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

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Dedicated to

My Loving parents

Beloved

Wife & Little Son

Certificate

This is to certify that the research work embodied in this thesis entitled ***"Effect of genetic variations of PADI4, PTPN22 and TIMP4 in the susceptibility of Rheumatoid arthritis in North Indian Population"*** has been carried out by **Mr. Vivek Kumar** under the joint supervision. He has fulfilled all the requirements for the degree of Doctor of Philosophy in Biotechnology of Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow (U.P.) India. The work stated in this thesis is original, carried out by the candidate himself and has not been submitted in part or full to any other University for any other degree.

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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 Nucleic 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
GSH-Px	Glutathione Peroxidase
GWAs	Genome Wide Association Studies
H_2O_2	Hydrogen Peroxide
Hb	Haemoglobulin
HCl	Hydro Chloric Acid
HDL	High Density Cholesterol
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 Cholesterol
MHC	Major Histocompability Constant
MMPs	Matrix Metalloproteinase
m^2	Metre square
MCP	Meta carpophalangeal
m-RNA	Messenger Ribo Nucleic 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 Glyceraldehydes
TBA	Thiobarbutyric Acid
TCA	Tri Carboxylic Acid
TDW	Triple Distilled Water
TIMP	Tissue Inhibitory Matrix Metalloproteinase
TC	Total Cholesterol
U/L	Unit/Litre
VLDL	Very Low Density Cholesterol
VAS	Visual Analog Scale
V	Volume
W	Tryptophan
Wt	Weight
Zn	Zinc

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1. INTRODUCTION

Immune system is the defence system of our body which protects us from invading pathogens. It is able to differentiate self from nonself components. However sometimes this mechanism fails resulting in inappropriate targeting of self components leading to a condition called auto (self) immunity (defence). Autoimmunity is a biological phenomenon which activates self reactive B and T lymphocytes. Autoimmune reactions due to the abnormal auto reactive T cells and auto-antibodies can cause organ specific or systemic diseases. Autoimmune diseases (AID) are characterized by the failure of immune-tolerance against self antigens that are regulated by complex genetic factors or infectious agents and/or environmental factors. Autoimmune diseases can be divided into two categries, organ-specific diseases like diabetes mellitus (DM) and intestinal bowel diseases (IBD), that only affect specific organ systems, and systemic diseases such as systemic lupus erythrematous (SLE), *Rheumatoid arthritis*, systemic sclerosis (SS) and Sjogren's syndrome (SJS) which involve the whole body.

Rheumatoid arthritis

According to a 2014 report of the UN non communicable diseases account for about 2/3rd of all deaths. *Rheumatoid arthritis* (RA) is a non communicable inflammatory disease which leads to progressive destruction of synovial joints (Bodman & Roitt, 1994). 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). Prevalence of RA in Indian population was reported to be about 0.75% (Malaviya *et al.*, 2003). *Rheumatoid arthritis* (RA) is diagnosed on the basis of American College of Rheumatology (ACR) criteria, presence of anti-cyclic citrullinated antibody (anti-CCP). ACR criteria involve radiological, clinical and serological parameters, where presence of three or four criteria confirms the diagnosis of RA. Measures of inflammation as C- reactive protein (CRP) and erythrocyte sedimentation rate (ESR) elevation are also considered impoatant for diagnosis of inflammatory disease as RA.

The inflammation in RA is measured by acute phase reactants. The disease activity measures of RA are expressed in disease activity score-28 (DAS28) which measures swelling and tenderness in 28 joints of affected person according to ACR criteria (Felson *et al.,* 1993; Prevoo *et al.,* 1995). In RA the most widely used inflammatory markers are erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) evaluations. The level of inflammation is measured by acute-phase plasma protein levels in blood that is indirectly measured by ESR. Since the inflammation causes the red blood cells to settle more promptly (Firestein *et al*., 2009). ESR is a non-specific acute phase reactant of systemic inflammation, but elevated levels has many reasons therefore only rheumatic disease may not be attributed for high ESR. ESR levels can be greatly influenced by malignancies, infections or abnormal size and shape of red blood cells (Kushner 1991). In affetcted rheumatic patients ESR tend to be higher in females as compared to males (Nestel AR, 2012; Radovits *et al.,* 2008; De Silva *et al.,* 2008). It also increases with age (Nestel AR 2012; Radovits *et al.,* 2008; De Silva *et al* 2008; Ranganath *et al*., 2005) and body mass index (BMI) (Firestein *et al.,* 2009). To overcome the limitations of ESR, another important component as CRP has been suggested as a substitute inflammatory marker for the disease activity evaluation in *Rheumatoid arthritis* (Fransen *et al.,* 2003). The use of CRP over ESR in assessing *Rheumatoid arthritis* inflammation estimation has been emphasized by (Crowson *et al.,* 2009).

In *Rheumatoid arthritis* elevated production of reactive oxygen species (ROS), reactive nitrogen species (RNS) and depletion of antioxidants are being implicated. The intracellular redox state may be an important determinant leading to the progression of the disease. The enhanced imbalance between oxidants and antioxidants may be enough to induce structural and functional changes in cells and tissues. This state is typically termed as oxidative stress. These ROS and RNS have both beneficial and toxic effects. Feldmann et al, 1996 reported that the generation of ROS (SOD, H_2O_2) in large amount by activated macrophages in RA leads to oxidative stress (Feldmann *et al*., 1996).

These ROS are capable of damaging membrane lipids, connective tissue and nucleic acids of the cell. Free radicals and their byproducts are essential mediators of inflammation. Synovial cavity is the main target of immune attack in RA which may be due to chemo-attractant property of synovial fluid. This leads to accumulation of leukocytes within the synovial tissue and causes respiratory burst characterized by increased oxygen consumption and increased anaerobic glycolysis leading to generation of superoxide, hydroxyl, hypochloric radicals etc. (Marshells & Bangert, 1995). Neutrophils are very active in synovial fluid of RA patients which augment inflammation and enhance damage of joint componants (Vasudevan and Sree Kumaris, 2001; Nurcomb *et al*, 1991).

Free radicals generation and enzymes degrading them are in tight homeostasis in the body, which prevent damage. However, imbalanced activity may be lead to free radical mediated tissue injury. The study by Blake et al. 1981 showed that enzymatic/non enzymatic antioxidant systems are highly deregulated and impaired in RA (Blake *et al*., 1981). Thus, there are chances of free radical mediated damage in the body of RA patients due to their higher production and impaired quenching. The analysis of activities of different antioxidant enzymes like superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and glutathione reductase (GR) may have effective therapeutic potential (Blake *et al*., 1981; Mazetti *et al*., 1996; Shah & Vohora, 2002).

The higher inflammation and oxidative stress not only affects joints but also affects various other systems leading to important systemic menifestations. Cardiovascular disease (CVD) is one of them. The higher mortality rate of RA patients is due to their exposure to greater risk for cardiovascular diseases (CVD), myocardial infarction (MI) and stroke (Wolfe *et al*., 1994). There is dyslipidemia observed in RA patients (Mishra *et al*, 2012) that may increase their morbidity and mortality from CVD. In recent years tremendous data reported the role of biochemical parameter which play an important role in RA (O’Dell, *et al*., 2007; Lucia *et al*., 2011; Pallinti *et al*., 2009; Fischman *et al*., 2010; Magnus *et al*., 2010; Chavan *et al*., 2015).

As RA has genetic predisposition and many loci have been implicated which are associated with the disease. The heritability of RA has been predicted in approximately 60% populations (Mac Gregor *et al.,* 2000, Deighton *et al*., 1989). Genetic susceptibility for this disease is very high where (i) according to R.M. Nakamura, first degree relatives of the RA patients are at four to six times higher risk for developing RA (Nakamura RM, 2000). The presence of some MHC-II encodinf allels in HLA-DR molecules as (HLADRB1 *0401 and HLA-DRB1*0404) are found to be associated with more severe disease condition (Mattey *et al*., 2007, Van Gaalen *et al., 2004).*

There are some gene which may play important role in the disease susceptibility to RA (van der D *et al*., 2010, Szodoray *et al.,* 2010). Linkage disequilibrium studies revealed susceptibility loci for RA within several chromosomes, with one consistently implicated the HLA-DRB1 gene. Peptidyl arginine deiminase type IV (PADI4) (EC 3.5.5.15) is one of member of PADI gene family located on chromosome 1p36.13. It encodes the enzyme which is responsible for the posttranslational conversion of arginine residues into citrulline in many mammalian tissues (Zhou & Menard 2002). A previous cloning analysis report has shown the presence of five isoforms in rodents: PAD I (Ishigami *et al* 2001), PAD II, PAD III (Nishijyo *et al*., 1997), PAD IV (Ishigami *et al*., 1998) and ePAD, which was provisionally named as PAD VI. PAD I has been identified in the epidermis (Guerrin *et al*., 2003), PAD II in sweat glands, PAD III in the hair follicle, while PAD IV has been detected in the precursors of neutrophils and macrophages (Vossenaar *et al*., 2004) and PAD VI mRNA has been detected in the testis, peripheral blood leucocytes and ovary**.**

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, involved in the protein–protein interactions.

Begovich *et al*. (2005) 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 or(1858 T/T genotype) (Harrison 2006).

The actual agents involved in joint degradation or the matrix metalloproteinases (MMPs). The matrix metalloproteinases (MMPs) are a family of zinc dependent endopeptidases, that regulate the breakdown of extracellular matrix (ECM), which is necessary for physiological processes of tissue remodeling, morphogenesis, embryonic development and resorption but it very importance in the pathological conditions including tumor growth, inflammation and metastasis (Lambert *et al.,* 2004). Extracellularly, the activity of MMPs is regulated by tissue inhibitor of metalloproteinases (TIMPs) (Visse and Nagase 2003). The TIMP family consists of four different members (TIMPs 1 to 4). All of these other than TIMP-4 are expressed in body fluids and most tissues. TIMP-4 has a tissue-specific distribution, being present in striated muscles, brain and ovaries. The expression of TIMPs is typically induced by external stimuli such as certain inflammatory cytokines (IL-6, IL-1β) and by certain growth factors.

Extracellularly, TIMPs forms high affinity noncovalent bonds with MMPs and inhibits its activities. Proteolytic activities of MMPs are inhibited by the binding of the amino-terminal domain of TIMP to the active site of MMPs. The carboxy-terminal domain of certain TIMPs, may also form complexes with proenzymes of MMPs (proMMPs) (Visse and Nagase 2003). However, limited differences in TIMPs' specificities have been recognized. Indeed, TIMP-1 is a special inhibitor of soluble MMPs, while TIMP-2 and TIMP-3 are efficient inhibitors of the membrane-bound MMPs. TIMP-3; inhibitory activity is enhanced by some members of the ADAMTs (a disintegrin and metalloproteinase with thrombospondin motifs) family which inhibits TNF-α-converting enzyme and aggrecanases. TIMPs have been shown to regulate the cell survival and apoptosis, stimulate cell proliferation participating in mitosis and tissue differentiation, and inhibit angiogenesis.

Tissue damage in RA is determined by the ratio of MMP:TIMP. According to a study TIMP gene polymorphism are also associated with *Rheumatoid arthritis* (Lee *et al.*, 2003). All these factor affects the protein of RA patients. Therefore it is very omportant for analyse proteomic and metabolic changes occurring in RA.

NMR analysis of metababololites allow rapid identification of metabolic perturbations in biological systems and is routinely used to evaluate systemic response to any subtle pathophysiological stimuli or stress. Over the years, it has been increasingly recognized as a valuable complementary approach to genomics, transcriptomics, and proteomics to achieve a complete understanding of the disease mechanism. It also provides opportunities for developing diagnostic/prognostic biomarkers for the disease. and. Practically, metabolomics approaches relies on multivariate statistical analysis of data collected with advanced analytical techniques -such as gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR) spectroscopy. Of them, NMR combined with multivariate analysis is an ideal platform and it has been extensively used in several metabolomic studies. Compared to other analytical and biochemical methods, NMR poses several advantages. First, it is applicable for a variety of biological and clinical samples, tissue extracts and even cell lines. Second, it is rapid, quantitative and offers the potential for high-throughput (>100 samples per day). Third, it is non-destructive, non-invasive and nonselective i.e. multiple metabolites are detected in as little as 50 µl of peripheral blood plasma/serum . And last it requires virtually no sample preparation and provides highly reproducible results.

Therefore it becomes very important to understand the process of the disease which could help in identifying the factors which are dearranged in the disease. Identifying of these would not only help in the diagnosis but would help also help in providing more thereupatic targets. Thus which can be utilized for diagnosis and better management of RA.

Identification of these would help in determing the potential of already existing parameter on our Indian participants. Analysis of other factor would help clinicians plan more targeted treatment to same tissue destruction and prevent ongoing inflammation in RA patients. The patients investigation was therefore planned to i) Analyze the existing diagnostic parameters and confermation of RA with help of clinical diagnosis, ii) Analysis of serological parameter possible changes, iii)evaluation of the status of oxidant-antioxidant pathway in RA patients iv) Genome wide association studies(GWAS) of PADI4, PTPN22 and TIMP4 genes for their role in susceptibility to RA.

Objectives

RA being systemic inflammatory disease involves attaractions in many physiological components therefore the present study was planned to analyze biochemical parameter and genetic association of a few genes for their role in susceptibilty 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 LITRATURE

2.1 Rheumatoid Arthritis

In the 4th century BC Rheumatic diseases were first recognized by Hippocrates. The term rheumatology has its origin from “rheuma,” which indicate flowing, and is mentioned in article titled Hippocratic corpus in which he observed that podagra was related to opulent lifestyle and termed it as “arthritis of the rich.” (Pasero and Marsen, 2004). 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, chronic autoimmune disease, characterized by inflammation in synovial membrane (synovitis). The affected joints become warms and swollen with tenderness and stiffness in the final stage which causes functional disability (Vandana *et al.,* 2012). Inflammation and systemic swelling of peripheral joints is the hallmark of RA (Michelle & Kahlenber, 2011). Inflammation leads to cartilage destruction, bone erosion and joint deformity. RA patients commonly report pain and stiffness in multiple joints. Although some of them experience symptoms at just one location but later the symptom emerge at other sites, accompanied by symptoms of dieting behavior, weakness, or fatigue. The joints which are always 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). RA preferentially affects women, in ratios around 4:1(Wendy Marder, 2015).

2.2 Prevalence

Worldwide the estimated prevalence of RA is 1 to 2% (Sudha *et al.,* 2012). Another Study of (Malemba *et al.,* 2012) reported that the prevalence of RA in African population was 0.6 to 0.9% in adults (Manish *et al.,* 2011). The prevalence of male populations differs significantly among different areas of the world. This variation maybe related to lower occurrence of the disease in developing countries or due to differences in the age distribution between the populations studied.

North American-Indian populations have the highest recorded occurrence of RA, with prevalence of 3.5 to 5.3% whereas low prevalence has been reported is rural South African blacks and in Japanese (0.1%) (Del *et al.*, 2003). The prevalence in Pima Indians was 5.3% (Del Puente et al., 1989) and Chippewa Indians was 6.8% (Harvey and Lotze, 1981).

2.3. Prevalence of India

The study of Sudha et al 2012 reported that 1% prevalence of RA. The prevalence reported from the developed countries was 0.75% which was quite similar to Indian population. Probably matched occurrence RA in of Indians and Caucasions may be due to the fact that the North Indian population is genetically closer to the Caucasians than to other ethnic groups (Malviya *et al.*, 1993). Recent Indian study has predicted that about 70% of patients with RA are women. It is 2 to 3 fold higher in women than men due to the hormonal or reproductive changes before menopause, (Mohana *et al.*, 2010).

2.4 Role of Age and Gender

Rheumatoid arthritis may affect at any age. However it is observed that RA affects at third to sixth decade with women twice as likely to establish the disease than men. Wolfe *et al* (1994) reported that the prevalence of RA is apparently higher in females than the male with the predicted ratio around 3:1. Lee and Weinblatt (2001) have reported that the 1% Caucasian population is affected with the male to female ratio of 1:2.5. Jonsson *et al.,* (1998) reported that the young women below the age of 50 years with RA have higher risk of developing fractures than women without *Rheumatoid arthritis.*

2.5 Diagnosis of *Rheumatoid arthritis*

2.5.1 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 & 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, where presences of four parameters confirm the diagnosis.

Box-1 Revised criteria for classification of RA (Arnett et al., 1987).

1. Morning stiffness - Morning stiffness in and around the joints lasting at least one hour before maximal improvement.

2. Arthritis of three or more joint areas - At least three joint areas simultaneously having soft tissue swelling or fluid observed by a physician (the 14 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 wrist, MCP or PIP joint (Fig-1A).

4. Symmetric arthritis - Simultaneous involvement of the same joint on both sides of the body (bilateral involvement of PIP, MCP or MTP -joints is acceptable without absolute symmetry) (Fig-1C).

5. Rheumatoid nodules -Subcutaneous nodules over bony prominences or extensor surfaces or in juxta - articular regions observed by a rheumatologist (Fig-1B).

6. Serum rheumatoid factor - Demonstration of abnormal amounts of serum rheumatoid factor by any method that is also positive in less than 5% of normal control subjects.

7. Radiographic changes - Changes typical of RA on hand wrist radiographs which must include erosions or unequivocal bony decalcification localized 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)

Figure: 1A.

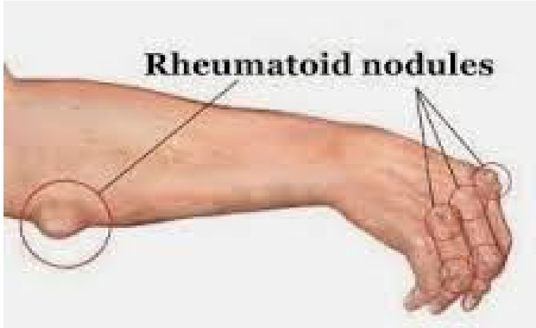


Figure: 1B

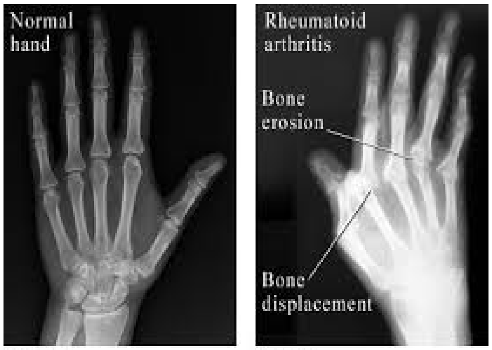


Figure: 1C

Figure: 1D

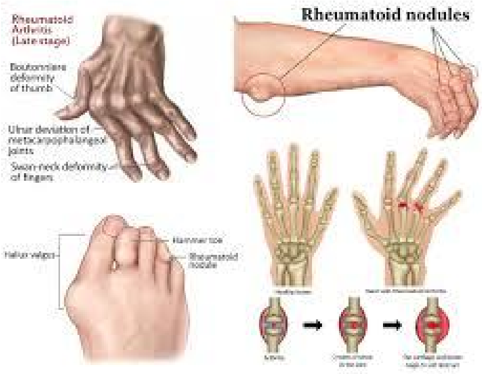


Figure: 1E

This classification has some limitations, because some of the criteria are generally not present or are not specific and sensitive enough for early diagnosis of RA for initiation of treatment. According to Nell VP, 2005 rheumatoid factors has been observed in many other autoimmune diseases, such as in primary sjogrens syndrome, systemic lupus erythematosus, mixed connective tissue disease and also some non-autoimmune conditions as chronic infections in old age (Nell *et al.,*2005).

2.5.2 X-ray imaging

Plain radiography has been employed as the gold standard diagnostic tool for evaluating disease progression and effectiveness of therapy in either individual patients or patients recruited under clinical trials. This method is used for the small joints like hands, wrist and foot. Joint space narrowing (JSN) is used for scoring in 42 joints for cartilage degradation (Bruynesteyn 2004) Erosion score (ES) is used for scoring in 44 joints for bone degradation (Figure-1C and 1D). Other evaluations clinical assessment like absence of pharmacological treatment, knowledge of etiological factors, some phenotypic studies, joint space narrowing etc. are help on to diagnose RA. The European League Against Rheumatism (EULAR) and ACR have collaborate to produced such classification criteria (Aletaha *et al*., 2010).

2.6 Risk Factors for *Rheumatoid arthritis*

According to Sangha (2000), important risk factors for the predisposition of *Rheumatoid arthritis* are genetics, age and sex and socio-economic status, education and stress are other contributing factors.

Some other factors that are responsible for increased risk for developing *Rheumatoid arthritis* are positive family history, silicate exposure, and smoking (Harris 1990), where as high vitamin D intake (Merlino *et al.,* 2004), oral contraceptive use (Goemacre *et al*.,1990) and tea consumption (Mikuls *et al*.,2002) are associated with decreased risk of RA. Tobacco smoking is the strongest and most consistent modifiable risk factor for RA. A history of smoking has been found to be associated with modest to moderate (1.3 to 2.4 times) increased risk of RA onset (Silman and Hochberg, 2001). This relationship between smoking and RA was strongest among people who were ACPA (Positive ACPA is a marker of auto-immune activity) (Scott *et al.*, 2010).

2.7 Management of *Rheumatoid arthritis*

Rheumatic diseases are a huge burden on the health care systems worldwide, and account for significant dysfunction, loss of productivity and reduction in quality of life (Sangha, 2000, Young *et al.,* 2007) have shown that approximately one third of RA patients are disable to work after 5 years of onset of the disease. Almost half of the patients have substantial functional disability within 10 years (Young *et al*., 2007). Therefore, RA imposes an important economic burden on society and loweres life expectancy.

Chronic inflammation in RA limits the joint activity which decreases the quality of life (Adam & Daniel., 2005).

Management of *Rheumatoid arthritis* is improved over the last 13 years. Reasons of the improvements are new drugs, and overall novel approach toward treating patients. Rheumatoid factor is not specific marker for RA and may be present in other disease, such as hepatitis C and in healthy older persons. Anti-citrullinated peptide antibody (ACPAd) is more specific marker for RA and plays an important role in disease pathogenesis of RA (Balsa *et al.*, 2010).

After onset of the disease patients should immediately consult rheumatologist for management of pain with appropriate physiotherapy, corticosteroids or disease-modifying anti-rheumatic drugs (DMARDs). DMARDs are the backbone of RA therapy. Drug therapy for RA may also involve Non-steroidal anti-inflammatory drug (NSAIDs), and oral, intramuscular, or intra-articular corticosteroids for controlling pain and inflammation. Ideally, NSAIDs and cortico-steroids are used only for short-term management (Amy & 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 recommend low dosage of steroids as these are highly effective for relieving symptoms of rheumatoid arthritis and can slow joint damage. In our study (Patel *et al*., 2015) occasional use of steroid have been shown to be helpful for control of the disease activity. Protein kinase inhibitors have also been developed, and are be called "molecular-targeting antirheumatic drugs" (MTARDs), as opposed to "disease-modifying anti-rheumatic drugs (Yamanaka *et al.*, 2012).

Single DMARD is not able to achieve the goal in a huge majority of patients therefore DMARDs are given sequentialy in patients for improvements (Ernest 2010).

Different cellular and cytokine targets have been identified in RAwhich are 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 depletor of B cells (Bingom 2008). New drugs have emerged with novel mechanism of action in recent years as drugs acting a competitive inhibitor of intracellular enzymes needed for de novo pyrimidine synthesis by activated lymphocytes in RA patients or IL-1 receptor antagonist (Anakinra), or tumor necrosis factor (TNF) antagonists. Anti–interleukin-6 receptor recombinant antibodies have also being evaluated for efficacy. Non-pharmacologic treatments for *Rheumatoid arthritis* have been also tried like therapeutic fasting, dietary supplementation of essential fatty acids, along with spa therapies and exercise.

2.8 Pathogenesis of *Rheumatoid arthritis*

In RA, many joints are affected as the knee, ankle, elbow, and wrist. Joints that are tagets of RA are usually tender, swollen, and with constrainst mobility.

2.8.1 Synovial joints

Synovial joints (also known as diarthroses or normal joint) are the most common type of joint in the body. Like other joints, synovial joints achieve movement at the point of contact of the articulating bones. Synovial joints consist of different types of tissue including bone, cartilage, synovium, synovial fluid and tensile tissues such as ligament and tendon. It protects and covers the bone ends and the articular capsule encloses the joint structure. Ligaments are fibrous thickenings of the articular capsule that provide stability to the articular cartilage.

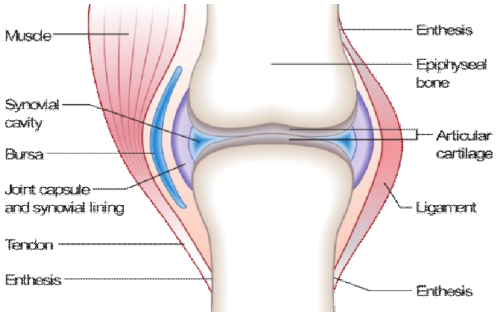


Figure: 2A Normal joint

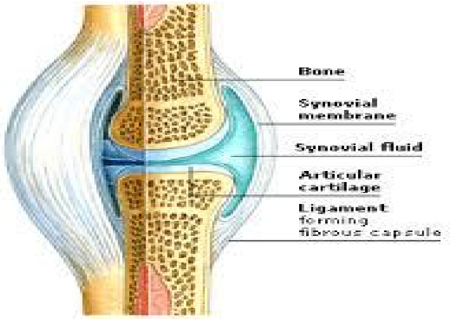


Figure: 2B Inflammed joint

The surfaces of the cells lining the synovium consist of the network of capillaries important for nutrient and gaseous exchange. The synovium is therefore permeable to water, gases, nutrients, small molecules and proteins, but not to large proteins, glycosaminoglycans and proteoglycans oligosaccharides. The viscous nature of synovial fluid is due to an important molecule that is hyaluronic acid (HA). This property of the synovium allows it to trap synovial fluid that is osmotically active. It loads resistant molecules within the cavity, with degradation products of matrix proteins and glycoproteins which are released in the synovial fluid due to circulation during normal and pathological turnover (Ali 2011).

The inflamed joints of the *Rheumatoid arthritis* can be divided into two categories: (i) one with reversible signs and symptoms related to aseptic inflammatory synovitis and (ii) the other with irreversible structural damage caused by synovitis. This concept is useful for disease staging, determining prognosis and medical or surgical treatment selection. The synovial membrane normally consists of relatively thin intimal linning with only one or a few cell layers (Firestein 2003). After disease onset hypocellular synovial membrane becomes hyperplastic, comprising of a superficial lining layer of synovial fibroblasts and macrophages (McInnes & Schett., 2007). At the lining overlies an interstitial zone with marked cellular infiltrates containing fibroblasts, macrophages, dentritic cells, mast cells, T cells and B cells (which differentiate locally into antibody-secreting plasma cells) (McInnes & Schett., 2007) (Figure 2B). The interaction between activated lymphocytes and monocytes, leads to 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, IL-1and TNF-α are suspected to stimulate synoviocytes and osteoclasts. Post activation of the cells leads to the irreversible destruction of bone and cartilage (Goldring 2006). Synoviocytes are also known to produce MMPs, which are normally inhibited by the TIMPs. In RA, the proportion of proteinases to their inhibitors 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 proteglycan and collagen fibres (McInnes & Schett 2007). The chondrocytes themselves synthesize or respond to local cytokines released by the synovial membrane such as IL-1β and TNF. This has a synergistic effect in cartilage destruction, although the effect of IL-1β seems more potent than that of TNF (McInnes and Schett., 2007). In addition, synovial fibroblasts, neutrophils and mast cells situated in the synovial membrane further release matrix-degrading enzymes (McInnes & Schett., 2007), which in turn contribute to cartilage degradation.

2.8.2 Articular cartilage

Articular cartilage is a thin layer of specialized connective tissue with special viscoelastic properties. The load-bearing function of articular cartilage depends on the structural design of the tissue and the interactions between its unique resident componanats, the chondrocytes and the extracellular matrix (ECM) that makes up the bulk of the tissue. Fibrillar and non-fibrillar collagens, proteoglycans, and non-collagenous proteins are the distinct classes of macro molecules which form the basic orchiteture and frame work of ECM. Articular cartilage contains the collagens type II, IX, and XI which form a febrile meshwork that gives cartilage tensile stiffness and strength (Buckwalter *et al.*, 2005).

2.9 Etiology

Although the exact etiology of RA remains elusive, a genetic basis for the disease has been emphasized in some studies (Smolen & Steiner 2003). The epitope of the HLA-DRB1*04 cluster was found in more than 80% of patients (Smolen *et al.*, 2007). The patients expressing two HLA-DRB1*04 alleles are at increased risk of joint destruction (Weyand *et al.*, 2002). Although 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 *et al.*, 2009). Environmental factors, like smoking and infection, may also influence the development of RA and also affect, rate of progression and severity of RA (Klareskog *et al.*, 2007, Getts & Miller 2010).

Signalling pathways and various immune modulators (cytokines and effector cells) are involved in the patho-physiology of RA (Smolen & Steiner 2003). Post inflammation synovial lining becomes hyperplastic, and the synovial membrane expands and forms villi (Smolen & Steiner 2003). The pathophysiology of *Rheumatoid arthritis* mediated by an inter-related 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. Particulary, IL-6 acts directly on neutrophils through membrane-bound IL-6 receptor(IL-6R), which contributes to inflammation and joint destruction by secreting proteolytic enzymes and reactive oxygen intermediates (Dayer & Choy 2010). Role of Il-6 has been demonstrated (Fig.3) in RA patients which promotes neutrophil and activate fibroblasts (Lally *et a*l., 2005).

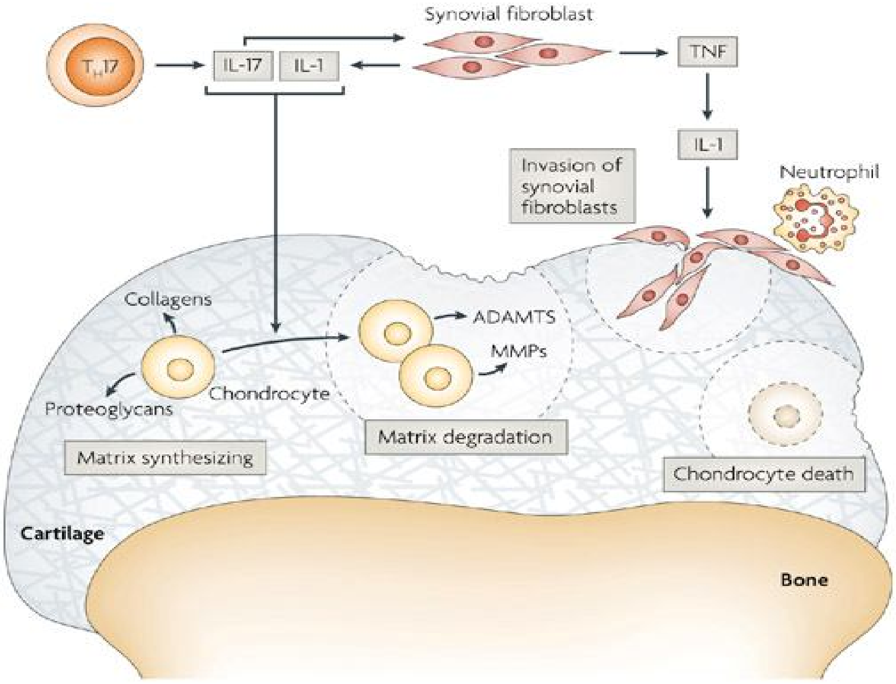


Figure: 3

Many of the studies have established the critical role of mast cells in the pathogenesis of *Rheumatoid arthritis* (David & Weinbaltt 2001). The study by Carcassi (1999) established the important role of HLA-DR4 and another HLA-DRBI alleles in the pathogenesis of RA which are also considere genetic markers associated with majority of Caucasian patients (Carcassi *et al*., 1999).

2.10 Early *Rheumatoid arthritis*

The sign of Early *Rheumatoid arthritis* are swelling and pain of the PIP and MCP joints, and later, the larger joints as knee, elbow and ankle get affected. Infiltration of large numbers of activated leukocytes in synovial membrane, causes hyperplasia and inflammation, which leads to destruction of cartilage and bone. Since RA is a systemic autoimmune inflammatory disease, other parts or organs of the body may get affected at the later stage. Development of rheumatoid nodule may peak in the fourth and fifth decades of life in RA patients (Ernest 2004).

2.11 Biomarkers

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 marker to diagnose RA at an early stage. Chronic condition of the disease requires, re-characterization of pathological and physiological process using biomarkers which can change the future of medicine (Nass & Moses 2007). Any parameter that can be objectively examined and measured indicating the disease progression is defined as biomarker. A biomarker may indicate normal biological processes, pathogenic processes and pharmacological response to a therapeutic intervention. Biomarkers are indicators including a wide range of biomolecules as nucleic acids, proteins, sugars, lipids, and metabolites. They may be whole cells or may encompass biophysical characteristics of tissues. Biomarkers can be detected, either individually or as larger sets or patterns. Their detection may be accomplished by a wide variety of methods ranging from biochemical analysis of blood or tissue samples.

2.11.1 Serological markers

2.11.1.1 C-reactive protein: Inflammatory markers such as C-reactive protein (CRP), tumour necrosis factor –α (TNF-α), interleukin-1 (IL-1) are highly expressed in synovial fluid and serum of *Rheumatoid arthritis* patients. CRP is an acute-phase protein produced by hepatocytes, upon stimulation by the cytokines TNF- α, IL-6 and IL-1. (Hanna *et al*., 2008; Shrivastava & Pandey 2013). CRP is a general marker of systemic inflammation. It is elevated in the patients with RA. Several cytokines are responsible for articular inflammation and destruction of cartilage in RA (Fox 2000). IL-6 is the most abundantly expressed cytokine in RA patients with biological activities regulating the immune responses, inflammations and haematopoiesis. IL-6 stimulates the secretion of immunoglobulin by plasmacytes and promotes the proliferation 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 CRP, fibrinogen, haptoglobin and serum amyloid-A. Which regulates the proliferation and differentiation of osteoclasts and induces bone resorption (McInnes & Schett 2007).

TNF-α is one of the pivotal pro-inflammatory cytokines responsible for inflammation and joint destruction in RA. The two receptors of TNF-α (p55 and p75 TNFR) are readily detected in both synovial fluid of patients with RA (Ferrero *et al*.., 2001). The severity of RA is correlated with the concentration of TNF-α in patients (Jenkins *et al*., 2002). TNF-α is a potent stimulator of mesenchymal cells such as synovial fibroblasts, osteoclasts, 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 actions is thought to leads to joint damage. Although, TNF-α and IL-6 have overlapping and synergic actions, some of the effects of these two cytokines are regulated by distinct mechanisms (Rahman *et al.*, 2005).

The increased CRP concentrations in serum samples of RA patients before the onset of symptoms of RA suggesting the changes in the patients before actual disease ensue. According to Singh *et al.,* (2013) CRP may also partly mediate complement activation in RA. The study of Nielen *et al.*, (2004) suggest that, serological abnormalities in patients occur before the onset of symptoms and they had slightly higher CRP concentrations. The hepatic acute phase protein response is an outstanding feature of many inflammatory diseases, including RA.

2.11.1.2 Erythrocyte sedimentation rate: The erythrocyte sedimentation rate (ESR) has also been the most widely used marker of inflammation in RA. According to Firestein *et al.*, (2009), ESR, is an indirect measure of the level of acute-phase plasma proteins in the blood, probably induced by inflammation because inflammation cause the red blood cells to settle more rapidly. The test is relatively easy and inexpensive to perform, but ESR levels respond slowly to inflammatory stimuli and changes in disease activity.

2.11.1.3 Autoantibodies: The first RA-associated antibodies are rheumatoid factors(IgG, IgM) (RFs) These antibodies bind to their receptor expressed on various cell types (Song, & Kan 2010). Fc receptors (FcγR I, FcγR II and FcγR III) are cell surface receptors expressed on various leukocytes specifically binds to IgG and IgG immune complexes (ICs), where crosslinking with these FcγRs activate leukocytes effector functions such as respiratory cellular burst, cytokine secretion, antibody-dependent cellular cytotoxicity and phagocytosis (Bolland & Raveth (2000).

Rheumatoid factor (IgG) has four subclasses and all of these have distinct biological properties (Bolland & Raveth 2000). IgG1 and IgG3 are able to activate all types of Fc receptors so it may be expected that IgG1 and IgG3 would be mainly involved in the immunopathology associated with IgG mediated autoimmune inflammatory conditions.

RA is normally accompanied by polyisotypic rheumatoid factor production in which the IgG RA specific and IgM contribute to the inflammatory reactions by activation of complement and phagocytes. Though IgM RF is measured in most studies, but its specificity for diagnosis of RA is limited. That may be because of a very low level of IgM-RF is also is present in the sera of normal people and a high concentration of IgM-RF is detected in individuals with viral and bacterial infections or chronic inflammations other than RA which can induce polyclonal stimuli also to B cells.

Anti-cyclic citrullinated peptide (anti-CCP) autoantibodies are also produced in RA patients. In the inflamed region of synovium, anti-CCP autoantibodies are found to be accumulated. Anti-citrullinated antibodies (anti-CCP) have specificity of 89–100% and sensitivity of 41–80% for the diagnosis of RA. Hoffman IEA 2005 showed a slightly lower sensitivity of than RF (66.4%) but they have much higher specificity (97.1%) for RA. This high specificity due the dysregulated mechanism of humoral immune response against citrullinated peptides in RA patients. Anti-CCP can be detected even in early RA (in 40-60% of the cases) and is also present in 34.5% of RF-negative patients particularly in the early phase of RA. The presence of anti-CCP early might indicate the later onset of severe joint destruction and progressive development of the disease.

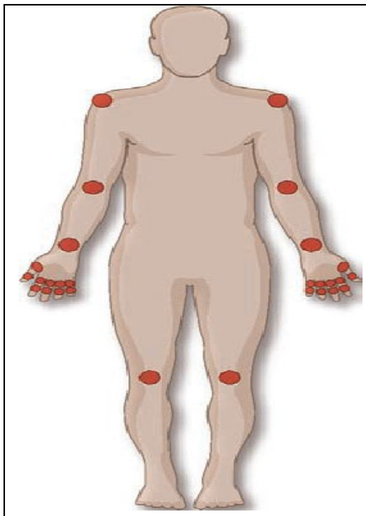
2.11.1.4 Liver function marker: Among patients with arthritis, hepatic involvement has been reported only in cases of *Rheumatoid arthritis* (RA) and its variants. The abnormal liver function may be due to the disease activity. Elevated alkaline phosphatase (ALP) level has been reported in 18 to 50% of patients with RA. It has been shown that 65% of patients with RA had abnormal liver biopsies with one-half having mild portal chronic inflammatory infiltrate of the portal tract and small foci of necrosis, and one in four having fatty liver (Ruderman *et al.*, 1997). Drug-induced liver injury is frequent in RA, especially with nonsteroidal anti-inflammatory drug (NSAID) and methotrexate treatments. Liver histology demonstrates diffuse lymphocyte infiltrate, periportal fibrosis with lymphocytic infiltration and portal hypertension. Liver enlargement and elevated aminotransferases have also been reported in adult-onset Still's diseases while liver biopsies have demonstrated a specific mild portal infiltrate of limited significance (Andres *et al.*, 2001).

2.11.2 Biomarkers for the monitorization of the disease activity

2.11.2.1 Disease activity score (DAS): DAS is scored according to involvement of joints as shown in the figure. The swelling and tenderness is scored depending on joint involvement in RA. DAS28 score of higher than 5.1 is indicative of high disease activity, whereas a DAS28 below 3.2 indicates low disease activity and score between 3.2-5.1 indicate moderate activity. A patient is considered to be in remission if they have a DAS28 lower than 2.6 (Van Der *et al.*, 1990, Van Der *et al.*, 1993)

$$\text{DAS 28} = 0.56 * \sqrt{}\,(\text{Number of tender joints}) + 0.28 * \sqrt{}\,(\text{Number of swollen joints}) + 0.7 * \text{In}\,(\text{ESR: 1hour}) + 0.014 * \text{VAS}.$$

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

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

2.11.2.2 Visual analog scale: 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. Therefore pain cannot be considered effectively treated or relived unless it is measured (Reville *et al*., 1977). Therefore, the only way to ensure that patients receive 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).

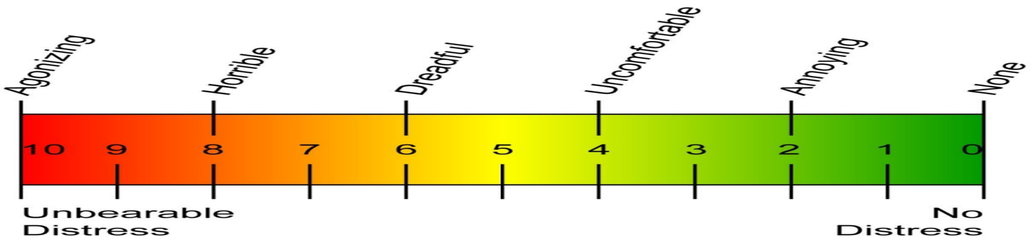


Figure-4 : Scale for the calculation of VAS

2.12 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 collectively known as oxidants. Interestingly, ROS have also been considered as risk factors that stimulate the autoimmune diseases (Okamoto 2005). Several studies have been suggested that reactive oxygen species (ROS) and oxidative stress are involved in progression of RA (Kamanli *et al.,* 2004; Sezgin *et al.,* 2005). Oxidative componants have the potential to damage biomolecules such as lipids, DNA and proteins in the affetcted tissues.

ROS are required to maintain the cells redox state and play an important role in cell signaling, differentiation, proliferation, apoptosis, cytoskeletal regulation, growth and phagocytosis in physiological conditions. However if the concentrations of ROS are increased beyond physiological concentrations they can damage cellular components, such as lipids, proteins and nucleic acids. The imbalance between oxidants and antioxidants 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 antioxidants deficiency or both (Valko *et al*., 2007).

In normal conditions, antioxidant defence system control and manage reactive oxygen and nitrogen species. The antioxidant system may work through enzymatic as well as non enzymatic mediators among enzymatic componants have enzymatic activity such superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GR) and glautathione-S-transferase (GST) are impotant quenchers while non- enzymatic antioxidant defences 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 equilliberium is disturbed, it can produce oxidative stress (Mitchell *et al*., 2003).

2.12.1 Malondialdehyde (MDA)

Overproduction of ROS increases oxidative stress, process that can be an important mediator of damage to membrane lipids, proteins and DNA including cell structures (Valko *et al*., 2007). Prime targets of ROS attack are the polyunsaturated fatty acids in the membrane lipids causing lipidperoxidation (LPO), which may lead to disorganization of cell structure and function. Further decomposition of peroxidized lipids yields a wide variety of end-products, including malondialdehyde (MDA) (Gambhir *et al*., 1997). Measurement of MDA is widely used as an indicator of lipid peroxidation (LPO) (Romero *et al*., 1998). MDA has an important role in pathogenesis of RA. Many studies have reported high MDA in the serum, plasma and synovial fluid of RA patients (Kamanli *et al*., 2004, Gambhir *et al*., 1997, Pallinti *et al*., 2009). There is growing awareness that reactive oxygen species and free radicals may play an important role in mediating cellular injury and tissue damage in rheumatoid arthritis. Thiele *et al.*, (2015) have been reported malondialdehyde-acetaldehyde (MAA) adduct formation is increased in RA. They appear to 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.

2.12.2 Superoxide dismutase (SOD)

In *vivo*, the biological effects of highly reactive and toxic compounds are controlled by a wide spectrum of oxidative defence mechanisms (Gutteridge 1994). Superoxide dismutase (SOD) is believed to play a key role in the enzymatic defence mechanism in the cell against oxygen toxicity (Petkau 1986). Among the actively generated ROS, superoxide anion ($O_2^{\cdot -}$)is the primary product that is liberated into 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 glutatione rductase.

Zinc and copper are constituents of antioxidative enzymes. Copper can act as an antioxidant and neutralizes free radicals and may also help 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 disease. (Araya *et al*., 2006).

Copper and zinc are components of SOD. Copper is a cofactor of Ceruloplasmin, which is an important antioxidant in serum (Honkanen *et al.*, 1991). On increase in the concentrations of ROS, lipid peroxidation increases and this increase, leads to enhanced damage in 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 & Baba 2006 SOD acts as an endogenous cellular defense system in oxidative stress to degrade superoxide ($O_2^{\cdot -}$) into oxygen and hydrogen peroxide which makes SOD as a potentially useful therapeutic agent for treatment of inflammatory disorders as RA (Yasui & Baaba 2006).

2.12.3 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, bacterial, and plant sources (Deisseroth & 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 & 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 oxidase 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 serum of RA patients. Decreased erythrocytes catalase activity is also being reported (Taysi *et al.*, 2002). The studies have reported lower catalase activity in serum of RA patients (Janina *et al.*, 2014, Kumar et al, 2016). Catalase expression affects expression of genes which influence inflammation (Benhamou *et al.*, 1998). Lower levels of catalase may be responsible for high inflammation in RA.

2.12.4 Glutathione reductase:

Glutathione reductase (GR,EC1.6.4.2) is a flavoenzyme dependent on NADPH that catalyzes the reduction of GSSH to GSH. Feijoo *et al.*, (2010) observed that myeloperoxidase levels are elevated in patients with chronic inflammatory disease, especially those with active disease and high myeloperoxidase levels are related to an increase in oxidative damage and the inflammatory response for myeloperoxidase and therefore GR seems to show a similar activity pattern based on the availability of NADPH. Glutathione reductase (GR), an oxidative stress inducible enzyme, plays a significant role in the peroxyl scavenging mechanism and in maintaining functional integration of the cell membranes.

2.13 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 TC, while others demonstrate increased levels of TC and LDL-C in patients with early RA (Georgiadis *et al.,* 2006). Although reports on lipid profiles in RA patients vary, 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 & 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 ant rheumatic medications (Georgiadis *et al.*,2006, Van Halm *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.*,2003, Di Micco *et al.,* 2009).

The clinical importance of lipid levels on CVD risk in RA 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 cardioprotective-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 erythomatosus, patients have non-protective “pro-inflammatory HDL” (piHDL) which promotes accumulation of oxidized phospholipids in LDL-C (Charles-Schoeman *et al.*, 2009).

2.14 Genetic Polymorphism

Recent GWAS in rheumatoid arthritis (RA; MIM180300) have unraveled many disease susceptibility loci. Most of these genes/loci have risk alleles of known immune functions (Hollis *et al*., 2010) justifying their involvement in RA. RA is a complex autoimmune disease characterized by chronic inflammation of the synovial joints followed by progressive articular damage leading to major functional disability (Firestein 2003).

Although the etiology of RA remains unsolved but genetic component are shown to be associated with susceptibility for developing RA from twin and family studies 60% or high heritability (Mac Gregor *et al*., 2000). The human leukocyte antigen (HLA) class II molecules are most widely recognized as genetic risk/susceptibilty factors for RA. 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).What so ever the genetic association studies have long implicated the human leukocyte antigen locus DRB1(HLA-DRB1) as the principle genetic factor conferring risk to RA (Stahl et al., 2010).

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 & 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.

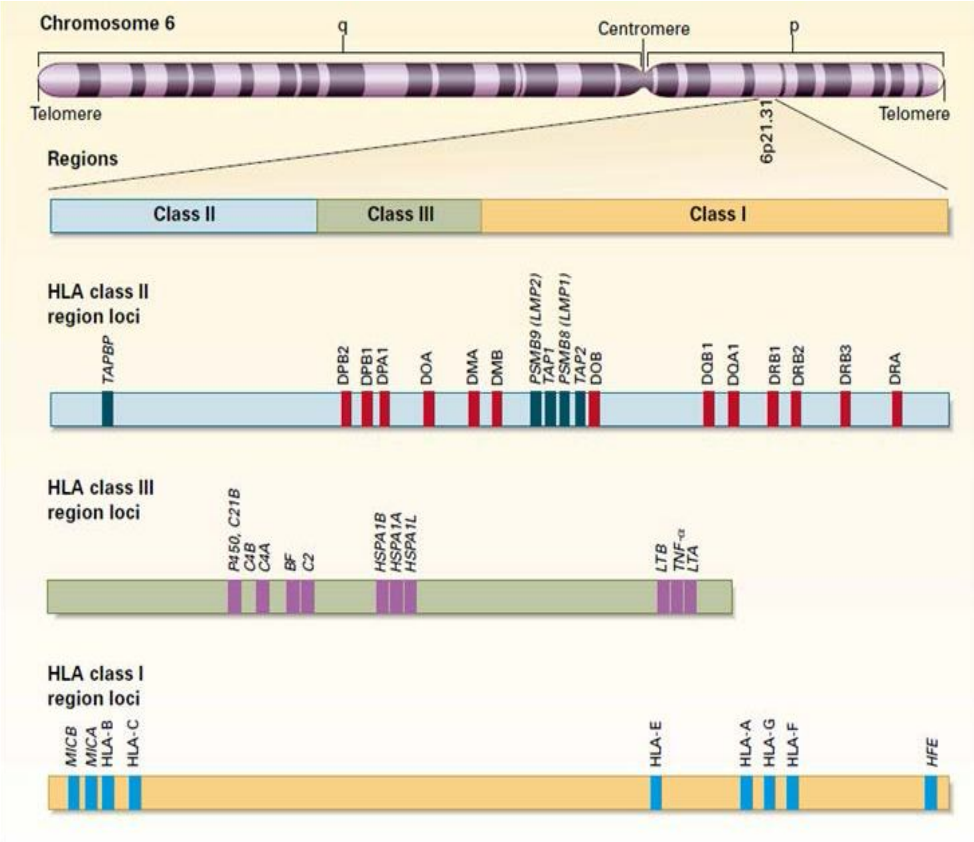


Figure-5: Figure show the organization and location of the HLA complex on chromosome 6 (Klien et al. 2000)

2.14.1 Peptidylarginine deiminase 4 (PADI4)

The genetic variant, PADI4 gene is located on chromosome 1 (1p36). The PADI4 gene encodes the type 4 peptidylarginine deiminase enzyme, which catalyses the posttranslational modification of arginine to citrulline, producing citrullinated proteins (Vossenaar *et al.*, 2004). 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.

The protein peptidylarginine deiminase (PAD 4) consists of 663 amino acid residues with a 74 kDa molecular weight (Luo *et al.*, 2006) and is the only isotype out of five described to be expressed in cell nucleus (Nakashima *et al.*, 2002). PAD 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).

PAD 4 is a calcium dependant enzyme. Therefore for it to function an increase in cytosolic Ca^{+2} concentration (2 µM) is required for citrullinated antigens to appear (Luo *et al.*, 2006). 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). 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. It has been reported that functional variant of the gene encoding PADI4 were 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 PADI4, which has 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 gene coding region of the exons 2–4 of PADI4 which . They found five haplotypes differing in four polymorphic sites; one denominated the susceptibility haplotype and was associated with RA. The SNPs involved are 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).

2.14.2 Protein tyrosine phosphatase non-receptor -22(PTPN22)

1858C->T single-nucleotide polymorphism (SNP) of protein tyrosine phosphatase non-receptor 22 (PTPN22) (rs2476601) is the `best examples of a non-HLA common susceptibility allele for autoimmunity (Siminovitch, 2004), (Gregersen 2005). PTPN22 gene is located on chromosome 1p13.3–p13.1 and encodes a intracellular tyrosine phosphatase (Canton *et al*., 2005). 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 protein (Rieck *et al*., 2007, Bottini *et al* 2006).

Lymphoid specific phosphatase is suggested to be 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 humans is yet to be demonstrated. The expression of Lyp protein is showm 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 centres and increased immunoglobulin levels, although autoantibodies were not detected in these animals. Changes in B-cell function was not found, suggesting that the abnormalities reflect a role of T-cell regulation on B-cell differentiation. SNP 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 signalling. The result of this missense mutation is a possible loss of negative regulation of T-cell signalling (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). Although there were several attempts to find different SNPs in the PTPN22 gene that may be associated with RA in the non-European populations, however no evidence of association with RA, were observed in their with haplotype analysis and re-sequencing of this region (Lee *et al*., 2009).

PTPN22 gene was first reported as a non-HLA RA risk factor in European populations, after an initial finding of 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 ethic 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 protein 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).

2.14.3 Tissue inhibitor of metalloproteinases 4 (TIMP4)

Destruction of cartilage is a common pathological feature of *Rheumatoid arthritis* (RA) and osteoarthritis (OA). 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 the arthritides (Nagase *et al.*, 1993, Nagase & Okada 1996, Firestein 1996). MMPs, a gene family of neutral Zn^{2+} metalloproteinases, are composed of at least 18 members, which are classified into five subgroups of structurally related MMPs: (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), stromelysin3 (MMP-11), metalloelastase (MMP-12), MMP-19,9 enamelysin (MMP-20),10 and MMP-23 (Bartlett *et al.*, 1996).

Tissue inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors of MMPs and so are important regulators of ECM turnover (Brew & Nagase 2010).

They play an important role in tissue remodelling and growth, in both physiological and pathological conditions (Maria & Leif 2005). TIMPs are the endogenous inhibitors that regulate the MMPs (Brew & Dinakaran 2000). Extracellularly, TIMPs inhibit MMP activity by forming high affinity noncovalent complexes with MMPs (Visse & Nagase 2003). The amino-terminal domain of TIMP binds the active site of MMPs, inhibiting their proteolytic activity. The carboxy-terminal domain of certain TIMPs also has the ability to form complexes with proenzymes (proMMPs) regulating the MMP activation process. Some MMPs, including MMP14, possess a conserved sequence of 10-12 amino acids between the propeptide and N-terminal domain that is recognised by the furin family of serine proteinases (Pei & Weiss 1995). 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 are 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 MMPs : 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 onset of RA and high level of MMPs show erosive effect in 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.

In recent studies RA seems to have derangement of mineral content like Mg, Zn, Cu and P. Their optimum concentration is required for normal functioning of the body. However alterations in level of these trace minerals as Mg, Zn and Cu (Copper) have been implicated in 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. The development and progression of RA was suggested due to marginal deficiencies of Zn and Cu based on their serum levels. Many of these trace elements are present in bones as iron, copper, zinc, manganese, fluoride, stroncium and boron. As the changes in the concentration of trace elements has been linked to inflammatory response therefore the presen study was undertaken to analyze) The concentration of Zn, Cu, Mg and P in female and male RA subjects along with activity of superoxide dismutase (SOD) and disease activity score (DAS-28-CRP). These may help in determination of possible roles of these in disease activity of female and male RA patients.

2.15 Nuclear Magnetic Resonance

Many metabolites have been reported to play a role in rheumatoid arthritis. Groups of metabolites are indicated whose role would be interesting to analyse the mechanism of and possible 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 provided an indication of biological processes related to metabolites alteration in RA. More clinical studies would be needed to elucidate the effects of vitamin supplementation on RA activity and progression. In addition, circadian rhythms in hormone production and other metabolite levels are important to consider. For instance, evaluating the timing of glucocorticoid treatment is for obtaining an optimal effect (Cutolo, 2008).

The NMR profiles of synovial fluid were markedly different from their matched serum samples. There were high levels of lactate in the synovial fluid compared to the serum 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 arthritic 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 have looked at the plasma of patients with RA (Lauridsen *et al.*, 2010). They found differences in the metabolites between patients with RA and healthy controls and differences between patients with active RA and controlled RA (Lauridsen *et al.*, 2010). The metabolites that they identified were cholesterol, lactate, acetylated glycoprotein and lipids. The lactate levels represented oxidative damage and thus indirectly reflect active inflammation.

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 were selected from outdoor patients department of Vinod Dixit hospital, Kannauj and Community Health Centre (CHC) Shivrajpur. Patients relatives and family friends having 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 haemoglobin 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 was 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 analysis. Fasting heparinized 5.0ml blood of each subject of 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 4.0ml 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 (meta carpophalangeal (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 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 1948, 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 controls were placed into 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. Macroscopically 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 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 controls were placed into 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 r.p.m. 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 patient's 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 100 mm in the appropriate box on Form B and DAS 28 score was calculated. A DAS28 score of higher than 5.1 is indicative of high disease activity, whereas a DAS28 below 3.2 indicates low disease activity. DAS 28 score between 3.2 to 5.1 indicates moderate activity. A patient is considered to be in remission if they have a DAS28 lower than 2.6.

3.7.6 Visual analog scale (VAS):

The Visual Analogue Scale (VAS) consists of a straight line with the endpoints defining extreme limits such as'no pain at all' and 'pain as bad as it could be' (Fig-5) ((2000) Glossary). 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 scale then defines the subject's pain. This tool was first used in psychology by Freyd in 1923. According to Jensen MP (1986) using a ruler, the score is determined by measuring the distance (mm) on the 10-cm line between the "no pain" anchor and the patient's mark, providing a range of scores from 0–100.

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 was upto 37 U/L at 37°C and females 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 was upto 37 U/L at 37°C and females was 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 pipetted 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 $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

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

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 (1,2,3,4) was read. Further for standard curve preparation, different amount of H_2O_2 ranging from 40 - 160μ moles was taken in small test tubes in increasing order of concentration like 40, 80, 120 & 160μmoles. 2ml of dichromate acetate was added to each and unstable blue precipitate of perchromic acid was obtained. It was heated for 10 minutes in a water bath, which changed colour of solution to green, due to formation of chromic acetate. There after, it was cooled at room temperature and volume of the reaction mixture was made to 3ml by distilled water. Absorbance was read at 570nm 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 & 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 480nm 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 follows :

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

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 follows :

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

3.8.5 Alkaline phosphatase (ALP)

The assay of ALP was carried out using commercially available kit (span Diagnostics Limited, India) based on Kind and King's method (1954).The principle of the method is that alkaline phosphatase converts phenyl phosphate to inorganic phosphate and phenol at pH 10. Phenol so formed reacts in alkaline pH with 4-aminoantipyrine in presence of the oxidizing agent potassium ferricyanide and forms an orange-red coloured complex, which can be measured colorimetrically. The colour 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 80- 140 µg/ dl and Females 80-155µg/dl.

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

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.

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

3.9.3 Zinc

Colorimetric method was used for the estimation of Zinc, that react 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.

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

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.

$$\text{Phosphorous + Ammonium Molybdate} \longrightarrow \text{Phosphorous molybdate complex}$$

Eq. ..3.4

3.10 Lipid Profile

3.10.1 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 Triglycerides

Span diagnostic kit was used for estimation of triglycerides, which followed end point colorimetry 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.

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

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

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

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

3.10.3 LDLcholesterol

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 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. Graphpad 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) anf RBC lysis buffer were taken and mixd well and incubated at room temperature for 30 min or 56^0C for 10 min. After incubation, the content was centifuged 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 froath was made by repeated pipetting. 100µl 5N NaCl was added to it and mixed by soft hand taping. To it 200µl water was added and the content were 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 were 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 4^0C. The pellet collected was washed twice with 500 µl 70% ethanol. The tubes were inverted and allow to dry at room temperature. The DNA was dissolved in TE and stored at -20^0C. 1% TAE agarose gel use to check DNA.

3.13 DNA Concentration Measurement

A 1 µl aliquot of the DNA in TE buffer was diluted with 995 µl of sterile deionised water (1 :200) in a 1.5 ml microcentrifuge 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 use for the final concentration of DNA in the sample

3.14. Check Gel Analysis for the Quality of DNA

Neat DNA (2 µl) was added to 8 µl of loading buffer composed of 0.25% bromophenol blue (Sigma- 0.25 mg) in 40% sucrose solution. The mixed sample (10 µl) was placed in to 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 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 L 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 H 2008). The PCR product were 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 were 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 400 µl 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 D2O 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 (D2O; 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 ^{1}H 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 ^{1}H 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 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, BioSpin). The region □ (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 □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 resultant 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 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 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 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 “gplot” 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 were not included in this analysis. The final list of altered metabolites were 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

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

4.1.1 Demographic characteristics of RA patients with control

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±0.99ns
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}

Table-1 showing the demographic characteristic related to sex, age, Body Mass Index and Blood Pressure of RA patients with control

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

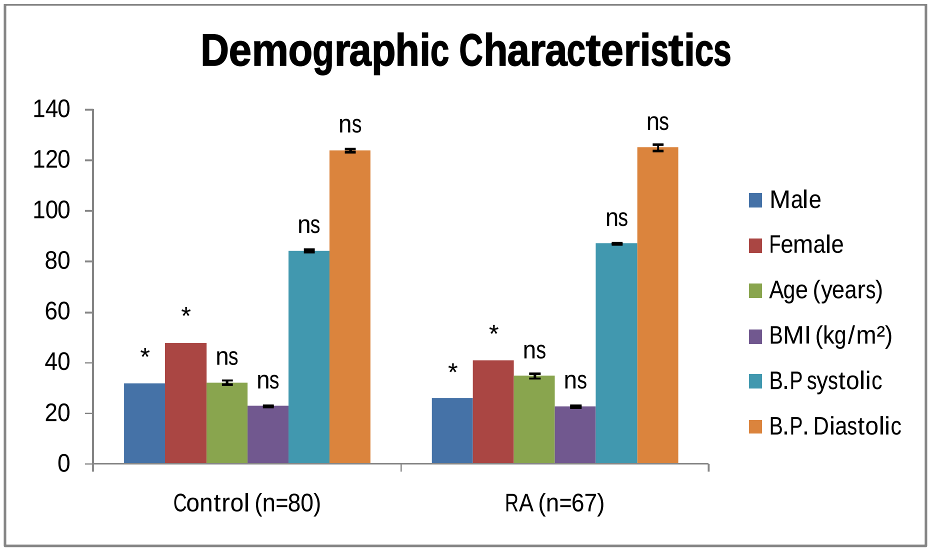


Figure-6

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 rheumatoid factors which is one of criteria of the ACR along with 3/4more ACR criteria. The 80 control and 67 age matched patients were selected.Amongst RA patients 51 were rheumatoid factor (RF) positive and 16 were RF negative. We could not observed 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 shown in table-1.

4.1.2 Demographic characteristics of female (RA and control) and Male (RA and control)

Characteristics		**Control Female (n = 48)**	**RA Female (n=41)**	**Control Male(n=32)**	**RA Male (n=26)**
Sex		48	41	32	24
Age (years)		35.77±1.20	$41.05\pm1.62^{*}$	29.50±0.89	$36.19\pm8.63^{**}$
BMI (kg/m^2)		22.70±0.21	22.33 ± 0.43^{ns}	23.22±0.237	23.56 ± 0.34^{ns}
BP	systolic	84.35±0.65	$88.44\pm1.16^{**}$	84.31±0.64	85.04 ± 1.05^{ns}
	diastolic	122.13±0.69	126±1.89*	126.38±0.94	124.73 ± 1.12^{ns}

Table-2 P** =P<0.005 P* =P<0.05 P^{ns} = Non significant

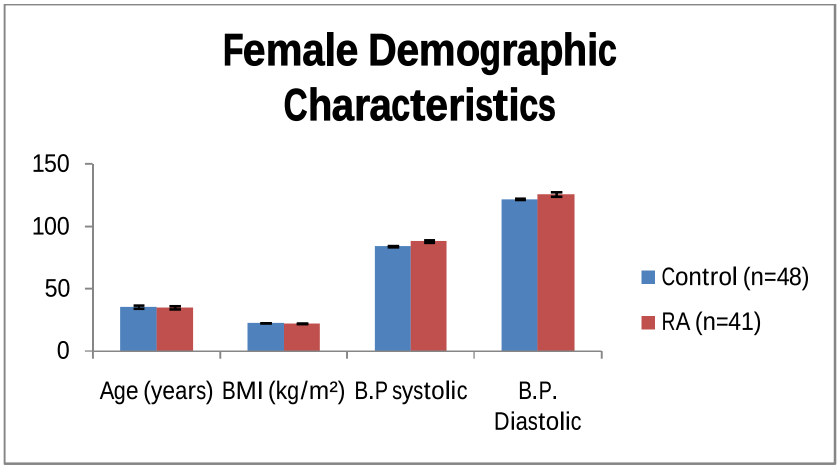


Figure-7A

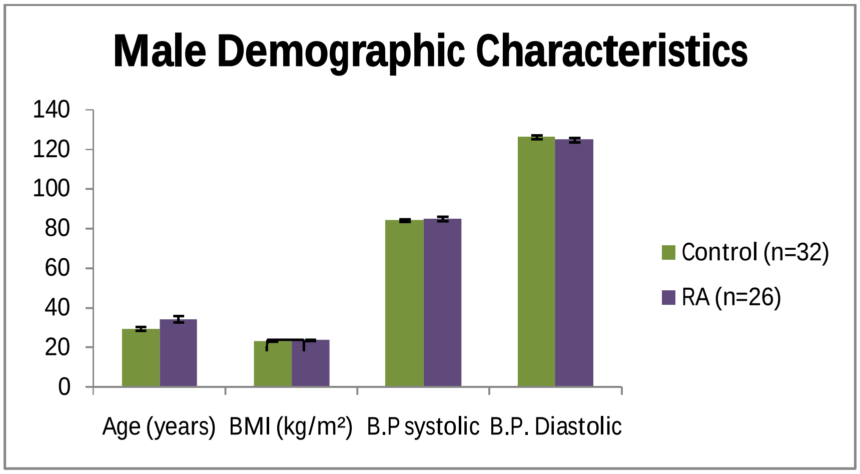


Figure-7B

The table-2 shows the comparison between control females vs RA females and control males vs RA males. BMI was nonsignificantly different in RA males and females as compared to control males and females. The of BP (Systolic and diastolic) of female RA patients was found significantly increased ($P<0.05$) as compared to control females however, nonsignificant difference was observed in RA males as compared to control males but these values of BP were within normal ranges (Table-2).

4.2 Clinical and Serological Variables

4.2.1 Clinical and serological variables of RA and controls

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***

Table.3 : The levels of serological variables of two groups were summarized

$P^{***} = P<.0001$

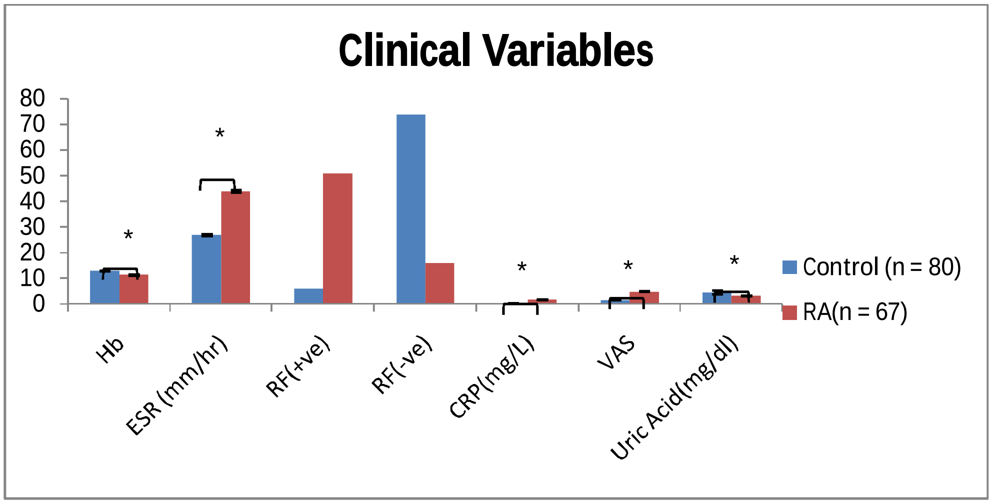


Figure-8

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 also significantly ($p<0.0001$) increased (2 fold) as compared to control. 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. Uric acid level was significantly decreased ($p<0.0001$) in RA as compared to control.

4.2.2. Clinical variables of female (RA and control) and male (RA and control)

Variables	Control Females (n = 48)	RA Females (n =41)	Control Males(n=32)	RA Males (n=26)
Hb	12.70±0.16	11.59±0.20***	13.58±0.22	11.11±0.40***
ESR (mm/hr)	27.37±0.47	44.45±0.85***	26.25±0.57	43.59±0.68***
DAS	2.3352±0.0123	4.32±0.051***	2.3062±0.0134	4.29±0.076***
CRP(mg/L)	0.423±0.027	1.74±0.137***	0.469±0.032	1.88±0.12***
VAS(*Pain on VAS (in holding and lifting activity)*	1.711±0.0929	5.29±0.273***	1.622±0.085	4.53±0.223***
Uric Acid(mg/dl)	4.58±0.0967	3.15±0.22***	4.81±0.11	3.61±0.148***

Table.4 The levels of serological variables of two groups were summarized.

P***= P<.0001

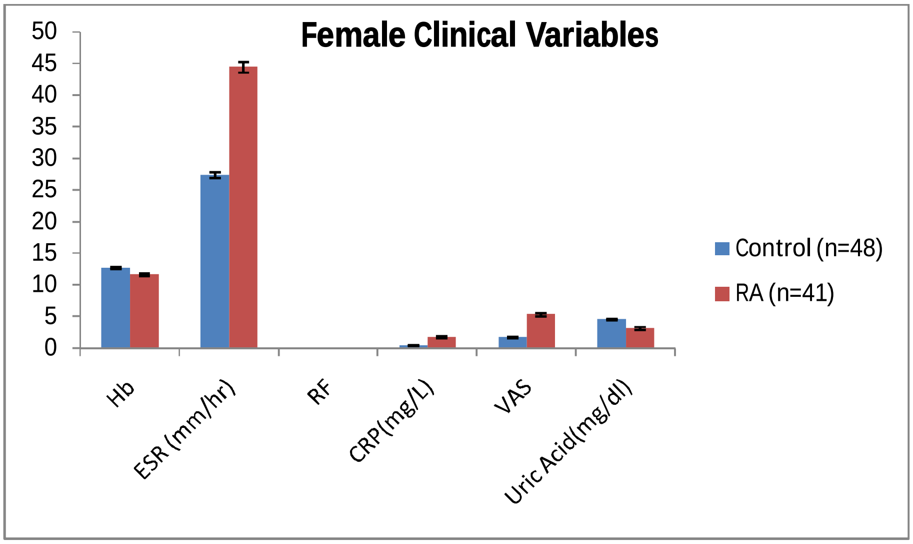


Figure-9A

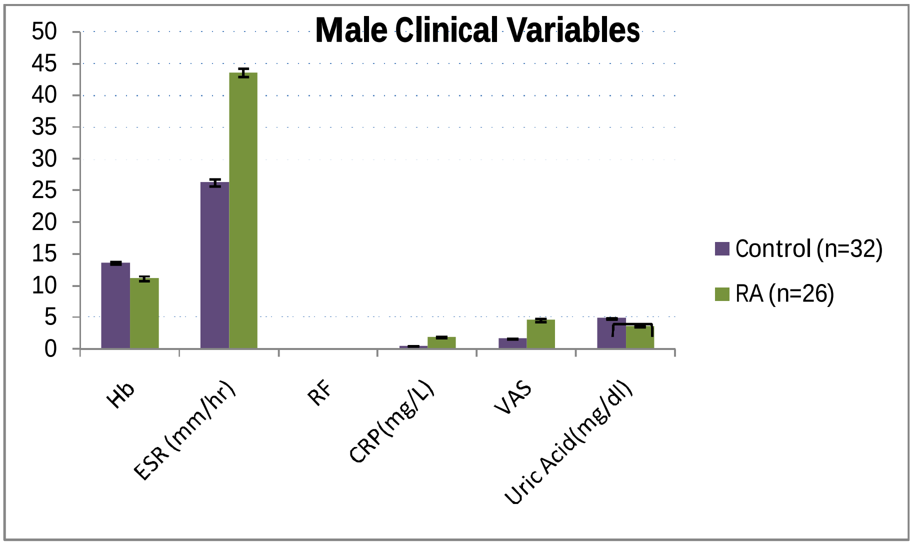


Figure 9B

Table-4 shows the comparison between control females vs RA females and control males vs RA males. Here the mean level of hemoglobin was significantly decreased (p<0.0001) in RA females and males participants compared to control participants. Mean level of ESR and DAS were significantly increased (p<0.0001) (2 fold RA) in female and male RA participants as compared to control (female and male). The mean level of CRP was significantly increased (p<0.0001) in both male and female RA patients as compared to males and females of control. VAS mean level were (4 fold) significantly increased in RA females as compared to control females and (3 fold) significantly increased ($p<0.0001$) in RA males as compared to control males. Uric acid mean level was significantly decreased ($p<0.0001$) in RA (female and male) as compared to control (female and male) (Table-4).

4.3 Liver Function

4.3.1 Liver function of RA and control

Variables	**Control (n = 80)**	**RA(n = 67)**
*Aspartate aminotransferase-AST(*SGOT*) (U/l)*	40.887±0.34	$24.58\pm1.162^{***}$
Alanine aminotransferase-ALT(SGPT*) (U/l)*	36.22±0.670	$26.158\pm0.967^{***}$

Table.5 The parameter which have been taken for the analysis of liver function test were SGOT and SGPT. $P^{***}= P<.0001$ vs. control

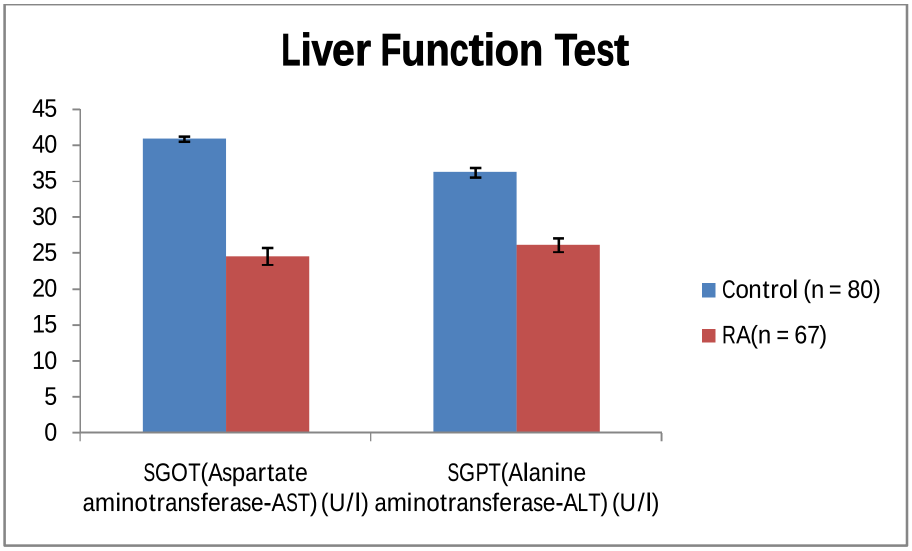


Figure-10

Table-5 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-5), but these values were within normal range.

4.3.2 Liver function of female (RA and control) and male (RA and control)

Variables	**Control Female (n = 48)**	**RA Female (n =41)**	**Control Male(n=32)**	**RA Male (n=26)**
SGOT*(Aspartate aminotransferase-AST) (U/l)*	40.24±0.452	25.77±1.82***	41.84±0.506	22.66±0.736***
SGPT*(Alanine aminotransferase-ALT) (U/l)*	34.155±0.710	27.69±1.38***	39.33±1.09	23.72±1.085***

Table.6 P^{***}= P<.0001 vs control

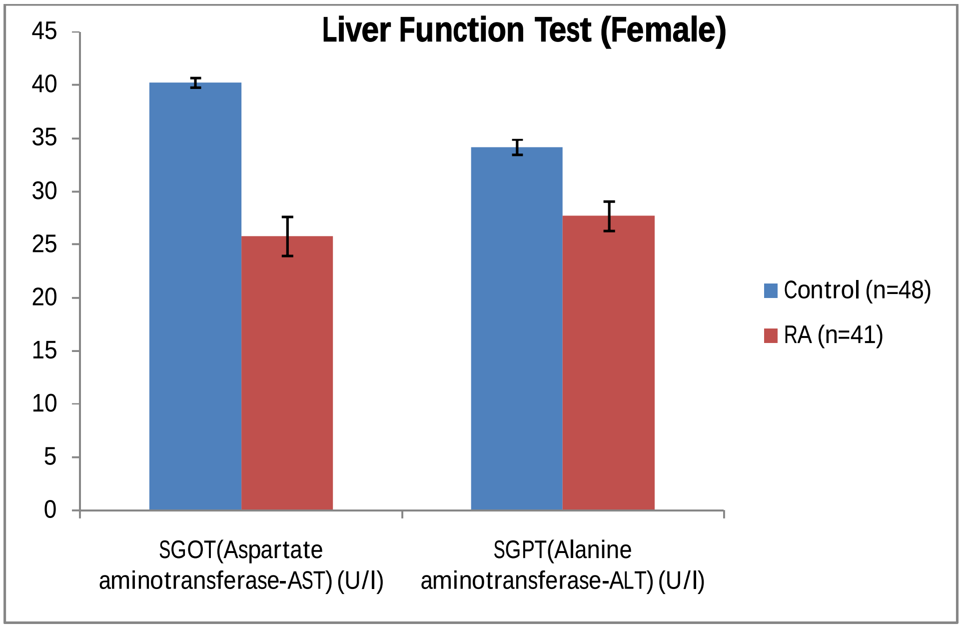


Figure-11A

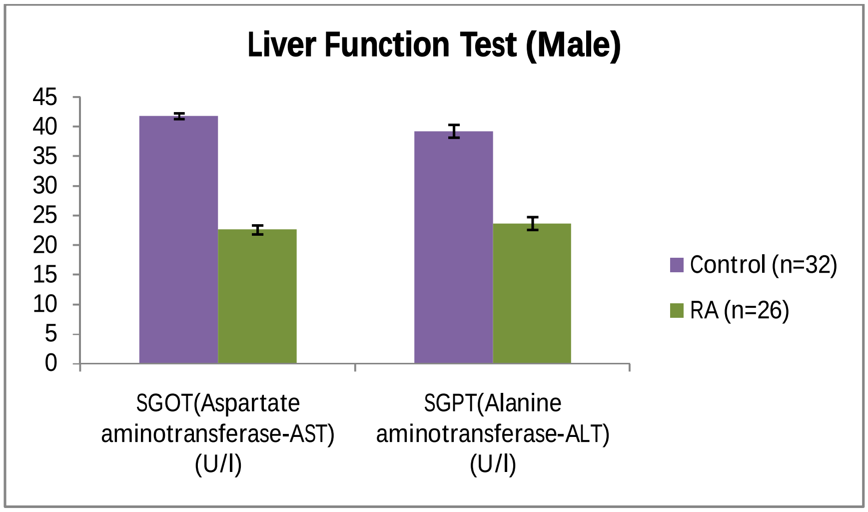


Figure-11B

The liver function variables are summarized in Table-6. This table shows the change in variables in RA (Male and female) and control (male and female). The mean level of SGOT is significantly decreased ($p<0.0001$) in RA (male and female) as compared to control (male and female). SGPT is significantly decreased ($p<0.0001$) in RA (male and female as compared to control male and female). However, these values were within normal range.

	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.

Table-7: Intercorrelations of variables of RA patients

Intercorrelation analysis showed positive and significant correlation (r=0.575; P<0.01) between SGOT and SGPT (Table-7). ESR and VAS also showed significant positive correlation(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 and Activity of Antioxidant Enzymes and ALP level

4.4.1 Lipid peroxidation and activity of antioxidant enzymes and ALP level in RA and control

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***

Table-8 Shows the parameters of pro-oxidant, antioxidants and enzyme in RA and Control P***= P<.0001

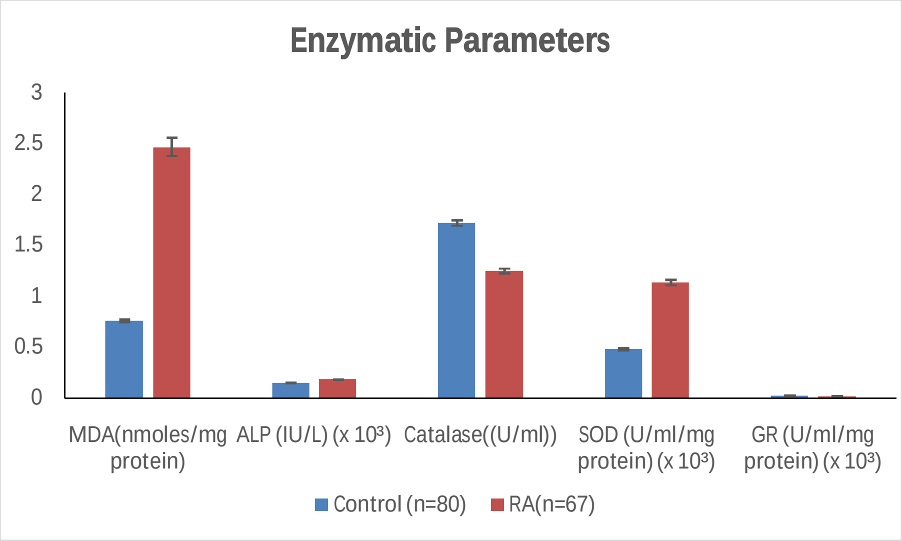


Figure-12

A significant increase in MDA level was observed (P<.0001) in RA patients as compared to control (Table-8). It may be due to increased ROS during chronic inflammation (table-8). In our study mean ALP activity was 1.3 fold increased (p<0.0001) in RA as compared to control (Table-8). 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 control probably due to chronic oxidative stress (Table-8).

4.4.2 Enzymatic parameter of female (RA and control) and male (RA and control)

Enzyme	**Control Female (n = 48)**	**RA Female (n =41)**	**Control Male(n=32)**	**RA Male (n=26)**
MDA(nmoles/mgprotien)	0.76±0.02	2.59±0.11***	0.755±0.022	2.294±0.14***
ALP(IU/L)	150.27±0.90	182.75±0.76***	149.03±1.17	179.05±1.52***
Catalase(U/ml)	1.70±0.04	1.29±0.027***	1.753±0.0313	1.196±0.03***
SOD(Dismutase(U/ml /mg protein))	486.97±12.83	1193.5±34.24***	470.431±13.33	1044.51±33.07***
GR(U/ml/mg protein)	25.463±0.71	21.148±0.41***	27.58±1.21	18.66±0.48***

Table-9 P^{***}= P<.0001 P^{**} =P<0.005 significant

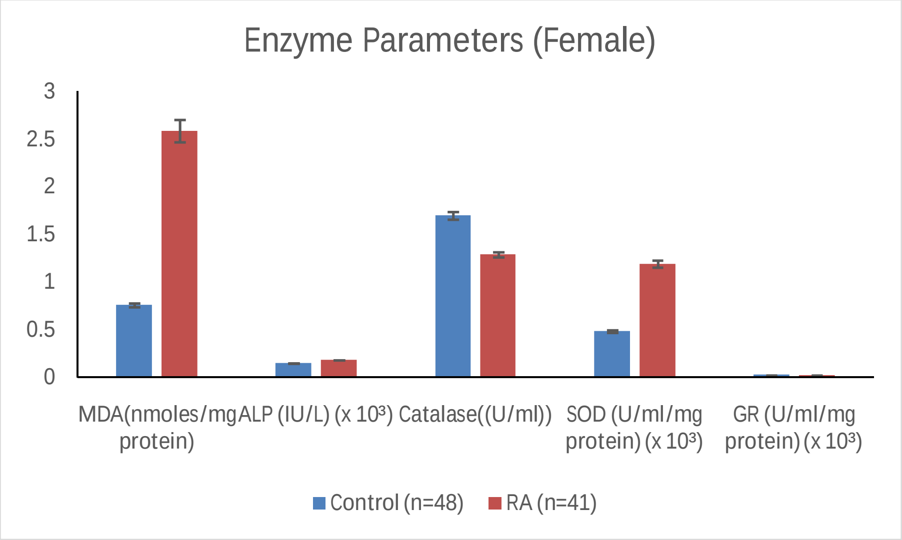


Figure-13A

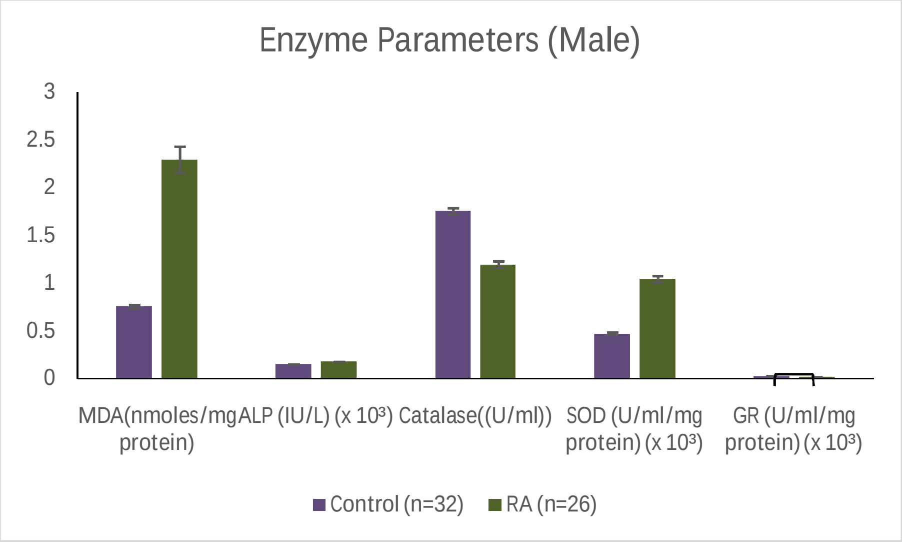


Figure-13B

In our comparative study (Table-9) between two groups MDA level was 3 fold increased in RA female and male patients as compared to control (female and male) which may be due to increased ROS during chronic inflammation. The mean level of SOD was significantly increased ($p<0.0001$) (2.5 times) in RA females and males. The mean a of ALP was significantly increased (1.4 times) ($p<0.0001$) in female and male RA patients compared to control. Activity of catalase was significantly decreased ($p<0.0001$) in male and female RA patients as compared to control due to chronic oxidative stress. Activity of GR of RA females was significantly decreased (0.3 fold) as compared to control females and in RA male mean level GR activity was significantly decreased (0.4 fold) as compared to control males.

	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

Table 10: Shows the correlation between various parameters in RA patients (N-45). The p is significant at *p<0.05, **p<0.01.

The interaction the enzymes and MDA showed significant positive correlation between MDA and GR (r = 0.29; P<0.05)(Table-10) and between GR and SOD (r = 0.396; P<0.01) (Table-10). ALP activity also showed significance positive correlation between SOD (r = 0.4928; P<0.01) and GR (r = 0.313; P<0.01) (Table-10)

4.5 Trace Metal

4.5.1 Trace metal of RA and Control

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

Table.11 P^{***}= P<.0001

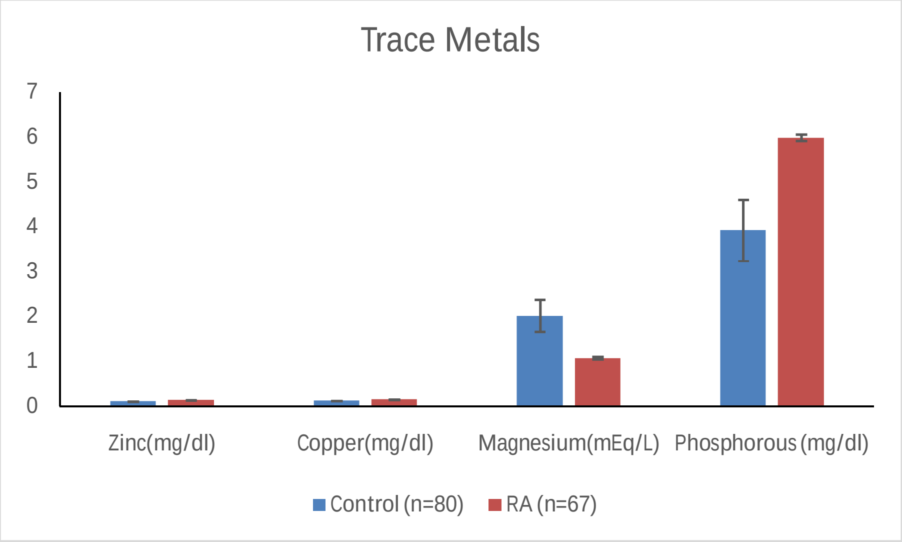


Figure-14

Zinc and copper are 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 key component of bone, 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-10).

4.5.2 Trace metal of female (RA and control) and male (RA and control)

Metals	**Control Female (n = 48)**	**RA Female (n =41)**	**Control Male(n=32)**	**RA Male (n=26)**
Zinc(µg/dl)	102.07±1.464	133.16±1.15***	99.704±1.40	137.84±1.15***
Copper(µg/dl)	127.07±3.09	144.508±2.05***	117.322±2.78	154.79±2.51***
Magnesium(mEq/L)	2.07±0.045	1.115±0.033***	1.95±0.060	1.031±0.048***
Phosphorous (mg/dl)	4.077±0.088	5.838±0.078***	3.703±0.097	6.090±0.120***

Table-11 P^{*}= P<.0001

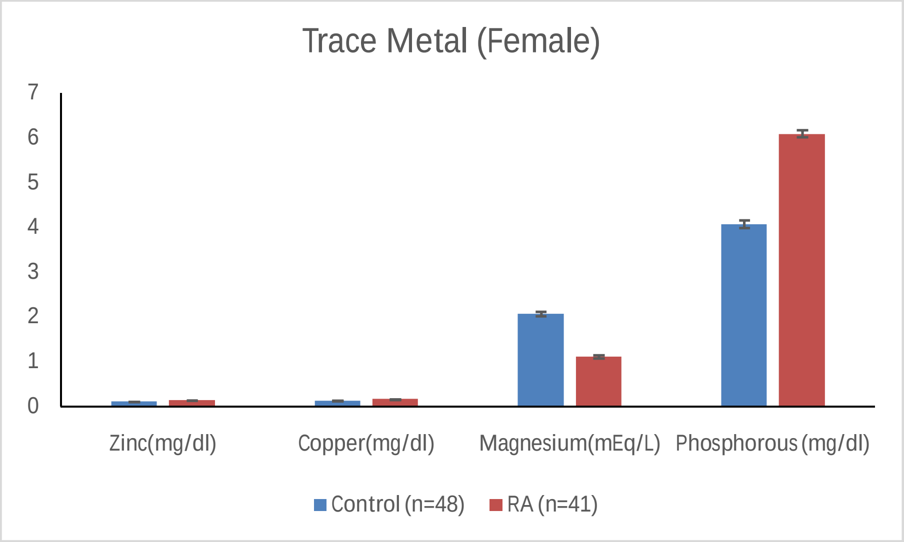


Figure-15A

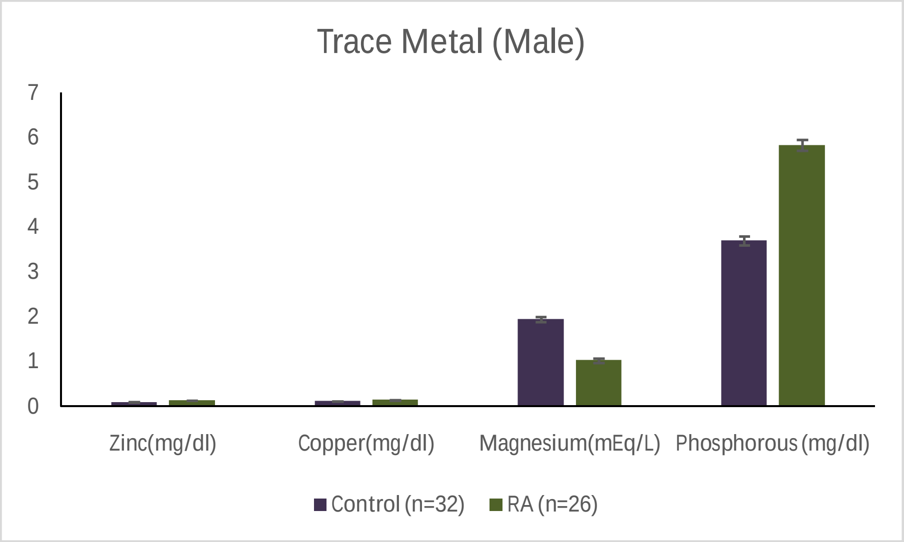


Figure-15B

The mean levels of zinc, copper and phosphorous were significantly increased (1.3 times) in RA patients (male and female) as compared to control (male and female). However, Mg concentration was significantly decreased ($p<0.0001$) (0.5 fold) in RA patients (male and female) as compared to control (male and female) (Table-11).

4.6 Lipid Profile

4.6.1 Lipid profile of RA and Control

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***

Table.12 P***= P<.0001

The levels of lipid variables of two groups are summarized in table-12.

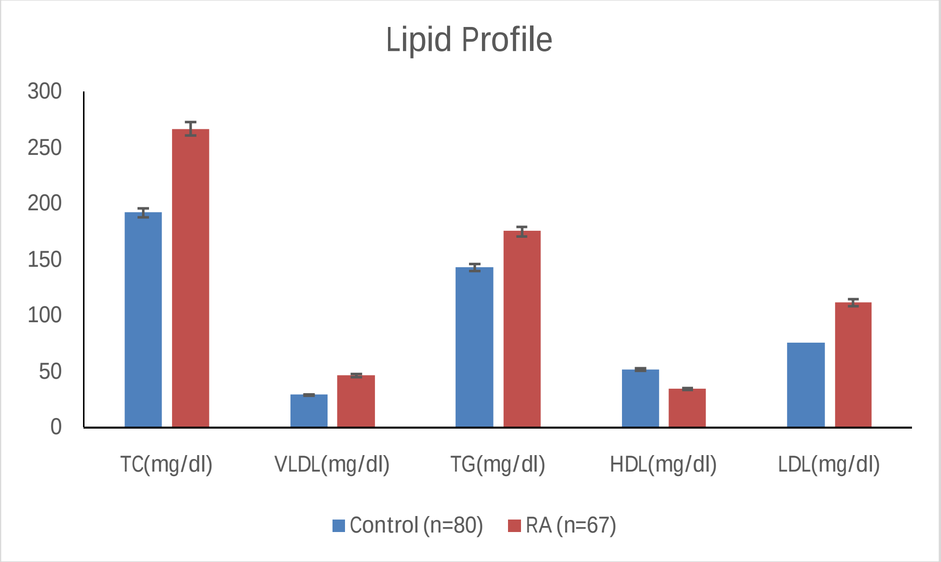


Figure-16

Mean level of TC and TG were significantly increased (P<.0001) (1.4 fold) in RA as compared to control. VLDL and LDL levels were significantly increased (1.7 times) in RA patients as compared to control. The mean level of HDL was significantly decreased by (0.5 fold) in RA patients as compared to control. These variables except HDL are responsible for arteriosclerosis and thus are risk factors for cardiovascular disease in RA patients (Table-12).

4.6.2 Lipid profile of female (RA and control) and male (RA and control)

Variables	Control Female (n = 48)	RA Female (n =41)	Control Male (n=32)	RA Male (n=26)
TC(mg/dl)	191.96±5.3	248.11±8.10***	191.98±5.39	296.72±5.35***
VLDL(mg/dl)	29.27±1.09	47.317±2.19***	29.63±1.22	46.22±1.91***
TG(mg/dl)	143.44±4.55	170.57±5.48***	142.70±4.44	182.85±7.23***
HDL(mg/dl)	51.25±1.37	34.66±0.89***	53.73±1.64	34.91±1.12***
LDL(mg/dl)	76.20±1.78	109.94±4.14***	75.417±1.64	114.60±4.75***

Table.13 The levels of lipid variables of two groups are summarized in Table-13.

P^{***}= P<.0001

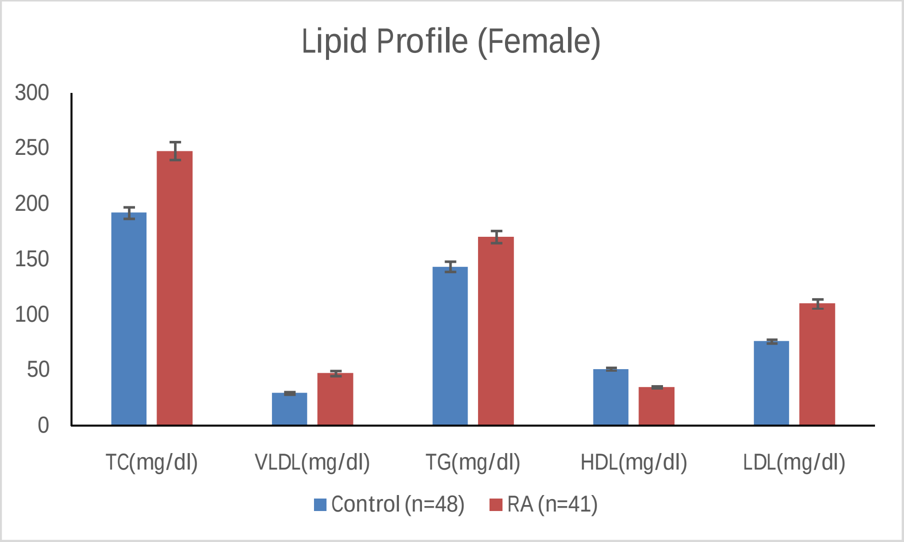


Figure- 17A

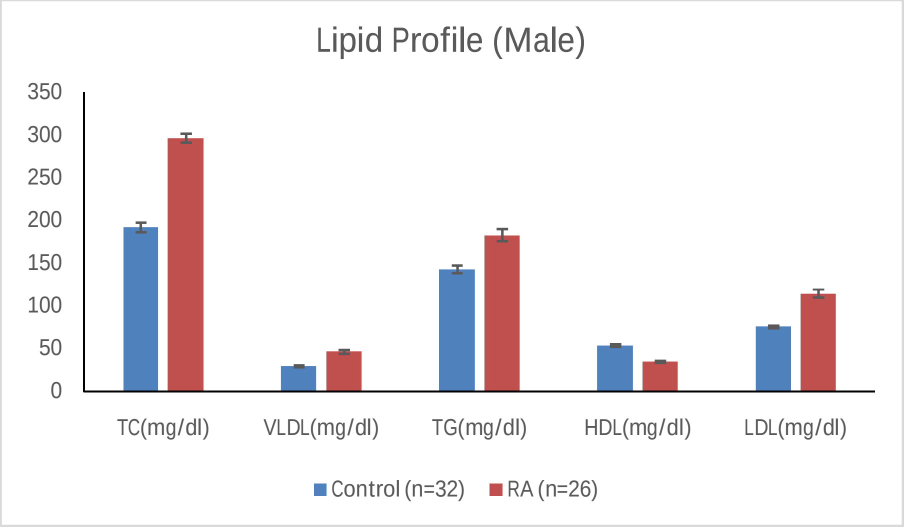


Figure-17B

The mean level of TG was significantly higher by (1.2 fold) in RA females as compard to control females and (1.5 fold) significantly increased ($p<0.0001$) in RA males as compared to control males. The mean value of VLDL was significantly increased ($p<0.0001$) (1.8 times) in RA males and females as compared to control males and females. The mean value of TG was significantly increased ($p<0.0001$) (1.3 times) in RA males and females as compared to control males and females. HDL in RA patients (females and males) was significantly lower ($p<0.0001$) (0.3 fold) as compared to control (females and males) while LDL, was significantly higher ($p<0.0001$) (1.5 fold) in RA patients (females and males) as compared to respective controls (females and males).

	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}	-0.029^{ns}	1						
P	0.096 ns	-0.013 ns	0.026 ^{ns}	-0.231^{ns}	0.084 ns	1					
TC	0.087 ns	0.183 ns	0.268**	-0.257^{ns}	-0.179 ns	-0.132 ns	1				
VLDL	0.024 ns	0.111 ns	0.0261^{ns}	-0.021^{ns}	-0.073 ns	0.248**	0.424**	1			
TG	0.163 ns	0.045 ns	0.1505^{ns}	-0.190^{ns}	-0.121 ns	0.063 ^{ns}	0.425**	0.407 **	1		
LDL	0.038 ns	0.310 **	0.0283^{ns}	0.002^{ns}	0.122 ns	-0.154 ns	0.257**	0.294**	0.018 ns	1	
HDL	-0.046 ns	0.150 ns	-0.018^{ns}	-0.036 ns	-0.076 ns	0.017 ^{ns}	-0.011 ns	0.087 ns	-0.054 ns	0.059 ns	1

Table-14: Intercorrelation of several variables of RA patients. 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.

Intercorrelation between CRP and lipid parameter showed inverse and significant correlation between and C-reactive protein ($r = -0.367$; $P<0.01$). However total cholesterol and Zinc showed significance 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 significance positive correlation with total cholesterol (r = 0.425; P<0.01) and VLDL (r = 0.294; P<0.01)

4.7 Genetic Polymorphism

4.7.1 Peptidylarginine deiminase type 4 (PADI4)

4.7.1.1 RS188_1

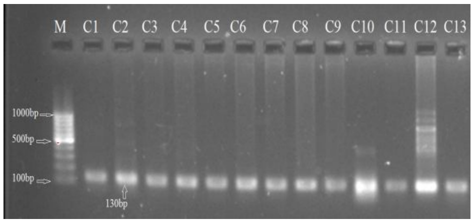


Figure-18A

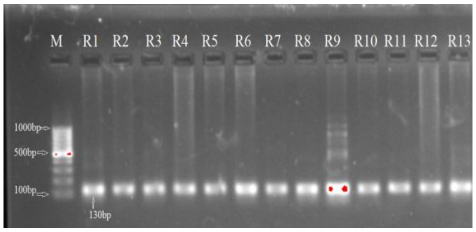


Figure-18B

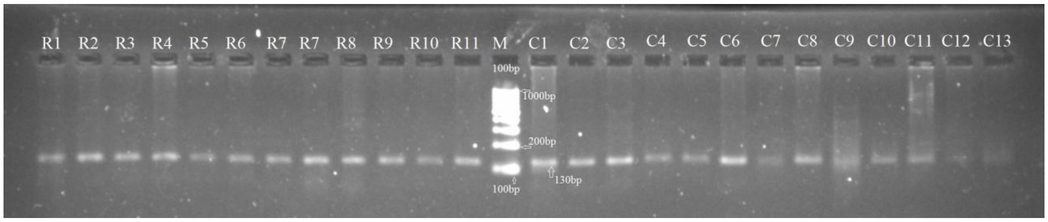


Figure-18C

Fig.-15 shows that PADI haplotype RS188_1 is not associated with RA. We could not observed CT or TT genotypes in either control or RA patients as no polymorphic bands were observed after digestion of PCR product with RsaI restriction enzyme. Therefore the loci PAD188_1 is non-polymorphic in our population and is not associated with RA.

4.7.1.2 RS188_2

RA and Control

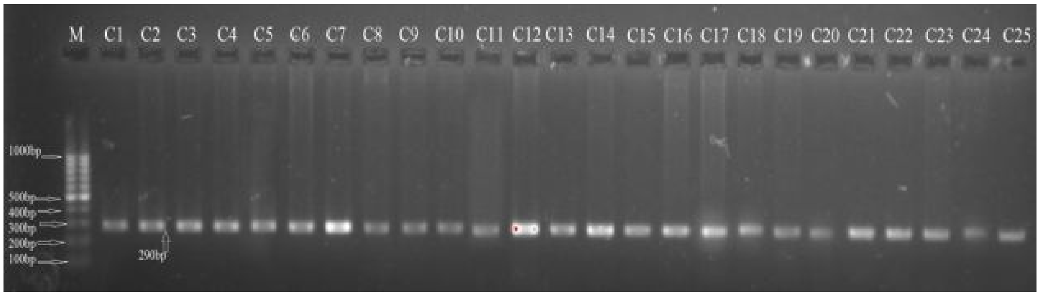


Figure-19A

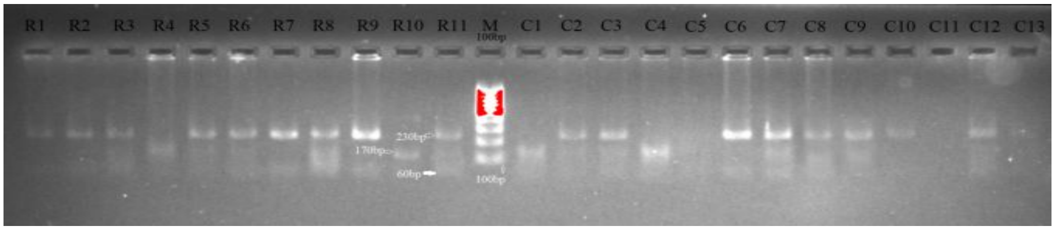


Figure-19B

RS188_2 (%)	Genotype distribution (%)			Allele frequency	
	CC	CG	GG	C	G
Control (80)	13(16.25)	46(57.5)	21(26.25)	72(45)	88(55)
RA (67)	24(35.8)	31(46.2)	12(17.9)	79(58.9)	55(41.1)

Table-15 (OR =0.546, 95% CI = 0.334-0.894, p=0.16).

Our results shows that wild type CC allele is present in 13 control and 24 RA, CG alleles are present in 46 control and 31 RA. While GG allele is present in 21controls and 12 RA. As we could not find prevalence of any specific allele of PADI RS188_2 with RA therefore this allele is also not associated with RA in north Indian Population.

Our results shows (table-15) no significant differences in allele and genotypic frequencies between RA patients and control for RS188_2 C/G polymorphism.

4.7.1.3 PADI_102

RA and Control

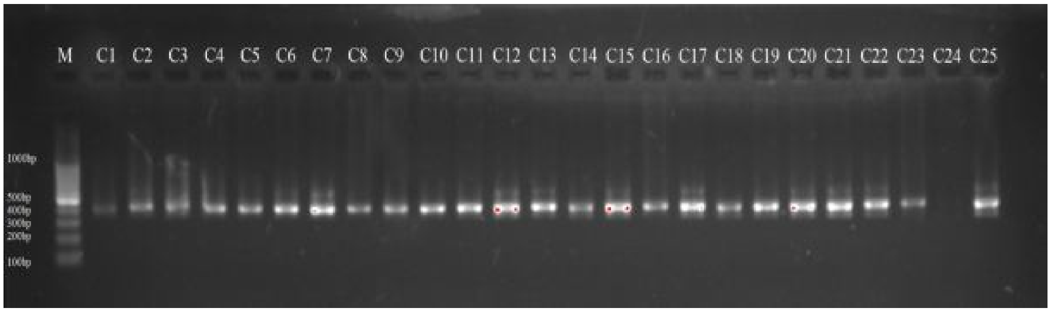


Figure-20A

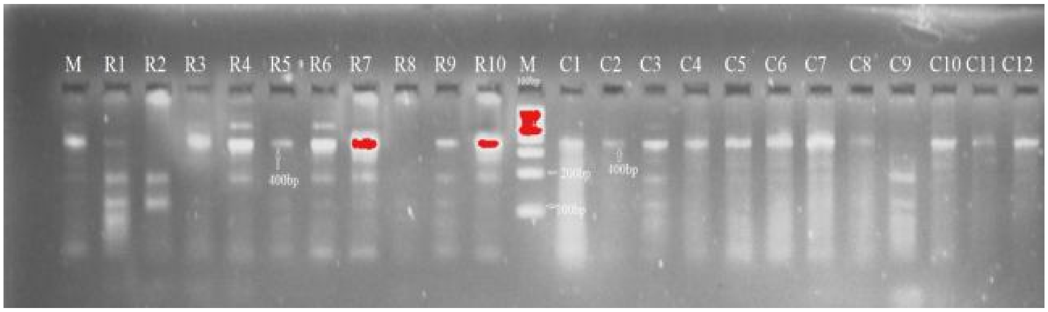


Figure-20B

PADI_102 (%)	Genotype distribution (%)			Allele frequency	
	CC	CT	TT	C	T
Control (80)	46(57.5)	22(27.5)	12(15)	113(70.6)	(29.4)
RA (67)	14(20.8)	40(59.7)	13(19.4)	68(50.7)	66(49.3)

Table-16 (OR =2.18, 95% CI = 1.354-3.510, p=0.0001).

Our results show that wild type CC allele is present in 46 control and 14 RA, CT allele is present in 22 control and 40 RA. While TT allele is present in 12 controls and 13 RA. Above table show the genotype and allele frequencies of PADI4 C/T genotype and allele frequencies in RA patients and controls. A significant difference between genotypic and allelic distribution of PADI_102 polymorphism was observed between RA and control group. However wild type allele CC was predominantly present in control. Whereas RA patients has significantly reduced presence of wild type CC allele. Hence the PADI4_102 C allele seems to be associated with the risk of RA in north Indian population.

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

4.7.2.1. A/G SNP TIMP-4

RA and control

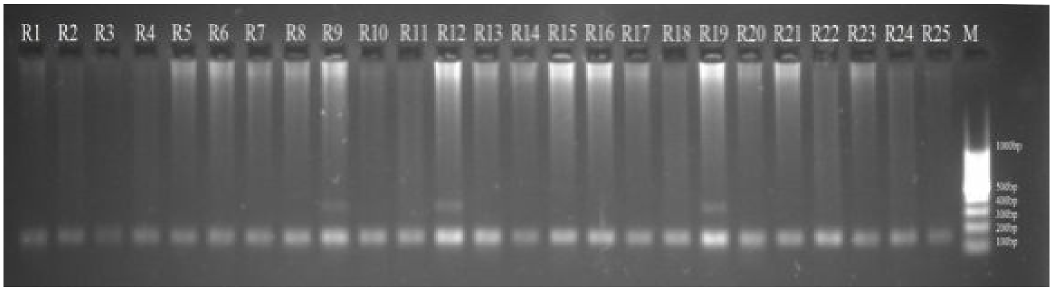


Figure-21A

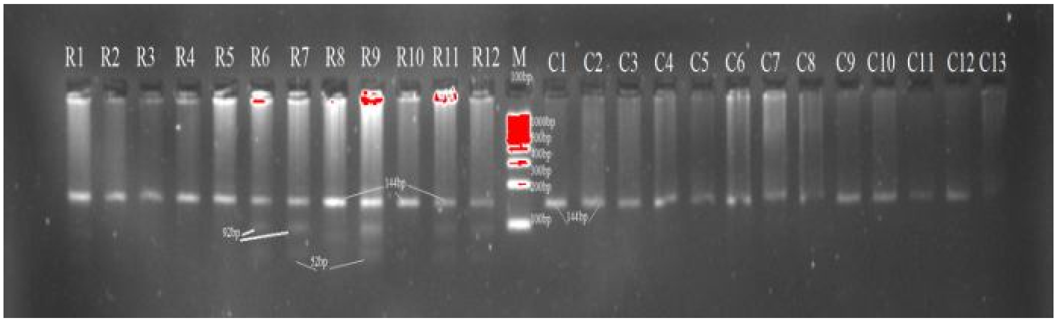


Figure-21B

Our results show that wild type AA allele is present in 80 control and 40 RA, AG alleles are present in 00 control and 27 RA. While GG allele is not present in controls and RA. No polymorphic bands were observed after digestion of PCR product 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.

4.7.2.2 C/T SNP

RA and Control

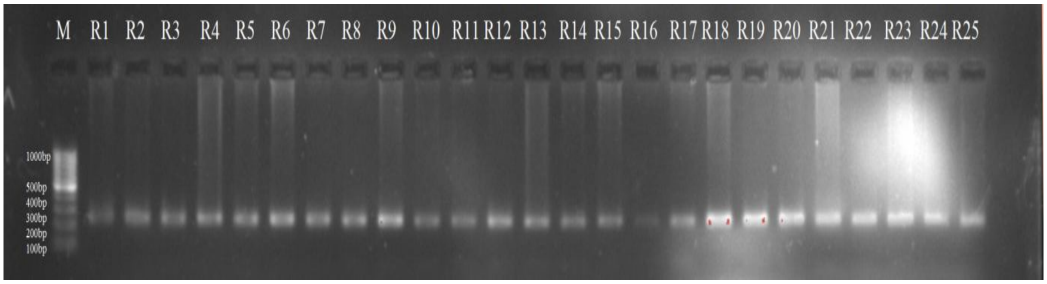


Figure-22A

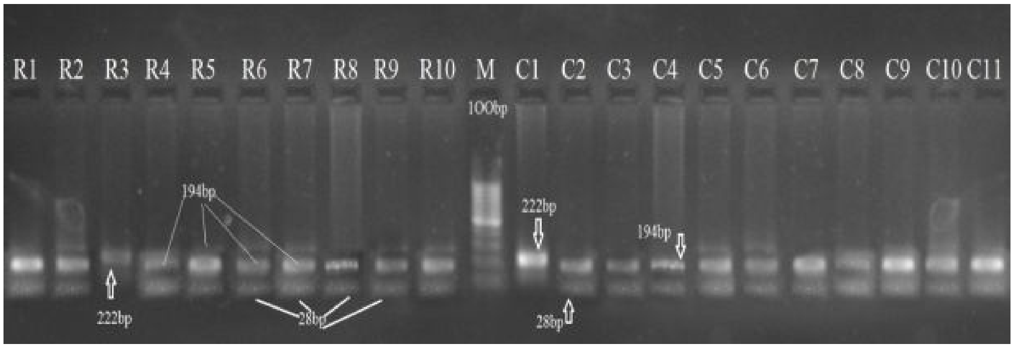


Figure-22B

TIMP-4 gene RA patients and normal control groups

TIMP-4 rs17035945	Genotype distribution (%)			Allele frequency (%)	
	CC	CT	TT	C	T
Control (80)	65(81.25)	12(15)	03(3.75)	142(88.75)	18(11.25)
RA (67)	48(71.6)	14(20.8)	05(7.4)	110(82.08)	24(17.92)

ORs (95% CI) = 1.534 (0.850-2.770), P = 0.154

Table-17

Our results show that wild type CC allele is present in 65 control and 48 RA, CT alleles are present in 12 control and 14 RA. While TT allele is present in 02 controls and 05 RA. Above table show the genotype and allele frequencies of TIMP4 C/T genotype and allele frequencies in RA patients and controls. Nonsignificant difference between genotypic and allelic distribution of TIMP4 C/T polymorphism was observed between RA and control group. However wild type allele CC was predominantly present in control. Whereas RA patients has significantly reduced presence of wild type CC allele.

4.7.3. PTPN22

C/T SNP

RA and Control

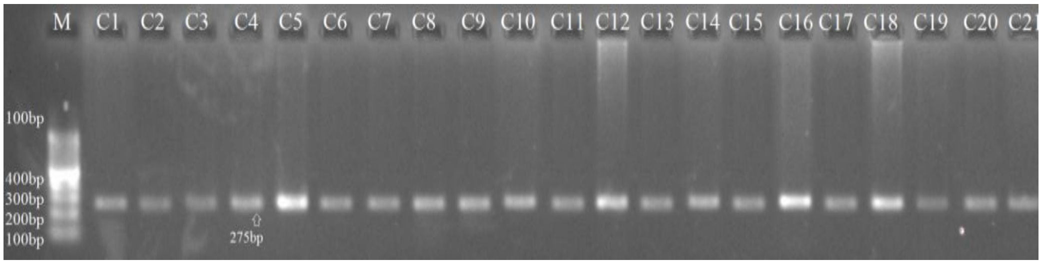


Figure 23A

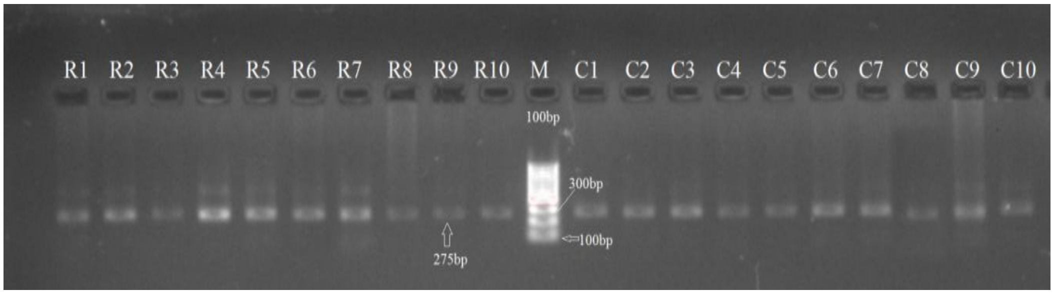


Figure 23B

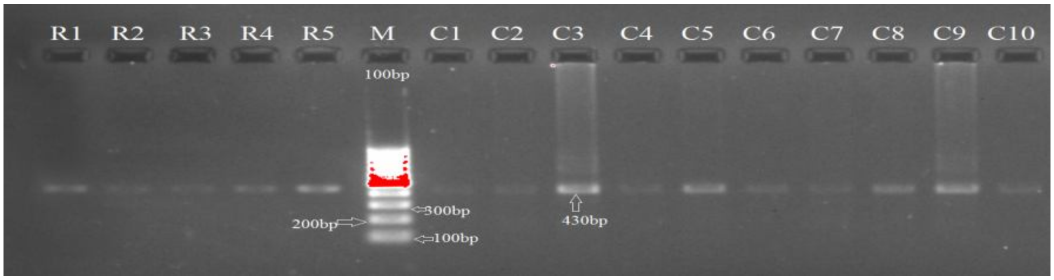


Figure 23C

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

4.8 Nuclear Magnetic Resonance

4.8.1 Metabolic alterations in RA:

The representative 1D ^{1}H CPMG NMR spectra of serum samples (one from each group) are shown in Figure 25. 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 recruitment judiciously to minimize the differences due to confounding variables as evident from Table 20.

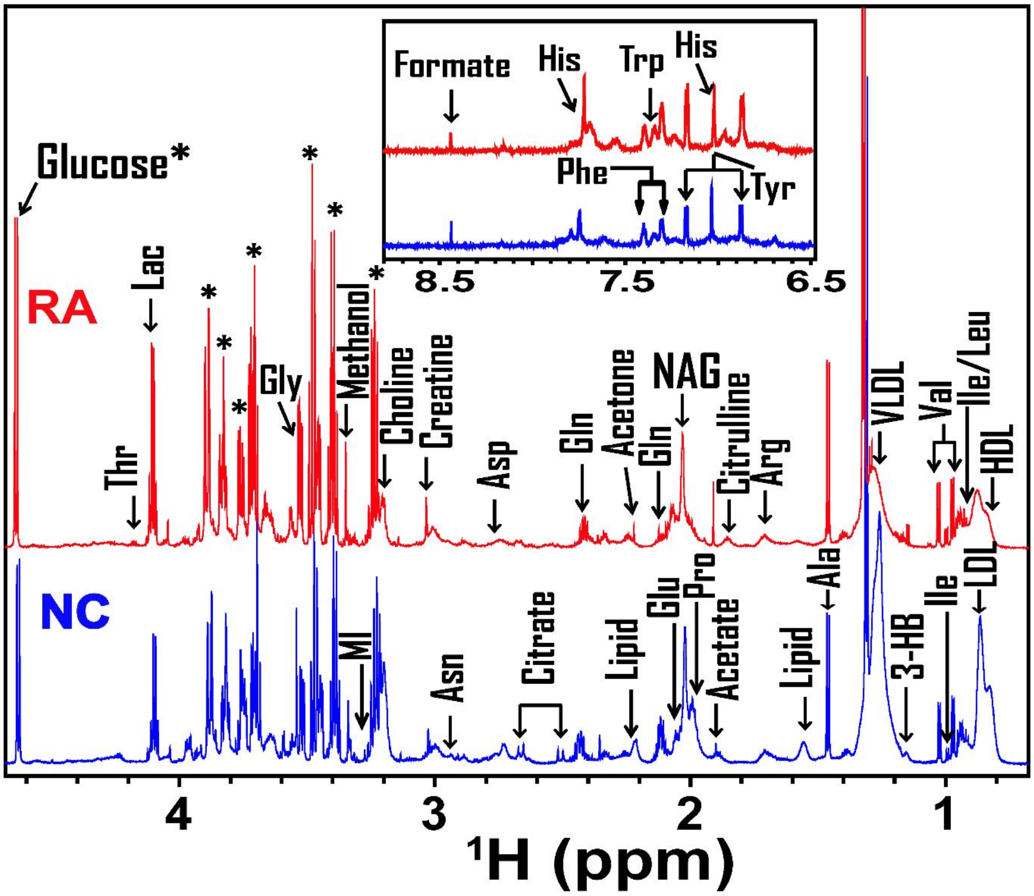


Figure 24: Stack plot of cumulative 1D ^{1}H CPMG NMR spectra (ranging from 4.6–0.7 and inset 8.5–6.5 ppm) obtained for sera of RA patients (red) and normal controls (blue). Key acronyms are: HDL; high density lipoproteins; LDL: low density lipoproteins; VLDL: very-low density lipoproteins; Val: Valine; 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 26A, 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 (Fig. 26) 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) as enlisted in **Table 2**.

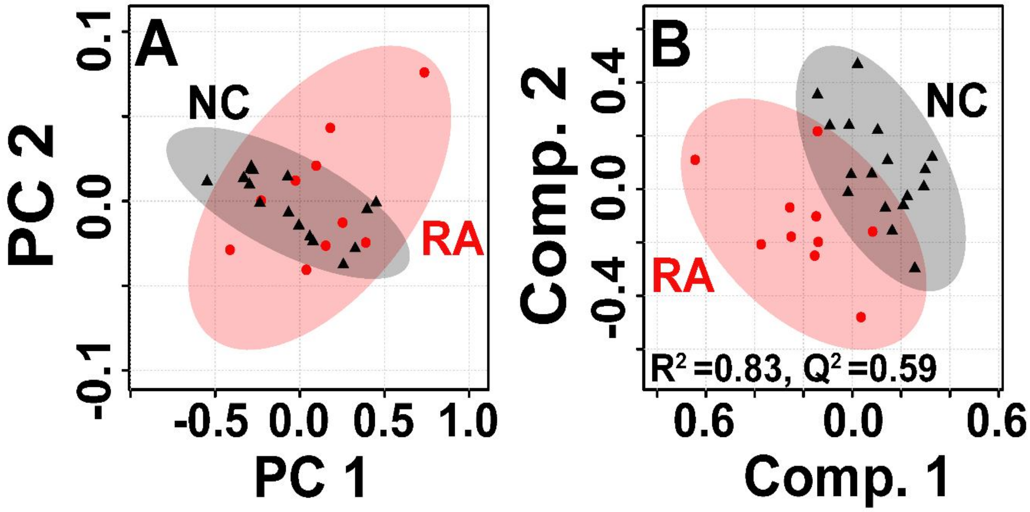


Figure 25: 2D PCA (A) and PLS-DA (B) score plots derived, respectively, from CPMG spectra showing clear statistical separation between RA (represented by red circles) and normal control (NC) samples (represented by black triangles). Each circle and triangle represents one subject. The validation parameters (R^2 and Q^2) corresponding to PLS-DA model are also displayed in their score plots.

Table 18: Details of metabolites best describing the variation between RA and normal controls. The up and down levels represent, respectively, increased and decreased metabolite levels in RA patients compared to normal controls.

S.No	**^{1}H ppm**	**Metabolite**	**VIP**	**AUC**	**p-Value**	**Level**
1	0.805	HDL	1.39	0.78	0.02	Down
2	0.855	LDL	2.21	0.74	0.03	Down
3	1.255	VLDL	4.42	0.78	0.01	Down
4	1.025	Valine	1.25	0.57	0.63	Down
5	1.315	Lactate	5.43	0.68	0.06	Up
6	1.555	Lipid	1.55	0.75	0.02	Down
7	1.985	Proline	1.50	0.74	0.04	Down
8	2.015	NAG	1.38	0.66	0.07	Down
9	2.115	Glutamate	1.70	0.82	0.00	Down
10	2.355	Pyruvate	1.51	0.76	0.02	Down
11	2.425	Glutamine	1.23	0.75	0.02	Down
12	2.525	Citrate	1.61	0.92	0.00	Up
13	2.545	Methylamine	1.18	0.88	0.00	Down
14	3.025	Creatine	1.07	0.70	0.14	Down
15	3.205	Choline	2.69	0.68	0.21	Up
16	3.895	Glucose	2.19	0.76	0.01	Up
17	4.035	Creatinine	1.18	0.82	0.00	Down
18	4.235	Threonine	1.04	0.92	0.00	Down
19	5.285	PUFA	1.79	0.75	0.02	Down
20	7.735	Histidine	1.48	0.91	0.00	Down

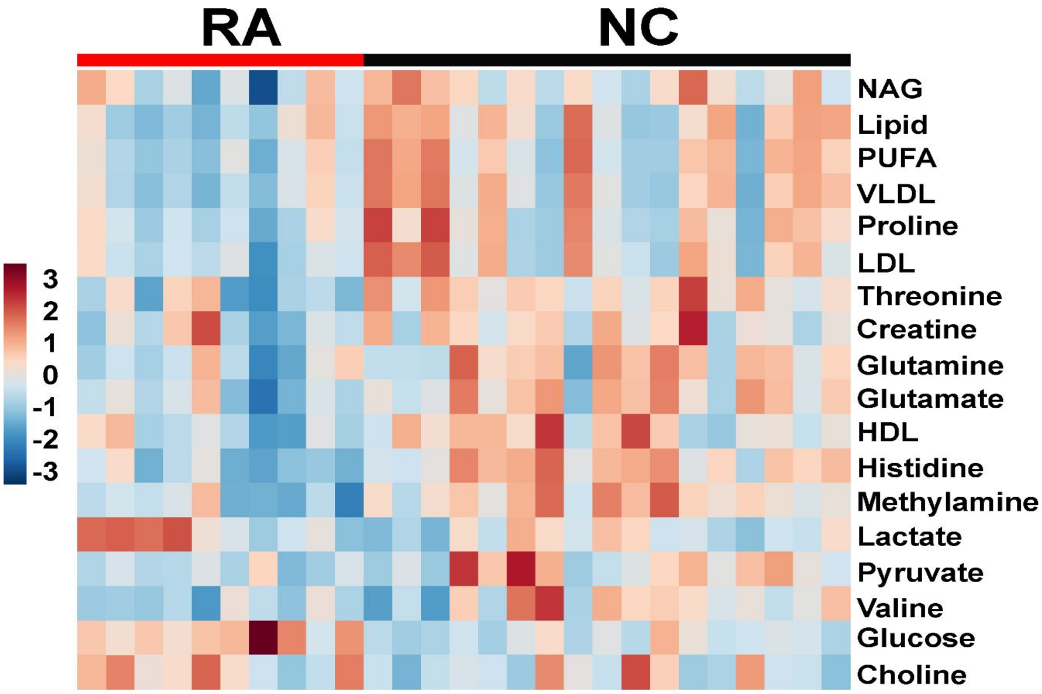


Figure 26: Heat maps showing z-scores of identified 20 statistically significant metabolite entities altered in RA patients compared to normal controls as shown in Table 20. The red and cyan here signify, respectively, elevation and reduction in metabolite concentration in RA patients.

These discriminatory metabolite entities were used to construct the heatmaps, commonly used for unsupervised clustering, Figure 27 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 28. 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.

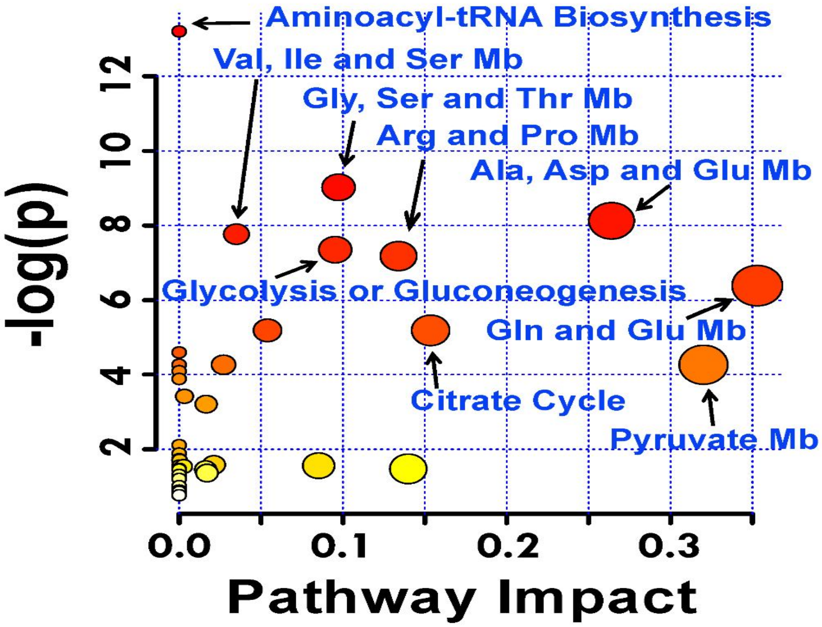


Figure 27: Identification of the perturbed metabolic pathways by overrepresentation analysis (ORA)using the significantly altered metabolites as identified in Table 2. The analysis was done by using a pathway library restricted to Homo sapiens (human), and p-values for ORA stand for hypergeometric test. Test p-value (vertical axis, intensity of colour) and impact factor (horizontal axis, size of circle); Key-Mb=Metabolism.

5. DISCUSSION

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease which affects symmetric and multiple joints of the body. RA causes significant morbidity and mortality as compared to general population. The disease has 0.8% prevalence with annual incidence of 0.5-1% in thw world (Makhdoom *et al.*, 2009, Gubler *et al.*, 1953). Like other autoimmune disease RA is more prevalent in females as compared to male()1-4. The difference sin over occurrence and development of aggressive disease in females is not clear but genetic and hormonal fectors are suggested to be involve (6-11).Joint destruction occurs as the disease progresses. The study was planned to the study the changes occurring in RA patients in their activity score (DAS-28), pain on visual analoge scale (VAS), inflammatory changes and changes related to oxidative stress. Their general well being and risk of patients developing cardiovascular disease was also analyzed by evaluation of their response ti therapy and analysis of lipid parameters.

The sample collection was done from hospitals, local clubs, neighbourhood patients and control were recruited after varifing inclusion and exclusion criteria as discussed in material and methods. The study was apoorved by Institutional Ethical Committee and written informed consent was obtained from all the participants.

In our study, there was no significant difference in the BMI and systolic blood pressure of patients and control (Table-2 and figure 7) and male and female RA subjects. The haemoglobin of the patients was significantly decreased in RA patients as 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 have also reported the severity of the disease accounts for systemic manifestations with effects on blood. Anaemic syndrome has been reported to be a common manifestation which may result in increased disease activity (Bloxham et al, 2011).

The patients showed increased ESR, pain on visual analog scale and CRP as compared to control. Though patients are on medication, still raised values indicate ongoing inflammatory process in their body leading to increased production of CRP. Female patients in our study had higher DAS and VAS as compared to Male RA subjects. Higher CRP is suggestive of reoccurrence of active disease sometimes along with ongoing medication or after withdrawal of the drug. DAS was significantly increased in patients along with the ESR and CRP level. Pain on visual analog scale (VAS) was significantly increased in patients (Table-4) with pain in females as compared to male RA subjects.

Our previous report has shown improves CRP, MDA, DAS-28 and VAS in RA patients treated with methotrexate at 6 months of followup (Patel *et al.*, 2015). The aggressive disease as RA is controlled by DMARDs, NSAIDS, glucocorticoid and biologics wich target TNF-alpha, IL-1 and IL-6 receptors (23.39). There is heterogeneity in the response of the drugs and various side effects on liver and kidney are reported. In our study we could not observed adverse effects of MTX therapy on liver or kidney function.

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 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 leflunamide treatment. Leflunamide 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 leflunamide. 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).**

The values for SGOT and SGPT which indicate functional status of liver are significantly lower in RA patients. Our treated patients showed significantly reduced levels of SGOT and SGPT as compared to control. In patients the values for SGOT positively coorelated with SGPT ($r=0.575; p<0.01$). Study by Iannone et al, 2014 has shown that RA patients may be successfully treated with MTX without increasing 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 supplementaion with drug dose of 15mg/week (intramuscular or subcutaneous) (Patel *et al*., 2015). However, patients refractory to low-dose MTX therapy may require larger doses of oral MTX. Several previous studies have demonstrated variability in bioavailability of oral MTX at high doses. This warrants a subsequent switch to parenteral MTX subcutaneous (SC). The pharmacokinetics of SC MTX is similar to intramuscular MTX but SC MTX may be preferred by most patients (Yadlapati & Efthmiou 2016). Our patients are also on SC or intramuscular MTX, which has better potential and effective at a dose of 15 mg/week.

In our study lipid per-oxidation in terms of MDA production was significantly increased in RA patients (Table 1) which may be due to increased ROS during chronic inflammation. Female patients had higher MDA as compared to male patients. Lipid peroxides are generated at the site of tissue injury due to inflammation and diffuses into blood and can be estimated in serum or plasma(Gutteridge JM (1995)) . Several studies (Ali *et al.*, 2014, Kamanli *et al.*,2004, Sarban *et al.*, 2005, Hassan *et al.*, 2001) have reported raised levels of MDA in the serum, plasma and erythrocytes of RA patients. In our study SOD levels are highly increased (Table 8) (Kumar *et al.* 2016). Superoxides anion (O$_{2-)}$ has important role in pathogenesis of many diseases. It is neutralized by SOD to hydrogen peroxide (H_2O_2). H_2O_2 is further quenched by activity of catalase and glutathione peroxidase. Transformation of O_2^- to H_2O_2 prevents the formation of aggressive compound as peroxynitrile (ONOO) and hydroxyl radical (OH^-)(Afonso *et al.*, 2007). The patients showed significantly higher activity of SOD and ALP (Table 8). There was strong positive correlation between SOD and ALP activity (Table). They showed reduced activities for catalase and glutathione reductase.

Reactive oxygen species and oxidative stress have a role in the pathogenesis of RA (Kamanli *et al.*, 2004). 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). Prime targets of ROS attack are the polyunsaturated fatty acids in the membrane (Table 8) lipids causing lipid peroxidation (LPO) which may lead to disorganization of cell structure and function. Further decomposition of peroxidized lipids yields a wide variety of end-products, including malondialdehyde (MDA) (Gambhir *et al.*, 1997). Malon dialdehyde (MDA) is one of an important lipid peroxide which is high in RA patients (Mishra *et al.*, 2012, Patel *et al.*, 2015). Measurement of MDA is widely used as an indicator of LPO.

Many studies have reported high MDA in the serum, plasma and synovial fluid of RA patients (Kamanli *et al.*, 2004, Gambhir *et al.*, 1997, Pallinti *et al.*, 2009). MDA has an important role in pathogenesis of RA. There is growing awareness that reactive oxygen species and free radicals may play an important role in mediating cellular injury and tissue damage in rheumatoid arthritis. Thiele *et al.*

(2015) has reported malondialdehyde-acetaldehyde (MAA) adduct formation is increased in RA. They appear to 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.

Higher levels may be the result of respiratory burst triggered by leucocytes. A study has shown activation of neutrophilic myeloperoxidase-hydrogen peroxide system in RA synovial tissue which may contribute to cyclic self-perpetuating inflammation (Nurcomb *et al.* 1991). Methotrexate treatment has been reported to increase Zn-SOD activity but it has no effects on GSH-Px in rats (Armagan *et al.*, 2008, Al-Saleh *et al.*, 2009).

But possibly increased activity of SOD (Vijayakumar *et al.*, 2006, Cimen *et al.*, 2000) may be attributed to increased O_2^- production by hyperactive cells leading to SOD induction (Gregory & Fridovich 1973). Another possibility may be excessive free radical production through the xanthine-xanthine oxidase system is the primary factors in RA, rather than an impaired antioxidant system (Cimen *et al.*, 2000). Else higher SOD levels may be a change to nullify excessive free radical production. Post treatment the antioxidants are increased which lead to lower plasma MDA and increased total antioxidant capacity (TAC) (Nourmohammadi *et al.*, 2010).

However lower SOD has also been reported in patients with RA on MTX therapy in comparison with RA without MTX therapy (Al-Youzbaki *et al.*, 2013). It has also being observed that MTX can suppress directly or indirectly the generation of active oxygen metabolites induced by IL-6, which is produced in response to TNF-α stimulation in synovial cells of RA(Sung *et al.*, 2000) as well as in polymorphnuclear cells. The increased levels serum Cu/Zn SOD may support the hypothesis of radical-mediated injury.

Over expression of extracellular SOD leads to dismutation of superoxide resulting in H_2O_2 accumulation. Analysis of H_2O_2 in different settings is being done and authors conclude, more SOD does not mean more H_2O_2 (Lin *et al.*, 2006). The formation of H_2O_2 due to dismutation of superoxide is limited by the amount of superoxide, not by the rate it is converted to H_2O_2. Accumulation of superoxide leads to the oxidation of NO forming peroxynitrite. There more H_2O_2 is unlikely to be toxic as this would amount to substituting a very mild cytokine (H_2O_2) for a potant (peroxynitrite) (Zaghloul *et al*., 2014).

Decreased activity of SOD in RA patients has also been reported (Mohamad A 2011). However our study is in line with (Kamanli *et al.*, 2004) who have reported increased SOD levels in RA patients. Mazetti *et al.* (1996) have reported higher serum copper/Zn superoxide in patients with RA. Igari *et al*. (1982) have reported 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 activity of superoxide dismutase need to be detoxified by glutathione peroxidase and catalase activity. Catalase plays an important role in preventing ROS mediated damage by using H_2O_2 and converting it to water and oxygen. In our RA patients, catalase activity is significantly decreased as compared to control. Lower catalasae activity may be due to interaction of catalase by hydrogen peroxide(Mohamad *et al*., 2011). Lowered activities of their enzymes may lead to conversion of H_2O_2 to hydroxyl radical by iron released from hemoglobin of lysed erythrocytes (Taysi *et al*.,2002). However unaltered catalase activity in RA patients has been reported (Veselinovic *et al*., 2014).

Catalase activity was not found in serum of RA patients. Decreased erythrocytes catalase activity is also being reported (Taysi *et al*., 2002). Our study is in accordance with and shows lower catalase activity in serum of RA patients. Catalase expression affects expression of genes which influence inflammation (Benhamou *et al*., 1998). Lower levels of catalase may be responsible for high inflammation in RA. Cimen *et al.* (2000) have reported higher SOD activity and MDA levels and unchanged catalase and GSH-Px activities in RA patients. The study by Gonzalez *et al.* (2015) 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 patients. Both GPx and catalase use H2O2 as substrate where catalase acts in the presence of high concentration of the substrate while GPx acts at lower concentrations. They also suggested that H2O2 concentration may be lower than in other chronic inflammatory diseases, with oxidative damage being mediated possibly by HO□ (Prego *et al*., 1997).

Glutathione reductase (GR), an oxidative stress inducible enzyme, plays a significant role in the peroxyl scavenging mechanism and in maintaining functional integration of the cell membranes. Glutathione reductase is a flavoenzyme dependent on NADPH that catalyzes the reduction of GSSH to GSH. Feijoo *et al.* (2010). observed that myeloperoxidase levels are elevated in patients with chronic inflammatory disease, especially those with active disease, and that high myeloperoxidase levels are related to an increase in oxidative damage and the inflammatory response, for myeloperoxidase and GR seem to show a similar activity pattern based on the availability of NADPH. Erythrocyte GSH and glutathione 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 glutathione reductase activity as observed in our study. The study by Aryaein *et al.* (2011) showed that GR, vitamin E, Beta-carotene was lower and MDA was higher in the patient group than in controls. Kamanli *et al. (*2004) observed significantly lower GSH-Px, catalase, levels of GSH in plasma of RA patients. However higher GR activity have also been reported in RA(Bazzichi *et al.*, 2002). Kerimova *et al.* (2000) also reported decreased catalase and unaffected GR activities in RA subjects. Low GR activities 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 patients alkaline phosphatase (ALP) activity is higher relative to control. ALP showed strong positive and significant relationship with SOD. ALPs role is implicated in osteoid formation and mineralization and expression of its isoform is in osteoblasts, leucocytes, liver, kidney, breast and brain (Weiss *et al.*, 1986, Gum *et al.*, 1990). The bone formation markers are measured in serum and about half of ALP in serum comes from bone. Several studies (Thompson *et al.*, 1990, Nanke *et al.*, 2002, Spooner *et al.*, 1982) have reported high serum ALP levels 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-cells is well documented in the pathogenesis of RA. Raised ALP may be due to its leakage from injured or killed cells.

Alkaline phosphatase has been implicated as marker in RA patients. It can provide diagnostic information by determination of isoform of ALP derived from liver or bone (Vaithialingam *et al*., 2013). Thus MDA and antioxidants systems work reciprocally to keep oxidative stress mediated damage in control. An inverse association between serum antioxidant levels and inflammation have been reported (Paredes *et al*., 2002).

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. Thus antioxidants may be helpful in control of clinical outcomes and oxidative stress in RA patients. In conclusion oxidative stress management may be considered a therapeutic option for RA along with DMARD. Supplementation of antioxidants along with catalase and/or GPX may confer more protection. In recent studies RA since to have derangement of mineral contents as magnesium copper, zinc, phosphorous, boron etc. They are required in optimum concentration in the body. However changes in their levels as Mg(17), Zn (18) Cu (copper).

There is scarity 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 & Bellonn, 1998). Decreased levels of Mg is considered as marker for RA (Lucia *et al*., 2011 Linos *et al*., 980). Magnesium has important functions in cardiovascular system, as an activator of sodium potassium ATPase, antiarrhythmic and is associated with cardio vascular disease susceptibility (Weisinger & Bellonn, 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; Durazzo *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 negative correlation with copper ($r=-0.412$; $p<0.05$) and MDA ($r=-0.345$; $p<0.05$). The studies suggest 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 induction of differentiation of vascular smooth muscle cells into osteoblast-like cells capable of extraskeletal mineralization which is important process in 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 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 inhibit albumin synthesis in 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 acute-phase response of inflammation and with treatment with NSAIDS (Balogh *et al*., 1980). It is suggested that these may be due to different 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. The studies have shown that hypercuperemia was associated with inflammatory response is due to oxidative stress (Ford 2000) as they found positive correlations between serum Cu levels and inflammatory markers serum CRP and ESR in RA patients (Liuzzo *et al*.,1994; Salomen 1991). In contrast to many other studies, we found inverse correlation between Cu and CRP level (r= -0.419, P<0.01). Cu is an environmental bioelement which play a key role in the cell's physiology, as a cofactor or component of the enzymes, participating in anti-oxidative process, or in detoxification of oxygen free radicals. RA patients have higher levels of copper as compared to control (Rainford 1982). Later on it was found that Cu complexes were effective in treating arthritis. Cu complexes have anti inflammatory properties and antarthritic drug in their active form are complexed with copper (Rainford 1982). The hypercupreuria that develops was suggested to be the outcome of dyslipidemia (Aaseth *et al*., 1978) or the cytokines have been reported to enhance the release of Cu thioneins 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 additional biochemical marker.

RA causes significant morbidity as a result of synovial inflammation, joint destruction and disability.Patients wuth RA have abnormal lipoprotein pattern (dyslipedemia). They have low level od HDL-c and high level of LDL-c in a pattern similer to inflammatory and infectious diseases (Rantappa *et al*., 1991, Filippatos *et al.,* 2013). Systemic inflammation in RA leads to significantly increased risk of cardiovascular diseases as compared to general population (Turesson *et al.*, 2004; Solomen *et al*., 2006 . Therefore control of inflammatrion mat have beneficial effects on cardiovascular risk and improvement in the lipoprotein profile.

The patients showed dyslipidemia with high total cholesterol, LDL-cholestrol, VLDL and low HDL cholesterol. Patients with RA have abnormal lipoprotein pattern. In our study also though dyslipidemia is observed as compared to control, but the valus of lipid parameters analyzed are either with in permissible limits or show borderline variations. These values may not be predictor of CVD risk in our RA patients. Several other studies did not showed any variation in lipid levels in RA patients with respect to healthy population (Dessein *et al*.,2002). Some other observed an overall reduction in all lipid sub fraction in case of active disease (Boers *et al*., 2003). The existing data has wide heterogeneity in the reporting of associated dyslipedemia. Studies have shown that in established RA, total cholesterol levels were only marginally raised irrespective of disease activity (Noumohamad 2007). High cholesterol induces oxidative stress leading to free radical generation that promotes lipid peroxidation (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 (Laurence *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 take 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

Although lipid levels are important risk factor, 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) and improvement in HDL-c levels post DMARD therapy (Watson *et al*., 2009). In our previous report (Patel *et al*., 2015) we observed increased HDL levels after 24 weeks of followup in MTX treated patients, but long term therapy does not have any favourable effects on HDL, nor does it increase TC, VLDL, TG and LDL. In RA there are reports showing either increased, 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). Larger cross-sectional study in 204 patients with RA, demonstrated an inverse association between elevated CRP and HDL-C-levels (White *et al*., 2006). Studies have shown presence of dyslipidemia at least ten year before the onset of clinical symptoms of RA (Van Halm 2006). In our study boarder line dyslipedemia 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 improved the atherogenic index(total/HDL cholestrol), an important prognostic CVD risk factor and it appears that the use of corticosteroid is not a risk factor for cardiovascular disease (Wallberg *et al*., 1997). Conventional DMARD (including corticosteroids) treatment has favorable effects on the lipid profile, as there is mounting evidence for favorable effects of DMARD treatment on the cardiovascular risk in RA (Van Halm *et al*., 2006) this might be (partially) mediated by favorable effects on the lipid profile..

Genetic Polymorphism

PADIs are involved in the post-translational deimination of arginine in proteins; the resulting citrullination partially un folds proteins via loss of the positive charge of the arginine moiety (Ikari *et al*., 2005, Yamada *et al*., 2005, Yamamoto *et al*., 2005). PADI4 is non-HLA genetic factors involved in RA by citrulline formation, which have been implicated in RA pathogenesis (Anzilotti 2010). Our results (table-16) showed significant association of PADI_102 C/T polymorphism in RA. These result were similar to the data of Somia (2012) who have reported the significant association between RA and control. 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) was also reported the significantly differences in frequencies of RA and control. In Asian population some variants of PADI4 genes have been reported in susceptibility of 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 several different populations, including Japanese, Korean and Chinese cohorts(Suzuki *et al*., 2003, Freudenberg *et al*., 2011, Fan *et al*., 2008). Potential explanation for the differences between different populations may be attributed to differences in the genetic variation in PADI4, or in gene-gene interaction or gene-environment

PTPN22 gene is negative regulator of signaling pathways of T and B cell receptor which encode the lymphoid protein tyrosine phosphatase (Lyp). (Cloutier & 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) Protein tyrosine phosphatase non receptor 22 (PTPN22) has recently been recognised 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). The 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) 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 locus of PTPN22 (rs3789607, rs12144309, rs3811021 and rs12566340) were genotyped and they found that was not associated with risk of RA. (Wan R. Wan Taib *et al.*, 2010) Independent of HLA, PTPN22 1858C>T gene polymorphism is best described genetic risk factor for RA. (Lee *et al.*, 2012) In central India a research finding revealed the association between PTPN22 polymorphism and RA while there was no association of Vitamin D receptor (VDR) polymorphism with RA susceptibility. (Shukla *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).

TIMP4

TIMP-4 is belongs to the TIMP gene family and located on 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 acids residues of C’ terminal, further three disulfide bonds provide conformational stability to the proteins. (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 & 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 (disintegrin and metalloproteinase with thrombospondin motifs). (Clark & Parker 2003) A previous study has reported that there were no significant differences of allele or genotypic frequencies of TIMP-4 C/T gene polymorphisms between RA and control. (Lee *et al*., 2008) An association study of TIMP-4 SNP provide no significant association to the development of schizophrenia and autism spectrum disorders (ASDs). (Yim *et al*., 2013).

Thus RA being an autoimmune disease is associated with disturbances in serum magnesium levels (Cortes *et al*., 2007). Inflammation is the primary cause for systemic alterations in the levels of metals and enzymes which is further modulating acute phase plasma proteins (Dean 2007). RA is associated with dyslipedemia 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 negative correlation between serum magnesium with TC, triglycerides, LDL-c and positive correlation with HDL-c. Thus lower serum magnesium may be associated with worsened lipid profile and increased CVD risk of RA patients. (Panoulas *et al*., 2007) reported increased serum uric acid is independently associated with CVD in RA patients but uric acid is lower in our patients as compared to controls.

Therefore RA is a complex disease which is associated with inflammation, dyslipedemia, hypomagnesia, higher levels of phosphorous, copper and zinc. Probably deregulated minerals are consequences of dyslipedemia and inflammation. We found lowed SGOT and SGPT and lower values for serum uric acid. Paanoulas *et al*., 2007 reported increased serum uric acid is independently associated with CVD in RA patients but uric acid is lower in our patients as compared to controls. Thus monitoring of kidney functions along with liver functions are recommended. Methotrexate is treatment of choice for management of RA in our patients.

NMR analysis of RA samples showed dyslipedemia and higher cittrulline 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 & Hárságyi, 1988).

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

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 peptidylarginine deiminase (Tarcsa E 1996). Because citrulline is a major antigenic determinant recognized in the RA. 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 S 2002). NMR analysis showed the abundances of citrulline and ornithine in the RA group than those in the controls.

6. SUMMARY AND CONCLUSIONS

Summary

Rheumatoid arthritis (RA) is an autoimmune, inflammatory disease which involves multiple synovial joints. RA causes significant disability and 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 found to be more prevalent in females as compared to males. Female to male ratio in RA is 3:1. The differences in over occurrence and development of worst and aggressive disease in females is not clear but genetic and hormonal factors are suggested to be involved.

Higher disease activity of RA is controlled by various treatments either given singly or in combination and include DMARDS, NSAIDS, glucocorticosteroids and biologics. However patients report differential response to the same therapy and side effects of these on liver and kidney are reported. RA also affects hemoglobin levels in affected individuals.

In RA, high inflammation is observed with high oxidative stress. Probably inflammatory and oxidative stressors together orchesterate the onset and progression of complex disease as RA. Immune cells and cytokines play an important role along with oxygen radicals as superoxide and hydrogen peroxide released by activated macrophages in the progression of rheumatoid arthritis. Oxidative stress is the condition when concentration of ROS and RNS becomes deleterious and damage the cells and biological macromolecules. Oxidative stress occurs due to disturbed balance between body's antioxidant mechanisms and oxidative stress production and has important role in the development of chronic disease as autoimmunity like RA, cancer etc. They are capable of damaging membrane lipids, connective tissue and nucleic acids of the cell. Free radicals and their byproducts are essential mediators of inflammation.

Synovial tissue is the main site which experiences very high activity during RA. Due to chemo-attractant property of synovial fluid, leukocytes accumulate with in the synovial tissue triggering a respiratory burst characterized by increased oxygen consumption and increased anaerobic glycolysis leading to generation of superoxide, hydroxyl, hypochloric radicals etc. Neutrophils have been shown to be very active in synovial fluid of patients with RA which leads to inflammation and damage. Studies show that enzymatic/non enzymatic antioxidant systems are highly deregulated and impaired in RA. Markers of protein and lipid oxidation have been found to be raised in arthritic animals. Therefore there are chances of free radical mediated damage to the body of RA patients due to their higher production or improper scavenging.

Epidemiological studies have shown an increased premature mortality in patients with RA compared with general population. Patients with RA have abnormal lipoprotein pattern, principally low levels of high density lipoprotein (HDL) and high levels of low density lipoprotein (LDL). The improvement in the lipoprotein profile in RA appears to be associated with suppression of inflammation. Dyslipidemia is often associated with normal or decreased LDL, HDL in a manner comparable to inflammatory and infectious diseases. Patients with systemic inflammation like Rheumatoid arthritis face a significantly increased risk of CVD compared with general population . Control of inflammation may have an effect on modifying cardiovascular risk.

There is little and conflicting data available for mineral content like Mg, Zn, Cu and P in RA patients. These are essential elements which are responsible for normal functioning of the body. However change in level of Mg, Zn and Cu have been implicated in pathogenesis of RA, a chronic inflammatory disease as they are the co-factor of important enzymes involved in collagen and bone metabolism, the antioxidant defense system and the immune system. In the previous studies, the development and progression of RA was suggested due to marginal deficiencies of Zn and Cu based on their serum levels. The alteration of these trace elements has been linked to inflammatory response.

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 which is product of lipid peroxidation and activities of enzymes of free radical scavenger system like superoxide dismutase (SOD), 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. These may help in determination of possible roles of these in damage to the 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 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 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. Patient‘s 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 test, 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.

In our study lipid per-oxidation in terms of MDA production was significantly increased in RA patients which may be due to increased ROS during chronic inflammation. Lipid peroxides are generated at the site of tissue injury due to inflammation and diffuses into blood and can be estimated in serum or plasma. There are reports of raised levels of MDA in the serum, plasma and erythrocytes of RA patients. In our study SOD activity were highly increased. Superoxides anion (O_2^-) has important role in pathogenesis of many diseases. It is neutralize by SOD to hydrogen peroxide (H_2O_2). H2O2 is further quenched by activity of catalase and glutathione peroxidase. The patients showed significantly higher activity of SOD and ALP. There was 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 level 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. Decreased Mg level is considered as marker for RA. The level of phosphorous and copper was non-significantly higher in male RA patients as ompared to females. In female RA patients, phosphorous showed inverse correlation with copper. There was strong association between elevated phosphorous and Ca and phosphorous products and the development of calciphylaxis. Both female and male RA patients had higher serum copper levels as compared to 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 level 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 and autoimmune injury. Our results are in accordance with other studies, suggesting that RA, is associated with serum magnesium disturbances. Mg is one of the essential nutrient of the body and studies suggest its role in reducing chronic inflammation. Decreased Mg level is considered as marker for RA. Magnesium is an activator of sodium potassium ATPase, is antiarrhythmic and is associated with cardio vascular disease susceptibility. Inflammation trigerrs its deficiency in animal models. In humans, low serum magnesium concentrations have been associated with high C-reactive protein (CRP) levels.

The patients show dyslipedemia 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 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 are being reported between serum magnesium with TC, triglycerides, LDL-c and positive correlation with HDL-c. Thus lower serum magnesium may be associated with worsened lipid profile and increased CVD risk of RA patients.

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

NMR study showed higher citrulline formation in RA which correlates well with 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 particularly hypomagnesium, dyslipedemia and lower hemoglobin. Magnesium supplementation and oxidative stress management may be considered important therapeutic option for RA along with DMARD.

Rheumatoid arthritis (RA) is chronic inflammatory autoimmune disease with unknown etiology. RA affects various symmetric joints of the body. The disease is more prevalent in female as compare to male. Women tend to have worst disease than male. The study was done on age and sex matched control and patients.

CONCLUSIONS

The final conclusions are

1. RA patient showed significantly lower haemoglobin 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 and SGOT and SGPT. As the patients are on methotrexate therapy supplemented with folic acid and vitamin C. The results show that the therapy with supplements is not worsening liver or kidney function in our patients.
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 suggesting their dependence to quench excessive free radicals in the body.

4. The serum level of zinc, copper and phosphorous where significantly elevated in RA patients as compared to control where as serum magnesium was significantly decreased in RA patients as compared to control.

5. RA patients showed dyslipedemia which may be due to ongoing process of inflammation and oxidative stress. Patient showed higher level of total cholesterol, very low density lipoprotein, triglycerides, low density lipoprotein and lower level of high density lipoprotein as compared to control. Dyslipedemia may increase their risk for arteriosclerosis and subsequent cardiovascular disease (CVD).

6. In our study peptidylarginine deiminase (PADI) alleles, RS 188_2 and PADI_102 showed some polymorphism in RA versus control. However allele occurrence was also seen in controls therefore it is difficult to predict their association with susceptibility to RA. Another gene PTPN-22 was found to the non-polymorphic in our population. However significant association of AG SNP of TIMP4 was observed in our patients.

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

Therefore our study shows that RA is more aggressive in females. Onset of RA in females is probably early as compared to males. RA patient had increased risk of oxidative stress, dyslipedemia, deranged minerals and higher inflammation as compared to controls. Their antioxidant enzymatic activities were compromised as compared to control. They showed some association with PADI (RS 188_2) and PADI_102 and TIMP4 (AG SNP). The serological changed were confirmed by NMR analysis. Probably including magnesium in therapy and inclusion of antioxidant along with regular medicine may help to control disease better than present treatments.

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8 LIST OF PUBLICATIONS

- **Kumar V**, Prakash J, Gupta V, Khan MY. Antioxidant Enzymes in Rheumatoid Arthritis. J Arthritis, 2016; 5:206doi: 10.4172/2167-7921.1000206.
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RESEARCH ARTICLE

SEX DIFFERENCES IN TRACE METALS AND DISEASE ACTIVITY IN PATIENTS WITH RHEUMATOID ARTHRITIS.

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Abstract

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Objectives: Rheumatoid arthritis (RA) is chronic autoimmune disease marked by tissue inflammation and joint destruction. Sex differences and essential element derangement in the incidences of RA are well described. **Methods:** The present study aims to determine serum mineral level, superoxide dismutase (SOD) activity and disease activity score (DAS) in female and male RA subjects and association of minerals with disease activity in both the sexes.

Results: Female RA patients had significantly reduced serum Zn as compared to male RA subjects. Females had non significantly low values for copper, magnesium and phosphorous as compared to male RA subjects. SOD and DAS were significantly increased in female RA subjects. The RA subjects had higher levels of serum zinc, copper and lower levels of serum Mg when compared with reference values. However none of them were found to be associated with disease activity.

Conclusions: The results suggest derangement of minerals in RA where mineral evaluation and supplementation especially of magnesium in RA patients would be helpful.

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Introduction:-

Rheumatoid arthritis (RA) is chronic inflammatory autoimmune disorder of unknown etiology affecting various symmetric joints of the body. Like other autoimmune diseases RA is also more prevalent in females as compared to males[1-4]. Female to male ratio in RA is 3:1[5]. The differences in over occurrence and development of aggressive disease in females is not clear but genetic and hormonal factors are suggested to be involved[6-11].

Several observational studies suggest that women with RA have worst disease as compared to men[12-16].

In recent studies RA seems to have derangement of mineral content like Mg, Zn, Cu and P. Their optimum concentration is required for normal functioning of the body. However alterations in level of these trace minerals as Mg[17], Zn[18] and Cu (Copper) have been implicated in pathogenesis of RA as they are the co-factor of important enzymes involved in collagen and bone metabolism, the antioxidant defense system[19] and the immune system[20]. The development and progression of RA was suggested due to marginal deficiencies of Zn and Cu based on their

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Research Article Open Access

Antioxidant Enzymes in Rheumatoid Arthritis

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Abstract

Joint destruction in rheumatoid arthritis (RA) is due to tissue injury in the area caused by inflammatory reactions, release of MMPs and free radicals produced by neutrophils and macrophages. The control of free radical production may have therapeutic roles thus the study was done to check the status of lipid peroxidation product malondialdehyde (MDA) and a few antioxidant enzymes in RA patients. 45 RA patients and 40 controls were selected. Controls were asymptomatic and RA patients were selected according to ACR criteria. RA patients had significantly high MDA, SOD and ALP and reduced activity of catalase and GR as compared to controls. SOD showed positive correlation with ALP. GR was positively related with MDA, SOD and ALP. The study shows that MDA is involved in the pathogenesis of RA. The system is trying to quench free radicals by high SOD activity. Higher production of H_2O_2 or some other mechanism is responsible for inhibition of catalase and GR. However system is trying to reduce the damage by neutralizing superoxide anion. Therapeutic intervention of the oxidative stress may be considered for effective control of inflammation in RA patients.

Keywords: Malondialdehyde; Superoxide anion; Superoxide dismutase; Catalase; Glutathione S-transferase

Abbreviations

RA: Rheumatoid Arthritis; MDA: Malondialdehyde; SOD: Superoxide Dismutase; GST: Glutathione-S-Transferase

Introduction

Rheumatoid arthritis (RA) is the inflammatory disease which leads to progressive destruction of multiple synovial joints [1]. T-cells and cytokines play an important role along with oxygen radicals as superoxide and hydrogen peroxide released by activated macrophages in the progression of rheumatoid arthritis [2]. These reactive oxygen species (ROS) and reactive nitrogen species (RNS) which are thus produced have both beneficial and toxic effects. Oxidative stress is the condition when concentration of ROS and RNS becomes deleterious and damage the cells and biological macromolecules [3,4] and thus the body. Oxidative stress occurs due to disturbed balance between body's antioxidant mechanisms and oxidative stress production and has important role in the development of chronic disease as autoimmunity like RA, cancer etc. [5-7].

They are capable of damaging membrane lipids, connective tissue and nucleic acids of the cell. Free radicals and their byproducts are essential mediators of inflammation. Due to chemo-attractant property of synovial fluid, leukocytes accumulate with in the synovial tissue triggering a respiratory burst characterized by increased oxygen consumption and increased anaerobic glycolysis leading to generation of superoxide, hydroxyl, hypochloric radicals etc. [8]. Neutrophils have been shown to be very active in synovial fluid of patients with RA which leads to inflammation and damage [9,10].

In the body free radical generation and enzymes degrading them are in tight homeostasis which prevents damage. However, studies [11,12] show that enzymatic/non enzymatic antioxidant systems are highly deregulated and impaired in RA. Markers of protein and lipid oxidation have been found to be raised in arthritic animals. Therefore there are chances of free radical mediated damage to the body of RA patients due to their higher production or improper scavenging. Thus analysis of activities of different antioxidant enzymes like superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px) and glutathione reductase (GR) may have effective therapeutic potential [11,13,14]. There is more interest in roles of these in the clinical outcomes of disease like RA, therefore, we were interested in analyzing the level of MDA which is product of lipid peroxidation and level of enzymes of free radical scavenger system like superoxide dismutase (SOD), GR, catalase and levels of alkaline phosphatase (ALP) in RA patients treated with MTX, Folic acid Vit-C and occasional corticosteroids.

Materials and Methods

45 samples (30 females, 15 males) were randomly selected from the OPD of Orthopaedics from different centers during the study period. Patients were recruited who fulfilled 4 or above criteria of American College of Rheumatology (ACR) [15]. Out of 45 patients with RA, 38 were tested positive for RF factor and anti-cyclic citrullinated protein (CCP) antibody. 40 asymptomatic independent controls (24 females, 16 males) were recruited from local clubs, neighbourhood and volunteers. Controls were asymptomatic (painless, no cripitation, no decrease of joint space on X-ray, nonobese and without any other systemic disease) and independent of the patients.

Patients were recommended MTX (15 mg once a week) along with folic acid (1 mg OD) and vitamin C to alleviate symptoms. Whenever