2. REVIEW OF LITERATURE

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 indicates flowing, and is mentioned in an 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 warm 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 in 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 autoimmune 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 lowers 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 anti-rheumatic 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 sequentially in patients for improvements (Ernest 2010).

Different cellular and cytokine targets have been identified in RA which 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 targets 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.

![Normal joint](image1)

**Figure: 2A Normal joint**

![Inflamed joint](image2)

**Figure: 2B Inflamed 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 lining 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, dendritic 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-1 and 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 proteoglycan 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 components, 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 architecture 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 al.*, 2005).

![Diagram of the pathophysiology of RA](image)

**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 considered 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)

\[
\text{DAS 28= 0.56} \times \sqrt{\text{Number of tender joints}} + 0.28 \times \sqrt{\text{Number of swollen joints}} + 0.7 \times \ln(\text{ESR: 1hour}) + 0.014 \times \text{VAS.}
\]
Box-2 : For the analysis of swollen and tender joints for DAS 28

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<tr>
<th>Joints</th>
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<tr>
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<td>Swollen</td>
<td>Tender</td>
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<td>Shoulder</td>
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<td>Elbow</td>
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<td>Wrist</td>
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<td>Metacarpophalangeal Joints (MCP)</td>
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<td>Proximal Interphalangeal Joints (PIP)</td>
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<td>Knee</td>
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<td>Total</td>
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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).
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 components have the potential to damage biomolecules such as lipids, DNA and proteins in the affected 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$_2^-$) is the primary product that is liberated into extracellular matrix as well as sequestered in lysosomes. Superoxide is then converted into hydrogen peroxide (H$_2$O$_2$) either spontaneously or catalytically by the catalase or glutathione reductase.

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 (Westermark 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$_2^-$) into oxygen and hydrogen peroxide which makes SOD as a potentially useful therapeutic agent for treatment of inflammatory disorders as RA (Yasui & Baaba 2006).

[27]
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 al., 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 erythematosus, 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_2 and 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 shown 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 1 to 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, strontium and boron. As the changes in the concentration of trace elements has been linked to inflammatory response therefore the present 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.