these would help in the understanding pathophysiology of the disease and provide more therapeutic targets, which can be utilized for better management of RA.

Objectives

RA being systemic inflammatory disease involves multiple organs and physiological components therefore the present study was planned to analyze biochemical parameter and genetic association of a few genes for their role in susceptibility of RA.

(I) To study the effect of RA on serum enzymes involved in inflammation.

(II) Genetic polymorphism of PADI4, PTPN22 and TIMP4 in the RA subjects and controls for their possible role in disease onset and/or progression of RA.

(III) Analysis and compilation of results obtained.

2. REVIEW OF LITERATURE

2.1 *Rheumatoid Arthritis*

Rheumatoid Arthritis (RA) is an autoimmune inflammatory rheumatic disease, which was first recognized by Hippocrates in the 4th century before Christ. Goemaere *et al.,* 1990 reported that the appearance and distribution of lesions in ancient skeletons and suggested that *Rheumatoid arthritis* may have existed in North America at least 3000 years ago.

Rheumatoid arthritis (RA) is a systemic autoimmune disease, characterized by inflammation of the synovial tissue (synovitis). The affected joints are the hallmark of RA (Michelle and Kahlenber, 2011) which become warm and swollen with tenderness and stiffness in the final stage, which causes functional disability (Vandana *et al.,* 2012). RA patients commonly suffer from pain, stiffness and disability in multiple joints. Although some of them may experience symptoms at just one location, later the symptom emerges at other sites, accompanied by symptoms of dieting behavior, weakness, or fatigue (Gurpreet *et al.,* 2017). Joints which are commonly involved are wrist joints, proximal interphalangeal (PIP) and metacarpophalangeal (MCP) joints. The distal interphalangeal (DIP) joints and sacroiliac joints are not affected (Harris *et al.,* 2005). Inflammation leads to cartilage destruction, bone erosion and joint deformity (Hayer *et al*., 2016).

2.2 Epidemiology

RA shows the worldwide distribution that affects all races. The symptoms of RA commonly appear at the age of between 30 to 50 years of life. The onset of RA over the age of 60 years is called Late-Onset RA (LORA) while below the age of 30 years is called Young-Onset RA (YORA) (Spinel *et al*., 2013). Worldwide the estimated prevalence of RA is 1 (Sudha *et al.,* 2012; Rudan *et al*., 2015). Malemba *et*

al., 2012 has reported that the prevalence of RA in African population was 0.6 to 0.9% in adults (Manish *et al.,* 2011). The prevalence in Pima Indians was 5.3% (Puente *et al*., 1989) and Chippewa Indians were 6.8% (Harvey *et al*., 1981). Probably, the matched prevalence of RA in Indians and Caucasians population may be due to the fact that the Indian population is genetically closer to the Caucasians than in other ethnic groups (Malaviya *et al*., 1993).

A recent Indian study has predicted that about 70% of patients with RA are women (Paul *et al*., 2017). It is 2 to 3 fold higher in women than men due to the hormonal or reproductive changes before menopause (Mohana *et al*., 2010; Wendy, 2015). Jonsson *et al*., 1998 has reported that the young women below the age of 50 years with RA have a higher risk of developing fractures than women without *Rheumatoid arthritis***.**

2.3 Diagnosis of *Rheumatoid arthritis*

2.3.1 ACR criteria

Current classification criteria were developed by the American College of Rheumatology (ACR) in the mid-1980s (Arnett *et al*., 1988) by replacing the earlier existing New York classification criteria (Bennett and Burch, 1967). Using these criteria, it is possible to distinguish *Rheumatoid arthritis* from other rheumatic conditions with a specificity of 89% and sensitivity between 91-94%. The revised criterion of ACR 1987 is represented in Box-1, wherein the presence of four parameters confirms the diagnosis.

Box-1 Revised criteria for the classification of RA (Arnett *et al*., 1988).

1. Morning stiffness - Morning stiffness lasting at least for one hour (≥ hour)

2. Arthritis of 3 or more joint areas - At least involvement of three or more joint areas having swelling of soft tissue or fluid accumulation as observed by a physician (the fourteen possible joint areas are (right to left) proximal interphalangeal joint (PIP), metacarpophalangeal joint (MCP), wrist, elbow, knee, ankle and metatarsophalangeal joint (MTP) (Fig- 1A).

3. Arthritis of hand joints - At least one joint area swollen in the wrist, MCP or PIP joint (Fig-1A).

4. Symmetric arthritis - Involvement of the simultaneous or similar joint, however, bilateral involvement of PIP, MCP or MTP -joints without absolute symmetry are acceptable) (Fig-1C).

5. Rheumatoid nodules –Appearance of subcutaneous nodules over bony structures (Fig-1B).

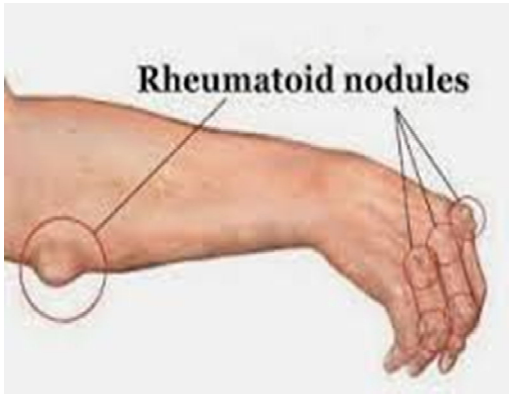
6. Serum rheumatoid factor - Presence of abnormal amounts of autoantibodies as IgG/IgM termed as serum rheumatoid factor (RFs).

7. Radiographic changes - Changes typical of autoimmune RA on wrist, hand radiographs with erosions or bony decalcification present adjacent to the involved joints. For classification purposes, a patient is said to have RA if he/ she has satisfied at least four of the seven criteria. Criteria, one through four must be present for at least 6 weeks (Fig-1C and D)

Many studies have reported that the rheumatoid factors have been observed in multiple autoimmune diseases (AIDs), such as in systemic lupus erythematosus (SLE), primary Sjogren's syndrome (pSS), mixed connective tissue disease and some non-autoimmune conditions as chronic infections occurring in old age (Nell *et al*., 2005; Patel and Shahane, 2014), therefore presence of ¾ criteria is considered confirmatory.

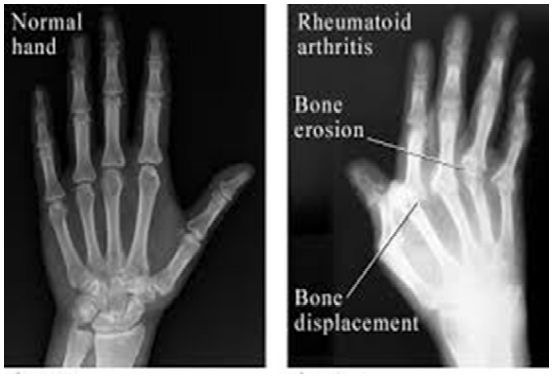
https://www.slideshare.net

Figure: 1A. Shows the changes in Early and Late RA in Patients hand joints



http://www.avenuephysio.co.uk

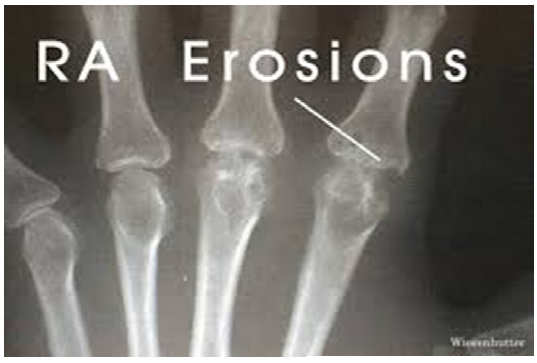
Figure: 1B



http://www.sites.google.com

Figure: 1C

1B shows the RA nodules in affected patient and 1C shows the radiographical changes in RA and normal individuals



http://www.cdaarthritis.com

Figure: 1D shows the erosions in joints of RA affected individuals

2.3.2 Imaging

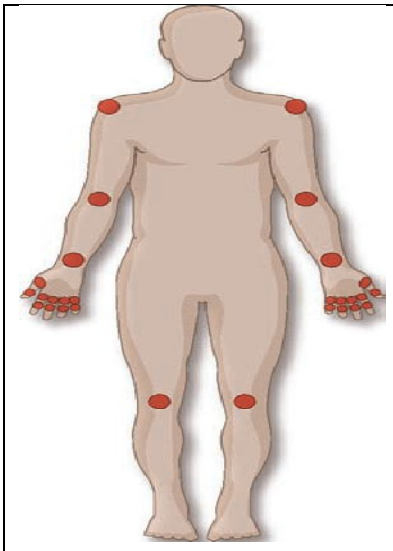
Plain radiography (X-Ray) is important for detection of erosions and determining the progression of joint damages, but there are many instances where ultrasound (US), magnetic resonance imaging (MRI) and computed tomography (CT) scanning provides added information. MRI and US are now used regularly by clinicians to help diagnose RA in the pre-radiographic stage as they offer improved visualization of joint erosions. Bruynesteyn, 2004 has seen joint space narrowing (JSN) that is used for scoring in 42 joints for cartilage degradation. The erosion score (ES) is used for scoring in 44 joints for bone degradation (Figure-1C and 1D). Other evaluations as clinical assessment, like an absence of pharmacological treatment, knowledge of etiological factors, some phenotypic studies, joint space narrowing etc. are helpful to diagnose RA. The European League Against Rheumatism (EULAR) and ACR have collaborated to produce such classification criteria (Aletaha *et al*., 2010).

2.3.3 Disease activity score (DAS):

DAS is calculated according to the involvement of joints as shown in the box. The swelling and tenderness are scored depending on joint involvement in RA (Terao *et al*., 2013). DAS28 score higher than 5.1 is indicative of high activity of disease, whereas a DAS28 below 3.2 indicates lower activity of disease and score between 3.2 and 5.1 indicate moderate activity (Jensen *et al*., 2017). A patient is considered to be in remission if they have a DAS28 lower than 2.6 (Van *et al.,* 1990; Van *et al.,* 1993; Anderson *et al.,* 2012; Jensen *et al*., 2017).

DAS 28= 0.56*√ (Number of tender joints) + 0.28*√ (Number of swollen joints) + 0.7* In (ESR: 1hour) + 0.014*VAS.

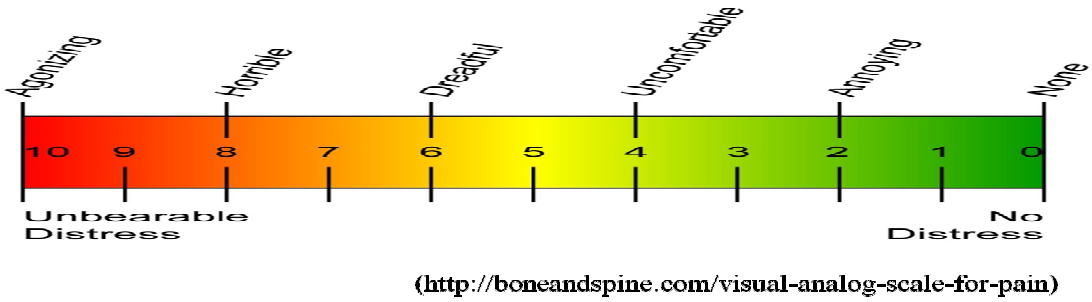
Box-2 : For the analysis of swollen and tender joints for DAS 28



Joints	Left		Right	
	Swollen	**Tender**	**Swollen**	**Tender**
Shoulder				
Elbow				
Wrist				
Metacarpophalageal Joints (MCP) 1 2 3 4 5				
Proximal Interphalangeal Joints (PIP) 1 2 3 4 5				
Knee				
Subtotal				
Total	0		0	

2.3.4 Visual analog scale:

VAS was first described in 1921 as the pain assessment measure (Hayes *et al*., 1921). Pain is a subjective experience that cannot be verified by traditional diagnostic methods. This nature of pain and its psyche involvement makes exact measurement difficult (Klimek *et al.,* 2017). Therefore, pain cannot be considered effectively treated or relieved unless it is measured (Revill *et al*., 1977). Therefore, the only way to ensure that patients receive an equally high quality of pain relief is to rely on the proven reliable indicator of pain. It can be achieved by the patient's self-report, which the patient can provide as it can be quantified only indirectly (Chapman *et al*., 1985).



(http://boneandspine.com/visual-analog-scale-for-pain)

Figure-2: Scale for the calculation of VAS

2.4 Risk Factors for *Rheumatoid arthritis*

Many environmental and genetic factors risk factors are known to be involved in the development of RA. Suggested environmental risk factors include smoking, vitamin D deficiency, infectious agents, silica exposure (Stolt *et al*., 2005; Karlson and Deane, 2012), obesity etc. (Harris, 1990; Sangha, 2000; Josef *et al*., 2018).

Optimum vitamin D levels (Merlino *et al*., 2004; Kim *et al.*, 2012), Goemacre *et al*., 1990 oral contraceptive use (Goemacre *et al*., 1990) and tea consumption (Mikuls *et al*., 2002) are associated with decreased risk of RA. Smokers have a high risk of *Rheumatoid arthritis* development as compared with non-smokers (Sugiyama *et al*., 2010; Vesperini *et al*., 2013). A history of smoking has been found to be associated with modest to moderate (1.3 to 2.4 times) increase in the risk of RA onset (Silman and Hochberg, 2001).

A study suggested that the firefighters and other emergency responders exposed to dust at the site of the 2001 World Trade Center collapse in New York, USA, had increased risk of systemic autoimmune diseases, including RA (Webber *et al*., 2015). The dust contained pulverized glass fibers, silica, cement, asbestos and other materials. Too *et al*., 2016 showed significantly increased the risk of developing RA in a population of Malaysian women by the occupational exposure to textile dust (Too *et al.*, 2016). The association was observed for both ACPA-positive RA and ACPA-negative RA.

2.5 Early *Rheumatoid arthritis*

Initially, the sign of early *Rheumatoid arthritis* is swelling and pain of the PIP and MCP joints, and later the larger joints like knee, elbow and ankle get affected. Sokolove *et al.,* 2013 reported that infiltration of activated leukocytes in synovial membrane causes hyperplasia and inflammation and finally the destruction of cartilage and bone (Sokolove *et al*., 2013). In RA, joints affected as the knee, ankle, elbow, and wrist are usually tender, swollen, and with constraints mobility.

2.6 Pathogenesis of *Rheumatoid arthritis*

2.6.1 Synovial joints

Synovial joint known as diarthrosis joins the bone with the fibrous joint capsule. The synovial joint is required for proper movement of any joint hence the smooth surface is provided by very thin and fine layering of smooth and strong hyaline cartilage, known as articular cartilage (Wang and Peng, 2015). The frictionless surface within a joint during movement is provided by the fibrous capsule which is covered with synovial membrane whose resident cells are responsible for secretion of fluid component into the cavity for lubrication known as synovial fluid. To support synovial joint soft tissues also surround them with tendons (attach muscle to bone) and ligaments (attach bone to bone).

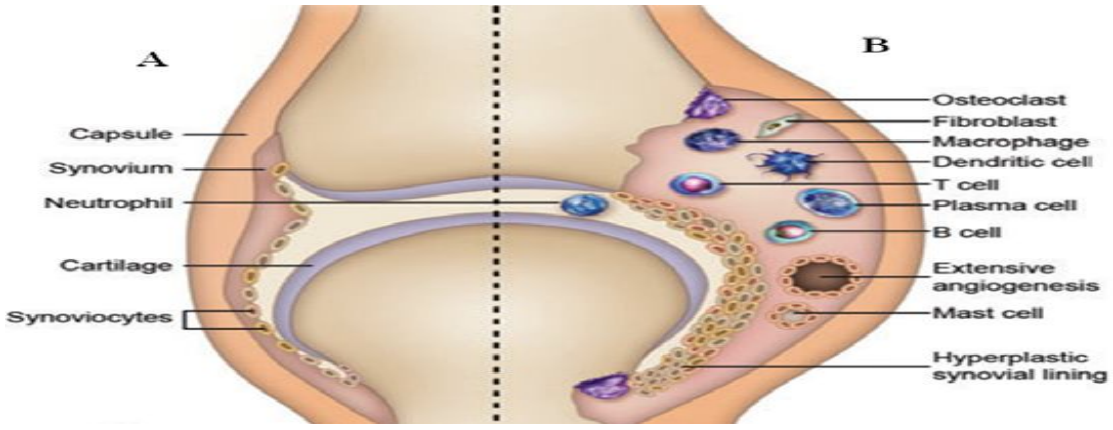


Figure: 3 Normal joint **(A)** v/s pathologic RA **(B)** (Strand, *et al.* 2007)

In the normal joint, the synovium consists of a network of capillaries that is important for nutrient and gaseous exchange. The synovium is therefore permeable to water, gases, nutrients, small molecules, and proteins, but not the large molecules such as proteins proteoglycans oligosaccharides and glycosaminoglycans. Synovial fluid is viscous in nature due to the presence of hyaluronic acid (HA). The inflamed joint of RA shows reversible signs (aseptic- inflammatory synovitis) and irreversible structural (structural damage). These signs are useful for disease staging, determining prognosis and medical or surgical treatment selection. McInnes and Schett, 2007 has shown that the after disease onset, the hypo-cellular synovial membrane becomes hyperplastic, comprising of a superficial lining layer of synovial fibroblasts and macrophages (McInnes and Schett, 2007; Ospelt, 2017). At the lining overlies an interstitial zone with marked cellular infiltrates containing fibroblasts, macrophages, dendritic cells, mast cells, T cells and B cells (which differentiate locally into antibody-secreting plasma cells) (McInnes and Schett., 2007) (Figure-3B). The interaction between activated lymphocytes and monocytes, leads to the production of pro-inflammatory cytokines, immunoglobulins and rheumatoid factors (RF) which are central to the immunological reaction. It is not yet fully understood how many mediators are involved and how they orchestrate the process, but IL-1β and TNF-α are suspected to stimulate synoviocytes and osteoclasts. Activation of the cells leads to the irreversible destruction of bone and cartilage (Goldring, 2006; Shi *et al.*, 2011). Synoviocytes are also known to produce MMPs, which are normally inhibited by the TIMPs. In RA, the proportion of proteinases to their inhibitors is not balanced. Chondrocytes switch from an anabolic matrix-synthesizing state to a catabolic state which is characterized by the activation of matrix-degrading proteases, (MMPs) the enzymes that cleave cartilage components such as proteoglycan and collagen fibers (McInnes and Schett, 2007)

2.6.2 Articular cartilage `

The mature human articular cartilages have four main layers; these are superficial, middle, deeper and calcified layers. The superficial layer is thin, aligning tangential and the middle is transitional in layering. Each layer has their specific amount of glycosaminoglycans, collagen, mineral, and water in chondrocyte morphology. This layer is responsible for protecting deeper layer from shear stress. This is necessary for proper weight distribution throughout the joint. It is reported that any structural change may lead to cartilage breakdown and initiates the disease (Kumar *et al.*, 2001). The outermost layer is acellular, that face the joint space. It contains low proteoglycan content and collagen fibers (I, II, III) are placed parallel to the articular surface. The deepest superficial layer with all type of collagen fibers also contains relatively high numbers of flattened chondrocytes (Kuettner *et al.*, 1992). This layer which is in contact with the synovial fluid is responsible for most of the tensile properties of cartilage. The middle and deeper layers provide an anatomic and functional bridge between superficial and deep layer.

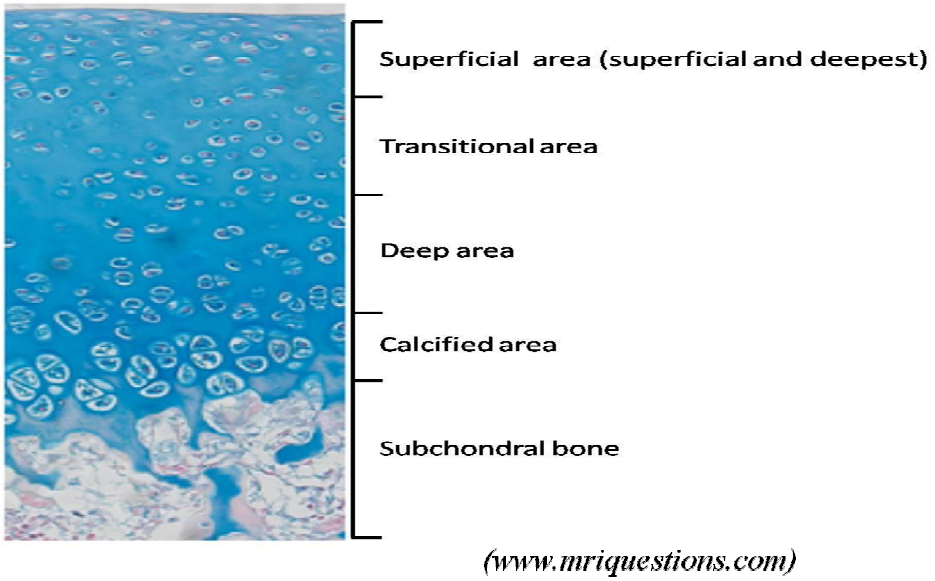


Figure-4: *Histological organization of articular cartilage*

The middle layer provides actual defense by providing resistance to compressive forces (Schumacher *et al.*, 1994). The deeper part of the cartilage has a calcified zone that is in indirect contact with the subchondral bone with a limited number of active chondrocytes involved in the synthesis of type X collagen. Ellipsoidal chondrocytes are present in the deepest superficial layer cellular region. These chondrocytes are larger, elongated and are oriented parallel to the articular surface in a vertical column (Schumacher *et al.*, 1994).

2.7 Etiology

The exact etiology of RA remains elusive, a genetic basis for the disease has been emphasized in some studies (Smolen and Steiner 2003). The epitope of the HLA-DRB1*04 cluster was found in more than 80% of patients (Smolen *et al.*, 2007). In particular, HLA–DRB1 alleles encoding a common amino acid sequence called the shared epitope (SE) and it identified on the third hypervariable region of the allele for RA (Weyand *et al.*, 1992; Amal *et al.*, 2016). These alleles are also associated with a more severe disease (Amal *et al.*, 2016). Non-MHC risk alleles may represent only 3-5% of the genetic burden of RA. These loci are PTPN22, PADI4, STAT4, TRAF1-C5 and TNFAIP3 (Plenge, 2009). Environmental factors, like smoking and infection, may also influence the development of RA and also affect the rate of progression and severity of RA (Klareskog *et al.*, 2007; Getts and Miller, 2010).

Signaling pathways and various immune modulators (cytokines and effector cells) are involved in the pathophysiology of RA (Smolen and Steiner, 2003; Choy, 2012). Post inflammation synovial lining becomes hyperplastic, and the synovial membrane expands and forms villi (Smolen and Steiner, 2003). The pathophysiology of RA is mediated by an interrelated network of cytokines, proteolytic enzymes and prostanoids. According to Smolen *et al.*, 2007 TNF-alpha, IL-6 and IL-1 are key mediators of cell migration and inflammation in RA. Particularly, IL-6 acts directly

on neutrophils through a membrane-bound IL-6 receptor (IL-6R), which contributes to inflammation and joint destruction by secreting proteolytic enzymes and reactive oxygen intermediates (Dayer and Choy, 2010). The role of IL-6 has been demonstrated (Figure-5) in RA patients, which promote neutrophil and activate fibroblasts (Lally *et al.*, 2005; Bing *et al.*, 2017). A study has established the critical role of mast cells in the pathogenesis of *Rheumatoid arthritis* (David and Weinbaltt, 2001).

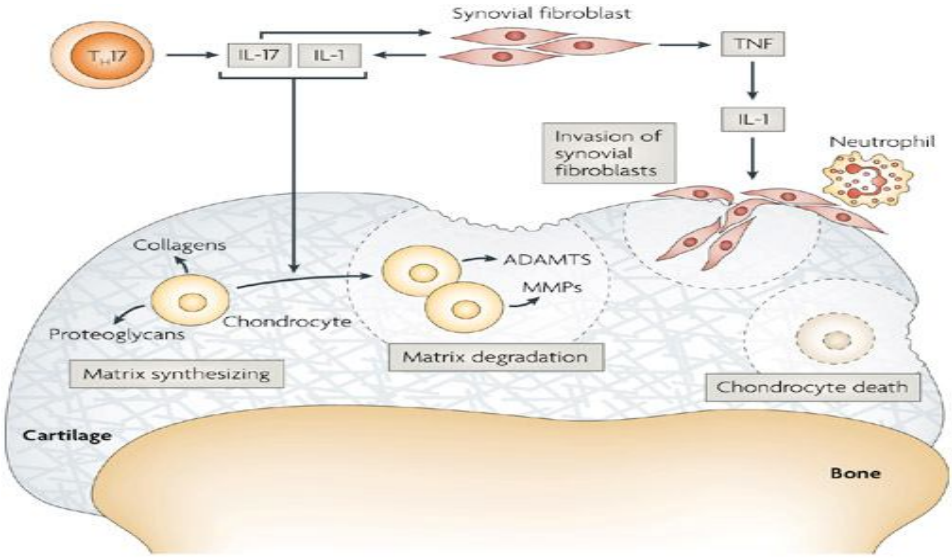


Figure-5: Role of IL-6 demonstration (Lally *et al.*, 2005)

2.8 Management of *Rheumatoid arthritis*

According to Sangha 2000, rheumatic diseases are a huge burden on the healthcare systems worldwide, and account for significant dysfunction, loss of productivity and reduction in quality of life (Sangha, 2000). Young *et al.*, 2006 has shown that approximately one-third of RA patients are disabled after 5 years of onset of the disease and patients have a substantial functional disability within 10 years (Young *et al.*, 2006). Therefore, RA imposes an important economic burden on society and lowers life expectancy.

Different cellular and cytokine targets have been identified in RA, which is targeted with specific inhibitors, including the tumor necrosis factor (TNF) antagonists, interleukin-1 (IL-1) antagonist, an inhibitor of T cell co-stimulation and a selective depletion of B cells (Bingham, 2008; Dinarello, 2010). New drugs are emerging that act as a competitive inhibitor of intracellular enzymes. Non-pharmacological treatments for *Rheumatoid arthritis* have also been tried, like therapeutic fasting, dietary supplementation of essential fatty acids, along with spa therapies and exercise**.**

After the onset of the disease, patients should immediately consult a rheumatologist for management of pain with appropriate physiotherapy, corticosteroids or disease-modifying anti-rheumatic drugs (DMARDs). DMARDs are the widely used as a therapy for RA. Drug therapy for RA sometimes also involve nonsteroidal anti-inflammatory drug (NSAIDs). Their route may be oral or intramuscular or intra-articular corticosteroids for controlling pain and inflammation. Ideally, NSAIDs and corticosteroids are for only short-term management of RA (Amy and Wasserman, 2011). DMARDs are the preferred therapy (Saag *et al.*, 2008; Deighton *et al*., 2009).

NSAIDs, salicylates, or cyclooxygenase-2 inhibitors are commonly used for initial treatment of *Rheumatoid arthritis,* to reduce joint pain and swelling. Rheumatologist recommends low dosage of steroids as these are highly effective for relieving symptoms of *Rheumatoid arthritis* and also reduce joint damage. In our study (Patel *et al*., 2015) occasional use of steroid has been shown to be helpful for control of the disease activity. Protein kinase inhibitors have also been developed, and are being called "molecular-targeting antirheumatic drugs" (MTARDs), as opposed to "disease-modifying anti-rheumatic drugs (Yamanaka *et al.*, 2013).

2.9 Serological and Biochemical Parameters

More sophisticated, effective and aggressive therapies are available, which can control the disease at an early stage by preventing irreversible damage. However, there is a need for the sensitive and specific serological parameters to diagnose RA at an early stage. The chronic condition of the disease requires, re-characterization of the pathological and physiological process using biomarkers which can change the future of medicine (Nass and Moses, 2007).

2.9.1 Serological parameters

2.9.1.1 Inflammatory protein: C-reactive protein (CRP), tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1) expression are high in synovial fluid and serum of *Rheumatoid arthritis* patients. CRP is an acute-phase protein produced by hepatocytes that stimulate the cytokines TNF- α, IL-6 and IL-1 (Hanna *et al.*, 2008; Shrivastava and Pandey, 2013). The increased CRP concentrations in serum samples of RA patients before the onset of RA, suggest the changes in the patients long before the actual disease ensue. According to Singh *et al.*, 2013 CRP may also mediate complement activation in RA.

Cytokines are important players that are responsible for inflammation and destruction of articular cartilage in RA subjects (Fox, 2000). IL-6 is the most abundantly expressed cytokine in RA patients, which regulate the biological activities like immune responses, inflammations and hematopoiesis. IL-6 stimulates antibody secretion by plasmacytes and promotes the multiplication of T and B cells (thus it is involved in the production of the rheumatoid factor). It further induces synthesis of acute-phase proteins such as C-reactive protein (CRP), haptoglobin, fibrinogen and serum amyloid-A, that regulates the multiplication and differentiation of osteoclasts and induce bone resorption (McInnes and Schett, 2007).

TNF-α is one of the pivotal pro-inflammatory cytokines responsible for inflammation and destruction of joint in RA. The two receptors of TNF-α (p55 and p75 TNFR) are readily detected in both synovial fluids of patients with RA (Ferrero *et al.*., 2001). The severity of RA correlates with the concentration of TNF-α in patients (Jenkins *et al*., 2002). TNF-α is a potent stimulator of mesenchymal cells, such as osteoclasts, synovial fibroblasts, and chondrocytes that release tissue-destroying MMPs. TNF-α also inhibits the production of tissue inhibitors of metalloproteinases (TIMPs) secreted by synovial fibroblasts. Its dual action is thought to lead to joint damage. Although, TNF-α and IL-6 have some overlapping and synergic actions, however, variety of the effects of these are regulated by different mechanisms (Rahman *et al*., 2005).

2.9.1.2 Erythrocyte sedimentation rate: The erythrocyte sedimentation rate (ESR) has been the widely used as the inflammatory marker in RA. According to Firestein *et al.*, 2009, it is is an indirect measure, reflecting the acute-phase plasma proteins level in the blood (e.g. fibrinogen) as these cause the red blood cells (RBCs) to settle rapidly. ESR levels can be greatly influenced by malignancies, infections, abnormally shaped or sized RBCs or serum protein concentrations (Kushner, 1991). The test is inexpensive and easy to perform, but ESR levels respond slowly to inflammatory stimuli and to changes in disease activity.

2.9.1.3 Autoantibodies: The first RA-associated antibodies are rheumatoid factors (IgG, IgM) (RFs). These antibodies bind to their receptor expressed in various leukocytes (Song and Kan, 2010) and activate their effector functions such as respiratory cellular burst, cytokine secretion, antibody-dependent cellular cytotoxicity and phagocytosis (Bolland and Raveth, 2000).

Anti-cyclic citrullinated peptide (anti-CCP) autoantibodies are produced in RA patients. In the inflamed region of synovium, anti-CCP autoantibodies are found to be accumulated. Anti-CCP antibodies have high specificity (89–100%) and sensitivity

(41–80%) for the diagnosis of RA. Anti-CCP can be detected in early RA in 40-60% of the RF-positive cases and is also present in 34.5% of the RF-negative cases particularly in the early phase of RA.

2.9.1.4 Role/Effects of Liver: Among patients with arthritis, hepatic involvement has been reported in cases of *Rheumatoid arthritis* (RA). The abnormal liver function may be due to the disease activity or therapeutic agent. The CRP is an acute phase protein, produced in the liver as a reaction to microbial pathogens or inflammatory changes (Firestein, 2009). It has been shown that 65% of RA patients have abnormal biopsies of the liver with one-half having mild portal chronic inflammatory infiltrate (of the portal tract and small foci of necrosis), and one in four reports fatty liver (Ruderman *et al*., 1997). Liver injury due to a drug is frequent in RA, especially with methotrexate (MTX) and nonsteroidal anti-inflammatory drug (NSAID) treatments. Liver histology demonstrates diffuse lymphocyte infiltrate, periportal fibrosis with lymphocytic infiltration and portal hypertension.

2.9.2 Biochemical parameters

Free radicals are defined as reactive species that contains an unpaired electron in an atomic orbital. These are highly reactive species, capable of damaging biologically relevant molecules and cellular components (Young *et al.,* 2001; Bansal and bilaspuri, 2011).

Box-3 Sources of free radicals (Ajuwon *et al*., 2015)

Endogenous sources	Exogenous sources
Mitochondrial electron transport chain	Radiation (UV light, X-ray and γ-radiation)
Neutrophils and macrophages during inflammation	Environmental pollutants and toxins
Xanthine oxidoreductase, NADPH oxidase	Cigarette smoke, alcohol, high-calorie diet
Microsomal oxidation in endoplasmic reticulum	Heavy metals
Myeloperoxidase (Phagocytes)	Infectious agent
Lipoxygenase, cyclooxygenases, prostaglandin synthase	Strenuous exercise

They can result from non-enzymatic reactions of organic compound having oxygen and ionizing reactions (Pham *et al.,* 2008). Basically divided into three types-

A) Reactive oxygen species (ROS) (Bansal and bilaspuri, 2011)
B) Reactive nitrogen species (RNS) (Dorge, 2011)
C) Reactive chlorine species (RCS) (Freidovich, 1999)

(http://www.bloodjournal.org/content/117/22/5816?sso-checked=true)

Figure-6: Common reactive oxygen species

Reactive oxygen species (ROS) are generated in the cells when stimulated by several physiological and environmental conditions such as infections, pollutants and ultraviolet radiation are collectively known as oxidants. Interestingly, ROS have also been considered as risk factors that stimulate the autoimmune diseases (Okamoto, 2005). Several studies have suggested that the reactive oxygen species (ROS) and oxidative stress are involved in the progression of RA (Kamanli *et al.,* 2004; Sezgin *et al.,* 2005). Oxidative components have the potential to damage biomolecules such as lipids, DNA and proteins in the affected tissues.

ROS are required to maintain the cell redox state and play an important role in cell signaling, differentiation, proliferation, apoptosis, cytoskeleton regulation, growth

and phagocytosis in physiological conditions. However, if the concentration of ROS increases beyond physiological concentrations they can damage cellular components, such as lipids, proteins and nucleic acids. The imbalance between oxidants and antioxidant levels cause disruption of redox signaling that is implicated in inducing damage. This cellular state is termed 'oxidative stress' (Filippin *et al*., 2008) which can result from an excess of oxidants or antioxidant deficiency or both (Valko *et al*., 2007).

In normal conditions, antioxidant defense system control and manage reactive oxygen and nitrogen species. The antioxidant system may work through enzymatic as well as non-enzymatic mediators. Among enzymatic components superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST) are important quenchers while non- enzymatic antioxidant defenses include vitamin A and C. According to Mitchell *et al*., 2003, there is equilibrium between free radical/reactive oxygen species formation and endogenous antioxidant defense mechanisms, but if this equilibrium is disturbed, it can produce oxidative stress (Mitchell *et al*., 2003).

2.9.2.1 Malondialdehyde (MDA): Overproduction of ROS increases oxidative stress that can be an important mediator of damage to membrane lipids, proteins and DNA, including cell structures (Valko *et al.,* 2007). The important targets of attack of ROS are the polyunsaturated fatty acids present in the membrane lipids leading to lipid peroxidation (LPO), ultimately resulting in disorganization of cell structure and function. Further peroxidized lipid decomposition, produces a wide variety of end-products, including malondialdehyde (MDA) (Gambhir *et al*., 1997). MDA estimation is widely used as an indicator of lipid peroxidation (LPO) (Romero *et al*., 1998; Siddique *et al.,* 2012). MDA has an important role in the pathogenesis of RA. Several studies have reported enhanced MDA in the plasma, serum and synovial fluid of RA patients (Kamanli *et al*., 2004; Gambhir *et al*., 1997; Pallinti *et al*., 2009; Mishra *et al*., 2012; Patel *et al*., 2015). Increasing evidences point towards the important role of

reactive oxygen species and free radicals in mediating cellular injury and tissue damage in *Rheumatoid arthritis.* Thiele *et al*., 2015 have reported malondialdehyde-acetaldehyde (MAA) adduct formation is enhanced in RA. These may result in robust antibody responses, which are strongly associated with anti-citrullinated protein antigens (ACPAs) suggesting that MAA formation may be a cofactor that drives tolerance loss, resulting in the autoimmune responses characteristic of RA.

Free radicals and other reactive species play an important role of super oxidant leading to oxidation of biomolecules like proteins, amino acids, lipids and DNA (Mirshafiey *et al*., 2008), which are ultimately responsible for cell injury and death (McCord, 2000).

2.9.2.2 Superoxide dismutase (SOD): Superoxide dismutase (SOD) is believed to play a key role in the enzymatic defense mechanism in the cell against oxygen toxicity (Petkau, 1986). Among, the actively generated ROS, superoxide anion (O_2^-**)** is the primary product that is liberated into the extracellular matrix as well as sequestered in lysosomes. Superoxide is then converted into hydrogen peroxide (H_2O_2) either spontaneously or catalytically by the catalase or glutathione reductase.

By increasing the concentrations of ROS, lipid peroxidation increases and this enhance the damage of tissues. Intracellular, localized Cu-Zn SOD scavenges the ROS and therefore, acts as an antioxidant enzyme. Several investigations have reported controversial activity of SOD in RA, with some reporting increased and some reporting decreased activity (Westermarck *et al*., 1987; Imadaya *et al*., 1988). According to Yasui and Baba, 2006, SOD acts as an endogenous cellular defense system in oxidative stress to degrade superoxide (O_2^-) into oxygen and hydrogen peroxide, which makes SOD as a potentially useful therapeutic agent for treatment of inflammatory disorders as RA (Yasui and Baaba, 2006).

2.9.2.3 Catalase: Catalase is an antioxidant enzyme ubiquitously present in mammalian and non-mammalian aerobic cells containing a cytochrome system. It was initially isolated from ox liver and later from blood, bacteria, and plant sources

(Deisseroth and Dounce, 1970). The enzyme contains four ferrihemoprotein groups per molecule. The enzyme has a molecular mass of 240 kDa. Catalase activity varies greatly between tissues. The activity is highest in the liver and kidney and lowest in connective tissues. In eukaryotic cells, the enzyme is concentrated in the subcellular organelles called peroxisomes microbodies (Zamocky and Koller, 1999).

Catalase catalyses the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen. Hydrogen peroxide is formed in the eukaryotic cell as a by-product of various oxides and superoxide dismutase reaction. Hydrogen peroxide is highly deleterious to the cell and its accumulation causes oxidation of cellular targets such as DNA, proteins, and lipids leading to mutagenesis and cell death (Bai *et a1*., 1999; Kowaltowski *et al*., 2000). Removal of the H_2O_2 from the cell by catalase provides protection against oxidative damage to the cell. Its role in oxidative stress-related diseases has been widely studied (Bai *et al*., 1999; Tome *et al*., 2001)

Catalase activity was not found in the serum of RA subjects. Low erythrocyte catalase activity is being reported (Taysi *et al*., 2002). The studies reported lower catalase activity in serum of RA patients (Janina *et al*., 2014; Kumar *et al*, 2016). Catalase expression affects the expression of genes which influence inflammation (Benhamou *et al*., 1998). Lower levels of catalase may be responsible for high inflammation in RA.

2.9.2.4 Glutathione reductase: Glutathione reductase (GR, EC1.6.4.2) is a flavoenzyme dependent on NADPH that catalyzes the reduction of the GSSH to GSH. Glutathione reductase (GR) is an oxidative stress-inducible enzyme, which plays a significant role in the peroxyl scavenging mechanism and in maintaining the functional integration of the cell membranes.

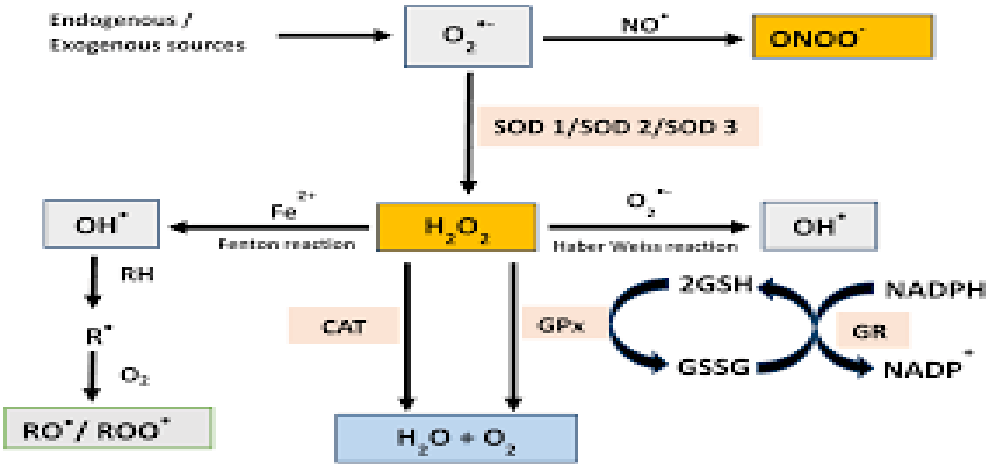


Figure-7: Generation of scavenging of free radicals. (Olawale *et al.*, 2015)

2.9.2.5 Lipid profile: Lipid levels appear to be altered as a result of RA disease activity. Data on total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels in RA patients are conflicting; some studies demonstrate similar (Park *et al.*,1999) or lower (Boers *et al.*, 2003) levels of total cholesterol, while others demonstrate increased levels of total cholesterol and LDL-C in patients with early RA (Georgiadis *et al.*, 2006). Although reports on lipid profiles in RA patients are varying, growing evidence suggests that patients with active untreated RA have reduced total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) levels (Boers *et al.*, 2003, Choy and Sattar, 2009, Myasoedova *et al.*, 2011). Regardless of the TC changes in RA patients, with a decrease in HDL-C, several studies support the notion that RA leads to a more atherogenic lipid profile (TC to HDL-C ratio) which is correlated with disease activity and improves after treatment with anti-rheumatic medications (Georgiadis *et al.*, 2006; Van *et al.*, 2006).

Inflammation is a common denominator in both RA and atherosclerosis. A growing body of evidence supports the involvement of common pro-inflammatory cytokines such as macrophage migration inhibitory factor (MIF), IL-1, IL-6, and

tumor necrosis factor-alpha (TNF-α) in the development and progression of both RA and atherosclerosis (Full *et al.,* 2009; Di Micco *et al.,* 2009).

There is a risk of CVD in RA, by lipid alteration is not completely understood. Recent evidence suggests that there may be a paradoxical effect of lipids on the risk of CVD in RA, where TC and LDL-C levels are associated with increased cardiovascular risk (Myasoedova *et al.,* 2011). Furthermore, although HDL-C is generally considered to be cardio-protective both through its ability to promote cholesterol efflux from artery cell walls and anti-inflammatory properties which protect LDL-C from oxidation. A growing body of evidence suggests that in inflammatory conditions such as RA and systemic lupus erythematosus (SLE), patients have non-productive "pro-inflammatory HDL" (piHDL) which promotes accumulation of oxidized phospholipids in LDL-C (Charles *et al.,* 2009).

Zinc and copper are constituents of anti-oxidative enzymes. Copper can act as an antioxidant and neutralizes free radicals that may help to prevent some of the damage caused by ROS (Araya *et al*., 2006; Davis, 2003; Rakel, 2007). Maintaining the proper dietary balance of Cu, along with other minerals such as zinc and manganese are important for management of the disease (Araya *et al*., 2006).

Their optimum concentration of minerals is required for normal functioning of the body. However, the alterations in level of these trace minerals as Mg, Zn and Cu (Copper) have been implicated in the pathogenesis of RA, as they are the co-factor of important enzymes involved in collagen and bone metabolism, the antioxidant defense system and the immune system. Copper and zinc are components of SOD. Copper is a cofactor of ceruloplasmin, which is an important antioxidant in serum (Honkanen *et al*., 1991). The development and progression of RA were suggested due to marginal deficiencies of Zn and Cu based on their serum levels (Simkin, 1976; Rainsford *et al.,* 1982). Many of these trace elements are present in the bones as iron,

copper, zinc, manganese, fluoride, strontium and boron (Sandstead *et al.*, 1998). These metals may help in determining of possible roles of these in disease activity of female and male RA patients.

2.10 Genetic Polymorphism

The occurrence of RA cases within close families has been a constant observation in many studies and can be estimated either as the sibling relative risk, or the relative risk to first degree relatives (parents, children, and siblings) (Frisell *et al*., 2016). Values for sibling relative risk or relative risk has been observed to exist between 2 and 10, consistent with an increased prevalence of RA within the families of affected persons as compared to the general population (Deighton and Walker, 1991; Del *et al*., 1984; Grant *et al*., 2001; Hemminki *et al.*, 2009; Frisell *et al*., 2016).

A common approach to estimate the inherited risk for developing RA is assessing the proportion of the variance of the disease which is explained by genetic variations (i.e., the genetic contribution to the disease), also called disease heritability (Frisell *et al.,* 2016). Multiple approaches have been proposed to analyze the heritability of RA, but all suffer from important shortcomings, leading to large variations in estimates.

Although the etiology of RA remains elusive, but genetic components are shown to increase the risk of developing RA from family studies. 60% or higher heritability is found in UK and Finnish populations (Aho *et al*., 1986; Mac Gregor *et al*., 2000), while a Danish study has published in 2002 found a heritability of 0% (Svendsen, 2002), revised to 12% when large cohort was used in a follow-up study in 2012 (Svendsen, 2013). The genetics of susceptibility to RA has already been covered in detail in many reviews (Kim *et al*., 2017; McAllister *et al*., 2011; Terao *et al*., 2016; Viatte *et al.*, 2013; Yamamoto *et al*., 2015; Yarwood *et al.*, 2016; Yarwood *et al.*, 2016). The most comprehensive meta-analysis of all available association studies

has now identified over 100 loci associated with RA susceptibility (Hollis *et al*., 2010; Okada *et al.*, 2014).

The human leukocyte antigen (HLA) class II molecules are most widely recognized as genetic risk/susceptibility factors for RA (Kampstra *et al.*, 2017). However, results of family studies suggest that this association accounts for only one-third of the genetic susceptibility, as non-HLA genes are also involved in disease susceptibility (Deighton *et al*., 1989). The genetic association studies have been implicating the human leukocyte antigen locus DRB1 (HLA-DRB1) as the principle genetic factor conferring risk of RA (Matzaraki et al., 2017).

The major histocompatibility complex (MHC) has been persistently associated with rheumatoid arthritis in different populations across the world. The MHC gene is located on chromosome 6p21.3, and spans over 3.6 MB (Klein *et al*., 2000). The MHC is a highly dense region containing ~200 defined HLA genes, which play an important role in immune function (Milner and Campbell, 2001). The HLA genes encode three distinct MHC classes as class I, class II and class III.

The class I components are encoded by the human leukocyte antigen (HLA) class I genes: HLA-A, HLA-B and HLA-C. HLA class I genes are expressed by all nucleated cells. They present antigens to CD8+ T cells, which are involved in cell-mediated immune response.

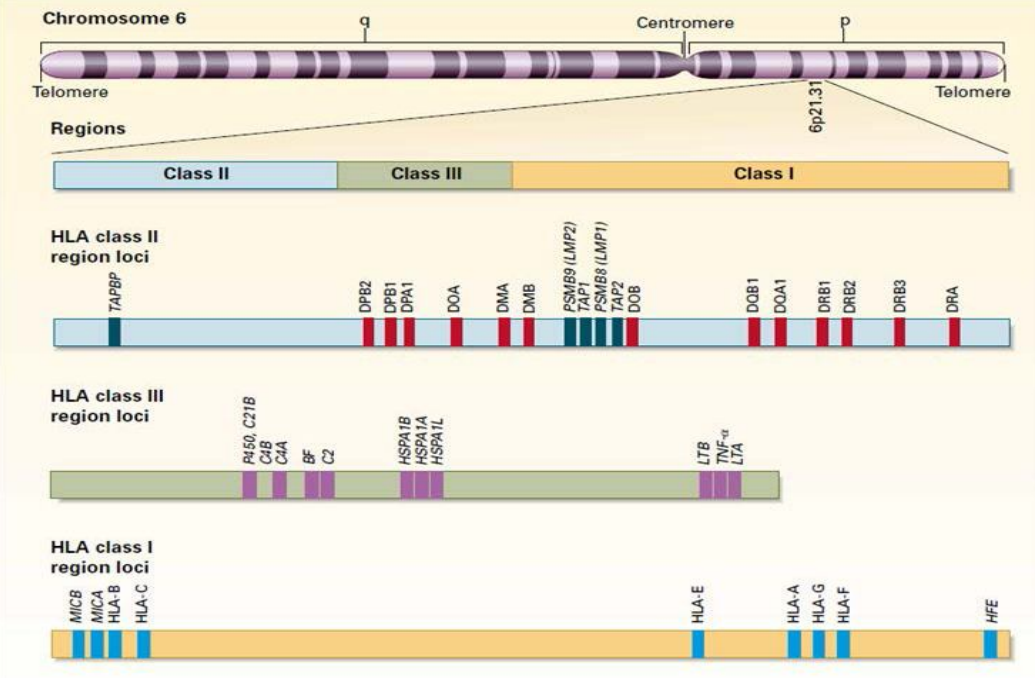
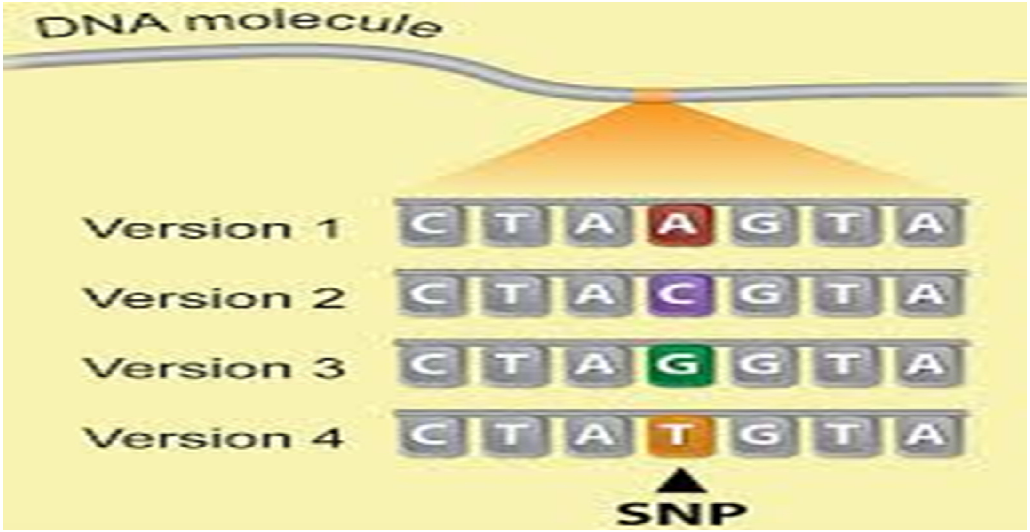


Figure-8: Figure shows the organization and location of the HLA complex on chromosome 6 (Klien *et al*., 2000)

Single Nucleotide Polymorphism (SNPs) are variations in DNA sequence that result when a single nucleotide (G, C, A or T) gets altered in the genome sequence. SNPs constitute about 90% of the variations in DNA sequence of the human genome. They frequently occur after every 100/300 bases along the 3 billion bases constituting the human genome. They are easier to type in population studies since they are evolutionary stable. These SNPs can influence the human response toward a disease or environmental adversities such as viruses, bacteria, toxic drug, chemical and other therapies. Many of these SNPs are present in the candidate genes conferring disease susceptibility and this has given way to numerous case-control studies in finding the association between a particular polymorphism and disease (Carlton, 2006). Thus they have emerged as genetic markers of choice owing to their even distribution and high density in the human genomes (Kruglyal, 1999; Sachidanadam *et al.,* 2001; Venter *et al.*, 2001). With the advancement of biotechnology, SNPs are increasingly being used in map-based cloning (Wang and Liu, 2006), Marker-assisted breeding (Flint *et al.,*

2003), study of evolutionary conservation between different species (Feltus *et al.*, 2004; Hillier *et al.*, 2007) and detection of risk associated alleles linked to human diseases (Eberle *et al.*, 2007).



(http://learn.genetics.utah.edu/content/precision/snips)

Figure-9 Single Nucleotide Polymorphism

2.10.1 Peptidylarginine deiminase 4 (PADI4)

The PADI4 gene encodes the type 4 peptidyl-arginine deiminase enzyme, which catalyses the posttranslational modification of arginine to citrulline, producing citrullinated proteins (Vossenaar *et al.*, 2004; Witalison *et al.*, 2015). Citrullinated epitopes are the most specific targets of RA-specific autoantibodies, well known as anti-citrullinated protein antibodies (ACPA), e.g., cyclic citrullinated peptide (CCP) antibody. A previous report has shown the presence of five isoforms in rodents: PADI I (Ishigami *et al.*, 2001), PADI II, PADI III (Nishijyo *et al.*, 1997), PADI IV (Ishigami *et al.*, 1998) and ePADI, which was provisionally named as PADI VI. PADI I have been identified in the epidermis (Guerrin *et al.*, 2003), PADI II in sweat glands, PADI III in the hair follicle, while PADI IV has been detected in the precursors of neutrophils and macrophages (Vossenaar *et al.*, 2004) and PADI VI mRNA has been detected in the testis, peripheral blood leucocytes and ovary.

The protein peptidyl arginine deiminase (PADI4) consists of 663 amino acid residues with a 74 kDa molecular weight (Luo *et al.*, 2006) and is the only isotope out of five described to be expressed in the cell nucleus (Nakashima *et al.*, 2002). PADI4 enzymes have diverse physiologic functions including aggregation of keratin during terminal differentiation in the epidermis (Senshu *et al.*, 1996), and gene expression regulation by chromatin modeling (Wang *et al.*, 2009).

PADI4 is a calcium-dependant enzyme. Therefore, its activation requires high cytosolic Ca^{+2} (2□μM) for citrullinated antigens to appear (Luo *et al.*, 2006; Yi-Liang *et al.*, 2017). Calcium ions induce conformational changes that create the active site in the catalytic domain of the enzyme. Intracellular calcium concentrations range from ~200□nM (resting cells) to ~1□μM (activated cells), calcium concentrations in the cytosol can be increased during apoptosis or necrosis, leading to PAD activation and protein citrullination (Stensland *et al.*, 2009).

The strongest genetic association of RA was observed in the HLA region on chromosome 6p21. This region extends over 3.6 MB, including the major histocompatibility complex (MHC) -class I, II, and III molecules, and contains many other genes with immunoregulatory functions. Previously, it was reported that HLA-DRB1 shared epitope (SE) alleles were associated with ACPA-positive RA, but not with ACPA-negative RA (Van *et al.*, 2004; Ding *et al.*, 2009).

The mechanism by which PADI4 genotype may influence RA susceptibility has not yet been annotated. Antibodies to these citrullinated peptides are extremely specific for RA and usually precede the development of disease, advocating their essential role in RA pathogenesis. PADI4 was the first non-HLA genetic risk factor known to be associated with RA, especially in Japanese population (Suzuki *et al.*, 2003). The association has also been observed in Korean and North American populations (Plenge *et al.*, 2005; Kang *et al.*, 2006). Studies in Spanish, Swedish and

UK populations provided no evidence for association of PADI4 with RA (Caponi *et al.,* 2005; Martinez *et al.,* 2005). A meta-analysis revealed a significant association between RA and the PADI4_94 SNP in Asian community (Takata *et al.,* 2008).

PADI4 may be considered as one of the strong loci for RA susceptibility (Mohamed *et al.,* 2018). It has been reported that a functional variant of the gene encoding PADI4 was associated with RA in Japanese individuals (Suzuki *et al*., 2003). RA-susceptible PADI4 haplotypic variant was shown to produce a more stable transcript than the non-susceptible variant, implying that the RA-susceptible variant enables increased production of cittrulline peptides, which also has been detected in RA synovial tissue (Suzuki *et al*., 2003). Suzuki *et al*., 2003 described 17 single nucleotide polymorphisms (SNPs), four of them located in a gene coding region of the exons 2–4 of PADI4. They found five haplotypes differing in four polymorphic sites; one denominated the susceptibility haplotype and was associated with RA. The SNPs involved is named RS188_1, RS188_2and PADI4 102; the first two determine an amino acid change, and the last one is a silent polymorphism (Suzuki *et al*., 2003; Vossenaar *et al*., 2004; Hoppe *et al*., 2006). In this same study, Suzuki *et al*., 2003 described that the functional haplotypes (RS188_1 and RS188_2) affected transcript stability, decreasing its degradation four times, and also demonstrated an association between haplotype homozygous individuals and ACPA positivity in patients with RA. In another study, this increase in PADI4 mRNA stability was confirmed when mononuclear cells of peripheral blood from patients with RA were analyzed (Harney *et al*., 2005).

2.10.2 Protein tyrosine phosphatase, non-receptor -22 (PTPN22)

PTPN22 gene is located on chromosome 1p13.3–p13.1 and encodes an intracellular tyrosine phosphatase (Canton *et al.,* 2005; Bin *et al.,* 2018). The best

associated genetic variant rs2476601, which affects amino acid 620, is an arginine (R) to tryptophan (W) missense polymorphism that alters the function of the protein (Rieck *et al*., 2007; Bottini *et al* 2006). 1858C->T single-nucleotide polymorphism (SNP) of protein tyrosine phosphatase non-receptor 22 (PTPN22) (rs2476601) is the best example of a non-HLA common susceptibility allele for autoimmunity (Siminovitch, 2004; Gregersen, 2005; Umemura *et al.,* 2016).

Lymphoid-specific phosphatase is suggested to be a negative regulator of T-cell signaling, as demonstrated in an animal model (Hasegawa *et al*., 2004) and in human cell lines (Bottini *et al*., 2004). The functional effect of the PTPN22 1858 C/T polymorphism on T-cells in human is yet to be demonstrated. The expression of Lyp protein is in other cell types: B-cells, monocytes, neutrophils, dendritic cells and natural killer cells (Bottini *et al*., 2004).

In a knockout mouse lacking the murine homologue of human PTPN22 (PES domain-enriched tyrosine phosphatase (PEP)), the threshold for T-cell receptor signaling was lowered and the number of effector and memory T-cells increased (Hasegawa *et al*., 2004). The knockout mice also showed an increased number of germinal centers and increased immunoglobulin levels, although autoantibodies were not detected in these animals. Changes in B-cell function were not found, suggesting that the abnormalities reflect a role of T-cell regulation of B-cell differentiation. SNP in PTPN22 (C1858T) change the amino acid which disrupts the binding of Lyp to an intracellular kinase, Csk (C-terminal Src kinase) which can then no longer inactivate another kinase, Lck (lymphocyte-specific protein tyrosine kinase), that is involved in T-cell signaling. The result of this missense mutation is a possible loss of negative regulation of T-cell signaling (Bottini *et al*., 2004).

The frequency of the associated PTPN22 risk variant rs2476601 differs among European individuals, showing a gradient of decreasing from northern to southern Europe, i.e. from 12.5% in the Swedish and Finnish to 2.5-7.4% in the Spanish and Italian populations, respectively (Gregersen *et al*., 2006). PTPN22 gene was first reported as a non-HLA RA risk factor in European populations, after an initial finding of the association with the related autoimmune disease type 1 diabetes (T1D) in 2004 (Begovich *et al*., 2004; Bottini *et al*., 2004). Till then the association with RA has been persistently documented in multiple ethnic populations of European descent (Plenge *et al*., 2005; Wesoly *et al*., 2005; Lee *et al*., 2005; Harrison *et al*., 2006).

The missense same SNP (C1858T) in the allele PTPN22 has recently been shown to be associated with 4 autoimmune diseases, RA (Begovich *et al*., 2004), SLE (Systemic lupus erythematosus) (Yogoku *et al*., 2004), autoimmune thyroid disease (Velaga *et al*., 2004) and type 1 diabetes milletus (Smyth *et al*., 2004).

2.10.3 Tissue inhibitor of metalloproteinases 4 (TIMP4)

Destruction of cartilage is a common pathological feature of *Rheumatoid arthritis* (RA). Cartilage destruction is the major cause of joint dysfunction, which results in impairment of the "quality of life" in these patients. Two pathways are followed for the destruction of the cartilage. Firstly, an intrinsic pathway by which chondrocytes themselves degrade cartilage extracellular matrix (ECM) and, secondly, an extrinsic pathway by which tissues or cells other than chondrocytes, such as inflamed synovium, pannus tissue, and infiltrated inflammatory cells, break down the ECM of cartilage. Most of the proteinases belonging to all classes of proteinases are expressed in joint tissues of patients with OA and RA. Among the proteinases, matrix metalloproteinases (MMPs) are believed to have a key role in the joint destruction in arthritis (Nagase *et al*., 1993; Nagase and Okada, 1996; Firestein, 1995). MMPs, a

gene family of neutral Zn^{2+} metalloproteinases, are composed of at least 18 members, which are classified into five subgroups (a) collagenases, including tissue collagenase (MMP-1), neutrophil collagenase (MMP-8), and collagenase-3 (MMP-13) (b) gelatinases such as gelatinase A (MMP-2) and gelatinase B (MMP-9); (c) stromelysins, including stromelysin 1 (MMP-3) and stromelysin 2 (MMP-10); (d) membrane-type MMPs (MT-MMPs), (Sato *et al*., 1994; Takino *et al*., 1995; Will & Hinzmann 1995; Pei 1999) including MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), MT4-MMP (MMP-17), and MT5-MMP (MMP-24); and (e) other MMPs such as matrilysin (MMP-7), stromelysin (MMP-11), metalloelastase (MMP-12), MMP-19, enamelysin (MMP-20), and MMP-23 (Bartlett *et al*., 1996).

Tissue inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors of MMPs and important regulators of ECM turnover (Brew and Nagase, 2010). They play an important role in tissue remodeling and growth, in both physiological and pathological conditions (Maria *et al.*, 2005). TIMPs are the endogenous inhibitors that regulate the MMPs (Brew and Dinakaran, 2000). Extracellularly, TIMPs inhibit MMP activity by forming high-affinity noncovalent complexes with MMPs (Visse and Nagase, 2003). The amino-terminal domain of TIMP binds the active site of MMPs, inhibiting their proteolytic activity while the carboxy-terminal domain of certain TIMPs can form a complex with the proenzymes (proMMPs) regulating the MMP activation process. The TIMP family consists of four distinct members (TIMPs 1to 4), TIMP-1 (Welgus *et al.*, 1979), TIMP-2 (Stetler-Stevenson *et al.*, 1989), TIMP-3 (Pavloff *et al.*, 1992) and TIMP-4 (Greene *et al.*, 1996). All of these, except TIMP-4 is expressed in most tissues and body fluids. TIMP-4 has a tissue-specific distribution, which is localized in brain, striated muscles, and ovaries and is also expressed in human heart and certain other tissues (Greene *et al*., 1996). The expression of TIMPs

is typically induced by external stimuli such as certain inflammatory cytokines (IL-6, IL-1β) and by certain growth factors. Tissue destruction is caused by several mechanisms, including the production of monokines and matrix metalloproteinases (MMPs) (Bresnihan, 1999). MMPs are the proteases that participate in the degradation and remodeling of the extracellular matrix.

The MMP: TIMPs ratio determines tissue damage in arthritis. Patients with RA have increased levels of MMPs, which are significantly higher, in the synovial tissues, than in the circulation (Ishiguro *et al*., 2001). According to Katrib *et al*., 2001, TIMPs are highly expressed in inflamed synovium during the onset of RA and high level of MMPs show an erosive effect in the early stage of RA (Cunnane *et al*., 2001). Importantly, high levels of MMPs have predictive value for the development of joint erosions in the early stage of RA.

2.11 Nuclear Magnetic Resonance

Many metabolites have been reported to play a role in *Rheumatoid arthritis*. Evaluation of these metabolites would be interesting to analyze the mechanism their possible role and open treatment options for *Rheumatoid arthritis*. Eicosanoids, fatty acids, lipids, trace elements, vitamins and several hormones are interesting candidates for elucidating the mechanism of RA. Pathway analysis may provide an indication of biological processes related to metabolite alteration in RA. The NMR profiles of synovial fluid were markedly different from their matched serum samples with high levels of lactate and low levels of glucose in the synovial fluid compared to the serum in RA patients. These changes were consistent with the hypoxic status of the rheumatoid joint (Naughton *et al.*, 1993). Serum from mice has been used to identify a metabolite biomarker pattern associated with RA (Weljie *et al*., 2007). Using NMR Weljie *et al*., 2007 found that uracil, xanthine and glycine could be used to distinguish

arthritis from control animals (Weljie *et al.*, 2007). The presence of the metabolites suggests that nucleic acid metabolism may be highly affected in RA and there may be an association with oxidative stress. More recently, a group in Denmark has looked at the plasma of RA patients (Lauridsen *et al.*, 2010), and found differences in the metabolites between RA subjects and healthy controls and differences between patients with active and controlled RA (Lauridsen *et al.*, 2010).

3. MATERIALS AND METHODS

3.1 Selection of Controls

The control group consisted of normal healthy persons of different age group of either sex. The normal healthy subjects were selected from medical college, staff members of college and departmental staff in age group of 23 to 49 years. Prior consent of the subjects was taken before including them in study. The study was started after approval from the Institutional ethical committee and written informed consent was obtained from all the participants.

3.2 Selection of *Rheumatoid arthritis* Subjects

The subjects that were affected with *Rheumatoid arthritis* were included in the present study. These subjects were in the age group of 23–49 years of either sex. Male or female was selected from outdoor patients department of a Vinod Dixit hospital, Kannauj and Community Health Centre (CHC) Shivrajpur. Patient's relatives and family friends having the disease were also included. Taking into account the inclusion and exclusion criteria, only those *Rheumatoid arthritis* subjects were included in the study, who had no other disease as cardiovascular, hepatic, renal, hypertension, osteoarthritis etc. Before including them in study their prior consent was taken. The patient history, physical activity and visual analogue score (VAS) were obtained by a personal interview of all the patients along with relevant clinical data and treatment history was collected using a health assessment questionnaire (HAQ). Only those patients were recruited for study that fulfilled 4 or above criteria of the American College of Rheumatology (ACR) (Arnett *et al.*, 1988).

3.3 Selection Criteria for the Study Population

3.3.1 Inclusion criteria

The selected *Rheumatoid arthritis* patients were in the age group of 23-49 years. The primary inclusion criteria were definite RA fulfilling 1987 ACR criteria and disease duration of ≥6 months. Their hemoglobin estimation, ESR estimation and agglutination based RA antibody detection was done apart from X-rays finding. For the control subjects the age group 23-49 years were taken. Controls were free from any disease related to articular cartilage, bone, liver or endocrine system.

3.3.2 Exclusion criteria for both the groups

Subjects with known infectious disease, diabetes mellitus, hypertension, thyroid dysfunction, neurological disorders, cancer and any other forms of arthritis were excluded from the study.

3.4 Collection of Blood Sample

Blood was drawn from overnight fasting patients for all the analyses. Fasting heparinised 5.0ml blood of each subject was collected early in the morning before the breakfast from the median vein of the forearm. A part of blood was used for ESR analysis and rest 4.0 ml blood without delay was centrifuged for 10 minutes at 15,000 rpm in the refrigerated centrifuge machine (0-5ºC) so as to collect plasma**.** Out of 67 samples (41 females, 26 males) 51 were tested positive for RF factor. 80 asymptomatic independent controls (48 females, 32 males) were recruited from local clubs, neighbourhood and volunteers.

3.5 Clinical Criteria for Selection

Controls were asymptomatic (painless, no criptation, no decrease of joint space on X-ray, non obese and without any other systemic disease) and independent of the patients. They were monitored for liver function test at every six months. The

follow up after 3 years for blood pressure and liver function test was done. Clinical characteristics of patients included symmetric arthritis with complaints of severe multiple joint pain along with morning stiffness (>1hr) of joint, presence of rheumatoid nodules along with radiographic changes like erosion, swelling (>3 joint, especially phalanges), multiple joint involvement and deformity of peripheral joint (metacarpophalangeal (MCP) and proximal interphalangeal joint (PIP) and decreased range of motion. All the patients had normal ligament stability.

Patients were recommended MTX (15mg once a week) along with folic acid (1mg OD) and vitamin C to alleviate symptoms. Patients were given the local steroid (triamcinolone acetonide 0.5ml) whenever they complain about swelling with the existing treatment (usually at the change of season) for the swollen joints. The usual requirement was 4-6 times /year. The patients did not have any renal disease and were non hypertensive.

3.6 Demographic Parameters

3.6.1 Body mass index (BMI)

Body mass index is a measurement which determines weight category of a person depending on their height and weight, a person can belong to one of the following weight categories (James, 2002). BMI is calculated as

BMI = (weight in kilograms) /height in meters^2

Underweight	BMI less than 18.5
Normal weight	BMI between 18.5 & 24.9
Overweight	BMI between 25.0 & 29.9
Obese	BMI 30.0 and above

3.6.2 Blood pressure (BP)

Blood pressure is a measurement of the force on the walls of the arteries as the heart pumps blood through the body. Normal BP range is 80-120.

3.7 Clinical Variables

3.7.1 Hemoglobin

Hemoglobin was estimated by taking 1/10 HCl in the Hb tube. The finger was pricked with needle and 20µl of blood sample was collected with a single mark pipette. The Hb tube in the hemometer was placed and N/10 HCl was added drop by drop until the colour of the solution in the Hb tube coincides with the glass plates of the hemometer. If the colour coincides with the glass plates of the hemometer, the reading was observed and recorded from the Hb tube. Normal values within different individuals are as follows (Sahli's method):

Males	14 to 18 gm/dl
Females	13 to 14 gm/dl

3.7.2 Erythrocyte sedimentation rate (ESR)

ESR was estimated within an hour of collection of blood by Westergren method (ICSH recommendations). The blood with anticoagulant was thoroughly mixed by using Pasteur pipette, and the Wintrobe's tube was filled upto '0' mark to avoid any gas bubbles in the blood. The tube was placed in ESR stand and left undisturbed for 1 hour. After 1 hour, results were recorded (Sinton, 1958; Terry, 1950).

3.7.3 C- reactive protein (CRP)

Qualitative analysis was done using all reagents at optimum temperature as the sensitivity of the test is reduced at low temperatures. 50 µl of the sample and one drop of each positive and negative control were placed in separate circles on the test slide. CRP-latex reagent is swirled gently before use and one drop (50 µl) was added next to

the samples to be tested. The drops were mixed with a stirrer and spread over the entire surface of the circle. Different stirrers are used for each sample. The slide was placed on a mechanical rotator at 80-100 rpm for 2 minutes. False positive results could appear if the test is observed after two minutes. Microscopically the presence or absence of visible agglutination was observed immediately after removing the slide from the rotator. The presence of agglutination indicates a CRP concentration equal or greater than 6 mg/l. CRP quantitative estimation was done by commercial kit from Merck

3.7.4 Qualitative estimation of rheumatoid factor (RF)

Qualitative determination of rheumatoid factors was done by EURO Diagnostic kit based on latex agglutination. 50 µl of the sample and one drop of each positive and negative control were placed in separate circles on the slide test. The normal temperature RF latex reagent was swirl gently before using and one drop (50 µl) was added next to the sample to be tested. The drops were mixed with a stirrer, with spreading them over the entire surface of the circle. Different stirrers were used for each sample. The slide was placed on a mechanical rotator at 80-100 rpm for 2 minutes. False positive results could appear if the test is read after two minutes.

3.7.5 Disease activity score (DAS28)

It was calculated using the universally accepted formula:

DAS 28= 0.56*√ (Number of tender joints) + 0.28*√ (Number of swollen joints) + 0.7* In (ESR: 1hour) + 0.014*VAS.290

A swollen and tender joint examination was performed for each patient. The observations of each affected joint were filled in Form A. When complete, all of the swollen and tender joints were added and recorded in the appropriate boxes on Form B. The patients' erythrocyte sedimentation rates were recorded in mm/h in the

appropriate box on Form B. The patient's general health was recorded on a visual analog scale (VAS) of 10 mm in the appropriate box on form B and DAS 28 score was calculated. DAS28 score > 5.1 is indication of high disease activity, whereas a DAS28 < 3.2 indicates low disease activity. DAS 28 score in between 3.2 to 5.1 indicates moderate activity. A patient is considered to be in remission if they have a DAS28 < 2.6.

3.7.6 Visual analog scale (VAS):

The visual analogu scale (VAS) consists of a ruler with 'no pain at all' at one/starting point and 'pain as bad as it could be' at the other side with maximum score (Burckhardt and Jones, 2003). The patient is asked to mark his pain level on the line between the two endpoints by performing some fixed activity as holding and balancing objects. The distance between the VAS scales, then defines the subject's pain. This tool was first used in psychology by Freyd, 1923. Jensen, 1986, proposed ruler based estimation, where the score is analyzed by measuring the distance (mm) on the ruler with 0 as "no pain" and 10 as "intolerable pain" while doing some physical activity.

3.7.7 Liver function test

3.7.7.1 SGOT : SGOT is performed by IFCC method kit (Coral clinical system-Tulip group India). Normal reference values for males were upto 37 U/L at 37°C and females were upto 31 U/L at 37°C.

3.7.7.2 SGPT: SGPT is performed by IFCC method kit (Coral clinical system-Tulip group India). Normal reference values for males were upto 37 U/L at 37°C and females were upto 31 U/L at 37°C.

3.8 Biochemical Analysis

3.8.1 MDA (Malonaldehyde) Level

Serum MDA level was estimated as per the method of Satoh, 1978. 200 µl of serum was added to 300 µl of trichloroaceticacid (TCA)-thiobarbituric acid (TBA)-hydrochloric acid (HCl) solution. The composition for the TCA-TBA-HCl solution was 15% (w/v) TCA, 0.375% (w/v) TBA and 1M HCl, which were mixed in equal volumes. Reaction mixture was incubated in a boiling water bath for 10 minutes. After incubation, the reaction mixture was cooled completely Centrifuged at 2000 rpm at 15 °C for 20 minutes. 100 µl of protein free-supernatant was pipette out and 25 µl of 1 M sodium hydroxide was added to eliminate the white precipitate formed. Normal saline was used instead of serum as blank. The absorbance of the reaction mixture was measured at 535 nm, and results were expressed as µmol/L/mg protein, using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

$$MDA\ (\mu mol/l) = OD_{535} \times 1.75 / 0.156$$

$O.D._{535}$ (optical density in λ) =532 nm and extinction = $1.56 \times 10^5\ M^{-1}\ cm^{-1}$

3.8.2 Catalase activity assay by the method of Sinha, 1972

Reagents required are dichromate/acetic acid. (5% potassium dichromate (w/v) 50 ml and 98 – 100% glacial acetic acid (w/v) 150 ml), hydrogen peroxide (0.2M) solution, phosphate buffer (0.01M) at, pH 7.0. For sample preparation, haemolysate was diluted with 20 parts of cold distilled water and used as such. 4ml of H_2O_2 solution was taken in a small beaker in which 5ml of phosphate buffer was added, 1ml of properly diluted sample (haemolysate) was mixed rapidly with gently swirling motion. 2ml of dichromate/acetic acid reagent was taken in test tubes, labeled as 1,2,3,4 1ml of reaction mixture made previously was added to the test tubes containing dichromate/acetic acid reagent at an interval of 60 second for each tube.

O.D. at 570nm of four tubes (1, 2, 3 and 4) was read. Further for standard curve preparation, different amount of H_2O_2 ranging from 40–160 µmoles was taken in small test tubes in increasing order of concentration like 40, 80, 120 & 160 µmoles. 2ml of dichromate acetate was added to each and unstable blue precipitate of per chromic acid was obtained. It was heated for 10 minutes in a water bath, which changed color of solution to green, due to the formation of chronic acetate. Thereafter, it was cool at room temperature and volume of the reaction mixture was made to 3ml by distilled water. Absorbance was read at 570 nm and standard curve was plotted between O.D. and amount of H_2O_2. The activity of catalase was expressed as µmoles of hydrogen peroxide consumed/min/gm Hb or units/gm Hb and calculated by using calibration curve.

3.8.3 Superoxide Dismutase (SOD) activity assay by Mishra and Fridovich, 1972

Reagents required are epinephrine or adrenaline (1.8mM), Sodium carbonate buffer (0.3M, pH=10.2) (Na_2CO_3 + $NaHCO_3$), 0.6 mM of EDTA (ethylene di-amine tetra acetic acid). Sample was prepared by mixing 0.1ml RBC Hemolysate and 0.9ml T.D.W. In a test tube 0.5ml diluted sample was taken, to which 0.5ml sodium carbonate buffer (0.3M, pH 10.2), 0.5ml EDTA (0.6mM), 1.0ml triple Distilled H_2O and 0.5ml epinephrine (1.8mM) was added. Increase in absorbance at 480 nm was measured every 30 seconds till 2.5 minutes. For blank in a test tube 0.5ml buffer (sodium carbonate) was taken 0.5ml sodium carbonate buffer (0.3M, pH 10.2), 0.5ml EDTA (0.6mM), 1.0ml triple Distilled H_2O and 0.5ml epinephrine (1.8mM) was added. Instead of sample, 0.5 ml buffer (Sodium carbonate) was added. Increase in absorbance at 480nm was measured every 30 seconds till 2.5 minutes.

Calculations were done as follow:

$$\text{Specific activity of enzyme (SOD)} = \frac{\text{Units per ml enzyme}}{\text{Hb gm/dl}}$$

Unit per ml enzyme = 50% inhibition/0.1 X 50

$$\text{Percentage inhibition (\%)} = \frac{X \times 100}{A}$$

50% inhibition = 100% inhibition/2

Where,

x = O.D. change in experimental reaction–O.D. change in control/blank reaction

A = O.D. change in experimental reaction

3.8.4 Glutathione reductase activity assay by Bergmeyer, 1963.

Reagents required were phosphate buffer (0.1M; pH 7.5) (Na_2HPO_4 + KH_2PO_4), 0.2M GSSG (oxidized glutathione), NADPH (0.12mM). For sample preparation, 1ml RBC hemolysate + 0.9ml T.D.W. i.e. 1:10 dilution was taken. In a test tube phosphate buffer (2100µl), GSSG (300µl), diluted haemolysate (300µl), NADPH (300µl) were added, then O.D. at 340nm was recorded.

Calculations were done as follow:

$$\text{Specific activity of GR} = \frac{\text{OD Change Per Minute}}{6.3 \times 10^3} \times \frac{\text{ml of reaction mixtre}}{\text{ml of sample volume}} \times 10^6$$

3.8.5 Alkaline phosphatase (ALP)

The assay of the ALP was carried out using commercially available kit (span Diagnostics Limited, India). The principle of the method is that alkaline phosphatase converts phenyl phosphate to inorganic phosphate and phenol at pH l0. Phenol so formed reacts in alkaline pH with 4-aminoantipyrine in the presence of the oxidizing agent potassium ferricyanide and forms an orange-red colored complex, which can be measured colorimetrically. The color intensity is proportional to the enzyme activity.

3.9 Trace Metals

3.9.1 Copper

Copper level was estimated by colorimetric method, Copper, released from ceruloplasmin in an acidic medium, reacts with Di-Br-P AESA to form a coloured complex. Intensity of the complex formed is directly proportional to the amount of copper present in the sample. Normal range of males was 80- 140 µg/ dl and females was 80- 155µg/dl.

$$\text{Copper} + \text{Di-Br-PAESAb} \xrightarrow{\text{Acidic Medium}} \text{Coloured complex} \qquad \text{Eq. ..3.1}$$

3.9.2. Magnesium

Level of magnesium was estimated by Calamagite method, Magnesium combines with calmagite in an alkaline medium to form a red coloured complex. Interference of calcium and proteins is eliminated by the addition of specific chelating agents and detergents. Intensity of the colour formed is directly proportional to the amount of magnesium present in the sample. Normal range in serum of adults is 1.3-2.5 mEq/L.

$$\text{Magnesium} + \text{Calamagite} \xrightarrow{\text{Alkaline medium}} \text{Red colour complex} \qquad \text{Eq. ..3.2}$$

3.9.3 Zinc

Colorimetric method was used for the estimation of Zinc that reacts in an alkaline medium with Nitro-PAPS to form a purple coloured complex. Intensity of the complex formed is directly proportional to the amount of zinc present in the sample. Normal range in serum is 60-120 µg/dl.

$$\text{Zinc} + \text{Nitro-PAPS} \xrightarrow{\text{Alkaline medium}} \text{Purple colour complex} \qquad \text{Eq. ..3.3}$$

3.9.4 Phosphorous

Phosphorous level was estimated by Molybdate U.V. method, phosphate ions in an acidic medium react with ammonium molybdate to form a phosphomolybdate complex. This complex has an absorbance in the ultraviolet range

and is measured at 340 nm. Intensity of the complex formed is directly proportional to the amount of inorganic phosphorus present in the sample. Normal range of serum of adults is 2.5 - 5.0 mg/dl.

Phosphorous + Ammonium molybdate $\longrightarrow$ Phosphorous molybdate complex Eq. ..3.4

3.10 Lipid Profile

3.10.1 Estimation of serum total cholesterol

Span diagnostic kit was used for the estimation of total cholesterol, which followed cholesterol oxidase/peroxidase (CHOD-POD) method. The enzyme, cholesterol esterase catalyzed hydrolysis of cholesterol esters to free cholesterol and fatty acid molecules. Then free cholesterol gets oxidized in the presence of cholesterol oxidase to form cholest-4en-3-one and H_2O_2. Liberated H_2O_2 reacts with phenol and 4 AAP in presence of peroxidase to form red colored quinoneinine complex the intensity of which was measured at 505 nm.

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol ester hydrolase}} \text{cholesterol} + \text{Fatty acid}$$

Eq. ..3.5

The 3-OH group of cholesterol is then oxidized to ketone in oxygen requiring reaction catalyzed by cholesterol oxidase.

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{cholesten} - 3 - \text{one} + H_2O_2$$

Eq. ..3.6

H_2O_2 one of the reaction products is measured in a peroxidase catalyzed reaction that forms a dye.

$$2H_2O_2 + \text{Phenol} + \text{AAP} \xrightarrow{\text{Peroxidase}} 4H_2O + \text{Quinoneimine dye}$$

Eq. ..3.7

3.10.2 Estimation of triglycerides

The span diagnostic kit was used for estimation of triglycerides, which followed end point calorimetry enzymatic test using glycerol-3-phosphate oxidase**.** The enzyme, lipoprotein lipase catalyzes hydrolysis of triglycerides to glycerol and fatty acids. Glycerol is then phosphorylated in an ATP-requiring reaction catalyzed by glycerophosphate oxidase. The formed glycerophosphate is oxidized to dihydroxyacetone and H_2O_2 in a glycerophosphate oxidase catalyzed reaction. H_2O_2 then reacts with 4-AAP and 4 -chlorophenol under the catalytic influence of peroxidase to form colored quinoneimine complex, the intensity of which was measured at 505nm.

$$\text{Triglyceride} + 3\,H_2O \xrightarrow{\text{Lipase}} \text{Glycerol} + 3 \text{ fatty acids} \qquad \text{Eq. . ..3.8}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerokinase}} \text{Glycero –3-phosphate} + \text{ADP} \qquad \text{Eq. ..3.9}$$

$$\text{Glycero –3-Phosphate} + O2 \xrightarrow{\text{Glyccerophospate oxidase}} \text{DHAP} + H_2O \qquad \text{Eq. ..3.10}$$

$$2\,H_2O_2 + \text{4-AAP} + \text{4 -chloro phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + HCl + 4\,H_2O \qquad \text{Eq. ..3.11}$$

3.10.3 Estimation of LDL cholesterol

LDL cholesterol was calculated by using the formula

LDL cholesterol=Total cholesterol –HDL cholesterol –triglyceride

LDL cholesterol level in plasma was expressed as mg/dl.

3.10.4 Estimation of HDL cholesterol

HDL, VLDL and chylomicron fractions are precipitated by additiom of PEG-6000. After centrifugation, the HDL fraction remains in the supernatant and was estimated using with CHOD-PAP method.

3.10.5 VLDL Cholesterol

VLDL Cholesterol was calculated by the formula

VLDL Cholesterol = Triglyceride /5

3.11 Statistical Analysis

The values of two independent groups were compared by one way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test. Before performing ANOVA, the homogeneity of variance among groups was tested by Hartley F max, Cochran C, and Bartlett v2 tests. Association among variables of both the patient groups was done by Pearson correlation analysis. The proportion of sex (male and female) among two groups was compared by v2 test. A two-tailed (a = 2) P\0.05 was considered to be statistically significant. Graph pad prism (version 3.0) and STATISTICA (version 6.0) were used for the analysis.

3.12 DNA Isolation

DNA isolation from blood tissue was done by using a standard phenol-chloroform protocol (Somasundaram *et al*., 2002). Blood (600µl) and RBC lysis buffer were taken and mixed well and incubated at room temperature for 30 min or 56^0C for 10 min. After incubation, the content was centrifuged at 14000 rpm for 10 min at 4^0C. The supernatant was discarded and the process was repeated till white pellet was obtained. The white pellet was added 500µl water and centrifuged for 5min. The supernatant was discarded. To the pellet 200µl proteinase-K and 10µl 10% SDS was added and froth was made by repeated pipetting. 100µl 5N NaCl added to it and mixed by soft hand taping. To it 200µl water was added and the content was mixed by inverting 2-3 times. Then 400µl tris-saturated phenol and 100µl Chloroform (In 4:1and mix well) was added and mixed. They were centrifuged for 10min at 14000rpm. Three layers appeared to be an upper aqueous upper layer was taken and

precipitation was done by adding 1ml absolute alcohol. The content were centrifuged for 10 min at 10000 rpm at 4^{o}C. The pellet collected was washed twice with 500 µl 70% ethanol. The tubes were inverted and allow drying at room temperature. The DNA was dissolved in TE and stored at -20^{o}C. 1% TAE agarose gel uses to check the DNA.

3.13 DNA Concentration Measurement

A 1µl aliquot of the DNA in TE buffer was diluted with 995µl of sterile deionised water (1:200) in a 1.5 ml micro centrifuge tube. The diluted sample was vortexed and left overnight at 4°C and then at room temperature for at least four hours before determining the concentration. Shimazdu spectrophotometer UV-1800 was used to estimate the DNA concentration of each sample. The spectrophotometer was set to measure absorbance at 260 and 280nm. The correction factor was used for the final concentration of DNA in the sample

3.14. Check Gel Analysis for the Quality of DNA

DNA (2µl) was added to 8µl of loading buffer composed of 0.25% bromophenol blue (Sigma- 0.25 mg) in 40% sucrose solution. The mixed sample (10 µl) was placed into the wells of the TAE agarose gel. The electrophoresed gel was analyzed on to a transilluminator (Uvtec SXT 20M) and viewed using ultra-violet light (254 nm wavelength). A protective face shield (Oberon UV absorbance) was used when viewing the UV transilluminated gel. Nitrile gloves were worn during preparation and handling of the gel.

3.15 PCR Amplification

The DNA was amplified using polymerase chain reaction (PCR) has indicated for different genes. PTC 100 thermal cycler was used to amplify template DNA in the experiments. Specific PCR primers and protocols for PADI4 (peptidylarginine deiminase 4), PTPN22 (protein tyrosine phosphatase 22) and TIMP4 (tissue inhibitor

metalloproteinase 4) were used to amplify the template DNA. PADI4, PTPN22 and TIMP4 PCR products were digested with appropriate restriction enzymes before electrophoresis. The PCR products of PADI4, PTPN22 and TIMP4 were electrophoresed on 2% agarose gel for 45 min. The digested PCR products of PADI4, PTPN22 and TIMP4 were genotyped with 3% agarose gel for 50 min.

3.16 PCR Protocols

The PCR protocols used for the analysis of all three genes were from the literature with some necessary modifications.

3.17 Primers

Both the reverse and forward primers were obtained from Invitrogen Life Technologies and Amersham Pharmacia Biotech in a lyophilised form and reconstituted to 100 µM stock solution in deionised water. A 1:10 dilution of the stock solution was made in deionised water and the resultant 10 µM solution was aliquoted in 50 µl volumes and stored at -20°C before use. The 10 µM aliquots of primers were preferably used within six months. Thawing and re-freezing cycles were kept to a minimum to reduce denaturation of primers.

3.17.1 PADI4

We examined the SNPs RS188_1 (position 456724), RS188_2(position 456806) one intronic PADI4 SNP padi4_102 (17546809C/T on chromosome 1, GenBank rs2240337). Restriction fragment length polymorphism (RFLP) genotyping was carried out in the RA and control. The following primers were used for the PCR. For RS188_1 forward primer was F 5-GTG TGG CTG AAA TGC AGT GAG GTA-3 and Reverse was 5-CTC CAG GCT CCC CAC GTT ACT T-3, for RS 188_2 forward primer was 5-GGG TCC CCT ACA GTC TGT TCT-3 and reverse primer was P5R 5-CCA GTG CAA TCG GTA CAA AG-3 and for PADI_102 the forward primer was 5-CTG GCC CAG GCA CCA CCA G-3 and reverse primer was 5-AGG GTT TCG

GCA GCT GTG CC-3 (Caponi *et al.,* 2005). Annealing temperature were 57.2, 59.8 and 54.0 respectively. PCR products were digested by enzymes RsaI, NIaIII and RsaI respectively (Thermo scientific Third Avenue Waltham, MA USA).

3.17.2 TIMP4

The polymorphism of TIMP4 A/G was detected by PCR using forward primer 5-ATG GCT GGC AAA GAA TAG A-3 and reverse primer 5-TGG GAT GAG AAA GCA ATA C-3. The polymorphism of TIMP4 C/T (rs 17035945) was detected by PCR. Sequence of primer used for amplification was forward primer 5-ATGATGCTGTCAAACCACCT-3 and reverse primer as 5-CTCCCAAACCC CCA TTAG TCT-3 (Lee *et al.*, 2008). The PCR product was digested by HpyCH4III (Thermo scientific Third Avenue, Waltham, MA USA). HpyCH4III-digested PCR products showed the genotype specific DNA fragments: 222bp (genotype T/T) 222bp, 194bp and 28bp (genotype T/C); 194bp and 28bp (genotype C/C).

3.17.3 PTPN22

Genotyping of the PTPN22-1858C/T SNP was performed by PCR-restriction fragment length polymorphism. The forward and reverse primers were, respectively, 5'-GATAATGTTGCTTCAACGGAATTT-3' and 5'-CCATCCCACACTTTATTTT ATACT-3' (Dieude *et al.,* 2005). The PCR products were digested with enzymes RsaI and XcmI. The digested product was checked on 3% TAE-agarose gel.

3.18 NMR

3.18.1 Sample Preparation

In each case, the 3.0 ml of blood sample was drawn and processed to extract the serum as per the established protocol. The extracted serum was transferred into a sterile 1.5 ml microcentrifuge tube (MCT) and stored at −80 °C immediately after the processing until the NMR experiments were performed. All serum samples were

thawed and centrifuged at 10,000 rpm for 5 minutes to remove precipitates just before acquiring the NMR data. A total 400µl of sample was used in 5 mm NMR tubes (Wilmad Glass, USA) for data acquisition:200 µl of serum was adjusted to a final volume by adding 200 µl of 0.9% saline sodium phosphate buffer of strength 20 mM and pH 7.4 prepared in D_2O and adding a co-axial insert containing the known concentration of TSP (Sodium salt of 3-trimethylsilyl-(2,2,3,3-d4)-propionic acid) i.e. 0.1% was used as external standard reference to aid metabolite quantification for NMR experiment. Deuterium oxide (D_2O use as a co-solvent and to provide a deuterium field/frequency lock) and the sodium salt of trimethylsilylpropionic acid-d4 (TSP) used for NMR experiments were purchased from Sigma-Aldrich (Rhode Island, USA).

3.18.2 NMR measurements

All NMR spectra were recorded at 298 K on Bruker Biospin Avance-III 800 MHz NMR spectrometer operating at proton frequency of 800.21 MHz, equipped with CryoProbe and an actively shielded gradient unit with a maximum gradient strength output of 53 G/cm. The raw NMR data were processed in Topspin-2.1 (Bruker NMR data Processing Software). For each serum sample, two types of 1D ^1H NMR spectra were recorded: (a) transverse relaxation-edited CPMG (Carr–Purcell–Meiboom–Gill) spectra and (b) diffusion-edited bipolar pulse pair longitudinal eddy current delay (BPP-LED) spectra. The 1D ^1H CPMG NMR spectra were recorded using the standard Bruker's pulse program library sequence(cpmgpr1d) with pre-saturation of the water peak through irradiating it continuously during the recycle delay (RD) of 5 sec. Each spectrum consisted of the accumulation of 128 scans and lasted for approximately 15 minutes. Each FID (free induction decay) was zero filled and Fourier-transformed to 64 K data points following manual phase and baseline-

correction using Bruker NMR data Processing Software Topspin-v2.1. A line broadening factor of 0.3 Hz and a sine–bell apodisation function was applied to FIDs before Fourier Transformation. After FT, the chemical shifts were referenced internally to methyl peak of L-lactate (at δ=1.33 ppm). All recorded spectra were, visually inspected for acceptability and subjected to multivariate statistical analysis to identify the altered metabolic patterns.

3.18.3 Identification of metabolite peaks

Chemical shifts were identified and assigned as far as possible, by comparing them with the chemical shifts available with the open access software program MetaboMiner with tolerances of 0.05 ppm (^{1}H) and 0.1 ppm (^{13}C). The metabolite peaks were identified if there was only one candidate in the database within the specified tolerances for an observed peak and its correlated shifts. The metabolite peaks in one-dimensional ^{1}H CPMG NMR spectra were identified and assigned as far as possible, by comparing them with the chemical shifts available with the software Chenomx (NMR Suite, v8.1, Chenomx Inc., Edmonton, Canada). The assigned resonances of the metabolite peaks were validated using: (a) previously reported NMR assignments of metabolites, data obtained from BMRB database (Biological Magnetic Resonance Data Bank) and HMDB (The Human Metabolome Database) and (b) Assigned resonances in two-dimensional spectra. For unambiguous assignment of the various peaks in these spectra, two-dimensional (2D) ^{1}H–^{1}H TOCSY (Total Correlation Spectroscopy) and ^{1}H-^{13}C HSQC (Heteronuclear Single Quantum correlation) NMR spectra were also acquired at 298 K for some of the serum samples using the parameters as described previously. The assignments of peaks from lipid moieties were obtained based on previous literature reports.

3.18.4 Data reduction

Before multivariate data analysis, all the NMR spectra were manually phased and baseline corrected. The CPMG (δ0.5−8.5 ppm) spectra were binned and automatically integrated using AMIX package (Version 3.8.7, Bruker, Bio Spin). The region δ (4.7-5.5) distorted due to water suppression were excluded from the CPMG data set to avoid the effects of imperfect water suppression. Finally, the selected regions were reduced to spectral bins of δ 0.01 ppm. Subsequently, the spectral bins were integrated and normalized to the sum of all integral regions for each spectrum to compensate for the differences in concentration of metabolites among individual serum samples. The resulting datasets were finally used for multivariate analysis using the open access web-based metabolomic data processing tool, named MetaboAnalyst**.**

3.18.5 Multivariate pattern recognition Analysis

After data binning and normalization, the data from CPMG and diffusion edited experiments were subjected to multivariate statistical analysis in MetaboAnalyst (a freely available, user-friendly, web-based analytical platform for high-throughput metabolomics studies from the University of Alberta, Canada). The normalized NMR data sets were Pareto scaled and subsequently, subjected to unsupervised principal component analysis, (PCA) for an initial overview of the grouping trend (i.e. Intrinsic clustering) and outliers within the data set. PCA was performed according to default settings on the MetaboAnalyst interface. After the initial overview and identifying the outliers, the supervised partial least-squares discriminant analysis (PLS-DA) was used as a diagnostic model to identify the distinguishing features and further to identify the marker metabolites that can differentiate the RA group from the control group. Model validation and significance

of class discrimination were assessed using permutation test statistics. PLS-DA tends to over fit the data and therefore the model needs to be rigorously validated to see whether the separation is statistically significant or is due to random noise. To avoid the over fitting of the PLS-DA model, 10-fold cross-validation algorithm was used to evaluate 100% classification accuracy based on top 5 latent variables. The goodness of the model and the model robustness were assessed by the cross-validation parameters, R^2 and Q^2, respectively. R^2 is the fraction of variance explained by a component, and cross validation of this component provides Q2, which describes the fraction of the total variation predicted by a component. The value of Q^2 ranges from 0 to 1 and typically a Q^2 value greater than 0.5 is considered a good model, and those with Q^2 values over 0.7 are robust. Interpretation of PLS-DA model was based on the score plot, regression coefficients and the variable importance in the projection plot (VIP). Significantly altered metabolite entities were identified based on their significantly higher values of VIP scores and coefficient values. The coefficient importance is based on the weighted sum of PLS-regression scores; whereas, the VIP score represents a weighted sum of squares of the PLS loadings and indicate the importance of the variable to the whole model and the corresponding coefficient values attribute its discriminatory potential. Generally, the variables (or metabolite peaks) with high VIP and coefficient scores indicate that it is important for class discrimination. The robustness of the PLS-DA model for discriminating the RA from control cohorts was further verified using receiver operating characteristic (ROC) analysis. The boxplot representation (evaluated through univariate analysis) was used to visualize the variation in the levels of significantly altered metabolites in RA patients identified in the multivariate analysis.

3.18.6 Hierarchical Clustering and Heat Map

Unsupervised hierarchical clustering was used to assess, how similar or different the RA samples are compared to normal control samples on the basis of their metabolite profiles. Hierarchical clustering was performed in R statistical package "plot" of MetaboAnalyst. Using Pearson's correlation based dissimilarity measures and a clustering method named Ward's linkage was used to produce a dendrogram-cum-heat map showing the overall similarity/dissimilarity between control and RA samples. The hierarchical clustering was performed with all the metabolite entities significantly altered in CPMG spectra of RA sera compared to normal control sera (identified based on the criterion PLS-DA VIP score ≥ 1).

3.18.7 Pathway analysis

MetaboAnalyst was used to identify metabolic pathways more likely associated with the metabolic alterations induced by RA. A file, in the appropriate format, containing the quantitative measures of metabolite entities (i.e. normalized characteristic bins) significantly altered in the sera of RA patients (identified through PLS-DA analysis) was subjected to pathway analysis and Metabolite Set Enrichment analysis (MSEA) in MetaboAnalyst. The MSEA function in MetaboAnalyst enables identification of altered metabolic pathways from its extensive HMDB-derived collection of more than 71 pathways and metabolite libraries. The lipid and membrane metabolites such as LDL, VLDL, HDL, and N-acetyl glycoprotein were not recognized by the program; thus these were not included in this analysis. The final list of altered metabolites was uploaded and analyzed by Over Representation, Analysis (ORA) in MetaboAnalyst. One-tailed *p*-values are provided after adjusting for multiple testing. The output of this program will mark a metabolic pathway as

significant if significantly more compounds involved in that pathway are present in the input list than would be expected by random chance.

4. RESULTS

Chapter I: Serological Studies

The study was initiated after approval from the institutional ethical committee and written informed consent was obtained from all the participants. The participants were enrolled after evaluation on the basis of inclusion and exclusion criteria. Blood was collected from overnight fasting participants.

4.1 Demographic Characteristics

Table-1: *Demographic characteristics of healthy controls and RA patients*

Characteristics		**Control (n = 80)**	**RA(n = 67)**
Sex: (male/female)		32/48	$26/41^{***}$ RF+ve= 51 RF–ve =16
Age (years)		33.26±0.87	$34.81±0.99^{ns}$
BMI (kg/m^2)		22.91±0.16	$22.81±0.308^{ns}$
BP	Systolic	84.33±0.46	$87.11±0.308^{ns}$
	Diastolic	123.82±0.60	$125±1.23^{ns}$

P^{***}= P<.0001 P^{ns} = Non significant

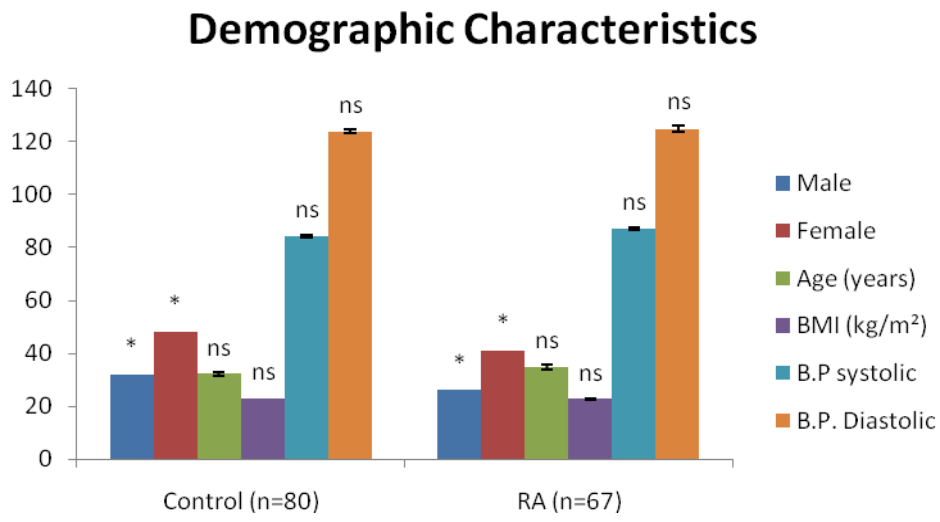


Figure-10: *Graphical representation of demographic characteristics of healthy control and patients*

Total 147 age and sex matched participants were selected, among these 80 asymptomatic controls and 67 rheumatoid patients were recruited (Table-1). The screening of patients was done on the basis of presence of 3/4more ACR criteria (including the presence of rheumatoid factor (RF). Amongst RA patients, 51 were rheumatoid factor (RF) positive and 16 were RF negative. We could not observe any significant changes in the age, BMI and blood pressure of RA patients as compared to control (Table-1). The demographic parameters of selected asymptomatic control and 67 *Rheumatoid arthritis* patients are shown in table-1.

4.2 Clinical and Serological Variables

Table-2: *Clinical variables (Mean±SD) in healthy controls and RA patients*

Variables	**Control (n = 80)**	**RA(n = 67)**
Hb	13.05±0.139	11.40±0.198***
ESR (mm/hr)	26.92±0.355	44.12±0.583***
RF	6(+ve)	51(+ve)/16(-ve)
DAS	2.32±0.0092	4.312±0.0429***
CRP (mg/L)	0.441±0.020	1.79±0.0959***
VAS(*Pain on VAS (in holding and lifting activity)*)	1.675±0.0641	5±0.196***
Uric Acid (mg/dl)	4.6775±0.74	3.331±0.150***

P*** = P<. 0001

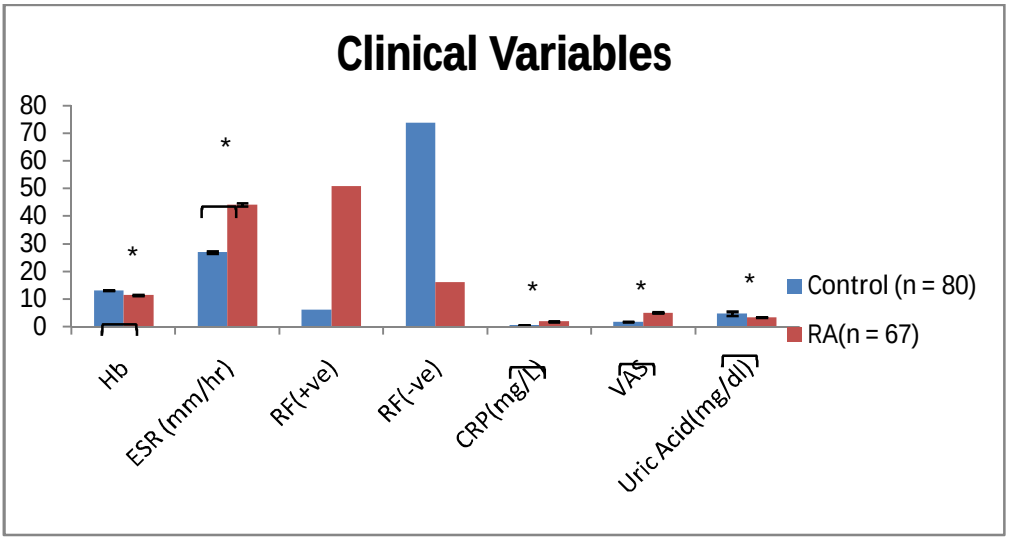


Figure-11: *Graphical representation of serological parameters in serum of healthy control and RA patients*

The mean level of hemoglobin was found significantly ($p<0.0001$) decreased in RA patients (0.2 fold) as compared to controls. Mean level of ESR and DAS-28 of RA patients was significantly ($p<0.0001$) increased (2 fold) as compared to control. The mean level of CRP was significantly increased ($p<0.0001$) (4 fold) in RA patients as compared to control. VAS levels were significantly increased (3 fold) in RA as compared to control. The uric acid level was significantly decreased ($p<0.0001$) in RA as compared to control.

4.3 Liver Function

Table-3: *Liver function parameters (SGOT and SGPT) (Mean±SD) in healthy controls and RA patients*

Variables	**Control (n = 80)**	**RA(n = 67)**
*Aspartate aminotransferase-AST(*SGOT*) (U/l)*	40.887±0.34	24.58±1.162***
Alanine aminotransferase-ALT(SGPT*) (U/l)*	36.22±0.670	26.158±0.967***

P*** = P<.0001 vs. control

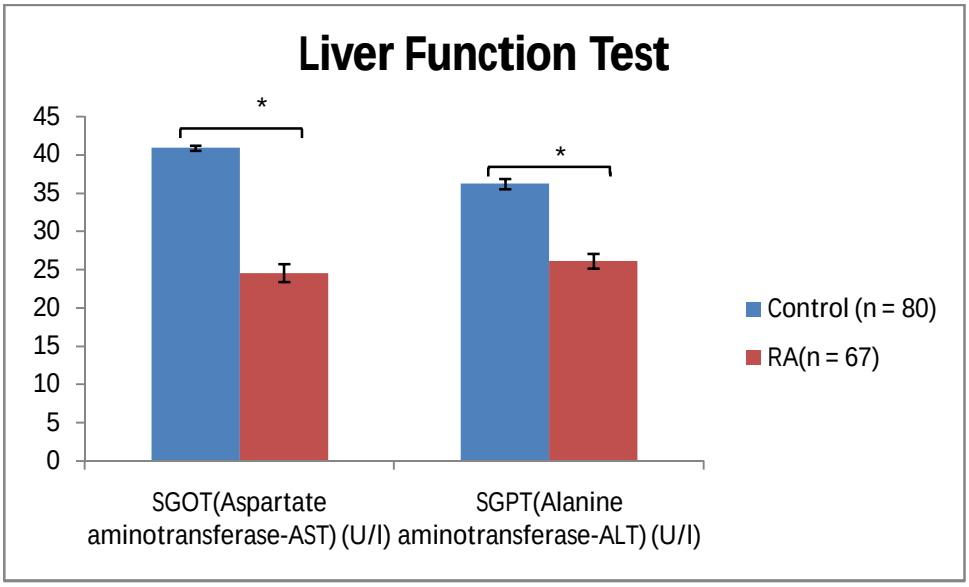


Figure-12: *Graphical representation of liver function parameters (SGOT and SGPT) in serum of healthy control and RA patients*

Table-3 shows the liver function of RA and control. SGOT and SGPT level of treated RA patients were significantly decreased (P<0.0001) as compared to control.

Table-4: *Intercorrelations of variables of RA patients.*

	ESR	**SGOT**	**SGPT**	**VAS**	**CRP**	**URIC ACID**
ESR	1					
SGOT	0.068^{ns}	1				
SGPT	0.069^{ns}	0.575^{**}	1			
VAS	0.3143^{*}	-0.0315^{ns}	-0.187^{ns}	1		
CRP	-0.1951^{ns}	-0.1827^{ns}	-0.1385^{ns}	0.0753^{ns}	1	
URIC ACID	0.3176^{*}	0.0226^{ns}	0.0239^{ns}	0.0409^{ns}	-0.0199^{ns}	1

Intercorrelations of variables of RA patients. Ns-Non significant, ** Correlation is significant at 0.01 level, * Correlation is significant at 0.05 level

Hb- hemoglobin; ESR- Erythrocyte sedimentation rate; SGOT- Aspartate aminotransferase(AST); SGPT- Alanine aminotransferase (ALT); VAS- pain of visual analog scale; CRP- C reactive protein.

Intercorrelation analysis showed significant positive correlation (r=0.575; P<0.01) between SGOT and SGPT (Table-4) and ESR and VAS (r=0.314; P<0.05) (Table-7). The results show the dependence of SGOT and SGPT and ESR with VAS.

ESR and VAS correlation show that inflammation may be responsible for pain in RA patients.

4.4 Lipid Peroxidation, Activity of Antioxidant Enzymes and ALP status

Table-5: *Levels of lipid peroxidation (MDA), antioxidant enzyme activities of superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) levels (Mean±SD) in healthy controls and RA patients*

Enzyme	Control (n = 80)	RA(n = 67)
MDA(nmoles/mgprotien)	0.76±0.014	2.47±0.091***
ALP(IU/L)	149.77±0.71	181.32±0.7795***
Catalase((U/ml))	1.72±0.026	1.25±0.0227***
SOD(Dismutase(U/ml/mg protien))	480.35±9.356	1135±25.990***
GR(U/ml/mg protien)	26.31±0.65	20.186±0.34***

P*** = P<.0001

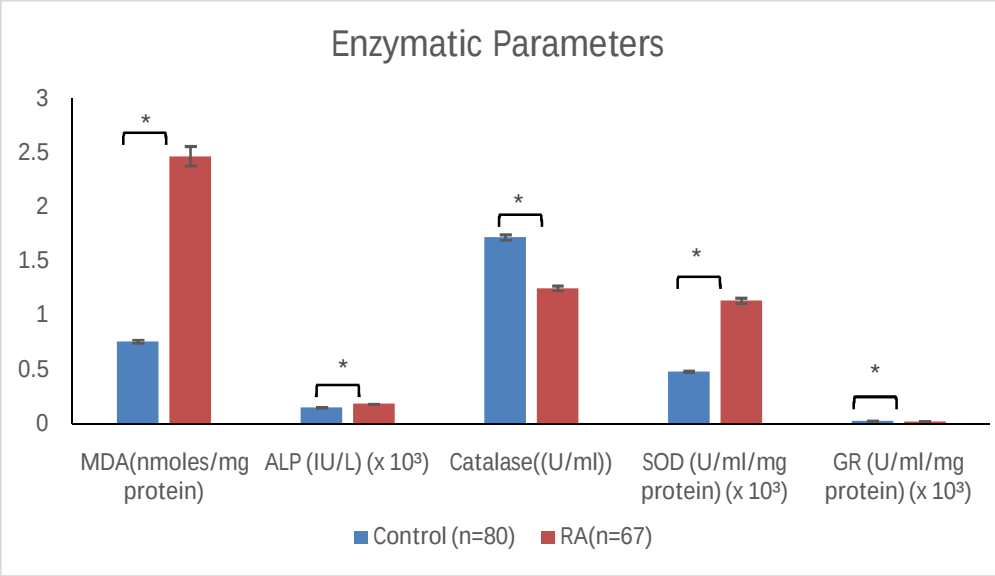


Figure-13: *Graphic representation of MDA and enzymes as SOD, catalase, ALP along with reduced glutathione in healthy controls and RA patients*

A significant increase in MDA level was observed (P<.0001) in RA patients as compared to control (Table-5). It may be due to increased ROS during chronic inflammation (Table-5). In our study mean ALP activity was 1.3 fold increased (p<0.0001) in RA as compared to control (Table-5). Moreover, the activity of antioxidant enzyme SOD was found to be significantly increased in RA patient when

compared to control. However, catalase and GR activities were significantly decreased (P<.0001) in RA patients compared to controls probably due to chronic oxidative stress (Table-5).

Table 6: *Shows the correlation between various parameters in RA patients.*

	MDA	**SOD**	**Catalase**	**GR**	**ALP**
MDA	1				
SOD	-0.0477^{ns}	1			
Catalase	-0.1415^{ns}	0.1908^{ns}	1		
GR	0.2969*	0.3961**	0.1355^{ns}	1	
ALP	0.0647^{ns}	0.4928**	0.0349^{ns}	0.3131**	1

*p<0.05, **p<0.01.

The interaction of enzymes and MDA showed significant positive correlation between MDA and GR (r = 0.29; P<0.05) (Table-6) and between GR and SOD (r = 0.396; P<0.01) (Table-6). ALP activity also showed significant positive correlation between SOD (r = 0.4928; P<0.01) and GR (r = 0.313; P<0.01) (Table-6).

4.5 Trace Metal

Table-7: *Levels of trace metals (Zn, Cu, Mg and P) in healthy controls and RA patients*

Metals	**Control (n = 80)**	**RA (n = 67)**
Zinc(µg/dl)	101.12±1.04	134.98±0.996***
Copper(µg/dl)	123.17±2.22	150.8±1.69***
Magnesium(mEq/L)	2.02±0.36	1.08±0.0276***
Phosphorous (mg/dl)	3.92±0.68	5.99±0.067***

P***= P<.0001

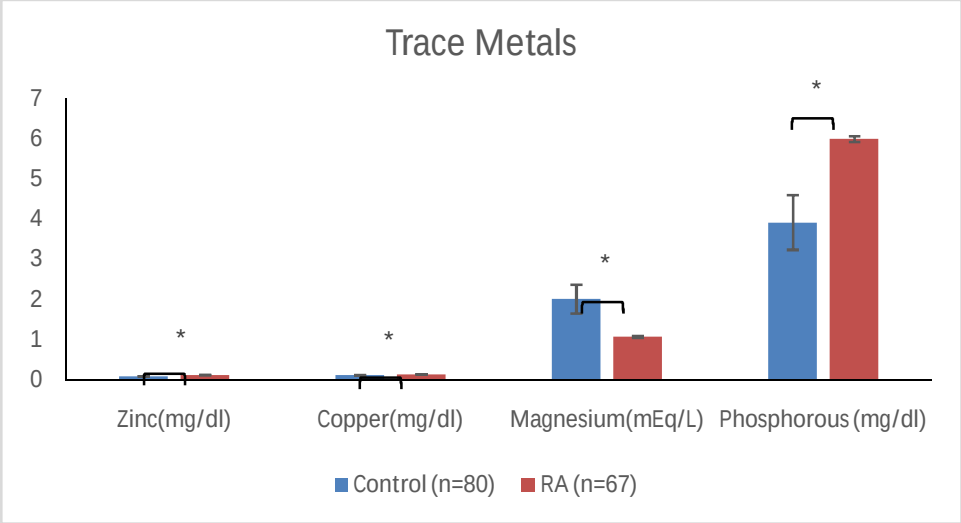


Figure-14: *Graphic representation of Zn, Cu, Mg and P levels in healthy controls and RA patients*

Zinc and copper are the main component of antioxidant enzyme SOD, the activity of SOD was significantly increased (P<0.0001) in RA patients. It was 1.3 times higher in RA patients as compared to control. Phosphorous is a key component of bone, the mean level of phosphorous was 2 fold increased in RA patients as compared to control. Mg levels were significantly decreased in RA patients as compared to control (Table-7).

4.6 Lipid Profile

Table-8: *Status of lipid profile (TC, VLDL,TG, HDL,LDL) in healthy control and RA patients*

Variables	**Control (n = 80)**	**RA (n = 67)**
TC (mg/dl)	191.97±3.843	266.97±6.08***
VLDL (mg/dl)	29.41±0.813	46.89±1.52***
TG (mg/dl)	143.14±3.24	175.33±4.4***
HDL(mg/dl)	52.24±1.05	34.76±0.69***
LDL (mg/dl)	75.89±	111.75±3.12***

P*** = P<.0001

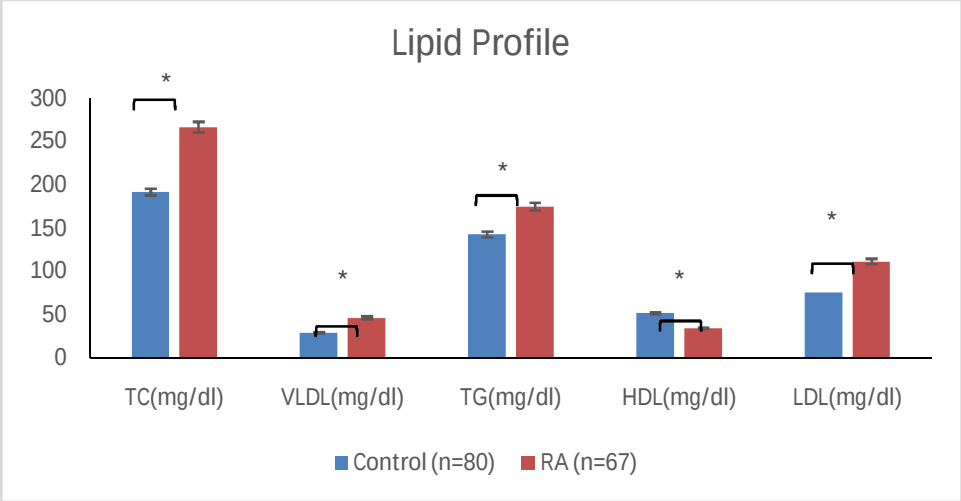


Figure-15: *Graphic representation of lipid profile (TC, VLDL,TG, HDL,LDL) levels in healthy controls and RA patients*

Mean level of TC and TG were significantly increased (P<.0001) (1.4 fold) in RA as compared to control and VLDL and LDL levels showed significant increase (1.7 times) in RA patients as compared to control (Table-8). The mean level of HDL was significantly decreased (by 0.5 fold) in RA patients as compared to controls. All these variables are responsible for arteriosclerosis and thus are risk factors for cardiovascular disease in RA patients (Table-8).

Table-9: *Intercorrelation of several variables of RA*

	CRP	**Uric acid**	**Zn**	**Cu**	**Mg**	**P**	**TC**	**VLDL**	**TG**	**LDL**	**HDL**
CRP	1										
Uric acid	-0.019 ns	1									
Zn	-0.026 ns	0.018 ns	1								
Cu	-0.367 **	0.068 ns	-0.072 ns	1							
Mg	-0.003 ns	0.062 ns	-0.123 ns	-0.029ns	1						
P	0.096 ns	-0.013 ns	0.026 ns	-0.231ns	0.084 ns	1					
TC	0.087 ns	0.183 ns	0.268 **	-0.257ns	-0.179 ns	-0.132 ns	1				
VLDL	0.024 ns	0.111 ns	0.0261ns	-0.021ns	-0.073 ns	0.248**	0.424**	1			
TG	0.163 ns	0.045 ns	0.1505ns	-0.190ns	-0.121 ns	0.063 ns	0.425**	0.407 **	1		
LDL	0.038 ns	0.310 **	0.0283ns	0.002ns	0.122 ns	-0.154 ns	0.257**	0.294**	0.018 ns	1	
HDL	-0.046 ns	0.150 ns	-0.018ns	-0.036 ns	-0.076 ns	0.017 ns	-0.011 ns	0.087 ns	-0.054 ns	0.059 ns	1

ns-Non significant, ** Correaltion is significant at the 0.01 level, * Correlation is significant at 0.05 level. CRP- C reactive protein, Zn-Zinc, Cu-Copper, Mg-Magnesium, P-Phosphorous, TC-Total cholesterol, VLDL-Very low density lipoprotein, TG-Triglycerides, LDL-Low density lipoprotein, HDL-High density lipoprotein.

CRP and serum copper showed significant inverse correlation ($r = -0.367$; $P<0.01$). Total cholesterol and Zinc showed significant positive correlation ($r = 0.268$; $P<0.01$). LDL showed significant direct correlation with uric acid ($r = 0.31$; $P<0.01$), total cholesterol ($r = 0.257$; $P<0.01$) and VLDL ($r = 0.294$; $P<0.01$). VLDL showed significant positive correlation with phosphorous ($r = 0.248$; $P<0.01$) and total cholesterol ($r = 0.424$; $P<0.01$). Triglycerides showed significant positive correlation with total cholesterol ($r = 0.425$; $P<0.01$) and VLDL ($r = 0.294$; $P<0.01$).

Chapter II: Genetic Polymorphism Analyses

4.7 Genetic Polymorphism

4.7.1 Peptidylarginine deiminase type 4 (PADI4)

4.7.1.1 RS188_1

Figure-16 shows that PADI haplotype RS188_1 is not associated with RA. We could not observe CT or TT genotypes in either control or RA patients as no polymorphic bands were observed after digestion of PCR products with RsaI restriction enzyme. Therefore the locus PAD188_1 is not associated with RA.

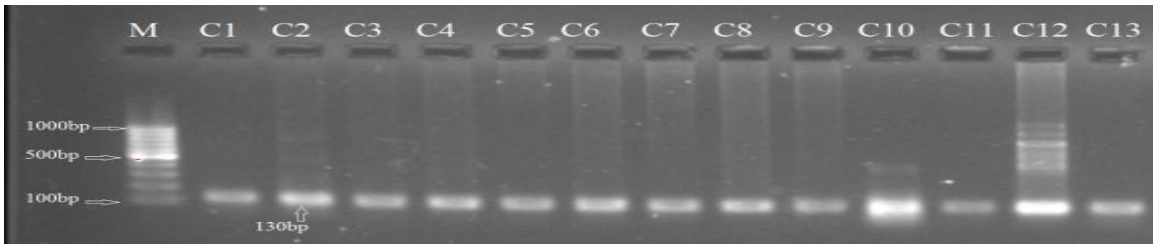


Figure-16A *Shows the PCR amplification of PADI4 RS188_1 of controls*

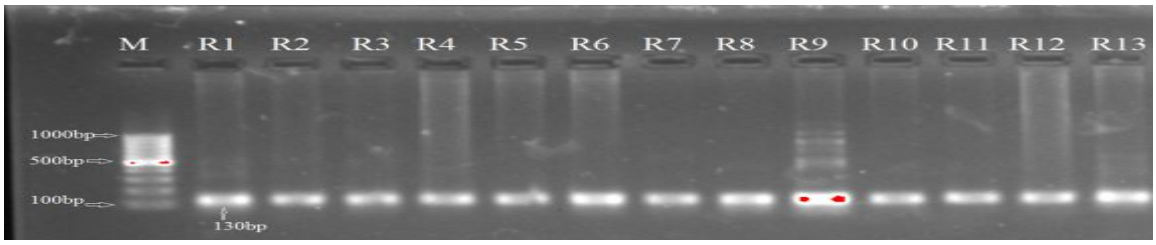


Figure-16B *Shows the PCR amplification of PADI4 RS188_1 of RA patients*

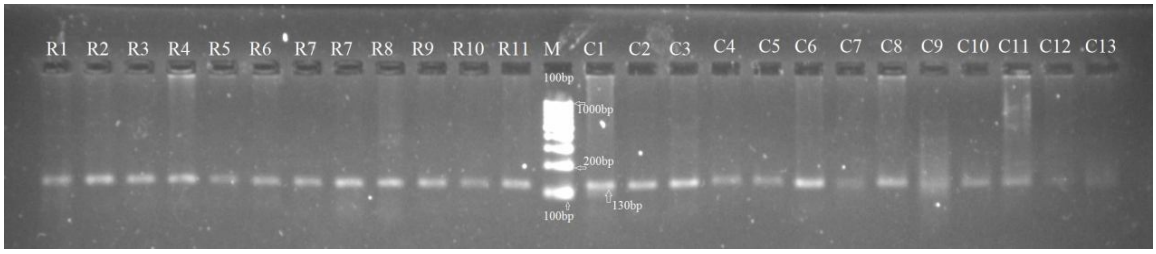


Figure-16C *shows thae PADI4 RS188_1 control and RA patients digestion of PCR products with RsaI restriction enzyme*

4.7.1.2 RS188_2

The odds ratio (OR) with 95% confidence interval (CI) was used to calculate the strength of association between the PADI4 polymorphism and RA susceptibility. We calculated the OR at 95% CI for PADI4 RS188_2: CC versus CG + GG in a dominant model, GG versus CC + CG in a recessive model, CC versus GG in co-dominant I model, CC versus CG in co-dominant II model and C versus G in the

allelic model. For PADI4 RS188_2 polymorphism the ORs were 2.88 (95% CI: 1.32-6.25, p<0.01) in dominant model, 1.63 (95% CI: 0.73-3.63, P>0.05) in recessive model, 3.2 (95% CI: 3.2-8.6, p<0.05) in co-dominant I model, 2.74 (95% CI: 1.21-6.18, p<0.05) in co-dominant II model and 1.76 (95% CI: 1.1-2.8, p<0.05) in allelic model. It is evident from the above results that the PADI4 RS188_2 C>G was significantly associated with the RA disease in dominant, codominant I & II, genotypic and allelic models. Our results have shown (Table-10 and 11) significant differences in allele and genotype frequencies between RA patients and control for RS188_2 C/G polymorphism.

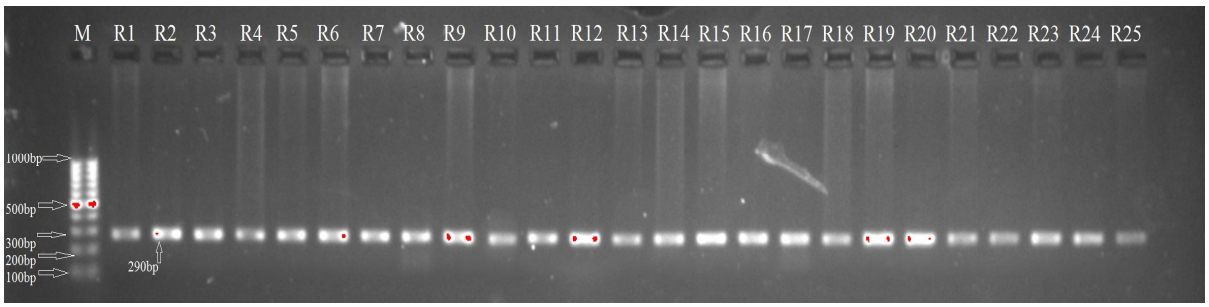


Figure- 17A *Shows the PCR amplification of PADI4 RS188_2 of RA patients*

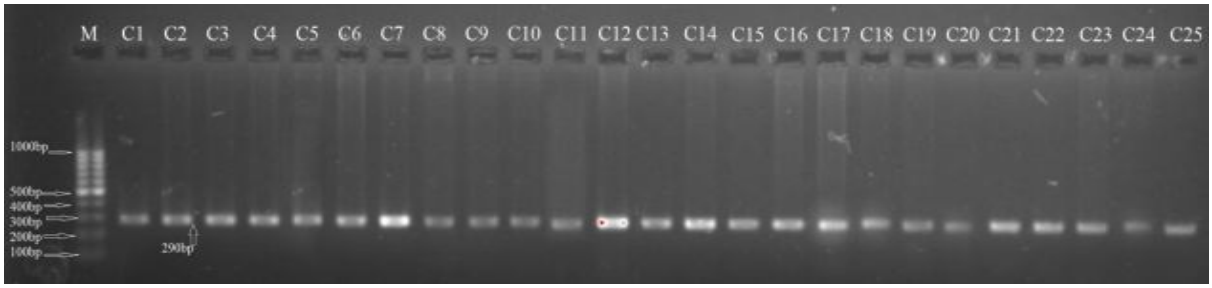


Figure-17B *Shows the PCR amplification of PADI4 RS188_2 of control*

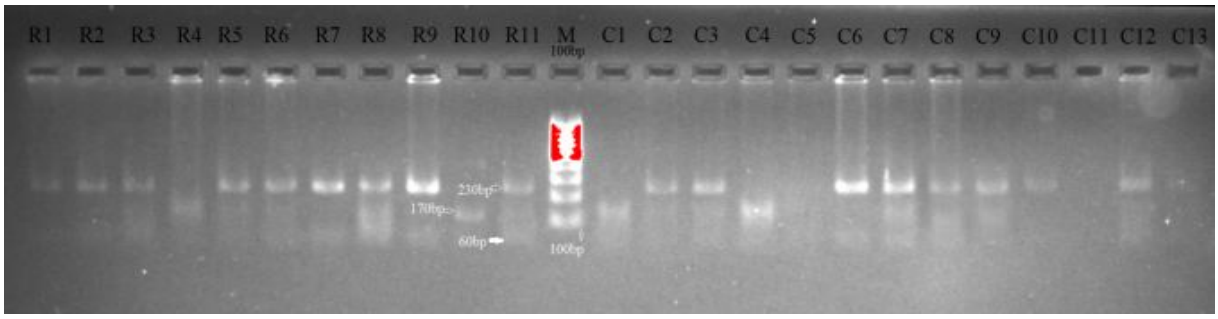


Figure-17B *shows the PADI4 RS188_2 control and RA patients digestion of PCR products with NIaIII restriction enzyme*

Table-10 PADI4 RS188_2: Polymorphic parameters of controls and patients sample

		Controls *N* (%)	Patients *N* (%)	Odds ratio at 95% CI(lower-upper)	*P* value	χ^2 value (Pearson)
Genotypic Distribution	CC	13(16.3)	24(35.8)		<0.05	7.556
	CG	46(57.5)	31(46.3)			
	GG	21(26.3)	12(17.9)			
Allelic Frequency	C	72(45.0)	79(58.9)			
	G	88(55.0)	55(41.1)	1.76(1.1-2.8)	<0.05	5.69

Table-11 PADI4 RS188_2: Logistic regression analysis

Genetic Models	Controls *N* (%)	Patients *N* (%)	Odds ratio at 95% CI(lower-upper)	*P* value	χ^2 value (Pearson)
CC : CG + GG (Dominant model)	13(16.2):67(83.8)	24(35.8):43(64.2)	2.88(1.32-6.25)	<0.01	7.41
CC + CG : GG (Recessive model)	59(73.7):21(26.3)	55(82.1):12(17.9)	1.63(0.73-3.63)	>0.05	1.46
CC : GG (Co-dominant model-I)	13(16.3):21(26.3)	24(35.8):12(17.9)	3.2(1.2-8.6)	<0.05	5.67
CC : CG (Co-dominant model-II)	13(16.3):46(57.5)	24(35.8):31(46.3)	2.74(1.21-6.18)	<0.05	6.06

4.7.1.3 PADI4_102

The odds ratio (OR) with 95% confidence interval (CI) was used to calculate the strength of association between the PADI4_102 polymorphism and RA susceptibility. We calculated the OR at 95% CI for PADI4_102: CC versus CT + TT in a dominant model, TT versus CC + CT in a recessive model, CC versus TT in co-dominant I model, CC versus CT in co-dominant II model and C versus T in the allelic model. For PADI4_102 polymorphism the ORs were 0.195 (95% CI: 0.093-0.41, p<0.0001) in dominant model, 0.73 (95% CI: 0.31-1.74, P>0.05) in recessive model, 0.28 (95% CI: 0.1-75, p<0.05) in co-dominant I model, 0.17 (95% CI: 0.08-

0.37, p<0.0001) in co-dominant II model and 0.42 (95% CI: 0.26-0.67, p<0.001) in allelic model. It is evident from the above results that the PADI4_102 C>T was significantly associated with the RA disease in dominant, codominant I, II and allelic models. A significant difference between genotypes and allelic distribution of PADI_102 polymorphism was observed between RA and control group. Wild type allele CC was predominantly presently in control, whereas, RA patients showed significantly reduced wild-type CC allele. Hence the PADI4_102 C allele seems to be associated with the risk of RA in north Indian population.

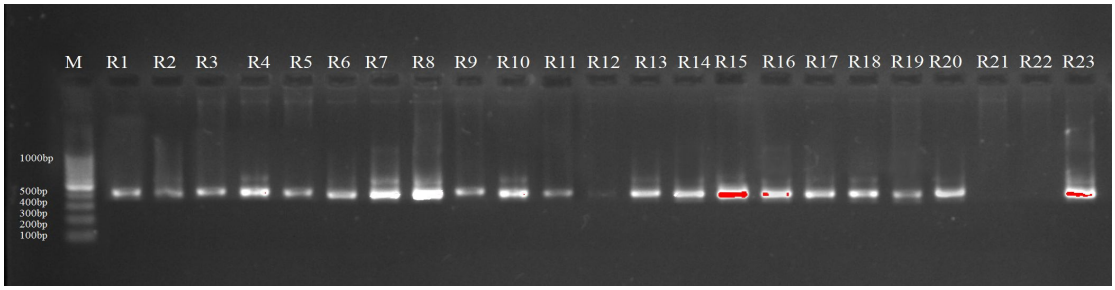


Figure 18 A *Shows the PCR amplification of PADI4_102 of RA patients*

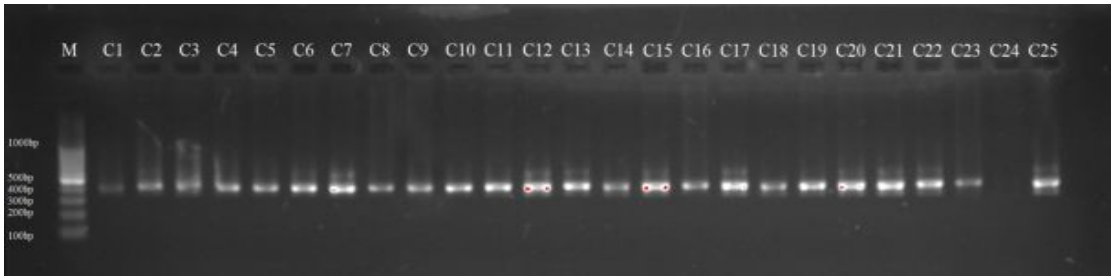


Figure-18B *Shows the PCR amplification of PADI4 _102 of controls*

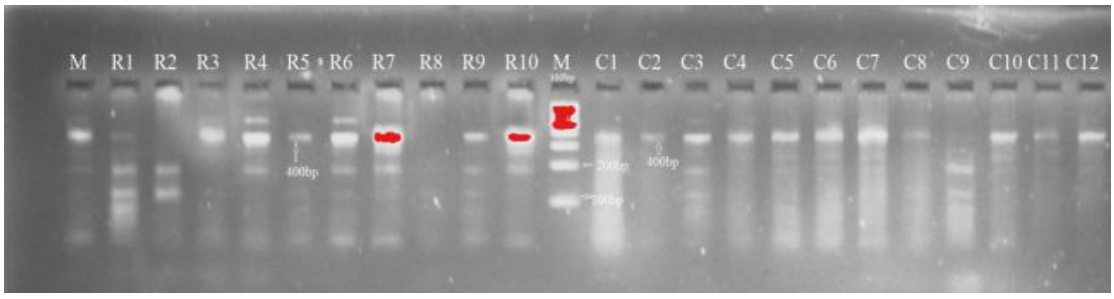


Figure-18C *shows the PADI4_102 control and RA patient's digestion of PCR products with RsaI restriction enzyme*

Table-12 PADI4_102: Polymorphic parameters of controls and patients sample

		Controls *N*(%)	Patients *N*(%)	Odds ratio at 95% CI(lower-upper)	*P* value	χ^2 value (Pearson)
Genotypic Distribution	CC	46(57.5)	14(20.9)		<0.0001	21.35
	CT	22(27.5)	40(59.7)			
	TT	12(15.0)	13(19.4)			
Allelic Frequency	C	114(71.25)	68(50.7)	0.42(0.26-0.67)	<0.001	13
	T	46(28.75)	66(49.3)			

Table-13 PADI4_102: Logistic regression analysis

Genetic Models	Controls *N* (%)	Patients *N* (%)	Odds ratio at 95% CI(lower-upper)	*P* value	χ^2 value (Pearson)
CC : CT + TT (Dominant model)	46(57.5):34(42.5)	14(20.9):53(79.1)	0.195(0.093-0.41)	<0.0001	20.22
CC + CT : TT (Recessive model)	68(85.0):12(15.0)	54(80.6):13(19.4)	0.73(0.31-1.74)	>0.05	0.5
CC : TT (Co-dominant model-I)	46(57.5):12(15.0)	14(20.9):13(19.4)	0.28(0.1-0.75)	<0.05	6.69
CC : CT (Co-dominant model-II)	46(57.5):22(27.5)	14(20.9):40(59.7)	0.17(0.08-0.37)	<0.0001	21

4.7.2 Tissue inhibitor of metalloproteinases-4 (TIMP-4)

4.7.2.1. A/G SNP TIMP-4

Our results showed that wild type AA allele is present in 80 controls and 67 RA; AG alleles are present in 00 control and 27 RA patients, while GG alleles are not present in controls and RA patients. No polymorphic bands were observed after digestion of PCR products with DdeI restriction enzyme. As we could not find prevalence of any specific allele of TIMP-4 A/G (rs308952) with RA therefore this allele is also not associated with RA in north Indian population.

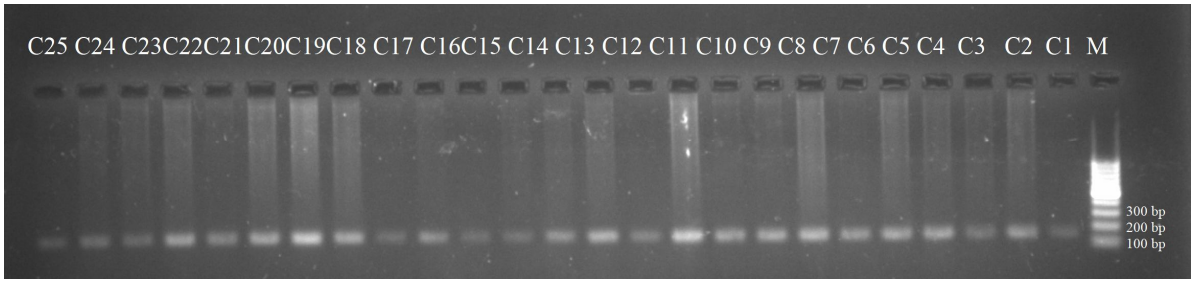


Figure 19A *Shows the PCR amplification of TIMP4 A/G of controls*

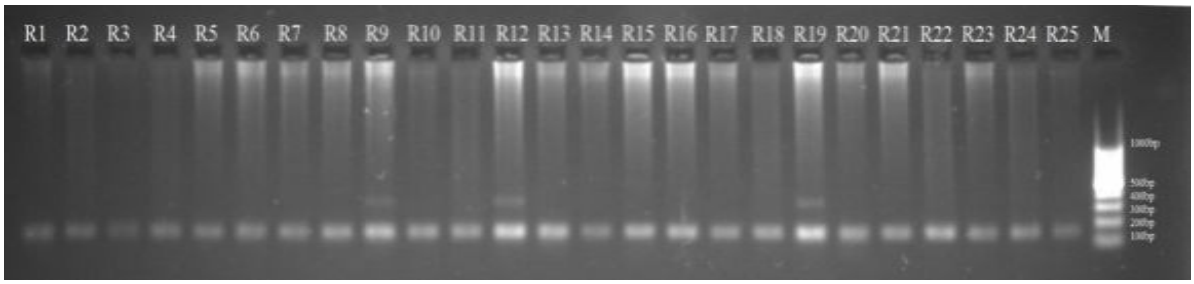


Figure-19B *Shows the PCR amplification of timp4 A/G of RA patients*

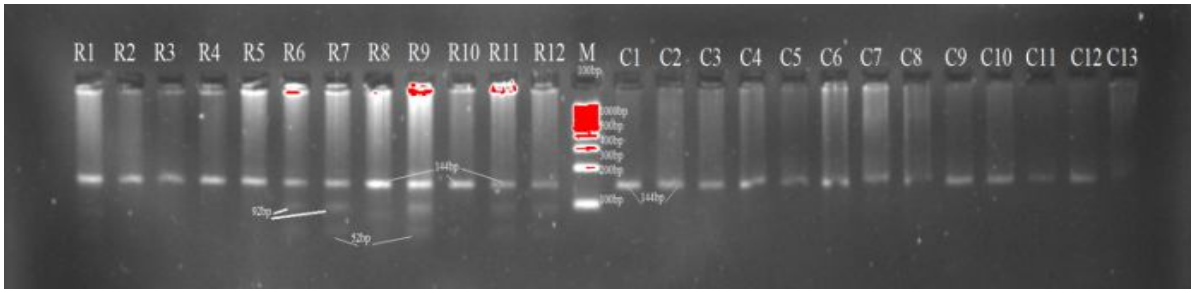


Figure-19C *shows the TIMP4 A/G controls and RA patients digestion of PCR products with DdeI restriction enzyme*

4.7.2.2 C/T SNP

The odds ratio (OR) with 95% confidence interval (CI) was used to calculate the strength of association between the TIMP4 C/T polymorphism and RA susceptibility. We calculated the OR at 95% CI for TIMP4 C/T: CC versus CT + TT in a dominant model, TT versus CC + CT in a recessive model, CC versus TT in co-dominant I model, CC versus CT in co-dominant II model and C versus T in the allelic model. For TIMP4 C/T polymorphism the ORs were 0.58 (95% CI: 0.27-1.26, p>0.05) in dominant model, 0.48 (95% CI: 0.11-2.1, p=NA) in recessive model, 0.44 (95% CI: 0.1-1.94, p=NA) in co-dominant I model, 0.63 (95% CI: 0.27-1.49, p>0.05) in co-dominant II model and 0.58 (95% CI: 0.30-1.12, p>0.05) in allelic model. . It is evident from the above results that the TIMP4 C/T was not significantly associated

with the RA disease in dominant, recessive, codominant I and II and allelic models. A non significant difference between genotype and allelic distribution of TIMP4 C/T polymorphism was observed between RA and control group. However, wild type allele CC was predominantly presently in control as compared to RA patients.

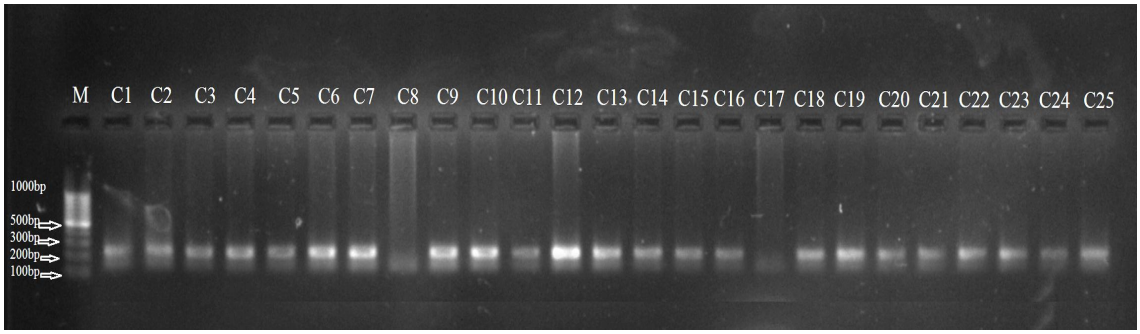


Figure 20A *Shows the PCR amplification of TIMP4 C/T of controls*

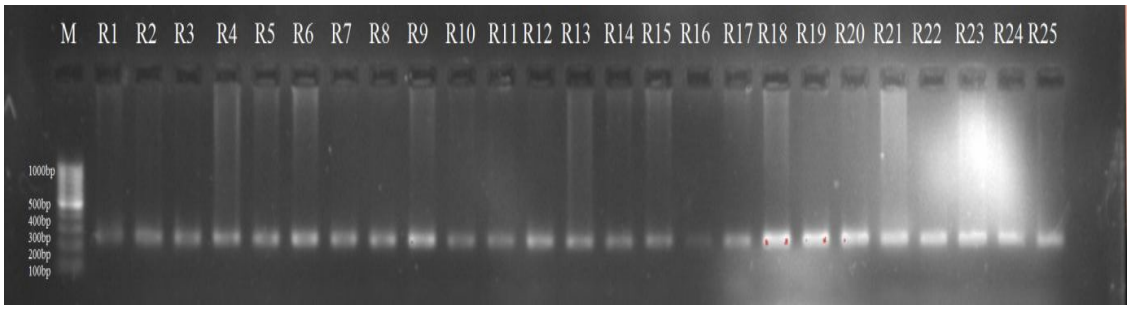


Figure-20B *Shows the PCR amplification of TIMP4 C/T of RA patients*

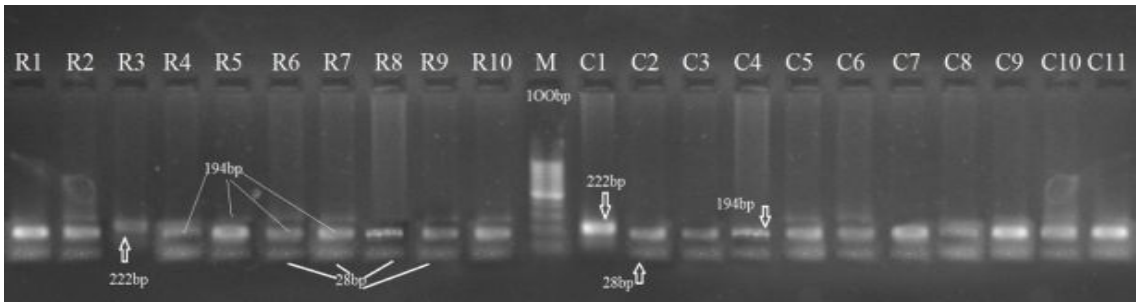


Figure-20C *shows the TIMP4 C/T control and RA patients digestion of PCR products with HpyCH4III restriction enzyme*

Table-14 TIMP-4: Polymorphic parameters of controls and patients sample

		Controls *N* (%)	Patients *N* (%)	Odds ratio at 95% CI(lower-upper)	*P* value	χ^2 value (Pearson)
Genotypic Distribution	CC	65(81.25)	48(71.6)	0.58(0.27-1.26)	>0.05	1.89
	CT	12(15.0)	14(20.9)			
	TT	3(3.75)	5(7.5)			
Allelic Frequency	C	142(88.75)	110(82.1)	0.58(0.3-1.12)	>0.05	2.64
	T	18(11.25)	24(17.9)			

Table-15 TIMP-4: Logistic regression analysis

Genetic Models	Controls *N* (%)	Patients *N* (%)	Odds ratio at 95% CI(lower-upper)	*P* value	χ^2 value (Pearson)
CC : CT + TT (Dominant model)	65(81.3):15(18.7)	48(71.6):19(28.4:	0.58(0.27-1.26)	>0.05	1.89
CC + CT : TT (Recessive model)	77(96.3):3(3.7)	62(92.5):5(7.5)	0.48(0.11-2.1)	NA	NA
CC : TT (Co-dominant model-I)	65(81.3):3(3.7)	48(71.6):5(7.5)	0.44(0.1-1.94)	NA	NA
CC : CT (Co-dominant model-II)	65(81.3):12(15.0)	48(71.6):14(20.9)	0.63(0.27-1.49)	>0.05	1.11

4.7.3. Protein tyrosine phosphatase non receptor 22

4.7.3.1. PTPN22 C/T SNP

The result of above figure shows that the PTPN22 C/T SNP is not associated with RA. No polymorphic bands were observed after digestion of PCR products with XcmI and RsaI restriction enzymes. Therefore the locus PTPN22 C/T is non-polymorphic in our population and is not associated with RA.

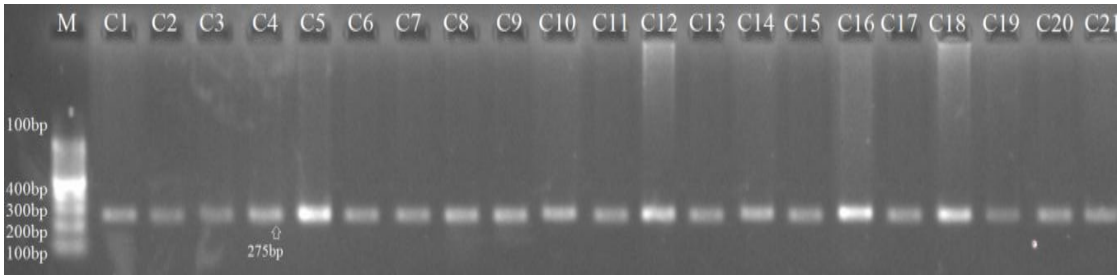


Figure 21A *Shows the PCR amplification of PTPN22- 1858 C/T of controls*

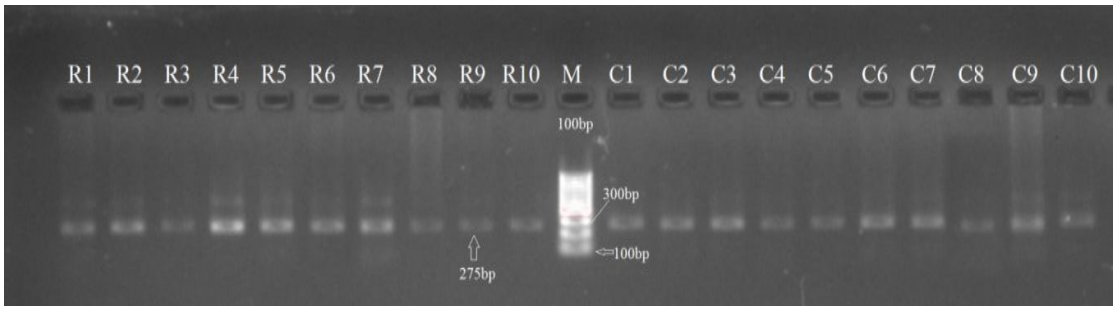


Figure 21B *Shows the PCR amplification of PTPN22- 1858C/T of RA patients*

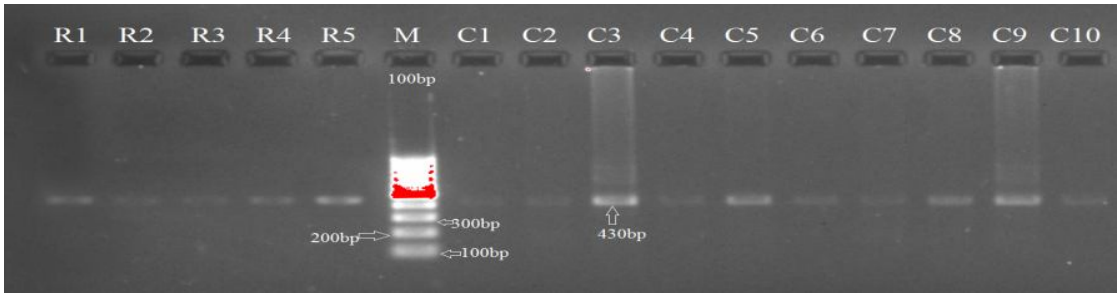


Figure 21C *shows the PTPN22- 1858C/T control and RA patients digestion of PCR products with XcmI restriction enzyme*

Chapter III- Study of expression of serum metabolites through NMR

4.8 Nuclear Magnetic Resonance

4.8.1 Metabolic alterations in RA:

The most representative 1D ^{1}H CPMG NMR spectra of serum samples (one from each group) are shown in Figure 22. The CPMG spectra showed ^{1}H NMR signals mainly from small metabolites with intense signals mainly from lipoproteins (including HDL, LDL, and VLDL), phospholipids, unsaturated lipids, choline metabolites, N-acetyl glycoproteins from glucose, lactate and amino acids like alanine, glutamate, glutamine, proline, and histidine etc.. The visual comparison of the ^{1}H-NMR spectra failed to identify any major differences between RA patients and control groups. Therefore, the NMR spectra were subjected to multivariate data analysis to identify RA induced serum metabolic changes. In the present study, we discriminated 10 RA patients from 20 age and sex matched normal controls, and established the serum metabolic patterns of RA. The study subjects were recruited judiciously to minimize the differences due to confounding variables as evident from figure.

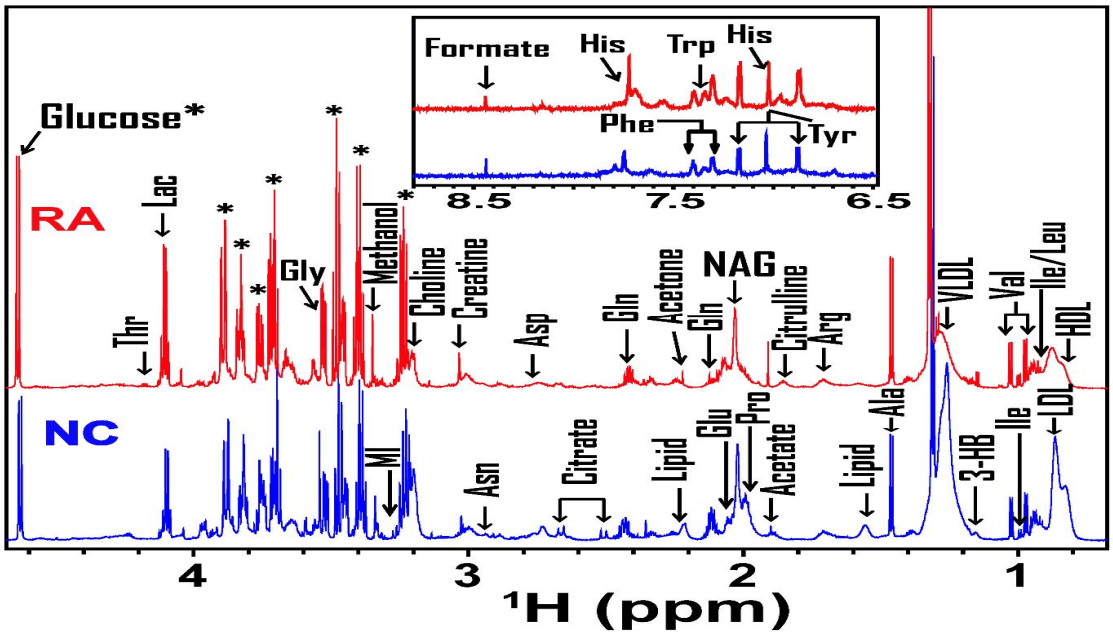


Figure- 22 *Stack plot of cumulative 1D ^{1}H CPMG NMR spectra of RA and controls*

Stack plot of cumulative 1D ^{1}H CPMG NMR spectra (ranging from 4.6–0.7 and inset 8.5–6.5 ppm) obtained for sera of RA patients (red) and normal controls (blue). Key acronyms are: HDL; high density lipoproteins; LDL: low density lipoproteins; VLDL: very-low density lipoproteins; Val: Valine; Ile: Isoleucine; Leu: Leucine; Ala: Alanine; NAG: N-acetyl glycoproteins, Glu: Glutamate; Gln: Glutamine; Asn: Asparagine; Asp: Aspartate; Cho: Choline, Gly: glycine; MI: Myo-Inositol; Pro: Proline; Thr: Threonine; His: Histidine; Phe: Phenylalanine; Tyr: Tyrosine, Trypthophan: Trp.

First, the ^{1}H CPMG NMR data were analyzed using unsupervised PCA method. As shown in Figure 23A, the RA group could be obviously discriminated from the normal control groups along the PC1 direction, indicating that the RA sera had diverse characteristics compared to the NC group. This method further allows the detection of outliers, defined as observations located outside the 95 % confidence region of the PCA model. Further, we performed supervised clustering method PLS-DA to investigate subtle metabolic differences among the groups. The parameters used to assess the quality of each model, including explained variation R^2 and the predictive capability, Q^2, are displayed in their respective score-plots in Figure 26B. The model quality parameters R^2 and Q^2, were significantly higher (R^2, Q^2>0.5), indicating that the PLS-DA models (constructed from CPMG and diffusion-edited spectra) possessed satisfactory fit with good predictive power. The two dimensional PLS-DA score plots derived from 1D ^{1}H CPMG spectra showed that the RA and control groups are well clustered and separated from each other indicating that the biochemical composition profiles of serum metabolites in RA patients are significantly different from normal controls. The metabolites responsible for the discrimination of two cohorts were identified using the VIP, coffecient, and p-value <

0.05. Overall, we identified 20 metabolites significantly perturbed in sera of RA patients (VIP score ≥1 and coffecient score ≥30).

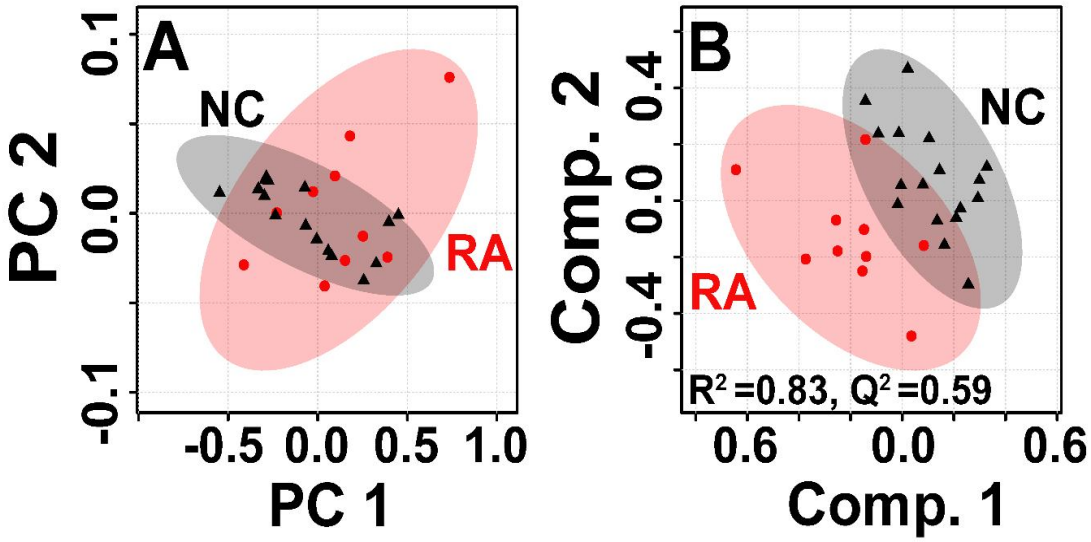


Figure-23: 2D PCA (A) and PLS-DA (B) score plots derived, respectively, from CPMG spectra showing clear statistical separation between RA (represented by red circles) and normal control (NC) samples (represented by black triangles). Each circle and triangle represents one subject. The validation parameters (R^2 and Q^2) corresponding to PLS-DA model are also displayed in their score plots.

The metabolites were perturbed in RA versus normal control. The RA patients showed lower HDL, proline, NAG, glutamate, pyruvate, glutamine, methylamine, creatine, creatinine, threonine, PUFA and histidine. While for others changes were not observed. Glucose, citrate and lactate were higher in RA patients.

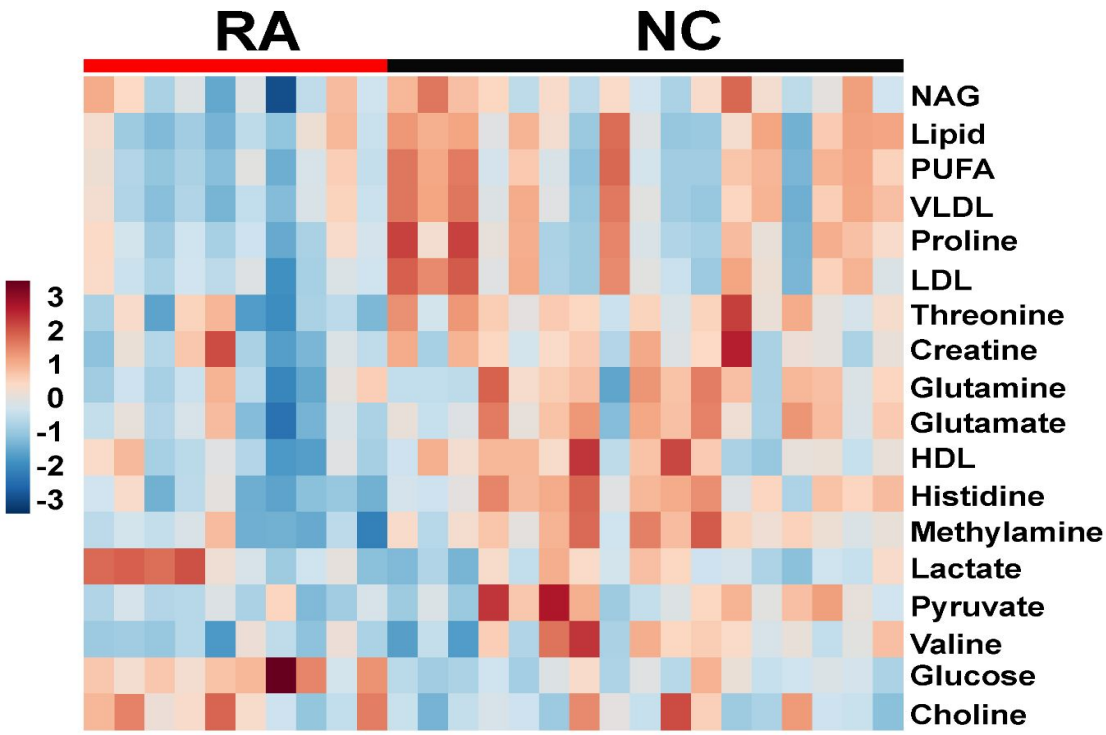


Figure-24: *Heat maps showing z-scores of identified 20 statistically significant metabolite entities altered in RA patients compared to normal controls. The red and cyan here signify, respectively, elevation and reduction in metabolite concentration in RA patients.*

These discriminatory metabolite entities were used to construct the heatmaps, commonly used for unsupervised clustering; Figure 24 which clearly shows that RA group is visually distinguishable from normal control group based on these significant metabolites (up-regulated and down-regulated metabolites are shown in red and cyan color, respectively). The combination of altered metabolites can provide an indication of metabolic pathways that may be more relevant in the context of RA. Therefore, we performed pathway analysis and metabolite set enrichment analysis (MSEA) in MetaboAnalyst to establish which pathways are affected in RA patients. The resulted summary of pathway analysis is shown in Figure 25. Mainly, five metabolic pathways of importance (protein biosynthesis, amino-acid metabolism, glucose-energy metabolism, lipid metabolism and choline metabolism) were found to be disturbed.

Although, MetaboAnalyst pathway analysis tool is extremely valuable, to do an overall analysis of metabolic data, the metabolic pathways associated with the identified combinations of metabolites are biased owing to limited metabolites identified by NMR in the serum.

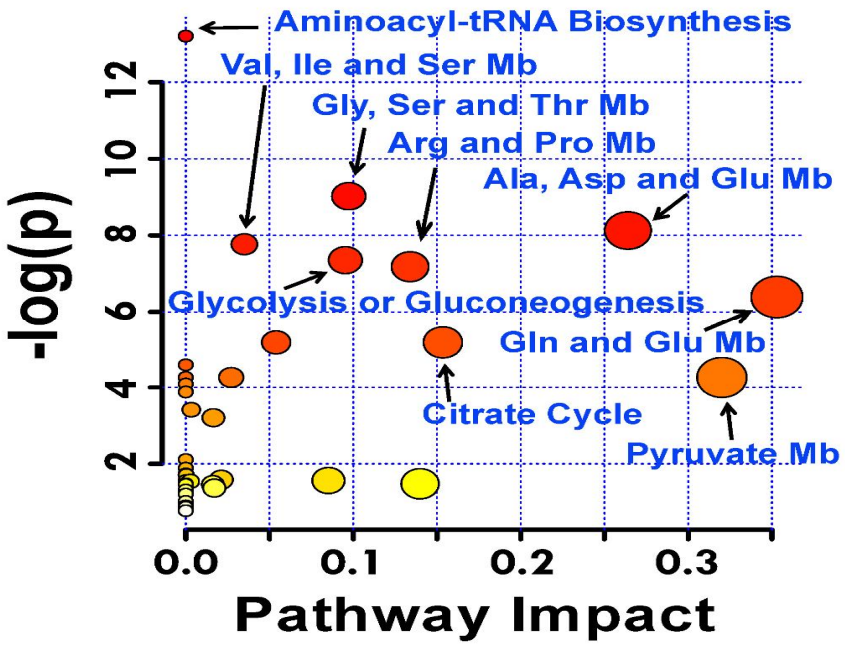


Figure-25 *Perturbed metabolic pathways*

Identification of the perturbed metabolic pathways by over representation analysis (ORA) was done and significantly altered metabolites were identified. The analysis was done by using a pathway library restricted to Homo sapiens (human), and p-values for ORA stand for hypergeometric test. Test p-value (vertical axis, intensity of colour) and impact factor (horizontal axis, size of circle)

5. DISCUSSION

RA is a joint disease with unknown etiology and is considered as a degenerative and inflammatory disease. However, increasing evidence support the role of inflammation, dyslipidemia, oxidative stress and genetic association in the pathophysiology of RA. The present study aims to evaluate changes occurring in RA by evaluation of serological parameters, inflammatory markers, pro and anti-oxidants, and metabolomic profiling by using NMR. The study also evaluated the association of PADI4, PTPN22 and TIMP4 genes with RA by genome-wide association studies.

The evaluation of the changes occurring in RA patients in their disease activity score 28 (DAS-28), pain on the visual analog scale (VAS), inflammation and changes related to oxidative stress were analyzed. Their general well being and risk of developing cardiovascular disease was also analyzed by evaluation of their response to therapy and analysis of lipid parameters.

Demographic Analyses

In our study, no significant difference was observed in the BMI and systolic blood pressure of patients and control (Table-1 and figure 10).

Clinical Analyses

In our study, the hemoglobin of patients were significantly (P<.0001) low when compared to control. Previous studies have reported (Ganna, 2014) low hemoglobin levels in RA which was related to disability and impairment. Choy and Panayi, 2001 reported that low haemoglobin levels account for systemic manifestations with the severity of the disease. The anemic syndrome has also been reported with similar manifestation which may result in increased disease activity (Bloxham *et al*., 2011).

RA patients showed significantly (P<.0001) increased ESR, pain on visual analog scale and CRP as compared to control. Though patients are on medication, still raised values indicate an ongoing inflammatory process in their body leading to increased production of CRP. DAS was significantly increased in patients along with ESR and CRP levels. Higher CRP is suggestive of recurrence of active disease sometimes along with ongoing medication or after withdrawal of the drug. In our study, female patients had higher DAS and VAS with respect to male RA patients.

Serological Analyses

In our study, serum uric acid was lower in RA patients as compared to control. Uric acid is the end product of purine metabolism and potent antioxidant. It has a protective effect against oxidative stress as an intracellular free radical scavenger. Choe and Kim, 2015 has reported reduced serum uric acid concentrations with leflunomide and methotrexate combination and methotrexate treatment alone with more pronounced changes with leflunomide treatment. Leflunomide may possibly affect urate transporters in renal epithelial cells (Emery *et al.*, 2000) and as MTX is also showing similar response thus it may also have effects similar to leflunomide. The lower uric acid may be suggestive of increased urinary secretion of uric acid but may not be related to disease activity status in RA patients (Choe and Kim, 2015).

Liver Function Analyses

The values for SGOT and SGPT which indicate functional status of the liver are significantly lower in RA patients. Our treated patients showed significantly reduced levels of SGOT and SGPT as compared to control. In RA patients, the values for SGOT positively correlated with SGPT ($r=0.575$; $p<0.01$). Iannone *et al.*, 2014 has shown that MTX can successfully treat RA patients without enhancing the risk of hepatotoxicity. However, Curtis *et al.*, 2010 have reported abnormal SGOT or SGPT

levels in their patients with RA on DMARD therapy (methotrexate >10mg/day). Our findings did not show elevated levels of these enzymes probably because of methotrexate administration with folic acid and vitamin C supplementation with drug dose of 15mg/week (intramuscular or subcutaneous) (Patel *et al.,* 2015). The pharmacokinetics of subcutaneous (SC) MTX are similar to intramuscular MTX but SC MTX may be preferred by most patients (Yadlapati and Efthmiou, 2016).

In our study, we found dyslipidemia, hypomagnesia and higher levels of phosphorous, copper and zinc. Probably deregulated minerals are consequence of dyslipidemia and inflammation. Methotrexate therapy improved CRP, DAS-28 and VAS in RA patients at 6 months follow up (Patel *et al.,* 2015). RA is controlled by DMARDs, NSAIDs, glucocorticoid and biologics, which target TNF-alpha, IL-1 and IL-6 receptors (Curtis *et al.,* 2010; Balogh *et al.,* 1980). There is heterogeneity in the response of the drugs and various side effects on the liver and kidney are reported (Curtis *et al*., 2010). Methotrexate is the treatment of choice for the management of RA in our patients but, monitoring of kidney functions along with liver functions is recommended. Though in our study, we could not observe adverse effects of MTX therapy on liver or kidney function.

Lipid and MDA Analyses

MDA production was statistically high in RA subjects (Table-5 and Figure-13). This increased MDA may be the result of increased reactive oxygen species (ROS) due to chronic inflammation. Female patients had higher MDA as compared to male patients. Lipid peroxides are produced at the tissue injury site because of inflammation and later diffuse into the blood. These can be estimated in serum or plasma (Gutteridge, 1995). Several studies (Ali *et al.,* 2014; Pallinti *et al.,* 2009;

Kamanli *et al.,* 2004; Sarban *et al.,* 2005; Hassan *et al.*, 2001) have reported higher levels of MDA in the plasma, serum and erythrocytes of RA subjects.

Thiele *et al.*, 2015 have observed malondialdehyde-acetaldehyde (MAA) adduct formation is enhanced in RA patients. These adducts appear to result in the robust response of antibody which may be tightly associated with anti-citrullinated protein antigens (ACPAs). These results suggest that formation of MAA may trigger loss of tolerance leading to the autoimmune response in RA.

Significant morbidity is observed in RA because of inflammation of synovium which leads to joint destruction and disability. Patients with RA have an abnormal lipoprotein pattern (dyslipidemia). They have low levels of HDL-c and high level of LDL-c, a pattern similar to inflammatory and infectious diseases (Rantappa *et al.,* 1991; Filippatos *et al.,* 2013). Systemic inflammation in RA leads to the significantly increased risk of cardiovascular diseases as compared to the general population (Turesson *et al.,* 2004; Solomon *et al.,* 2006. Therefore, control of inflammation may have beneficial effects on cardiovascular risk and improvement in the lipoprotein profile.

The patients showed dyslipidemia with high total cholesterol, LDL-cholesterol, VLDL and low HDL cholesterol. In our study also though dyslipidemia is observed in RA subjects as compared to control, but the values of lipid parameters analyzed are either within permissible limits or show borderline variations. These values may not be predictive of CVD risk in our RA patients. Several other studies have not shown any variation in lipid levels in RA patients with respect to healthy population (Dessein *et al.,* 2002; Erum *et al.,* 2017). Some other observed an overall decrease in all lipid sub-fractions of lipid in the case of active disease (Boers *et al.,* 2003). The existing data has wide heterogeneity in the reporting of associated

dyslipidemia. Studies have shown that in established RA, there were only marginally raised levels of total cholesterol irrespective of disease activity (Nurmohamed, 2007). High cholesterol induces oxidative stress leading to free radical generation that promotes lipid peroxidation (Lee and Prasad, 2003). In hypercholesterolemia, high levels of lipids and phospholipids are accumulated resulting in increased production of arachidonic acid and prostaglandins with the help of phospholipase A2 and cyclooxygenase enzyme (Lawrence *et al.,* 2001). MDA is the end product of lipid peroxidation; therefore, its measurement gives indirect evidence of LDL oxidation. Under intense oxidative stress, aldehyde level increases and takes part in numerous pathological conditions such as cancer, arthritis, atherosclerosis, and cardiac disease (Uchida *et al.,* 2003). Patients with RA showed higher accumulation of MDA.

RA is associated with disturbances in serum magnesium levels (Cortes *et al.,* 2007). Inflammation is the primary cause of systemic alterations in the levels of metals and enzymes, which is further modulated by acute phase plasma proteins (Dean, 2000). RA is also associated with dyslipidemia, as observed by higher LDL-c, TC, cholesterol and lower levels of HDL-c. The study by Mahalle *et al.*, 2012; Chavan *et al.,* 2015 have shown a negative correlation between serum magnesium (Mg) with total cholesterol, triglycerides (TG), low-density lipoprotein cholesterol and positive correlation with high-density lipoprotein HDL-c. Thus, lower serum magnesium may be associated with a worsened lipid profile and enhanced CVD risk of RA patients.

Although lipid levels are important risk factors, especially high-density lipoprotein, other studies have observed changes in TC, LDL-c and HDL-c after MTX monotherapy and combination therapy (Pincus *et al.,* 2003, Navarro *et al.,* 2013) and improvement in HDL-c levels post DMARD therapy (Filippatos *et al.,* 2013). In our

previous report (Patel et al., 2015) we observed increased HDL levels after 24 weeks of follow up in MTX treated patients, but long-term therapy does not have any favorable effects on HDL, nor does it increase TG, VLDL, TC and LDL with respect to healthy control. In RA there are reports showing either enhanced, decreased or similar levels for TC, LDL-c and HDL-c in comparison to control subjects (Heldenberg *et al.,* 1983; Lorber *et al.,* 1985; Lakatos & Harsagyi 1988; Kavanaugh 1994; Asanuma *et al.,* 1999). White *et al.,* 2006 demonstrated reciprocal association between HDL-c and CRP on a large cross-sectional done on 204 RA patients (White *et al.,* 2006). Van, 2006 showed the presence of dyslipidemia at least 10 years before the appearance of clinical symptoms of RA.

In our study borderline dyslipidemia is present in subjects with RA who are on DMARD treatment. Our patients are given local corticosteroid, when they complain of severe pain and swelling. Prednisolone rapidly improves an important prognostic CVD risk factor, the atherogenic index (log total/HDL cholesterol) (Niroumand et al., 2015). Therefore corticosteroid may not be the risk factor for CVD (Wallberg *et al.,* 1997). Evidences show the favorable effect of conventional DMARD (including corticosteroids) on lipid profile, and therefore, on CVD risk in RA subjects (Van *et al.,* 2006).

Oxidative Stress Analyses

In our study, superoxide dismutase activity is highly enhanced (Table-5) (Kumar *et al.,* 2016). Superoxides anion (O_2^-) plays an important role in the pathogenesis of several diseases. SOD activity neutralizes superoxide anion to hydrogen peroxide (H_2O_2). Activities of catalase and glutathione peroxidase further quench H_2O_2 preventing the formation of aggressive peroxynitrite ($ONOO^-$) and hydroxyl radical (OH^-) (Afonso *et al.,* 2007). Our RA patients showed the

significantly enhanced SOD and ALP activity (Table-5). There was a strong positive correlation between SOD and ALP activity (Table-6). However low activities were observed for catalase and GR. Enhanced activities may be the result of respiratory burst induced by leucocytes. The activation of neutrophilic myeloperoxidase-hydrogen peroxide system in synovial tissue of RA, may result in cyclic self-perpetuating inflammation (Nurcomb *et al.,* 1991). MTX therapy has been reported to increase Zn-SOD activity, but has no effects on glutathione peroxidase (GSH-Px) in rats (Armagan *et al.,* 2008; Al-Saleh *et al.,* 2009), but possibly increased the SOD activity (Vijayakumar *et al.,* 2006; Cimen *et al.,* 2000) this may be attributed to enhanced O2 - production by hyperactive cells resulting in SOD induction (Gregory and Fridovich, 1973). Another possibility may be high free radical production through the xanthine-xanthine oxidase system in RA, rather than defective antioxidant system (Cimen *et al.,* 2000). This enhanced SOD activity may also be an alteration to nullify high free radical production. Post-treatment the antioxidants are high which may result in changes in plasma MDA and high total antioxidant capacity (TAC) (Nourmohammadi *et al.,* 2010).

However, some studies report reduced SOD activity in RA subjects on MTX therapy as compared to RA subjects without MTX therapy (Al-Youzbaki *et al.*, 2013). Sung *et al.,* 2000 have observed that MTX can directly or indirectly suppress the production of active oxygen metabolites induced by IL-6, (which is produced in response to TNF-α stimulation) in RA synovial cells (Sung *et al.,* 2000) as well as in polymorphonuclear cells. The high levels of serum Cu/Zn SOD may support the hypothesis of radical-mediated injury.

Overexpression of extracellular SOD results in dismutation of superoxide, resulting in H_2O_2 accumulation. After evaluation of H_2O_2 in different experimental, it

was concluded that, high SOD does not mean high H_2O_2 (Lin *et al.,* 2006). H_2O_2 formation due to dismutation of superoxide is restricted by the amount of superoxide, not by the rate it is converted to H_2O_2. Accumulation of superoxide results in the oxidation of NO forming peroxynitrite. Thus more H_2O_2 is not likely to be as toxic as this would amount to replacing a very mild cytokine (H_2O_2) for a potent (peroxynitrite) (Zaghloul *et al.,* 2014). Reduced activity of SOD in RA patients has also been reported (Mohamad, 2011).

However, our study is in line with Kamanli *et al.,* 2004, who have reported increased SOD levels in RA patients. Mazzetti *et al.,* 1996 have reported higher serum copper/zinc superoxide in patients with RA. Igari *et al.,* 1982 have reported the correlation between the overall synovial SOD activity and both the clinical severity of the disease and the CRP levels. Mazetti *et al.,* 1996 have concluded that exercise-induced hypoxic reperfusion mechanism within the inflamed joint in RA may lead to increased production of Cu/Zn SOD. Mateen *et al.,* 2016 have shown that increase of oxidative stress increases with the progression of RA.

H_2O_2 formed due to the activity of superoxide dismutase need to be detoxified by glutathione peroxidase and catalase activity. Catalase plays a critical role in preventing ROS mediated damage by using H_2O_2 and converting it into water and oxygen. In our RA patients, catalase activity was significantly low as compared to control. Reduced catalase activity may be due to the interaction of catalase by hydrogen peroxide (Mohamad *et al.,* 2011). Reduced activities of their enzymes may lead to the conversion of hydrogen peroxide (H_2O_2) to hydroxyl radical by iron released from the hemoglobin of lysed erythrocytes (Taysi *et al.,* 2002). Unaltered catalase activity in RA patients has been reported (Veselinovic *et al.,* 2014). Catalase activity was not found in the serum of RA patients. Decreased erythrocyte catalase

activity is also being reported (Taysi *et al.,* 2002). Our study is in accordance with the studies showing reduced catalase activity in serum of RA subjects. Catalase affects the expression of genes which influence inflammation (Benhamou *et al.,* 1998). Low levels of catalase may be responsible for high inflammation in RA. Cimen *et al.,* 2000 have reported enhanced SOD activity and MDA levels and unaltered catalase and GSH-Px activities in RA patients. Gonzalez *et al.,* 2015 has observed the positive correlation between antioxidant GPx and lipid peroxidation levels. Their results suggest that GPx activity is involved in the primary mechanisms against oxidative stress in RA subjects. Both GPx and catalase use hydrogen peroxide (H_2O_2) as the substrate where catalase acts in the presence of high concentration of the substrate while GPx acts at lower concentrations. They also suggested that H_2O_2 concentration may be reduced than in other chronic inflammatory diseases, with oxidative damage being mediated possibly by HO^- (Prego *et al.,* 1997).

Glutathione reductase (GR) is an oxidative stress-inducible enzyme, which plays a significant role in the peroxyl scavenging mechanism and is important in maintaining the functional integration of the cell membranes. GR is a flavoenzyme dependent on NADPH that catalyzes the reduction of the GSSH to GSH. Feijoo *et al.,* 2010 has observed, that myeloperoxidase levels are enhanced in patients with chronic inflammatory disease, especially those with active disease, and that high myeloperoxidase levels are related to augmentation in oxidative damage and the inflammatory response, for myeloperoxidase and glutathione reductase seem to show a similar activity pattern based on the availability of NADPH. Erythrocyte GSH reductase levels rise in healthy individuals, exposed to chronic oxidative stress (Evelo *et al.,* 1992). These findings suggest that GSH levels may be inappropriate in patients with active *Rheumatoid arthritis*, perhaps reflecting impaired GR activity as observed

in our study. The study by Aryaein *et al.,* 2011 showed that GR, beta-carotene, vitamin E was reduced and MDA was enhanced in the patient group than in controls. Kamanli *et al.,* 2004 observed significantly attenuated GSH-Px and catalase activity and low levels of GSH in plasma of RA subjects. However, enhanced GR activity has also been reported in *Rheumatoid arthritis* (Bazzichi *et al.,* 2002). Kerimova *et al.,* 2000 has reported reduced catalase and unaltered GR activities in RA subjects. Reduced GR activity in the red blood cells and polymorphonuclear leucocytes of patients with RA was reported by Mulherin *et al.,* 1996. Vanella *et al.,* 1987 described reduced EGR activity in 15 patients with *Rheumatoid arthritis* and Tarp has reported a similar finding in nine patients with *Rheumatoid arthritis* (Tarp, 1992).

In our RA subjects, alkaline phosphatase (ALP) activity was enhanced relative to control. ALP showed strong positive and significant relationship with SOD. ALPs role is implicated in the osteoid formation and mineralization. Its isoforms are expressed in osteoblasts, liver, leucocytes, breast, kidney, and brain (Weiss *et al.,* 1986, Gum *et al.,* 1990). The bone formation markers are measured in serum and interestingly about half of ALP in serum comes from the bone. Several studies (Thompson *et al.,* 1990, Nanke *et al.,* 2002, Spooner *et al.,* 1982) have reported high serum ALP activity in RA patients. The increased activity may be due to inflammatory cytokines as interleukin-1 (IL-1) which has been correlated with the acute phase reactants (Thompson *et al.,* 1990) and CRP levels. The role of T-cell is well documented in the pathogenesis of RA subjects. Enhanced ALP activity may be due to its leakage from injured or killed cells. Alkaline phosphatase has been implicated as a marker in RA subjects. It can provide diagnostic information by determination of an isoform of ALP derived from bone or liver (Vaithialingam *et al.,*

2013). An inverse association between serum antioxidant levels and inflammation has been reported (Paredes *et al.,* 2002).

A study by Jalili *et al.,* 2014 showed that antioxidants may significantly improve disease activity, but do not affect the number of painful and swollen joints. Antioxidants and MDA work reciprocally to keep oxidative stress-mediated damage in control and may be helpful in the control of oxidative stress and clinical outcomes in RA subjects. Management of oxidative stress may be an important therapeutic option for RA along with DMARD. Supplementation of antioxidants along with catalase and/or GPX may confer more protection.

Trace Metal Analyses

In recent studies, RA seems to have derangement of mineral contents as magnesium, copper, zinc, phosphorous, boron. They are required in essentially required in the body. There is a scarcity of data related to minerals and their role in RA. Magnesium levels in our treated RA patients were lower as compared to controls. Mg (Talal, 1992) is one of the essential nutrient of the body and studies suggest its role in chronic inflammation (Weisinger and Bellorin, 1998; Mahalle *et al.,* 2012). Decreased level of Mg is considered a marker for RA (Lucia *et al.,* 2011; Linos *et al.,* 1980). Magnesium has important functions in the cardiovascular system, as an activator of sodium, potassium ATPase. It is antiarrhythmic and is associated with cardiovascular disease susceptibility (Weisinger and Bellorin, 1998; Chiuve *et al.,* 2011; Mahalle *et al.,* 2012; Makhdoom *et al.,* 2009). In humans, low serum magnesium concentrations have been associated with high C-reactive protein (CRP) levels (Guerrero & Rodriguez 2002; Rodriguez & Guerrero, 2008). Several cross-sectional studies have reported inverse relationships between magnesium intake and

some inflammatory markers, including high sensitive CRP (hs-CRP) and IL-6 (King *et al*., 2005; Song *et al.,* 2005; Bo *et al.,* 2006; Song *et al*., 2007; Chacko *et al*., 2010).

The level of phosphorous was significantly higher in RA patients as compared to controls (6.13± 0.101 Vs 4.08± 0.122; $p<0.05$). In RA patients phosphorous showed positive correlation with catalase ($r=0.396$; $p<0.05$) and zinc levels ($r=0.344$; $p<0.05$) and a negative correlation with copper ($r=-0.412$; $p<0.05$) and MDA ($r=-0.345$; $p<0.05$). The studies suggest a strong association between elevated phosphorous and Ca and phosphorous products and the development of calciphylaxis. Phosphorous influences a number of pathways involved in vascular calcification. It also has a role in the induction of differentiation of vascular smooth muscle cells into osteoblast-like cells capable of extraskeletal mineralization which is an important process in the development of vascular calcifications. Thus phosphorous may have a role in augmenting inflammation.

In our study, the levels of zinc and copper are higher in RA patients as compared to controls. This clearly shows that RA patients are not deficient in Zinc or copper (Milanino *et al.,* 1993). As zinc is considered anti-inflammatory with studies showing a negative correlation between zinc and levels of IL-1 and TNF-α. Our study is in accordance with findings of Mierzecki *et al*., 2011 who have reported nonsignificant but higher levels of zinc in serum. Though zinc levels should have been lower considering the role of proinflammatory cytokines as IL-1 and TNF- α in inhibiting albumin synthesis in the liver and lower their zinc-binding capacity, which should, in turn, reduce the plasma zinc levels. However, lower values of zinc in other studies may be due to pharmacological treatments or other effects which also need to be considered. Serum zinc levels have been shown to decrease during the acute - phase response of inflammation and with treatment with NSAIDs (Balogh *et al.,*

1980). It is suggested that these may be due to variations in disease activity and treatments. Probably alterations in inflammation may have some role in the levels of essential minerals.

In our study levels of copper are also higher. Their levels have been shown to increase in all inflammatory processes including RA. Our findings are consistent with Scudder *et al.,* 1978 and Tuncer *et al.,* 1999 who reported high copper in RA patients. The studies have shown that hyper-cupremia associated with the inflammatory response is due to oxidative stress (Ford, 2000) as they found positive correlations between serum Cu levels and inflammatory markers as high serum CRP and ESR in RA patients (Liuzzo *et al.*, 1994; Salonen, 1991). In contrast to many other studies, we found an inverse correlation between Cu and CRP levels ($r= -0.419$, $P<0.01$). Cu is an environmental bio element which plays a key role in the cell's physiology, as a cofactor or a component of the enzymes, participating in the anti-oxidative process, or in detoxification of oxygen free radicals. RA patients have higher levels of copper as compared to control (Rainford, 1982). It was found that Cu complexes were effective in treating arthritis. Cu complexes not only have anti-inflammatory properties but some anti-arthritic drugs in their active form are complexed with copper (Rainford, 1982). The hypercupremia that develop was suggested to be the outcome of dyslipidemia (Aaseth *et al.*, 1978) or due to cytokines which are reported to enhance the release of Cu thionins during the oxidative burst of polymorphonuclear cells (Balogh *et al.,* 1980). As many studies have reported higher levels of copper in active RA, thus copper may be used as an additional biochemical marker.

Genetic Polymorphism Analyses

PADI4

PADIs are involved in the post-translational deimination of arginine in proteins; the resulting citrullination partially unfolds proteins via loss of the positive charge of the arginine moiety (Ikari *et al.,* 2005; Yamada *et al.,* 2005; Yamamoto and Yamada, 2005). PADI4 is non-HLA genetic factors involved in RA responsible for citrulline formation, which have been implicated in RA pathogenesis (Anzilotti, 2010). Our results showed no association between RS188_1 polymorphisms with RA while there was a significant association between RS188_2C/G and PADI_102C/T polymorphisms in RA patients.

PADI4-102, SNP is present in the intron of PADI4 gene and affects the stability of mRNA. This PADI4 locus is significantly associated with susceptible haplotype and augments the citrullination leading to high ACPA production (Suzuki *et al.,* 2003; Vossenar *et al.,* 2004). However, PADI4 haplotype (PADI4-89, 90, 92) was associated with disease development but does not seems to affect erosion. Whereas it is associated with erosion in caucasian population (Bang *et al.,* 2010; Hoppe *et al.,* 2009).

Our results (table-12 and 13) showed a significant association of PADI_102 C/T polymorphism in RA. These results were similar to the data of Somia, 2012 who has reported the significant association of this locus with RA. PADI gene has been suspected in the prognosis, activity, and severity of RA (Suzuki *et al.,* 2013; Ceccarelli *et al.,* 2012). Ikari *et al.,* 2005 also reported the significant differences in frequencies of RA and control. In Asian population, some variants of PADI4 genes have been reported in susceptibility to RA (Ikari *et al.,* 2005; Suzuki *et al.,* 2003; Hoppe *et al.,* 2006). No significant association was observed between RS188_2 C/G

polymorphism of PADI in RA and control. Many studies have failed to confirm this association in various European populations (Martinez *et al.,* 2005; Burr *et al.,* 2010).

PADI4 haplotypes have been demonstrated to be associated with RA in different populations, including Japanese, Korean and Chinese cohorts (Suzuki *et al*., 2003; Freudenberg *et al*., 2011; Fan *et al*., 2008).

PADI2 and PADI4, these two genes are largely expressed in hemopoietic cells (Vossenaar et al., 2003). The expression of PADI2 and PADI4 was related to high inflammation, where both the enzymes were shown to be present inside or near the citrullinated fibrins deposits (Foulquier et al., 2007). The association studies show variations in different cohorts, thus the variation between Caucasian and Asian populations might be due to heterogeneity of both genetic and environmental factors or gene-environmental interaction may be important in its susceptibility for RA (Too *et al.,* 2012).

TIMP4

TIMP-4 belonged to the TIMP gene family and is located on the 3p25.2 chromosome (NCBI). In general, all mammalian TIMPs have two domains, one with 125 amino acid residues of N' terminal and one with 65 amino acid residues of the C terminal, with three disulfide bonds providing conformational stability to the protein (Williamson *et al.,* 1990). Matrix metalloproteinases (MMPs) are the major catabolic proteinases includes collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, which break ECM of joints (Murphy and Nagase, 2008). The degradation of ECM is the characteristic feature of arthritic diseases in which the structural parts of the cartilage, proteoglycan aggrecan, and type II collagen are degraded by MMPs (matrix metalloproteinases) and ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) (Clark and Parker, 2003).

TIMP4 expression is significantly low in OA cartilage (Clements *et al.,* 2006; Kevorkian *et al.,* 2004) and it is shown that TIMP-4 is important in inhibiting adjuvant-induced arthritis (AIA) in a rat model. It probably acts via suppression of the release of TNF-α, an important pro-inflammatory cytokine (Celiker *et al.,* 2002).

A previous study has reported that there were no significant differences of an allele or genotype frequencies of TIMP-4 C/T gene polymorphisms between RA and control (Lee *et al*., 2009). Although an imbalance in the MMP: TIMP ratio in favour of MMP appears to be an important determinant of tissue damage in both RA and OA, we found no significant associations between the TIMP-2 and TIMP-4 polymorphisms with RA (Lee et al., 2008).

PTPN22

PTPN22 gene is a negative regulator of signaling pathways of T and B cell receptor which encode the lymphoid protein tyrosine phosphatase (Lyp) (Cloutier and Veillette, 1999; Stanford *et al.,* 2010). At the position of 620 in PTPN22 1858C>T polymorphism arginine changed to tryptophan residue and 1858T variants are found to be associated with diabetes (Bottini *et al*., 2004).

In immune cells, phosphorylation/dephosphorylation cascade of signalling intermediates (Stoker, 2005) is important response for extracellular stimulus as antigens or cytokines (Mustelin *et al.,* 2005; Rhee and Veillette, 2012; Dolton *et al.,* 2006; Vang *et al.,* 2008; Mustelin *et al.,* 2002). These reversible reactions of phosphorylation and dephosphorylation are mediated by different protein kinases and protein phosphatizes (Hunter, 2009). Protein tyrosine phosphatases (PTPs) are essential in the regulation of crucial signaling pathways required for cell division, cell differentiation and survival (Tonks, 2006).

Protein tyrosine phosphatase, non-receptor 22 (PTPN22) has recently been recognized as a missense SNP, associated with RA (Begovich *et al.,* 2004). Both RA and type I diabetes (T1D) show strong association with Trp620 allele (rs2476601) polymorphism (Lee *et al.,* 2007; Smyth *et al.,* 2008).

A replicated study of PTPN22 revealed the association with juvenile idiopathic arthritis (JIA) and RA (Anne *et al.,* 2005). Different studies in Japanese and Russian populations reported that there was no direct association of PTPN22 R620W polymorphism with RA (Ikari *et al.,* 2006; Zhebrun *et al.,* 2011). However, the studies of Zahra *et al.,* 2017 and Yeniley, 2017 have shown that the RA patients have a significantly higher frequency of 1858T risk alleles as compared to the healthy controls and it could play an important role in disease susceptibility.

An Australian case-control study recently reported the association of PTPN22 rs2476601polymorphism with JIA in females only (Chiaroni *et al.,* 2015). In European population, other loci of PTPN22 (rs3789607, rs12144309, rs3811021 and rs12566340) were genotyped and they found that, they were not associated with risk of RA (Wan *et al.,* 2010). Independent of HLA, PTPN22 1858C>T gene polymorphism is described as a genetic risk factor for RA (Lee *et al.,* 2012). In central India, a research finding, revealed the association between the PTPN22 polymorphism and RA while there was no association with the Vitamin D receptor (VDR) polymorphism with RA susceptibility (Smriti *et al.,* 2014).

A recent study in Iran showed that only C allele is present and there was no association with autoimmune disease susceptibility, including RA in the population (Ahmadloo *et al.,* 2015) but a study in South West of Iran reported that PTPN22 may play an important role in susceptibility of autoimmune diseases (Abbasi *et al.,* 2016). The same SNP reported in JIA is sex-specific where females are reported to be more

susceptible (Goulielmos *et al.,* 2016). The risk of RA in Asian populations is not associated with PTPN22 1858C/T polymorphism, but a meta-analysis reported that susceptibility to RA is associated with PTPN22 1858C/T polymorphism in Caucasian populations (Gowher *et al.,* 2016).

NMR Analyses

NMR analysis of RA samples showed dyslipidemia and higher citrulline in RA patients as compared to control. Lakatos reported lower TG and higher TC and LDL-c levels and reported lower HDL-c levels (Lakatos and Hárságyi, 1988).

High lactate was observed in our study. Yang *et al.,* 2015 and Lauridsen *et al.,* 2010 suggested that an elevated level of lactate could be one of the biomarkers for the diagnosis of chronic inflammatory conditions (Yang *et al.,* 2015; Van, 2012; Lauridsen *et al*., 2010). Indeed, lactate is reported to have a pathogenic role (Yang, 2015; Van, 2012; Lauridsen *et al.,* 2010). The higher lactate concentration in RA may be related to low oxygen levels prevalent in an inflammatory environment (increased NAC—N -acetylated glycoprotein) with the induction of hypoxia, promoting anaerobic respiration (Yang *et al.,* 2015; Lopez *et al.*, 2012; Gu *et al.,* 2012).

NMR analysis showed the abundances of citrulline and ornithine in the RA group than those in the controls. Citrulline synthesized from ornithine and carbamoyl phosphate is a key intermediate of the urea cycle. Cittruline is generated by posttranslational modification of arginine residues by peptidyl arginine deiminase (Tarcsa, 1996). Because citrulline is a major antigenic determinant recognized in the RA subjects. Therefore, ACPAs have been used for the diagnosis of RA and have been established as a useful tool to discriminate RA from other arthritic diseases (Bas, 2002).

6. SUMMARY AND CONCLUSIONS

Summary

Rheumatoid arthritis (RA) is an autoimmune, inflammatory disease which involves multiple synovial joints. RA causes significant disability and economic burden as a result of synovial inflammation and joint destruction. Patients with RA face difficulty in performing their daily activities. RA, like other autoimmune diseases, is three times more prevalent in females as compared to males. The differences, over the occurrence and development of worst and aggressive disease in females, are not clear, but genetic and hormonal factors are suggested to be involved.

As RA is a complex disease, therefore it is very important to analyze the major players involved in the pathogenesis of RA. The present study was therefore planned to analyze i) demographic and serological parameters in the RA patients with liver and kidney function monitoring; ii) the level of MDA, a product of lipid peroxidation and activities of free radical scavenger system enzymes as superoxide dismutase (SOD), glutathione reductase (GR), catalase and levels of alkaline phosphatase (ALP) in RA patients treated with MTX, folic acid. Vit-C and occasional corticosteroids, iii) Analysis of mineral content in RA patients versus control, iv) evaluation of lipid parameters in RA and control subjects, v) genetic association of RA with a few loci for analyzing their association with our Indian RA patients, vi) NMR analysis of metabolites for their possible role in RA. They may help in determining possible roles of these in damage to RA patients and may open therapeutic opportunities for better management of *Rheumatoid arthritis.*

There was no significant difference in the BMI of female and male patients. Females have more aggressive and painful disease than males. As female patients in our study had less average age than males, suggesting the early onset of RA in them

as compared to males. There are differences in functional capacity in female and male subjects with RA where females have a more functional impairment than males. In our study also DAS-28-CRP is higher in females as compared to males. These differences may be due to general strength of bones and muscles, bone mineral density (BMD), hormones etc.

The patients had reduced hemoglobin as compared to controls. ESR, pain during holding and lifting of objects on VAS and C-reactive protein levels were significantly higher as compared to control. The values for liver function tests, i.e., SGOT and SGPT of RA subjects were significantly lower than control. As compared to control, the uric acid of RA patients was significantly reduced suggesting no adverse effects of MTX therapy on liver and kidney function.

In our study MDA production was significantly high in RA patients. It is lipid peroxidation end- product, which may be due to high ROS during chronic inflammation. Lipid peroxides are generated at the site of tissue injury due to inflammation and diffuse into the blood and can be estimated in serum or plasma. There are reports of high levels of MDA in the plasma, serum and erythrocytes of RA patients.

In our study activity of SOD was highly enhanced. Superoxides anion (O_2^-) plays an important role in pathophysiology of many diseases. Superoxides anion (O_2^-) is neutralized by SOD to hydrogen peroxide (H_2O_2). H_2O_2 is further quenched by the activity of catalase and glutathione peroxidase. The patients showed significantly higher activity of SOD and ALP. There was a strong positive correlation between SOD and ALP activity. They showed reduced activities for catalase and glutathione reductase. The GR activity was positively correlated to MDA, SOD and ALP.

In the present study, we found decreased levels of serum magnesium in female and male RA subjects as compared to the reference range, though no significant difference was observed between the two sexes in serum magnesium levels. The decreased Mg level is considered as a marker for RA. The level of phosphorous and copper was non-significantly higher in male RA patients as compared to females. In female RA patients, phosphorous showed an inverse correlation with copper. There was a strong association between elevated phosphorous and Ca and phosphorous products and the development of calciphylaxis. Both female and male RA subjects had raised serum copper levels as compared to the reference values. RA patients are shown to have high levels of copper. Their levels have been shown to increase in all inflammatory processes including RA.

We found decreased levels of serum magnesium in female and male RA subjects as compared to reference range, though no significant difference was observed between the two sexes in serum magnesium levels. Chronic inflammatory conditions in RA may alter the levels of magnesium and possible mechanism of reduced magnesium may be due to chronic inflammation induced autoimmune injury. Our results are in accordance with other studies, and suggest that RA is associated with serum magnesium disturbances. Mg is one of the essential nutrient in the body and studies suggest its role in reducing chronic inflammation. The decreased Mg level is considered as a marker for RA. Magnesium is an activator of sodium, potassium ATPase, is antiarrhythmic and is associated with cardiovascular disease susceptibility. In humans, low serum magnesium concentrations have been associated with high C-reactive protein (CRP) levels.

The patients show dyslipidemia with significantly higher total cholesterol, triglycerides, low-density lipoprotein and very low-density lipoprotein as compared to

control, but changes were within or at borderline of the reference range. The HDL of the patients was significantly reduced as compared to control. Inflammation may be the primary cause for systemic alterations in the levels of minerals and enzymes which further modulate acute phase plasma proteins. Negative correlation is being reported between serum magnesium with TC, triglycerides, LDL-c and positive correlation with HDL-c. Thus, lower serum magnesium may be associated with a worsened lipid profile and increased CVD risk of RA patients.

Genome-wide association study (GWAS) revealed an association of RA with many genes. In our study, PADI alleles RS188_2 and PADI_102 showed some association with RA. But these allelic combinations of the SNP were also observed in controls, suggesting the need to recruit a much larger cohort for the analysis. A significant association was found with A/G SNP of TIMP4 with RA.

The NMR study showed higher citrulline formation in RA which correlates well with the presence of anti-citrullinated antibodies in RA patients. Lipid parameters were also deranged in RA.

Therefore, RA in the present study has been shown to have marked oxidative stress, high inflammation, deranged minerals, decrease magnesium levels, dyslipidemia and lower hemoglobin. Magnesium supplementation and oxidative stress management may be considered an important therapeutic option for RA along with DMARD.

CONCLUSIONS

The final conclusions are

1. RA patients showed significantly lower hemoglobin and higher ESR, DAS, CRP and VAS as compared to controls. Significant differences were observed in ESR, DAS, CRP and VAS in RA female versus males.
2. RA patient had normal uric acid, SGOT and SGPT. MTX was reported to adversely affect liver profile but MTX supplementaion with folic acid and vitamin C did not result in worsening of liver profile of RA patients in long-term followup.
3. RA patients had high oxidative stress as they had high MDA. They had significantly higher activity of superoxide dismutase and alkaline phosphatase, but lower activity of catalase and glutathione reductase. RA females experienced more oxidative stress, however, they also have better activities of antioxidant enzymes as compared to RA males. Positive correlation was observed between GR and SOD and SOD and ALP, suggestive of their dependence to quench excessive free radicals in the body. The serum levels of zinc, copper and phosphorous were significantly elevated in RA patients as compared to control, while serum magnesium was significantly decreased in RA patients as compared to control. Probably including magnesium in therapy and inclusion of antioxidant along with regular medicine may help to control disease better than present treatments.

4. RA patients showed dyslipidemia which may be due to the ongoing process of inflammation and oxidative stress. The patient showed higher levels of total cholesterol, very low-density lipoprotein, triglycerides, low-density lipoprotein and lower level of high-density lipoprotein as compared to control. Dyslipidemia

may increase their risk for atherosclerosis and subsequent cardiovascular disease (CVD).

5. In our study peptidyl arginine deiminase (PADI) alleles, RS 188_2 and PADI_102 showed some polymorphism in RA versus control. Another gene PTPN-22 was non-polymorphic in our population. However, no significant association of A/G SNP of TIMP4 was observed in our patients.

6. NMR analysis showed that lipid parameters are deranged in RA patients as compared to control.

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8. LIST OF PUBLICATIONS

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