Genetic Variations and Epigenetic Modifications of TNF-α in Rheumatoid Arthritis

By

Naziha Khalid

(NUST201361706MASAB92513F)

Atta-ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences and Technology (NUST)

Islamabad, Pakistan

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A thesis submitted in partial fulfillment of the requirement for the degree of Masters of Science

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Naziha Khalid

(NUST201361706MASAB92513F)

Supervisor: Dr Peter John

Atta-ur-Rehman School of Applied Biosciences (ASAB)

National University of Science and Technology (NUST)

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DEDICATION

I dedicate this work to my late grandfather and my beloved parents whose prayers have made this possible.

Acknowledgement

I would like to pay my highest respect to the omnipotent creator of the universe. **Allah Almighty,** Lord of the worlds, the Most Beneficial and the Most Merciful. Without His help, blessings, mercy, and forgiveness, I would have never been able to achieve any success in the research work. With the greatest humility I would like to thank Allah for answering my prayers and helping throughout my life.

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Naziha Khalid

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List of Abbreviations

ACPA	Anti-citrullinated protein antibodies
ACR	American College of Rheumatology
ASAB, NUST	Atta-ur-Rehman School of Applied Biosciences, National University of Science and Technology
bFGF	Basic fibroblast growth factor
BLAST	Basic Local Alignment Search Tool
CNS	Central nervous system
CRP	C-reactive protein
DMARDs	Disease-modifying anti-rheumatic drugs
DNA	Deoxyribonucleic Acid
dNTPs	Nucleotide trisphosphates
DR3	Death receptor 3
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
ESR	Erthrocyte sedimentation rate
EtBr	Ethidium Bromide
FADD	Fas-associated protein with death domain
FLS	Fibroblast like synoviocytes
GM-CSF	Granulocyte-macrophage colony stimulating factor
HLA	Human Leukocyte Antigen
Ig	Immunoglobulin
IGL	Immunogenetics Lab
IL	Interleukin
$MgCl_2$	Magnesium Chloride
MHC	Major Histocompatibility Complex

MSP	Methylation specific primer
NaCl	Sodium Chloride
NCBI	The National Center for Biotechnology Information
OA	Osteoarthritis
OASF	Osteoarthritis synovial fibroblasts
PAD	Peptidylarginine deiminases
PADI4	Peptidyl Arginine Deiminase, Type IV
PCR	Polymerase Chain Reaction
PDGF	Platelet derived growth factor
PGE2	Prostaglandin E2
RA	Rheumatoid Arthritis
RANK	Receptor Activator of nuclear factor kappa-B
RANKL	Receptor Activator of nuclear factor kappa-B ligand
RASF	Rheumatoid arthritis synovial fibroblasts
RF	Rheumatoid Factor
RFLP	Restriction Fragment Length Polymorphism
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulphate
SNP	Single Nucleotide Polymorphism
TACE	TNF-α converting enzyme
TAE Buffer	Tris–Acetic Acid–EDTA Buffer
TE Buffer	Tris- EDTA Buffer
TGF-β	Transforming growth factor-β
Th cells	T-helper cells
TNFR	Tumor necrosis factor receptor
TNFα	Tumor Necrosis Factor α

TRADD	Tumor necrosis factor receptor type 1-associated death domain
TRAF-2	TNFR-associated factor-2
WBCs	White Blood Cells

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Abstract

Background: Preceding studies have shown positive association of TNF- α polymorphism has been found in Caucasian population for RA. Similarly studies have been done to find association in inflammatory bowel disease in Caucasian population and osteoarthritis in Chinese population which has come up to be negative. Age related loss in methylation pattern has been observed in TNF promoter region while other studies have shown varied methylation pattern of genes including DR3, IL6, IL10 in rheumatoid arthritis.

Methods: In this study, two polymorphisms (rs361525 and rs1800750) of TNFα located in the promoter region were studied in Pakistani population as case-control study through restriction fragment length-polymerase chain reaction (RFLP-PCR). Methylation status of TNF-α promoter was also investigated using Methylation specific and Un-methylation specific PCR after bisulfite conversion of genomic DNA.

Results: The results showed that TNFα SNP rs361525 genotype had no association with disease susceptibility while significant association was found in the SNP rs1800750, the allele A was found to have a protective role in disease. Results showed statistically significant association of methylation status of the promoter to RA. Additional studies with larger and diverse populations are required to confirm current findings.

Keywords: rs361525, rs1800750, RFLP-PCR, Rheumatoid arthritis, methylation specific PCR, TNFα.

CHAPTER 1

INTRODUCTION

Rheumatoid arthritis is a fairly common autoimmune disease that has far reaching implications on the health of the individual and socioeconomic costs. As the disease progresses it is associated with systemic complications, progressive disability, and early death (McInnes *et al.,* 2011). The treatment available is very aggressive but lacks predictive biomarkers. Understanding the underlying mechanisms that cause pathogenesis and progression is a promising avenue for developing effective treatment strategy. It is a multi-factorial disease caused by interplay between genetics and environmental factors. Characteristic features include synovial inflammation, self-destruction of cells via production of autoantibodies (rheumatoid factor and anti-citrullinated protein antibody [ACPA]), bone and cartilage destruction and various other systemic complications (McInnis *et al.,* 2011).

1.1 Disease Pathology

Rheumatoid arthritis can be best described as an amalgam of various disease conditions (van der Helm-van Mil *et al.,* 2008), several of which include inflammatory cascades ultimately leading to persistent inflammation and damage to cartilage and bone.

One of the key inflammatory cascades includes increased production and expression of tumor necrosis factor (Feldmann *et al.,.,* 1996). Normally TNF is correlated with cellular responses including apoptosis, cell survival and inflammation (Keystone *et al.,* 2010).

This pathway brings about both synovial inflammation and joint destruction. Over-production of TNF can be ascribed to a number of causes, including interactions between T and B lymphocytes, synovial-like fibroblasts, and macrophages (Scott *et al.,* 2010).

1.2 Role of synovial and cartilage cells in RA development

The prevailing cell populations in diseased joints are synovial and cartilage cells. Synovial cells can further be divided into fibroblast-like and macrophage-like synoviocytes. Macrophage-like synoviocytes drive the overproduction of pro-inflammatory cytokines. It has been seen that joint destruction can be related to fibroblasts invading cartilage (Tolboom *et al.,* 2005). Furthermore substantial information has been collected about joint destruction and the role of osteoclast activation as a key process leading to bone erosion. Synovial fibroblasts are chiefly found in the synovial sub-lining of joints which is multilayered and hyperplastic in this disease. Activated RA-SFs show abnormally aggressive and invasive behavior that is characteristic of metastatic cancer cells. They also have distinctive morphological features such as round shape and large pale nuclei with prominent nucleoli (Karouzakis *et al.,* 2006). However one the most characteristic functional feature of RA-SF is its ability to adhere to cartilage and to initiate the degradation of extracellular matrix (ECM). The major pathological changes in the synovial tissue in RA development are shown in Figure 1.1.

Synovial hyperplasia could be theorized to be due to an increased rate of proliferation of the RA-SF. This hypothesis is supported by the increased expression of transcription factors, markers of proliferation and growth factors, including platelet derived growth

factor (PDGF), basic fibroblast growth factor (bFGF) and transforming growth factor-β (TGFβ) (Qu *et al.*,. 1994, Allen *et al.,* 1990).

Osteoclasts are the main cause of bone destruction. In the synovial fluid of RA patients, large amounts of RANKL have been detected. RANKL, in combination with GM-CSF, promotes the differentiation of osteoblasts and monocyte progenitor cells into osteoclasts (Gravallese *et al.,* 2000). This is established by specific inhibition of osteoclast activation can reduce joint destruction yet not affect joint inflammation (Cohen *et al.,* 2008).

There is ambiguity concerning the start site of arthritis whether it initiates in the bone and moves to the joints or vice versa (Schett *et al.,* 2010).

1.3 Autoantibodies in RA

Rheumatoid factors (RF) are autoantibodies directed against the Fc portion of IgG. They can be found in 75-80% of RA patients at some time during the course of their disease. In relation to chronic polyarthritis high-titre of IgM RF is fairly specific for the diagnosis of RA. However the presence of IgM RF has little predictive value since the presence may antedate the clinical development of RA (Nielsen *et al.,* 2012). There is convincing evidence that in particular genetic context, smoking is a potential trigger for rheumatoid arthritis (Taylor., 2014). In addition, when these two factors occur together, they are associated with an autoantibody response directed against highly citrullinated peptides that may considerably antedate the onset of clinical features of RA. Citrulline is derived from the amino acid arginine after peptide translation under the influence of the enzyme peptidyl arginine deiminase (PADI4). Identifying the citrullinated target RA antigen(s) is

of importance for understanding aetiopathogenesis. Proposed candidates include citrullinated fibrinogen, citrullinated vimentin, citrullinated a-enolase and citrullinated type II collagen (Taylor., 2014). Testing for anti-citrullinated peptide antibodies (ACPA) has become common in the evaluation of patients for RA. Since ACPA is now recognized as a component of the new criteria for diagnosis of RA (Aletaha *et al.*, 2010) measurement of IgM RF might be considered to be redundant.

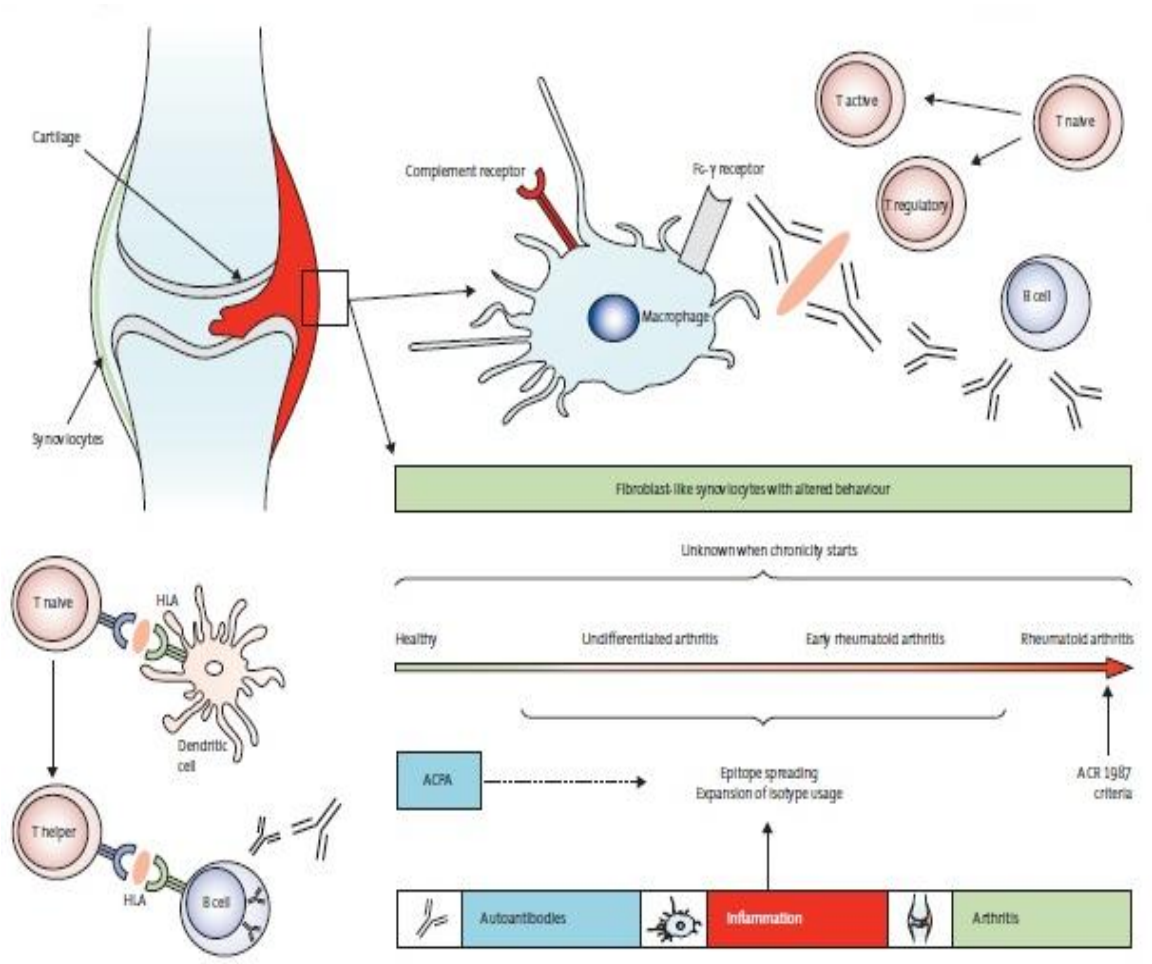

Figure 1.1: Pathological changes in synovium in RA. The joint comprised of two bones protected by cartilage and aligned by a capsule. The inner surface of the capsule is made of fibroblast-like synoviocytes that produce synovial fluid. In a joint affected by RA, the synovium is swollen due to an infiltrate comprising of fibroblast-like and macrophage-like synoviocytes, macrophages, several populations of T cells, and B cells. Macrophages are activated to produce all kind of pro-inflammatory products (eg, tumour necrosis factor) partly by immune complexes binding to Fc-γ receptors and complement receptors on their surface (Scott *et al.*, 2010).

1.4 Diagnosis criteria

An early diagnosis can ensure a better chance at disease management. Since the initial symptoms of disease are relatively non-specific it becomes difficult to diagnose at early stage. These symptoms include weakness, sudden weight loss, low grade fever, fatigue (Chen *et al.,* 2006). Specific criteria have been set by the American College of Rheumatology which is used for diagnosis and classification (Aletaha *et al.,* 2010). These include:

- Morning stiffness in affected joints
- Arthritis of 3 or more joint areas
- Arthritis of hand joints
- Symmetric arthritis
- Rheumatoid nodules
- Rheumatoid factor (RF)
- Radiographic changes

The diagnosis of rheumatoid arthritis is mainly clinical. The typical presentation is poly-articular, with pain, stiffness, and swelling of multiple joints in a bilateral, symmetric pattern (Majithia *et al.,* 2007) while some patients may present oligo-articular involvment. Disease onset coincides with joint pains spanning weeks or months often in conjunction with anorexia, weakness and fatigue.

The commonly involved joints are wrists, and proximal interphalangeal, metacarpophalangeal, and metatarsophalangeal joints. The distal interphalangeal joints

and spinal joints are usually not affected (Harris., 2005). Upon examination common findings include swelling, tenderness and warmth of, with atrophy of muscles near the involved joints (Majithia *et al.,* 2007).

There is no one single confirmatory test for rheumatoid arthritis. Preliminary laboratory tests should include a complete blood cell count with differential, rheumatoid factor, and erythrocyte sedimentation rate or C-reactive protein (Majithia *et al.,* 2007). For patients with mono-articular presentation of disease joint aspiration may be required to rule out infectious arthritis.

Increased level of erythrocyte sedimentation rate (ESR) and serum C reactive protein (CRP) associates with disease activity in RA (Van-Leeuwen *et al.,* 1993). Nevertheless, fewer joints related to disease might present a low/normal acute-phase response in spite of significant inflammation. Positive serology for rheumatoid factor (RF) and anti-citrullinated peptide antibodies (ACPA) in early inflammatory arthritis can help in determination of patients most likely to benefit from early aggressive therapy (Jeffery., 2014). RF can help with diagnosis, but it is more prognostic in nature in early inflammatory arthritis, helping in the prediction of the possibility of other more severe disease and extra-articular features. ACPA on the other hand offers a more specific test for early RA with a specificity of 94-97% and a sensitivity of 62-72% (Nishimura *et al.,* 2007). ACPA is specific to RA, often appearing before RF and may be present even before clinical disease develops. It is more diagnostic in nature than RF and has strong predictive value for more severe disease and poorer prognosis (Syversen *et al.,* 2008). Almost 30% of RA patients are anti-nuclear antibody positive without evidence of

connective tissue disease therefore it is important to consider alternative diagnosis for example joint fluid analysis may be helpful in excluding crystal arthropathy and infection (Jefferey., 2014).

Conventional radiographs play a partial role in the diagnosis of early RA. The earliest stage of inflammation can be seen on radiographs of soft-tissue swelling and periarticular osteopenia. Later changes include cortical irregularity, established erosions (Machold *et al.,* 2007), loss of joint space and joint malalignments. Images are usually more useful after 6 weeks of joint symptoms. Erosions are hard to detect and can be taken as a severity marker, they are mostly subjected to be seen on hands and feet where they occur earliest at the MCPs, MTPs, PIPs and ulnar styloid (Lindqvist *et al.,* 2003).

A baseline chest X-ray can be considered before beginning of DMARD therapy. USS and MRI are typically used to detect soft tissue inflammation and visualize the presence of erosions at an earlier stage, and may detect inflammatory changes that are not clinically apparent (Keen *et al.,* 2005).

1.5 Role of cytokines in RA

Cytokines act as molecular messengers between cells and there are numerous studies revealing that numbers of cytokines have been involved in the pathogenesis of RA by triggering or regulating the inflammatory responses (Burska *et al.,* 2014). An extensive range of pro-inflammatory cytokines can be identified to have an impact in RA synovial samples, despite differences in disease duration, severity or drug therapy. Cytokine dysequilibrium is observed within the chronic inflammatory situation in rheumatoid

synovium showing that multiple anti-inflammatory mediators are also up-regulated, but the levels are insufficient to suppress synovitis (Taylor., 2010) the dysequilibrium is shown in Figure 1.2.

Over-expression of certain cytokines, such as IL-1, IL-6, IL-8, IL-17, IL-21, tumor necrosis factor (TNF)-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been studied in RA patients (Jorgensen *et al.,* 2008, Niu *et al.*, 2010, Niu *et al.,* 2011, Gradaigh *et al.,* 2004, Maini *et al.,* 2000). These cytokines could promote synovial membrane inflammation and osteocartilaginous resorption via stimulation of osteoclastic mediators (Chen., 2010)

Autoreactive T cells, such as Th1 and Th17 cells, are thought to play important roles in autoimmune pathology of RA (Dong *et al.,* 2000, Harrington *et al.*, 2005, Park *et al.,* 2005). IL-12, a Th1 cytokine, was increased in serum and synovial fluid in RA and its level correlated with disease activity score (Petrovic-Rackov *et al.,* 2006). The discovery of Th17 cells enriched our knowledge and understanding of RA pathogenesis. Th17 profile therefore has shown pathogenic role in RA (Qu *et al.,* 2013), the same study also reported increased IL-17 in the sera and synovial fluid of RA patients.

IL-21 and IL-22 were also observed at a high level in RA patients compared to the healthy controls or osteoarthritis (OA) patients (Ikeuchi *et al.,* 2005). Both IL-23 and IL-17 have been reported to promote each other's production and have been detected in RA synovial fibroblasts, the various pathways implicated in production include PI3-kinase/Akt-, NF- κB-, and p38-MAPK-mediated pathways (Aggarwal *et al.,* 2003, Kim

et al., 2007). RANK/RNAKL signaling controls the differentiation of multinucleated osteoclasts from their precursors along with their activation and survival in normal bone remodeling and in several pathological conditions (Boyce *et al.,* 2007). In RA synovial tissue, p19 subunit and RANKL have been reported to have positive correlation and might contribute to bone destruction in RA (Sato *et al.,* 2006, Pope *et al.,* 2013).

IL-1 is produced by an assortment of cells that are part of the innate system and mediates bone resorption and cartilage destruction. The IL-1β-NF-κB axis is central in the production of pro-inflammatory mediators in the inflamed synovium (Schiff., 2000, Lee *et al.,* 2009).

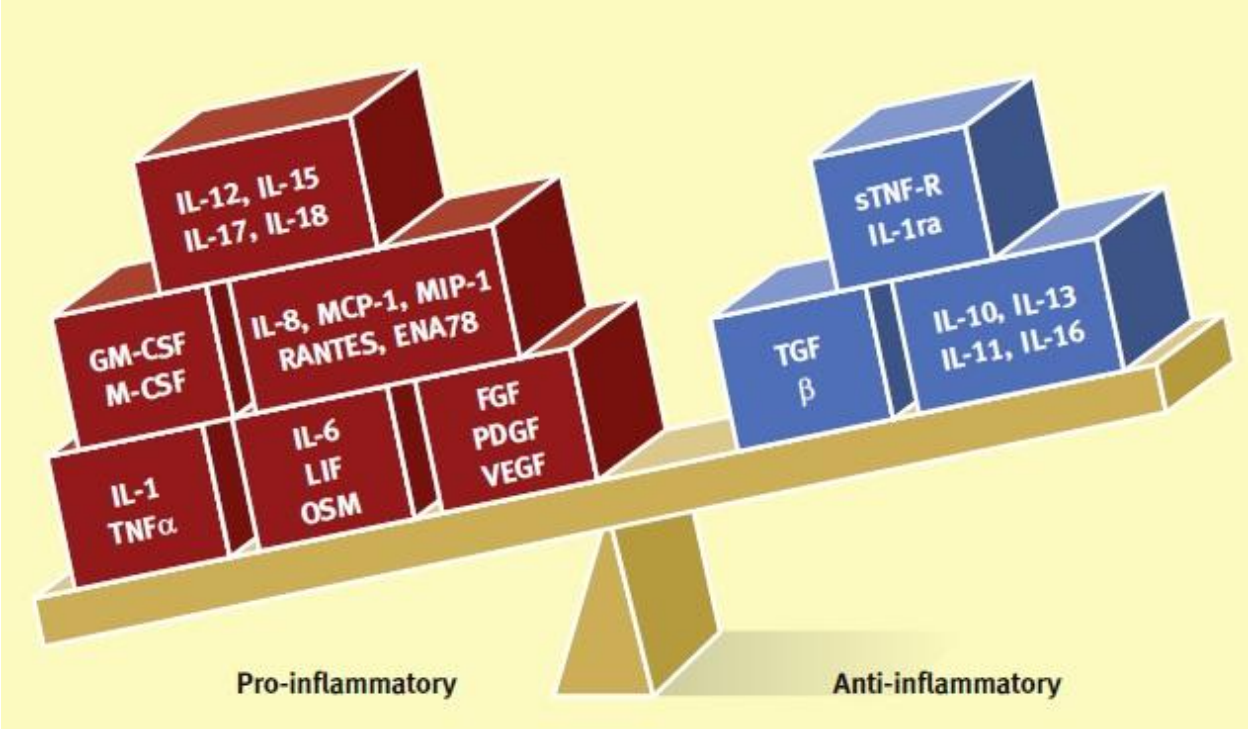

Figure 1.2: Cytokine Disequilbrium

Additionally, pro-inflammatory cytokine, IL-6, has important effects on the differentiation and activation of B and T cells, macrophages, osteoclasts, chondrocytes, and endothelial cells and broad effects on hematopoiesis in the bone marrow (Chen., 2010). IL-6 level has been significantly associated with clinical symptoms (Wang., 2013).

Other cytokines/chemokines, such as IL-8, IL-9, IL-15, leukemia inhibitory factor (LIF), granulocyte colony-stimulating factor (G-CSF), chemo-attractant protein-1 (MCP-1), growth-related oncogenes GRO-α and GRO-β, CCL19, CXCL12, and CXCL13, were up-regulated in preclinical or clinical period of RA development by induction of IL-17 or TNF-α and IL-1 synergically (Hughes-Austin *et al.,* 2013, Deane *et al.,* 2010, Koenders *et al.,* 2006, Sellam *et al.,* 2013).

1.6 Tumor necrosis factor α and its role in Disease Pathogenesis

TNF-α has shown to be involved in the pathogenesis of RA at early stage. It is produced locally in the joint by synovial macrophages and lymphocytes infiltrating the joint synovium (Niu *et al.,* 2014). TNF has been documented as a key pathogenic cytokine that drives a pathogenic cytokine milieu leading to tissue damage (Choy *et al.,* 2001).

It is a trimeric protein comprised of three 157 amino acid subunits, each 17 kDa. Studies have shown the important role of TNF-α in RA by its ability to degrade cartilage and bone (Jia *et al.,* 2013). Activated macrophages in the inflamed synovial membrane tissue produce TNF-α and this is up-regulated in sera and synovial fluid. Macrophages are triggered by signals from Th cells and by immune complexes (Brennan *et al.,* 2008).

TNF-α controls the secretion of other pro-inflammatory cytokines including IL-1, IL-6, IL-8 and GM-CSF. Furthermore, TNF-α promotes the recruitment of leukocytes into the joint through up-regulation of cell adhesion molecules (Bingham *et al.,* 2002). Over-expression of TNF-α also stimulates the development of osteoclasts and the activation of metalloproteinases in the nearby articular cartilage, leading to tissue destruction (Lubberts *et al.,* 2003). Even though there is no clear evidence of a direct TNF cytotoxic effect on the synovial cells, the possibility cannot be rejected as part of the deleterious processes involved in RA physiopathology (Aguillon *et al.,* 2006).

TNFα has been shown to play a vital role in fighting infections in animal models (Parks *et al.,* 2011). TNFα is a type II transmembrane protein, cleaved by TNF-α converting enzyme (TACE) to a soluble form. It acts as a ligand for two receptors, TNFR1 and TNFR2, to enable transduction of anti-apoptotic, pro-inflammatory signals (Fuggle *et al.,* 2014). TNF-α mediated functions include phagosome maturation, autophagy inhibition and apoptosis following activation of caspase 8 by TNFR1 (Harris and Keane., 2010).

Three major observations have been integral for determining the role of TNF-α in RA pathogenesis (Feldmann *et al.,* 2001), these include:

1. High concentrations of TNF in plasma, synovial fluid, and tissue from RA patients (Vinasco *et al.,* 1997; Cuenca *et al.,* 2003);
2. Mouse animal model that have been engineered to over-express human TNF gene have been shown to develop polyarthritis similar to RA (Keffer *et al.,* 1991),

while administering human TNF monoclonal antibodies, prevents articular lesions and diminish the incidence of murine arthritis (Williams *et al.,* 1992), and

3. Clinical benefits shown in RA patients that have been treated with chimeric as well as human anti-TNF monoclonal antibody (Elliot *et al.,* 1993, Kempeni. 1999) or with soluble TNF receptors (Weinblatt *et al.,* 1999; Moreland *et al.,* 1997)

CHAPTER 2

LITERATURE REVIEW

2.1 Epidemiology and Prevalence of Rheumatoid Arthritis

RA is the disease of persistent inflammation affecting the synovium, leading to joint damage and bone destruction causing disability and increases mortality (Alamanos *et al.*, 2005). Previous studies relating incidence and prevalence of RA have been shown to suggest variance in disease occurrence in different populations. Each study based on RA epidemiology has certain methodological differences, which may be due to the variation in case identification, case recording and prevalence rates, therefore the criteria that may have existed for one study isn't applicable for another. These constraints may limit the comparison of descriptive studies and their interpretation.

Other likely explanation for disease variance in populations might include regional disparity in behavioural factors, climate, environmental exposures, RA diagnosis and genetic factors (Costenbader *et al.,* 2008; Gilbert *et al.,* 2003). It has been stated that RA is less recurrent in some regions than others, and more frequent reports of prevalence is found in US and UK based studies (Carmona *et al.,* 2010). A greater part of prevalence studies carried out in Northern European and North American areas estimate a prevalence of 0.5– 1.1% (Aho *et al.,* 1998) while studies from Southern European areas have reported a prevalence of 0.3–0.7% (Drosos *et al.,* 1997). The developing countries report comparatively lower disease prevalence somewhere between 0.1 and 0.5% (Spindler *et al.,* 2002; Akar *et al.,* 2004; Lau *et al.,* 1993; Dai *et al.,* 2003; Senna *et al.,* 2003).

Incidence rates of RA vary between 20 and 50 cases per 100,000 inhabitants in North American and North European countries annually (Riise *et al.,* 2001; Aho *et al.,* 1999; Doran *et al.,* 2002; Soderlin *et al.,* 2002). Little is known about incidence rate in Southern Europe areas and almost no studies are based in developing countries. The prevalence rates of RA in the different regions of the world are shown in Table 1 (Carmona *et al.,* 2010).

Table 2.1: Prevalence rate of RA in various countries

S.No	Country	Total Prevalence %	Male	Female
1	Japan	0.20	0.14	0.25
2	China	0.20-0.34	0.76	2.23
3	India	0.19-2.50	0.19	1.24
4	Pakistan	0.14	1.08	2.86
5	Bangladesh		0.0-0.20	0.56-1.20
6	Indonesia	0.20-0.30		
7	Spain	0.20-2.70	0.20	0.80
8	Ireland	5.00		
9	United Kingdom	0.30	0.02-2.18	0.12-2.99
10	Saudi Arabia		0.19	0.25
11	Iran		0.10	0.55
12	Kuwait	1.26		
13	USA(incl. Alaskans)	0.73	0.43-1.52	3.79

There is comparatively limited data available on changing trends in the incidence and prevalence of RA over time. Some studies from North American, North European, and Japanese populations propose a declining trend in prevalence and the incidence of the disease after the 1960s but this can be attributed to the methodological issues which have since been revised (Aho *et al.,* 1998; Doran *et al.,* 2002; Shichikawa *et al.,* 1999).

As general rule of thumb mortality rates are higher among RA patients than in the general population but they vary widely among studies. They are higher in hospital-based studies and comparatively lower in population based studies (Alamanos *et al.,* 2005). The expected survival of RA patients is most likely to decrease by 3–10 years based on the severity of the disease and the age of disease onset. However the causes of death in diseased condition don't generally differ from general population (Liao *et al.,* 2011; Gabriel *et al.,* 2003).

2.2 Factors affecting Disease Progression

Molecular explanation for rheumatoid arthritis are emerging with help of the studies carried out on animal models of inflammation, which shows linkage between the hypothalamic–pituitary–adrenal axis and cytokine production (Capellino *et al.,* 2010). Normally the central nervous system (CNS) regulates immunological responses and brings about homeostasis, however in diseased conditions these interactions regulate development of disease either locally via neurotransmitters or centrally via cytokines. Utilizing these observations to successful treatment options in rheumatoid arthritis has however challenging.

Autoantibodies, such as rheumatoid factor and ACPA, are often used as diagnostic markers in patients before the development of arthritis. It is plausible that biological features of the targeted autoantigen may also contribute to disease devlopment. Other possible factors include local microvascular, neurologic, bio-mechanical, and microtrauma-related mechanisms (McInnes *et al.,* 2011). Considering the multi-factorial

nature of disease susceptible genes and disrupted signaling pathways are not the only factors contributing to pathogenesis, environmental aspects and epigenetic regulation need also be considered, Figure 2.1 provides a schematic diagram for disease pathogenesis.

2.3 Genetics involved in RA Development

MHC (major histocompatibility complex) has been the region consistently shown to associate with RA pathogenesis. It is localized on chromosome 6 (6p21.3), containing approximately 220 genes, and is known to have immune function in about 40% of the genes. The MHC is divided into three regions, class I, II and III. It is the human leukocyte antigens (HLA) DR, DP and DQ situated in the class II region and these antigens that are known to play a role in RA.

It has been hypothesised that an arthritogenic peptide might bind to the RA-susceptible HLA_DR molecules and cause pathological response however such peptides have yet to be identified (Harney *et al.,* 2003).

In a study on Thai RA patients by sequencing the DRB 1, 3, 4 and 5 it was shown DRB1 *01:01, *04:01, *04:05 and *10:01 had higher allele frequency than healthy controls while the allele frequency of DRB3 *03:01 was lower in patients.

Sequencing of the HLA-DRB1 showed that the subtype DR4 varies in incidence in population, giving rise to the shared epitope hypothesis (Gregersen *et al.,* 1987), which states that a conserved epitope spanning 70-74 amino acid residues in the β chain of DR

molecule. The alleles cannot be discriminated by restriction fragment length polymorphism which suggests that susceptibility is conferred by polymorphism rather than linked gene (Harney *et al.,* 2003)

Zanelli *et al.,* (2000) have implicated HLA-DQ in RA pathogenesis, suggesting that HLA-DQ3 and DQ5 are positively associated with RA while DRB1 alleles confer protection. This is an alternate theory to the shared epitope hypothesis called the RA-protection hypothesis (Huizinga *et al.,* 2003).

Recent study by Luckey *et al.,* (2014) suggest that DRB1 *0401 and *0402 molecules can produce cytokines however the conditions to produce pro-inflammatory cytokines may vary. The results suggest that DRB1 *0402 molecule can't entirely be dismissed as to not being associated with RA.

Presence of anti-citrullinated protein antibodies (ACPA) is a classification criterion for the determination of RA. It was seen that HLA-DRB1 *0404 was associated with anti-citrullinated fibrinogen (Auger *et al.,* 2005). It is suggested that DR molecules that are encoded by shared epitope alleles are implicated in the presentation of citrullinated peptides to T cells (Kochi *et al.,* 2014).

2.4 Environmental Factors affecting Disease Pathogenesis

There is need to able to accurately predict the course of disease once it has been identified and determine how it will progress. Various predictors of prognosis have been

identified that can be effectively divided into genetic and non-genetic (environmental) categories.

The environmental determinants of disease can be further divided into other categories e.g. diet, smoking, age, hormonal factors etc. Conventionally RA can be divided into older onset and younger onset depending upon the age the patient develops the disease. Cross-sectional studies have shown that older patients have a better outcome suggesting that the treatment is less aggressive (Symmons., 2003). While another study by (Young., 1995) suggest the opposite i.e. the younger patients are more likely to enter spontaneous remission. The same study also suggests that men enter spontaneous remission more likely than women. RA is more common in women than in males the ratio being 3:1 (Tobón, Youinou *et al.,* 2010). (Mikuls, Saag *et al.,* 2002) suggest that excess mortality in women patients maybe limited to those who are seropositive.

2.4.1 Smoking Associated with Higher Risk

Numerous studies have established higher risk of RA associated with cigarette smoking, especially in patients that are rheumatoid factor (RF) or anti-citrullinated protein antibody (ACPA) positive (Hoovestol *et al.,* 2011). The risk is greater in men as compared to women shown by epidemiological studies (Sugiyama *et al.,* 2010). The same epidemiological study also showed that the risk to develop disease is approximately 1.3-times higher in smokers than for non-smokers in females. Another study showed similar results stating that smoking increases risk of developing RA in men as compared to women and the effect was seen in RF positive RA patients (Krishnan *et al.,* 2003).

Another epidemiological study shows an increasing risk of developing RA in heavy smokers (Stolt *et al.,* 2003), while a recent meta-analysis to summarize the association of lifelong exposure to smoking suggests that even light smoking can be linked to RA (Di Giuseppe *et al.,* 2014).

2.4.2 Antioxidants: Protective role in RA

Oxidative stress is known to promote inflammatory condition thereby causing the production of excess reactive oxygen species (ROS) generating a continuous cycle of oxidation, inflammation. Studies have been known to suggest that oxidative stress is elevated in RA patients compared to those of the control subjects (Sung *et al.,* 2013). The likely causes of increased oxidative stress in RA are not clear due to methodological limitations of cross-sectional case–control studies.

Studies have discussed possible role of dietary antioxidants intake in the development of the disease. Reduced intake of antioxidant nutrients by RA patients may add to the disease development. The antioxidant nutrient intake of patients with RA was determined and it was found that daily intake of total vitamin A and β-carotene was notably lower as compared to control (Bae *et al.,* 2003). Importance of vitamin E was studied in an animal model by feeding mice with vitamin E deficient diet followed by RA induction, the results showed that the serum had higher titer of inflammatory molecules like PGE2, nitric oxide (Choi *et al.,* 2009).

The efficacy of selenium supplementation in improving biochemical alterations and clinical symptoms of RA has been assessed. One study report that selenium

supplementation increased lowered serum and red blood cell GPx activity in RA patients (Hasen *et al.,* 1996).

A randomized, double-blind placebo-controlled trial to using conjugated linoleic acid (CLA) and vitamin E was reported to improve clinical symptoms (Aryaeian *et al.,* 2009).

2.4.3 Hormonal factors in RA prevalence

The prevalence of RA in females might suggest a role for hormonal factors. Additionally, estrogens stimulate the immune system. Lower levels of testosterone have been reported in men with RA (Cutolo *et al.,* 2002, Heikkila *et al.,* 1998). No changes have been observed in the levels of sex hormones in women with RA and healthy controls (Heikkila *et al.,* 1998)

In patients with RA, pregnancy often causes remission, followed by a flare-up after delivery suggesting that child-bearing might protect against RA (Tobon *et al.,* 2010, Nelson *et al.,* 1993). Hormone replacement therapy (HRT) may decrease RA susceptibility in women who have HLA-DRB1*01and/or*04 alleles (Salliot *et al.,* 2009).

There is significant controversy regarding oral contraceptives decreasing the risk of developing RA, while some studies have found clear association (Berglin *et al.,* 2010, Doran *et al.,*), while others have not shown a lower incidence of RA in women treated with oral contraceptives (Pedersen *et al.,* 2006, Pikwer *et al.,* 2009).

2.4.4 Infectious agents implicated in RA development

A number of infectious agents have been studied and implicated in disease development on the basis of higher frequency of positive viral serology (presence of specific antibody) in synovial fluid of RA patients. Possibly these agents might be involved in development of disease in patients who have genetic susceptibility and not in isolation (Esquide *et al.,* 2012).

There is evidence supporting a role for parvovirus B19 which includes the incidence of viral DNA in the synovial fluid, synovial cells, and/or synovial tissue of RA patients (Tobon *et al.,* 2010). Sera from RA patients have been known to contain high titres of Epstein-Barr virus (EBV) additionally EBV RNA has been identified in B cells in synovial tissue from RA patients (Meron *et al.,* 2009).

Porphyromonas gingivalis is a major causative agent of periodontitis which has been associated with RA (de Pablo *et al.,* 2008). It is known to express peptidylarginine-deiminase (PAD) which is responsible for citrullination of proteins (Lundberg *et al.,* 2010) and causing chronic inflammation and erosive destruction of periodontal bone (Rosenstein *et al.,* 2004). Despite such findings, verification that microorganisms are implicated in the development of RA remains inconclusive.

2.5 Epigenetic role in RA

The association of rheumatoid arthritis (RA) with numerous genetic loci is well documented; however, development of disease is not just based on genetics.

Environmental factors considerably contribute to the pathogenesis. In comparison to the genetic background, epigenetic factors are responsive to external stimuli and might alter gene expression (Kyburz *et al.,* 2014). Therefore, epigenetic mechanisms can function as intermediaries between genes and environmental factors.

2.5.1 DNA methylation

DNA methylation is usually linked with transcriptional suppression in CpG-rich genomic region (Jones., 2012). The mechanism of which can either be by direct inhibition of transcription factor binding or by specific proteins that bind to methylated DNA and thereby block the access of DNA polymerases and transcription factors (Kyburz *et al.,* 2014).

Neidhart *et al.,* (2000) provided the first proof of the presence of DNA methylation pattern in RA by showing the expression of LINE-1 retrotransposons in RA synovial fibroblasts. Retrotransposons are mobile DNA sequences that are normally repressed by DNA methylation. Further evidence stating that DNA was hypomethylated in RASFs as compared to OASFs was shown by Karouzakis *et al.,* (2009).

An overall genome study to identify the signature methylome in RASFs with OASFs as control was carried out by Nakano *et al.,* (2012) showing various differentially methylated genomic regions, a total of 203 genes that were differentially methylated were identified, with roles ranging from inflammation to leukocyte recruitment. Another genome wide study by de la Rica *et al.,* (2013) also confirmed the results of altered methylome pattern in RASFs.

The investigation of DNA methylation in RA have also been identified in small gene studies showing changed methylation status of single gene promoters (usually at a single CpG site), these include DR3, IL1R2, and CXCL12 both in peripheral blood mononuclear cells and FLSs (Klein *et al.,* 2013, Karouzakis *et al.,* 2011, Takami *et al.,* 2006). Altered methylation patterns have also been seen in genes like IL-10 (Lin *et al.,* 2012, Li-houng *et al.,* 2011) showing hypomethylation, similarly hypomethylation of IL-6 promoter region was observed in mono-nuclear cells of RA patients (Nile *et al.,* 2008) with another study carried out by Ishida *et al.,* (2012) showing similar results. Previous studies have analysed the promoter of CD40L in CD4+ positive cells (Liao *et al.,* 2012).

Evidence of epigenetic role in RA with regard to gender was shown in a study with results that showed an increased inactivation pattern of the X-chromosome in female RA patients (Chabchoub *et al.,* 2009).

It is yet to be confirmed whether DNA methylation might explain the changes in HLA expression. Glant *et al.,* have studied the expression of chromatin-modifying enzymes in B- and T-cells in RA patients and also worked on animal model of mice, the results showed an increased expression of genes: Aurora kinase A and B that play a role in phosphorylation (Glant *et al.,* 2013).

2.5.2 Histone modifications

Chromatin biology is another field of epigenetic study (Bannister *et al.,* 2011). Chromatin structure is made up of DNA tightly wound around an elaborate protein network forming the nucleosome. Transcriptional activation and repression is dependant on the post-

translational modification of histones, these modifications change nucleosomal structural conformation and allow access of transcriptional activators.

The first study linking histone methylation with RA involved histone methyltransferase EZH2, which was seen to be over-expressed in RASF when compared to OASF as control (Trenkmann *et al.,* 2011). The tumor suppressor gene secreted frizzled-related protein 1 (SFRP1), an inhibitor of the Wnt pathway, was shown to be a target of EZH2 in RASF. However, EZH2 over-expression had no impact on global histone trimethylation in RASF (Trenkmann *et al.,* 2011). The association of H3K27me3 in other autoimmune disorder has been seen (Dai *et al.,* 2010). Further studies in RA are needed to elucidate the role of histone methylation marks in RA.

Studies on the activity of HDAC in RA have resulted in contradictory results. A decreased activity of HDAC1 and HDAC2 in RA synovial tissue compared with OA synovial tissues as control have been reported (Huber *et al.,* 2007). While increased HDAC activity and increased HDAC1 expression dependant on TNF-α was reported by Kawabata *et al.*, (2010).

Studies utilizing inhibitors of HDAC (HDACi) support the hypothesis of increased activity of HDACs in RA. Trichostatin A (TSA) was used in a study and used to block IL-6 production in patients with RA using peripheral mono-nuclear cells and macrophages (Grabiec *et al.,* 2010). Recently another study used Largazole (class I HDACi) to evaluate its effect on TNF-α induced expression of ICAM-1, VCAM-1 and

MMP-2 and it was seen that largazole activates p38 and Akt pathway (Ahmed *et al.*, 2013).

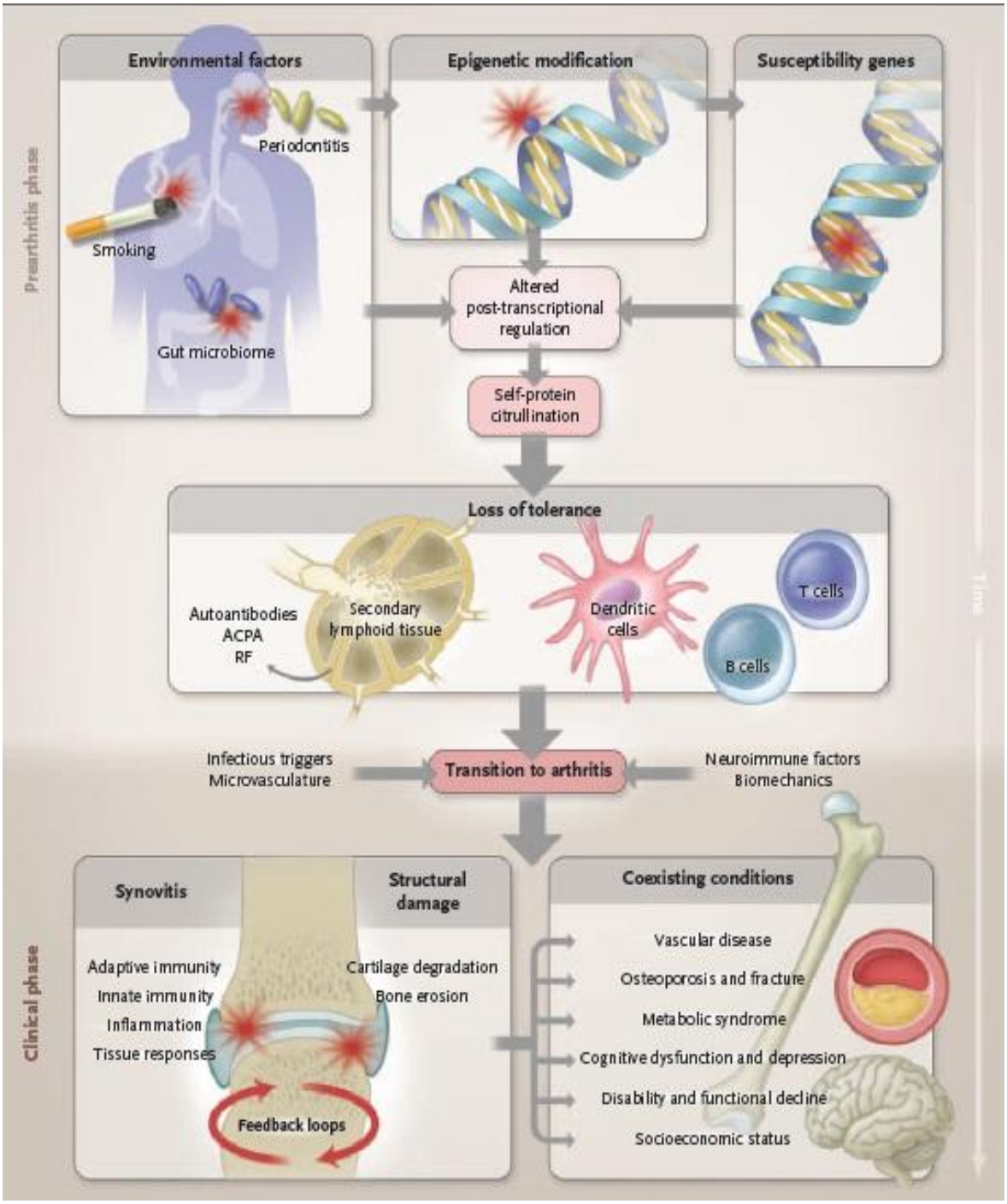

Figure 2.1: Progression of Disease

2.6 TNF-α SNPs and rheumatoid arthritis

Non-HLA genes within the MHC have also been studied for linkage with RA. Tumour necrosis factor (TNF) gene lies in the class III region of MHC. TNF production can be regulated at the levels of transcription, post-transcription, and translation.

The numerous biological responses to TNFα are mediated via two structurally diverse receptors: type 1 (TNFR1) and type II (TNFR2) both of which are trans-membrane glycoproteins with various cysteine rich repeats in the extracellular N-terminal domains. Despite the similar structural and functional homology, the intracellular domain for the receptor is different, and signaling is carried out via both overlapping and distinct pathways. One of the primary characteristic that discriminate the intracellular domains of these receptors is the presence of a death domain in TNFR1. The death domain is primarily a sequence of approximately 70 amino acids that plays a pivotal role in cellular apoptosis. This intracellular death domain provides a docking site for numerous accessory proteins, including Fas-associated death-domain-containing protein **(**FADD), TNFR1- associated death-domain-containing protein (TRADD), and TNFR-associated factor-2 (TRAF-2). TRADD and TRAF2 furthermore subdivide into pro-apoptotic and inflammatory signaling pathways that are characteristic of TNFR1 as shown in figure 2.2 (Palladino *et al.,* 2003).

Variation on the promoter and coding sequence of TNF gene can alter the extend of response in terms of secretion of cytokine (Bouma *et al.,* 1996). Thus far, a total of 5 polymorphisms have been documented, four of which have a guanine to adenine

transition at position –376, –308, –238 and –163 (Wilson *et al.*, 1993), while the fifth polymorphism has a cytosine (C) insertion in a C-stretch starting at position +70 (Brinkman *et al.*, 1995). All of these polymorphisms are located in the transcriptional regulation region which means that changes in bases may affect gene expression (Matsuda *et al.*, 1992). Analysis of 50 Irish multi-case families showed a susceptibility gene in or near the TNF locus conferring susceptibility to RA, which was contributed to the presence of microsatellites (Mulcahy *et al.*, 1996).

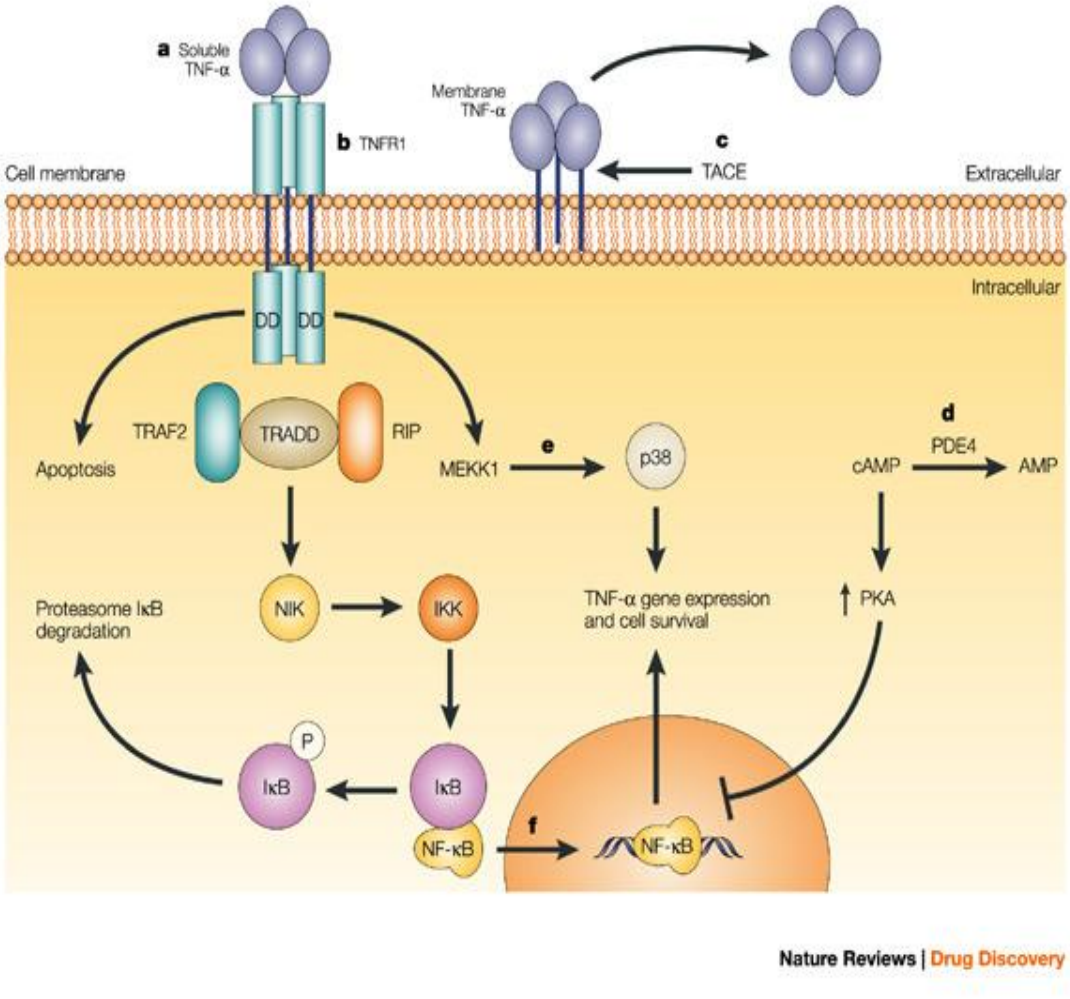

Figure 2.2: TNFα signaling pathway

Of all SNPs known to affect the TNF promoter gene, the one positioned at position -308 seems to be the most related to disease susceptibility. The -308 (rs1800629) TNF SNP involves the substitution of guanine (G) for adenine (A), generating two genotypic (G/A or A/A) alleles (Wilson *et al.,* 1992). The polymorphic A/A allele leads to an increased rate of TNF gene transcription compared to wild-type G/A allele in in-vitro expression studies, it has also been linked with increased risk for a variety of illnesses (Verwij., 1999; Hajeer and Hutchinson., 2000). Meta analysis involving European, Latin American and Asian populations by Lee *et al.,* (2007) showed that the polymorphism -308A/G has contradictory results regarding disease association. Danis *et al.,* (1995) has described that the frequency of polymorphic A/A allele in Caucasian population is 3 times more in RA patients compared to healthy controls. While Cuenca *et al.,* (2003) have shown the presence of A/A allele is twice as more in Chilean RA patients than in normal controls.

Furthermore, the heterozygous G/A allele form has been associated with the development of severe form of disease with early onset in Swedish patients (Cvetkovic *et al.,* 2002), while similar results have been shown in RA Turkish patients with a significant association with bad prognosis of the disease (Ozen *et al.,* 2002).

It can be noted that the frequency of -308 TNF polymorphism is higher in the healthy Caucasian population compared to other ethnic groups such as Latin-American, Chinese, Japanese or black African populations (Aguillon *et al.,* 2006).

TNFα -238 (rs361525) polymorphism is a G to A change in the promoter region. A study by Trakov *et al.,* (2009) reported protective association with the disease, the results

showed that the G allele has a higher risk in development of RA in Macedonians compared to A allele. There have been studies that conclude the absence of association between TNFα polymorphism and the severity of disease (Wilson *et al.,* 1995) however the contradicting results imply that the TNF-α allele may or may not play a limited part in predisposition to RA and the difference in results might not only be linked to genetic background but also the variation in climate, food, and parasite exposure.

Another study also showed that polymorphisms -238 G/A and +489 G/A were independent of the HLADRB1 shared epitope (Verweij *et al.,* 1998). Several studies done on the TNF locus might possibly suggest a genetic link in RA independent of the DRB1 locus, but at the same time there is also some that hypothesize that it might be secondary to linkage disequilibrium.

Another polymorphism reported in the promoter of TNFα is at the position -376 (rs1800750) which is a G to A change. In a study by Waldron-Lynch *et al.,* (2001) on families with RA showed -376A allele in only a single individual carrying the rare TNF H4 haplotype. This frequency correlates with another study by Brinkman *et al.,* (1997) signifying that this allelic polymorphism might occur rarely to play a major role in RA susceptibility.

Aims and Objectives:

1. To investigate genetic susceptibility of TNF- α promoter polymorphisms
2. To detect DNA methylation status of TNF- α promoter

CHAPTER 3

MATERIALS AND METHODS

3.1 Genotyping

Genotyping is the procedure of studying the genetic makeup of an individual by investigating their DNA sequence. Genotyping can be done on a broad range of organisms, including microorganisms.

In humans, DNA is isolated from tissue sample e.g. saliva or blood; it is then copied several times to make amplicons through a process called polymerase chain reaction in a thermal cycler. In the following study genotyping was done through restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) which utilizes restriction enzymes to cut genome into various fragment lengths depending on the allele present that can be detected on gel.

3.1.1 Study Subjects

The study was a case control based study. Patients were recruited from Rehmaat Noor Clinic, Rawalpindi and Military Hospital, Rawalpindi. The individuals were diagnosed with RA after satisfying the ACR criteria for RA and confirmed on the basis of serological diagnosis.

All subjects were recruited in the study after signing an informed consent form.

3.1.2 Sample Collection

Venous blood for patients and healthy controls were collected in EDTA tubes (BD vacutainer TM, Frankin Lakes, New Jersey, USA) using 5mL clean and sterilized syringes (BD 0.6mm* 25mm G* 1 TW). The samples were properly labeled with identification number of sample, date of collection and name, age and gender of individual.

3.1.3 Sample Storage and Transport

Blood samples collected in the vacutainers were stored on ice and dispatched to IGL, ASAB, NUST. They were then stored at 4^{o}C before further processing.

3.1.4 Genomic DNA Extraction Solutions

Four main extraction solutions were used for the extraction of genomic DNA from collected blood samples. The composition and function of each have been described in Table 3.1.

- **Solution A**

This consisted of 0.32M Sucrose, 10mM Tris (pH 7.5) and 5mM $MgCl_2$ dissolved in distilled water and raised to required volume and autoclaved. Triton X-100 (1% volume) was then added.

- **Solution B**

This consisted of 10mM Tris (pH 7.5), 400mM Sodium Chloride (NaCl) and 2mM Ethylenediaminetetraacetic acid (EDTA pH 8.0) added to added distilled and raised upto required volume by distilled water.

- **Solution C**

Phenol makes Solution C. This was used in a mixture with Solution D.

- **Solution D**

It consisted of a mixture of chloroform and isoamyl alcohol in a 24:1 ratio by volume.

Table 3.1: Solutions required in DNA extraction

S.No	Solutions	Components	Function
1	Solution A	• 0.32M Sucrose • 10 mM Tris (pH 7.5) • 5 mM Magnesium Chloride ($MgCl_2$) • Triton X-100 1% (v/v)	For lysis of white blood cells (WBCs)
2	Solution B	• 10 mM Tris (pH 7.5) • 400 mM Sodium Chloride (NaCl) • 2 mM EDTA (pH 8.0)	For DNA precipitation and separation of proteins
3	Solution C	• Phenol	Help separation of DNA
4	Solution D	• Chloroform (24 volume) • Isoamyl Alcohol (1 volume)	Forms the aqueous layer in which the DNA is separated

- **1X TAE Buffer**

TAE buffer was used in gel electrophoresis and was originally prepared as a stock solution of 10X concentration. For 10X concentration 48.5g of Tris, 11.4mL glacial acetic acid and 20mL 0.5M EDTA (pH 8.0) was added to distilled water and dissolved then raised to make 1 litre TAE and stored at room temperature.

To prepare 1X concentration required volume was taken from the stock solution and raised to desired volume by distilled water using the formula $M_1V_1 = M_2V_2$.

3.1.5 Genomic DNA Extraction

DNA was extracted using phenol-chloroform extraction method. This cost effective method is a two day protocol and gives high yield of DNA. A total of 4 solutions are used in the extraction each serving different purpose.

For extraction of DNA 750µL of blood was transferred in a 1.5mL eppendorf tube and equal amount (750µL) of Solution A was added, the tube was then closed and inverted 4 to 6 times and kept at room temperature for 5-10 minutes. The tube was then centrifuged at 13,000 rpm for 1 minute in centrifuge machine (Spectrafuge 24D Labnet, Edison, New Jersey, USA). Supernatant was discarded and the nuclear pellet was re-suspended in 400µL of Solution A. Another round of centrifuge followed at 13,000rpm for 1 minute. The step was repeated again until the supernatant was clear. The nuclear pellet was then re-suspended in 400µL Solution B, 12 µL 20% SDS and 5 µL Proteinase K and incubated at 37^{o}C overnight.

A fresh mixture of Solution C and Solution D were prepared and 500 µL of the mixture was added to the tube. The tube was then centrifuged at 13,000 rpm for 10 minutes. After centrifugation the upper aqueous layer was carefully collected into a fresh tube. To this layer equal volume (500 µL) of Solution D was added and centrifuged at 13,000 rpm for 10 minutes. The upper layer was collected again in a new tube and 55 µL of 3M Sodium Acetate (pH 6.0) and equal volume (500 µL) isopropanol were added, the tube was

inverted few times to precipitate DNA and then centrifuged at 13,000 rpm for 10 minutes. The supernatant was discarded and 70% ethanol was added to wash the DNA pellet and centrifuged for 7 minutes at 13,000 rpm. Ethanol was then discarded and the pellet dried at 37^{o}C until all the ethanol has evaporated.

The DNA was suspended in 50 µL TE buffer, completely dissolved and later stored at -20^{o}C until further use.

The extracted DNA was qualitatively analyzed on 1% agarose gel and appropriate dilutions using nuclease-free water were made for use in PCR.

3.1.6 Primer Designing

The SNPs selected for association studies via restriction digestion in the study were located in the promoter region of the TNFα gene. Primer sequences were taken from literature that amplified a specific region of TNFα promoter in both diseased and healthy controls. Forward and reverse primers were checked for complementarity with the desired region of interest using NCBI primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome).

Other properties were evaluated using Oligocalc, an online oligonuceotide properties calculator (Kibbe, 2007) (http://www.basic.northwestern.edu/biotools/oligocalc.html). The sequence of primers and the resulting amplicon size are given in Table 3.2.

Table 3.2: Primers for SNPs located in the promoter region of TNFα gene

S.No	dbSNP	Position	Change	Primer Sequence (5'-3')	Temp	Size
1	rs361525	238	G/A	**Forward:** AGAAGACCCCCCTCGGAACC **Reverse:** ATCTGGAGGAAGCGGTAGTG	57°C	155 bp
2	rs1800750	376	G/A	**Forward:** TTTCTGAAGCCCCTCCCAGTTC **Reverse:** TACCCCTCACACTCCCCATCC	57°C	224 bp

3.1.7 Restriction Fragment Length Polymorphism-Polymerase Chain Reaction (RFLP-PCR)

PCR was carried out to amplify a specific region on the TNF gene, the PCR product was later digested with restriction enzymes to check the presence of the polymorphism in question.

The reaction mixture had a total volume of 20 µL was prepared in 0.2 mL tubes (Biologix). 2 µL DNA template was added in 2 µL 10X PCR buffer (Thermo Scientific, EU, Lithuania), 2 mM $MgCl_2$, 2 µL 2 mM deoxyribonucleotide triphosphates (dNTPs) (Thermo Scientific, EU, Lithuania), 2 µL of each forward and reverse primers for particular SNP (10 µM), 0.25 µL Taq Polymerase (Thermo Scientific, EU, Lithuania). The volume was raised to 20 µL by adding 7.75 µL nuclease-free water. The reaction mixture was briefly centrifuged for 5 seconds for thorough mixing and any air bubbles were tapped out.

The reaction was carried out in a thermocycler (Biorad). The optimized conditions consisted of an initial denaturation at 95°C for 5 minutes followed by 35 repeated cycles for amplification; denaturation at 95°C for 40 seconds, annealing for both primer pairs at 57°C for 40 seconds and extension at 72°C for 40 seconds. The final extension was done at 72°C for 10 minutes. The PCR products were then stored at 4°C. The PCR profile is schematically depicted in Figure 3.1.

The presence of product was analyzed on 2% gel electrophoresis then further processed for restriction digestion immediately.

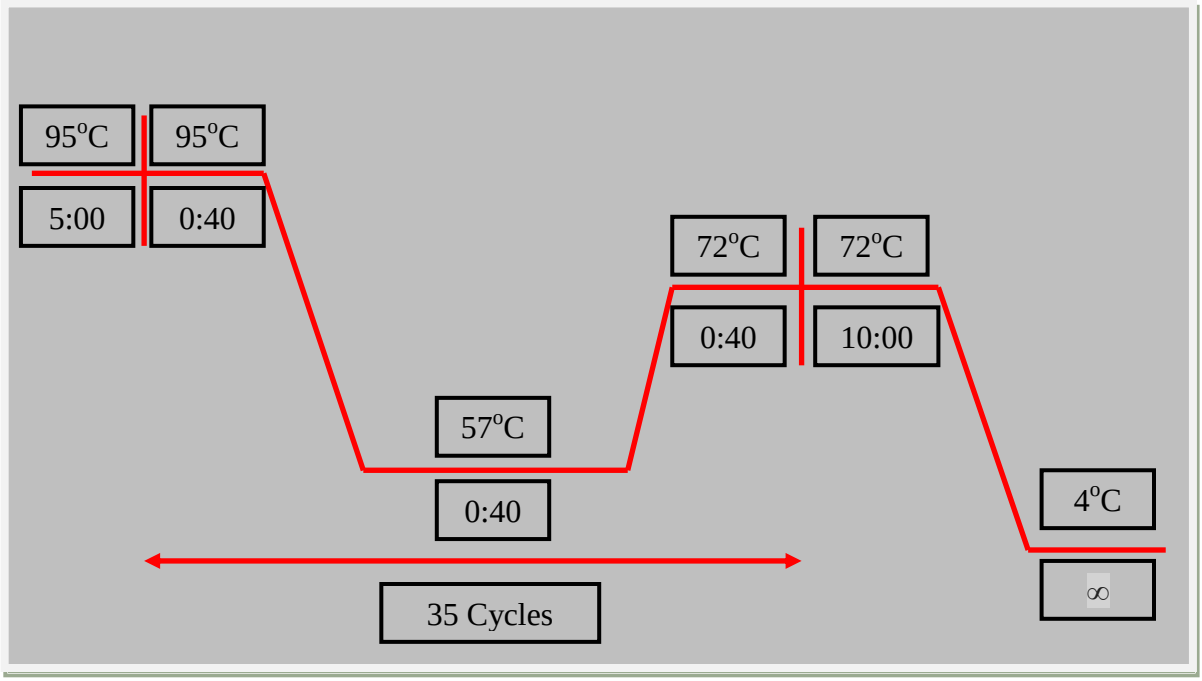

Figure 3.1: Polymerase Chain Reaction profile for both primer sets.

3.1.8 Restriction Digestion

Two SNPs were analyzed in the given study therefore two restriction enzymes were used to study the presence of SNP by the phenomena of restriction digestion. The presence or absence of a particular allele would affect the pattern of digestion of the PCR product by restriction enzyme giving various fragment lengths that could be seen on agarose or polyacrylamide gels. Table 3.3 shows the restriction enzymes used for each SNP studied and the various fragment size obtained.

Msp1 enzyme (Thermo Scientific, EU, Lithuania) was used to analyze the TNFα -238 polymorphism by adding 2 µL Tango Buffer (Thermo Scientific, EU, Lithuania) and 0.25 µL Msp1 enzyme to 10 µL of PCR product and incubated at 37^{o}C overnight.

Tas1 enzyme (Thermo Scientific, EU, Lithuania) was used to analyze the TNFα -376 polymorphism by adding 2 µL Buffer B (Thermo Scientific, EU, Lithuania) and 0.25 µL Tas1 enzyme to 10 µL of PCR product and incubated at 65^{o}C overnight.

Restriction site for both the enzymes are shown in Figure 3.2. After the overnight incubation enzymes were denatured to stop the reaction. Msp1 was denatured at 80^{o}C for 20 minutes while Tas1 was denatured by adding 0.8 µL 20 mM EDTA. The digestion products were seen on 3% agarose gel.

Table 3.3: Restriction enzymes used and fragment lengths

S.No	SNP	Primer Sequence (5'-3')	Restriction Enzyme	Product Size after digestion and allele
1	rs361525	**Forward:** AGAAGACCCCCCTCGGAACC **Reverse:** ATCTGGAGGAAGCGGTAGTG	Msp1	**GG:** 132 and 20 bp **AA:** 155 bp **AG:** 132, 20, and 155 bp
2	rs1800750	**Forward:** TTTCTGAAGCCCCTCCCAGTTC **Reverse:** TACCCCTCACACTCCCCATCC	Tas1	**GG:** 224bp **AA:** 169 and 55 bp **AG:** 169, 55 and 225 bp

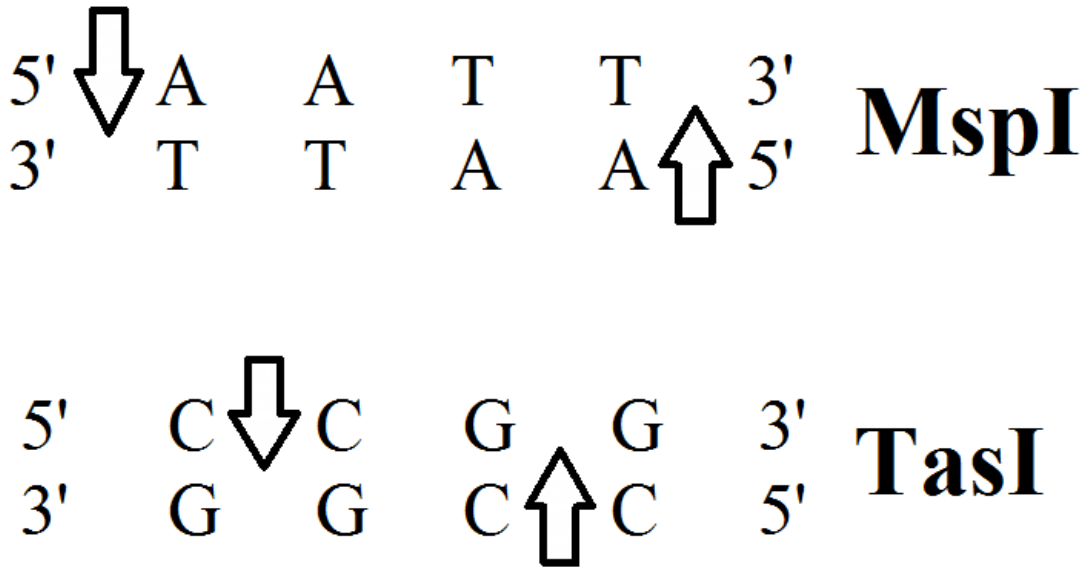

Figure 3.2: Digestion site for restriction enzymes used

3.1.9 Gel Electrophroesis

DNA was run of 1% agarose gel, for which 0.5 g agarose was dissolved in 50 mL 1X TAE buffer and heated till the agarose dissolved. PCR product was run on 2% agarose; 1g in 50 mL 1X TAE buffer, and digestion product was run on 3% agarose; 1.5g agarose in 50 mL 1X TAE buffer. 3 µL Ethidium Bromide (EtBr, 0.5 µg/mL) was added as a stain. The gel was then allowed to solidify at room temperature in a gel casting tray.

Two µL DNA or 5 µL PCR product was mixed with 3 µL loading dye (0.25% Bromophenol blue in 40% sucrose solution). The mixture was loaded in the wells taking care to avoid air bubbles. Length of PCR product or the fragments produced by restriction digestion were compared with 50 bp DNA ladder (Thermo Scientific, EU, Lithuania).

Electrophoresis was done at 100 Volts for 30 minutes in 1X TAE buffer. The gel was analyzed on Dolphin-Doc plus documentation system (Wealtech).

3.1.10 Statistical Analysis

Statistical anaylsis was preformed by Graphpad Prism. For the calculation of genotype and allele frequencies Hardy-Weinberg equation was used. Furthermore Chi-squared (X^2) test was applied for association analysis.

3.2 METHYLATION ANALYSIS

Methylation is the addition of methyl group on a substrate, in cell biology it contributes to epigenetic inheritance via DNA methylation. DNA methylation alters the function of the DNA, usually to suppress gene transcription.

In vertebrates it is characteristically present at CpG sites. The process occurs at the cytosine residue causing the conversion of cytosine to 5-methylcytosine. Majority of CpG sites in human DNA are methylated but certain areas, known as CpG islands that are GC-rich which are un-methylated. These islands are associated with the promoters of genes.

3.2.1 DNA Quantification:

Genomic DNA was quantified using ThermoScientific Nanodrop 2000 Spectrophotometer and NanoDrop™ 2000 software. After the selection of application of interest i.e. nucleic acids and further dsDNA, steps were followed for instrument initialization. A Blank was established using the TE buffer since DNA is re-suspended in TE Buffer. 1-2 µl of blanking buffer is used after which 1-2 µL of sample. The step is repeated thrice and average calculated.

3.2.2 Bisulfite Conversion:

For DNA methylation analysis, DNA was subjected to bisulfite conversion using Thermo Scientific EpiJET Bisulfite Conversion Kit (Cat #K1461).

3.2.2.1 Reagent Preparation:

Reagents used in Bisulfite conversion of DNA were supplied by the manufacturer in dry mixtures or concentrated form which then needed to be dissolved or diluted before use.

- **Modification Reagent:**

Modification Reagent was a dry mixture and needed to be dissolved before use. The amount of Modification Reagent in each vial given was sufficient for 10 DNA conversion reactions. Each vial was therefore prepared by the addition of 0.9 mL of molecular biology grade water, 200 µl of Modification Solution I and 60 µl of Modification Solution II. The contents were then dissolved by inverting for about 10 minutes.

- **Wash Buffer:**

Wash buffer was provided in concentrated form and prepared to use via dilution using 25 ml of 96-100% ethanol.

- **Desulfonation Buffer:**

Desulfonation buffer was supplied in concentrated form and prepared for use through dilution using 10 mL of 96-100% ethanol.

3.2.2.2 Conversion:

500 ng of purified genomic DNA into a PCR tube and volume raised to 20 µL by addition of nuclease free water. 120 µL of prepared Modification Reagent solution was then added and thoroughly mixed. The PCR tubes were then placed in a thermal cycler and the following protocol was used to perform denaturation and bisulfite conversion of DNA.

The resulting product (converted DNA) was added to 400 µL of Binding Buffer in a DNA Purification Micro Column placed in a collection tube. The micro column placed into the collection tube was centrifuged at 12,000 rpm for 30 seconds and flow-through discarded. Placed the micro column into the same collection tube and added 200 µL of

Wash Buffer to the micro column and centrifuged at12,000 rpm for 30 seconds. Discarded the flow-through again. Placed the micro column into the same collection tube and added 200 µL of Desulfonation Buffer, to the micro column and incubated at room temperature for 20 min. Centrifuged the micro column placed into the collection tube at 12,000 rpm for 30 seconds and discarded flow-through. Washed the product in micro column by adding 200 µL of Wash Buffer and centrifuged at12,000 rpm for 30 seconds. Discarded the flow-through and washed again using 200 µL of Wash Buffer and centrifuged at 12,000 rpm for 60 seconds. The column was then placed in a clean 1.5 mL micro-centrifuge tube and 10 µL Elution Buffer was added. Centrifuged at 12,000 rpm for 60 seconds. Repeated this step by adding 10 µL of elution buffer to the micro column. Eluted DNA was then stored at -20 ºC for downstream analysis.

3.2.3 Methylation Specific Primer Designing:

To find the sequence of promoter region of the gene, Ensembl Genome Browser (http://www.ensembl.org/index.html) was used to get sequence of target gene.“Human” as desired specie was chosen and TNF-α searched and the best matched result clicked to open gene summary page. Under “Gene Summary”, “Sequence” was selected, the sequence of the gene including 5' flanking, exons, introns and flanking region was displayed. The exons are highlighted in pink background and red text, the sequence in front of the first exon was the promoter sequence. From the option of "Configure this page" changed the input the size of 5' Flanking sequence (upstream) to 1000 bp and saved the configuration.

To ensure that the selected region was the promoter sequence, the promoter sequence was copied. Opened UCSC BLAT search (https://genome.ucsc.edu/cgi-

bin/hgBlat?command=start), chose "Human" as desired genome and pasted the copied gene promoter sequence. On the result page, clicked the first hit that opened the genome browser Page. The query sequence was now aligned with UCSC genome sequence. After zooming out a bit, the selected promoter sequence was determined to match the UCSC annotation. Moreover, CpG island feature was turned on, and observation of CpG Island in the query sequences further confirmed it to be a promotor sequence.

Methylation Specific Promoters were designed using an online designing tool known as MethPrimer (Li and Dahiya, 2002) accessed through http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi.

Two sets of primers were used for analysis for methylation status. One pair for methylated DNA and other for unmethylated DNA. Table 3.4 shows the primer sequences for the two sets of primers used and their product size.

Table 3.4: Primer sequences used in Methylation Specific PCR

S.No	Primer	Sequence (5' – 3')	Product Size
MSP1	Left Methylated-Specific primer	GAGATAGAAGGTGTAGGGTTTATTATC	130 bp
	Right Methylated-Specific primer	TACCTTTATATATCCCTAAAACGAA	
	Left Un-methylated-Specific primer	TAGAAGGTGTAGGGTTTATTATTGT	126 bp
	Right Un-methylated-Specific primer	TACCTTTATATATCCCTAAAACAAA	
MSP2	Left Methylated-Specific primer	TTAGAAGATTTTTTTCGGAATC	130 bp
	Right Methylated-Specific primer	TATCTCGATTTCTTCTCCATCG	
	Left Un-methylated-Specific primer	GGTTTAGAAGATTTTTTTTGGAATT	120 bp
	Right Un-methylated-Specific primer	TCTATCTCAATTTCTTCTCCATCAC	

3.2.4 Methylation Specific PCR Amplification:

The Bisulfite converted DNA was subjected to PCR amplification using Methylation Specific primers in order to analyze the methylation status of promoter region of TNFα. For each sample, two reaction tubes were prepared. One contained Methylated-Specific primer pair while the other contained Un-methylated-Specific primer pair. Reaction mixture (20 µL) was prepared in 0.2 mL PCR tubes (Biologix, USA) by the addition of 2 µL of Bisulfite converted DNA, 2 µL each of Left and Right Methylated or Un-

methylated-specific primers, 4 µL nuclease free water and 10 µL of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, EU, Lithuania).

The thermocycling conditions consisted of initial template denaturation at 95^{o} C for 10 minutes followed by 38 cycles of PCR amplification. Each amplification step further consisted of 3 steps: denaturation at 95^{o}C for 30 seconds, annealing at 57^{o}C for 1 minute and 1 minute at 72^{o}C for extension. Final extension was at 72^{o}C for 7 minutes. The PCR products were kept at 4^{o}C and analyzed on 2% agarose gel.

3.2.5 Gel Electrophoresis:

Gel Electrophoresis was carried out to analyze the methylation status of the TNFα promoter. 2% agarose gel was prepared by dissovling 1.6 g of agarose in 80 mL of 1X TAE buffer and heated in microwave oven for 2 minutes. 3 µL Ethidium bromide (0.5 ug/ml) was added for staining of DNA. The gel was allowed to solidify at room temperature in a gel casting tray. On solidification, gel was transferred to buffer tank containing 1x TAE. 7 ul of the amplified product was loaded on to gel. The gel was the run at 100 volts for 35 minutes. The results were analyzed through Gel Documentation System (Dolphin Doc).

CHAPTER 4

RESULTS

4.1 Genotyping

In the association study of two SNPs for TNFα located in the promoter region a total of 125 RA cases and 125 controls were studied. The patients and healthy individuals were analyzed for the occurrence of polymorphism by restriction fragment length polymorphism- polymerase chain reaction (RFLP-PCR). The resulting data was statistically analyzed for determining significance between occurrence of SNP and susceptibility to disease.

4.1.1 Study Subjects

A total of 250 subjects were involved in the study, 125 individuals diagnosed with RA and 125 healthy controls. The individuals represented a small group in Pakistani population to investigate the association of disease and SNP occurrence.

Percentage of females recruited in the RA case group was 87.3% while male comprised of 12.7% of the total cases. The mean age of individuals in case group was 43.88±14.03. On the other hand percentage of females in control group was 72.8% and males were 27.2% with mean age of 37.09±13.36.

The characteristics of human subjects in RA patients and control group are surmised in Table 4.1.

Table 4.1: Characteristics of individuals involved in the study

S.No		Cases		Controls
1	**Anti-CCP**	125		0
2	**RA Factor**	125		0
3	**Females (%)**	87.3		72.8
4	**Males (%)**	12.7		27.2
5	**Age (Mean ± SD)**	43.88±14.03		37.09±13.36
6	**Duration of Disease**	>6 Months	31.5%	0
		>2 Years	14.6%	
		>4 Years	53.9%	

4.1.2 Association Study of SNP rs361525

The polymorphism rs361525 is located in the promoter region of TNFα. 125 RA patients and 125 healthy controls were analyzed via RFLP-PCR (Figure 4.1). All the likely combinations of alleles were observed in the study subjects using restriction digestion (Figure 4.2).

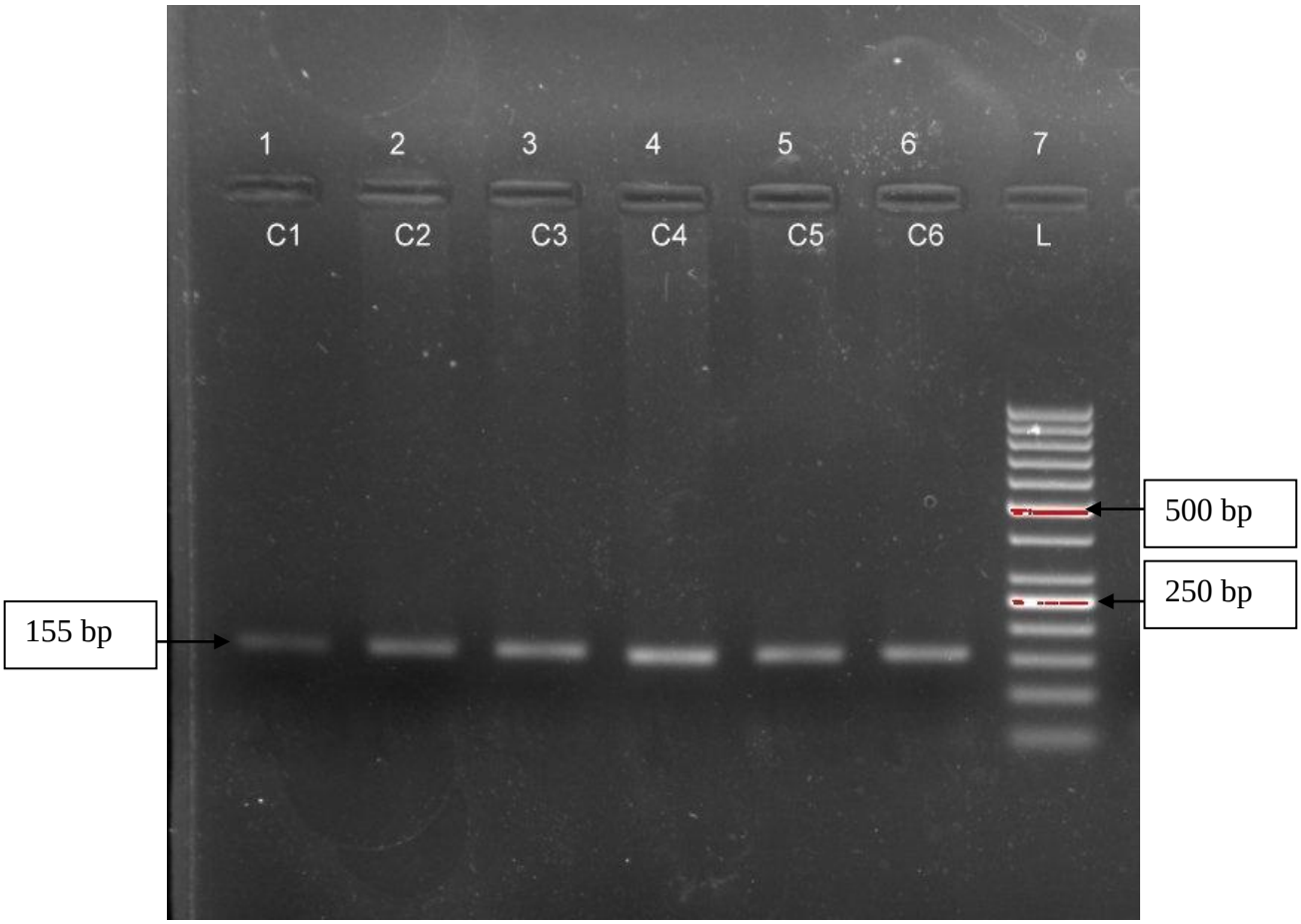

Figure 4.1: Electropherogram of EtBr stained 2% gel of 6 control subjects for rs361525 RFLP-PCR. A band for 155bp was observed in all RA cases and control groups showing amplification of a specific region of TNFα gene.

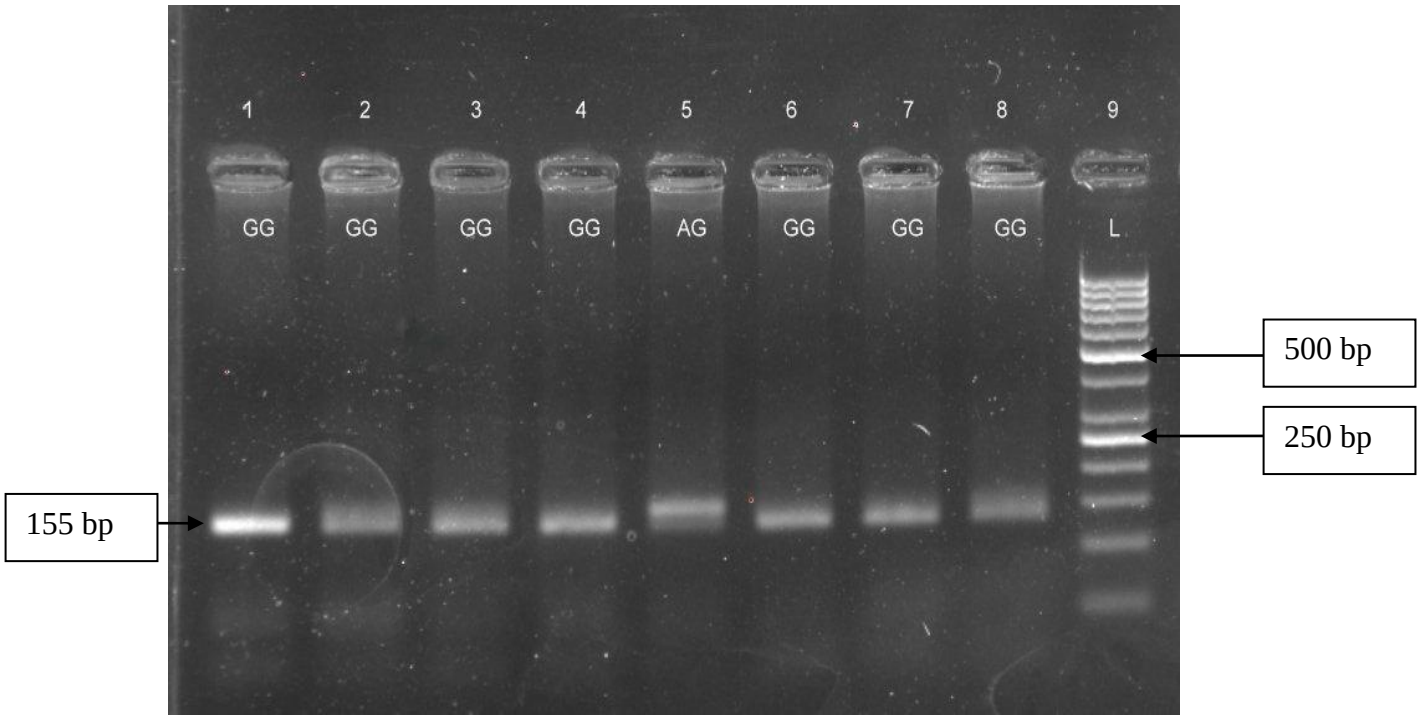

Figure 4.2: Electropherogram of EtBr stained 3% agarose gel for restriction digestion by MspI. The fragments obtained after digestion conferred to the genotype present. Lane 1 till 8 presented GG genotype; fragments obtained 135 bp and 20 bp except lane 5 which had AG allele; fragments seen 155 bp, 135 bp and 20 bp. Genotype AA is not shown in the figure but only one fragment will be obtained; 155bp.

Genotype frequency was calculated in the patients and matched with frequency of healthy controls (Figure 4.3). The data was statistical analyzed by Graphpad Prism 5 and Chi-square (χ^2) test employed for association analysis of genotype with rheumatoid arthritis. The value of association was found to be 4.667 with probability of error (*p* value) 0.09 (Table 4.2). Allele frequency was also calculated between cases and controls using Fischer test, two tailed analysis shown in Figure 4.4. The genotype distribution data showed no significant association however allelic distribution between cases and control

showed *p* value of 0.0056 (Table 4.3) which is statistically significant suggesting that the allele A may be associated with disease susceptibility.

The polymorphism was also verified for any deviation from Hardy-Weinberg equilibrium (HWE). The calculations were performed on each group and substantial difference was seen in the frequencies of genotype between observed and expected values. *p* value for both cases and control group was 0.0001 shown in Table 4.4.

The values showed that there was no statistical significant association between genotype of SNP with RA; however the allele A might confer susceptibility to disease in Pakistani population.

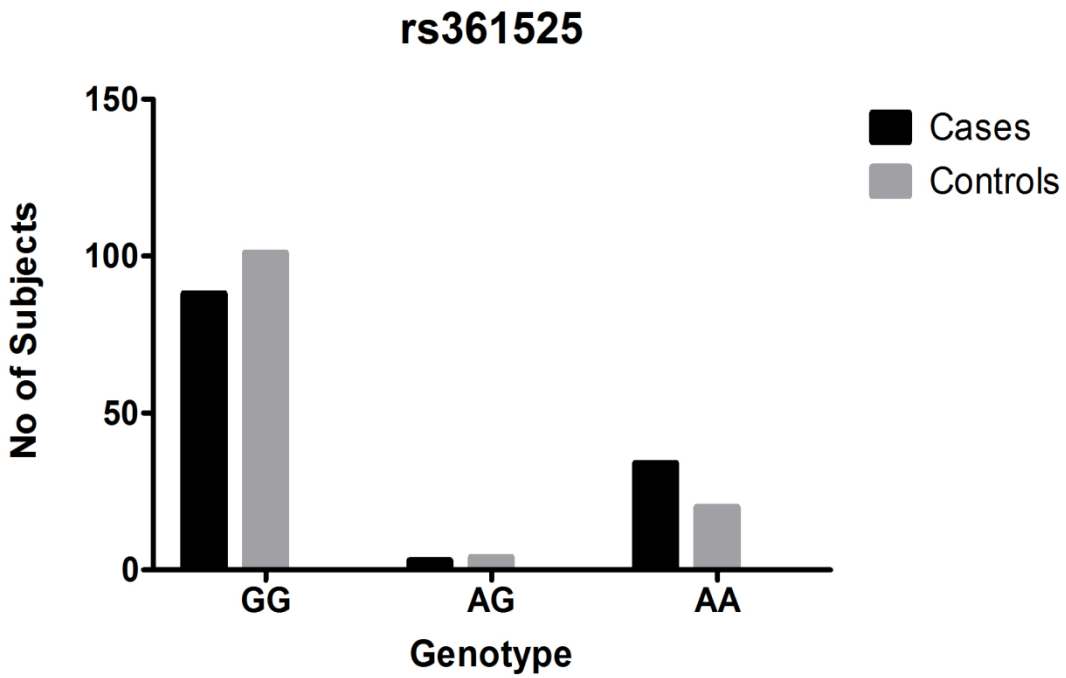

Figure 4.3: Distribution of subjects across different genotypes for rs361525

Table 4.2: Genotype frequency distribution of rs361525

S.No	Cases/Controls	Genotype Frequency Distribution			χ^2 statistics		
		AA	AG	GG	χ^2	*df*	*p* value
1	**RA Cases (n=125)**	34 (27.6%)	3 (1.7%)	88 (70.7%)	4.667	2	0.09
2	**Controls (n=125)**	20 (15.8%)	4 (3.5%)	101 (80.7%)			

Table 4.3: Allele Frequency of rs361525

S.No	Cases/Controls	Allele Frequency Distribution		Odds Ratio (95% CI)	*p* value	χ^2
		A	G			
1	**RA Cases (n=125)**	71 (28.4%)	179 (71.6%)	1.857 (1.213-2.844)	0.0056	8.23
2	**Controls (n=125)**	44 (17.6%)	206 (82.4%)			

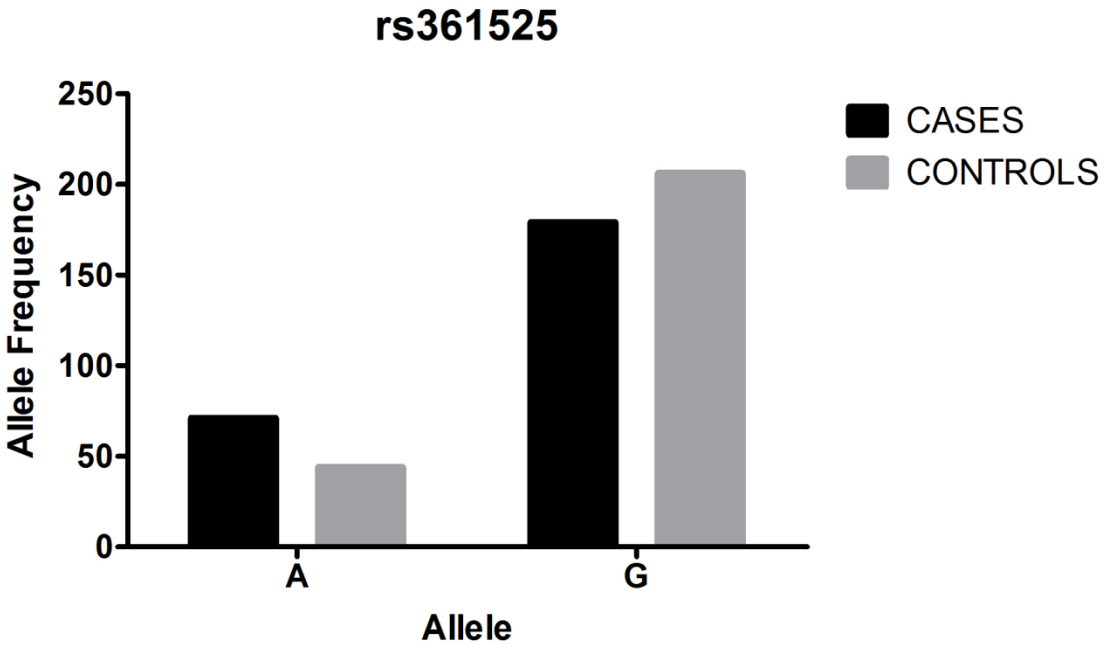

Figure 4.4 Allelic distribution of rs361525

Table 4.4: Hardy-Weinberg calculations for rs361525

S.No	Cases / Controls	Observed Genotype Percentage			Expected Genotype Percentage			χ^2	*p* value
		AA	AG	GG	AA	AG	GG		
1	**RA Cases (n=125)**	27.6	1.7	70.7	8.1	41	51	49.47	0.0001
2	**Controls (n=125)**	15.8	3.5	80.7	3.1	29	68	28.81	0.0001

4.1.3 Association Study of SNP rs1800750

The polymorphism rs1800750 is located in the promoter region of TNFα. 125 RA patients and 125 healthy controls were analyzed via RFLP-PCR (Figure 4.5). All the likely combinations of alleles were observed in the study subjects via restriction digestion (Figure 4.6).

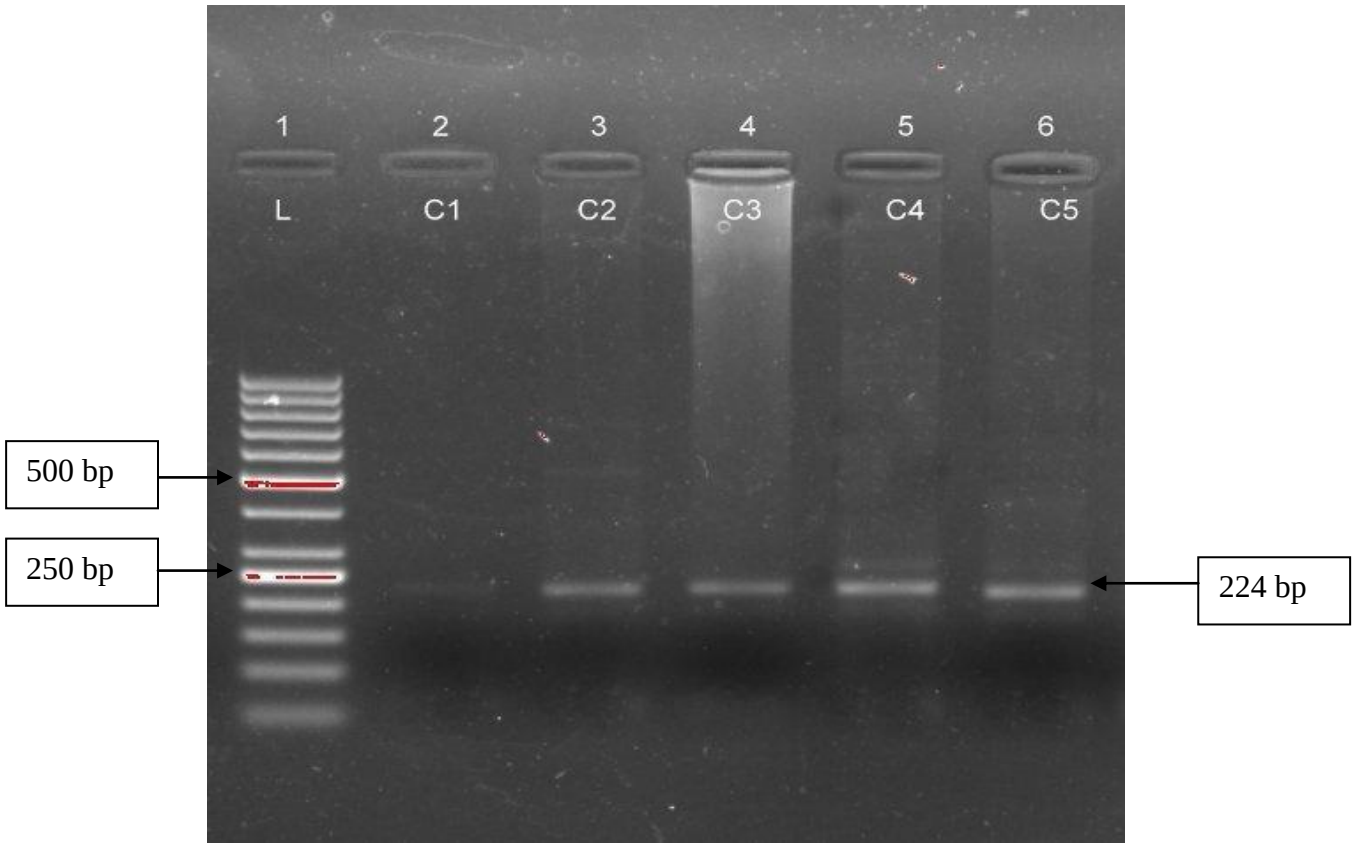

Figure 4.5: Electropherogram of EtBr stained 2% gel of 5 control subjects for rs1800750 RFLP-PCR. A band for 224bp was observed in all RA cases and control groups showing amplification of a specific region of TNFα gene.

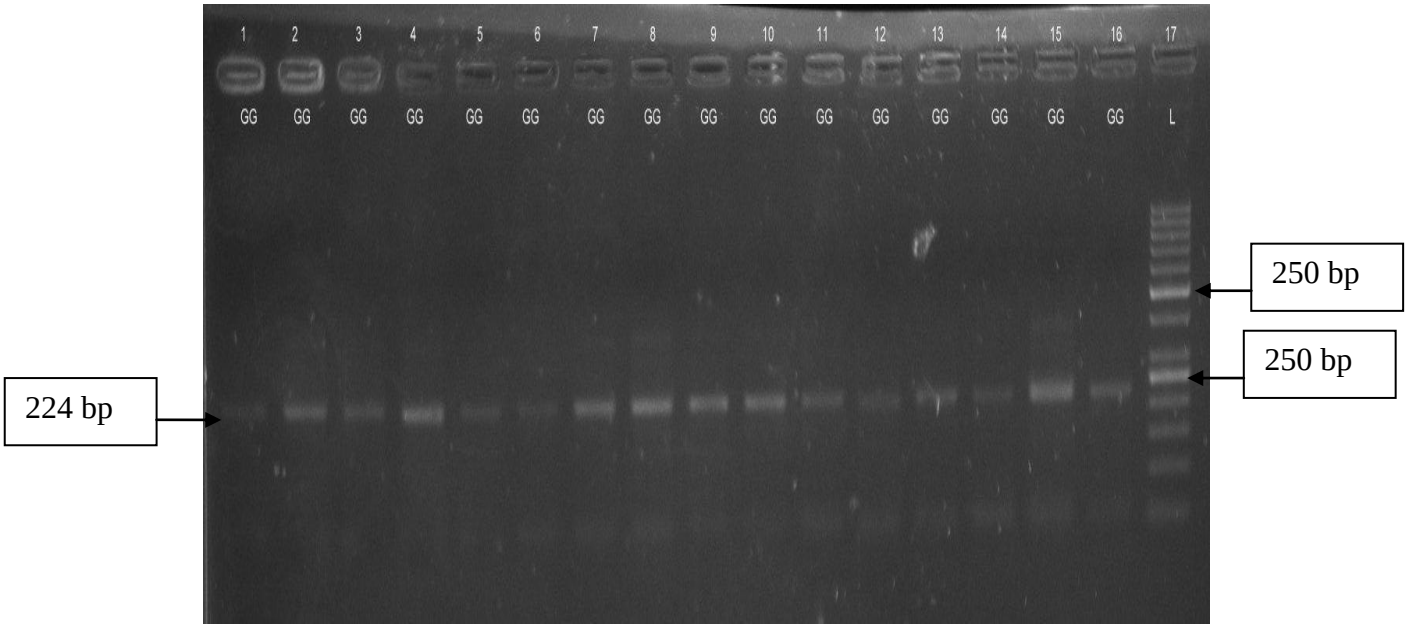

Figure 4.6: Electropherogram of EtBr stained 3% agarose gel for restriction digestion by TasI. The fragments obtained after digestion conferred to the genotype present. Lane 1 till 16 presented GG genotype; fragments obtained 224 bp. Genotype AA is not shown in the figure but fragments obtained will 169 and 55bp, genotype AG isn't shown either fragments obtained in that case will be 224, 169 and 55 bp.

Genotype frequency was calculated in the patients and matched with frequency of healthy controls (Figure 4.7). The data was statistical analyzed by Graphpad Prism 5 and Chi-square (χ^2) test employed for association analysis of genotype with rheumatoid arthritis. The value of association was found to be 9.232 with probability of error (*p* value) 0.0099 (Table 4.5). Allele frequency was also calculated between cases and controls using Fischer test, two tailed analysis shown in Figure 4.8. The genotype distribution data showed significant association as did allelic distribution between cases and control showing *p* value of 0.0004 (Table 4.6).

The polymorphism was also verified for any deviation from Hardy-Weinberg equilibrium (HWE). The calculations were performed on each group and substantial difference was seen in the frequencies of genotype between observed and expected values in the control group. *p* value for cases was 0.512 and for control group it was 0.0007 shown in Table 4.7.

The values showed that there is statistical significant association between genotype of SNP rs1800750 with susceptibility RA in Pakistani population.

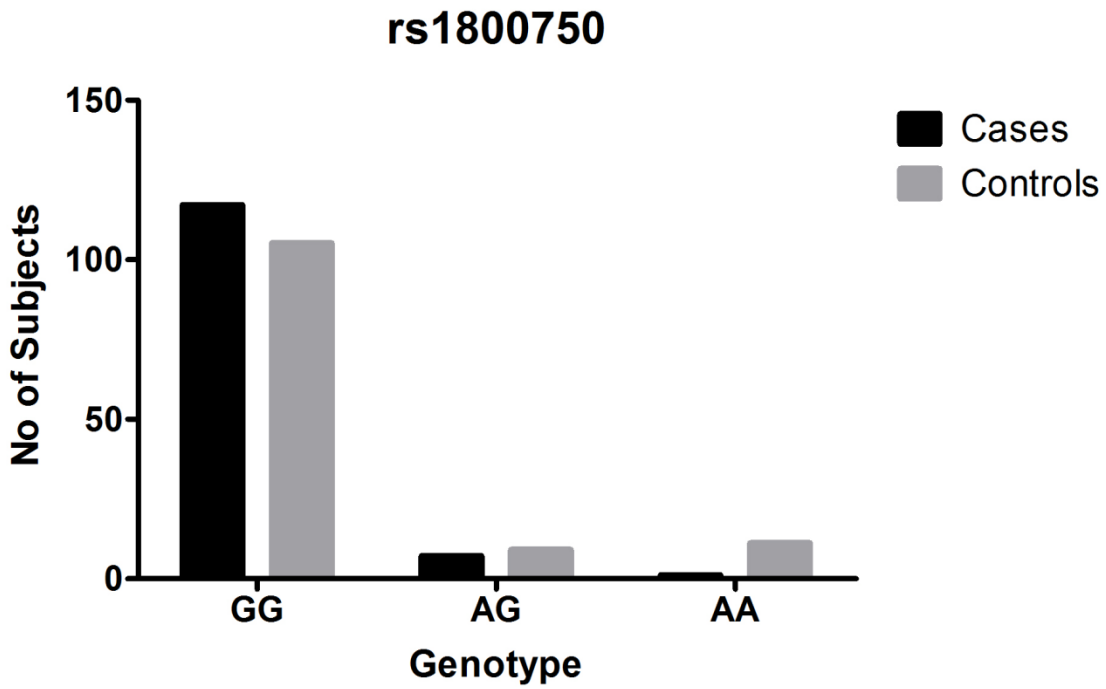

Figure 4.7: Distribution of subjects across different genotypes for rs1800750

Table 4.5: Genotype frequency distribution of rs1800750

S.No	Cases / Controls	Genotype Frequency Distribution			χ^2 Statistics		
		AA	AG	GG	χ^2	*df*	*p* value
1	RA cases (n=125)	1 (0.9%)	7 (5.4%)	117 (93.7%)	9.232	2	0.0099
2	Controls (n=125)	11 (8.8%)	9 (7.4%)	105 (83.8%)			

Table 4.6: Allele Frequency of rs1800750

S.No	Cases / Controls	Allele Frequency Distribution		Odds Ratio (95% CI)	*p* value	χ^2
		A	G			
1	**RA Cases (n=125)**	9 (3.6%)	241 (96.4%)	0.2638 (0.1228-0.5667)	0.0004	13.15
2	**Controls (n=125)**	31 (12.4%)	219 (87.6%)			

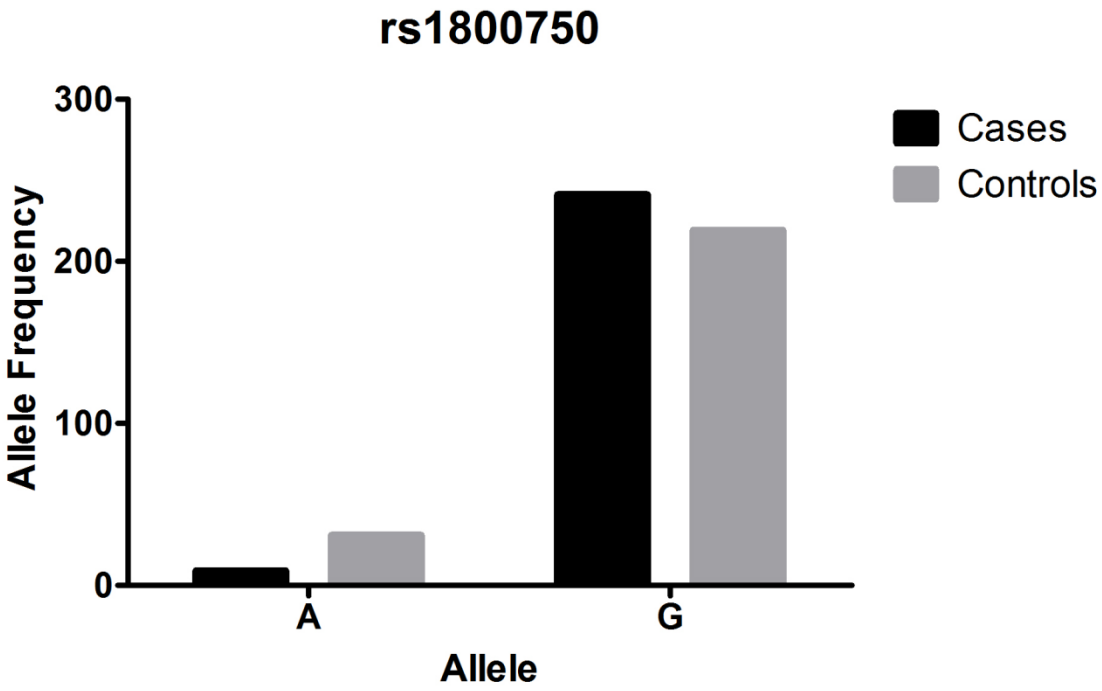

Figure 4.8: Allelic Distribution of rs1800750

Table 4.7: Hardy-Weinberg calculations for rs1800750

S.No	Cases / Controls	Observed Genotype Percentage			Expected Genotype Percentage			χ^2	*p* value
		AA	AG	GG	AA	AG	GG		
1	**RA Cases (n=125)**	0.9	5.4	93.7	0.13	6.9	93	1.339	0.512
2	**Controls (n=125)**	8.8	7.4	83.8	1.5	22	77	14.46	0.0007

4.2 Methylation Analysis

Methylation analysis was performed on 10 RA cases and 5 controls using methylation specific PCR after bisulfite conversion (Figure 4.9). Individuals were analyzed on the basis on methylation status of TNFα promoter region at two specific sites using two sets of primers. The data observed was statistically analyzed.

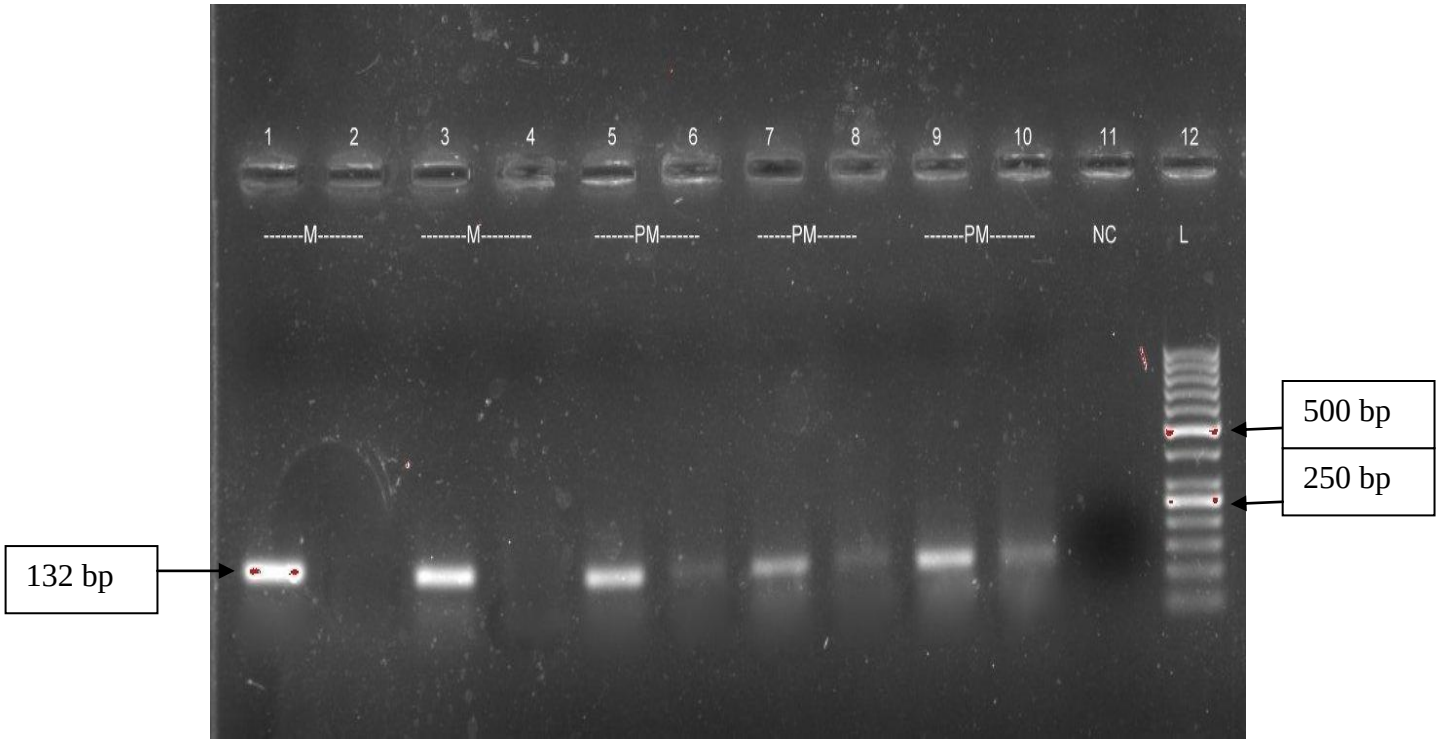

Figure 4.9: Electropherogram of EtBr stained 2% agarose gel of methylation specific PCR. Methylation status was determined by visualizing PCR product band in the methylated or un-methylated wells. Lanes 1, 3, 5, 7, and 9 represent the methylated specific primer while Lane 2, 4, 6, 8, and 10 show un-methylated specific primer. Sample in lane 1 and 3 were methylated and those in 5, 7 and 9 were partially methylated as seen due to the presence of bands in both wells. NC is negative control.

Table 4.8 summarizes the individual methylation status of controls and RA patients for both set of primers used as examined by methylation-specific and un-methylation specific PCR. *p* value for both primer sets was <0.0001 suggesting significant association of methylation with rheumatoid arthritis.

The percentage of individuals and their methylation status is shown in Figure 4.10 for each primer set. Thus data suggests significant association of un-methylation of TNFα promoter with RA.

Table 4.8: Methylation Status of TNFα promoter

S.No			Methylation Status			*p* value
			Methylated	Partially Methylated	Un-methylated	
1	**MSP1**	RA Cases	2 (20%)	5 (50%)	3 (30%)	0.0001
		Controls	0	5 (100%)	0	
2	**MSP2**	RA Cases	4 (40%)	5 (50%)	1 (10%)	0.0001
		Controls	0	5 (100%)	0	

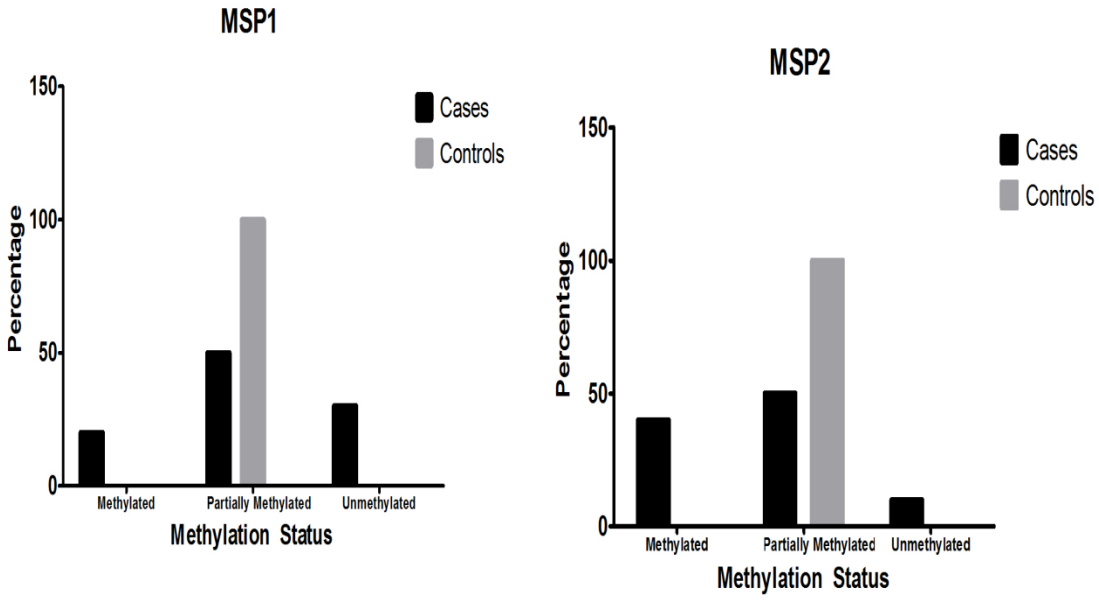

Figure 4.10: Methylation status for MSP1 and MSP2

CHAPTER 5

DISCUSSION

Rheumatoid arthritis is a multi-factorial inflammatory disease that affects humans around the globe. It causes pain, swelling of joints leading to stiffness and ultimately loss of function in the affected patients. The presence and expression of RA can be visualized as the results of an undefined stochastic process on a genetic basis of particular HLA genes, gender and unknown numerous genes. Each of these genetic factors has a discrete effect on the susceptibility and severity of RA. It has been observed to be present 2-4 times more in the female population as compared to males (Kvien *et al.,* 2006) which is in accordance with what was observed in the given study, out of the 125 cases 87.3% presented the female population while 12.7% were male.

The autoimmune component of the disease cause chronic inflammation of joints and destruction of bone and cartilage thereby suggesting a regulatory of pro-inflammatory cytokines such as TNFα.

Numerous studies have been focused on the association of TNFα promoter polymorphisms and RA susceptibility. Of all the reported polymorphisms some have been extensively studied while others still need more studies to distinctly relate the presence with disease. The data present so far represents contradictory outcomes which can be attributed to a lot of factors but it is highly probable that promoter variants for TNFα are indeed related to disease severity and response to treatment (Fonseca *et al.,* 2007).

There has been strong evidence that in osteoarthritis (OA), TNF-α is one of the crucial mediators of disease altering the delicate equilibrium of cartilage matrix degradation and repair, leading to cartilage breakdown (Sellam *et al.,* 2008). However it was observed that rs361525 SNP did not confer susceptibility to the disease and also did not contribute to the phenotype of OA in the Han Chinese population (Ji *et al.,* 2013). Similarly, no association between TNF rs361525 and TNF inhibitors (Swierkot *et al.,* 2014) was seen in Caucasian population.

Another study based on Caucasian population showed that the variant TNF-α -238 allele had no significant effect in inflammatory bowel disease (Ferguson *et al.,* 2008). However some publications report positive correlation in Japanese, Hungarian and Korean groups, even though its with small numbers of patients. Kim *et al.,* (2003) showed in a study on Korean patients a significantly higher frequency of -238A allele of TNF-α. While Vatay *et al.,* (2003) also showed an increased risk in Hungarian subjects.

It was seen after statistical scrutiny that rs316525 had no linkage of genotype to the incidence of RA even though Hardy-Weinberg calculations showed difference between observed and expected values. There was however significance in allele frequency suggesting that the variant allele A might infer susceptibility to the risk of RA.

It should be however be taken into consideration that study carried out by Munoz-Valle *et al.,* (2012) had smaller cases to control ratio making it complicated to detect the genotype AA of TNF-α -238, since it is the minor leading to the potential of false results. Therefore a larger study group should be analyzed to minimize the possibility of false results.

The frequency of the TNFα promoter SNP rs1800750 A allele found in the RA multiplex families in a study by (Waldron-Lynch *et al.,* 2001) was seen in only one individual carrying a rare haplotype which correlates to other studies by Hamann *et al.,* (1995) and Brinkman *et al.,* (1997) signifying that this alleles occur too rarely to play a major role in RA susceptibility in these populations.

In the present study the SNP rs1800750 was seen to be significantly associated with RA risk susceptibility shown by the genotypic and allelic discrepancies between cases and controls. The calculations via Hardy-Weinberg Equilibrium also managed to show significant difference in the occurrence of polymorphism.

The analysis of SNPs in the TNF-α promoter may point to both pharmacogenomic and nutrigenomic advances to combat the pathway defect. Stio *et al.,* (2007) observed that a Vitamin D analogue, TX 527 could have an immunosuppressive effect on TNF-α production in Crohns disease patients mediated by the down-regulation of NF-κB via the activation of the Vitamin D receptor (VDR). This suggests that natural or synthetic vitamin D analogues may provide a basis for therapeutic interventions that could be especially helpful in individuals carrying variant alleles in the TNF-α promoter.

Additionally genetic susceptibility isn't that only factor contributing to disease susceptibility and severity. Other factors such as epigenetic modifications at DNA and histone levels also play a significant role in pathogenesis. Various genes have been implicated in disease development by virtue of epigenetic modifications. Preceding

studies have shown that numerous CpG motifs exist in the promoter region of apoptosis-inducing TNF receptor super-family genes (Rudert *et al.,* 1995).

TNFα is known to be over-expressed in metabolic disorders and other inflammatory diseases. Variations in its secretion by macrophages are mediated by epigenetic modifications that occur during the monocyte differentiation (Sullivan *et al.,* 2007). Determining the methylation status of RA patients and controls showed that methylation might play a detrimental role in pathogenesis of RA.

Although the changes between controls and patients were comparatively reticent it could be that similar changes in the genes encoding other important regulators of inflammation could have synergistic effects leading to larger biological consequences leading to the development of age-related inflammatory rheumatic diseases such as RA (Gowers *et al.,* 2011). The recent advancements in ultra high throughput DNA sequencing technology will facilitate studies to determine the role of epigenetics in the development of RA.

CONCLUSION

In this study, the results showed that there is a lack of association of rs361525 promoter polymorphism with RA while there is association of rs1800750 with the disease in Pakistani population. Other polymorphism may have impact on disease risk and also play a role in disease severity but this requires a large sample size to eliminate the limitations of this study. However, the significant change in methylation status of TNF-α promoter region shows that genetic variations are not the only cause of pathogenesis suggesting

that better therapeutic interventions can be made when the multi-factorial nature of the disease is considered.

CHAPTER 6

REFERENCES

Aggarwal, S., N. Ghilardi, *et al.,* (2002). Interleukin-23 Promotes a Distinct CD4 T cell Activation State Characterized by the Production of Interleukin-17. *Journal of Biological Chemistry, 278(3):* 1910-1914.

Aguillón, J. C., A. Cruzat, *et al.,* (2006). Could single-nucleotide polymorphisms (SNPs) affecting the tumor necrosis factor promoter be considered as part of rheumatoid arthritis evolution? *Immunobiology, 211(1-2):* 75-84.

Aguillon, J. C., Cruzat, A., Aravena, O., Salazar, L., Llanos, C., Cuchacovich, M. (2006). Could single-nucleotide polymorphisms (SNPs) affecting the tumor necrosis factor promoter be considered as part of rheumatoid arthritis evolution? *Immunobiology, 211*: 75-84.

Ahmed, S., S. Riegsecker, *et al.,* (2013). Largazole, a class I histone deacetylase inhibitor, enhances TNF-α-induced ICAM-1 and VCAM-1 expression in rheumatoid arthritis synovial fibroblasts. *Toxicology and Applied Pharmacology, 270(2)*: 87-96.

Aho, K., O. Kaipiainen-Seppanen, *et al.,* (1998). Epidemiology of rheumatoid arthritis in Finland. *Seminars in Arthritis Rheumatism, 27(5)*: 325-334.

Akar, S., M. Birlik, *et al.,* (2004). The prevalence of rheumatoid arthritis in an urban population of Izmir-Turkey. *Clinical Experimental Rheumatology, 22(4)*: 416-420.

Alamanos, Y. and A. Drosos (2005). Epidemiology of adult rheumatoid arthritis. *Autoimmunity Reviews, 4(3)*: 130-136.

Aletaha, D., T. Neogi, *et al.,* (2010). 2010 Rheumatoid arthritis classification criteria: An American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis & Rheumatism, 62(9)*: 2569-2581.

Aryaeian, N., Shahram, F., Djalali, M., *et al.,* (2009) Effect of conjugated linoleic acids, vitamin E and their combination on the clinical outcome of Iranian adults with active rheumatoid arthritis. *International Journal of Rheumatic Diseases, 12*: 20–28.

Auger, I., M. Sebbag, *et al.,* (2005). Influence of HLA-DR genes on the production of rheumatoid arthritis-specific autoantibodies to citrullinated fibrinogen. *Arthritis & Rheumatism, 52(11)*: 3424-3432.

Bae, S. C., Kim, S. J., Sung, M. K., (2003). Inadequate antioxidant nutrient intake and altered plasma antioxidant status of rheumatoid arthritis patients. *Journal of the American College of Nutrition, 22*: 311–315

Bannister, A. J. and T. Kouzarides (2011). Regulation of chromatin by histone modifications. *Cell Research, 21(3)*: 381-395.

Berglin, E., H. Kokkonen, *et al.,* (2010). Influence of female hormonal factors, in relation to autoantibodies and genetic markers, on the development of rheumatoid arthritis in northern Sweden: a case–control study. *Scandinavian Journal of Rheumatology, 39(6):* 454-460.

Bingham Iii, C. O. (2002). The pathogenesis of rheumatoid arthritis: Pivotal cytokines involved in bone degradation and imflammation. *Journal of Rheumatology, 29(SUPPL. 65)*: 3-9.

Bouma, G., J. B. A. Crusius, *et al.*, (1996). Secretion of Tumor Necrosis Factor α and Lymphotoxin α in Relation to Polymorphisms in the TNF Genes and HLA-DR Alleles. Relevance for Inflammatory Bowel Disease. *Scandinavian Journal of Immunology, 43(4)*: 456-463.

Boyce, B. F., Xing, L (2007). Biology of RANK, RANKL, and osteoprotegerin. *Arthritis Research and Therapy, 9(1).*

Brennan, F. M. and I. B. McInnes (2008). Evidence that cytokines play a role in rheumatoid arthritis. *Journal of Clinical Investigation, 118(11)*: 3537-3545.

Brinkman, B. M. N., Huizinga, T. W. J., Kurban, S. S. et al (1997). Tumor necrosis factor α gene polymorphisms in rheumatoid arthritis: association with susceptibility to, or severity of disease? *British Journal of Rheumatology, 36*: 516–521.

Brinkman, B. M., E. L. Kaijzel, *et al.*, (1995). Detection of a C-insertion polymorphism within the human tumor necrosis factor alpha (TNFA) gene. *Human Genetics, 96(4)*: 493.

Burska, A., M. Boissinot, *et al.*, (2014). Cytokines as Biomarkers in Rheumatoid Arthritis. *Mediators of Inflammation,* 1-24.

Capellino, S., M. Cosentino, *et al.*, (2010). Catecholamine-producing cells in the synovial tissue during arthritis: modulation of sympathetic neurotransmitters as new therapeutic target. *Annals of Rheumatic Disease, 69(10)*: 1853-1860.

Carmona, L., M. Cross, *et al.*, (2010). Rheumatoid arthritis. *Best Practice & Research Clinical Rheumatology, 24(6)*: 733-745.

Chabchoub, G., E. Uz, *et al.*, (2009). Analysis of skewed X-chromosome inactivation in females with rheumatoid arthritis and autoimmune thyroid diseases. *Arthritis Research & Therapy, 11(4)*: R106.

Chen, G. (2010). Immunotherapy of rheumatoid arthritis targeting inflammatory cytokines and auto reactive T cells. *Archivum Immunologiae et Therapiae Experimentalis, 58(1)*: 27-36.

Chen, J. Y., C. M. Wang, *et al.*, (2006). Association of rheumatoid factor production with FcγRIIIa polymorphism in Taiwanese rheumatoid arthritis. *Clinical & Experimental Immunology, 144(1)*: 10-16.

Choi, E. J., Bae, S. C., Yu, R., Youn, J., Sung, M. K., (2009). Dietary vitamin E and quercetin modulate inflammatory responses of collagen-induced arthritis in mice. *Journal of Medicinal Food, 12*: 770–775.

Choy, E. H. S., Panayi, G. S (2001). Cytokine Pathways and Joint Inflammation in Rheumatoid Arthritis. *New England Journal of Medicine, 344(12)*: 907-916.

Cohen, S. B., R. K. Dore, *et al.,* (2008). Denosumab treatment effects on structural damage, bone mineral density, and bone turnover in rheumatoid arthritis: A twelve-month, multicenter, randomized, double-blind, placebo-controlled, phase II clinical trial. *Arthritis & Rheumatism, 58(5)*: 1299-1309.

Costenbader, K. H., S. C. Chang, *et al.,* (2008). Geographic variation in rheumatoid arthritis incidence among women in the United States. *Archives of Internal Medicine, 168(15)*: 1664-1670.

Cuenca, J., Cuchacovich, M., Perez, C., Ferreira, L., Aguirre, A., Schiattino, I., Soto, L., Cruzat, A., Salazar-Onfray, F., Aguillon, J.C. (2003). The -308 polymorphism in the tumor necrosis factor gene promoter region and ex vivo lipopolysaccharide-induced TNF expression and cytotoxic activity in Chilean patients with rheumatoid arthritis. *Rheumatology, 42*: 308-313.

Cutolo, M., Villaggio, B., Craviotto, C., Pizzorni, C., Seriolo, B., Sulli, A (2002). Sex hormones and rheumatoid arthritis. *Autoimmunity Reviews,* 284-289.

Cvetkovic, J. T., S. Wallberg-Jonsson, *et al.,* (2002). Susceptibility for and clinical manifestations of rheumatoid arthritis are associated with polymorphisms of the TNF-alpha, IL-1beta, and IL-1Ra genes. *Journal of Rheumatology, 29(2)*: 212-219.

Dai, S. M., X. H. Han, *et al.,* (2003). Prevalence of rheumatic symptoms, rheumatoid arthritis, ankylosing spondylitis, and gout in Shanghai, China: a COPCORD study. *Journal of Rheumatology, 30(10)*: 2245-2251.

Dai, Y., Zhang, L., Hu, C., Zhang, Y (2010). Genome-wide analysis of histone H3 lysine 4 trimethylation by ChIP-chip in peripheral blood mononuclear cells of systemic lupus erythematosus patients. *Clinical and Experimental Rheumatology, 28*:158-168.

Danis, V. A., M. Millington, *et al.*, (1995). Increased frequency of the uncommon allele of a tumor necrosis factor alpha gene polymorphism in rheumatoid arthritis and systemic lupus erythematosus. *Disease Markers, 12(2)*: 127-133.

de la Rica, L., Urquiza, J. M., Gomez-Cabrero, D., *et al.*, (2013) Identification of novel markers in rheumatoid arthritis through integrated analysis of DNA methylation and microRNA expression. *Journal of Autoimmunity, 41*: 6e16.

de Pablo, P., T. Dietrich, *et al.*, (2008). Association of periodontal disease and tooth loss with rheumatoid arthritis in the US population. *The Journal of Rheumatology, 35(1)*: 70-76.

Deane, K. D., C. I. O'Donnell, *et al.*, (2010). The number of elevated cytokines and chemokines in preclinical seropositive rheumatoid arthritis predicts time to diagnosis in an age-dependent manner. *Arthritis & Rheumatism, 62(11)*: 3161-3172.

Di Giuseppe, D., Discacciati, A., Orsini, N., Wolk, A. (2014) Cigarette smoking and risk of rheumatoid arthritis: A dose-response meta-analysis. *Arthritis Research and Therapy, 16*: R61.

Dong, C., Flavell, R. A (2000). Cell fate decision: T-helper 1 and 2 subsets in immune responses. *Arthritis Research, 2(3)*: 179-188.

Doran, M. F., C. S. Crowson, *et al.*, (2004). The effect of oral contraceptives and estrogen replacement therapy on the risk of rheumatoid arthritis: a population based study. *The Journal of Rheumatology, 31(2)*: 207-213.

Doran, M. F., G. R. Pond, *et al.*, (2002). Trends in incidence and mortality in rheumatoid arthritis in Rochester, Minnesota, over a forty-year period. *Arthritis Rheumatology, 46(3)*: 625-631.

Drosos, A. A., Alamanos, I., Voulgari, P. V., Psychos, D. N., Katsaraki, A., Papadopoulos, .I, et al (1997) Epidemiology of adult rheumatoid arthritis in northwest Greece 1987–1995. *Journal of Rheumatology, 24*: 2129– 33.

Elliot, M. J., Maini, R. N., Feldmann, M., Long-Fox, A., Charles, P., Katsikis, P., Brennan, F. M., Walker, J., Bilj, H., Ghrayeb, J., Woody, J. N. (1993). Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor alpha. *Arthritis & Rheumatism, 36*: 1681-1690.

Feldmann, M. (2001). Pathogenesis of arthritis: recent research progress. *Nature Immunology, 2(9)*: 771-773.

Feldmann, M., Brennan, F. M., Maini, R. N (1996). Rheumatoid arthritis. *Cell, 85*: 307-310.

Ferguson, L.-R. (2008). Single nucleotide polymorphism in the tumor necrosis factor-alpha gene affects inflammatory bowel diseases risk. *World Journal of Gastroenterology, 14(29)*: 4652.

Fonseca, J. E., J. Cavaleiro, *et al.,* (2007). Contribution for new genetic markers of rheumatoid arthritis activity and severity: sequencing of the tumor necrosis factor-alpha gene promoter. *Arthritis Research Therapy, 9(2).*

Fu, L.-h., C.-l. Ma, *et al.,* (2011). Hypomethylation of proximal CpG motif of interleukin-10 promoter regulates its expression in human rheumatoid arthritis. *Acta Pharmacologica Sinica, 32(11)*: 1373-1380.

Fuggle, N. R., F. A. Howe, *et al.,* (2014). New insights into the impact of neuro-inflammation in rheumatoid arthritis. *Frontiers in Neuroscience, 8*.

Gabriel, S. E., C. S. Crowson, *et al.,* (2003). Survival in rheumatoid arthritis: a population-based analysis of trends over 40 years. *Arthritis Rheumatism, 48(1)*: 54-58.

Gibert, M. and A. Sanchez-Mazas (2003). Geographic patterns of functional categories of HLA-DRB1 alleles: a new approach to analyse associations between HLA-DRB1 and disease. *European Journal of Immunogenetics, 30(5)*: 361-374.

Glant, T. T., T. Besenyei, *et al.,* (2013). Differentially Expressed Epigenome Modifiers, Including Aurora Kinases A and B, in Immune Cells in Rheumatoid Arthritis in Humans and Mouse Models. *Arthritis & Rheumatism, 65(7)*: 1725-1735.

Gowers, I. R., K. Walters, *et al.,* (2011). Age-related loss of CpG methylation in the tumor necrosis factor promoter. *Cytokine, 56(3)*: 792-797.

Grabiec, A. M., S. Krausz, *et al.,* (2010). Histone Deacetylase Inhibitors Suppress Inflammatory Activation of Rheumatoid Arthritis Patient Synovial Macrophages and Tissue. *The Journal of Immunology, 184(5):* 2718-2728.

Gravallese, E. M., C. Manning, *et al.,* (2000). Synovial tissue in rheumatoid arthritis is a source of osteoclast differentiation factor. *Arthritis & Rheumatism, 43(2)*: 250-258.

Gregersen, P. K., Silver, J., Winchester, R. J (1987) The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. *Arthritis and Rheumatism, 30*:1205e13.

Hajeer, A. H. and I. V. Hutchinson (2000). TNF-alpha gene polymorphism: clinical and biological implications. *Microscopy Research and Technique, 50(3)*: 216-228.

Hamann, A., C. Mantzoros, *et al.,* (1995). Genetic variability in the TNF-alpha promoter is not associated with type II diabetes mellitus (NIDDM). *Biochemical and Biophysical Research Communications, 211(3)*: 833-839.

Harney, S. (2003). Molecular genetics of rheumatoid arthritis. *Current Opinion in Pharmacology, 3(3)*: 280-285.

Harrington, L. E., Hatton, R. D., Mangan, P. R., Turner, H., Murphy, T. L., Murphy, K. M., Weaver, C. T (2005). Interleukin 17–producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nature Immunology, 6*: 1123-1132.

Harris, E. D. (2005). Clinical features of rheumatoid arthritis. *Kelleys Textbook of Rheumatology*, 1043-1078.

Harris, J., Keane, J. (2010). How tumor necrosis factor blockers interfere with tuberculosis immunity. *Clinical Experimental Immunology, 161*: 1-9.

Hasen, G. V., Nielsen, L., Kluger, E., *et al.*,, (1996) Nutritional status of Danish rheumatoid arthritis patients and effects of a diet adjusted in energy intake, fish-meal, and antioxidants. *Scandinavian Journal of Rheumatology, 25*: 325–330.

Heikkila, R., Aho, K., Heliovaara, M., Knekt, P., Reunanen, A., Aromaa, A., et al (1998). Serum androgen-anabolic hormones and the risk of rheumatoid arthritis. *Annals of Rheumatic Disease, 57*:281–5

Hoovestol, R. A. and T. R. Mikuls (2011). Environmental exposures and rheumatoid arthritis risk. *Current rheumatology reports, 13(5)*: 431-439.

Huber, L. C., M. Brock, *et al.*, (2007). Histone deacetylase/acetylase activity in total synovial tissue derived from rheumatoid arthritis and osteoarthritis patients. *Arthritis & Rheumatism, 56(4)*: 1087-1093.

Hughes-Austin, J. M., K. D. Deane, *et al.*, (2013). Multiple cytokines and chemokines are associated with rheumatoid arthritis-related autoimmunity in first-degree relatives without rheumatoid arthritis: Studies of the Aetiology of Rheumatoid Arthritis (SERA). *Annals of the Rheumatic Diseases, 72(6)*: 901-907.

Huizinga, T. W. J. (2003). Genetics in rheumatoid arthritis. *Best Practice & Research Clinical Rheumatology, 17(5)*: 703-716.

Ikeuchi, H., T. Kuroiwa, *et al.,* (2005). Expression of interleukin-22 in rheumatoid arthritis: Potential role as a proinflammatory cytokine. *Arthritis & Rheumatism, 52(4)*: 1037-1046.

Ishida, K., T. Kobayashi, *et al.,* (2012). Interleukin-6 Gene Promoter Methylation in Rheumatoid Arthritis and Chronic Periodontitis. *Journal of Periodontology, 83(7)*: 917-925.

Jeffery, R. C. (2014). Clinical features of rheumatoid arthritis. *Medicine, 42(5)*: 231-236.

Ji, B., J. Shi, *et al.,* (2013). Association analysis of two candidate polymorphisms in the Tumor Necrosis Factor-α gene with osteoarthritis in a Chinese population. *International Orthopaedics, 37(10)*: 2061-2063.

Jia, T., Y. Pan, *et al.,* (2013). Strategies for active TNF-α vaccination in rheumatoid arthritis treatment. *Vaccine, 31(38)*: 4063-4068.

Jones, P. A (2012) Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nature Review Genetics, 13(7)*: 484e92.

Jørgensen, K. T., A. Wiik, *et al.,* (2008). Cytokines, autoantibodies and viral antibodies in premorbid and postdiagnostic sera from patients with rheumatoid arthritis: case–control study nested in a cohort of Norwegian blood donors. *Annals of the Rheumatic Diseases, 67(6)*: 860-866.

Karouzakis, E., Y. Rengel, *et al.*, (2011). DNA methylation regulates the expression of CXCL12 in rheumatoid arthritis synovial fibroblasts. *Genes and Immunity*, *12(8)*: 643-652.

Kawabata, T., K. Nishida, *et al.*, (2010). Increased activity and expression of histone deacetylase 1 in relation to tumor necrosis factor-alpha in synovial tissue of rheumatoid arthritis. *Arthritis Research & Therapy, 12(4)*: R133.

Keen, H. I., A. K. Brown, *et al.*, (2005). MRI and Musculoskeletal Ultrasonography as Diagnostic Tools in Early Arthritis. *Rheumatic Disease Clinics of North America*, *31(4)*: 699-714.

Keffer, J., Probert, L., Cazlaris, H., Georgopoulos, S., Kaslaris, E., Kioussis, D., Kollias, G. (1991). Transgenic mice expressing human tumor necrosis factor: a predictive genetic model of arthritis. *EMBO J, 10*: 4025-2031.

Kempeni, J. (1999). Preliminary results of early clinical trials with the fully human anti-TNFalpha monoclonal antibody D2E7. *Annals of Rheumatic Diseases, 58 (Suppl I)*: 170-172.

Keystone, E. C. and C. F. Ware (2010). Tumor Necrosis Factor and Anti-Tumor Necrosis Factor Therapies. *The Journal of Rheumatology Supplement, 85(0)*: 27-39.

Kim, H. R., M. L. Cho, *et al.*, (2007). Up-regulation of IL-23p19 expression in rheumatoid arthritis synovial fibroblasts by IL-17 through PI3-kinase-, NF- B- and p38 MAPK-dependent signalling pathways. *Rheumatology, 46(1)*: 57-64.

Kim, T. H., B. G. Kim, *et al.,* (2003). Tumor necrosis factor-alpha and interleukin-10 gene polymorphisms in Korean patients with inflammatory bowel disease. *Korean Journal of Gastroenterology, 42(5)*: 377-386.

Klein, K. and S. Gay (2013). Epigenetic modifications in rheumatoid arthritis, a review. *Current Opinion in Pharmacology, 13(3)*: 420-425.

Kochi, Y., A. Suzuki, *et al.,* (2014). Genetic basis of rheumatoid arthritis: A current review. *Biochemical and Biophysical Research Communications, 452(2)*: 254-262.

Koenders, M. I., E. Lubberts, *et al.,* (2006). Interleukin-17 Acts Independently of TNF-α under Arthritic Conditions. *The Journal of Immunology, 176(10)*: 6262-6269.

Krishnan, E. (2003). Smoking, gender and rheumatoid arthritis – epidemiological clues to etiology. Results from the behavioral risk factor surveillance system. *Joint Bone Spine, 70*:496–502.

Kvien, T. K., T. Uhlig, *et al.,* (2006). Epidemiological aspects of rheumatoid arthritis: the sex ratio. *Annals of New York Academy of Sciences,* 212-222.

Kyburz, D., E. Karouzakis, *et al.,* (2014). Epigenetic changes: The missing link. *Best Practice & Research Clinical Rheumatology, 28(4)*: 577-587.

Lau, E., D. Symmons, *et al.,* (1993). Low prevalence of rheumatoid arthritis in the urbanized Chinese of Hong Kong. *Journal of Rheumatology, 20(7)*: 1133-1137.

Lee, Y. H., J. D. Ji, *et al.*, (2007). Tumor necrosis factor-alpha promoter-308 A/G polymorphism and rheumatoid arthritis susceptibility: a metaanalysis. *The Journal of rheumatology, 34(1)*: 43-49.

Lee, Y.-R., S.-H. Kweon, *et al.*, (2009). Inhibition of IL-1β-mediated inflammatory responses by the IκBα super-repressor in human fibroblast-like synoviocytes. *Biochemical and Biophysical Research Communications, 378(1)*: 90-94.

Liao K. P, Karlson E.W (2011). Rheumatoid arthritis and other synovial disorders: classification and epidemiology. In: Hochberg MC, Silman AJ, Smolen JS, Weinblatt ME, Weisman MH, editors. *Rheumatology, 5rd ed*. Mosby. p. 823-827.

Liao, J., Liang, G., Xie, S., *et al.*, (2012) CD40L demethylation in CD4(+) T cells from women with rheumatoid arthritis. *Clinical Immunology, 145(1)*:13e8.

Lin, S. Y., S. C. Hsieh, *et al.*, (2011). A whole genome methylation analysis of systemic lupus erythematosus: hypomethylation of the IL10 and IL1R2 promoters is associated with disease activity. *Genes and Immunity, 13(3)*: 214-220.

Lindqvist, E., K. Jonsson, *et al.*, (2003). Course of radiographic damage over 10 years in a cohort with early rheumatoid arthritis. *Annals of the Rheumatic Diseases, 62(7)*: 611-616.

Lubberts, E., van den Berg, W. B. (2003). Cytokines in the pathogenesis of rheumatoid arthritis and collagen-induced arthritis. *Advances in Experimental Medicine and Biology, 520*: 194-202.

Luckey, D., M. Behrens, *et al.,* (2014). DRB1*0402 may influence arthritis by promoting naive CD4+T-cell differentiation in to regulatory T cells. *European Journal of Immunology, 44(11)*: 3429-3438.

Lundberg, K., N. Wegner, *et al.,* (2010). Periodontitis in RA—the citrullinated enolase connection. *Nature Reviews Rheumatology, 6(12)*: 727-730.

Machold, K. P., T. A. Stamm, *et al.,* (2007). Very recent onset rheumatoid arthritis: Clinical and serological patient characteristics associated with radiographic progression over the first years of disease. *Rheumatology, 46(2)*: 342-349.

Maini, R. N. and P. C. Taylor (2000). Anti-Cytokine Therapy for Rheumatoid Arthritis. *Annual Review of Medicine, 51(1)*: 207-229.

Majithia, V. and S. A. Geraci (2007). Rheumatoid Arthritis: Diagnosis and Management. *The American Journal of Medicine, 120(11)*: 936-939.

Matsuda, M., N. Sakamoto, *et al.,* (1992). Delta-thalassemia caused by disruption of the site for an erythroid-specific transcription factor, GATA-1, in the delta-globin gene promoter. *Blood, 80(5)*: 1347-1351.

McInnes, I. B., Schett, G. (2011). The Pathogenesis of Rheumatoid Arthritis. *New England Journal of Medicine, 365:* 2205-2219.

Meron, M. K., H. Amital, *et al.,* (2009). Infectious Aspects and the Etiopathogenesis of Rheumatoid Arthritis. *Clinical Reviews in Allergy & Immunology, 38(2-3)*: 287-291.

Mikuls, T. R., Saag, K. G., Criswell, L. A., Merlino, L. A., Kaslow, R. A., Shelton, B. J., Cerhan, J. R (2002). Mortality risk associated with rheumatoid arthritis in a prospective cohort of older women: results from the Iowa Women's Health Study. *Annals of the Rheumatic Diseases, 61*: 994-999.

Moreland, L., Baumgartner, S., Schiff, M., Tindall, E., Fleischmann, R., Weaver, A., Ettlinger, R., Cohen, S., Koopman, W., Mohler, K., Widmer, M., Blosch, C. (1997). Treatment of rheumatoid arthritis with a recombinant human tumor necrosis factor receptor (p75)-Fc fusion protein. *New England Journal of Medicine, 337:* 141-147.

Mulcahy, B., F. Waldron-Lynch, *et al.,* (1996). Genetic variability in the tumor necrosis factor-lymphotoxin region influences susceptibility to rheumatoid arthritis. *American Journal of Human Genetics*, *59(3)*: 676-683.

Munoz-Valle, J. F., E. Oregon-Romero, *et al.,* (2014). High expression of TNF alpha is associated with -308 and -238 TNF alpha polymorphisms in knee osteoarthritis. *Clinical Experimental Medicine, 14(1):* 61-67.

Nakano, K., J. W. Whitaker, *et al.,* (2012). DNA methylome signature in rheumatoid arthritis. *Annals of the Rheumatic Diseases, 72(1)*: 110-117.

Neidhart, M., Rethage, J., Kuchen, S., Kunzler, P., Crowl, R. M., Billingham, M. E., Gay, R. E., Gay, S. (2000). Retrotansposable L1 Elements expressed in Rheumatoid Arthritis synovial tissue association with genomic DNA hypomethylation and influence on gene Expression. *Arthritis & Rheumatism, 43(12)*: 2634-2647.

Nelson, J. L., K. A. Hughes, *et al.*, (1993). Maternal-fetal disparity in HLA class II alloantigens and the pregnancy-induced amelioration of rheumatoid arthritis. *New England Journal of Medicine, 329(7)*: 466-471.

Nielsen, S. F., S. E. Bojesen, *et al.*, (2012). Elevated rheumatoid factor and long term risk of rheumatoid arthritis: a prospective cohort study. *British Medical Journal, 345:* e5244.

Nile, C. J., R. C. Read, *et al.*, (2008). Methylation status of a single CpG site in theIL6promoter is related to IL6 messenger RNA levels and rheumatoid arthritis. *Arthritis & Rheumatism, 58(9)*: 2686-2693.

Nishimura, K., D. Sugiyama, *et al.*, (2007). Meta-analysis: Diagnostic Accuracy of Anti–Cyclic Citrullinated Peptide Antibody and Rheumatoid Factor for Rheumatoid Arthritis. *Annals of Internal Medicine, 146(11)*: 797-808.

Niu, X. and G. Chen (2014). Clinical Biomarkers and Pathogenic-Related Cytokines in Rheumatoid Arthritis. *Journal of Immunology Research*, 1-7.

Niu, X., D. He, *et al.*, (2010). IL-21 regulates Th17 cells in rheumatoid arthritis. *Human Immunology, 71(4)*: 334-341.

Niu, X., D. He, *et al.*, (2011). Regulatory immune responses induced by IL-1 receptor antagonist in rheumatoid arthritis. *Molecular Immunology, 49(1-2)*: 290-296.

O' Gradaigh, D. (2004). Joint erosion in rheumatoid arthritis: interactions between tumor necrosis factor, interleukin 1, and receptor activator of nuclear factor kB ligand (RANKL) regulate osteoclasts. *Annals of the Rheumatic Diseases, 63(4)*: 354-359.

Ozen, S., M. Alikasifoglu, *et al.*, (2002). Tumor necrosis factor alpha G>A -238 and G>A -308 polymorphisms in juvenile idiopathic arthritis. *Rheumatology, 41(2)*: 223-227.

Palladino, M. A., F. R. Bahjat, *et al.*, (2003). Anti-TNF-α therapies: the next generation. *Nature Reviews Drug Discovery, 2(9)*: 736-746.

Park, H., Li, Z., Yang, X. O., Chang, S. H., Nurieva, R.,Wang, Y., Wang, Y., Hood, L., Zhu, Z., Tian, Q., Dong, C (2005). A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nature Immunology, 6(11)*: 1133-1141.

Parks, E. L., Geha, P. Y.,Baliki, M. N., Katz, J., Schnitzer, T. J., Apkarian, A. V. (2011). Brain activity for chronic knee osteoarthritis: dissociating evoked pain from spontaneous pain. *European Journal of Pain, 15*: 843e841-843e814.

Pedersen, M., S. Jacobsen, *et al.*, (2006). *Arthritis Research & Therapy, 8(4)*: R133.

Petrovic-Rackov, L. and N. Pejnovic (2005). Clinical significance of IL-18, IL-15, IL-12 and TNF-α measurement in rheumatoid arthritis. *Clinical Rheumatology, 25(4)*: 448-452.

Pikwer, M., U. Bergström, *et al.*, (2009). Breast feeding, but not use of oral contraceptives, is associated with a reduced risk of rheumatoid arthritis. *Annals of the Rheumatic Diseases, 68(4)*: 526-530.

Pope, R. M., Shahrara, S (2013). Possible roles of IL-12-family cytokines in rheumatoid arthritis. *Nature Reviews Rheumatology, 9(4)*: 252-256.

Qu, N., M. Xu, *et al.*, (2013). Pivotal Roles of T-Helper 17-Related Cytokines, IL-17, IL-22, and IL-23, in Inflammatory Diseases. *Clinical and Developmental Immunology*, 1-13.

Qu, Z., Garcia, C. H., O'Rourke, L. M., Planck, S. R., Kohli, M., Rosenbaum, J. T (1994). Local proliferation of fibroblast-like synoviocytes contributes to synovial hyperplasia. Results of proliferating cell nuclear antigen/cyclin, c-myc, and nucleolar organizer region staining. *Arthritis & Rheumatism, 37*: 212-220.

Riise, T., B. K. Jacobsen, *et al.,* (2000). Incidence and prevalence of rheumatoid arthritis in the county of Troms, northern Norway. *Journal of Rheumatology, 27(6)*: 1386-1389.

Rosenstein, E. D., Greenwald, R. A., Kushner, L. J., Weissmann, G. (2004) Hypothesis: the humoral immune response to oral bacteria provides a stimulus for the development of rheumatoid arthritis. *Inflammation, 28*:311–8.

Rudert, F., E. Visser, *et al.,* (1995). Identification of a silencer, enhancer, and basal promoter region in the human CD95 (Fas/APO-1) gene. *DNA Cell Biology, 14(11)*: 931-937.

Ruiz-Esquide, V., Sanmarti, R (2012). Tobacco and Other Environmental Risk Factors in Rheumatoid Arthritis. *Reumatologia Clinica, 8(6)*: 342-350.

Salliot, C., C. Bombardier, *et al.*, (2010). Hormonal replacement therapy may reduce the risk for RA in women with early arthritis who carry HLA-DRB1 *01 and/or *04 alleles by protecting against the production of anti-CCP: results from the ESPOIR cohort. *Annals of the Rheumatic Diseases, 69(9)*: 1683-1686.

Sato, K., Suematsu, A., Okamoto,K., Yamaguchi, A., Morishita,Y., Kadono, Y., Tanaka, S., Kodama,T., Akira, S., Iwakura, Y., Cua, D. J., Takayanagi, H (2006). Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. *Journal of Experimental Medicine, 203(12)*: 2673-2682.

Schett, G., Firestein, G. S. (2010). Mr Outside and Mr Inside: classic and alternative views on the pathogenesis of rheumatoid arthritis. *Annals of Rheumatic Diseases, 69:* 787–789.

Schiff, M. H. (2000). Role of interleukin 1 and interleukin 1 receptor antagonist in the mediation of rheumatoid arthritis. *Annals of the Rheumatic Diseases, 59(1)*: i103-i108.

Scott, D. L., Wolfe, F., Huizinga, T. W. J. (2010). Rheumatoid arthritis. *Lancet, 376:* 1094-1108.

Sellam, J. and F. Berenbaum (2010). The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. *Nature Reviews Rheumatology, 6(11)*: 625-635.

Sellam, J., S. Rouanet, *et al.,* (2013). CCL19, a B Cell Chemokine, Is Related to the Decrease of Blood Memory B Cells and Predicts the Clinical Response to Rituximab in Patients With Rheumatoid Arthritis. *Arthritis & Rheumatism, 65(9)*: 2253-2261.

Senna, E. R., A. L. De Barros, *et al.,* (2004). Prevalence of rheumatic diseases in Brazil: a study using the COPCORD approach. *Journal of Rheumatology, 31(3)*: 594-597.

Shichikawa, K., K. Inoue, *et al.,* (1999). Changes in the incidence and prevalence of rheumatoid arthritis in Kamitonda, Wakayama, Japan, 1965-1996. *Annals of Rheumatic Diseases, 58(12)*: 751-756.

Soderlin, M. K., O. Borjesson, *et al.,* (2002). Annual incidence of inflammatory joint diseases in a population based study in southern Sweden. *Annals of Rheumatic Diseases, 61(10):* 911-915.

Spindler, A., Bellomio, V, Berman A, Lucero E, Baigorria M, Paz S, *et al.* (2002) Prevalence of rheumatoid arthritis in Tucuman, Argentina. *Journal of Rheumatology, 29:* 1166– 70

Stio, M., M. Martinesi, *et al.,* (2007). The Vitamin D analogue TX 527 blocks NF-kappaB activation in peripheral blood mononuclear cells of patients with Crohn's disease. *Journal of Steroid Biochemical and Molecular Biology, 103(1)*: 51-60.

Stolt P, Bengtsson C, Nordmark B, Lindblad S, Lundberg I, Klareskog L, *et al.,* (2003) Quantification of the influence of cigarette smoking on rheumatoid arthritis: results from a population based case-control study, using incident cases. *Annals of Rheumatic Diseases, 62:*835–41.

Sugiyama, D., K. Nishimura, *et al.,* (2010). Impact of smoking as a risk factor for developing rheumatoid arthritis: a meta-analysis of observational studies. *Annals of the Rheumatic Diseases, 69(01)*: 70-81.

Sullivan, K. E., A. B. Reddy, *et al.,* (2007). Epigenetic regulation of tumor necrosis factor alpha. *Molecular Cell Biology, 27(14):* 5147-5160.

Sung, M. K. and S. C. Bae (2013). Dietary Antioxidants and Rheumatoid Arthritis. *Bioactive Food as Dietary Interventions for Arthritis and Related Inflammatory Diseases,* 515-527.

Swierkot, J., K. Bogunia-Kubik, *et al.,* (2015). Analysis of associations between polymorphisms within genes coding for tumor necrosis factor (TNF)-alpha and TNF receptors and responsiveness to TNF-alpha blockers in patients with rheumatoid arthritis. *Joint Bone Spine, 82(2)*: 94-99.

Symmons, D. P. M. (2003). Environmental factors and the outcome of rheumatoid arthritis. *Best Practice & Research Clinical Rheumatology, 17(5)*: 717-727.

Syversen, S. W., P. I. Gaarder, *et al.,* (2008). High anti-cyclic citrullinated peptide levels and an algorithm of four variables predict radiographic progression in patients with rheumatoid arthritis: results from a 10-year longitudinal study. *Annals of the Rheumatic Diseases, 67(2)*: 212-217.

Takami, N., K. Osawa, *et al.,* (2006). Hypermethylated promoter region of DR3, the death receptor 3 gene, in rheumatoid arthritis synovial cells. *Arthritis & Rheumatism 54(3):* 779-787.

Taylor, P. C. (2014). Aetiopathology of rheumatoid arthritis. *Medicine, 42(5):* 227-230.

Tobón, G. J., P. Youinou, *et al.,* (2010). The environment, geo-epidemiology, and autoimmune disease: Rheumatoid arthritis. *Journal of Autoimmunity, 35(1)*: 10-14.

Tolboom, T. C. A., A. H. M. van der Helm-Van Mil, *et al.,* (2005). Invasiveness of fibroblast-like synoviocytes is an individual patient characteristic associated with the rate of joint destruction in patients with rheumatoid arthritis. *Arthritis & Rheumatism, 52(7):* 1999-2002.

Trajkov, D., S. Mishevska-Perchinkova, *et al.,* (2009). Association of 22 cytokine gene polymorphisms with rheumatoid arthritis in population of ethnic Macedonians. *Clinical Rheumatology, 28(11)*: 1291-1300.

Trenkmann, M., Brock, M., Gay, R. E., Kolling, C., Speich, R., Michel, B.A., Gay, S., Huber, L. C (2011). Expression and function of EZH2 in synovial fibroblasts: epigenetic repression of the Wnt inhibitor SFRP1 in rheumatoid arthritis. *Annals of Rheumatic Diseases, 70*: 1482-1488.

van der Helm-van Mil, A. H. M. and T. W. J. Huizinga (2008). Advances in the genetics of rheumatoid arthritis point to subclassification into distinct disease subsets. *Arthritis Research & Therapy, 10(2)*: 205.

Van-Gaalen, F. A., Linn-Rasker, S. P., van Venrooij, W. J (2004). Autoantibodies to cyclic citrullinated peptides predict progression to rheumatoid arthritis in patients with undifferentiated arthritis: a prospective cohort study. *Arthritis & Rheumatism, 50(3):* 709-715.

Vatay, A., L. Bene, *et al.,* (2003). Relationship between the tumor necrosis factor alpha polymorphism and the serum C-reactive protein levels in inflammatory bowel disease. *Immunogenetics, 55(4)*: 247-252.

Verweij, C. L. (1999). Tumor necrosis factor gene polymorphisms as severity markers in rheumatoid arthritis. *Annals of Rheumatic Diseases, 58(1)*: I20-26.

Verweij, C. L. and T. W. Huizinga (1998). Tumor necrosis factor alpha gene polymorphisms and rheumatic diseases. *British Journal of Rheumatology, 37(9)*: 923-6.

Vinasco, J., Berau´ n, Y., Nieto, A., Fraile, A., Mataran, L., Pareja, E., Martı´n, J. (1997). Polymorphisms at the TNF loci in rheumatoid arthritis. *Tissue Antigens, 49*: 74-78.

Waldron-Lynch, F., C. Adams, *et al.,* (2001). Tumor necrosis factor 5' promoter single nucleotide polymorphisms influence susceptibility to rheumatoid arthritis (RA) in immunogenetically defined multiplex RA families. *Genes Immunity, 2(2)*: 82-87.

Wang, J., Platt, A., Upmanyu, R., Germer, S., Lei, G., Rabe, C., Benayed, R., Kenwright, A., Hemmings, A., Martin, M., Harari, O. (2013). IL-6 pathway-driven investigation of response to IL-6 receptor inhibition in rheumatoid arthritis. *BMJ Open, 3(8)*.

Weinblatt, M. E., Kremer, J. M., Bankhurst, A. D., Bulpitt, K. J., Fleischmann, R. M., Fox, R. I., Jackson, C. G., Lange, M., Burge, D. J. (1999). A trial of etanercept, a recombinant tumor necrosis factor receptor: Fc fusion protein, in patients with rheumatoid arthritis receiving methotrexate. *New England Journal of Medicine, 340:* 253-259.

Williams, R. O., Feldmann, M., Maini, R. N. (1992). Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. *Proc Natl Acad Sci USA, 89*: 9784-9788.

Wilson, A. G., F. S. di Giovine, *et al.,* (1992). Single base polymorphism in the human tumor necrosis factor alpha (TNF alpha) gene detectable by NcoI restriction of PCR product. *Human Molecular Genetics, 1(5)*: 353.

Wilson, A. G., N. de Vries, *et al.,* (1995). A tumor necrosis factor alpha polymorphism is not associated with rheumatoid arthritis. *Annals of Rheumatic Diseases, 54(7)*: 601-603.

Wilson, A., N. De Vries, *et al.,* (1993). An allelic polymorphism within the human tumor necrosis factor alpha promoter region is strongly associated with HLA A1, B8, and DR3 alleles. *The Journal of experimental medicine, 177(2)*: 557-560.

Young, A. (1995). Short-term outcomes in recent-onset rheumatoid arthritis. *Rheumatology, 34(suppl 2)*: 79-86.

Zanelli, E., Breedveld, F. C., de Vries, R. R. (2000). HLA class II association with rheumatoid arthritis: facts and interpretations. *Human Immunology, 61*: 1254-1261